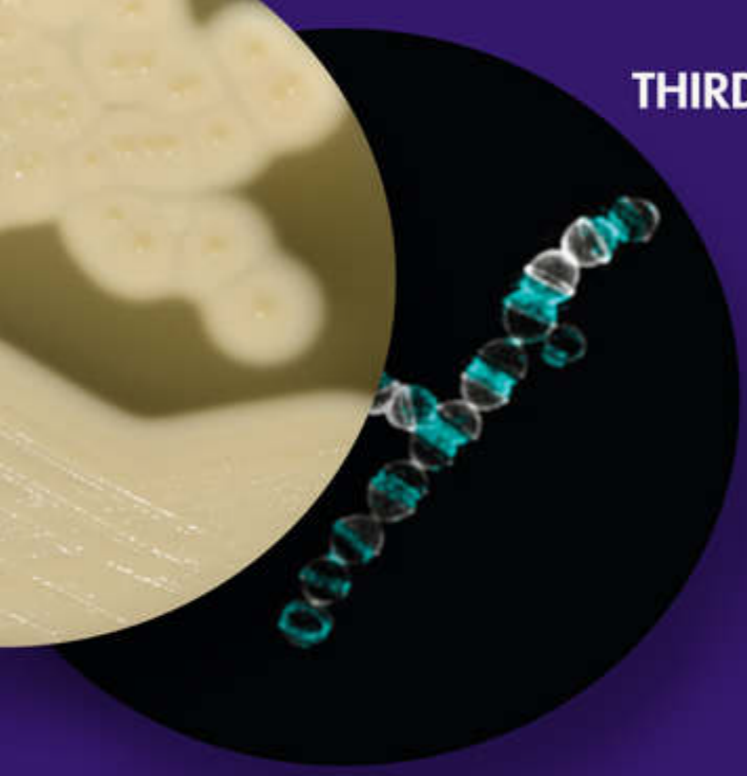
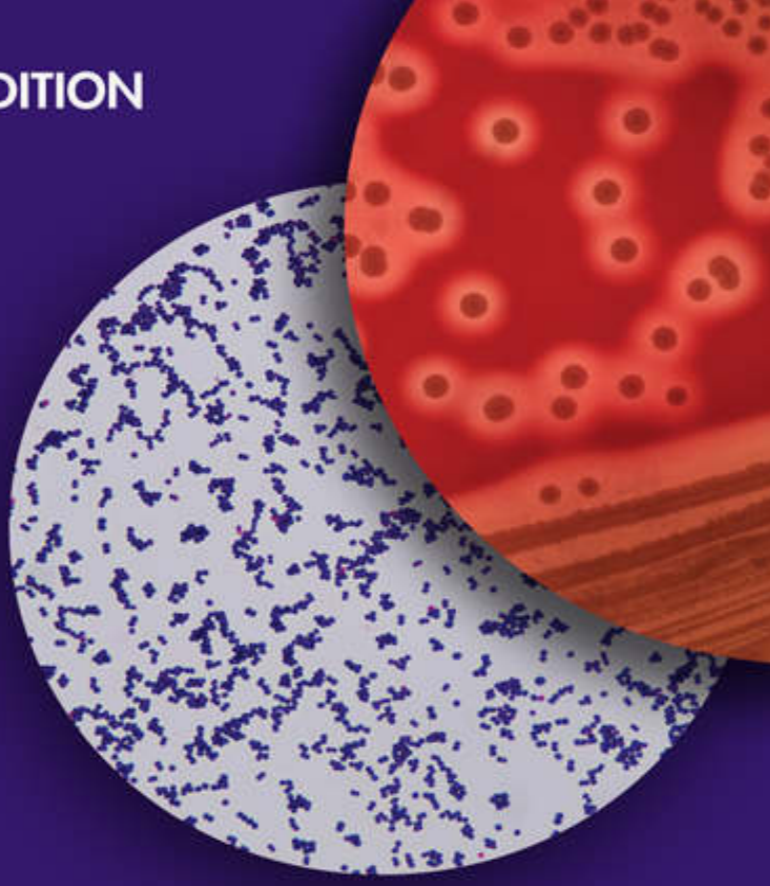
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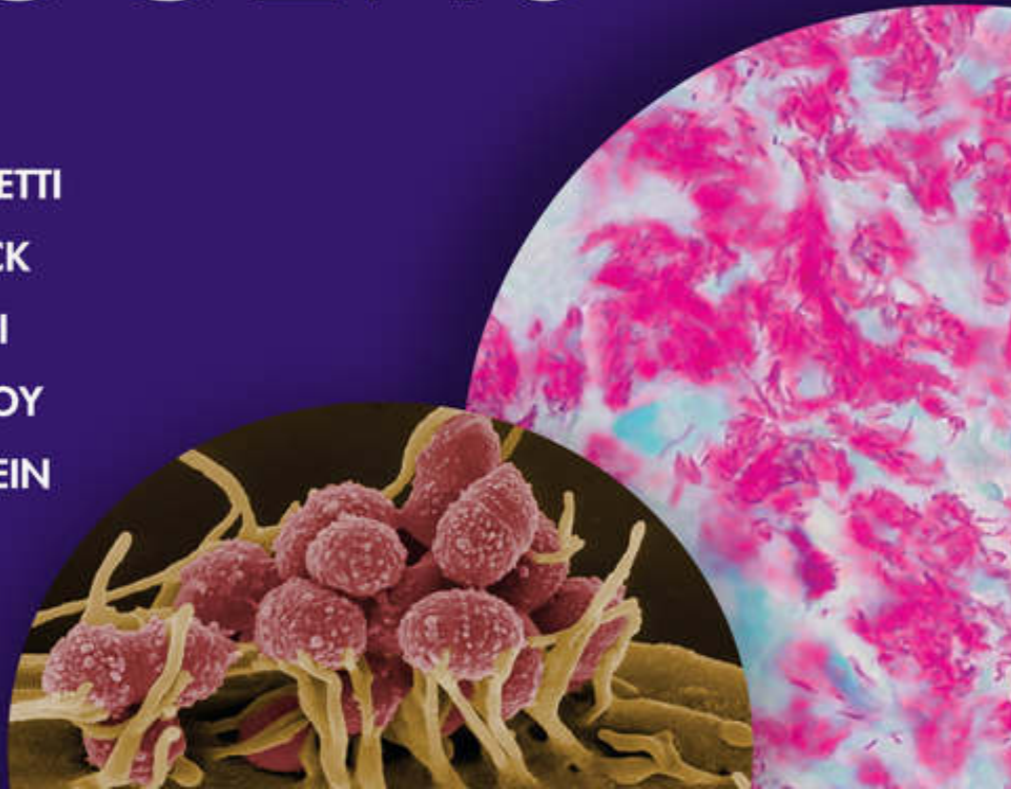
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Clostridium perfringens grown on egg yolk agar. Courtesy Steven Morton and Jackie Cheung, Monash University.

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Contents

Dedication / ix
Contributors / xi
Preface / xix
About the Editors / xxi

SECTION I
THE GRAM-POSITIVE CELL WALL / 1
SECTION EDITOR: Vincent A. Fischetti

1 The Gram-Positive Cell Wall / 3
MANFRED ROHDE

2 Surface Proteins on Gram-Positive Bacteria / 19
VINCENT A. FISCHETTI

SECTION 2
THE STREPTOCOCCUS / 33
SECTION EDITORS: Vincent A. Fischetti and Joseph J. Ferretti

A. GROUP A STREPTOCOCCI

3 Intracellular Invasion by *Streptococcus pyogenes*: Invasins, Host Receptors, and Relevance to Human Disease / 35
BEINAN WANG AND P. PATRICK CLEARY

4 Capsular Polysaccharide of Group A Streptococci / 45
MICHAEL R. WESSELS

5 Toxins and Superantigens of Group A Streptococci / 55
BLAKE A. SHANNON, JOHN K. McCORMICK, AND PATRICK M. SCHLIEVERT

6 Genetics of Group A Streptococci / 67
KYU HONG CHO, GARY PORT, AND MICHAEL CAPARON

7 Molecular Mimicry, Autoimmunity, and Infection: The Cross-Reactive Antigens of Group A Streptococci and Their Sequelae / 86
MADELEINE W. CUNNINGHAM

8 Extracellular Matrix Interactions with Gram-Positive Pathogens / 108
SVEN HAMMERSCHMIDT, MANFRED ROHDE, AND KLAUS T. PREISSNER

9 Group A *Streptococcus*-Mediated Host Cell Signaling / 125
VIJAY PANCHOLI

10 Vaccine Approaches To Protect against Group A Streptococcal Pharyngitis / 148
VINCENT A. FISCHETTI

11 The Bacteriophages of *Streptococcus pyogenes* / 158
W. MICHAEL McSHAN, KIMBERLY A. McCULLOR, AND SCOTT V. NGUYEN

12 Molecular Epidemiology, Ecology, and Evolution of Group A Streptococci / 177
DEBRA E. BESSEN, PIERRE R. SMEESTERS, AND BERNARD W. BEALL

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B. GROUP B STREPTOCOCCI

13 Surface Structures of Group B *Streptococcus* Important to Human Immunity / 204
LAWRENCE C. PAOLETTI AND DENNIS KASPER

14 Group B *Streptococcus* (*Streptococcus agalactiae*) / 228
VANESSA N. RAABE AND ANDI L. SHANE

C. GROUP C AND G STREPTOCOCCI

15 Genetics and Pathogenicity Factors of Group C and G Streptococci / 239
HORST MALKE

16 Pathogenicity Factors in Group C and G Streptococci / 264
CLAIRE E. TURNER, LAURA BUBBA, AND ANDROULLA EFSTRATIOU

17 Infections Caused by Group C and G *Streptococcus* (*Streptococcus dysgalactiae* subsp. *equisimilis* and Others): Epidemiological and Clinical Aspects / 275
GIO J. BARACCO

D. *STREPTOCOCCUS PNEUMONIAE*

18 The Cell Wall of *Streptococcus pneumoniae* / 284
WALDEMAR VOLLMER, ORIETTA MASSIDDA, AND ALEXANDER TOMASZ

19 *Streptococcus pneumoniae* Capsular Polysaccharide / 304
JAMES C. PATON AND CLAUDIA TRAPPETTI

20 *Streptococcus pneumoniae*: Invasion and Inflammation / 316
ALLISTER J. LOUGHRAN, CARLOS J. ORIHUELA, AND ELAINE I. TUOMANEN

21 Phase Variation of *Streptococcus pneumoniae* / 331
JING LI AND JING-REN ZHANG

22 Genomics and Genetics of *Streptococcus pneumoniae* / 344
FRANCESCO SANTORO, FRANCESCO IANNELLI, AND GIANNI POZZI

23 Pneumococcal Vaccines / 362
DAVID E. BRILES, JAMES C. PATON, RESHMI MUKERJI, EDWARD SWIATLO, AND MARILYN J. CRAIN

E. ENTEROCOCCI

24 Pathogenicity of Enterococci / 378
ELIZABETH FIORE, DARIA VAN TYNE, AND MICHAEL S. GILMORE

25 Enterococcal Genetics / 398
KEITH E. WEAVER

F. ORAL STREPTOCOCCI

26 Biology of Oral Streptococci / 426
JJACQUELINE ABRANCHES, LIN ZENG, JESSICA K. KAJFASZ, SARA PALMER, BRINTA CHAKRABORTY, ZEZHANG WEN, VINCENT P. RICHARDS, L. JEANNINE BRADY, AND JOSE A. LEMOS

27 The Biology of *Streptococcus mutans* / 435
J.A. LEMOS, S.R. PALMER, L. ZENG, Z.T. WEN, J.K. KAJFASZ, I.A. FREIRES, J. ABRANCHES, AND L.J. BRADY

28 Genetics of *sanguinis*-Group Streptococci in Health and Disease 449
ANGELA NOBBS AND JENS KRETH

G. LACTOCOCCI

29 Genetics of Lactococci 461
PHILIPPE GAUDU, YUJI YAMAMOTO, PETER RUHDAL JENSEN, KARIN HAMMER, DELPHINE LECHARDEUR, AND ALEXANDRA GRUSS

SECTION 3
THE STAPHYLOCOCCUS / 483
SECTION EDITOR: Richard P. Novick

30 Staphylococci: Evolving Genome / 485
JODIE A. LINDSAY

31 Staphylococcal Plasmids, Transposable and Integrative Elements / 499
NEVILLE FIRTH, SLADE O. JENSEN, STEPHEN M. KWONG, RONALD A. SKURRAY, AND JOSHUA P. RAMSAY

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32 Temperate Phages of *Staphylococcus aureus* / 521
HANNE INGMER, DAVID GERLACH, AND CHRISTINE WOLZ

33 Pathogenicity Islands and Their Role in Staphylococcal Biology / 536
RICHARD P. NOVICK

34 Respiration and Small Colony Variants of *Staphylococcus aureus* / 549
RICHARD PROCTOR

35 Noncoding RNA / 562
EMMA DESGRANGES, STEFANO MARZI, KAREN MOREAU, PASCALE ROMBY, AND ISABELLE CALDELARI

36 The Staphylococcal Cell Wall / 574
RITA SOBRAL AND ALEXANDER TOMASZ

37 Staphylococcal Protein Secretion and Envelope Assembly / 592
OLAF SCHNEEWIND AND DOMINIQUE M. MISSIAKAS

38 Surface Proteins of *Staphylococcus aureus* / 599
TIMOTHY J. FOSTER

39 Immune Evasion by *Staphylococcus aureus* / 618
NIENKE W. M. de JONG, KOK P. M. van KESSEL, AND JOS A. G. van STRIJP

40 *Staphylococcus aureus* Secreted Toxins and Extracellular Enzymes / 640
KAYAN TAM AND VICTOR J. TORRES

41 Regulation of *Staphylococcus aureus* Virulence / 669
CHRISTIAN JENUL AND ALEXANDER R. HORSWILL

42 Virulence and Metabolism / 687
ANTHONY R. RICHARDSON

43 Staphylococcal Biofilms / 699
MICHAEL OTTO

44 Fulminant Staphylococcal Infections / 712
YVES GILLET, THOMAS HENRY, AND FRANÇIOS VANDENESCH

45 *Staphylococcus aureus* Colonization of the Human Nose and Interaction with Other Microbiome Members / 723
CLAUDIA LAUX, ANDREAS PESCHEL, AND BERNHARD KRISMER

46 *Staphylococcus aureus* in Animals / 731
ANDREAS F. HAAG, J. ROSS FITZGERALD, AND JOSÉ R. PENADÉS

47 Antibiotic Resistance and the MRSA Problem / 747
MARTIN VESTERGAARD, DORTE FREES, AND HANNE INGMER

48 Immunity to *Staphylococcus aureus*: Implications for Vaccine Development / 766
RICHARD A. PROCTOR

49 Nonconventional Therapeutics Against *Staphylococcus aureus* / 776
CAROLINE M. GRUNENWALD, MONIQUE R. BENNETT, AND ERIC SKAAR

SECTION 4
THE LISTERIAE / 791
SECTION EDITOR: Daniel A. Portnoy

50 Epidemiology and Clinical Manifestations of *Listeria monocytogenes* Infection / 793
WALTER F. SCHLECH III

51 Innate and Adaptive Immune Responses during *Listeria monocytogenes* Infection / 803
SARAH E. F. D'ORAZIO

52 Regulation of *Listeria monocytogenes* Virulence / 836
JÖRGEN JOHANSSON AND NANCY E. FREITAG

53 *Listeria monocytogenes*: Cell Biology of Invasion and Intracellular Growth / 851
JAVIER PIZARRO-CERDÁ AND PASCALE COSSART

54 Metabolism of the Gram-Positive Bacterial Pathogen *Listeria monocytogenes* / 864
JOHN-DEMIAN SAUER, ANAT A. HERSKOVITS, AND MARY X. D. O'RIORDAN

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SECTION 5
SPORE-FORMING PATHOGENS / 873
SECTION EDITOR: Julian I. Rood

55 The *Bacillus cereus* Group: *Bacillus* Species with Pathogenic Potential / 875
MONIKA EHLING-SCHULZ, DIDIER LERECLUS, AND THERESA M. KOEHLER

56 Sporulation and Germination in Clostridial Pathogens / 903
AIMEE SHEN, ADRIANNE N. EDWARDS, MAHFUZUR R. SARKER, AND DANIEL PAREDES-SABJA

57 Clostridial Genetics: Genetic Manipulation of the Pathogenic Clostridia / 927
SARAH A. KUEHNE, JULIAN I. ROOD, AND DENA LYRAS

58 Genomics of the Pathogenic Clostridia / 940
ROBERT J. MOORE AND JAKE A. LACEY

59 Virulence Plasmids of the Pathogenic Clostridia / 954
SARAH A. REVITT-MILLS, CALLUM J. VIDOR, THOMAS D. WATTS, DENA LYRAS, JULIAN I. ROOD, AND VICKI ADAMS

60 Enterotoxic Clostridia: *Clostridium perfringens* Enteric Diseases/ 977
ARCHANA SHRESTHA, FRANCISCO A. UZAL, AND BRUCE A. McCLANE

61 Enterotoxic Clostridia: *Clostridioides difficil*e Infections / 991
S. MILETO, A. DAS, AND DENA LYRAS

62 Histotoxic Clostridial Infections / 1012
MASAHIRO NAGAHAMA, MASAYA TAKEHARA, AND JULIAN I. ROOD

SECTION 6
MYCOBACTERIA AND CORYNEBACTERIA / 1027
SECTION EDITOR: Miriam Braunstein

63 Mycobacteriophages / 1029
GRAHAM F. HATFULL

64 Immunology of *Mycobacterium tuberculosis* Infections / 1056
JONATHAN KEVIN SIA AND JYOTHI RENGARAJAN

65 Unraveling the Structure of the Mycobacterial Envelope / 1087
MAMADOU DAFFÉ AND HEDIA MARRAKCHI

66 The Dream of a Mycobacterium / 1096
CATHERINE BARANOWSKI, E. HESPER REGO, AND ERIC J. RUBIN

67 *Mycobacterium tuberculosis* Metabolism / 1107
GABRIEL T. MASHABELA, TIMOTHY J. de WET, AND DIGBY F. WARNER

68 Protein Export into and across the Atypical Diderm Cell Envelope of Mycobacteria / 1129
VINCENT J. C. van WINDEN, EDITH N. G. HOUBEN, AND MIRIAM BRAUNSTEIN

69 *Corynebacterium diphtheriae*: Diphtheria Toxin, the *tox* Operon, and Its Regulation by $Fe2^+$ Activation of apo-DtxR / 1154
SADIYA PARVEEN, WILLIAM R. BISHAI, AND JOHN R. MURPHY

Index / 1165

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Dedication



The editors dedicate this third edition of *Gram-Positive Pathogens* to Olaf Schneewind (1961–2019) in commemoration of his outstanding contributions to the molecular genetics and pathobiology of the staphylococci and other Gram-positive pathogens.

At the time of his death, Olaf was the Louis Block Professor and chair of the Department of Microbiology at the University of Chicago and one of the world's leading authorities on the human pathogens *Staphylococcus aureus*, *Bacillus anthracis*, and *Yersinia pestis*; his extensive contributions to the world of microbiology are represented by over 250 published papers.

Olaf received his MD/PhD degree in 1988 at the University of Cologne in Germany. He then moved to the United States to work as a postdoctoral associate in Vincent Fischetti's laboratory at the Rockefeller University in New York from 1988 to 1992. During this time, Olaf built on the finding that surface proteins on Gram-positive bacteria contained a common LPXTG sequence at their C-terminus. He found that this sequence was essential for anchoring surface proteins to the wall peptidoglycan, setting the stage for his future discoveries. He moved to UCLA in 1992, where he showed that surface proteins are cleaved between the threonine and the glycine of the LPXTG motif by a molecule he termed sortase, a unique enzyme with transpeptidase activity. He subsequently identified four classes of sortases (A to D) that catalyze unique chemical reactions such as the promotion heme-iron scavenging, the formation of infectious spores, and the assembly of pili. These major discoveries, which solved the long-standing mystery of how surface proteins are attached to the bacterial envelop, propelled him to professor by 2001, when he moved to the University of Chicago.

As a faculty member at the University of Chicago and subsequent chair of the Department of Microbiology, Olaf established that surface proteins of *S. aureus* are essential for the pathogenesis of staphylococcal infectious diseases, owing to their role in nutrient acquisition, bacterial agglutination, or downregulation of host immune responses. This work led to the exploration of immune responses and a collaborative research effort with Novartis AG to develop a vaccine against *S. aureus*. In 2003, Olaf established the Great Lakes RCE for Biodefense & Emerging Infectious Diseases and, with the help of an NIH construction grant, built the Howard Taylor Ricketts Laboratory at Argonne National Laboratory, a state-of-the-art biosafety level 4 facility. As director of the Biodefense Center, Olaf expanded his research to study multiple bacterial pathogens, including *Corynebacterium diphtheriae*, pathogenic *Bacillus* species (*B. anthracis*) and *Yersinia* species (*Y. pestis*). He examined the contributions of *Yersinia* type III secretion in pathogen-mediated depletion of host immune cells and developed a subunit vaccine that protects animals against pneumonic plague. Olaf and colleagues discovered the genes and biosynthetic pathway for bacterial lipoteichoic acid as a target for the development of new antibiotics. His research program on *B. anthracis* also focuses on characterizing the assembly of the S-layer, a two-dimensional array of proteins that associates noncovalently with bacterial secondary cell wall polysaccharides, and the development of conjugative anthrax vaccines involving the capsules of these microbes. In his studies of staphylococcal pathogenesis, he developed and analyzed an elegant and highly informative model of how the classic staph abscess forms and evolves within mammalian tissues—a major contribution to the understanding of staphylococcal disease.

Olaf earned multiple honors and awards. Among these are fellowships of the American Association for the Advancement of Science and the American Academy of Microbiology plus recent election to the National Academy of Sciences. Olaf was an inspiring teacher and mentor, and many of his former students and post-docs have established distinguished careers in microbiology and infectious diseases. He had a warm personality, many friends, and a wide international circle of collaborators. He loved to talk science and loved doing it in a group with some good German beer. He will be sorely missed.

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Preface

Gram-positive bacteria are structurally unique from their gram-negative counterparts by lacking an outer membrane and related secretory systems and have a thick peptidoglycan. Because of this and other distinctions, they have developed novel approaches to pathogenesis by acquiring (among others) a unique family of surface proteins, toxins, enzymes, and prophages. In this new edition, the editors have enhanced this compendium of gram-positive bacterial pathogens by including new data generated from genomic sequencing as well as the latest knowledge on gram-positive structure and mechanisms of antibiotic resistance and theories on the mechanisms of gram-positive bacterial pathogenicity. This edition emphasizes streptococci, staphylococci, listeria, and spore-forming pathogens, with chapters written by leading researchers in these areas. The chapters systematically dissect these organisms biologically, genetically, and immunologically, in an attempt to understand the strategies used by these bacteria to cause human disease. It is hoped that the insights gained from understanding these strategies will lead to the rational design of novel therapeutics and vaccines to control infections caused by gram-positive bacteria.

Vincent A. Fischetti
Richard P. Novick
Joseph J. Ferretti
Daniel A. Portnoy
Miriam Braunstein
Julian I. Rood

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About the Editors



Vincent A. Fischetti is Professor and Head of the Laboratory of Bacterial Pathogenesis and Immunology at the Rockefeller University in New York. He received his Ph.D. in Microbiology with honors from New York University School of Medicine and has over 45 years of experience in the anti-infectives field, nearly all of which have been spent at Rockefeller. His laboratory was the first to identify phage lytic enzymes as novel therapeutics to treat infections caused by the major antibiotic-resistant bacterial pathogens. Several of these lysins are now in the clinical development pipeline. His group has also engineered lysins and IgG immunoglobulins to create chimeric high affinity antibodies directed to bacterial wall carbohydrates for passive immune protection from infection. Dr. Fischetti is a fellow of the American Academy of Microbiology and the National Academy of Inventors and has received two NIH MERIT awards. He has been editor-in-chief of *Infection and Immunity* for 10 years and serves as advisory editor for the *Journal of Experimental Medicine* and *Trends in Microbiology*, among others.



Richard P. Novick received his M.D. with honors in microbiology from New York University. Following postdoctoral studies at the National Institute for Medical Research in London and at Rockefeller University, he joined the Public Health Research Institute in New York. There he developed the fields of staphylococcal molecular genetics and plasmid biology, founding the plasmid biology department at PHRI and the journal *Plasmid*. After 10 years as Director of the PHRI, he returned to full-time lab work at the NYU School of Medicine where, among other important contributions to the plasmid field, he discovered the *agr* quorum-sensing system that regulates staphylococcal virulence and the phage-inducible staphylococcal pathogenicity islands (SaPIs). He is a Fellow of the AAAS and of ASM and was elected to the National Academy of Sciences in 2006. He is currently the Recanati Family Professor of Science, Emeritus, at NYU and is engaged in developing the SaPIs as a nonantibiotic antibacterial platform.



Joseph J. Ferretti is an Emeritus Professor in the Department of Microbiology and Immunology at the University of Oklahoma Health Sciences Center in Oklahoma City. He has worked in the area of *Streptococcus pyogenes* research for over 50 years since his early days at the University of Minnesota, where he trained under the tutelage of Lewis Wannamaker, Elia Ayoub, and Ernest Gray. Following a postdoctoral fellowship at The Johns Hopkins University, where he worked with Philip E. Hartman in the study of microbial genetics, he joined the University of Oklahoma College of Medicine in 1969. His main research contributions, supported by years of support from the National Institutes of Health and other agencies, have been in the areas of streptococcal genetics, regulation of virulence factors, bacteriophages, and genomics. Dr. Ferretti is the author or coeditor of six books, including both previous editions of *Gram-Positive Pathogens.*



Daniel A. Portnoy received his Ph.D. in 1983 under the tutelage of Stanley Falkow at the University of Washington and at Stanford University. After postdoctoral training at the Rockefeller University, he moved first to Washington University, where he began working with *Listeria monocytogenes*, and then to the University of Pennsylvania. In 1997, Dr. Portnoy moved to the University of California–Berkeley, where he currently holds joint appointments in Molecular and Cell Biology and Plant and Microbial Biology and teaches a course in bacterial pathogenesis to approximately 200 undergraduates. His lab works on a wide range of topics, including the cell biology of infection and innate immunity, and has collaborated with the private sector on the development of recombinant *L. monocytogenes*-based vectors for cancer immunotherapy. Dr. Portnoy's contributions were recognized by his election to the National Academy of Sciences in 2013 and the National Academy of Inventors in 2017.

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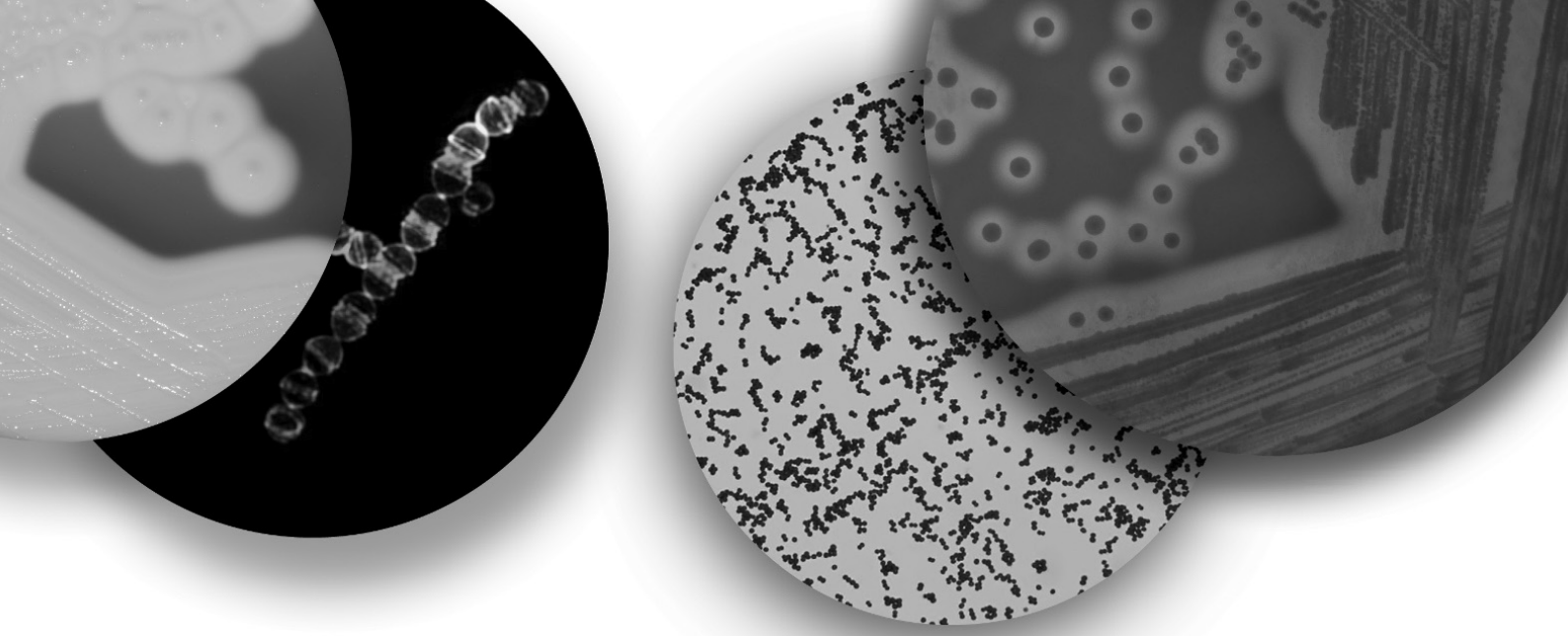


Miriam Braunstein is a Professor of Microbiology and Immunology at the University of North Carolina School of Medicine. She received her Ph.D. from Princeton University, where she carried out graduate research on histone acetylation with James Broach. After completing her degree, she became a Life Sciences Research Foundation Fellow working at the Albert Einstein College of Medicine with William Jacobs, Jr. It was during her postdoctoral training that she began studying *Mycobacterium tuberculosis*. Dr. Braunstein's laboratory studies the mechanisms of protein secretion pathways and secreted virulence effectors of *M. tuberculosis*. Her laboratory also works on developing inhaled therapies to treat tuberculosis. Dr. Bruanstein is the recipient of a Burroughs Welcome Fund Investigators in the Pathogenesis of Infectious Disease Award.



Julian Rood is Professor of Microbiology at Monash University in Melbourne, Australia. He graduated with a Ph.D. from the University of Melbourne and did postdoctoral work at the University of Wisconsin and the Australian National University. After starting his own laboratory in 1980 at Murdoch University in Western Australia, he moved to Monash University in 1985, where he was awarded a Personal Chair in Microbiology in 1998. His current research interests are centered upon *Clostridium perfringens*, focusing on the role of toxins in disease pathogenesis, the regulation of toxin and virulence gene expression, and the mechanism by which conjugative antibiotic resistance and toxin plasmids can move between *C. perfringens* strains. He is a Fellow of the American Academy of Microbiology, a Fellow and Life Member of the Australian Society for Microbiology, and a past president of the Australian Society for Microbiology. He is the Editor-in-Chief of Plasmid.

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SECTION 1

The Gram-Positive Cell Wall

Section Editor: **Vincent A. Fischetti**

The Gram-Positive cell wall structure differs in many respects from its gram-negative counterpart. While much has been reported regarding the complexity of the gram-negative envelope,until recently similar information has not been available for the gram-positive wall. The following chapters provide the reader with new insight into the gram-positive cell wall structure and the increasing complexity of its surface protein coat, as well as our increasing knowledge of its polymeric nature and the location of its periplasmic constituents, offering the most comprehensive understanding to date of the gram-positive envelope.

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The Gram-Positive Bacterial Cell Wall

MANFRED ROHDE

1

HISTORICAL BACKGROUND

In 1884, the Danish bacteriologist Hans Christian Gram developed a staining procedure to view stained bacteriaunder the light microscope (1). His staining method, nowadays simply called Gram staining, discriminated between a Gram-positive and Gram-negative bacterial cell wall. He introduced a dye, gentian violet, which penetrates the cell wall and cytoplasmic membrane, thus staining the cytoplasm of the heat-fixed bacteria. After addition of iodine, an insoluble complex is formed which is retained by the Gram-positive bacterial cell wall upon addition of a decolorizer such as ethanol. Therefore, Gram-positive bacteria appear almost purple, while Gram-negative bacteria retain the dye to a lesser extent or not at all and have to be counterstained with a second dye, safranin or fuchsine, appearing pink or reddish. It is noteworthy that some mycobacteria showed an indifferent staining behavior when Gram stained, suggesting that the cell wall of mycobacteria might be somehow different from the other two types. In the following decades, it became obvious that cell walls/cell envelopes are more diverse and that Gram staining alone often could lead to misinterpretations of the cell wall composition.

Before the early 1950s, when the chemical composition of bacterial cell walls was not known, it was speculated that chitin or cellulose, polymers recognized as providing rigid structures to other organisms, might also represent the building material of the bacterial cell wall. In 1951, experiments with a phenol-insoluble material from *Corynebacterium diphtheria* (2) revealed glucosamine and diaminopimelic acid as components of the bacterial cell wall which are associated with polysaccharides. Chemical examination of streptococcal cell wall layers highlighted the presence of amino acids and hexosamines in the cell wall extract, as well as rhamnose as a main component in Gram-positive bacteria (3, 4). Systematic analyses of a number of Gram-positive bacteria identified the hexosamines glucosamine and muramic acid as major components together with three prevalent amino acids, namely, d-alanine, lysine or diaminopimelic acid, and glutamic acid. By then, a typical basic basal unit in Gram-positive cell walls was also recognized in which glucosamine and muramic acid are linked with three amino acids via a peptide bond (5, 6). Gram-negative bacteria express the identical basal unit. Numerous analyses of other bacteria revealed that each bacterial genus or even species is often characterized by a distinctive pattern of amino acids, amino sugars, and sugars connected to the basic basal unit. It was believed that these differences should provide a valuable pattern to discriminate between bacterial genera/species (7, 8). Over the following years other compounds of the Gram-positive cell wall were recognized, such as teichoic acids (TAs), which are polyribitol phosphates (9), and lipoteichoic acid (LTA). Furthermore, numerous proteins were found to be linked to the cell wall.

Methods of staining bacteria for light microscopic examinations have limitations since the resolution is not high enough to reveal structural details. With the advent of transmission electron microscopes (TEMs) in the 1930s and the parallel development of preparation methods for biological samples, electron microscopic imaging of ultrathin sections of embedded bacteria became the method of choice to study bacterial cell walls in detail at high resolutions (10–13). With this methodology, it was possible for the first time to discriminate between the structures of Gram-positive and Gram-negative bacteria based on morphological differences in an image. First, electron microscopic preparation protocols developed for eukaryotic cells or tissues were also applied for bacteria. The most fruitful era started when embedding protocols were customized for bacteria and new kinds of embedding resins became available, for example, the Lowicryl resins for low-temperature embedding, which allowed the introduction of the progressive lowering of temperature method (14–16). This development was paralleled by technical inventions, especially cryo-methods in which bacteria are physically instead of chemically fixed, and it opened up a new horizon in understanding bacterial cell walls. It should be mentioned that even today new methodologies are arising and pushing morphological studies toward vitrified and unstained bacteria in a fully hydrated status and therefore in a close-to-nature condition. It is noteworthy that major developments in electron microscopic methodology required a long period of invention and testing before the technique was introduced to the market. For example, three-dimensional (3D) electron microscopy was developed about 30 years after the invention of the TEM. Invention and precommercial development of cryo-electron tomography (CET) occurred another 30 years later. Due to the rapid development of computer performance and the progress in specialized software nowadays, one can estimate that new imaging techniques are introduced faster. For example, the introduction of cryo-focused ion beam (cryo-FIB) combined with a scanning electron microscope (cryo-FIB-SEM) as a new close-to-nature approach was sold a few years after the

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first advent of FIB-SEMs for conventional resin-embedded biological samples.

THE BACTERIAL CELL WALL

Bacteria are mostly unicellular organisms which can be found in a wide variety of environments. Therefore, bacterial cell walls deserve special attention because they (i) provide the essential structure for bacterial viability by protecting against the often hostile environment, (ii) are composed of unique components found nowhere else in nature, (iii) are responsible for the shape of the bacteria, (iv) provide a halt for ligands and proteins for adherence to host cells, (v) expose receptor sites for drugs or viruses, (vi) represent the most important sites for attack of antibiotics, (vii) provide structures for immunological distinction and variation, and (viii) can cause symptoms of disease in animals and humans.

Chemistry of the Bacterial Cell Wall Backbone

The major backbone of the bacterial cell wall is the peptidoglycan, also called murein, which consists of repeating linear units of the disaccharide *N*-acetylglucosamine (NAG) linked to *N*-acetylmuramic acid (NAM). The disaccharides are cross-linked via often flexible pentapeptide amino acid chains forming a mesh-like framework (17). Chemically, the peptidoglycan consists of alternating β-1,4-linked *N*-acetylglucosamine (GlcNAc; NAG) and *N*-acetylmuramic acid (MurNAc, NAM, a variant of GlcNAc with a D-lactate attached to the C-3 by an ether bond). Termination of a peptidoglycan strand is achieved at the reducing end by a 1,6-anhydroMurNAc residue, in which the C-1 and C-6 of the sugar backbone are bound through an ether linkage. The appearance of the unusual 1,6-anhydroMurNac is used to determine the end of the strands. The peptide stems are covalently linked to the glycan strands with an amide bond to the carboxyl carbon of the D-lactyl group of MurNAc. One hallmark of the peptidoglycan is that the glycans are conserved across bacterial species, whereas the peptide stem is often modified and diverse, containing D-amino acids. An L-alanine is usually found in the first position of the pentapeptide stem from the lactyl group of MurNAc, which can be replaced by glycine or L-serine in some rare exceptions. The second amino acid is mostly occupied by a D-isoglutamic acid (D-iGlu). In *Streptococcus pneumoniae* this D-iGlu is amidated to yield a D-isoglutamine (18). The γ-carbon of D-iGlu is bound to the third amino acid. This amino acid in the third position of the peptide stem has the highest diversity among bacteria. Generally, one can summarize that in most Gram-negative bacteria and some Gram-positive bacteria, like in the bacilli and mycobacteria, this third position is occupied by the unusual amino acid *meso*-diaminopimelic acid. In contrast, in most other Gram-positive bacteria it is usually an L-lysine (see Fig. 1). The peptide stem is finally terminated by two D-alanines, although different D-amino acids can be found in this place, too (17).

In summary, one hallmark of Gram-positive bacteria is the observed differences in the types of cross-links in which the peptides are connected to the peptidoglycan. Today more than 100 chemotypes can be distinguished, and their differences are based on different linking units and substituents in the peptide chain (19).

Due to its unique chemical structure, the peptidoglycan sacculus forms a large polymer that can be isolated and viewed even with a light microscope (see Fig. 2). The difference between Gram-negative and Gram-positive bacteria is the thickness of the peptidoglycan layer surrounding the cytoplasmic membrane. Gram-positive bacteria exhibit a layer of peptidoglycan strands which can reach a size of between 30 and 100 nm or even thicker, whereas Gram-negative bacteria have a layer of only a few nanometers (see Fig. 3). While the chemical composition of peptidoglycan and the family of proteins for assembling is known for a number of different bacteria, the overall arrangement of these components in Gram-positive cell walls is not fully solved. For Gram-negative bacteria it has been shown with CET that individual very thin densities, probably representing glycan strands, run circumferentially around the long axis of the bacterial cell (20). In contrast, the 3D arrangement of peptidoglycan in Gram-positive bacteria is still under discussion (21). Three models have been proposed over the years. The first model suggests that glycan strands run circumferentially around the long axis as in Gram-negative bacteria. This model is called the "circumferential" or "layered" model (22). In the second model the glycan strands are supposed to run perpendicular to the bacterial cell wall in a hexagonal lattice, so this is called the "perpendicular" or "scaffold" model (23, 24). This model was proposed on the basis of nuclear magnetic resonance studies applying a synthetic 2-kDa fragment of the peptidoglycan formed from NAG-NAM (pentapeptide)-NAG-NAM (pentapeptide). Nuclear magnetic resonance revealed that this fragment forms a right-handed helix with a periodicity of three NAG-NAMs per helix turn. The first two amino acids can adopt a limited number of conformations (24). Atomic force microscopy (AFM) studies with gently disrupted sacculi of *Bacillus subtilis* established the so-called coiled cable model, in which bundles of glycan strands form thicker moieties of around 50 nm that run around the cell (25). It should be noted that a model with glycan strands running parallel to the long axis has never been considered, because it was unclear how such a sacculus could elongate. In addition, the coiled cable model is not considered nowadays since CET did not show any cable-like structures in the thick peptidoglycan layer. These earlier observations might have been based on the fact that isolated peptidoglycan was harvested by boiling bacteria, opened up by a French press cell, diluted in water, and air dried on mica before AFM imaging was performed.

Biochemical Synthesis of the Peptidoglycan Layer

Synthesis of the peptidoglycan is a three-step mechanism, which is localized at three locations within a bacterium. The sequential Mur ligase pathway is involved in biosynthesis of the peptidoglycan. The early steps of synthesis start in the bacterial cytoplasm, where precursors linked to undecaprenyl pyrophosphate (UDP), such as UDP-NAM-pentapeptide (UDP-NAM) and UDP-NAG, are formed. In the second step UDP-NAM is bound to another cytoplasmic membrane-bound UDP functioning as a transport lipid. This complex is called lipid I and is located at the inner cytoplasmic face of the membrane. Covalent attachment of the second precursor UDP-NAG forms the transport lipid complex lipid II. Then, for example, in the case of *Staphylococcus aureus*, a peptide cross-bridge is attached to the third amino acid in the pentapeptide, consisting of five glycine residues. Next, the entire lipid II complex is flipped over the cytoplasmic membrane to the extracellular side by a flippase. The precise biochemical process of the flipping mechanism is not yet fully understood. Lipid II is incorporated into nascent growing peptidoglycan by penicillin binding proteins (PBPs) on the extracellular side of the membrane. This third step involves, first, a transglycosylation and, second, a transpeptidation reaction performed by PBPs for incorporating new glycans with flexible

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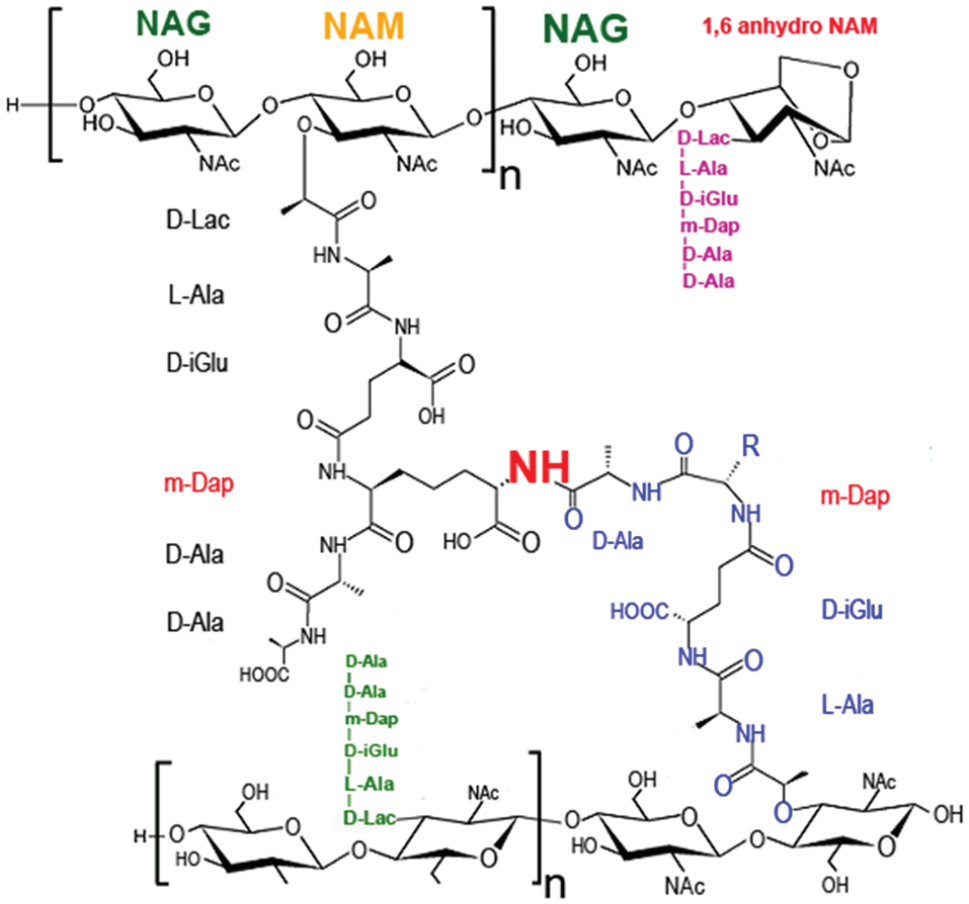


FIGURE 1 The bacterial cell wall backbone, peptidoglycan; shown are the two glycan strands (in black). Peptide stems are depicted in black (left side), and the second peptide stem, in blue. Note the cross-linking NH (in red) via the two unusual amino acids m-diaminopimelic acid (m-Dap, in red) and the presence of D-alanine in the peptide stems. Two more peptide stems (green and pink) are depicted which can interact to build the next cross-linking between glycan strands.

peptides into the existing peptidoglycan layer (18, 26–31). For detailed reading about chemical reactions and enzymes involved in this process, please refer to the review by Teo and Roper (18).

Turnover of Peptidoglycan

The first report describing the bacterial cell wall turnover concerned the Gram-positive bacterium *Bacillus megaterium* and was published more than 50 years ago (32). Later, pulse-chased experiments demonstrated with radioactively labeled cell wall precursors that all studied Gram-positive bacteria as well as Gram-negative bacteria carry out a cell wall turnover (33–36). The model for peptidoglycan growth in Gram-positive bacteria implies an inside-to-outside growth in which newly synthesized peptidoglycan is delivered to the cytoplasmic membrane face of the peptidoglycan layer in a relaxed form. With polymerization and cross-linking, peptidoglycan moves to the outside of the cell wall and is stretched due to the high turgor pressure within the bacterial cell (37). Once the maximally stretched peptidoglycans in the outer layers start to age, they are subsequently hydrolyzed by autolysins (38). It was estimated that around 50% of the total cell wall mass is turned over within one generation. This would have been a massive loss of resources for the bacteria, and it was speculated that hydrolyzed constituents of the cell wall might be recycled by bacteria. Indeed, this was found to be the case in Gram-negative bacteria such as *Escherichia coli*, and the biochemical pathways are well understood (39, 40). It remained unclear if Gram-positive bacteria would also recycle cell wall material. It was found that large amounts of cell wall fragments could be detected in growth medium of exponentially grown Gram-positive bacteria such as *Bacillus*,

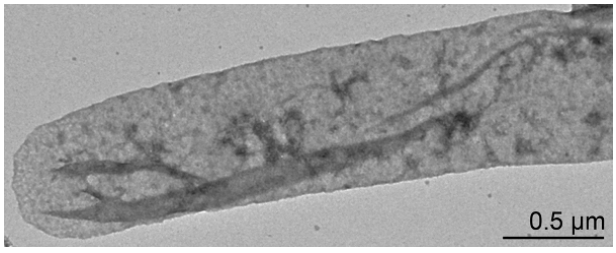


FIGURE 2 TEM image taken at an acceleration voltage of 80 kV of a peptidoglycan sacculus of *E. coli* after boiling for 3 h in 10% SDS. The mesh-like sacculus was negatively stained with 1% aqueous uranyl acetate, air-dried, and observed in a normal TEM.

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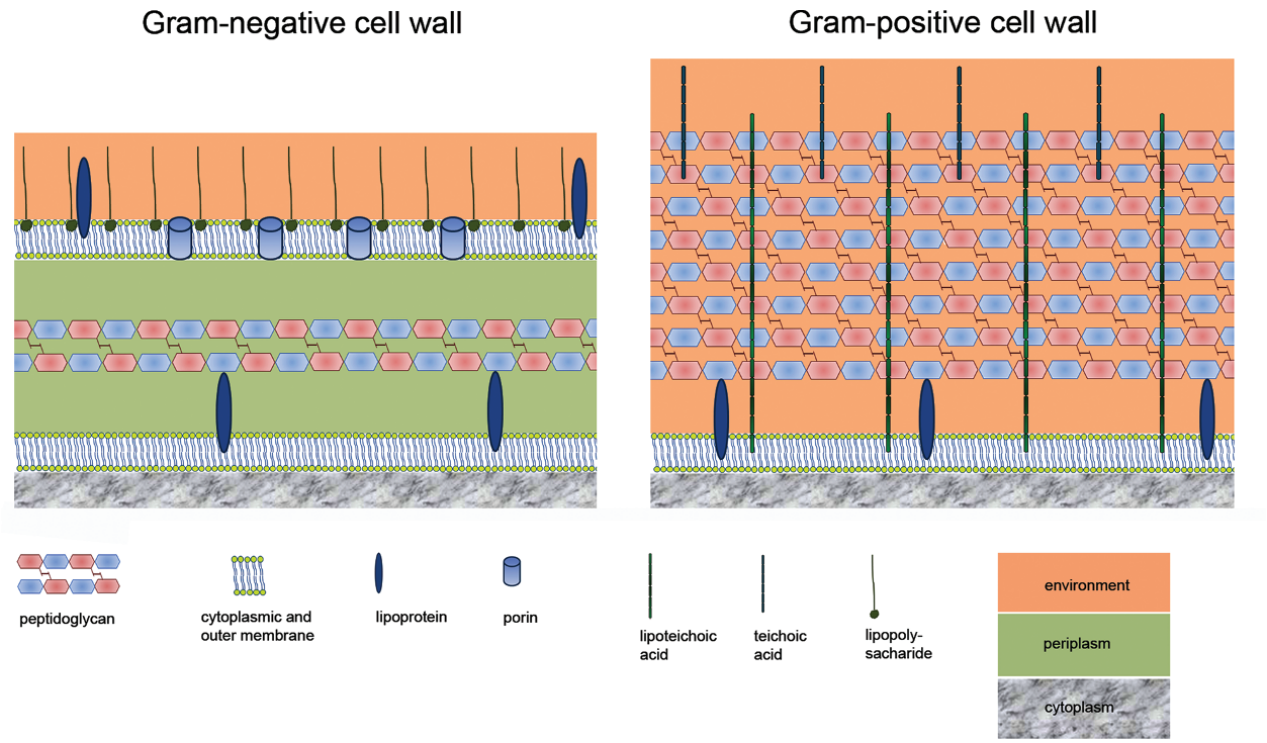


FIGURE 3 Schematic drawing of Gram-negative and Gram-positive cell walls. A characteristic of Gram-negative cell walls is the presence of two membranes, the cytoplasmic membrane and the outer membrane. Between these membranes is the periplasmic space, in which a very thin layer of peptidoglycan is found; lipopolysaccharides are attached to the outer membrane, and porins are inserted in the outer membrane. A thick layer of peptidoglycan and the lack of an outer membrane are the main characteristics of Gram-positive cell walls; instead of lipopolysaccharides, Gram-positive bacteria have lipoteichoic acid and teichoic acid localized in the cell wall. The periplasmic space is not shown since the existence of such a periplasm in Gram-positive bacteria is still being studied.

Lactobacillus, *Listeria*, and *Staphylococcus* strains (39). Thus, a robust turnover of cell wall components was established, but it was only shown recently that this correlates with high recycling rates of such compounds. In the early 2010s it was demonstrated that Gram-positive bacteria recycle hydrolyzed cell wall material, as does *E. coli*, although key steps and particular aspects of the involved pathways are less well understood. Meanwhile, orthologs of *E. coli* enzymes involved in the biochemical process of cell wall recycling were found in several Gram-positive bacteria (41). Interestingly, new observations suggest that cell wall recycling is turned on when Gram-positive bacteria reach the transition to the stationary phase of growth, not in the exponential growth phase, explaining why massive hydrolyzed cell wall constituents were detected in the exponential phase. Furthermore, it has been reported that in Gram-positive bacteria cell wall recycling is a crucial step for survival in the stationary phase and/or in a resting or persistence phase for pathogenic bacteria (39, 42). In addition, it should be noted here that the process of delivering newly synthesized peptidoglycan to the inner side of the cell wall and hydrolyzing "older" peptidoglycan at the outside of the wall has to be in an extremely strongly regulated dynamic equilibrium to ensure bacterial cell growth and division. If this equilibrium is somehow altered and more older peptidoglycan is shed from the outside of the cell wall than newly synthesized peptidoglycan can be delivered to the inner side of the peptidoglycan layer, the layer gets thinner, with the consequence that the entire bacterial cell can be lysed by intracellular turgor pressure. In addition, antibiotics such as penicillin and cephalosporins might bind better to PBPs and further inhibit the delivery of newly synthesized peptidoglycan, resulting in an even thinner peptidoglycan layer (43).

LIPOTEICHOIC ACID AND WALL TEICHOIC ACID AS MAJOR CONSTITUENTS OF THE CELL WALL

TA, which represents an important cell wall polymer and is found in many Gram-positive bacteria, was first described in 1958 (44, 45). Today TAs enclose two abundant bacterial cell wall polymers: (i) LTAs, which are anchored via lipid domains in the cytoplasmic membrane, and (ii) wall TAs (WTAs), which are covalently bound in the peptidoglycan layers. TAs consist of a diverse family of bacterial cell surface glycopolymers chemically defined as phosphodiester-linked polyol repeat units, or glycerol phosphate or ribitol phosphate residues. Structurally, WTA and LTA expose highly negatively charged properties due to the multiple phosphate groups in the structure and less positively charged groups due to inserted d-alanine residues. LTA shows relatively low differences in the structures of different bacteria, whereas WTA is extremely versatile in its structural groups. Why Gram-positive bacteria produce so much LTA and WTA at the same time is not well understood. For example, LTA can be deleted, but only if bacteria are grown below 30°C, whereas WTA is dispensable in *B. subtilis* and *S. aureus* under laboratory conditions (46). Deletion of LTA and WTA together is lethal because both cannot compensate for one another anymore (47, 48). Therefore, WTA and LTA have to fulfil vital functions, and indeed, for *S. aureus* it was shown that loss of WTA results in less colonization and infection in

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in vivo experiments in the rabbit endocarditis model (49). Based on the chemical structures, LTAs are grouped into at least five LTA types. Despite their role in infection, other major functions of TA have been established over the past years: (i) WTA and LTA can protect against environmental stress (50); (ii) LTA protects against harmful molecules such as antimicrobial peptides and cationic antibiotics (interestingly, in the structure of LTA a d-alanine is inserted which is responsible for the protection) (51–53); (iii) WTA and LTA are the main controlling mechanisms for enzyme activities, especially for autolysins and cation concentrations in the peptidoglycan layer (54); (iv) WTA is responsible for receptor binding and binding to surfaces in pathogenicity (49); (v) LTA directs the right placement of cell division machinery (50, 55); (vi) WTA serves as a phage receptor (56); and (vii) WTA and LTA mediate biofilm formation and binding to medical devices (57).

Different pathways, despite the fact that LTA and WTA show similarities in structure, perform biosynthesis of LTA and WTA. It is known that the *S. aureus* system needs at least 12 genes for WTA synthesis of the backbone structure of poly-Rbo-P (polyol ribitol phosphate). In contrast, only three genes are involved in synthesis of the LTA backbone poly-Gro-P (polyol glycerol phosphate) (58). For modification of the backbones, numerous other genes are involved in incorporation, for example, of d-alanine or hexoses. Synthesis of WTA is accomplished in *S. aureus* in five steps. Interestingly, the WTA synthesis pathway shares UDP as a common lipid carrier. As mentioned above, UDP is also involved in the first steps of peptidoglycan synthesis. Most genes involved are arranged in gene clusters (58–61). On the other hand, LTA synthesis starts directly from phosphatidylglycerol, which is located in the cytoplasmic membrane. Synthesis initiates on the glycolipid, which also serves as the membrane anchor for LTA. Therefore, no nucleotide activated precursor like UDP is needed. Again, between different Gram-positive bacteria, extensive differences exist in the amount of genes involved, the lengths of the LTA and WTA molecules, and modification of the synthesized backbones. For further detailed information on WTA and LTA biosynthesis, I strongly recommend the reviews by Brown et al. (58) and Percy and Gründling (62).

In summary, new studies will shed light on the structural and functional relationships of different LTA and WTA types and their impact on cell division, bacterial cell surfaces, and interaction with host receptors and should allow for the identification of new receptors in the bacteria-host interplay. Furthermore, the role of LTA in inducing immune stimulatory effects will be further developed. In particular, the question of cell tropism should be addressed, since the various differences observed in the chemical structures of LTA and WTA between Gram-positive bacteria might reveal evidence that these molecules play an important role in cell tropism recognition reactions.

CAPSULE

The capsule represents the outermost layer of a Gram-positive bacterial cell wall. It consists of a gelatinous polymer composed of polysaccharides or polypeptides, or both, and surrounds the entire bacterium with a thick layer. The capsule of *Bacillus anthracis*, for example, is composed of polymeric D-glutamic acid, whereas the capsule of group A streptococci consists of hyaluronic acid, a repeat polymer of disaccharides composed of D-glucuronic acid, and *N*-acetyl-D-glucosamine. In a proper capsule the polymer is firmly attached to the cell wall, whereas if the polymer is only loosely attached, the structure is often referred to as a slime layer, which has no repeated pattern. Major functions of capsules in pathogenic bacteria are (i) the protection against phagocytosis by neutrophils or macrophages due to the smooth capsular surface and the highly expressed negative charges, (ii) the prevention of complement-mediated bacterial lysis, and (iii) the contribution to virulence determinants. Furthermore, the capsule composition is responsible for different serotypes, which are observed particularly in certain species of streptococci. In the case of pneumococci, the different capsule polysaccharides are used as vaccine antigens. The capsule is synthesized and assembled at the cytoplasmic membrane and then extruded or secreted through the cell wall to the outside (63).

Since the major component of the capsule is water, it is not easy to preserve such polymeric polysaccharides in a natural state for electron microscopic observation. A method of choice is to introduce a kind of a scaffold within the capsule layers to prevent collapsing through chemical fixation and dehydration. As has been explicitly described several times in this chapter, fully hydrated and vitrified samples give the best close-to-nature view of the dimensions of a given bacterial capsule. Nevertheless, the incorporation of lysine as a positively charged component, which reacts by van der Waals forces with the capsule layer negative charges from the polysaccharides, provides a reasonable method to preserve the capsular ultrastructure in ultrathin sections. For a rapid visualization of capsules in a TEM, the capsule can be stained with cationic-coated gold nanoparticles (lysine-coated gold nanoparticles). For *S. pneumoniae* it has been demonstrated that fixation with 1% formaldehyde further supports the capsule structure, whereas fixation with glutaraldehyde results in loss of the capsule structure (64). The bound gold particles around the bacterium show indirectly the presence of the capsule and its dimensions (see Fig. 4). For ultrathin sections, bacteria are incubated with lysine acetate. In the second step the bound lysine reacts with ruthenium red, which subsequently is precipitated by osmium tetroxide. The precipitates formed in the capsular layers give reasonable support for preservation of the capsules in various Gram-positive bacteria, which allow for measurements of capsule dimensions or for imaging of the *in vivo* expressed capsules in blood or organs (52) (see Fig. 5).

EXTRACELLULAR VESICLES OF GRAM-POSITIVE BACTERIA

The extracellular vesicles formed by Gram-negative bacteria have been known of since the 1960s, whereas reports about extracellular vesicles from Gram-positive bacteria first appeared in the early 1990s (65, 66). The entire process of forming extracellular vesicles is nowadays referred to as vesiculogenesis (67). One reason for the lack of studies of extracellular vesicles of Gram-positive bacteria might be that it was believed that such vesicles simply could not be generated, due to the thick peptidoglycan layer in such bacteria. No decent model was able to explain mechanistically the formation and passage through such a thick peptidoglycan mesh at that time. Extracellular vesicles from Gram-negative bacteria are often referred to as outer membrane vesicles (OMVs) since they generate by pinching off as vesicles from outer membranes (68, 69). In contrast, extracellular vesicles of Gram-positive bacteria are often called membrane vesicles (MVs). It was reported that OMVs serve as a cargo vehicle for DNA, RNA, virulence factors, immunomodulators, and adhesions for pathogenic bacteria. Therefore, OMVs are considered to play an important part in the pathogenesis of Gram-negative pathogens (70, 71). Both types of extracellular

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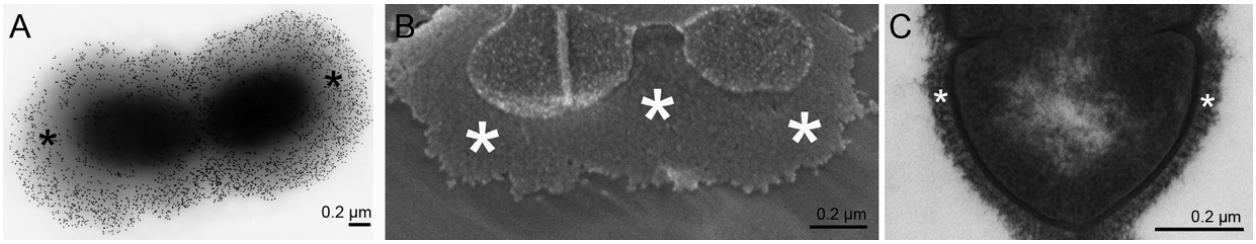


FIGURE 4 Visualization of Gram-positive bacterial capsules. (A) Cationic gold nanoparticles (lysine-coated 15-nm gold nanoparticles) label the thick capsule of *Streptococcus pneumoniae* after fixation with 1% formaldehyde at low pH (stars). (B) Cryo-FESEM at close-to-nature conditions reveals the thick capsule layer of *S. pneumoniae* marked with white stars. The thickness is comparable to the labeled capsule in (A); samples were nitrogen-slush-frozen, freeze-fractured at –105°C, freeze-etched at –105°C for 30 sec, and sputter-coated with gold/palladium. (C) For ultrathin sections, capsules can be preserved with the lysine-ruthenium-red osmium embedding protocol (see reference 52). Following embedding in LRWhite resin, *Streptococcus suis* is surrounded by a dense capsule layer (white stars).

vesicle have in common that they are formed by a lipid bilayer that forms an inner lumen in which the varying cargos are engulfed. Vesicle size ranges from 20 to 500 nm, and vesicles are best viewed using a TEM with negative staining or by field emission SEM (FESEM). In 2009 the first report about MVs from *S. aureus* was published. The study found that MVs were more or less identical to the described OMVs from Gram-negative bacteria, with the exception that MVs exhibit a mostly smaller vesicle size of up to about 200 nm compared to OMVs (72). It is noteworthy that MVs from *S. pneumoniae* and *Listeria monocytogenes* are considerably smaller than those from other Gram-positive bacteria, suggesting that bacteria synthesize and regulate MVs in different ways (72–74).

Generation, translocation, and secretion are the main unresolved issues about MVs, considering that they need to be released through the thick peptidoglycan layer into the environment. Three hypothetical models have been reported or discussed: (i) vesicles generate from the cytoplasmic membrane in a process comparable to the pinching off of OMVs from the outer membrane and are then pushed by the interior turgor pressure through the peptidoglycan layers. This suggests that MVs' size is small since the diameter is regulated by the known particle size exclusion of the given peptidoglycan layer for a specific bacterium. (ii) Cell wall-modifying enzymes are released or transported by the MVs, thus facilitating transmigration through the peptidoglycan layers possibly by losing the mesh at the site of travel. (iii) Transport occurs through channels in the layer, which suggests that MVs are extremely flexible in morphology, i.e., not always spherical vesicles, and can be released through much smaller channels then the actual size of the spherical MVs found in the environment (72). Therefore, further investigations are needed to clarify the enigmatic release of MVs through the Gram-positive peptidoglycan layers. CET could serve as the method of choice to clarify the transmigration through the peptidoglycan layer since bacteria are snap-frozen and even fast events can be fixed. However, the search for such events in tomograms can be very time consuming.

Nevertheless, more and more evidence is emerging that MVs of Gram-positive bacteria play an important role in pathogenesis, since for *S. aureus* it is reported that MVs contain PBPs, which can block the activity of β-lactam antibiotics. Furthermore, the global regulator MsrR, which is involved in methicillin resistance, was also detected in *S. aureus* MVs. MVs can be considered Trojan horses involved in passing on resistance genes among Gram-positive bacteria (75). In addition, several toxins have been found in MVs, such as listerolysin O in *L. monocytogenes* and pneumolysin in *S. pneumoniae*. Both toxins induce pore formation in the host cells and are therefore important virulence factors for colonization and invasion (73, 74). MVs have also been described in group A streptococci and their content characterized in detail (see Fig. 6). In summary, not only were virulence-associated proteins such as M1 protein,

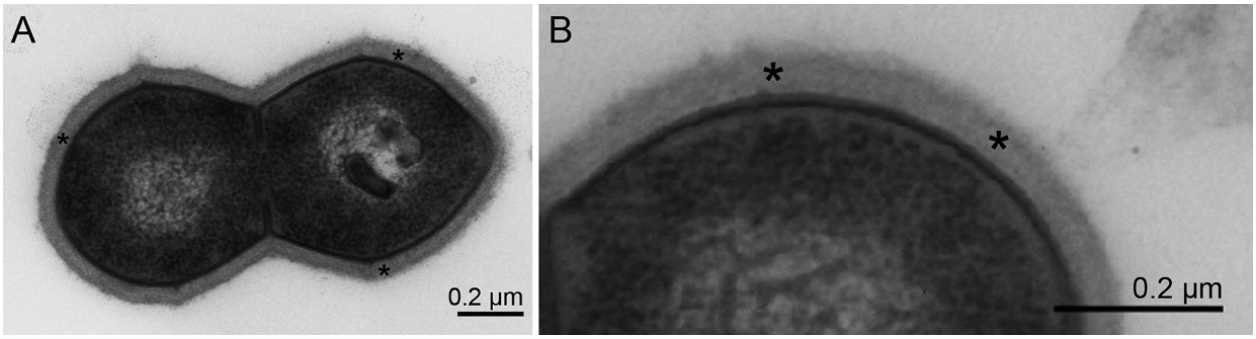


FIGURE 5 Good preservation of streptococcal capsules under *in vivo* conditions. (A) Streptococcal capsules (*Streptococcus pyogenes* administered intravenously) are well preserved (black stars) in spleen under *in vivo* conditions in the mouse model even after fixation with glutaraldehyde and formaldehyde, dehydration with acetone, and embedding in epoxy resin and ultrathin sectioning. (B) Enlargement of another bacterium depicting nicely preserved capsule (black stars). Most likely, proteins in the blood have covered and preserved the capsule and prevented loss of capsule during aldehyde fixation.

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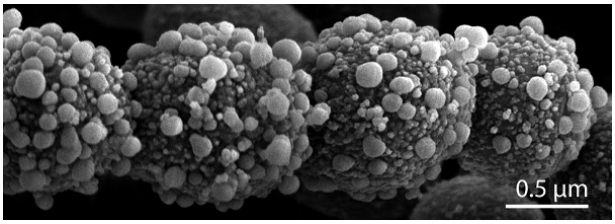


FIGURE 6 Formation of membrane vesicles on the surface of *Streptococcus pyogenes* M1 serotype imaged with FESEM after chemical fixation with aldehydes, dehydration with acetone, critical-point drying, and sputter-coating with gold/palladium.

streptolysin O, and serine protease HtrA detected, but numerous metabolic proteins residing in the streptococcal cytoplasm were also identified, as well as surface-exposed proteins including anchorless surface proteins, lipids, and RNA. Furthermore, the involvement of the virulence-associated two-component regulator CovRS was demonstrated, and loss of CovRS resulted in increased vesicle formation (76).

A SPECIALIZED CELL WALL IN MYCOBACTERIA

Mycobacteria are classified as Gram-positive bacteria, although they are also referred to as acid-fast bacteria due to the high density of lipids in the cell wall, which prevents accurate Gram staining. Thus, the staining is performed with Ziehl-Neelsen stain. The complexity of mycobacteria cell walls is a distinct feature that is not found in other bacteria. Three major macromolecules—peptidoglycan, arabinogalactan, and mycolic acids—are the building blocks of the mycobacterial cell wall. The structure of the mycobacterial cell wall was described in the 1960s and 1970s, and electron microscopy played an important role in describing the unusual morphological structures of the cell wall. The current accepted model of the cell wall is based on studies which identified the mycolyl-arabinogalactan-peptidoglycan complex as the core structure of mycobacteria (77). This unique arrangement that includes lipids and proteins is responsible for the characteristically important and efficient permeability barrier of the mycobacterial cell wall, particularly against drugs, and provides the basis for the potent pathogenicity of mycobacteria (see Fig. 7). Due to the presence of a large amount of lipids in the cell wall, earlier studies were confronted with the difficulty of extracting the lipids during the dehydration protocol. Therefore, for a long time whether the lipids formed a lipid bilayer in the cell wall, as suggested by Minnikin in 1982 (77), was discussed. He suggested an asymmetrical membrane to which the mycolic acids are covalently attached as an inner leaflet. The presence of such a lipid bilayer was confirmed by freeze-fracture studies, which clearly defined a second fracture plane, which is typical for a lipid bilayer. These findings supported the hypothesis of a second bilayer outside the cytoplasmic membrane, even though these studies were performed with corynebacteria (78, 79).

However, the existence of a bilayer outside of the cytoplasmic membrane was still heavily criticized because the proposed bilayer has never been clearly identified in ultrathin sections due to artefact-producing embedding preparations such as chemical fixation and dehydration with acetone. Instead, a more or less lucent zone, an outer layer, probably representing lipids and mycolic acids, was detected dividing the mycobacterial cell wall into a triple-layer structure composed of the cytoplasmic membrane, cell wall, and outer layer (see Fig. 8). This translucent zone is covered with a very thin stainable layer consisting of capsule and attached proteins. Freeze substitution revealed more or less similar images, even though the cell wall appeared thinner (80–82). A major step forward in elucidating the mycobacterial outer cell wall structure was performed with close-to-nature imaging applying cryo-ultrathin sections and CET of fully hydrated and vitrified samples (83). These studies revealed important changes to the current model. First, a lipid bilayer was detected covering the outside of the cell wall, indicating that mycobacteria express a similar outer membrane resembling Gram-negative bacteria. Second, no evidence was found for an asymmetrical membrane; instead, a symmetric membrane and an additional periplasmic space was postulated (79). In addition, it is clear that extractable lipids play a dominant role in mycobacterial membrane integrity and properties.

The mycobacterial peptidoglycan is synthesized as observed in other bacteria, in the cytoplasm, using UDP and is then flipped over the cytoplasmic membrane and inserted in the growing peptidoglycan network by the action of hydrolases and PBPs. Nevertheless, mycobacteria exhibit a number of differences compared to model bacteria: (i) mycobacterial peptidoglycan is extremely cross-linked, (ii) the cross-links are based on up to 80% of the total peptidoglycan on 3-3 peptide cross-links instead of the 4-3 peptide cross-links found in other bacteria, and (iii) the peptidoglycan backbone shows modifications such as glycolylations of NAM and amidation of D-glutamic acid and *meso*-diaminopimelic acid (84–88). Furthermore, the mycobacterial peptidoglycan is surrounded by a layer of arabinogalactan, a disaccharide, which has long arabinan polymers attached. It is noteworthy that some galactans remain free of arabinan polymers, and most important, the arabinan chain ends are branched. These branched ends are the binding partners for the long carbon chains of mycolic acid. These incorporated fatty acids are responsible for the extremely thick waxy coat of mycobacteria and make the mycobacterial cell wall mostly impermeable, contributing to pathogenicity. For detailed reactions and enzymes involved in the process, please refer to the review by Jankute et al. (84).

ELECTRON MICROSCOPIC TECHNIQUES APPLIED TO STUDY THE MORPHOLOGY OF GRAM-POSITIVE BACTERIA CELL ENVELOPES

Since the early 1950s TEM has been applied for studying the morphology and ultrastructure of Gram-positive bacterial cell walls. Surprisingly, even today a single unique method that would allow study of the ultrastructural details of all the different Gram-positive bacteria under close-to-nature conditions with an electron microscope is still needed. The newly developed CET might be the current method of choice, though CET has some drawbacks and restrictions, especially when bacteria with a width of more than 0.4 to 0.5 µm have to be imaged. The new super-resolution light microscopy methods such as stimulated emission depletion, photoactivated localization microscopy, structured illumination microscopy, and total internal reflection fluorescence were all very promising, but a breakthrough was hindered by the limitations when imaging immune fluorescent-labeled structures. The expression of fluorescence tags or fluorescence proteins, such as green fluorescent proteins, might alter the *in vivo* biological activities to a certain extent, therefore giving rise to inaccurate localizations within the bacterial cell. If the reader is interested in pursuing this topic in detail, I recommend the literature regarding the MreB protein (involved in the bacterial division process) over the past years. Depening

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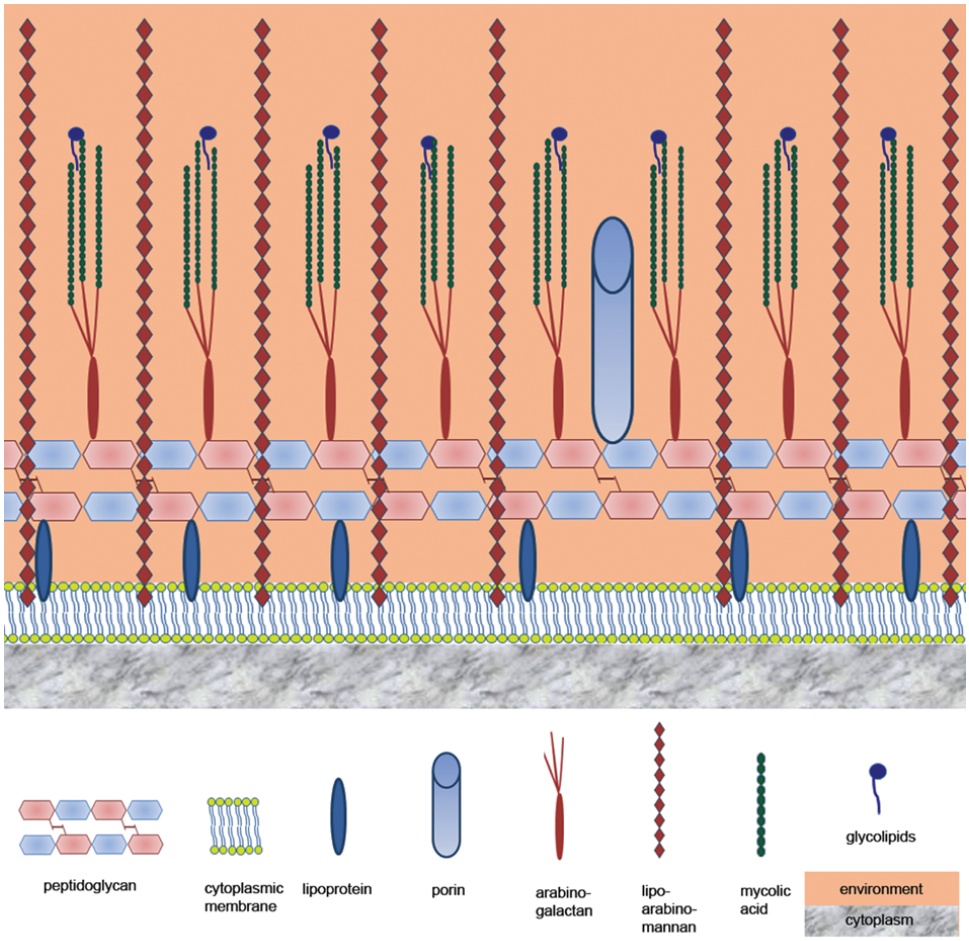


FIGURE 7 Schematic drawing of a mycobacterial cell wall. A thin layer of peptidoglycan and arabinogalactan, to which large amounts of mycolic acids are attached, is characteristic. An unusual compound is lipoarabinomannan, which is attached to the cytoplasmic membrane. On the outermost outside, glycolipids are attached to the mycolic acids; transport is facilitated by inserted porins. The mycobacterial outer membrane is not included in the scheme since the presence of such an outer membrane is still under discussion.

on the high-resolution imaging method applied, different asumptions about its distribution, arrangement, and localization in *B. subtilis* were made, i.e., looking at if MreB forms helices in the bacterial cell (89).

Several attempts have been undertaken to elucidate the ultrastructure of Gram-positive cell walls. In early TEM imaging of embedded and ultrathin cut bacteria, the preparation scheme included chemical fixation with aldehydes, introduction of heavy metals, dehydration with acetone/ethanol, and embedding in suitable resins. All these preparation steps had to be done to cope with the "hostile" environment created by the electron microscope, namely, high vacuum and bombardment with high-energy electrons resulting in heating up the section. Thus, it is obvious that these treatments might not result in proper preservation of the native cell wall. To overcome some of these detrimental effects in the preparation of bacteria, cryo-methods were introduced, such as freeze-substitution or hydrated cryo-ultrathin sections. With the advent of high-pressure-freezing techniques, preservation of bacterial cell wall structures was pushed further in the direction of close-to-nature conditions. Nowadays, CET is the best method to perform imaging in a frozen vitrified hydrated state of the bacteria (90). However, the drawbacks of this technique are that it is only available in certain institutes and it needs a sophisticated infrastructure and time for performing in-depth analysis. The future will show if the newly developing cryo-FIB-SEM micromachining will advance the deciphering of the ultrastructural details of the bacterial cell wall since the examined bacteria are in their fully hydrated condition and physically frozen, overcoming the problem of chemical fixation with aldehydes. This technique allows the observation of lamellae (about 10 to 20 nm thick) cut out of the bacterium, thus gaining access to small ultrastructural details. It should also be mentioned that other techniques, such as X-ray diffraction and X-ray lithography, have been unsuccessful because the bacterial cell wall is not crystalline. AFM has also been implemented, but with AFM only the surface of a sample can be imaged, and therefore, only limited ultrastructural information was obtainable.

The same restriction holds true for SEM. With the advent of FESEM it became possible to study bacterial structures at very high magnification (up to 400,000-fold) and resolution. Nevertheless, FESEMs have never been able to provide the amount of ultrastructural details observed with TEMs on

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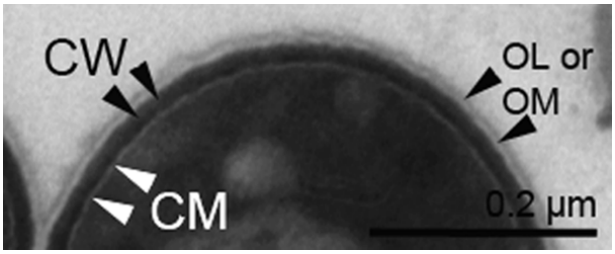


FIGURE 8 Typical appearance of a triple-layer structure of the mycobacterial cell wall of *Mycobacterium avium* ssp. *paratuberculosis* after special embedding applying the osmium-thiocarbohydrazide (TCH)-osmium method; this method especially preserves lipids much better than other methods because after the first osmium tetroxide step TCH binds to the sample bound osmium, and in the second osmium step more osmium is bound to TCH, therefore stabilizing lipids. In addition bacteria were embedded by applying the progressive lowering of temperature method down to –50°C, and bacteria were then embedded in the hydrophobic Lowicryl resin HM20; this protocol makes it possible to clearly define the triple-layer structure of the mycobacterial cell, which is lost in most other embedding methods, despite the cryo-based protocols. CM, cytoplasmic membrane; CW, cell wall; OL, outer layer; OM, outer membrane.

ultrathin sections. This is simply because FESEM reveals only the surface topography of a bacterial cell. It is noteworthy that FESEM does not allow discrimination between Gram-positive and Gram-negative bacteria (see Fig. 9). In addition, FESEM samples need to be coated to be conductive. This so-called sputter-coating is often the last step in an SEM preparation protocol. Samples are usually sputter-coated with a thin 5- to 8-nm film of either gold, gold palladium, or platinum. Even though these layers are very thin, they might cover some fine ultrastructural details of interest when observed at high magnifications. Surprisingly, high-resolution images of the Gram-positive cell wall do not exist. However, FESEM has been very useful in studying the interactions of pathogenic bacteria with host cells.

The following subsections give a general description of most of the electron microscopic methods applied for studying bacterial surface structures. If the reader is interested in detailed protocols, please refer to specialized text books on electron microscopic methods.

Heavy Metal Coating or Shadowing

When biological samples were examined for the first time under a TEM, it became obvious that the contrast of the biological material is fundamental and that methods had to be developed to increase the contrast for TEM images. One of the first approaches applied was coating with heavy metals (91, 92). A shadow line behind the exposed structures appeared when the metal coating was performed at a certain angle. If the coating angle and the measured length of the resulting shadow were known, the height of the structure could be determined. In earlier years, metal coating became the method of choice for the ultrastructural description of regularly patterned cell wall structures, named S-layers, attached to the bacterial cell wall (44, 93–95).

Negative Staining

The metal coating approach has restrictions when macromolecules or protein complexes have to be imaged. For such

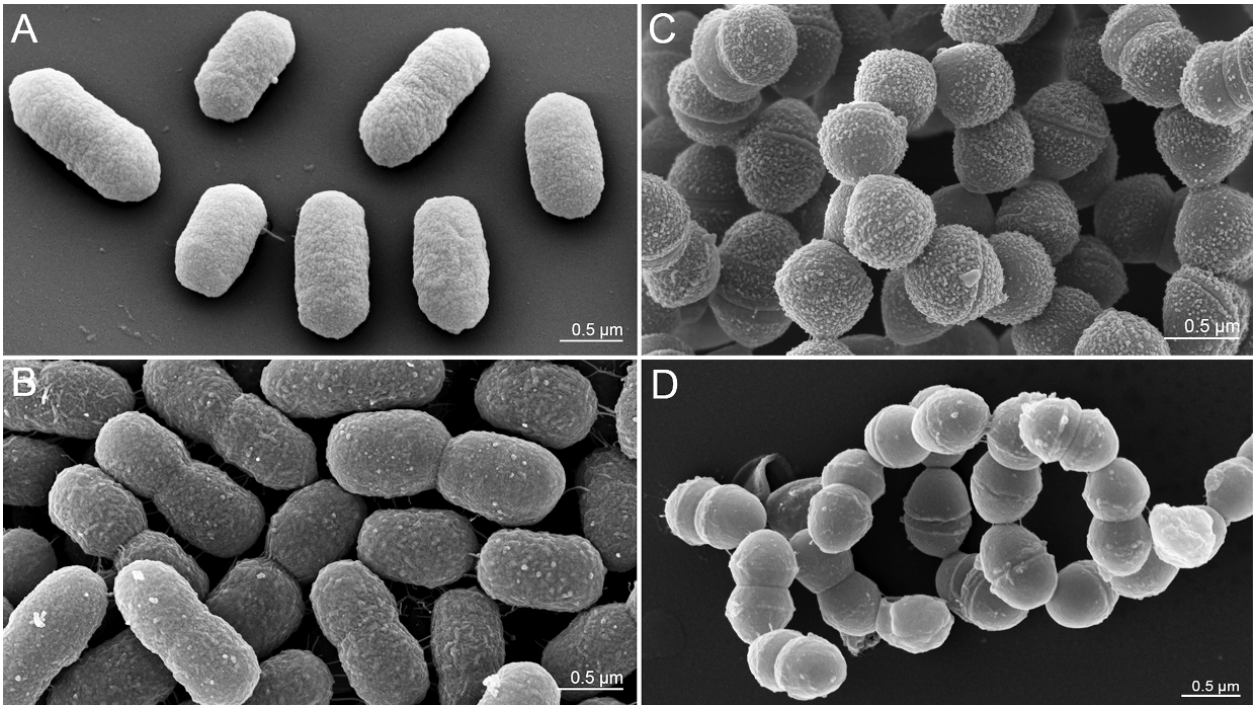


FIGURE 9 FESEM of aldehyde-fixed, acetone-dehydrated, critical-point-dried, and gold/palladium sputter-coated samples. (A) *Escherichia coli* (Gram-negative), (B) *Acinetobacter baumannii* (Gram-negative), (C) *Streptococcus pyogenes* (Gram-positive), and (D) *Enterococcus faecium* (Gram-positive). Since FESEM reveals only the surface structures and no information from inside the bacteria, FESEM does not distinguish between Gram-negative and Gram-positive bacteria.

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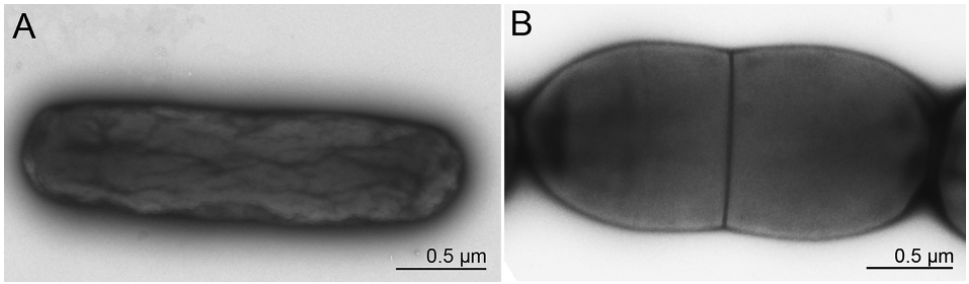


FIGURE 10 TEM images of negatively stained bacteria. (A) *Escherichia coli* (Gram-negative) and (B) *Streptococcus gordonii* (Gram-positive). Negative staining with 1% aqueous uranyl acetate cannot discriminate between Gram-negative and Gram-positive bacteria because the different peptidoglycan thicknesses cannot be resolved.

purposes the idea of embedding macromolecules into heavy metal salts such as Na-K-phosphotungstate, and later, uranyl acetate and others, was considered. The advantages of negative staining are manifold: it is (i) reliable and repeatable, (ii) fast, (iii) avoids flattening of macromolecules on the support film when air-drying, (v) stabilizes the protein in the electron beam, and (vi) allows determination of the shape and quaternary structure of an enzyme complex at around 1.3-nm resolution. In addition, the use of different heavy metal salts results in higher or lower contrasts (96–98). It is noteworthy that negative-stained viruses and larger enzymes opened the door for 3D microscopy and image processing beginning in the 1970s (99, 100). Negative staining was the method of choice when isolated peptidoglycan sacculi or pole caps were analyzed (68). Nevertheless, negative staining is not suitable to differentiate between Gram-negative and Gram-positive bacteria (see Fig. 10).

Conventional Embedding

In the 1950s, the embedding technique was introduced to analyze the ultrastructural details of bacteria (10) because intact bacteria were unsuitable for these studies. Therefore, ultrathin sectioning of bacteria was needed to gain access to internal morphological structures. With the invention of ultramicrotomes, it was possible to obtain ultrathin sections of biological samples, thus facilitating detailed studies of bacterial cell walls (see Fig. 11).

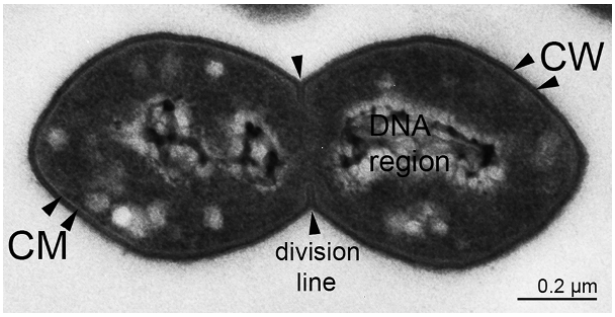


FIGURE 11 Typical image of a conventional embedded Gram-positive bacterium, *Streptococcus pneumoniae*, after aldehyde fixation, contrasting with osmium tetroxide and uranyl acetate, dehydration, and embedding in epoxy resin. In ultrathin sections the DNA region is typically aggregated and forms a translucent area within the bacterial cell. The thick peptidoglycan layers are dark featureless structures. CM, cytoplasmic membrane; CW, cell wall.

After that, morphological studies of bacterial cell walls started to blossom. Ultrathin sections have a thickness of around 50 to 80 nm, meaning that a single bacterium measuring 1 µm in length can be cut into nearly 15 sections. Again, one was directly confronted with the problem of the low contrast of biological samples. Thus, the early embedding protocols usually included fixation with aldehydes; contrasting with heavy metals such as osmium tetroxide, ruthenium red, and uranyl acetate; and dehydration with acetone/ethanol depending on the resin used for embedding. At that time epoxy or methacrylate resins were used the most, and polymerization was carried out at 60 to 70°C. Nowadays, many different resins are available, and every class of resin offers a slightly different image of the embedded bacterial ultrastructure, depending on the embedding protocol (see Fig. 12). In addition, the counterstaining of ultrathin sections before TEM examination influences the appearance of ultrastructural details in the sections. These protocols revealed unequivocally the visible differences between Gram-positive

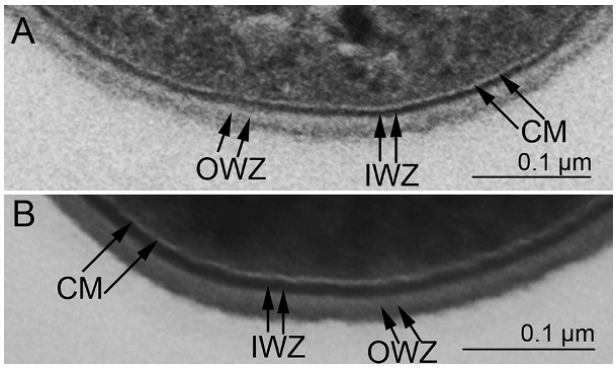


FIGURE 12 Comparison of embedding in different embedding resins. (A) *Staphylococcus aureus* embedded in low-viscosity (LV) resin. (B) *S. aureus* embedded in LRWhite resin. *S. aureus* was fixed, stained with osmium and uranyl acetate during dehydration with ethanol, and embedded in the epoxy LV resin, a replacement for the widely used Spurr resin, and the aromatic acrylic resin LRWhite. Both protocols show similar features, namely, a clearly defined cyoplasmic membrane (CM) and a peptidoglycan with two distinct zones, the dark inner wall zone (IWZ) and the outer wall zone (OWZ), which is more translucent in appearance. LRWhite resin preserves the OWZ much better compared to LV resin. In addition, the cytoplasm of both identically treated bacterial cells looks quite different. LRWhite has proven to be a reliable resin for studying bacterial ultrastructures.

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and Gram-negative bacterial cell walls. In most of the ultrathin sections, the Gram-positive cell wall appears as an amorphous structure and, depending on the resin and applied embedding protocol, some structural details can be detected, such as the periplasmic space in Gram-positive cell walls (101, 102). It should be clearly stated here that these embedding protocols are prone to inducing artifacts in the samples and therefore influence the interpretation of the observed ultrastructural details (103). Nevertheless, these methods served as a basis for most of the descriptions of bacterial ultrastructure, and they are widely available in nearly all life science electron microscopy units.

Cryo-Methods

Conventional embedding approaches lack the accuracy required to investigate tiny ultrastructural details because of the potential adverse effects of chemical fixatives, the introduction of heavy metals, and dehydration during preparation. The development of cryo-methods began in the early 1980s, when vitrification of water in biological samples for electron microscopic studies was applied for the first time by Mayer and Brüggeller (104) and Dubochet et al. (105–107). Earlier, freeze-fracturing was introduced in the 1960s (108).

Freeze-fracturing and freeze-etching

One of the earliest cryo-methods applied was freeze-fracturing and freeze-etching. Samples are frozen in nitrogen slush, and thereby, water in the samples is brought into its vitrified state. Then the samples are fractured, sometimes etched, and subsequently coated with metal or carbon or both. From this sample a replica is produced which exhibits the surface topography. The depth of the topographical structures depends on the etching time (109, 110). Usually, during freeze-fracturing, the fracture line is in the hydrophobic region of a membrane, i.e., in the bacterial cytoplasmic membrane, thus exposing transmembrane or membrane-bound proteins. Only rarely does a fracture line run across the cell wall or inside a cell wall. Areas which are exposed give a more or less featureless matrix or, such as in cross-fractures, show a polymeric network which cannot be further resolved. These findings did not succeed in producing considerable new understandings of the Gram-positive cell wall (111).

High-pressure freezing and freeze-substitution

The development of freeze-substitution of quickly frozen samples, which was paralleled by the invention of low-temperature embedding of resins such as the Lowicryl series of methacrylate resins, began in the 1980s (14, 16). Bacteria are snap-frozen in liquid propane or ethane and then rapidly transferred into a substitution medium containing osmium and/or uranyl acetate in acetone. Remarkably, it was demonstrated that a certain water content in acetone (up to 4%) resulted in much better visibility of membranes (112). Samples are then kept for 2 days at –80°C, warmed up to –50°C and then –20°C and left for 1 day at each step. The following embedding can be performed with low-temperature resins, such as Lowicryl resins, or samples are brought to ambient temperature and embedded with conventional resins (81, 103, 113). During substitution, bacteria are stained and dehydrated, resulting in visibly better preservation of ultrastructural details. Most freeze-substituted bacteria in ultrathin sections are recognizable by the fact that no distinct DNA region can be observed, whereas in conventional embedding, DNA mostly aggregates and forms the typical lucent DNA region in the middle of the bacterial cell (see Fig. 13).

Currently, hundreds of substitution protocols exist which are customized to fulfill the needs of the examined biological samples and to address the study purpose, e.g., for ultrastructural studies or immune cytochemical localization studies. Freeze-substitution was pushed forward even more by the high-pressure freezing of bacteria. This method was developed in the 1960s (108). At ambient temperatures adequate freezing of bacteria is reached with cooling rates of more than 10,000 Kelvin/second to vitrify the water content in the sample. High pressure is a potent physical cryo-protectant because it lowers the freezing point of water considerably, and thicker samples can be vitrified. The currently available equipment freezes samples at about 200,000 kPa. At this pressure, samples of up to 200 µm can be frozen without formation of ice crystals (108, 114–116). The combination of these two methods is now considered to be the best approach for ultrastructural studies of bacteria when no access to CET is possible. One result of such studies is the above-mentioned appearance of a periplasmic space in Gram-positive bacteria (79, 117, 118). Nevertheless, CET observations have raised questions about these assumptions.

Cryo-sections of hydrated vitrified bacteria and Cryo-FIB-SEM

Even though high-pressure freezing and freeze-substitution have been steps toward close-to-nature conditions, it is without doubt that ultrastructural details and the organization of macromolecules are still changing to a certain degree. This occurs because replacement of vitrified water and introduction of heavy metals during the substitution process for staining bacterial components—even when performed in cold conditions—are expected to change native structures. For real close-to-nature observations, the water content of samples has to be vitrified to guarantee a fully hydrated status, and samples should be observed in the vitrified frozen status below –80°C. Hydrated cryo-ultrathin sections fulfill these two criteria (see Fig. 14).

The expression CEMOVIS (cryo-electron microscopy of vitreous sections) was introduced for this cryo-method (119). The technique of cutting cryo-ultrathin sections at –110°C or below is very demanding and has been realized only by some laboratories for studying bacterial cell walls (117, 118, 120). As with nearly every method, cryo-ultrathin sectioning of bacteria has its drawbacks, such as knife marks and compressions. For example, forces working during the cutting process, i.e., compression in the cutting direction, can be harmful to the preservation of cell walls or lipid membranes (121–123). A method which overcomes the thinning of bacteria without introducing any compression on the sample is highly desirable. Dual-beam imaging with an SEM (FIB-SEM) with an attached ion gun (focused gallium gun) was introduced in the 1990s, particularly for material sciences. The ion gun is used to mill away material from a sample, which then is imaged by the electron beam with suitable detectors, often called back-scattered electron detectors. This is followed by the next cutting step, imaging step, and so on. This process is also known as serial block face sectioning. Thus, a series of sections is generated which can be aligned and further processed with suitable software to reveal 3D structures of the imaged regions. The use of ion beam milling was later extended to plastic-embedded biological samples such as bacteria, eukaryotic cells, and tissues (124, 125). As early as 2006 a cryo-version of FIB-SEM was invented and was tested for cutting frozen vitrified bacteria with ion milling (126, 127). Soon after, cryo-FIB-SEM was introduced to the life sciences and is now optimized for 2D and 3D imaging. Studies highlighted the fact that ion beam milling is an

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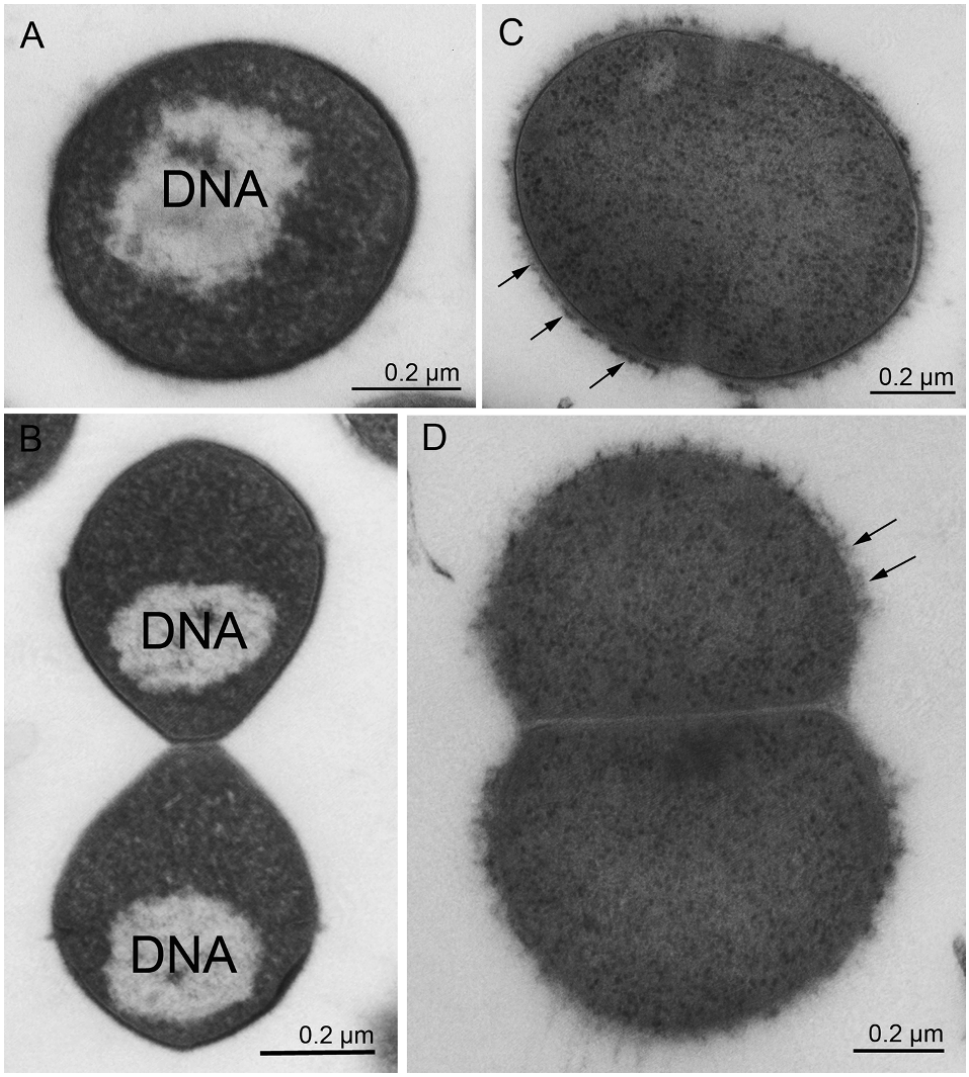


FIGURE 13 Comparison of conventional embedded and high-pressure frozen and freeze-substituted group G streptococci. (A and B) Cross-section and longitudinal section of dividing conventional embedded bacteria. Note the prominent translucent DNA region and the absence of any material attached to the outside of the bacterial cell wall. (C and D) Cross-section and longitudinal section of high-pressure frozen and freeze-substituted bacteria. No translucent DNA region is detectable, and the bacterial cytoplasm appears as a uniform structure throughout. Some material attached on the outer side of the bacterial cell wall is also preserved.

artifact-free method without any compression to cut vitreous frozen fully hydrated biological samples (128, 129). Today, the technique is so advanced that lamellae of a thickness of around 20 to 30 nm can be cut out of the vitrified bacteria, transferred to a cryo-TEM, and imaged under vitrified conditions. This modern invention allows the performance of CET on such lamellae and 3D analysis of the cut-out lamellae. For this, the next modern electron microscopic imaging technique, namely CET, is applied.

Cryo-electron tomography

CET, also called electron cyrotomography, reveals structures within a bacterial cell in essentially close-to-nature conditions. 3D reconstruction with a TEM can be achieved with a tomographic approach. For this, the sample is tilted usually between –70° to +70° around an axis. Every 1- or 2-degree tilting step is recorded to collect a 3D data set. The data set is aligned and the tomogram, a density map, is calculated. Since a 3D view is available, one can scan through every slice in the tomogram and can view structures in that specific slice. The basis for CET has been known since the early 1960s, but technical progress for cryo-TEMs had to be invented, such as automatization of electron microscopes, electron energy filtering, introduction of highly sensitive slow charged-couple-device cameras for recording images, 300-kV cryo-TEMs, imaging plates, and phase-plates for recording very low signals (130–135). One of the major drawbacks is the "bad" signal-to-noise ratio in CET imaging; thus, again, missing contrast in the images is a problem which had to be circumvented.

CET is perfectly suitable for bacteria that are not thicker than about 300 to 400 nm and for studying bacterial ultrastructures under close-to-nature conditions. If thicker bacteria need to be imaged, cutting lamellae from the sample

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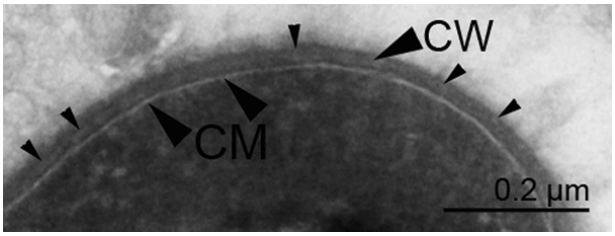


FIGURE 14 Cryo-electron microscopy of a vitreous ultrathin section (CEMOVIS) of *Streptococcus pyogenes*. Note that the very well-preserved cytoplasmic membrane (CM) and some structural details can be detected in the peptidoglycan layers of the cell wall (CW, arrow heads) when compared to the dark appearance of cell walls in conventional embedded bacteria (compare to Fig. 11).

by cryo-FIB-SEM is the most promising approach. Since the image in CET is generated solely by the density of the biological material by itself, most imaging is performed at reasonable underfocused settings to obtain contrast in the images. Therefore, the peptidoglycan layer is in most cases seen as a solid structure without any more details. For example, the membrane bilayer is not represented as conventional embedding images have shown. Nevertheless, by changing and adjusting imaging conditions, the outer membrane and cytoplasmic membrane of *E. coli* were clearly visible in reconstructions, and new insights were gained into the architecture of mycobacterial cell walls (20, 83).

OUTLOOK

The advent of the new cryo-approaches described in this article should allow researchers to examine Gram-positive cell walls under close-to-nature conditions and will certainly help us to achieve a better understanding of the micromorphology of the peptidoglycan together with its attached components. In particular, the discussion of a periplasmic space in Gram-positive bacteria comparable to the one in Gram-negative bacteria should be resolved by cryo-methods. It is expected that new and unanticipated structures, which have not been seen before, will be made visible. This should occur despite the fact that most cryo-studies performed to date used model Gram-positive bacteria laboratory strains of *S. aureus*, *S. pneumoniae*, and *B. subtilis*. Once imaging of newly isolated Gram-positive bacteria from nature is performed, it should reveal new scenarios of the Gram-positive cell wall. This occurred very recently when Dobro et al. (136) conducted studies of uncharacterized Gram-negative bacterial structures. These structures will certainly open up a new avenue for research. The downside of the implementation and further development of these cryo-techniques is that the equipment required for these studies is very expensive and therefore such electron microscopic platforms might be available in only a few institutions. In addition, the new technology requires experienced personnel, special software, and a certain computing environment. Plus, researchers have to be convinced that studying the Gram-positive bacterial cell wall is a fascinating research goal, and more cryo-approaches are needed to decipher the secrets of the peptidoglycan layers. Nowadays, electron microscopy and fluorescence light microscopy are joining forces in a correlative light and electron microscopy approach in which fluorescent-labeled structures can be made visible and then studies with CET at high resolution under vitrified conditions. Therefore, correlative light and electron microscopy opens up another new field of imaging in which fluorescence-labeled structures of interest, even mobile structures in the bacterial cell, can be followed by live imaging, be snap-frozen at a certain time point, and then be imaged at high resolution by CET in close-to nature conditions.

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Surface Proteins on Gram-Positive Bacteria

VINCENT A. FISCHETTI

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In the same way arms, legs, hair, and fur are used in higher species for their survival in the environment, bacteria use surface appendages for similar purposes. Surface molecules in bacteria range from complex structures, such as flagella that propel the organism in aqueous environments, to less sophisticated polysaccharides and proteins. All of these molecules benefit the organism for survival in a hostile environment, such as the waters of a rushing stream, the blood of an infected animal, the surface of an object, or the surface of a mucosal epithelium. Although it was previously believed that bacteria were simple single-cell organisms with little complexity, it is now apparent that they are highly evolved, advanced particles that possess a wide array of surface molecules that manipulate the organism in its environment. For human pathogens, surface molecules have been finely tuned to allow adherence and colonization of host surfaces, invasion of cells, evasion of the host's immune response, and persistence in infected tissues.

In an effort to emphasize the complexity of bacterial surface molecules and their use in the everyday life of the bacterium, this article focuses on the surface proteins found on Gram-positive bacteria. For an extensive review of the subject, see references 1 and 2.

THE GRAM-POSITIVE CELL WALL

From electron microscopic analysis of the Gram-positive cell envelope and from a number of elegant chemical, structural and immunological analyses (also, see Chapter 1 of this volume), a picture of the Gram-positive cell wall has emerged (Fig. 1). The structure differs significantly from the Gram-negative cell wall in two ways: (i) the presence of a thicker and more cross-linked peptidoglycan and (ii) the lack of an outer membrane. Because of these differences, surface molecules on Gram-positive organisms vary from those on Gram-negative organisms, which requires specialized systems to transport and anchor molecules through the outer membrane (3, 4). In general, surface proteins in Gram-positive bacteria can be separated into three categories: (i) those that anchor at their C-terminal ends (through an LPxTG motif), (ii) those that bind by way of charge or hydrophobic interactions, and (iii) those that bind via their N-terminal region (lipoproteins) (Fig. 1).

C-TERMINAL-ANCHORED PROTEINS

Decades ago, relatively harsh treatments of extraction were used to remove surface proteins from Gram-positive bacteria, resulting in the isolation and characterization of molecular fragments rather than complete molecules. It was not until the early 1980s that the genes for surface proteins on Gram-positive bacteria were first published (protein A from *Staphylococcus aureus* [5] and M protein from *Streptococcus pyogenes* [6]). Although at first glance these molecules had little in common, it was soon realized that the C-terminal end of the protein A sequence contained a sequence error. Upon correction (5), it became apparent that this region of protein A exhibited very close sequence homologies with the M protein molecule. As other protein sequences from Gram-positive bacteria were published in the ensuing years, it was obvious that a common theme existed within the structure of these C-terminal-anchored molecules. Since then, hundreds of proteins from Gram-positive bacteria that anchor through their C-terminal region, based on the presence of an LPxTG motif (7), have been reported (see Table 1 for a short representative list). Because of the number of molecules reported and their varied functions, the M protein molecule will be used here as a model for surface proteins anchored via an LPxTG motif.

M Protein Structure

Of the C-terminal-anchored proteins, the M6 protein may be considered an archetypical molecule since it was the first Gram-positive surface protein to be completely sequenced (6). A unique characteristic of this molecule is the presence of regions with sequence repeats and a conserved anchor region. As shown in Fig. 2, the M6 protein is composed of four repeat blocks, each differing in size and sequence (6). The A-repeats are each composed of 14 amino acids in which the central blocks are identical and the end blocks diverge slightly from the central consensus repeats. The B-repeats, composed of 25 amino acids each, are arranged in the same way. The C-repeats, composed of 2.5 blocks of 42 amino acids each, are not as similar to each other as are the A- and B-repeats. There are also four short D-repeats that show some homology. These repeat segments make up the central helical rod region of the M molecule because of the high helical potential ascribed to the amino acids found within this segment, as determined by conformational analysis (8, 9).

A close examination of the sequence within the M molecule revealed a repeating seven-residue periodicity of nonpolar amino acids, a basic characteristic of α-helical coiled-coil proteins such as mammalian tropomyosin. In

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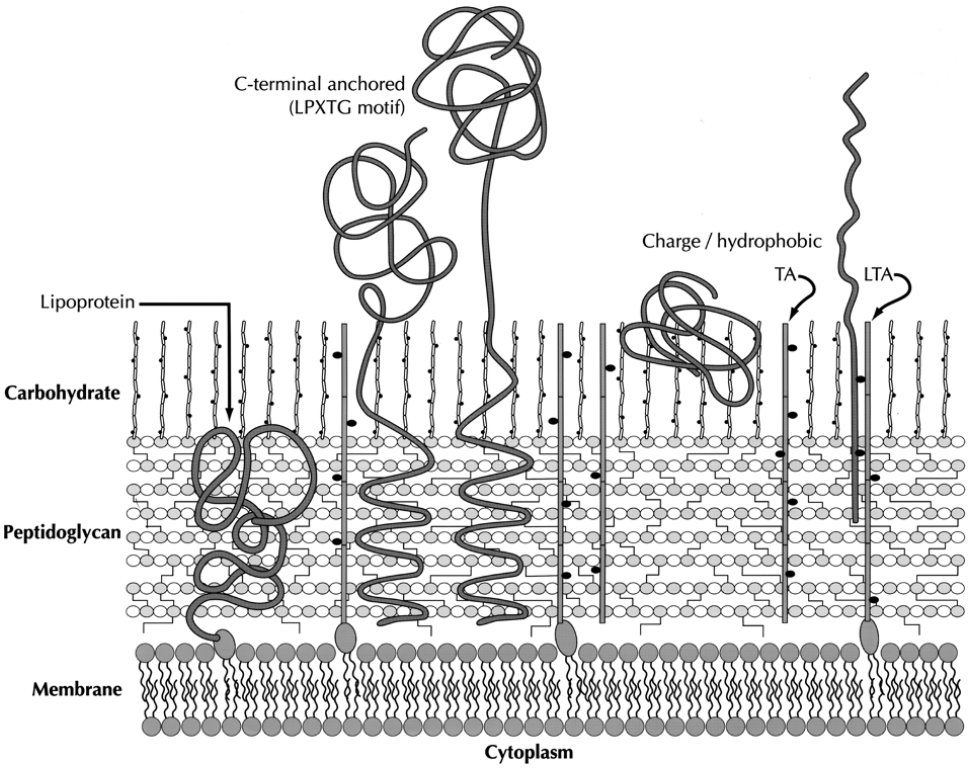


FIGURE 1 Major surface structures of the cell wall of Gram-positive bacteria. Linked to the surface of the peptidoglycan, many Gram-positive organisms have polysaccharide structures that in some cases are used for their immunological classification. Surface proteins are linked by three mechanisms. (i) Lipoproteins have a lipid linked through a cysteine at the N terminus. (ii) C-terminal-anchored proteins are attached and stabilized in the peptidoglycan through a C-terminal complex containing an LPxTG motif. (Most surface proteins are anchored this way.) (iii) Certain surface proteins are attached through hydrophobic and/or charge interactions to the cell surface. (Some proteins are bound ionically to the lipoteichoic acid.) The teichoic acids (TA) are a common feature of the Gram-positive cell wall. TA is usually composed of a repeating carbohydrate-phosphate polymer linked through a phosphodiester linkage to the peptidoglycan. Lipoteichoic acid (LTA) is composed of a similar polymer linked to the cytoplasmic membrane through a fatty acid.

general, α-helical coiled-coil proteins are constructed from a reiterating seven-residue amino acid pattern (a-b-c-d-e-f-g)*n* in which residues in positions a and d are hydrophobic and form the core residues in the coiled coil, and the intervening residues are primarily helix-promoting. The arrangement of amino acids of the M6 protein within the seven-residue pattern is shown in Fig. 3. The seven-residue periodicity from amino acids 12 through 362 strongly suggests that this region is in a coiled-coil conformation and forms the helical central rod region of the molecule. Discontinuities in the heptad pattern (seen especially in the B-repeat region), which have been found in the sequence of other M proteins and other coiled-coil molecules, probably account for the flexibility of the M molecules observed in electron micrographs (9). Based on these irregularities in the heptad pattern, the central rod region is divided into three subregions that correlate with the A-, B-, and C-repeat blocks (8).

Subsequent sequence data on a variety of M protein molecules revealed that while all M proteins retain a common structural organization, they vary in the N-terminal and central domains while keeping the conserved C-terminal region (10–25) (Fig. 4). One of the striking features observed in these various M molecules is their size variation (see below), which is affected by the presence, number, and size of the sequence repeat regions (particularly the A and B repeats) and the length of the N-terminal nonhelical region. Based on the nine M proteins in Fig. 4, the A repeats are more the exception than the rule. The biological meaning of this is unclear, since all the M types depicted are found on clinical isolates of *S. pyogenes*. Though they may vary somewhat in number, the C-repeats are present in all M proteins and are surface exposed. This conserved sequence among different M molecules has vaccine implications (refer to Chapter 10 in this volume). For a different perspective on the M molecule please refer to reference 26.

Electron and Fluorescence Microscopy

Although early electron micrographs of the surface of Gram-positive bacteria revealed certain structures, it was not known whether they were proteinaceous or polysaccharides. It was not until Swanson et al. (27) published the first electron micrographs of the M protein on the surface of group A streptococci that it was realized that the molecule was an elongated structure. Although other proteins were also reported to be on the streptococcal surface, they were not apparent in these electron micrographs. Thus, direct visualization of surface molecules is limited to molecules with

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TABLE 1 Sequenced surface proteins from Gram-positive bacteria linked to the cell wall through an LPxTG motif—a representative list of molecules from a range of Gram-positive pathogens[a]

Name/gene	Name/function	Organism	LPxTG[b]	GenBank accession no.
1. emm6	M protein	*S. pyogenes*	**LPSTG**	M11338
2. emm5	M protein	*S. pyogenes*	**LPSTG**	M20374
3. Mrp4	IgG/fibrinogen binding	*S. pyogenes*	**LPSTG**	M87831
4. Prot H	Human IgG Fc binding	*S. pyogenes*	**LPSTG**	M29398
5. T6	Protease resistant protein	*S. pyogenes*	**LPSTG**	M32978
6. sof22	Serum opacity factor	*S. pyogenes*	**LPASG**	U02290
7. Sfb	Fibronectin binding	*S. pyogenes*	**LPATG**	X67947
8. ZAG	Binds γ_2 M, Alb, IgG	*Streptococcus zooepidemicus*	**LPTTG**	U02290
9. Prot L	Light chain binding	*Peptostreptococcus magnu*	**LPKAG**	M86697
10. bac	IgA binding protein	Group B streptococci	**LPYTG**	X58470
11. fnbA	Fibronectin binding	*Streptococcus dysgalactiae*	**LPQTG**	Z22150
12. SzPSe	M-like	*Streptococcus equi*	**LPQTS**	U73163
13. Prot G	IgG binding protein	Group G streptococci	**LPTTG**	X06173
14. MRP	Surface protein[c]	*Streptococcus suis*	**LPNTG**	X64450
15. spaP	Surface protein	*S. mutans*	**LPNTG**	X17390
17. fruA	Fructosidase	*S. mutans*	**LPDTG**	L03358
19. Prot A	IgG binding protein	*S. aureus*	**LPETG**	X00342
23. inlA	Internalization protein	*Listeria monocytogenes*	**LPTTG**	M67471
26. Hyal1	Hyaluronidase	*S. pneumoniae*	**LPQTG**	L20670
27. InlC2	Internalin-like	*L. monocytogenes*	**LPTAG**	U77368
28. RSP	Adhesin	*Erysipelothrix rhusiopathiae*	**LPN/QTG**	AB052682

[a]From references 6, 10, and 84–96.
[b]Bold indicated the LP and TG of the LPxTG motif.
[c]Surface protein: proteins that have been identified to have a C-terminal anchor motif, but function is unknown.

certain physicochemical characteristics. In the case of the M protein, it is the α-helical coiled-coil structure that allowed its visualization by electron microscopy (8).

In experiments designed to answer questions about the synthesis and placement of M protein on the cell wall, Swanson et al. (27) found that after trypsinization to remove existing M protein on living streptococci, newly synthesized M protein was first seen by electron microscopy on the cell in the position of the newly forming septum. In similar experiments designed after the classical experiments of Cole and Hahn (28) using fluorescein-labeled anti-M antibodies, trypsinized streptococci placed in fresh medium for 10 min revealed M protein first at the newly forming septum (Fig. 5). On organisms examined after 40 min of incubation or longer, M protein was not observed in the position of the old wall, suggesting that the M molecule is produced only where the new cell wall is synthesized. This confirmed the observation of Swanson et al. (27).

In their pioneering work in 2006, Carlsson et al. showed that the sequence of the signal peptide which contains a YSIRK-G/S motif (SP_{+YSIRK}), found in M protein, directs the molecule to the division septum, while proteins lacking this motif (SP_{-YSIRK}), such as that found in streptococcal protein F (also called SfbI), are directed to the poles (29). Modern microscopic techniques were used to better visualize this sorting phenomenon on the bacterial surface (30). The data show that M protein rapidly appears and accumulates at the septum during bacterial growth, while SfbI slowly gathers at the poles, where the peptidoglycan is older and more heavily cross-linked (Fig. 6). Data showing decreased M protein (but not SfbI) expression using methicillin or the division regulator DivIVA strongly suggest a close connection between the regulation of cell division and protein anchoring.

C-Terminal Anchor Region

An examination of the C-terminal end of surface molecules that anchor at that end revealed, without exception, that all have a similar arrangement of amino acids. Up to seven charged amino acids are found at the C terminus which are composed of a mixture of both negatively and positively charged residues. Immediately N-terminal to this short charged region is a segment of 15 to 22 predominantly hydrophobic amino acids sufficient to span the cytoplasmic membrane of the bacterium, placing the charged end in the cytoplasm (Fig. 2). In all these proteins, the sequences found in the hydrophobic and charged regions are not necessarily the same, but the chemical characteristics of the amino acids used to compose them are conserved. Beginning about eight amino acids N-terminal from the hydrophobic domain is a heptapeptide with the consensus sequence LPxTG, which is extraordinarily conserved among the C-terminal-anchored proteins examined (31). Although several amino acid substitutions are seen in position 3 of the heptapeptide (predominantly A, Q, E, T, N, D, K, and L), positions 1, 2, 4, and 5 are nearly completely conserved (~100% in the L and P positions and >85% in the T and G positions). This conservation is also maintained at the DNA level. The preservation of this heptapeptide and the high homology within the hydrophobic and charged regions suggest that the

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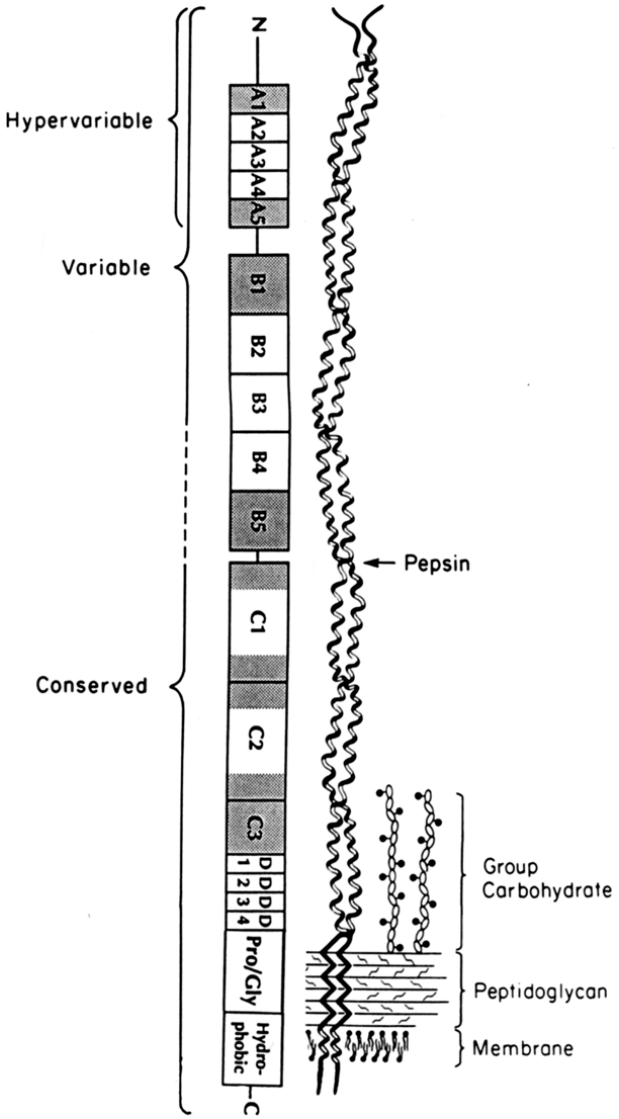


FIGURE 2 Characteristics of the complete M6 protein sequence. Blocks A, B, C, and D designate the location of the sequence repeat blocks. Block C3 is half the size of blocks C1 and C2. Shadowed blocks are those in which the sequence diverges from the central consensus sequence. Pro/Gly denotes the proline- and glycine-rich region likely located in the peptidoglycan. The membrane anchor is a 19-hydrophobic-amino-acid region adjacent to a 6-amino-acid charged tail. Pepsin identifies the position of the pepsin-sensitive site after amino acid 228. The C-terminal end is located within the cell wall and membrane.

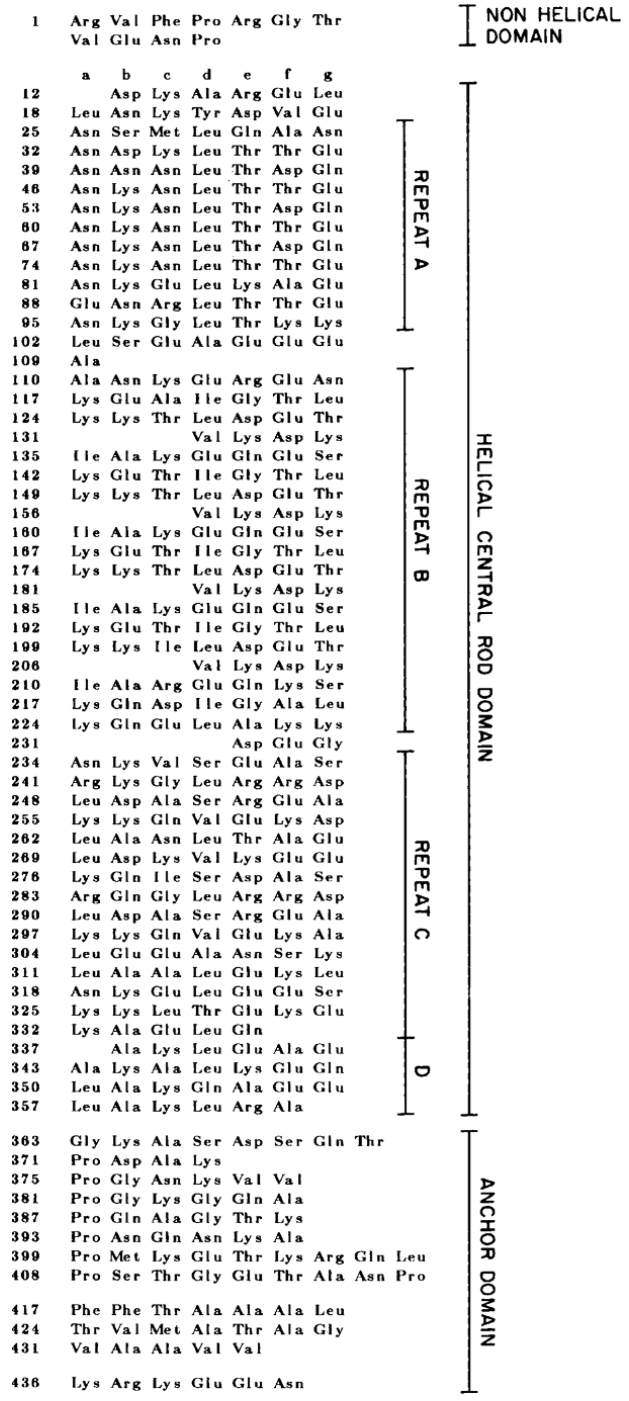


FIGURE 3 Complete M6 protein sequence arranged to highlight the seven-residue periodicity found in the helical central rod region. Region assignments are based on sequence and conformational analyses. Arrangement of the sequence is based on the position of amino acids in a seven-residue periodicity designated by letters a through g beginning at residue 12 and continuing through residue 362, with interruptions at residues 109, 131, 156, 181, 206, 231, and 337. Alignment from residues 363 to 416 is used essentially to highlight the regularity of the position of prolines in the sequence. No periodicity is found from residue 417 to the end. Three major regions are indicated (nonhelical, helical, and anchor). The pepsin-sensitive site is between Ala-228 and Lys-229.

method of anchoring these molecules within the bacterial cell is also highly conserved, and the LPxTG motif may serve as an enzyme recognition sequence.

Thus, C-terminal-anchored surface proteins in Gram-positive bacteria (which could number >25 different molecules in a single organism) that contain an SP_{+YSIRK} leader sequence, are synthesized and exported at the septum, where new cell wall is also being produced and translocated to the surface (8, 28). Therefore, to coordinate the export and anchoring of all these proteins, the C-terminal hydrophobic domain and charged tail function as a temporary stop,

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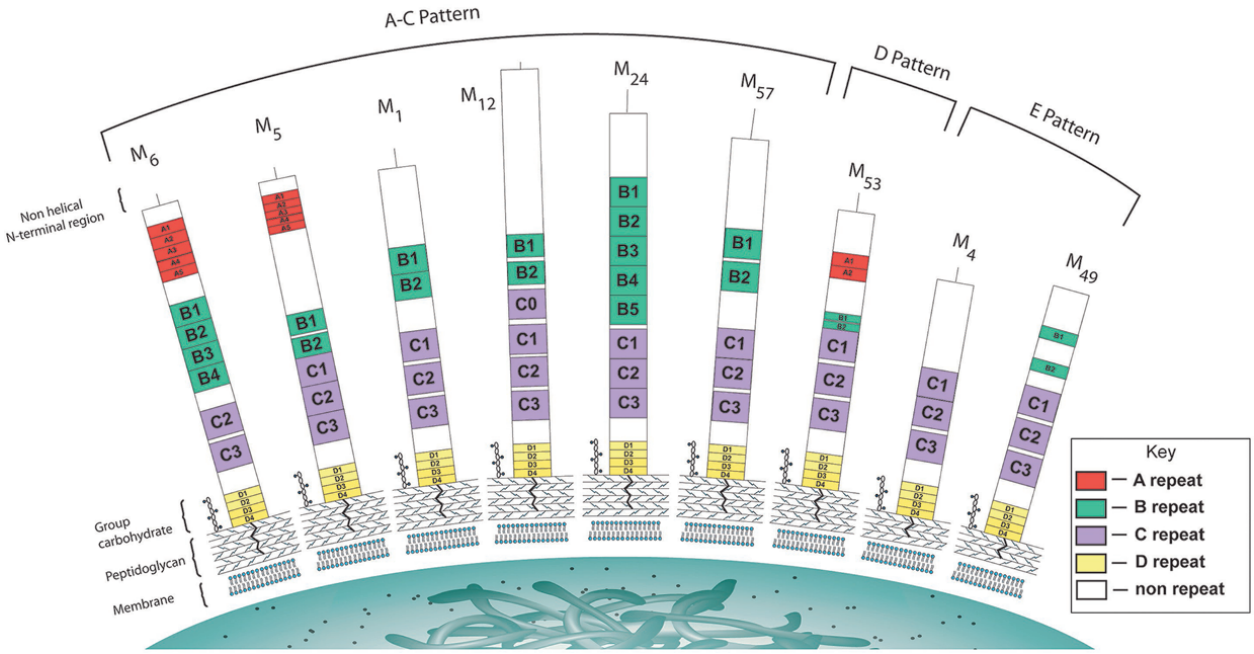


FIGURE 4 Schematic representation of the organization of nine well-described M proteins showing the presence and location of the sequence repeat regions and pattern arrangement. Figure reused from reference 26 with permission.

precisely positioning the LPxTG motif at the outer surface of the cytoplasmic membrane (Fig. 7). This motif is then cleaved by the transpeptidase sortase, ultimately resulting in the attachment of the surface-exposed segment of the protein to the peptidoglycan (32). For an extensive review see reference 33. This idea is supported by studies indicating that the C-terminal hydrophobic domain and charged tail are missing from the streptococcal M protein extracted from the streptococcal cell wall (32, 34). Studies indicate that the substrate in the peptidoglycan to which the protein is linked is the cross bridge (e.g., glycine in *S. aureus*, alanine in *S. pyogenes*) of the pentapeptide (35).

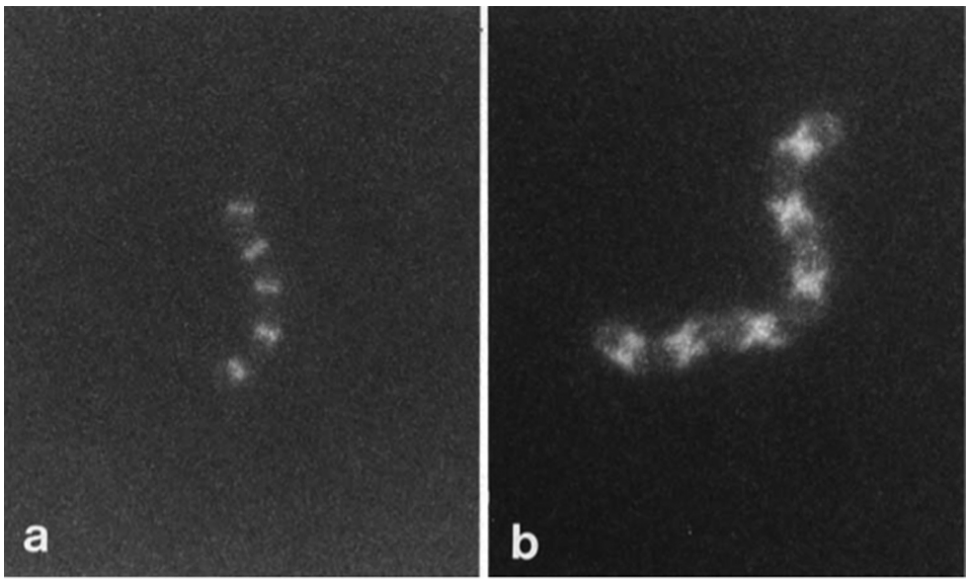


FIGURE 5 Fluorescein-labeled anti-M6 antibody analysis of the appearance of M6 protein on streptococcal cell walls. M6 streptococci were treated with trypsin to remove surface M protein. Cells, reincubated at 37°C, were removed at intervals, fixed, and stained with fluorescein-labeled anti-M6 antibody. (a) After 10 min of incubation the M protein is located within a thin band at the position of the newly forming septum. Magnification, ×5,000. (b) Location of the M protein after 40 min of incubation. Note that no fluorescein label is seen in the position of the old wall. Magnification, ×6,000.

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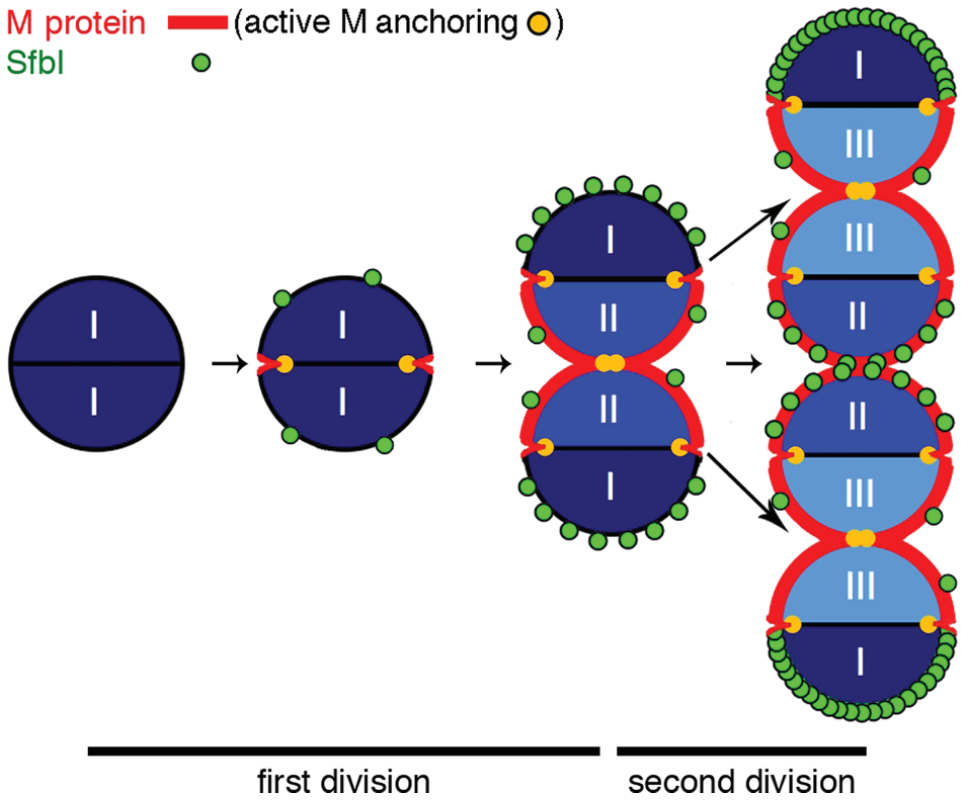


FIGURE 6 A model representation of the anchoring of M protein and SfbI. The regeneration of surface proteins during two division cycles is presented. M protein (red) is anchored exclusively to newly synthesized peptidoglycan at the septum (sites of active anchoring are labeled yellow). Anchoring of M protein at daughter septa begins before the mother septum is completely closed, resulting in simultaneous anchoring at both locations. Following two generations, M protein is anchored to all newly synthesized, but not pre-existing, peptidoglycan. SfbI (green) is anchored over time in patches to peripheral peptidoglycan, with some preference to the poles. Following two generations, the oldest poles (I) display the most intense SfbI labeling, while one-generation-old poles show less labeling (II), and newly formed poles (III) show little SfbI labeling with permission reference 30.

Enzymes That Cleave the LPxTG Motif

Sortase

Sortase is a membrane-bound transpeptidase found in Gram-positive bacteria (2, 36–42), with the capacity to both cleave the LPxTG motif and anchor the newly formed C terminus to the nascent cross bridge in the peptidoglycan (39, 43) (for a detailed review see reference 44). Mutants in the sortase gene (*SrtA*) have been shown to be defective in their ability to surface display proteins (36–38, 45). Because surface proteins are essential for the infectivity and survival of Gram-positive bacteria in the host, SrtA mutants have been shown to be attenuated in colonization experiments (46) and animal infection models (45). A genome-wide analysis of sortases revealed that sortase-like proteins were present in almost all Gram-positive bacterial species and that in every Gram-positive bacterium in which one sortase-like protein was identified, at least one other was also found, and in most cases several others (36, 47). Further analysis indicates that genes encoding sortase-like proteins are usually found to be clustered along with genes encoding their probable substrates. Because of their importance in surface protein display, sortase molecules could be a target for antibiotic development. However, unlike current antibiotics, which affect bacterial viability, antibiotics that block the action of sortases would compromise the pathogenicity of a pathogen, yielding a new class of anti-infectives.

LPxTGase

Recently, a new enzyme was identified from *S. pyogenes* that differs significantly from sortase but also cleaves within the LPxTG motif of surface proteins (48, 49). This unique enzyme, termed LPxTGase, has no aromatic amino acids, is rich in alanine (particularly D-Ala [50]), is heavily glycosylated, and is 30% composed of uncommon amino acids, suggesting that it may be constructed independently of ribosomes. The enzyme, which is also found in *S. aureus*, is also important in cell surface protein display, as well as in cell wall assembly. Because the cleavage activity of LPxTGase is at least two orders of magnitude greater than that of sortase (43), LPxTGase may play an essential role in the rapid display of thousands of surface protein molecules during a short (20- to 30-min) bacterial division cycle.

Wall-Associated Region

Immediately N-terminal from the LPxTG motif is the wall-associated region, which spans about 50 to 125 amino acid

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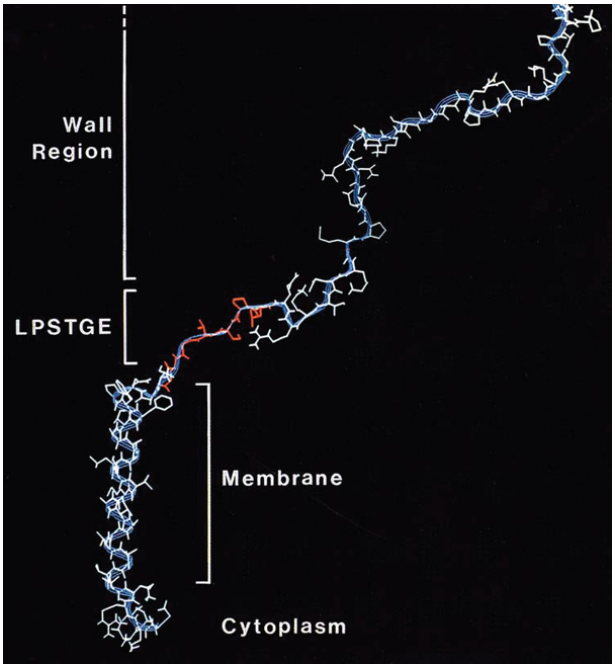


FIGURE 7 Computer-generated model of the C-terminal end of the M protein sequence (residues 371 to 441). A comparable region is found in all C-terminal-anchored surface proteins from Gram-positive bacteria (see Table 1). The predicted location of this segment of the molecule is shown in the cytoplasm, membrane, and peptidoglycan. The space between the membrane and peptidoglycan (wall region) may be considered the "periplasm" of the Gram-positive bacterium. The figure was generated on a Steller computer using the Quanta 2.1A program for energy minimization.

residues and is found in nearly all the proteins analyzed. Although this region does not exhibit a high degree of sequence identity among the known surface proteins, it is characterized by a high percentage of proline/glycine and threonine/serine residues. For some proteins (such as the M protein) the concentration of proline/glycine is significantly higher than that of threonine/serine, while in others this relationship is either reversed or nearly equal. Because of its proximity to the hydrophobic domain (which is located in the cell membrane), this region would be positioned within the peptidoglycan layer of the cell wall (34) (Fig. 1). The reason for the presence of these particular amino acids at this location is unknown. One hypothesis suggests that the prolines and glycines, with their ability to initiate turns and loops within proteins, allow the peptidoglycan to become more easily cross-linked around these folds, thus stabilizing the molecule within the cell wall (34). The function of the threonines and serines within this region is not immediately apparent, despite the fact that these amino acids are substrates for glycosylation.

Surface-Exposed Region

As the C-terminal region (buried within the cell wall/membrane) is characteristically conserved among the C-terminal-anchored surface proteins, the surface-exposed region is characteristically unique. Despite their differences, this region falls into three distinct groups: (i) those with several sequence repeat blocks, (ii) those with repeat blocks located close to and within the cell wall region, and (iii) those without any repeat sequences (Fig. 8). Streptococcal M protein and protein G may be considered molecules representative of those containing several sequence repeats. However, the most commonly found structures are those with repeats located close to the cell wall and an extended nonrepetitive region N-terminal to this segment.

Conformational analysis of 12 representative surface molecules by the algorithm of Garnier et al. (51) revealed that, in general, proteins containing repeat sequences were predominantly helical within the region containing repeat segments (Fig. 9). Conversely, regions and molecules without repeat blocks were predominantly composed of amino acids exhibiting high β-sheet, β-turn, and/or random coil potential. Thus, within most of these molecules, the presence of repeat segments usually predicts the location of a helical domain. Perhaps the pressure to maintain repeat blocks is to preserve the helix potential within specific regions of these molecules, the presence of which may determine an extended protein structure, as has been shown for the M protein (9).

Seven-Residue Periodicity

As shown above, most of the surface molecules containing repeat sequences were found to be α-helical in regions composed of repeats, while molecules without repeat blocks exhibited a high degree of β-sheet, β-turn, and random coil. However, little information is available to indicate that, except for M6 protein, any of these surface proteins are also in a coiled-coil conformation. To attempt to answer this question, an algorithm (Matcher) was developed (31) and used to determine if a seven-residue periodicity also exists in molecules containing α-helical regions. When this algorithm was applied to representative sequences, extended regions of seven-residue periodicity were found in Arp4, FcRA, protein H, PAc, and spaP proteins (Fig. 9). This strongly suggested that these molecules may attain a coiled-coil conformation within these α-helical segments. It is apparent that only proteins that contain sequence repeats and exhibit high α-helical potential show the presence of a seven-residue pattern of hydrophobic amino acids. On the other hand, even though protein G exhibits extensive helix potential, only a limited portion was found to contain the heptad pattern. Interestingly, however, the PAc protein from *Streptococcus mutans* has only one small segment between residues 120 and 520 that displays repeat blocks, and this segment exhibits strong helix potential. This is precisely the region that was also found to contain a regular seven-residue heptad

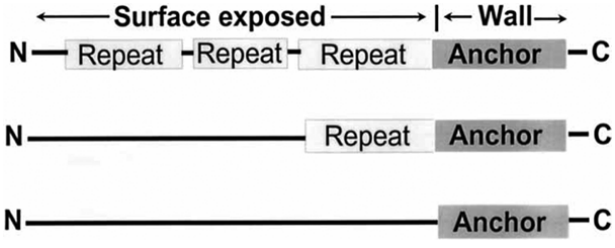


FIGURE 8 Characteristics of C-terminal-anchored surface proteins. These proteins fall into three basic categories: those with multiple repeats, single repeats, or no repeats. Proteins with a single repeat located close to the cell wall are the most common. The anchor is the region of the molecule located within the cell wall carbohydrate and peptidoglycan.

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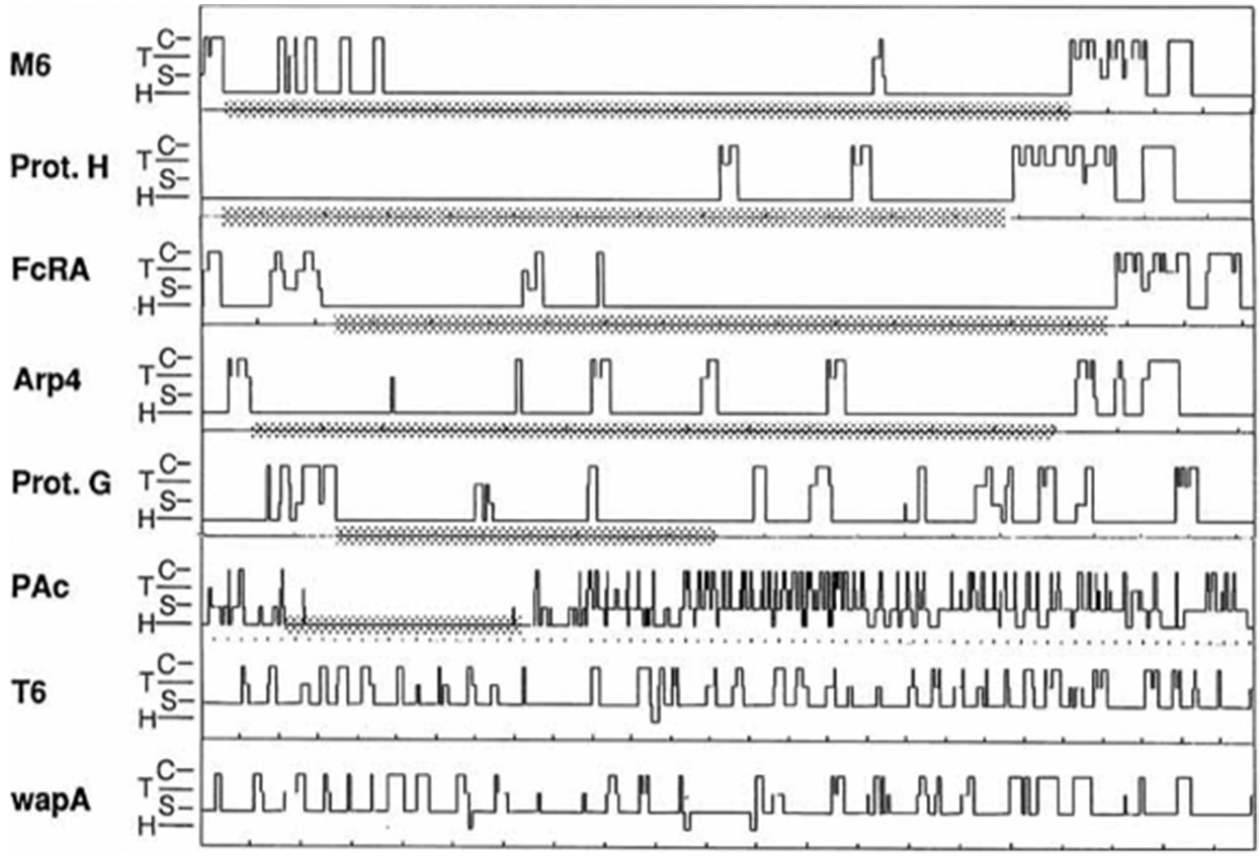


FIGURE 9 Conformational characteristics of the surface molecules from Gram-positive bacteria. The sequences were analyzed by the Garnier-Robson algorithm supplied with the EuGene protein analysis package. The location within these molecules of regions exhibiting random coil (C), β-turn (T), β-sheet (S), and α-helix (H) are designated. Sequences were derived from the references listed in Table 1. Shaded areas are those containing a seven-residue periodicity based on the Matcher program. Proteins T6 and wapA exhibit no extended helical regions or seven-residue periodicity.

pattern. Taken together, these data strongly suggest that the coiled-coil conformation may also be a common characteristic of surface proteins found on Gram-positive organisms.

Size Variation

To fully appreciate the diversity among certain surface proteins on Gram-positive organisms, it is important to understand a property of the M protein molecule, namely, size variation. Using an M protein-specific but broadly cross-reactive monoclonal antibody as a probe, the size of the M protein, extracted by solubilizing the cell wall with phage lysin from a number of streptococcal strains, was examined by Western blotting. M protein derived from 20 different serotypes exhibited variations in size ranging from 41 to 80 kDa in molecular mass, depending on the strain (8). Subsequent sequencing of the M protein genes (*emm*) from other serotypes revealed that though the overall arrangement of the M molecules was the same, differences in the overall size of the repeats and the presence or absence of certain repeat blocks resulted in the observed size variation (see Fig. 4). This variation may be explained by the long reiterated DNA sequence repeats likely to serve as substrates for recombinational events or replicative slippage, generating deletions and duplications within the *emm* gene leading to different sizes of M proteins (52). Based on these specific characteristics, M proteins were grouped into four distinct constructions, called *emm*-patterns A, B, C, and D (53). Interestingly, it was discovered that *emm*-patterns A, B, and C were predominantly associated with pharyngeal infections, and D-pattern strains were associated with skin infections (26, 53).

Sequence analysis of the *emm6* gene from small streptococcal outbreaks (i.e., classroom or school) revealed that they are clonally related and not the result of separate acquisitions of unrelated M6 strains during the course of the infection (data not shown). The observation that size variants occur among separate clinical isolates within the same outbreak suggests that the size mutant constituted the major organism in the streptococcal population at the time of isolation and that a given size mutant has a selective advantage under those clinical conditions (54). Perhaps serological pressure as a result of a local immune response forces the appearance of the size variant.

Although sequence repeats have been reported in several surface proteins on Gram-positive bacteria, size variation has not been reported or tested for all these proteins. However, in addition to M protein, size variation has been observed in PspA from *Streptococcus pneumoniae* (55), HagA from *Porphyromonas gingivalis* (56), protein A from *S. aureus* (44), and Sfb1 from *S. pyogenes* (57).

Multifunctional Proteins

Of the >200 proteins identified on the Gram-positive bacterial surface, the great majority contain domains that bind

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molecules found in body secretions (Table 1), including immunoglobulins (IgA and IgG), albumin, fibronectin, and fibrinogen. Other surface proteins have been identified because of the presence of enzymatic domains. Based on current data, it is clear that the majority of these proteins are multifunctional, and the identified function is limited to only one segment of the molecule. In most cases, the function of the other domains have not been identified. For example, as described above, it appears that many surface proteins contain a repeat region located close to the cell wall and a nonrepetitive segment N-terminal to it (Fig. 8). In most cases the binding domain (e.g., fibronectin, immunoglobulin) has been localized to the repeat segment, while the N-terminal region exhibits no known function. In the case of streptococcal opacity factor, both domains have been defined (58); the repeat region binds fibronectin, while the N-terminal domain has the catalytic function that cleaves ApoA1 of high-density lipoprotein, resulting in the opacity of serum. In the case of highly repetitive proteins such as M protein and protein G, each repeat domain has a specific function. In M protein, the A-repeats bind albumin, mucin, and C4 binding protein, while the B-repeats bind fibrinogen (59–63), and the C-repeats as well as the hypervariable region bind factor H of complement (64, 65). The C repeats also bind keratinocytes (66). In protein G, one repeat domain binds IgG and the second binds albumin. The multifunctional characteristic of surface proteins is not limited to molecules anchored via their C-terminal region. Proteins bound through hydrophobic/charge interactions and N-terminally anchored proteins also exhibit this characteristic (see below).

Thus, in general, surface proteins on most Gram-positive organisms are multifunctional molecules with at least two and in some cases three or more independent functions. Given that up to 25 or more of these surface proteins may ultimately be found on a single cell surface, the potential complexity associated with the bacterial surface becomes enormous.

PROTEINS ANCHORED BY CHARGE AND/OR HYDROPHOBIC INTERACTIONS

Although the great majority of proteins identified on the surface of Gram-positive bacteria anchor through their C termini, a few have been identified to bind through less-defined charge and/or hydrophobic interactions (Table 1).

Surface Glycolytic Enzymes

Through an analysis of the individual proteins identified in a cell wall extract of group A streptococci, it was discovered that some proteins were enzymes normally found in the glycolytic pathway located in the cytosol (67). In all, five enzymes have been identified (triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase [GAPDH], phosphoglycerate kinase, phosphoglycerate mutase, and α-enolase) that form a short contiguous segment of the glycolytic pathway. Interestingly, they are a complex of enzymes involved in the production of ATP as well as other functions (67–70). Thus, given the right substrate, streptococci have the capacity to produce ATP on the cell's surface, which further increases surface complexity. These glycolytic enzymes have been identified on the surface of nearly all streptococcal groups, and certain enzymes have been found on some other pathogens such as *Candida albicans*, trypanosomes, and schistosomes (Table 2). Support for the specific translocation of these enzymes to the cell surface instead of nonspecific release and cell surface attachment through cell lysis comes from the inability to identify cytoplasmic markers or other glycolytic enzymes in the growth medium or on the cell surface.

TABLE 2 Glycolytic enzymes found on the surface of microorganisms

Enyzme[a]	Bacteria	Fungus	Parasite
GAPDH	Streptococci *S. pneumoniae* *S. aureus* *Mycobacterium avium*	*Candida albicans*	Schistosome Trypanosome
PGK	Streptococci	*C. albicans*	Trypanosome
TPI	Streptococci		Trypanosome
PGM	Streptococci		
α-Enolase	Streptococci	*C. albicans*	

[a]GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; TPI, triosphosphate isomerase; PGM, phosphoglycerate mutase.

Two of the best-characterized surface glycolytic enzymes on streptococci are GAPDH and α-enolase (66, 68). Like other surface proteins on Gram-positive bacteria, GAPDH is a multifunctional protein with binding affinities for fibronectin and lysozyme as well as for cytoskeletal proteins such as actin and myosin. GAPDH has also been shown to have ADP-ribosylating activity in addition to its GAPDH activity. α-Enolase is also multifunctional in its ability to specifically bind plasmin(ogen) (71). It is the major plasmin-binding protein for streptococci. Evidence has shown that there is one gene for each of these enzymes, which are produced in the cytosol. A proportion (roughly 30% to 40%) is then translocated to the surface. Because these molecules are not synthesized with an N-terminal signal sequence, it is unclear how they are transported to the cell surface. Also, because they do not contain an apparent anchor motif, it is not known how they are attached to the cell surface. Because they can be removed with chaotropic agents, it is reasonable to assume that they are bound through charge and/or hydrophobic interactions (72). Pancholi and coworkers (97) produced a GAPDH mutant that could not be secreted to determine the importance of this molecule's export to the cell surface. Results revealed downregulation of most genes involved in *S. pyogenes* virulence and genes belonging to carbohydrate and amino acid metabolism and upregulation of those related to lipid metabolism. This mutant also affected virulence in a mouse model, indicating that the GAPDH export process is also involved in regulating *S. pyogenes* virulence.

Other Proteins

PspA, a surface protein and a virulence determinant for *S. pneumoniae* (73), has many of the structural characteristics found in M protein, such as sequence repeats and α-helical coiled-coil conformation, but it does not contain a C-terminal-anchor motif. It has been shown that after surface translocation, PspA anchoring requires charge interactions between the membrane-associated choline-containing lipoteichoic acid and the C-terminal repeat region of the PspA molecule (74) (Fig. 1).

N-TERMINAL-ANCHORED LIPOPROTEINS

Although lipoproteins (proteins containing a lipid covalently linked to an N-terminal cysteine) have been described

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in Gram-negative bacteria, they have also been identified in Gram-positive bacteria (for reviews, see references 75 and 76). While most are predicted to be lipoproteins by genetic analysis based on the consensus sequence for lipoproteins (77), they usually comprise molecules that are not surface-exposed but are located between the cytoplasmic membrane and the peptidoglycan, equivalent to the periplasm of Gram-negative bacteria. These proteins are involved in transport systems such as the *mal* and *ami* operons of *S. pneumoniae* and the Msm system of *S. mutans*. A few of these lipoproteins have been identified by their ability to act as adhesins, allowing these organisms to adhere to a variety of substrates. SsaB, a lipoprotein from *Streptococcus sanguis* that shares sequence homology with other streptococcal adhesins, has been implicated in interaction with a salivary receptor and the coaggregation with *Actinomyces naeslundii*. Many of these so-called adhesins have also been found to participate in various transport systems for the streptococcus. Lipoproteins have also been implicated in the spore cell cycle of *Bacillus subtilis* (78), conjugation in *Enterococcus faecalis* (79), and enzymatic functions (80, 81).

Thus, it is expected that these proteins are located predominantly on the surface of the cell membrane, with the majority of the molecule located within the peptidoglycan (Fig. 1). In some cases certain domains would be surface exposed, allowing them to function as adhesins or even surface enzymes. The *Mycobacterium tuberculosis* cell wall consists predominantly of mycolic acids, which is atypical, but its hydrophobicity could serve as an anchoring point for lipoproteins (82).

CONCLUSION

Based on current information, the surface of the Gram-positive bacterial cell wall is highly complex (Fig. 10) and could even be considered to be a distinct organelle because it is composed of proteins with specific binding functions and enzymatic activity combined with the ability to generate energy. Considering the fact that there could be more than 25 different proteins on a given bacterial cell surface (83), each with the potential of up to three functional domains, more than 75 independent activities could potentially be present on the cell surface, much more than had ever been previously anticipated.

Because extensive cytoplasmic domains are not present within the C-terminal region of surface proteins thus far identified in Gram-positive bacteria, it is unlikely that the binding of these molecules to specific ligands induces a cytoplasmic signal to activate a gene product. It is more likely that binding initiates a conformational signal on the bacterial cell surface to perform a specific function. For example, streptococci usually infect the pharynx, particularly the tonsils, through contact with contaminated saliva. Upon entering the oral cavity, the organism first encounters the mucus that coats the mucosal epithelium. Not being a motile organism, it must navigate the mucus to reach the tonsilar epithelial surface to initiate infection. Soluble components found in the mucus, such as IgA, IgG, albumin, fibronectin, etc., are able to interact with their respective binding proteins on the streptococcal surface. This binding may initiate a set of conformational events within these proteins to drive the organism through the mucus to the epithelial surface. Perhaps the energy required for this is derived from the surface glycolytic enzymes necessary to generate ATP, or perhaps an enzyme is activated to solubilize the mucin. This must all occur quickly, or the organism will be swept into the gut and eliminated by ciliary action. Thus, the molecules necessary to initiate infection are poised and ready on the bacterial surface. Therefore, before arriving on the mucosal epithelium, the organism may be considered an inert particle with a wide array of surface proteins capable of driving the organism to the mucosal surface. When stable contact is made with the epithelial surface, the organism has time to initiate the infection process. Attempting to sort out when and how these proteins function during the infection process will be the challenge for future studies. With this understanding, however, new strategies could be developed in controlling diseases caused by this class of bacteria.

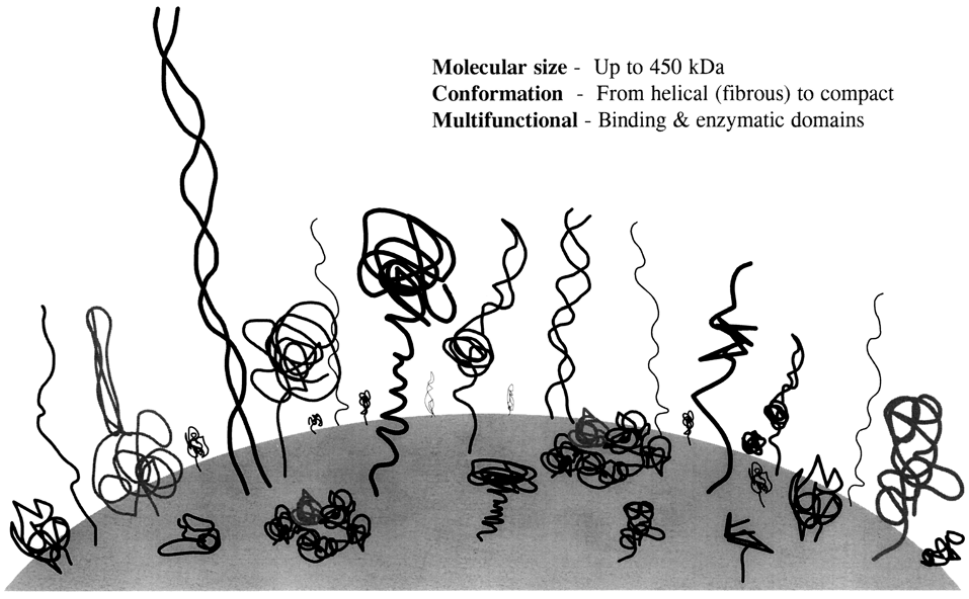


FIGURE 10 Appearance of the surface of Gram-positive bacteria exhibiting a wide array of protein molecules. Each molecule depicted may have thousands of identical copies densely packed on the surface.

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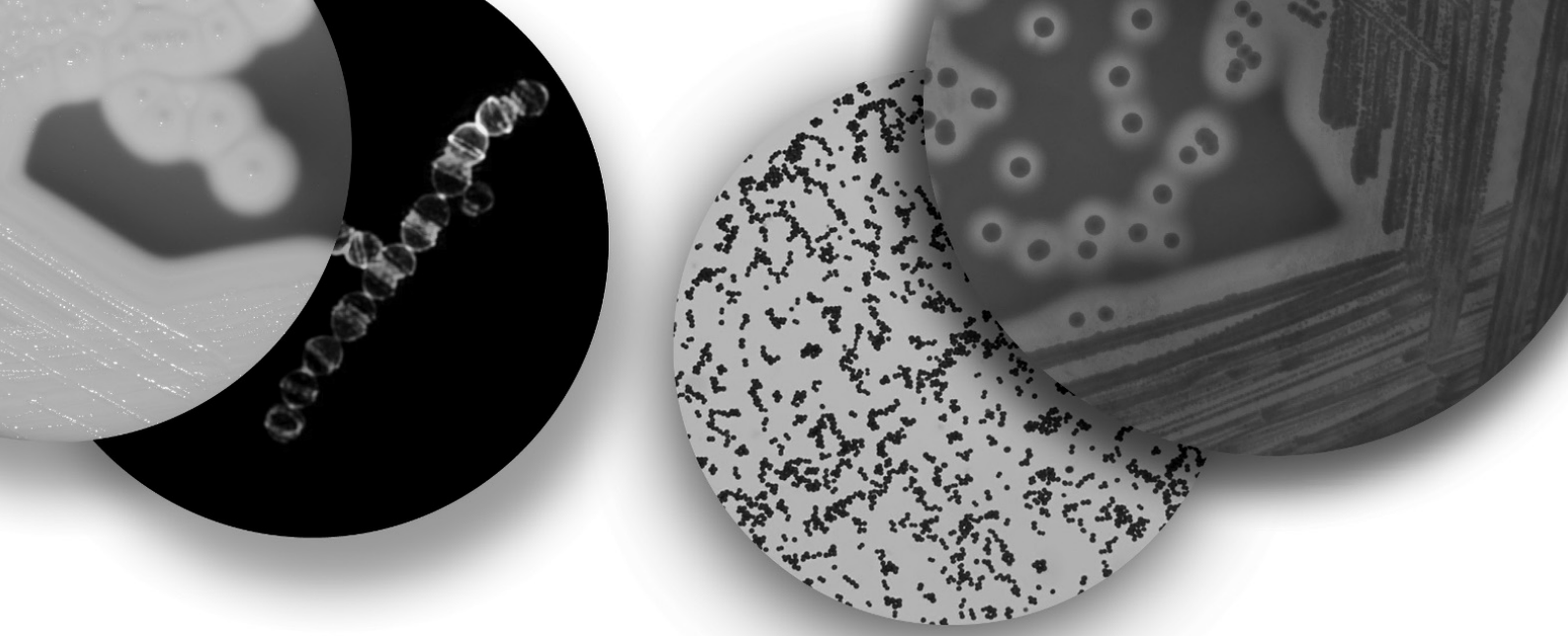
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SECTION 2

The Streptococcus

Section Editors: **Vincent A. Fischetti and Joseph J. Ferretti**

As a species, the streptococci are a diverse group, ranging from commensal organisms which occupy various niches of the body to pathogens having the capacity to infect a wide range of tissue sites. They are also diverse in their resistance to current antibiotic therapy, from the multi resistant enterococci to the barely resistant *S. pyogenes*. It is thus not surprising to find, when looking back over the past century of research on pathogenic organisms, that the streptococcus appears as one of the best studied of the gram-positive pathogens.

Although published results have steadily increased during the first three quarters of this period, the introduction of molecular techniques in the latter quarter of the century has seen a nearly logarithmic increase in new information. Despite this wealth of knowledge, however, numerous questions still remain unanswered. The first part of the century relied on the biology of the organism to answer questions of pathogenesis; in the latter part, however, molecular techniques overshadowed the biology in a

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mad scramble to understand the genetic complexity of these bacteria. Since the last edition, the complete genome sequences of eleven different species of streptococci have been completed. Analyses of multiple genome strains in the same species have shown that significant differences may occur, even in the same species. A common feature of all the streptococci is the presence of horizontal gene transfer and recombination systems, allowing for enhanced survival of an organism in its ecological niche, often by the emergence of new strains with increased virulence properties.

The chapters to follow are the best of a mixture of genetics, biology, and immunology designed to both ask and answer questions pertaining to the pathogenicity of the streptococci. The information gained from these studies and the ideas they stimulate for future experiments will allow the development of new tools not only to prevent infection by these organisms specifically but perhaps by gram-positive bacteria in general.

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Intracellular Invasion by *Streptococcus pyogenes*: Invasins, Host Receptors, and Relevance to Human Disease

BEINAN WANG AND P. PATRICK CLEARY

3

Streptococcus pyogenes has evolved a variety of both surface-bound and extracellular factors that alter the inflammatory response and impair phagocytic clearance of the bacteria. The more than 150 genotypes use both similar and different strategies at the biochemical level to colonize their host and avoid protective defenses. These are reviewed elsewhere (5). Intracellular invasion is dependent on at least two classes of surface proteins, the M proteins (6, 7) and fibronectin (Fn)-binding proteins (3, 8). The M proteins serve many functions in the pathogenesis of *S. pyogenes*, including resistance to phagocytosis, adherence, and intracellular invasion (5, 9). The Fn-binding protein F (PrtF) and the allelic variant SfbI are also adhesins and invasins, produced by 50 to 60% of the M genotypes. The function of these proteins in the context of intracellular invasion is described below.

IMPACT OF INTRACELLULAR INVASION ON *S. PYOGENES* INFECTION

Many geographic and temporal clusters of sepsis and toxic shock, caused by a few serotypes of *S. pyogenes*, have been reported (10, 11). An M1 subclone was associated with many such clusters (10, 12). Surprisingly, devastating soft tissue infections were often reported in patients who had not experienced previous trauma or wounds that might initiate systemic spread of the organism (11). Cleary and coworkers (2, 13) considered the possibility that intracellular invasion of mucosal surfaces provides a window for streptococci to reach deeper tissue, even the bloodstream. Indeed, a globally disseminated M1 subclone invaded A549 epithelial cells at significantly higher frequency than did other subclones of M1 streptococci. The source of M1 subclones, i.e., uncomplicated disease or more invasive disease (blood isolated), did not correlate with efficiency of ingestion by epithelial cells (13). Only the M1 subclone that had acquired 70 kb of DNA sequence from two distinct prophages (13) was associated with high-frequency invasion of cultured cells. To date, no relationship to prophages and intracellular invasion has been discovered.

Although an association between high-frequency intracellular invasion and systemic streptococcal disease is still uncertain, the intracellular state may significantly increase the capacity of this bacterium to disseminate in human populations. When strains from carriers and patients with uncomplicated pharyngitis and sepsis were compared, those from carriers were observed to be internalized by HEp2 cells at the highest frequency (14). A highly variable M1 culture could be enriched for more invasive streptococci by *in vitro* serial passage through human epithelial cells (15); therefore, *in vivo* cycling of streptococci between the interior and exterior of the mucosal epithelium may select for variants that are more efficiently internalized. From 10 to 40% of children continue to shed streptococci after treatment with penicillin (16). Brandt et al. reported 80% relapse, by the same initial strain following vigorous penicillin therapy, when 40 consecutive isolates from 18 patients were characterized (17). Treatment failures and subsequent carriage of streptococci were reported to be more common when the infecting strain produced PrtF (also termed SfbI), an invasin that promotes high-frequency ingestion of streptococci by epithelial cells (18). This correlation was not, however, confirmed by a smaller study (17). *In vitro*, intracellular *S. pyogenes* can resist at least 100 μg/ml of penicillin (Cleary, P. unpublished data); therefore, penicillin treatment may further select for strains that can be efficiently internalized by epithelial cells. High-frequency intracellular invasion may increase the rate of antibiotic therapy failure and therefore increase the size of the human reservoir that can disseminate the organism to others in the population. As the reservoir enlarges, the probability of serious, systemic infection also increases. Thus, strains or serotypes that are less able to acquire an antibiotic-free niche may be less often associated with severe disease. Facinelli and colleagues observed an association between genetic resistance to erythromycin and more efficient uptake of *S. pyogenes* by epithelial cells (19). They suggested the frightening possibility that erythromycin resistance had become genetically linked to efficient invasion of human cells and thus coselected by antibiotic therapy. A genetic link was not established, however, nor did the authors exclude the possibility that a single subclone had disseminated in the study population. A thorough analysis of a cluster of toxic shock in southern Minnesota showed that nearly 40% of the school children in nearby communities

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were carriers of a serotype M3 clone that was responsible for systemic disease in adults (20). These investigators suggested that school children served as the reservoir for *S. pyogenes* that produced toxic shock in adults with underlying physical disabilities.

ARE TONSILS A RESERVOIR FOR *S. PYOGENES*?

The best and most direct evidence that intracellular bacteria are an important source for dissemination of streptococci and the cause of recurrent tonsillitis is based on microscopic studies of surgically excised tonsils (16). This study found that 13 of 14 tonsils from children plagued by recurrent tonsillitis harbored streptococci within epithelial cells in the tonsillar crypts. *S. pyogenes* was also observed in macrophage-like cells at high frequency in these specimens. Tonsils from control subjects who had tonsils removed for other reasons did not contain streptococci. Intracellular streptococci were also observed in cultured tonsils infected with *S. pyogenes* (21). The invasive M1 subclone was confirmed *in vitro* to efficiently invade primary keratinized tonsillar epithelial cells (22). In a murine intranasal infection model this M1 subclone and an M49 strain were shown to specifically invade nasal-associated lymphoid tissue, tissue known to be functionally homologous to human tonsils (23). Initially, streptococci were associated with M-like cells, sporadically located along the base of the nasal epithelium, and by 24 h postinoculation had formed microcolonies throughout the lymphoid tissue. From 1 to 10% of the streptococci, depending on the mouse, were intracellular. This study suggests that M-like cells are the primary portals for transport of *S. pyogenes* across the nasal mucosa into lymphoid tissue. Subsequent studies also demonstrated that nasal-associated lymphoid tissue is the initial, primary location for antigen-specific T cell priming and activation following intranasal infection by *S. pyogenes* (24, 25).

S. PYOGENES INVASINS AND HOST RECEPTORS

The intracellular state has many advantages for a bacterium's survival in a host, such as evading phagocytic killing, escaping neutralization by antibodies, and avoiding antibiotics. In many respects *S. pyogenes*' invasion of human cells is analogous to that of *Yersinia pseudotuberculosis*, which involves a bacterial outer membrane protein, InvA, binding to integrins (26). Integrins are a mammalian family of heterodimeric, transmembrane glycoproteins that bind to cell-secreted extracellular matrix (ECM) proteins, a collection of extracellular molecules, such as collagen, Fn and laminin (Ln), secreted by mammalian cells (27). Engagement of integrins by ECM proteins results in activation of host signal transduction pathways that lead to rearrangements of host cell cytoskeleton, cell membrane movement, and other cellular activities (28). Bacterial invasins are a subclass of bacterial adhesin molecule required for ingestion by host cells. Typically, invasins are proteins expressed on the surfaces of bacterial cells that directly or indirectly recognize specific host cell receptors (26). In general, invasins are capable of inducing reorganization of the host cell's cytoskeleton by generating specific signals from receptors with which they interact. Integrins are often exploited for microbial entry into mammalian cells. This may be due to their ubiquitous expression and their ability to affect cytoskeletal arrangement (27).

S. pyogenes has evolved multiple surface molecules that bind to various ECM proteins, which tether them to and activate integrin signaling pathways required for invasion. In addition, this bacterium has also evolved multiple mechanisms for invasion of a wide variety of mammalian cells. The host cell receptors, bacterial ligands, and biochemical pathways vary, depending on the strain of streptococcus and host cell type, adding another layer of complexity (6).

Streptococcal adhesins are a variety of surface molecules which can be grouped into four families, depending on their association with their surfaces: (i) those that are covalently linked by their C-terminus to the cell wall peptidoglycan through an LPxTz motif, (ii) those that are tethered to the bacterial cell membrane through N-terminal modifications of lipids to form lipoproteins, (iii) those that are bound to the bacterial surface by noncovalent interactions, and (iv) those that are expressed and retained on the surface by an as yet unknown mechanism. The most prominent adhesins belong to the family of cell wall-anchored proteins that are covalently linked to the peptidoglycan by a membrane-associated transpeptidase called sortase A (29).

ZIPPER AND OTHER MECHANISMS OF STREPTOCOCCAL INGESTION

Cytoskeletal events accompanying the uptake of a variety of pathogenic bacteria have been studied. Ruffling of the membrane precedes the entry of *Shigella* and *Salmonella* spp. (26). In contrast, internalization by a zipper mechanism is mediated by interactions between surface invasins, ligands, and host cell receptors. These interactions lead to pseudopod formation, which requires the extension of actin filaments beneath the host cell membrane (7).

Scanning electron microscopy revealed that adherent $M1^+$ bacteria are often in close contact with host cell microvilli, suggesting that this association may be an initial step in internalization (Fig. 1A). Microvilli frequently extend across bacterial surfaces and appear to entrap streptococci. Microvilli increase in number near adherent streptococci and appear to morphologically expand into pseudopodia-like structures, although these structures may arise independently (Fig. 1B). Membrane engulfment of streptococci suggests a zipper mechanism of uptake, as shown in Fig. 1C. The M1 strain of streptococcus, like *Listeria* and *Yersinia* spp., can be internalized by this zipper-like mechanism (7, 26). In contrast to $M1^+$ streptococci, $M1^-$ bacteria are rarely observed to interact with microvilli, and no pseudopodia-like structures were seen (7). *De novo* actin polymerization is essential for uptake of these streptococci, whether M^+ or M^- strains, because invasion is blocked by cytochalasin D, an inhibitor of actin polymerization (2). Confocal immunofluorescent microscopy confirmed that vacuoles containing internalized streptococci are surrounded by polymerized actin. Actin is occasionally associated with membrane-spanning chains and seems to disappear soon after entry.

Other microscopic studies demonstrated that physical events leading to ingestion of *S. pyogenes* vary dramatically between strains and serotypes (30, 31). Strain A40 induces the accumulation of small-membrane omega-shaped cavities in the host cell membrane near adherent streptococci. Shortly thereafter, membrane invaginations are formed into which streptococci enter. The integrity of the membrane is not disrupted, and streptococci end up in a cytoplasmic vacuole. These cell membrane changes are dependent on Fn and $\alpha_5\beta_1$ integrins, but focal adhesion complexes were not observed to contain polymerized actin (30). Further studies showed that these membrane structures are caveolae, structures previously found to mediate entry of both viruses and parasites into cells. In contrast, invasion of HEp2 cells by

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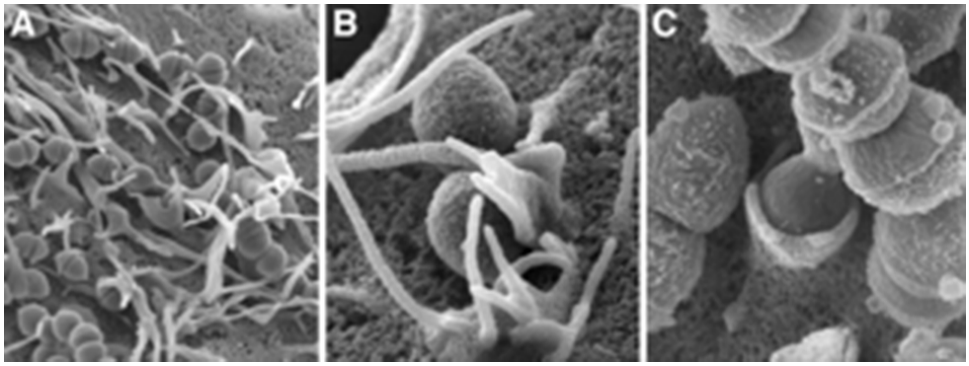


FIGURE 1 Scanning electron micrographs showing different stages of streptococci-HeLa cell interactions. (A) A high density of microvilli surrounding and in contact with adherent streptococci. (B) A common morphological change that microvilli undergo. The cup-like structure appears to gradually engulf the bacteria and ultimately pull them into a vacuole. (C) A streptococcal chain has been partially ingested by a mechanism that morphologically resembles a receptor-ligand interaction. It is not known whether microvilli are involved in the latter or whether streptococci are ingested by two physically different cytoskeletal rearrangements.

strain A8 (M40 Sfb1$^-$) was not inhibited by an Fn peptide analogue, nor by anti-$\alpha_5\beta_1$ antibody. This strain induced dramatic aggregation of microvilli near and around attached streptococci, and ingestion of this strain was independent of caveolae and lipid rafts. However, invasion of HEp2 and A549 epithelial cells by the M1 strain is dependent on Fn and $\alpha_5\beta_1$ integrins (22, 32). Comparison of strains A40 (M12 Sfb1$^+$) with A8 (M40 Sfb1$^-$) revealed striking differences. It is unclear whether these differences reflect yet another mechanism of streptococcal uptake or merely depend on different experimental conditions. Irrespective of differences, this study further implicated participation of microvilli in the uptake of streptococci by epithelial cells.

M PROTEINS AND OTHER Fn-BINDING PROTEINS ARE INVASINS

S. pyogenes expresses an array of cell surface proteins that facilitate bacterial colonization of a variety of human tissues (33). Some are adhesins that also function as invasins. The proteins SfbI and F1 (PrtFI) are closely related streptococcal adhesins that bind Fn with high affinity. Approximately 50% of *S. pyogenes* isolates carry the gene encoding SfbI/PrtFI, although many do not express the gene under laboratory conditions (3, 14, 18). Prebinding of soluble Fn to PrtFI-bearing streptococci can promote bacterial adherence to cultured cells and dermal tissue. This and experimental evidence from several laboratories are consistent with SfbI/PrtFI promoting intracellular invasion via a similar mechanism.

Molinari et al. (3) demonstrated that SfbI can mediate invasion of HEp2 cells. Streptococcal invasion can be ablated by antiserum raised against SfbI or by preincubation of host cells with recombinant SfbI. Latex beads coated with recombinant SfbI readily adhere to and are efficiently ingested by epithelial cells, demonstrating that the interaction of SfbI with host cells is sufficient for internalization. Fn binding by SfbI is required for invasion, because beads coated with a recombinant SfbI peptide lacking the Fn-binding domains do not efficiently adhere to HEp2 cells. Ozeri et al. (8) reported that invasion of HeLa cells by PrtFI$^+$ bacteria is dependent on addition of serum or purified Fn. In both studies, antibodies against Fn inhibited invasion. Also, PrtFI peptides, containing at least one Fn-binding domain, were found to inhibit PrtFI-mediated invasion. PrtFI binds to a 70-kDa N-terminal fragment of Fn. This region of Fn differs from the region bound by epithelial cells. The 70-kDa fragment can inhibit Fn-mediated invasion by competing with intact Fn for PrtFI binding; however, only intact Fn molecules are capable of supporting bacterial invasion (8). Antibody directed against the integrin $\beta1$ subunit specifically blocks Fn-PrtF1-mediated invasion of HeLa cells (8). This result suggests that one or more $\beta1$-containing integrins are involved in bacterial internalization. In contrast, invasion of GD25 (embryonic mouse stem) cells appears to be mediated by integrin $\alpha v\beta3$ (the major Fn-binding integrin of this cell line), since invasion is inhibited by a peptide that specifically blocks Fn binding to this integrin (8). Collectively, these results demonstrate that Fn functions as a molecular bridge between SfbI/PrtFI and host cell Fn receptors. At least 11 Fb-binding proteins of *S. pyogenes* have been identified (34), indicating that *S. pyogenes* has evolved multiple invasins to utilize the Fn-integrin mechanism for internalization of host cells.

Expression of Sfb1/PrtF1 is not a prerequisite for high-frequency intracellular invasion. For example, serotype M1 strains typically lack the gene encoding SfbI/PrtFI, but some can invade human epithelial cells with high efficiency (13, 18). Invasion of cultured cells by the globally disseminated M1 strain 90-226 is primarily dependent on expression of M1 protein. Inactivation of the M1 protein gene, *emm1*, decreased invasion of HeLa, A549, and HEp2 cells by 50-fold (7, 32), and latex beads coated with M1 are readily ingested by HeLa cells (7). These results indicate that invasion of epithelial cells by strain 90-226 is mediated in large part by M1 protein.

Expression of M1 protein is not sufficient for invasion, however, because bacterial internalization is dependent on exogenous serum, the ECM proteins, Fn or Ln (32). M1protein appears to be the major Fn-binding protein expressed by strain 90-226, because inactivation of *emm1* reduces binding by 88%. Also, purified M1 protein can bind Fn *in vitro* (32). This protein is also able to bind collagen VI, an ECM molecule that is distributed underneath the respiratory epithelium in the upper and lower airways (35). However, this is not a general property of all M proteins; only three, M1, M6, and M3 proteins are known to bind Fn (32, 36; Cleary P. unpublished data). Interestingly, M3 protein was also found to promote serum-dependent invasion of HEp2 cells (6).

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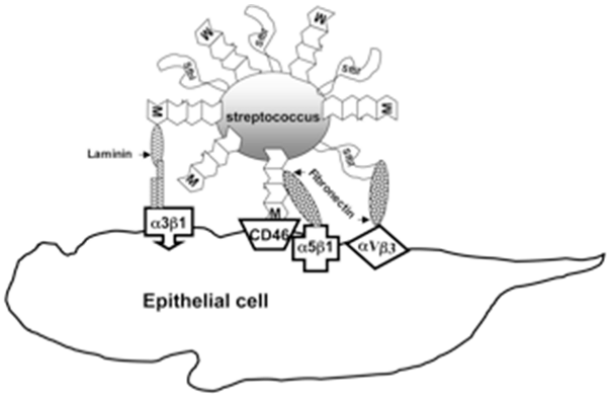


FIGURE 2 The various agonist and cellular receptors which streptococci commandeer to promote their own phagocytosis by epithelial cells. Both the M protein and high-affinity fibronectin proteins SfbI/PrtF1 are known invasins which depend on formation of a fibronectin bridge between the bacterial surface and integrin receptors α5β1 or αVβ3. Although less well studied, laminin can also serve as an agonist for ingestion of streptococci by linking them to α3β1 integrins. For some strains fibronectin-mediated invasion of epithelial cells also requires interaction between M protein and the complement regulatory protein CD46.

In the presence of Fn, internalization of M1 streptococci is dependent on the interaction of M1, Fn, and the epithelial cell Fn receptor α5β1 (Fig. 2). A monoclonal antibody that specifically blocks Fn binding to this integrin can ablate invasion of A549, HeLa, and human tonsillar epithelial cells (22, 32). Low-molecular-weight nonpeptidyl α5β1 antagonists are also effective inhibitors of invasion of A549 and human tonsillar epithelial cells (22). The inhibitory effects of α5β1 antagonists are not due to generalized effects on either bacterial or host cells, because the inhibitors do not abrogate Ln-mediated invasion. Rather, the inhibitory effects of α5β1 antagonists are observed only when bacteria are exposed to either serum or purified Fn. Thus, as in the case of Sfb1/PrtF1$^+$ strains, Fn appears to function as a bridging molecule in promoting invasion by M1$^+$ bacteria. Ln can replace Fn as the agonist in invasion assays (Fig. 2). Failure of Ln to promote invasion of A549 cells by M$^-$ mutants suggests that this ECM protein binds sufficiently to M1 protein to serve as a bridge between streptococci and α3β1 integrins.

The invasion efficiencies of M1 strains are not dictated solely by the presence of invasion agonists. LaPenta et al. (2) and Cleary et al. (15) reported that M1 isolates exhibit widely varying invasion efficiencies, even when experiments are performed in the presence of serum. These variations are likely due, at least in part, to varying levels of M1 protein expression. Transcription of *emm1* can vary considerably between isolates and even between different cultures of the same isolate (15). While this possibility has yet to be thoroughly investigated, it is consistent with the finding of Ozeri et al. (8) that the number of integrin-binding sites on bacteria can greatly affect invasion efficiency. Expression of different M proteins, M6, M3, or M18, in a common streptococcal background demonstrated that M proteins can differ widely in their capacity to promote ingestion of streptococci. Serum and the cellular receptor CD46 (Fig. 2) were required for uptake of streptococci that expressed M3 protein, whereas neither were required when the recombinant expressed M6 protein (6). Efficient invasion of A549 cells by strain 90-226 also requires that the M1 protein engage both CD46 and, indirectly, α5β1 integrins (37). Thus, it appears that M proteins of different serotypes often recognize different receptors on surfaces of mammalian cells. Berkower's results suggest that the invasion potential is actually determined by the primary amino acid sequence of the respective M protein (6). While it is clear that several types of M protein can facilitate invasion by *S. pyogenes*, there is no single mechanism underlying M protein-mediated ingestion of streptococci.

STREPTOCOCCAL FOCAL ADHESION AND SIGNAL TRANSDUCTION

The complex networks of intracellular events that lead to ingestion of several microbial pathogens have begun to be unraveled. Studies have not only identified potential targets for therapeutics but in some cases revealed insight into basic cell biology questions. Uptake by host cells requires streptococci to manipulate normal outside-inside signals spawned from cellular receptors. Interactions of Fn-binding-proteins and integrins trigger a cascade of signals that cause cytoskeleton rearrangement leading to ingestion of streptococci by nonphagocytic epithelial cells. Analyses of streptococci-induced signaling pathways have focused on two major invasins and Fn-binding proteins, SfbI/PrtF1 and M1 protein. Ozeri et al. reported that infection of epithelial cells with an M6 SfbI/PrtF1$^+$ strain caused integrins to be recruited to sites of bacterial adhesion and colocalization of phosphorylated focal adhesion kinase (FAK) and paxillin, common signaling and structural components of focal adhesion complexes (38). Invasion of epithelial cells by the M6 strain was significantly reduced when host cells were pretreated with genistein, a general inhibitor of protein tyrosine kinases, such as Src kinases and FAK. Invasion was also reduced by inhibitors of the small GTPases Rac and Cdc42. Moreover, internalization of this strain by FAK$^{-/-}$ and Src$^{-/-}$ cells and cells expressing dominant negative forms of Rac1 or Cdc42 was less efficient. From these findings, Ozeri et al. proposed a model for SfbI/PrtF1-mediated *S. pyogenes* invasion which is incorporated into Fig. 3: Fn bound to surface SfbI/PrtF1 protein associates with integrins to cause integrin clustering, followed by recruitment of paxillin, FAK, and other focal adhesion proteins at the site of entry. This initiates autophosphorylation of FAK, which creates a docking site for Src kinases. Paxillin present in this complex and additional sites on FAK is then phosphorylated by Src. Ozeri et al. proposed that Rac and Cdc42 are required to orchestrate signaling events initiated by Fn bound to SfbI/PrtF1 protein. Then the focal adhesion complex provides an anchor for actin polymerization and cytoskeleton rearrangements required for internalization of streptococci. To date, however, the relationship between integrin-generated signals, formation of focal adhesion complex, and Rac and Cdc42 is poorly understood and is unsupported experimentally. It is also possible that streptococci interact with additional receptors which in turn activate other mechanisms of signaling and pathways of ingestion.

M1$^+$ strains of *S. pyogenes* invade epithelial cells efficiently without expression of SfbI/PrtF1 protein. M1-mediated invasion of epithelial cells was shown to require phosphatidylinositol 3-kinase (PI3K) (39), a heterodimer consisting of the regulatory subunit p85 and the catalytic subunit p110. Activation of PI3K catalyzes phosphorylation of membrane-associated phosphatidylinositol, which binds to downstream targets, including integrin-linked kinase (ILK) (40). Experiments that disrupted activity found that ILK is also required for *S. pyogenes* invasion of HEp2 cells (41). ILK is known to be a crucial link between integrins and the cytoskeleton, is capable of phosphorylating the β_1 integrin cytoplasmic domain, and can be coimmunoprecipitated with β_1 integrin

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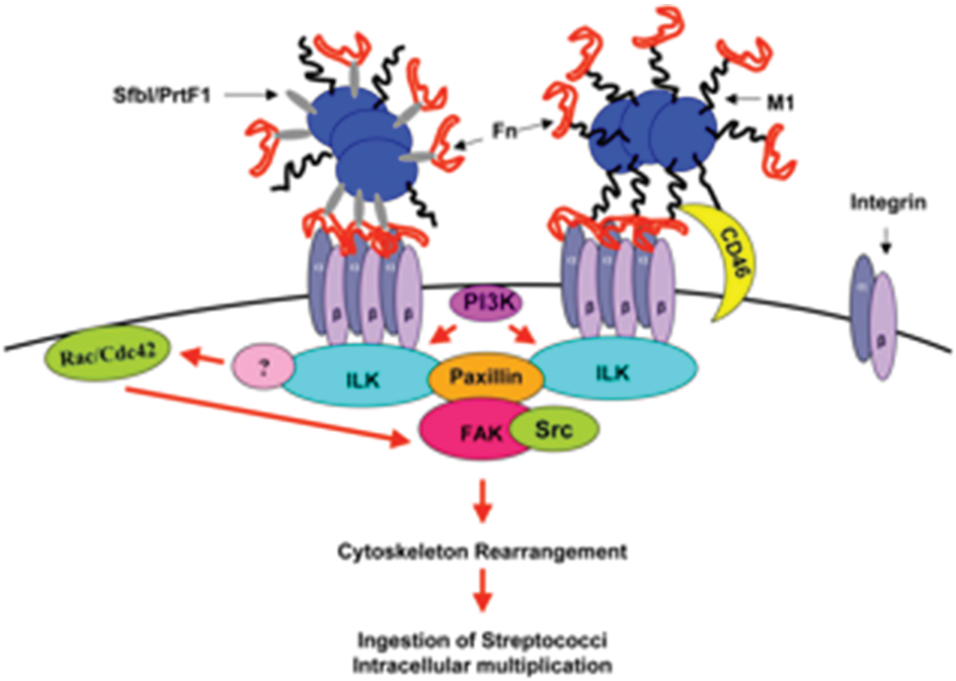


FIGURE 3 Postulated and confirmed components of streptococci-induced focal adhesion complexes and signaling molecules required for formation of those complexes and subsequent cytoskeletal changes that lead to ingestion of streptococci by epithelial cells. Fibronectin (Fn) bound to surface M1 or SfbI/PrtF1 proteins associates with integrins to cause integrin clustering leading to ILK activation in a PI3K-dependent manner. For some strains contact between M protein and CD46 is also required for high-frequency invasion. The function of CD46 in this process, however, is unknown. Following recruitment of paxillin, FAK, and other focal adhesion proteins, autophosphorylation of FAK creates a docking site for Src kinases to phosphorylate recruited paxillin and additional sites on FAK. Alternatively, Rac and Cdc42 are activated by ILK through an intermediate to participate in formation of focal adhesion complexes, which provide an anchor for actin polymerization and cytoskeleton rearrangement that ultimately results in uptake of *S. pyogenes*.

in lysates of mammalian cells (42). Invasion of epithelial cells by $M1^+$ group A streptococci was impaired up to 80% by a specific ILK inhibitor. Inhibition of ILK expression with small interfering RNA or inhibition of activity by transfection with a kinase-inactive ILK gene also significantly reduced GAS invasion. Controls, assays of the non-integrin-dependent invasion of epithelial cells by *Salmonella*, confirmed that inhibitors of either PI3K or ILK did not have a general negative impact on the host cell's ability to ingest bacteria but were specific for Fn-mediated uptake of *S. pyogenes*. ILK is known to be activated upon integrin engagement in a PI3K-dependent manner and to require that its pleckstrin homology-like domain be bound to phosphatidylinositol 3,4,5-triphosphate, a product of PI3K. Therefore, it is possible that PI3K indirectly provides the cytoplasmic membrane anchor for ILK.

Invasion of epithelial cells by M6 $SfbI/PrtF1^+$ streptococcus was also reduced 70 to ~80% by inhibitors of PI3K and ILK. In addition, interaction of M49 streptococci with plasminogen facilitates integrin-mediated and ILK-dependent internalization of bacteria into keratinocytes (43), also suggesting that ILK is a critical signal component involved in integrin-mediated invasion. This is consistent with the possibility that ILK indirectly activates Rac and Cdc42 (44, 45), which can regulate actin cytoskeleton rearrangement (46, 47), leading to various forms of cell adhesion complexes and stress fibers (41). Therefore, ILK may be an intermediate between integrins and Rac and/or Cdc42. Although M1-Fn and SfbI/PrtF1-Fn complexes may induce cellular changes by shared mechanisms, M1-mediated invasion is significantly less sensitive to genistein (41), again indicating that different mechanisms of signaling likely exist.

Paxillin, a cellular signaling molecule involved in cytoskeleton rearrangement downstream of ILK, is phosphorylated in response to Fn-bound streptococci that express either M1 or PrtF1/SfbI protein but is not phosphorylated in response to a mutant streptococcus deficient in both proteins. Inhibition of paxillin phosphorylation significantly reduced invasion by $M1^+$ but did not affect ingestion of $PrtF1/SfbI^+$ strains, indicating that paxillin phosphorylation is induced by both invasins but only required for M1-mediated invasion (48). M3 streptococci lack the major epithelial invasins SfbI/PrtF1 and M1 protein but have the potential to cause invasive disease. Experiments demonstrate that Src kinase and Rac1 but not PI3Ks are essential for internalization of these streptococci (49). There appears to be a bifurcation point downstream of ILK leading to PI3K or Src/Rac1, and it appears that *S. pyogenes* has evolved different invasins, which utilize various cellular signaling pathways to promote ingestion, pathways that lead to the distinct morphological changes observed in electron microscopic studies.

M1 protein can interact with multiple cellular receptors, either directly or indirectly (Fig. 2). For example, CD46, a cofactor in factor I-mediated inactivation of complement

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proteins C3b and C4b (50) and expressed on the surface of all nucleated human cells, enhances invasion of epithelial cells by $M3^+$ and $M1^+$ streptococci (6, 37). Overexpression of a mutant form of CD46 with a deletion in the cytoplasmic domain results in partial reduction of invasion. Feito et al. analyzed CD46 binding to clinical isolates of *S. pyogenes* using a soluble construct encompassing the extracellular domain and showed strong binding to M types 1, 3, 8, 18, 24, 28, 29, 31, and 78, weak binding to M6 and M29 strains, and no binding to M types 11, 12, 27, or 30. Although only a limited number of strains have been studied for the interactions between CD46 and streptococci, it is likely that the well-conserved C repeats of M proteins expressed by these different clinical isolates also contain the CD46-binding sites (51). CD46's role in intracellular signaling and identification of its docking partners are poorly defined; however, these preliminary studies suggest that M1 protein-mediated ingestion of *S. pyogenes* requires costimulation from two cellular receptors (37).

Diverse invasins, agonists, and cellular receptors predict that the signaling networks which lead to ingestion of streptococci by epithelial cells may also be varied and multiple. Although our knowledge of the signaling mechanisms responsible for ingestion of *S. pyogenes* is rudimentary, Fig. 3 attempts to interpret what is known. Association of multimeric Fn-binding protein-Fn complexes with $\alpha5\beta1$ integrins on the bacterial surface cause integrin clustering that leads to activation of ILK in a PI3K-dependent manner and recruitment of paxillin and FAK. Autophosphorylation of FAK creates a docking site for Src kinase resulting in phosphorylation of paxillin and additional sites of FAK. Rac and Cdc42 proteins are likely activated by ILK signaling, building focal adhesion complexes which ultimately regulate localized actin depolymerization and polymerization and the cytoskeletal changes that engulf the bacteria. Many gaps in the pathway exist, but the tools are now available to further dissect the mechanisms.

OTHER INVASINS

A few serotypes, including M1 strain 90-226, produce the Fn-binding protein Fba. Like M protein, the C5a peptidase (SCPA) and Fba expression are positively controlled by Mga, a multigene transcriptional activator. Mutation and complementation experiments demonstrated that Fba promotes adherence and uptake of streptococci by epithelial cells in the presence of Fn (52). More recent studies found that Fba binds the complement regulatory factor FHL-1 and that this agonist also promotes uptake of streptococci in $M1^-$ mutants of strain 90-226 (53). Elsner et al. reported that mutants which lack the lipoprotein Lbp/Lsp showed reduced adherence and invasion of epithelial cells (54). However, others found that Lbp/Lsp functions as an adhesin but reported that mutations which eliminate expression of Lpb/Lsp had no impact on invasion of HEp2 cells (55). Our laboratory also found that mutation of Lpb/Lsp protein had no effect on invasion of A549 cells by strain 90-226. Expression of that protein on the surface of lactococcus imparted the organism with the potential to adhere to A549 cells but did not promote bacterial uptake. It is possible that this lipoprotein varies between strains or that differences in experimental conditions account for the different conclusions. Thus, binding Fn to the streptococcal surface is not in itself sufficient to promote invasion of epithelial cells. Fn density and/or conformation in the context of the streptococcal surface more than likely influences its potential to promote integrin aggregation or the signals generated by the occupied integrin.

The surface-bound protease SCPA was demonstrated to be the primary, Fn-independent invasin for *Streptococcus agalactiae* (56). Although M1 protein is the primary invasin for strain 90-226, SCPA accounts for 15 to 20% of bacteria internalized by A549 cells (57). Complete deletion or amino acid substitutions that block protease activity also reduced the capacity of SCPA to promote ingestion of *S. pyogenes*. Cheng et al. postulated that autocatalytic removal of the propeptide during protease maturation might uncover the protein's cell-binding site (56).

Serum opacity factor is known to mediate invasion through binding of Fn to its C-terminal domain (58). Experiments revealed that the N-terminal, opacification domain of serum opacity factor also contributes to epithelial cell invasion independently of the C-terminal Fn-binding domain (59). Although the host invasion agonist and cellular mechanisms have not been identified, it appears that like M1 protein, serum opacity factor can use different pathways for cellular internalization.

Invasins that are independent of Fn have also been identified. Human plasminogen that is present in plasma and extracellular fluids has been linked to streptococcal invasion of human cells. Several plasminogen-binding proteins have been identified; however, the plasminogen-binding M-like protein (PAM) and PAM-related protein (Prp), which have high affinity for human plasminogen, are the most studied and are associated with an *S. pyogenes* strain isolated from a severe invasive infection (60, 61). Plasminogen-mediated invasion is significantly reduced after preincubation of bacteria with either $\alpha_1\beta_1$ or $\alpha_5\beta_1$ integrins but not after preincubation with $\alpha_v\beta_5$ integrin. Uptake of streptococci via plasminogen was also shown to be dependent on ILK signaling (43), indicating that *S. pyogenes* can use a variety of agonists to instigate integrin-mediated internalization by human cells.

M-like proteins bind C4BP, an inhibitor of the classical pathway of complement activation that blocks opsonization by C3b and phagocytosis of *S. pyogenes* by polymorphonuclear leukocytes (PMNs). Recent work demonstrated that a highly virulent M1 strain expresses protein H, a member of the M protein family that binds C4BP and enhances invasion of endothelial cells, independent of M1 protein (62). In addition, the proline-rich protein in human saliva associates with GrpE, a chaperone protein primarily expressed at cell division sites on the surface of *S. pyogenes* and significantly promotes internalization of streptococci, independent of M1 and PrtF1/SfbI proteins (63). Whether ingestion of streptococci, mediated by C4BP or proline-rich protein requires integrin signaling, is dependent on cytoskeleton rearrangement should be investigated.

FATE OF INTRACELLULAR STREPTOCOCCI

Listeria spp. can escape from vacuoles, and subsequent formation of actin tails allows the bacteria to spread to neighboring cells (26). Immunofluorescent microscopy did not reveal actin tails associated with streptococci at 5 or 7 h postinfection (7). Transmission electron microscopy showed streptococci enclosed in vacuoles within host cells (1, 2). Both *Mycobacterium tuberculosis* and *Listeria* are able to block the killing mechanisms of macrophages, providing a safe cellular compartment for persistent infection. The ultimate fate of internalized streptococci has, however, been controversial. Several studies failed to observe growth of intracellular streptococci, and infected cell lines eventually clear most of the bacteria (1, 64). Consistent with these findings are transmission electron micrographs taken 8 h postinfection showing degraded streptococci inside phagolysosomes

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(1). An early study described a unique strain of *S. pyogenes*, strain A8, that survived and multiplied within HEp2 cells (14), but to date this observation has not been confirmed or expanded. Thulin et al. found live *S. pyogenes* within macrophages at the noninflamed edge of soft tissue infections sampled by biopsy (65) and suggested that phagocytes containing intracellular streptococci contribute to the expansion of these rapid and sometimes lethal wound infections. Microscopic analysis of PMNs from intravenously infected mice contained intracellular streptococci in vacuoles, and mice inoculated with these purified infected PMNs died within 5 days, suggesting that the intracellular bacteria were not only viable but more virulent than those grown in culture medium (66). Staali et al. showed that M protein and M-like protein H are required for *S. pyogenes* to survive ingestion by PMNs. They discovered an undefined mechanism that prevents fusion of azurophilic granules with the phagosome (67). Although Cywes et al. originally suggested that uptake of *S. pyogenes* by epithelial cells was a host defense mechanism (4), their more recent experiments seem to show the opposite, i.e., intracellular invasion provides a safe haven for *S. pyogenes* (68). They demonstrated that two extracellular toxins, NADase and streptolysin O, produced by the highly invasive M1 clone of streptococcus, work together to prevent trafficking of intracellular streptococci to lysosomes, allowing 15% of bacteria to survive within immortalized keratinocyte lines. We reported many years ago that M1T1 streptococci isolated before 1980 did not efficiently invade epithelial cells (2), whereas the highly invasive M1T1 clone isolated in the 1990s invaded A549 cells at high frequency. The highly invasive M1T1 strain that Sharma et al. (68) used harbored a 36-Kb genetic element that encoded the *nga* and *slo* genes, providing a mechanism for coexpression of NADase and SLO, which in turn promoted intracellular survival. One wonders whether this genetic element is related to the prophage which we reported to distinguish the invasive M1 clone from less invasive M1 strains (13).

Historically, a primary focus of *S. pyogenes* pathogenesis has been the M protein and its capacity to block phagocytic uptake by PMNs. Now the field faces the dichotomy that this pathogen can not only resist engulfment by professional phagocytes but also survive ingestion by PMNs, which may then become a Trojan horse for dissemination of bacteria through human tissues. Finally, the outcome of streptococcal uptake by epithelial cells or phagocytes depends on the strain and the spectrum of proteins expressed on their surface.

OTHER MECHANISMS

The levels and distribution of cellular agonists are important determinants of the frequency of ingestion of *S. pyogenes* by human cells. The growth factor TGF-β1 is known to regulate the expression of Fn and other ECM proteins and positively control the integrin signaling pathway. Wang et al. (25)

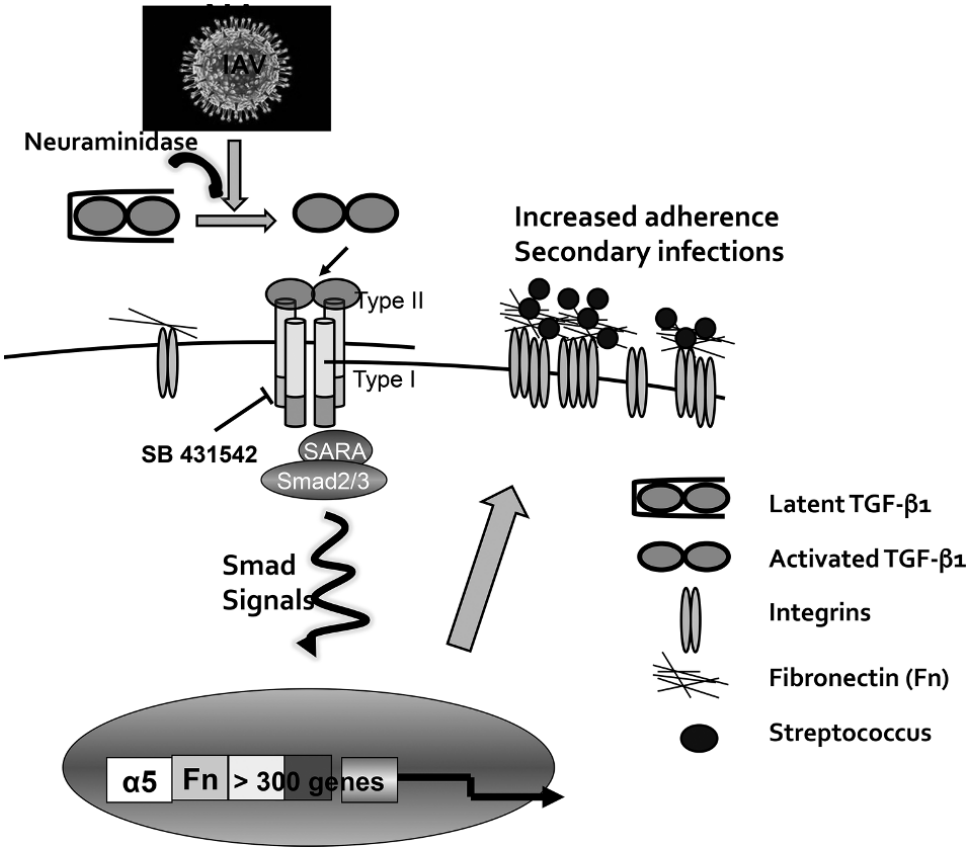


FIGURE 4 IAV facilitates bacterial adherence through the activation of the TGF-β signaling pathway. IAV neuraminidase activates latent TGF-β, which launches Smad signals that locally upregulate integrin and Fn expression by infected cells, providing increased numbers of receptors for adherence of streptococci. This in turn could lead to increased susceptibility to more frequent and more serious streptococcal infections.

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reported that infection of HEp2 cells or mice with *S. pyogenes* induces TGF-β1 expression by host cells. Moreover, pretreatment of HEp-2 cells with TGF-β1 increases expression of α5 integrin and Fn by HEp2 cells and therefore increases the frequency of ingestion of streptococci by these cells. The increased ingestion is blocked by inhibition of TGF-β1 signaling or by antibodies directed against α5 integrin or Fn. More recently, Li et al. reported that the neuraminidase of influenza A virus (IAV) enhances expression of α5 integrin and Fn on the surface of lung epithelial cells through activation of TGF-β1 (Fig. 4). This promotes FnBP-dependent adherence to these cells *in vitro* and secondary infections of lungs following inoculation with *S. pyogenes* (69). They found that inhibition of TGF-β1 or the activity of viral neuraminidase impeded upregulation of these cellular adhesins and IAV-enhanced bacterial adherence. Further study with $Smad^{-/-}$ mice, which are TGF-β1 signaling deficient, revealed that significantly larger numbers of streptococci were recovered from the lungs of IAV-infected $Smad3^{-/-}$ mice than from $Smad3^{+/+}$ littermates. Flow cytometry demonstrated that IAV infection of $Smad3^{+/+}$ mice increased the number of lung cells with high levels α5 integrin/Fn expression, whereas the number of these cells in lungs from $Smad3^{-/-}$ mice did not change after IAV infection. These results indicate that loss of TGF-β1 signaling prevents the upregulation of α5 integrin/Fn expression in IAV-infected mice and reduces host susceptibility to *S. pyogenes* coinfection. These finding are consistent with the possibility that neuraminidase in the presence of *S. pyogenes* (70) could enhance TGF-β1 activity, leading to global upregulation of integrins and ECM proteins, and increased invasion of lung epithelium, leading to systemic streptococcal infections subsequent to or during IAV infections.

SUMMARY

S. pyogenes has evolved multiple mechanisms for invading epithelial cells. Strains of this species differ substantially in invasion frequency and their dependence on invasion agonists. High-frequency internalization is mediated by ECM proteins, such as Fn, Ln, and collagen proteins that form bridges between a surface invasin and the appropriate integrin receptor on the target mammalian cell. Because adherence and invasion can be independent events, mediated by distinct surface proteins and distinct integrins, a two-receptor process is proposed for high-efficiency internalization. The spectrum of target host cells, invasion efficiency, and the ECM-binding proteins displayed on the bacterial surface determine the requirement for serum agonists. No single invasin or surface protein accounts for high-efficiency invasion of epithelial cells by all strains of streptococci. These findings suggest that intracellular invasion may be triggered by different agonists in different tissues or at different stages of infection. Intracellular streptococci appear to account for the persistence of streptococci after penicillin therapy and to be one source of streptococci that are responsible for recurrent tonsillitis. Therefore, the capacity to invade epithelial cells may influence the prevalence of a given strain of *S. pyogenes* in human populations. Multiple invasins may be differentially expressed in response to varying environmental cues at a specific tissue site. Invasins can bind the same, different, or multiple host proteins and then trigger different cellular signaling pathways. These variations may permit the bacteria to colonize various sites of a host and help explain the diverse manifestation of streptococcal diseases. A better understanding of streptococcal invasins, host cell receptors, and downstream signals may reveal the underlying mechanisms, which could lead to new and improved treatment strategies.

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Capsular Polysaccharide of Group A *Streptococcus*

MICHAEL R. WESSELS

4

INTRODUCTION

In her early studies of group A streptococci (*Streptococcus pyogenes*), Rebecca Lancefield noted an association between virulence and a distinctive appearance of the bacterial colonies on solid media. Isolates that were highly virulent for mice and that grew well in fresh human blood typically formed large colonies with a translucent, liquid appearance (mucoid) or an irregular, collapsed appearance (matte). By contrast, avirulent isolates that grew poorly in human blood formed compact, opaque colonies (glossy). Strains that grew as mucoid or matte colonies usually produced large amounts of M protein, which Lancefield gave the designation "M" because of this association with colony morphology (1, 2). Later work by Armine Wilson demonstrated that the mucoid or matte appearance of such strains was in fact due to elaboration of capsular polysaccharide, not M protein (3). Wilson showed that the mucoid or matte colony type was converted to a nonmucoid or glossy colony by growth on medium containing hyaluronidase which digested the hyaluronic acid capsule, whereas growth on medium containing trypsin, which digested M protein, had no such effect. Furthermore, while many strains that produced abundant capsular polysaccharide also were rich in M protein, expression of the two surface products was not always linked; certain mucoid or highly encapsulated strains produced little or no M protein, and certain strains rich in M protein produced little or no capsule (1, 4).

Both M protein and capsule have since been shown to contribute independently to *S. pyogenes* virulence. The association of mucoid strains of *S. pyogenes* with both invasive infection and acute rheumatic fever suggested a role for the capsular polysaccharide in virulence. Anecdotal observations that linked the capsule to pathogenesis of streptococcal disease were supported by a study that characterized the colony morphology of more than 1,000 *S. pyogenes* clinical isolates received by a streptococcal reference lab in the 1980s. In that study, only 3% of *S. pyogenes* isolates from patients with pharyngitis had a mucoid colony morphology, whereas 21% of isolates from patients with invasive disease syndromes such as bacteremia, necrotizing fasciitis, or streptococcal toxic shock syndrome and 42% of isolates associated with acute rheumatic fever were mucoid (5). The overrepresentation of mucoid strains among isolates associated with invasive infection and rheumatic fever suggested that the capsule contributes to enhanced virulence. The occurrence of outbreaks of acute rheumatic fever associated with mucoid strains of *S. pyogenes* at several locations in the United States in the 1980s supported the same inference (6–8). These clinical and epidemiologic observations implicating the capsule in pathogenesis have been corroborated by extensive experimental studies that have demonstrated a definite role of the hyaluronic acid capsule as a virulence factor in *S. pyogenes* infection.

BIOCHEMISTRY AND GENETICS OF CAPSULE PRODUCTION

Hyaluronic Acid Biosynthesis

The *S. pyogenes* capsular polysaccharide is composed of hyaluronic acid, a high-M_r (>10^6 kDa) linear polymer made up of β(1→4)-linked disaccharide repeat units of D-glucuronic acid(1→3)-β-D-*N*-acetylglucosamine. The polysaccharide is synthesized from the nucleotide sugar precursors UDP-glucuronic acid and UDP-*N*-acetylglucosamine by a membrane-associated enzyme, hyaluronan synthase. High-M_r hyaluronic acid (or hyaluronan) can be produced by incubation of cell-free membrane extracts of *S. pyogenes* with the two substrate UDP-sugars in the presence of divalent cations (9–11).

A genetic locus required for hyaluronic acid production in *S. pyogenes* was located independently by three laboratories using transposon mutagenesis to produce acapsular mutants from a mucoid *S. pyogenes* strain. Transposon insertions that produced the acapsular phenotype were mapped to a locus that was highly conserved among *S. pyogenes* strains (12–14). Characterization of this region of the *S. pyogenes* chromosome revealed a cluster of three genes, *hasABC*, whose products are involved in hyaluronic acid biosynthesis. The first gene, *hasA*, encodes hyaluronan synthase, a protein with a predicted M_r of 47.9 kDa that includes at least four predicted membrane-spanning domains, consistent with evidence that the enzyme is membrane associated (15, 16). The *hasA* gene product shares significant similarity with the NodC protein of *Rhizobium meliloti*, which is required for synthesis of an *N*-acetylglucosamine-containing polysaccharide, with chitin synthases, and with hyaluronan synthases from other microbial and higher animal species (17, 18). The *S. pyogenes* hyaluronan synthase has been expressed in *Escherichia coli* as a His_6-fusion protein. Activity of the purified enzyme is strongly stimulated in the presence of certain phospholipids, particularly cardiolipin (diphosphatidylglycerol), which appears to

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be associated with the enzyme in *S. pyogenes* (19). Cardiolipin molecules may contribute to formation of a transmembrane pore for export of the hyaluronic acid polymer, since the hyaluronan synthase itself contains only two relatively small extracellular domains (20).

The second gene, *hasB*, encodes UDP-glucose dehydrogenase, a protein with a predicted M_r of 45.5 kDa that catalyzes the oxidation of UDP-glucose to UDP-glucuronic acid (21). The third gene in the cluster, *has*C, encodes a predicted 33.7-kD protein identified as UDP-glucose pyrophosphorylase (22). The function of this enzyme is to catalyze the condensation of UTP with glucose-1-phosphate to form UDP-glucose. Thus, the reaction catalyzed by the *has*C product yields a substrate for UDP-glucose dehydrogenase encoded by *hasB*, whose reaction product is, in turn, a substrate for hyaluronan synthase encoded by *hasA* (Fig. 1). While the enzyme protein encoded by *has*C is enzymatically active, it is not required for hyaluronic acid synthesis by *S. pyogenes*. Selective inactivation of *has*C resulted in no decrement in hyaluronic acid synthesis by a highly encapsulated strain of *S. pyogenes*; the implication is that another source of UDP-glucose is available within the cell (23). Furthermore, expression of recombinant *hasA* and *hasB* (without *has*C) resulted in synthesis of hyaluronic acid in *E. coli* and *Enterococcus faecalis* (13, 15). Together, these observations have led to the conclusion that *hasA* and *hasB* are the only two genes uniquely required for bacterial synthesis of hyaluronic acid. The three genes *hasABC* are transcribed as a single message of approximately 4.2 kb from a promoter immediately upstream of *hasA* (24). The identification of a potential *rho*-independent terminator at the 3′ terminus of *has*C suggests that no additional genes are included in the operon. A paralog of *hasB*, *hasB2*, is present at a chromosomal location remote from the *has* operon. Its product is able to support a low level of hyaluronic acid production in the absence of *hasB* (25).

The simplicity of the capsule gene cluster in *S. pyogenes* contrasts with the size and complexity of capsule synthesis loci in other encapsulated Gram-positive bacteria, which generally consist of at least 12 genes (26–30). One exception is the type 3 pneumococcus, whose capsule production is strikingly similar to that in *S. pyogenes* with respect to the structure of the polysaccharide and the capsule synthesis genes; in type 3 pneumococci, capsule synthesis involves three genes that are homologs of *hasABC* (31, 32). In both *S. pyogenes* and type 3 pneumococci, it seems likely that the capsule synthesis gene cluster was acquired from another species by horizontal gene transfer. This hypothesis is also suggested by genome comparisons of *S. pyogenes* with group B *Streptococcus*: with the exception of the *hasABC* cluster that

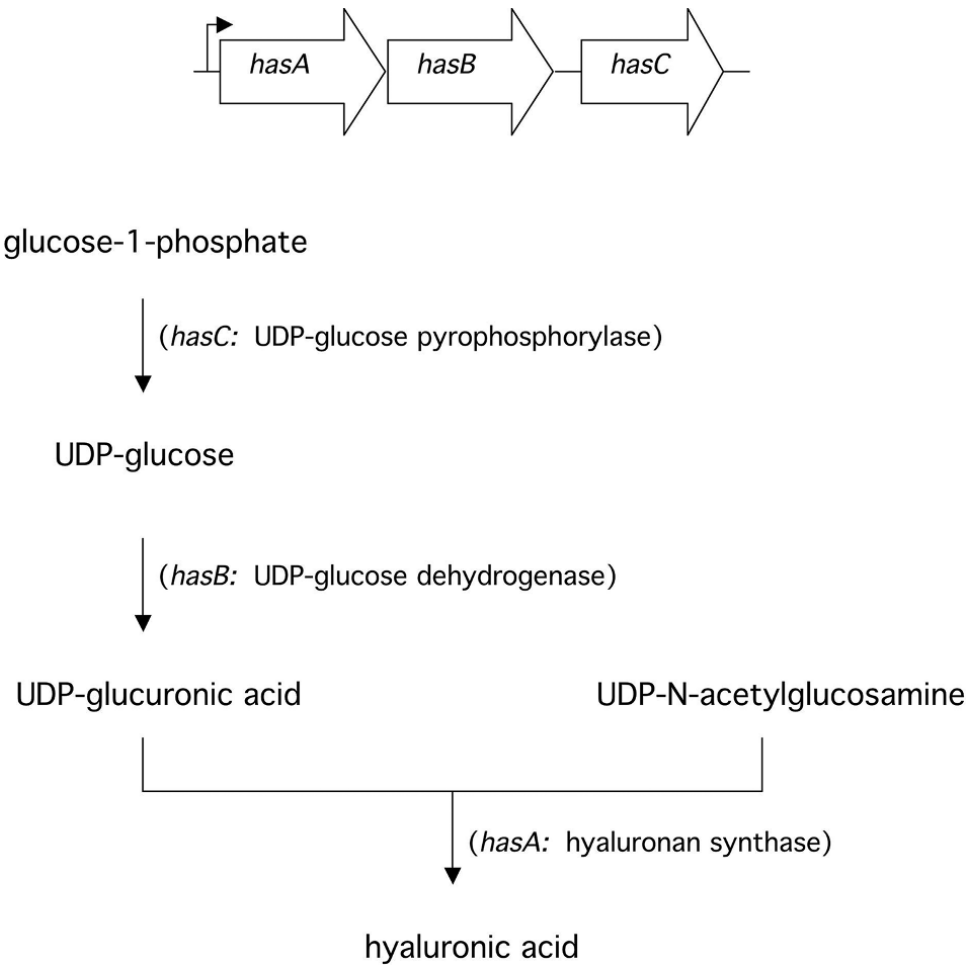


FIGURE 1 Schematic diagram of the *has* operon and the function of each gene product in the biosynthetic pathway for synthesis of hyaluronic acid in *S. pyogenes*.

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is absent in group B *Streptococcus*, there is a high degree of gene synteny over more than 25 kb of the corresponding chromosomal region in *S. pyogenes* and group B *Streptococcus*.

While the *has* operon is highly conserved, it is not universally present in *S. pyogenes* clinical isolates. In particular, strains of M types 4 and 22 and some isolates of M 89 lack the capsule synthesis operon and do not produce a hyaluronic acid capsule (33–36). Interestingly, the absence of the capsule synthesis genes in M types 4 and 22 is associated with the presence of enzymatically active hyaluronidase, whereas in serotypes that produce capsule, the chromosomal *hylA* gene contains a point mutation that renders it catalytically inactive. These divergent patterns of gene expression suggest that *S. pyogenes* has evolved two mutually exclusive survival strategies: production of an antiphagocytic hyaluronate capsule or production of hyaluronidase, presumably as a spreading factor in host tissues and/or to degrade host hyaluronic acid as a source of nutrition.

Regulation of Hyaluronic Acid Biosynthesis

The *has* operon is highly conserved among *S. pyogenes* strains. A study of nine *S. pyogenes* isolates that came from diverse settings and whose dates of isolation spanned 75 years found only six mutations within a 300-nucleotide central region of the *hasA* gene (17); all were single nucleotide substitutions, and none resulted in a change in the amino acid sequence of the protein. Despite the high degree of conservation of the hyaluronan synthase gene, individual *S. pyogenes* strains vary widely in the amount of capsule they produce. Since the *has* genes are conserved, differences in capsule expression among strains are likely to reflect differences in the regulation of *has* gene transcription. Experimental support for this hypothesis comes from the observation that *has* gene transcripts are detected during exponential growth of well-encapsulated strains of *S. pyogenes* but not in strains that do not produce detectable hyaluronic acid (24). Analysis of the *has* operon promoter using a reporter system in which the promoter was fused to a promoterless chloramphenicol acetyl transferase (CAT) gene showed that full activity of the *has* promoter required no more than 12 nucleotides of chromosomal DNA upstream of the promoter's –35 site (37). This observation is consistent with the finding that many strains of *S. pyogenes* (10 of 23) contain the insertion element IS*1239′* approximately 50 nucleotides upstream of the –35 site without any apparent effect on capsule production (Figure 2) (23).

One factor that influences the amount of capsule produced by a particular strain is the structure of the *has* operon promoter itself. Experiments using CAT reporter gene fusions revealed 3-fold higher activity of the *has* promoter from a highly encapsulated M type 18 strain than that from a poorly encapsulated M type 3 strain (37). Construction of hybrid promoters by site-directed mutagenesis demonstrated that nucleotide substitutions in two regions accounted for the differences in promoter activity between the two serotypes: three nucleotides in the −35, −10 spacer region and four nucleotides in the +2 to +8 positions relative to the start site of *hasA* transcription. The characteristic fine structure of the *has* operon promoter in type 18 strains may account in part for the observation that M18 strains are typically mucoid (i.e., highly encapsulated) since the precise sequence of the *has* promoter appears to be serotype-specific.

Capsule production varies not only among strains but also in an individual strain under different circumstances. For example, during growth in liquid batch culture, *S. pyogenes* organisms produce large capsules during the exponential phase and then shed their capsules into the medium during the stationary phase. Studies using a *has* promoter-CAT fusion integrated into the *S. pyogenes* chromosome demonstrated a rapid increase in promoter activity to a maximal level during the exponential phase of growth, with a sharp fall as the organisms entered the stationary phase. The production of cell-associated capsular polysaccharide followed an identical but slightly delayed pattern, consistent with the expected lag for changes in the translation of enzyme proteins that are subsequently reflected in the rate of polysaccharide synthesis. These results indicate that the changing levels of encapsulation observed during different phases of growth are due to transcriptional regulation of *has* operon expression. Further studies of the effect of growth rate on capsule expression in a chemostat continuous culture system found that levels of CAT activity and capsule production were at least three times higher in cells maintained at a constant rapid growth rate (doubling time of 1.4 hours) than in cells maintained at a slow growth rate (doubling time of 11 hours) (S. Albertí, J.L. Levin, and M.R. Wessels, unpublished observations).

Upregulation of capsule production is likely to occur during infection when the organism moves from the external environment into the pharynx or deep tissues, where both temperature and nutrient availability are optimal for bacterial growth. This hypothesis was supported by experiments using a reporter strain of *S. pyogenes* in which expression of green fluorescent protein was controlled by the *has* operon promoter. Introduction of the strain into the mouse peritoneum or into the baboon pharynx triggered a rapid increase in fluorescence, reflecting transcription from the *has* promoter (38). Thus, the regulation of capsule expression may be a useful adaptation to survival in host environments in which the capsule is required to defend the organism against complement-mediated phagocytic killing.

The dynamic changes in *S. pyogenes* capsule expression with changes in growth rate and the observation that some strains of *S. pyogenes* appear more highly encapsulated after animal passage have suggested that capsule gene transcription is regulated by cellular mechanisms in addition to those attributable to the intrinsic structure of the *has* operon promoter. Mga, the *trans*-acting regulatory protein that influences expression of several *S. pyogenes* proteins, appears not to regulate capsule expression, at least in most strains of



FIGURE 2 Map of the region of the *S. pyogenes* chromosome that includes the *has* operon encoding enzymes required for hyaluronic acid synthesis. The other genes shown appear not to be involved in capsular polysaccharide synthesis or surface expression. In some strains insertion sequence IS*1239′* is present approximately 50 nucleotides upstream of the *has* operon promoter (adapted from the genome sequence of M1 strain SF370, GenBank accession number AE004092 [93]).

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S. pyogenes. Inactivation of Mga was associated with reduced *has* gene transcription in an M1 strain (39); however, no effect on capsule production and/or *has* transcription was observed upon Mga inactivation in strains of M6, M18, or M49 (40–42). RofA, a regulatory protein involved in control of protein F expression, also is not known to affect capsule production (43).

Transposon mutagenesis of a poorly encapsulated M3 strain of *S. pyogenes* resulted in the identification of a novel regulatory locus whose inactivation dramatically increased capsule production (42). The locus consists of two genes, *csrR* and *csrS* (also called *covR* and *covS*), whose predicted products resemble the regulator and sensor proteins, respectively, of bacterial two-component regulatory systems (Fig. 3). Targeted inactivation of *csrR* by allelic exchange mutagenesis resulted in a 6-fold increase in capsule production. The increase in hyaluronic acid synthesis was accompanied by a parallel increase in *has* gene transcription—evidence that CsrR regulates transcription of the capsule synthesis genes. Mutation of *csrR* appeared not to affect expression of M protein or hemolytic activity, but subsequent work in several laboratories has demonstrated that the system also represses expression of several other virulence factors, including streptokinase (*ska*), streptolysin S (*sag* operon), mitogenic factor/streptodornase (*speMF*), an integrin-like protein/IgG protease (*mac* or *ideS*), and in some strains, cysteine protease (*speB*) (44–47). Microarray transcriptional profiling experiments suggested that CsrRS is involved directly or indirectly in regulation of approximately 10% of *S. pyogenes* genes (44, 48–50). The membrane-associated CsrS protein is a histidine kinase thought to respond to at least two environmental signals: an elevated extracellular Mg^{2+} concentration is associated with increased phosphorylation of the cognate regulator CsrR, presumably as a result of stimulating CsrS autokinase activity and/or inhibiting CsrS phosphatase activity for CsrR (51). The human antimicrobial peptide LL-37, at concentrations below those that inhibit *S. pyogenes* growth, has an effect on CsrR phosphorylation opposite to that of elevated magnesium (52, 53). Phosphorylation of CsrR increases its affinity for binding to the promoter region of the *has* operon and most other regulated genes to repress transcription (46, 54, 55). The likely importance of this regulatory system in virulence is supported by the markedly greater resistance to *in vitro* phagocytosis displayed by a (highly encapsulated) *csrR* mutant than by the wild-type parent strain and a 500-fold increase in the mutant's virulence for mice after intraperitoneal challenge (42). Up to 40% of invasive *S. pyogenes* isolates have inactivating mutations in CsrS or CsrR, whereas such mutations are rare among isolates from pharyngitis patients (56–58).

Capsule production is also controlled by RocA, a protein first identified as a regulator of CsrR expression and later shown to influence phosphorylation of CsrR (59, 60). Mutations in *rocA* are associated with increased expression of the *has* operon and of other genes regulated by the CsrRS system. *S. pyogenes* strains of *emm* types 18 and 3 each harbor a mutation in *rocA* that results in expression of a truncated RocA protein and increased capsule production (61, 62).

THE CAPSULE AND HOST IMMUNE DEFENSES

Immunogenicity of Hyaluronic Acid

In 1937, Kendall et al. reported the purification of capsular polysaccharide from mucoid strains of *S. pyogenes* (63). Their analysis revealed that an apparently identical capsular material was produced by different M types of *S. pyogenes*. The *S. pyogenes* polysaccharide contained equal amounts of *N*-acetylglucosamine and glucuronic acid as exclusive components of monosaccharides; this finding suggested that it might be structurally identical to a polysaccharide isolated from bovine vitreous humor and human umbilical cord. Immunization of rabbits and horses with mucoid *S. pyogenes* cells failed to elicit antibodies detectable by precipitin reactions with the polysaccharide. Later studies have confirmed that the "serologically inactive" polysaccharide produced by *S. pyogenes* is hyaluronic acid, a high M_r polysaccharide with a repeating unit structure identical to that of hyaluronic acid isolated from animal sources. Presumably because it is recognized as a "self" antigen, hyaluronic acid is poorly immunogenic in several animal species. Fillit and colleagues evoked antibodies to hyaluronic acid by immunization of rabbits with encapsulated streptococci of group A or C and by immunization of mice with hyaluronidase-treated hyaluronic acid linked to liposomes (64, 65). Such antibodies, however,

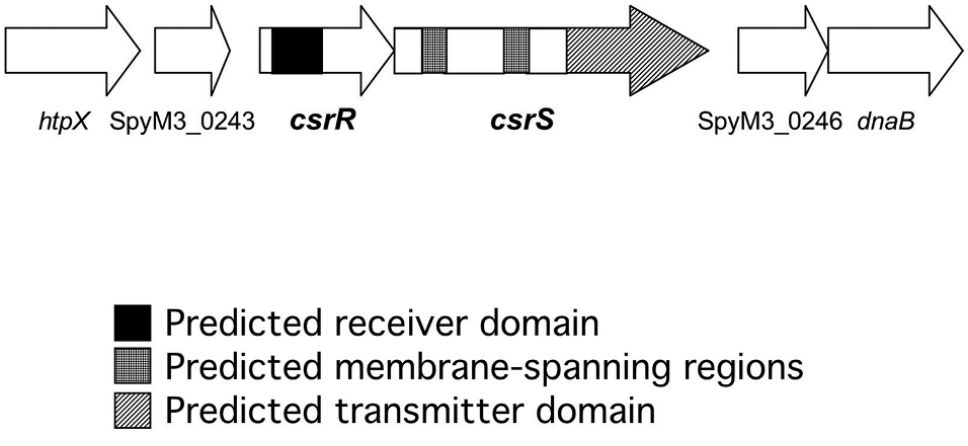


FIGURE 3 Diagram of the *csrRS* chromosomal locus encoding a two-component regulatory system that regulates hyaluronic acid synthesis. Sequences corresponding to regions of the predicted proteins with properties characteristic of the response regulator (CsrR) or sensor (CsrS) components are indicated (adapted from the genome sequence of M3 strain MGAS315, GenBank accession number AE014074 [94]).

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have not been shown to have any opsonic activity against *S. pyogenes in vitro* or to confer protection against *S. pyogenes* infection *in vivo*.

Capsule and Resistance to Phagocytosis

S. pyogenes, like other Gram-positive pathogens, is resistant to direct bacteriolysis by complement and antibodies. Therefore, clearance of the organisms from the blood or deep tissues depends largely on uptake and killing by phagocytes. This concept was supported by the observations of Todd, Lancefield, and others that virulence of *S. pyogenes* isolates in both clinical settings and in experimental animal infections was closely paralleled by the capacity of the strains to resist killing by human blood leukocytes. *S. pyogenes* strains that produced large amounts of M protein multiplied in fresh human blood, while those that lacked M protein did not (66, 67). In addition, however, early studies demonstrated a relationship between surface expression of the hyaluronic acid capsule and resistance to phagocytosis. Mucoid or highly encapsulated strains tended to be resistant, and hyaluronidase treatment increased their susceptibility to phagocytosis *in vitro*, a result that suggested a protective effect of the capsule (68–70). More recent work with acapsular mutants has provided definitive evidence that the capsule is a major determinant of resistance to phagocytic killing. In several assays measuring complement-dependent phagocytic killing by human blood leukocytes, acapsular mutants of type 18 and type 24 *S. pyogenes* were significantly more susceptible than the corresponding encapsulated wild-type strains (14, 71, 72).

One mechanism through which capsular polysaccharides may confer resistance to phagocytosis is the inhibition of complement activation. The type III capsular polysaccharide of group B *Streptococcus*, for example, prevents deposition of C3b on the bacterial surface through inhibition of alternative complement pathway activation by sialic acid residues present in the polysaccharide (73, 74). In *S. pyogenes*, however, the presence of the capsular polysaccharide appears to have no effect on the amount of C3b deposited on the bacterial surface. Similar amounts of C3 were detected on acapsular mutants and on encapsulated wild-type strains after incubation of the organisms in serum (71). The increased resistance of well-encapsulated *S. pyogenes* strains to complement-dependent phagocytic killing, therefore, must reflect the capacity of the capsule to block access of neutrophils to opsonic complement components bound to the bacterial surface rather than inhibition of C3b deposition (Fig. 4).

Resistance of *S. pyogenes* to phagocytosis has been assessed most often with the bactericidal test of Lancefield, in which a small inoculum of the test strain is rotated for 3 hours in heparinized fresh human blood (67). Strains that exhibit a significant increase in CFU during rotation in blood are considered resistant. An effect of the capsule is evident in this assay (acapsular mutants show less net growth than encapsulated wild-type strains), but the effect of M protein is more striking (14, 72). M protein-deficient strains not only fail to grow but typically are reduced in number after a 3-hour incubation. By contrast, when resistance to phagocytosis is assessed with a different assay, the effect of capsule is more dramatic than that of M protein. In this second assay system, a larger bacterial inoculum is rotated for 1 hour with human peripheral blood leukocytes in the presence of 10% normal human serum as a complement source (75, 76). In the latter assay, acapsular mutant strains and poorly encapsulated wild-type strains are killed (~90% reduction in CFU) despite the presence of M protein, while well-encapsulated strains do not change or increase in number. A mutant that is deficient in M protein but that produces wild-type amounts of capsule is killed less efficiently than a capsule-deficient mutant in the same background (72). In a study of isogenic mutants deficient in capsule, M protein, or both in the background of a mucoid type 18 *S. pyogenes* strain, mouse virulence correlated better with results in the second assay system than with the Lancefield whole-blood assay (72).

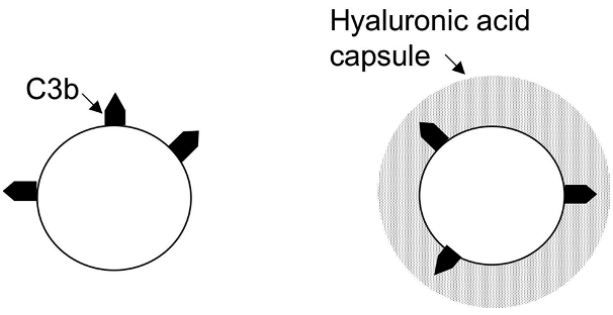


FIGURE 4 The hyaluronic acid capsule and resistance to complement-mediated phagocytosis. The capsule does not prevent deposition of C3b on the bacterial cell wall but, rather, interferes with the interaction of bound C3b with phagocyte receptors.

CAPSULE AS A VIRULENCE FACTOR

Experimental Infection Models

In the 1940s, investigators attempted to determine experimentally whether the hyaluronic acid capsule contributed to the virulence of *S. pyogenes*. Hirst found that decapsulating *S. pyogenes* by treatment with leech extract containing hyaluronidase had no effect on virulence of the bacteria in mice (77). However, Kass and Seastone found reduced virulence of decapsulated *S. pyogenes* in mice, perhaps because the latter authors administered hyaluronidase to the infected mice at frequent intervals to prevent regeneration of the capsule after challenge (78). Subsequently, more direct proof that the capsule contributes to virulence has come from several studies using acapsular mutant strains of *S. pyogenes* derived by transposon mutagenesis or by targeted inactivation of gene(s) required for hyaluronic acid synthesis (Table 1).

Because the capsule is required for *S. pyogenes* resistance to complement-mediated phagocytic killing (see above), it is not surprising that capsule expression enhances virulence in systemic infection models in which the animals succumb to overwhelming bacteremia. In addition, however, the hyaluronic acid capsule contributes in a pivotal fashion to the capacity of the organisms to produce invasive soft tissue infection. A mouse model of invasive *S. pyogenes* soft tissue

TABLE 1 Animal models in which the hyaluronic acid capsule has been shown to enhance virulence of group A streptococci

Type of infection	Animal species	Reference(s)
Systemic infection	Mouse	14, 76
	Chicken (embryo)	95
Pharyngeal colonization	Mouse	81, 82
	Baboon	83
Pneumonia	Mouse	82
Soft tissue infection	Mouse	79, 80

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infection has been developed in which the bacterial inoculum is delivered by superficial injection just below the skin surface in a small volume and with minimal trauma to the tissues (79, 80). Mice challenged with the highly encapsulated M24 strain Vaughn developed dermal necrosis with underlying purulent inflammation and secondary bacteremia, while mice challenged with the acapsular mutant strain 24-4 developed no lesion at all or minor superficial inflammation and no bacteremia (79). Similar results were observed in the same model with a moderately encapsulated M3 strain of *S. pyogenes* (originally isolated from a patient with necrotizing fasciitis) which was compared with two isogenic acapsular mutants derived by allelic exchange mutagenesis of the *has*A (hyaluronan synthase) gene. In the latter experiments, the majority of animals challenged with the encapsulated M3 parent strain developed spreading, necrotic soft tissue infection and died, whereas the acapsular mutants produced only mild local infection and no deaths (80). The dramatic impact of the capsule on virulence in this model is correlated with equally striking histopathologic findings: In animals challenged with an acapsular mutant, a small focus of neutrophilic inflammation and necrosis is confined by a well-formed abscess. By contrast, after challenge with the encapsulated wild-type strain, acute inflammatory cells extend throughout the subcutaneous tissue and are associated with thrombosis of blood vessels, with infarction and necrosis of the overlying dermis—a histopathologic picture very similar to that in patients with *S. pyogenes* necrotizing fasciitis. Examination of the histologic sections by immunofluorescence microscopy with an *S. pyogenes*-specific antibody showed that the acapsular *S. pyogenes* cells were confined to the abscess cavity, while the encapsulated wild-type organisms were widely dispersed throughout the subcutaneous tissues.

Capsule and Mucosal Colonization

Studies by at least two groups have demonstrated a critical role for the hyaluronic acid capsule in pharyngeal colonization in mice. BALB/c mice inoculated intranasally with the stable (transposon insertion plus chromosomal deletion) acapsular mutant strain 24-4 rapidly cleared the organism from the pharynx, and no mice died (81). In contrast, throat cultures of mice challenged with the revertible (simple transposon insertion) acapsular mutant 24-72 yielded encapsulated revertants, and the rate of persistent throat colonization and lethal systemic infection was similar to that in animals challenged with the encapsulated type 24 parent strain. A study of an acapsular mutant derived from the mouse-virulent M50 strain, B514, yielded similar results: intranasal challenge with the wild-type strain resulted in chronic throat colonization in C57BL/10SnJ mice. After intranasal challenge with an acapsular mutant derived by plasmid insertion mutagenesis of the *has*A gene, encapsulated revertants accounted for all *S. pyogenes* recovered from throat cultures 4 days after challenge (82). Intratracheal inoculation of C3HeB/FeJ mice with the wild-type strain produced pneumonia and secondary systemic infection in approximately 50% of the animals within 72 hours, while only 2 animals challenged with the acapsular mutant became ill, 5 and 6 days after challenge, respectively. In both cases only encapsulated revertants were recovered from cultures of the throat, lungs, and blood (82). The findings in both studies of impaired colonization by the acapsular mutant strains and the rapid emergence of encapsulated revertants together provide strong evidence that encapsulation confers a powerful survival advantage for *S. pyogenes* in the upper airway. This conclusion was supported also by experiments in a primate model of *S. pyogenes* pharyngeal colonization: an acapsular mutant derived from an M3 strain was cleared from the pharynx of baboons more quickly than the wild-type parent strain (83). While these results suggest that capsule production enhances initial survival and persistence of *S. pyogenes* in the pharynx, studies of long-term carriage in a primate model and in human carriers have shown that carriage isolates acquire mutations that result in reduced or absent hyaluronic acid production (84, 85). Taken together, the results of these studies suggest that the hyaluronic acid capsule enhances transmission and initial colonization but that prolonged carriage in human beings may be favored by subsequent downregulation or loss of capsule production.

Capsule and *S. pyogenes* Attachment to Epithelial Cells

The influence of the hyaluronic acid capsule on the capacity of *S. pyogenes* to colonize the pharyngeal epithelium may be explained, at least in part, by the protective effect of the capsule in preventing opsonophagocytic killing, although the importance of resistance to opsonophagocytosis at a mucosal site is less well-defined than in blood or deep tissues. In addition, the capsule is an important factor in the process of *S. pyogenes* attachment to epithelial cells, both by modulating interactions of other bacterial surface molecules with the epithelium and by direct interaction of *S. pyogenes* capsular hyaluronic acid with eukaryotic cells. The capsule may influence adherence by interfering with the interaction of any of a variety of *S. pyogenes* surface molecules that have been implicated as adhesins: M and M-like proteins, fibronectin-binding proteins, lipoteichoic acid, and glyceraldehyde-3-phosphate dehydrogenase. In general, acapsular mutant strains of *S. pyogenes* attach in higher numbers to skin or throat keratinocytes than do the corresponding encapsulated wild-type strains. For type 24 *S. pyogenes*, the capsule prevents M protein-mediated adherence to keratinocytes, since inactivation of the *emm24* gene reduced adherence in the background of an acapsular mutant but not in the background of the same strain expressing wild-type amounts of capsule (86). It is likely that the capsule exerts a similar modulating influence on bacterial adherence mediated by other *S. pyogenes* surface molecules.

Not only does the capsule influence the binding interactions of other *S. pyogenes* surface structures, but there is evidence that the capsule itself can serve as a ligand for attachment of *S. pyogenes* to CD44 on epithelial cells. CD44 is a hyaluronic acid-binding glycoprotein found on a variety of human cells, including epithelial and hematopoietic cells. The interaction of CD44 with hyaluronic acid appears to have important functions in cell migration, organization of epithelia, lymphocyte homing, and tumor metastasis. Because CD44 is expressed on the surface of tonsillar epithelial cells, it may serve as a receptor for binding of hyaluronic acid on the surface of *S. pyogenes*. This hypothesis was supported by the finding that a monoclonal antibody to CD44 reduced attachment of wild-type strains of *S. pyogenes* to human pharyngeal keratinocytes by ~90% but had no effect on attachment of acapsular mutant strains (86). Further evidence that CD44 acts as a receptor for *S. pyogenes* came from the observation that transfection of K562 cells with cDNA encoding either of two epithelial isoforms of CD44 conferred on the cells the capacity to bind *S. pyogenes*. Cells expressing CD44 bound a variety of *S. pyogenes* wild-type strains, including several in which the level of capsule expression was below the limit of detection in a dye-binding assay; however, inactivation of the hyaluronan synthase gene in such a poorly

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encapsulated *S. pyogenes* strain completely abrogated binding (86). A study of transgenic mice with defective expression of epithelial CD44 found reduced pharyngeal colonization compared to that in wild type mice after intranasal challenge with *S. pyogenes*, evidence that CD44 can function as a receptor for *S. pyogenes* binding to the pharyngeal epithelium *in vivo* (87).

Capsule and CD44-Mediated Tissue Invasion

CD44 is a transmembrane protein, and its ligation by hyaluronic acid can result in signal transduction and induction of cellular events involved in cell migration. Binding of *S. pyogenes* to CD44 on human pharyngeal keratinocytes *in vitro* has been shown to trigger a similar pattern of cellular responses, including the formation of lamellipodia or membrane ruffles at the site of *S. pyogenes* attachment and disruption of intercellular junctions (88). These events were associated with enhanced translocation of encapsulated *S. pyogenes* compared to an acapsular mutant across epithelial cell layers *in vitro*. The results suggested that interaction of the *S. pyogenes* capsule with CD44 manipulates a host signaling pathway to disrupt epithelial integrity and enable bacterial penetration into underlying tissues.

Capsule and *S. pyogenes* Entry into Epithelial Cells

Several laboratories have documented the entry of *S. pyogenes* into human epithelial cells, a process that has been proposed to represent a virulence mechanism—either a step in bacterial invasion into deep tissues or a means of escape of the organism into an intracellular sanctuary shielded from antibiotics and host immune defenses (79, 89–91). However, while there is irrefutable evidence that the capsule plays a key role in invasive infection *in vivo*, there is equally clear evidence that the capsule prevents *S. pyogenes* entry into epithelial cells. In studies of *S. pyogenes* entry into cultured human keratinocytes from pharyngeal epithelium or external skin, poorly encapsulated strains entered cells far more efficiently than well-encapsulated strains, regardless of M type or clinical source of the isolates (79). Acapsular mutants entered more than 1,000 times more efficiently than corresponding encapsulated parent strains. The apparently opposite effects of the capsule on invasive infection *in vivo* compared to those on epithelial cell "invasion" *in vitro* suggest that bacterial entry into epithelial cells is not a step in productive infection but rather a host response that limits the spread of poorly encapsulated strains. By contrast, virulent encapsulated strains resist ingestion by eukaryotic cells, both epithelial cells and professional phagocytes, and invade deep tissues by an extracellular route, a process that appears to be facilitated by CD44-mediated disruption of cell-cell junctions. This hypothesis is supported by the finding that clinical isolates from patients with invasive *S. pyogenes* infection "invade" epithelial cells at much lower rates than throat or skin isolates (92). After internalization, the viability of *S. pyogenes* within epithelial cells *in vitro* falls rapidly after bacterial entry (79, 90). It is possible, however, that transient intracellular survival of *S. pyogenes*, perhaps in a cyclic fashion, contributes to persistent carriage.

CONCLUSIONS

Careful observations by microbiologists and clinicians in the 1920s and 1930s pointed to a link between the hyaluronic acid capsule and *S. pyogenes* disease pathogenesis. More recent studies have defined the genetic locus that directs hyaluronic acid biosynthesis and have characterized the molecular mechanisms through which the capsule enhances *S. pyogenes* virulence. It is now appreciated that the *has* capsule synthesis operon is both highly conserved and widely, but not universally, distributed among *S. pyogenes* strains, attesting to the adaptive role played by the hyaluronic acid capsule in the coevolution of *S. pyogenes* with the human host. As a poorly immunogenic "self" antigen, the capsular polysaccharide appears to have persisted in an invariant form in *S. pyogenes*, presumably because it has not been subject to the selective pressure of an effective, polysaccharide-specific, humoral immune response in the infected host that has led to emergence of multiple capsular types in other pathogenic bacteria. Studies of acapsular mutant strains have demonstrated that the capsule protects *S. pyogenes* from complement-mediated phagocytic killing and is essential for full virulence in a variety of experimental infection models. The *S. pyogenes* capsule also influences attachment of the bacteria to human epithelial cells, both by modulating the interaction of M protein and other potential adhesins and by itself serving as a ligand for attachment of *S. pyogenes* to CD44 on epithelial cells. The latter mechanism may be of particular importance in *S. pyogenes* colonization of the pharynx, because it appears to participate in the adherence of diverse *S. pyogenes* strains, including those that produce only small amounts of capsule. Finally, the successful adaptation of *S. pyogenes* to survival in the human host involves regulation of capsule expression that is dependent on the fine structure of the *has* operon promoter, on the CsrRS two-component regulatory system, and likely, on additional mechanisms yet to be uncovered.

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Toxins and Superantigens of Group A Streptococci

BLAKE A. SHANNON, JOHN K. McCORMICK AND PATRICK M. SCHLIEVERT

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Streptococcus pyogenes (i.e., the group A *Streptococcus*) is a human-restricted and versatile bacterial pathogen that produces an impressive arsenal of both surface-expressed and secreted virulence factors. *S. pyogenes* exists primarily as an asymptomatic colonizer of the skin and mucous membranes of the nasopharynx, and despite being universally sensitive to β-lactam antibiotics *in vitro*, this bacterium continues to generate significant morbidity and mortality on a global scale. Human diseases induced by *S. pyogenes* usually occur as relatively uncomplicated manifestations such as pharyngitis and skin infections, but it may also cause more problematic diseases including erysipelas and scarlet fever. In addition, *S. pyogenes* can cause devastating invasive diseases including puerperal sepsis, bacteremia, necrotizing fasciitis, and streptococcal toxic shock syndrome (TSS). This bacterium is further recognized as a very important cause of postinfection sequelae including acute rheumatic fever and rheumatic heart disease, acute glomerulonephritis, and potentially, pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections. Although surface-expressed virulence factors are clearly vital for colonization, establishment of infection, and the development of disease, the secreted virulence factors are likely the major mediators of tissue damage and toxicity seen during active infection. The collective exotoxin arsenal of *S. pyogenes* is rivaled by few bacterial pathogens and includes extracellular enzymes, membrane active proteins, and a variety of toxins that specifically target both the innate and adaptive arms of the immune system, including the superantigens; however, despite their role in *S. pyogenes* disease, each of these virulence factors has likely evolved with humans in the context of asymptomatic colonization and transmission. In this article, we will focus on the biology of the true secreted exotoxins of the group A *Streptococcus*, as well as their roles in the pathogenesis of human disease.

THE CYTOLYTIC TOXINS OF *S. PYOGENES*

β-hemolytic activity on blood agar is a hallmark feature of the group A *Streptococcus*, and this phenotype has been used clinically for over a century. The streptolysin S (SLS) exotoxin is a small, oxygen stable hemolysin that is responsible for the characteristic β-hemolysis of *S. pyogenes* (1). SLS is a cytolytic toxin that has features of the ribosomally synthesized and posttranslationally modified lantibiotic bacteriocins from other Gram-positive bacteria; however, lantibiotic bacteriocins mostly function as antibacterials without cytotoxic activity for eukaryotic cells (2). Production of SLS is encoded by the SLS-associated gene (*sag*) cluster, which contains genes encoding the structural SLS protein (*sagA*) as well as enzymes responsible for the posttranslational modification and transport of the toxin and, potentially, an immunity function (*sagB* through *sagI*) (3). Furthermore, the *sag* locus is conserved among group A *Streptococcus* strains regardless of M protein serotype (3). SLS has been shown to target and disrupt the function of multiple host cells, including erythrocytes, macrophages, neutrophils, and keratinocytes (2, 4–6). The exact mechanism of action of SLS is not completely clear, although it is thought to act by forming a pore in host cell membranes (7). However, a recent study has demonstrated that SLS lyses erythrocytes by disrupting the function of anion transporters to cause an influx of chloride anions (Cl^-). The influx of Cl^- is followed by an influx of water, resulting in colloidal-osmotic rupture of erythrocytes (8). Furthermore, SLS has been shown to work in conjunction with the host protease calpain to facilitate the destruction of intracellular junction proteins, promoting paracellular invasion of the bacteria across the epithelial barrier (9). To examine the role of SLS in invasive models of disease, infection of an *S. pyogenes* strain deleted for SLS function was tested in a murine model of necrotizing soft tissue infection, and SLS was determined to be essential for pathogenesis (3). A similar study using transposon mutagenesis created a mutant deficient in SLS production that remained normal with respect to other exoprotein expression and was also shown to be markedly less virulent in a mouse model of subcutaneous infection than in the isogenic wild-type strain (10). Therefore, SLS likely contributes to *S. pyogenes* pathogenesis through a combination of inhibiting phagocyte function and damaging epithelial barriers.

Streptolysin O (SLO) is a well-characterized exotoxin that functions as a thiol-activated cytolysin and is secreted from nearly all group A *Streptococcus* isolates (11). The *slo* gene encodes SLO monomers that oligomerize on eukaryotic membranes in a cholesterol-dependent manner, resulting in the formation of a large pore. This produces significant damage and ultimately results in host cell apoptosis (12). The SLO toxin is active in a reduced state and is rapidly inactivated in the presence of oxygen; thus, activity is not thought to be visible on routine blood agar plates (13). Previous studies have shown that the host targets of SLO include erythrocytes, macrophages, neutrophils, and keratinocytes (14–17). SLO has also been shown to inhibit neutrophil degranulation at sublytic concentrations (16). In addition to SLO, the *slo* gene

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resides in an operon that additionally encodes *S. pyogenes* NADase (SPN). The NADase functions by hydrolyzing cellular NAD^+ and by depleting cellular ATP to decrease the host energy sources (18). Multiple studies have concluded that the entry of SPN into host cells is dependent on SLO pore formation (19, 20), and SPN and SLO in conjunction increase epithelial cell apoptosis, compared to SLO alone (21). Using immortalized human keratinocytes, strains lacking SLO were attenuated for intracellular survival compared to the wild-type strain (20). Furthermore, in a murine model of necrotizing soft tissue infection, *S. pyogenes* strains lacking SLO were deficient in causing myositis, bacteremia, and soft tissue infection (22). Another study demonstrated lower infectivity of SPN mutant strains in models of necrotizing fasciitis, although the deletion of SLO led to an inability to cause disease (22). Therefore, SLO likely promotes the pathogenesis of *S. pyogenes* in invasive skin and soft tissue infection through the destruction of phagocytes, as well as the cytolysin-mediated translocation of SPN into target cells.

Independent studies investigating the role of both SLO and SLS in murine models of necrotizing fasciitis and systemic infection each demonstrated that both cytolytic toxins significantly contribute to the development of murine necrotizing fasciitis (23, 24). Although SLO and SLS have been extensively studied and shown to be important contributors to the pathogenesis of invasive *S. pyogenes* infections, their roles during colonization and acute nasopharyngeal infection have yet to be defined.

STREPTOCOCCAL SUPERANTIGENS

S. pyogenes is recognized as one of the few bacterial pathogens that produces superantigen exotoxins. Streptococcal superantigens are ribosomally synthesized, relatively low molecular mass (~22 to 28 kDa) proteins that contain classical signal peptides that are cleaved after secretion to release the mature toxin. Superantigens function by activating T cells and are among the most potent known activators of T cells. The term "superantigen" was first used to describe the massive primary T cell response to these bacterial toxins (25). However, the *S. pyogenes* superantigens are also historically known as the erythrogenic toxins or scarlet fever toxins, due to their role in causing a red rash in the context of scarlet fever (26–28). In fact, the characteristic rash seen in scarlet fever is likely due to an amplified hypersensitivity reaction resulting from superantigen activity (28). Since the appearance of a rash was the defining feature of the "erythrogenic" toxins, Watson (27) first proposed the name "streptococcal pyrogenic exotoxin" (Spe) owing to the fever-producing ability, because he initially believed that the Spes belonged to a separate family of toxins. We now know that these represent the same group that belongs to a larger group of structurally conserved exotoxins that are also produced by *Staphylococcus aureus* (29–32) and some Lancefield group C and G β-hemolytic streptococci (33–37). There are now at least 14 characterized and genetically distinct *S. pyogenes* superantigens, and many of them are encoded within lysogenic bacteriophage, or putative bacteriophage, elements. Therefore, different strains often encode different repertoires of typically between 3 and 6 distinct superantigen genes (38–42). Although nomenclatural inconsistencies exist in the literature, a recent comprehensive review of the streptococcal superantigens has clarified many of these issues (43). Using this updated nomenclature, the currently known repertoire includes streptococcal pyrogenic exotoxin (Spe) types A, C, G, H, I, J, K, L, M, N, O, and P, as well as the streptococcal superantigen and streptococcal mitogenic exotoxin Z (SmeZ) (Fig. 1A). The SpeN, SpeO, and SpeP superantigens were first identified in *Staphylococcus equi* subsp. *zooepidemicus* (originally defined as SzeN, SzeF, and SzeP, respectively) (36), but to our knowledge, these particular superantigens have not been identified in *S. pyogenes*. Additionally, *S. equi* subsp. *equi* can encode SeeH, SeeI, SeeL, and SeeM, which are highly orthologous to SpeH, SpeI, SpeL, and SpeM, respectively (37). Although most of the streptococcal superantigens are encoded on bacteriophage elements, SpeG and SmeZ are exceptions in that they appear to be encoded within the core chromosome (35, 38–42). SpeJ is also not found in association with bacteriophage genes (40), yet it is not present in the many sequenced strains. SmeZ, in particular, is known to have many allelic variants, and most of the sequence changes are located on the surface of the toxin and rarely found within the predicted receptor binding domains. This indicates that SmeZ is likely under significant immunological pressure to alter antigenic characteristics as a possible immune evasion strategy (35). Despite the fairly weak primary amino acid sequence homology (Fig. 1B), crystal structures determined for these toxins share the "generic" superantigen fold (Fig. 1C) (44). Each structure includes an N-terminal α-helix which leads into a β-barrel domain, also known as the oligosaccharide/oligonucleotide binding fold (45). A central α-helix then joins this domain to a β-sheet structure known as the β-grasp domain. Despite the clear similarities to the overall fold, there are also important differences in how these toxins engage their host receptors.

Superantigens function by causing excessive T cell activation through simultaneous engagement of both the variable region of the T cell antigen receptor (TCR) β-chain (referred to as "Vβ") on T cells and to different regions of major histocompatibility complex (MHC) class II molecules on antigen-presenting cells (44, 46). Thus, superantigens are remarkable in that they have evolved to target two critical and extremely diverse receptors of adaptive immunity. In superantigen-mediated T cell activation, the toxin binds directly to MHC class II molecules, and this occurs without processing by the antigen-presenting cell. Superantigens do not undergo major modifications after release from the cell or major structural alterations upon binding to their ligands. The interaction with the TCR is dependent on binding to different Vβ regions, and this occurs, generally, away from the regions of the TCR that are critical for peptide-specific recognition. Due to the unconventional contacts created by these interactions, superantigen-mediated T cell activation is also not MHC restricted, and furthermore, both $CD4^+$ and $CD8^+$ T cells are stimulated in a Vβ-specific manner. Thus, superantigens can "force" an excessive primary response that is not seen with conventional peptide antigens. In the most severe case, this excessive activation of T cells results in the massive release of proinflammatory cytokines from both T cells and antigen-presenting cells (47) that can result in a serious and potentially lethal disease defined as TSS (32).

Cocrystallization structures have been determined for SpeA and SpeC in complex with the mouse Vβ8.1 and human Vβ2.1 chains, respectively (48). Earlier cocrystal structures exist for the staphylococcal superantigens staphylococcal enterotoxin B (SEB) and staphylococcal enterotoxin C (SEC) in complex with the mouse Vβ8.1 chain (49, 50). Although highly homologous superantigens, such as SEB, SEC, and SpeA, may have similar architectures for their Vβ chain interactions, the β-chain-SpeC complex involved a significantly larger contact surface, a different orientation, and contacts with the complementarity determining region (CDR) 3 loop, whereas SEB, SEC, and SpeA had no intermolecular

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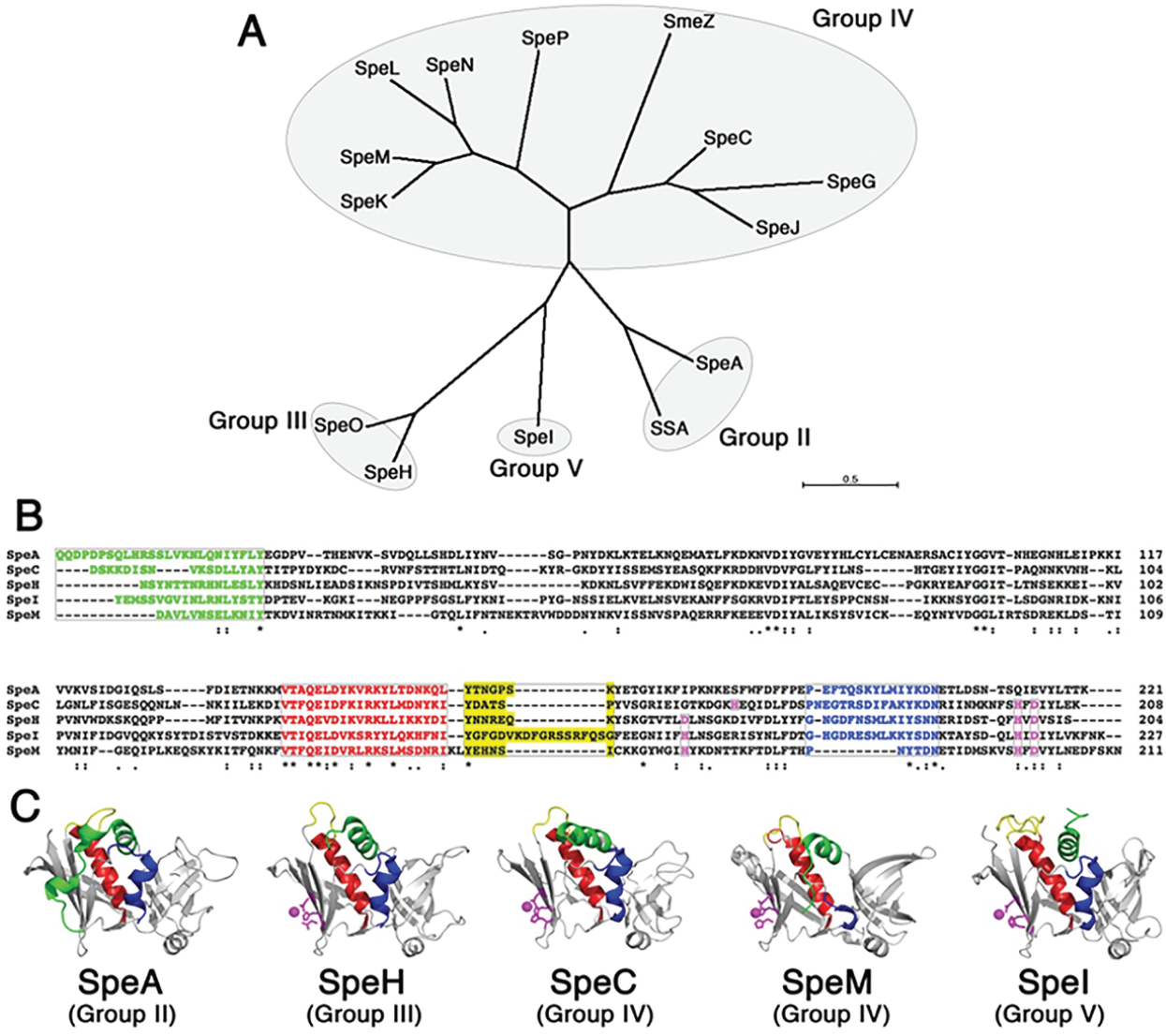


FIGURE 1 Phylogenetic relationships and structural conservation of the streptococcal superantigens. (A) Unrooted neighbor-joining tree showing phylogenetic relationships of known streptococcal superantigens. The unrooted tree was based on the alignment of amino acid sequences using CLUSTAL W (166) and constructed using MEGA7 (167). The groups indicate a prior classification scheme for the superantigen family (32). (B) Amino acid alignment of five representative streptococcal superantigens. The colors designate distinct domains in the superantigen structure, including the N-terminal α-helix (green), the central α-helix (red), the α3-β8 loop that is unique to the group V superantigens (168), and a C-terminal α-helix that is lacking in a subgroup of group IV. Residues involved in the coordination of a zinc atom important for binding to the MHC class II β-chain are colored magenta. (C) Crystal structures of representative streptococcal superantigens are colored as in panel B.

contacts with this loop (48). Nevertheless, it appears that the critical component for functional recognition of the TCR is the CDR 2 loop of the β-chain (51).

The interaction of superantigens with MHC class II can also show considerable diversity, because superantigens have two independent binding domains. One binding face occurs through the N-terminal oligosaccharide/oligonucleotide binding fold domain and is of relatively low affinity ($\sim 10^{-5}$ M). Although somewhat controversial (52–55), it appears likely that most, if not all, streptococcal superantigens contain this low-affinity MHC binding domain. A second, zinc-dependent MHC binding face occurs on the β-grasp domain and is of relatively high affinity ($\sim 10^{-7}$). The zinc ion, shown in magenta in Fig. 1C, is coordinated through three conserved residues, whereas the fourth ligand is from a conserved residue on the MHC β-chain.

Figure 2 presents the complexes for a conventional TCR-MHC class II interaction, as well as how SpeA (which lacks a high-affinity MHC class II binding domain) likely engages TCR Vβ and MHC class II and SpeC, which crosslinks MHC class II (56). The SpeA model is analogous to the standard "wedge" model of superantigen-mediated T cell activation (49), whereas the SpeC model is analogous to the proposed model for staphylococcal enterotoxin A (57, 58). In these models, the mechanism by which superantigens subvert the normal T cell activation process is apparent, whereby the

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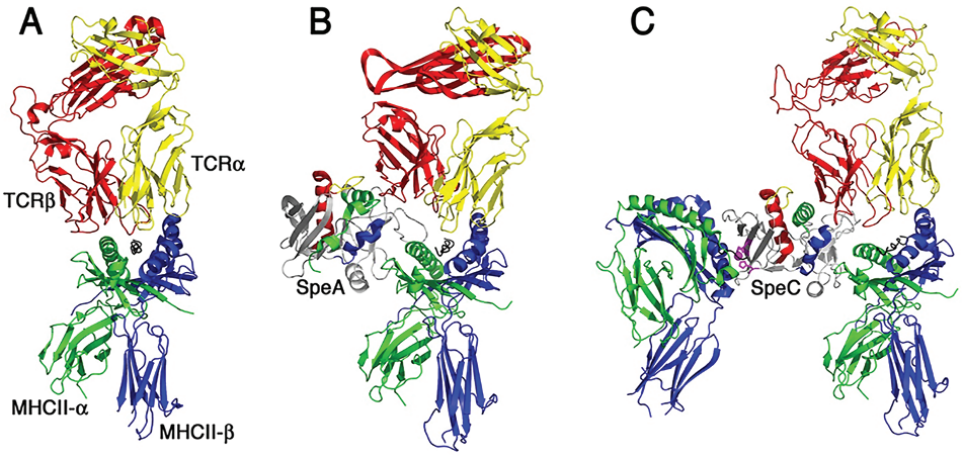


FIGURE 2 Models of T cell activation complexes for streptococcal superantigens. Ribbon diagrams demonstrating typical antigen-mediated T cell activation (A) and modeled T cell activation complexes for SpeA (B) and SpeC (C). The cocrystal structures of SpeA and SpeC in complex with their respective TCR β-chains (48) and of SpeC in complex with the MHC class II through the zinc-dependent high-affinity binding domain have been determined (169). SpeC also activates T cells in a mode similar to the staphylococcal enterotoxin A model (58) where SpeC engages MHC class II α-chain through a generic low-affinity binding domain (170) and engages the MHC class II β-chain through a zinc-dependent, high-affinity binding domain (169). The binding architecture for the generic low-affinity MHC class II binding to SpeA and SpeC is modeled using the staphylococcal enterotoxin B-MHC class II cocrystal structure (171). Note the presence of the zinc ion (magenta) coordinated in the high-affinity binding site for SpeC and that SpeA lacks this zinc site. The TCR α-chain (shown in gray) for both the SpeA and SpeC diagrams is modeled for clarity by superimposition of the α/β TCR shown on the left of the respective TCR β-chains for both superantigens. The figure was generated using Pymol.

antigenic peptide is displaced from the TC, and the specificity of the reaction is governed by superantigen recognition of the Vβ CDR2 loop (51).

Since early in the past century, the incidence of serious streptococcal disease greatly decreased, likely owing to the widespread use of antibiotics (59). However, it was not until the late 1980s that descriptions of well-defined cases led to the recognition of a severely toxic streptococcal superantigen-mediated disease called streptococcal TSS (60, 61). Indeed, since the mid-1980s there has been a sustained resurgence in the frequency and severity of streptococcal TSS and other invasive diseases caused by group A streptococci (62–64). This led to the hypotheses that there was an emergence of an excessively virulent strain or strains or, alternatively, that there had been major changes in host susceptibility to these infections. Indeed, many of the contemporary invasive infections have been attributed to the M1T1 clone (62), which is now recognized to have acquired an $SpeA_2$- and DNAse-encoding bacteriophage, as well as a horizontally acquired region encoding the SLO and SPN, likely from an M12 serotype (65). These acquisitions increase the virulence of this clone (66). Over the past decade, there has also been a dramatic resurgence in scarlet fever in mainland China and Hong Kong, South Korea, the United Kingdom, and Germany (67–70). Although it is not yet entirely clear, these large-scale outbreaks are likely linked to the acquisition of superantigen-producing bacteriophage elements in *S. pyogenes* (71–73).

Multiple lines of experimental evidence indicate that the streptococcal superantigens are significant virulence factors for severe streptococcal disease. The prototypical streptococcal superantigens are SpeA and SpeC, and these two toxins have been recognized as the likely causative agents in most cases of streptococcal TSS (61, 62, 74). Indeed, SpeA and SpeC are produced by many *S. pyogenes* strains isolated from patients with streptococcal TSS (29, 74–78). Due to the association of SpeA and SpeC with streptococcal TSS, two toxoid vaccines have been generated for these toxins that, when used in the rabbit model of TSS, provided complete protection to lethal quantities of the respective superantigen (53, 79). There are at least six naturally occurring alleles of *speA* (80, 81), and serotype M1 and M3 strains expressing the $speA_2$ and $speA_3$ alleles caused the majority of streptococcal TSS in the 1980s (81). Of the streptococcal superantigens that have been tested, the toxins share the properties of inducing fever (pyrogenicity) and lethal shock in the rabbit model. For example, recombinant SpeA, SpeC, and SpeJ can induce symptoms of streptococcal TSS without necrotizing fasciitis/myositis in this model (53, 79, 82), and SpeA administered to rabbits as a vaccine also conferred significant protection from TSS with necrotizing fasciitis/myositis after challenge with viable M1 and M3 streptococci (64). Circulating superantigens have also been found in patients with streptococcal TSS (83), and the lack of humoral immunity to the superantigens is a risk factor for the development of streptococcal TSS (84, 85).

Although significant immunological research with bacterial superantigens has been conducted in mouse models, conventional mice are no longer considered appropriate models to evaluate the biological effects of these toxins. Lethal effects are not seen at very high relative doses without the coadministration of a liver-damaging agent, whereas the rabbit model is susceptible to fairly low doses when administered continuously (86). It has further been demonstrated that HLA-DQ transgenic mice were more susceptible to SpeA-induced lethality whether administered as a pure toxin or during infection with $SpeA^+$ *S. pyogenes*, whereas nontransgenic mice

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did not show an obvious effect (87, 88). Consistent with this, it has been established that in humans, MHC class II allelic variation contributes to the differences in severity seen for invasive streptococcal infections (89). This is understood to be due to differential cytokine production triggered by streptococcal superantigens, and MHC class II α-chain polymorphisms affect superantigen responses (90, 91). Recently, using infection models with transgenic mice expressing human MHC class II alleles, we demonstrated that the SpeA superantigen is a critical bacterial factor that is necessary to induce an acute nasopharyngeal infection (92). Using this model, SpeA was shown to activate specific Vβ-subsets *in vivo* (93), and strikingly, functional Vβ-specific T cell subsets were required for the infection phenotype (94). Furthermore, humoral immunity to the SpeA superantigen dramatically inhibited infection by *S. pyogenes* (92, 94). We believe that these findings support the further development of toxoid superantigens as vaccines to target the transmission and colonization by *S. pyogenes*.

STREPTOCOCCAL PYROGENIC EXOTOXIN B

SpeB is highly conserved in virtually all *S. pyogenes* isolates (95, 96), although despite its original name as a streptococcal pyrogenic exotoxin, SpeB is known to function as a broad-spectrum protease and is secreted as a 40-kDa zymogen. The pro-domain is auto-catalytically cleaved to generate an active 28-kDa proteinase (97, 98). Additionally, an SpeB inhibitor located downstream of *speB* is cotranscribed with the protease. The inhibitor, termed Spi, has sequence and predicted structural homology with the pro-domain of SpeB (99). The inhibitor is resistant to cleavage by active SpeB, making it an effective inhibitor of protease activity within the bacterial cytoplasm (99). Although SpeB is highly conserved across most *S. pyogenes* strains, its expression may vary depending on the type of infection (100). For example, when analyzing isolates from patients with pharyngitis, impetigo, invasive disease, or acute rheumatic fever, 40% of isolates from patients with acute rheumatic fever produced SpeB, compared to only 5.5% of isolates from impetigo patients (100). Therefore, the variability in SpeB expression suggests that SpeB is more important for certain forms of disease progression.

SpeB is considered an important virulence factor most likely due to the immunomodulating effects of its protease activity (101, 102). SpeB has broad proteolytic activity against a number of human proteins, including interleukin-1β (102), immunoglobulins (101), fibrinogen (103), fibronectin (104), kininogens (105), and a metalloprotease (106). Although SpeB has been shown to cleave certain immunoglobulins, a study investigating the cleavage of IgG under physiologic conditions found that IgG is only cleaved in a reduced form, whereas physiologic IgG is active in an oxidized form (107). Therefore, SpeB likely does not contribute to *S. pyogenes* pathogenesis through the cleavage of IgG. The innate host immune system is also weakened through proteolytic destruction of complement factors such as C3 and the membrane attack complex (108, 109). SpeB also has the capacity to cleave many human chemokines (110), further disrupting the immune response. In addition to the many immune modulating factors degraded by SpeB, the cysteine protease also degrades tight-junction proteins such as E-cadherin and desmogleins, facilitating the paracellular invasion of the bacteria across the epithelial barrier (111, 112). Therefore, the broad spectrum of SpeB targets likely ameliorates the virulence of *S. pyogenes* by perturbing the host immune system and compromising the epithelial barrier.

Given the broad spectrum of SpeB, *S. pyogenes* proteins can also be proteolyzed (113). SpeB can directly degrade multiple superantigens, such as SmeZ, which affects the ability of the superantigen to induce proliferation of human lymphocytes (114, 115). In addition, the cleavage of protein F1, an *S. pyogenes* cell wall-attached fibronectin-binding protein, decreased the fibronectin-dependent internalization of *S. pyogenes* into human cells, suggesting that SpeB also plays a role in the internalization process (116). Likewise, SpeB-deficient mutants were shown to have enhanced *in vitro* internalization into human epithelial and endothelial cells (117). Therefore, an inactive SpeB may be favored later during infection to promote the dissemination of the bacteria (116). Furthermore, earlier work identified a phase shift in an invasive M1T1 strain *in vivo* favoring a nonfunctional cysteine protease to conserve the function of the multiple virulence factors normally degraded by SpeB (118, 119). This phase shift is linked to inactivating mutations within the two-component CovRS sensor kinase, which results in a hypervirulent phenotype in the serotype M1 background (120). An inverse relationship has also been identified between SpeB expression and the severity of invasive group A streptococci infections, further suggesting that *S. pyogenes* may need to differentially regulate SpeB expression, depending on the site of infection and events occurring during infection (121).

In vivo studies supporting the role of SpeB as a virulence factor have not always been congruent. For example, an *S. pyogenes* strain with decreased SpeB expression exhibited a decreased capacity to cause murine necrotizing fasciitis, and complementation of SpeB restored the wild-type phenotype (122). Alternatively, isogenic gene replacement strains and a mouse model of invasive soft tissue infection showed that *speB* mutants had no apparent effect on the ability of group A streptococci to cause local tissue injury and invasive infection (123). Overall, the diversity of SpeB targets may contribute to the protease being both a virulence factor and a form of virulence regulation through the degradation of bacterial proteins.

DNases OF *S. PYOGENES*

The DNases of *S. pyogenes* constitute an additional mechanism that contributes to the pathogenicity of the bacterium. Through the analysis of all known *S. pyogenes* genomes, eight DNases have been identified (124): *spnA*, *spdB*, *sda1*, *sda2*, *spd1*, *spd3*, *spd4*, and *sdn*. Of the known DNases, *spnA* and *spdB* are chromosomally encoded and are found across all *S. pyogenes* isolates (125, 126). The six other DNases are prophage associated, and therefore they are only found in certain *S. pyogenes* strains (124). Furthermore, the DNase *spnA* is the only known cell wall-anchored DNase of *S. pyogenes* (124, 126).

DNases contribute to the pathogenesis of *S. pyogenes* by facilitating innate immune evasion of the pathogen. During infection, neutrophils release antibacterial granule proteins and chromatin to create neutrophil extracellular traps (NETs) (127). NETs bind and trap bacteria and degrade bacterial virulence factors, ultimately resulting in bacterial death (127). The secreted DNase Sda1 and the cell wall-anchored DNase SpnA are both able to degrade the chromatin backbone of NETs, allowing the bacteria to avoid the degradation of their virulence factors and promote bacterial survival (128–130). *S. pyogenes* DNases further contribute to pathogenesis through the degradation of bacterial DNA (131). By degrading CpG-rich bacterial DNA, the TLR9-mediated immune response is weakened due to the

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degradation of the TLR9 activating signal (131). In a model of mouse necrotizing fasciitis, Sda1 was shown to suppress TLR9-dependent tumor necrosis factor-α and tumor necrosis factor-α induction, promoting evasion of the innate immune system (131). Additionally, a murine model of skin and soft tissue and a cynomolgus macaque model of pharyngitis infection showed that isogenic mutants of *S. pyogenes* lacking DNase production were less virulent than the wild-type strain, highlighting their importance in pathogenesis (129). The ability of DNases to degrade NETs and bacterial DNA, along with the decreased virulence of DNase-deficient mutants *in vivo*, emphasize the importance of *S. pyogenes* DNases as extracellular virulence factors.

STREPTOCOCCAL INHIBITOR OF COMPLEMENT

The streptococcal inhibitor of complement (SIC) is a 31-kDa protein secreted by certain *S. pyogenes* strains, most notably M1 serotypes (132). SIC is highly variable, with large numbers of variants arising *in vivo* during epidemic spread of the organism (133, 134). Such high variation suggests that the phenomenon offers selective advantage to the microbe. Most of the variants are single amino acid changes, insertions, or deletions. Other M types may make related proteins, some of which have minimal effect on the complement system (135). SIC was originally identified as a protein that inhibits the membrane attack complex of the complement system, with its mechanism being the inhibition of C567 insertion into the membrane (136). Furthermore, SIC is rapidly internalized by neutrophils and binds specifically to ezrin and moesin, proteins that link the actin cytoskeleton to the host cell surface, interfering with polymorphonuclear opsonophagocytosis and intracellular killing (136). SIC also directly inhibits other components of the innate immune system, including lysozyme, secretory leukocyte proteinase inhibitor, human defensins, and cathelicidin LL-37, all of which can be toxic to *S. pyogenes* (137–140). Additionally, SIC inhibits the release of bradykinin, a potent vasodilator, which could function to decrease inflammation at the site of infection, allowing the bacteria to persist (141). More recently, SIC also inhibited the bactericidal activity of histones, which are found in NETs (142).

In vivo studies suggest that SIC increases streptococcal virulence. Mice that were inoculated intranasally with M1 SIC-negative streptococci had a lower incidence of throat colonization than mice inoculated with the wild-type M1 strain (143). SIC-negative strains also exhibited a decrease in survival in macrophages (137). Therefore, SIC likely promotes bacterial survival and dissemination by allowing the organisms to avoid innate immune defenses in the extracellular environment.

IgG-TARGETING ENZYMES OF *S. PYOGENES*

S. pyogenes has evolved multiple mechanisms to target IgG antibodies, a major effector molecule of the humoral immune system. The immunoglobulin G-degrading enzyme (IdeS, also known as Mac) is a protease that removes the Fc region of IgG antibodies and can thus inhibit opsonophagocytosis of *S. pyogenes* (144, 145). This virulence factor is specific for IgG and does not target IgM, IgE, or IgD antibodies (144). However, *in vivo* studies using a mouse invasive infection model failed to demonstrate a contribution of IdeS to virulence (146). Endoglycosidase S (EndoS) is an ~108-kDa secreted enzyme that hydrolyzes the β-1,4 linkage between the first two *N*-acetylglucosamine residues on the glycan linked to Asn297 within the C_H2 domain of the IgG Fc region (147). Deglycosylation of IgG through this mechanism impairs antibody effector functions, including binding to the Fc-gamma receptor and complement activation (148). Nevertheless, similar to IdeS, deletion of the gene encoding EndoS in the M1 5448 background had no obvious phenotype in an invasive intraperitoneal mouse infection model (149). In both cases, the lack of an *in vivo* phenotype may reflect a more subtle role for these enzymes that is not apparent through acute, invasive infections. In addition to EndoS and IdeS, which show high levels of specificity for IgG, the broad-spectrum SpeB cysteine protease is also capable of cleaving IgG in the hinge region, similar to protease papain (147); however, the *in vivo* relevance of this has been questioned (107).

STREPTOKINASE

Streptokinase is a single-chain 414-amino acid protein that is able to activate the host protein plasminogen (150). Streptokinase forms a complex with the inactive zymogen plasminogen, or a trimolecular complex with plasminogen and fibrinogen, to generate the active serine protease plasmin (151, 152). These activator complexes bind to the bacterial surface, allowing the bacteria to acquire additional protease activity. Plasmin is responsible for degrading fibrin clots, connective tissue, extracellular matrix components, and adhesion proteins (153). Therefore, the improper activation of plasmin could result in widespread tissue damage and dissemination of the bacteria (154, 155). Transgenic mice expressing human plasminogen had an increased mortality rate compared to mice without the transgene. This susceptibility was directly related to streptokinase expression, highlighting the importance of streptokinase in *S. pyogenes* virulence (154). Furthermore, increased levels of bacteria-bound plasmin correlated with a decrease in C3b deposition and a decrease in C3b-mediated neutrophil killing (156). Additionally, bacteria-associated plasmin has been shown to prevent histone-mediated killing by NETs (157). In addition to activating plasminogen, streptokinase has also been shown to activate the contact system, resulting in the release of bradykinin (158). The release of bradykinin triggers vascular leakage, which could further promote the dissemination of the bacteria (159, 160). The release of streptokinase by *S. pyogenes* ultimately promotes the dissemination of the bacteria by facilitating the conversion of plasminogen to plasmin.

FUTURE PERSPECTIVES

The increased incidence of severe invasive group A streptococcal diseases, and the recent resurgence of scarlet fever, still remain incompletely explained; however, the streptococcal superantigens are clearly implicated in both illnesses and thus are key virulence factors driving the evolution of *S. pyogenes* pathogenesis. Indeed, the majority of genetic diversity in different strains and serotypes of *S. pyogenes* occurs due to the presence or absence of large mobile genetic elements, including lysogenic bacteriophage and integrative conjugative elements (161, 162). In particular, many of the superantigens, as well as DNases, and some other select toxins are encoded within bacteriophage elements, and this provides *S. pyogenes* with an extra level of adaptability. The apparent excessive redundancy of these toxins has yet to be explained, although we believe this allows *S. pyogenes* to avoid neutralizing antisuperantigen antibodies and may also provide additional means to efficiently target polymorphic MHC class II molecules from different populations.

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Although most research on streptococcal exotoxins has focused on their role in severe streptococcal diseases, the established niche for *S. pyogenes* is a state of colonization in the throat or on the skin of humans. Also, multiple *S. pyogenes* virulence factors do not operate efficiently in mouse models (92, 154, 163–165). To understand the basic biology of this organism, which is not related to severe and invasive disease, better models will be necessary to evaluate specific virulence factors, therapies, and vaccines. Thus, most, if not all, of these remarkable exotoxins that can alter normal immune system function, damage tissue, and promote disease have each likely evolved in the context of streptococcal persistence and transmission. We hope a clearer understanding will lead to further rationales to design vaccines capable of targeting the colonization state of *S. pyogenes*.

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Genetics of Group A Streptococci

KYU HONG CHO, GARY C. PORT AND MICHAEL CAPARON

6

INTRODUCTION

Streptococcus pyogenes (the group A streptococcus) is remarkable in terms of the large number of very different diseases it can cause in humans. These range from superficial and self-limiting diseases of the pharynx (e.g., pharyngitis, commonly known as strep throat) and skin (impetigo) to infections that involve increasingly deeper layers of tissue and are associated with increasing degrees of destruction of tissue (e.g., erysipelas, cellulitis, necrotizing fasciitis, and myositis). The organism has the ability to spread rapidly through tissue and to penetrate into the vasculature to cause lethal sepsis. Other diseases result from the production of toxins that spread through tissue or spread systemically from a site of local infection (scarlet fever and toxic shock syndrome). Still other diseases are the result of an immunopathological response on the part of the host that is triggered by a streptococcal infection. These diseases include rheumatic fever, acute glomerulonephritis, certain types of psoriasis, and potentially even some forms of obsessive-compulsive syndrome disorder (pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections).

S. pyogenes is even more remarkable in terms of the large number of factors that have been identified as potential virulence determinants for these various diseases. These include surface proteins (M proteins, fibronectin-binding proteins, surface dehydrogenases, C5a peptidase), the hyaluronic acid capsule, secreted degradative enzymes (several distinct DNases, a cysteine protease, NAD^+-glycohydrolase, hyaluronidases,) and many secreted toxins (streptolysin S, streptolysin O, the pyrogenic exotoxins, streptococcal superantigens, streptokinase). This is only a partial list of the potential virulence factors this bacterium can produce, and many of these are considered in more detail elsewhere.

Until recently, the function that any of these potential virulence factors contributed to the pathogenesis of any specific streptococcal disease was only poorly understood. A major reason for this deficiency was the lack of sophisticated genetic systems that could be applied to the analysis of a specific virulence factor according to modern molecular criteria. These criteria were succinctly outlined by Falkow in his "Molecular Koch's Postulates" (1). These state that (i) the phenotype under investigation should be associated with pathogenic members of a species, (ii) the gene(s) associated with the virulence trait should be identified and isolated by molecular methods, (iii) specific inactivation of identified gene(s) should lead to a measurable loss in pathogenicity, and (iv) reintroduction of the unmodified wild-type gene should lead to a restoration of pathogenicity. An exciting development is that, over the past several decades, the work of many groups has contributed to the development of sophisticated group A streptococcal genetic systems that now allow the many diseases that this organism can cause to be studied at this level of molecular resolution. The modern *S. pyogenes* genetic modification systems are supported by the numerous complete genome sequences now available. Since the first complete genome sequence of an *S. pyogenes* strain in 2001 (2), 61 complete genome sequences and approximately 300 draft sequences have been deposited as of March 2018 (3). The average *S. pyogenes* genome is approximately 1.85 Mb and encodes ~1,800 genes, the function of many of which remains to be elucidated (4). The other major advance has been in the development of a variety of genetic tools aimed at manipulating the *S. pyogenes* genome. The goal of this article is to present an overview of these tools, including the methods of genetic modification and their applications.

GENETIC EXCHANGE

Transduction

A key element of any genetic system involves some system of genetic exchange between bacterial hosts, allowing the construction of an altered genome in the target host which can then be subjected to an analysis of its virulence phenotypes. Unlike several other species of streptococci, the group A streptococci are not naturally competent for the uptake of exogenous DNA, even though their genomes encode many of the genes required for transformation (see below). Conjugative DNA transfer does occur in group A streptococci; however, this is restricted to the transfer of conjugative plasmids and conjugative transposons (see below), and there is no evidence of mobilization of chromosomal markers. There is also no evidence that any important virulence traits are encoded by these types of mobile elements, although they are undoubtedly important in the transmission of resistance to various antibiotics.

Even though there is no evidence to support genetic exchange by transformation or by conjugation, analysis of several polymorphic loci, most notably the genes which encode the M proteins and the fibronectin- and collagen-binding proteins and T antigen (FCT)/pilus region have provided considerable evidence of horizontal transfer of

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genetic material among natural populations of *S. pyogenes* (5). In this regard, considerable attention has been focused on the contribution of phage to genetic transfer (5). *S. pyogenes* strains are rich in phage, and several of these have been demonstrated to encode virulence factors, including the pyrogenic exotoxins SPE-A and SPE-C (6). Thus, it is not surprising that transduction was the first mechanism of genetic exchange that was exploited in the manipulation of the *S. pyogenes* genome (7). The most highly developed of these are derivatives of phage A25. This lytic phage is classified as a Bradley group B phage that recognizes the peptidoglycan of groups A, C, and G streptococci as its cellular receptor. It has a 35-kb double-stranded genome with circular permutation and terminal repetition (8), and it is proficient in transducing markers *in vitro* (for a detailed description of a method of transduction, see reference 9). The most useful derivatives of A25 for use in transduction are those developed by Malke and colleagues, who constructed a derivative with two distinct temperature-sensitive lesions ($A25_{ts1\text{-}2}$) that becomes defective for growth at 37°C (10). This feature is useful for transduction, since it allows the production of a transducing lysate of the donor host at the permissive temperature (30°C) but prevents the killing of transduced hosts when infection is performed at the nonpermissive temperature. The A25 phage was used in some of the first mutagenesis studies in group A streptococci, including the original identification of Mga, the master regulator of M protein (7). In spite of the early use of this method, genetic manipulation via phage transduction is inherently limited in application since it requires preexisting antibiotic resistance markers within or near genes of interest. Additionally, some phage possess narrow host specificities, thereby limiting their utility to only a few strains. Furthermore, phage transduction can be laborious. Fortunately, simpler and more rapid genetic engineering methods, including electroporation of shuttle plasmids and saturating transposon mutagenesis, have largely replaced phage transduction (see below).

Transformation

One of the most significant advances in genetic technology for *S. pyogenes* has been the development of methods for transformation. This has allowed the introduction of heterologous DNA into an *S. pyogenes* host and has opened the door to a large number of techniques for mutagenesis and allelic exchange that are described in greater detail below. The breakthrough technology that began the era of transformation for *S. pyogenes* was the introduction by several companies of reasonably priced instruments for the introduction of DNA by electroporation. As is true for most methods of transformation, success with electroporation-based transformation results from careful attention to growth conditions. Electroporation of streptococci generally requires that the cell walls be weakened. Most successful methods have adapted the techniques originally developed by Dunny and colleagues for electroporation of the enterococci (11). This method uses cells from the early exponential stages of growth in medium supplemented with glycine. The addition of glycine is thought to contribute to a decreased level of cross-linking in the cell wall, and the exact stage of growth and concentration of glycine is determined empirically (for a detailed description of the method, see references 9, 12–14).

It is of interest to note that the genomic sequences of several *S. pyogenes* strains of the M1, M3, and M5 serotypes have revealed that while they have genes required for DNA uptake by natural transformation and that while these genes are expressed in response to a peptide pheromone, they remain nontransformable (15). In addition, a cluster of genes associated with the ability to cause disseminated infection, called the Sil locus, contains genes highly homologous to the *comAB* competence locus (16). To date, the Sil genes have only been found among serotype M14 and M18 strains, and since their G+C content is markedly lower than the rest of the *S. pyogenes* chromosome, they may have been more recently acquired by these lineages from horizontal transmission (17).

Plasmid Technology

The development of efficient transformation systems has allowed for the rapid genetic modification of *S. pyogenes* hosts through the introduction of plasmids into *S. pyogenes*. Applications include (i) construction of chromosomal allelic replacements, gene deletions, and site-specific mutations; (ii) complementation of chromosomal mutations *in trans*; (iii) analysis of reporter gene expression; and (iv) delivery of transposable elements to construct random mutant libraries for forward genetic screens. Common Gram-negative plasmid replicons (including pUC, pBR322, and pSC101) are unable to replicate in Gram-positive species and can therefore serve as "suicide" delivery vectors for transposon delivery or recombination-based chromosomal integrative elements (see below). Also, many of the plasmids which have proven useful for genetic analysis in other Gram-positive species, including pC194, pE194, and pUB110, appear unable to replicate reliably in *S. pyogenes*. Fortunately, a handful of plasmids have been identified that do replicate in *S. pyogenes*, and these plasmids have been engineered over the past 20 years to give rise to a growing set of tools aimed at manipulating the *S. pyogenes* chromosome (Table 1).

The most widely used plasmid for *S. pyogenes* genome engineering is derived from pWV01 (18). This plasmid was isolated from a lactococcal species and is the prototype member of a family of "promiscuous replicons" that have the remarkable property that they can replicate in both Gram-negative (e.g., *Escherichia coli*) and Gram-positive (e.g., *S. pyogenes*) hosts. pWV01 replicates using a rolling-circle type mechanism characteristic of many single-stranded bacteriophages and likely exists in a single-stranded conformation for extended periods of time in replicating cells. This can have consequences for plasmid structural integrity, segregation instability, and expression of open reading frames inserted for complementation or ectopic or orthogonal expression (19). Common derivatives of pWV01 include plasmids such as pABG5 (Fig. 1), which include an *S. pyogenes* promoter for ectopic or orthogonal expression (20), and the high-copy variant pBAV1kT5, which lacks a set of inverted repeats and an open reading frame thought to be responsible for copy number regulation (21). A versatile and widely used derivative of pWV01 known as pG+host4 was developed by Maguin and coworkers to contain a temperature-sensitive (ts) replicon (22). The plasmid contains four amino acid substitutions in the RepA protein, which is responsible for nicking one DNA strand at the plus origin to initiate replication, thus rendering the process temperature sensitive. Subsequent derivatives of this plasmid include pJRS233 (23) and pGCP213 (24), which add low copy (pSC101) or high copy (ColE1/pUC) thermo-stable Gram-negative replication origins, respectively, to facilitate various applications for subcloning and plasmid propagation in *E. coli*. In addition, pGCP213 includes a full-length gene encoding LacZ containing the multiple cloning site from pCR2.1 (Invitrogen), allowing for blue/white screening of insertions via

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TABLE 1 Common plasmids utilized for *S. pyogenes* genome engineering

Capability	Name	Ori G+/G–	Ab cassette(s)	Features	Reference(s)
Site-directed mutagenesis	pJRS233	pWV01 ts/pSC101	*erm*C	Low copy (*E. coli*) thermosensitive shuttle vector	23
	pGCP213	pWV01 ts/pUC (pBR322)	*erm*C	High copy (*E. coli*) thermosensitive shuttle vector	24
	pCRK	pWV01 ts/pUC (pBR322)	*aphA3*	High copy (*E. coli*) thermosensitive shuttle vector	25
	pCRS	pWV01 ts/pUC (pBR322)	*aad9*	High copy (*E. coli*) thermosensitive shuttle vector	25
	pHY304	pWV01 ts/NA	*erm*C	Thermosensitive vector, must be grown at 30°C	111
	pSinS/pHlpK	pCRS (ΔrepA) pCRK (ΔpUC)	*aad9*/*aphA3*	Conditional suicide plasmid pSin stabilized by temperature-sensitive helper plasmid	42
Complementation	pABG5	pWV01/NA	*aphA3*/*cat*A	Thermostable shuttle vector, ProfA promoter	20
	p7INT, p7INT[a] (pGCP365)	NA/pUC (pBR322)	*erm*C	Integrates into prophage tmRNA attB site	33
	pEU7742	pAMβ1/NA	*aad9*	Single copy with toxin/antitoxin, contains theophylline-inducible riboswitch and *gusA* reporter	32
	pGCP189	pWV01 ts/pSC101	*erm*C	Insert gene of interest downstream of GuaB in synthetic polycistron	45
Transposon	pMGC57-TnErm	NA/pHSG396 (pBR322)	*cat*A/*erm*C (for Tn)	Tn*4001*	80
	EZ-Tn*5*	NA/NA	*erm*C	Tn*5*	43
	pOSKAR	pWV01 ts/pUC (pBR322)	*aad9*/*aphA3* (for Tn)	Mariner Kan-Tn flanked by T7 For microarrary TraSH	25
	pKRMIT	pWV01 ts/pUC (pBR322)	*aad9*/*aphA3* (for Tn)	Mariner Kan-Tn flanked by MmeI For TnSeq	42
Reporter	pGCP070	pWVO1/NA	*aphA3*/*cat*A	TetR inducible sfGFP	100
	pWAR303	pWV01	*erm*C	Luciferase LuxAB	112

[a]Common antibiotic markers used in group A streptococcus mutagenesis include *aad9* (spectinomycin resistance, Sp^r), *cat*A (chloramphenicol resistance, Cm^r), *aphA3* (kanamycin resistance, Km^r) and *ermC* (erythromycin resistance, Em^r). Because group A streptococcus remains sensitive to penicillin and is a preferred clinical treatment, the use of β-lactamase for selection during group A streptococcus mutagenesis is both unethical and prohibited.

α-complementation in *E. coli*. Plasmids pCRS and pCRK are similar to pGCP213, differing primarily in their antibiotic resistance cassettes (25). The modifications to the standard electro-transformation conditions described by Scott's group greatly increase the transformation efficiency of these ts plasmids (23). The main application of the pG+host-derived ts plasmids has been in the construction of in-frame deletions and allelic replacement to introduce site-specific mutations of chromosomally encoded genes and in the delivery of transposable elements for mutagenesis (see below).

These plasmid-based methodologies can be adapted for functional studies to validate the contribution a given gene confers to a given phenotype via expression in a heterologous host. The heterologous host can be an *S. pyogenes* strain which naturally lacks the phenotype under investigation or can be another streptococcal or enterococcal species. In one of the first examples of this strategy, Scott and Fischetti's group introduced a copy of the gene which encodes the M6.1 protein (*emm6.1*) on a mobilizable plasmid vector, used this to transform a naturally competent streptococcal species, and then mobilized the plasmid by conjugation into an *emm*-deficient *S. pyogenes* host (26). Analysis of the resulting strain demonstrated that the introduction of *emm6.1* converted the host strain from being sensitive to killing in a bactericidal assay to being resistant to killing in the assay. This study was the first formal application of Falkow's molecular Koch's postulates to *S. pyogenes* pathogenesis (26). Additional applications of this approach have included the demonstration that protein F is sufficient to confer a fibronectin-binding phenotype to non-fibronectin-binding *S. pyogenes* and enterococcal hosts (27) and that the *has* operon is sufficient to confer the ability to produce hyaluronate to both acapsular *S. pyogenes* and enterococcal hosts (28).

The second class of commonly used vectors in *S. pyogenes* was based on the low-copy pAMβ1 replicon originally isolated from *Enterococcus faecalis* (29). Unlike pWV01, the pAMβ1 replicon cannot replicate in *E. coli*, so derivatives have been engineered to contain an origin of replication that is proficient in an *E. coli* host (19). The plasmid pAT28 developed by Courvalin's group (30) is typical of the pAMβ1-derived vectors and contains the high-copy pUC replicon derived from ColE1 for replication in *E. coli* and several other useful features including the multiple cloning site and the *lacZα* reporter gene of pUC18,

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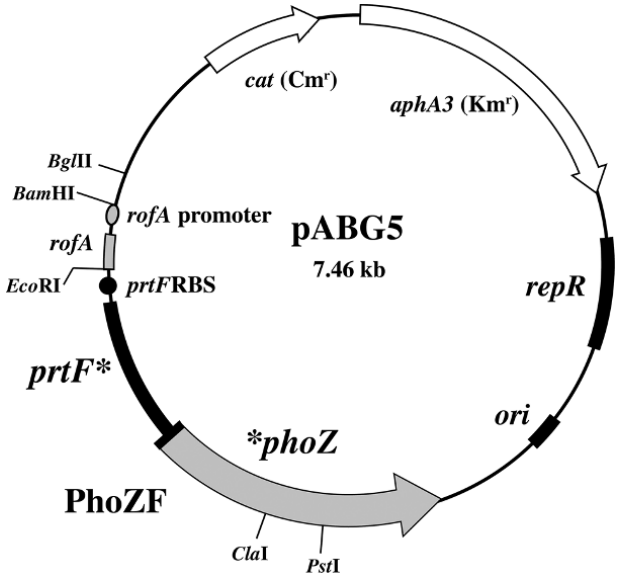


FIGURE 1 A chimeric secreted alkaline phosphatase reporter gene. The plasmid pABG5 is a pWV01-based *E. coli*-streptococcal shuttle vector which encodes resistance to chloramphenicol (*cat*) and kanamycin (*aphA3*). The plasmid contains a promoterless reporter gene formed by the fusion of the N-terminal region of the cell wall-associated protein F (*prtF**) to the enzymatic domain of the enterococcal alkaline phosphatase (**phoZ*). Since the chimeric protein (secreted protein F-PhoZ reporter, or PhoZF) lacks the C-terminal cell wall attachment domain of protein F and the N-terminal lipoprotein tethering domain of PhoZ, it is freely secreted from the cell. The PhoZF chimera retains the enzymatic activity of PhoZ and can be easily quantitated in culture supernatants. Restriction sites for *Bgl*II or *Bam*HI and *Eco*RI can be used to place the promoter of interest in an orientation to direct transcription of *phoZF*, which is then translated using the ribosome-binding site of *prtF* (RBS). Insertion of an open reading frame of interest between the *Eco*RI and either the *Cla*I or *Pst*I site places the open reading frame under the control of the strong *rofA* promoter for ectopic expression and/or complementation analyses.

which allows screening for insertion of cloned DNA by α-complementation in *E. coli* (30). In contrast to the rolling circle replication of pWV01, pAMβ1 replicates via a double-stranded θ-type mechanism which typically leads to greater plasmid stability. Also, as a general rule, pWV01-based vectors transform *S. pyogenes* at a much higher efficiency than do pAMβ1-derived vectors. Because of their different modes of replication, this difference may involve how the restriction system of *S. pyogenes* recognizes the two plasmids. However, it should be noted that very little is currently understood about restriction in *S. pyogenes* and that, in general, it has not posed a significant barrier to the introduction of DNA purified from any number of different *E. coli* K12 hosts. Despite its lower transformation efficiency, the pAMβ1-derived vectors have proven very useful in obtaining expression of cloned genes that were only poorly expressed by pWV01-based vectors (31). A useful derivative of pAMβ1 is pEU7742 (32), which includes a toxin-antitoxin cassette (axe-txe) from an *Enterococcus faecium* plasmid for enhanced plasmid stability.

An alternative to using replicating plasmids for expression of heterologous genes or genes *in trans* is the vector system known as p7INT, which contains an *E. coli* origin of replicon to facilitate manipulation in *E. coli*, along with the integrase gene (*int*) and attachment site (*attP*) from the T12 temperate bacteriophage of *S. pyogenes* (33) (Fig. 2). Following its introduction into *S. pyogenes*, *int* catalyzes the site-specific recombination of the entire circular molecule into the chromosomal phage T12 attachment site (*attB*) located at the 3′ end of the transfer-messenger RNA gene (33) by a mechanism resembling integration of lambda phage (34). Because the plasmid lacks the phage excisionase, integration is highly stable. Derivatives of p7INT have been used to study Rgg-dependent gene regulation through use of a *luxAB* luciferase-based reporter system (35). It should be noted that integration of p7INT is strain specific, because two alleles of transfer-messenger RNA (tmRNA) exist among the published sequences, which are distinguished by a single nucleotide polymorphism in *attB*. Common strains, including NZ131 and HSC5, have an *attB* site compatible for p7INT integration, while others, including JRS4, have an incompatible site. However, p7INT can be modified by a single base change, generating p7INT*, which can integrate into the alternative *attB* site (G.C. Port and M.G. Caparon, unpublished) (Fig. 2).

DIRECTED MUTAGENESIS

The techniques developed for directed mutagenesis (reverse genetics) in *S. pyogenes* have proven invaluable for the analysis of the contributions that specific genes make to pathogenesis. In this approach, a defined mutation is constructed to inactivate, or in some cases modify, a specific gene to construct an isogenic mutant strain for analysis. These techniques have become particularly useful with the availability of the complete or draft genome sequences of *S. pyogenes* (3). In the following, we consider methods to alter or inactivate a targeted gene on the *S. pyogenes* chromosome using (i) allelic replacement using linear DNA, (ii) directed insertional integration, and (iii) construction of in-frame deletion alleles.

Allelic Replacement Using Linear DNA

This method of mutagenesis is straightforward and involves first cloning the gene of interest into any suitable plasmid vector in *E. coli*. With the available genome information, this has become a simple task of analyzing the sequence and designing suitable primers for PCR amplification, followed by insertion of the amplification product into an *E. coli* cloning vector. As a general rule, *S. pyogenes* DNA is most stable when cloned on low-copy vectors in *E. coli*, and several vectors have been used with great success. The standard PCR methodology is then used to insert a selectable marker into the open reading frame to inactivate it. The markers that have been most successful are those derived from Gram-positive organisms that can also be used for selection in *E. coli* and include *erm*C (resistance to erythromycin; GenBank accession no. NG_047805.1), *aphA3* (resistance to kanamycin; GenBank accession no. V01547.1), *tet*M (resistance to tetracycline; GenBank accession no. X92947.2), *aad*9 (resistance to spectinomycin; GenBank accession no. M69221.1), and *cat*A (resistance to chloramphenicol; GenBank accession no. NG_047564.1). It should be noted that because resistance to β-lactam antibiotics does not naturally exist among clinical *S. pyogenes* isolates, for ethical reasons, genes encoding any type of β-lactamase must not be used for this application. Strong polar mutations can be introduced through the use of dedicated insertional mutagenesis cassettes,

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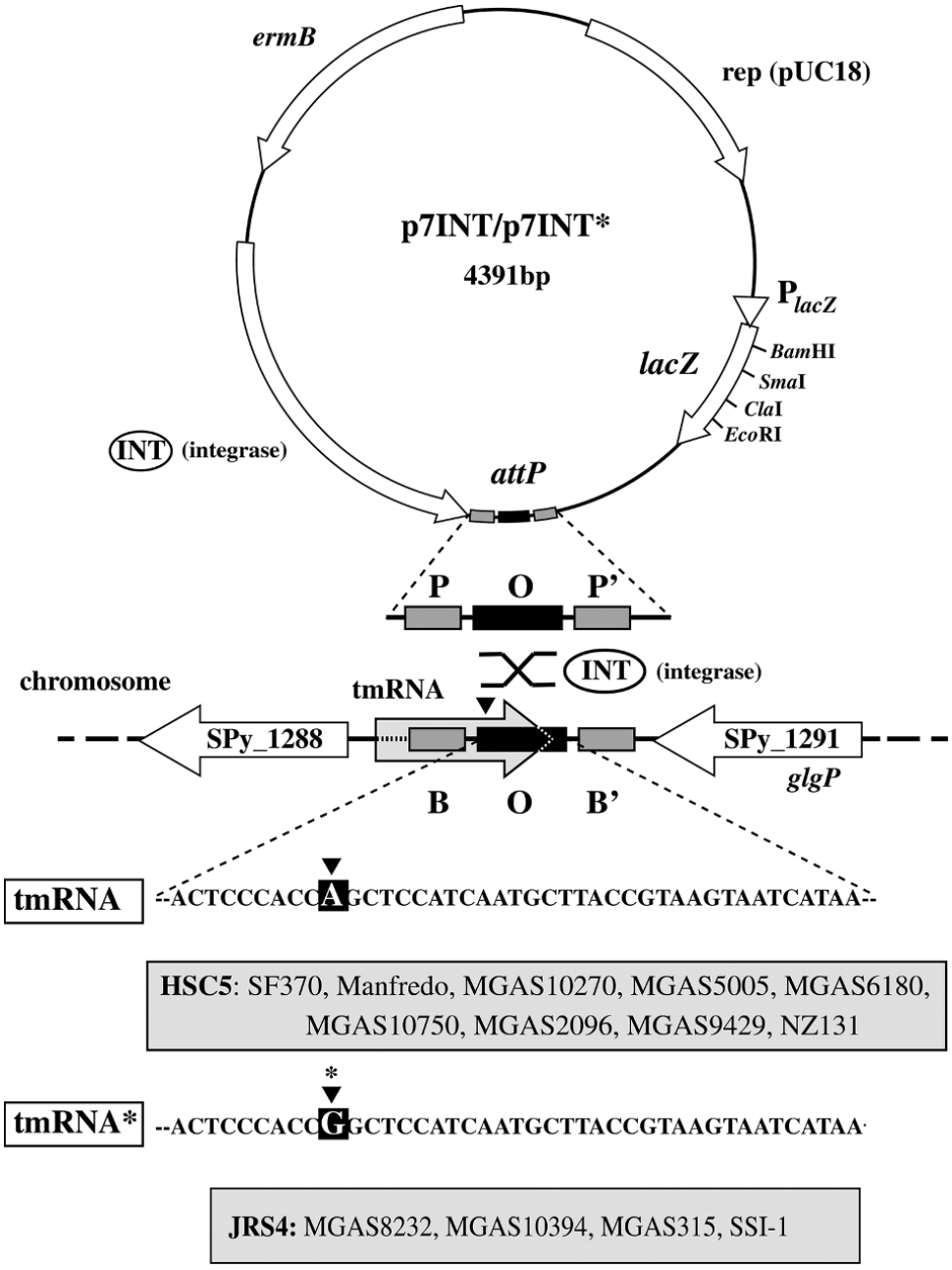


FIGURE 2 p7INT and p7INT*. The plasmids p7INT and p7INT* are pUC18-based *E. coli* vectors that encode resistance to erythromycin (*erm*). Restriction sites for *Bam*HI, *ClaI*, *SmaI*, and *Eco*RI within *lacZ* can be used to insert any promoter and gene of interest for ectopic expression and/or complementation analyses. The plasmids also contain a phage integrase (INT) and an *attP* sequence which targets integration into the *S. pyogenes* chromosome at the 3′ end of the tmRNA locus within the 96-bp "O" site of POP′ and BOB′ of *attP* and *attB*, respectively. With respect to O sites, *S. pyogenes* has been found to contain one of two alleles with either an A or G (see text). Plasmid p7INT contains an A, whereas p7INT* has been mutated to contain a G, and the appropriate plasmid must be used depending on the allele found within the *S. pyogenes* strain of interest. Following introduction of the appropriate plasmid into *S. pyogenes*, selection for the antibiotic-resistant determinant of the plasmid (*erm*) selects for chromosomes in which the plasmid has integrated into the chromosome at the *attB* site. The distribution of tmRNA and tmRNA* between a selection of commonly used strains is shown, with GenBank accession numbers as follows: HSC5, CP006366.1; SF370, AE004092.2; Manfredo, AM295007.1; MGAS10270, CP000260.1; MGAS5005, CP000017.2; MGAS6180, CP000056.1; MGAS10750, CP000262.1; MGAS2096, CP000261.1; MGAS9429, CP000259.1; NZ131, CP000829.1; JRS4, CP011414.1; MGAS8232, AE009949.1; MGAS10394, CP000003.1; MAGS315, AE014074.1; SSI-1, BA000034.2.

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such as the Omega interposon (ΩKm-2), which contains strong transcription and translation termination signals (36). Following its construction, the insertionally inactivated allele is converted to a linear molecule by PCR amplification or restriction digestion of the backbone plasmid. The resulting linear molecule is then used to transform the target *S. pyogenes* host with selection for the inserted resistance marker. Transformation of linear DNA is best conducted using highly purified salt-free DNA (for example, see 37). Because the introduced molecule is linear, preservation of the circular chromosomal structure requires that all resistant transformants arise by two homologous recombination events flanking each side of the inserted resistance marker. The end result is the exchange of the wild-type allele for the inactivated allele (Fig. 3). While this type of recombination does not occur at high frequency, the method is usually successful because the frequency of nonhomologous recombination is typically extremely low. Insertion into the expected locus is confirmed using PCR-based analyses and/or genome sequencing, and the resulting mutant can be subjected to functional studies. However, in interpreting the resulting functional data, it should be kept in mind that this method of mutagenesis generates strong polar mutations. Thus, expression of distal genes should be examined using quantitative real-time reverse transcriptase PCR (qRT-PCR) or other methods suitable for analysis of gene expression.

A variation of this technique can be used to subject a large cloned region to rapid high-resolution insertional mutagenesis, which can be useful for analyses of complex phenotypes and pathways whose genes are often clustered together on the chromosome. This method uses a version of the *E. coli* transposon mini-γδ (mγδ-200) that has been modified to contain a kanamycin-resistance gene that can be selected for in both *E. coli* and *S. pyogenes*. Called "shuttle mutagenesis," this technique uses the ability of m-γδ to easily and efficiently generate a large number of random transposon insertions into a segment of DNA cloned on a plasmid to obtain a series of mutations along the entire length of the cloned streptococcal DNA. These sets of nested insertions are then crossed into the streptococcal chromosome as described above. This method has been successfully used to identify regulatory genes linked to a target gene of interest (38). For a detailed description of this technique, consult Hanski et al. (39).

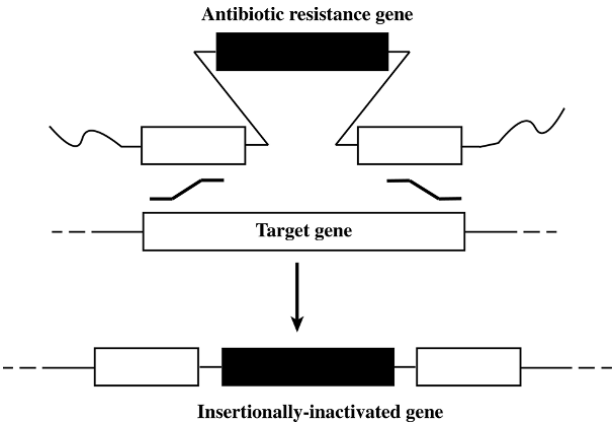


FIGURE 3 Strategy for allelic replacement mutagenesis using linear DNA. Segments of DNA homologous to the 5′ and 3′ ends of the target gene are placed flanking a gene encoding a selectable antibiotic resistance gene, typically on a standard plasmid vector using *E. coli*-based molecular cloning technology. The resulting *E. coli* plasmid vector is converted to a linear molecule by PCR or by digestion using restriction enzymes with sites outside of the cloned streptococcal gene (vector DNA is represented by the curved lines) and introduced into *S. pyogenes* with selection for resistance to the antibiotic encoded by the introduced resistance gene. Recombination between the two homologous sequences (indicated by the lines between the introduced DNA and chromosome) results in the replacement of the insertionally inactivated allele for the native chromosomal allele (shown below the arrow).

Directed Insertional Inactivation

A commonly used technique for directed mutagenesis employs mutations constructed as the result of a single homologous recombination event. For this technique, an internal segment of the target gene lacking the 5′ and 3′ ends is cloned into a Gram-negative restricted plasmid. A selectable marker for *S. pyogenes* is also included on the plasmid located adjacent to the cloned segment, but this marker should not be a β-lactamase (see above). As a general rule, the larger the fragment that is amplified, the greater the frequency at which allelic exchange occurs, although this method has been successful with fragments in the 250- to 500-bp range. The resulting plasmid is purified from *E. coli* and used to transform *S. pyogenes* with selection for resistance to the introduced marker. Because the commonly used *E. coli* replicons do not replicate in *S. pyogenes*, resistant transformants most frequently arise as a result of a single homologous recombination event between the internal segment of the gene introduced on the plasmid and the identical sequence on the streptococcal chromosome. The resulting chromosomal structure is generated by integration of the entire plasmid and consists of a partial duplication of the target gene, which now flanks the integrated plasmid sequences (Fig. 4A). As a consequence of using only an internal fragment of the target gene, one of the duplicated copies lacks its 3′ end and the other lacks its 5′ end such that both partial copies should be inactive. Thus, the end result is the directed insertional inactivation of the target gene. A number of modified *E. coli* plasmids have been utilized for this mutagenesis technique, often by replacing a β-lactamase ampicillin cassette for an appropriate selection marker. These include pCIV2 (40) and pSPC18 (41), which contain a kanamycin and spectinomycin resistance cassette, respectively.

The principal advantage to this method is that single recombination events are relatively quick and easy to generate because they require a single selection step rather than the multiple passages and screening required for construction of in-frame deletions using ts shuttle vectors (see below). This approach is also simple because it requires the cloning of only a single segment of the chromosome and can work with fragments as small as 0.25 kb. However, there are several disadvantages to the method. These include the possibility that the mutations are not necessarily stable because a second recombination event between the duplicated gene segments can result in excision of the plasmid vector and regeneration of a wild-type structure. Also, because the incoming plasmid does not replicate, it has only a limited period of time with which to undergo recombination for insertion into the chromosome. This latter limitation was addressed by Le Breton et al., who devised a two-plasmid system whereby a suicide plasmid (pSinS) lacking the pWVO1 RepA is complemented by a helper plasmid (pHlpK) that provides a ts RepA. The two plasmids are used to transform *S. pyogenes* at a permissive temperature, and a subsequent shift to a higher temperature prevents plasmid replication, selecting for plasmid integration

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(42). A final limitation to discuss is that the mutations generated by this technique are strongly polar. However, it is possible to modify the technique to directly assess the impact of polarity on the phenotype under investigation. In this case, an additional control strain is constructed, where instead of cloning a segment internal to the target gene, a segment is cloned that has its 5′ end anchored internal to the gene, but its 3′ end is anchored at a location distal to the 3′ end of the coding region of the gene. Integration of this construction into the target gene in a wild-type strain also results in a partial duplication. However, because the cloned segment overlaps the 3′ end of the gene, the first copy of the gene is regenerated

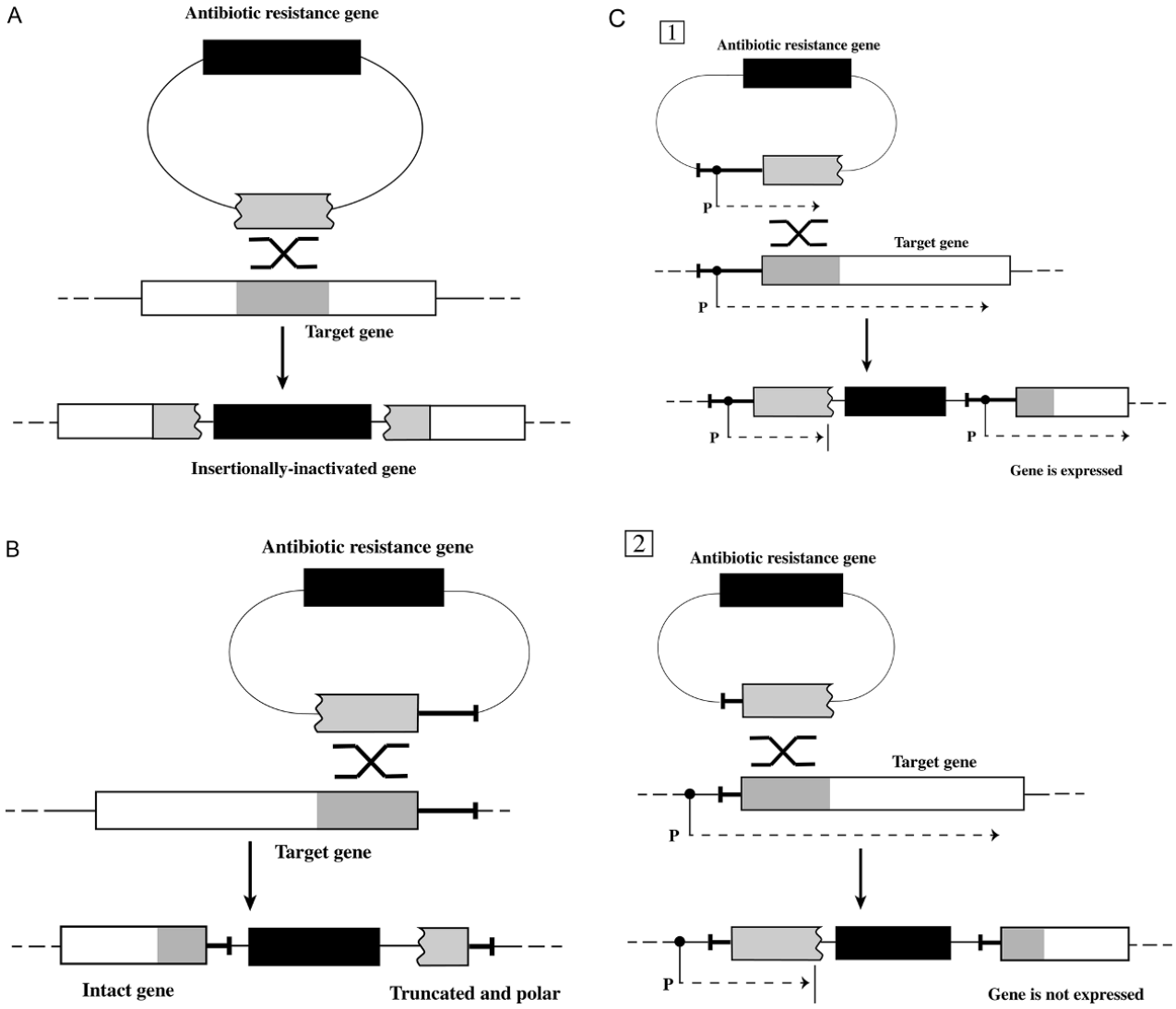


FIGURE 4 Directed insertional mutagenesis of targeted genes using circular DNA. (A) Insertional inactivation of the target gene. A DNA segment internal to the target gene (shown by the gray box enclosed by wavy lines) is cloned onto an *E. coli* plasmid which cannot replicate in *S. pyogenes*. The plasmid is introduced into an *S. pyogenes* host as a circular molecule (top of figure) with selection for a resistance marker on the plasmid. A single homologous recombination event between the chromosome and the circular molecule (shown by the "X") results in the integration of the plasmid into the chromosome, a partial duplication of the gene (the two regions shaded in gray) in which neither of the two copies are complete, resulting in gene inactivation. (B) Generation of a polar insertion 3′ to the target gene. If the segment of DNA cloned on the integrational plasmid includes the 3′ terminus and sequences downstream of the target gene (shown by the regions shaded in gray and the thick bar, respectively), homologous recombination (shown by the "X") results in a partial gene duplication in which the 5′ copy is intact but the 3′ copy is truncated, producing a polar effect on any downstream gene(s) which may share a promoter in common with the target gene. When compared with an insertion mutation that inactivates the target gene (Fig. 1 or Fig. 2), this strategy tests whether a phenotype is due to the loss of target gene function or to a polar effect on a distal gene. (C) Mapping the *cis*-acting control regions of the target gene. The technique is modified by constructing several plasmids representing a nested set of the chromosomal region extending various distances 5′ of the target gene. The plasmids are integrated into the target locus as described above, and the end-products are a partial duplication of the target gene in which the proximal copy is truncated and inactive. If the duplicated region includes the *cis*-acting control regions (represented by the broken arrow and the closed circle labeled "P"), the distal copy of the target gene will be expressed (scenario 1). In contrast, if the cloned segment does not include the *cis*-acting control regions, then the distal copy will not be expressed (scenario 2).

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and the integrated vector is located adjacent to this intact copy such that it is still polar on expression of any distal genes (Fig. 4B). If the phenotype under analysis is the result of a polar effect and the loss of expression of a distal gene, then this control strain should also demonstrate the mutant phenotype, even though it has an intact and functional copy of the target gene. On the other hand, an unaltered wild-type phenotype in the control strain indicates that the mutant phenotype is solely the result of insertional inactivation of the target gene (for example, see 43).

An additional modification of this method can be used to map the promoter and *cis*-acting control regions of a target operon. This is based on the technique developed by Moldover and colleagues (44) for analysis of promoters in *Bacillus subtilis* and is similar in concept to the method described above for placing a polar insertion downstream of a target gene. However, in this technique, the segment of DNA cloned into the integrational vector is anchored within the coding region at its 3′ end but is anchored upstream of the start of the gene at its 5′ end. Integration of this construct into the target locus again generates duplication. However, since the 3′ end of the cloned segment ends within the gene, the first copy is truncated and is inactive. This is followed by the integrated vector and then by the second intact copy of the target gene, which is now preceded exactly by the 5′ flanking region cloned into the vector. If this 5′ flanking region includes the promoter and other *cis*-acting control regions, the target gene will be expressed (Fig. 4C-1). If this segment lacks these elements, the target gene will not be expressed (Fig. 4C-2). By using a nested set of insertions containing different lengths of the upstream control region, it is possible to map the sequences required for expression and regulation of the target gene with a high degree of precision. This method has been used to examine regulation of *mga*, the regulator of the genes which encode the M proteins and the C5a peptidase (40).

In-Frame Deletion

Despite the increase in time and effort, the construction of in-frame deletions has become the standard for directed mutagenesis because they are stable and nonpolar. In-frame deletions have been constructed in the genes for many *S. pyogenes* virulence factors, including the C5a peptidase (16), the M protein, the cysteine proteinase (45), the *has* operon (46), and streptolysin O (47). Most in-frame deletion mutations were constructed by the method pioneered in the Cleary lab (16) that utilizes a derivative of the ts plasmid pG+host4. In this method, the gene of interest is cloned into the vector in *E. coli*. Standard molecular techniques are then used to delete a large central region of the gene, but at the same time preserve its reading frame. Alternatively, a set of four primers are designed to perform splicing by overlap extension (48) to directly generate the deletion construct and insert it into the shuttle plasmid, all via PCR and without the use of restriction digestion and ligation (49). The resulting construct is introduced into an *S. pyogenes* host at a temperature that is permissive for replication of the plasmid. The culture is then shifted to the nonpermissive temperature while maintaining selection for the resistance determinant of the plasmid. This selects for chromosomes into which the nonreplicating plasmid has been inserted by homologous recombination between the in-frame deletion allele and the resident wild-type allele. At this stage, the chromosome contains both the wild-type and deletion alleles. At some frequency, a second homologous recombination occurs which results in the excision of the integrated plasmid. Depending on the recombination junctions, either the wild-type or the deletion allele remains behind in the chromosome (Fig. 5). Shifting the culture back to the permissive temperature enriches for chromosomes from which the plasmid has been excised, since replication of the integrated plasmid creates a second origin of replication for the chromosome, which is usually deleterious to growth. The culture is once again shifted to the nonpermissive temperature, but this time in the absence of selection for the plasmid. This enriches for segregants, which have lost the excised and now nonreplicating plasmid. These segregants are plated as single colonies, which are then examined by streaking each colony onto agar plates in the presence or absence of antibiotic selection to identify clones which have lost the plasmid and thus are sensitive to the antibiotic used for selection. These are typically subjected to further screening by PCR and/or genome sequencing to identify chromosomes which contain the in-frame deletion allele. For a detailed description of this technique see Le Breton and McIver (12).

Counterselection Markers

The requirement for multiple passages at nonpermissive and permissive temperatures can make this method of in-frame deletion time-consuming. However, counterselection strategies that select against the retention of the plasmid can be used to expedite the recovery of chromosomes that have undergone plasmid excision and loss (50). One such strategy involves phenylalanyl tRNA synthetase (alpha-subunit) encoded by *pheS*. A single amino acid substitution in PheS (PheS*) confers relaxed substrate specificity and allows for the misincorporation of a toxic derivative of phenylalanine, 4-chloro-phenylalanine (4CP), into proteins. Here, a plasmid containing *pheS** is integrated into the chromosome. Subsequent culture in the presence of 4CP results in the death of cells that retain the *pheS**-containing plasmid, while those that have excised the plasmid survive (51). The addition of a second amino acid substitution in PheS* (defined here as PheS**) (52) confers even greater misincorporation of 4CP and higher counterselection efficiency and has been successfully used in *Streptococcus mutans* (53) and in *S. pyogenes* (Port and Caparon, unpublished) (Fig. 6). An alternative counterselection strategy involves introduction of the *rpsL* marker into the ts vector (54). This gene encodes ribosomal protein S12, and point mutations in *rpsL* which render the ribosome resistant to the antibiotic streptomycin arise spontaneously in *S. pyogenes* at a frequency of $\sim 10^{-9}$ cell^{-1} generation^{-1} (26). However, *in trans*, the wild-type allele is dominant, so when a ts vector containing wild-type *rpsL* is integrated into the chromosome of a streptomycin-resistant strain, the resulting partial diploid will be sensitive to streptomycin. Thus, the inclusion of streptomycin during the final passage at the nonpermissive temperature directly selects for chromosomes from which the integrated plasmid has excised and segregated. This method has been used to introduce mutant alleles of the virulence regulator Mga into the chromosome for functional analyses (54).

In-Frame Deletion Using CRISPR/Cas9

A drawback to the standard in-frame deletion method mentioned above is that it can be time-consuming, usually taking more than a week for the multiple passages that are required to enrich for chromosomes that contain the deletion allele (Fig. 5). This time period can be reduced through the application of a system using clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated

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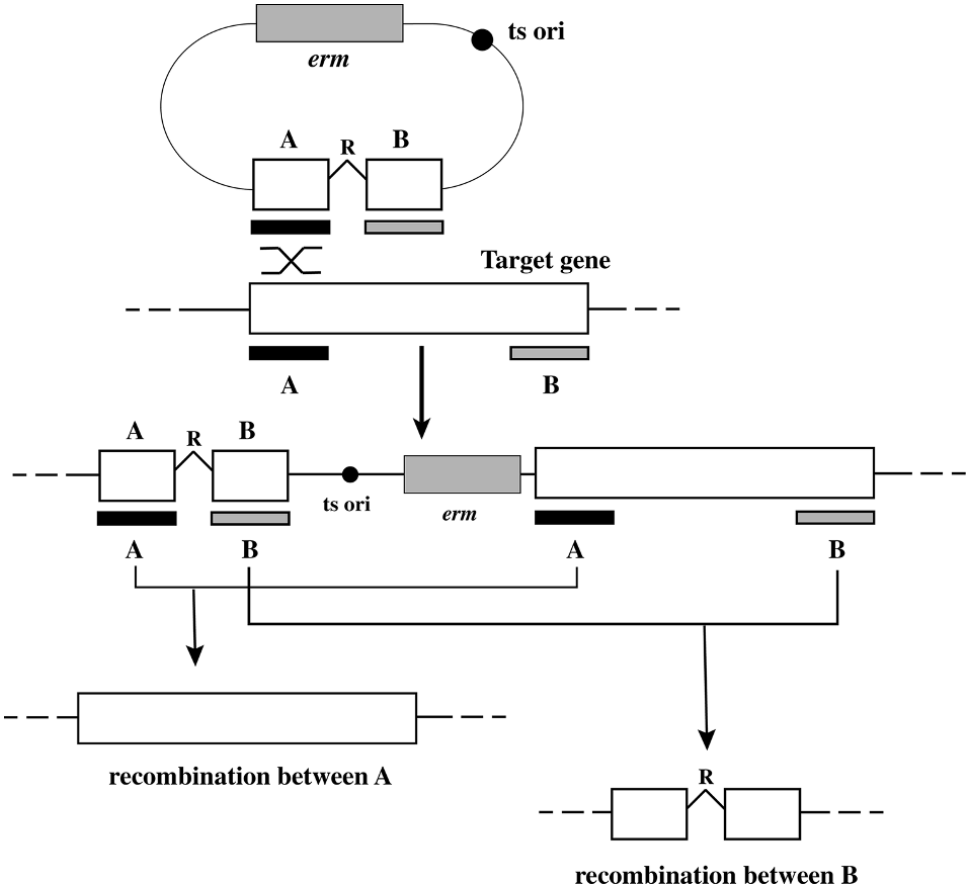


FIGURE 5 Construction of an in-frame deletion. Standard PCR-based methods are used to generate a deletion of the internal region of a copy of the target gene that has been cloned on an *E. coli*-streptococcal shuttle vector that is temperature-sensitive for replication (indicated by "ts ori"). The deletion is constructed so as to maintain the reading frame of the gene (represented as the bent line labeled "R," connecting the 5′ region labeled "A" and the 3′ region labeled "B"). Following its introduction into *S. pyogenes*, growth at a temperature that is nonpermissive for replication of the plasmid with selection for the antibiotic-resistant determinant of the plasmid (*erm*) selects for chromosomes in which the plasmid has integrated by homologous recombination (indicated by the "X"). The two regions of homology flanking the deletion are represented by the solid and gray bars labeled "A" and "B." Recombination between the A bars or, alternatively, between the B bars (shown by the brackets and arrows) results in excision of the plasmid and either the restoration of the wild-type structure or allelic exchange for the deletion allele (these products are illustrated below the second set of arrows). Growth at a temperature permissive for replication of the plasmid enriches for excisant chromosomes, presumably because if integrated, the plasmid origin of replication becomes active, resulting in misregulation of chromosomal replication. Presence of the wild-type or deletion allele in any isolate is easily determined by assay for the unique restriction site engineered into the deletion allele (indicated by the "R" above the bent line) or by PCR with primers flanking the region of interest.

protein 9 (Cas9). CRISPR/Cas9 was originally described in *S. pyogenes*, where it functions to confer an adaptive immunity against invading genetic elements, especially bacteriophages (55, 56). It does this by introducing double strand breaks at specific sequences that the Cas9 enzyme is programmed to recognize by incorporation of a small targeting RNA molecule (CRISPR RNA [crRNA]) that is encoded in the CRISPR array (55, 56).

To cleave a specific DNA site, the Cas9 nuclease associates with both a crRNA that has a short sequence complementary to the DNA target sequence (known as the protospacer) and with a separate trans-activating crRNA (trRNA, sometimes called tracrRNA) (57). The trRNA is partially complementary to the crRNA and is required for crRNA maturation from a primary transcript encoding pre-crRNAs. The crRNA and trRNA form a based-paired structure that is incorporated into Cas9 and targets the complex to a protospacer region located adjacent to a short motif known as the protospacer-adjacent motif, which for *S. pyogenes* Cas9 is NGG. Jinek et al. simplified the system by merging trRNA and crRNA into a single synthetic molecule known as the single guide RNA (sgRNA) (57). Due to its simplicity, requiring only Cas9 and sgRNA, the type II CRISPR/Cas9 system from *S. pyogenes* has found wide application for genome editing in many organisms, including higher eukaryotes (58). A derivative, "dead" Cas9 (dCas9), modified by site-

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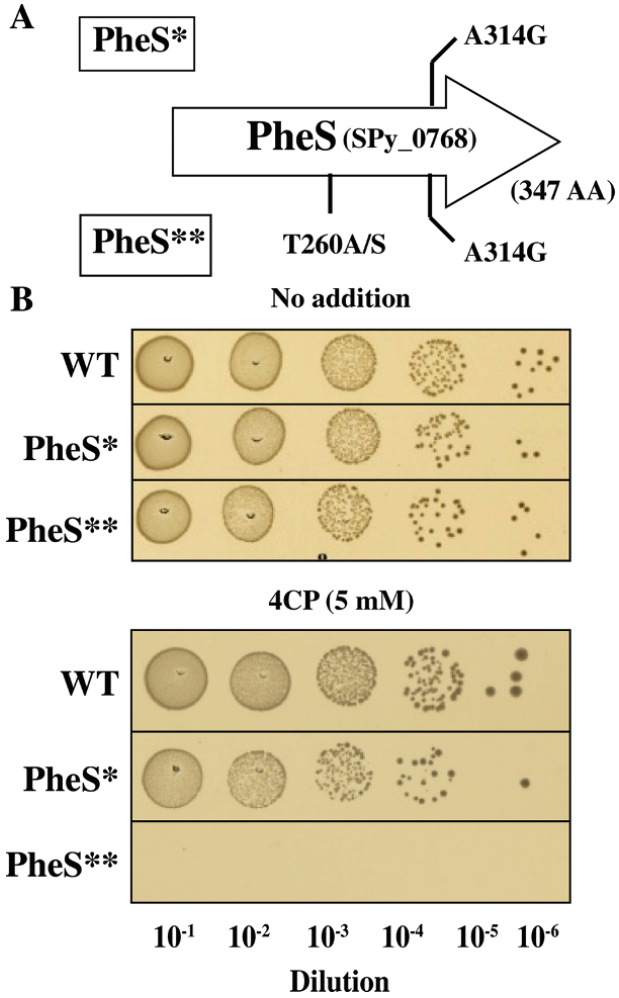


FIGURE 6 PheS**, a counterselectable marker. The phenylalanyl tRNA synthetase (alpha subunit) PheS catalyzes the attachment of phenylalanine onto its cognate transfer RNA. (A) Introduction of a single amino acid substitution (PheS*, A314G) confers relaxed substrate specificity and allows for the misincorporation of a toxic derivative of phenylalanine, 4-chloro-phenylalanine (4CP), into proteins. Addition of a second substitution at position 260 (PheS**, either T260A or T260S in combination with A314G) facilitates increased misincorporation of 4CP. (B) *S. pyogenes* containing a multicopy plasmid (pABG5) either as an empty vector (WT) or engineered to express PheS*, or PheS** was serial diluted and plated onto solid media in the absence (top panel) or in the presence (bottom panel) of 5 mM 4CP. Strains expressing PheS* show moderate sensitivity to 4CP toxicity, whereas strains expressing PheS** show enhanced sensitivity.

specific mutations that inactivate its two nuclease activities, can be used in a variety of approaches for silencing expression of specific genes (the CRISPRi method) (56, 59).

Cas9 can also be adapted for the efficient construction of in-frame deletions in *S. pyogenes* by providing a direct counterselection against strains that retain the wild-type allele. This method requires modification of the standard ts plasmid to contain Cas9 and an sgRNA in addition to the in-frame deletion allele (K.H. Cho, unpublished). The sgRNA should include a short ~20-nucleotide sequence complementary to a sequence in the targeted gene that will be eliminated by the deletion, which is adjacent to a suitable protospacer-adjacent motif. When the plasmid is introduced into *S. pyogenes* with the wild-type gene allele, only the strain with the in-frame deletion allele in its chromosome created by double-crossover survives, because it has eliminated the protospacer sequence. In contrast, any strain retaining the wild-type allele is killed due to the introduction of a double strand break of its chromosome by the Cas9 nuclease. This process is highly efficient, because unlike most eukaryotic organisms that can repair a double strand DNA break through nonhomologous end joining and homology-directed recombination (60), many prokaryotes use only homology-directed recombination to repair the double strand break. Thus, if no homologous DNA is available for repair, the double strand DNA break can be lethal (61). This method has been successfully used to create in-frame deletions in *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *E. coli* (62, 63). It is anticipated that this method will be even more successful in *S. pyogenes*, because more than half of the *S. pyogenes* genomes (14 out of 20 completely sequenced and annotated strains available in GenBank at the time of this writing) already possess *cas9*. For these strains, just providing an sgRNA may be sufficient for selection of in-frame deletion mutants.

Considerations for Directed Mutagenesis

The multiple *in vitro* passages required for directed mutagenesis techniques have a tendency to enrich for spontaneous mutants with reduced expression of virulence factors (64), including the M protein and the hyaluronic acid capsule (65). It is therefore important to isolate and phenotypically test multiple independently derived mutants to confirm that they have a consistent phenotype, as well as to genetically complement any mutants to confirm that the targeted mutation is the cause of the observed phenotype. If the gene targeted for mutagenesis lies upstream of an essential gene, it may be difficult to select for the initial single crossover due to a polar effect. To counter this point, a larger DNA fragment encompassing the entire downstream gene can be utilized to ensure continued gene expression following the initial crossover (66). Finally, some mutants are less fit and have a slower growth rate than the wild type. Therefore, any deletion mutants that are generated are quickly outcompeted by their wild-type counterparts, making them difficult to recover following *in vitro* passage. In this case, an allelic replacement mutant can be made whereby a gene of interest is replaced by an antibiotic cassette, which allows for direct selection of the mutated chromosome (66).

TRANSPOSON MUTAGENESIS

The techniques described above have been extremely useful for the analysis of virulence in *S. pyogenes*. However, they require a detailed pre-existing knowledge of the target gene and thus are more suited for reverse genetics approaches applied to testing hypotheses which address the functions of previously identified and characterized genes or genes identified from whole-genome sequence information whose function is suggested by homology with a characterized gene. This approach is not well suited for a forward genetics approach for the identification of novel genes that contribute to virulence. This is most successfully done using a traditional unbiased genetic approach, in which a virulence phenotype is established and random mutations are generated to identify genes which influence this phenotype. In *S. pyogenes*, the

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most successful method of random mutagenesis has used the insertion of a transposable element at multiple loci in the chromosome. This section considers the various types of transposons that have been successfully used to identify novel genes in *S. pyogenes*, with particular focus on recently adapted transposon systems that allow for the characterization of high-diversity libraries, which include hundreds (signature tagged mutagenesis) or even thousands (TnSeq) of mutants during infection.

Tn*916*

The first transposable element used to identify novel virulence genes in *S. pyogenes* was Tn*916* (Fig. 7A). This element is the prototype of the family of transposons discovered by Clewell's group (29), which are now known as the conjugative transposons. They have the remarkable property that they are self-transmissible and transpose from a locus in a donor chromosome to a different locus in a recipient chromosome when these chromosomes are located in different cells. The conjugal transfer event requires cell-cell contact and can occur between very distantly related species and even between Gram-positive and Gram-negative hosts. This biology of these unique elements has been extensively investigated and is the subject of several excellent reviews (for example, 67) and thus will only be briefly mentioned here.

The advantage to using Tn*916* is that it can be applied to probably any isolate of *S. pyogenes*, including those which may be difficult to transform. The disadvantages are that Tn*916* is a large element for a transposon (over 18 kb in size), does transpose into preferred sites, and frequently generates chromosomes with multiple insertions. The transposon has served as a useful mutagenesis agent and has been used to identify novel genes involved in regulation (e.g., *mga* [7]), expression of streptolysin S (68–70), and production of the capsule (71, 72), among others.

Tn*917*

This transposable element was also originally described by Clewell's group in *E. faecalis* (73), highly engineered by Youngman and colleagues, and applied with great success to the mutagenesis of *Listeria monocytogenes* and *B. subtilis*. One characteristic of this family is that its members contain fairly large terminal repeats, although they are much smaller in size than the conjugative transposons. They produce stable insertions, generate a 5-bp target site duplication upon insertion, and transpose at high frequency and with a high degree of randomness. Their movement involves a replicative pathway, which requires that donor and recipient molecules undergo replication, and proceeds through the cointegration of donor and host molecules which contain two directly repeated copies of the transposon at the fusion junctions. Resolution of the cointegrate occurs via site-specific recombination catalyzed by a transposon-encoded enzyme called resolvase that recognizes a specific site in each copy of the transposon (the *res* site). The end result is that a single copy of the transposon is located at a random site in the recipient molecule. Scott's group (74) developed a mutagenesis vector (pJRS290) (75) that is based on pG+host4 and contains a highly engineered derivative of Tn*917* developed by Youngman and coworkers (76) (Tn*917*-LTV3) (Fig. 7B). This derivative of Tn*917* has several useful features, including an *E. coli* plasmid origin of replication that facilitates the direct cloning of chromosomal DNA adjacent to the inserted transposon. Using this vector, Tn*917*-LTV3 was found to transpose efficiently and with a high degree of randomness in *S. pyogenes*.

Tn*4001*

The transposable element Tn*4001* was originally isolated as an agent of transmissible gentamycin-resistance in *S. aureus* (77). It is a composite-type transposon and a member of the Tn*5* family. These transposable elements share a common structure in which directly repeated copies of an insertion sequence flank an antibiotic resistance gene. Each insertion sequence itself is bounded by short inverted repeats that flank a transposase gene, which is the only transposon-encoded gene required for movement. The individual insertion sequences themselves are usually capable of transposition independent of the entire element. For Tn*4001*, two copies of the insertion sequence IS*256* flank a central gentamycin-resistance gene. The transposition pathway for this class of elements involves a "cut and paste" mechanism catalyzed by the transposase, which recognizes as its substrates the short inverted repeats of the insertion sequences. Studies have shown that Tn*4001* has a broad host range, including staphylococci, oral streptococci, and mycoplasmas, and that it chooses its targets for insertion with a high degree of randomness. Since the cut and paste pathway does not require replication of the donor molecule, the element can easily be delivered into the host of choice via a nonreplicating suicide vector. However, a number of problems have limited its use. The most significant of these is that transposition of the entire element occurs at a somewhat low frequency relative to the frequency of transposition of the individual insertion sequences. As a result, a chromosome with a Tn*4001* will frequently also have multiple copies of IS*256* inserted at other loci. To address this problem, derivatives of Tn*4001* have been constructed that essentially contain only the inverted repeats of IS*256* oriented to flank the gene for transposase and a selectable marker (78). The resulting elements are relatively small in size (~2 kb) and transpose at a high frequency characteristic of an individual copy of IS*256*. The organization of the element also prevents independent transposition of the insertion sequence and ensures that the resulting population of insertions is homogeneous, although the resulting mutants may have insertions at two loci. A spectinomycin-resistant version of this transposon (called TnSpc for simplicity) (Fig. 7C) was used to identify novel genes required for expression of the SpeB cysteine protease of *S. pyogenes*, including a transcriptional regulator (*ropB*) and a chaperone (*rop*A) (78). Additional variants include the introduction of an erythromycin resistance gene and 1 of 12 unique 34-bp signature tags for application in signature-tagged mutagenesis studies. In this method, 12 independent pools of mutants are generated. Then, one mutant is taken from each of these pools, and they are combined to generate an inoculum with 12-fold complexity that is used as the input into an assay of virulence. The output pool is then analyzed by a PCR assay to conduct a census of the recovered sequence tags, which is then compared with the input pool to determine the relative fitness of individual mutants. Mutants with reduced fitness are putative virulence genes, and this method has been successfully applied to the identification of genes that promote pathogenesis in a zebrafish model of *S. pyogenes* infection (79, 80).

Tn*FuZ*

Since the terminal inverted repeats of IS*256* are quite short (26 bp), several specialized derivatives of the transposon have

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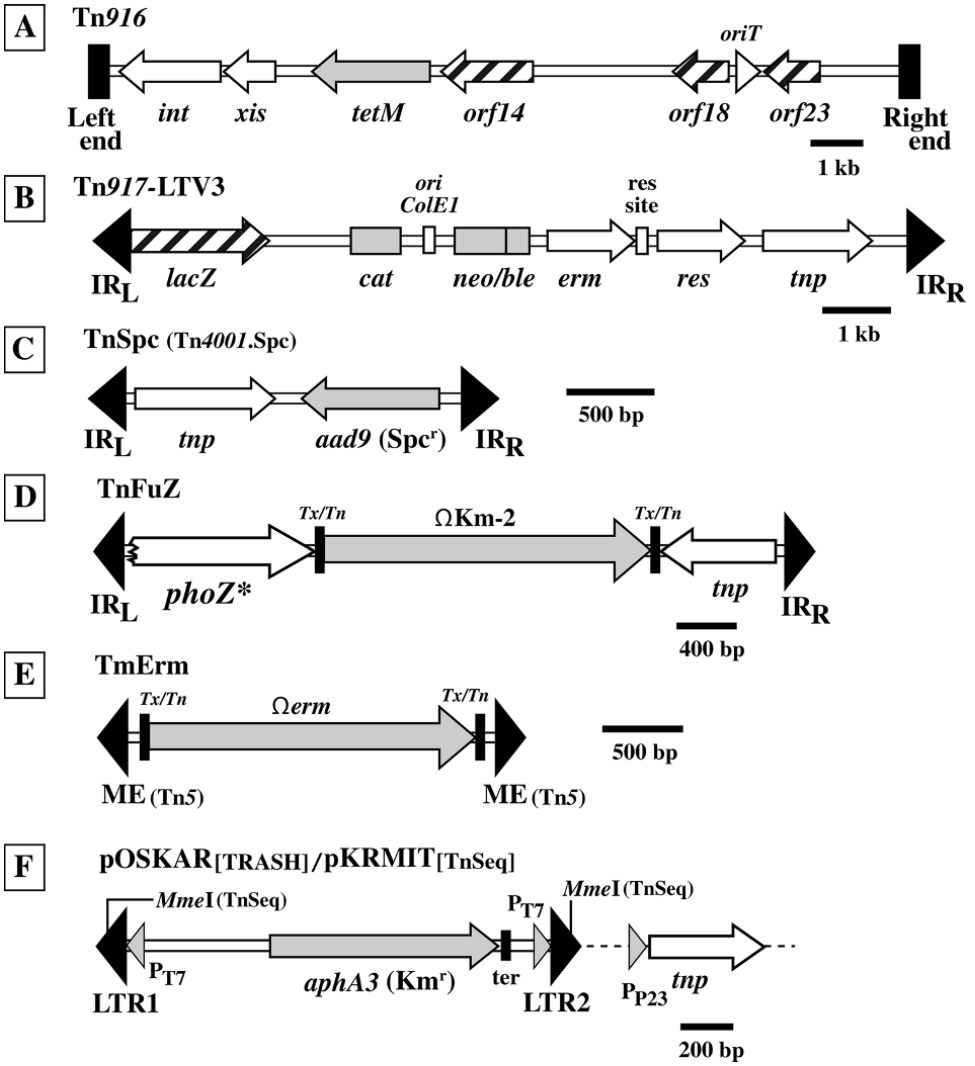


FIGURE 7 Transposon mutagenesis of *S. pyogenes*. The several transposons that have been used for mutagenesis of *S. pyogenes* are shown. Antibiotic resistance genes are represented by the gray bars, transposon ends and/or terminal inverted repeats are shown in black, genes that are essential for transposition are shown by white bars, and genes that are nonessential for transposition are represented by the stripped bars. (A) Tn*916* (GenBank accession no. U09422) is the prototype conjugative transposon and contains at least 24 different open reading frames. Of these, one is required for resistance to tetracycline, two are essential for transposition, and the rest are likely involved in conjugal transfer. (B) Tn*917*-LTV3 is a highly engineered derivative of the Tn*3*-like transposon Tn*917*. This element transposes via a replicative mechanism and has been modified to include a promoterless *lacZ* reporter gene to generate random transcriptional fusions and an *E. coli* ColE1 plasmid origin of replication to facilitate the cloning and analysis of inactivated loci. (C) TnSpc is a derivative of the Tn*5*-like transposon Tn*4001*, which transposes via a cut and paste mechanism and consists of the left and right inverted repeats and transposase of IS*256* and a spectinomycin resistance gene. (D) TnFuZ introduces the gene for the *E. faecalis* alkaline phosphatase (*pho*Z) altered by removal of the region encoding its signal sequence (broken line at the 5′ end of *phoZ**). TnFuZ acts as an "export signal sequence trap." Insertions into genes that encode a protein export sequence promote the secretion of PhoZ* enzymatic activity, which can be detected by a number of high-throughput assays for alkaline phosphatase activity. (E) TmErm is an engineered element that contains the right and left mosaic ends (ME) of Tn*5* flanking an erythromycin resistance marker (Ω*erm*) that includes strong transcription and translation stop sites (*Tx/Tn*). To apply transposome mutagenesis to *S. pyogenes*, the DNA fragment containing TmErm is purified, such that the resulting preparation does not include the ampicillin resistance determinant contained on the pMOD-2::Ω*erm* vector backbone. The purified DNA fragment containing TmErm is then reacted with transposase (EZ::TN, Epicentre Technologies) *in vitro* to form the transposome that is then used to transform *S. pyogenes* using electroporation. (F) pOSKAR and pKRMIT are transposon systems incorporated into the ts pWV01-based *E. coli*-streptococcal shuttle vector that utilize the highly active *mariner Himar1* transposable element (MarC9) under control of the P23 promoter from *Lactococcus lactis*. Both transposon systems include a kanamycin resistance gene (*aphA3*) flanked by long terminal repeats (LTR) whose promiscuous target insertion site requires only a TA dinucleotide sequence, leading to a highly random library of insertions. pOSKAR can be utilized for TraSH analysis through the use of outward-facing T7 promoters, while pKRMIT also includes an outward-cutting *MmeI* site for use with TnSeq analysis.

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been developed that place a reporter gene adjacent to the IS*256* end. The Tn*FuZ* (fusion to *phoZ*) reporter places a version of the *E. faecalis phoZ* alkaline phosphatase adjacent to the transposon inverted repeat (Fig. 7D). This reporter gene has been modified by removal of its endogenous export signal sequence to construct an "export signal sequence trap." In this method, insertion of the element into an open reading frame can result in the formation of a translational fusion with PhoZ (the use of PhoZ as a reporter gene is discussed below). If the donor open reading frame contains a secretion export signal, the chimeric PhoZ protein will be secreted. Since PhoZ is only active following its export across the cytoplasmic membrane, a simple and rapid colorimetric screen can be used to identify colonies arising from a mutant that contains an insertion in a gene that encodes a secreted protein. The Tn*FuZ* element has been used to identify secreted proteins whose expression is enhanced during aerobic growth (81). Another example of the versatility of IS*256*-based elements is those that have been modified to include genes encoding a bacterial luciferase (use of luciferase as a reporter gene is discussed below). Insertion into a transcription unit that directs transcription of the luciferase genes allows gene expression *in vivo* to be monitored noninvasively using specialized imaging cameras that can capture photons emitted by the streptococci in living host tissue (82).

Transposome Mutagenesis

In transposome mutagenesis, a stable complex between transposase and transposon DNA is formed *in vitro* and then introduced into a bacterium. The elevated concentration of Mg^{2+} in the bacterial cytoplasm activates transposase that then catalyzes transposition of the element to some random location in the bacterial chromosome. This method has been adapted for efficient mutagenesis of *S. pyogenes* (43). For this, a streptococcal erythromycin-resistance gene was introduced between the inverted repeats of a derivative of Tn*5* (EZ-TN5^{tm}) on the *E. coli* plasmid pMOD-2 (Epicentre Technologies) to construct TmErm (Fig. 7E) (43). Following digestion with a restriction endonuclease, the transposon (TmErm)-containing linear DNA fragment is reacted with a modified hyperactive transposase (EZ::TN, Epicentre Technologies) to generate the transposome. Following transformation of *S. pyogenes* using electroporation, erythromycin-resistant colonies were obtained at frequencies between 10^3 and 10^4 CFU/μg DNA (43). Mutations generated by this method have proven to be very stable even in the absence of erythromycin selection, possibly because the inserted transposon does not encode its own transposase.

Himar1-Based Mariner Transposon Systems

While the generation of a complex transposon library is relatively straightforward, mapping the location of the insertion site in the chromosome of any single mutant has historically been quite laborious. Prior to the development of rapid and relatively low-cost whole-genome sequencing technology, arbitrary PCR (78) was usually utilized to map the site of transposon insertion. An adaptation of this method for rapid screening using pools of mutants was a form of microarray-based analysis known as transposon site hybridization, utilizing a transposon with outward-facing T7 promoters at flanking ends (pOSKAR) (Fig. 7F) (83). Later, this transposon platform was modified to allow for analysis by high-throughput next-generation sequencing through the use of an outward-cutting restriction enzyme known as *Mme*I for the technique known as TnSeq (pKRMIT) (Fig. 7F) (84). Both of these transposon platforms utilize the highly active and promiscuous *mariner Himar1* transposable element, whose target insertion site requires only a thiamine-adenine (TA) dinucleotide sequence, leading to a highly random library of insertions.

Le Breton and McIver's group developed a Mariner-based system for use in *S. pyogenes* by introducing the *mariner* transposon into the ts pWV01 shuttle vector. Because the transposon is highly active, propagation of the shuttle vector in *E. coli* is unstable and requires careful screening of preparations of purified plasmid to ensure that the correct plasmid structure has been maintained prior to electroporation into *S. pyogenes* (12). Furthermore, transposition likely occurs quite rapidly relative to the rate of plasmid loss following growth at restrictive plasmid temperatures. Therefore, additional screening is required for the subsequent *S. pyogenes* mutant pools prior to phenotypic testing. Despite these technical limitations, multiple labs have used this transposon system for transposon site hybridization analysis utilizing the plasmid pOSKAR to screen for genes required for growth in human blood (25), to identify regulators of the Rgg quorum sensing system (85, 86), and for TnSeq analysis utilizing the plasmid pKRMIT for analysis of core essential genes required for growth *in vitro* (42) and for murine soft-tissue infection (87).

Analysis of Transposon Mutants

Regardless of the type of transposable element utilized, there are a number of additional tests that should be performed to ensure that the mutant phenotype is the direct result of insertion of the transposon. This involves determining the sequence of the locus into which the transposon has inserted via arbitrary PCR, inverse PCR after enzyme digestion and ligation, or whole-genome sequencing. With this data in hand, the next step is to construct a mutant in the locus in the wild-type parental strain using one of the strategies for directed mutagenesis outlined above. If the original insertion is responsible for the mutant phenotype, then it is expected that all isolates obtained via directed mutagenesis will also be mutants. It should also be kept in mind when interpreting results that transposon-generated mutations are also polar, so the gene(s) responsible for the observed phenotype may lie downstream of the interrupted gene.

ANALYSIS OF GENE EXPRESSION

Gene regulation phenomena play a key role in pathogenesis. The interaction between the host and the microbe is dynamic and often is a progression through a number of discrete steps. Each of these steps is characterized by the expression of specific sets of bacterial genes required for survival and multiplication and the host's response to the action of the products of these microbial genes. As a consequence, the microorganism is continually challenged to adapt to new and changing environmental conditions. Pathogens have taken advantage of the dynamic nature of this interaction and have evolved to recognize changes in specific environmental conditions as markers that define a particular host compartment or stage of infection. The pathogen uses this information to modulate expression of virulence genes required for survival in this host compartment. Thus, an understanding of the *in vitro* conditions that regulate expression of a specific virulence gene provides insight into how the gene contributes to virulence *in vivo* (for review see 88). Regulation of virulence genes often involves control at the level of transcription. For *S. pyogenes*, transcriptional

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regulation has often been analyzed through the use of reporter genes, particularly in studies designed to determine the specific conditions that activate or repress expression of a gene of interest.

The basic strategy for using reporter genes to analyze gene expression involves fusing the promoter for the virulence gene of interest to a reporter gene that encodes a gene whose product can easily be quantitated. This feature is of particular utility when the assays for quantitation of the product of the streptococcal gene under analysis are time-consuming, expensive, and/or cumbersome. The reporter gene lacks its own promoter, so its product becomes an accurate relative indicator of the steady state level of transcription initiation from the target promoter. It should be kept in mind that because the message is a chimera between the initiation signals of the target gene and the reporter gene, it is not subject to the same posttranscriptional and posttranslational controls. Furthermore, the half-life of the reporter gene's translation product does not reflect that of the native polypeptide. Thus, reporter genes are most appropriately used to quantitate the strength of initiation of transcription of the target promoter.

The reporter genes that have been successfully used in *S. pyogenes* include chloramphenicol acetyltransferase (Cat), β-galactosidase (Lac), β-glucuronidase (Gus), alkaline phosphatase (Pho), and bacterial luciferase (LuxAB). The products of all these genes are stable, highly active enzymes that are highly specific for their substrates. Their utility is enhanced by the availability of synthetic substrates with isotopic, colorimeteric, fluorogenic, or light-emitting properties that allow very sensitive detection. Also, the reporter genes encoding these enzymes are derived from Gram-positive bacteria or have been extensively modified to optimize their expression in Gram-positive hosts and differ extensively from their counterparts used for analysis in *E. coli*.

The most successful applications of Cat have employed *cat*86, originally isolated from *Bacillus pumilus*, which has been modified by the deletion of an attenuator sequence and the substitution of an adenine-thiamine-guanine (ATG) start codon for the native gene's thiamine-thiamine-guanine (TTG) start codon. The advantages of Cat are that *cat*86 is very stably maintained in *S. pyogenes* and that the assays for its enzymatic acetylation of chloramphenicol are very sensitive (89, 90). It has several disadvantages: the most sensitive assays involve radioactivity, are expensive, are somewhat labor-intensive relative to other reporter genes, and require the preparation of cell-free extracts. Both Lac and Gus utilize *E. coli* genes that have been modified by the introduction of ribosome binding sites recognized by Gram-positive bacteria (91, 92). The advantages to using these as reporters are that assays for their enzymatic activities are easy to perform and rapid and many samples can be analyzed simultaneously, that a wide variety of substrates is available, and that it is possible to analyze both permeabilized cells and cell extracts. Disadvantages are that they are not as sensitive or as stably maintained in *S. pyogenes* as *cat*86. Nevertheless, *gusA* has proven to be very useful in numerous applications, including identification of regulatory sites in the *hasA* promoter (93) and to find a regulator (*rocA*) that modulates transcription of the CovRS two-component global regulator of virulence and stress resistance (94).

The LuxAB bacterial luciferase, originally from *Vibrio fischeri*, has found utility in dissecting the mechanism of Rgg-associated signaling peptides in *S. pyogenes* (35). An advantage of a luciferase reporter is that the assay is extremely sensitive and quantitative, because exposure of bacterial cultures to volatile decyl aldehyde (decanal) for less than one minute can result in a measurable light output. A disadvantage of this reporter is that luciferase activity is growth phase sensitive due to the reaction's requirement for reduced flavin mononucleotide as a cofactor (95) and has been shown to fail in some bacteria during the stationary phase (82) or under conditions of low pH or oxidative stress (96).

A chimeric reporter protein based on fusion of two naturally secreted proteins has also been developed. This reporter contains as its enzymatic partner, the alkaline phosphatase of *E. faecalis* (PhoZ) (97). This enzyme is secreted from *E. faecalis* and is a lipoprotein that becomes tethered to the outer leaflet of the cell membrane. Fusing the enzymatic C-terminal domain of PhoZ, not including its lipoprotein secretion signal, to the nonlipoprotein N-terminal secretion domain of protein F of *S. pyogenes* results in a very stable, highly enzymatically active chimeric protein (PhoZF) (Fig. 1) which is freely secreted from the streptococcal cell into the surrounding medium (20). This feature makes quantitative analysis of the secreted chimeric protein in culture supernatant very simple and requires neither permeabilization nor preparation of cytoplasmic extracts. Due to the widespread popularity of alkaline phosphatase enzymes, an excellent selection of sensitive substrates with a variety of useful characteristics, including those with fluorescent and light-emitting properties, are inexpensive and are readily available.

Green fluorescent protein (GFP) has become a widely used reporter of gene expression because of its ease of detection and its utility for application in real-time assays for analyses *in vitro* and *in vivo* during infection. Numerous versions of GFP are available that have been modified to alter codon usage, stability, and spectral properties (98). The GFPuv variant has been used to examine regulation of the promoter for the hyaluronic acid biosynthesis gene cluster in streptococci recovered directly from infected tissues (99). This latter study also reported an important consideration for interpreting data derived from GFP-based reporters in that fluorescence is often not uniform among streptococcal cells in any population, including many cells that express no detectable fluorescence (99). It is likely that the microaerophilic or anaerobic conditions that are typically used for culturing *S. pyogenes* are not ideal for GFP maturation, since oxygen is required for GFP chromophore formation. However, a more stable variant known as superfolder GFP has shown utility in several applications in *S. pyogenes*, including monitoring subcellular localization of proteins (100).

Numerous strategies have been used to introduce reporter fusion constructs into *S. pyogenes* for analysis. Plasmid vectors have the advantage that they are easy to manipulate and can be used to generate and analyze a large number of permutations and variations of promoter structure to probe the *cis* and *trans*-acting control regions. A plasmid-based reporter is present in multiple copies, and this amplification increases the sensitivity of detecting the activity of the promoter under analysis. This latter feature can also make plasmid-based systems sensitive to multiple-copy-number-derived artifacts, such as titration of *trans*-acting regulatory components, when those components are only present in limiting quantities. Furthermore, any sequence-directed local effects of chromosomal structure on expression of a given promoter will not likely be replicated in a plasmid environment. Integration of reporter constructs into the chromosome can alleviate many of these latter types of potential artifacts but with a trade-off in ease of use and sensitivity. Integration is usually performed by introducing the reporter construct on a nonreplicating *E. coli*-based plasmid to target integration into the target gene's native locus by homologous recombination promoted by the

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cloned promoter segment. Alternatively, use of the integrating p7INT/p7INT* plasmid can reproduce a more native chromosomal environment through integration at the *attB* site (Fig. 2).

Next-generation sequencing approaches such as RNAseq for global analysis of the transcriptome coupled with quantitative real-time RT-PCR methods have recently become the standard for analysis of gene expression in *S. pyogenes*. Although it requires preexisting knowledge of the genome sequence, this disadvantage is more than offset by the facts that RNAseq does not require the laborious construction of reporter gene fusions, that it is fast and easy to perform, that it can directly analyze the entire transcriptome, including novel RNA species, and that it is not limited to the genes for which probes were designed. RNA analysis has been greatly facilitated by the development of technology for the simple and rapid purification of high-quality RNA from relatively small samples (101). In practical terms, quantitative real-time RT-PCR is used for validation expression of individual genes of interest from data developed from RNAseq analyses (102). In quantitative real-time RT-PCR, total RNA is purified and then converted to first-strand complementary DNA (cDNA) using reverse transcriptase. These cDNAs then serve as a template for real-time PCR using primers specific for the gene in question. Real-time thermocyclers are modified with a detection system that can continuously monitor the fluorescence of a probe that is sensitive to the quantity of DNA that is being synthesized by PCR, and the amplification cycle at which a detectable amplification product is first detected is a quantitative measure of the concentration of that message in the input cDNA pool. Data are typically reported as a ratio to a reference gene such as *recA* or *gyrA* whose expression is relatively constant under analysis conditions.

Techniques for Ectopic Expression

Structure-function studies of the role of putative virulence factors require techniques for ectopic expression, both to control the timing and relative levels of expression of a given gene and to study the structure-function relationships of specific domains of a given virulence-associated protein. The former case requires the availability of an ectopic promoter whose activity can be tightly, easily, and quantitatively controlled by some external factor. The latter case requires a method for the expression of various domains of the polypeptide under analysis into the context of an unrelated polypeptide. This allows an independent analysis of the functionality of that domain separate from other regions of the original polypeptide. Methods to accomplish both of these expression strategies have been developed for analysis of virulence in *S. pyogenes*.

An initial approach for using a regulated heterologous promoter to direct expression of the gene under analysis was based on the *nisA* promoter of *Lactococcus lactis*. In lactococci, the NisR/K two-component system controls the expression PnisA in direct response to nisin concentration. The method for using this regulated promoter involves the introduction of *nisK* and *nisR* into an *S. pyogenes* strain, while the gene of interest is introduced under control of the *nisA* promoter, which makes expression of the gene of interest sensitive to the concentration of exogenous nisin that is added to the culture. Tightly regulated expression of a *gusA* reporter gene and induction of up to 60-fold over background using a broad range of nisin concentrations has been reported for *S. pyogenes* (74). The NisR/K system has some drawbacks, including the stability of nisin (103) and a relatively high basal level of expression in the uninduced state, which makes conditional expression of putative essential genes difficult. A more tightly controlled inducible promoter is based on the tetracycline-inducible promoter P_{tet}. In this system, the *tet* repressor (TetR), binds to a 19-nucleotide palindromic sequence, referred to as the *tet* operator (*tetO*), between the -35 and -10 site of P_{tet}. In the presence of inducer, commonly tetracycline or nonantimicrobial analogs, including anhydrotetracycline (aTc), TetR binds to the inducer, reducing its binding affinity for *tetO* and thereby allowing for expression of the downstream open reading frame. Originally from *E. coli*, this system has been adapted for use in Gram-positive bacteria by the use of optimized promoter sequences and ribosome-binding sites and through addition of multiple *tetO* sites for enhanced dynamic range (104). A reverse TetR has been developed that has an enhanced, rather than reduced, affinity for *tetO* in the presence of inducer, and thereby the presence of inducer represses gene expression (105). P_{tet} has been used in *S. pyogenes* to control the expression of reporter genes (100) and to experimentally verify the essentiality of ribonucleases J1 and J2 (106). A third, and perhaps more widely used, conditional expression system is based on a theophylline-inducible riboswitch (32). In this system, a ribosome-binding site (RBS) for expression of the target gene has been engineered to become occluded and unavailable due to mRNA secondary structure. However, the binding of the theophylline ligand to the mRNA induces the formation of an alternative secondary structure, such that the RBS is free to bind ribosomes and translation is initiated. The native RBS located upstream of a gene of interest is thus replaced by the riboswitch for conditional expression studies. Two advantages of the theophylline-inducible riboswitch are less leaky expression than P_{tet} and the fact that riboswitches do not require accessory proteins. The theophylline riboswitch has been used to study the essentiality of the histone-like protein gene *hlp* (107), *gacA* (108), and several genes predicted to be essential from a saturating TNseq transposon screen (42).

Structure-function dissection of virulence protein function can benefit from ectopic expression methods that allow the analysis of defined subdomains of the polypeptide. This has proven particularly valuable for identification of the ligand-binding domains of adhesins that direct attachment of bacteria to various host cell receptors. In this approach, the subdomain under examination is expressed and displayed on the bacterial cell surface in the context of a heterologous protein. A technique developed for this strategy, which uses the framework of the M6.1 protein, was based on the work of Pozzi and coworkers (109), who demonstrated that the central domain of the M protein could be replaced with a heterologous sequence without affecting expression and surface presentation of the resulting chimera. Essentially, the heterologous domain is placed between the N-terminal secretion and C-terminal attachment domains of M protein to display the heterologous domain on the streptococcal cell surface. A successful application of this strategy involved analysis of the two distinct fibronectin-binding domains of protein F (110). For a more detailed explanation of this technique see Hanski et al. (39).

CONCLUDING REMARKS

Rapid progress has been made in recent years in the development of sophisticated techniques for genetic analysis in *S. pyogenes*. Much of this effort has been directed at the development of methods for the mutagenesis of known genes.

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Considerable progress has also been made in the development of strategies for the identification of novel genes. It is likely that the widespread application of these techniques to the virulence properties of *S. pyogenes* will enrich our understanding of streptococcal pathogenesis with insight at the molecular level and will help to establish and clarify the contributions of specific genes. Additional use and development of methods for the analysis of gene expression and heterologous expression will continue and will allow analyses of virulence factors at much higher levels of resolution than previously possible.

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Molecular Mimicry, Autoimmunity, and Infection: The Cross-Reactive Antigens of Group A Streptococci and their Sequelae

MADELEINE W. CUNNINGHAM

7

INTRODUCTION

Cross-reactive antigens are molecules on the group A streptococci that mimic host molecules and during infection or immunization induce an autoimmune response against host tissues, leading to the autoimmune group A streptococcal sequelae (1–25). "Molecular mimicry" is the term used to describe immunological cross-reactivity between the host and bacterial antigens. Immunological cross-reactions between streptococcal and host molecules have been identified involving antibodies or T cells that react with streptococcal components and tissue antigens (6, 7, 22, 24, 25). The advent of monoclonal antibodies (mAbs) and T cell clones/hybridomas has greatly advanced the identification of the host and streptococcal antigens responsible for immunological cross-reactions associated with immunization, infection, and autoimmune sequelae. The identification of cross-reactive antigens in group A streptococci and important in our understanding of the pathogenesis of autoimmune sequelae, such as rheumatic fever including carditis (8, 11) and Sydenham chorea (7), which may follow group A streptococcal infection (3).

Molecular mimicry between host and bacterial antigens was first defined as identical amino acid sequences shared between different molecules present in tissues and the bacterium (26–28). The investigation of molecular mimicry through the use of mAbs has identified other types of molecular mimicry. The second type of mimicry involves antibody recognition of similar structures such as alpha-helical coiled-coil molecules such as streptococcal M protein, and the host proteins myosin, keratin, tropomyosin, vimentin, and laminin that share regions containing 40% identity or less and cross-reactive sites are not completely identical (4, 29–43). A third type of molecular mimicry is revealed in immunological cross-reactions between molecules as diverse as DNA and proteins (36, 40, 44) or carbohydrates and peptides (45–47). The three types of molecular mimicry are summarized in Table 1. Studies of the cross-reactive antigens of the group A streptococci have contributed greatly to our knowledge about molecular mimicry, autoimmunity, and infection and the antibody molecules involved. The antibody molecules described as polyreactive or cross-reactive indicate recognition of multiple antigens. As described, the basis of the immunological cross-reactions may be identical or homologous amino acid sequences shared between two different proteins or may be epitopes shared between two entirely different chemical structures.

A theme in molecular mimicry suggests antibody recognition of an internal biomarker, often a structural protein, which cross-reacts with the streptococcal antigen and a cell surface antigen leading to cytotoxicity or to functional cell signaling (6, 7). Through these mechanisms, tissue is altered and T cells are recruited to the site of attack (48).

HISTORICAL PERSPECTIVE

Cross-reactive antigens and antibodies were first found to be associated with acute rheumatic fever (ARF) and group A streptococci when it was discovered that rheumatic fever sera or anti-group A streptococcal antisera reacted with human heart or skeletal muscle tissues (15, 17, 19). Antibodies against group A streptococci in the serum of rheumatic fever patients were shown to be absorbed from the sera with human heart extracts, and in contrast, the antiheart antibodies were shown to be absorbed with group A streptococci or streptococcal membranes (49, 50). Rabbit antisera produced against group A streptococcal cell walls reacted with human heart tissue, and antibodies produced against human heart tissue reacted with group A streptococcal antigens (49, 51, 52). In rheumatic fever, heart-reactive antibodies appeared to persist in patients with rheumatic recurrences, and there appeared to be a relationship between high titers of antiheart antibodies and recurrence of rheumatic fever (15, 19). Heart-reactive antibodies were reported to decline within 5 years of the initial rheumatic fever attack.

Subsequent research on this early work by Kaplan and Suchy (49) implicated the streptococcal cell wall containing the M protein as the cross-reactive antigen recognized by the antiheart antibodies, while studies by Zabriskie and Freimer (15, 17) implicated the streptococcal membrane as the site of the cross-reactive antigen. Beachey and Stollerman (53) and Widdowson and Maxted (54–56) reported an M-associated non-type-specific antigen and an M-associated protein, respectively, which were thought to be associated with immunological cross-reactions in ARF. In 1977, van de Rijn

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TABLE 1 Types of immunological mimicry (cross-reactivity)

Identical amino acid sequences in different proteins
Similar protein structures shared among different proteins
Diverse molecules such as DNA, carbohydrates, and proteins

et al. demonstrated that highly purified peptides from group A streptococcal membranes reacted with the antiheart antibodies in rheumatic fever serum (57). Taken together, this evidence suggested that cross-reactive antigens were located in both the cell wall and membrane of the group A streptococci.

Evidence also supported the hypothesis that the group A polysaccharide was a cross-reactive antigen (58). Goldstein and colleagues showed that glycoproteins in heart valves contained the *N*-acetyl-glucosamine determinant that they proposed to be responsible for immune cross-reactivity of group A streptococci with heart tissues. Dudding and Ayoub demonstrated the persistence of anti-group A carbohydrate antibody in patients with rheumatic valvular disease (59). Lyampert in Russia also published studies suggesting that the group A streptococcal polysaccharide antigen induced responses against host tissues (60–62). McCarty suggested that the terminal O-linked *N*-acetyl-glucosamine might cross-react with antibodies against the group A carbohydrate and host tissues (63). Additional evidence suggested that the hyaluronic acid capsule of the group A streptococcus also might induce responses against joint tissues (64, 65). Administration of a peptidoglycan-polysaccharide complex prepared from group A streptococci to rats induced carditis and arthritis (66–71). The studies in the 1960s and 1970s left little doubt that group A streptococci induced autoantibodies against the heart and other host tissues and that streptococcal components could induce inflammatory lesions resembling arthritis and carditis in animal tissues.

CROSS-REACTIVE mAbs RECOGNIZE CARDIAC MYOSIN AND ALPHA-HELICAL COILED-COIL MOLECULES IN HEART AND BRAIN

mAbs cross-reactive with group A streptococci and human heart tissues were produced from mice immunized with streptococcal cell wall and membrane components (4, 33, 40, 72) and from rheumatic carditis patients (35). Figure 1 illustrates the cross-reactivity of an antistreptococcal mAb with myocardium. In 1985, Krisher and Cunningham identified myosin as a cross-reactive host tissue antigen that provided the link between streptococci and the heart (4). In 1987, it was demonstrated that cardiac myosin could induce myocarditis in genetically susceptible mice (73). Myosin is an alpha-helical coiled-coil molecule that was shown to be an important tissue target of the cross-reactive mAbs (72). Other host tissue antigens recognized by antistreptococcal mouse and human mAbs included the alpha-helical coiled-coil molecules tropomyosin (29, 40), keratin (29, 45, 74), vimentin (36), laminin (6, 30, 75), DNA (36), and *N*-acetyl-β-D-glucosamine (45–47), the immunodominant epitope of the group A polysaccharide. A summary of the cross-reactive antistreptococcal mAb host tissue targets is provided in Table 2. The tissue targets identified by the cross-reactive mAbs may be important in the manifestations of group A streptococcal rheumatic fever sequelae of arthritis, carditis, chorea, and erythema marginatum (76, 77). A summary of

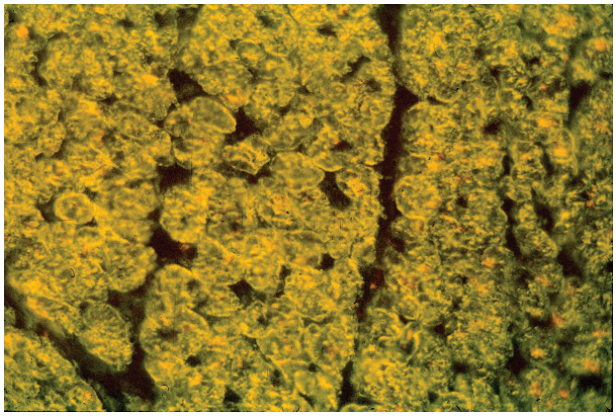


FIGURE 1 Reaction of mouse antistreptococcal mAb with a human tissue section of myocardium in a indirect immunofluorescence assay. Mouse IgM (20 μg/ml) was unreactive (not shown). mAbs were tested at 20 μg/ml. (Taken from reference 40 with permission from the *Journal of Immunology*.)

the cross-reactive human and mouse mAb specificities has been published in previous reviews (78–81).

The cross-reactive antibodies were divided into three major subsets based on their cross-reactivity with (i) myosin and other alpha-helical molecules, (ii) DNA, or (iii) *N*-acetyl-glucosamine. Figure 2 illustrates the subsets of cross-reactive antistreptococcal/antimyosin mAbs. All three subsets were identified among mAbs from mice immunized with group A streptococcal components, but in humans, the predominant subset reacted with the *N*-acetyl-glucosamine epitope and myosin and related molecules. This result is not surprising, since rheumatic fever patients do not develop antinuclear antibodies during the course of their disease. Elevated levels of poly-reactive antimyosin antibodies found in ARF sera (35) and in animals immunized with streptococcal membranes or walls (17, 49, 50, 52) most likely account for the reactivity of these sera with myocardium and other tissues. Similar types of cross-reactive mouse and human antibodies have been investigated by Lange and colleagues (82) and by Young and colleagues (83), respectively.

Poly-specific or cross-reactive autoantibodies have emerged as a theme in autoimmunity and molecular mimicry (36, 84–86). The V-D-J region genes of the human and mouse cross-reactive mAbs have been sequenced (29, 30, 87), but there is no consensus sequence to explain the molecular basis of poly-specificity and cross-reactivity. It is worth noting that the three groups of reactivities in mice do not have specific antibody V gene families or VH and VL gene combinations that correlate with a specific reactivity. However, higher-avidity cross-reactive antimyosin mAb (87) was associated with reactivity with laminin and

TABLE 2 Cross-reactive monoclonal antibody host tissue targets

Cardiac myosin
Skeletal myosin
Tropomyosin
Keratin
Vimentin
Laminin

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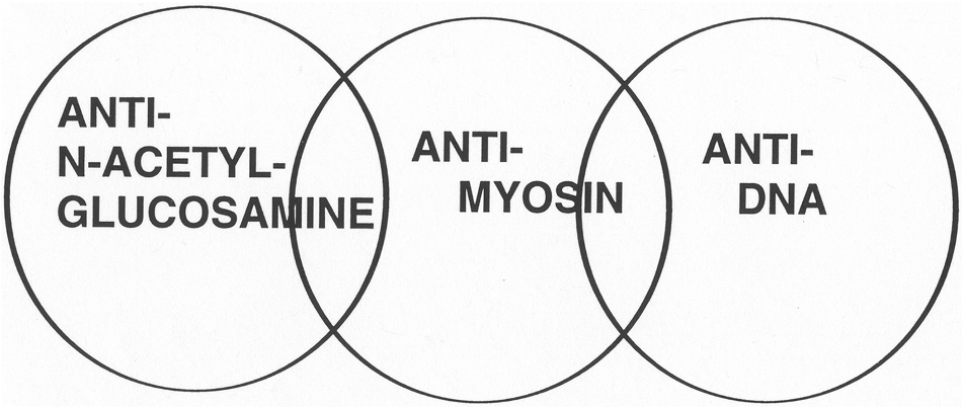


FIGURE 2 Human and murine antistreptococcal/antimyosin mAbs were divided into three subsets based on their reactivity with myosin and *N*-acetyl-glucosamine, the dominant group A carbohydrate epitope, with DNA and the cell nucleus, a property found among murine mAbs, and reactivity with myosin and a family of alpha-helical coiled-coil molecules. (Taken from reference 203 with permission from Indiana University School of Dentistry Press.)

complement-mediated heart cell cytotoxicity (30, 75). The V genes of cross-reactive human mAbs from rheumatic fever were encoded by a heterogeneous group of VH3 family genes (VH-3, VH-8, VH-23, VH-30) and a VH4-59 gene segment (29). Wu et al. reported a similar group of V gene sequences for their human antistreptococcal/antimyosin antibodies produced from Epstein-Barr virus-transformed B cell lines (83). Many sequences were found to be either in germline configuration or were highly homologous with a previously sequenced germline V gene. It has been proposed that a germline antibody may be poly-reactive due to conformational rearrangement and configurational change, permitting binding of diverse molecules (88).

Cytotoxic mouse and human mAbs have identified the extracellular matrix protein laminin as a potential tissue target present in basement membrane surrounding myocardium and valve surface endothelium (6, 30, 75, 89, 90). Cross-reactive antibodies may become trapped in extracellular matrix that may act like a sieve to capture antibody and lead to inflammation in host tissues. The cross-reactive antibodies in rheumatic heart disease, even though they react with cardiac myosin, target valve surface endothelium and laminin (6) as shown in Fig. 3 and 4 and initiate the inflammation at the endocardium, where T cells then target the activated valve endothelium and infiltrate the valve (48) as shown in Fig. 5 and lead to eventual scarring and neovascularization of the normally avascular valve. Vascular cell adhesion molecule-1 (VCAM-1) expression has been found on the valve surface endothelium that attracts very late antigen-4 (VLA-4) on the activated T cell (48).

mAbs IDENTIFY STREPTOCOCCAL CROSS-REACTIVE ANTIGENS

Introduction

The discovery leading to the development of mAbs (91) was transformative for the investigation of autoantibodies and their specificities, including the cross-reactivities of the antibodies of the group A streptococci (4). Because a single mAb can represent a minor specificity and not the antibodies represented in the disease, the sera from patients with the disease should be investigated to prove that the mAbs reflect the disease and potentially its pathogenesis. Antimyosin mAbs identified cross-reactive antigens in streptococcal membranes (36, 92) and walls (36). The streptococcal M protein was shown to react with the heart or myosin cross-reactive mAbs (40, 92). In addition, a 60-kDa protein present in the cell membrane (93, 94) was cross-reactive with myosin in the heart, and a 67-kDa protein that was immunologically similar to class II major histocompatibility molecules was also identified (95). The data suggested that cross-reactive antigens were present in both the wall and membrane of the group A streptococci. The data support previous evidence from both Kaplan (49–52, 96) and Zabriskie (15, 17, 19). Although these data were once thought to be conflicting, it is clear that the cross-reactive antibodies recognize more than one antigen in the streptococcal cell. The data

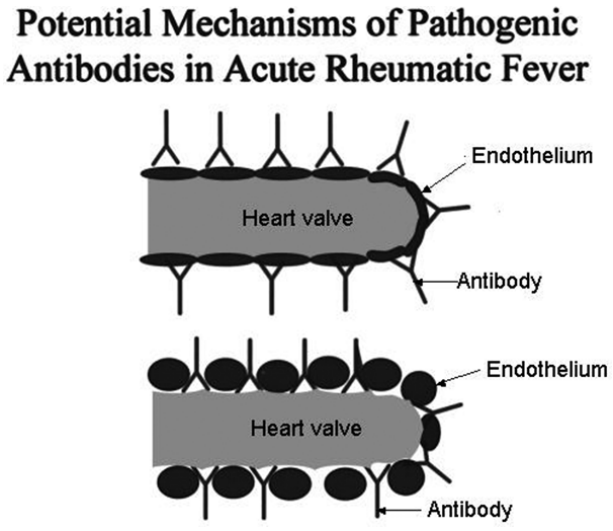


FIGURE 3 Diagram illustrating the potential mechanism of antibody in the pathogenesis of rheumatic heart disease. Cross-reactive antibody is shown binding directly to endothelium (top diagram) or binding to basement membrane of the valve (bottom diagram) exposed due to shear stress or damage by antibody and complement. (Reproduced with permission from ASM press).

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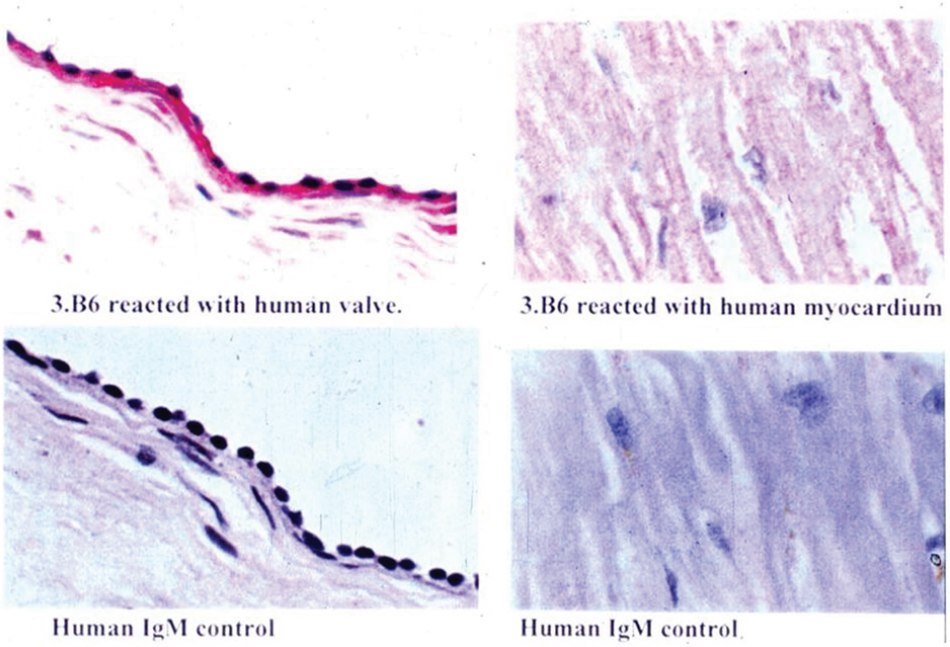


FIGURE 4 Reactivity of antistreptococcal/antimyosin mAb 3.B6 with normal human valve endothelium and myocardium. Formalin-fixed human mitral valve (A) and myocardium (B) were reacted with mAb 3.B6 at 10 μg/ml. mAb 3.B6 binding was detected using biotin-conjugated anti-human antibodies and alkaline phosphatase-labeled streptavidin followed by fast red substrate. Control sections did not react with human IgM at 10 μg/ml (C,D). (Reproduced with permission from Lippincott Williams and Wilkins).

previously reported by Goldstein (58) are also supported by the evidence that a subset of cross-reactive mAbs recognized *N*-acetyl-β-D-glucosamine, the immunodominant epitope of the group A polysaccharide. *N*-acetyl-glucosamine is a major epitope of some of the cross-reactive mouse mAbs and virtually all of the human cross-reactive mAbs investigated (6, 45–47). The studies link together the cross-reactivity of the group A carbohydrate epitope, GlcNAc, human cardiac myosin, and streptococcal M protein. Antigenic redundancy due to cross-reactivity may be an important factor in triggering disease in a susceptible host. The cross-reactive antigens are now seen as separate entities recognized by mAbs that recognize more than one antigen molecule. Thus, the previous studies, which before seemed to be conflicting, were all correct. The use of mAbs has allowed dissection of the group A streptococcal cross-reactive antigens that would not have been possible using polyclonal sera. The following sections provide more evidence about the identification and analysis of the cross-reactive antigens of the group A streptococci. Table 2 summarizes the host tissue protein antigens targeted by antistreptococcal antibodies in disease and following immunization with group A streptococcal components. Table 3 summarizes the group A streptococcal cross-reactive antigens, of which the group A carbohydrate (6) and the M proteins (8, 97–101) are the most well investigated.

M Proteins and Rheumatic Fever

Investigation of the M proteins has provided important information about the sequence and primary structure of the molecule. The hypothesis that M proteins and myosin have immunological similarities was supported by the structural studies of Fischetti and colleagues (102–105) that demonstrated the seven-amino-acid-residue periodicity that is common among group A streptococcal M proteins and shared with proteins such as tropomyosin, myosin, desmin, vimentin, and keratin. Figure 6 illustrates the seven-residue periodicity and the homology characteristic of alpha-helical coiled-coil proteins such as tropomyosin and myosin. The cross-reactivity between group A streptococci and myosin was first identified by Krisher and Cunningham using mouse mAbs from mice immunized with group A streptococcal antigens (4). Subsequent studies by Dale, Beachey, Bronze, and colleagues using polyclonal sera and affinity purified antibodies further demonstrated immunological cross-reactivity between M proteins and myosin (37–39, 106). Studies using the cross-reactive mAbs also identified immunological cross-reactivity between streptococcal M proteins (both PepM [the N-terminal half of the molecule] and recombinant molecules) and cardiac and skeletal myosins (31, 36, 40).

Since streptococcal M proteins have been investigated for potential epitopes that were recognized by the cross-reactive mAbs, affinity purified antimyosin antibodies from ARF sera were reacted with peptides of streptococcal M5 protein. Affinity purified antimyosin antibodies from ARF reacted with an M5 amino acid sequence (residues 184 to 188) near the pepsin cleavage site (near the center of the molecule) in M5 and M6 proteins (40). The epitope, located in the B repeat region of M5 and M6 proteins, appeared to be a B cell epitope for antibody-mediated cross-reactivity with myosin (32, 40). An M5 peptide, containing the B repeat region epitope Gln-Lys-Ser-Lys-Gln (QKSKQ) was shown to inhibit antimyosin antibodies in ARF (40). Furthermore, an M5 peptide that contained the QKSKQ sequence induced antibodies in BALB/c mice against cardiac or skeletal myosins and the light meromyosin (LMM) fragment of myosin. This evidence further supported the previous findings

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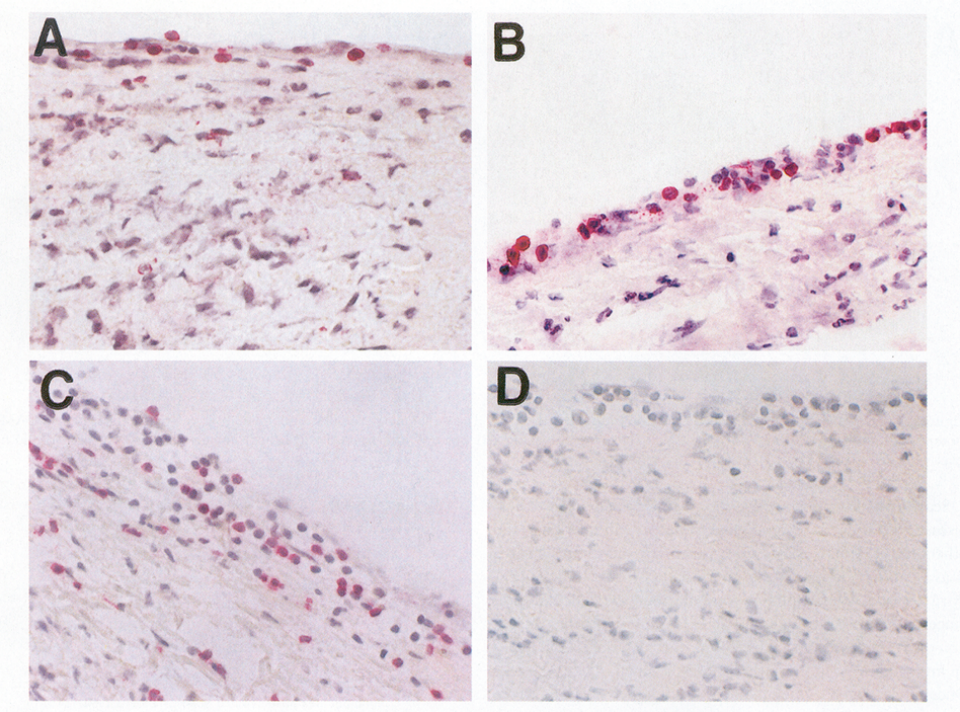


FIGURE 5 Adhesion and extravasation of T lymphocytes into an ARF valve in valvulitis. (A,B) Extravasation of $CD4^+$ lymphocytes (stained red; original magnification, 200× and 400×, respectively). (C) Extravasation of $CD8^+$ lymphocytes (stained red) into the valve through the valvular endothelium (magnification, 200×). An IgG_1 isotype control mAb (IgG_1) did not react with the same valve (not shown). (With permission from the *Journal of Infectious Diseases*, Chicago Press).

indicating that the QKSKQ sequence is important in the antibody-mediated cross-reactions with myosin in ARF (40). M5 residues 164 to 197 were demonstrated to induce antibodies against sarcolemmal membrane of heart tissue (107), and M5 residues 84 to 116 induced heart-reactive antibody and reacted with antimyosin antibody purified from ARF sera (40, 107). Studies by Kraus and colleagues (42) demonstrated a vimentin cross-reactive epitope present in the M12 protein, and M protein peptides containing brain cross-reactive epitopes were localized to the M5 protein sequence 134 to 184 and the M19 sequence 1 to 24 (98, 99). A summary of the currently known myosin cross-reactive B cell epitopes in M5 protein is shown in Fig. 7, which illustrates the A, B, and C repeat regions of M protein.

Immunization of BALB/c mice with each of 23 overlapping synthetic peptides of M5 protein has revealed that the M5 peptides NT3-NT7, B2B3B, and C1A-C3 induce antihuman cardiac myosin antibodies as shown in Fig. 8. Similar overlapping synthetic peptides spanning the M5 protein molecule have been reported elsewhere (32). Titers of the anti-M5 peptide sera were 10 times greater against cardiac myosin than against skeletal myosin, tropomyosin, vimentin, and laminin (32). Certain M5 peptides appeared to induce a cardiac myosin-directed response in BALB/c mice. Furthermore, BALB/c mice developed mild myocarditis when immunized with M5 peptides NT4, NT5, NT6, B1A, and B3B (32). Peptides from the C-repeat region of M5 protein did not produce myocardial lesions when administered to BALB/c mice.

TABLE 3 Cross-reactive antigens of group A streptococci

M proteins
Hyaluronic acid capsule
N-acetyl-glucosamine/group A polysaccharide
60-kDa wall-membrane antigen
67-kDa antigen

The C-repeat region of class I M proteins contains the class I epitope, which is identified by reactivity with anti-M protein mAb 10B6 (108–111). Class I M protein serotypes were streptococcal strains associated with pharyngitis and rheumatic fever. Responses against the class I epitope were stronger in patients with rheumatic fever than in those with uncomplicated disease (112, 113). mAb 10B6, which recognizes the C-repeat epitope, also reacted with skeletal and cardiac myosins and the heavy meromyosin subfragment of myosin (112). The C-repeat class I epitope in M5 contains the amino acid sequence KGLRRDLDASREAK, which shares homology with RRDL, a conserved amino acid sequence found in cardiac and skeletal myosins. The myosin sequence RRDL is located in the heavy meromyosin subfragment of the cardiac and skeletal myosin heavy chain (112).

It was reported that reactivity of IgG from ARF sera was greater to the class I peptide sequence than IgG from uncomplicated pharyngitis (112). This was confirmed in a study by Mori and colleagues (114). However, the lower immune response to the class I epitope in uncomplicated pharyngitis most likely occurred because the patients with uncomplicated pharyngitis had been treated with penicillin (115). Antibodies against the class I epitope were affinity purified from ARF sera and shown to react with myosin (112).

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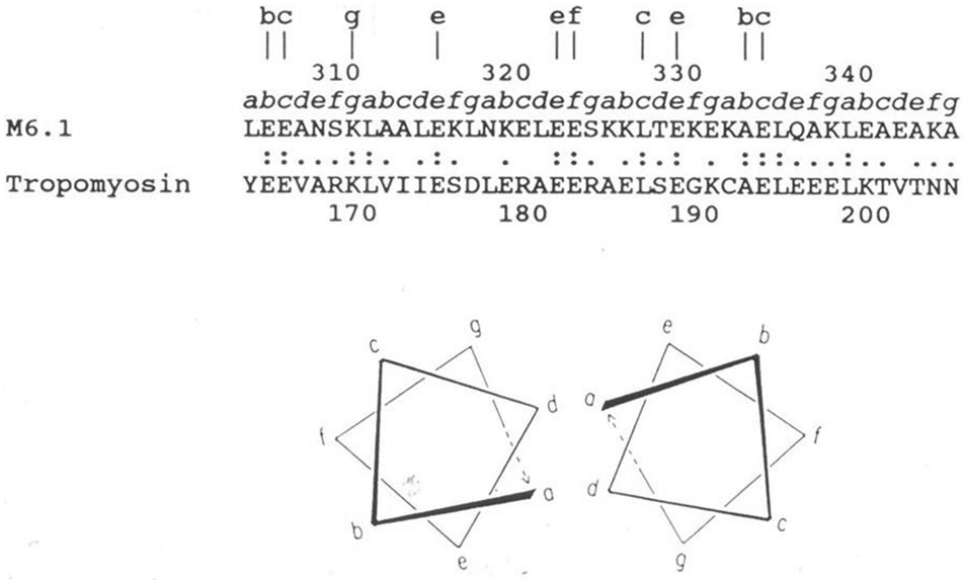


FIGURE 6 Sequence alignment of streptococcal M6 protein and human cardiac tropomyosin in a region exhibiting significant homology. Lowercase letters a to g directly above the sequence designate the position of these amino acids within the seven-residue periodicity in both segments. Lowercase letters at the top of the figure designate identities at external locations in the heptad repeat. Double dots indicate identities, and single dots indicate conservative substitutions. Within this segment of the streptococcal M6 molecule, 31% homology is observed with tropomyosin. Since both molecules are alpha-helical coiled-coil proteins, they contain the seven-residue repeat pattern where positions a and d are usually hydrophobic. Similar homologies are seen between M proteins and myosin heavy chains and any of the three laminin chains. (Reproduced from reference 40 with permission from the *Journal of Immunology*.)

Studies by Huber and Cunningham demonstrated that streptococcal M5 peptide NT4 containing the amino acid sequence GLKTENEGLKTENEGLKTE could produce myocarditis in MRL/++ mice, an autoimmune-prone strain (116). The studies demonstrated that the myocarditis was mediated by $CD4^+$ T cells and class II major histocompatibility complex molecules. Antibodies against the IA^k major histocompatibility molecule or antibody against $CD4^+$ T cells abrogated the myocarditis (116). Amino acid sequence homology between the NT4 peptide of M5 and human cardiac myosin demonstrates 80% identity. Figure 9 illustrates the homology between the M5 peptide NT4 sequence and the cardiac myosin sequence. The mimicking sequence in NT4 is repeated four times in the M5 protein and is present in myosin only once. Repeated regions of the M protein that mimic cardiac myosin may be important in breaking tolerance in the susceptible host and producing autoimmune disease. The data support the hypothesis that epitopes in streptococcal M5 protein that mimic cardiac myosin may be important in breaking tolerance to this potent autoantigen. Table 4 summarizes the M protein amino acid sequences observed to induce inflammatory myocardial lesions in mice.

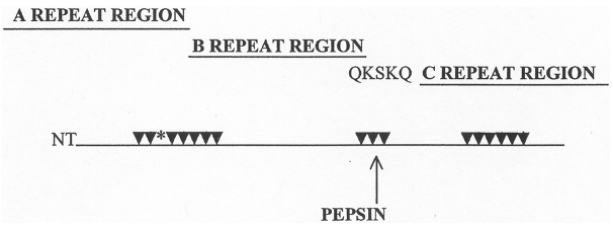


FIGURE 7 Antimyosin antibody cross-reactive sites in M5 protein A, B, and C repeat regions of the molecule. Arrows point to sites determined to induce human cardiac myosin cross-reactive antibody (32). The asterisk (*) marks the location of peptide NT4 containing several repeats of an epitope in cardiac myosins that causes myocarditis in MRL/++ and BALB/c mouse strains (32, 116). Site QKSKQ is the epitope determined to react with antimyosin antibody in ARF sera (40). (Reproduced from *Effects of Microbes on the Immune System* with permission from Lippincott Williams and Wilkins.)

Streptococcal M proteins not only mimic epitopes in cytoskeletal proteins, but they mimic epitopes in other strong bacterial and viral antigens, namely, heat shock protein (Hsp-65) (117) and coxsackie viral capsid proteins (31, 116, 118, 119). These immunological cross-reactions may be vital to the survival of the host and suggest that antibody molecules may recognize and neutralize more than a single infectious agent. Such antibodies may be an important first line of defense and would be highly advantageous for the host. Multiple pieces of evidence from experiments using synthetic peptides have shown that sites in the M protein mimic a site(s) in the VP1 capsid protein of coxsackie virus (31, 120). Further evidence demonstrated that some of the antistreptococcal/antimyosin mAbs neutralized enteroviruses and were cytotoxic for heart cells (31). This was extremely interesting because coxsackie viruses cause autoimmune myocarditis in susceptible hosts (121–123). The Hsp-65 antigen has been shown to play a role in the development of arthritis and diabetes (124, 125). Cross-reactive epitopes shared between streptococcal M proteins and Hsp-65 play a role in arthritis sequelae. Antibodies against heat shock protein 60

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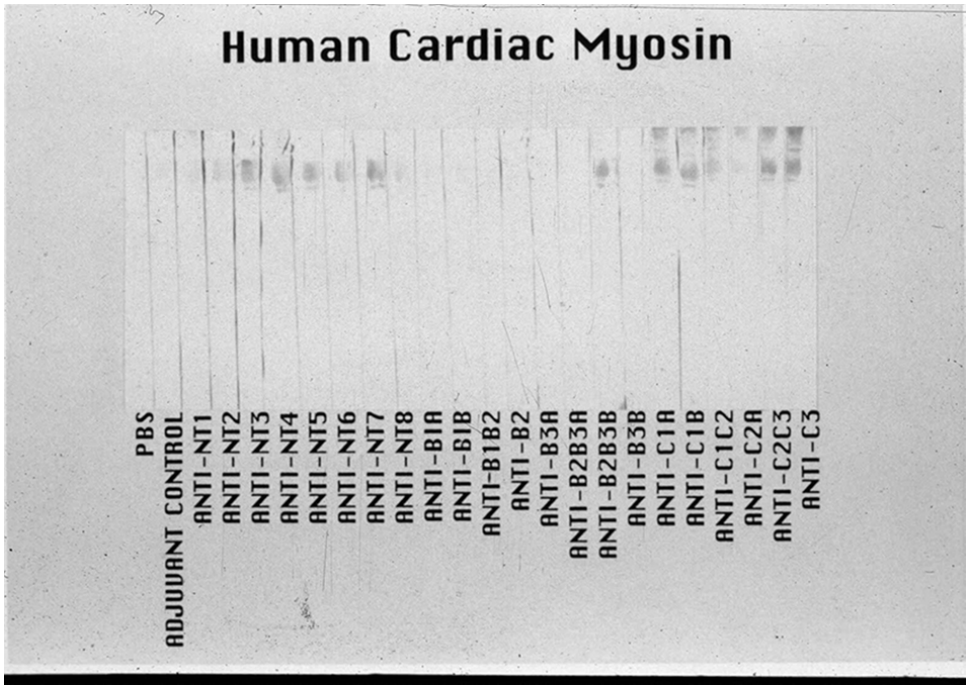


FIGURE 8 Reactivity of the antistreptococcal M5 peptide sera in a Western immunoblot of human cardiac myosin. The 200-kDa protein band of the purified human cardiac myosin, shown in the stained portion of the Western blot, reacted most strongly with antipeptide sera from mice immunized with the M5 peptides NT3-7, B2B3B, C1A, C1B, C2C3, and C3. Sera were tested at a 1:1000 dilution. A control antimyosin mAb CCM-52 (a gift from William Clark, Cardiovascular Research Institute, Michael Reese Hospital and Medical Center, Chicago, IL) reacted with the 200-kDa band present in our purified preparation of human cardiac myosin heavy chain. Purification of the human cardiac myosin heavy chain to homogeneity was previously described by Dell et al. (204). The Western blot confirms the data seen in the enzyme-linked immunosorbent assay with human cardiac myosin. (Reproduced from reference 32 with permission from ASM Press.)

have been implicated in cytotoxicity against endothelium (126). Shared epitopes among pathogens may be important in molecular mimicry, may break tolerance to cryptic host molecules, and may influence the development of autoimmune diseases. Table 5 summarizes bacterial and viral proteins that have immunological similarities with M protein.

Identification of the Molecular Basis of Mimicry in the Streptococcal M Protein

Studies were undertaken to crystallize a portion of the streptococcal M1 protein (127) to evaluate its virulence properties when portions of the amino acid sequence were mutated, but also to investigate regions and structures responsible for eliciting or reacting with cross-reactive antibodies that participate in molecular mimicry. The structural study revealed irregularities and instabilities in the coiled coil of the M1 fragment crystal. Similar structural instabilities and irregularities occur in myosin and tropomyosin that had previously been demonstrated to cross-react with the streptococcal M proteins (37–40). Stabilization of the irregularities of the M protein structure, by creating mutants of the M protein gene to alter specific amino acids, enhanced the stability of the coiled coil and diminished the virulence properties of the M1 protein, including decreased cross-reactivity with autoantibodies that cross-reacted with heart tissues, cardiac myosin, and group A streptococcal M proteins (127). Loss of the heart or tissue cross-reactivity of the streptococcal M protein improves its potential for use as a vaccine antigen.

M Proteins and Cross-Reactive T Cells

M proteins have also been shown to stimulate human T lymphocyte responses (97, 128–135), including cytotoxic T lymphocytes from ARF patients (128, 136). PepM protein

```
Streptococcal M Protein M5 Peptide NT4      GLKTENEG
                                             :  :::
                          cardiac myosin     KLQTENGE
```

FIGURE 9 Sequence homology between human cardiac myosin and peptide NT4 that causes myocarditis (32, 119). The homologous sequence repeats four times in the streptococcal M5 protein and in NT4 and once in cardiac myosin. Repeated sequences in M proteins that mimic cardiac myosin may be important in inducing inflammatory heart disease. (Adapted from reference 119 with permission from the *Journal of Immunology*.)

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TABLE 4 M5 protein sequences that produce myocarditis in mice[a]

Peptide	Amino acid sequence	Residues
A repeat region		
NT4	GLKTENEGLKTENEGLKTE	40-58
NT5	KKEHEAENDKLKQQRDTL	59-76
NT6	QRDTLSTQKETLEREVQN	72-89
B repeat region		
B1A	TRQELANKQQESKENEKAL	111-129
B3B	GALKQELAKKDEANKISD	202-219

[a]NT4 produced myocarditis in BALB/c (32) and MRL/++ (116) mice. The other peptides shown produced myocarditis in BALB/c mice. (Reproduced from *Effects of Microbes on the Immune System* with permission from Lippincott Williams and Wilkins.)

(pepsin-extracted M protein fragment) has been reported to be a superantigen that stimulated Vβ2, Vβ4, and Vβ8 bearing T cell subsets (133–135, 137). Other reports show that neither recombinant nor native forms of M proteins are superantigenic (138–140). A superantigenic site reported for the M5 protein has been localized to the amino acid sequence located in M5 residues 157 to 197 (KEQENKETIGTLKKILDETVKDKLAKE QKSKQNIGALKQEL) in the B3 repeat region (137). The site has a significant amount of sequence homology with other superantigens. The role of M protein or other superantigens in ARF may be to activate large numbers of T cells, including some that are cross-reactive and may lead to ARF.

Studies of T cell epitopes in rheumatic fever and in animal models focus on the M5 protein molecule because serotype M5 has often been associated with ARF outbreaks (141). T and B cell epitopes of the M5 protein were defined in earlier studies by Robinson and colleagues (131, 132), by Good and colleagues (129, 130), and in my laboratory (32).

Guilherme et al. isolated cross-reactive T cells from mitral valves, papillary muscle, and left atrium of rheumatic fever patients previously infected with M5 group A streptococci (97). T cell lines were responsive to several peptides of the streptococcal serotype M5 protein and proteins from heart tissue extracts. Sequences from the A and B repeat regions of M5 protein that stimulated the valvular T cells are shown in Table 6. In BALB/c mice using synthetic peptides, myosin-cross-reactive T cell epitopes from the A, B, and C repeat regions of the M5 protein were identified (32). Six dominant myosin cross-reactive T cell epitopes were located in the M5 molecule (32). Table 6 summarizes (i) the dominant myosin cross-reactive T cell epitopes of M5 protein in BALB/c mice (32), (ii) the M5 sequences recognized by T cell clones from rheumatic heart valves (97), and (iii) the M5 peptides reported by Good and colleagues to stimulate human T cells from normal controls and rheumatic fever patients that were responsive to myosin peptides (129, 130). An important correlation seen in Table 6 indicates that M5 peptides NT4/NT5 (GLKTENEGLKTENEGLKTE and KKEHEAENDKLKQ QRDTL) and B1B2/B2 (VKDKIAKEQENKETIGTL and TIGTLKKILDETVKDKIA), which were dominant cross-reactive T cell epitopes in the BALB/c mouse, contain sequences similar to those reported to be recognized by T cells from rheumatic valves (97). Peptides NT4 and NT5 produced inflammatory infiltrates in the myocardium of animals immunized with those peptides (32). The collective evidence on cross-reactive T cells suggests that amino acid sequences in M5 protein that share homology with cardiac myosin may break tolerance and promote T cell-mediated inflammatory heart disease in animals and humans (32).

TABLE 5 Bacterial and viral antigens with immunological similarities with group A streptococcal M proteins

Heat shock protein 65
Coxsackievirus VP1, 2, 3 capsid proteins
Group A streptococcal carbohydrate

T cells isolated and cloned from the peripheral blood or from human rheumatic mitral valves of human rheumatic heart disease patients recognized streptococcal M5 peptides and LMM peptides (21, 22, 25). The cross-reactive T cells taken from ARF and RHD rheumatic heart disease heart valves mainly recognize three regions (residues 1 to 25, 81 to 103, and 163 to 177) of the amino-terminal region of M5 protein, several valve-derived proteins, and peptides of the β-chain of the human cardiac LMM region, which is the smaller of two subunits produced by tryptic digestion of human cardiac myosin (21, 22, 25, 97). Mimicry between M proteins and cardiac myosin stimulates cross-reactive T cells in the peripheral blood of the host (25, 142, 143). The T cells then travel to the valve once the endothelium of the valve becomes activated and inflamed (48). This extravasation event at the valve endocardium allows the cross-reactive T cells to enter the valve, where they recognize and proliferate to valvular proteins such as vimentin (89), which may include cardiac myosin from papillary muscle. T cells that remain in the valve survive if they continue to be stimulated by host alpha helical proteins within the valve. The activated cross-reactive T cells when continually stimulated within the valve become pathogenic for the host and produce the TH1 mechanism of pathogenesis with scarring in the valve. Figure 10 illustrates the events in rheumatic carditis that result from immune responses against the cross-reactive antigens of the group A streptococci (144).

The granulomatous Th1 cell response and the presence of interferon-γ (IFNγ) have been reported as dominant in rheumatic valves (145). Studies of rheumatic heart disease have suggested that Th17 cells in peripheral blood are elevated in rheumatic heart disease with concomitant down-regulation of the Treg phenotype (146). In ARF and rheumatic heart disease, elevated numbers of Th17 cells, high interleukin-17A (IL-17A) levels, and decreased T regulatory cells were reported in peripheral blood (146). Although less is known about Th17 cell responses in rheumatic heart disease, Th17 cells are important in group A streptococcal infections and have been identified in nasopharyngeal and tonsillar lymphoid tissues in streptococcal infection animal models (147–149). Different routes of bacterial infection may induce long-lived Th1 memory cells and short-lived Th17 cell responses (148). IL-17 triggers effective immune responses against extracellular bacteria, such as promoting neutrophil responses (150–153).

The Lewis rat model of rheumatic heart disease also suggests the presence of IL-17A in the pathogenesis of experimental valvulitis induced by immunization with group A streptococcal antigens (13, 101). This finding does not preclude Th1, which is often found in tandem with Th17 responses in autoimmune disease models and in human autoimmune diseases (148). Although both Th1 and Th17 are proinflammatory, Th17 responses have been found to be more damaging in the chronic stages of disease leading to

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TABLE 6 Summary of myosin or heart cross-reactive T cell epitopes of streptococcal M5 protein[a]

Peptides	Sequence[a]	Origin of T cell clone or response[c]
1–25	TVTRGTISDPQRAKEALDKYELENH[b]	ARF/valve (142)
81–96	DKLKQQRDTLSTQKETLEREVQN[b]	ARF/valve (142)
163–177	ETIGTLKKILDETVK[b]	ARF/valve (142)
337–356	LRRDLDASREAKKQVEKAL	Normal/PBL (130)
347–366	AKKQVEKALEEANSKLAALE	Mice (129)/normal PBL (130)
397–416	LKEQLAKQAEELAKLRAGKA	ARF/PBL (130)
NT4 40–58	GLKTENEGLKTENEGLKTE	BALB/c/Lymph node[d]
NT5 59–76	KKEHEAENDKLKQQRDTL	
B1B2 137–154	VKDKIAKEQENKETIGTL	
B2 150–167	TIGTLKKILDETVKDKIA	
C2A 254–271	EASRKGLRRDLDASREAK	
C3 293–308	KGLRRDLDASREAKKQ	

[a]Taken from reference 32 with permission from ASM press.
[b]The amino terminal TVTRGTIS sequence was taken from the M5 amino acid sequence published by Manjula et al. (201) and deviates from the M5 sequence published by Miller et al. (202) at positions 1 and 8. Other marked sequences ([81–96] and [163–177]) were taken from the PepM5 sequence as reported by Manjula (201). These two sequences are found in the sequence as 67–89 and 174–188, respectively, as reported by Miller et al. (202). All other sequences shown above are from the M5 gene sequence reported by Miller et al. (202).
[c]PBL, peripheral blood lymphocytes, ARF, acute rheumatic fever.
[d]BALB/c mice immunized with purified human cardiac myosin and the recovered lymph node lymphocytes were stimulated with each of the peptides in tritiated thymidine uptake assays.

fibrosis, and IL-17A was dispensable in the earlier proinflammatory stages of myocarditis in mice (154) but promoted fibrosis in later stages and was associated with heart failure in humans (155).

A New Model of Valvular Heart Disease Induced by Group A or Group G Streptococcal M Proteins in the Lewis Rat

A new model of experimental autoimmune valvulitis and rheumatic heart disease was developed by Quinn et al. by immunization of the Lewis rat model with the recombinant M6 protein (23). The lesions that developed demonstrated characteristics of rheumatic heart disease in humans with verrucae (Fig. 11C) (23), Anitschkow cells, and edema with cellular infiltration into the endocardium of the valve (Fig. 11A and B) (8, 23). T cell lines from the model proliferated to both the rM6 protein and cardiac myosin. The model appeared to be very similar in appearance to the VCAM-1 activation (Fig. 11D) in humans and infiltration of $CD4^+$ T cells into the VCAM-1 positive endothelium/endocardium (not shown) of the valve in rheumatic heart disease (48) (Fig. 12). More recent studies identified A and B region peptide epitopes of M5 protein that induced valvulitis in the Lewis rat (8). Repeat region peptides NT4, NT5/6, and NT7 (see Table 6) induced valvulitis similar to the intact pepsin fragment of M5 protein, PepM5. T cell lines from rats with valvulitis proliferated to M5 peptides NT5/6 and NT6. Passive transfer of an NT5/6-specific T cell line into naive rats produced valvulitis characterized by infiltration of $CD4^+$ cells and upregulation of VCAM-1 in valvular endothelium, while another NT6-specific T cell line did not target the valve (8). Thus, the infiltrating T cell line appeared to be important in the induction of inflammation in the valve.

Recent studies have used the Lewis rat model to demonstrate the induction of valvular heart disease by the C repeat region peptides of group A streptococcal M5 protein (12, 13). T cells from the model proliferated to M protein and cardiac myosin. Further studies by Ketheesan and colleagues demonstrated that exacerbation and increasing severity of valvulitis followed repeated group A streptococcal immunizations with group A streptococcal M protein (156). Lewis rats were immunized with group A streptococcal recombinant M5 protein and boosted once, twice, or three times. Anti-cardiac myosin antibodies and T cell responses increased with the booster immunizations. More severe lesions were observed in the repetitively immunized Lewis rats (156). Thus, the model demonstrates increased cardiac damage as seen in humans after repeated streptococcal infections. Immunization of the Lewis rat model with M protein from group G streptococci also led to valvular lesions (101). In the experimental autoimmune valvulitis model, both group A and group G M proteins and cardiac myosin induced proliferation of the T cells. IL-17A and IFNγ were found to be upregulated in the model (101). Thus, roles for IL17A in ARF and rheumatic heart disease are supported by the studies of patients with carditis.

Human Cardiac Myosin Peptide Epitopes in Rheumatic Heart Disease in Human Populations and Their Use in Monitoring Progression of Disease

Studies of humans with rheumatic fever and rheumatic heart disease have identified disease-specific epitopes of human cardiac myosin in the development of rheumatic carditis in humans (24). Immune responses to cardiac myosin were similar in rheumatic carditis among a small sample of worldwide populations, in which serum IgG targeted disease-specific human cardiac myosin epitopes in the S2 subfragment hinge region within S2 peptides containing amino acid residues 842 to 992 and 1164 to 1272 (Fig. 13) (24). Analysis of the anti-cardiac myosin autoantibodies in rheumatic carditis in a Pacific Islander family further confirmed the specific responses against cardiac myosin in rheumatic heart disease and confirmed the presence of potential rheumatogenic epitopes in the S2 region of human cardiac myosin (24). The study suggested that cardiac myosin epitopes targeted in rheumatic carditis were in the S2 hinge region of cardiac myosin and that the anti-cardiac myosin responses were

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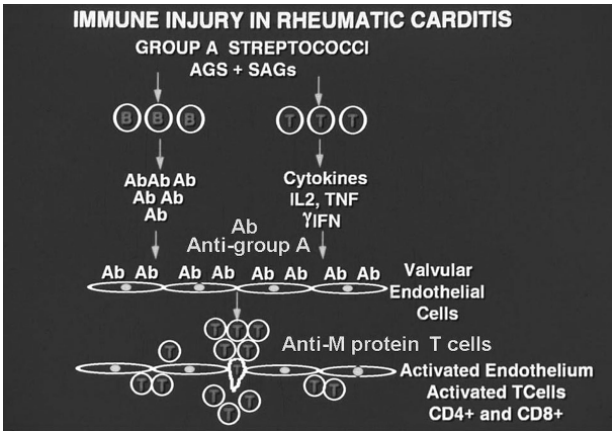


FIGURE 10 Diagram representing the proposed immunopathogenesis of poststreptococcal rheumatic heart disease. Initially, B and T cells are activated by specific streptococcal antigens and superantigens, leading to strong immune responses against streptococcal and host antigens. The development of pathogenic clones of B and T lymphocytes is important in the development of the disease. Initially, antibodies develop against the group A carbohydrate, which are cross-reactive with the valve surface, and glycoproteins such as laminin and bind to the valve surface endothelium (endocardium), leading to inflammation and upregulation of cell adhesion molecules such as VCAM-1 on activated surface endothelium of the valve. M protein-reactive T cells enter the valve through the surface endothelium by binding to cell adhesion molecules such as VCAM-1 and extravasate into the valve (48). The formation of scar tissue in the valve followed by neovascularization allows the disease to continue in the valve. The specificity of the T cells in blood (25) and T cells entering the valve have been shown to react to M protein (22, 97, 144). T cell subsets include Th1 (IFNγ) (145) in the pathogenesis of proinflammatory responses and the development of the scarred and fibrotic valve. IL-17A has also been associated with rheumatic heart disease in humans and animal models, suggesting that Th17 cells are involved in disease (101, 146, 149).

similar among populations with rheumatic carditis worldwide regardless of the infecting group A streptococcal M protein serotype.

Further study of cardiac myosin peptide epitopes revealed that Australian patients with active rheumatic heart disease reacted significantly more to peptides from the S2 or LMM regions of human cardiac myosin than did healthy controls (11). Comparison of serum from active rheumatic heart disease patients to serum from patients treated for over a year with antibiotics revealed that the treatment led to a reduction in the anti-cardiac myosin peptide autoantibodies (11). Thus, identifying the immune response against the cardiac myosin peptides may monitor progression of rheumatic heart disease and may also determine effective treatment with a concomitant decline of the autoantibodies to cardiac myosin.

M Proteins and Poststreptococcal Acute Glomerulonephritis

Kefalides and colleagues showed that sera from patients with acute poststreptococcal glomerulonephritis contained antibodies against laminin, collagen, and other macromolecules found in the glomerular basement membrane (157). The epitope recognized in collagen was identified and shown to be located in the 7-S domain of type IV collagen (158). Streptococcal antigens, immunoglobulins, and complement were detected in the kidney glomeruli in acute poststreptococcal glomerulonephritis (159). Studies by Lange and Markowitz suggested that the glomerular basement membrane shared antigens with streptococcal M12 protein (160) and/or between the group A streptococcus and glomeruli (41, 161–163). Kraus and Beachey discovered a renal autoimmune epitope (Ile-Arg-Leu-Arg) in M protein (43). Evidence has suggested that certain M protein serotypes are associated with nephritis and that molecular mimicry or immunological cross-reactivity between glomeruli and M protein could be one mechanism by which antiglomerular antibodies are produced during infection. In animal models of nephritis induced by nephritogenic streptococci (M type 12), antiglomerular antibodies eluted from kidney glomeruli reacted with the type 12 streptococcal M protein (164). Furthermore, an antiglomerular mAb reacted with the M12 protein (163). These studies support immune-mediated mechanisms in poststreptococcal acute glomerulonephritis (AGN). Streptococcal and renal antigens may be an important source of mimicking antigen present in the kidney and play a role in binding immunoglobulin and complement with production of nephritis.

Novel Cross-Reactive Protein Antigens in Group A Streptococci

Antimyosin antibodies from ARF were used to identify group A streptococcal cross-reactive antigens other than M proteins. The cross-reactive antigens were detected in a *Streptococcus pyogenes* strain that had spontaneously lost its *emm* gene. The affinity purified antimyosin antibodies were used to screen gene libraries and the antigen preparations from this M protein-deficient streptococcus. A 60-kDa streptococcal actin-like protein (165), a 60-kDa wall-membrane protein (93, 94), and a 67-kDa protein from a cloned gene product (95) were identified. All of the antigens were unique and interesting. The 60-kDa actin-like molecule behaved biochemically like an actin and produced actin-like filaments observed by electron microscopy (165). The 67-kDa cloned gene product recognized by antimyosin antibody from ARF sera was novel in that it shared enough sequence homology with major histocompatibility molecules that it reacted with sera against mouse major histocompatibility complex class II molecules and exhibited a hydrophilicity plot almost identical to that of major histocompatibility complex class II molecules (95). The gene for the 67-kDa protein was present only in pathogenic streptococcal groups A, C, and G (95). This gene was not found in other streptococcal groups, *Escherichia coli*, or staphylococci when they were screened for hybridization with the gene probe. The role of the 67-kDa protein as a potential virulence factor in the pathogenesis of streptococcal infections or sequelae is not yet known.

IMMUNE RESPONSES TO *N*-ACETYL-B-D-GLUCOSAMINE, DOMINANT EPITOPE OF GROUP A POLYSACCHARIDE, IN THE PATHOGENESIS OF RHEUMATIC HEART DISEASE AND SYDENHAM'S CHOREA IN ARF

Immunological Cross-Reactivity Between Carbohydrates and Peptides

The structure of the group A polysaccharide is a polymer of rhamnose with *N*-acetyl-β-D-glucosamine linked to the rhamnose backbone. The *N*-acetyl-glucosamine is the immunodominant epitope of the group A carbohydrate that

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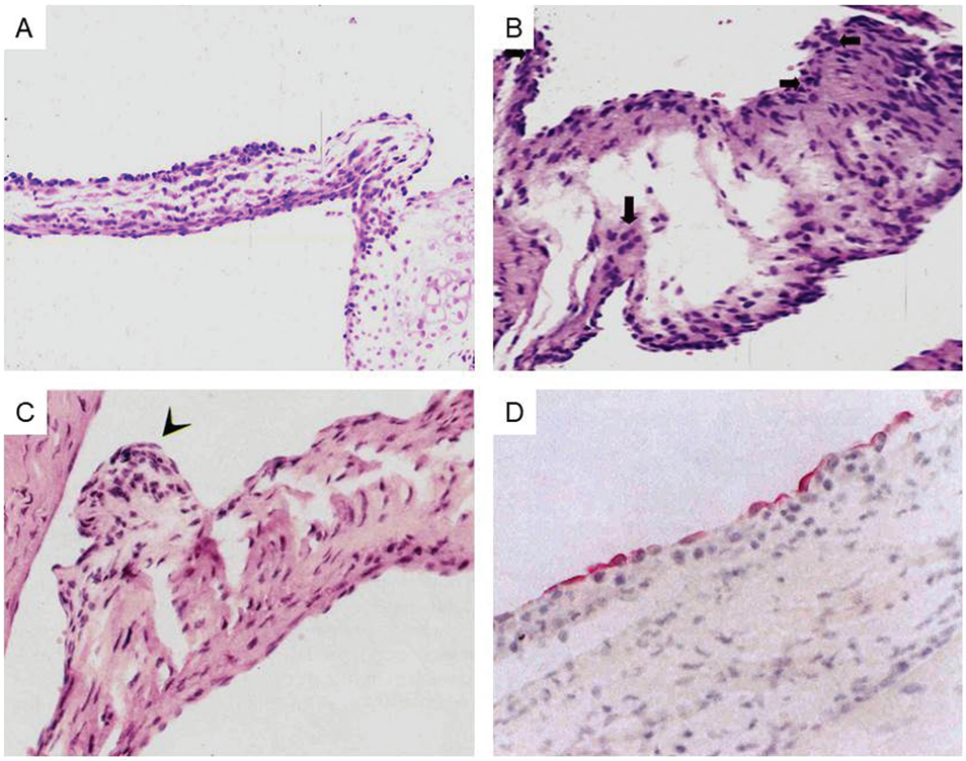


FIGURE 11 (A) Induction of valvulitis and cellular infiltration in hematoxylin- and eosin-stained heart valves from Lewis rats immunized with group A streptococcal M5 peptides NT1 to NT4/5 (AVTRGTINDPQRAKEALD amino acid [aa] residues 1 to 18; NT-2 KEALDKYELENHDLKTKN aa residues 14 to 31; NT-3 LKTKNEGLKTENEGLKTE aa residues 27 to 44; NT-4 GLKTENEGLKTENEGLKTE aa residues 40 to 58; NT-4/5 GLKTEKKEHEAENDKLK aa residues 54 to 70). (Taken from Kirvan et al. [8] with permission from the *Journal of Translational Cardiology*.) (B) Induction of valvulitis, edema, and cellular infiltration in hematoxylin- and eosin-stained heart valves from Lewis rats immunized with group A streptococcal M5 peptides NT1 to NT4/5 (AVTRGTINDPQRAKEALD aa residues 1 to 18; NT-2 KEALDKYELENHDLKTKN aa residues 14 to 31; NT-3 LKTKNEGLKTENEGLKTE aa residues 27 to 44; NT-4 GLKTENEGLKTENEGLKTE aa residues 40 to 58; NT-4/5 GLKTEKKEHEAENDKLK aa residues 54 to 70). (Taken from Kirvan et al. [8] with permission from the *Journal of Translational Cardiology*.) (C) Verrucous nodule observed on Lewis rat valve after immunization with group A streptococcal rM6 protein. (Taken from Quinn et al. [23] with permission from *Infection and Immunity*.) (D) VCAM-1 expressed on rheumatic valve. (Taken from Roberts et al. [48] with permission from the *Journal of Infectious Diseases*.)

distinguishes *S. pyogenes* from other streptococcal species. The *N*-acetyl glucosamine and the group A polysaccharide have been identified in cross-reactions between streptococci and heart tissues (58, 61, 62). Recently, Shikhman, Adderson, and others reported that a subset of cross-reactive antimyosin/antistreptococcal mAbs from immunized mice and virtually all human mAbs from rheumatic carditis patients reacted strongly with an *N*-acetyl-glucosamine epitope of the group A streptococcal carbohydrate (29, 45–47). Furthermore, synthetic peptides from keratin (45, 47), coxsackie virus (46), and human cardiac myosin (29) reacted with mAbs against *N*-acetyl-glucosamine and myosin as previously described. In addition, a synthetic cytokeratin peptide, SFGSGFGGGY, mimicked *N*-acetyl-glucosamine in its reaction with antibodies to *N*-acetyl-glucosamine and lectins. Mimicry between the group A carbohydrate epitope and peptide molecules is a form of mimicry between chemically diverse molecules. Based on results with peptides altered at a single amino acid residue, it was deduced that aromatic and hydrophobic interactions were important in cross-reactive anticarbohydrate antibody binding to the peptide. Our data as well as that of others (166, 167) clearly link anticardiac myosin and anti-*N*-acetyl-glucosamine responses with antiheart cross-reactivity.

Rheumatic Heart Disease

Dudding and Ayoub described the elevation and persistence of anti-group A polysaccharide antibody in rheumatic valvulitis (59). Studies of the human antistreptococcal/antimyosin mAbs that reacted strongly with *N*-acetyl-glucosamine have revealed that one of the mAbs was cytotoxic for human endothelium and reacted with valvular endothelium in tissue sections of a human valve (6). The cytotoxic mAb recognized the extracellular matrix protein laminin that is part of the basement membrane underlying the valvular endothelium. These data suggest that human antibody cross-reactivity with valve tissues is established through antimyosin/anti-*N*-acetyl-glucosamine/antilaminin reactivity. Antibodies that react with valve endothelium may lead to inflammation at the valve surface and promote T cell infiltration of the valve in rheumatic heart disease. Based on our current knowledge as

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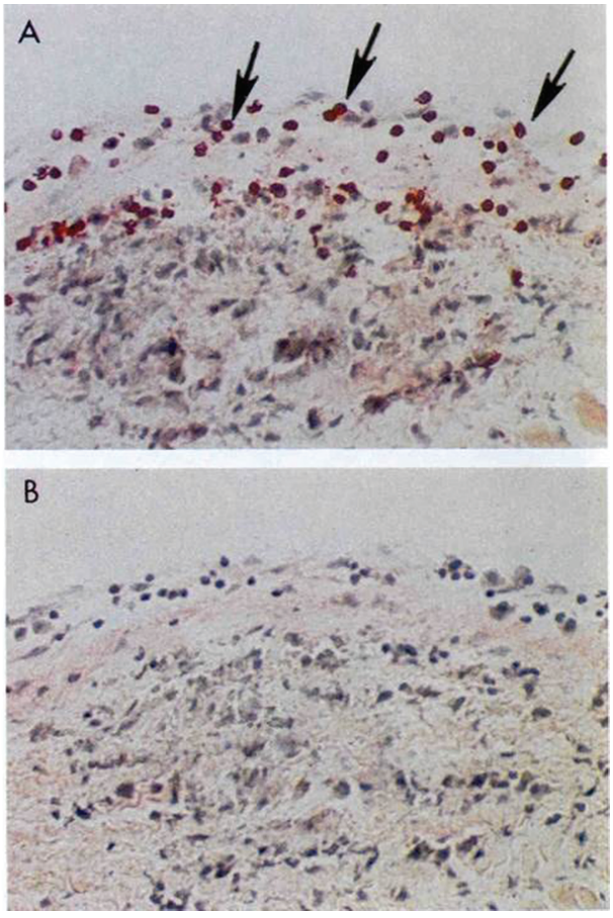


FIGURE 12 Extravasation of $CD4^+$ lymphocytes into valve above Aschoff's body in the subendocardium of the left atrial appendage. Original magnification, 200×. (A) Stained with anti-CD4 Mab; (B) stained with antibody isotype control. (Taken from Roberts et al. [48]).

illustrated in Fig. 10 (143, 144), the proposed events in rheumatic valvulitis/carditis involve both antibodies and T cells. Cross-reactive antibodies target the valve and are believed to act against the group A carbohydrate and valve endothelium based on several lines of evidence described herein (6, 58, 59, 89, 96). T cells are cross-reactive with streptococcal M proteins and homologous alpha helical protein antigens such as myosin, laminin, tropomyosin, or vimentin and become activated and extravasate through activated endothelium into the valve, where they differentiate into $CD4^+$ TH1 cells producing IFNγ and valvular scarring (21, 48, 97, 142–144, 168). VCAM-1 was upregulated on valve endothelium (endocardium) in rheumatic carditis, indicating that the endothelium of the valve in rheumatic carditis was activated (48). Evidence strongly supports the cross-reactive T cell component of the disease, where T cells are shown to penetrate the valve endothelium into an originally avascular valve (21, 32, 48, 97, 136, 142, 143, 168–174). T cells in peripheral blood of patients (24) and the valve (21, 22, 97, 175) have been shown to be cross-reactive with M protein and cardiac proteins, including cardiac myosin epitopes, and they are both $CD4^+$ and $CD8^+$ phenotypes, but the $CD4^+$ phenotype and Th1 cells dominate. The development of scarring and fibrosis in the valve is part of the pathogenesis caused by IFNγ (3, 145, 175) production and IL-17A (101, 146), since the scarring promotes neovascularization and development of a blood supply into normally avascular valve tissue. T cells can subsequently enter the valve through blood vessels developed in the scar. The valve becomes predisposed to cellular infiltration through both the activated valve endocardial surface and the neovascularized scar tissue. Although antibodies develop against collagen in rheumatic heart disease (176, 177), they have not been found to be cross-reactive but may contribute to the pathogenesis of disease (178) once mimicry damages the valve and collagen is exposed to the immune system.

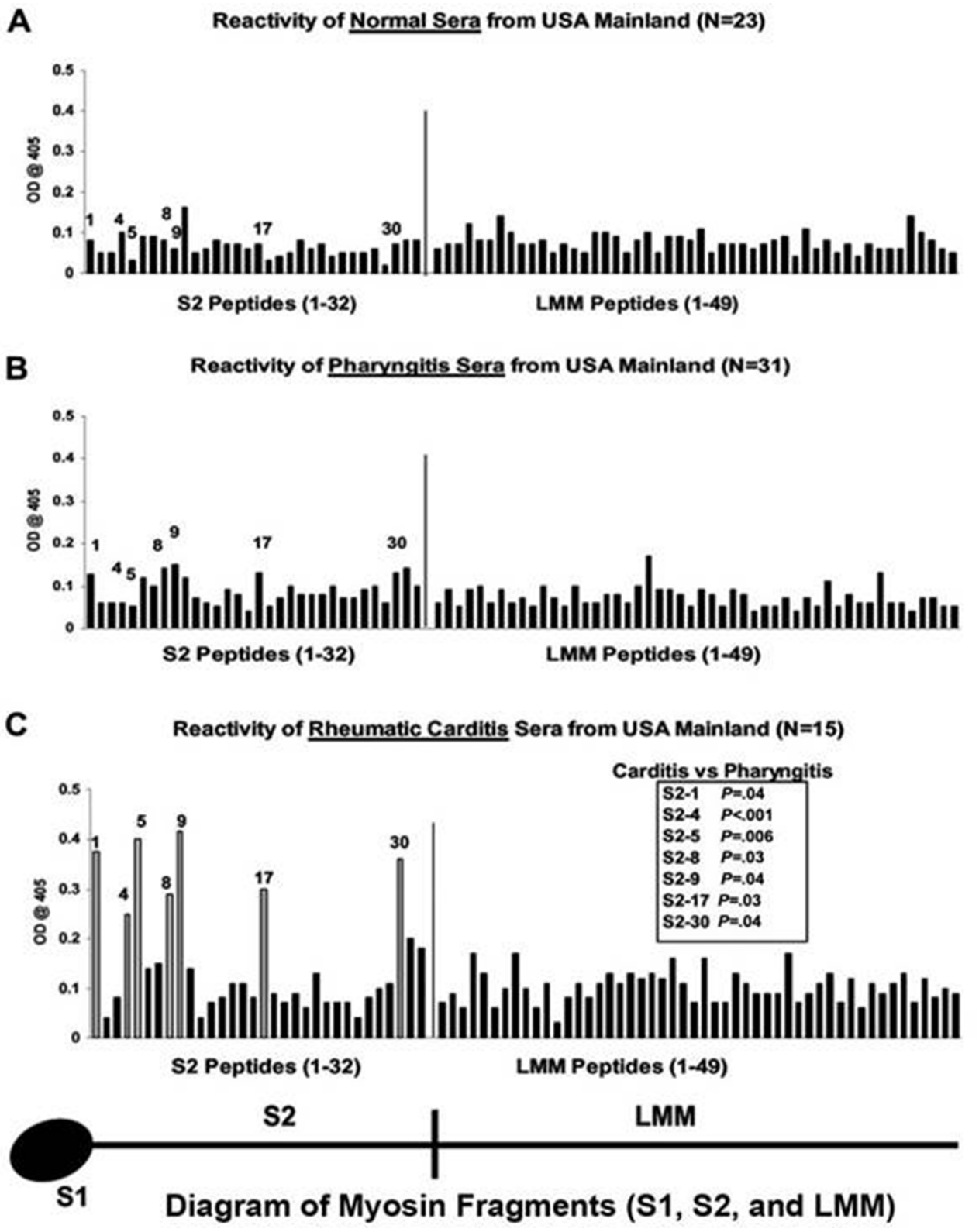
Recent reviews on rheumatic heart disease describe the collective studies and proposed pathogenesis of molecular mimicry in the disease (1–3, 179–184). Initial damage at the chordae tendineae would be sufficient to begin the process of damage to the endothelium of the valve (25) where T cells infiltrate and congregate as they recognize laminin and other valvular proteins that are part of the basement membrane (185). These mechanisms lead to the upregulation of inflammatory molecules such as VCAM-1 (48), which allows the valve to be infiltrated by the T cells, and then the continual damage of the valve with every repetitive streptococcal infection. Treatment with antibiotic prophylaxis as part of the guidelines from the American Heart Association for treatment of rheumatic fever is an important deterrent to the development of rheumatic fever and heart disease in children.

Sydenham Chorea and Pediatric Autoimmune Neuropsychiatric Disorder Associated with Streptococcal Infection (PANDAS)

Sydenham chorea is the major neurological manifestation and movement disorder of rheumatic fever. Human mAbs produced from disease were compared with sera from Sydenham chorea and used to identify the potential antibody targets as well as provide a better understanding of pathogenesis. Human Sydenham chorea-derived mAbs and serum IgG demonstrated specificity for mammalian lysoganglioside and the group A streptococcal carbohydrate epitope *N*-acetyl-β-D-glucosamine (7). Chorea-derived mAbs and acute serum antibodies, as well as cerebrospinal fluid from Sydenham chorea, reacted with lysoganglioside and targeted the surface of human neuronal cells. These antibodies also induced elevated calcium/calmodulin-dependent protein kinase II activity (CaM kinase II) (7). Although the mAbs were IgM, the serum antibodies that triggered the cell signaling in human neuronal cells were IgG. IgG can penetrate the blood-brain barrier when breached by bacterial components or other molecules such as epinephrine (186–188), and the IgG can reach brain tissues, where it can target specific cells in the brain tissue and produce disease.

Thus, the human chorea mAb 24.3.1 IgG was shown to penetrate the brain when the genetic information for the chorea antibody V genes were expressed in a transgenic mouse model (189). The antibody was expressed by B cells in the transgenic model and was produced by B cells and specific IgG accumulated in mouse serum. Once the blood-brain barrier was broken, the antibody that was tagged with a specific mouse allotype was observed in the basal ganglia, where the allotype-tagged chorea antibody IgG penetrated dopaminergic neurons in the ventral tegumental area or the substantia nigra as shown in Fig. 14 (189). The chorea antibodies were then shown in tritiated thymidine experiments to lead to production of excess dopamine by neuronal cells that could lead to the symptoms of chorea. Other animal models demonstrated that antibodies induced by streptococcal immunization led to deposition in the basal ganglia and the

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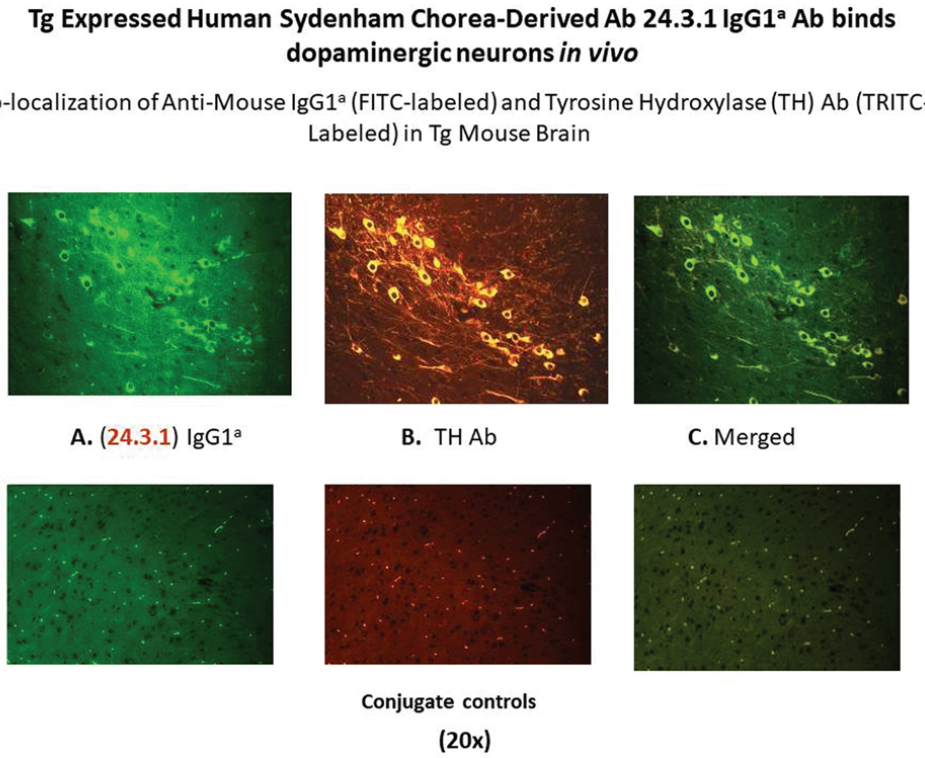


FIGURE 14 The human Sydenham chorea MAb 24.3.1 V gene expressed as the human V gene-mouse IgG1a constant region in transgenic (Tg) mice targets dopaminergic neurons. Chimeric Tg24.3.1 VH IgG1a antibody expressed in Tg mouse sera penetrated dopaminergic neurons in Tg mouse brain *in vivo*. Colocalization of Tg 24.3.1 IgG1a (anti-IgG1a Ab; left panel) and tyrosine hydroxylase antibody (anti-TH Ab; middle panel). TH is a marker for dopaminergic neurons. The left-hand panel shows IgG1a (labeled with fluorescein isothiocyanate [FITC]), the center panel shows TH Ab (labeled with tetramethylrhodamine isothiocyanate [TRITC]), and the right-hand panel shows a merged image (FITC-TRITC). Brain sections (basal ganglia) of VH24.3.1 Tg mouse (original magnification, 320×), showing (A) FITC-labeled anti-mouse IgG1a, (B) TRITC-labeled anti-TH Ab, and (C) the merged image. Controls treated with secondary antibody are negative. (Figure taken with permission from Cox et al. [189].)

development of behaviors characteristic of Sydenham chorea, such as holding a food pellet or holding on to a beam or demonstrating obsessive compulsive grooming after water spray to fur (190, 191). Passive transfer of the autoantibodies to animals also led to behaviors characteristic of neuropsychiatric symptoms and obsessive compulsive disorders (192–194).

Studies collectively demonstrated that the IgG from Sydenham chorea targeted a group of cross-reactive antigens in the brain (7–9, 195, 196). These include lysoganglioside, as mentioned above, and they also target tubulin, an intracellular protein abundant in the brain. More importantly, the cross-reactive antibodies have been shown to react with and signal the dopamine receptors D1 and D2 that have led to the characterization of Sydenham chorea as a dopamine receptor encephalitis where the autoantibodies signal the D2 receptor (189). The autoantibodies have been found in both serum and CSF of patients with Sydenham chorea, and the IgG autoantibodies as well as human mAbs signal human neuronal cells and activate CaMKII, which is an important signaling molecule in the brain. These cross-reactive antistreptococcal chorea-derived antibodies lead to the production and release of excess dopamine by neuronal cells in the basal ganglia that ultimately leads to chorea.

FIGURE 13 Reactivity of serum samples originating from the United States with human cardiac myosin peptides from the S2 and LMM regions in the enzyme-linked immunosorbent assay (1:100 serum dilution). (A) Mean reactivity of normal serum samples from control donors with no evidence of streptococcal infection or heart disease on the U.S. mainland against S2 and LMM peptides. These samples rarely reacted with any S2 or LMM peptide at an optical density of 0.2. (B) Mean reactivity of serum samples from patients with streptococcal pharyngitis in the United States against S2 and LMM peptides. These samples rarely reacted with any S2 or LMM peptide at an optical density of 0.2. (C) Serum immunoglobulin G from patients with rheumatic carditis in the United States. Serum samples from patients with rheumatic carditis from the United States reacted predominantly with peptides S2-1, S2-4, S2-5, S2-8, S2-9, S2-17, and S2-30, compared with the reactivity of serum samples from patients with pharyngitis in the United States against those same peptides (B). (C) Unadjusted Mann-Whitney *P* values for the comparison between carditis and pharyngitis on the United States mainland. The comparison for S2-4 is statistically significant on the basis of a two-sided alpha level adjusted to preserve the false-discovery rate at 5%. (Taken from Ellis et al. [24] with permission from the *Journal of Infectious Diseases*).

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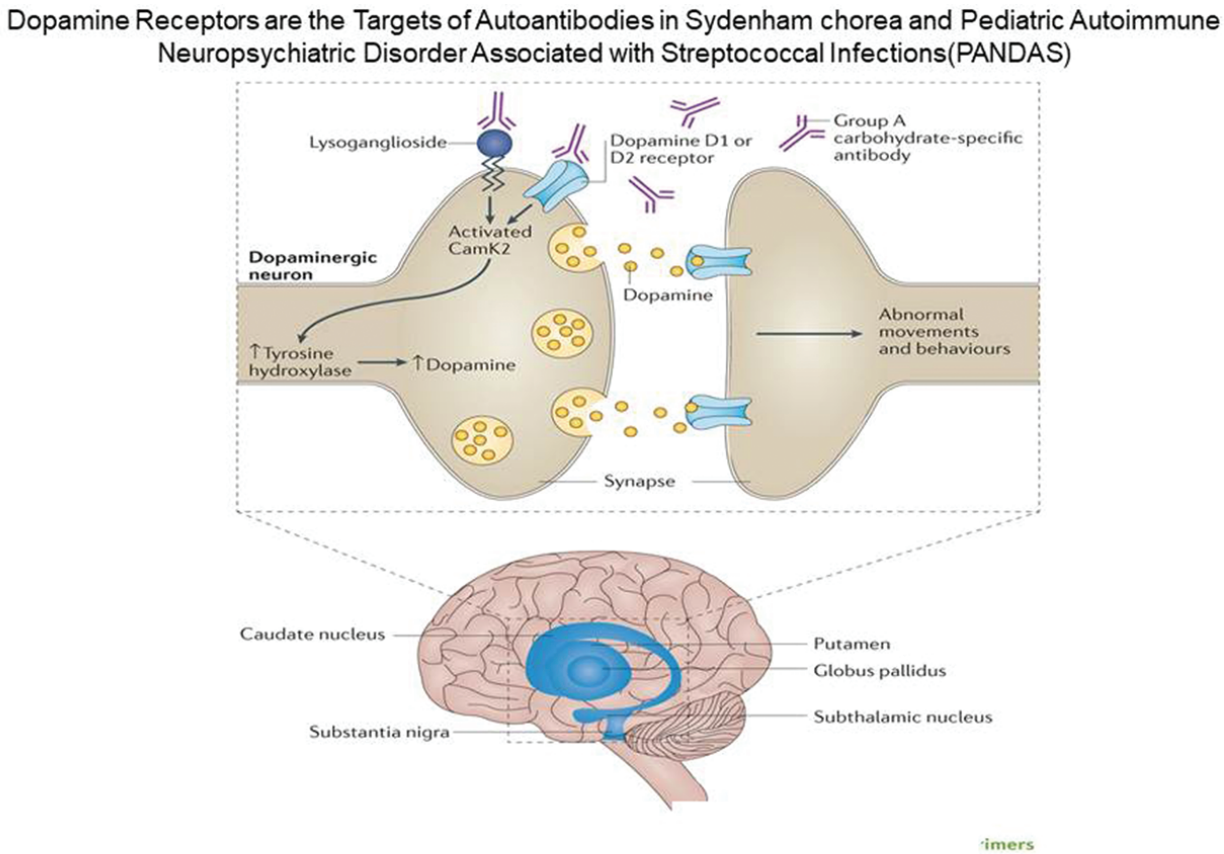


FIGURE 15 Diagram of proposed events leading to the group A streptococcal sequelae Sydenham chorea and PANDAS. Autoantibodies against brain tissues in Sydenham chorea and PANDAS with piano-playing choreiform movements cross-react with the group A streptococcal carbohydrate, lysoganglioside, and dopamine receptors D1R and D2R. The antibodies in Sydenham chorea and PANDAS react with the surface of neuronal cells and trigger cell signaling events, leading to upregulation of calcium/calmodul-independent protein kinase II (CaMKII) and excess dopamine release that leads to the involuntary movements in Sydenham chorea or PANDAS with piano-playing choreiform movements. Both Sydenham chorea and PANDAS are likely to be a dopamine receptor encephalitis based on our data (189, 205) and those of Dale et al. (197, 198). (Taken from *Nature Reviews Disease Primers* with permission).

Similar antineuronal autoantibodies and autoantibody-mediated neuronal cell signaling are elevated in serum and CSF from PANDAS with the choreiform movements. In both Sydenham chorea and PANDAS, the presence of abnormally elevated antidopamine receptor autoantibodies suggests a dopamine receptor encephalitis (197, 198). Studies of mice infected intranasally with group A streptococci illustrated the importance of infection and its role in opening the blood-brain barrier that must be broken to allow IgG to penetrate the brain. Studies by Agalliu and Cleary and colleagues show that intranasal group A streptococcal infection leads to activated Th17 cells that traverse the olfactory bulb and open the blood-brain barrier to proteins such as IgG (199, 200). Upon opening the blood-brain barrier, IgG antineuronal autoantibodies can penetrate the brain and potentially lead to Sydenham chorea and PANDAS.

The studies suggest (i) that the antibodies against streptococci and brain in Sydenham chorea and related diseases produce central nervous system dysfunction through a neuronal signal transduction and subsequent excess dopamine release mechanism and (ii) that the molecular targets of the chorea antibodies include lysoganglioside and the dopamine receptors in neuronal cell membranes. The antineuronal autoantibodies also target the group A streptococcal carbohydrate epitope *N*-acetyl-β-D-glucosamine present on the rhamnose backbone of the carbohydrate and present in the cell membrane and wall of the group A streptococci as well as the intracellular brain protein tubulin. The diagram in Fig. 15 illustrates proposed events of how antineuronal autoantibodies against lysoganglioside and the dopamine receptors D1 and D2 may function in Sydenham chorea and PANDAS (3).

CONCLUSIONS

The theme in mimicry, as found in the above-described studies, suggests that cross-reactive antibodies target an intracellular biomarker such as myosin in the heart and tubulin in the brain, but for the antibodies to be pathogenic, they must target a cell surface antigen such as the extracellular matrix protein laminin in the valve endocardial basement membrane or dopamine receptors present at the surface of neu-

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ronal cells, leading to the initiation and manifestations of disease. In general, the autoantibodies present as a group of specificities in disease include the cross-reactive antigens recognized by the autoantibodies in disease sera. The cross-reactivity found in individual sera from the disease has been characterized using human mAbs that reflect the serum IgG in the disease.

Cross-reactive antibodies and cross-reactive T cells against group A streptococci are important in the pathogenesis of autoimmune sequelae that characterizes rheumatic fever following streptococcal infection. Cross-reactive antibodies have been defined as those that recognize host tissue alpha-helical coiled-coil antigens such as myosin, tropomyosin, keratin, vimentin, and laminin in the heart (79) and tubulin, lysoganglioside, and dopamine receptors in the brain (9). Cross-reactive antistreptococcal antibodies recognize peptide sequences in alpha-helical proteins such as the M protein virulence determinant of the group A streptococci as well as the *N*-acetyl-glucosamine molecule, the dominant epitope of the group A streptococcal carbohydrate (45–47). Glycosylated proteins are expected to be targets of the cross-reactive antistreptococcal antibodies. Historically, Goldstein et al. determined autoantibody reactivity with carbohydrates on heart valves (58), and Dudding and Ayoub demonstrated that the group A carbohydrate autoantibodies were an indicator of poor prognosis in valvular heart disease (59). This evidence supports our findings and confirms the importance of the autoantibodies against streptococcal antigens in the development of cross-reactivity that is the basis of the initial disease processes in rheumatic fever and rheumatic heart disease.

In rheumatic heart disease, laminin, an extracellular matrix molecule present in the basement membrane of the valve, may trap cross-reactive anticarbohydrate autoantibodies at the endocardial cell surface and lead to damage or inflammation of the endothelium. Activated endothelium leads to subsequent extravasation of streptococcal M protein/myosin-cross-reactive T cells into the valve (8, 48, 97, 142, 168). Cross-reactive group A streptococcal antigens with apparent roles in the pathogenesis of rheumatic heart disease include the M proteins and the group A polysaccharide. Amino acid sequences of streptococcal M5 protein that have been shown to be pathogenic in animals have also been reported to be recognized by T cells from rheumatic heart valves (21, 97, 142, 143, 168). The identification of human cardiac myosin cross-reactive B and T cell epitopes of M5 protein has been a step forward in understanding the cross-reactive epitopes that produce disease in animals and humans (32) and are hallmarks of molecular mimicry.

In Sydenham chorea, mimicry between the *N*-acetyl-glucosamine molecule of the group A carbohydrate and brain gangliosides potentially leads to antibodies that bind to the surface of neuronal cells and triggers induction of dopamine in the disease (7). In the pathogenesis of chorea, the development of IgG responses and higher-affinity cell surface reactive antibody that can bind strongly enough to alter host tissues may lead to disease. In carditis, T cells can enter the valve, but only those that are consistently stimulated with local antigens retain a strong immune response. The Th1 T cell subset and its proinflammatory IFNγ cytokine are dominant in valves with a potential role also for IL-17A and the Th17 subset of T cells in fibrosis and scarring.

Although the identity and pathogenic mechanisms of cross-reactive antigens and their cross-reactive antibodies and T cells is much clearer now than in the past, there will always be new lessons to be learned about the pathogenesis of the autoimmune sequelae of ARF and how cross-reactivity and mimicry of host tissues by the group A streptococci leads to autoimmune sequelae following streptococcal disease.

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Extracellular Matrix Interactions with Gram-Positive Pathogens

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8

The adherence to and invasion of eukaryotic cells are the main strategies pathogenic bacteria use for colonization, evasion of immune defense, survival, and causing disease in their mammalian hosts. Most Gram-negative bacteria use pili to achieve adherence and invasion (1), while only a few Gram-positive species are equipped with these structural elements (2, 3). Hence, Gram-positive pathogens express specific cell surface components called adhesins, or MSCRAMM (microbial surface components recognizing adhesive matrix molecules), which directly or indirectly facilitate the adherence of the bacteria to host tissues, thereby mediating not only colonization but also invasion of microbes (4, 5). Bacterial adhesins of several Gram-positive pathogens, including *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Streptococcus pneumoniae*, are of utmost importance to enable their attachment to adhesive glycoproteins of the host extracellular matrix (ECM) as well as their adherence to host epithelial or endothelial cells, utilizing this strategy also to trigger immune evasion mechanisms (5–7).

The structure and composition of the eukaryotic ECM as a major noncellular component of the host is rather diverse in structure to provide the connective tissue with tensile strength and elasticity and to meet the functional demands at various sites in the body. In addition, the ECM affects the cellular physiology of the host organism and is vital for adhesion, migration, proliferation, and differentiation of many cell types. The ECM not only serves as a structural scaffold for host cells but also provides support for infiltrating pathogens to colonize and invade, particularly under conditions of injury and trauma. Cell-surface adhesive components of the host are often recognized by pathogenic bacteria in a tissue- or cell-specific manner. Pathogens also bind a variety of humoral adhesive components in body fluids that serve as molecular glue for microbial binding to the host tissue, leading to the activation of cellular mechanisms to promote the invasion of pathogens.

The diverse interactions of bacteria with host ECM components therefore represent important pathogenicity mechanisms in microbial infection. The identification and characterization of host ECM molecules and complementary bacterial adhesins would contribute not only to a better understanding of the molecular aspects of pathogenesis but also to the design of novel strategies to control and manage infectious diseases (8). In this article we describe the interactions of ECM components with adhesins of Gram-positive pathogens. The first section introduces the structure and function of ECM proteins, and the second part deals with the interactions of Gram-positive pathogens with such ECM components and the biological consequences thereof. Examples of these interactions with *S. pneumoniae* and *S. aureus* are depicted in Tables 1 and 2.

EXTRACELLULAR MATRIX

In mammals the ECM comprises more than 300 proteins, designated as the core matrisome (9), whereby a large number of ECM-modifying enzymes, growth factors, and other glycoproteins are associated with these protein polymer networks of varying compositions. The different ECM scaffolding proteins and ECM-associated components cooperate to assemble and remodel connective tissues at various sites in the body in close contact to cells through adhesion receptors, particularly integrins (10). Together with receptors for ECM-bound growth factors, they provide multiple inputs into cells to control the survival, proliferation, differentiation, shape, polarity, and motility of cells, culminating in the quote "Without this bit of glue, you wouldn't look like you."

The major structural components of eukaryotic ECMs are different types of collagens that form various interstitial and basement membrane networks (11). The interstitial ECM is largely comprised of fibril-forming collagens (types I, II, and III) together with fibril-associated collagens (types IX and XII), whereas basement membranes contain a two-dimensional collagen type IV network that is often interconnected with a laminin network. Other components, such as collagenous and noncollagenous glycoproteins, elastin, proteoglycans, hyaluronan, growth factors, and proteases, become associated with ECM, giving rise to their specialized structure and function at various tissue locations in the body (12). The main portion of interstitial ECM is produced and deposited by embedded connective tissue cells such as fibroblasts in the dermis, smooth muscle cells and fibroblasts in the vasculature, and osteoblasts and chondroblasts in bone and cartilage. In addition, melanocytes, fat, skeletal muscle, nerve and epithelial cells, and circulating blood cells, such as macrophages, granulocytes, and lymphocytes, participate in the synthesis and secretion of ECM material and thereby determine the specific character of a given tissue or organ.

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TABLE 1 *Streptococcus pneumoniae* surface proteins that recognize ECM molecules, plasminogen, and C4b-binding protein (C4bBP)

Bacterial protein	Acronym	ECM ligand(s)
Choline-binding proteins		
Choline-binding protein E	CbpE/Pce	Fibrinogen, laminin
Choline-binding protein F	CbpF	Elastin
Choline-binding protein I	CbpI	Elastin
Choline-binding protein L	CbpL	Collagen, elastin
Pneumococcal surface protein C	PspC	Vitronectin, thrombospondin-1
LPxTG-anchored proteins		
Complement factor H-binding protein	Hic/PspC 11.4	Vitronectin, thrombospondin-1
Neuraminidase A	NanA	Fibrinogen, collagen IV
Plasmin- and fibronectin-binding protein A	PfbA	Fibronectin, plasmin
Plasmin- and fibronectin-binding protein B	PfbB	Fibronectin, plasmin
Pneumococcal adhesion and virulence factor B	PavB	Fibronectin, plasminogen, Thrombospondin-1
Pneumococcal protease A	PrtA	Collagen IV
Pilus 1 subunit	RrgA	Fibronectin, collagen I, laminin
Zinc metalloprotease	ZmpB	Collagen IV
Nonclassical surface proteins		
Pneumococcal adhesion and virulence factor A	PavA	Fibronectin
Endopeptidase O	PepO	Fibronectin, plasminogen, C4bBP
Enolase-1	Enolase	Plasminogen, C4bBP
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	Plasminogen
Phosphoglycerate kinase	PGK	Plasminogen

During embryonic development, the ECM is constantly rearranged, which is necessary for regulating morphogenesis in an active and dynamic fashion. Likewise, the time-dependent modification and rebuilding of ECM at various locations in the body is essential for inflammation and wound healing. At these sites, the provisional ECM may provide bacterial entry and colonization, and soluble proteolytic fragments of ECM proteins are indicators of matrix turnover, particularly at sites of tissue remodeling or tumor cell invasion (11). Specific circulating fragments of type XVIII collagen (endostatin) (13) or type IV collagen (tumstatin) (14) were shown to exert potent antiangiogenic activities on vascular cells (15). Their respective interactions with microbes, however, have not been explored.

Structure and Function of ECM Molecules

Collagens

Twenty-eight types of collagen, encoded by more than 45 genes, have been defined on a molecular basis. Collagens are divided into (i) fibril-forming collagens, (ii) fibril-associated

TABLE 2 *Staphylococcus aureus* surface proteins that recognize ECM molecules

Bacterial protein	Acronym	ECM ligand(s)
Adhesins (MSCRAMM[a])		
Bone sialoprotein-binding protein	Bbp	Bone sialoprotein, fibrinogen
Clumping factor A	ClfA	Fibrinogen
Clumping factor B	ClfB	Fibrinogen
Collagen adhesin	Cna	Collagen
Fibronectin-binding protein A	FnBPA	Fibronectin, fibrinogen, elastin
Fibronectin-binding protein B	FnBPB	Fibronectin, elastin
Iron-regulated surface determinant protein A	IsdA	Fibronectin, fibrinogen
Protein A	SpA	von Willebrand factor
Membrane protein		
Elastin-binding protein	EbpS	Elastin

[a]MSCRAMM, microbial surface components recognizing adhesive matrix molecules.

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collagens with interrupted triple helices, (iii) network-forming collagens, and (iv) transmembrane collagens. A major subfamily of collagens is formed by the fibril-forming types, such as I, II, III, V, and XI, that are produced and assembled, e.g., during wound healing processes (16). These collagens are characterized by large stretches of triple-helical protein strands and are distinct from the fibril-associated collagens and the third subfamily, the network-forming collagens, of which types IV and X are prominent examples. All collagen types are expressed in a tissue- and/or cell-specific manner and contribute to the appearance and mechanical properties of a given connective tissue, thereby also determining its cellular environment.

Network-forming collagens, such as different isoforms of type IV, determine the structure of basement membranes as a specialized ECM, present at all tissue borders in connection with the diverse forms of epithelial cells. An intercalated laminin network that can exist independently is linked to type IV collagen via nidogen/entactin proteins. Basement membranes serve quite distinct functions in the body, such as linking epithelia to the underlying interstitial ECM, covering skeletal myotubes with basal lamina, or serving as a solute filtration barrier in the kidney glomerulae or at sites of gas exchange in the lung alveolae. Heparan sulfate proteoglycans, usually perlecan, and adhesive glycoproteins such as fibronectin are embedded into these supramolecular arrays as well (11). The majority of structural components are in direct contact via (adhesion) receptors with epithelial, endothelial, striated muscle, fat, and nerve cells, which produce the individual components and deposit them in a polarized fashion toward the cellular basolateral side of a given epithelium.

Laminins

Laminins are major constituents of basement membranes and are the first ECM proteins to be produced during embryogenesis. Because of their specific interactions with type IV collagen, proteoglycans, and other ECM components, as well as with several adhesive cell types, laminins play an important structural and functional role within basement membranes. Laminins are heterotrimeric molecules and have a characteristic flat, cross-shaped appearance with a molecular mass of 900 kDa. They are composed of three chains, α (400 kDa), β (200 kDa), and γ (200 kDa), which are linked to each other by disulfide bridges. There are five α chain, three β chain, and three γ chain isoforms identified in humans. In total, 15 laminin molecules (heterotrimeric isoforms) are classified on the basis of different combinations of the chains (17–19), which are expressed in a tissue-specific manner. Laminins are heavily glycosylated proteins with a 12 to 27% carbohydrate content that is often represented by complex-type carbohydrates (20). Laminins are in close contact with a variety of cell types, including muscle, adipocytes, neurons, and endothelial and epithelial cells, and are involved in the promotion of cell proliferation, attachment, and chemotaxis, as well as neurite outgrowth and enhancement of angiogenesis (21).

Elastin

Another specialized structural protein of the ECM in specific tissues is elastin, which is the main component of elastic fibers. These fibers are responsible for the elasticity of lung, skin, and other tissues, as well as blood vessels. Elastin is a highly cross-linked polymer of the nonproteolytically modified, hydrophobic precursor tropo-elastin. During fibrogenesis, secreted tropo-elastin is transformed into insoluble polymeric elastin by intermolecular covalent bonds between lysines into desmosine and isodesmosine cross-links through the copper-dependent enzyme lysyl oxidase (22, 23). The rubber-like mechanical properties of elastin result from repetitive hydrophobic domains of tropo-elastin subunits.

Matricellular Glycoproteins

Adhesive glycoproteins constitute specialized structural and functional components of the ECM. For example, upon vessel wall injury, particularly at sites of wound healing, the initial adhesion of platelets and their subsequent aggregation are dependent on the adhesive glycoproteins von Willebrand factor, fibronectin, thrombosponin-1, and vitronectin that are present in the subendothelial ECM and stored inside platelets before they are secreted during the initial phase of hemostatic plug formation. Adhesive glycoproteins not only promote the attachment and spreading of cells via their transmembrane integrin receptors (10), but also perform multiple functions by interacting with other ligands, such as heparan sulfate, collagens, proteases or mediators of humoral defense mechanisms (24). These proteins also exist as soluble forms in the blood circulation, which may differ from those in the subendothelium as well as in α-granules of platelets owing to alternative splicing, differences in the state of polymerization, different conformations, or the transition into self-aggregating proteins. The partitioning of these "matricellular" proteins between humoral and cell surface or ECM phases, together with inducible receptor sites on platelets or inflammatory cells, indicates that these adhesive glycoproteins are important players in tissue remodeling (such as in tumors) or defense mechanisms (25). Thus, the matricellular adhesion proteins not only provide versatile molecular links mediating the adhesive processes and responsive reactions at localized tissue sites, but are also of major importance for the initial adherence phase of pathogens to the host tissue.

Fibronectin

Fibronectin is a ubiquitous adhesive protein that is essential for the adhesion of almost all types of cells. It is abundant at various ECM sites as well as in the blood circulation and has a concentration in the range of 200 to 700 μg/ml (26). This high-molecular-weight adhesive glycoprotein has a broad spectrum of functions ranging from its contribution in ECM formation, cell growth, and differentiation to wound healing and tumor progression. It exists in two forms in the human body: as a soluble 440-kDa compact dimer in plasma (so-called cold-insoluble globulin) and as a multimeric fibrillar form in connective tissue and basement membranes (26, 27). The characteristic dimeric form of the molecule in solution is generated by disulfide bridging at the carboxy-termini of two similar subunits, each with a molecular mass of about 220 kDa (28). In addition to approximately 30 intrachain disulfide bonds, two free sulfhydryl groups per subunit are involved in the formation of high-molecular-weight polymers of fibronectin, which are predominantly found in tissues. The heterogeneity observed in fibronectin molecules isolated from plasma or tissue is due to a variation in both the amino acid sequence and posttranslational modifications. Although only one fibronectin gene has been identified, variations in the carbohydrate content or structure, phosphorylation, sulfation, and acetylation are responsible for the additional heterogeneity of the protein.

The multidomain fibronectin molecule contains three types of repetitive domains with independent three-dimensional modular structures, whereby each monomer is typically composed of 12 type I repeats (40 amino acids each), 2 type II repeats (60 amino acids each) and 15 to 17 type III repeats (~90 amino acids each) (29). Owing to the

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presence of intramolecular disulfide bonds, type I and type II repeats are structurally rigid, in contrast to the conformationally flexible β-barrels of type III repeats, which are devoid of these linkages, thus enabling them to be involved in fibronectin polymerization (30). The sensitivity of fibronectin to proteolytic degradation has been used to identify the structure-function relationships of independent domains. The 30-kDa amino-terminal fragment contains the major acceptor site for factor XIIIa (transglutaminase)-mediated cross-linking and bears the binding sites for heparin, fibrin, and bacteria, including *S. aureus* (31). The well-known property of fibronectin to bind collagen or gelatin and to complement C1q is contained within the adjacent 40-kDa fragment, while the central portion of the molecule has no well-defined binding functions.

The versatile recognition sequence Arg-Gly-Asp (RGD) for integrins was first recognized in the type III-10 repeat of fibronectin (32) and has been found in a large number of adhesive and nonadhesive proteins as well. Additional heparin-binding and fibrin-binding domains are located within the carboxy-terminal portion of the fibronectin molecule. Together with the RGD-containing cell attachment site, the heparin-binding domain is crucial for the establishment of integrin-mediated stable focal adhesions in many cell types, as has been demonstrated in fragment complementation assays. The transformation from the compact soluble to the extended fibrillar form of fibronectin, a highly regulated process termed fibrillogenesis, requires the application of mechanical forces generated by cells: these bind and exert forces on fibronectin through their integrin receptors, which mechanically couple the intracellular actin cytoskeleton to the ECM via an elaborate adhesion complex (33). The accumulation of fibronectin into an insoluble form is potentiated by disulfide bridge formation, as well as by covalent cross-linking, catalyzed by transglutaminase/factor XIIIa.

Vitronectin

Another adhesive glycoprotein, vitronectin, circulating in human plasma as a folded monomer of 75 kDa, exists either as a single-chain protein or as a two-chain disulfide-linked polypeptide (65 + 10 kDa) and exerts multiple functions in the complement, fibrinolytic, and blood coagulation systems (34, 35). In contrast, in its multimeric, unfolded form, vitronectin can efficiently bind to glycosaminoglycans such as heparin and becomes incorporated into the ECM of various human tissues, supporting cell adhesion and differentiation as well as regulating ECM composition and stability. Several immunofluorescent and histochemical studies suggest the deposition of vitronectin in a fibrillar pattern in loose connective tissue in association with dermal elastic fibers in skin and with renal tissue (36, 37). The interaction sites for several binding proteins are located in different vitronectin domains: the N-terminal part comprises a somatomedin B domain and an RGD motif that allows attachment of vitronectin to the integrin receptors $\alpha_v\beta_3$, $\alpha_v\beta_1$, and $\alpha_v\beta_5$ (38). In addition to four hemopexin-type repeats, vitronectin consists of three heparin-binding domains (HBD1: amino acids 82–137; HBD2: amino acids 175 to 219; HBD3: amino acids 348 to 361), the latter of which mediates binding to proteoglycans and is involved in ligand-induced multimerization of the protein (34, 39).

Moreover, the accumulation of terminal complement complexes along elastic fibers later in life, the association of vitronectin with keratin bodies during keratinocyte programmed cell death (apoptosis), and colocalization of vitronectin with deposits of the terminal complement complex in kidney tissue from patients with glomerulonephritis suggest a role for the complement inhibitor vitronectin in preventing tissue damage in proximity to local complement activation. Although the mechanism of deposition of exogenous matricellular vitronectin alone or in association with other proteins into different tissues remains unclear, it may occur in a fashion similar to that for fibronectin; thus, both proteins are prominent candidates for promoting the adherence of bacteria at various accessible ECM sites. The interaction of vitronectin with glycosaminoglycans or different types of native collagens and basement membrane-associated osteonectin and the cross-linking of vitronectin by transglutaminase/factor XIIIa are reactions that are likely to occur in the ECM *in vivo* as well (40, 41). Finally, several Gram-negative and -positive pathogenic bacteria recruit plasma vitronectin as a humoral complement inhibitor of the lytic membrane attack complex to prevent its assembly and to protect the microbes against lysis (42).

Fibrinogen

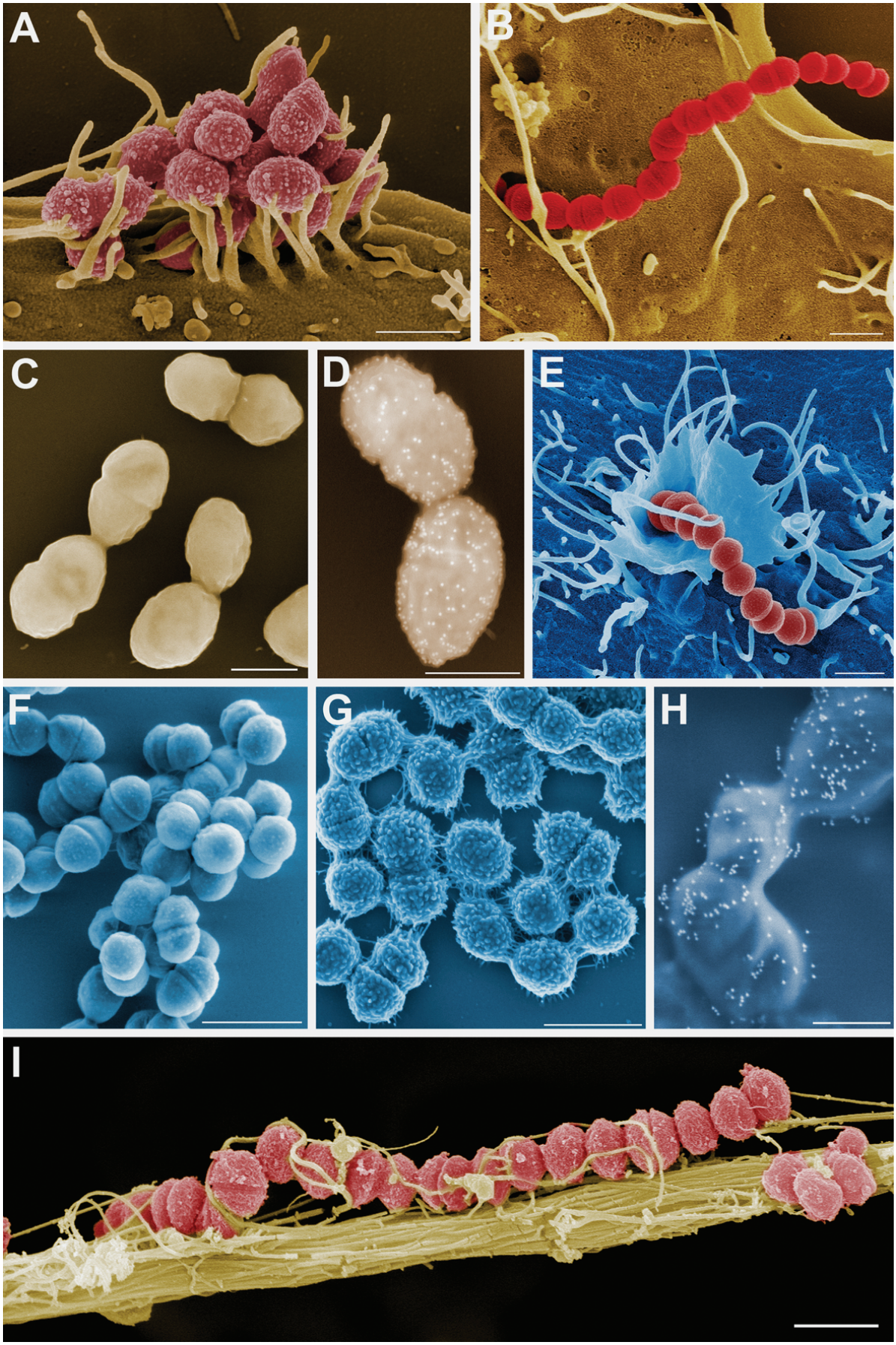
The adhesive glycoprotein fibrinogen is a major plasma glycoprotein that mediates the aggregation of activated platelets via their $\alpha_{IIb}\beta_3$-integrins in the early phase of hemostasis and is converted into insoluble fibrin by the key protease of the blood clotting cascade, α-thrombin. The primary structure of fibrinogen (43) together with biophysical studies indicate that the 350-kDa molecule is composed of two identical sets of three polypeptide chains, Aα, Bβ, and γ, which are disulfide-bridged and organized in an antiparallel fashion, representing a symmetrical dumbbell-shaped quaternary structure (44). Upon selective and specific proteolytic attack by α-thrombin, two pairs of fibrinopeptides, A and B, are sequentially released, and the appearance of these peptides in the circulation is an indicator of thrombin activity *in vivo*. Covalent stabilization of the forming fibrin clot is mediated by factor XIIIa-dependent cross-linking between γ and α chains of fibrin. Together with invading cells and aggregating platelets, the fibrin clot constitutes the majority of the initial provisional ECM network during the wound healing process (45).

Fibrinogen is a multifunctional protein capable of binding to collagen, fibronectin, components of the fibrinolytic system, and a variety of eukaryotic cells, as well as to bacteria. While the fibrin clot is being organized during normal wound healing, it may already serve as a cofactor surface for tissue plasminogen activator-dependent plasminogen formation, ultimately leading to clot lysis. Cell surface integrin receptors for fibrinogen have been identified on platelets ($\alpha_{IIb}\beta_3$) (required for platelet aggregation) and on mammalian cells (46). Integrin $\alpha_M\beta_2$ (complement receptor 3) on phagocytes may also recognize fibrinogen during wound healing and defense when phagocytic clearance of fibrin(ogen)-associated clot or cell fragments is required. The recognition of the distal end of the γ-chains of fibrinogen and of two RGD-containing sites by different integrins indicates that fibrinogen may serve as a bridging component between surface receptors on different cells or other extracellular sites once they become exposed (47).

Thrombospondin

Thrombospondins are multifunctional 420-kDa homotrimeric, multidomain glycoproteins with multiple functions that are synthesized and secreted by various cell types such as endothelial cells, smooth muscle cells and macrophages (48–50). In addition, thrombospondin-1 is stored in platelet α-granules to become secreted in the initial phase

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of hemostasis, whereby the released thrombospondin may strengthen fibrin network formation and becomes incorporated into the repair thrombus. The plasma concentration of circulating thrombospondin-1 ranges between 20 and 300 ng/ml and increases temporarily upon activation-induced platelet α-granule release up to 30 μg/ml (50). The level of thrombospondins in the circulation and other body fluids (produced by vascular cells or secreted by activated platelets) and their distribution in tissues appears to vary in correlation with different pathological states.

Thrombospondins belong to a family of structurally unrelated members of the tenascin protein family, including osteonectin and osteopontin. These matricellular proteins regulate divergent cellular functions including cell adhesion and signaling, proliferation of different cell types, as well as angiogenesis and immune responses (51–53). Owing to their highly regulated biosynthesis, induced by various hormones and cytokines during embryonic development, nerve regeneration, and vascular remodeling related to angiogenesis, these proteins become sequestered in the ECM and thereby mediate cell recruitment via different cell surface receptors, including integrins. Thrombospondin specifically promotes cell attachment and spreading via α_v-integrins in the vascular system but may also lead to destabilization of focal adhesions (54). Human thrombospondin-1 monomers each consist of a globular N-terminal, a heparin-binding domain, a von Willebrand factor-binding domain, three properdin-like type I repeats, three epidermal growth factor-like type II repeats, eight calcium-binding type III repeats, and a globular C-terminal domain (48, 55). These domains interact with a wide range of host molecules in the ECM (designated the "interactome"), including structural proteins, cytokines, and proteases (56), resulting in scaffolding of these factors and modulating their functions.

INTERACTIONS OF GRAM-POSITIVE PATHOGENS WITH ECM MOLECULES

Collagens and Gram-Positive Pathogens

Many staphylococcal and streptococcal species specifically interact with collagenous proteins (Fig. 1), and several examples of these specific interactions will be discussed. It has been proposed that these interactions might be important in the pathogenesis of various diseases, such as osteomyelitis and infective arthritis. For example, Switalski et al. (57) isolated a surface protein of 135 kDa from *S. aureus* and tentatively identified it as a collagen receptor, while Patti et al. (58) cloned and sequenced a gene, *cna*, encoding a collagen-binding protein from *S. aureus*. This collagen-binding protein exhibits all the main features of a sortase-anchored surface protein of Gram-positive bacteria. The ligand-binding domain of the *S. aureus* protein is localized in the A region of collagen (58). So far, only a single gene has been identified for staphylococcal collagen-binding protein, although proteins of different molecular sizes have been reported from different strains, probably owing to strain-to-strain variation in the number of repeats. The collagen-binding protein from *S. aureus* is involved in the adherence of bacteria to cartilage. This interaction is specific for collagen because synthetic beads coated with collagen mediate the adherence to cartilage, whereas the beads coated with fibronectin show no adherence (57).

Animal pathogenic streptococci interact with different collagens to promote serious pathologies. For example, in infectious endocarditis, which is most commonly caused by streptococci and characterized by the formation of septic masses of platelets on the surfaces of heart valves, collagen-like platelet aggregation-associated protein of *Streptococcus sanguis* and direct interactions with host ECM collagens enhance platelet accumulation and subsequent bacterial colonization. *Streptococcus anginosus* isolated from an endocarditis specimen also binds collagen I and IV, and in addition to SspA and SspB, which are members of the antigen I/II family, CshA and FbpA are involved in the binding of collagen I and II (58).

A study showing the involvement of collagen in acute rheumatic fever, a serious autoimmune sequela of *S. pyogenes*, underlines the pathogenetic relevance of this interaction (59). *S. pyogenes* serotypes M3 and M18 isolated during outbreaks of rheumatic fever have the unique capability to bind and aggregate collagen type IV. While M18 isolates bind collagen through capsule structures, M3 protein was identified as a collagen-binding factor of M3 serotypes, recognizing collagen IV via the PARF (peptide associated with rheumatic fever) domain (60, 61). Immunization of mice with purified M3 protein resulted in the generation of anticollagen type IV antibodies. These findings suggest a link between collagen binding and the presence of collagen reactive antibodies, which may provide a pathomechanistic basis for rheumatic fever. Furthermore, M1 of pneumococci bind to collagen VI in the lamina propria underneath the epithelial basement membrane of the upper and lower airways and plays a relevant role for host-pathogen interplay during respiratory tract infection (62).

As another example, *Streptococcus mutans* interacts with collagen type I via a 16-kDa binding protein with high affinity, as shown by the adherence of bacteria to a collagen-coated surface. Furthermore, collagen mediates the attachment of *S. mutans* to dentin in the oral cavity, thereby playing a role in the pathogenesis of root surface caries (57). The collagen-binding protein of *S. mutans*, designated Cnm protein, represents a new strain-specific member of the collagen-binding adhesin family (63). Moreover, the binding of collagen to the antigen I/II family of polypeptides of oral streptococci has been reported to facilitate the invasion of bacteria into root dentinal tubes (64), which further

FIGURE 1 (A) Adhesion of SfbI (streptococcal fibronectin binding protein I)-expressing GAS to an epithelial cell (HEp-2) mediated by fibronectin, which serves as a bridging molecule between streptococci and host cell integrins. (B) Invasion of SfbI-expressing streptococci (red) into an epithelial cell through the formation of invaginations by coopting host cell caveolae, fibronectin, and integrins. (C) *Streptococcus gordonii* expressing SfbI protein without exposure to fibronectin; this serves as a control to (D) *S. gordonii* expressing SfbI after incubation with fibronectin and subsequent labeling with antifibronectin antibodies and protein A gold-nanoparticles; note the strong binding of fibronectin to the surface of the bacteria via SfbI protein (white gold-nanoparticle dots). (E) Invasion of non-SfbI-expressing streptococci (red) into an epithelial cell (HEp-2) through signal induction of membrane ruffling of the host cell. (F) *S. gordonii* expressing SfbI on its surface after incubation with type IV collagen alone; note the smooth bacterial surface. (G) Preincubation of SfbI-expressing *S. gordonii* with fibronectin, followed by exposure to type IV collagen, results in a drastic accumulation of collagen on the bacterial surface. (H) Detection of type IV collagen on the surface of SfbI-expressing *S. gordonii*, following incubation with fibronectin and collagen IV (white gold-nanoparticle dots). (I) Direct binding of M3 serotype streptococci (red) to mouse type I collagen (derived from the mouse tail). Bars represent 1 μm in panels A, B, and E to I and 0.5 μm in panels C and D.

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underlines the importance of collagen-bacteria interactions for tissue site-specific diseases.

Some *S. pyogenes* strains, which do not interact with collagen directly, are able to recruit collagen by prebound fibronectin and the SfbI protein, a major fibronectin-binding protein (FnBP) (59). Fibronectin-mediated recruitment to sites of ECM-collagen thereby represents a mechanism for the aggregation, colonization, and immune evasion of *S. pyogenes*. A similar process is hypothesized for collagen I and its interactions with M proteins (65). Cpa, an adhesin of the group A streptococci (GAS) pilus and located at its tip, is a collagen I-binding protein, but the exact role in the infection process is still elusive (3, 66). However, the discovery of internal thioester bonds in the pilus tip adhesin Cpa of *S. pyogenes* raises the possibility of pathogen-encoded covalent adhesion (67, 68), such as for the host opsonin complement C3b. A subset of pneumococci express type I pili and the adhesin RrgA that both interact with collagen I as well (2), stressing the principle of bacterial adhesin expression for effective microbial colonization of host tissues.

Laminin and Gram-Positive Bacteria

Exposure of laminin to pathogenic bacteria is usually seen in damaged or inflamed tissues, as specifically detected for the binding of *S. pyogenes* to laminin (69). Human monoclonal antibodies, which have been developed from rheumatic heart disease patients, have also been found to recognize laminin, among other proteins. These autoantibodies are generally cross-reactive with a variety of *S. pyogenes* components and are poly-specific in nature. It has been proposed that autoantibodies recognizing myosin in the heart muscle and laminin in the heart valve are centrally involved in the pathogenesis of rheumatic heart disease (70). Thus, laminin plays an important role at least in the pathogenesis of *S. pyogenes*-mediated autoimmune diseases. Streptococcal-induced damage of the epithelial cell surfaces or entry through skin sores may thereby provide access to basement membrane laminin and the surrounding tissue for colonizing bacteria. To this end, two streptococcal laminin-binding proteins (Lbp) have been described, including streptococcal pyrogenic exotoxin B. This protein, which is ubiquitously expressed by *S. pyogenes* strains, is a secretable cell surface cysteine protease, degrades host ECM components, and activates interleukin-1β (71). This 34-kDa Lbp was found to be present and highly conserved in all *S. pyogenes* strains examined and mediates microbial attachment to HEp2 cells (72). Moreover, Lmb protein from *Streptococcus agalactiae* has been identified as another Lbp, and the structures of these binding proteins of group B streptococci and GAS have been elucidated (73, 74). Their structures indicate that these proteins belong to the ABC-transporter family of bacterial metal-binding receptors and are the substrate-binding proteins for zinc and iron ions. Lmb-mediated attachment of *S. agalactiae* to laminin may be essential for the bacterial colonization of damaged epithelium and translocation of bacteria into the blood stream (75). In addition, Lmb expression is upregulated during meningitis, implying that binding of laminin is crucial for group B streptococcal meningitis (76). ShrA of GAS is another Lbp and contributes to GAS adherence and subsequent infection (77). In addition, the streptococcal collagen-like protein-1 of *S. pyogenes* binds to laminin and fibronectin, and these interactions were proposed to facilitate bacterial internalization as well (78).

While the binding of laminin and other ECM proteins to *S. mutans* and *S. anginosus* has been reported (79), a more specific interaction between laminin and a *Streptococcus gordonii* strain isolated from a patient with infective endocarditis was mediated by a 145-kDa cell wall protein whose production was regulated in the presence of laminin (80). Moreover, sera from patients with infective carditis contained antibodies against this protein, whereas no significant recognition was seen with sera from patients with valvulopathies, suggesting an increased expression of this Lbp during infective endocarditis. Although a few streptococcal strains isolated from the oral cavity, including viridian streptococci, expressed this protein, the majority of strains isolated from patients with endocarditis expressed binding protein(s) for laminin, indicating that this ECM component might be an important factor in the pathogenesis of viridans endocarditis.

Elastin and Gram-Positive Bacteria

The specific binding of elastin was described for a number of *S. aureus* strains, whereby a major high-affinity-binding component was identified as a 25-kDa staphylococcal surface protein termed EbpS. The particular binding to elastin is mediated by a discrete domain defined by a short peptide sequence in the amino terminal extracellular region of EbpS (81). EbpS is located at the bacterial surface as an integrated membrane protein, and its expression was correlated with the ability of bacteria to grow to a higher density in liquid cultures, indicating that EbpS may have a role in regulating bacterial cell growth (82). It has also been postulated that EbpS might contribute to infection in organs containing elastin, such as lung, skin, and blood vessels, yet no specific studies have been performed so far. Finally, *S. aureus* FnBPs can also bind to elastin, which may be relevant in bacterial adhesion to connective tissue *in vivo*. In addition, several bacteria can capture plasminogen, which can be activated to plasmin by host and bacterial plasminogen activators, whereby bacterial cells become armed with a host protease which destroys ECM proteins such as elastin, opsonins, and other proteins that may contribute to immune evasion and promote microbial spreading and invasion during skin infection (83).

Fibronectin and Gram-Positive Pathogens

The adhesive glycoprotein fibronectin not only acts as a major substrate for eukaryotic cell adhesion but also serves as the prototype of adhesive ECM proteins to specifically bind to microorganisms (84) (Fig. 1). The molecular interactions, structural requirements, and functional consequences of fibronectin binding to Gram-positive bacteria have been extensively studied in several bacterial species. In particular, most *S. aureus* isolates, as well as various streptococcal strains, specifically bind and adhere to fibronectin, mediated by several different bacterial adhesins (85–87) with highly homologous recognition motifs for the host protein. Two genes, *fnbpA* and *fnbpB*, that encode the FnBP of *S. aureus* have been described. The gene products exhibit typical features of Gram-positive surface proteins, and the binding domain was localized to a 38-amino-acid repeat sequence of these proteins. FnBPA has been shown to induce the aggregation of platelets as well and may play a role in *S. aureus*-associated infective endocarditis (88).

A number of FnBPs, which serve as pleiotropic virulence factors in streptococci (89), have been identified in *S. pyogenes*, including the SfbI, also referred to as protein F, encoded by the gene *prtF*, which mediates the adherence of GAS to epithelial cells (90, 91). SfbI protein consists of

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638 amino acids and comprises five structurally distinct domains (92). The N-terminal signal peptide is followed by an aromatic domain and four proline-rich repeats, which are flanked by nonrepetitive spacer sequences. A second repeat region, distinct from the proline repeats, is located in the C-terminal part of the protein. Fibronectin binding was located in the C-terminal repeat region as well as in the nonrepetitive spacer sequence. Functional analysis showed that the repetitive sequence is essential for adherence, whereas both repeat and spacer sequences are required for the invasion of streptococci into eukaryotic cells (93). SfbI therefore represents a highly evolved prokaryotic protein that exploits fibronectin not only for extracellular targeting but also for its subsequent activation that leads to efficient cellular invasion.

Consequently, synthetic beads coated with SfbI serving as a model for bacteria were readily internalized by epithelial cells, indicating that SfbI alone is sufficient for microbial invasion (4, 94). SfbI triggers cellular invasion through caveolae and participates in organelle formation to avoid intracellular killing (95). This process may also contribute to the mechanisms of intracellular survival and persistence of *S. pyogenes*. The functional domains of SfbI were highly conserved in isolates belonging to different serotypes (92), such that pathogenic bacteria are attached to human fibronectin through tandem β-zippers (96), indicating that SfbI is the first defined invasin of GAS. The cellular receptor responsible for SfbI (protein F)-mediated microbial invasion has been identified as host $\alpha_5\beta_1$-integrin capable of recognizing fibronectin-bound bacteria (97). Unlike streptococcal M protein, SfbI does not show any cross-reactivity with host proteins (98).

Besides its involvement in adherence and invasion, SfbI has also been shown to be a strong mucosal adjuvant (94), since mice immunized intranasally with ovalbumin coupled to SfbI evoked a substantially higher immunoglobulin A response in lung lavage than did mice immunized with ovalbumin alone. SfbI also evoked a protective immune response in mice because animals immunized with SfbI protein were protected against the lethal challenge of GAS belonging to different serotypes (99). These findings underline the importance of SfbI in vaccine development.

"Serum opacity factors" of GAS (termed SfbII and SOF22) also specifically interacted with fibronectin, whereby their fibronectin-binding domain was located in the C-terminal repeat region of each protein that is not involved in the serum opacity factor activity. Yet, the role of its interaction with fibronectin is not yet clear, since as a soluble protein it may compete with bacteria-integrated FnBP for binding to fibronectin. In *in vivo* experiments, however, synthetic beads coated with the repeat region of serum opacity factor showed significant adherence to epithelial cells (100).

Another FnBP, Sffbp12, was described in GAS with no homology to SfbI or SfbII (101), but Sffbp12 shares a high degree of homology with fibronectin- and fibrinogen-binding proteins of *Streptococcus dysgalactiae* and *S. aureus*, respectively. An additional FnBP, designated F2, functions as a major adhesin in a subset of *S. pyogenes* strains and has been reported to be an important factor for both early and late pathogenic stages of superficial infections (102). Two other FnBPs from *S. pyogenes*, Fba and FbaB, have been identified (72); Fba has been shown to promote bacterial entry into epithelial cells, while FbaB is etiologically involved in the development of invasive streptococcal disease. *S. agalactiae* also binds fibronectin via a C5a peptidase, which cleaves the complement anaphylatoxin C5a to destroy its chemotactic function, which is necessary for the recruitment of inflammatory host cells (103).

The diversity of fibronectin-bacteria interactions can be further extended: fibronectin also binds to animal-pathogenic streptococci, facilitating their adherence to host vascular endothelial cells and promoting the infectious process via the blood circulation (104). In this regard, the cattle pathogen *S. dysgalactiae* expresses two binding proteins, FnbA and FnbB (105), whereby their fibronectin binding domains are located in the repeat region of both proteins that do not show any sequence homology. Despite the differences in the amino acid sequences of repeats for various FnBPs, they were capable of cross-inhibiting the binding of fibronectin to *S. aureus*, *S. dysgalactiae*, and *S. pyogenes*, respectively (106). The repeat motif of SfbI therefore conforms to the consensus sequence previously reported for FnbA, FnbB, and FnbpA. In *S. aureus* the FnBPA triggers invasion of bacteria into host cells, and the multiple repeats ($n = 11$) are essential to trigger invasion (107). Within the stretch of repeats the *in vivo* relevance of six high-affinity repeats was indirectly documented by the presence of antibodies in patients with *S. aureus* infections, recognizing complexes of these repeats together with bound fibronectin (108).

Fibronectin binding has also been observed with coagulase-negative staphylococci (109), but unlike other Gram-positive cocci, they only interact with immobilized fibronectin, allowing them to colonize artificial devices such as intraocular lenses, prosthetic cardiac valves, vascular grafts, prosthetic joints, and intravascular catheters. Fibronectin incorporated into fibrin thrombi is also the cause of infection at sites of blood clots or damaged tissue (110). Moreover, the ECM-binding protein (Embp) of *S. epidermidis* mediates a specific interaction between the bacteria and fibronectin to immobilize the adhesive glycoprotein, thereby establishing the first step of biofilm formation. Here, the binding of microbial Embp to fibronectin is assigned to its type III repeat 12 in the C-terminal region (111).

S. pneumoniae also produces an arsenal of FnBPs for interactions both with soluble fibronectin and with the fibrillar multimeric form of the host protein (112, 113). In contrast to GAS or *S. aureus*, but similar to *S. epidermidis*, pneumococci recognize the C-terminal heparin-binding domain of fibronectin (86, 113). Here, the virulence factor A (PavA) was the first FnBP identified among pneumococci to be relevant for bacterial adherence. PavA is located on the pneumococcal surface, despite lacking a signal peptide and a motif for anchoring (114). PavA also plays a direct role in the pathogenesis of pneumococcal infections, since isogenic PavA mutants are substantially attenuated in virulence (less than 1% of wild type) in a mouse sepsis model or in experimental meningitis (114, 115). This FnBP shares about 70% sequence identity with the FnBPs FBP54 of *S. pyogenes* and FbpA of *S. gordonii* (87, 116). Interestingly, PavA lacks the typical fibronectin-binding repeats described, e.g., for SfbI of *S. pyogenes* or for FnBPA of *S. aureus* (117–119). Antibodies targeting the 42-kDa N-terminal part of PavA did not block pneumococcal binding to immobilized fibronectin, suggesting that the C-terminal part of PavA recognizes fibronectin (115). Moreover, pneumococci lacking PavA still possess 50% of wild-type fibronectin-binding activity, pointing to the expression of additional FnBPs on these bacteria. In fact, the choline-binding protein CbpG, the sortase-anchored pilus adhesin RrgA, and the binding protein for plasmin and fibronectin (PfbA) have been characterized as FnBP as well, the latter serving as a bacterial adhesion and antiphagocytosis factor (120).

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In addition, the pneumococcal adherence and virulence factor B (PavB) was shown to specifically bind to fibronectin (2, 112, 121, 122). PavB is highly conserved among pneumococci, and orthologous sequences to PavB were confirmed in other streptococci such as *S. agalactiae*, *S. gordonii*, and *S. mitis*, whereas an orthologue is absent in *S. mutans*, *S. aureus*, enterococci, and other relevant human pathogens. The core domain of PavB consists of approximately 150-amino-acid-long repetitive sequences, referred to as streptococcal surface repeats (SSUREs). The number of SSUREs in PavB proteins among pneumococcal isolates is strain dependent but serotype independent (8, 112). PavB interacts via its SSURE domain with fibronectin but also with other host proteins such as plasminogen or thrombospondin-1 (112, 123). Importantly, the efficiency of PavB binding to immobilized fibronectin or plasminogen increases with the number of SSUREs. This finding is in agreement with the interaction model established for other FnBPs, demonstrating that repetitive units independently interact with type III repeats of fibronectin.

Thus, a high-affinity interaction between a particular FnBP and fibronectin is only achieved via synergistic interactions of repetitive units, while individual repeats only provide low-affinity binding. To demonstrate the mode of interactions between PavA or PavB and fibronectin, binding studies have been conducted using type III fibronectin domains. Although the two FnBPs differ in amino acid sequence and overall structure, they interacted with at least 13 out of 15 type III repeats of fibronectin (124). It appears that such binding mechanisms are somehow related to those described for classical FnBPs of GAS or *S. aureus*, in which the interactions are mediated via tandem β-zippers (96). Remarkably, the interactions of pneumococcal proteins PavA and PavB are different from those of *S. epidermidis* Embp, although both bacterial species only interact with the C-terminal part of fibronectin. While PavA and PavB bind to several fibronectin type III repeats, Embp preferentially recognizes fibronectin type III repeat 12 (111).

Vitronectin and Gram-Positive Bacteria

Vitronectin has equivalent effects on host cell adhesion and bacterial binding as was demonstrated for fibronectin, but owing to the limited expression of vitronectin and its deposition in the periphery, specific interactions with bacteria are likely to occur in damaged or altered tissues (Fig. 1). Specific binding of vitronectin to various strains of staphylococci, pneumococci, and groups A, C, and G streptococci have been identified (125). The binding of vitronectin to group G streptococci and pneumococci but not to group A and C streptococci, is inhibited by heparin, indicating the diversity of vitronectin-binding proteins among streptococci. As with fibronectin, binding to vitronectin mediates the adherence of Gram-positive bacteria to host cells and the ECM (104, 126, 127). Bacterial vitronectin-binding proteins therefore represent additional adhesins of Gram-positive bacteria. The adhesin of *S. aureus* responsible for vitronectin has striking similarity to a heparan sulfate-binding protein from the same strain, suggesting that the host protein undergoes multiple interactions with different bacterial surface recognition sites as well (128).

Of the four vitronectin-binding proteins expressed by *S. epidermidis* (129), a dominant vitronectin-binding protein of *S. epidermidis* and of *S. aureus* is their major autolysin Atl (130). This bacterial adhesin constitutes a surface-associated protein, whereby AtlE of *S. epidermidis* and AtlA of *S. aureus* share sequence homology and domain organization. The full-length proteins are proteolytically cleaved in their amidase and glucosaminidase domains. Importantly, the amidase domain contains two repetitive regions, termed R1ab and R2ab, which are responsible for the interaction with vitronectin and thrombospondin-1 (130). Also, interactions of vitronectin with multiple recognition sites of the *S. epidermidis* wall surface may strengthen bacterial colonization (129). This is supported by the fact that AtlE contributes to the adherence of *S. epidermidis* to a polystyrene surface, to heat shock cognate protein Hsc70, or to other plasma proteins (131). Distant sequence homology exists between vitronectin and the heme-binding plasma protein hemopexin, whose ligand-binding properties strongly depend on its conformational flexibility. In addition to RGD- and heparin-dependent interactions with Gram-positive bacteria, hemopexin-type repeats in vitronectin and in hemopexin itself have been proposed to function as target sites for group A streptococci (132), mediating microbial adherence and colonization in the blood vessel wall.

Pneumococci exploit host-cell-bound vitronectin for their adherence to cells of the respiratory tract and probably also to endothelial cells, although this has not been demonstrated experimentally. Pneumococcal adhesins interact with vitronectin bound to $\alpha_v\beta_3$-integrin and thereby activate signaling pathways, which induce a reorganization of the actin cytoskeleton. This in turn leads to the formation of micro-spikes and tight binding of pneumococci to the host cells (126). The multifunctional adhesin PspC, which earlier was shown to interact with the ectodomain (secretory component) of the polymeric immunoglobulin receptor (pIgR) and complement inhibitor factor H, has been identified as pneumococcal vitronectin-binding protein as well (133). Vitronectin binds preferentially to PspC subtypes, which contain two R (repetitive) domains, formerly shown to be relevant for binding of the secretory component of pIgR. The C-terminal heparin-binding domains of vitronectin serve as interaction sites for PspC, thus confirming that heparin inhibits pneumococcal interactions with the multimeric form of vitronectin.

Since vitronectin is an important inhibitor of the terminal complement cascade, it was not surprising that vitronectin bound to pneumococcal PspC is functionally active and inhibits complement-mediated membrane lysis (133), thereby protecting these bacteria against the host innate immune system. PspC is produced by almost all pneumococcal serotypes, with the exception of serotype 3 strains, which produce a PspC-like protein referred to as Hic (factor H-binding inhibitor of complement) (class II PspC, PspC11.4). Hic, originally identified as a factor H-binding protein, was also shown to bind to vitronectin (130), whereby the central core of Hic (amino acids 151 to 201) was identified as a target domain for vitronectin. This region is different from the factor H binding domain (amino acids 38 to 92) of Hic, and consequently, Hic is able to bind two inhibitors of the complement cascade at once, thereby evading immune responses and facilitating invasive infectious disease caused by serotype 3 pneumococci (130).

Fibrinogen and Gram-Positive Bacteria

Together with invading cells and aggregating platelets, the fibrin clot represents the predominant initial provisional ECM network for the early phase of wound sealing (45). At sites of trauma and injury, fibrinogen therefore serves as a major host substrate for bacterial adhesion. Various

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fibrinogen-binding proteins mediating bacterial colonization in wounds or catheters have been described, of which those expressed by *S. aureus* are the best characterized (134). In particular, "clumping factor" serves to recognize the carboxy-terminal portion of fibrinogen γ-chain in a manner analogous to $\alpha_{IIb}\beta_3$-integrin binding. Moreover, the homology between metal ion-dependent adhesion sites of integrin α_{IIb}- and α_M-subunits or clumping factor and an integrin-like protein from *Candida albicans* (135) indicates common mechanisms of fibrinogen binding to mammalian cells, lower eukaryotes, and prokaryotes. Staphylocoagulase serves as an additional fibrinogen-binding factor that is not involved in bacterial clumping but, owing to prothrombin binding and proteolytic conversion, helps to promote fibrin formation or bacterial attachment onto fibrinogen-coated surfaces (136). As another example, the adherence of *S. aureus* to endothelial cells is mediated predominantly by fibrinogen as a bridging molecule leading to acute endovascular infections (137). Although platelet aggregation via a fibrinogen-dependent mechanism can be induced by this pathogen, this process is independent of the aforementioned principal platelet $\alpha_{IIb}\beta_3$-integrin binding to fibrinogen (138). *S. aureus* colonizes skin lesions in more than 90% of patients with atopic dermatitis, and fibrinogen appears to play a major role in the enhanced binding of *S. aureus* to atopic skin tissue.

In GAS, M protein is a cell surface structure principally responsible for binding fibrinogen, contributing to the known antiopsonic property of M protein (139). Fibrinogen binding to streptococci of groups C and G also leads to the inhibition of complement fixation and subsequent phagocytosis, indicating a relevant role of this interaction for bacteria to evade immune defense (140, 141). Furthermore, bacterial colonization was reduced in a mouse mastitis model by vaccination with *S. aureus* fibrinogen-binding proteins (142), providing a new concept for antimicrobial therapy. Another fibrinogen-binding protein, FbsB, has been reported in *S. agalactiae*. This protein plays an essential role in the overall process of host cell entry by these bacteria, whereby the treatment of lung epithelial cells with FbsB fusion protein blocked *S. agalactiae* invasion of epithelial cells in a dose-dependent fashion, indicating that FbsB may serve as a target for antimicrobial intervention (143).

Alongside its role as a major FnBP in mediating fibronectin-related adhesion of *S. pyogenes* to different tissues, SfbI also interacts with fibrinogen. Upon binding, a cryptic thioester of SfbI can undergo trans-esterification such that SfbI can form a covalent bond with fibrinogen (reminiscent of the reaction between complement C3b and bacterial surfaces) (144). The mutated form of SfbI lacking the internal thioester was unable to interact with fibrinogen or human cells. Interestingly, *in silico* analysis identified a high number of bacterial proteins (including proteins of the human pathogen *S. pneumoniae*) containing cryptic thioesters as well. These data point to the fact that certain bacterial adhesins function as chemical harpoons and can irreversibly link microbes to host molecules. Likewise, using atomic force microscopy, the mechanical stability of the interaction between the prototypical staphylococcal adhesin SdrG and the short peptide epitope of the fibrinogen B β-chain resembles the strength of a covalent bond. Thus, adhesins can attach to their target host proteins with exceptionally resilient mechano-stability (145).

In addition to staphylococcal or streptococcal surface proteins that interact with ECM components, proteins released by these bacterial species can directly influence fibrin formation or dissolution and thereby influence the wound healing process. While staphylocoagulase binds and activates prothrombin to thrombin, staphylokinase and streptokinase interact with plasminogen by forming a stoichiometric complex to produce plasmin, eventually resulting in thrombolysis. These strategies apparently allow effective fixation and subsequent penetration of these bacteria within wound areas of the host.

Thrombospondin and Gram-Positive Bacteria

The high-affinity interactions of human thrombospondin-1 with *S. aureus* strains and pneumococci, as well as other Gram-positive bacteria such as *S. epidermidis* (130, 146, 147), have been explored in the context of wound repair as well. Binding to thrombospondin-1 mediates the adherence of staphylococci to tissues via activated platelets during inflammation or infection. A large number of protein A-negative and protein A-positive staphylococcal isolates bind to thrombospondin-1-coated artificial surfaces, indicating that microbial adherence is significantly promoted as a function of immobilized thrombospondin-1. Moreover, bacterial adhesion to epithelial and endothelial cells is increased in the presence of host cell-bound thrombospondin-1, which functions in this scenario as a molecular bridge between the microbe and the host cell (147). Human thrombospondin-1 also facilitates the interaction between *S. aureus* or pneumococci and platelets, resulting in their irreversible activation (123, 148–150). Strikingly, the vitronectin-binding proteins AtlE, AtlA, and Eap from *S. epidermidis* and *S. aureus* and pneumococcal PspC and Hic have also been identified as thrombospondin-1-binding proteins in this regard. Moreover, the pneumococcal adhesin PavB, which interacts via its SSURE domains with plasminogen and fibronectin, was shown to act as a binding partner for thrombospondin-1 (123, 130, 148). The importance of pneumococcal PavB and PspC in this regard was confirmed by using PavB- and PspC-negative strains, with the result that thrombospondin-1-mediated pneumococcal adherence to human epithelial cells was significantly reduced (123).

The binding of thrombospondin-1 to coagulase-negative streptococci may contribute to bacterial adherence on biomaterial surfaces (151), and specific binding of thrombospondin-1 to groups A, C, and G streptococci has been reported in this regard. As for *S. aureus*, the binding component in streptococci is resistant to trypsin. Thus, Gram-positive bacteria, particularly pathogenic microorganisms, produce a plethora of proteinaceous adhesins that interact with human thrombospondin-1 or in combination with other ECM proteins, thereby enhancing the interaction of bacteria with platelets and nonprofessional host cells, e.g., for effective wound infection.

Degradation of ECM by Gram-Positive Pathogens

The invasion of bacteria into tissues requires the degradation of matrix proteins for proper microbial colonization to occur. This is accomplished by various bacterial enzymes as well as by host-derived plasmin that becomes generated preferentially on the surface of bacteria with the help of host plasminogen activators (Fig. 2). In extravascular tissues, urinary-type plasminogen activator (uPA) is the predominant enzyme responsible for pericellular plasmin formation, while endothelium-derived tissue-type plasminogen activator (tPA) is the major intravascular plasmin-generating enzyme and acts in a fibrin-dependent manner. A number of bacteria, such as groups A, C, and G streptococci and

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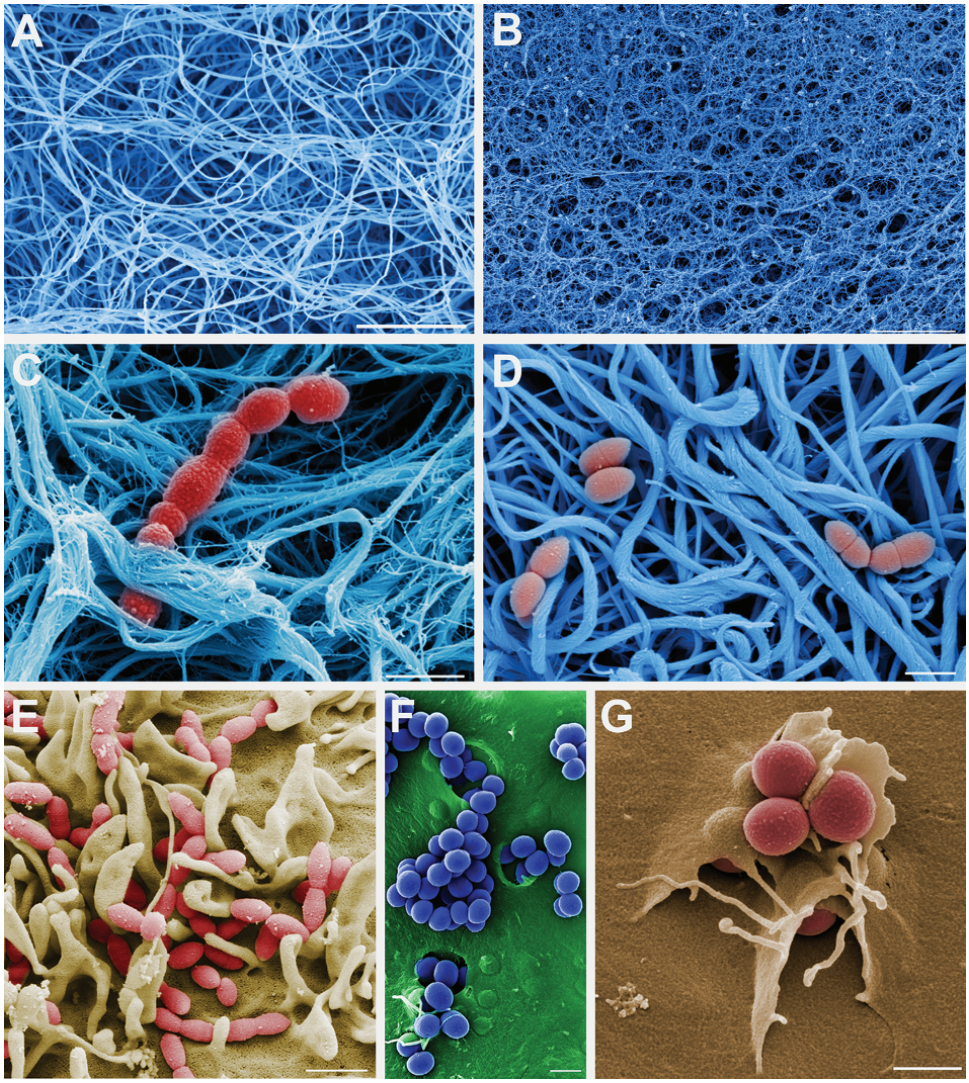


FIGURE 2 (A to D) Dissolution of a fibrin clot by *Streptococcus pneumoniae* (D39, serotype 2). (A) *In vitro* fibrin clot after fixation with aldehydes, dehydration with acetone, and critical-point drying that was subsequently exposed to *S. pneumoniae* in the presence of host-derived plasminogen activator and plasminogen; no dissolution of the fixed fibrin clot. (B) Following binding of plasminogen to its bacterial binding protein α-enolase and addition of plasminogen activator and plasminogen, the formed plasmin degrades native fibrin bundles. (C) Higher magnification of the process shown in panel B to demonstrate the dissolution of thick fibrin bundles (compare with panel D). (D) The same process as shown in panel C, except that a plasmin inhibitor was added to prevent the degradation of fibrin bundles. (E) Adhesion and initial invasion of *S. pneumoniae* (serotype 35A) to a Detroit 562 human nasopharyngeal carcinoma cell, mediated by bacterial binding of vitronectin and its interaction with host cell integrins, inducing a signaling cascade via integrin-linked kinase. (F, G) Two invasion mechanisms of methicillin-resistant *Staphylococcus aureus* into epithelial cells (HeLa): (F) via the formation of large invaginations and (G) by inducing rearrangements of the actin cytoskeleton of the host cell, i.e., membrane ruffling. Bars represent 10 μm in panel A, 200 μm in panel B, and 1 μm in panels C to G.

pneumococci express surface receptors for plasmin(ogen) that facilitate pericellular proteolysis and microbial invasion (152–154). A plasmin-binding protein (PAM) has been isolated from GAS, which belongs to the M protein family and binds to the characteristic kringle domains of plasminogen. This binding was blocked by a lysine analog, indicating that lysine residues in the M-like protein significantly contribute to the interaction (155).

As another example, plasminogen binding to *S. pyogenes* is responsible for bacterial adherence and pericellular invasion of human pharyngeal cells (154). Plasminogen binding has also been associated with the skin infections caused by *S. pyogenes* (156). The critical role of plasminogen in GAS infections has further been demonstrated in a study using transgenic mice overexpressing human plasminogen with markedly increased mortality when infected with GAS (157).

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Moreover, the structural similarity of plasminogen kringle motifs to neutrophil-derived polypeptides ("defensins") (158) suggests that their antimicrobial activity can be related to interference with plasmin formation, thereby preventing the spreading of infection. Finally, once bound to the streptococcal surface, plasmin was found to be protected from inactivation by the host serine protease inhibitor α_2-antiplasmin.

Another group of bacterial plasminogen-binding proteins, including the glycolytic enzymes α-enolase, glyceraldehyde-3-phosphate dehydrogenase, and phosphoglycerate kinase, fulfill their job as "moonlighting factors" and essentially contribute to plasminogen recruitment on the surface of *S. pneumoniae* and hence to pneumococcal pathogenesis (12, 152, 159, 160). The subsequent activation of surface-bound plasminogen via host-derived tPA or uPA endows pneumococci with proteolytic activity, which is then exploited by these microbes for degradation of fibrin thrombi, components of the ECM (including basement membrane proteins), and activation of matrix-metalloproteinases (152). Moreover, during the initial steps of bacterial colonization, host cell-bound plasminogen may facilitate penetration of microbes into tissues. In contrast, recruitment of soluble plasminogen to microbes reduced their attachment to epithelial or endothelial cells but increased their interaction with neutrophil granulocytes (153). These mechanisms are reminiscent of eukaryotic α-enolase-dependent recruitment of plasminogen to host immune or tumor cells, which thereby become equipped for effective tissue invasion (161).

Several bacteria express and secrete enzymes that interact with plasminogen to convert it into active plasmin. These include group A, C, and G streptococci and *S. aureus* that produce streptokinase and staphylokinase, respectively. These proteases form complexes with plasminogen, and the promoted conformational changes in the enzymogen induce plasmin formation. Moreover, staphylokinase requires fibrin as a cofactor and may induce immunogenic antibody responses, thereby provoking proliferation of T lymphocytes (152). Although staphylokinase and streptokinase had been applied as thrombolytic agents for use in thrombolysis of patients with, e.g., acute myocardial infarctions and arterial thrombosis (160), these treatments are no longer feasible and safe, also due to the unwanted immunogenicity of the bacterial enzymes.

As an acute-phase reactant in host defense, the circulating broad-spectrum proteinase inhibitor α_2-macroglobulin serves to eliminate spent proteinases via receptor-mediated endocytosis and may do so with microbial enzymes as well (162). Interestingly, various strains of streptococci exhibit specific binding to α_2-macroglobulin (163). A protein of 78 kDa was purified from GAS that interacted specifically with native α_2-macroglobulin. Although this protein does not possess any proteolytic activity, its interaction with α_2-macroglobulin provoked a conformational change similar to that observed in α_2-macroglobulin-protease complexes (164). Through their interaction with α_2-macroglobulin via the streptococcal surface protein GRAB, streptococci might gain access to host tissues, possibly via the α_2-macroglobulin receptor (165). In fact, mice infected with a GRAB mutant exhibited a longer survival and expressed less bacterial dissemination, emphasizing the role of α_2-macroglobulin in the virulence of *S. pyogenes* (166).

CONCLUSIONS

Infections by Gram-positive pathogens are still a major cause of serious human diseases. The rapid emergence of resistance to antibiotics in various Gram-positive cocci and (with the exception of pneumococci) the fact that no vaccine is available against most of these microorganisms make these Gram-positive pathogens a major health hazard. A better understanding of the pathogenic mechanism of Gram-positive infections is a prerequisite to improve existing suboptimal vaccines or to develop effective new ones. In addition, knowledge of the pathophysiologic processes of microbe-host interactions at different phases of infection is essential to design novel strategies for the prevention and treatment of these diseases.

The adherence and invasion of microbes are the first crucial, disease-causing mechanisms of Gram-positive pathogens. Since diverse molecular interactions of pathogens with the components of host ECM and cell surfaces are involved in both bacterial adherence and invasion, an understanding of the underlying mechanisms is of the utmost importance to design novel antimicrobial and therapeutic strategies. For example, bacterial adhesins, including the pleiotropic virulence factors of streptococci (89) that specifically interact with host ECM components, can be considered as drug targets for an antiadherence or anti-invasion strategy, e.g., utilizing the knowledge of specific interaction sites documented in this chapter. However, the problems with this strategy are that not all adhesin genes are present in every bacterial strain, there is antigenic variation of these adhesins, and environmental factors can also affect the expression of these genes. Moreover, since each microorganism can employ multiple adhesins and attachment mechanisms to initiate infection, effective antiadhesion drugs may contain cocktails of various inhibitors.

A classic example of the potential use of bacterial cell-surface components interacting with ECM is documented by the FnBP SfbI from *S. pyogenes*. SfbI protein acts as both an adhesin and an invasin of GAS. Mice immunized with this protein not only show reduced pharyngeal colonization but are also protected against lethal streptococcal challenge. Since SfbI is highly conserved in its functional domains in streptococcal isolates from different geographical regions, it does not cross-react with the host proteins, is highly immunogenic, and represents a promising vaccine candidate. Besides its role in adhesion and invasion, SfbI is a strong mucosal adjuvant, and because of its nontoxic nature, it has an advantage over the toxin-based adjuvants. In addition, immunization with *S. aureus* fibrinogen-binding proteins leads to reduced colonization in a mouse mastitis model. Synthetic peptides representing the fibronectin-binding domain of *S. aureus* adhesins as well as antibodies against this protein domain were successful in blocking *S. aureus* colonization on implanted foreign material. These examples clearly demonstrate that components involved in the interactions of ECM with Gram-positive pathogens can serve as potential drug targets and justify further research in this field.

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Group A *Streptococcus*-Mediated Host Cell Signaling

VIJAY PANCHOLI

9

INTRODUCTION

Bacteria must adhere to, colonize, and invade the target tissue while evading host protective immune responses to be successful pathogens. A pathogen sequentially engages (adheres to) its surface-exposed adhesins with complementary host receptors to successfully colonize and infect the targeted cell. Group A *Streptococcus* (*Streptococcus pyogenes*) is one of the most successful pathogens and is a model organism used to understand various aspects of pathogenesis. The latter may be related to toxins, horizontal gene transfer, mammalian protein binding, cellular invasion, intracellular proliferation, immune evasion, innate immunity, programmed cell death, and/or autoimmunity. *S. pyogenes* displays a remarkable tissue tropism for the human pharynx and the skin, causing a variety of diseases (1–3). Initial *S. pyogenes* infection can easily be treated with penicillin, and there is no known emergence of penicillin-resistant strains. Despite important advances in hygiene and modern methods of prevention, *S. pyogenes* still ranks among the 10 deadliest human pathogens, causing an estimated more than 500,000 deaths annually (4, 5).

The surface of *S. pyogenes* presents an array of proteins that perform a variety of functions (6–8). Beyond this multifunctional nature, the complex makeup of *S. pyogenes* pathogenesis is attributed to several other factors. These include the prevalence of several *S. pyogenes* serotypes, strain-specificity within one serotype, individual-based differential immune responses, the ability of several proteins in concert to perform one particular function, and cultural and host (extracellular and intracellular) environment-based differential gene expression levels (3, 9–11). With the availability of complete genome sequence analyses of several hundred *S. pyogenes* strains (https://www.ncbi.nlm.nih.gov/genome/?term=streptococcus+pyogenes), it is easy to predict the number of different surface proteins, which may serve as potential adhesins during initial interactions with host cells. However, it is unknown how many of them remain expressed during the adherence process and serve as adhesins for specific cell-surface receptors. The complex dynamic relationship among these proteins is unknown because the expression of many of these proteins is environmentally regulated (3, 9–11). Further, many of the surface proteins are differentially regulated only when the bacteria reach the intracellular environment (12–14). Similarly, the interaction of bacteria with their host cells may up- or downregulate the expression of host cell receptors and may accordingly modulate intracellular signaling events (13, 15, 16). Bacterial adhesion-host receptor interactions studied *in vitro* may vary with polarized cells versus nonpolarized cells, normal versus established cells, and professional phagocytes versus nonprofessional phagocytic cells (17–19). The dynamics of these interactions in the early events of streptococcal infection are multifactorial and extremely complex.

Despite many obvious missing links in our understanding of the precise signal transduction pathways evoked by these organisms in their host cells, the available literature suggests a common paradigm (20). Through their surface proteins, pathogenic Gram-positive cocci interact with specific receptors on the target cell and induce a series of biochemical signals. These signals, which are characterized by the induction of phosphokinase enzymes and phosphorylation of several intracellular proteins, ultimately target the nucleus and lead to either generalized or specific gene activation. Activation of some of these genes may result in the modulation of interleukins or other cytokines, which may then initiate a proinflammatory response. These induced signals, and subsequently the resulting products, could have several effects on the invasion of bacteria. *S. pyogenes* can produce infections in virtually every human organ and tissue. Mild to severe inflammation is the key immune response during infection. Thus, although initial interactions may involve pharyngeal and skin cells, subsequent interactions of *S. pyogenes* after evasion or invasion of host innate immune responses invariably involve neutrophils as the first responders of the host defense and other host cells, including macrophages, mast cells, dendritic cells, and endothelial and other myeloid cells. Numerous studies have laid a sound foundation for our understanding of the cross talk between Gram-negative bacteria and host cells, thereby unraveling their basic virulence mechanisms (20–23). The information available on the cell biology of *S. pyogenes*-infected human/mouse pharyngeal cells, skin cells, neutrophils, macrophages, and endothelial cells in the past decade has contributed substantially to a better understanding of the *S. pyogenes* pathogenesis. These studies highlight the fact that unlike many Gram-negative pathogens, which take advantage of deficient or failed immune systems of immunocompromised hosts, *S. pyogenes* causes invasive diseases in the immunocompetent host because it is endowed with the ability to thwart human innate immune responses (24–27). This ability encompasses evading host complement deposition and activation, impairing phagocyte recruitment and activation, resisting the microbicidal activities of antimicrobial peptides (AMPs) and reactive

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oxygen species (ROS), escaping neutrophil extracellular traps (NETs), and causing programmed cell death (autophagy, apoptosis, pyroptosis, or necroptosis). Several excellent reviews on these aspects of *S. pyogenes* inflammation and sepsis have been published in recent years (24, 26–34). The field of the cellular microbiology of pathogenic streptococcal infection is, however, continuously expanding as the newer CRISPR/CAS9 (clustered regularly interspaced short palindromic repeat)-based technology, reagents, and knockout mouse strains have become available. In this article, the past and recent reports on signaling events initiated primarily by *S. pyogenes* infection are summarized along with relevant complementery information on other Gram-positive streptococci, where necessary.

INITIAL INTERACTIONS OF *STREPTOCOCCUS* WITH MUCIN ON PHARYNGEAL CELL MUCOSA

Situated on top of the human respiratory epithelial cilia is a viscous layer of mucus that continuously flows toward the pharynx. The anatomical disposition of respiratory mucus requires that all mucosal pathogens interact first with mucin before they adhere to a host cell receptor on the epithelial cell. Mucins are a highly complex class of glycoproteins found in both secretory and bound forms and are expressed by several genes (35–37). The structural diversity among the mucin family increases due to the posttranslational O-glycosylation and sulfation modifications that contribute to more than 50% of the molecular mass of the mucin molecule (35). Although mucin may serve as an initial protective barrier from invading pathogens, overproduction of mucin has been shown to exacerbate the pathological symptoms of diseases such as bacterial pneumonia and cystic fibrosis (38–40). While several reports have described the role of mucin in the pathogenesis of Gram-negative bacterium-associated cystic fibrosis and, similarly, of oral streptococcus-mediated diseases (38, 39, 41), the information available about the interaction of mucin with pathogenic Gram-positive bacteria and its implication in the disease process is very limited (42–45). The mucin-binding property of *S. pyogenes* is regulated by the *mga* gene (46). The M protein and a 39-kDa *S. pyogenes* cell wall-associated surface protein (streptococcal surface dehydrogenase [SDH]) are the first reported surface proteins that mediate streptococcal binding to salivary/tracheobronchial mucin (47). The binding of *S. pyogenes* to mucin also increases streptococcal adherence to and invasion of cultured human pharyngeal cells (47). Pullulanase, a 129-kDa *mga*-regulated *S. pyogenes* surface protein also binds to submaxillary mucin and serves as a surface adhesin (48). Pullulanase possesses carbohydrate-degrading activities and may facilitate direct bacterial interaction with host cells (48). *S. pyogenes* also adheres to the salivary proline-rich protein of pharyngeal epithelial cells via the GrpE chaperon protein (49). Additional mucolytic enzymes such as streptococcal neuraminidase (45) or other extracellular proteases (50–52) may play a similar role in facilitating bacterial migration through the viscous mucin layer and allowing them to target the epithelial surface-specific receptor before the invasion.

Certain types of mucins such as MUC2 and MUC5AC are overexpressed in human bronchial explant and epithelial cell cultures in response to interactions with culture supernatants of Gram-negative (e.g., *Pseudomonas aeruginosa*) and Gram-positive (*Staphylococcus aureus*, *S. pyogenes*, *Staphylococcus epidermidis*) bacteria (53). This expression is found to be tyrosine-phosphorylation dependent (53), indicating that a specific type of mucus production may be the result of the induction of specific intracellular signaling events. Although respiratory cell-bound mucins, such as MUC1, are thought to be involved in cell adhesion (54–56), their cytoplasmic domains have been shown to play a role in cytoskeletal rearrangements in certain disease conditions (57). An asymptomatic carrier stage of *S. pyogenes* infection with little or no immune response in school-age children is very common (58). During this stage, the interaction of *S. pyogenes* with pharyngeal mucosal cells demands special attention. In adults, the mucins MUC5B (originally named MG1) and MUC7 (originally MG2) have been detected in saliva from the minor salivary glands and the submandibular and sublingual glands (59, 60). MUC5AC is considered to be the predominant mucin in airway mucus (61). During the first year of infancy, the salivary concentration of MUC5B increases and MUC7 decreases (62). MUC7 seems to play a role in mediating the interaction between bacteria and neutrophils (63). Similarly, the amount of mucins also seems to decline as people age (64). This observation may have a clinically important underlying reason for the lower frequency of *S. pyogenes* infection in infancy and old age. Thus, the tight regulation of mucin production is critical to maintaining a required balance between beneficial and detrimental outcomes. While not much information is available on mucin-associated signaling events during *S. pyogenes* infection, the suppression of the p21-activated kinase-Janus kinase signaling pathway has been shown to act as an inducer for *S. pneumoniae* pneumolysin-induced MUC5AC mucin transcription (65).

BINDING OF STREPTOCOCCAL ADHESINS TO HOST CELL RECEPTORS AND INTRACELLULAR SIGNALING EVENTS IN TARGET CELLS

The role of staphylococcal and streptococcal exotoxins, which function as superantigens, in the induction of mitogenic and inflammatory responses is well established and is characterized by the release of a variety of chemokines and cytokines from peripheral blood monocytes (30, 66–70). Although the release of a variety of inflammatory cytokines/inflammatory mediators, including AMPs (31), prostaglandin E2, leukotrienes B4, and chemokines, triggered by streptococcal superantigens plays an important role in invasive *S. pyogenes* disease, individuals infected with the same strain may develop very different manifestations (70). Kotb et al. (67) proposed that leukocyte antigen class II allelic variation contributes to differences in severity of invasive streptococcal infection through the ability to regulate the production of the cytokines tumor necrosis factor-β (TNF-β), interferon-γ (IFN-γ), and interleukin 4 (IL-4) mediated by streptococcal superantigens. In particular, patients with the DRB1*1501/DQB1*0602 haplotype mount significantly reduced responses and hence are less likely to develop severe systemic disease (67). Beyond the ability of superantigens to directly activate T cells, resulting in overzealous inflammatory responses, it was recently recognized that the essential role of *S. pyogenes* superantigens is to promote nasopharyngeal colonization and infection in a Vβ-specific T-cell-dependent manner. Thus, in the absence of accessible T cell receptor or the presence of neutralized superantigens, *S. pyogenes* infection cannot be established (71, 72). A similar study with *S. aureus* superantigen, which also activates mucosa-associated invariant CD8+ T cells as a substantial source of initial cytokine responses has indicated that only in the presence of

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such initial cytokine responses does colonization of mucosa by *S. pyogenes* or *S. aureus* occur (73).

In addition to superantigens, an important role of the M protein in excessive plasma leakage from vasculature leading to life-threatening hypotension and multiorgan failure was recently recognized (74). Herwald et al. (74) reported that the ability of the M protein to bind fibrinogen (75, 76) allows the M protein/fibrinogen complex to interact with β2-integrins and activate neutrophils. As a result, neutrophils release heparin-binding protein, an inflammatory mediator that induces vascular leakage and a massive inflammatory response, leading to toxic shock. More evidence is available that confirms the ability of target epithelial cells or blood monocytes to release certain interleukins and TNF as a result of their interaction with intact Gram-positive bacteria in a CD14-independent manner (77, 78). The examination by Wang et al. (78) of keratinocyte responses to adherent versus nonadherent *S. pyogenes* revealed a distinct pattern of expression of several proinflammatory molecules. This study further reported that both adherent and nonadherent streptococci are capable of inducing IL-6 and prostaglandin E_2; however, only adherent streptococci are able to induce IL-1α, IL-1β, and IL-8. Similarly, only adherent streptococci are capable of damaging the keratinocytes. Since the production of cytokines and the damage to the keratinocytes are considerably reduced in streptolysin O (SLO)-deficient *S. pyogenes* mutants, it is rational to conclude that streptococci also utilize some of their secretory products to induce a proinflammatory response (78).

The C-repeat region of streptococcal M protein binds to CD46, a membrane-bound complement regulatory protein related to factor H, which serves as a cellular receptor for *S. pyogenes* on human keratinocytes (79). CD46 belongs to a family of proteins that contain four structurally related short consensus repeat domains and regulate the activation of the complement components C3b and C4b (80). A major M protein-binding site is located within short consensus repeats 3 and 4, probably at the interface of these two domains (81). The hyaluronic acid capsule also modulates the M-protein-mediated adherence and acts as a ligand for attachment of *S. pyogenes* to CD44 on human keratinocytes (82). CD44 is also reported to be a receptor for *S. pyogenes* colonization of the pharynx (83). It was also proposed that this streptococcal-host cell intimate contact, mediated by the M protein CD46, may initiate proinflammatory responses from keratinocytes during infection (78). In fact, CD44-dependent *S. pyogenes* binding to polarized monolayers of human keratinocytes induces marked cytoskeletal rearrangements, resulting in membrane ruffling and disruption of intercellular junctions. The signal transduction induced after binding of *S. pyogenes* to the keratinocyte surface via CD44 involves a small signaling GTPase (Rac1) and the cytoskeleton linker protein ezrin, as well as tyrosine phosphorylation of cellular proteins (84). Studies of bacterial translocation in two models of the human skin have indicated that cell signaling triggered by the interaction of the hyaluronic capsule of *S. pyogenes* with CD44 opens intercellular junctions and promotes tissue penetration by the bacteria through a pericellular route (84).

The evidence that streptococci may regulate intracellular signaling events upon contact with their target cells, such as pharyngeal cells, was first suggested when a novel multifunctional protein, SDH was identified on the surface of streptococci (85). SDH, which shows binding activity to a variety of mammalian proteins such as fibronectin, myosin, plasmin, and actin, is also a cysteine-residue targeting the ADP-ribosylating enzyme (86, 87). ADP-ribosylation plays an important role in intracellular signaling events and is the mechanism by which many bacterial toxins show their deleterious effects on target cells (88). SDH does not serve as a toxin, but its direct contact with human pharyngeal cells (both polarized and nonpolarized) results in DNA condensation and apoptosis (89, 90). Interestingly, SDH belongs to a novel class of surface-expressed, anchorless housekeeping enzymes and is now well established as a virulence factor for pathogens (90, 91). Like SDH, another metabolic enzyme, streptococcal surface enolase (SEN), has also been shown to be present on the surface of *S. pyogenes* and *Streptococcus pneumoniae* functioning as a strong plasminogen receptor (92, 93). Human enolase has been shown to be expressed on the surface of a variety of cells, such as hematopoietic, neuronal, endothelial, and epithelial cells, and serves as a receptor for plasminogen (94–97). SDH binds to four membrane-associated proteins (89), two of which (47 kDa and 55 kDa) have been characterized as the human enolase and urokinase plasminogen activator receptors (uPAR), respectively (98). The report describing increased adherence of *S. pyogenes* to human pharyngeal cells in the presence of plasminogen indicates that this molecule may serve as a molecular bridge between human enolase expressed on the surface of pharyngeal cells and the SEN molecule expressed on the surface of *S. pyogenes* (97). Binding of plasminogen by *S. pyogenes* through SEN or other enolase-binding proteins facilitates the pericellular invasion of the host cell by *S. pyogenes* (97). Since SDH binds directly to human enolase and SEN (98) and is associated with the M protein and M-like protein (99), it is conceivable that both human enolase and uPAR can serve as potential receptors for *S. pyogenes*-mediated signaling events. uPAR is the native receptor of uPA, which is primarily responsible for recruiting plasmin during the infection process. uPAR is a glycosylphosphatidylinositol-anchored protein that is internalized via endocytosis in a clathrin-independent manner (100–102). It is likely that the binding of SDH to uPAR may contribute to the induction of downstream signaling events (103–105), the activation of the fibrinolytic system, and *S. pyogenes* internalization and invasion. Mice expressing human plasminogen *in trans* are significantly more susceptible to invasive *S. pyogenes* diseases than the $uPA^{-/-}$ mice, since in the latter the dissemination of *S. pyogenes* is significantly poor due to the lack of plasmin accumulation (106).

The putative receptor of SDH also includes two additional proteins, the 30- to 32-kDa protein 14-3-3, and the 80-kDa protein moesin (98). The 14-3-3 protein is an important regulatory protein that plays a diverse regulatory role in cell signaling (107–109) and serves as a gatekeeper of phosphorylation (110). While the roles of SDH and the 14-3-3 protein in *S. pyogenes* infection have not been explored, the 14-3-3 protein has been shown to play a determining role in parasitic and chlamydial infections (111, 112). Moesin is a member of the ezrin, radixin, moesin (ERM) protein family and plays a key role in membrane cortex integration and epithelial organization; thus, this protein is involved in endothelial barrier integrity and neutrophil functions (113–117). Similar to SDH, the *S. pyogenes* streptococcal inhibitor of complement (Sic) protein has also been shown to bind to moesin (118). By binding to moesin, the Sic protein enhances bacterial survival by enabling *S. pyogenes* to avoid the intracellular environment, resulting in pathogen abundance at the site of infection (118). Like Sic, SDH may contribute to *S. pyogenes* abundance during human infection and the subsequent dissemination of *S. pyogenes* to the community. Similar to the secreted Sic protein, the cell-associated streptococcal C5a peptidase (ScpA) protects *S. pyogenes* from

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phagocytosis by enzymatically cleaving complement C5a and thus interrupting host defenses (119, 120). Terao et al. (121) reported that unlike ScpA, which binds C5a with low affinity (equilibrium dissociation constant [K_D] = 7.25 mM) and cleaves C5a, SDH binds to C5a on neutrophils with a very high affinity (K_D = 0.409 μM) and inhibits its activation, thus contributing to *S. pyogenes* evasion from human neutrophils. The ability of ScpA to cleave C5a may contribute to its low binding affinity. Thus, both ScpA and SDH are required for the inactivation of C5a. On the other hand, the extracellularly released glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *S. pneumoniae* binds to C5a with a much higher affinity (K_D = 3.4 nM); however, this binding activates C5a (122). It is not clear whether this contrasting finding is caused by the absence of ScpA in *Pneumococcus* and is associated only with the secreted form of pneumococcal GAPDH (121). The mechanism that underlies the pneumococcal GAPDH-mediated activation of C5a is unknown. SDH, like whole streptococci, is capable of activating both tyrosine and serine/threonine kinases that phosphorylate several proteins in human pharyngeal cells (89). A recently characterized low-molecular-weight protein, tyrosine phosphatase (SP-PTP), secreted from *S. pyogenes* has a dual specificity for phosphorylated serine/threonine and tyrosine and plays a significant role in virulence (123). *S. pyogenes* mutants lacking SP-PTP are completely attenuated and when cocultured with human respiratory cell lines show a significantly high number of tyrosine-, as well as ser/Thr-phosphorylated proteins compared to the wild type and the *sp-ptp*-complemented *S. pyogenes* strains (123). The increased number of phosphorylated proteins indicates the ability of SP-PTP to interact with host cells directly and to interfere with intracellular signaling events (123).

ROLE OF INTEGRINS IN STREPTOCOCCAL-MEDIATED SIGNALING EVENTS

Integrins are large αβ heterodimeric membrane proteins found on the surface of a wide variety of mammalian cells (23, 124). The integrins seem to be involved in promoting adhesion to extracellular matrix proteins such as fibronectin, collagen, and laminin. Several fibronectin-binding proteins on the surface of *S. pyogenes* have been described and include protein-F/Sfb1 (125, 126), protein F2 (127), serum opacity factor (128), protein H (129), streptococcal glyceraldehyde-3-phosphate dehydrogenase (surface dehydrogenase, SDH) (85), Fbp54 (130), and M1 (131). Sfb1, protein F, and protein F1 have been found, at least in part, to mediate streptococcal invasion of HEp2 or HeLa cells (126, 132). Similarly, the M1 protein efficiently binds to the human lung epithelial cell lines, A549, in the presence of fibronectin via α5β1 integrin and mediates *S. pyogenes* invasion (131). This integrin-mediated interaction seems to play a critical role in signaling events that regulate streptococcal invasion. While recognizing the importance of α5β1 integrin-mediated streptococcal invasion of epithelial cells, the study emphasizes that the ability of fibronectin to mediate invasion varies considerably depending on the source of the integrin preparation (130, 131). *S. pyogenes* invasion is also reported to be mediated by laminin (133), which recognizes α1β1, α2β1, α3β1, α6β1, or α7β1 integrins on the cell surface (23, 124, 134). Both entry of *S. pyogenes* via protein F1 and uptake of fibronectin-coated beads into HeLa cells are blocked by anti-B1-antibody (132, 135). Thus, during the invasion of epithelial and endothelial cells, the streptococci and many other Gram-positive pathogens may attach to different components of extracellular matrix and use one or more integrins. By creating invasion-resistant xylose-transferase-deficient Chinese hamster ovary cell lines using CRISPR/Cas9 technology, Esko's group recently showed that the cellular invasion of *S. pyogenes*, *S. agalactiae*, and *S. aureus* is mediated primarily through the L4A domain of laminin α2 subunits (136).

Bacterial invasion of a target epithelial cell is a dynamic process which involves the active participation of the cytoskeletal structure (21, 22, 137). It is thus reasonable to predict that binding to integrins allows streptococci to communicate with the cytoskeletal structure of the cell and regulates streptococcal entry into the cell. Studies showing the inhibition of streptococcal invasion of Detroit pharyngeal cells in the presence of the tyrosine- and serine/threonine kinase inhibitors, genistein, and staurosporine indicate that the rearrangement of cytoskeletal structure, which facilitates bacterial entry, is substantially regulated by the activation of these intracellular signaling enzymes (89). In HeLa cells, the endocytosis of a highly invasive type M1 *S. pyogenes* strain is associated with polymerized actin and Lamp1 glycoprotein of the lysosomal vacuole (138). The M1 protein either directly or indirectly via bound fibronectin initiates signals that depend on the phosphatidylinositol 3-kinase (PI-3) pathway as in the presence of PI-3 inhibitors, (such as LY294002, wortmannin) and the actin polymerization, the streptococcal invasion of host cells is significantly decreased (139). Similarly, protein F1 also mediates recruitment of *de novo* tyrosine-phosphorylated focal adhesion kinase (140). Src kinases such as yes, paxillin, and Fyn proteins enhance the efficiency of *S. pyogenes* uptake but are not an absolute requirement. Also, Rac and Cdc42 are also activated, indicating that there are at least two independent pathways, which converge to trigger actin rearrangement and endocytosis/bacterial uptake (140). The fibronectin-binding M type-12 *S. pyogenes* strain A40 invades following membrane invagination at the level of the bacterium-cell (HEp-2) contact area. The non-fibronectin-binding M type-5 strain A8, on the other hand, recruits elongated microvilli upon bacterial contact, which then fuse to surround bacteria, leading to the formation of a large pseudopod-like structure that engulfs bacteria (135). The invasion of HEp-2 cells by strain A40 but not A8 is inhibited by treatment of cells with cholesterol-depleting agents, such as methyl-B-cyclodextrin and filipin, indicating that caveolae may act as an entry port for strain A40 (141). Whether caveolin protein serves as a receptor for streptococci is not known.

Unlike type M1 *S. pyogenes*, type M3 *S. pyogenes*, which lacks the SFB1 and PrtF1 proteins, activates Src family PTKs (protein tyrosine kinase) and GTPase Rac1, and accumulates actin nucleation complex Arp2/3 at the point of entry in endothelial cells in a PI3-kinase-independent manner (84, 142). In all published reports, the fibronectin-binding property of *S. pyogenes* is said to have a primary role in initial adherence to, colonization of, and subsequent invasion of respiratory epithelial cells (126, 131, 143, 144). It is reasonable to speculate from these studies that integrin-mediated signaling events play a critical role in streptococcal dissemination in the tissue. In contrast to this view, it seems that dissemination of *S. pyogenes* occurs more efficiently only in mice lacking plasma fibronectin, because F1-defective/negative *S. pyogenes* strains and transgenic mice lacking plasma fibronectin are less virulent as a result of limiting bacterial spread (145). Thus, the fibronectin-binding ability may be viewed as the bacterium's deliberate attempt to decrease its virulence and form an unassuming but more advantageous coexistence with its human host.

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Rac1 GTPAses that are activated by the *S. pyogenes* type M3 strain bind to and activate the mixed-lineage kinases (MLK1,2,4), a group of mitogen-activated protein kinases that contain in their N-terminal region a GTPase recognition, a Cdc42/Rac interactive binding motif (CRIB) (142, 146, 147). This event leads to activation of mitogen-activated protein kinase kinases which regulate the c-Jun N-terminal kinase. The activation of p21-activated kinase by Rac allows the activation of inhibitory kappa B kinase, which phosphorylates IKB (the inhibitor of the nuclear factor NF-κB), promoting IKB ubiquitination and its degradation by the proteasome and allowing NF-κB to translocate into the nucleus and target gene expression (148, 149) (Fig. 1). In humans, the activated NF-κB dimers consist mainly of the Rel family protein p50 and p65 subunits, which then bind to responsive κB sites in the promoters and enhancers of the target genes (150). Like NF-κB, STAT molecules (signal transducer in the cytoplasm and activator of transcription in the nucleus) are also latent cytoplasmic transcription factors activated by cytokine stimulation via tyrosine phosphorylation (151). The binding of cytokines to their receptors results in autophosphorylation of receptor-associated JAK kinases that phosphorylate and activate STATs (152). The simple external binding of *S. pyogenes* has been shown to be sufficient to induce NF-κB activity in human epithelial and hematopoietic cells (27, 70, 153–155). This is consistent with the fact that neither cytochalasin B (156) nor protein synthesis inhibitor (157), which prevents bacterial internalization, abolishes NF-κB production. *S. pyogenes* also induces STAT1, STAT3, and IFN regulatory factor-1 DNA-binding activity and the formation of IFN-α-specific transcription factor complex and IFN-stimulated gene factor-3 in human primary macrophages (157). Streptococcus-mediated STAT activation is, however, cycloheximide sensitive (157), suggesting that prior activation and production of cytokines is required for STAT activation. These earlier studies thus shed light on the molecular mechanisms of interaction between streptococci and host cells that lead to different inflammatory responses.

Initial binding of *S. pyogenes* to host epithelial cells *in vitro* and *in vivo* through the activation of several intermediary signaling events as described above induces the secretion of several proinflammatory cytokines such as IL-1α, IL-1β, IL-2, IL-6, TNF-α, RANTES/CCL5 (Regulated upon activation, normal T cell expressed and secreted/chemokine [C-C motif] ligand 5) and IFN-β (70, 71, 158, 159). The induction of these proinflammatory responses in nasal-associated lymphoid tissue is the outcome of initial transforming growth factor-beta-, IL-6-, IL-21-, IL-1β-, and IL-23-dependent differentiation of Th17 cells from naive T cells (160). IL-1α or IL-1β in synergy with IL-23 promotes IL-17 secretion from memory T cells (161). The main function of *S. pyogenes*-induced nasal lymphoid tissue-associated IL-17-secreting T cells is to mediate inflammation by stimulating the production of inflammatory cytokines, such as TNF- α, IL-1β, and IL-6, and a variety of inflammatory chemokines that promote the recruitment of neutrophils and macrophages (160). The cytokines released from infected epithelial cells and resident macrophages, similar to nasal lymphoid tissues induce the expression of P, E, and L selectins of endothelial cells. Blood leukocytes (mainly neutrophils) then temporally bind to individual selectins (first P and E selections, followed by L selectins) via their surface integrins and rolls along the endothelial layer to populate the site of infection through transendothelial migration (162, 163). Selectins play an active role in the initiation of the intracellular signaling pathway (TNF-α, IL-8, respiratory burst) in leukocytes and cell-cell interaction. Vascular dysfunction and ischemic destruction during *S. pyogenes* necrotizing fasciitis, characterized by the platelet/neutrophil complex, are mediated essentially by platelet-P selectin (164–166).

INITIAL INTERACTION OF *S. PYOGENES* WITH NEUTROPHILS AND NEUTROPHIL EXTRACELLULAR TRAPS (NETS)

Neutrophils recruited at the site of *S. pyogenes* infection through the extravasation process, as described above, phagocytose the invading pathogen. The *S. pyogenes* is then engulfed by a phagosome and killed by AMPs, such as cathelicidins (LL-37), α-defensins, neutrophil elastase, lysozyme, or ROS (167). AMPs, which are hydrophobic molecules composed of ~10 to 50 amino acid residues with net positive charge, interact with the net negative charge of the bacterial surface. This strong electrostatic interaction allows AMP to insert into the cytoplasmic membrane, causing membrane rupture (168). ROS kill bacteria by oxidizing microbial proteins and lipids through the action of induced cytosolic NOX2, NADPH enzymes, and the conversion of the resulting reactive oxygen (superoxide $O_2^{\bullet-}$) to deleterious hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) (169). *S. pyogenes* still overcomes this hostile environment and successfully colonizes 5 to 25% of humans through its endowed stealth behavior (29, 31).

A relatively newly discovered neutrophil innate immune function is attributed to the extracellular discharge of fibril matrix and formation of extracellular traps, known as neutrophil extracellular traps (NETs) (170). The NETs contain DNA fibrils along with histone proteins, neutrophils granules, neutrophil elastase, myeloperoxidase, lactoferrin, gelatinase, and cytoplasm but not actin or tubulin. NET activation and release allows neutrophils to kill extracellular pathogens and minimize damage to the host cells; the process is also called NETosis (171). Bacterially induced NETosis involves activation of NADH oxidase (Nox), which generates superoxide ion ($O_2^{\bullet}$) and hence is also called Nox-dependent NETosis. A similar process that occurs in mast cells is called METosis, or MCETs (172). Like neutrophils, other myeloid cells, including mast cells (172), macrophages (173), basophils (174), and eosinophils (175), also extrude DNA/histone-based extracellular traps. Hence, the common terminology for these phenomena observed in many myeloid cells types, ETosis, has been suggested as the novel cell death pathway (176). The accumulation of histone H4 and CXCL10 detected in biopsies from patients with necrotizing fasciitis caused by *S. pyogenes* has have been reported (177). The histone H4 specifically targets monocyte in human blood and evokes the mobilization of Toll-like receptor 4 (TLR4)-dependent chemotactic chemokines CXCL9 and CXCL10 and helps maintain the proinflammatory response (177, 178). The significance of histone in the NETs is specifically attributed to its antibacterial activity, which was first observed by Hirsch on several Gram-negative and Gram-positive bacteria, including *S. pyogenes* (179). *In vitro* and *in vivo* mouse skin infection models have shown that mast cells also play an important role in *S. pyogenes* skin infection (172). The *in vivo* skin infection model using mice deficient in mast cells showed a lack of LL-37, an important component of mast cell extracellular traps (172). A characteristic of mast cell activation is the release of histamine. SLO and SpeB of *S. pyogenes* have been shown to induce histamine from mast cells upon *in vitro* infection (172). NETosis is a highly dynamic process

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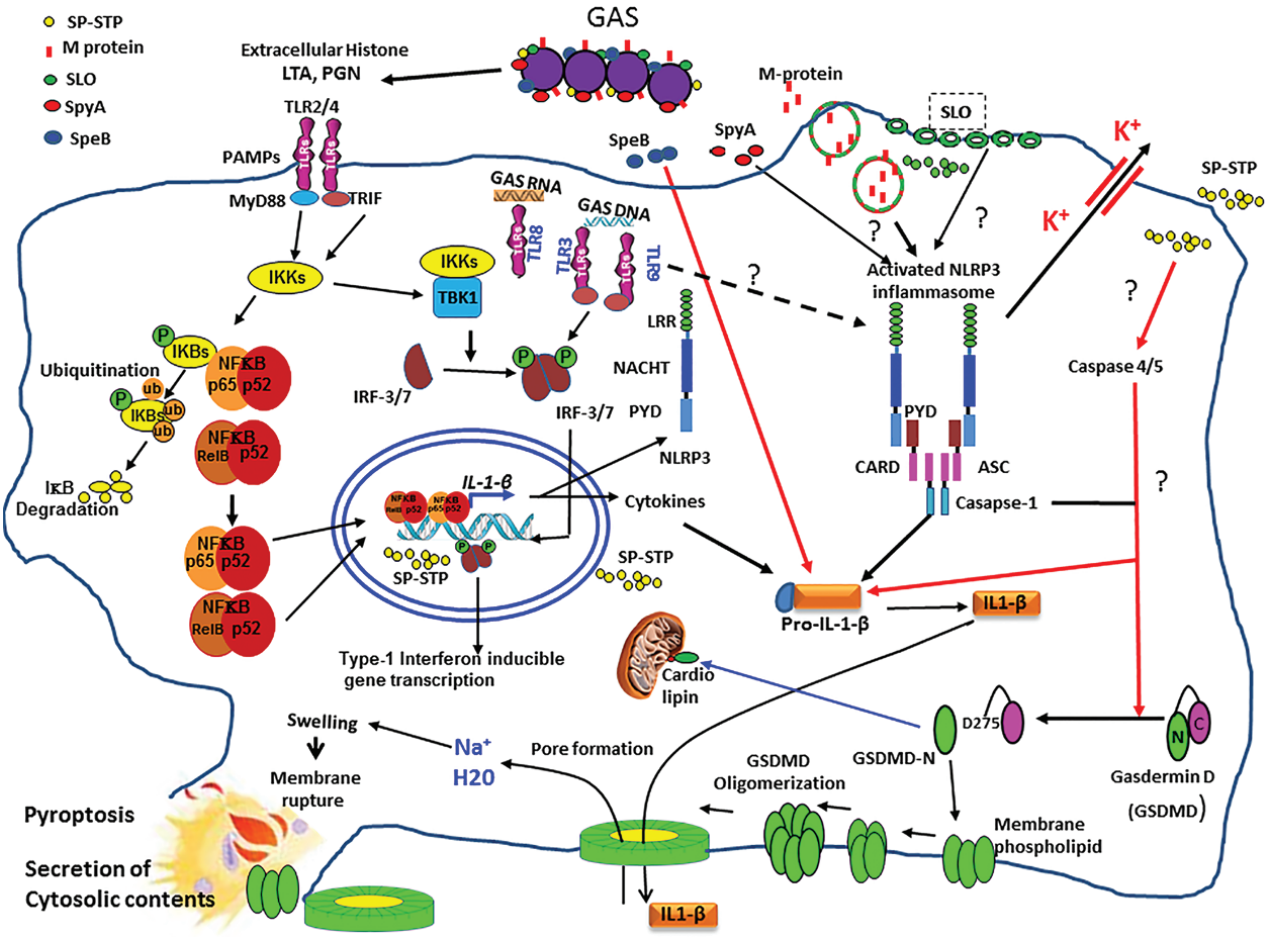


FIGURE 1 Group A *Streptococcus*-mediated signaling events responsible for inflammation. A group of cytosolic protein complexes called inflammasomes contributes to *S. pyogenes*-mediated inflammation. *S. pyogenes* activates NLRP3 inflammasomes. The activation of NLRP3 inflammasomes occurs in two steps. The first step involves transcriptional priming of inflammation. There are at least four well-studied receptors (uPAR, enolase, CD44, CD46) that directly interact with the surface proteins of *S. pyogenes*. Fibronectin, fibrinogen, and other lectin-like proteins play a crucial bridging role in the binding of *S. pyogenes* surface proteins to integrins of eukaryotic cells. These initial interaction-mediated signaling events help *S. pyogenes* invade the host cells. *S. pyogenes* lipoteichoic acid and peptidoglycan fragments, which serve as PAMPs/DAMPs, initiate inflammation by binding to the Toll-like receptors TLR-2/4, which serve as an external pattern-recognition receptor (PRR). *S. pyogenes* DNA and RNA can initiate inflammation via binding to intracellular PRRs (TLR3, TLR8, TLR9). These PRR/PAMP interactions occur in a MyD88-dependent or independent manner via Toll/interleukin-1 receptor-domain-containing adapter-inducing interferon-β (TRIF). MyD88-dependent signaling activates NF-κB and the NF-κB-mediated induction of IL-1β, NLRP3, and other cytokines. TRIF activates type 1 IFN and the IFN-induced chemokines. The second step (activation), not known of until recently in *S. pyogenes*-mediated inflammasome activation, is initiated by the SpeB-removed or secreted M-protein, which enters the cell via clathrin-coated pit-mediated endocytosis followed by activation of NLRP3 inflammasomes. Other factors, such as SLO and NADase may cooperatively make holes in eukaryotic cells and inject bacterial product in them to exploit intracellular signaling events and activate inflammasomes. Streptococcal ADP-ribosyl transferase (SpyA) seems also to activate NLRP3 inflammasomes. The assembly of the NLRP3 inflammasome complex is formed by NLRP3 (NACHT, LRR, and PYD domain-containing protein 3), the adaptor ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), and caspase-1. The activated NLRP3 inflammasome cleaves and converts inactive pro-IL-1β to active and proinflammatory IL-1β. This signaling is called canonical IL-1β-dependent inflammasome activation. The mediators of noncanonical IL-1β-activation (red arrows) include SpeB, which may enter the cell after SLO/NADase-mediated pore formation and cleave Pro-IL-1β to active IL-1β in an NLRP3-independent manner. SP-STP may have a role in activation of the human homolog of caspase-11, the caspase-4/5 which triggers both caspase-1-dependent and -independent production of the inflammatory cytokine IL-1β. The inflammasome activation is associated with K+ efflux. The activation of inflammasome resulting in proinflammatory responses ultimately leads to pyroptosis, a phenomenon characterized by membrane blebbing, swelling and lysis of the cell, and release of cytosolic content. Although not shown in *S. pyogenes* infection, pyroptosis is caused by the cleavage of gasdermin D (GSDSMD) to its N-terminal P30 fragment, which migrates to the membranes, binds to phospholipid, oligomerizes, and forms pores. The activated IL-1β is secreted out, and salts and water enter the cell through these pores, leading to swelling and rupture of the cell. The released contents then start another cycle of proinflammatory responses in adjacent cells.

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considering the short life (minutes to hours) of neutrophils. As the NETosis activation pathway has emerged, it has become clear that the level of transcription activity ("transcriptional firing") reflects the degree of DNA condensation occurring during NETosis since inhibition of transcription and the translation suppresses both types of NETosis (180). This is true for both NADPH oxidase (NOX2)-dependent (suicidal) and NOX2-independent NET formation. NOX2-independent NETs are induced by calcium ionophore and are also called vital NETosis. Extensive citrullination of histone in multiple loci occurs only during calcium-mediated NOX2-independent NETosis (180). Vital NETosis is completed in minutes, unlike suicidal NETosis, which takes hours. During vital NETosis, the neutrophils are not dead but are without DNA and are still capable of phagocytosing and killing the engulfed bacteria. Suicidal NETosis is characterized by the rupture of the plasma membrane along with extrusion of DNA content into the extracellular space.

Recent *in vitro* and *in vivo* findings from the Nizet group elegantly revealed how *S. pyogenes* evades NETosis-mediated proinflammatory signaling events in and killing by neutrophils and macrophages (27). Streptodornase degrades DNA in the NETs, allowing *S. pyogenes* to escape NETosis (181). SclA, a surface protein, inactivates LL-37 and inhibits myeloperoxidase release (182). High-molecular-weight hyaluronan capsule engages human immunoglobulin-like transmembrane protein Siglec-9 lectin possessing an immunoreceptor tyrosine-based inhibitory motif, dampening neutrophil activation (183–185). Encapsulated *S. pyogenes* resists phagocytosis and contributes to H_2O_2 resistance. Superoxide dismutase, NADPH oxidase, and alkyl hydroperoxide reductase reduce the H_2O_2 accumulation and detoxify H_2O_2 (29). Recent findings have shown that the resistance of *S. pyogenes* to NETs is also due to antiphagocytic M protein, because its N-terminal portion strongly binds to histone and inactivates the antimicrobial activity of histones (186). The B-repeats of the M protein also bind to immature LL-37 (hCAP-18), or murine cathelicidin-related AMP (mCRAMP), and sequester them from being active (187).

IL-1β/INFLAMMASOME SIGNALING PATHWAYS AND *S. PYOGENES* INFECTION

Inflammatory responses are meant to resolve inflammation, limiting tissue damage. Such immune responses are rapid and destructive but self-resolving and specific as seen for most, if not all, mild extracellular *S. pyogenes* infections. However, upon tissue invasion and establishment of an intracellular niche in epithelial cells and macrophages, some *S. pyogenes* strains survive intracellularly and propagate within the cytoplasm, resulting in damage and destruction of the host tissues, sepsis, organ failure, and often death (188, 189). The orchestration of such calculated inflammatory responses, which determine the fate of infection, is initiated with the binding of pattern recognition receptors (PRRs) on the host and pathogen (danger)-associated molecular patterns (PAMPs/DAMPs) on the pathogens (190, 191). IL-1 plays a pivotal role in inflammation caused by *S. pyogenes* and other Gram-positive streptococcal pathogens (34). The inflammation, in general, is mediated by inflammasomes. The inflammasome is a high-molecular-weight protein complex that requires assembly of PRRs and PAMPs/DAMPs, which in turn activate inflammatory caspases and cytokines of the IL-1 family (IL-1 and IL-18) and the induction of inflammation-associated cell death (pyroptosis) (192). IL-1 exists in two forms: membrane-bound IL-1α and the soluble form, IL-1β, both of which initiate a variety of signaling events via binding to the IL-1 receptor (193). The role of the induced level of IL-1α in *S. pyogenes* infection is not clear. However, IL-1β plays a significant role in the defense against *S. pyogenes* and infection by other Gram-positive pathogens by attracting neutrophils at the site of infection (34). Increased levels of another cytokine of the IL-1 family, IL-18, which contributes to the induction of IFN-γ and natural killer cells, also has been reported during *S. pyogenes* infection (194, 195), but its precise function as a protective or pathological agent is not clear. The conversion of IL-1 to IL-1β is not dependent solely on the inflammasome, because several other pathways are also responsible for its activation. *S. pyogenes*'s SpeB (cysteine protease) and proteases of other pathogens which are capable of cleaving IL-1β may contribute to the alternative (noncanonical type; see details below) inflammasome-independent pathways (34) (Fig. 1).

There are 13 mammalian Toll-like glycoprotein receptors (TLRs) that have been recognized for their ability to sense a wide variety of microbial PAMPS at the cell surface and within endosomes (196). Additional PRRs include nucleotide-binding and oligomerization domain-like receptors (NLRs) and the retinoid acid-inducible gene-I-like receptors (RIG1) that recognize PAMPS/DAMPS intracellularly (197, 198). The presence of both extracellular and intracellular PRRs suggests that even though microbes may evade surveillance by extracellular PRRs, they are subsequently encountered by another line of PRRs in the cytosol. The detection of PAMPS/DAMPS by these PRRS activates multiple proinflammatory signaling pathways or the inflammasome pathway. The ability of *S. pyogenes* to activate inflammasomes is dependent on the cell type, M type *S. pyogenes* strains, as well as *in vitro* versus *in vivo* experimental conditions. Fundamentally, inflammasome activation in monocytes differs from that in macrophages (193, 199). In monocytes, including neutrophils, caspase-1 is constitutively expressed, leading to activation of pro-IL-1 and release of active IL-1β after single stimulation with a bacterial ligand. On the other hand, macrophages (THP-1 cells) require two distinct stimuli, one (priming step) that stimulates transcription and translation of pro-IL-1β and a second stimulus such as ATP, and other factors that are required for caspase-1 activation with bioactive IL-1β production and its secretion (193). Thus, irrespective of the cell type, IL-1β production is dependent on the assembly of an inflammasome complex. The first step of priming could be mediated by TLRs, the IL-1 receptor, or the TNF receptor. While TLR-mediated priming occurs via the myeloid differentiation primary response gene (MyD88) (196), the NLR-RIP2 (receptor-interacting-serine/threonine-protein kinase 2, RIPK2 or RIP2) complex resulting in the NF-κB activation to induce IL-1β, the IL-1 receptor, and the tumor necrosis factor receptor is activated via binding to secreted soluble IL-1β and TNF-α (197) (Fig. 1).

Streptococci commonly recognize only two main inflammasome complexes: NLRP3 and AIM2 (absent in melanoma 2) multi-protein complexes (34). NLRP3 stands for NACHT, LRR and PYD domains-containing protein 3, wherein NACHT stands for NAIP [nucleotide-binding and oligomerization domain-like protein family apoptosis inhibitor protein], CIITA [C2TA or major histocompatibility complex (MHC) class II transcription activator], HET-E [incompatibility locus protein from *Podospora anserina*], and TEP1 [TP1 or telomerase-associated protein]) domain; LRR stands for leucine-rich repeat; and PYD stands for pyrin domain)-containing protein 3. *S. pyogenes* (M1 strain SF370)

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has been shown to activate type 1 interferons and induces p38 mitogen-activated protein kinase, NF-κB, TNF-α, and IL-6 in bone marrow-derived mouse macrophage in a TLR2 (receptor for lipoteichoic acid)-, TLR4 (receptor for lipopolysaccharide)-, and/or TLR9 (receptor for bacterial DNA containing unmethylated CpG sequence)-independent but MyD88-dependent manner (200). The fact that this *S. pyogenes*-mediated induction occurs even in the absence of *S. pyogenes* SLO or streptolysin S (SLS) indicates that these virulence factors do not participate and regulate inflammatory responses in mice. However, an encapsulated *S. pyogenes* strain, 950771, induces NF-κB in mouse bone-marrow-derived macrophage in an SLO-dependent manner (201).

S. pyogenes strain- and host cell-dependent variations as described above are also observed for inflammasome activation. Thus, *S. pyogenes* strain SF370 and the isogenic mutants lacking SLO and SLS have revealed the induction of IL-1β, IL-10, TNF-α, and CXCL-10 via activation of the NALP3/NLRP3 inflammasome complex in *S. pyogenes*-infected human peripheral blood-derived macrophage (202). Encapsulated live *S. pyogenes* strain 950771 and its SLO, however, activate caspase 1 and NLRP3 in bone marrow-derived macrophages of C57BL/6J mice in a TLR2-independent manner (201). In general, the cells that are exposed to TLR ligands require the second stimulation of purinergic receptor P2X7R by ATP to activate NLRP3 inflammasomes (193). However, *S. pyogenes*/SLO-mediated NLRP3 stimulation occurs independently of P2X7R and ATP (201). The mechanism of NLRP3 activation remained unknown until the recent report from the Nizet laboratory which unraveled the second stimulus initiated by the M1 protein. *S. pyogenes* M1 protein alone can promote IL-1β signaling in THP-1 macrophages but not in epithelial cells, neutrophils, or monocytes. The ability of M proteins from various M types to promote IL-1β signaling specifically in macrophages parallels their ability to cause cell death. Thus, an M28 protein, which does not cause cell death/pyroptosis, is unable to induce IL-1β. Although in this study THP-1 cells were stimulated using lipopolysaccharide, an SLO mutant and an M1 mutant were unable to activate NLRP3 or IL-1β *in vitro*. These findings were substantiated by revealing the induction of IL-1β in mice after injecting the purified M1 protein in a thioglycollate-induced experimental peritonitis model. Interestingly, the B-repeat of the M protein which plays a key role in various other immune evasion functions (186, 187) has been shown to be specifically involved in the induction of inflammasomes, because the M1 protein lacking a B-repeat is unable to propagate IL-1β signaling in both *in vitro* and *in vivo* experiments (203). The specificity of the M1 protein-mediated second stimulus directed to its B-repeat indicates that a host-specific receptor likely recognizes the M1 protein located in clathrin-coated pits since the internalization of the M protein, and subsequent induction of NLRP3 inflammasome, is inhibited by a specific inhibitor of the clathrin-mediated endocytosis process (203). This study confirms the previous report on the inhibition of *S. pyogenes*-induced inflammasomes by dynamin-dependent endocytosis using an endocytosis inhibitor, Dynasore (202). Dynamin is a GTPase protein and plays an essential role in membrane fission during clathrin-mediated endocytosis and caveolae from the cell membrane and formation of polymerization of actin (204). *S. pyogenes* ADP-ribosyl transferase toxin SpyA has also been shown to simulate protective caspase-dependent IL-1β production in mouse bone marrow-derived macrophages, ultimately causing their apoptosis (194). This stimulation and apoptosis are mediated through the activation of the NLPR3 inflammasome (194). In the absence of SpyA, such a protective role of IL-1β is abrogated, resulting in increased intracellular survival within the macrophages and higher mortality in a mouse infection model. SpyA thus acts independently of TLRs.

Purified DNA isolated from *S. pyogenes* can induce IFN-β and TNF-α, indicating the potential role of TLR9 in its activation from the cytoplasm (205, 206). INF-α plays an important role in protecting against Gram-positive bacterial infection in general (207). *S. pyogenes* streptodornase-mediated DNase activity diminishes intracellular TLR9-dependent IFN-α and TNF-α secretion by murine bone marrow-derived macrophages. This promotes resistance to the intracellular macrophage killing both *in vitro* and *in vivo*, thus providing another mechanism of microbial innate immune evasion and promoting necrotizing skin infection (205). Like DNA, *S. pyogenes* ribosomal RNA (23S) is also recognized by mouse TLR13 upon phagocytosis by murine macrophages (208). TLR13 is, however, present in mice and a few mammals but absent in humans. TLR8 recognizes *S. pyogenes* 23S RNA following phagocytosis of *S. pyogenes* by human monocyte-derived macrophages (209). Together, these findings indicate that *S. pyogenes* nucleic acids may play an important role in immune recognition during infection.

NONCANONICAL INFLAMMASOME

In addition to caspase-1, caspase-11 (human caspase-4 and caspase-5) has also been shown to process IL-1β via NLRC4 and NLRP1 (210–212). The original caspase-$1^{-/-}$ mouse strain that was used in many earlier studies to understand the function of caspase-1 in the production of IL-1β during Gram-negative bacterial infections was later identified as the caspase-$1^{-/-}$-$11^{-/-}$ double knockout genotype (210). These findings kindled questions about the possible role of casapse-11 in the pathogenesis of bacterial infections. Caspase-$11^{-/-}$ mice are resistant to lipopolysaccharide-induced toxic shock. Mice deficient in both caspase 1 and caspase-11 are more resistant to salmonella and shigella infections (212). The importance of caspase-11-dependent pyroptosis thus becomes more obvious to restrict intracellular bacterial replication. It is, however, believed that caspase-11 forms a noncanonical inflammasome in response to Gram-negative bacteria but not Gram-positive bacteria, although this has not been thoroughly tested. Caspase-4 and caspase-5 (which are absent in mice) are the human orthologs of caspase-11 in mice (211). From the available knowledge, the secreted PP2C-type eukaryote-like serine/threonine phosphatase (SP-STP) from *S. pyogenes* is the only protein which has been reported to induce 2- to 4-fold mRNA expression of caspase-4 and caspase-5 in the Detroit 562 human pharyngeal carcinoma cell line after treatment for 24 h (13) (Fig. 1). It is noteworthy that SP-STP is the only known streptococcal protein that enters the human respiratory epithelial cells and transmigrates to the nucleus of host cells and causes apoptosis of the Detroit pharyngeal cells (13). Since there has been no follow-up study regarding the role of these caspases in the induction of IL-1β *in vitro* or *in vivo* in *S. pyogenes* infection models, it is currently unknown whether such an induction involves a novel noncanonical inflammasome and whether the induced mRNA expression alone is sufficient for autoactivation. Further, an M1T1 *S. pyogenes* mutant lacking SP-STP is completely attenuated, and the lung alveolar epithelial cells of mice infected with this mutant show a significant decrease in apoptosis (13). Further studies of this protein in relation to the activation of caspase-4, -5, and -11 may reveal a new mechanism of *S. pyogenes* pathogenesis.

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Although earlier reports indicated that caspase-11 was not induced during *Listeria monocytogenes* infection and had no role in *L. monocytogenes*-mediated inflammation (213), the lipoteichoic acid of *L. monocytogenes* and *S. aureus* was recently shown to promote caspase-11 cleavage and activation via NLRP6 activation (214). Similarly, recombinant *S. pneumoniae* pneumolysin and *S. aureus* α-toxin have also recently been shown to activate caspase-4 and caspase-11 in A549 (human lung adenocarcinoma) cell lines (215). Another example of noncanonical inflammasome activation, which occurs in parallel with a classical inflammasome during *S. pyogenes* infection, is the ability of *S. pyogenes* SpeB to directly cleave pro-IL-1β into functionally active IL-1β via its cysteine protease activity (216). Expression levels of SpeB vary among different M types due to naturally occurring mutations in the CovR/S regulatory system and host environmental regulation (25, 217). Thus, differentially regulated SpeB expression, and other secondary stimuli such as M protein, SLO, and SpyA, activates or inactivates canonical or noncanonical inflammasomes that mediate IL-1 activation in a tightly regulated manner, resulting in the resolution of infection, hyperinflammation, and uncontrolled sepsis.

CAUSE OF PYROPTOSIS

Recently, the ultimate cause of inflammasome-activated caspase-1, -4, -5, and -11 leading to pyroptosis via activation of IL-1β was reported to be the cleavage of gasdermin D (GSDMD) (218, 219), but activation of GSDMD has not been reported in *S. pyogenes*-infected monocytes/macrophages. GSDMD is a member of the gasdermin family, which regulates epithelial cell proliferation and acts as a tumor suppressor (220). Upon cleavage of cytoplasmic GSDMD at Asp275, its N-terminal portion translocates and binds to phospholipids of the cell membrane as well as cardiolipin of mitochondria (221) (Fig. 1). Multimeric GSDMD-N then assembles in the membrane in the form of pores, allowing the efflux of IL-1β as well as an influx of water, resulting in cell swelling and eventual rupture and the release of cytosolic contents (222, 223). The extracellular release of the GSDMD-N may also bind to the outer membrane lipid of extracellular bacteria, causing extracellular *in trans* bactericidal activity, but without harming neighboring mammalian cells. The lack of plasma membrane disruption is the result of lipid-binding specificity. GSDMD does not bind to nonphosphorylated phosphatidylinositol, phosphatidylethanolamine, or phosphatidylcholine, found on the inner leaflet of the cell membrane (224).

S. PYOGENES-MEDIATED AUTOPHAGY (XENOPHAGY) AND AUTOPHAGIC SIGNALING

The group A *Streptococcus* has evolved with several strategies for its perpetual existence as a successful pathogen. Like many intracellular pathogens (225–229), *S. pyogenes* also survives and often multiplies intracellularly, especially within macrophages (230). Intracellular *S. pyogenes* is either killed by the endosomal vacuole content of the host cell or survives and causes cell death. Autophagy is a cellular homeostatic mechanism by which cytoplasmic contents are engulfed by a characteristic double-membrane autophagosome. The autophagosomes subsequently fuse with lysosomes (autophagolysosome), and the contents are eventually degraded and recycled in an orderly fashion. Autophagy thus is a homeostasis mechanism that contributes significantly to the innate immune system (231).

The role of autophagy in *S. pyogenes* infection was first demonstrated in HeLa cells infected with strain JRS4, an M6 *S. pyogenes* (232). This report revealed the temporal increases in the colocalization of *S. pyogenes* with the LC3 protein and lysosome-associated membrane protein 1, a marker for autophagosomal vacuoles and vacuolar membrane proteins. Autophagy also is known as xenophagy (autophagy of infectious particles), which also involves membrane traffic and fusion. Further kinetic studies of M6 *S. pyogenes* strain-mediated autophagy also showed that Rab GTPases/Rab proteins localize at distinct intracellular membranes and vesicles and function as GTP-bound "on" or GDP-bound "off" forms. During the membrane fusion process, Rab proteins (GTPase) play a significant role in membrane trafficking and autophagosome formation in *S. pyogenes*-infected HeLa cells (233). Among many Rab GTPases, the autophagosome-associated Rab5 and Rab7 determine *S. pyogenes* invasion, endosome fusion, and intracellular fate. Additional Rab GTPase regulatory proteins, Rab9a, Rab23, and Rab17, have also been shown to participate in the later stages of *S. pyogenes*-containing vacuolar enlargement, lysosomal fusion, and membrane recycling (233, 234) (Fig. 2). The fusion of recycling endosomes with *S. pyogenes*-containing autophagosome vacuoles was recently revealed to be mediated by the SNARE (soluble *N*-ethyl-maleimide-sensitive factor attachment protein) receptor complex formed by STX6 and VTI1B (vesicle transport through interaction with t-SNAREs homolog 1B)-VAMP3 (vesicle-associated membrane protein 3) proteins (235). Beclin 1, another protein encoded by the BECN1 gene, is a mammalian ortholog of the yeast autophagy protein, Atg6, which plays a critical role in autophagy and cell death (236). Upon engagement of pathogen receptor CD46 with *S. pyogenes* (M types 6 and 49), possibly via the M protein (79), Beclin1/VPS34-mediated autophagic killing occurs (237). The possible pathway leading to autophagy likely occurred through Cyt-1/GOPC signaling (237). GOPC is a trans-Golgi-associated protein that interacts with its single PDZ domain (post synaptic density protein [PSD95], Drosophila disc large tumor suppressor [Dlg1], and zonula occludens-1 protein [zo-1]) with a variety of cell surface receptors, such as CD46, that possess a cytoplasmic tail containing tyrosine residues (Cyt-1) (238). Recently, the mechanistic role of Beclin-1 in *S. pyogenes*-mediated autophagic killing was defined more clearly. In *S. pyogenes*-infected cells, the antiapoptotic prosurvival proteins Bcl-2 and Bcl-xL as well as class III PI3 kinase directly interact with Beclin-1 and suppress the Beclin-1-regulated autophagy (239). Beclin-1, on the other hand, binds to the UV resistance-associated gene protein (UVRAG) and ATG14L, which together stimulate autophagy (240). UVRAG also interacts with the vacuolar protein complex (VPS11, VPS16, and VPS18) and facilitates the activation of Rab7- and Rab5-tagged vacuoles during maturation of endosomes and autophagosomes (241). Upon endocytosis of *S. pyogenes* into epithelial cells, Bcl-xL inhibits *S. pyogenes*-induced autophagy directly by suppressing autophagosome-lysosome fusion and indirectly by suppressing internalization of *S. pyogenes* through interaction with Beclin-UVRAG (242). NLRP4 also has been shown to directly interact with Beclin1 via the VPS-UVRAG complex (243). The association of *S. pyogenes* with autophagosomal vacuoles and the autophagic process increases when NLRP4 but not NLRC4 is knocked down (243). Interaction of NLRP4 in *S. pyogenes*-infected cells revealed that NLRP4 is accumulated in *S. pyogenes*-containing phagosomes and transiently dissociates from Beclin 1 after *S. pyogenes* infection and thus modulates

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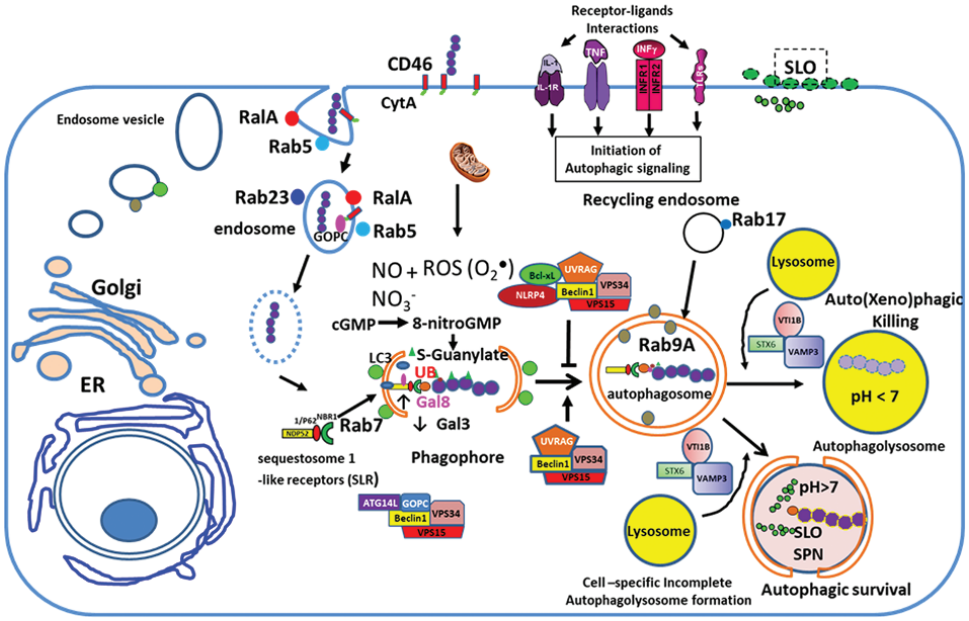


FIGURE 2 Intracellular signaling associated with autophagy/xenophagy of *S. pyogenes*. *S. pyogenes* invades host epithelial cells by one of two mechanisms, invagination at the point of bacterial contact with host cells or massive induction of microvilli, which form membrane ruffling for engulfment of bacteria (filopodia). Cytoskeletal rearrangements involve induction of the PI-3 kinase pathway or RAS/CDC42(Rat Sarcoma oncogene receptor family small GTPases/cell division control protein 42)/tyrosine kinase activation. Autophagy (self-eating) or xenophagy (eating of a nonself particle, e.g., bacteria) is controlled by Ser/Thr kinases and by the lipid kinase activity of PI-3 kinase and ATG14-like protein (ATG14L). Initial entry of *S. pyogenes* happens through endocytosis, which is tightly regulated by several Rab GTPases. Typical donors of endosome membranes are endoplasmic reticulum, Golgi apparatus, mitochondrion, and plasma membrane. For physiological autophagy Rab1, 5, 7, 24, and 33B act in a regulated fashion to form autophagolysosome, which is the stepwise culmination of the fusion of a double-membrane phagophore with endosome with *S. pyogenes* (phagosome) to form autophagosome followed by fusion of a double-membrane autophagosome with a lysosome. Upon endocytosis of *S. pyogenes*, the infection-specific Rab GTPases Rab23, Rab7, Rab9a, and Rab17 act in a sequential manner for the initiation, elongation/closure, maturation, and maintenance of autophagic/xenophagic vacuole to degrade intracellular *S. pyogenes*. *S. pyogenes* is killed only when autophagosome is fused with a lysosome. During the initial phase of *S. pyogenes* autophagy, the lipid kinases VPS34, Beclin1, GOPC (Golgi-associated PDZ and coiled-coil motif-containing protein), and ATG14L are involved in forming phagophore. Subsequently, ATG14L is replaced with UVRAG (UV resistance-associated gene protein) in the VPS34-Beclin1 complex to form autophagosome. During the final maturation of autophagosome containing *S. pyogenes* to autophagolysosome, the SNARE protein STX6, VTI1B, and VAMP3 are involved. NLRP4 and Bcl-XL serve as a negative regulator of the autophagy of *S. pyogenes*. Additional factors that are involved in the autophagy of *S. pyogenes* are a sequestosome-1-like protein complex constituted by nuclear dot protein (NDP52), neighbor of BRCA1 gene 1 protein, and 1/P62 proteins that decorate the inner membrane of phagophore via binding to the LC3 protein. One of these SLR proteins, NDP52, directly interacts with E3 ligase, which ubiquitinates bacteria. Galectin 8 also plays an important role in the attachment of SLR with which ubiquitinated *S. pyogenes* is associated. Phosphorylated SLR protein then multimerizes within phagophore and matures into autophagosome and retains *S. pyogenes* within the autophagosomes. In keratinocytes infected with *S. pyogenes*, autophagosomes do not mature to autophagolysosome, possibly because of the secretion of SLO/NADase, while in some cells, such as HeLa and HEP-2 cells, complete autophagolysosome occurs and *S. pyogenes* is killed. Incomplete formation of autophagolysosome remains defective in acidic pH, which does not allow *S. pyogenes* to be killed. A failure to maintain low pH may differentially regulate virulence gene expression, which in turn may affect bacterial survival. In response to *S. pyogenes* entry, host cell systems also generate second messenger cyclic GMP and nitric oxide as well as ROS. Oxygen radical and nitric oxide together may form reactive peroxynitrite, which may then target cysteine residues of the *S. pyogenes* surface protein to S-guanylate *S. pyogenes*. As indicated, only S-guanylated *S. pyogenes* is ubiquitinated and subsequently subjected to xenophagic killing. Endothelial cells are defective in the S-guanylation process. Endothelial cells also express relatively less specific galectin-8 than galectin-3, which results in abrogation of xenophagic killing and intracellular multiplication of *S. pyogenes*. Xenophagic killing and survival of *S. pyogenes* are strain specific (with variable SpeB expression and related protease activity, which degrades ubiquitin and SLR protein complex) and cell type specific (normal versus established cancer cell line, epithelial, macrophage versus endothelial).

the autophagic bactericidal process (243). Cumulative studies have revealed that in HeLa cells, autophagy plays an important role in the intracellular degradation of type M6 *S. pyogenes*, and a number of autophagy-associated proteins dynamically play a role as well.

However, in contrast to the M6 *S. pyogenes* strain JRS4, which is defective in SpeB production, an M1T1 *S. pyogenes* strain, which produces SpeB, has been shown to survive and multiply within the host cells (Hep-2 cells) and avoid autophagy and endosomal degradation (244). This observation is attributed to the ability of the M1T1 strain to avoid the ubiquitination process of the autophagy protein. For selective autophagy, ubiquitination of *S. pyogenes* via LC3-adaptor proteins, p62, neighbor of BRCA1 gene 1 protein, NDP52

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(sequestosome 1/p62-like receptors, or SLRs), and recognition by the autophagy marker LC3 is a critical step (245). SpeB degrades host ubiquitin-LC3-adaptor proteins and ubiquitinated proteins of the host cells (244). Thus, the M1T1 mutant lacking SpeB that is associated with ubiquitin-LC3-adaptor protein and found in autophagosomes is killed similarly to the JRS4 strain (244). Likewise, the JRS4 M6 *S. pyogenes* strain complemented with the *speB* gene behaves like the M1T1 *S. pyogenes* strain and avoids autophagy (244). The role of SLO in the autophagic killing of *S. pyogenes* by HeLa cells (232), keratinocytes (246), or endothelial cells (247, 248) is continuously evolving. While earlier reports (232) indicated that the autophagic killing in HeLa cells is dependent on SLO, results obtained using keratinocytes (buccal palate-derived primary cells) infected with unencapsulated M3 and JRS4 M6 strains and their isogenic mutants lacking SLO, SLS, and NAD glycohydrolase (NADase) have revealed that SLO enhances *S. pyogenes* survival (246).

The survival of S. *pyogenes* within autophagosomes is also dependent on the intracellular pH of the cell. Low pH quickly suppresses the growth of pathogens, including *S. pyogenes*. In a low-pH environment, the expression of many virulence factors (SLO and SLS) is significantly downregulated, and at high pH the expression of SpeB is upregulated (249). Thus, although SpeB-defective M6, M49, and M89 strains are efficiently targeted by autophagy and effectively cleared by epithelial cells, they do multiply within autophagosomes of human microvascular endothelial cells (HMEC-1) that are defective in acidification (247). It seems that survival and elimination of *S. pyogenes* within autophagosomes is more relevant to the pH environment than is the ability of bacteria to express SpeB (247). SpeB contributes only moderately (~25%) to the survival and growth of *S. pyogenes* within host cells. Even increasing the pH of endothelial cells or using an *S. pyogenes* mutant lacking SpeB does not alter the growth (247). *S. pyogenes* streptolysin-O or NAD glycohydrolase (NADase) prevents phagolysosome acidification and thus enhances *S. pyogenes* survival (250).

A recent report attributed the differences in the *S. pyogenes* multiplication patterns in epithelial cells and those in endothelial cells to the relative presence of galectin-3 in these cells (251). Galectins are beta-galactosidase-binding proteins. GAL-8 and GAL-3 have been identified as two of the proteins associated with invasive bacteria (i.e., *Salmonella* spp. and *Neisseria meningitis*) for autophagy targeting (252, 253). Although the direct role of galectin in autophagy is unknown, it has been found that higher expression of Gal-3 and lower expression of Gal-8 in endothelial cells compared to epithelial cells may contribute to the observed differential *S. pyogenes* multiplication (251). Gal-3 also promotes *S. pyogenes* replication and suppresses the recruitment of Gal-8 and ubiquitin. This study also revealed that upon knockdown of Gal3 in endothelial cells, the colocalization of Gal8, ubiquitin E3-ligase (parkin), and ubiquitin-decorated *S. pyogenes* are significantly increased. Similarly, upon inhibition of Gal-8 in epithelial cells, recruitment of parkin, ubiquitin, and *S. pyogenes* elimination are severely affected.

The activation of inflammasomes invariably generates ROS that activate autophagy (254–256). Like ROS, nitric oxide (NO) is a short-lived reactive species that plays a significant role in cell signaling (257). Superoxide and NO together form peroxynitrite, which nitrosylates many molecules. ROS/NO-mediated nitrosylation of cGMP (8-nitro-cGMP) has been found to be an inducer of autophagy (258). In particular, in the presence of this endogenous nitrated nucleotide, selective clearance of invasive *S. pyogenes* occurs via polyubiquitylation. The Lys-63-linked polyubiquitination has been found to be targeted specifically to S-guanylated *S. pyogenes*, which is internalized into autophagosomes and subsequently eliminated. Interestingly, one of the guanylated and ubiquitinated surface proteins of intracellular *S. pyogenes* is SDH (85, 258). The increased multiplication of *S. pyogenes* within endothelial cells compared to epithelial cells is also attributed to its intrinsically defective xenophagic mechanism (248). In particular, endothelial cells, despite being capable of inducing autophagy (i.e., association with the autophagosome LC3 marker) upon *S. pyogenes* endocytosis, are unable to form double-membrane structures, a crucial step required for xenophagy. Further, endothelial cells are not efficient enough to ubiquitinate *S. pyogenes* because of poor recruitment of Atg proteins as well as in the induction and production of 8-nitro-GMP as described above (248). Together, these findings indicate that NOS-mediated posttranslational modification and ubiquitination of *S. pyogenes* within the host cell plays a pivotal role in pathophysiology associated with xenophagy (Fig. 2).

In the host cell, mono- and poly-ubiquitylated proteins are deubiquitinated by CYLD (cylindromatosis) deubiquitinases (259–261). Normal cells express CYLD in much higher quantities than cancer cells or established cell lines. Hence, bacteria may remain less ubiquitinated. SLS mediates xenophagic recognition of *S. pyogenes* in keratinocytes via galectin-8 but in a ubiquitin-independent manner (246). It is not known whether, in addition to the SLS activity of *S. pyogenes*, the increased quantity of CYLD also plays a role in the lack of association of *S. pyogenes* with ubiquitin. The regulatory role of CYLD-mediated deubiquitination in protection against lethal infection by *S. pneumoniae* (262) and *Haemophilus influenzae* (263) and other pathogens (262, 264) is now well established. Whether CYLD-mediated modulation of posttranslational modification plays a role in *S. pyogenes* needs further verification. Thus, the observed differences in autophagy-mediated intracellular killing can be attributed to the intracellular expression levels of deubiquitinase enzymes, in addition to the other factors described above.

GROUP A *STREPTOCOCCUS*-MEDIATED APOPTOSIS AND APOPTOTIC SIGNALING

Streptococci can also induce apoptosis, or programmed cell death (265, 266). Apoptosis is crucial for proper organismal development, maintenance of cell number homeostasis, and elimination of diseased or otherwise harmful cells (267, 268). Apoptotic cell death in response to bacterial infection is believed to delete infected and damaged epithelial cells and to restore epithelial integrity, which is distorted during infection (269). Thus, apoptosis may contribute to the resolution of bacterial infection. The ability of host cells to undergo apoptosis is considered the host defense strategy to keep the pathogen at a disadvantage by not providing a target for adherence, invasion, and subsequent proliferation. However, the ability of the bacterium to direct the host cell to undergo apoptotic cell death, thus allowing it to escape from phagocytic activity, may be an important virulence factor. Apoptosis of few epithelial cells directed by bacteria not only helps these bacteria escape from one of the host's lines of defense, but it also facilitates penetration to the next cellular barrier. Tsai et al. (265) and Nakagawa et al. (266) showed that M type 1, 6, and 49 *S. pyogenes* strains induce apoptosis in 10 to 20% of the upper and lower respiratory cell lines, HEp-2 laryngeal and A-549 lung alveolar, respectively. In particular, the cells with internalized bacteria show increased expression

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of the Bax protein, which translocates to mitochondria (266), resulting in cytochrome C release. At the same time, the expression of prosurvival gene products, such as Bcl-2 and Bcl-X_L, is significantly decreased in mitochondria (Fig. 3). Caspase-9 activation and inhibition of apoptosis of these cells in the presence of caspase inhibitors indicate that caspase activation is likely an important mediator which triggers apoptosis (265, 266).

SpeB may play a direct role in causing this limited apoptosis by activating matrix metalloproteinase and release of first apoptosis signaling and TNF-α (265). Another study showed that NAD+-glycohydrolase of an M type-3 clinical isolate gains access to the keratinocyte cytoplasm by enhancing SLO-dependent damage of keratinocyte cell membranes (270). Once inside the cell, NAD+ glycohydrolase inhibits *S. pyogenes* internalization by keratinocytes and

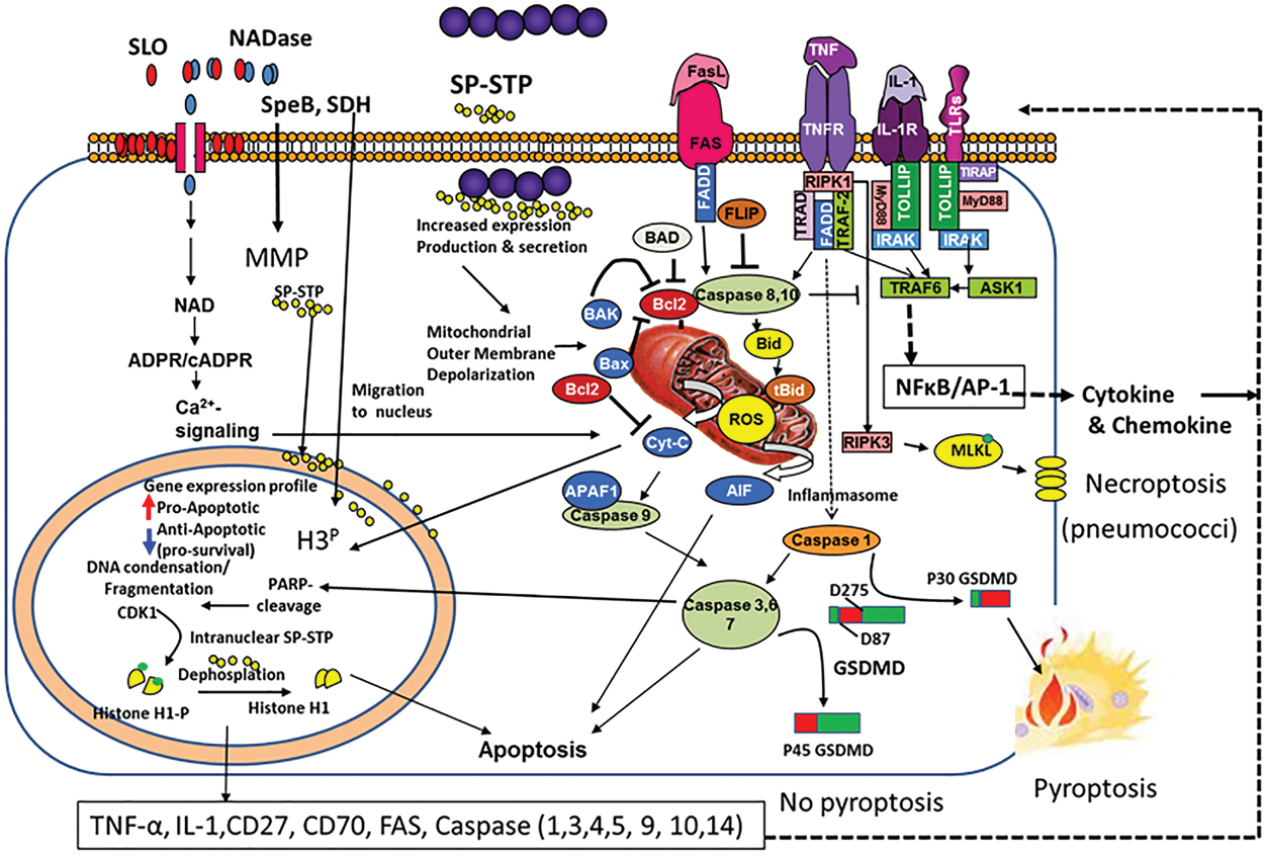


FIGURE 3 Cell-signaling events that regulate *S. pyogenes*-mediated programmed cell death. *S. pyogenes* infection-mediated cell death of the host cells depends on the cell type and is triggered extracellularly and intracellularly as shown. Epithelial cells infected with *S. pyogenes* undergo apoptotic cell death characterized by DNA fragmentation, cytochrome release, BAX (BCL2-Associated X apoptosis regulator protein) translocation to mitochondria, and inhibition of BcL2, Bcl-XL (B-cell lymphoma-extra large). *S. pyogenes*-induced apoptosis is also characterized by increased expression of IL-1β; caspase-1, -9, -3, -4, -5, -6, and -14; cytochrome C oxidase; and protein involved in Ca^{++}-signaling. At least five purified proteins have been shown to contribute to *S. pyogenes*-mediated apoptosis of host cells. SpeB causes apoptosis of Hep2-cells by activating matrix metalloproteinase (MMP) and induction of first apoptosis signal molecule (FAS) and TNF-α. SLO facilitates *S. pyogenes*-mediated apoptosis by allowing NADase into the cell and cleaving NAD to ADP-ribose (ADPR) and possibly cyclic ADPR, which in turn activates calcium signaling, and mitochondrial membrane depolarization, releases reactive oxygen radical, IL-1β, and activates caspases in human and mouse macrophages. SDH and SP-STP induce apoptosis of human respiratory epithelial cells (Detroit 562 and A549) in part by inducing nuclear condensation via histone H3 phosphorylation and/or H1 dephosphorylation and by upregulating mRNA expression of several proapoptotic genes and downregulating prosurvival genes. SP-STP induces caspase-4 and caspase-5, crosses two membrane barriers, and transmigrates to the nucleus, where it dephosphorylates CDK1 phosphorylated histone H1 protein. SP-STP induces apoptosis both extracellularly and intracellularly. SP-STP expression increases several-fold in intracellular *S. pyogenes* and is secreted in the cytoplasm and causes apoptosis possibly by modulating several intracellular signaling events within the cytoplasm and in the nucleus. Apoptosis and pyroptosis are both modulated by GSDMD. Caspase-3 activation inhibits pyroptosis, while caspase-1-mediated cleavage of GSDMD results in P30 GSDMD that multimerizes in the form of pore in the membrane that causes pyroptosis (see Fig. 1). GSDMD P45 resulting from the cleavage activity of caspase-3 is inactive and prevents pyroptosis but allows apoptosis. GSDMD is ultimately responsible for pyroptosis. Caspase-8 inhibition can lead to receptor-interacting serine/threonine-protein kinase 1 (RIPK1)-dependent activation of RIPK3, which upon phosphorylation of mixed-lineage kinase domain-like pseudokinase (MLKL), leads to necroptosis, the phenomenon which is observed in pneumococcus-infected or purified pneumolysin-treated eukaryotic respiratory cells and human cardiomyocytes.

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together with SLO induces apoptosis (270, 271). The precise signaling events are not known, but it is postulated that NAD+ glycohydrolase may enzymatically hydrolyze NAD, resulting in the accumulation of nicotinamide, ADP-ribose (ADPR). The cyclic ADPR may stimulate Ca^{2+} signaling pathways, including those leading to apoptosis (270–272). SLO alone can cause apoptosis of murine macrophage, possibly through mitochondrial membrane depolarization and the disruption of phagolysosomal membrane, resulting in uninterrupted *S. pyogenes* multiplication and eventual caspase-1 dependent apoptosis (273). Global transcriptional regulation in neutrophils after phagocytosis of *Borrelia hermsii*, *L. monocytogenes*, *Burkholderia cepacia*, *S. aureus*, and *S. pyogenes* has shown that *S. pyogenes* induces unique changes in neutrophil gene expression (274). More specifically, phagocytosis of *S. pyogenes* results in up- and downregulation of 393 genes (168 upregulated, 225 downregulated), 71 of which are apoptosis-related genes that include upregulation of AP-1, FOSL1, FOSB, JUNB, and TNFRSF5 (CD40), and downregulation of at least 26 genes involved in the response to IFN. The downregulated genes include IFN-responsive genes (IFIT1, IFIT4, IFITM2, IFI16, IFN regulatory factor-1, MX1, and ADAR) and transcription factor-related genes (STAT1, STAT3, STAT5B, STAT6, JAK1, RELA, and RELB) representing the JAK-STAT survival pathway. Interaction of streptococcal SDH with human pharyngeal cells (Detroit 562) resulting in nuclear condensation indicates that SDH may play a role in *S. pyogenes*-mediated apoptosis (89, 275). Interaction of SDH with pharyngeal cells also induced *de novo* phosphorylation of histone H3 protein. Phosphorylation of H3 plays a decisive role in transcription regulation during growth and development (276–278). *S. pyogenes* interaction mediated H3 phosphorylation, and nuclear condensation is an important marker of cells undergoing apoptosis (275, 279). An application-targeted gene array study showing the upregulation of proapoptotic genes and downregulation of important anti-apoptotic genes along with 30 to 40% of apoptosis in human pharyngeal cells after 12 h coculturing with SDH indicated that it significantly contributes to the *S. pyogenes*-mediated apoptosis of pharyngeal cells (280). Eukaryotic GAPDH is known to localize into the nuclear compartment and cause apoptosis (281–283). GAPDH of group B *Streptococcus* (*S. agalactiae)* causes apoptosis of murine macrophages (284).

Recently, SP-STP, a novel virulence factor, has been found to cause apoptosis of human respiratory cells (13). SP-STP is secreted in the *S. pyogenes* culture supernatant and is highly expressed in the intracellular *S. pyogenes* population of Detroit cells infected with *S. pyogenes*. SP-STP shares significant homology with eukaryotic PP2C (Ser/Thr phosphatase), which is known to promote apoptosis of neuronal and endothelial cells (13). Purified SP-STP has also been found to cause apoptosis of human respiratory epithelial cells when exogenously added or when ectopically expressed within the cytoplasm. Enzymatically active STP from *Enterococcus faecalis* is, however, unable to cause apoptosis when added exogenously and cocultured with respiratory epithelial cells. Interestingly, enzymatically active STP of *E. faecalis* and enzymatically inactive SP-STP have a similar tertiary structure based on circular dichroism (CD)-spectra analysis (13). Enzymatically inactive SP-STP is unable to cause apoptosis, indicating that SP-STP enters the cell in a receptor-specific manner. Ectopically expressed SP-STP causes apoptosis in >95% of epithelial cells within 12 h of incubation. Microarray and reverse transcription-quantitative PCR analyses of SP-STP-treated human pharyngeal cell lines have revealed upregulation of several proapoptotic genes (TNF-α; IL-8; caspase-1, -3, -4, -5, and -9; GADD45a [growth arrest and DNA-damage-inducible protein]; CD40; and CD27) and downregulation of antiapoptotic prosurvival genes (BCl2, BCLxL, MCL-1). Exogenously added or ectopically expressed SP-STP also has been shown to transmigrate to the nucleus and dephosphorylate CDK-phosphorylated H1. Transmigration of SP-STP in the nucleus results in apoptosis of the cell. Dephosphorylation of the phosphorylated histone H1 is known to be associated with cell apoptosis, indicating that SP-STP causes apoptosis through a novel mechanism (285). Together, these studies indicated that host cell death caused by *S. pyogenes* occurs through various mechanisms (Fig. 3).

During pneumococcal meningitis, neuronal injury arises indirectly from the bacteria and excessive immune response by the host. In experimental meningitis, concentrations of several caspases are significantly elevated, and active caspase-3 is present in the dentate gyrus (286). However, intrathecal treatment with broad-spectrum caspase inhibitor rescues only around 50% of the neurons of experimental mice with acute pneumococcal meningitis (287). In a microglial neuronal cell tissue culture system, pneumococcal infection results in rapid damage of mitochondria followed by the release of cytochrome C and apoptosis-inducing factor (288). Anti-apoptosis-inducing factor antibody also inhibits apoptosis in a significant number of cells infected with pneumococci, indicating that neuronal damage during pneumococcal meningitis occurs both as caspase-dependent and caspase-independent (mitochondrial pathway) manners (287, 288). Unlike in neuronal cells, pneumococci induce apoptosis of human alveolar and broncho-epithelial cells by caspase-6 and noncaspase protease but not by caspase-3 (289). *S. pneumoniae* also translocates into the myocardium and causes necroptosis via the RIPK1/RIPK3/MLKL signaling pathway, which is activated as a result of the suppression or downregulation of caspase-8 (290–293). *S. pneumoniae* strains that cause low activation of NF-κB in macrophage have been shown to cause severe pneumonia associated with necroptosis (294, 295).

The term "apoptosis" is defined essentially based on the morphology. As the biochemical changes occurring during this event leading to cell death have become evident, they have been differentially and appropriately recognized with corresponding differential microscopic morphological characteristics. Inflammasome-associated cell death (pyroptosis) and apoptosis are distinct yet related phenomena and engage in bidirectional cross-talk in macrophages (296). Active caspase-1, -4, and -5 typically cleave GSDMD at the Asp 275 residue, releasing p30 GSDMD, which causes rapid cell death or pyroptosis. However, the active caspase-1-activated caspase-3 and caspase-7 cleave GSDMD at Asp87, rendering it inactive, which prevents pyroptosis and causes apoptosis (296). Thus, pyroptotic stimuli can activate apoptosis, but apoptotic stimuli inactivate pyroptosis (Fig. 3). A recent study showed that DPP8/9 (postproline cleaving serine protease) inhibition by Val-boroPro (Talabostat-PT-100) stimulates the mammalian immune system and causes pro-caspase-1-dependent pyroptosis of macrophages and monocytes (297). Interestingly, this inhibitor does not cleave apoptotic caspase-3 or caspase-7 and renders GSDMD uncleaved, yet causes pyroptosis and not apoptosis in an active pro-caspase-1-dependent manner (297). Inflammasomes can also cause GSDMD-independent secondary pyroptosis and IL-1 release even in the absence of caspase-1 protease activity (298).

In conclusion, *S. pyogenes* manipulates the host cell to survive, proliferate, and spread to other cells, and the

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programmed cell death (noninflammatory autophagic, apoptotic, and inflammatory pyroptotic, and necroptotic) process controls pathogen replication and host inflammation (299). However, recent studies using transgenic mice overexpressing the prosurvival Bcl2 protein found minimal impact on the clearance of *S. pyogenes* and *Legionella* during *in vitro* bone-marrow-derived macrophage infection and *in vivo* intranasal/subcutaneous infection (300). Hence, it is rational to conclude that the most important role of programmed cell death during *S. pyogenes* infection is to minimize inflammation and increase host resilience and not to help clear or prevent *S. pyogenes* proliferation (300).

CONCLUDING REMARKS

The direct and indirect evidence of the consequences of streptococcal interaction with target epithelial cells suggest that streptococcal adherence is not merely a static process but is highly regulated, dynamic, and complex. The foundation of this dynamic process is intracellular signaling events that ultimately determine the fate of the targeted cell and subsequently that of adjacent cells. While together, these events allow streptococci to disseminate in tissues, the biochemical changes resulting from cascades of signaling events in affected cells determine the clinical picture of streptococcal disease. Thus, *S. pyogenes* and other streptococcus-mediated intracellular signaling events play a critical role in the disease process and outcomes.

The study of *S. pyogenes*-mediated signaling events is so far based on established and known cell lines *in vitro*, and hence such studies may not provide an answer to the severity of *S. pyogenes* infections *in vivo*. Limited attention has been given to the genetic basis of populations that are prone to develop frequent and severe *S. pyogenes* infections. Given the easily accessible and powerful CRISPR/CAS9, genome sequencing technologies, and the human genome database, the future holds promise to develop *in vitro* and *in vivo* infection models reflecting the targeted population to help us understand with precision why certain populations are more susceptible than others to *S. pyogenes* infection. Such studies may help develop novel host-directed therapeutics. Therapeutic targeting of the relevant host cell signaling events may hold promise to counteract severe, invasive, and often fatal *S. pyogenes* diseases more effectively. Approaches to reprogramming cellular functions have already been implemented for many septic shock and inflammatory diseases, despite persistent obstacles (301–306).

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Vaccine Approaches To Protect against Group A Streptococcal Pharyngitis

VINCENT A. FISCHETTI

10

INTRODUCTION

Streptococcus pyogenes (Lancefield group A) is a human pathogen responsible for a wide range of diseases, the most common of which are nasopharyngeal infections (or strep throat) and impetigo. More than 25 million cases of group A streptococcal infections occur each year in the United States at a cost of over $1 billion to the public in addition to losses in productivity. In recent years, an increase in streptococcal toxic shock and invasive infections (particularly, necrotizing fasciitis) has been reported with certain strains of group A streptococci, resulting in rapid fatalities in up to 30% of the cases (1, 2). Since there have been no reports of penicillin resistance in group A streptococci, streptococci-related diseases can be successfully treated with this antibiotic; however, with erythromycin, the second drug of choice for these bacteria, resistance is currently observed. Before treatment, group A streptococcal infections are usually associated with fever, significant discomfort, and generalized lethargy mostly due to the wide range of toxic substances secreted. About 3% of individuals with untreated or inadequately treated streptococcal pharyngitis develop rheumatic fever and rheumatic heart disease, a sequela of the streptococcal infection resulting in cardiac damage, particularly of the mitral valve. While in the United States outbreaks of rheumatic fever are usually sporadic and infrequent, affecting only local areas of the country (3), in developing countries as many as 1% of school-age children are estimated to have rheumatic heart disease (4). Because of this, and the concern that penicillin-resistant strains may appear, there is a strong incentive to develop a safe and effective vaccine against group A streptococcal pharyngitis.

Although more prevalent during the winter months, at any given time up to 30% of asymptomatic humans carry group A streptococci in their pharynx. Except under unusual circumstances and one serotype, there is no animal reservoir for these organisms. Thus, the eradication or significant reduction in the carriage of group A streptococci in the human pharynx would have a profound effect on the dissemination of this organism in the population and on the initiation of streptococcal disease. Even in the case of the highly invasive strains of streptococci, there is no evidence that they have their origins in other than pharyngeal sources.

Lancefield (5) showed more than 50 years ago that the surface M protein would be a prime candidate for a vaccine to protect against streptococcal infection. However, we have learned since that time that protection incurred by serum immunoglobulin G (IgG) to the M molecule is type specific, and antibodies directed to the N-terminal type-specific region induce protection against only that M type among the >250 M protein types (refer to the CDC website: https://www.cdc.gov/streplab/groupa-strep/index.html). More recent studies have revealed that broad protection against streptococcal infection may be achieved by the induction of a local secretory response to exposed conserved sequences found within the M molecule. The approach prevents streptococcal colonization of the upper respiratory mucosa in a mouse model.

It has been known for decades that patients with rheumatic fever have serum IgG to human smooth muscle tissue at levels three to four times that found in normal serum. While these antibodies have also been found to react with streptococcal cell wall, membrane, and M protein N-terminal determinants, their role, if any, in the pathogenesis of rheumatic fever has not yet been proven. The fact that rheumatic fever and further cardiac damage occur around the time of the streptococcal infection, and not between infections when cross-reactive antibodies are still elevated (6), argues against the direct involvement of these antibodies in the disease process. Despite this, and until proved otherwise, it will be important to minimize the induction of cross-reactive antibodies in streptococcal vaccine preparations. This article concentrates on the progress to date toward the development of a vaccine to protect against streptococcal nasopharyngeal infection. For a more detailed review of the subject see reference 7.

M PROTEIN STRUCTURE AND FUNCTION

Protective immunity to group A streptococcal infection is achieved through antibodies directed to the M protein (8), a major virulence factor present on the surface of all clinical isolates. M protein is a coiled-coil fibrillar protein composed of three major segments of tandem repeat sequences that extends nearly 60 nm from the surface of the streptococcal cell wall (8) (Fig. 1). The A- and B-repeats located within the N-terminal half are antigenically variable among the >250 known M protein types, with the N-terminal nonrepetitive region and A-repeats exhibiting hypervariability. The more C-terminal C-repeats, the majority of which are surface

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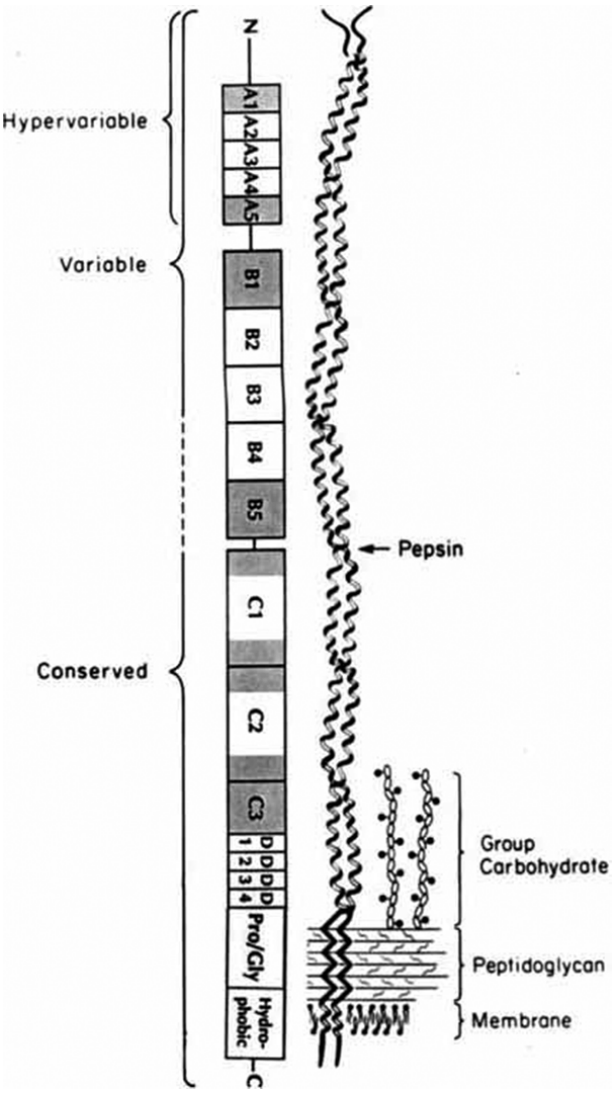


FIGURE 1 Proposed model of the M protein from M6 strain D471 (29, 85). The coiled-coil rod region extends about 60 nm from the cell wall with a short nonhelical domain at the NH2 terminus. The Pro/Gly-rich region of the molecule is found within the peptidoglycan (86). The membrane-spanning segment is composed of predominantly hydrophobic amino acids, and a short charged tail extends into the cytoplasm. Data suggest that the membrane anchor may be cleaved shortly after synthesis (86). The A-, B-, and C-repeat regions are indicated along with those segments containing conserved, variable, and hypervariable epitopes among heterologous M serotypes. Pepsin designates the position of a pepsin-susceptible site near the center of the molecule.

exposed, contain epitopes that are highly conserved among the identified M proteins (9). Because of its antigenically variable N-terminal region, the M protein provides the basis for the Lancefield serological typing scheme for group A streptococci, the M serotype (8), and a more contemporary molecular approach to typing group A streptococci, the *emm* type (10), referring to the sequence of the first 80 or so amino acids at the N-terminus of the M protein gene.

The M protein is considered the major virulence determinant because of its ability to prevent phagocytosis when present on the streptococcal surface and thus, by this definition, all clinical isolates express M protein. This function may in part be attributed to the specific binding of complement factor H to both the conserved C-repeat domain and the fibrinogen bound to the B-repeats (11), preventing the deposition of C3b on the streptococcal surface. It is proposed that when the streptococcus contacts serum, the factor H bound to the M molecule inhibits or reverses the formation of C3b,Bb complexes and helps to convert C3b to its inactive form (iC3b) on the bacterial surface, preventing C3b-dependent phagocytosis. Studies have shown that antibodies directed to the B- and C-repeat regions of the M protein are unable to promote phagocytosis. This may be the result of the ability of factor H to also control the binding of C3b to the Fc receptors on these antibodies, resulting in inefficient phagocytosis. Antibodies directed to the hypervariable N-terminal region are opsonic, perhaps because they cannot be controlled by the factor H bound to the B- and C-repeat regions. Thus, it is possible that through evolution the streptococcus has devised a method to protect its conserved region from being used against itself by binding factor H to regulate the potentially opsonic antibodies that bind to these regions.

TYPE-SPECIFIC PROTECTION

The M protein has been a prime vaccine candidate to prevent group A streptococcal infections since Lancefield showed clearly that M protein-specific human and animal antibodies have the capacity to opsonize streptococci in preparation for phagocytic clearance. In general, serum IgG directed to the hypervariable amino-terminal portion of M protein leads to complement fixation and phagocytosis by polymorphonuclear leukocytes of streptococci of the homologous serotype (12). Antibodies directed to other antigens on the surface of group A streptococci do not allow for phagocytosis, suggesting that besides the M protein, no other streptococcal surface antigen is able to induce antibodies to override the antiphagocytic effect of the M protein. Fox (13) has reviewed the early attempts at M protein vaccine development. Since the early 1970s few human trials have been realized (14, 15). This is partially because of problems with hypersensitivity reactions found with the acid-extracted M protein preparations of the time and the fact that only type-specific protection was observed. In addition, repeated attempts to separate heterologous protein contaminants from the type-specific determinants proved unsuccessful.

In 1979, Beachey et al. (16) used pepsin-extracted M24 protein (PepM24, the N-terminal half of the native M24 molecule) (Fig. 1) to immunize human volunteers. Unlike earlier acid-extracted products (13), this highly purified fragment was found to be free of non-type-specific reactivity and did not induce delayed-type hypersensitivity tests in the skin of the 37 adult volunteers. Immunization with alum-precipitated PepM24 protein led to the development of type-specific opsonic antibodies in 10 of 12 volunteers, none of whom developed heart-reactive antibodies as determined by immunofluorescence. These studies clearly indicated that M protein vaccines free of sensitizing antigens could be produced but further emphasized the type specificity of the immune response.

Using these studies as a starting point, Beachey and coworkers (17) began to develop a type-specific epitope-based vaccine strategy to protect against streptococcal disease (for a recent review see 18). It was soon learned that the complete PepM24 fragment was not necessary, but peptides representing the first 20 or so N-terminal amino acids of the M24

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protein also evoked type-specific opsonic antibodies to M24 streptococci. Experiments with synthetic peptides of the N-terminus of the M1, M5, M6, and M19 proteins resulted in the same conclusions. When a hybrid peptide was chemically synthesized representing the N-terminal sequence of both the M24 and M5 proteins and injected into animals with complete Freund's adjuvant, opsonic antibodies were produced to both M5 and M24 streptococci (19). Opsonic antibodies to three M proteins were obtained when the N-terminal sequences of three M protein sequences (M5-M6-M24) were synthesized in tandem and injected into rabbits.

MULTIVALENT TYPE-SPECIFIC VACCINE

After the untimely death of Edwin Beachey, James Dale in the Beachey laboratory improved on Beachey's approach and used recombinant technology to prepare a tandem oligonucleotide array representing the N-terminal sequence of four serotypes (M24-M5-M6-M19) (Table 1) (20). When the recombinant tetravalent fusion protein was purified and used to immunize rabbits, antibodies were raised against all four M proteins with variations in both the enzyme-linked immunosorbent assay titer and opsonic activity to the four respective streptococcal types. These studies were subsequently repeated using an octavalent construct (21); however, in these studies, opsonic antibodies were produced against six of the eight serotypes used in the vaccine construct (less reactivity being observed with the serotypes represented at the ends). To help overcome this, a 26-valent vaccine was subsequently developed comprising four peptides in which the serotype at one end was duplicated at the other (22). It was designed to contain the N-terminal region of M proteins from epidemiologically important serotypes, including those responsible for pharyngitis in children and those historically responsible for rheumatic fever. The antibodies produced in rabbits to this mixture of peptides were found to be 69% opsonic in one of the three rabbits immunized. Using a 50% reduction in growth of streptococci as an endpoint in a bactericidal assay, 92% (24/26) of the serotypes induced an opsonic response. Whether a 50% reduction is sufficient to block human infections still remains to be tested. As with the other vaccine studies using N-terminal sequences, none of the rabbits developed tissue cross-reactive antibodies to human tissues, including cardiac tissues.

TABLE 1 List of vaccine candidates

Vaccine type	Antigen used	Primary reference(s)
M-protein, type specific	N-terminal multivalent	27, 87
M-protein, conserved region	Whole C-repeat region	38, 88
	Single epitope	89
	T- and B-cell epitopes	49
Group carbohydrate	*N*-acetyl-glucosamine	90
Others (non-M protein)	C5a peptidase	79
	Serum opacity factor	83
	T-antigen (pili)	91
	Fibronectin binding (SfbI)	84
	Serine protease	92, 94
	Conserved proteins	93

In a subsequent phase I safety study, a hexapeptide was generated containing the N-terminal region of M1, M3, M5, M6, M19, and M24 proteins and used to immunize 28 human volunteers intramuscularly (23). After three immunizations, significant antibody levels were produced to all six proteins. After a 1-year follow-up, the vaccine was well tolerated with no evidence of tissue cross-reactive antibodies. Based on epidemiological data (24–26), Dale et al. subsequently engineered a 30-valent vaccine that would represent 98% of pharyngitis cases in the United States and Canada and >80% of invasive disease in these countries (27). The vaccine was shown to induce antibodies to all vaccine serotypes present in the vaccine, which was an improvement over the 26-valent construct (22). These studies confirm and extend the earlier experiments of Beachey and colleagues and reveal that such an approach may be useful for the prevention of infection by specific streptococcal serotypes.

An important factor to consider in the development of a type-specific epitope-based vaccine is the potential of the streptococcus to generate new M serotypes by changing the amino-terminal portion of the M protein. For example, in the type 6 M protein of strain D471, type-specific opsonizing antibodies are directed against epitopes located both at the amino-terminal end (residues 1 through 21) and within the A-repeat block (28), which begins at amino acid 27 and continues to residue 96 in the 441-amino acid M6 molecule (29). High-frequency, intragenic recombinational events within the A-repeat block can lead to a significant loss in the opsonizing ability of monospecific antibodies directed to this region (30). For instance, an opsonic antibody generated to the D471 parental strain showed some or no opsonizing activity to size-variant derivatives of this strain or other M6 streptococci isolated from patients. In addition, Harbaugh et al. (31) showed that sequence variation also occurs within the nonrepeating N-terminal end of the M protein sequence from strains of the same serotype and that these changes ultimately affect the binding of opsonic antibodies. More recently, Penney et al. (32) showed a significant degree of sequence variation within the N-terminal hypervariable region of several strains identified serologically as M1. In all, these findings strongly indicate that opsonic antibodies induced by M protein N-terminal sequences from a given vaccine strain may prove to be ineffective or weakly effective against other strains of the same serotype as a result of antigenic drift. Therefore, a type-specific vaccine necessary to protect against a streptococcal infection would require a multivalent antigen corresponding to stable immunodeterminants on serotypes that together account for the majority of the nasopharyngeal isolates prevalent within the population at a given time. Thus far, this region has not been identified. For a detailed review of the type-specific approach and other approaches see Dale et al. (33).

MUCOSAL VACCINE FOR NON-TYPE-SPECIFIC PROTECTION

At its peak incidence, 50% of children between the ages of 5 and 7 years suffer from streptococcal infection each year. Furthermore, the siblings of a child with a streptococcal pharyngitis are 5 times more likely to acquire the organism than one of his or her parents. This decreased occurrence of streptococcal pharyngitis in adults might be explained by a nonspecific age-related host factor resulting in a decreased susceptibility to streptococci. Alternatively, protective antibodies directed to antigens common to a large number of group A streptococcal serotypes might arise as a consequence of multiple infections or exposures experienced during

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childhood. This could result in an elevated response to conserved M protein epitopes. This latter hypothesis is partly supported by studies of the immune response to the M protein in which it was found that the B-repeat domain (Fig. 1) was clearly immunodominant. When rabbits were immunized with the whole M protein molecule, the first detectable antibodies were directed to the B-repeat region, which rose steadily with time. It was only after repeated M protein immunizations that antibodies were produced against the hypervariable A- and conserved C-repeat regions.

Early human trials by Fox and colleagues (15, 34) strongly suggested that mucosal vaccination with M protein was protective. Using highly purified acid-extracted M protein (which contains type-specific and conserved region fragments of M protein), volunteers were immunized either intranasally or subcutaneously. When they were challenged orally with virulent streptococci, the intranasally immunized volunteers displayed lower rates of both nasopharyngeal colonization and clinical illness compared with placebo controls. Volunteers immunized subcutaneously displayed only a reduction in clinical illness and showed no reduction in nasopharyngeal colonization.

Unlike antibodies to the N-terminal region of the M molecule, it was clear that antibodies directed to the exposed C-repeat region were not opsonic. Because of this, experiments were performed to explore whether mucosal antibodies directed only to the conserved region of M protein could be responsible for protection against streptococcal infection. Taking advantage of the pepsin site in the center of the M molecule (separating the variable and conserved regions) (Fig. 1), the recombinant M6 protein was cleaved, and the N- and C-terminal fragments were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting. When the blots were reacted with different adult human sera, all adults tested had antibodies to the C-terminal conserved region, while as expected, only sera that were opsonic for the M6 organisms reacted with the N-terminal variable region. Similar studies performed with M protein isolated from five common serotypes (M3, M5, M6, M24, M29) revealed that sera from 10 of 17 adults tested did not have N-terminal-specific antibodies to these M types, while only two sera reacted with two serotypes and the remaining five sera reacted with only one serotype. However, all sera tested reacted to the C-terminal fragment of the M molecule. Similar results were seen when salivary IgA from adults and children were tested in an enzyme-linked immunosorbent assay against the N- and C-terminal halves of the M6 molecule (V.A. Fischetti, unpublished data). In all, this is further evidence that the relative resistance of adults to streptococcal pharyngitis is clearly not due to the presence of type-specific antibodies to multiple M serotypes of streptococci but may be due to the presence of antibodies to conserved determinants.

From these findings it was reasoned that an immune response to the conserved region of the M molecule might afford protection by inducing a mucosal response to prevent streptococcal colonization and ultimate infection. In view of the evidence that the conserved C-repeat epitopes of the M molecule are immunologically exposed on the streptococcal surface (35), it should be possible to generate mucosal antibodies that are reactive to the majority of streptococcal types, using only the conserved region antigen for immunization.

PASSIVE PROTECTION

Secretory IgA (sIgA) is able to protect mucosal surfaces from infection by pathogenic microorganisms despite the fact that its effector function differs from those of serum-derived immunoglobulins. When streptococci are administered intranasally to mice, they are able to cause death by first colonizing and then invading the mucosal barrier, resulting in dissemination of the organism to systemic sites. Using this model, a study was conducted to determine if antigen-specific sIgA, delivered directly to the mucosa, plays a role in protecting against streptococcal infection. Live streptococci were mixed with affinity-purified M protein-specific sIgA or IgG antibodies and administered intranasally to the animals (9). The results clearly showed that the anti-M-protein sIgA protected the mice against streptococcal infection and death, whereas the opsonic serum IgG administered in the same way was without effect. This indicated that sIgA can protect at the mucosa and may preclude the need for opsonic IgG in preventing streptococcal infection. This study was one of the first to compare purified, antigen-specific sIgA and serum IgG for passive protection at a mucosal site.

In the Dale laboratory, passive protection against streptococcal pharyngeal colonization was also shown by the oral administration of purified lipoteichoic acid (LTA) but not deacylated LTA before oral streptococcal challenge in mice (36). The addition of anti-LTA by the same route also protected mice from oral streptococcal challenge. While several *in vitro* studies showed the importance of M protein and LTA in streptococcal adherence, these *in vivo* studies together with those presented above using sIgA suggest that both M protein and LTA may play a key role in the colonization of the mouse pharyngeal mucosa. However, it is uncertain whether this is also true in humans.

ACTIVE IMMUNIZATION WITH CONSERVED REGION PEPTIDES

To determine whether a local mucosal response directed to the conserved exposed epitopes of M protein can influence the course of mucosal colonization by group A streptococci, peptides corresponding to these regions were used as immunogens in a mouse model (37). Overlapping synthetic peptides of the conserved region of the M6 protein were covalently linked to the mucosal adjuvant cholera toxin B subunit (CTB) and administered intranasally to the mice in three weekly doses. Animals were challenged intranasally with live streptococci (either homologous M6 or heterologous M14) 30 days later, and pharyngeal colonization by the challenge organism was monitored via throat swabs for 10 to 15 days. Mice immunized with the peptide-CTB complex showed a significant reduction in colonization with either M6 or M14 streptococci compared with mice receiving CTB alone (38) (Fig. 2). Thus, despite the fact that conserved-region peptides were unable to evoke an opsonic antibody response (12), these peptides have the capacity to induce a local immune response capable of influencing the colonization of group A streptococci at the nasopharyngeal mucosa in this model system. These findings were the first to demonstrate protection against a heterologous serotype of group A streptococci with a vaccine consisting of the widely shared C-repeat region of the M6 protein.

Confirmation of these findings was later published independently using a different streptococcal serotype as the immunizing and challenge strains (39, 40). In a separate study, Pruksakorn et al. (41), using a different criterion for streptococcal opsonization than those previously published (i.e., stationary-phase organisms were used instead of organisms in logarithmic phase [42, 43]), found that when a peptide derived from the conserved region of the M protein, termed 145, was used to immunize mice, it induced antibodies capable of opsonizing type 5 streptococci and streptococci

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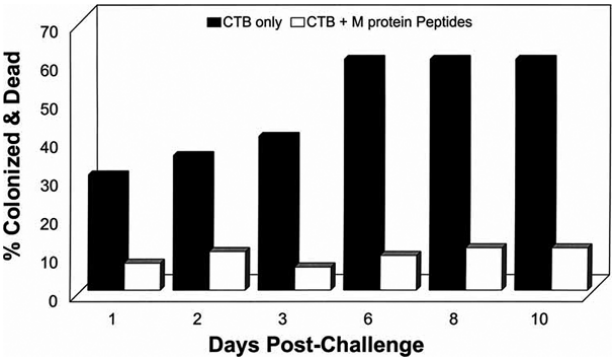


FIGURE 2 The extent of colonization and death of mice challenged with group A streptococci after oral immunization with M-protein conserved-region M6 peptides linked to CTB. The throats of orally immunized mice were swabbed each day after challenge with M14 streptococci, and the specimens were plated on blood plates to determine the extent of colonization compared with that of mice vaccinated with CTB only. Plates showing group A streptococci were scored as positive.

isolated from Aboriginal and Thai patients with rheumatic fever. These findings are in sharp contrast to those from the studies of Jones and Fischetti (12), who showed that antibodies to the conserved region of M protein are not opsonic. To further reduce the size of the 145 peptide to avoid possible tissue cross-reactivity, a 12-amino acid B-cell epitope was constructed called J8 (44). In an attempt to maintain the alpha-helical conformation of the J8 peptide as found in the native M molecule, the flanking regions were substituted with nonstreptococcal sequences with high helix potential (45). In an immunogenicity study this chimeric peptide was linked to diphtheria toxoid (J8-DT) and found to be immunogenic in mice immunized nasally, resulting in a mucosal IgA response and a systemic IgG response (46–48). When these peptides were used to immunize mice intranasally in a protection study, significant protection was observed after streptococcal challenge by the same route (40), resulting in similar protection as unmodified peptides (38). Thus, in addition to a mucosal response, if these conserved-region chimeric peptides do in fact induce an opsonic response in humans (41, 42), they may offer added protection against streptococcal infection. While it was reported that the J8-DT peptide completed phase 1 clinical trials in human volunteers (the safety phase) in 2013, the results of that trial have yet to be reported. The developers of this vaccine found that delivery by either the subcutaneous or intranasal routes was effective in protecting mice from streptococcal infection (49, 50). Another candidate vaccine, called StreptInCor, is a 55-amino acid peptide derived from the conserved C-repeat region of the M5 protein (51). While the smaller J8 peptide described above contains a single B-cell epitope, the StreptInCor vaccine incorporates both a T- and B-cell epitope. Also, being larger, it retains the alpha-helical conformation of the native molecule, resulting in proper T-cell activation (49, 52). In a study of *S. pyogenes* strains isolated in Sao Paulo Brazil, the authors estimated that the StreptInCor vaccine would cover about 71% of the infections (53, 54).

PILUS-BASED VACCINE

One of the major immunogenic proteins on *S. pyogenes* described by Lancefield is termed the T-antigen (55), a molecule used to subclassify *S. pyogenes* into immunologically distinct T types (56, 57). Subsequently, the T-antigens were determined to be pili, flexible rod-like structures on the surface of *S. pyogenes* (58). Pili are heteropolymers made up of a major backbone subunit-forming the shaft of the pilus and two minor subunits at either end of the shaft (AP1 and AP2), the genes of which are located on the FCT (fibronectin-collagen-T-antigen) pathogenicity island (59). A detailed analysis of *S. pyogenes* disease strains and the sequence of T-antigens they carried revealed that 12 T types could represent >90% of the *S. pyogenes* causing disease in Europe and the United States (60, 61). As with the multivalent M protein vaccine, the modular structure of the T-molecule (62) may allow for a similar design of a pili-based vaccine for *S. pyogenes*.

VACCINIA VIRUS AS A VECTOR

To further verify the validity of using the M protein conserved region as a streptococcal vaccine, experiments were repeated in a vaccinia virus vector system. In these studies, the gene coding for the complete conserved region of the M6 molecule (from the pepsin site to the C terminus) was cloned and expressed in vaccinia virus producing the recombinant VV::M6 virus (63). Tissue culture cells infected with this virus were found to produce the conserved region of the M6 molecule. Animals immunized intranasally with only a single dose of recombinant virus were significantly protected from heterologous streptococcal challenge compared with animals immunized with wild-type virus (Fig. 3). When the extent of colonization was examined in animals immunized with the wild type or the VV::M6 recombinant, the VV::M6-immunized animals showed a marked reduction in overall colonization, indicating that mucosal immunization reduced the bacterial load on the mucosa in these animals. Animals immunized intradermally with the VV::M6 virus and challenged intranasally showed no protection.

The approaches described above again proved that induction of a local immune response was critical for protection against streptococcal colonization and that the protection was not dependent on an opsonic response. However, in the event that the streptococcus was successful in penetrating

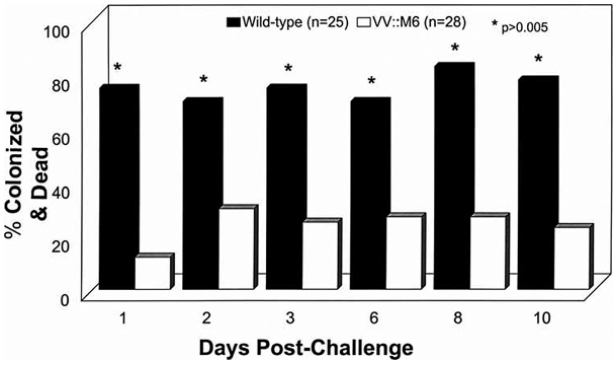


FIGURE 3 The extent of colonization and death of mice challenged with group A streptococci after oral immunization with recombinant vaccinia virus containing the gene for the whole conserved region of the M6 protein. The throats of orally immunized mice were swabbed each day after challenge with M14 streptococci, and the specimens were plated on blood plates to determine the extent of colonization compared with that of mice vaccinated with wild-type vaccinia only. Plates showing group A streptococci were scored as positive.

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the mucosa and establishing an infection, only then would type-specific antibodies be necessary to eradicate the organism. This idea may explain why adults sporadically develop a streptococcal pharyngitis. The success of these strategies not only forms the basis of a broadly protective vaccine for the prevention of streptococcal pharyngitis but may also offer insights for the development of other vaccines. For instance, a vaccine candidate previously shown to be ineffective by the parenteral route may prove to be successful by simply changing the site of immunization. Furthermore, these results emphasize the fact that in some cases antigens need to be presented to the immune system in a specific fashion to ultimately induce a protective response.

THE USE OF GRAM-POSITIVE COMMENSALS AS VACCINE VECTORS

The CTB-linked peptide and vaccinia systems mentioned above, although successful for protection against streptococcal infection, are not ideal. The CTB system requires large quantities of purified peptide and a linkage protocol that allows no more than two peptides per CTB molecule to enable the proper binding of CTB to GM_1 ganglioside (38). Although possible to achieve, these requirements would make this type of vaccine relatively expensive to produce, even if recombinant technology were used to prepare the fusion molecules. Given the fact that such a vaccine ultimately must be administered in developing countries, the cost would likely be prohibitive. The vaccinia virus vector, on the other hand, is inexpensive but unlikely to gain approval from the Food and Drug Administration since oral/intranasal administration, which could result in serious complications, is required for effectiveness. Because of these limitations, a safer and cheaper mucosal vaccine delivery system was developed.

While several nonliving systems of delivering antigen to mucosal sites have been developed (37, 64–66), live vectors may afford a better and more natural response without the need to reimmunize to gain higher antibody titers. In most instances, live antigen delivery vectors are derived from bacteria (usually Gram negative) (67) or viruses (68) that are normally considered mammalian pathogens. Perhaps this is due in part to our better understanding of these organisms, making genetic manipulations easier. Usually, these organisms have been extensively engineered to reduce their pathogenicity yet maintain certain invasive qualities (e.g., to invade the M cells of the gut mucosa) to induce a mucosal immune response. To circumvent some of the safety and environmental issues inherent in the wide-scale dissemination of engineered pathogens, a nonpathogenic Gram-positive bacterial vaccine vector was developed (69). In this system, foreign antigens are displayed on the surface of Gram-positive human commensal organisms that colonize the niche invaded by the pathogen (oral, intestinal, or vaginal). Colonization generates both an enhanced local IgA response to the foreign antigen and systemic IgG and T-cell responses. Unlike many other live bacterial systems, in which the foreign antigen is either retained in the cytoplasm, translocated to the periplasm, or in some cases secreted, the Gram-positive vector anchors the foreign antigen to the cell for surface display (69). Since the cell wall peptidoglycan of the Gram-positive cell is a natural adjuvant, an enhanced response is obtained when the engineered organisms are processed for antibody induction. The ability to accomplish this is based on the discovery that surface molecules from Gram-positive bacteria that anchor via their C termini (of which >200 have now been sequenced) have a highly conserved C-terminal region that is responsible for cell attachment (70, 71).

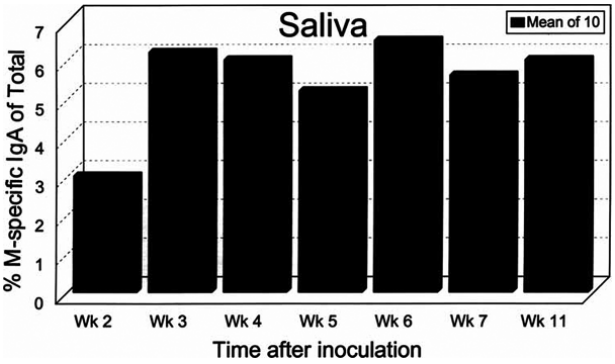


FIGURE 4 M protein-specific salivary IgA in rabbits colonized with *S. gordonii* expressing the conserved region on the cell surface. Salivary samples were taken after pilocarpine induction and tested in an enzyme-linked immunosorbent assay against the M protein.

A NEW-GENERATION STREPTOCOCCAL VACCINE

Based on successful mouse studies examining the immune response to a recombinant antigen expressed on the surface of *Streptococcus gordonii*, a recombinant *S. gordonii* was engineered that comprised the C-terminal half of the M protein containing the exposed conserved region of the molecule. This segment was similar to that used successfully in the vaccinia virus experiments (see above) (72). *S. gordonii* expressing this segment of the M protein on its surface successfully colonized for up to 12 weeks all of the 10 rabbits that were immunized. During this time, the animals raised a salivary IgA (Fig. 4) and serum IgG (Fig. 5) response to the intact M protein. The amount of M protein-specific sIgA was up to 5% of the total IgA in the saliva of these animals. The IgA and IgG induced by this method did not cross-react with human heart tissue, as determined by an immunofluorescence assay, or with tropomyosin, myosin, or vimentin as determined by Western blotting. As an added feature of the live vector system, Byrd et al. constructed *S. gordonii* which coexpressed immunomodulatory molecules such as interleukin-2 and interferon-gamma along with the M protein conserved region (73). They found that in the coexpression constructs, the cytokines modulated the systemic immune response compared to control constructs without cytokines.

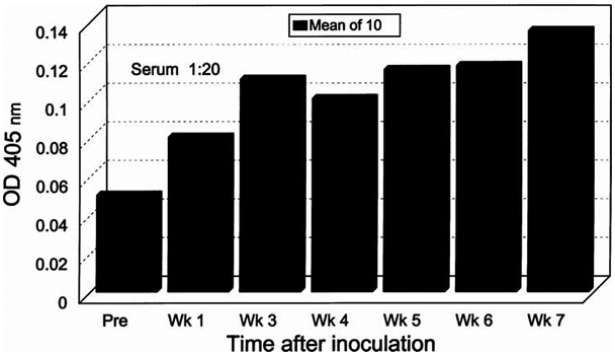


FIGURE 5 M protein-specific serum IgG in rabbits colonized with *S. gordonii* expressing the conserved region on the cell surface. Blood samples were taken at weekly intervals and tested in ELISA against the M protein.

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The commensal delivery system would be ideal for developing countries if it proved to be effective. Because it is a live vector, it would be easy to administer and not likely to require additional doses. Also, since Gram-positive bacteria are stable for long periods in the lyophilized state, a cold chain would not be required. Early studies showed that, when reintroduced into the human oral cavity, *S. gordonii* is capable of persisting for over 2 years and is transmitted to family members. For a developing country this factor could be ideal, since rarely is the whole population able to be immunized. However, it remains to be determined if this approach will induce a protective immune response in humans (74).

NON-M-PROTEIN APPROACHES TO PROTECT AGAINST STREPTOCOCCAL INFECTION

Other approaches have been developed recently that may serve an alternative to using M protein as a vaccine candidate (75). Cleary and coworkers (76) have identified a group A streptococcal protease that specifically cleaves the human serum chemotaxin C5a, preventing its binding to polymorphonuclear neutrophils. It was suggested that the surface-exposed streptococcal C5a peptidase prevents the influx of phagocytes to a streptococcal infection by destroying this chemotaxin. As proof, cleavage of C5a has been shown to reduce the influx of inflammatory cells at the site of a streptococcal infection (77). Expanding this finding, it was found that delivery of a defective form of the streptococcal C5a peptidase molecule intranasally to mice showed promise in protecting against challenge from heterologous M serotypes (78). In these studies, immunized animals cleared the challenge streptococci from the throat more rapidly than did control animals. To support this approach, human sera were tested for the presence of C5a peptidase antibodies in acute and convalescent sera from children with streptococcal pharyngitis (79). The authors found that C5a peptidase was highly immunogenic.

Using a different strategy, Musser and his group (80) have been working on the group A streptococcal cysteine protease known as streptococcal pyrogenic exotoxin B (SpeB). The gene for this protein is found in virtually all strains of group A streptococci, and in most cases its product is secreted from these organisms. Since cysteine proteases have been implicated in bacterial pathogenicity, Kapur et al. (80) found that a protease-negative SpeB mutant lost nearly all of its ability to cause death in mice when compared with wild-type organisms. They showed that mice passively immunized with rabbit IgG to cysteine protease exhibited a longer time to death than did control animals. Active immunization with cysteine protease gave the same result. Thus, while this vaccine approach prolonged the time to death, it did not prevent death.

N-acetyl-glucosamine, a polysaccharide component of the streptococcal cell wall, is the group-specific antigen for group A streptococci. Since most people infected by streptococci develop anti-*N*-acetyl-glucosamine antibodies, Zabriskie and colleagues attempted to determine if these antibodies have any effect on protecting against streptococcal infection. Using a modified *in vitro* phagocytic assay and a low bacterial inoculum, they found that anticarbohydrate antibodies specific for the *N*-acetyl-glucosamine were phagocytic. Comparing human and rabbit opsonic sera, they concluded that very high titers of anti-*N*-acetyl-glucosamine-specific antibodies were necessary to be effective in opsonization and phagocytosis of *S. pyogenes* (81).

As for other candidates, serum opacity factor, a cell wall-associated and -secreted protein of class II group A streptococci (82), has been shown to produce a protective immune response to heterologous streptococcal serotypes (83). The fibronectin binding domain of SfbI, a fibronectin binding protein of *S. pyogenes*, when used to immunize the upper respiratory mucosa of mice, was found to induce a protective mucosal response after challenge with streptococci by the same route (84).

In conclusion, while several viable vaccine antigens exist as candidates to protect against *S. pyogenes* infections (Table 1), no single antigen has yet risen to be commercially developed. Some of the reasons include (i) the inability to conclusively identify the antigen responsible for inducing the heart cross-reactive antibodies, (ii) the expense involved in determining if the vaccine antigen responsible for protection will not induce rheumatic fever because of the low frequency of the disease, and (iii) the lack of resistance seen in *S. pyogenes* to penicillin-based antibiotics. All this complacency will change the first time an *S. pyogenes* strain is isolated that is found to be resistant to penicillin.

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The Bacteriophages of *Streptococcus pyogenes*

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11

OVERVIEW

The year 2015 was the centenary of the discovery of bacteriophages (phages) by Frederick Twort (1), and the first associations of phages with *Streptococcus pyogenes* (group A streptococcus) were made by Cantacuzene and Boncieu in 1926 (2) and by Frobisher and Brown in 1927 (3), both groups showing that the "scarlatina toxin" (erythrogenic toxin, pyrogenic exotoxin A) could be passed to a new strain after exposure to a sterile filtrate from a toxigenic isolate. Additional studies by Evans in the 1930s and 1940s documented the prevalence of group A streptococcal phages, linked them with virulence, and provided early additional evidence for lysogeny (4–8). More studies followed of both lysogenic and lytic streptococcal phages (9–15), but the link between lysogeny and the transmission of the erythrogenic toxin was not made until the landmark study by Zabriskie in 1964 (16); the phage-encoded toxin structural gene (*speA*) was subsequently identified by Weeks and Ferretti (17, 18) and independently by Johnson and Schlievert (19). The history and contributions of these and other early workers to our knowledge of the bacteriophages of *S. pyogenes* are covered elsewhere (20). Following these studies, a significant body of research has shown that phages contribute to the success and virulence of their host streptococci by acting as vectors for genes encoding virulence factors and through their ability to disseminate streptococcal genes from one cell to another through transduction. Since group A streptococci are not thought to be normally competent for transformation, except possibly under certain conditions (21), and since Hfr-type conjugal transfer of chromosomal genes has not been observed, it is easy to argue that the bacteriophages of *S. pyogenes* play a major role in the genetics and molecular biology of this pathogen.

LYTIC PHAGES OF GROUP A STREPTOCOCCI

Biology and Distribution of Lytic Phages

Bacteriophages typically may be grouped by their life cycle into two categories: lytic phages and lysogenic (temperate) phages. Lytic phages infect their host cell and begin the viral replicative cycle within a short time frame. At the end of replication and assembly, the host bacterial cell typically lyses and releases the newly formed bacteriophage particles. The five decades following the discovery of phages saw numerous investigations of lytic phages of *S. pyogenes*, which included studies of their host range, their basic biology, and their ability to mediate general transduction. In contrast to lysogenic phages, lytic phages do not alter the phenotype of the host streptococcal cell by a long-term genetic relationship, but they can shape the host population by eliminating susceptible cells in a population or by facilitating genetic exchange by transduction.

Bacteriophage A25

The best-studied lytic phage of *S. pyogenes* is bacteriophage A25, which was originally isolated in the early 1950s from Paris sewage (11, 12) and was found to mediate generalized transduction in *S. pyogenes* (22). Phage A25 is also referred to in the literature as phage 12204, its designation by the American Type Culture Collection (ATCC 12204). The genome sequence of A25 has been determined to be 33,900 bp with a GC content of 38.44%, very close to the predicted size (23) and GC content (24) reported previously. The sequence is available from GenBank (accession no. KT388093.1). Electron microscopy shows that it belongs to the *Siphoviridae* with an isometric, octahedral head measuring 58 to 60 nm across and a long flexible tail that measures 180 to 190 nm in length and 10 nm in diameter (25, 26). The tail of this phage is composed of 8-nm circular subunits and terminates in a transverse plate with a single projecting spike that is about 20 nm long (26, 27). In contrast to many lysogenic phages, the genome sequence shows that A25 does not encode a hyaluronidase (hyaluronate lyase) as part of its tail fiber. It does, however, encode a lysin that has activity against groups A, C, G, and H streptococci (28).

One-step growth experiments showed that phage A25 has an average burst size that may vary depending upon the host strain, with reported average burst sizes of 30 PFU/cell when propagated with strain K56 (29) and 12 CFU/cell using strain $T25_3$ (30). Peptidoglycan is the cell receptor for A25, and treatment of the cells with the group C streptococcus phage C1 lysin (PlyC) destroyed the receptor binding (31). Phage A25 has a broad host range, being adaptable to group G streptococci after passage (31). This broad host range is reflected by the mosaic nature of the A25 genome, which contains genetic modules shared with bacteriophages from *S. pyogenes*, *Streptococcus pneumoniae*, *Streptococcus suis*, *Streptococcus dysgalactiae* subsp. *equisimilis*, *Streptococcus iniae*, and *Streptococcus equi* subsp. *zooepidemicus* (32).

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Wannamaker and coworkers showed that A25 also could infect 48% of group C strains tested (33). Other *S. pyogenes* lytic phages in the same study could also infect group C strains at frequencies ranging from 34 to 47%. The possibility that A25 and other lytic *S. pyogenes* phages can infect multiple species of streptococci may contribute to horizontal transfer of both host and prophage genes via transduction. Phylogenetic analysis of the terminase suggests that A25 uses *pac* style packaging of its genome (32), and low-stringency *pac* site recognition has been associated with high-frequency transduction in phages (34). The efficiency of transduction by A25, discussed below, suggests that its packaging of DNA sequences is not highly stringent.

The genome sequence of A25 revealed that, remarkably, this bacteriophage was very likely an escaped lysogen containing a remnant lysogeny module that retained a *cro*-like antirepressor and operator sequence but no longer contained a cI-like repressor or integrase (32). The genome has a modular genetic arrangement similar to many *S. pyogenes* genome prophages (Fig. 1) with regions associated with regulation, DNA replication, endonucleolytic cleavage, DNA packaging, head and head morphogenesis genes, tail genes, and lysis. In contrast to most genome prophages, however, no identifiable virulence genes were found. An extended region of high DNA homology with the lysogeny module of a number of genome prophages is present (shaded gray in Fig. 1), and the shared operator sequence between these prophages and A25 appears to be a mechanism of A25 resistance for these prophages, allowing their Lambda-like repressor proteins to silence A25 expression and abort its replication. The key role of this repressor was confirmed by transferring a plasmid-encoded copy of this gene into susceptible strains of *S. pyogenes*, leading to high-level resistance to A25 infection (32). The remainder of the A25 genome shows little homology to known *S. pyogenes* prophages, instead having regions of homology to phages from other streptococcal species as described above. Streptococcal phage Str01, sequenced after A25, showed near identical homology to the A25 genome except for the presence of a cI-like repressor and integrase gene. Interestingly, homology to this portion of the Str01 genome was found to prophages from group G streptococci. This suggests that lysogenic escape was a more distant event then rescued by the acquisition of integrase and cI-like repressor from a group G streptococcal phage, further implicating multispecies genetic transfer within streptococci and streptococcal phages.

Transduction in *S. pyogenes*

Transformation, conjugation, and transduction are common means of genetic exchange in bacteria. In *S. pyogenes*, natural transformation may occur when the cells live in a biofilm (35), but such exchange has not been seen under typical laboratory conditions. Conjugative transposons are frequent elements in *S. pyogenes*, but they are not associated with transfer events seen with the F plasmid of *E. coli*. Generalized transduction, by contrast, occurs in *S. pyogenes* and is mediated by both lytic and lysogenic phages.

Transduction in *S. pyogenes* was first reported in 1968, detailing how five phages, three lytic and two lysogenic, were able to transduce streptomycin resistance (22). Of this group of phages, phage A25 (phage 12204) was able to transduce antibiotic resistance at the highest frequency (1×10^{-6} transductants per PFU). Transfer was DNase resistant but sensitive to antiphage serum, supporting generalized transduction as the mechanism of genetic exchange. The capsule of *S. pyogenes* is composed of hyaluronic acid, which was

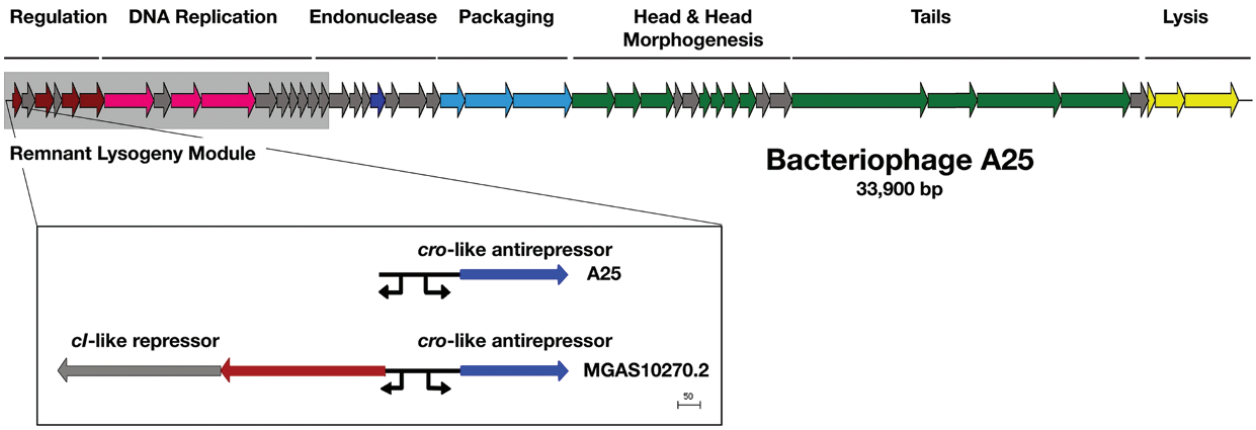


FIGURE 1 The genome of bacteriophage A25 reveals an escape from lysogeny. The 33,900-bp generalized transducing phage A25 is shown. The portion of the chromosome included in the shaded box is the high homology region that contains the remnant lysogeny module and other genes that A25 shares with prophages from genome strains MGAS10270 (M2), MGAS315 (M3), MGAS10570 (M4), and STAB902 (M4). Unlike these complete lysogens, A25 only has the operator and antirepressor from the lysogeny module (shown in expanded view below the map), apparently having lost the integrase and repressor for lysogeny some time in the past. This A25 expanded region is compared to the homologous region from genome prophage MGAS10270.2, which contains these elements as well as the upstream genes including the *cI*-like repressor. Promoters are shown as directional arrows. Introduction of the MGAS10270.2 repressor into an A25-sensitive *S. pyogenes* strain results in its conversion to a high level of A25 resistance (32). The genome follows a typical modular arrangement, with the predicted function for A25 genes indicated by color: regulation, dark red; DNA replication, pink; encode endonucleases, dark blue; genome packaging, light blue; structural, green; and lysis, yellow. The figure is redrawn from McCullor et al. (32).

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found to be a barrier to A25 infection (11). Lysogenic phages often encode a hyaluronidase, but phage A25 lacks such a gene since this enzymatic activity has not been associated with nor was a gene identified by genome sequencing (32). The state of *S. pyogenes* encapsulation can vary during the growth phase (36), and some strains, such as the recently described M4 isolate from Australia (37), do not express capsule at all. Therefore, the susceptibility of cells to phage-mediated transduction probably varies by growth state and genetic background, both of which could influence horizontal transfer.

A number of strategies have been used to improve transduction frequencies. Malke showed that transduction frequencies could be improved by using specific A25 antiserum to block unabsorbed or progeny phages from infecting transductants resulting from the initial adsorption (38). In theory, this phenomenon leading to higher transduction frequencies can occur in nature, particularly with infections of strains containing resident prophages with high homology to A25 within the lambda-type regulatory region. As discussed above, superinfection immunity represses A25 gene expression but does not impede initial phage adsorption and injection of DNA material. This mechanism protects the infected cell from lysis by A25 infection and allows for survival of transduced cells. Increased levels could also be obtained by the use of temperature-sensitive mutants of phage A25 (29), as could UV irradiation of transducing lysates prior to adsorption to the host streptococci (38–40).

Lysogenic bacteriophages of *S. pyogenes* are capable of mediating transfer of antibiotic resistance by transduction. Strains with bacteriophage T12-like prophages can produce transducing lysates capable of transferring resistance to tetracycline, chloramphenicol, macrolides, lincomycin, and clindamycin following lysogen induction. Generalized transduction transfer of erythromycin and streptomycin resistance following mitomycin C treatment of endogenous prophages has also been observed (41).

Transduction may play a role in the dissemination of genes among related streptococcal species. Some bacteriophages isolated from groups A and G streptococci can infect serotype A, C, G, H, and L strains; some were capable of infecting multiple serotypes (42). The same study showed that phage A25 could transduce streptomycin resistance to a group G strain. Wannamaker and coworkers further showed that streptomycin resistance could be transferred to *S. pyogenes* strains by a temperate transducing phage isolated from group C streptococcus (33). The wealth of *S. pyogenes* genome data supports the idea that horizontal gene transfer has been important in the evolution of this pathogen (43), and transduction is assumed to play an important role in this process. However, the molecular mechanisms that would drive this process are little understood. The majority of the studies into streptococcal transduction were done before the advent of modern techniques of molecular biology and genomics, and therefore this may be an opportune time to reexamine this phenomenon. A better understanding of streptococcal transduction may prove key to understanding the flow of genetic information in natural populations of *S. pyogenes* and the horizontal transfer of information from other genera. In addition, better understanding of gene transfer mediated by phage transduction could translate to better molecular tools for manipulation of *S. pyogenes* genomes, which currently remains a more challenging feat compared to other streptococcal species. More efficient genetic manipulation, in turn, could further our understanding of gene function within *S. pyogenes*.

LYSOGENIC PHAGES OF GROUP A STREPTOCOCCI

Genome Prophages, their Distribution, and Attachment Sites

Lysogenic bacteriophages are defined by their ability to integrate their DNA into the host bacterium's chromosome via site-specific recombination, becoming a stable genetic element that can be passed to daughter cells following cell division. Studies from the pregenomics era suggested that lysogeny was common in *S. pyogenes* (9, 10, 44–47), but it was genome sequencing, starting with the first sequence completed and confirmed by almost every subsequent sequenced genome, that demonstrated that toxin-carrying prophages were not only common but prominent genetic features that shaped the fundamental biology of this bacterial pathogen (Table 1). The number of lambdoid prophages or phage-like chromosomal islands found in a given genome strain has ranged from a low of one (MGAS15252) to as many as eight (MGAS10394), with three to four elements being common. These prophages are found integrated into multiple sites on the *S. pyogenes* genome, being found in each quadrant (Fig. 2 and Table 2). The majority of the genome prophages (72%) are found integrated into genes encoded on the lagging strand (relative to *ori*C). No prophages have been found to target genes in the hypervariable regions that include the M-protein (*emm)* or the streptococcal pilus. Some sites are frequent targets for prophage integration; the genes for DNA binding protein HU, transfer messenger RNA (tmRNA), and DNA mismatch repair (MMR) protein MutL are commonly occupied by a prophage or prophage-like chromosomal island.

Integration of prophages occurs via homologous exchange between sequences shared between the phage and host chromosomes (*attP* and *attB*, respectively); this process is mediated by a phage-encoded integrase. These duplications between the phage and host DNA can be as little as a few nucleotides to over 100 bp, often including coding regions of the bacterial genome (48–50). In *S. pyogenes*, the identifiable duplications between *attB* and *attP* range from 12 bp (MGAS10394.1) to 96 bp (T12). An extensive survey of bacterial genome prophages found that prophages usually integrate into a gene open reading frame (ORF) (69% of identified prophages), while integration into an intragenic region, such as seen in coliphage Lambda, is less common, accounting for only 31% of prophages (50). Gene targets included tRNA genes (33%), tmRNA (8%), and various other genes (28%). Examples of each target site are observed in the *S. pyogenes* genome prophages (Table 2). Most commonly, the duplication occurs between the phage and the 3′ end of the host gene, and integration leaves the ORF intact via the duplicated sequence (50, 51). In *S. pyogenes*, however, the 5′ ends of genes are frequently targeted for integration, which potentially could lead to altered expression of the host gene (Table 2). The best-characterized system of altered host gene expression is the control of MMR in strain SF370 by *S. pyogenes* chromosomal island, serotype M1 (SpyCIM1), where the expression of genes for MMR, multidrug efflux, Holliday junction resolution, and base excision repair are controlled by this phage-like chromosomal island in response to growth (51–54). Similarly, the phage-like transposon MGAS10394.4 (also known as Tn*1207.3* [55]) separates the DNA translocation machinery channel protein ComEC operon proteins 2 and 3, potentially creating a polar mutation that silences protein 3. A number of other

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TABLE 1 Assembled and annotated genomes of *S. pyogenes* that are hosts to prophages

Strain	M type	Prophages (CI)[a]	Origin	Disease[b]	Genome size (bp)	Accession no.	Reference
SF370	M1	3 (1)	USA	Wound	1,852,441	NC_002737	113
MGAS5005	M1	3	Canada	Invasive (CSF)	1,838,554	NC_007297	114
M1 476	M1	3	Japan	STSS	1,831,128	NC_020540.2	115
A20	M1	3	Taiwan	Necrotizing fasciitis	1,837,281	NC_018936.1	116
MGAS10270	M2	4 (1)	USA	Pharyngitis	1,928,252	NC_008022	117
MGAS315	M3	6	USA	STSS	1,900,521	NC_004070	118
SSI-1	M3	6	Japan	STSS	1,894,275	NC_004606	119
MGAS10750	M4	3 (1)	USA	Pharyngitis	1,937,111	NC_008024	117
Manfredo	M5	4 (1)	USA	ARF	1,841,271	NC_009332	120
MGAS10394	M6	7 (1)	USA	Pharyngitis	1,899,877	NC_006086	118
MGAS2096	M12	2	Trinidad	AGN	1,860,355	NC_008023	117
MGAS9429	M12	3	USA	Pharyngitis	1,836,467	NC_008021	117
HKU16	M12	3	Hong Kong	Scarlet fever	1,908,100	AFRY00000001	121
HSC5	M14	3	USA	Not known	1,818,351	NC_021807.1	122
MGAS8232	M18	5	USA	ARF	1,895,017	NC_003485	123
M23ND	M23	4	USA	Invasive	1,846,477	CP008695	109
MGAS6180	M28	2 (2)	USA	Puerperal sepsis	1,897,573	NC_007296	124
NZ131	M49	3	New Zealand	AGN	1,815,785	NC_011375	125
Alab49	M53	3 (1)	USA	Impetigo	1,827,308	NC_017596	126
MGAS15252	M59	0 (1)	Canada	SSTI	1,750,832	NC_017040	127
MGAS1882	M59	1 (1)	USA	AGN, pyoderma	1,781,029	NC_017053	127

[a]Number of lambdoid prophages (number of phage-like chromosomal islands [CIs]).
[b]AGN, acute glomerulonephritis; ARF, acute rheumatic fever; CSF, cerebrospinal fluid; SSTI, skin or soft tissue infection; STSS, streptococcal toxic shock syndrome.

streptococcal genes are targeted at their 5′ ends by prophages, including genes encoding the recombination protein RecX, a HAD-like hydrolase, and DNA-binding protein HU (Table 2). Other prophages integrate into the promoter region preceding the ORF in dipeptidase Spy-713, *yesN*, and a gamma-glutamyl kinase. In the case of dipeptidase Spy0713 that is targeted by members of the SF370.1 family, integration separates the ORF from the predicted native promoter and may replace it with a phage-encoded promoter found immediately upstream of the coding region following integration (Fig. 3). This phage-encoded promoter is preceded by a canonical CinA box (56), suggesting that this putative alternative promoter may also change the transcriptional program of the gene. In another example, two serotype M3 prophages were found integrated into a clustered regularly interspaced short palindromic repeat (CRISPR) type II system direct repeat; remarkably, this event may have led to the loss of CRISPR function in these cells. In all of these examples, the integration of a phage or phage-like element into the 5′ end of a gene has the potential to alter streptococcal gene expression by blocking transcription or providing an alternative promoter, and the frequency of such transcription-altering prophages may be an important regulatory strategy in *S. pyogenes*.

Not all *S. pyogenes* prophages inactivate host genes following integration. Those that integrate into the 3′ ends of genes typically preserve gene function through the shared DNA sequence between the DNA molecules, and a number of examples are found in the genome prophages (Table 2). Bacteriophage T12 integrates by site-specific recombination into what was initially identified as a gene for a serine tRNA (57) but was correctly identified as a tmRNA gene after the completion of genome sequencing. Genes encoding tmRNA are frequently used as bacterial attachment sites (*attB*) for prophages that infect a range of bacterial species, including *Escherichia coli*, *Vibrio cholerae*, and *Dichelobacter nodosus* (50, 58). Besides the tmRNA gene, other 3′ gene targets used by genome prophages include the histone-like protein HU, dTDP-glucose-4,6-dehydratase, a putative SNF family helicase, and recombination protein *rec*O.

Morphology and Genome Organization

Tailed phages with double-stranded DNA genomes (*Caudovirales*) are abundant in the biosphere, perhaps being the most frequent form of life on Earth (59). Of the *Caudovirales*, the phage subset *Siphoviridae* (icosahedral heads with long, noncontractile tails) make up about 60% of the total (60) order. A bacteriophage survey from the pregenomics era found that 92% of phages of the genus *Streptococcus* were *Siphoviridae* by the current classification (61), and the few early electron micrographs published reflect this prevalence (13, 25, 26, 38). Figure 4 shows the typical *Siphoviridae* morphology of two well-studied *S. pyogenes* phages, SF370.1 and T12. The tail fibers of SF370.1, which contain the hyaluronidase (hyaluronate lyase) used for capsule penetration during phage infection (62), are seen in the micrograph. The lytic transducing phage A25 also has typical *Siphoviridae* morphology (25, 38). While lysogenic phages may be found to be mostly members of the *Siphoviridae*, given their probable common pool of genetic modules (see below), other phage morphotypes may be found in the lytic phages, such as the *Podoviridae* C1 phage of the related group C streptococci

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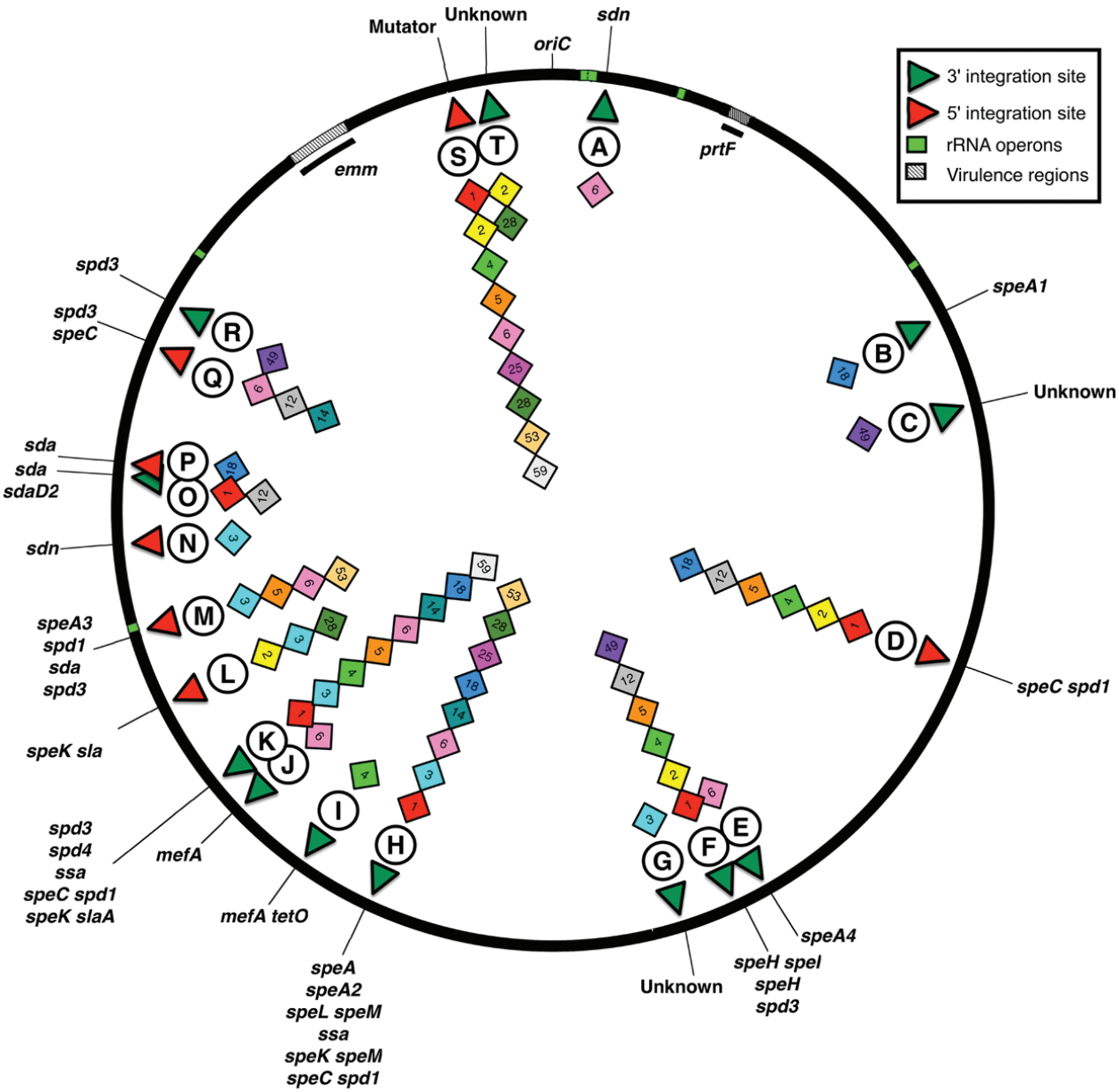


FIGURE 2 Prophage attachment sites in the *S. pyogenes* genome. The locations of the genome prophages are shown as a generalized chromosome backbone based on the SF370 M1 genome; each diamond represents a genome prophage identified at that site. The M type of the host for each prophage is indicated by the number within the diamond, and the circled letter is the identifier linked to Table 2 for *attB* gene identification, the integration target within that gene (5′ or 3′), and associated prophage virulence genes. The rRNA operons are indicated as green blocks, and the hypervariable regions containing virulence genes associated with *emm* or *prtF* are hatched. The origin of replication is indicated (OriC).

(63). The coliphage Lambda has been the prototype for lysogenic prophages, and the genetic organization of most group A streptococcal genome prophages follows a similar general plan (64, 65), having identifiable genetic modules for integration and lysogeny, replication, regulation, head morphogenesis, head-tail joining, tail and tail fiber genes, lysis, and virulence (Fig. 5).

Lysogeny module

Temperate phages are defined by their carriage of genes that establish and maintain a stable condition within a host cell, usually via site-specific integration. Minimally, lysogeny requires genes encoding an integrase (recombinase) and excisionase to mediate prophage DNA integration and excision as well as genes encoding repressor and antirepressor proteins to direct and control this process following the pattern seen in coliphage Lambda (66). Phage integrases typically mediate a recombination event between an identical sequence shared between the circular form of the prophage genome (*attP*) and the bacterial chromosome (*attB*), and the recognition of these DNA sequences is inherent in a given integrase protein (49, 67, 68). Most lambdoid phages usually have integrases that

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belong to the tyrosine integrase family, and the integrases of genome *S. pyogenes* prophages belong to this group. The excisionase gene in Lambda and many other Gram-negative host phages is positioned upstream of integrase, but in the lactic acid bacteria and other Gram-positive bacteria its genome location is variable (69, 70). Indeed, since excisionase proteins often show little conservation (71), the identification of the correct ORF in a given prophage is often difficult. Some excisionase genes may be provisionally identified in the *S. pyogenes* genome prophages by homology to other phages (64); however, to date, none have been confirmed experimentally.

DNA replication and modification

Following the lysogeny module is a region that shows considerable diversity between individual prophages and that encodes genes involving DNA replication and modification. Homologs of DNA polymerases, replisome organizer, restriction-modification systems, and primase genes are present in these regions as well as potential sequences that may function as origins of phage DNA replication (64, 65). Inspection of the genome annotations of these regions also shows that while many genes are unique to group A streptococcal phages, others have close homologs in phages from other streptococcal species such as *Streptococcus thermophilus* or *S. equi*, suggesting that a pool of genetic material is shared by a diverse group of phages (discussed below).

DNA packaging, capsid structural genes, and host lysis genes

The next region of prophage genomes is typically dedicated to the genes encoding the proteins for the assembly of phage heads and tails as well as the proteins needed to package the phage DNA into the heads and join this complex to the tails. The function of many of these genes has been inferred by homology to known phage proteins or sequences or by presumption of function due to their relative order in the chromosome. However, with the exception of the hyaluronate lyase (hyaluronidase) gene found in some *S. pyogenes* phage tail fibers (45, 62, 72), most of these genes have not been characterized experimentally, and these function assignments remain provisional. Following the capsid genes are the typical holin-lysin genes that are employed at the end of the lytic phase to lyse the infected bacterial cell and release the newly formed phage particles.

Virulence genes

Finally, at the distal end of the phage chromosome are the genes for host conversion that encode a range of virulence factors; prominent are exotoxins that are often superantigens and DNases such as streptodornase (Table 2). The origin of phage-encoded toxins remains unclear, but the fact that these toxin genes play no known role in the replication of the phage suggests that such genes were acquired at some point late in the phage's evolutionary history. It has been proposed that virulence factors may be acquired by phages by imprecise excision events (73), but finding known phage-associated virulence genes independently on the bacterial chromosome has not occurred. Some superantigen genes are not associated with prophages (74, 75), but whether these genes are a genetic source of prophage superantigens is unclear. Lateral gene capture of virulence genes has undoubtedly been important in their dissemination (76), and such exotoxins may have evolved *de novo* as elements to increase bacterial host cell fitness (77). Decayed prophage remnants with superantigen domains may be seen in the *S. pyogenes* genome (65), and these regions may serve as a genetic reservoir for virulence genes. Similarly, host-range variants of phages from different bacterial species or even genera are another potential reservoir for toxin genes; for example, the *speA* gene of *S. pyogenes* and the enterotoxins B and C1 of *Staphylococcus aureus* show a significant degree of homology and thus may share a common origin (18).

Diversity of Lysogenic Phages

Structural genes dominate phylogenic relations

Botstein, in a seminal 1980 paper, proposed that the product of bacteriophage evolution is not the individual virus but a pool of interchangeable genetic modules, each of which carries out a biological function in the phage lifecycle; natural selection therefore acts at the level of these individual modules (functional units) (78). Phylogenetic analysis of the Lambdoid *S. pyogenes* prophages predicts that several prominent groups exist (Fig. 6). Within each branch, considerable group diversity may exist in terms of targeted bacterial attachment site and encoded virulence factors (Table 2). Inspection of each group shows that phylogeny is driven by large shared blocks of genes encoding proteins for DNA packaging, heads and tails, and host lysis (Table 3); for example, see the analysis of the group of prophages containing phage T12, where all members of the group minimally share these structural genes (Fig. 7). However, others, such as the SF370.1 group, are much more clonal, all sharing the same virulence and lysogeny modules. Therefore, the phylogeny of individual functional modules (such as toxin genes) may be unrelated to other regions of the genome (such as the lysogeny module or capsid genes). The apparent shuffling of the individual modules may be driven by both homologous and nonhomologous recombination (64, 79–82). The actual driving force behind this engine of prophage diversity in *S. pyogenes* remains poorly understood, but some clues have emerged. In a survey of 21 toxin-carrying *S. pyogenes* strains, 18 were found to carry a highly conserved ORF adjacent to the toxin genes (83). This ORF, which was named paratox, may help promote homologous recombination between phages so that toxin genes may be exchanged, leading to phage diversity. Some genome phages or phage-like elements do not readily fall into any group: MGAS10394.2, HKU16.1, NZ131.1, m46.1, and MGAS10394.4 are phylogenetic outliers that have little commonality with other prophages. When the prophages are clustered by the M type of their host cell, some patterns do emerge (Figure 8). There appears to be a pool of phages shared by M1 and M12 *S. pyogenes* strains, for example. M3 strains have some phages that appear more frequently within this serotype, but the current sample size is too small to draw any definite conclusions. Indeed, more genomic data will be required to get a clearer picture of prophage distribution by serotype.

Prophage hyaluronidase (hyaluronate lyase)

A subset of genome prophages encode a hyaluronidase (hyaluronate lyase, hyaluronoglucosaminidase) gene (Table 3). Two alleles (*hylP* and *hylP2*) of this gene were originally identified (45, 72), and subsequent studies show that this gene exists in multiple alleles mainly distinguished by single nucleotide polymorphisms and by collagen-like domain indels in some variants (84, 85). The phage hyaluronidase gene found in the genome sequences also shows considerable diversity that may have resulted from recombination (45, 72, 84, 85). It had been suggested that phage hyaluronidase was a

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TABLE 2 Prophages of *S. pyogenes* and their integration sites and associated virulence genes

Target gene (attB)	Gene target for integration (*attB*)	Associated phages (virulence genes[a])	Identifier[b]
Single-stranded DNA binding protein recO	3′	MGAS10394.1 (*sdn*)	A
RNA helicase snf	3′	MGAS8232.1 (*speA1*)	B
Promoter of hypothetical Spy49_0371	5′	NZ131.1 (none identified)	C
Dipeptidase	5′	SF370.1 (*speC-spd1*) MGAS10270.1 (*speC-spd1*) MGAS10750.1 (*speC-spd1*) Man.4 (*speC-spd1*) MGAS2096.1 (*speC-spd1*) MGAS9429.1 (*speC-spd1*) MGAS8232.2 (*speC-spd1*) ND23.1 (*speC-spd1*)	D
tRNAarg	3′	MGAS10394.2(*speA4*)	E
dTDP-glucose-4,6-dehydratase	3′	SF370.2 (*speH-speI*) MGAS10270.2 (*spd3*) MGAS10750.2 (*spd3*) Man.3 (*speH-speI*) MGAS9429.2 (*speH-speI*) HKU16.3 (*speH-speI*) ND23.3 (*ssa*) NZ131.2 (*speH*)	F
CRISPR type II system direct repeat sequence	5′	MGAS315.1 (none identified) SPsP6 (none identified)	G
tmRNA	3′	T12 (*speA*) MGAS5005.1 (*speA2*) A20.1 (*speA2*) M1 476.1 (*speA2*) HSC5.1 (*speL-speM*) MGAS315.2 (*ssa*) SpsP5 (*ssa*) MGAS10394.3 (*speK-slaA*) MGAS8232.3 (*speL-speM*) ND23.4 (*spd3*) MGAS6180.1 (*speC-spd1*) Alab49.1 (*speL-speM*)	H
trmA	3′	m46.1 (mefA)	I
comE	ND[c]	MGAS10394.4 (mefA)	J
DNA-binding protein HU	5′	SF370.3 (*spd3*) MGAS5005.2 (*spd3*) A20.2 (*spd3*) M1 476.2 (*spd3*) MGAS315.3 (*spd4*) SPsP4 (*ssa*) MGAS10750.3 (*ssa*) Man.2 (*spd4*) MGAS10394.5 (*speC-spd1*) MGAS8232.4 (*spd3*) ND23.2 (*speI*) HSC5.2 (*spd3*) Alab49.2 (*speC-spd1*) MGAS1882.1 (*speK-slaA*)	K
Promoter of *yesN*	5′	MGAS10270.3 (speK-sla) MGAS315.4 (speK-sla) SPsP3 (speK-sla) MGAS6180.2 (speK-slaA)	L

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TABLE 2 (*Continued*)

Target gene (attB)	Gene target for integration (*attB*)	Associated phages (virulence genes[a])	Identifier[b]
Regulatory protein *recX*	5′	MGAS315.5 (speA3) SPsP2 (speA3) Man.1 (spd1) MGAS10394.6 (sda) Alab49.3 (spd3)	M
Putative gamma-glutamyl kinase	5′	MGAS315.6 (sdn) SPsP1 (sdn)	N
tRNAser	3′	MGAS5005.3 (sda) A20.3 (sdaD2) M1 476.3 (sdaD2) HKU16.2 (sdaD2) MGAS2096.2 (sdaD2) MGAS9429.3 (sda)	O
HAD-like hydrolase	5′	MGAS8232.5 (sda)	P
Excinuclease subunit *uvrA*	5′	HSC5.3 (spd3) HKU16.1 (ssa speC) MGAS10394.7 (spd3)	Q
Conserved hypothetical protein Spy49_1532	5′	NZ131.3 (spd3)	R
DNA MMR protein *mutL*	5′	SpyCIM1 (SF370.4) SpyCIM2 (MGAS10270.4) SpyCIM4 (MGAS10750.4) SpyCIM5 (man.5) SpyCIM6 (MGAS10394.8) SpyCIM25 SpyCIM28 (MGAS6180.3) SpyCIM53 (Alab49.4) SpyCIM59 (MGAS15252.1) SpyCIM59.1 (MGAS1882.1)	S
SSU ribosomal protein S4P	3′′	MGAS10270.5 (none identified) MGAS6180.4 (none identified) MGAS15252.2 (none identified)	T

[a]Prophage associated genes: superantigens: *speA*, *speC*, *speH*, *speI*, *speK*, *speL*, *speM*, *ssa*, and their alleles; DNases (streptodornases): *sda*, *spn*, *spd*, and their alleles; phospholipase: *sla* and alleles; macrolide efflux pump: *mefA*.
[b]Identifier for the phylogenetic tree in Fig. 5.
[c]ND, not determined.

potential virulence factor, but the crystallization and structural analysis of HylP1 from phage SF370.1 suggested that the function of this enzyme is to introduce widely spaced cuts in the bacterial hyaluronic acid to cause a local reduction in capsule viscosity and aid phage invasion during infection (62). Similar structural properties were observed in the hyaluronidase proteins encoded by prophages SF370.2 and SF370.3 (86).

Horizontal transfer of genes from other species

One observation to come from extensive genome sequencing is that the lysogenic phages of *S. pyogenes* share a gene pool with other streptococcal species, including those that are closely related (e.g., *S. equi*) as well as those more distantly related (*S. pneumoniae*). These shared genes include ones essential to the basic phage life cycle, such as capsid proteins, and virulence genes such as exotoxins and superantigens. For example, *S. equi* prophages share many superantigens or virulence genes with *S. pyogenes* phages, including *slaA*, *speL*, *speM*, *speH*, and *speI* (87). This study found that phage Seq.4 from *S. equi* strain Se4047 was very closely related at the DNA level to *S. pyogenes* Manfredo phage Man.3, including the two phage-encoded exotoxins. Similarly, *Streptococcus agalactiae* phage JX01, isolated in milk from cattle with mastitis, shares extensive homology with *S. pyogenes* prophage MGAS315.2 in the modules controlling DNA replication, tail, head-tail connector, head capsid, and DNA packaging, with over 97% amino acid identity between their terminase subunits; however, no virulence-associated genes were identified in this phage (88). The temperate phage MM1 of *S. pneumoniae* also draws from a common pool of structural genes, sharing the DNA packaging, the head-to-tail joining, and the tail genes with phage SF370.1 (89). Further, this phage and MGAS315.4 share tail and tape measure genes. Some of the oral streptococci also have phages that contribute to this common pool. The portal, terminase, major capsid protein, major tail protein, and tape measure protein of *Streptococcus mitis* phage SM1 share homology with prophage SF370.3 and a number of other phages of low-GC Gram-positive hosts (90). While acquisition of these phage structural genes from other streptococcal species may not directly impact virulence the same way that a novel exotoxin would, these capsid genes may help to expand the host range within the group A organisms. The dairy

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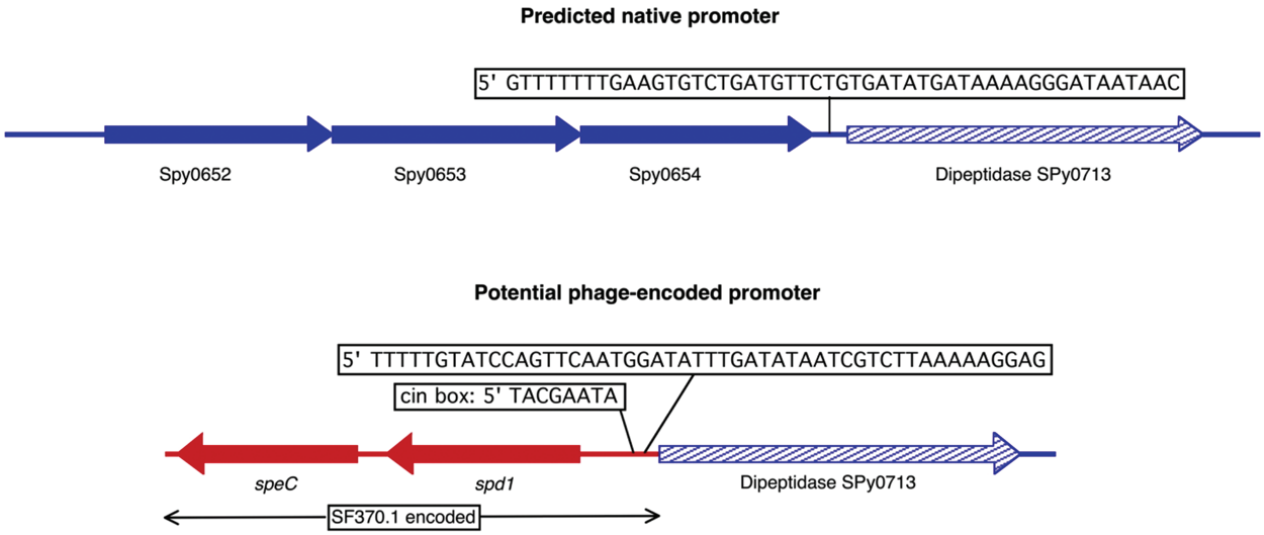


FIGURE 3 Integration of prophage SF370.1 may provide an alternative promoter for dipeptidase Spy0713. In strains lacking an integrated prophage at this site, the native promoter for dipeptidase Spy0713 is downstream of the uncharacterized gene Spy0654; the predicted sequence is shown above. Integration of phage SF370.1 into Spy0713 separates this gene from that of the native promoter, and a predicted promoter encoded by the prophage is now positioned in front of the dipeptidase ORF. This phage-encoded promoter is preceded also by a canonical CinA box (56), which is not part of the native promoter. Transcription of prophage virulence genes *spe*C and *spd1* is from the opposite strand and should not influence transcription of Spy0713. Promoter predictions were done using the online tool at http://www.fruitfly.org/seq_tools/promoter.html (110).

species *Lactococcus lactis* also is included in this pool of shared phage genes since prophage SF370.3 closely resembles the cos-site temperate phage r1t of *L. lactis* (64, 91).

S. PYOGENES PROPHAGES AND THE HOST PHENOTYPE

Prophages as Vectors for Virulence Genes

As described above, the link between bacteriophages and virulence in *S. pyogenes* may be traced to the earliest days of bacteriophage research. A considerable range of virulence-associated genes are carried by these prophages and prophage-like elements, including superantigens (*spe*A, *spe*C, *spe*G, *speH*, *speI*, *speJ*, *speK*, *speL*, SSA, and variants), DNases (*spd1*, MF2, MF3, and MF4), phospholipase A2 (*sla*), and macrolide resistance (*mef*A). It is quite common for a given prophage to carry more than one virulence gene, such as phage SF370.1, which contains both *spe*C and *spd1*. The number and diversity of prophage-associated virulence genes in *S. pyogenes* argues that these frequently play an important role in pathogenesis.

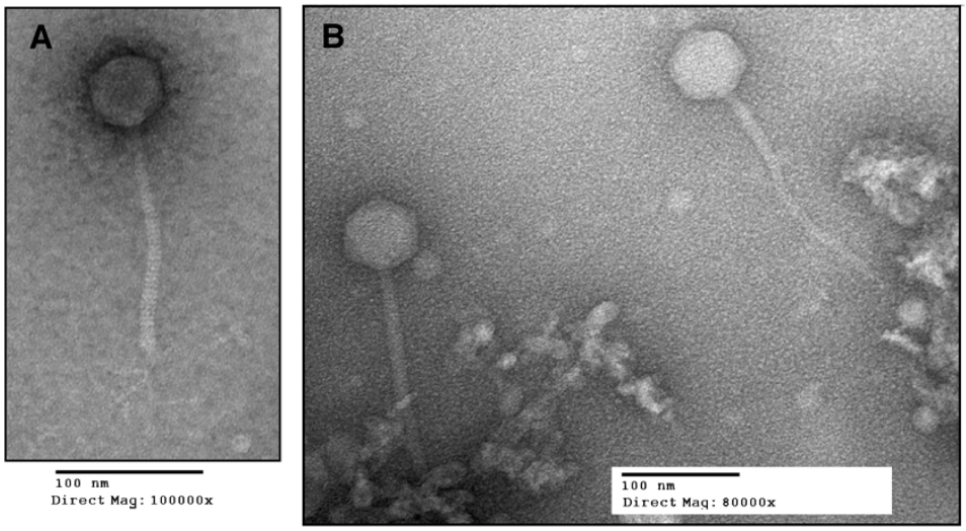


FIGURE 4 Morphology of streptococcal lysogenic phages. Prophages SF370.1 (A) and T12 (B) release typical *Siphoviridae* virions following induction. The SF370.1 head is about 55 nm across and the tail is 168 nm in length in this micrograph. In this image, the tail fibers that contain hyaluronate lyase (hyaluronidase) are visible. The T12 capsid has similar dimensions, with the head being about 66 nm and the tail length 196 nm. Electron micrographs provided by W.M. McShan and S.V. Nguyen.

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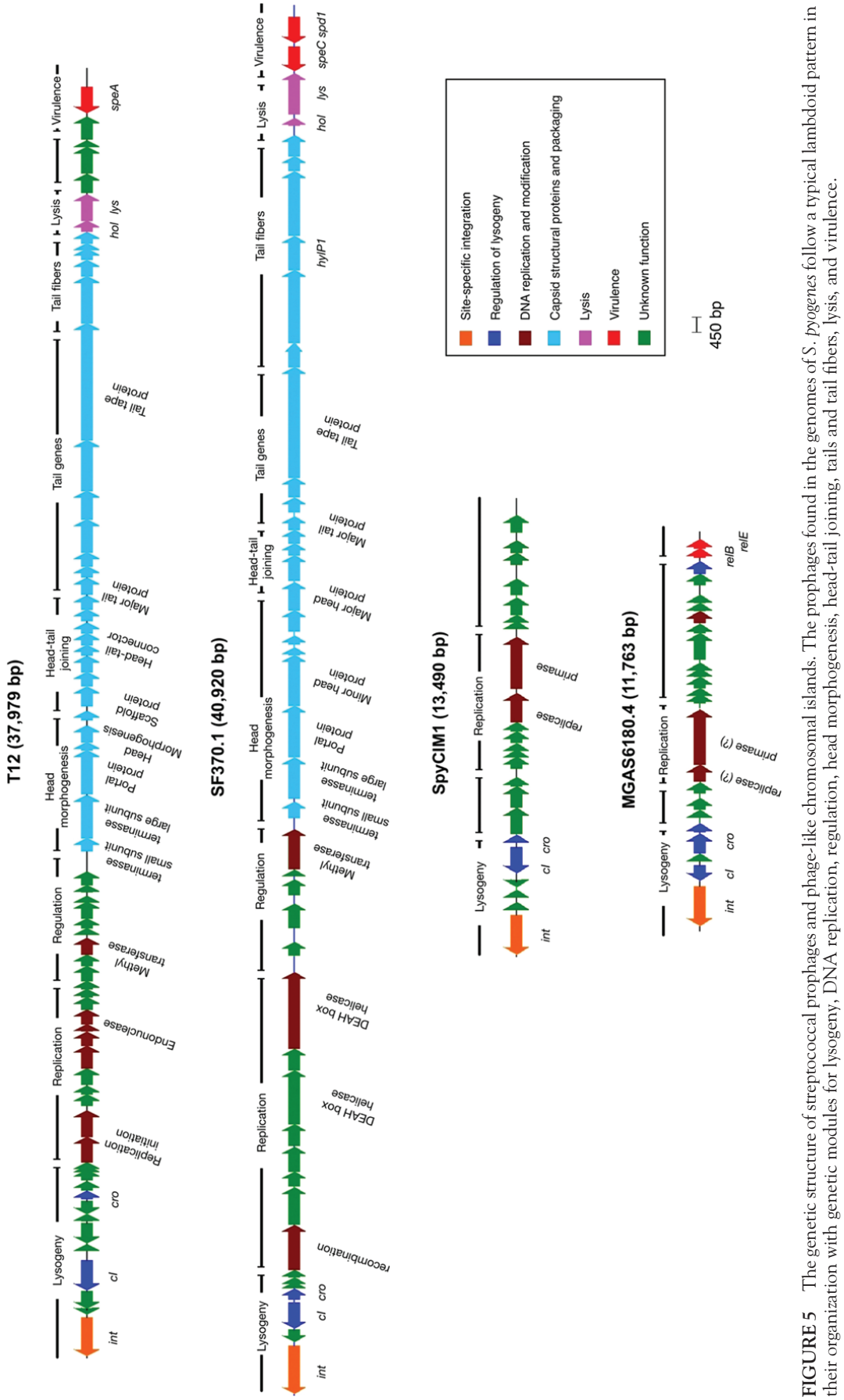
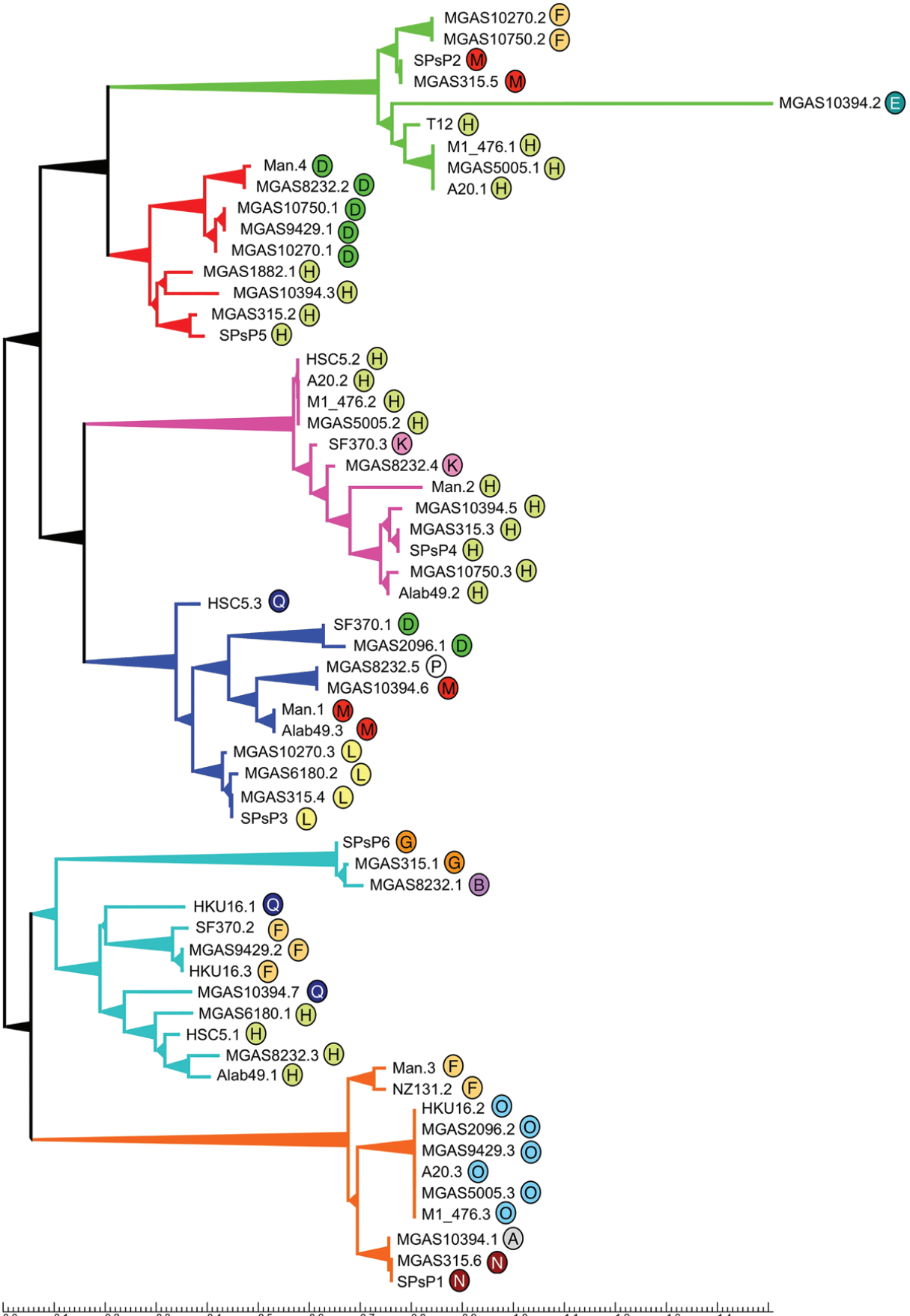


FIGURE 5 The genetic structure of streptococcal prophages and phage-like chromosomal islands. The prophages found in the genomes of *S. pyogenes* follow a typical lambdoid pattern in their organization with genetic modules for lysogeny, DNA replication, regulation, head morphogenesis, head-tail joining, tails and tail fibers, lysis, and virulence.

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TABLE 3 *S. pyogenes* prophages grouped by shared structural modules

T12 group	SF370.1 group	SF370.2 group	SF370.3 group	MGAS315.1 group	MGAS315.2 group	MGAS315.6 group	MGAS6180.4 group
MGAS5005.1	SF370.1[a]	SF370.2[a]	SF370.3[a]	MGAS315.1*	MGAS10270.1[a]	MGAS5005.3	MGAS10270.5
M1 476.1	MGAS10270.3[a]	MGAS10394.7[a]	MGAS5005.2	SPsP5*	MGAS315.2[a]	M1 476.3	MGAS6180.4
A20.1	MGAS315.4[a]	MGAS9429.2[a]	M1 476.2	MGAS8232.1*	SpsP6[a]	A20.3	MGAS15252.2
MGAS10270.2	SPsP3[a]	HKU16.3	A20.2		MGAS10750.1[a]	MGAS315.6[a]	
MGAS315.5	Man.1[a]	HSC5.1[a]	MGAS315.3[a]		Man.4[a]	SPsP1[a]	
SPsP2	MGAS10394.6[a]	MGAS8232.3[a]	SPsP4[a]		MGAS10394.3[a]	Man.3[a]	
MGAS10750.2	MGAS2096.1[a]	MGAS6180.1	MGAS10750.3[a]		MGAS9429.1[a]	MGAS10394.1[a]	
T12	HSC5.3[a]	Alab49.1[a]	Man.2[a]		MGAS8232.2[a]	MGAS2096.2[a]	
	MGAS8232.5[a]		MGAS10394.5[a]		MGAS1882.1[a]	MGAS9429.3[a]	
	MGAS6180.2[a]		HKU16.1[a]			HKU16.2	
	Alab49.3[a]		HSC5.2[a]			NZ131.2[a]	
			MGAS8232.4[a]				
			NZ131.3[a]				
			Alab49.2[a]				

[a]Contains a hyaluronate lyase (hyaluronidase) as a component of the tail fiber.

The expression of phage-encoded virulence genes, rather than an autonomous event, may be linked to the host streptococcal cell genetic background (92) or physiological state (93). For example, the reception of signals from co-cultured human cells influences *S. pyogenes* prophage virulence gene expression. Human pharyngeal cells release a soluble factor that stimulates expression of pyrogenic exotoxin C (SpeC) and phage DNase Spd1 as well as induction and release of phages by *S. pyogenes* grown in coculture (94, 95). Similar results were observed independently where the expression of prophage-encoded toxins SpeK and Sla was enhanced by coculture with pharyngeal cells (96). Eukaryotic cells also provide an environment that promotes the transfer of toxin-producing phages from a lysogen to a new host; this phenomenon can occur in either *in vitro* culture or a mouse model (97). However, the genetic background of the streptococcus influences whether the acquisition of a new prophage expressing DNase results in enhanced pathogenic potential of the resulting lysogen (92). Some of these bacterium-phage interactions appear to be linked to the cellular regulatory networks; for example, the *S. pyogenes* global regulator Rgg can control the expression of the phage-encoded DNase Spd3 by interacting with phage promoters of that gene (93). Alteration of streptococcal gene expression may also impact the levels of phage toxins released during an infection. Employing a murine subcutaneous chamber model, Aziz and coworkers showed that the expression of *S. pyogenes* protease SpeB diminished after extended colonization in the mouse; this loss of protease activity occurred with simultaneous enhancement of phage-encoded exotoxin SpeA and streptodornase expression (98). Interestingly, these altered expression patterns were independent events.

Phage-Like Elements that Carry Antimicrobial Resistance Genes

S. pyogenes harbors a number of genetic elements that appear to have phage sequences combined with sequences from transposons or plasmids and are vectors for antibiotic resistance. One example is Φm46.1, the main *S. pyogenes* element carrying the *mef*A and *tet*O genes (99). The chromosome integration site of Φm46.1 is a 23S rRNA uracil methyltransferase gene, and this phage has high levels of amino acid sequence similarity to Φ10394.4 of *S. pyogenes* strain MGAS10394.4 and λSa04 of *S. agalactiae* A909 (100, 101). The antibiotic resistance cassette of the PhiM46.1 family may be a recent acquisition: the lysogeny module appears split due to the insertion of a segment containing *tet*O and *mef*A into the phage DNA (99). Phage Φm46.1, besides being found frequently in *S. pyogenes*, has a broad host range that allows it to transduce antibiotic resistance to strains of *S. agalactiae*, *Streptococcus gordonii*, and *S. suis* (102). All of these species share a highly conserved *attB* site, which undoubtedly facilitates dissemination of this phage. Further, within GAS, Φm46.1 appears to be able to infect a wide range of M types (103). The ability of this phage family to mediate transfer of antibiotic resistance shows again how frequently *S. pyogenes* prophages modify the phenotypes of their host to improve fitness.

Prophages, Phage-Like Elements, and Regulation of Host Gene Expression

The impact of prophages on the host phenotype extends beyond toxigenic conversion. One early report noted the possible control of M protein and serum opacity factor expression

FIGURE 6 Phylogenetic relationships of *S. pyogenes* prophages. An unrooted phylogenetic tree was created by DNA alignment of the genome prophages. Prophages MGAS10394.2, HKU16.1, NZ131.1, m46.1, and MGAS10394.4 were so dissimilar from the other prophages that each occupied an independent branch; consequently, for clarity, they are not shown on the tree. The alignment organized the remaining prophages into six major branches, and the encircled letter identifier by each prophage refers to its associated attachment site (*attB*) described in Table 2; each identifier is colored to facilitate viewing. The groups are defined by shared modules for structural genes (Table 3). The tree was created using the software packages Clustal-omega and TreeGraph 2 (111, 112).

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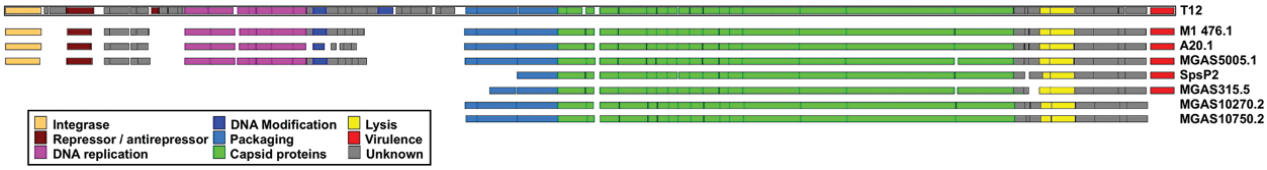


FIGURE 7 Shared genetic modules of the T12-related prophage family. The top line is the simplified genetic map of bacteriophage T12 colored by gene or genetic module for the integrase, repressor-antirepressor, DNA replication, DNA modification, DNA packaging, capsid proteins, lysis, and virulence (*speA*). Regions of unknown or uncertain function are colored gray. Beneath T12 is shown the genetic maps of the other genome prophages that share the extended region dedicated to packaging, capsid proteins, and lysis. DNA regions that are divergent from T12 are not shown. The figure illustrates that a structural gene module can be associated with divergent attachment sites or virulence genes. The alignment was derived from the phylogenetic tree presented in Fig. 5.

by a prophage or other extrachromosomal element that could be cured upon passage. In light of our current understanding of global transcriptional regulators, such a change suggests that the identified prophage could be interacting with or substituting for a regulator to mediate the changes reported in the older literature. The regulator Rgg has been shown to interact with several prophage-encoded genes. In addition to the DNase Spd3 mentioned above (93), Rgg has been shown to bind prophage-encoded promoters controlling genes for an integrase and a surface antigen (104). Recent studies suggest that the two-component regulatory system CovRS may also be influenced by prophage carriage. Many invasive strains of *S. pyogenes* often have mutations in *covRS*, and a subset of these invasive strains produce no detectable capsule at 37°C but produce capsule at sub-body temperatures. Brown and coworkers reported that the presence of prophage PhiHSC5.3 could rescue capsule production independent of temperature in a *covR*-defective strain but that this spontaneous capsule production was eliminated by curing of this prophage (105). Thus, in this case a prophage appears to act as a thermoregulator, possibly repressing capsule production at 37°C.

A mobile element frequently found in *S. pyogenes* genomes is the phage-like SpyCI, which integrates into the 5′ end of the MMR gene *mutL* (Fig. 9A) (52–54). The SpyCIs share integrase modules with related phage-like chromosomal islands from *Streptococcus anginosus*, *Streptococcus canis*, *S. dysgalactiae* subsp. *equisimilis*, *Streptococcus intermedius*, and *Streptococcus parauberis* (Fig. 9B) (52). The DNA replication module is even more widespread among other species, and in addition to the ones named above, is found in related chromosomal islands found in *S. agalactiae*, *S. mitis*, *S. pneumoniae*, *Streptococcus pseudopneumoniae*, *S. suis*, and *S. thermophilus* (52). The remarkable defining characteristic of SpyCIs is how they regulate MMR and the other genes in the operon (major facilitator family efflux pump *lmrP*, Holliday junction resolvase *ruvA*, and base excision repair glycosylase *tag*). The best-studied member of this family, SpyCIM1 from strain SF370, is a dynamic element that excises from the bacterial chromosome during early logarithmic growth and replicates as a circular episome (54). As the bacterial population reaches the end of the logarithmic phase and enters the stationary phase, SpyCIM1 reintegrates into the unique attachment site at the beginning of *mutL* ORF (Fig. 9A, insert). The result of this cycle is that SpyCIM1 acts as a growth-dependent molecular switch to control the expression of MMR, causing SF370 to alternate between a mutator and wild-type phenotype in response to growth: during rapid cell division and DNA replication, the integrity of the genome is maintained by an active MMR system, while during periods of infrequent cell division, mutations may accumulate at a higher rate (53, 54). Preliminary studies suggest that the SpyCIs, which lack identifiable structural genes, may employ a helper prophage for packaging and dissemination in a fashion similar to the well-characterized *S. aureus* pathogenicity islands (106).

More recent studies have shown that SpyCIM1 and related phage-like chromosomal islands also act as global regulators for host genes. The elimination of SpyCIM1 shifted the expression of over 100 bacterial genes involved in virulence and metabolism (107). These shifts in global expression occurred at both the logarithmic and stationary phases, upregulating some genes while repressing the expression of others. Some of the virulence genes with increased expression included the antiphagocytic M protein (*emm1*), streptolysin O (*slo*), capsule operon (*hasABC*), and streptococcal pyrogenic exotoxin (*speB*) (107). Other genes, such as the regulator *ropB*, arginine deiminase, and l-lactate oxidase, were depressed in expression in either early or late logarithmic growth or both. Several uncharacterized SpyCIM1 genes are predicted to encode proteins with helix-turn-helix domains, which could possibly act as DNA regulatory elements to control host genes. Further work will be needed to understand the molecular mechanism of SpyCIM1 as a regulator. Thus, phage-like chromosomal islands in *S. pyogenes* appear not only to mediate a growth-dependent mutator phenotype but also to be global regulators. This remarkable evolutionary adaption may help promote survival of *S. pyogenes* when faced with changing environments.

Prophages as Agents of Genome Plasticity

Our understanding of the role of prophages in shaping the biology of *S. pyogenes* continues to evolve. In *S. pyogenes* and many other bacterial species, symmetric genome rearrangements have been observed, and often these genome rearrangements from *S. pyogenes* patient isolates are associated with invasive infections. A hypervirulent M23 serotype strain, M23ND, was found to have a unique and extensive asymmetric rearrangement in its genome, these rearrangements being mediated by competence genes (rRNA-*comX*), transposons (IS*1239*), and conserved prophage genes (*hylP*) (108, 109). While these rearrangements did not affect genome integrity, some rearrangements did recluster a broad set of CovRS regulated genes to the same leading strands, suggesting a potential selective advantage to *S. pyogenes* by clustering genes for virulence, growth, and/or survival. An approximately 400,000-bp inversion was found to have occurred through shared hyaluronidase genes (*hylP*) between two different genome prophages; this inversion was predicted to prevent normal excision of either prophage due to the spatial disruption of the attachment site sequences. Remarkably, induction with mitomycin C led to reversal of the large inversion, allowing induction of these prophages to the lytic cycle. A subpopulation of survival cells was generated following induction that no longer had the large-scale inversion (108). Thus, genome prophages appear not only to

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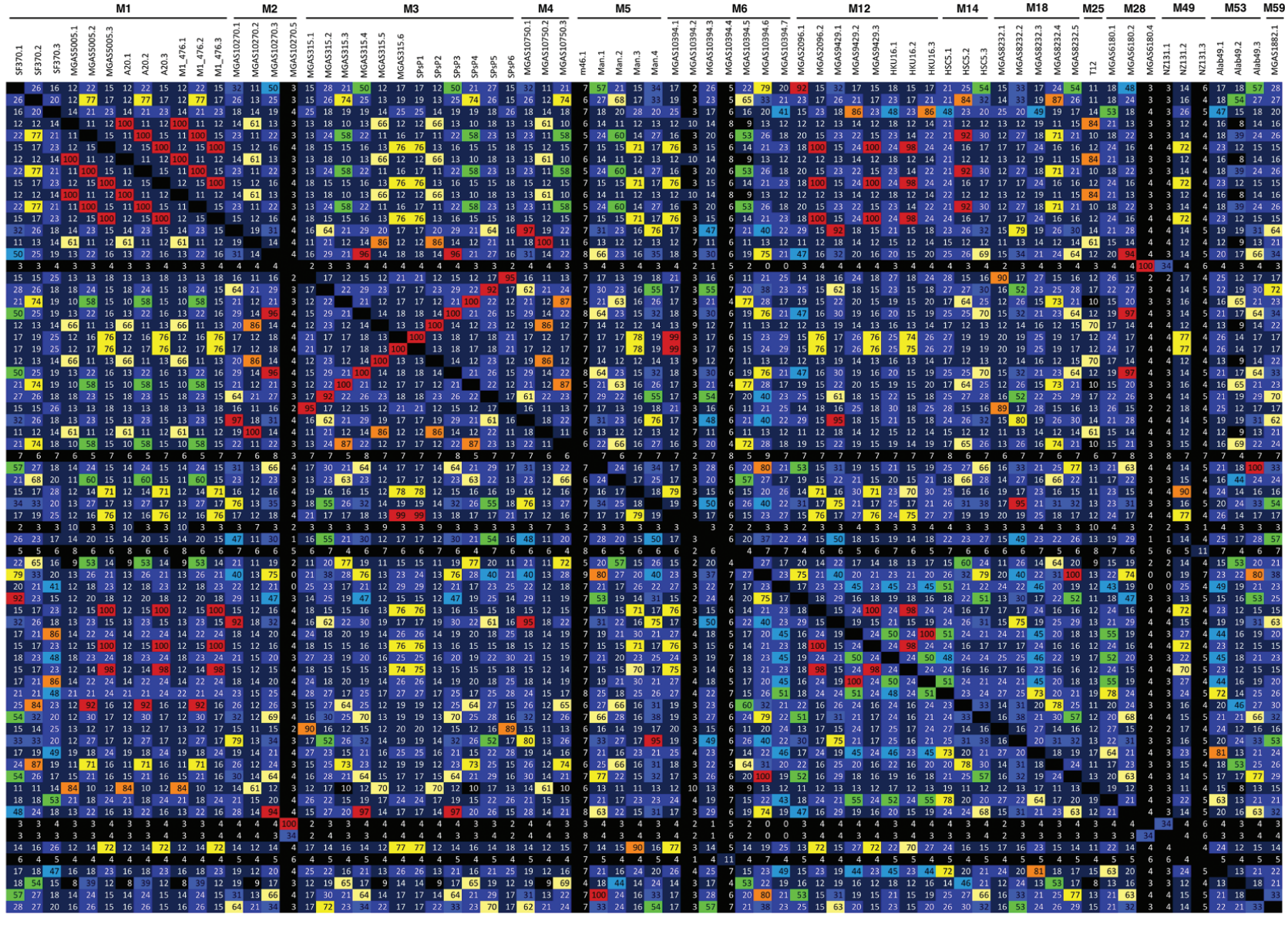


FIGURE 8 Identity matrix of genome prophages grouped by M type. The identity matrix presents the Clustal-omega DNA alignment from Fig. 5 as the percentage identity between genome prophages, which are grouped by the M type of their host streptococcus. The numbers within each cell represent the identity rounded to the nearest whole number, and the cell colors show the range into which each identity falls by increasing percentages of 10.

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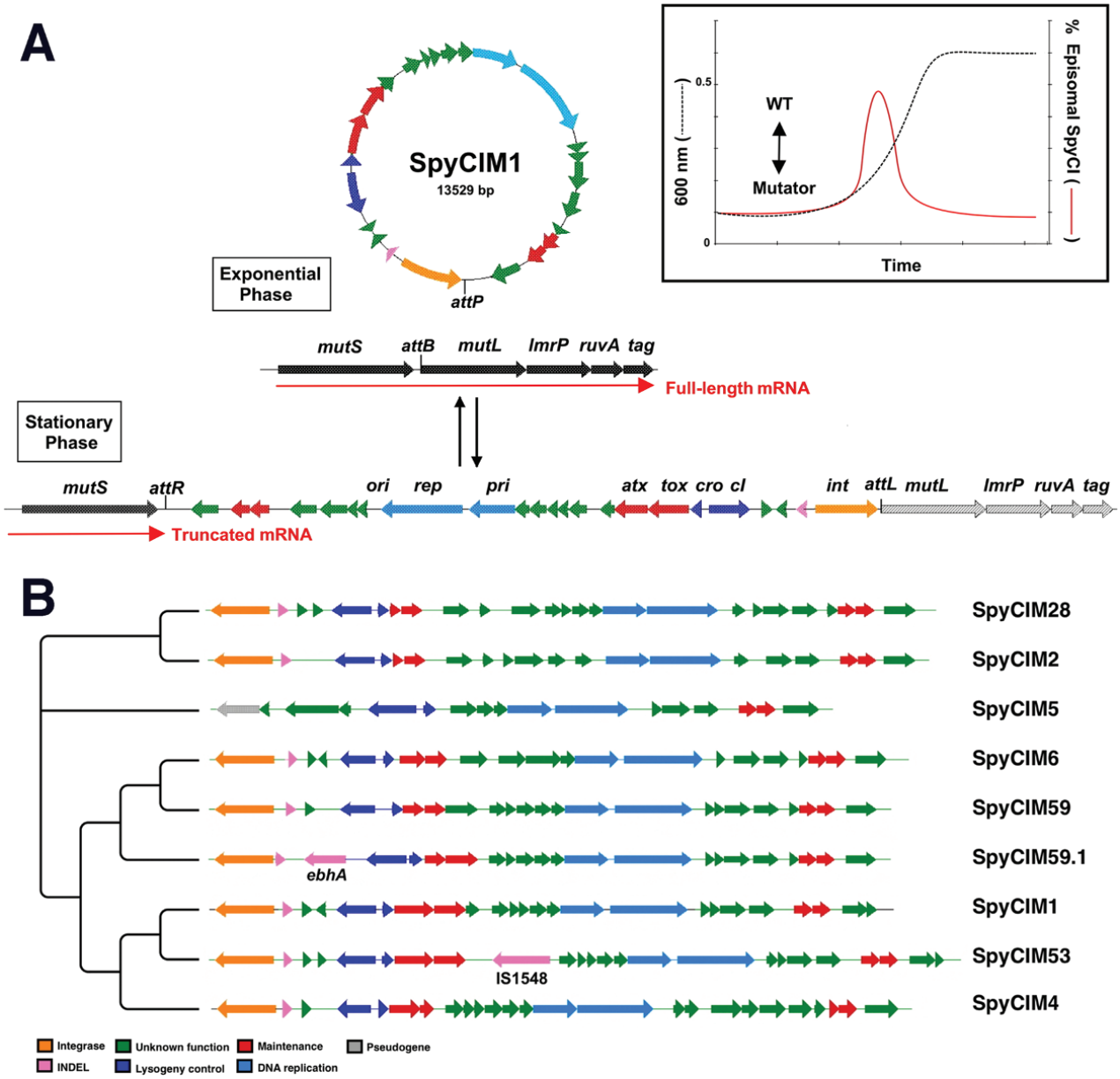


FIGURE 9 (A) SpyCIM1 regulation of the MMR operon through dynamic site-specific excision and integration. The MMR operon of *S. pyogenes* groups the genes encoding DNA MMR (*mutS* and *mutL*), multidrug efflux (*lmrP*), Holliday-junction resolvase (*ruvA*), and base excision repair glycosylase (*tag*). The orientation of this chromosomal region is shown here from the lagging strand to emphasize the MMR operon transcription. During exponential phase, SpyCIM1 excises from the chromosome, circularizes, and replicates as an episome, restoring transcription of the entire DNA MMR operon (WT). Excision and mobilization occur early in logarithmic growth in response to as yet unknown cellular signals (insert; adapted from Scott et al. [53]). As logarithmic growth continues, SpyCIM1 reintegrates into *mutL* at *attB*, and by the time the culture reaches the stationary phase, the integration process has completed, again blocking transcription of the MMR operon. WT, wild-type phenotype associated with unimpeded expression of the MMR operon. Reproduced from *Frontiers in Microbiology* (52) under the Creative Commons Attribution License (CC-BY 4.0). (B) The phylogenetic tree of the SpyCI DNA sequences is presented. The tree was created with TreeGraph 2 (111) using previously analyzed data (52).

improve the fitness of their host cell through the carriage of virulence genes, but also to play a role in genome plasticity.

CONCLUSIONS

The association of *S. pyogenes* with its bacteriophages is a major factor in the biology of this human pathogen, influencing the distribution of virulence genes, the spread of antibiotic resistance, the horizontal transfer of host genes, and the population distribution of cells. These relationships can range from simple predator-prey models to complex symbiotic associations and genome plasticity that promote the evolutionary success of both cell and phage. Further, in prophages the choice of integration site into the bacterial

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chromosome may alter the streptococcal genotype through either gene inactivation or replacement of normal promoter elements with phage-encoded ones. The similarity that prophages have to pathogenicity islands can hardly be overlooked, and the range of prophage-mediated characteristics that add to host survival or virulence can be easily predicted to increase as new investigations are reported. Genome sequencing has contributed greatly to our understanding of prophage distribution and genetic composition, and this bank of knowledge has been and will be an important foundation for future biological studies of the interactions of *S. pyogenes* and its phages that will be certain to reveal many novel and perhaps surprising relationships.

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Molecular Epidemiology, Ecology, and Evolution of Group A Streptococci

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12

ECOLOGY: HOST TISSUES AND DISEASES

The group A streptococcus (GAS; *Streptococcus pyogenes*) is a free-living organism. Its ecological niche appears to be quite narrow, and its only known biological host of import is humans. There may be occasional or very rare natural infections in non-human primates and other mammals (1, 2).

The primary sites for GAS colonization of the human host involve two tissues: the oropharyngeal mucosal epithelium of the upper respiratory tract (URT) and the superficial layers of the epidermis. GAS have no known environmental reservoir of significance, and transmission is almost exclusively person to person, via either respiratory droplets or direct contact. Thus, the throat and skin of the human host are the primary habitats for GAS. It is at these two tissue sites that GAS undergo successful reproductive growth and transmission of progeny to new hosts (Fig. 1).

Streptococcal pharyngitis and impetigo are superficial, self-limiting infections that usually cause a mild illness that resolves within 2 weeks, even if left untreated. Both are primarily diseases of childhood. Once colonization is established at the URT, the organism can cause a symptomatic pharyngitis, with or without tonsillitis. Symptomatic infection caused by GAS is typically characterized by a copious purulent exudate. Oropharyngeal infection can be cleared via a protective antibody that is produced by the host in response to infection. Alternatively, a "clinically inapparent" infection can arise wherein the infected individual lacks obvious clinical symptoms of illness, yet a specific immune response is mounted and directed to antigens of the infecting organism (3).

Another type of GAS-human interaction that can occur at the URT is asymptomatic carriage, where a vigorous host immune response to many/most GAS antigens is lacking (4). The carrier state can persist for weeks or months, and the organism (presumably) remains capable of transmission to a new host. However, relative to acute pharyngitis, the number of organisms present during carriage may be vastly reduced, and GAS are often in an altered dormant-like state signified by a relative lack of M protein expressed on the cell surface. Intracellular invasion of epithelial cells by GAS may contribute to persistent infection (5). Microcolony and biofilm formation, and the bacterial communities that arise, may also be a key part of the ecology of the GAS carrier state (6).

At the skin, GAS survive and replicate below the dry cornified layer, provoking a strong inflammatory response that gives rise to the purulent lesions of nonbullous impetigo, a form of pyoderma. Recovery of GAS from normal skin can precede impetigo by ~10 days (7, 8). However, it is not entirely clear whether there exists stable GAS colonization and/or a true long-term carrier state at the skin; uncertainty stems from the duration of GAS association with normal human skin in the absence of inflammation (i.e., disease), which may be transient (9). Breaks in the stratum corneum, which can be quite minor and barely noticeable, are a requirement for GAS to establish a firm foothold at this tissue site and cause disease. The scabies mite infests the epidermal layer just below the stratum corneum; scabies can cause intense itching and is a key risk factor in the development of GAS impetigo in many tropical communities (10–12). Also, it is not uncommon for *Staphylococcus aureus* to be a co-pathogen along with GAS in impetiginous skin lesions (13, 14). At least some strains of GAS that cause impetigo can subsequently spread from skin lesions to colonize the URT, peaking at 2 to 3 weeks post-skin infection (7, 8) (Fig. 1).

The relative incidence of GAS disease varies throughout the world in accordance with both season and locale. In the temperate regions of North America and Europe, pharyngitis is highly prevalent during the cold winter months, whereas impetigo thrives during warm, humid weather and is far less prevalent. In many tropical regions, such as the Northern Territory of Australia, GAS impetigo is far more common than pharyngeal infection among the indigenous population, and there are no discrete seasonal peaks in the incidence of disease (10). Climate and the level of hygiene strongly influence the prevalence of impetigo, whereas indoor crowding is a major risk factor for URT infection. These risk factors largely reflect the primary modes of transmission.

Worldwide there are an estimated >616 million cases of GAS pharyngitis per year (15), and >162 million cases of impetigo at any given time (16). In a study conducted ~40 years ago in the United States, for children between the ages of 5 and 7 years, it was estimated that an average of half experience one GAS infection annually (17). During the peak season, asymptomatic carriage rates at the URT range from (very) roughly 15 to 20% or higher; this value depends on the exact cohort (18). Bacteria tend to attain high numbers and densities during purulent infection. Large bacterial

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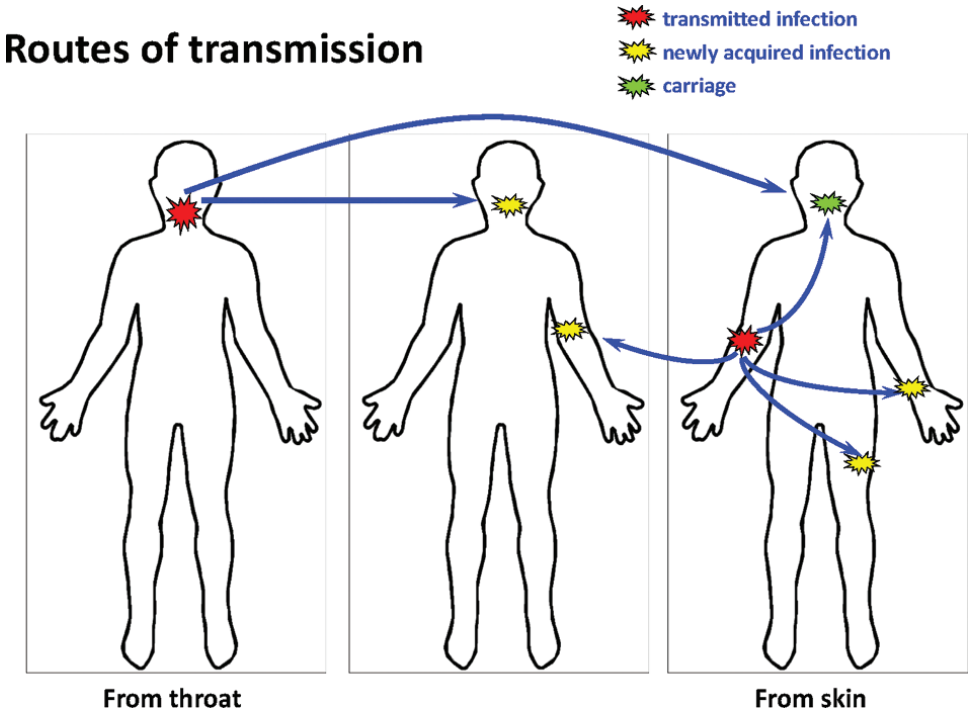


FIGURE 1 Transmission routes for GAS. From the throat, GAS can transmit via the respiratory route to a new host, where it either causes pharyngitis or persists in a quiescent carrier state. In a carrier state, the organism is presumed to be only weakly transmissible. Transmission from the throat to skin is relatively rare. From an impetiginous skin lesion, the organism can be transmitted by direct contact to the slightly damaged skin of a new host or to other damaged skin sites on the same host, causing multiple skin lesions. GAS can also be transmitted from a skin lesion to the throat of the same host, but it is widely assumed to enter a carrier state and is only weakly transmissible. From either the throat or skin, the organism can invade normally sterile deeper tissue, but this is rare relative to superficial infections.

counts yield large infective doses, which in turn can lead to the exposure of several susceptible hosts with the minimum infective dose, thereby augmenting transmission and spread.

A primary reason why GAS is a major public health problem is because of its ability to cause invasive (iGAS) disease and trigger nonsuppurative (i.e., non-pus-forming) sequelae. The latter diseases are of an autoimmune (acute rheumatic fever [ARF]) or immune complex (acute glomerulonephritis) nature. Collectively, the severe GAS diseases are responsible for >500,000 deaths each year (15).

In iGAS disease, bacteria gain access to deep tissue that is normally sterile. Consistent with pharyngitis providing the major GAS reservoir in temperate regions, iGAS disease is also more prevalent in these regions during the winter months (19). Intracellular invasion or paracellular transport of GAS at the URT may facilitate its access to the bloodstream. iGAS infection can be particularly severe in cases of necrotizing fasciitis and toxic shock syndrome. Although iGAS disease is associated with elevated rates of morbidity and mortality, its frequency of occurrence is relatively low. Population-based surveys of iGAS disease over the past 2 decades estimate the annual incidence at ~3 to 4 per 100,000 people per year in the United States and Europe, although more recently, the incidence of iGAS disease in the United States has increased to ~5 per 100,000 people per year (20); worldwide, there are an estimated 663,000 new iGAS cases per year (15). Thus, the overall incidence of iGAS disease is ~1,000-fold lower than the incidence of new pharyngitis and impetigo infections. Invasion of normally sterile tissue is often a result of "bacteremia without focus." When a lack of evidence for a superficial infection is coupled to GAS growth being largely restricted to deep tissue, and the patient is sequestered and nonambulatory, iGAS disease becomes a transmission dead end for the infecting bacterium.

From the ecological perspective of long-term evolution, GAS fitness is largely shaped by adaptations to its primary tissue reservoirs; acquisition of traits that enhance fitness in iGAS disease is probably coincidental. In North America, the composition of the iGAS strain population largely overlaps with the population of GAS strains recovered from superficial (pharyngitis) infections (21), although a few strains appear to have invasive indices at the more extreme high or low ends (22).

Cutaneous wound infections can be considered a mild form of iGAS disease, wherein the histopathology is distinct from nonbullous impetigo. Depending on the study, wound infections are occasionally classified under the broader umbrellas of "pyoderma" or "skin lesions." Ecthyma is a severe form of impetigo with extensive dermis involvement. Impetigo largely afflicts younger children and is mostly prevalent in communities with a warm humid climate (tropics, summer), whereas in contrast, GAS wound infections occur in all age groups and in all geographic regions, including those where URT disease predominates.

Transmission of GAS leading to cutaneous wound infections can occur from one tissue to another site within an individual (e.g., URT → broken skin), through shared skin-penetrating devices (e.g., needles), and possibly by direct

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person-to-person contact via open wounds. Skin contact may be an important mode of transmission in a recent GAS outbreak among adults that include intravenous drug users (IVDUs) and homeless people in a temperate zone (23, 24).

ECOLOGY: TRANSMISSION

The superficial nature of pharyngitis and impetigo provides GAS with an easy exit from infected hosts and an easy entrance into new hosts. Transmission is strongly influenced by the GAS agent and human host, coupled with both microenvironmental and macroenvironmental factors. The dynamics of both the GAS and human host populations largely dictate the scope of GAS disease outbreaks and epidemics.

Macroenvironment Risk Factors

As discussed above ("Ecology: Host Tissues and Diseases"), the primary environmental risk factors for GAS pharyngitis and impetigo are cold winters and tropical conditions, respectively. Copathogens that may elevate the risk for GAS infection include the scabies mite and *S. aureus* for impetigo and, possibly, undefined infectious agents for pharyngitis.

GAS-Microenvironment Factors

The microenvironments at the throat and skin differ from one another in numerous ways. Also, each tissue undergoes fluctuations in local conditions as a GAS infection evolves. To initiate infection, the GAS organism needs to be reasonably well adapted to the local tissue microenvironment. Distinctions between classic throat and skin strains of GAS (see "Epidemiology: Global Trends" below) are probably rooted, at least in part, in host tissue-specific adaptations. GAS cell surface components implicated in transmission and/or mediating colonization in a new host include the hyaluronic acid capsule and pili, among numerous other extracellular proteins (25–29). Yet the recent emergence and successful spread of *emm*89 iGAS strains is coupled to a loss in capsule (30, 31) (see "Epidemiology: Building Evolutionary Models" below). Conceivably, GAS transmission factors are strain specific.

At the URT, GAS initially encounter saliva and mucus secretions that contain mucins, secretory IgA, digestive enzymes, and antimicrobial peptides. At the broken surface just below the cornified layer of the skin, GAS face the early stages of wound healing: an inflammatory response in the epidermis is characterized by infiltration of low numbers of polymorphonuclear leukocytes and small amounts of plasma containing Igs and complement, plus antimicrobial peptides. As the infection evolves, the bacteria deploy virulence factors that are able to thwart host defenses, allowing GAS to undergo a net increase in growth, with a concomitant increase in infiltration by inflammatory cells leading to the accumulation of a purulent exudate (32).

Competition with normal flora is a key determinant of GAS colonization at the URT. In general terms, competition can be stiffest between highly related organisms. The closest genetic relative of GAS/*S. pyogenes* that is also a common human URT colonizer is *Streptococcus dysgalactiae* subspecies *equisimilis* (SDSE). SDSE usually have cell wall-associated group C or G group carbohydrate and are largely restricted to humans, with >60% genome sequence homology to *S. pyogenes* (33, 34). A few uncommon lineages of SDSE have group A (CHO-A) or group L carbohydrate (thus, the term "GAS" is largely equivalent to "*S. pyogenes*," and in this report, the two terms are completely equivalent unless otherwise noted). Recently, a widely disseminated and long-standing iGAS lineage of SDSE was described that appears to have arisen from a recombination event involving CHO-A biosynthesis genes from *S. pyogenes* (35).

SDSE bacteria are largely commensals of the oropharynx but are capable of causing many of the diseases attributed to *S. pyogenes* (36, 37). Like its close cousin, SDSE expresses capsules composed of hyaluronic acid, cell surface proteins that include M protein and T-antigens, and exotoxins such as streptolysin O; however, SDSE lacks some critical *S. pyogenes* virulence factors, such as the secreted cysteine proteinase SpeB. Besides the potential sharing of human host receptors for adherence to the epithelium, relatively little is known about how these two species—GAS and SDSE—compete for nutrients and other critical resources (38). Signaling between GAS, SDSE, and other streptococcal species has been demonstrated (39), and GAS are susceptible to killing by bacteriocins produced by other streptococcal species (40). Much remains to be uncovered regarding the role of the microbiome in shaping the success of GAS in colonizing and/or causing infection at the URT.

GAS physiology is heavily influenced by the nature and concentration of carbon sources (e.g., glucose), oxygen, and its toxic byproducts, among numerous other factors present in different tissues under shifting conditions (41, 42). Changes in the local microenvironment, coupled with increased bacterial density and nutrient depletion as the organisms reproduce and yield new progeny, impact the transcriptional program of the GAS cell, which in turn directs activation or repression of numerous genes associated with pathogenesis, and presumably transmissibility as well (43).

Acquisition of GAS at the URT does not necessarily lead to an infection, and in the asymptomatic carrier state, the local microenvironment is substantially different from the purulent environs. In the carrier state, virulence factor gene expression and the requirements for adaptation appear to be quite different (e.g., 44, 45).

Human (Host) Factors

In general terms, successful transmission of a pathogen depends on the availability of susceptible hosts, as well as the ratio of susceptible to resistant hosts in the context of the number of person contacts and potential exposures (46). Host genetic susceptibility (or resistance) to superficial GAS infections has not been characterized, and evidence for its existence is largely lacking.

Highly efficacious immunological protection against GAS infection is strain specific (i.e., M type-specific) (47), although a host immune response directed toward numerous other antigenic targets can confer at least partial protection (48, 49). Herd immunity is probably a critical host determinant for circumscribing the degree to which a given GAS strain can successfully transmit and spread within a human population. Thus, the possibility of future immunization with an M type-based vaccine is likely to reshape the composition of the GAS strain population (see "Epidemiology: Strategies for M Protein-Based Vaccines" below).

GAS (Agent) Factors

A key open question is whether GAS engage in a form of evolutionary bet-hedging via transmitting a bolus of organisms with a diverse array of transcriptional profiles, to increase the odds that at least one physiological form will colonize and thrive within an individual host. Alternatively, perhaps there exists a singular transcriptional profile that is

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deemed optimal for transmission within the wider human host population. Or perhaps a mixture of both strategies is used. Mutations in transcriptional regulatory genes and/or promoter regions may enhance the transmissibility of GAS (see "Evolution: Short Term" below).

GAS exhibit very high diversity in their cell surface proteins, in terms of both the presence or absence of a given protein, and in the sequence heterogeneity among products of the same locus. Several of the heterologous cell surface proteins may impart tissue-specific adaptations (reviewed in reference 50, 51). They also serve as key epidemiological markers (see next section). Genetic changes that lead to alterations in the antigenic composition of the GAS cell surface may aid the organism in escaping herd immunity.

EPIDEMIOLOGY: MOLECULAR MARKERS

The use of serological typing to categorize the pathogens of the *Streptococcus* genus (i.e., cell wall group carbohydrate), as well as the strains within a pathogenic streptococcal species, has a long and deep history. Unlike pneumococci, GAS lack a structurally diverse and immunogenic polysaccharide capsule. Instead, GAS express several cell surface proteins which collectively impart a broad repertoire of antigenic diversity. Underlying the serological phenotype is an extensive history of horizontal gene transfer (HGT) and recombination among the cell surface protein genes, plus accumulated point mutations, that has been shaped (at least in part) by the strong diversifying selection pressures of the host immune response. The antigenic structure of GAS cell surface proteins, and the underlying genotype of both the cell surface protein and core housekeeping genes, is highly complex.

Numerous typing schemes have been used to aid in understanding the epidemiology and underlying organization of the *S. pyogenes* species. A summary of typing (and related) schemes, and their applications and limitations, is provided in Table 1. Each epidemiological molecular marker is discussed in detail below.

M Protein

During the 1920s, work began that aimed at understanding the basis for protective immunity to GAS infection. Antibodies raised to extractable surface antigens, called M proteins, led to opsonophagocytosis of the strain from which the M protein was derived (52). However, antibody directed to the M protein of one organism often failed to confer protection against many other GAS isolates tested. A serological typing scheme arose through the development of antibodies directed to M proteins of different isolates. More than 80 distinct M types were identified, and protective immunity to GAS infection is, in large part, M type specific.

Copurifying with the M type-specific material was M-associated protein (MAP), which has two major antigenic forms (53, 54). The MAP I antigen was largely associated with ARF isolates, and MAP II was highly prevalent among serum opacity factor (SOF)-producing organisms. The MAP antigens were subsequently identified as part of the conserved C repeat region (CRR) of M protein (55), which occupies the COOH-terminal half of the surface-exposed portion of M protein. The CRR has two major antigenic forms known as class I and class II (56). The structure of M protein is depicted in Fig. 2A.

emm types

A nucleotide sequence-based *emm* typing scheme that closely parallels the M serotype scheme was developed ~2 decades ago (57). *emm* typing is based on the extensive nucleotide sequence differences at the 5′ end of the *emm* gene, whereby a unique *emm* type is defined as having <92% nucleotide identity to any other *emm* type, over the sequence corresponding to the first 30 codons of the mature M protein (Fig. 2A). Virtually all contemporary epidemiological studies define GAS isolates according to their *emm* type. Currently, there are ~243 recognized *emm* types (58).

Identification of the *emm* gene is not always obvious, and the problem can be due to paralogous *emm*-like genes mapping immediately upstream (*mrp*) or downstream (*enn*) of *emm* in numerous GAS strains (see next section; Fig. 2B). Confusion about *emm* type assignments has arisen wherein *enn* is directly fused to the 5′ end region of *emm* as the result of DNA excision arising from recombination between the *emm* and *enn* genes.

Among the ~243 *emm* types recognized to date, there are ~1,632 distinct allelic forms of the *emm* type-specific region, known as *emm* subtypes (58). The *emm* subtype alleles are based on sequence differences relative to the typing reference, within a 180-bp segment corresponding to the last 10 codons of the predicted signal peptide coding region and the first 50 codons corresponding to the NH_2-terminus of the mature M protein molecule (59).

emm patterns

Many strains of GAS have *emm*-like genes (*mrp*, *enn*) positioned immediately upstream and/or downstream of *emm* on the chromosome (60–63). Near the 3′ end of each *emm* and *emm*-like gene lies a region that encodes one of four divergent cell wall-spanning domains that can be distinguished by oligonucleotides (60, 64). Using a PCR-based mapping approach, the content of *emm* and *emm*-like genes and their arrangement on the chromosome was determined for GAS isolates of many different *emm* types. The resulting *emm* region maps are referred to as "*emm* patterns"; three major *emm* pattern groupings are recognized (Fig. 2B). M proteins of *emm* pattern E tend to be class II, whereas the *emm* patterns A-C and D groupings largely overlap with the class I CRR (60).

To date, the *emm* pattern genotype has been ascertained for GAS isolates of 170 of the ~243 recognized *emm* types (Table 2). With only two exceptions uncovered thus far (*emm54*, *emm218*), multiple isolates sharing the same *emm* type also share the same *emm* pattern grouping. The majority of *emm* types are represented by *emm* pattern groups D and E, accounting for >75% of the *emm* types analyzed.

emm clades and clusters

An *emm* cluster typing system was recently developed, based on the entire surface-exposed portion of M protein and its capacity to bind six human serum proteins (65). Phylogenetic analysis of >1,000 *emm* genes corresponding to 174 *emm* types reveals 2 main clades (X and Y), and 16 well-supported clusters, accounting for 82% of the *emm* types analyzed. The relationships between *emm* clades, clusters, and patterns for 174 *emm* types are illustrated in Table 3. Nearly all pattern E *emm* types (98%) belong to clade X, whereas 92% of pattern A-C *emm* types are clade Y.

In contrast to patterns A-C and E *emm* types, the pattern D *emm* types are present in both clades X and Y (Table 3) (65). The highly specialized plasminogen-binding *emm* cluster D4 of clade Y represents the largest *emm* pattern D grouping (n = 30 *emm* types). Within clade X, *emm* clusters E5 and E6 constitute a second group of pattern D *emm* types (n = 14); these two clusters include *emm* types of other pattern groups as well. Most of the remaining pattern D *emm*

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TABLE 1 Epidemiological typing methods for group A streptococci[a]

Scheme	Description of method	Applications	Limitations
M serotyping	Immunoprecipitation, using hyperimmune rabbit antisera	No longer in wide use	
emm typing	Sequencing, yielding >92% nt identity over the first 90 bases encoding the processed (mature) M protein of an *emm* type reference strain	Most commonly used typing method; highly reproducible; low cost; large database at CDC; can be used to infer *emm* clusters and *emm* pattern groups with reasonable accuracy	Based on oligonucleotide primers (via PCR); needs better integration with whole-genome sequencing projects
emm subtyping	Sequencing of 180 nt corresponding to the 10 COOH-terminal codons of the signal sequence and first 50 codons of the mature M protein	Further stratifies *emm* types into unique alleles; performed in parallel with *emm* typing; large database and BLAST server are maintained at the CDC	
emm pattern grouping	PCR-based mapping, based on 3′ ends of *emm* and *emm*-like genes	Assignment of organisms of known *emm* types into groupings that correspond reasonably well to tissue site preferences for superficial infection at the throat and/or skin	Inferences based on *emm* type may occasionally be incorrect; ~30% of *emm* types await *emm* pattern assignment; PCR-based mapping is laborious and limited by design of "universal" oligonucleotides
emm cluster typing	Based on phylogeny and four bioinformatic criteria, related to surface-exposed portion of M proteins; corresponds to binding by several human serum proteins	Used to extend information on *emm* typing; can be easily inferred from *emm* type; binding motifs can be used to infer binding properties of M proteins	Inferences based on *emm* type may occasionally be incorrect; ~29% of *emm* types await *emm* cluster assignment
SOF typing	Inhibition of the serum opacity reaction by hyperimmune rabbit serum	No longer in wide use	
sof typing	Sequencing of ~700 bp region near 5′ end of *sof*	Data can be generated though sequencing of PCR products or whole-genome sequencing	*sof* type assignment needs to undergo standardization; evidence for extensive recombination within the *sof* type region
T serotype	Bacterial cell agglutination, using hyperimmune rabbit antisera	In limited use by specialized laboratories, to supplement *emm* typing	Inconsistent results; labor-intensive; hyperimmune serum is limited and difficult to standardize
FCT region typing	PCR-based mapping; whole genome sequence	Can be used as an initial step in further characterization of pilin genes	Assignment of the FCT region needs to undergo further definition and standardization
FCT gene typing	Sequenced-based		Under development for all pilin subunit and adhesin genes
MLST	Nucleotide sequence of seven (partial) housekeeping genes, from PCR products or whole-genome sequences	Used to define "clones" (i.e., STs) and clonal complexes (i.e., CCs); often coupled with *emm* type; alleles are assigned via simple web-based user queries and curation; database of alleles, STs, and isolates is maintained on PubMLST server (Oxford)	Provides only a small snapshot of the core genome; isolate database is skewed toward unique genotypes (i.e., not population-based)
Whole-genome sequencing	Complete or partial genome assembly	Phylogenetic relationships of closely related organisms via the core genome sequence; investigations of local outbreaks and widespread epidemics; routine surveillance; pipelines for analysis of typing and antibiotic resistance genes	Core genome definition lacks standardization for the diverse range of strains in the species; assembly problems in regions of extensive sequence diversity and/or recombination; bioinformatic analysis can be time-consuming

[a]nt, nucleotide.

types are interspersed throughout clade Y (n = 16). Overall, the *emm* cluster scheme, based on the phylogeny of the entire surface-exposed portion of M protein, is highly consistent with the *emm* pattern scheme that is based on the COOH-terminal peptidoglycan-spanning domain of *emm* and *emm*-like flanking genes.

The *emm* cluster typing system is easy to implement since the *emm* cluster can be inferred from the *emm* type (66–69). Importantly, the *emm* type → *emm* cluster scheme is also highly informative of the functional capacity of the M protein molecule, based on consensus binding motifs for numerous human host plasma proteins (65).

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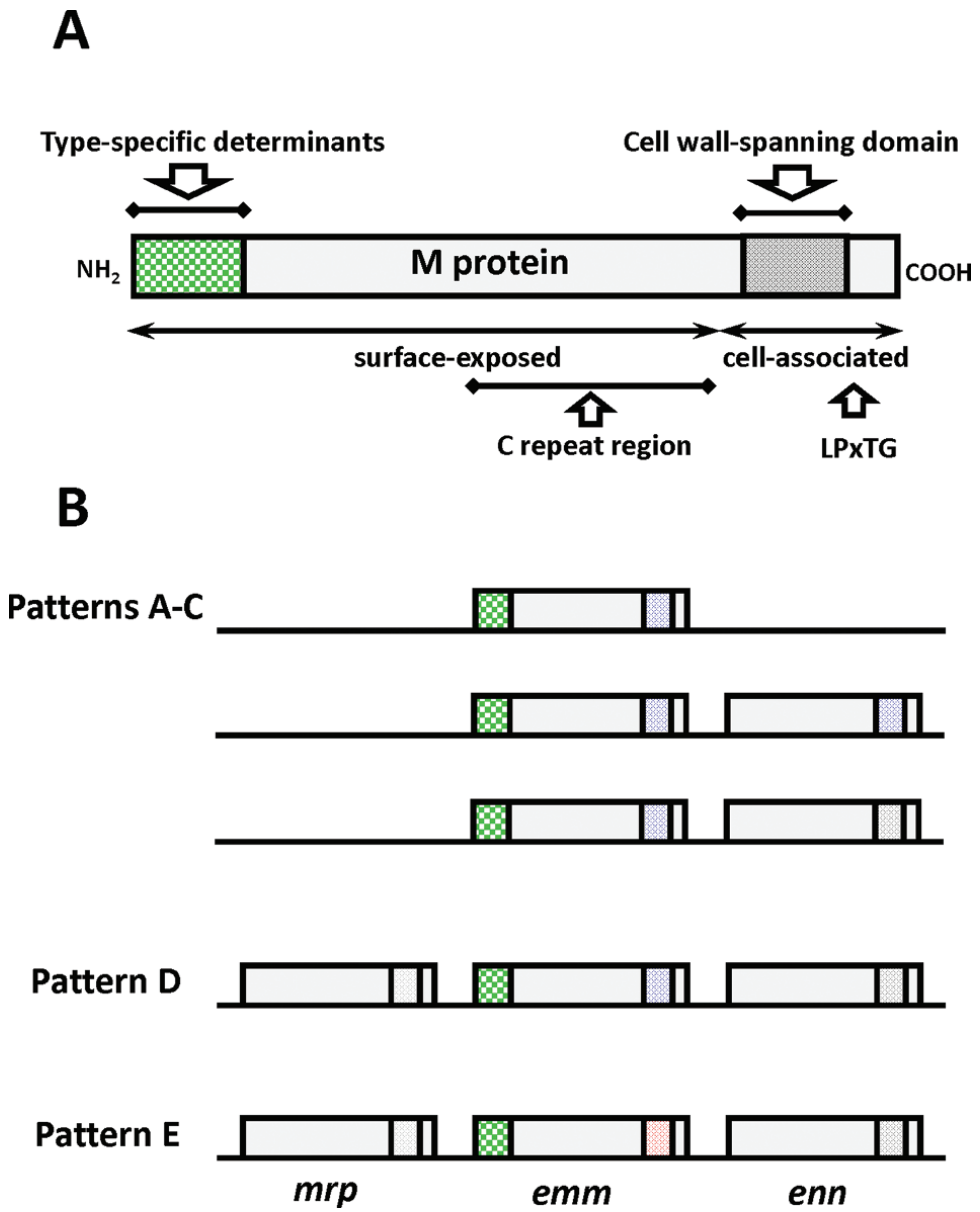


FIGURE 2 M protein structure and *emm* pattern gene arrangements. (A) Key features of M protein are shown, including the type-specific determinants, cell wall-spanning domain, and C repeat region. (B) Chromosomal arrangement of *emm* and the flanking *emm*-like genes (*mrp*, *enn*) gives rise to five *emm* patterns, which form three main groupings (A-C, D, E). Transcription of *emm* and *emm*-like genes is positively regulated by Mga, which is encoded by *mga*, which lies immediately upstream of the *emm* region.

M protein functional domains

As summarized in recent reviews (63, 70), the surface-exposed non-type-specific portion of M proteins (i.e., central region) contains functional domains that bind human host proteins such as IgG (various subclasses), IgA, plasminogen, and fibrinogen. The M type-specific determinants at the NH_2-terminus bind two complement regulators, C4b-binding protein (C4BP) and factor H-like 1.

Binding capacities strongly correlate with *emm* cluster (65). For example, binding of human IgG is mostly restricted to M proteins of *emm* clusters E1 through E6. Similarly, binding of C4BP is limited to M proteins of *emm* clusters E1, E3, E4, and E6. IgA binding is evident only in *emm* clusters E1, E4, and E6 products, whereas fibrinogen binding is restricted to M proteins of clade Y, and plasminogen binding is restricted *emm* cluster D4 (51, 65).

Although much less is known about the M-like proteins Mrp and Enn (Fig. 2B), binding properties shared with M protein have been described (reviewed in reference 63), and in some instances, they may use similar binding motifs. Additional investigation of the structure and functional binding properties of the M-like proteins, and structure of the *mrp* and

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TABLE 2 Distribution of *emm* types among *emm* pattern groups for 170 *emm* types.

emm pattern group	No. of *emm* types represented	% of total
A–C	35	20.6
D	64	37.6
E	67	39.4
Rearranged (REA)	2	1.2
A–C and D	2	1.2

enn genes, may eventually lead to a higher-resolution typing scheme that includes the entirety of the *emm* chromosomal region.

Serum Opacity Factor

SOF promotes the opacification of serum, wherein the serum opacity reaction can be neutralized by hyperimmune antiserum produced in animals (71). Not all GAS strains produce SOF, but SOF typing was often used as an adjunct for strains that posed challenges for M typing. Of note, SOF producers have a strong association with strains that harbor the MAP II antigen (72). In addition, SOF-producing strains tend to exhibit diminished beta-hemolysis (73).

More recent studies show SOF to be a proteinaceous virulence factor that is either bound to the GAS cell surface or secreted. SOF has an enzymatic domain that interacts with high-density lipoprotein, leading to the opacification of serum, plus a fibronectin-binding domain (74–77). The genes encoding M protein (*emm*) and SOF (*sof*) map ~15 kb apart on the GAS chromosome; as with *emm* and *emm*-like genes, transcription of *sof* is positively regulated by the Mga regulon (78, 79). *sof* types have been defined based on ~600 to 750 bp positioned near the 5′ end of the *sof* gene (80). However, *sof* typing is far less developed than *emm* typing, and attempts to generate a well-supported phylogeny for the *sof* type-specific determinants have largely failed due to extensive intragenic recombination (81).

The relationships between the presence or absence of SOF activity, or the *sof* gene, and the *emm* pattern and *emm* clade as inferred from the *emm* type, are summarized in Table 4. Based on the strong associations between the MAP II material, class II M proteins, and *emm* pattern E (60), it is not surprising that there are strong correlations between *emm* pattern E and the presence of the *sof* gene and/or a positive serum opacification reaction (50, 80, 82, 83). Exceptions include several *sof*-positive strains within *emm* cluster E6 that are assigned to the *emm* pattern D grouping via PCR-based mapping (*emm59*, *emm81*, *emm85*); *emm12* isolates (pattern A-C) have an inactive *sof* gene. Despite the close genome map positions of *sof* and *emm*, a single *emm* type can be found in association with more than one *sof* type and vice versa (80), indicative of HGT of *emm* or *sof* to new genetic backgrounds and intraspecies recombination within the greater *emm* region.

T-Antigens

T-typing is based on agglutination of trypsin-treated GAS cells with hyperimmune rabbit serum (84, 85). The T-typing antisera is produced by immunization with trypsin-treated GAS cells, followed by adsorption of antisera to undigested GAS cells of different T types; ~22 T types have been serologically defined. The T6 antigen gene (*tee6*) was the first one

TABLE 3 Distribution of *emm* clusters relative to *emm* pattern groups, for 174 *emm* types

emm cluster	*emm* clade	No. of *emm* types	No. of *emm* types: pattern A–C[a]	No. of *emm* types: pattern D	No. of *emm* types: pattern E	Other *emm* pattern (no. of *emm* types)[b]
E1	X	5	0	0	5	
E2	X	15	0	0	15	
E3	X	19	0	0	18	ND (1)
E4	X	17	0	0	17	
E5	X	7	2	3	0	REA (2)
E6	X	18	1	11	6	
singletons	X	4	0	1	3	
D1	Y	3	0	2	0	A–C and D (1)
D2	Y	5	0	5	0	
D3	Y	2	0	2	0	
D4	Y	31	0	30	0	ND (1)
D5	Y	3	1	2	0	
A-C1	Y	2	2	0	0	
A-C2	Y	2	2	0	0	
A-C3	Y	5	5	0	0	
A-C4	Y	5	5	0	0	
A-C5	Y	3	1	0	0	ND (2)
Singletons	Y	22	15	5	0	A–C and D (1); ND (1).
Outliers	Outlier	6	2	3	1	

[a] *emm* pattern assignments are based on references 65, 126, and 127.
[b] ND, not determined; REA, rearranged.

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TABLE 4 Relationships between SOF/*sof* and *emm* patterns and clades[a]

emm type-based grouping	No. of *emm* types	% SOF activity positive	% *sof* gene positive	SOF/*sof* positive *emm* types	SOF/*sof* negative *emm* types
Pattern A–C	22	0	5.6	12[b]	All others
Pattern D	42	7.3	7.1	59, 81, 85	All others
Pattern E	48	97.9	97.9	All others	15
Clade X	57	87.7	87.7	All others	15, 34, 42, 51, 65, 67, 99
Clade Y	56	0	1.8	12	All others

[a]Based on analysis of 113 *emm* types, as reported in reference 83.
[b]The SOF product of *sof12* is typically inactive.

cloned (86) and was subsequently mapped to a region of the GAS genome that had been previously characterized as having genes encoding microbial surface components recognizing adhesive matrix molecules that bind fibronectin and collagen (87–89). The acronym FCT (fibronectin-binding, collagen-binding, T-antigen) is widely used to describe this genome region (87). SDSE isolates also express similar T-antigens (90), as do group B streptococci (91, 92).

T-antigens are equivalent to pili. Within the FCT region lie several genes that contribute to the production of pili whose assembly is mediated by sortases (48) (reviewed in references 93–95). Seven FCT region forms are depicted in Fig. 3 (82, 92); the FCT-3 and FCT-4 regions are associated with the vast majority of *emm* types (82). For the most prevalent FCT region forms (i.e., FCT-1, 3, 4, and 5) found among GAS strains of different *emm* types, the pilin backbone (BP) proteins and many of the ancillary pilin (AP) proteins that act as adhesins display extensive sequence heterogeneity (87, 92, 96).

The relationships between T-typing sera and recombinant pilin proteins has been examined (48, 92, 97). In perhaps the most thorough study to date, using 15 T-typing sera, agglutination of trypsin-treated GAS cells corresponds most closely to reactivity with both the BP and AP1 (presumptive adhesin) subunits by immunoblot (92). For a T3/13/B3264 strain (Alab49; FCT-3), the recombinant AP1

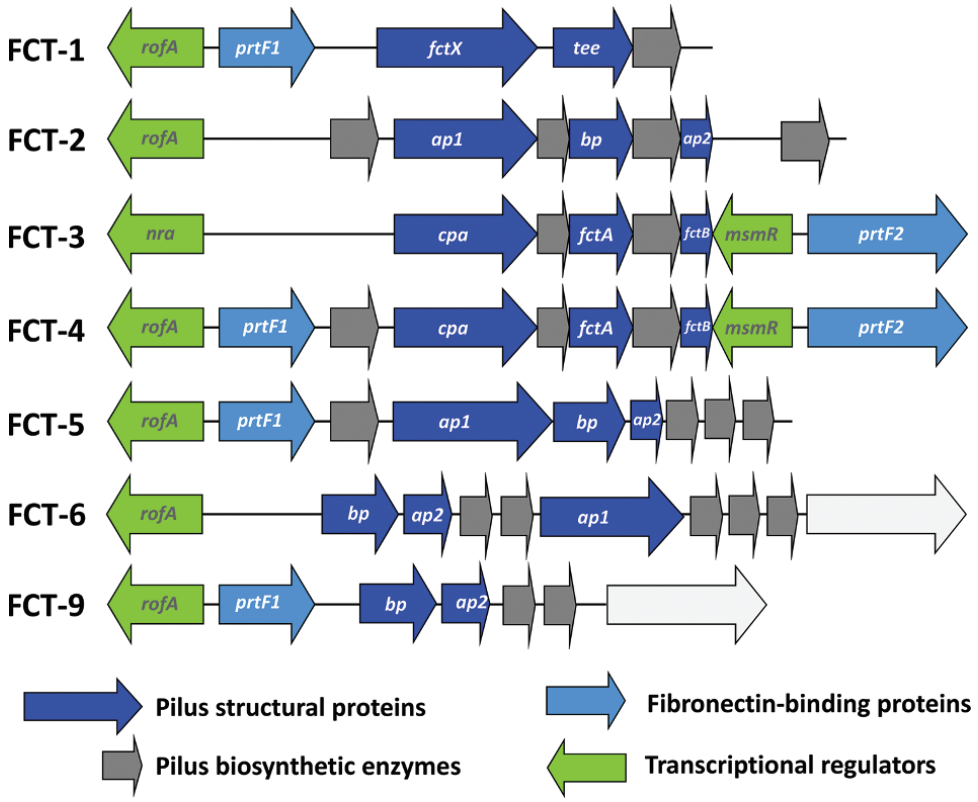


FIGURE 3 Structure of the pilus-encoding FCT region. Seven forms of the FCT region are shown. For pilus structural protein genes: *bp*, *tee*, and *fctA*, backbone pilins; *ap1*, *ap2*, *fctX*, *cpa*, and *fctB*, ancillary pilins. In FCT-2, *ap1*, *bp*, and *ap2* have sequence homology with *cpa*, *fctA*, and *fctB*, respectively, although divergence is high (82). Pilin adhesins include FctX from FCT-1, AP1 from FCT-2, and Cpa from FCT-3 and FCT-4. Pilin subunits that anchor the pilus shaft to the cell wall include AP2 and FctB (249). Fibronectin-binding protein genes include *prtF1* (sometimes designated *sfbI*), *prtF2* (sometimes designated *pfbp*), and possibly others that remain to be classified.

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subunit (i.e., Cpa) is strongly reactive by immunoblot with each of the anti-T3, anti-T13, and anti-B3264 sera, whereas the BP (i.e., FctA) is bound by anti-T3 sera only (97). However, there is very little amino acid sequence homology between Cpa and FctA, as is also the case for the other adhesin-backbone pilin subunit pairs (82, 98).

That some T-typing sera display immunoreactivity to multiple recombinant pilin proteins seems likely due to incomplete adsorption of the antisera and/or the presence of antigenic epitopes shared among pilin subunits belonging to the variety of GAS strains used for immunization. Possible unmasking of immunological determinants via the trypsin pretreatment used in the cell agglutination assay may further complicate efforts to draw direct connections between T-serotypes and specific pilin protein variant forms. Adding even further complexity to the T-typing scheme are the numerous GAS isolates that agglutinate with multiple T-typing sera, such as T3/13/B3264 or T8/25/Imp19; many other T-serotype combinations (>60 in total) are also observed (83). Overall, the T-typing sera displaying the least cross-reactivity with multiple pilin forms are T2, T4, and T6 (92); these appear to be best represented by the FCT-6, FCT-5, and FCT-1 regions, respectively (Fig. 3), although within these FCT regions lie at least some extensive pilin gene diversity (see, e.g., reference 99 for FCT-1).

T-serotype and M-serotype and/or *emm*-type data for GAS isolates ($n > 20{,}000$) from two large collections (the CDC and the University of Minnesota reference laboratory), recovered in the United States from about the 1980s to the mid-2000s (83), was collated and is summarized in Table 5. There are no strict sampling criteria for this assemblage; it seems reasonable to assume that it largely consists of a combination of isolates causing iGAS disease and strains with high prevalence. In this skewed, non-population-based sample, the dominant T types among *emm* pattern A-C strains are T1, T11 and/or T12, and T3, T13, and/or B3264, reflecting the highly prevalent M/*emm* types M1, M12, and M3, respectively.

T-non-typeable (TNT) GAS probably represent isolates that fail to produce pili; they account for ~9% of total isolates but >20% of *emm* pattern D isolates (Table 5). The relatively large numbers of TNT isolates among pattern D strains may reflect the disproportional presence of *nra* (82); Nra functions as a repressor or activator of pilus gene expression in a strain-dependent manner (88, 100). Nearly 60% of *emm* pattern D isolates are T3, T13, and/or B3264 (Table 5), which appears to be largely represented by the FCT-3 region form (82, 87, 92, 97). The most frequent M/*emm* types among pattern E isolates of this collection are M4 and M28 (83), which largely reflects the dominance of the T4 and/or T28 serotype grouping (Table 5). Several correlations between individual loci within the FCT region and *emm* pattern and/or *emm* type have been noted (50, 82, 92, 101).

Within an *emm* cluster group, there can be a very wide array of T types (based on Table 5; detailed data not shown) (83). For example, the *emm* cluster E3 isolates (13 *emm* types) are found in association with T5, T27, and/or T44 (28%); TNT (16%); T8, T25, and/or Imp19 (11%); T14 (11%); T9 (9%); T4 and/or T28 (9%); T3, T13, and/or B3264 (6%); and T11 and/or T12 (4%), among others. In contrast, cluster D4 isolates (21 *emm* types, all encoding plasminogen-binding M proteins) are far more homogeneous, with 72% associated with T3, T13, and/or B3264 (another 16% are TNT). Although *emm* genes within a cluster are more closely related to each another than to *emm* of other clusters, the extent to which surface pili contribute to the overall antigenic cell surface repertoire appears to vary widely by cluster.

For GAS sharing the same *emm* type, individual isolates often differ in their T-serotype (80, 83). For example, while a substantial majority of M/*emm* type 4 isolates from the Table 5 study are T4, there are other M/*emm4* isolates that have T types T4/28, T8/25/Imp19, T3/13, and TNT (83) (detailed data not shown). M/*emm4* isolates typically have *sof4*, but there are exceptions here as well (80). The unique combinations of M/*emm*, SOF/*sof*, and T types is indicative of HGT of the epidemiological marker genes among GAS.

Unlike M type-specific antibodies, there is no known human host protective effect mediated via T type-specific serum; this may be a consequence of immunoreactivity directed toward trypsin-treated target antigens that are not encountered during natural infection. However, antiserum

TABLE 5 Relationships between *emm* pattern and T types for >20,000 GAS isolates[a]

T type	% Total	% Pattern A–C	% Pattern D	% Pattern E
T1	18.73	34.64	0.93	0.14
T2	3.30	0.00	0.00	7.85
T6	6.19	11.09	1.97	0.34
T9	0.82	0.00	1.39	1.81
T14	0.88	0.11	4.29	1.55
T15	0.00	0.00	0.00	0.00
T18	0.59	1.10	0.00	0.00
T22	0.02	0.00	0.00	0.04
T23	0.07	0.12	0.00	0.00
T3, 13, and/or B3264	16.10	17.14	58.63	10.75
T4 and/or 28	15.88	0.15	3.59	37.24
T5, 27, and/or 44	4.36	4.90	0.70	4.03
T8, 25, and/or Imp19	6.60	1.13	2.90	13.82
T11 and/or 12	18.17	20.80	4.52	16.17
Nontypeable	8.29	8.83	21.09	6.25

[a]Based on analysis of T-serotyping data (83) according to *emm* pattern groups or clades inferred from M or *emm* type (65, 126, 127), for 21,231 isolates, whereby 271 isolates had >1 grouping and were counted more than once. M or *emm* types ranged from types 1 through 124.

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raised to recombinant pilin subunits or intact pili can be protective in experimental assays (48, 102). Shortcomings of the T-typing cell agglutination assay include a general lack of good resolution, the time-consuming process, and the requirement for standardized rabbit serum. Because T-typing sera display high levels of immunological cross-reactivity to different recombinant pilin polypeptides, and many GAS isolates agglutinate with multiple T-typing sera, it can be difficult to predict the FCT region genotype form based solely on the T-agglutination pattern.

At present, there is no single widely recognized genotyping scheme for the FCT region form and pilin genes (35, 82, 92, 96), but the need for one seems quite compelling given the importance of the FCT region in strain diversity as well as virulence.

Multilocus Sequence Typing and Core Housekeeping Genes

The first study to examine the population structure of GAS utilized multilocus enzyme electrophoresis (103). Isolates recovered from cases of severe iGAS disease correspond to several distinct lineages as defined by electrophoretic type. The multilocus enzyme electrophoresis approach was replaced by the more standardized multilocus sequence typing (MLST). For GAS, MLST is based on the nucleotide sequences of seven (partial) core housekeeping genes (104). MLST data are maintained at a user-interactive website (www.pubmlst.org/spyogenes) and currently lists 966 distinct sequence types (STs) (105); numerous investigators from throughout the world have generously contributed to this rich data set. Using goeBURST (106), 133 clonal complexes (CC) as defined by single locus variants (SLVs) have been detected. Figure 4 provides a chromosomal map of the relative positions of the core housekeeping genes and the *emm* and FCT regions.

A given pattern A-C *emm* type has a high tendency to be associated with a single ST or CC (104, 107, 108). However, the relationships between *emm* type and ST are not strict, and *emm* types of pattern groups D and E are often found in association with distant STs (see "Lateral Movement of *emm*" below). These data parallel findings on the complex relationships between M/*emm* types, *sof* types, and T types (80, 83) and, taken together, provide supporting evidence for the HGT of *emm* from one genetic background to another.

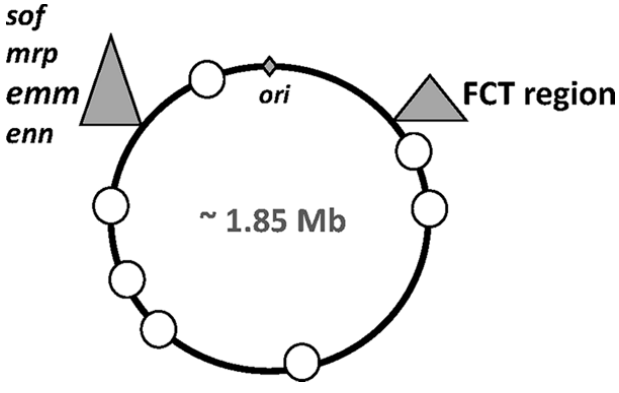


FIGURE 4 Chromosomal map of the epidemiological marker genes of GAS. Open circles represent the seven core housekeeping genes used in MLST (104). The *sof* locus, when present, lies ~15 kb upstream of *emm*. The FCT region, which encodes pilus structural proteins and pilus biosynthetic enzymes plus other adhesins and transcriptional regulators, ranges in size from ~11 to 16 kb. The FCT region lies ~250 to 300 kb from the *emm* region, on the opposite side of the origin of replication (*ori*). GAS genomes range in size from ~1.8 to 1.9 Mb.

Whole-Genome Sequences

The first completely assembled GAS genome was that of an *emm1* isolate (109). In the years that followed, numerous complete GAS genomes were reported for many of the most prevalent *emm* types found in resource-rich nations (updated listings are presented in recent reviews [50, 110]). Via next-generation sequencing technologies, many thousands of genomes of GAS isolates have undergone nucleotide sequence determination (e.g., 24, 30, 111–115). In nearly all studies, core genome sequences are compared for isolates sharing the same *emm* type, and numerous key insights on genetic changes that underlie enhanced transmission and/or virulence have been uncovered. The core genome data can be used to build evolutionary models of outbreaks and epidemics (see "Epidemiology: Building Evolutionary Models" and "Evolution: Short-Term" below).

A core genome MLST has not yet been developed for GAS. Although it is a reasonable concept for some bacterial species, its utility for GAS would probably be rather limited due to the ultrafine resolution this approach would provide for GAS, coupled with the extensive genetic recombination that characterizes this species. Core genome-based phylogenies of GAS of different *emm* types and/or CCs tend to yield lineages with deep branches, indicative of large genetic distances (50, 110, 111, 116).

The use of genome sequence data for surveillance of GAS has begun (35, 117–120). The CDC recently implemented a whole-genome sequence-based typing scheme for population-based iGAS strain surveillance conducted through the Active Bacterial Core program (35). For large-scale strain surveillance, the ability to obtain detailed information on typing and resistance features within a single output, from a single raw data input source, has largely precluded the need for multiple typing protocols and antibiotic susceptibility testing. The GAS typing pipeline automatically restricts an *emm* type to the sequence linked to *emm* consensus primer 1 (121) to improve accurate identification of *emm* and assigns an *emm* subtype (i.e., allele) as well. Partial sequences of BP genes and *sof* are also captured. In addition to *emm* subtype, the *emm*-like genes and MLST alleles and STs are identified, as is *gacI*, which is specific to CHO-A biosynthesis. Among numerous other genes, antimicrobial resistance determinants are also assessed.

A key feature of genome sequence-based active iGAS surveillance is the capacity to detect temporally and geographically related GAS clusters. For GAS isolates that are nearly identical at the single nucleotide polymorphism (SNP) level, the circumstances of where and when the isolates were recovered can be examined to help establish whether there exist disease clusters that may be well suited for an intervention (e.g., health care facilities, vulnerable patient populations such as the homeless, and IVDUs). Thus, next-generation sequencing coupled with an appropriate bioinformatics pipeline can be used as an autodetection tool to help guide public health interventions.

EPIDEMIOLOGY: GLOBAL TRENDS

Infection at the Epithelium of the Throat and Skin

Past studies show that GAS strains bearing certain M types have a strong tendency to cause pharyngitis but not impetigo.

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TABLE 6 *emm* pattern assignments for GAS recovered in 29 population-based surveys for pharyngitis or impetigo[a]

Disease association	No. of studies	Mean average % pattern A–C	Mean average % pattern D	Mean average % pattern E	Paired t-test[b] (% pattern A–C versus D)	Paired t-test (% pattern A–C versus E)[c]	Paired t-test (% pattern D versus E)
Pharyngitis	23	40.5 ± 19.8	5.8 ± 12.0	53.7 ± 16.0	<0.001	N.S.	n/a
Impetigo	6	9.3 ± 6.7	47.4 ± 8.1	43.4 ± 7.0	<0.001	n/a	N.S.

[a]Based on references 50 and 131. The 29 studies meet the following criteria: (i) isolates are clearly defined for recovery from cases of pharyngitis (or tonsillitis) or impetigo, and (ii) at least 25 isolates can be assigned to an *emm* pattern group based on *emm* type.
[b]Paired t-tests are two-tailed.
[c]N.S., nonsignificant; n/a, not applicable.

Similarly, there are M types often recovered from impetigo skin lesions but rarely from cases of tonsillopharyngitis (8, 122, 123). This observation gave rise to the important concept of classical throat and skin strains that are largely distinct from one another in their epidemiological and disease associations.

There are strong correlations between *emm* pattern groups A-C and D and recovery from cases of pharyngitis versus impetigo, respectively (50, 124, 125). Since the *emm* type is highly predictive of the *emm* pattern group for nearly all *emm* types examined (126, 127), reasonable inferences can be made about *emm* pattern grouping based on knowledge of *emm* types. Such inferences were made on an expanded scale for >5,000 GAS isolates derived from cases of pharyngitis or impetigo in 29 population-based surveillance studies from throughout the world (Table 6). In 23 studies on pharyngitis, *emm* pattern A-C and D isolates constituted a mean average of 41% and 6%, respectively. In six studies of impetigo, the mean average for *emm* pattern A-C and D isolates was reversed, at 9% and 47%. For pharyngitis versus impetigo groups, the difference between the fraction of A-C and D isolates in each surveillance study is highly significant (Table 6). *emm* pattern E isolates account for almost equal fractions of throat and skin infections; the mean average percentage of *emm* pattern E isolates shows no significant difference from pattern A-C isolates at the throat or pattern D isolates at the skin.

Based on the strong clinico-epidemiologic associations, the *emm* pattern A-C strains are designated "throat specialists," whereas the pattern D strains are "skin specialists." As a group, *emm* pattern E strains are considered "generalists." Importantly, the *emm* pattern serves as a useful genotypic marker for the preferred tissue site of infection by GAS (32, 50, 124, 125, 128).

The *emm* cluster typing scheme can also be applied to the 29 population-based surveillance studies on GAS pharyngitis and impetigo (Table 7). Both pharyngitis and impetigo isolates are almost evenly split between clades X and Y, reflected by fractional ratios of percent pharyngitis to percent impetigo approaching 1. However, within clade Y, the distribution of pharyngitis and impetigo isolates are highly skewed among the clusters, with clusters A-C1-5 disproportionately represented by pharyngitis isolates, and clusters D1-5 disproportionately represented by impetigo isolates.

Several predicted binding motifs within M protein strongly correlate with *emm* clusters (65). Based on the 29 population-based surveys, the fractional ratio of pharyngitis to impetigo isolates is highly skewed for the fibrinogen-binding clusters of clade Y (high ratio value, with pharyngitis dominant) and the plasminogen-binding cluster D4 (low ratio value, with impetigo dominant) (51). The differential binding properties for human host proteins, by M proteins in accordance with disease, are potential determinants of tissue tropism. How modulation of the coagulation-fibrinolytic pathways (129) may translate into GAS tissue tropisms is an active line of inquiry.

Invasive Disease

As detailed above ("Ecology: Host Tissues and Diseases"), iGAS disease is extremely rare relative to GAS infection at

TABLE 7 *emm* cluster and clade assignments for GAS recovered in 29 population-based surveys for pharyngitis or impetigo[a]

emm clade	*emm* cluster	No. of isolates	No. of pharyngitis isolates	No. of impetigo isolates	% of total pharyngitis	% of total impetigo	Fractional ratio of pharyngitis to impetigo[b]	Fractional ratio of impetigo to pharyngitis
ALL	ALL	5,439	4,674	765	100	100	n.a.	n.a.
X	ALL	2,804	2,433	371	52.1	48.5	1.07	0.93
Y	ALL	2,608	2,239	369	47.9	48.2	0.99	1.01
Outlier	ALL	27	2	25	0.04	3.3	n.d.	n.d.
X	E1-6	2,802	2,432	370	52.0	48.4	**1.08**	**0.93**
X	Singletons	2	1	1	0.02	0.1	n.d.	n.d.
Y	A-C1-5	1,849	1,817	32	38.9	4.2	**9.29**	**0.11**
Y	D1-5	344	49	295	1.1	38.6	**0.03**	**36.78**
Y	Singletons	415	373	42	8.0	5.5	1.45	0.69

[a]Based on references 50, 51, and 135 and Table 6.
[b]Calculated as the ratio of the percentage of total pharyngitis isolates to the percentage of the total impetigo isolates.
[c]n.a., not applicable; n.d., not determined.
Key ratios are highlighted (bold).

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the two primary ecological niches. When iGAS disease does occur, it is largely a transmission dead end, with the possible exception of direct contact transmission via open cutaneous wounds among underserved adult populations. Thus, iGAS infections have minimal impact on shaping the long-term evolution of GAS. The occurrence of iGAS disease is strongly affected by host risk factors and to a lesser extent by the genotype of the infecting GAS strain.

In the United States and Europe, most iGAS disease depends on transmission of GAS via a respiratory route (Fig. 1). Whole-genome sequencing of hundreds of GAS isolates has provided important details on genetic changes that enhance transmission and/or invasiveness (see "Evolution: Short-Term" below). The invasive index value provides insight on whether a GAS strain has an inherent ability to better gain access to deep tissue relative to its overall prevalence in the host population (22, 130). The *emm* types associated with iGAS disease have been intensively studied and are well described (e.g., 131–133).

Nonbullous impetigo caused by GAS usually arises in young children in tropical or subtropical climates. GAS can also cause slightly deeper cutaneous infections that are considered "pyoderma," and while they are not considered part of the iGAS disease spectrum, these cutaneous infections are distinct from typical impetigo in terms of patient demographics (i.e., adult, temperate regions) and host risk factors (e.g., IVDU, homeless, military) (23, 24, 134–136). Of interest is the strong association between certain pattern D *emm* types (specifically, *emm59* and *emm81*) and recent infections and outbreaks of deeper cutaneous infections. The *emm59* and *emm81* types are notable for their grouping in cluster E6 and tend to harbor *sof* as well; conceivably, these are "super-specialist" strains containing a hybrid mix of virulence factors that shift the modes of transmission and GAS disease course. Most other GAS-causing skin infection outbreaks among IVDUs are *sof*-positive *emm* pattern E strains (137, 138).

Acute Rheumatic Fever

Acute rheumatic fever (ARF) follows an inadequately treated GAS infection of the URT in a genetically predisposed host by ~3 to 6 weeks and can lead to rheumatic heart disease (RHD) via autoimmune attack of the heart valves (139–143). With >500,000 annual deaths, half attributed to RHD, GAS ranks high among all infectious causes of morbidity and mortality. Recent estimates of the prevalence of RHD are ≥15 to 30 million cases worldwide (15, 144–146); RHD is the leading cause of mortality from acquired heart disease in individuals <50 years old (147) and of all cardiovascular disease in children in resource-poor nations (148). Today, ARF and RHD are largely diseases of poverty, with an occasional resurgence in disease elsewhere (149).

Early epidemiological studies of ARF outbreaks in institutional settings showed that only a subset of GAS strains trigger ARF (11, 55, 140, 150–156). Historically, "rheumatogenic" GAS strains were referred to by their M protein serotypes, with M3, M5, M6, and M18 being highly prevalent. The concept of "nonrheumatogenic" strains is exemplified by the RHD patients of Irvington House during the preantibiotic era, who experienced a GAS outbreak (M type 4) in the absence of recurrent ARF attacks (155). Of ten M types often associated with ARF (M types 1, 3, 5, 6, 14, 18, 19, 24, 27, and 29 [157]), nine are *emm* pattern A-C (one is pattern E); of five relatively common M types that are rarely associated with ARF (M types 2, 4, 12, 22, and 28 [157]), four are *emm* pattern E (one is *emm* pattern A-C) (126, 127).

ARF-associated isolates recovered in Hawaii differ from the classic rheumatogenic *emm* types (158). Atypical *emm* types are also found in tropical Australia, where the prevalence of RHD is among the highest in the world (159). Over time, it has become much less clear which *emm* types are best assigned the "rheumatogenic" label, even though it is widely accepted that not all GAS strains trigger ARF in a genetically susceptible host (150, 155).

The potential role of GAS skin infection in triggering ARF and RHD in certain settings (e.g., noninstitutional, tropical) has been an ongoing debate since the 1960s (160). For example, the indigenous population of Australia suffers from a high incidence of ARF and RHD, yet GAS is far more frequently recovered from impetigo lesions than from the throat. Moreover, the strains recovered from the URT in this host population often belong to the *emm* pattern D skin specialist group or pattern E generalists. The epidemiological co-occurrence of GAS skin infections and RHD supports the idea that impetigo may contribute to this chronic disease. More extensive studies are needed to better understand skin infections and their potential role in ARF and RHD. The possibility that the closely related species SDSE might also trigger episodes of ARF or worsen RHD deserves deeper consideration as well (161, 162).

EPIDEMIOLOGY: DIVERSITY WITHIN HOST COMMUNITIES

Within a human host community over a narrow time frame, numerous GAS strains are in circulation. For the 29 population-based collections of GAS recovered from cases of pharyngitis and impetigo, the number of *emm* types and the Simpsons diversity index was calculated (Table 8). The number of *emm* types for the impetigo collections averaged nearly twice that of the pharyngitis collections ($t < 0.01$, unpaired t-test, 2-tailed). The Simpson's diversity index (D), which incorporates the number of recovered organisms into the calculation, also shows highly significant differences between the impetigo and pharyngitis collections, wherein the impetigo group has a higher mean average D value (a D value of 1 reflects maximal diversity). An analysis that includes all GAS, irrespective of clinical association, also shows fewer numbers of *emm* types accounting for a large proportion of isolates in resource-rich countries, where URT infections tend to predominate (131). The large numbers of circulating *emm* types is likely the consequence of expansive GAS strain migration, even into geographically remote communities (108). The biological basis for the difference in diversity among GAS in host populations experiencing pharyngitis versus impetigo may be tied to the mechanisms for transmission: respiratory droplets versus direct contact. It may be that the respiratory route is more highly efficient, such that a single infected individual can infect many more new hosts (i.e., higher basic reproductive rate), and this in turn leads to dominance by fewer strains, resulting in a lower D value.

EPIDEMIOLOGY: EVOLUTION OF MARKERS (*EMM*)

Although *emm* type diversity within a host community is relatively low for the *emm* pattern A-C throat specialists compared to the pattern D skin specialists (Table 8), there is relatively high diversity among *emm* pattern A-C organisms in terms of the number of *emm* subtypes (i.e., alleles based on the 5′ end of the *emm* gene) that evolved worldwide (Table 9). The average mean number of *emm* subtypes identified for pattern A-C strains is 22.3 subtypes per *emm*

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TABLE 8 *emm* type diversity within communities for the 29 population-based surveys for pharyngitis and impetigo[a]

Disease association	No. of studies	Mean average no. of *emm* types	Mean average *D* index[b]
Pharyngitis	23	17.4 ± 7.8	0.873 ± 0.065
Impetigo	6	33.8 ± 8.1	0.959 ± 0.012
	Unpaired t-test, 2-tailed	$t = 0.0049$	$t < 0.001$

[a]Based on references 50, 51, and 135 and Tables 6 and 7.
[b]Simpson's diversity index (*D*) (248) is based on *emm* types.

type, compared to only 4.0 and 6.9 for pattern D and E strains, respectively, based on current data available in the CDC online database (58), which receives numerous deposits from investigators throughout the world.

The >5-fold difference in the number of *emm* subtypes for throat specialists versus skin specialists may be partly due to sampling bias that skews in favor of GAS invasive and pharyngitis isolates in resource-rich nations, such as the highly prevalent *emm1* type (pattern A-C). In addition, the tandem sequence repeats that overlap the *emm* subtype-determining region of *emm5* and *emm6* genes (pattern A-C) are a mutational hotspot due to DNA slipped-strand mispairing (163). However, biological factors such as host immune selection promoting immune escape (164) may also play a role.

Nucleotide sequence alignment of *emm* subtype alleles for each *emm* type-specific region, derived from >500 global GAS isolates ($n = 105$ *emm* types), reveals average ratios of nonsynonymous substitutions per nonsynonymous site (Ka) and synonymous substitutions per synonymous site (Ks) of 4.9, 1.5, and 1.3 for *emm* types of the *emm* pattern A-C, D, and E groups, respectively (107). Thus, diversifying (positive) selection acting on the *emm* type-specific sequence appears to be strongest for *emm* pattern A-C strains.

A likely source of diversifying selection acting on the *emm* type region is the protective host immune response, which can drive immune escape. Conceivably, the immunological pressures at the oropharynx (pattern A-C throat specialists) are more intense than those at the skin (pattern D skin specialists), although an underlying molecular mechanism is

TABLE 9 Relationships between *emm* pattern or cluster and *emm* type and subtype

emm grouping category	Name of group	No. of *emm* types represented	No. of *emm* subtypes represented[a]	Average no. of *emm* subtypes per *emm* type
Pattern	A-C	35	781	22.31
Pattern	D	64	253	3.95
Pattern	E	67	460	6.85
Clade	X	85	518	6.09
Clade	Y	89	1,014	11.39
Cluster	E1	5	40	8.0
Cluster	E2	15	84	5.6
Cluster	E3	19	119	6.3
cluster	E4	17	150	8.3
Cluster	E5	7	15	2.1
Cluster	E6	18	98	5.4
Cluster	Clade X singletons	4	12	3.0
Cluster	A-C1	2	7	3.5
Cluster	A-C2	2	26	13.0
Cluster	A-C3	5	118	23.6
Cluster	A-C4	5	111	22.2
Cluster	A-C5	3	140	46.7
Cluster	D1	3	13	4.3
Cluster	D2	5	22	4.4
Cluster	D3	2	4	2.0
Cluster	D4	31	144	4.6
Cluster	D5	3	6	2.0
Cluster	Clade Y singletons	28	423	15.1
Clade Y singletons	Pattern A-C	16	385	24.1
Clade Y singletons	Pattern D	8	16	2.0
Clade X singletons	Pattern E	2	6	3.0

[a]Based on the CDC online database (58).

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not presently obvious. Perhaps herd immunity builds up more quickly with a respiratory mode of transmission compared to direct skin contact; as herd immunity builds, the number of susceptible hosts dwindles, leading to more frequent occurrences of immune escape. In further support of a role for herd immunity in shaping the *emm* type content of GAS populations, newly acquired URT infections in older children are skewed toward less common *emm* types, consistent with exposure and protective immunity to the more prevalent *emm* types in early childhood (165).

The collection of M protein types assigned to a single *emm* cluster have high sequence similarity relative to M proteins of other clusters (65). Indeed, an M type-specific region sharing the same cluster as other M types often elicits cross-protective antibodies (see "Epidemiology: Strategies for M Protein-Based Vaccines" below). Ultimately, this cross-protective effect may aid in the design of highly efficacious vaccines that have broad coverage but contain only a limited selection of type-specific epitopes. Whereas 90% of pattern D and E *emm* types belong to 1 of the 16 *emm* clusters, nearly half of pattern A-C *emm* types are standalone and do not cluster with any other *emm* type, highlighting once again a distinct dynamic for the evolution of many pattern A-C *emm* types.

Another possible factor at play in shaping the evolution of *emm* type-specific sequences is functional constraints, such as those imposed by binding C4BP via the type-specific region of numerous M proteins (166–168). Whole GAS organisms that bind C4BP tend to harbor M types associated with *emm* pattern groups D and E (167). Thus, there may be limits to diversification within M proteins that bind C4BP for the GAS strains that utilize this virulence strategy to enhance survival. Also, potentially related to selection pressures acting on GAS is the finding that engineered mutants with *emm* gene deletions have a (near) complete loss in the antiphagocytic effect for pattern A-C strains, whereas the loss is only partial for pattern D and E strains (169).

Overall, the throat specialists are characterized by fewer distinct *emm* types within a human host community but high diversification worldwide within the type-specific region (i.e., more *emm* subtypes). The skin specialists have about twice the number of *emm* types within a community, but diversification within the type-specific region is ~5-fold lower based on the number of *emm* subtypes or Ka/Ks ratios. The *emm* pattern E generalists are most similar to the skin specialists in these values.

The "*emm* type" and "*emm* cluster" definitions are categorical, and the noted differences between throat and skin specialists may partly reflect the placement of the categorical "cutoff" value along a continuum of sequence divergence. Factors that shape the GAS population structure, as defined by *emm* type, likely include the route of transmission, environmental risk factors, the host immune response, tissue-specific host factors, binding functions of M protein, and intrinsic GAS factors that influence mechanisms for genetic change.

EPIDEMIOLOGY: STRATEGIES FOR M PROTEIN-BASED VACCINES

Numerous vaccine strategies for protection against GAS infections were recently reviewed (170–172). Briefly, vaccine strategies can be divided into M protein and non-M protein target antigens, with the latter including streptolysin O, C5a peptidase, CHO-A, pili, and the enzyme arginine deiminase (102, 173–178). However, only the vaccine candidates that incorporate M protein as the target antigen have attained human clinical trial status (179).

M protein vaccines based on the conserved antigens of the CRR (Fig. 2A) have undergone extensive investigation (49, 180–185). Since all M proteins have a CRR, a CRR-based vaccine has the theoretical advantage of providing broad coverage against all GAS strains; several formulations are currently, or will soon be, in clinical trials. Potential drawbacks of a CRR-based vaccine include immune escape. The CRR region of M protein, which includes MAP I/II and the class I/II antigenic epitopes, is not 100% conserved. For example, among 175 *emm* types from a worldwide strain collection, there are 22 alleles of the CRR-based J8 vaccine candidate (65). Importantly, some of the amino acid residues within the CRR of the M protein display signatures of diversifying selection. Immune escape is a possibility for any vaccine target, and current knowledge indicates that it deserves careful consideration for a CRR-based vaccine.

The second category of M protein-based vaccine candidates targets the NH_2-terminal type-specific determinants (179, 186). Pioneering work in the 1940s and 1950s showed that type-specific serum is responsible for protective immunity against the homologous *emm* type (187–190); in contrast, minimal to no protective effects were observed for infection by heterologous GAS types. A 26-valent M type-specific recombinant polypeptide vaccine is the only GAS vaccine to have reached phase II clinical trials, whereupon it was found to be safe and highly immunogenic (179). The "type-specific protection" paradigm of GAS immunity has led to concerns about the potential for low coverage by the multivalent vaccine, especially in tropical settings, where the number of GAS *emm* types can be very high (131, 191–195).

Recent data indicate that cross-protective immunity between closely related *emm* types can induce broader protection via multivalent vaccines than type-specific immunity might otherwise predict. An initial hypothesis on cross-protective immunity was raised following genetic analysis of GAS strains collected in Brazil (196, 197). Cross-protective opsonization by hyperimmune rabbit antiserum generated via immunization with M type-based vaccines was subsequently demonstrated using a 30-valent vaccine (186) and an experimental 4-valent vaccine designed to protect against the 17 *emm* types belonging to *emm* cluster E4 (198).

Superficial skin infection can stimulate a robust antibody response. A recent study of humoral immunity acquired after impetigo infection in Fijian schoolchildren demonstrates that 38% of subjects had a ≥4-fold increase in anti-M-type-specific IgG titers that match the M type of the infecting strain (199). Importantly, cross-protective *emm* cluster-specific immunity was evident for clusters E4, E6, and D4, although the levels of cross-protection were variable depending on both the patient and *emm* cluster. That the cross-reactive immune responses frequently align with *emm* cluster groups holds promise for the design of M type-based vaccines having broad coverage. More data are needed to further solidify the clinical and experimental findings obtained to date; identifying immunoassays to help define correlates of protection (CoPs) is a high-priority goal (200). Serotype replacement may potentially be an issue for GAS following widespread immunization with M type-based vaccines, as is the case for *Streptococcus pneumoniae* and serotype-based capsule vaccines (201, 202).

EPIDEMIOLOGY: BUILDING EVOLUTIONARY MODELS

MLST data can be used to build models of evolutionary descent via eBURST or goeBURST, which are clustering

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algorithms based on SLVs (and/or double locus variants) among the seven core housekeeping genes (106, 203). The single mismatched allele that defines an SLV pair can arise by either point mutation or homologous recombination, whereby the latter may introduce >1 SNP.

For GAS, the combined total length of the seven housekeeping (partial) alleles is 3,134 bp. However, 3,134 bp represents only a fraction of the GAS core genome; for example, *emm3* type iGAS have a core genome of ~1,645 kb (115), which is ~524-fold longer in sequence than the MLST alleles combined. Based on these values, it can be estimated that an MLST-defined SLV pair of GAS would differ by an average minimum of >500 SNPs within its core genome if genetic change were strictly due to point mutation or intraspecies recombination between very closely related organisms. However, if there was extensive HGT and recombination between more distally related GAS, the number of SNPs that differentiate the SLV pair is expected to be even higher. Given that the core genome of *emm3* type iGAS strains has an estimated 29 to 133 SNPs per isolate (i.e., short-term evolution) (115), the models for evolutionary descent based on the SLVs of MLST using only seven core housekeeping genes may best capture intermediate stages of evolution, which can be quite informative.

MLST data in combination with epidemiological marker genotypes can be compared to whole-genome sequence data. A good example for comparison is the *emm89* strains causing iGAS disease in Europe and the United States, which underwent a marked increase in prevalence during the 2010s (30, 31, 111, 204). Using the ST and GAS isolate data deposited at www.pubmlst.org/spyogenes (105), a goeBURST plot that includes the recent epidemic *emm89* strains is shown in Fig. 5A. The newly emerged epidemic clade of *emm89* corresponds to ST101 (and its descendants). Based on demographic information (i.e., dates of recovery), it seems reasonable to assume that ST407 is ancestral to ST101. Notably, ST407 is recovered in association with two distinct *emm* types: at least one other ST407 GAS isolate in the PubMLST database has *emm78* in place of *emm89*, as do several other SLVs and double locus variants of ST407. Alignment of the 180-bp *emm* subtype regions of *emm78.0* (ST253) and *emm89.0* (ST407, ST101) reveals <60% identity for both nucleotide and amino acid sequences, indicative of substantial divergence (ClustalW; data not shown) and supporting a model wherein *emm78* and/or *emm89* underwent HGT and recombination onto an ST407-related genetic background. Both *emm78* and *emm89* are *emm* pattern E but belong to different *emm* clusters (E1 and E4, respectively).

Examination of the FCT regions of ST407 and ST101 *emm89* type GAS isolates reveals highly divergent Cpa and FctA proteins, corresponding to the AP1 and BP subunits of pili, respectively (Fig. 5B). The distinct Cpa and FctA forms appear to coincide with T serotypes T11 (ST407) and T13 (ST101) (35). The housekeeping gene locus that distinguishes ST407 and ST101 is *yqiL*, which lies adjacent to the FCT region (Fig. 4); *yqiL01* (ST401) differs from *yqiL03* (ST101) by three nucleotide substitutions, highly suggestive of diversification via recombination. In contrast to the eBURST analysis of MLST data, whole-genome sequencing and analysis of the core genomes of *emm89* isolates provides a much higher level of resolution (30, 35, 111, 112). Importantly, several SNPs and indels that distinguish the *emm89* clade 2 → clade 3 transition lead to alteration of virulence factor expression: loss of the hyaluronic acid capsule (Δ*has*A) and transcriptional upregulation of toxin genes encoding Nga and streptolysin O.

The genetic relationships among GAS strains harboring *emm89* and ~20 additional *emm* types were inferred based on ~75,000 SNPs of the core genome (111). The phylogeny shows that the *emm89* isolates of the three clades detailed above, and corresponding to CC407 (ST101, ST407), cluster within the same major lineage; ST142 isolates (double locus variants of ST407; Fig. 5A) also cluster on that same deep branch. However, GAS of other *emm* types, plus *emm89*-ST380 isolates belonging to another CC (204), each cluster along distinct lineages with deep branches (111). ST380 differs from both ST101 and ST407 at all seven housekeeping alleles, indicative of HGT of *emm89* to a distant genetic background.

The MLST data for *emm89* strains captures two different modalities of HGT involving *emm*: replacement of a nascent *emm* by a new *emm* type on the original genetic background (i.e., recipient-new clone pairs, with two *emm* types and one ST) and lateral movement of an *emm* type to a new genetic background (i.e., donor-new clone pairs, with one *emm* type and two STs) (107). Within CC407 (recipient) there is evidence for the former process, involving replacement of *emm78* with *emm89* or vice versa (Fig. 5A). A possible donor of *emm89* to CC407 is *emm89*-ST380 (latter scenario); alternatively, ST380 may have been the recipient of *emm89* from a CC407 donor. Comparisons of completely assembled genomes of possible donor-recipient-new clone pairings may aid in establishing the directionality of horizontal *emm* gene movements.

Based on the connections within CC407, the seven housekeeping genes of MLST appear to recapitulate the more expansive core genome sequence data reasonably well. However, MLST fails to capture SNPs arising in core virulence-related genes that may alter the trajectory of GAS disease in a host population. What MLST may be best tuned to, when combined with typing data on *emm*, pilin genes, and/or *sof*, are HGT events wherein a typing gene is present on distant STs/CCs or multiple typing genes are recovered in association with the same ST/CC (such as divergent CpaFctA in *emm89*-CC407; Fig. 5B). For GAS, MLST relationships can provide an intermediary link between short- and long-term evolution and may help delineate the genetic roots of the emergence of new strains that have unique repertoires of cell surface antigens and their linked virulence functions.

EVOLUTION: MOLECULAR MECHANISMS OF GENETIC CHANGE

Core genes of GAS contain a historical record of numerous homologous recombination events (111, 205–208). Intraspecific gene exchange between GAS strains most likely occurs during cocolonization of the URT or coinfection of impetigo lesions with multiple strains (209). GAS can grow as biofilms or form microcolonies during infection within both tissue niches (210, 211), and the high bacterial density that is attained likely facilitates HGT. There is also ample evolutionary evidence for gene exchange between GAS and other species, such as SDSE (212–218).

A major unresolved puzzle of GAS biology is the molecular mechanisms that govern HGT, leading to the high rates of homologous recombination that is inferred from its evolutionary history. In principle, nonspecific packaging of chromosomal fragments into bacteriophage capsids could provide a vehicle for HGT of bacterial DNA (i.e., generalized transduction). In addition to bacteriophage, GAS are also rich in integrative and conjugative elements (91). The

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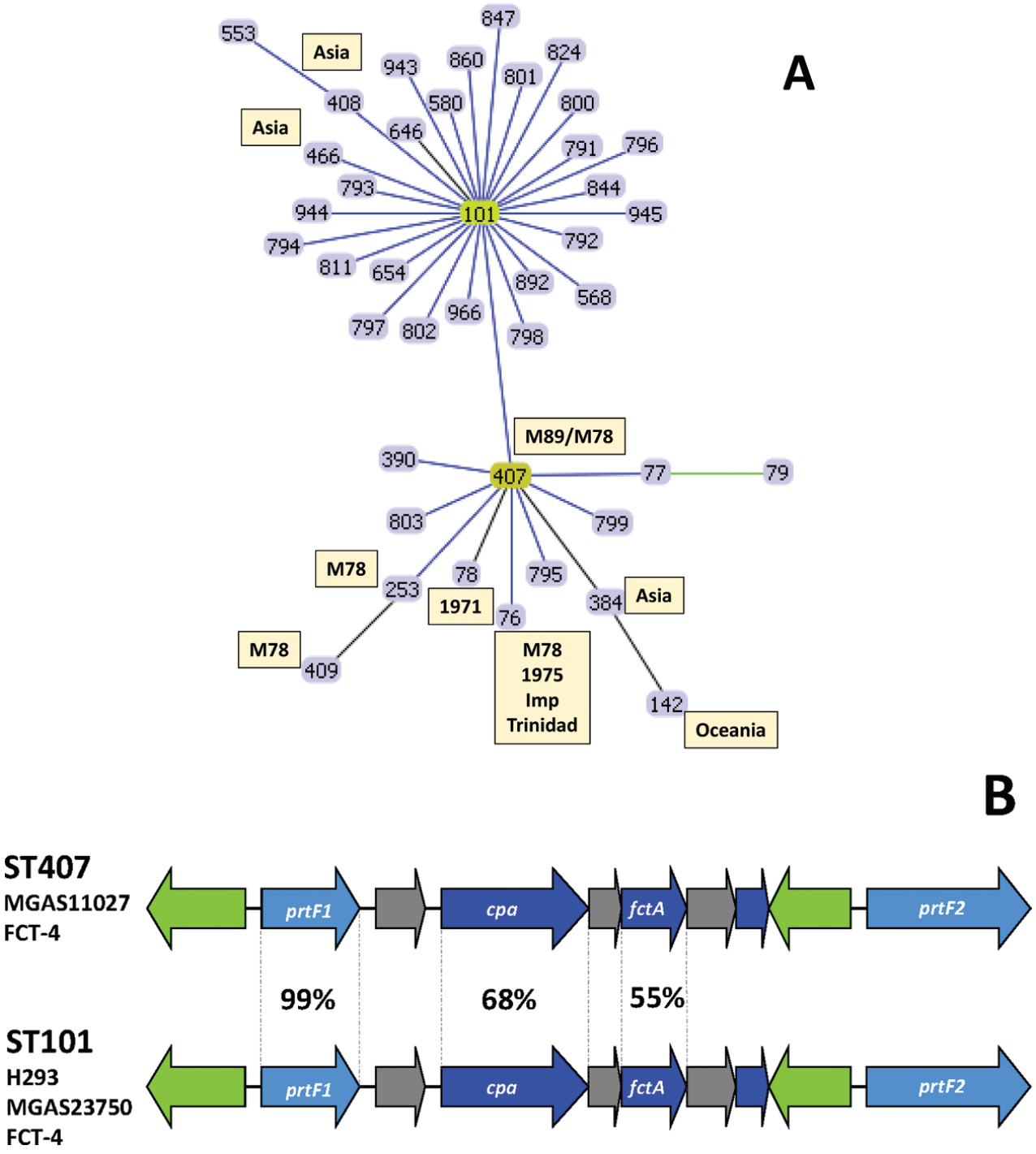


FIGURE 5 Model for evolutionary descent (CC407). (A) goeBURST diagram of CC407, which includes single representatives of 41 STs and is based on data from www.pubmlst.org/spyogenes (105). Unless noted otherwise (orange shaded boxes), all isolates (n = 114) are *emm*89 and were recovered from (i) the 1990s through 2010s, (ii) Europe or North America, and (iii) URT infections or iGAS disease. For evolutionary analysis of *emm*89 (formerly PT4245 [250]) isolates based on the core genome sequence (31, 111), clade 1 strain MGAS11027 is ST407, whereas clade 2 and epidemic clade 3 strains (MGAS23530 and MGAS27061, respectively) are ST101; the epidemic *emm*89 strain H293 from the United Kingdom is also ST101 (30). Note that ST407 corresponds to GAS with either *emm*89 or *emm*78 (105). (B) Amino acid sequence identities among predicted FCT region gene products of ST407 (MGAS11027) and ST101 (H293, MGAS23530) strains (all *emm*89) via ClustalW alignments.

degree to which random DNA fragments may slip through during the conjugative process remains unclear. A conserved RNA uracil methyltransferase (*rum*) gene provides an integration hotspot for both bacteriophage and integrative and conjugative elements in GAS, as well as other related species (219). Natural transformation via high-frequency uptake of naked DNA has not been demonstrated for GAS, although competence gene homologues are present in the GAS

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genome and (very) low efficiency transformation is detectable in biofilms (220–222). However, GAS secrete numerous DNases that degrade extracellular DNA, which may render the transforming substance unavailable for uptake by a competent cell.

A mutator phenotype that results in an increased mutation rate has been characterized for GAS. This defect in DNA mismatch repair is controlled by excision and reintegration of a streptococcal phage-like chromosomal island (SpyCI) at a site between the *mutS* and *mutL* loci (223) and is present in the genomes of numerous GAS strains (50). SpyCI yields an ~100-fold increase in the rate of spontaneous mutation, to as high as $\sim 10^{-7}$ to 10^{-8} mutations per generation. A possible relationship between the presence of SpyCI and CCs with a relatively low ratio of recombinational to mutational events remains to be explored.

EVOLUTION: LONG TERM

Core Housekeeping Genes

As previously stated, core genome-based phylogenies of GAS of different *emm* types and/or CCs tend to yield lineages with deep branches, indicative of large genetic distances (50, 110, 111, 116). Whole-core genome phylogenies depicted as SplitsTree graphs (neighbor-net analysis) show recombination between GAS of different *emm* types located deep within the network. Furthermore, there is no obvious clustering of the major lineages in accordance with *emm* pattern grouping or by any other recognized parameter.

Numerous methods have been used to assess the relative amount of recombination versus mutation that contributes to genetic change in GAS, based on the seven core housekeeping genes used in MLST (205–208). Together, the findings support the notion that GAS exhibit relatively high levels of recombination which, at least by some measures, is roughly comparable to the levels observed for the pneumococcus.

Using MLST data, the relative amount of recombination versus mutation among the *emm* pattern-defined subgroups of GAS was examined (107, 224). Findings based on several methodologies all point to the same general trend, wherein the pattern A-C throat specialists have the highest tendency to diversify by mutation, and pattern D skin specialists and pattern E generalists have a greater tendency to diversify by recombination. The data on a larger role for mutation, relative to recombination, in genetic diversification among the classical throat strains is consistent with the molecular evolutionary analysis of *emm* subtypes (see "Epidemiology: Evolution of Markers (*emm*)" above; Table 9) (107). Differences among the *emm* pattern-defined groups of GAS in terms of their mechanisms for genetic change underscore different dynamics in shaping their long-term population structures.

GAS strains may encounter ecological separation due to their tissue site preferences for infection (throat versus skin), geographic partitioning (temperate versus tropical) and temporal distances (winter versus summer). Ecological barriers can give rise to allopatric speciation, and therefore, signatures of early stages of speciation within the GAS population may be evident. However, analyses of housekeeping alleles provide strong support for highly fluid HGT and homologous recombination of housekeeping genes among all three *emm* pattern groupings (101, 128, 224). These findings extend to isolates known to be recovered from the URT versus impetigo lesions, regardless of *emm* pattern group (224).

In summary, despite wide spatial-temporal distances between many GAS strains, there is no clear evidence for diminished opportunities for HGT of core housekeeping genes between organisms of the different *emm* pattern groups. Numerous statistical measures all point to a population genetic structure for the *S. pyogenes* species that is highly nonclonal (205–208). Against a background of highly random genetic change in core housekeeping genes, loci that exhibit strong linkage with *emm* pattern genotypes are reasonably good candidates for playing a direct role in the adaptations leading to throat versus skin infection (e.g., 50, 101).

Lateral Movement of *emm*

For the same global set of >500 GAS isolates used to calculate Ka/Ks ratios within the *emm* type-specific region (see "Epidemiology: Evolution of Markers (*emm*)" above), the number of HGT events as a function of *emm* type was measured (107). Movement of *emm* to a distal ST is quantified as recovery of an *emm* type in association with >1 ST differing by ≥5 of the 7 housekeeping alleles; data show an average of 0.17, 0.60, and 0.75 lateral transfer events per *emm* type for the pattern A-C, D, and E groups, respectively. This finding supports the notion that for the classical throat strains, but less so for skin strains or generalists, *emm* type is often a reasonably accurate marker for ST or CC (104, 107, 108). In another measure of HGT and recombination, the number of *emm* types associated with *emm* type-variable STs (i.e., STs recovered in association with >1 *emm* type) was calculated; the number of *emm* type replacements per ST was 0.12, 0.15, and 0.02 for pattern A-C, D, and E isolates, respectively (107). Overall, there were three times as many events captured for HGT of *emm* to a distant ST ($n = 63$) than for replacement of *emm* on the same ST ($n = 21$).

Taken together, these data shed light on GAS strain competition surrounding the narrow time-space wherein the donor, recipient, and newly emerged clone all coexist. Strain competition can be mediated through host/herd immunity (225) and perhaps via other host-pathogen interactions as well. For the throat specialist strains, competition appears to be most intense between strains sharing the same *emm* type, because organisms of the original ST that served as the (presumptive) *emm* donor are rarely recovered. In contrast, for generalists, competition at the level of *emm* type appears to be considerably weaker, as evidenced by the frequent recovery of the same *emm* type in association with multiple distant ST backgrounds. How other cell surface antigens, such as SOF and/or pili, might contribute to the competitive sphere will require study of lateral movements of their genes and alleles within the GAS population.

Full Complement of Epidemiological Markers Considered

How do the host's specific immune defenses perceive the incoming antigenic repertoire present on the GAS cell surface? Except during carriage, the Mga regulon that upregulates the expression of *emm* and *emm*-like genes and *sof* is typically active. The pilus T-antigens appear to be expressed by >90% of the GAS population. The expression/nonexpression of the hyaluronic acid capsule can potentially mask/unmask some or all the GAS cell surface proteins. Setting aside the variance in the transcriptional program, which can have a profound effect on transmission and virulence (see "Evolution: Short Term" below), the collection of epidemiological markers provides a useful construct for defining GAS "strains."

Figure 6 summarizes the relative diversity or presence/absence of each major epidemiological marker in accordance with the *emm* pattern-defined groupings of throat specialists, skin specialists, and generalists. Importantly, *emm* type

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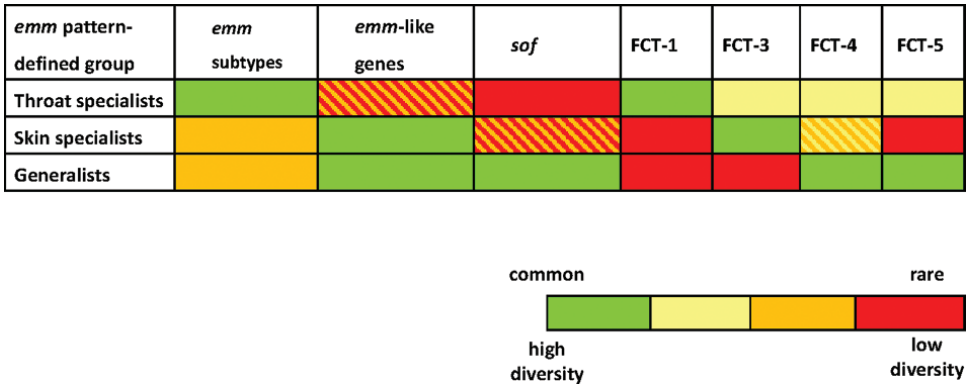


FIGURE 6 Trends in the distribution of epidemiological markers. An overview of the diversity and/or occurrence of epidemiological marker genes among three clinico-epidemiologic phenotype groupings of GAS. The relative distribution of FCT region forms is in accordance with one representative isolate for 94 unique *emm* types, as reported in reference 82; however, given the extensive HGT of *emm* and/or FCT genes to distant genetic backgrounds, the present analysis of the FCT region distribution may be far from complete.

is rarely recovered in association with multiple *emm* pattern groupings (Table 2), thereby allowing reasonably valid inferences to be drawn. The pattern- and cluster-defined *emm* subtype alleles reveal stark differences between the throat specialist, skin specialist, and generalist groupings regarding diversification (Table 9). Major distinctions also lie in the relative distributions of the *sof* locus (Table 4), *emm*-like genes (see Fig. 2B), and FCT region forms (Fig. 3) (82).

Do highly diverse *emm* subtypes compensate for the dearth of *emm*-like genes and *sof* among the throat specialists—and vice versa for the generalists? The diversity among *mrp* and *enn* alleles is modest, but they too have undergone intraspecies HGT (226); selection may be tied to functional binding attributes of the Mrp or Enn proteins (e.g., IgA, IgG) (63, 227–230). The *sof* gene also undergoes rather extensive intraspecies HGT (80); however, *sof* gene movement is nonrandom in that it is largely restricted to the *emm* pattern E generalists (Table 4). Yet the relationships between individual *emm* types and *sof* types are highly varied, with many unique combinations arising via HGT to distant genetic backgrounds (35).

Among the throat specialist group is a wide variety of FCT region forms (Fig. 6) (82). The FCT-1 region is the most prevalent form among representative pattern A-C organisms of unique *emm* types; FCT-1 is characterized by an AP1 adhesin (FctX) and major BP (Tee) that are markedly divergent from the highly variable Cpa and FctA pilins of FCT-3 and FCT-4 (82, 92). Not shown in Fig. 6 is FCT-2, which is (almost) exclusive to the highly prevalent *emm1* organisms; the AP1 and BP pilins of FCT-2 are akin to a distant lineage of Cpa and FctA, respectively (82). Thus, the unusually high success (i.e., fitness) of *emm1* strains may be due, in part, to its antigenically unique pilus structure. Overall, the absence of *sof* and *emm*-like genes among the pattern A-C strain grouping may be compensated for by the antigenic and structural diversity among its pili.

The skin specialist group appears to be the most homogeneous regarding epidemiological marker loci content, largely lacking *sof* but having both *mrp* and *enn*, and an FCT-3 region skewed heavily toward T3/13/B3264 (Table 5); this genotype is coupled to the lowest diversity among *emm* subtypes (Table 9). Also, the skin specialist group is dominated by *emm* cluster D4, encoding the plasminogen-binding M protein (51, 65). However, past lateral movement of *emm* type to distant genetic backgrounds is rather abundant among the skin specialists and is ~2-fold greater than that of *emm* type replacement on the same ST (107) (see "Lateral Movement of *emm*" above); there is also ample HGT of housekeeping alleles. Conceivably, the *emm* pattern D skin specialists represent a more recent subpopulation of GAS (i.e., younger) that has less diversity within any one gene, but the genes themselves are highly recombinatorial. Alternatively, the cell surface protein repertoire of skin specialists may be subject to less intense pressures for diversifying selection, perhaps due to its microenvironment and/or the host immune response at the skin.

The relative lack of pattern D and E *emm* types recovered in association with FCT-1 or FCT-2 regions may be indicative of functional constraints that would otherwise lower fitness, such as inadvertent adherence to a suboptimal tissue via specific host receptors. As a group, the *emm* pattern E generalists display all the *emm* region epidemiological markers and tend to have either the large FCT-4 region or FCT-5 (Fig. 6), both of which harbor the *prtF1* gene that encodes a fibronectin-binding protein (Fig. 3). Pattern E strains are also characterized by relatively high levels of recombination, involving both *emm* and the seven core housekeeping genes of MLST (50, 231, 232). Conceivably, within the generalist grouping are discrete throat or skin specialist strains whose phenotype is masked by the organizational framework (i.e., *emm* pattern group) that is imposed.

Historically GAS "strains" have been defined (primarily) by M serotype and secondarily by T and/or SOF serotype in combination with M type. Over the past 2 decades, *emm* typing has largely replaced GAS serotyping. The multitude of combinations of *emm* type and ST underscores the HGT of *emm* type, which is also consistent with the diverse array of M, T, and SOF serotype combinations observed. Even within a CC, STs are found in association with multiple *emm* types and/or divergent pilin genes (e.g., Fig. 5). Therein lies a conundrum for studying the population biology of GAS: How does one best define "strain?" This may or should be one of the next new challenges to be addressed by the GAS field.

EVOLUTION: SHORT-TERM

The monophyletic grouping of GAS strains in accordance with *emm* type and/or MLST-defined CC readily allows for

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inference of ancestral-descendent pairs within a branch, especially when coupled to epidemiological collections of strains with known dates of recovery. Whole-genome sequences of hundreds or thousands of GAS isolates sharing the same *emm* type and collected over a narrow spatial-temporal distance have captured epidemic waves of clones that are distinct from presumptive ancestral clones (24, 30, 111, 113–115, 233). An important finding is that within an *emm* type (specifically, *emm1* and *emm3*), iGAS isolates arise from all major lineages occupied by URT isolates and are genetically similar to the circulating pharyngitis strains from which they evolved (114, 234).

Comparative genomics of GAS recovered within a narrow spatial-temporal distance, and in association with a defined bacteria-host ecology (i.e., pharyngitis, asymptomatic carriage, and/or iGAS disease), have revealed high levels of diversifying selection at certain loci, particularly those involved in regulating the phenotypic expression of virulence factors (24, 30, 44, 111, 113–115, 233–240). Affected loci include *has*ABC (hyaluronic acid capsule biosynthesis), *covRS* (two-component system that acts as a global regulator), *rgg/ropB* (standalone response regulator), *mga* (response regulator and activator of *emm* transcription), *lia* (sensor kinase), and *nga-slo* (exotoxins). There are some recurrent themes, wherein downregulation of *emm* enhances URT carriage, downregulation of *speB* enhances iGAS disease, and upregulation of *nga-slo* enhances URT transmission. The contribution of capsule to GAS ecology seems more complex, and perhaps it is GAS strain dependent. In fact, in general terms, all mutations that alter fitness are context dependent and, accordingly, may be strain dependent. In addition to SNPs in critical virulence loci, GAS genomes are profoundly shaped by mobile genetic elements (which are often not included as part of the core genome); bacteriophage and integrative and conjugative elements can harbor critical virulence genes as well (e.g., 114).

EVOLUTION (OR NOT): RESURGENCE OF SCARLET FEVER

Scarlet fever (SF) was a dreaded GAS disease through the early 1900s; it manifests as a URT infection coupled to a pathognomonic scarlatina rash triggered by the action of erythrogenic toxin, which was subsequently characterized as a T-lymphocyte superantigen. The occurrence of SF in the United States and Europe declined mid-century and was largely sporadic in recent decades. However, in 2011 there was an alarming increase in the number of reported cases of SF in Hong Kong and in China soon thereafter (241–243). This upsurge was largely attributed to *emm12* GAS organisms that were resistant to macrolides and tetracycline. Comparative genome sequence analysis with other *emm12* strains revealed a novel prophage harboring the superantigen genes *spe*C and *ssa* (113, 244). However, the Hong Kong SF outbreak was multiclonal in origin.

A resurgence of SF cases also occurred in the United Kingdom, beginning in ~2013 (245). Comparative genomics failed to reveal a single clone or a single gene (e.g., a superantigen gene on a mobile genetic element) associated with those disease cases. As in Asia, the increase in SF incidence was associated with multiple lineages of GAS (246, 247).

Taken together, the recent upsurge in SF appears to be multifactorial and likely includes host-related factors. Numerous GAS lineages are responsible for SF, although resistance to antibiotics may have fueled the spread of some clones. Possible shifts in the content of superantigen genes may collectively have had an effect. The two cohorts—children in Asia and Europe and largely nonrural—possibly had a history of past exposure to a similar set of prevalent *emm* types and strains (131), and their herd immunity may be similar despite the geographic distance. Conceivably, an unknown copathogen may affect host susceptibility to SF and development of the scarlatina rash. Heightened clinician awareness and better reporting and surveillance, and/or changes in clinical practice, can also contribute to a higher disease incidence. The mysterious underpinnings of the recent upsurge in SF cases in Asia and Europe underscore the contributory role of factors other than genetic changes in GAS in shaping the epidemiology of GAS disease.

EVOLUTION AND POPULATION DYNAMICS

A central problem concerning GAS is the difficulty in connecting the evolutionary histories of different *emm* type-defined strains. There are large genetic gaps between extant organisms—but also rampant gene flow. So, what became of the evolutionary intermediates? Was there an extended series of population bottlenecks whereby new clones outcompeted their progenitors? And perhaps each successful new clone bore a new *emm* subtype plus changes in other genes, until a very unique and highly fit organism with a far more divergent *emm* type emerged and gained a strong foothold in the human host population. Perhaps the genetic intermediates went extinct due to low fitness in the context of shifting host-environmental conditions, or perhaps they remain extant, lurking in low numbers in a semiquiescent state and have yet to be sampled.

When did GAS emerge as a human pathogen? Was the GAS progenitor a human commensal? Or was the progenitor an animal pathogen that has yet to be recovered via sampling? Did the new GAS pathogen acquire key genes from a trading partner as a pivotal step in its emergence? What are the genotypes that confer adaptation of GAS to the primary ecological niches of the throat and skin? Are GAS commensals at heart, and should one view their ecology primarily through that lens?

What was the structure of the human population at the time GAS emerged as a human pathogen—highly migratory with intermingling or isolated tribes? What is the minimum host population size necessary to sustain GAS transmission? What becomes of a GAS strain if/when it runs out of susceptible hosts? Does the organism persist in an altered carrier state? Why do there exist ~243 *emm* types? And why are there even far larger numbers of unique combinations of M, SOF, and T antigens? Is the number of unique combinations of cell surface proteins related to characteristics of the human host population? If so, how?

What are the selective pressures shaping the GAS population of today? Is the ability to cause a clinically inapparent infection a more recent adaptation, driven by social behavior in the form of seeking antibiotics when ill, thereby giving the stealth invader a survival advantage? Why did SF recently re-emerge in Asia and the United Kingdom? Why did acute rheumatic fever undergo a renaissance in the United States during the 1980s? How might a future vaccine alter its evolutionary trajectory? A better understanding of the ecology and evolution of GAS, and its population genetic structure, may ultimately lead to interventions that limit its impact on human health.

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The authors dedicate this work to Susan K. Hollingshead (1953-2017), a coauthor of earlier editions of this chapter and a pioneer in the field of group A streptococcal genetics and evolution.

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Surface Structures of Group B *Streptococcus* Important in Human Immunity

LAWRENCE C. PAOLETTI AND DENNIS L. KASPER

13

With an uncanny ability to interpret results obtained with a handful of immunologic techniques that would be considered simplistic and insensitive by today's standards, Rebecca Lancefield and her coworkers in the 1930s set the stage for decades of research on *Streptococcus agalactiae*, also known as group B *Streptococcus* (GBS). In contrast to group A *Streptococcus*, which was isolated mainly from humans, the first strains of GBS studied by Lancefield were isolated from cows with mastitis or from "normal milk." At that time, GBS was a well-known cause of bovine mastitis and a concern mainly of the dairy industry and veterinary medicine (1).

The first report of GBS disease in humans appeared in 1938 (2). However, not until the 1960s, when several reports appeared in the clinical literature on the presence of hemolytic GBS among human newborns with sepsis and meningitis, did this pathogen receive serious attention from researchers (3–5). By the 1970s, GBS had eclipsed *Escherichia coli* as the most common bacterial cause of sepsis among newborns, with an incidence of early-onset disease of 2.9 per 1,000 live births and a case fatality rate of 46% (6). Despite variability among surveillance sites, rates of early-onset GBS disease remained high throughout the 1970s and 1980s (7), prompting the Institute of Medicine to declare in 1985 that the prevention of perinatal GBS disease was a national health priority (8).

Today, colonization with GBS—a risk factor for neonatal illness—has been reported in pregnant and nonpregnant adults (including the elderly), not only in the United States (7, 9) but also worldwide (10, 11). Concern about an expanding spectrum of GBS-related disease, in terms of both age and geography, accentuates the need to translate vaccine development efforts against this life-threatening opportunistic pathogen into global vaccine actions (12). This article highlights critical advances in our understanding of the roles of GBS surface antigens—specifically, the group B carbohydrate, the type-specific capsular polysaccharides (CPSs), and proteins—in immunity and of their application as components of experimental vaccines.

CARBOHYDRATE ANTIGENS OF GBS

Group B Carbohydrate

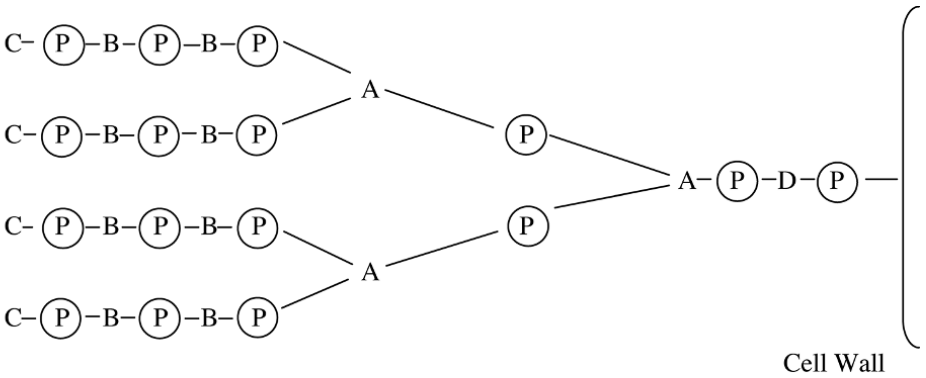
The group B carbohydrate is a cell wall-associated molecule common to all strains and serotypes of GBS. Positioned on the external surface of the cell wall and linked through a phosphodiester bond to the peptidoglycan (13), the group B carbohydrate is composed of rhamnose, galactose, *N*-acetylglucosamine, and glucitol. These sugars form four structurally unique oligosaccharide units (14) linked by phosphodiester bonds to create a complex and highly branched tetraantennary structure (Fig. 1). The terminal position and abundance of rhamnose suggested that this sugar would constitute or be part of an immunodominant epitope; this hypothesis was supported by inhibition studies using a number of derivative oligosaccharides with polyclonal and monoclonal antibodies specific to the group B carbohydrate (15).

Lancefield et al. (16) were the first to demonstrate the inability of antibody to the group B carbohydrate to protect mice from lethal challenge with viable GBS. Indeed, the presence of maternal antibody to the group B carbohydrate was associated with poor neonatal outcome despite good agreement between levels of specific IgG in maternal and cord sera (17). This result confirmed in humans what was already known in animals: that antibodies to the group B carbohydrate were not important to natural immunity.

Still, investigators wondered whether a group B carbohydrate vaccine would be effective in eliciting functional antibody if the carbohydrate's immunogenicity were enhanced. Rainard (18) demonstrated an improved immune response in cows when the group B carbohydrate was coupled to ovalbumin. Antibody induced to this conjugate vaccine was of the immunoglobulin G (IgG) class, was specific for the major sugars of the group B carbohydrate, and was opsonically active against a nonencapsulated GBS strain of bovine origin (18). That high-titered IgG specific for the group B carbohydrate was less active against highly encapsulated GBS strains of human origin was discerned in studies

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Oligosaccharides

A. α-L-Rha*p*-(1→3)- α-D-Gal*p*-(1→3)-ß-D-Glc*p* NAc-(1→4)- α-L-Rha*p*-(1→2)-[α-L-Rha*p* (1→3) α-D- Gal*p*-(1→3)-ß-D-Glc*p* NAc-(1→4)-]- α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→1')-D Glucitol-(3'-1)- α-L-Rha*p*.

B. α-L-Rha*p*-(1→2)-[α-L-Rha*p*-(1→3) α-D- Gal*p*-(1→3)-ß-D-Glc*p* NAc-(1→4)-]-α-L-Rha*p* (1→2)- α-L-Rha*p*-(1→1')-D-Glucitol-(3'-1)- α-L-Rha*p*.

C. α-L-Rha*p*-(1→2)- α-L-Rha*p*-(1→2)- α-L-Rha*p*-(1→1')-D-Glucitol-(3'-1)- α-L-Rha*p*.

D. α-L-Rha*p*-(1→3)- α-D-Gal*p*-(1→3)-ß-D-Glc*p* NAc-(1→3)- α-L-Rha*p*-(1→3)- α-L-Rha*p* (1→3)- α-L-Rha*p*-(1→3)-ß-L-Rha*p*-(1→4)-D-GlcNAc.

P = phosphate

FIGURE 1 Proposed structure of the streptococcal group B carbohydrate, as modified from reference 15. The group B carbohydrate is linked to *N*-acetylmuramic acid of the cell wall (162).

of a group B carbohydrate-tetanus toxoid conjugate vaccine (19). Adsorption studies using rabbit antisera to this vaccine with GBS strains of human origin that were highly encapsulated or with an isogenic mutant strain that lacked CPS showed CPS interference with specific antibody binding (19); this finding confirmed the inability of group B-specific antibody to access and bind to the group B antigen on highly encapsulated GBS cells. Not surprisingly, antibody to bovine isolates that possess low levels of CPS may be effective in combating GBS disease in the cow, whereas the same antibody would not be effective against more highly encapsulated GBS isolates of human origin. The ability of group B antibody to bind to poorly encapsulated GBS deserves further attention. The expression of CPS by a GBS strain (of human origin) is regulated by the cell's rate of growth (20), whereas the expression of the group B carbohydrate is not influenced by growth rate (21). Since slow growth rates, such as those of microbes colonizing the intestine (a common habitat of GBS), are associated with little capsule production, it might be that antibodies to the group B carbohydrate have a role in limiting colonization with this organism.

Capsular Polysaccharides

With few exceptions, all strains of GBS isolated from humans are encapsulated (Fig. 2, upper left) and can be classified on the basis of serology and CPS structure. Ten distinct GBS serotypes have been identified: Ia, Ib, II, III, IV, V, VI, VII, VIII, and IX. In the past, serotypes Ia, Ib, II, and III were equally prevalent in normal vaginal carriage and early-onset sepsis (i.e., that developing at less than 7 days of age) (9). However, type V is now an important cause of GBS infection (22), and strains of types VI and VIII have become prevalent among Japanese women (23). A pan-European study conducted between 2008 and 2010 showed that GBS serotypes Ia, III, and V together accounted for 88%, 96%, and 67% of strains isolated from neonates with early-onset disease, neonates with late-onset disease, and vaginal-rectal swabs of colonized pregnant women who delivered healthy babies,

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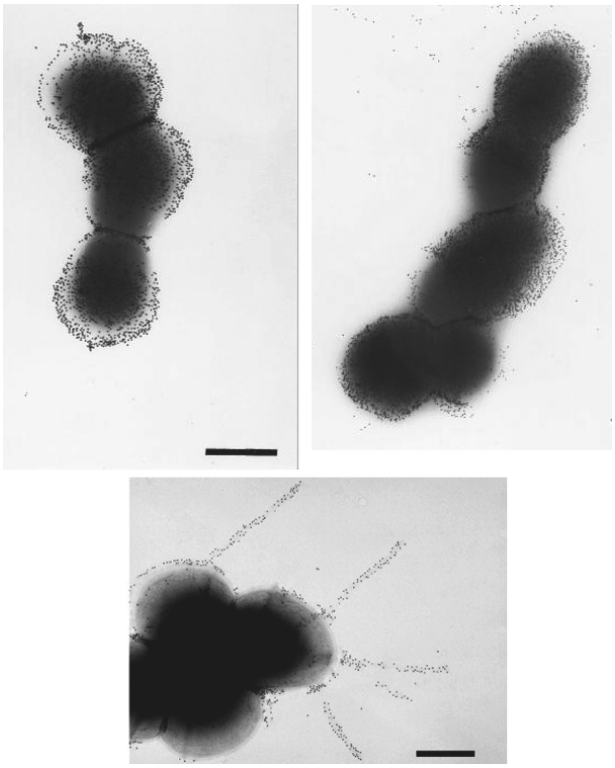


FIGURE 2 Electron micrographs of immunogold-labeled GBS showing surface localization of CPS, a protein antigen, and pili. Bars, 500 nm. (Upper left) CPS of GBS type Ia strain A909 was visualized after incubation with rabbit antiserum to the CPS followed by 20-nm-diameter gold-labeled protein A. (Upper right) The alpha C protein of GBS A909 was visualized after incubation with rabbit antiserum to the purified one-repeat alpha C protein followed by 15-nm-diameter gold-labeled protein A. Both upper images are reproduced from *Infection and Immunity* (163) with permission. (Bottom) Pilus structures of GBS type VIII strain JM9130013 were visualized with immunogold labeling using GBS80 antisera. The bottom image is reproduced with permission from *Science* (121).

respectively (12). These results reaffirm the clinical importance of types Ia and III and demonstrate that type V is an important serotype responsible for neonatal GBS disease in many parts of the world (24).

Remarkably, the serotype-specific polysaccharides of GBS are composed of three or four of only five different sugars: glucose, galactose, *N*-acetylglucosamine, rhamnose, and sialic acid. In marked contrast, other common pathogens such as *Streptococcus pneumoniae* and *Neisseria meningitidis* have different serotype polysaccharide structures that can contain different monosaccharides. The structural chair models in Fig. 3 highlight the heterogeneity among GBS CPSs. Chemical analyses revealed that some of the sialic acid residues were *O*-acetylated (at position C7, C8, or C9) in addition to being *N*-acetylated at C5 (25). Although not exhaustively studied, the percentage of *O*-acetylation on 10 GBS strains tested ranged from ~5 to ~55% (25), a result that should be considered when viewing the structures in Fig. 3. Nuclear magnetic resonance (NMR) proton profiles for 9 of the 10 GBS serotypes can serve as standards that can be evaluated side by side for identification and vaccine quality-control purposes (26).

Types Ia and Ib

Lancefield (27) was the first to notice serologic cross-reactions between strains bearing Ia and Ib antigens, although she did not know what cellular structure contained the differing epitopes. NMR analysis, performed 45 years after Lancefield's observation, showed that the native CPSs of GBS types Ia and Ib are indeed structurally similar, differing only in the linkage of the side chain galactose to *N*-acetylglucosamine (28); the type Ia CPS has a β-(1→4) linkage, and the type Ib CPS has a β-(1→3) linkage in this position. These linkages are critical to the immunospecificity of these CPSs. The repeating unit of Ia and Ib CPSs is a pentasaccharide with a disaccharide backbone and a trisaccharide side chain. Like all GBS CPSs, each possesses an α-(2→3)-linked sialic acid as a terminal side chain saccharide.

Type II

The native type II CPS repeating unit structure is composed of galactose, glucose, *N*-acetylglucosamine, and sialic acid in a 3:2:1:1 molar ratio (29). The repeating-unit structure of this CPS has two side chains—a sialic acid residue and a galactose residue—linked separately to the pentasaccharide backbone.

Type III

The type III CPS has a trisaccharide backbone of glucose, *N*-acetylglucosamine, and galactose and a disaccharide side chain of sialic acid and galactose linked β-(1→4) to the backbone *N*-acetylglucosamine (30). Although sialic acid *per se* is not immunodominant, the negatively charged carboxylate group on this sugar influences the conformation and thus the immunodeterminant epitopes of the entire polymer (31, 32). Removal of the terminal sialic acid from the repeating unit of the type III CPS with mild acid or neuraminidase results in a CPS with structural and immunologic identity to the pneumococcal type 14 CPS (33)—a finding that has enhanced our understanding of the roles of sialic acid in GBS immunity, as discussed further below.

Type IV

The GBS type IV CPS contains galactose, glucose, *N*-acetylglucosamine, and sialic acid in a 2:2:1:1 molar ratio (34). The presence of sialic acid does not appear to be a critical immunodeterminant for type IV CPS as it is for other GBS CPSs (34).

Type V

The repeating-unit structure of serotype V CPS is composed of seven sugars. The CPS has a trisaccharide backbone consisting of glucose, galactose, and glucose as well as two side chains. One side chain consists of glucose linked β-(1→3) to the backbone galactose. The other side chain is a trisaccharide of *N*-acetylglucosamine, galactose, and sialic acid. This side chain is linked β-(1→6) to a backbone glucose and terminates with sialic acid (35).

Type VI

The type VI CPS repeating unit comprises galactose, glucose, and sialic acid in a 2:2:1 molar ratio (36, 37). Type VI was the first GBS CPS described that lacked *N*-acetylglucosamine.

Type VII

The GBS type VII repeating unit contains a trisaccharide backbone and a trisaccharide side chain with a molar ratio of 2:2:1:1 for galactose, glucose, *N*-acetylglucosamine, and sialic acid (38).

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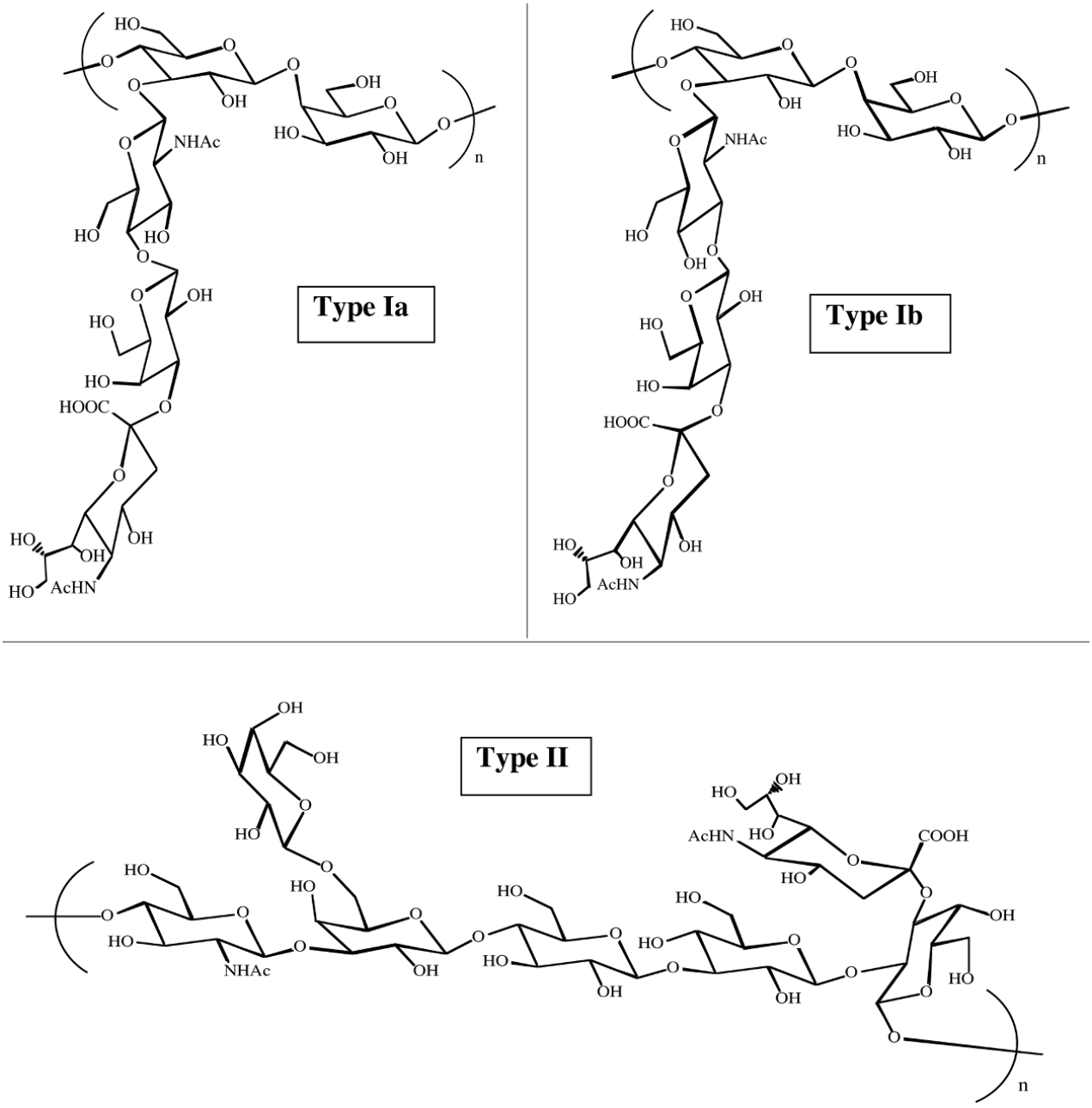
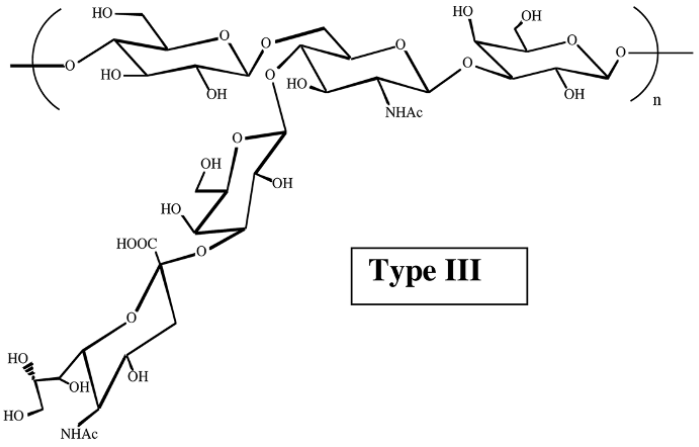
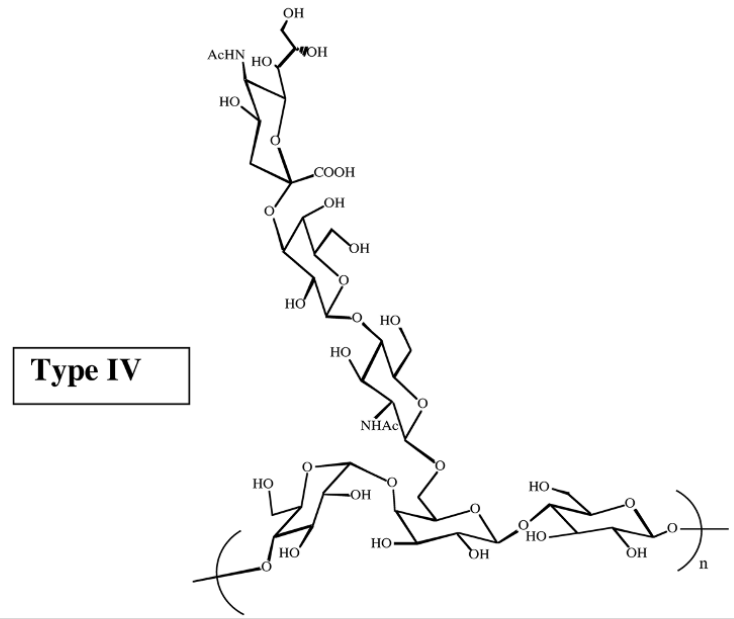


FIGURE 3 Chair models of the repeating units of GBS capsular polysaccharides.

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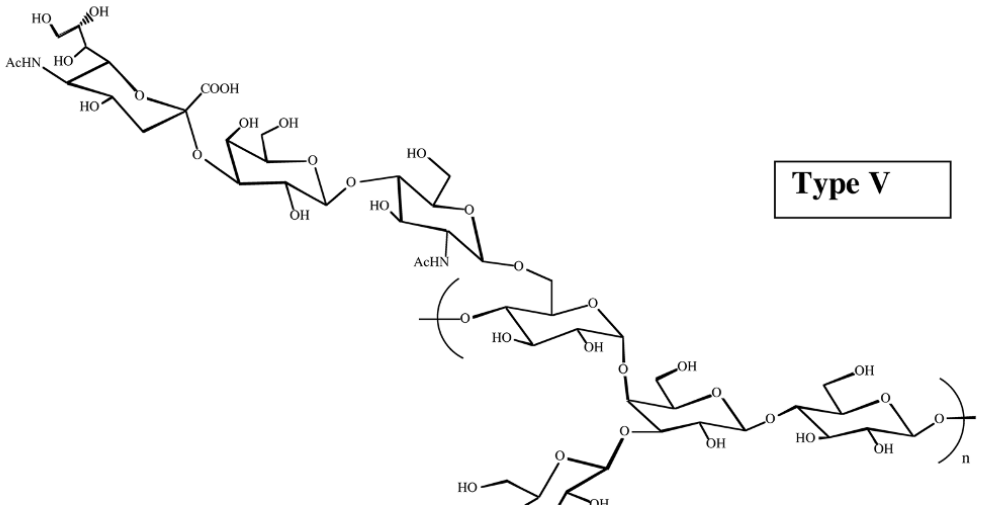


FIGURE 3 *(continued)*

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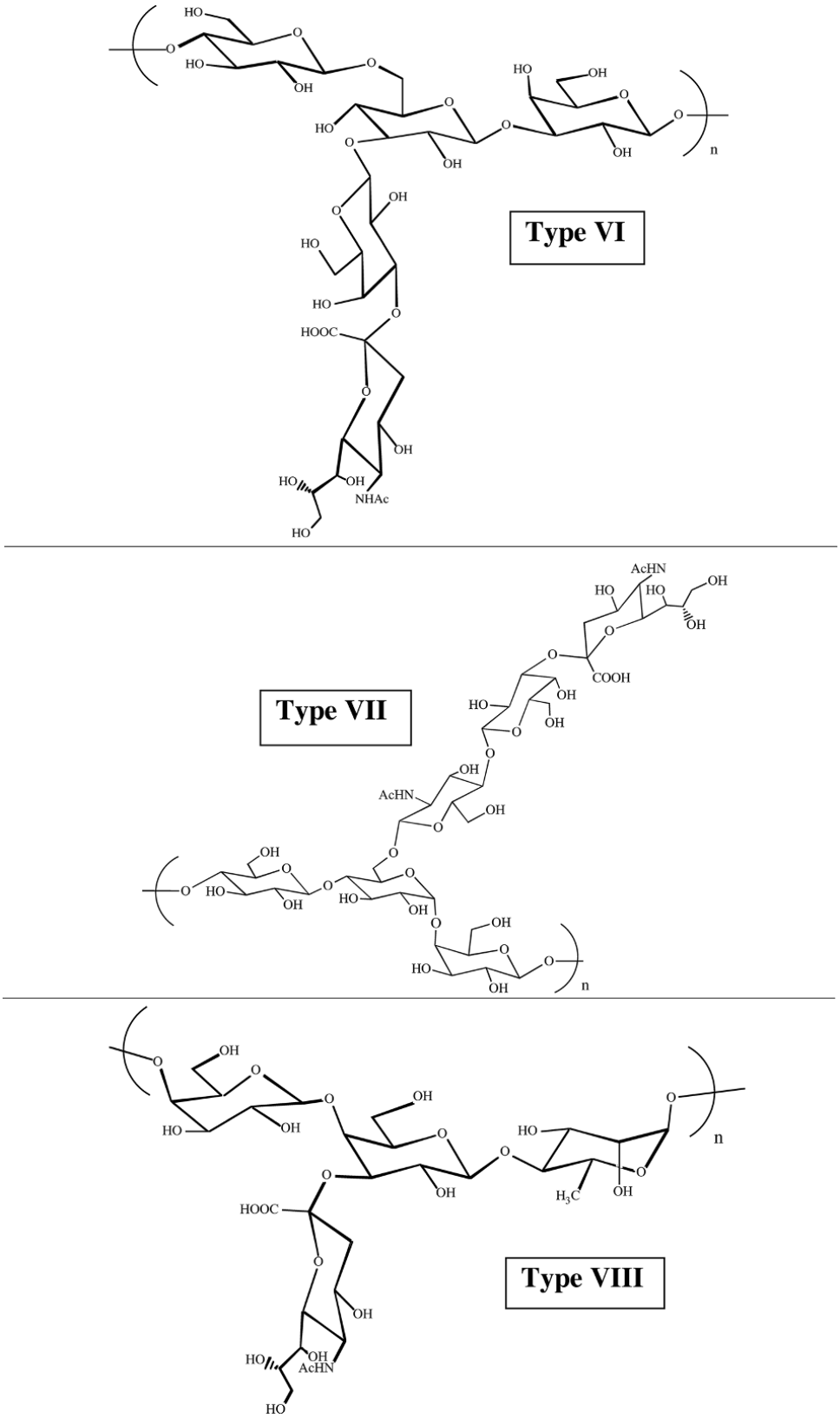


FIGURE 3 *(continued)*

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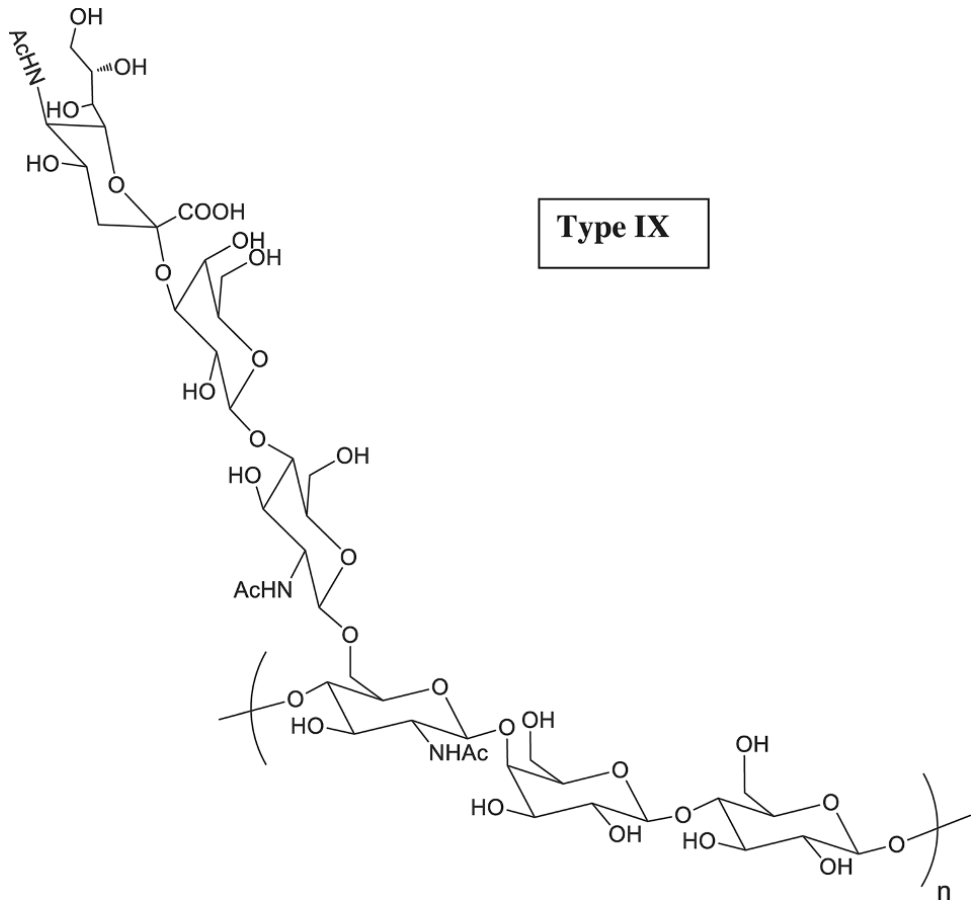


FIGURE 3 *(continued)*

Type VIII

The structure of GBS CPS type VIII is the mos unusual and simplest of all GBS serotypes. Like type VI CPS, the type VIII CPS lacks *N*-acetylglucosamine, yet unlike any other GBS CPS, this tetrasaccharide contains rhamnose, a sugar previously associated exclusively with the group B carbohydrate. The type VIII repeating unit contains glucose, galactose, rhamnose, and sialic acid in a 1:1:1:1 molar ratio, with sialic acid as the monosaccharide side chain (39).

Type IX

The most recently identified GBS serotype, type IX (40), contains a capsule with a repeating-unit structure comprising galactose, glucose, *N*-acetylglucosamine, and sialic acid in a 2:1:2:1 ratio. Structurally, the CPS has a trisaccharide backbone and a trisaccharide side chain that terminates with sialic acid (41). It is the only GBS serotype discovered thus far with two *N*-acetylglucosamine residues per hexasaccharide repeating unit.

Sialic Acid as a Structural Feature, a Virulence Factor, and a Site for Protein Coupling

Early studies of GBS capsule structures by Lancefield and Freimer (42) used hot HCl or cold trichloroacetic acid (TCA) (17) extraction methods and resulted in polysaccharide compositional information that was chemically incomplete. In addition, these early extraction techniques yielded only partial information about the antigenicity of the capsules of GBS. These investigators surmised that an acid-labile component contributed to the serologic specificity and negative charge of the CPS antigen. That GBS type-specific CPSs contain sialic acid was cited as a personal communication to Lancefield by Liu in 1972—a discovery verifying that sialic acid indeed "represents the missing antigenic component of the carbohydrate in the TCA antigen" (43). The acidic nature of the TCA extraction procedure resulted in the purification of an incomplete antigen because the α-(2→3) ketosidic linkage between sialic acid and galactose—a bond that is universal among all GBS type-specific CPSs—is acid labile (44).

Subsequent purification techniques avoided acid extraction methods and yielded polysaccharides whose complete structural information was obtainable. For example, structural analysis of GBS type III CPS showed that the acid-extracted or desialylated polysaccharide (core) structure was identical to that of *S. pneumoniae* type 14 CPS (45). This serendipitous connection launched a number of chemical, biochemical, and immunologic studies (46–48) of the fine structural features of this bacterial surface carbohydrate. An early study revealed that carboxylate groups on sialic acid residues form intramolecular hydrogen bonds with the galactose residues in the backbone of the type III CPS (31). Reduction of the carboxylate groups to hydroxymethyl groups of sialic acid destroyed CPS antigenicity with antisera to the native type III CPS, while oxidation of the carboxylate

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groups with sodium *meta*-periodate to remove carbons 8 and 9 did not affect antigenicity. The finding that GBS CPS retains its antigenicity upon oxidation with sodium periodate became important in the development of a coupling strategy to prepare GBS conjugate vaccines. The requirement for sialic acid to impart antigenicity on GBS CPSs is not universal, however. Whereas the sialic acid residue on type Ia CPS is required for complete antigenicity, the binding of specific antisera to the structurally related type Ib CPS does not depend on sialic acid (49).

Oligosaccharides enzymatically generated from native type III CPS not only defined the pentasaccharide repeating unit but also led to studies that revealed that specific antibody bound in a manner that increases along with the length of the structure (30, 32). NMR analyses and molecular dynamic modeling suggested an extended helix conformational structure of the native, fully sialylated type III CPS (50). In 1999, affinity-binding studies with monoclonal antibodies specific to GBS type III CPS supported earlier findings of a conformational epitope with extended helical segments (51). Almost two decades later, Carboni et al. (using current NMR technology; a new, functionally active, specific rabbit monoclonal antibody; x-ray crystallography; and synthetically and chemically obtained type III oligosaccharides) (52) described a linear, sialic acid-dependent functional epitope "that is fully contained within four consecutive sugars deriving from the PSIII backbone and one branched disaccharide present in this sequence."

Sialic acid is a key regulator of complement deposition on the surface of microbes. Surfaces of activating particles for the alternative complement pathway have little sialic acid (like many nonpathogenic bacteria), whereas nonactivating surfaces are relatively rich in sialic acid (like GBS). Mechanistically, surface-associated sialic acid greatly increases the binding affinity of β-1H for C3b, thereby accelerating intrinsic decay of the C3 convertase, whereas the decreased affinity of β-1H for the surface of an activating particle favors formation of the convertase on bound C3b (53). Therefore, the presence of sialic acid on GBS creates a surface that does not activate the alternative pathway of complement (54). Because the activation of the major particle initiating the alternative complement pathway is inhibited, the host now requires antibodies to activate complement deposition via the classical pathway. Marques et al. (55) reported lower amounts of C3 deposition on GBS organisms that possessed a sialylated rather than a desialylated CPS. In all, these findings suggested a critical role for sialic acid in evading the host's natural immune mechanisms and mechanistically explained a requirement for antibodies to opsonize GBS with C3b and facilitate phagocyte-mediated uptake and killing—i.e., a clear role in GBS virulence.

The role of the complement pathway in the immune response to sialylated antigen (GBS type III CPS) and desialylated antigen (*S. pneumoniae* type 14 CPS) was examined in untreated mice and in mice treated with cobra venom factor to deplete complement (56). Complement depletion did not affect the immune response to the sialylated antigen, but this condition markedly reduced the specific antibody response to desialylated CPS. These results demonstrated the importance of the CPS structure to both complement activation and generation of a specific antibody response (56).

The role of sialic acid as a virulence factor of GBS was confirmed in studies with a transposon mutant of type III strain COH 31r/s that lacked the ability to synthesize sialylated CPS (57). GBS strain COH 31-21 produced an asialo type III CPS that bound to pneumococcal type 14 capsular antisera but not to native type III CPS antisera. This single genetically mediated alteration in capsular structure was found to decrease virulence by 2 orders of magnitude from that of the wild-type parent strain. In fact, the asialo GBS strain was similar in loss of virulence to another transposon mutant, COH 31-15, that completely lacked CPS (57).

Sialic acid is the terminal, or sole, sugar of the side chains of all GBS CPSs (Fig. 3). In addition to its role as a virulence factor, sialic acid has been a convenient site for directed protein coupling in the development of GBS vaccines. Selective periodate oxidation of GBS CPSs removes C9 and creates aldehydes on the capsule's sialic acid at C8 and/or C7, which can serve as binding sites for the ε-amino group on lysine moieties in carrier proteins (the Schiff base reaction). Stable, covalent linkages are formed between the CPS and protein antigens through reductive amination chemistry. While conjugate vaccines prepared with CPSs of GBS types Ia, Ib, II, and III induced in humans the desirable antibody isotype IgG at levels greater than IgM, vaccines prepared with GBS type V did not (58). As discussed further below, the sialic acid moiety had an unexpected key role in the response of the immune system of nonhuman primates and humans to conjugate vaccines prepared with GBS type V CPS.

Genetics of CPS

The transposon mutants described above were the first used to map the genes that are important in GBS CPS expression (59). Since then, complete genomic sequences for the CPS biosynthesis regions of all known serotypes of GBS have been reported (Fig. 4) (41, 60). As in many encapsulated bacteria, genes conserved across diverse capsular serotypes flank genes encoding enzymes unique to a specific capsule serotype. In GBS, a central group of serotype-specific glycosyltransferases and polymerases is flanked on one side (Fig. 4) by genes encoding enzymes that synthesize and activate sialic acid, the terminal sugar on the side chain of all nine serotypes (61, 62). Flanking the other side of the serotype-specific glycosyltransferases (Fig. 4) is a group of genes hypothesized to function in export of the polysaccharide capsule (61). In analyzing the GBS capsular biosynthesis clusters of the different GBS capsular types, Cieslewicz and colleagues found strong evidence for horizontal gene transfer resulting in both intra- and interspecies recombination events (60). While diversity in GBS capsules was thought to be due to immune pressure, the relatively limited number of distinct capsule types might be related to structural elements required for pathogenicity.

The recent emergence of GBS type IX led Berti and coworkers not only to elucidate the chemical CPS structure of this new serotype but also to propose a phylogenetic relationship between it and two other recently identified GBS serotypes, V and VII (41). As shown in Fig. 4, serotype IX is genetically more similar to type V than to type VII, and the differences within the *cps* operon may reflect divergent evolution between serotypes VII and IX (41).

PROTEIN ANTIGENS OF GBS

Milestones in the discovery of GBS protein antigens can be divided into two time periods that reflect advances in molecular and genomic technologies. That surface proteins of GBS are important to immunity was initially recognized in 1975 when Lancefield and coworkers (16) described the cross-serotype protection afforded by rabbit antiserum to protease-sensitive epitopes. This publication led to an appreciation of the diversity of GBS surface proteins and to an increased

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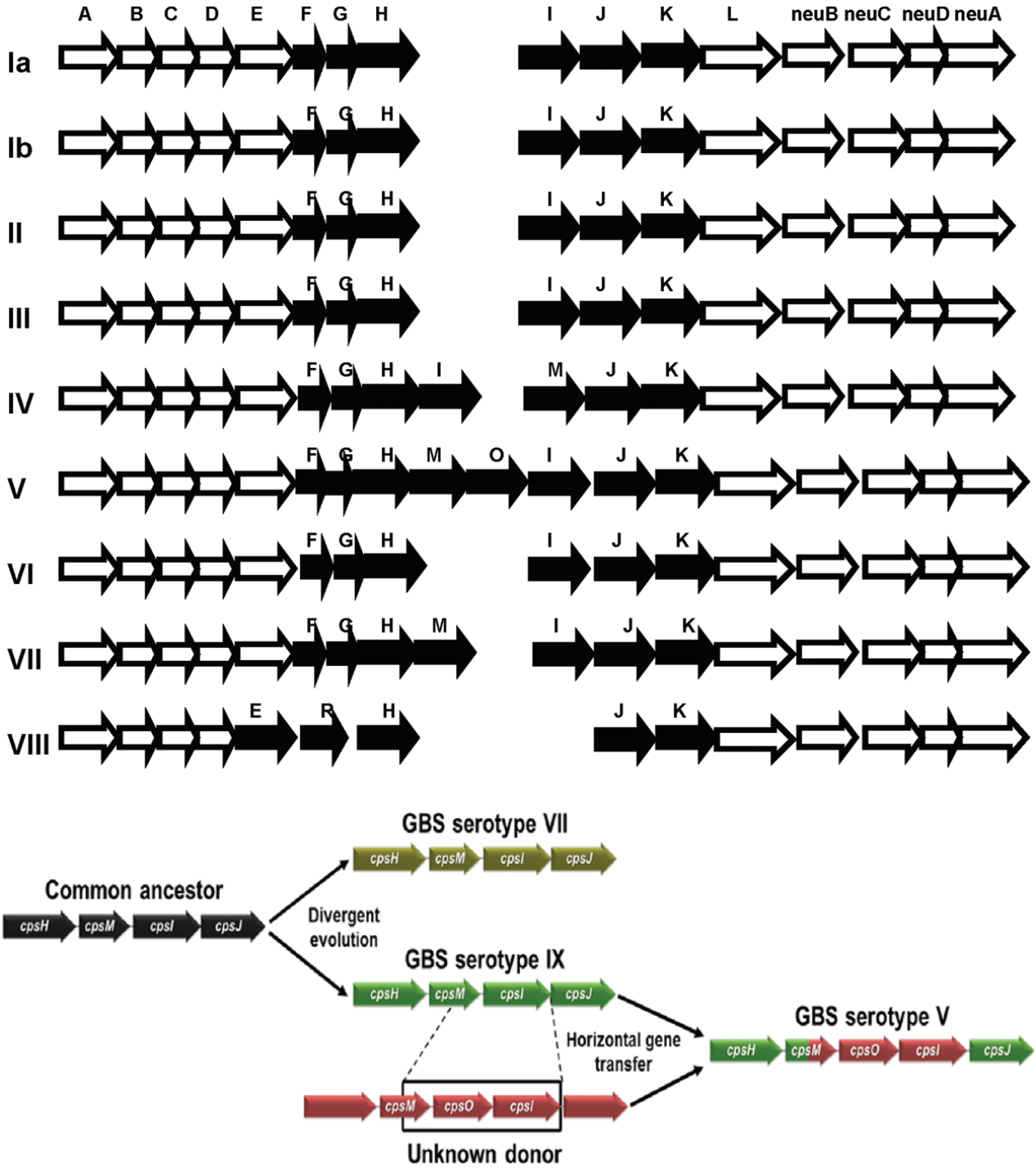


FIGURE 4 (Top) Schematic of *cps* gene clusters in nine GBS serotypes. Conserved genes are depicted by white arrows; variable genes are depicted by black arrows. The variable gap in the center of the *cps* clusters was introduced to permit alignment of homologous genes (60). (Bottom) Hypothetical genetic relationship between CPSs of serotypes V, VII, and (newly described) IX. Reproduced with permission from the *Journal of Biological Chemistry* (41).

awareness of their roles not only in immunity but also in pathogenicity.

The availability of complete genomic sequences for GBS isolates allowed systematic exploration of surface-expressed proteins that may function as protective immunogens (63). Sequences for GBS type III and type V isolates revealed an AT-rich genome with ~2 million nucleotides and ~2,100 open reading frames. As expected, the GBS genome possesses great similarity to those of *Streptococcus pyogenes* and *S. pneumoniae*; the GBS type V genome shares 1,060 of its 2,144 predicted genes with both organisms and 401 genes with one—but not both—of these species. Approximately 650 proteins were predicted to be membrane associated or secreted in the type V genome, including 24 proteins containing an LPXTG motif

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TABLE 1 GBS proteins evaluated as protective antigens

Designation(s)	Associated GBS serotype(s)	Mouse model[a]	Tested as CPS carrier protein?	Reference(s)
Alpha C protein	Ia, Ib, II	MPV-NP	No	78
MAV-NP	Yes	156		
Alp2	V, VIII	MPV-NP	No	84
Alp3	V, VIII	MAV-NP	Yes	157
Beta C proteins[b]	Ib	MAV-NP	Yes	71, 98–100, 155
BibA	Ia, Ib, II, III, IV, V	MAV-NP	No	118
BPS	All[c]	AMAV-C	No	104
FbsA	Ib, III, V	AMAV-C	No	117
LrrG	Ia, Ib, II–VIII	AMAV-C	No	112
Ornithine carbamoyltransferase	Ia, Ib, II–V	PNP	No	116
Phosphoglycerate kinase	Ia, Ib, II–V	PNP	No	116
Rib protein (R4)	III	AMAV-C	No	112
ScpB (C5a peptidase)	All[c]	AMAV	Yes	106, 110
Sip (GBS322, SAG0032)	Ia, Ib, II, III, V, VI	MAV-NP	No	63, 115
SAN_1485	III	MAV-NP	No	113
Pilus proteins[d]				
BP-1 (PI-1)	Ia, Ib, II, III, V	MAV-NP	No	122
AP1-2a (PI-2a)	Ia, Ib, II–V, VIII	MAV-NP	No	122
BP-2b (PI-2b)	Ia, Ib, III, IV, V	MAV-NP	No	122
GBS67 (AP1-2a, SAG1408)	Ia, Ib, II, III, V	MAV-NP	No	63
GBS80 (BP-1, SAG0645)	Ia, Ib, II, III, V	MAV-NP	Yes	63, 125
GBS104 (SAG0649)	Ia, Ib, II, III, V	MAV-NP	No	63

[a]MPV-NP, mouse maternal passive vaccination-neonatal pup challenge model; MAV-NP, mouse maternal active vaccination-neonatal pup challenge model; AMAV-C, adult active vaccination challenge model; AMAV, adult active vaccination; PNP, passive neonatal pup challenge model.
[b]Includes purified native beta C protein, recombinant beta C protein, and recombinant beta C protein with IgA-binding site.
[c]Not known for recently described serotype IX.
[d]BP, backbone protein (major pilus subunit); PI, pilus island; AP, ancillary protein (pilus subunit).

(which predicts surface exposure via cell-wall anchoring), 51 lipoproteins, and 177 proteins carrying a signal sequence. Approximately 300 genes were successfully expressed and used to raise antibodies in mice; of these, 139 recognized a predominant band in Western blots and 55 appeared (on the basis of fluorescence-activated cell sorting) to be surface expressed in GBS. In the type III genome, 21 genes encoded LPXTG proteins and, on the basis of characteristic sequences, 36 lipoproteins are also likely to be surface exposed (64, 65).

The combination of genomics and proteomics, along with *in vitro* and *in vivo* functional assays, has revealed a wide range of surface-expressed antigens that may play a role in natural immunity or serve as protective antigens for inclusion in GBS vaccines. Presented in this section and summarized in Table 1 are GBS proteins that have been evaluated as vaccine antigens in animal models of GBS disease.

Alpha C Protein

Wilkinson and Eagon (66) described a protein extract obtained from a serotype Ic strain of GBS. On the basis of this description, Lancefield recognized that the previously designated serotype Ic comprised the Ia CPS and the Ibc protein. In seminal experiments, Lancefield and coworkers demonstrated that antibodies to the protein component protected mice from lethal challenge with either Ia or Ib capsular serotype GBS strains that bore the Ibc proteins (16). Even in their initial work, Wilkinson and Eagon recognized that the protein extract from the Ic GBS strain contained two moieties, one sensitive to digestion with trypsin and the other resistant (66). Bevanger et al. described the trypsin-resistant component as the alpha antigen of the Ibc protein and the trypsin-sensitive component as the beta antigen (67). The term "C proteins" was coined to clarify the distinction between the proteins and the carbohydrate antigens; the presence of the C protein(s) was denoted by the letter following the capsular serotype (e.g., Ia/c) (68). Later work by a number of investigators identified the alpha and beta components as distinct proteins, independently expressed in different strains and encoded by separate genes (69–73). Generally present in strains of serotypes Ia and Ib and frequently present in strains of type II, these antigens are occasionally found in GBS of all serotypes. The trypsin-resistant antigen is now referred to as the alpha C protein (Fig. 2, upper right) and the trypsin-sensitive antigen as the beta C protein. In one survey, these proteins were found in 59% of strains in a collection of 785 clinical isolates from the University of Minnesota (74). All Ib strains expressed C proteins, and 84% expressed both alpha and beta proteins; 91% of Ia strains and 81% of type II strains expressed the alpha C protein. In a similar survey, Bevanger found that 52% of human isolates of GBS expressed the alpha C protein and 25% expressed the beta C protein (75).

Monoclonal antibodies have been used to identify the alpha C protein and to define it as a mouse-protective epitope in GBS (70). In a Western blot analysis, monoclonal

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antibody reacted with the vast majority of strains of GBS type Ia (all of 14 strains evaluated), type Ib (11 of 15 strains evaluated), and type II (all of 12 strains evaluated). Western blots of surface extracts from GBS strains also demonstrated an unusual phenotypic characteristic of this protein: a pattern of regularly spaced bands with a periodicity of ~8 kDa. Also evident on Western blots was size heterogeneity of the expressed protein from strain to strain, with the size of the largest band varying from <70 kDa to >160 kDa in a panel of 37 isolates. An interesting observation was that the protein sizes did not appear to be randomly distributed but clustered around 100 kDa, with relatively few isolates expressing either a very large or a very small alpha C protein.

The gene encoding the alpha C protein was cloned from the prototype Ia/c strain A909 from the Lancefield collection (76). This gene, *bca*, consists of 3,063 nucleotides and encodes several identifiable regions. A signal sequence with homology to other Gram-positive surface proteins was 56 amino acids long (77). This sequence was followed by an amino-terminal domain of 170 amino acids with a predicted molecular mass of 18.8 kDa. The most striking feature of the gene was a series of nine long tandem repeats that had 246 nucleotides each (encoding 82 amino acids, or 7.9 kDa) and that were identical at the nucleotide level. The repeat region composed >75% of the mature protein, and the correspondence between the size of the repeat region and the distance between the bands on a Western blot suggested that the repeats gave rise to the ladder pattern seen on Western blots. Antibodies to both the repeat region and the amino-terminal domain have been shown to be protective in an animal model and to mediate opsonophagocytic killing *in vitro* (78). The carboxyl terminus showed homology to those of other Gram-positive surface proteins, including an LPXTG domain (identified as a peptidoglycan-binding motif), a hydrophobic membrane-spanning region, and a charged tail thought to be involved in orienting and anchoring the protein to the cell wall (76).

A size discrepancy between the alpha C proteins of GBS isolates from mothers and those from their neonates suggested that a loss of repeats within the protein gene may occur during colonization or infection with GBS (79). This phenomenon was confirmed experimentally in an immune mouse model. While spontaneous deletions within the alpha C protein occur at a low rate ($\sim 10^{-4}$), passage through mice given antiserum to the alpha C protein gave rise to deletion rates approaching 50%. Moreover, the mutants bound poorly to the antiserum and were more poorly opsonized for phagocytic killing than the wild-type strain. *In vitro* experiments showed that purified alpha C proteins containing 9 or 16 repeats bound with high affinity to antibodies to wild-type (9-repeat) alpha C protein, whereas 1- and 2-repeat alpha C proteins bound with lower overall affinity (80). These differences were not due simply to a reduced number of antibody-binding sites but appeared to reflect a conformational dependence of the protein on repeat number. In contrast, antibodies to the one-repeat protein appeared to bind to all of the different constructs with similar overall affinity. Antibodies to the single- or double-repeat proteins showed greater protective efficacy *in vivo* and tended to allow fewer deletion mutants to escape. Further studies demonstrated that immunogenicity in mice was inversely related to repeat number (81): 9- and 16-repeat alpha C proteins evoked lower titers of antibody, particularly to the amino-terminal domain, than did the 1- and 2-repeat variants. These findings suggested that modulation of repeat number may be a mechanism for interaction with and evasion of the host's immune system.

Natural exposure to GBS containing alpha C protein induced functionally active, specific IgG and IgM but only in acute-phase sera from women with invasive GBS disease; colonization with GBS containing alpha C protein did not elicit a systemic immune response (82).

Rib protein

While the alpha C protein is present mainly in GBS strains of types Ia, Ib, and II, a phenotypically similar but immunologically distinct protein, designated Rib, has been discovered in a type III GBS strain (77). This protein, present in 31 of 33 clinical type III isolates studied, showed a laddering phenotype on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), resistance to trypsin digestion, and variability in molecular size among clinical isolates. Antiserum to the protein protected between 62 and 90% of mice from challenge with GBS but did not cross-protect the animals against infection by alpha C protein-positive GBS. Amino-terminal sequencing of the protein demonstrated homology to the alpha C protein, with identity in 6 of 12 residues. When cloned and sequenced, the gene encoding this protein showed structural as well as sequence similarity to the alpha C protein (83). Like *bca*, the *rib* gene contains a signal sequence, an amino-terminal region, a series of tandem repeats, and a carboxy-terminal membrane anchor region. Thus, a family of genetically similar, tandem repeat-containing proteins was identified in GBS. Rib has been shown to be identical to the previously recognized protein R4 described below.

Other alpha-like proteins

During the 1980s, the previously obscure GBS serotype V emerged as an important pathogen in the United States. A novel protein identified in many type V strains was phenotypically similar to the alpha C and Rib proteins (84). Antiserum to the purified protein recognized a homologous protein of variable size in 25 of 41 clinical isolates. This antiserum protected 78% of neonatal mice from GBS challenge. Thus, the protein exhibited the characteristic features of this family of proteins: (i) protective efficacy against infection with a homologous GBS strain in a mouse model, (ii) a laddering phenotype on SDS-PAGE with variable molecular size among clinical isolates, and (iii) resistance to trypsin digestion. In addition, this protein displayed amino acid sequence similarity both to the alpha C protein (17 of 18 evaluable residues) and to the Rib protein (5 of 10 residues). Further analysis revealed two more alpha-like genes, *alp2* and *alp3*, found most often in GBS of serotypes V and VIII. These displayed mosaicism, each containing elements of other GBS proteins while preserving the overall structure found in alpha C protein and Rib: an amino-terminal domain, a series of long tandem repeats, and a highly conserved carboxy-terminal domain (85). Alp3 was identical to R28, a protein reported to act as an adhesin in group A streptococci (86). Both proteins contained amino-termini with high degrees of similarity to alpha C protein and Rib protein and repeating units identical to those found in Rib. These findings strongly suggested inter- and intraspecies recombination of genetic material, a phenomenon subsequently noted in the capsular biosynthesis gene cluster.

R proteins

R proteins, also identified by their resistance to trypsin digestion, were initially recognized in *S. pyogenes* by Lancefield and Perlmann (87). Wilkinson (88) identified four immunologically distinct R proteins (R1, R2, R3, and R4) occurring in various combinations in streptococci of groups A, B,

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and C. Flores and Ferrieri (89) found R proteins of subtypes R1 and R4 in 49 (37%) of 131 GBS clinical isolates. The proteins were present predominantly in type II and III strains. Lindén et al. (90) showed that rabbit antiserum to an affinity-purified R protein from type III GBS protected mice against invasive infection by type II R-positive strains but not against that by type III R-positive strains. The presence of R protein antibodies in human serum correlated with the absence of invasive infection by R protein-expressing GBS strains in human neonates (91).

The R proteins appear to share two important phenotypic characteristics with the members of the tandem repeat-containing family of GBS surface proteins: trypsin resistance and protective capacity. Bevanger et al. (67) isolated a protein from a serotype III strain, designated it an R-associated protein, and found that it was biochemically and immunologically highly similar to both R4 and Rib. Similarly, Lachenauer and Madoff (84) showed that Alp2 from a GBS type V strain was reactive with antiserum to R1. Further characterization of the R proteins will probably reveal that they are members of the same family as the other tandem repeat-containing GBS proteins and that the genes encoding them possess a similar structure. Indeed, Smith and colleagues concluded on the basis of partial amino acid and genetic sequences that Rib was identical to R4 (92).

Beta C Protein

The beta C protein was initially recognized as the trypsin-susceptible component of the C protein (66). Subsequent work has demonstrated its expression on the surface of ~10 to 25% of GBS strains, with predominance in Ib strains (69, 74, 75). Russell-Jones et al. (72) recognized that this antigen was a 130-kDa protein and that it bound specifically to human IgA. This binding occurs via the Fc portion of the IgA heavy chain and is of high affinity, with an affinity constant of $3.5 \times 10^8\ M^{-1}$ (93). The affinity is considerably higher for serum than for secretory IgA. The gene for beta C protein was cloned by several groups (71, 94–96), and antibody to the cloned gene product was demonstrated to have protective capacity against GBS infection in mice (71). Two complete nucleotide sequences for the gene (*bac*) have been published and differ minimally; one is 1,134 amino acids in length (95) and the other, 1,164 amino acids (96). Other than a single amino acid difference, the two sequences differ only in the number of short XPZ repeats found in the carboxy-terminal half of the protein. Both contain consensus Gram-positive signal sequences and carboxy-terminal membrane anchor regions that have extensive homology to other Gram-positive surface proteins (including the GBS tandem-repeating proteins discussed above). Both groups of researchers studying the beta C protein detected IgA-binding capacity in the amino-terminal half of the molecule; however, Jerlström et al. (96) detected IgA binding within two separate non-overlapping subclones, whereas Hedén et al. (95) detected only a single IgA-binding region. Jerlström and colleagues (97) later localized IgA binding to a 73-amino-acid region containing an MLKKIE sequence and found that this sequence was required for IgA binding. These observations suggest that only one IgA-binding region exists (as is the case with other immunoglobulin-binding proteins) and that Jerlström's earlier finding may have resulted from the use of polyclonal IgA, which reacted in an immune fashion with a portion of the protein not involved in IgA binding.

In mice, antibodies elicited to beta C protein appear to be highly protective (71, 98, 99). Immunization of mice with this protein protected their neonatal pups from invasive GBS infection at >90% efficacy, and antibodies facilitated bacterial opsonization for phagocytic killing by polymorphonuclear leukocytes (99). One group has found that antibodies to beta C protein—unlike those to any other GBS antigen—are bactericidal *in vitro*; i.e., they are able to kill GBS in the absence of polymorphonuclear leukocytes (98). Beta C protein has also served as an effective carrier protein in conjugate vaccines. A glycoconjugate containing equal quantities of beta C protein and type III CPS linked by reductive amination was broadly protective against invasive GBS infection in neonatal mice challenged with either type III GBS or a type Ia/c beta-positive strain (100). Rabbit antiserum to the conjugate vaccine was opsonic against multiple strains containing either type III CPS or beta C protein. Similar conjugates with beta C protein have been made with types Ia, II, and III CPSs and have elicited antibody titers comparable to those elicited by CPS-tetanus toxoid (TT) conjugates (101). Lachenauer et al. (102) compared naturally acquired beta C protein-specific IgG levels in mothers colonized with beta C protein-positive GBS to levels in mothers who gave birth to infants who developed invasive illness with beta C protein-positive strains. In contrast to findings with antibody to several type-specific CPSs, no correlation was found between the beta C protein-specific IgG level and protection from invasive disease; however, only a small number of cases were available for study (102). Invasive infection but not colonization with beta C protein-containing GBS resulted in IgM and IgG specific to this trypsin-sensitive surface antigen (103). This finding suggests that this antigen has potential for inducing antibodies in humans if administered in sufficient doses parenterally.

BPS Protein

Erdogan and colleagues described a 105-kDa protein in a GBS strain and initially designated it R5 since it was derived from an R-positive GBS isolate (104). Although initially classified as an R-like protein, it bears no phenotypic relationship to other R proteins and has been renamed BPS protein (group B protective surface protein). In a mouse protection assay, BPS protein elicited antibodies that protected >80% of mice from lethal challenge with a single GBS strain; the survival rate was 20% among controls.

C5a Peptidase (ScpB)

C5a peptidase, a surface localized protease of ~125 kDa present in both group A and group B streptococci, has been shown to inactivate human C5a, an important component of complement-mediated neutrophil chemoattraction (105), but also serves as an anaphylatoxin. In addition, C5a peptidase may play a role in bacterial invasion of epithelial cells and may bind to fibronectin (106, 107). There is evidence that human serum contains antibodies to this protein (108); in group A streptococci, immunization with C5a peptidase elicited antibodies in mice and reduced colonization of the nasopharyx by several group A streptococcal strains (109). While C5a peptidase antibodies do not appear to be opsonic against group A streptococci, there is evidence of opsonic activity against GBS (110) when C5a peptidase is used as a carrier protein. The potential for the use of C5a peptidase in a GBS vaccine is discussed below. Recombinantly expressed C5a peptidase induced opsonically active antibody when delivered intranasally to mice in the presence of cholera toxin or CpG-ODN 1826 adjuvant (111). That recombinant forms of

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C5a peptidase and Sip (see below) induced mucosal IgA as well as IgG in murine serum and in vaginal tissue suggests that these proteins may have a role in vaccines designed to stem GBS vaginal colonization and thus reduce horizontal transfer of GBS to the newborn.

LrrG Protein

A genetic screen for exported proteins revealed *lrr*G, a gene that is highly conserved among all 17 clinical GBS isolates (representing 9 serotypes) tested (112). This gene encodes a novel leucine-rich repeat (LRR) motif common to bacterial invasins. Recombinant LrrG, shown to function as a GBS adhesin, was highly immunogenic in mice, eliciting antigen-specific IgG1. Active vaccination of inbred mice with recombinant LrrG in the presence of alum protected 10 of 11 mice (91%) from an ordinarily lethal challenge with GBS type III strain 97/0099, whereas only 5 of 12 mice (42%) that received recombinant Rib protein survived (112). These results, in combination with the conserved presence of *lrr*G among almost all GBS serotypes, make LrrG an attractive protein to consider in the quest for a universal GBS vaccine or as a carrier protein for CPS antigens.

SAN_1485

SAN_1485 was one of 43 surface-associated proteins identified by treatment of whole cells of the GBS type III COH1 strain with proteases and subsequent analysis of the recovered peptides by mass spectrometry (113). When administered to female outbred mice, recombinant SAN_1485, which consisted of a 550-amino acid fragment chosen by mass spectrometric analysis of the 1,085-amino acid protein, protected 82% and 87% of pups against challenge with an ordinarily lethal dose of GBS type III strain M781 and COH1, respectively (113).

Sip

This surface-localized protein of ~45 kDa is present in GBS of serotypes Ia, Ib, II, III, V, and VI (114). Immunization of mice with native Sip (surface immunogenic protein) elicited protection against challenge with several GBS serotypes (114). In a neonatal mouse protection assay, Martin and colleagues used recombinantly derived Sip to immunize mouse dams (115). Survival of neonatal pups whose dams had been immunized ranged from 75 to 98% (compared with rates of 0 to 12% among controls) across a broad range of serotypes. Recombinant Sip in combination with rScpB (C5a peptidase, described above) has been tested in mice as a mucosal vaccine (111).

Ornithine Carbamoyltransferase and Phosphoglycerate Kinase

Hughes and colleagues applied proteomics and reverse genetic methods to identify two surface-exposed enzymes of interest from strains of GBS serotypes Ia, Ib, II, III, IV, and V (116). Rabbit antisera to recombinantly expressed GBS ornithine carbamoyltransferase and phosphoglycerate kinase, admixed with GBS type III strain M732, resulted in 46% and 35% survival, respectively, of neonatal mouse pups; in contrast, only 2% of pups that received bacteria and normal rabbit sera survived (116).

BibA and FbsA

Combining genomics with targeted immune responses, ANTIGENome technology identified four conserved proteins on the GBS surface: two hypothetical proteins (Gbs0233 and Gbs1309) and two previously identified proteins (BibA and FbsA) (117). BibA is a well-conserved, cell wall-anchored protein presented as four allelic variants: variant I in type V strain 2603 (V/R) and type II strain 18RS21; variant II in type III strain NEM316 and type Ia strain 515; variant III in type V strain CJB111, type Ib strain H36B, and type Ia strain A909; and variant IV in serotype III strain COH1 (118). Active immunization of female mice with BibA variant I or a BibA variant I N-terminal fragment with Freund's adjuvant protected ~70% of newborn pups from an ordinarily lethal challenge with GBS type II strain 3050 or GBS type III strain D136C (119). Hyperimmune rabbit sera to BibA passively protected mice against challenge with strains of serotype Ia or serotype Ib (117).

FbsA is a GBS fibrinogen receptor protein with repeating units that are each 16 amino acids in length (120). Active vaccination of mice with recombinant FbsA, administered alone or with BibA, did not provide complete (100%) protection against lethal challenge with strains of GBS types Ib, III, or V despite the high degree of human antibody binding to the repeat regions of this protein (117).

Pilus Proteins

The remarkable discovery, through the application of a reverse vaccinology approach, that GBS contained pili (Fig. 2, bottom) (121) subsequently led to studies to determine whether pilus proteins were viable vaccine candidates. Each of three pilus variants encoded by separate pathogenicity islands—PI-1, PI-2a, and PI-2b—consists of five genes (Fig. 5). These well-conserved genes encode for pilus assembly: pilus backbone protein, two ancillary proteins, and two sortase proteins (122). All GBS strains tested harbor one or more of the three pathogenicity islands, a finding that supports the use of pili proteins as vaccine antigens in light of the number of nontypable GBS isolates from invasive disease states (123). In addition, levels of naturally occurring antibody to PI-1 and PI-2a, but not to PI-2b, were found to be lower in mothers of babies with early-onset GBS disease than in controls; GBS from babies with late-onset disease harbored a high frequency of hypervirulent type III of the ST-17 lineage with PI-1 and PI-2b phenotypes (12).

GBS67, GBS80, and GBS104

Again by means of a reverse vaccinology approach, genomes of eight GBS isolates representing serotypes Ia, Ib, II, III, and V were sequenced, and genes encoding for putative surface or secreted proteins were identified by computational analysis (63). Of the 589 proteins identified, 312 were cloned and expressed in *E. coli* and then evaluated as protective immunogens in the mouse maternal immunization-neonatal pup challenge model of GBS disease (Fig. 6). This industrial-scale approach yielded four proteins of great interest, one of which, GBS322, had been identified previously as Sip (see above). When administered to female mice, GBS322 in combination with the other three proteins (GBS67, GBS80, and GBS104) protected 445 (89%) of 498 pups challenged with 12 GBS strains representing six serotypes (Ia, Ib, II, III, V, and VIII); in contrast, only 18% of pups born to unvaccinated dams survived (63).

Evidence is mounting that, of the several GBS pilus proteins identified, GBS80 (also called BP-1 and SAG0645) and GBS67 (also called AP1-2a and SAG1408) are potentially important vaccine components not only because they play a role in attachment of and invasion by GBS *in vitro*

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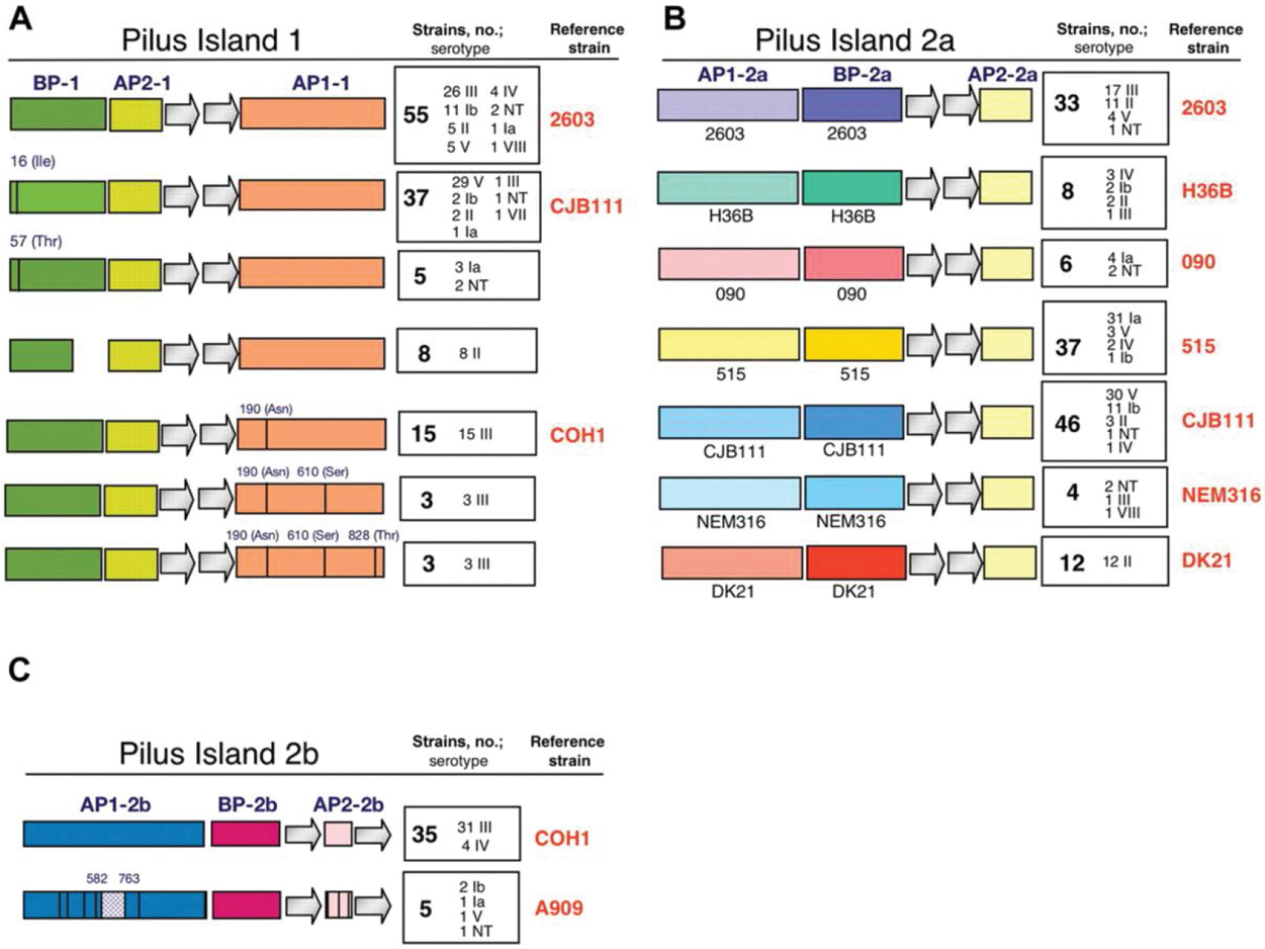


FIGURE 5 Pilus islands (PI)-1 (A), PI-2a (B), and PI-2b (C) of GBS: conservation of pilus island genes among 186 GBS clinical isolates. Sortase genes (gray arrows) are sequences with identity of 100% (same color) or <90% (different colors). Vertical bars are single mutations. The number above each vertical bar indicates the position or substitution of the mutated residue. Reproduced with permission from the *Journal of Infectious Diseases* (122).

(124) but also because levels of IgG to these proteins were significantly lower in sera from mothers whose babies suffered early-onset GBS disease than in controls (12). Indeed, GBS80 has also been shown to be an effective carrier protein as well as a stand-alone immunogen in mice (125).

GBS VACCINES

CPS Vaccines

Clinical data and experimental observations support an important role for CPS-specific antibodies in the prevention or control of GBS disease. These data include the following: (i) GBS produces type-specific CPSs that are targets of protective immunity (27, 33). (ii) A low level of CPS-specific maternal antibody is a risk factor for disease in infants; conversely, high levels of CPS-specific maternal IgG are associated with protection of neonates (126). (iii) Type-specific antibodies are opsonically active *in vitro* (127) and are protective in animal models of infection (100). (iv) IgG to type-specific CPSs can cross the placenta (128), a critical ability in a maternal vaccine against neonatal GBS disease (6).

Native types Ia, Ib, II, and III CPSs were the first GBS antigens tested as vaccines in adults. The first clinical trial with GBS vaccine enrolled 33 healthy adults to evaluate the safety and immunogenicity of type III CPS extracted by two methods (6). Although both preparations were well tolerated, the EDTA extraction method produced a type III CPS that was more immunogenic than that obtained by TCA extraction. By the 1980s, more than 300 healthy adults, including 40 third-trimester pregnant women, had been safely vaccinated with type III CPS or other GBS CPS vaccines at CPS doses ranging from 10 to 150 μg (128, 129). Although well tolerated, these antigens failed to induce a strong specific antibody response in the target population—i.e., individuals with low levels of preexisting CPS-specific antibody. However, type-specific antibody levels among responders (i.e., mainly those with preexisting specific antibody levels >2 μg/ml) increased sharply 4 weeks after CPS vaccination. High levels of CPS-specific antibody persisted for at least 2 years, and declining levels were restored to their peak upon revaccination with CPS. The class of human antibody among responders to GBS type III CPS (129) and to GBS type II CPS (33) was predominantly IgG. The response to CPS antigens differed with the serotype tested; native type II CPS was the most immunogenic (88% rate of response), and type Ia CPS was the least (40%) (129). GBS CPS vaccines induced antibodies that were active against homologous GBS serotypes in opsonophagocytic assays and were passively protective in animal studies (129). By the late

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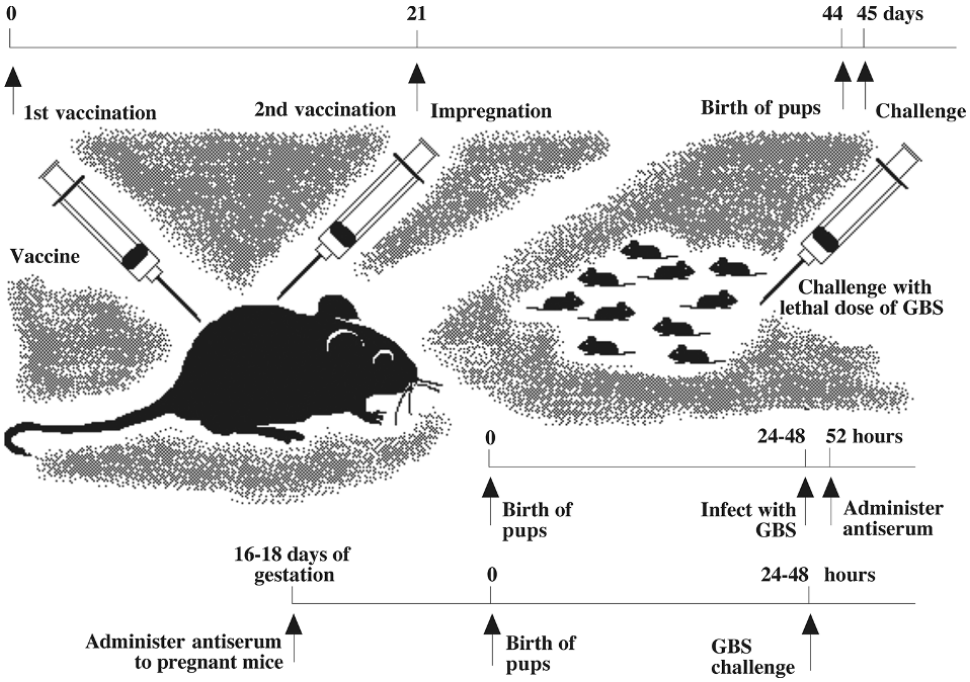


FIGURE 6 Mouse models of GBS disease. The immunogenicity and efficacy of GBS vaccines have been evaluated with the maternal vaccination-neonatal mouse model (top timeline) by actively vaccinating female mice, mating these mice, and subsequently challenging their offspring with GBS. In a passive vaccination-protection model (bottom timeline), newborn pups born to dams that received immune sera during pregnancy are challenged with GBS as a means of measuring the functional capacity of IgG. The therapeutic potential of GBS vaccine-induced antiserum (middle timeline) has been ascertained by inoculating naive pups with GBS 4 h before administration of immune serum. In all three models, pups are infected or challenged with GBS within 24 to 48 h of birth, and survival is assessed 48 h after challenge. Active vaccination of adult mice followed by an intraperitoneal challenge with viable GBS several weeks later has also been used to evaluate vaccines.

1980s, preclinical and clinical results showed that, although the CPS structures were critical vaccine components, they were not sufficiently immunogenic by themselves to be effective GBS vaccines. Researchers then began to focus on the development of GBS CPS vaccines whose immunogenicity was enhanced by methods that preserved their native antigenic structures.

Animal models are useful and often vital in the development of new vaccines. A mouse model of maternal vaccination and neonatal GBS disease (Fig. 6) has been used extensively to evaluate the efficacy of actively administered GBS vaccines or of passively administered type-specific immune sera (130). This model, which focuses primarily on neonatal survival, has been adapted to test the therapeutic potential of human antisera to GBS vaccines (131).

CPS-Protein Conjugate Vaccines

Conjugation technology has yielded an abundance of materials for use as reagents in experimental immunology or as potential vaccines. Without doubt, the clinical success of conjugate vaccines against *Haemophilus influenzae* type b infections spurred interest in adapting this technology to improve the immunogenicity of other poorly immunogenic carbohydrates, including those of GBS.

The first GBS CPS conjugate vaccines were prepared in 1990 by three coupling strategies (Table 2). Native type III CPS was coupled to TT (III-TT) either with adipic acid dihydrazide as a spacer molecule (132) or directly via aldehydes formed on a selected number of sialic acid residues (133). In a different approach, coupling of a type III oligosaccharide of 14 pentasaccharide repeating units to TT with a synthetic 6-C spacer molecule resulted in a single-site attachment of the reducing end of the oligosaccharide (134). The chemical methods used to create these and other bacterial conjugate vaccines have been reviewed in detail by Jennings and Sood (135). Despite differences in coupling chemistries, CPS size, purification methods, and the adjuvant used in the development and administration of these vaccines, all three conjugates were better immunogens in laboratory animals than was uncoupled type III CPS. Moreover, antibodies elicited by these GBS conjugate vaccines were of the IgG class and were functionally active when tested *in vitro* and *in vivo*.

With the coupling chemistry well in hand, progress was then made in developing conjugate vaccines against GBS serotypes other than type III (136, 137). Immunity in mice to multiple GBS serotypes was demonstrated in studies of a mixture of Ia-TT, Ib-TT, II-TT, and III-TT conjugates administered as a single tetravalent vaccine (138). The lack of interference of one serotype with another in the tetravalent vaccine opened the way to the development of a multivalent GBS vaccine for use in humans. However, clinical observations of an increased prevalence of serotype V dictated its inclusion in a multivalent vaccine (139). Preclinical studies of a serotype V-TT conjugate vaccine have paved the way for this innovation (140).

Because protective antibodies to type III GBS CPS appear to recognize a conformational (helical or linear) epitope, all

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TABLE 2 Characteristics of GBS conjugate vaccines

Capsular serotype(s)	Carrier protein(s)	Spacer	Coupling method	Linkage	Reference(s)
Preclinical					
Ia	TT[a]	None	RA[b]	Multisite, direct	137
Ia, II, III	TT and β-C[c]	None	RA	Multisite, direct	101
Ib	TT	None	RA	Multisite, direct	138
II	TT	None	RA	Multisite, direct	136
III oligosaccharides[d]	TT	6-C[e]	RA	Single, end-linked	134, 164
III	TT	ADH[f]	CR[g]	Multisite, random	132
III	TT	None	RA	Multisite, direct	133
III	β-C	None	RA	Multisite, direct	100
III	α-C[h]	None	RA	Multisite, direct	130
III	rCTB[i]	None	RA	Multisite, direct	165
III	rdHBcAg[j]	None	RA	Multisite, direct	165
III	C5a peptidase	None	RA	Multisite, direct	110
III	$rBCP^{\Delta IgA,k}$	None	RA	Multisite, direct	155
III	rAlp3[l]	None	RA	Multisite, direct	157
III	$rAlp3–rBCP^{\Delta IgA}$	None	RA	Multisite, direct	157
IV	TT	None	RA	Multisite, direct	166
V	TT	None	RA	Multisite, direct	140
VI	TT	None	RA	Multisite, direct	167
VII	TT	None	RA	Multisite, direct	166
VIII	TT	None	RA	Multisite, direct	167
Clinical					
Ia	TT	None	RA	Multisite, direct	144
Ib	TT	None	RA	Multisite, direct	144
Ia, Ib, III	CRM_{197}[m]	None	RA	Multisite, direct	149, 150, 168, 169
II	TT	None	RA	Multisite, direct	145
II, III	TT	None	RA	Multisite, direct	170
III	TT	None	RA	Multisite, direct	142
V	TT	None	RA	Multisite, direct	58, 147
V	CRM_{197}	None	RA	Multisite, direct	58

[a]Tetanus toxoid monomer.
[b]Reductive amination using sodium cyanoborohydride.
[c]GBS beta C protein.
[d]6, 14, or 25 pentasaccharide repeating units.
[e]6-aminohexyl-1-β-D-galactopyranoside.
[f]Adipic acid dihydrazide.
[g]Carbodiimide reduction.
[h]GBS alpha C protein.
[i]Recombinant cholera B toxin.
[j]Recombinant duck hepatitis B virus core antigen.
[k]Recombinant beta C protein with deleted IgA binding site.
[l]Recombinant alpha-like protein 3.
[m]Cross-reactive material, a diphtheria mutant toxoid isolated from *Clostridium diphtheriae*.

conjugate vaccines for clinical use were prepared with a coupling method known to preserve both the native repeating-unit structure and the conformation of the CPS (31). This conjugation method (141) is essentially a two-step process: (i) GBS CPS is subjected to mild oxidation with sodium periodate, which creates free aldehyde groups on a limited number of sialic acid residues on the CPS. (ii) The newly created aldehyde groups on the CPS serve as sites for irreversible coupling to free amino groups present on carrier proteins by a process called reductive amination. The end result is a conjugate vaccine that contains CPS covalently linked directly to the protein. A covalent linkage between CPS and the carrier protein was necessary for the formation of efficacious GBS vaccines.

Conjugate vaccines for use in phase 1 and phase 2 clinical trials have been individually prepared by the reductive amination method with GBS CPSs Ia, Ib, II, III, and V. Each CPS was individually coupled to TT, and a second type V conjugate vaccine was prepared with the mutant diphtheria toxoid CRM_{197}. Uncoupled GBS CPS preparations (types Ia, Ib, II, III, and V) were used as control vaccines in these clinical trials.

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Clinical trials with III-TT vaccine

The initial clinical trials with the second-generation (i.e., conjugate) GBS vaccines were designed to compare the safety and immunogenicity of conjugated and uncoupled type III CPS and to determine an optimal immunogenic dose (142). Both III-TT (at any CPS dose: 58, 14.5, or 3.6 μg) and III CPS were well tolerated, with minimal reactogenicity reported among the 90 female recipients. The geometric mean concentration (GMC) of III CPS-specific IgG among the 30 recipients of the 58-μg dose (as CPS) of III-TT rose from 0.09 μg/ml before immunization to 4.89 μg/ml 2 weeks thereafter; during this same interval, the GMC of specific IgG among the 30 recipients of III CPS increased from 0.21 μg/ml to only 1.30 μg/ml ($P < 0.05$). The relative immunogenicity of III-TT appeared to be dose dependent, with a peak GMC of type-specific IgG of 2.72 μg/ml in the 14.5-μg group and 1.10 μg/ml in the 3.6-μg group (142). Moreover, antibodies elicited by immunization with III-TT recognized a conformationally dependent epitope of the CPS, promoted *in vitro* opsonophagocytosis and killing of GBS, and were functional *in vivo* in a neonatal mouse protection model (142).

Preliminary results indicate that a III-TT vaccine is also safe and immunogenic when administered to healthy women at 30 to 32 weeks of gestation (143). Maternally derived type III CPS-specific IgG measured in serum from 1- and 2-month-old infants was functionally active *in vitro*.

Clinical trials with Ia-TT and Ib-TT vaccines

The Ia-TT and Ib-TT conjugate vaccines were also well tolerated and were more immunogenic than the respective uncoupled homologous CPSs in nonpregnant, healthy women (144). The GMC of type Ia CPS-specific IgG among recipients of a 60-μg dose (as CPS) of Ia-TT rose from 0.5 μg/ml to a peak of 26.2 μg/ml 8 weeks after vaccination, whereas during the same interval antibody levels among recipients of Ia CPS rose from 0.2 to only 2.4 μg/ml. Levels of type Ia CPS-specific IgG remained high in recipients of the Ia-TT vaccine at 1 and 2 years after vaccination, a result demonstrating the durability of the response. The response to Ia-TT vaccines was dose dependent: 60-μg and 15-μg doses elicited higher antibody levels than did a 3.75-μg dose (144).

Like the other GBS conjugate vaccines, Ib-TT vaccine was safer and more immunogenic than uncoupled homologous CPS (144). The prevaccination GMC of type-specific antibody among recipients of Ib-TT was 0.4 μg/ml, a level that rose to a peak of 14.2 μg/ml 4 weeks after vaccination with a single 63-μg dose (as CPS). In contrast, among recipients of uncoupled type Ib CPS, the prevaccination type-specific antibody GMC of 0.4 μg/ml rose to only 4.4 μg/ml 4 weeks after vaccination. The response to Ib-TT vaccine was dose dependent, with the highest (63-μg) and intermediate (15.75-μg) doses resulting in the greatest magnitude of antibody response (144).

Clinical trials with II-TT vaccine

Coupling of purified GBS type II CPS to TT also resulted in a safe and highly immunogenic vaccine in healthy, nonpregnant adults (145). Responses to the conjugate vaccine were dose dependent, with the lowest CPS dose of 3.6 μg evoking a peak type-specific IgG level of ~15 μg/ml 2 weeks after vaccination. As expected, immune sera were functionally active in an *in vitro* opsonophagocytosis assay against viable type II GBS. Interestingly, high concentrations of type II CPS-specific IgM and IgA were also elicited in a vaccine dose-dependent manner. These antibody isotypes were not elicited in humans who received the other (Ia-TT, Ib-TT, and III-TT) GBS CPS conjugate vaccines, and although one can speculate that the difference may lie in the structural features (described above) of type II CPS, confirmation awaits further experimentation.

Clinical trials with V-TT and V-CRM_{197} vaccines

In response to the emergence of serotype V, conjugates were prepared with purified type V CPS. Two conjugate vaccines—one prepared with GBS type V CPS and coupled to TT, the other coupled to CRM_{197} (Table 2)—were randomly administered to healthy nonpregnant women 18 to 45 years of age (58). Both conjugate vaccines were well tolerated, with no serious adverse reactions reported. Immune responses to V-TT and V- CRM_{197} were similar to those observed for other GBS conjugates, with type-specific IgG reaching peak GMCs of 6.5 to 8.9 μg/ml 2 to 4 weeks after vaccination. No statistical differences were observed between these two preparations.

Because of the increased rate of GBS infection among the elderly, Palazzi and coworkers examined the immune response of a V-TT vaccine in 22 healthy adults 65 to 85 years of age (146). The antibody isotype distribution of V-TT in this age group was similar to that measured in 18- to 45-year-old adults, and GMCs of type V CPS-specific IgG, IgA, and IgM ranged from 2 μg/ml to 3 μg/ml 4 weeks after vaccination; concentrations fell by only ~50% over 1 year. This was the first study that demonstrated the potential of GBS conjugate vaccines to stem disease in the elderly.

In the exploration of a multivalent GBS conjugate vaccine, a dose-ranging study was performed in healthy adults with GBS type V-TT vaccine to determine the amount of antigen that would elicit robust levels of specific IgG (147). While all three V-TT vaccine doses (38.5, 9.6, and 2.4 μg of coupled type V CPS) elicited significantly higher levels of type V CPS-specific IgG than did uncoupled type V CPS, the GMCs of specific IgG elicited by the three doses did not differ statistically—a result that may have been due to the small number of participants. Therefore, a multivalent GBS conjugate vaccine that contains ~2– to 10 μg of coupled type V CPS could lead to optimal immunity to this GBS serotype (147).

Surprisingly, GBS type V conjugate vaccines elicited high GMCs of type V CPS-specific IgM and IgA, a response pattern similar to that measured in recipients of II-TT vaccine (58). Both type II and type V CPSs have two side chains, one of which is composed of a single monosaccharide (Fig. 3). Could this or other CPS structural feature(s) influence the immune response to conjugates prepared with these antigens?

Unlike the well-studied type III CPS, whose structural conformation is influenced by the presence of the side-chain sialic acid (50), the structure of type V CPS is not affected by the presence of this 9-C sugar (148). This key finding led to studies in rhesus macaques with conjugate vaccines prepared with native type V CPS or desialylated type V CPS (dV-TT). Unlike mice, rhesus macaques elicited the same IgM-to-IgG responses to III-TT and V-TT as those measured in healthy humans. A higher IgG-to-IgM ratio observed in rhesus macaques immunized with dV-TT suggested that the terminal side-chain sialic acid impeded critical isotype switching, a result that corroborated structural analyses (148).

Clinical trials with trivalent GBS CPS conjugate vaccines

Clinical trials have progressed to include evaluation of a trivalent (Ia, Ib, III) GBS CPS conjugate vaccine in pregnant women with or without HIV disease (149, 150). For a more complete review of the current status of GBS conjugate vaccines, see Kobayashi et al. (151).

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GBS CPS-GBS Protein Conjugate Vaccines (Preclinical)

The drive toward a multivalent GBS vaccine occurred in response to current trends, such as the shift of GBS disease from newborns to elderly adults (152, 153) and the emergence of serotypes such as type IV and type V (154). The findings thus far broaden the target population that would receive a GBS vaccine and strengthen the call for a multivalent vaccine. Toward that goal, vaccines that combine CPSs with various GBS surface-expressed proteins have been generated in an effort to broaden coverage against multiple GBS serotypes. To date, these proof-of-principle vaccines have been evaluated only in laboratory animals.

Conjugate vaccines with ScpB (C5a peptidase)

Conjugate vaccines were prepared by combining a native and a truncated form of ScpB with type III CPS. Both vaccines reduced the severity of pneumonia in mice inoculated intranasally with serotype VI GBS. This result suggests that protective responses independent of GBS serotype could be achieved with ScpB-containing vaccines (106).

Conjugate vaccines with alpha C protein and beta C protein

Beta C protein of GBS (discussed in detail above) has been used both as a carrier for type III CPS and as an immunogen in a conjugate vaccine eliciting protective antibodies to GBS strains of serotype III as well as to non-type III GBS strains that contain this protein (100). Despite this advance, there was little enthusiasm for the use of beta C protein in a GBS vaccine because this protein bound human IgA (97)—a potentially undesirable property for a vaccine antigen. In 2007, Yang and coworkers engineered a recombinant beta C protein (rBCP) that lacked the human IgA binding site ($rBCP^{\Delta IgA}$); this new antigen was reevaluated as a standalone immunogen and as a carrier protein for type III CPS (155). Deletion of the IgA binding site did not negatively alter the antigenicity or the immunogenicity of rBCP, which also served as an effective carrier protein—equal to TT, which had become the gold standard carrier—for GBS type III CPS (155). When administered to female mice, the III-$rBCP^{\Delta IgA}$ vaccine protected offspring from an ordinarily lethal challenge of beta C protein-negative GBS type III strain M781 or beta C protein-positive type Ia strain A909. These results suggest that expanded coverage against GBS disease can be achieved with the use of relevant GBS surface proteins as carrier antigens (155).

Although results in animals vaccinated with III-$rBCP^{\Delta IgA}$ proved that broader coverage could be attained with a relevant carrier protein, a better carrier for GBS CPSs would be the alpha C protein (see above) since it is present on a larger number of GBS serotypes (130). A GBS type III-alpha C protein conjugate vaccine could potentially provide coverage against up to 90% of disease-causing strains of GBS. Conjugate vaccines using both the full-length nine-repeat alpha C protein and a two-repeat alpha C protein as carriers for type III CPS elicited antibodies in female mice that protected neonatal pups against lethal intraperitoneal challenge with either alpha-positive or type III GBS (156).

Building on earlier studies with alpha C protein and rBCP as viable carriers and recognizing the emergence of newer GBS serotypes (e.g., types V and VIII), researchers generated a trivalent GBS conjugate vaccine. The experimental vaccine coupled both recombinant alpha-like protein 3 (rAlp3) and $rBCP^{\Delta IgA}$ to GBS type III CPS; the resulting III-rAlp3-$rBCP^{\Delta IgA}$ vaccine elicited high-titer antigen-specific IgG in mice (157). When given to adult female mice, this vaccine protected offspring from challenge with a GBS type III strain and a beta C protein-bearing GBS type Ia strain but not against an Alp3-containing type V strain or a type VIII strain—this despite the finding that high-titer rabbit sera to Alp3 opsonized and killed the latter strains *in vitro* (157). It is worth noting that rabbit antiserum to Alp3 was also functionally active *in vitro* against six clinical GBS type III isolates, presumably because of cross-reactivity with the Rib protein (157).

GBS Protein Vaccine

A recombinantly expressed fusion protein of the nonimmundominant N-terminal domains of both alpha C protein and Rib (158) showed promise as a vaccine in mice against GBS serotypes containing these surface antigens. This advance led to the first clinical trial in healthy women of a non-CPS-containing GBS protein vaccine (NCT02459262, clinicaltrials.gov).

SUMMARY

The seminal observations by Rebecca Lancefield and coworkers on the role of carbohydrate and protein antigens in GBS immunity have led a generation of researchers not only to a better understanding of these antigens in immunity but also toward the development of effective vaccines. Today, GBS remains a major bacterial cause of disease among neonates and is increasingly common in the adult population, especially among the elderly and those with underlying illnesses (11, 139, 159, 160).

Now, more than 80 years since the first report of GBS infection in humans (2), we have sufficiently unraveled the complexities of the surface structures of this opportunistic pathogen, and we have developed the technologies necessary to produce and formulate effective vaccines against GBS disease (161).

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Group B *Streptococcus* (*Streptococcus agalactiae*)

VANESSA N. RAABE AND ANDI L. SHANE

14

INTRODUCTION

Streptococcus agalactiae, also known as group B *Streptococcus* (GBS), was first differentiated from other streptococci by Rebecca Lancefield in the 1930s after it was isolated from milk and cows with bovine mastitis (1). Lancefield described GBS colonization of the vaginal tract of asymptomatic women, but human pathogenicity was not described until 1938 when three reports of fatal postpartum infection were published (2, 3). Invasive GBS disease was rarely identified in humans until the 1960s, when increasing reports of adult and neonatal invasive infections were published (4–7). The incidence of invasive GBS disease continues to increase, and it remains a significant pathogen among both infants and adults.

S. agalactiae has historically been divided into nine serotypes (Ia, Ib, II, III, IV, V, VI, VII, VIII) based on the capsular polysaccharide (8). A tenth serotype (IX) was described in 2007 (9). Among nonpregnant adults in the United States, the most common serotype causing invasive disease is group V (29% in 2005 to 2006), followed by serotypes Ia, II, and III (10). A study from Alberta, Canada, of serotypes associated with invasive GBS disease from 2003 to 2013 demonstrated that the most frequent serotype was III (20%), followed closely by serotypes V (19%), Ia (19%), Ib (13%), and II (11%) (11). The dominant serotypes causing disease vary regionally and differ by invasive and colonizing isolates.

CLINICAL MANIFESTATIONS

The most common syndromes due to invasive GBS disease in adults are bacteremia without a focus and skin/soft tissue infections (10, 12–16). The former often presents with altered mental status, chills, and fevers (8). Bacteremia may also occur secondary to a focal source of infection; polymicrobial bacteremia, most commonly with *Staphylococcus aureus*, may be seen in 26 to 45% of cases (17, 18).

S. agalactiae bacteremia may lead to seeding of the cardiac valves and endocarditis. Vegetations in GBS endocarditis may become extremely large with a high risk of embolization (19, 20). In the 1930s and the 1940s, GBS endocarditis predominantly was associated with acute disease in pregnant and postpartum women, primarily involving the mitral valve (20). Rheumatic heart disease was a common predisposing factor, and embolization of vegetations from valves to the kidneys and spleen was common (20). The epidemiology of GBS endocarditis has evolved over time; a case series beginning in the 1960s described endocarditis affecting men and nonpregnant women, involvement of the aortic and tricuspid valves, and subacute presentations (20). The percentage of GBS infections manifesting as endocarditis is in decline, from 9% in one study conducted in Atlanta in 1982 to 1983 to approximately 3% of GBS infections in the United States in a study from 1990 to 2007 (10, 15). Although cardiac valve abnormalities are a risk factor for GBS endocarditis, cases have been described in structurally normal hearts (21–23). Mortality from GBS endocarditis may be quite high despite medical and surgical interventions, with 41% mortality in one case series of 27 patients from 1984 to 2004 (21).

Skin and soft tissue infections attributed to GBS may manifest as cellulitis, abscesses, foot infection, or decubitus ulcers (24, 25). Diabetes mellitus is a common underlying condition in patients with GBS skin and soft tissue infections (13). Necrotizing fasciitis and pyomyositis attributed to GBS has occasionally been described (18, 25, 26).

Acute and chronic osteomyelitis attributed to GBS has been reported in neonates, children, and adults (27). Infection may arise from direct inoculation from overlying skin/soft tissue infection, skin breakdown such as a decubitus ulcer, or via hematogenous seeding (27). Septic arthritis due to GBS is usually monoarticular, although multiple joints may be involved (28, 29). Any joint may be infected, but commonly affected joints include the knee, ankle, and shoulder (28, 29). Septic arthritis may involve prosthetic joints, with the majority of infections occurring at least 3 months after prosthesis placement; some GBS prosthetic joint infections require prosthesis removal in addition to antimicrobial therapy for successful treatment (30–32).

Group B streptococci, concurrently with other organisms, have been implicated in pneumonia (33). Imaging findings may include unilateral or bilateral lobar infiltrates (33). Dementia, neurological disease, and tracheoesophageal fistulae are associated with GBS pneumonia, likely due to increased risk of aspiration of the organism (34). Colonization of the airway with GBS is infrequent in patients with cystic fibrosis and when it occurs, it does not appear to be associated with worse clinical outcomes (35).

Meningitis is a severe manifestation of invasive GBS disease. It is uncommon in adults but is a common manifestation of late-onset GBS infection in neonates (36). Infants who survive the acute phase of the GBS meningitis infection may have significant cognitive or neurological sequelae; 32 to 44% of infants who survived GBS meningitis have neurodevelopmental impairment, with severe impairment occurring in up to 19% of surviving infants (37, 38).

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Another manifestation of GBS disease includes urinary tract infections. Urinary tract infections may be associated with a variety of GBS serotypes (39). In men, GBS may be associated with prostatitis (34, 40). GBS bacteriuria in women during pregnancy is a risk factor for late gestational maternal colonization with GBS and early-onset neonatal GBS infection (41, 42).

PREGNANT WOMEN

Pregnancy has been associated with a high incidence of invasive GBS disease. In a multistate evaluation from 2007 to 2009, the incidence of invasive disease due to GBS was twice as high in pregnant women (0.04/1,000 woman-years) compared to nonpregnant women (0.02/1,000 woman-years) (43). Although the majority of GBS infections are detected during labor and delivery, women in the postpartum period are also at an increased risk for invasive GBS disease even in the absence of other predisposing conditions (43, 44). Rates of maternal invasive GBS disease in the United States vary by state, with rates ranging from 0.1 per 1,000 deliveries to 0.8 per 1,000 deliveries (45). Worldwide, the incidence of systemic invasive GBS disease in pregnant women is estimated at 0.38 cases per 1,000 pregnancies, with a case fatality rate of 0.2% (44). Serotypes Ia and III account for over half of maternal systemic GBS disease, followed by serotypes V, Ib, and II (44). Systemic maternal GBS disease is associated with increased odds of premature delivery and sepsis in infants (44).

Asymptomatic rectovaginal colonization rates among pregnant women vary widely worldwide (Fig. 1), although the majority of estimates fall between 5 and 30% (46–97). A recent review estimated that 18% of pregnant women worldwide are colonized with GBS, and 98% of isolates were serotypes I to V, although there was regional variability in serotype distribution (98). In this study, colonization rates were highest among pregnant Caribbean women at 35% and lowest among pregnant East Asian women at 11% (98). Estimates of GBS colonization in the United States range from 10 to 30% (94–96). Colonization of GBS may be intermittent throughout pregnancy, although 17 to 28% of women with vaginal or rectal colonization in the first trimester have sustained colonization in the second and third trimesters or at term (99). Women with GBS colonization during pregnancy were recently found to have a 1.21 risk ratio for preterm birth compared to women without GBS colonization, with a higher risk ratio (1.98) observed in women with GBS bacteriuria (100).

Risk factors associated with maternal GBS intrapartum vaginal or rectal colonization include GBS bacteriuria during the current pregnancy and a history of GBS colonization in a previous pregnancy (41, 101, 102). In one study of pregnant women, no significant difference in the composition of the vaginal and rectal flora between GBS-colonized and -noncolonized women was observed (99). Studies examining the role of the composition of the vaginal microbiota in relation to colonization with GBS in nonpregnant women have similarly demonstrated no differences in the α-diversity or carriage of *Lactobacillus* between women with and without GBS colonization (103). The role of probiotic administration in GBS carriage remains unclear. One study of *Lactobacillus rhamnosus* and *Lactobacillus reuteri* administered to 110 women with detectable GBS colonization at 35 to 37 weeks gestation until delivery demonstrated that 43% of women in the probiotic group subsequently had negative GBS screens at delivery, compared to 18% in the placebo group; however, there were no significant differences in fetal outcome (104). However, in another placebo-controlled study of 34 women who received a 21-day course of *L. rhamnosus* and *Lactobacillus fermentum/L. reuteri* probiotics after a positive GBS screen at 36 weeks, no difference in GBS colonization rates after completion of the supplementation and no difference in infant health were observed between the placebo and probiotic recipients (105).

NONPREGNANT ADULTS

Although traditionally thought of as a disease afflicting infants and pregnant women, the incidence of GBS disease is increasing among nonpregnant adults, from 3.6 cases/100,000 people in 1990 to 7.3 cases/100,000 people in 2007,

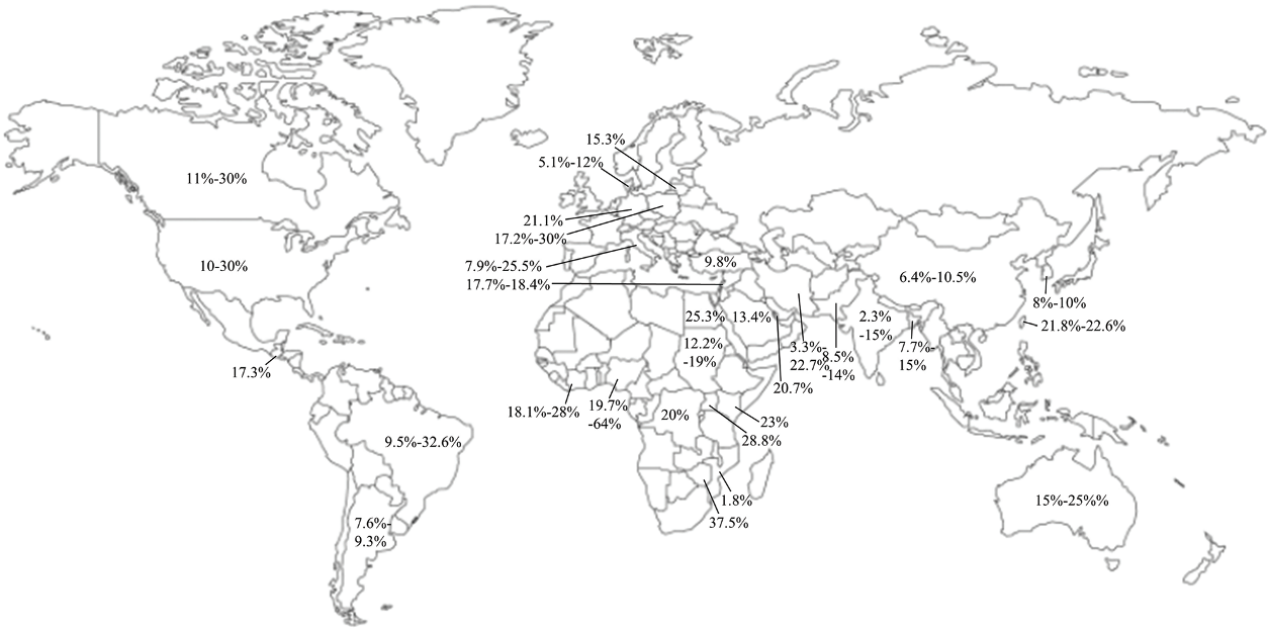


FIGURE 1 *Streptococcus agalactiae* colonization rates in pregnant women.

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based on surveillance in 10 U.S. states (10). Comorbidities are present in most adults with invasive GBS disease, including heart disease, neurological disorders, kidney disease, liver disease, alcohol abuse, smoking, obesity, malignancy, and immunocompromising conditions (10, 13, 106). Diabetes mellitus is a common comorbidity, particularly among patients with skin/soft tissue infections (10, 13, 106). Higher incidences and mortality rates from GBS disease have been observed among those of black race compared to white race, although the reason for this difference remains unclear (10, 13, 106).

OLDER ADULTS

Higher incidence rates of invasive GBS disease among people age 65 or older compared to the general nonpregnant adult population have been described in the United States (Fig. 2), Canada, the United Kingdom, and multiple European countries (13, 107–111). Annually, invasive GBS affects 25/100,000 adults age 65 and older in the United States (94). The elderly population accounted for approximately 40% of invasive cases reported in the United States in 2015, and they had a higher incidence of deaths (1.08 to 4.73 deaths/100,000 people) compared to the incidence of death in the general population (0.54 deaths/100,000 people) (111). An estimated 12% of elderly long-term care facility residents are colonized with GBS, while an estimated 22% of community-dwelling elderly adults in Houston in one study were colonized with GBS, with 47% carrying serotype V (24, 112). Invasive GBS disease in the elderly commonly presents as bacteremia, urinary tract infection, skin infection, and pneumonia (110, 113). Risk factors for GBS disease in the elderly population include being bedridden and residence in a long-term care facility (107, 110).

NEONATAL DISEASE

GBS is the leading etiology of culture-confirmed neonatal bacterial infection in the United States and results in significant mortality (114). Invasive neonatal GBS disease may be divided into early-onset disease (EOD), occurring at <7 days of life, and late-onset disease (LOD), occurring between 7 and 90 days of life. Serotypes I to V account for 97% of invasive neonatal GBS disease, with serotype III accounting for nearly half (43%) of EOD and 73% of LOD (36). Estimates of the incidence of EOD GBS disease range from 0.7 cases/1,000 live births in 1997 to 0.21 to 0.25 cases/1,000 live births in 2014 and 2015 (Fig. 3) (111, 114–133). Disease due to *S. agalactiae* has been found in 0.58% of neonates with encephalopathy who meet criteria for cooling, a more than 10-fold increase compared to all live full-term infants. These infants have double the risk of fatality compared to those with neonatal encephalopathy without associated GBS disease (134). Worldwide, estimates of EOD cases per 1,000 live births vary from country to country, with incidences of 0.09 in Japan, 0.58 in Panama, 0.76 in Hong Kong, 0 to 1.5 in South Africa, and 2.35 in the Dominican Republic, with a pooled estimate of 0.49 cases per 1,000 live births (36, 135–137). Overall, rates of EOD are highest in Africa and lowest in Asia (36). Worldwide, the pooled incidence of LOD is 0.26 cases per 1,000 live births (36). In the United States, the estimated incidence of LOD is 0.32 cases per 1,000 live births (Fig. 3) (111, 138).

Risk factors for EOD disease include maternal vaginal or rectal GBS colonization, GBS bacteriuria during pregnancy, prolonged labor, prolonged rupture of membranes, low birth weight, prematurity, intrapartum fever, and systemic maternal GBS disease (42, 44, 139). HIV exposure does not appear to be a risk factor for EOD (140). The risk factors for the development of LOD are not as well understood as those for EOD; known risk factors include prematurity and black race (138, 141). While maternal GBS colonization increases the risk of developing LOD, it does not appear to be as strong a risk factor as for EOD (138, 141). Infants exposed to HIV have a 4.43 higher likelihood of developing LOD compared to unexposed infants (140).

EOD is attributed to vertical transmission of GBS from the mother due to either ascending infection from the

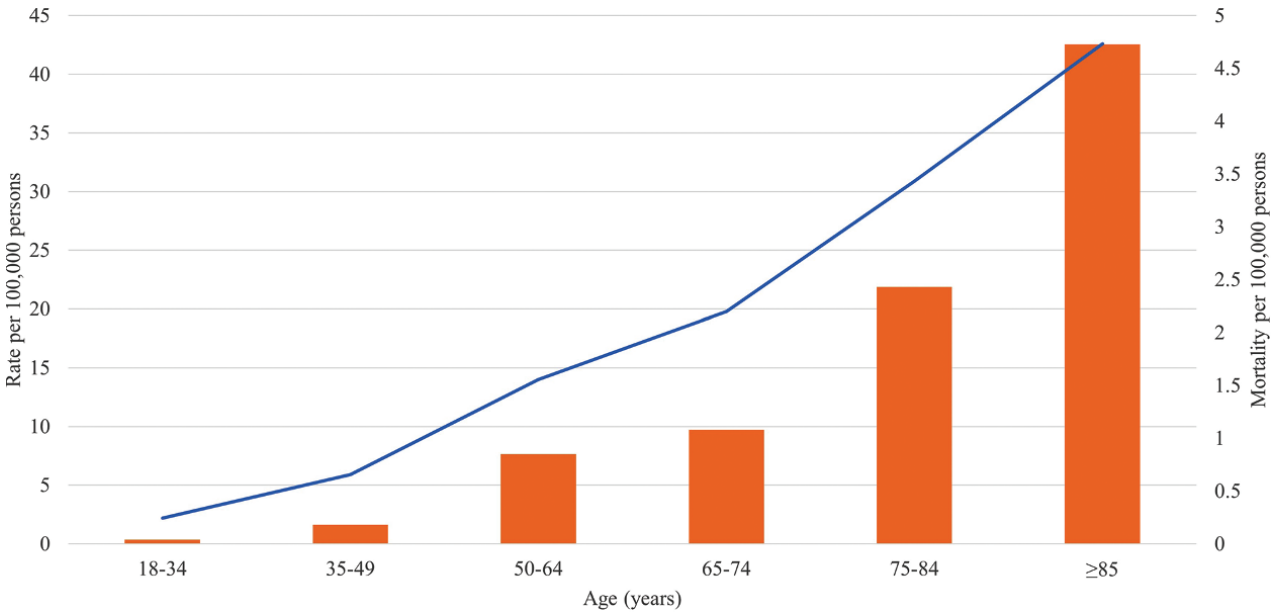


FIGURE 2 Invasive group B *Streptococcus* disease and mortality in nonpregnant adults, United States, 2015. Incidence shown in blue; mortality shown in orange.

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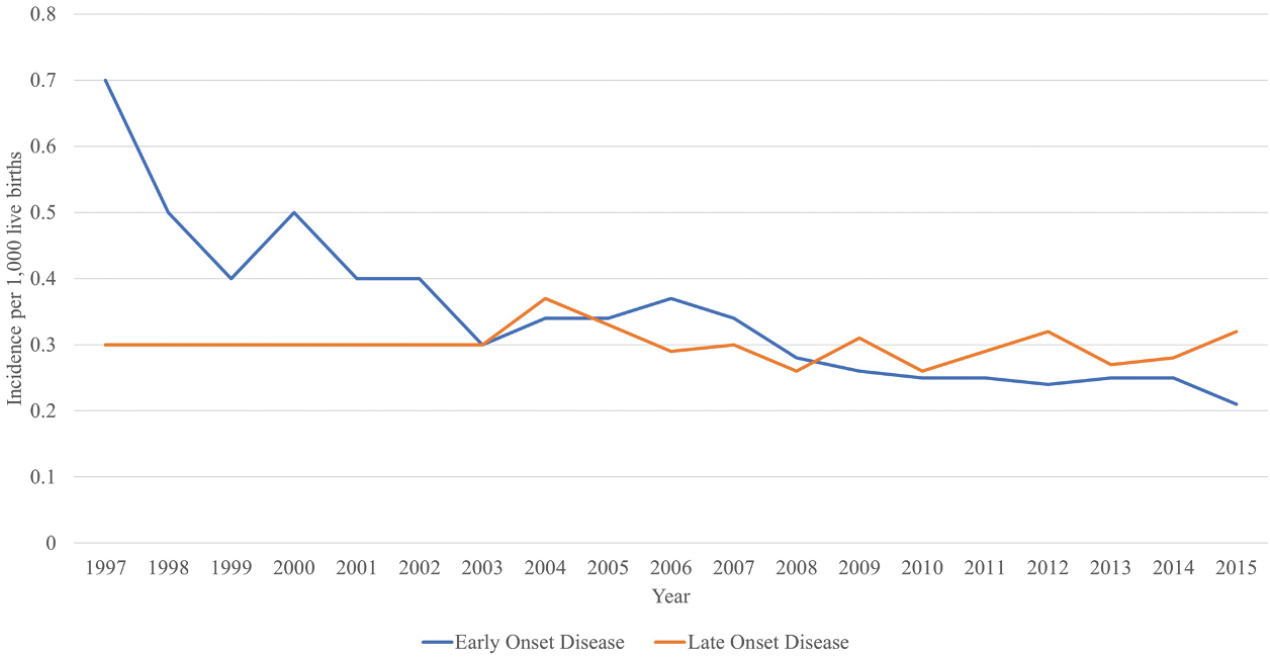


FIGURE 3 Incidence of invasive group B *Streptococcus* disease in neonates, United States, 1997 to 2015.

genital tract or infection obtained during passage through the vaginal canal at delivery; symptom onset may be subtle and may be noted within 24 hours after birth (36, 142). Typical manifestations of EOD include bacteremia and pneumonia, while meningitis, bone and joint, and soft tissue infections are less common (114). LOD is attributed to horizontal acquisition of GBS. Rarely, LOD has been attributed to GBS transmission from human milk among women with GBS mastitis (143). Meningitis is more common in LOD than EOD, although LOD may present as bacteremia, urinary tract infection, bone/joint infection, pneumonia, or soft tissue infections (36, 138, 139).

Neonatal invasive GBS disease overall results in case fatality rates ranging from 1 to 8.4% in full-term infants to 5 to 20% in preterm infants (36, 139). For EOD, mortality rates vary worldwide; in resource-endowed countries the estimated case fatality risk is 5%, but a much higher risk, up to 27%, has been observed in Africa (36). Mortality from EOD in the United States is estimated at 7%, with higher mortality in low-birth-weight and premature infants (114). Worldwide, LOD is estimated to have a case fatality rate of 7% (36). In addition to early- and late-onset invasive disease, *in utero* GBS disease accounts for approximately 1% of stillbirths worldwide and up to 4% of stillbirths in Africa (144).

PREVENTION OF NEONATAL DISEASE

Due to the burden of GBS disease in neonates, preventative measures have been developed to minimize invasive disease. Maternal antibiotic administration to prevent neonatal GBS disease began in 1973 (145). Intrapartum antibiotic prophylaxis reduces EOD among infants born to GBS-colonized women by 86 to 89% (42). In countries without a national policy on intrapartum antibiotic prophylaxis, approximately 1.1% of infants born to GBS-colonized mothers develop EOD due to GBS, whereas in countries with intrapartum antibiotic prophylaxis recommendations, the risk decreases to 0.03% (146). Penicillin or ampicillin administered at least 4 hours prior to delivery is the first-line therapy for intrapartum antibiotic prophylaxis, while cefazolin may be used for penicillin-allergic patients without anaphylaxis, angioedema, respiratory distress, or urticaria (42). In patients with severe penicillin allergy, testing of the GBS isolate for clindamycin susceptibility is recommended with use of intrapartum clindamycin for susceptible isolates and vancomycin for resistant isolates (42). Both risk-based and culture-based models for determining when to administer intrapartum antibiotic prophylaxis are currently in use worldwide and vary by geographic region (147). The United Kingdom does not recommend routine GBS screening cultures for pregnant women and uses risk-based algorithms to guide intrapartum antibiotic prophylaxis, whereas the United States, Canada, Australia, and New Zealand recommend universal screening with GBS cultures (42, 97, 148, 149). Adherence to intrapartum antibiotic prophylaxis guidelines varies widely; estimates of adherence in countries with universal culture-based screening programs range from 20 to 95%, while adherence estimates range from 10 to 50% in countries with risk-based screening programs (147).

Guidelines regarding intrapartum antibiotic prophylaxis for GBS in the United States were first issued in 1996 and have undergone several updates, most recently in 2010. The current 2010 guidelines recommend a screening culture from the vagina and rectum of all pregnant women between 35 and 37 weeks gestation to assess for colonization with GBS and offering intrapartum prophylaxis to all women with positive screening cultures, a history of GBS bacteriuria during the current pregnancy, or a previous infant affected by EOD GBS disease (42). Since the institution of intrapartum antibiotic prophylaxis in the United States, the incidence of invasive early-onset GBS disease has declined from 1.7 cases/1,000 live births in 1993 to 0.76 to 0.77 cases/1,000 live birth in 2005 to 2008 and an estimated 0.21 cases/1,000 live births in 2015 (111, 150, 151). However, while 87% of women in the United States undergo cultures to screen for GBS, only an estimated 36% of women in 2016 received care that was fully

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compliant with intrapartum antibiotic prophylaxis guidelines; challenges relate to appropriate timing of screening culture collection, appropriateness of intrapartum antibiotic selection, and appropriateness of intrapartum antibiotic dosages and timing (152).

An alternative strategy under development to prevent neonatal and maternal GBS disease is vaccination of mothers in the third trimester against GBS. Lower levels of capsular polysaccharide antibodies in mothers of children with invasive GBS disease compared to mothers of healthy infants have been noted since the 1970s (153, 154). Initial trials of unconjugated GBS polysaccharide vaccines elicited moderate serotype antibody responses (155). More robust antibody responses have been elicited with protein-polysaccharide conjugate vaccines using tetanus toxoid and additional surface protein target vaccines, such as Rib and Alpha C (156–158). Multiple GBS vaccine candidates, including a trivalent protein-polysaccharide conjugate vaccine targeting serotypes Ia, Ib, and III, are currently in development (159). Modeling based on 2015 worldwide disease estimates predicts that a GBS vaccine with 80% efficacy and 90% maternal coverage could prevent 107,000 infant deaths and stillbirths (160).

TREATMENT

S. agalactiae is uniformly regarded to be susceptible *in vitro* to penicillin, although reduced penicillin susceptibility has been detected in isolates from Japan, thought to be secondary to reduced penicillin-binding protein 2× expression (161, 162). However, penicillin G remains the mainstay of treatment for invasive disease. Generally, GBS is susceptible to other beta-lactam antibiotics, including ampicillin; first-, second-, and third-generation cephalosporins; and carbapenems, although the level of activity varies among different agents. In patients who demonstrate an anaphylactic or severe allergy to beta-lactam antimicrobials, alternative therapies include clindamycin, erythromycin, fluoroquinolones, and vancomycin. However, rising levels of GBS resistance to clindamycin, erythromycin, and fluoroquinolones have been noted in a multitude of studies, and these antibiotics should only be used for treatment if penicillin or cephalosporin is not appropriate and susceptibility of the organism has been determined (162–166). Resistance to vancomycin in clinical isolates has been reported (167).

Penicillin G is the first-line treatment for invasive GBS disease in adults (8). The duration of therapy depends on the clinical presentation. Ten days of therapy is generally acceptable for bacteremia, pneumonia, pyelonephritis, and skin/soft tissue infections. Longer durations of treatment are recommended for meningitis (minimum 14 days) and for osteomyelitis, endocarditis, and ventriculitis (minimum 4 weeks) (8). The addition of gentamicin for the initial 2 weeks of therapy is recommended for endocarditis (168).

In neonates with presumptive EOD, empiric therapy with ampicillin combined with an aminoglycoside is the standard of care (139). Once GBS is isolated, penicillin G monotherapy is recommended for treatment of invasive GBS infection in infants. Recommended dosing of penicillin G for infants up to 7 days of age is 250,000 to 450,000 units/kg/day and 450,000 to 500,000 units/kg/day for infants older than 7 days (139). Ten days of appropriate therapy is recommended for uncomplicated bacteremia and 14 days for uncomplicated meningitis, while complicated infections may require a longer duration of antimicrobial therapy. Septic arthritis or osteomyelitis is treated for 3 to 4 weeks, and at least 4 weeks of therapy is recommended for endocarditis or ventriculitis (139).

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Genetics and Pathogenicity Factors of Group C and G Streptococci

HORST MALKE

15

The genus *Streptococcus* comprises more than 70 species (some talk of more than 100) classified into eight phylogenetic groups, with Lancefield group C streptococci (GCS) and group G streptococci (GGS) being discovered in four of them, including one unknown group. GCS and GGS carry the immunedeterminant residues *N*-acetylgalactosamine and rhamnose, respectively, on the oligosaccharide side chains of their cell wall carbohydrate antigens. These organisms are distributed in both humans and animals. They are isolated as opportunistic commensals from the skin, nose, throat, vagina, and gastrointestinal tract but may also be associated with clinically important infections of these sites and with hospital outbreaks. Serious diseases caused by human GCS and GGS often resemble those due to their closest genetic relatives, the group A streptococci (GAS, carrying the immunodominant *N*-acetylglucosamine side chains of their cell wall carbohydrate antigens), and include septicemia, pharyngitis, cellulitis, otitis media, septic arthritis, meningitis, infective endocarditis, multiple organ abscesses, necrotizing fasciitis, and toxic shock syndrome (1–11). At one time, the genetic relationships of GCS and GGS strains were complicated and incompletely resolved due to the diversity of species within the serogroups and the uncertainties in assigning species names, particularly to the human-specific strains now classed with the phylogenetic Anginosus group (12). The current classification (Table 1) arrived at by numerous early (13–33) and more recent (34–43) studies relies on habitats, pathogenicity properties, physiological characteristics, and relationships of informational macromolecules, with serological grouping being useful for differentiating infraspecific biotypes. Before the advent of large-scale DNA sequencing and gene cloning technologies, GCS and GGS had rarely been subjected to genetic studies. However, studies of the host range of bacteriophages isolated from GAS, GCS, and GGS, and of their transducing potential, provided evidence for intergroup phage reactions and intergroup transduction between strains belonging to different Lancefield groups, thus amending the original notion of the strict group specificity of streptococcal phage-host interactions and emphasizing the importance of lateral gene transfer for the evolution of GCS and GGS (44, 45). An attempt to elucidate the genetic relationships between GAS, GCS, and GGS based on the nucleotide sequence of selectively neutral housekeeping genes supported events of interspecies gene transfer that predominantly occurred from GAS donors to GCS and GGS recipients (46). In *S. dysgalactiae* subsp. *equisimilis*, recently reviewed studies show that both mobile genetic elements and integrative conjugative elements are involved in horizontal gene transfer, with the latter playing the prominent role in the evolution of this subspecies (47). This applies also to the interspecies transfer of integrative and conjugative elements (ICEs) between *S. salivarius* (Salivarius group) and *S. parasanguinis* (Mitis group; see Table 1), in the case of which components of the mobile subfamily ICE*St3* element were very recently detected in their conjugation modules at high nucleic sequence identities (48). The application of recombinant DNA techniques and phylogenomic studies have advanced our understanding of GCS and GGS in diverse areas, and this article concentrates on the molecular genetics and the structure and function of pathogenetically relevant genes and proteins studied at the molecular level in recent years.

MOLECULAR TAXONOMIC APPROACHES TO THE CLASSIFATION OF GCS AND GGS

Currently, the four GCS and GGS species groups harbor a total of 12 individual species, based on 16S rRNA comparative sequence analysis (Table 1). At least four of them can be subdivided into subspecies, with the possibility of individual isolates within a common phylogenetic group exhibiting a diverse Lancefield group antigen pattern or none at all. The cladogram shown in Fig. 1 resolves the genetic relationships of all 12 species and (i) identifies *S. canis* as the closest relative of *S. pyogenes*, (ii) places *S. massiliensis*, thus far exhibiting an uncertain group assignment, in the Anginosus group, (iii) assigns *S. alactolyticus*, thus far of uncertain grouping as well, close to the *S. equi* clade, and (iv) leaves *S. acidominimus* in the state of unknown grouping and, based on its single-leafed clade, as the species least related to any other species. Of further interest, *S. sanguinis*, not included in Table 1 because it is so far unknown to comprise either group C or G strains (24), turns out not to fall within the Mitis group (Fig. 1), thus confirming that *S. sanguinis* and

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TABLE 1 Properties of Lancefield group C and G *Streptococcus* species

Phylogenetic group[a]	Species/subsp.[a]	Group antigen[a]	Hemolytic reaction[a]	Hosts[a]	Relative 16S rRNA sequence homology[b]		
					Maximum score	Identity (%)	Accession no.
Pyogenic	*S. canis*	G	β	Various animals, humans	2,555	98	AJ413204.1
	S. dysgalactiae subsp. *dysgalactiae*	C (L)	α, β, γ	Various animals	2,573	97	AB002487.1
	subsp. *equisimilis*	C, G (A, L)	β	Humans	2,529	97	NR_043661.1
	S. equi subsp. *equi*	C	β	Horses, donkeys	2,475	95	NC_012471.1
	subsp. *ruminatorum*	C	β	Various animals, humans	2,242	96	AJ605748.1
	subsp. *zooepidemicus*	C	β	Various animals, human	2,503	96	NC_011134.1
	S. parauberis	C	α, γ	Fish, cattle	2,575	97	NC_015558.1
	S. phocae subsp. *salmonis*	C (F, -)	β	Pinnipedia, salmon	2,303	97	NR_133868.1
	S. uberis	C (D, E, P, U, -)	α, β, γ	Cattle	2,627	97	NC_012004.1
Anginosus	*S. anginosus*	C, G (A, F, -)	α, β, γ	Humans, animals	2335	94	CP012805.1
	S. constellatus subsp. subsp. *constellatus*	C, G (A, F, -)	α, β, γ	Humans	2,211	96	JN787134.1
	subsp. *pharyngis*	C	β	Humans	2,390	94	NC_022245.1
	S. massiliensis	G	γ	Humans	2,218	94	NR_043173.1
Mitis	*S. parasanguinis*	C, G (B, F, -)	α	Humans	2,357	95	AF003933.1
Unknown	*S. acidominimus*	G (-)	α	Domestic animals, humans	2,285	94	NR_104972.1
	S. alactolyticus	G (D)	α	Mammalia, fowl	2,270	95	NR_041781.1

[a]Adapted from references 13–43. The order of the group antigens reading across may not correspond to their epidemiological prevalence.
[b]Based on nucleotide BLAST alignments (http://ncbi.nlm.nih.gov) of the specified 16S rRNA sequences relative to that of *S. pyogenes* NZ131 (accession no. NC_011375).

S. parasanguinis are not sister species (14, 22). Current methods for classification of clinically important *Streptococcus* isolates were also explored by determining internal features of their 16S rRNA, with the result of identifying short sequence segments unique to specific species (39). In the above context, such signature sequences were found to be highly conserved in the 16S rRNA genes of *S. dysgalactiae*, *S. equi*, and *S. anginosus* (and *S. pyogenes*), thus rendering identification procedures for them very simple.

Chromosomal DNA-DNA hybridization studies combined with biochemical tests indicate that the Pyogenic streptococci previously named *S. dysgalactiae* and "*S. equisimilis*," together with those belonging to the large-colony-forming GGS and serogroup L strains, exhibit high levels of DNA sequence identity and, on the basis of the commonly accepted species level hybridization (≥70%), constitute a single species, *S. dysgalactiae* (49, 50). Whole-cell protein electrophoresis revealed two subpopulations within this species, leading to differentiation between *S. dysgalactiae* subsp. *equisimilis*, comprising GCS and GGS strains of human origin, and *S. dysgalactiae* subsp. *dysgalactiae*, which harbors animal GCS and serogroup L strains (32). GGS isolated from animals always appear to qualify as *S. canis*. On the grounds of DNA sequence similarity, "*S. zooepidemicus*" is closely related to *S. equi*, warranting a similar subdivision of this species into *S. equi* subsp. *zooepidemicus* and *S. equi* subsp. *equi*, with the latter having evolved as a clonal descendent of the former (51, 52). Recent epidemiological studies have increasingly relied on genotypic classification methods such as restriction endonuclease digestion of total cell DNA followed by conventional (53) or pulsed-field gel electrophoretic analysis of macrorestriction fragment patterns (4, 6, 54), analysis of restriction fragment length polymorphism of rDNA (ribotyping) (27, 28), randomly amplified polymorphic DNA (RAPD) fingerprinting (29, 51, 52), multilocus enzyme electrophoresis (MLEE) (30, 50, 53), and typing based on comparative sequence analyses of the fast-evolving nonfunctional 16S-23S rRNA intergenic spacer region (51, 55, 56). Fluorescent *in situ* hybridization has also been used for the fast identification of GCS associated with necrotizing fasciitis (57). Finally, sequencing an internal fragment of the superoxide dismutase gene has yielded a phylogenetic tree, the topology of which agreed with that obtained from 16S rRNA sequences, rendering this method suitable for species-level identification of various streptococci (26). Taken together, the results obtained corroborate the species classification given in Table 1 and, moreover, testify to the utility of these methods for subspecific typing. For example, RAPD fingerprinting has proved useful in epidemiological investigations designed to delineate new and persistent *S. dysgalactiae* strains that caused intramammary infections in dairy cows (58). Comparison of the discriminatory power of different genomic typing methods revealed that RAPD exceeds MLEE for GGS (and GAS) typing (59) and that pulsed-field gel electrophoresis is even more efficacious than MLEE and RAPD as indicated by its potential to identify 93 distinct types among a total of 41 GAS and 58 GCS or GGS strains, with no types common to strains of different species (54). Although a comprehensive battery of species-specific gene probes for the identification of GCS and GGS has yet to be developed,

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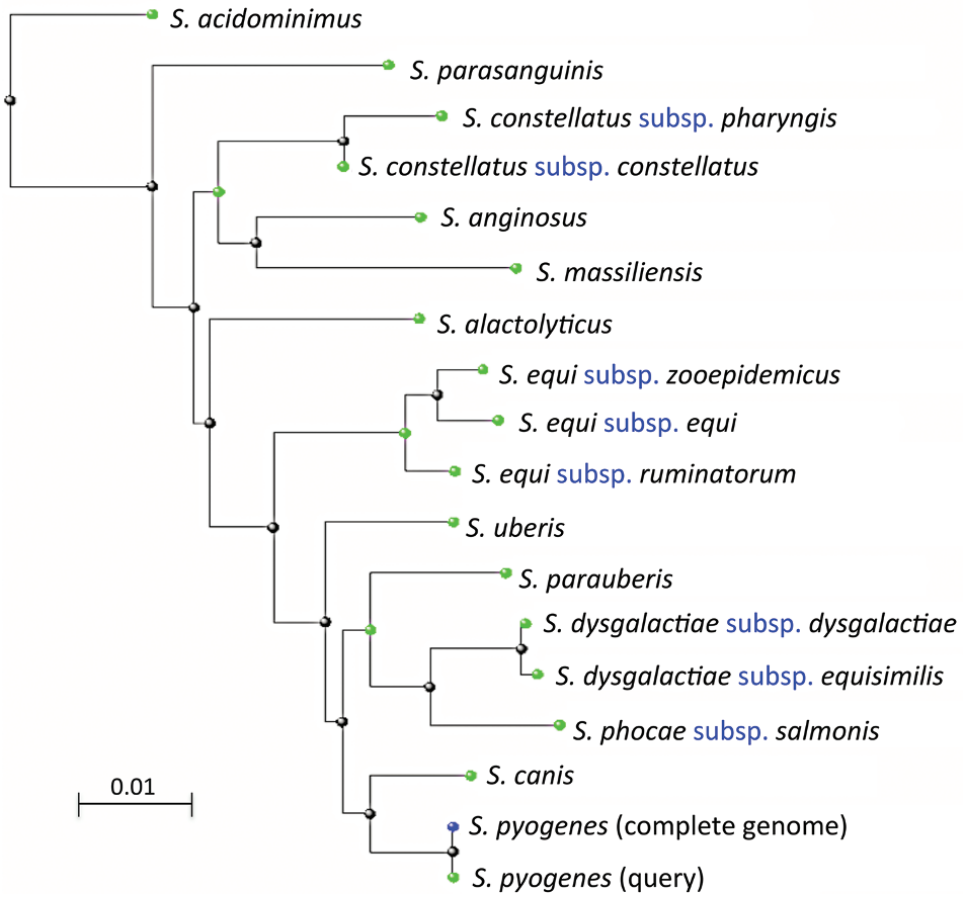


FIGURE 1 Phylogenetic relationships between the GCS and GGS species/subspecies included in Table 1. The cladogram is based on 16S rRNA gene sequences employed in BLAST alignments (http://ncbi.nlm.nih.gov) using the 16S rRNA sequence of *S. pyogenes* NZ131 as a query. The scale bar indicates 0.01 nucleotide substitutions per nucleotide position.

additional attempts to approach this goal have focused on oligonucleotides designed from 16S and 23S rRNA sequences (60). Alternatively, future differential genome analysis applied to GCS and GGS might identify genes that are responsible for species-specific features.

Relying exclusively on serological grouping for identifying GCS and GGS can be misleading, since numerous *S. dysgalactiae* subsp. *equisimilis* strains verified by 16S rRNA sequence analysis turned out to express the Lancefield group A antigen (61–63). Several of these "hybrid strains" were studied more closely and were found to carry the 12-gene *gacA-gacL* linkage group from *S. pyogenes* encoding the Lancefield group A carbohydrate consisting of a polyrhamnose backbone with the immunodominant *N*-acetylglucosamine side chain determined by *gacI* coding for glycosyltransferase (63). Obviously, these strains originate from a recombination event between *S. pyogenes* and a progenitor *S. dysgalactiae* subsp. *equisimilis* strain(s) comprising the entire *gacA-gacL* cluster.

PHYLOGENOMIC FINDINGS IMPLICATING GCS AND GGS

The exponential increase of available streptococcal genomes in recent years coupled with their comparative analysis led to the realization that they constitute dynamic entities undergoing continuous changes. The cladogram in Fig. 1 is concordant with the two latest independently derived phylogenies of the available genome sequences in 2014 for the genus *Streptococcus* when broken down to the clades related to GCS and GGS (37, 42). Both studies relied on, among others, highly revealing gene clustering methods using Markov clustering algorithms, hierarchal clustering, and assigned gene ontology terms to all available genomes (42) or used population structure analysis to investigate the relationships of the genus *Streptococcus* to the phylogenetic species groups (37). Of particular interest are gene gain/loss analyses showing that for most GCS and GGS species groups, their genomes evolve from an initial interval of gene gain (branches preceding those leading to the eight phylogenetic groups) through an interval of gene loss during diversification of the species groups, which ends with final genome expansion as indicated by a positive-gain negative-loss balance for the terminal branches of the present extant species. The exception is *S. uberis* within the Pyogenic group, which shows an overall gene loss at the tip of its branch. This evolutionary streamlining ("reductive evolution") and expansion process (42) is strongly supported by evidence indicating an open *Streptococcus* pan-genome which increases with the number of newly sequenced strains and is characteristic of species striving in multiple habitats open to lateral gene transfer (37, 64). In contrast, the core genome of the genus decreases with increasing genome numbers and finally reaches a plateau accounting for less than a fifth of the average gene content (ca. 2,000 genes [37]).

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Leaving the genus level and considering the phylogenetic species groups, the corresponding analyses reveal similar trends, although pan-genome sizes are smaller and core genome sizes larger than those at the genus level after new additions. When individual GCS and GGS species are examined, *S. parauberis* of bovine origin and *S. equi* subsp. *ruminatorum* excel at showing a greater gene gain/loss ratio than any other taxon (gain/loss = 6.0 and 3.4, respectively [42]). *S. parauberis* recently isolated as a pathogen from farmed fish (65) showed a considerable amount of gene turnover in this new habitat (gain/loss = 1.3 versus 6.0). It remains to be seen whether intensive aquaculture can account for the restructuring of its genome. Otherwise, in the biggest phylogenetic group, the Pyogenic group, there is little correlation between evolutionary relationships and host species (42). The best examples are the three subspecies of *S. equi*, which have short terminal branch lengths but infect a broad range of hosts. A similar situation is true of the two *S. dysgalactiae* subspecies, suggesting that a combination of reduced intergroup lateral gene transfer and group-specific reductive evolution has shaped the phylogenomic cohesiveness of the phylogenetic groups (42).

Within the phylogenetic groups relevant to GCS and GGS, gene ontology terms, i.e., assessments of the evolution of biochemical characteristics, show differential enrichments in two groups (42). Specifically, in the Mitis group, containing *S. parasanguinis*, genes annotated with the proteolysis term occur in the highest frequency. This reflects the human environments of the group in which proteases have prominent roles in colonization, dental plaque formation, utilization of salivary proteins, interfering with host defenses, or spreading through tissue. The importance of this characteristic is underscored by the observation that it is not subject to extensive reductive evolution. An additional feature found in this group is the enrichment for *N*-acetyltransferases, some of which are responsible for resistance to aminoglycoside antibiotics to which the group is intrinsically resistant. For the second group, the Pyogenic group, most of the enriched terms involve the carbohydrate metabolism, including the phosphoenolpyruvate-dependent sugar phosphotransferase system. This again is due to the wide host and environmental ranges inhabited by this group, as emphasized by the observation that these genes already arose in the branch leading to the phylogenetic group. As could be expected, also enriched in the Pyogenic group is the pathogenesis term, with *S. dysgalactiae* subsp. *equisimilis* and *S. equi* subsp. *equi* sharing 12 genes with each other. Of further interest, the pathogenesis gene ontology term does not occur in the branch leading to the Pyogenic group, suggesting acquisition by lateral gene transfer during evolution of the terminal branches.

In conclusion, evolutionary cohesiveness of the species in the phylogenetic groups generally includes GCS and GGS (42). Population structure analyses of streptococcal strains reveal two distinct evolutionary lineages, which when broken down to GCS and GGS include the Pyogenic group, on one hand, and the Mitis, Anginosus, and Unknown groups, on the other (37). Aside from the central topic of the foregoing considerations, the coccoid morphology of the genus *Streptococcus* contrasts with its molecular dynamics by presenting a "near-dead-end" state in its phylogeny (66, 67). Mapping prokaryotic cell shape onto the phylogenetic tree reveals spherical cells only at the tips of the branches, neither returning to other shapes nor occurring at primordial branches but having a polyphyletic origin. Thus, the coccus has selective value and deserves increased consideration in efforts directed at understanding this multifaceted genus. Investigations into the latter topic will also need to focus on the coordinated regulation of metabolism and virulence as implicated by the omnipresent three metabolite-responsive global CcpA, CodY, and Rex regulators that determine the relationship between streptococci and their hosts (68).

CELL SURFACE-ASSOCIATED PROTEINS

One hallmark of the Gram-positive pathogens is the synthesis of specific cell wall-associated proteins which enable them to interact in various ways with proteins present in the body fluids or extracellular tissue matrix of their mammalian hosts. Such interactions may facilitate colonization, lead to molecular host mimicry, or interfere with various host defenses against invasion.

M and M-Like Proteins

The major bona fide virulence determinant of GAS, M protein, is generally accepted to comprise polymorphic, fibrillar surface-exposed polypeptides that share common structural and functional features, among them a high propensity to form an alpha helix with a periodicity indicative of a coiled-coil structure, the capability of inhibiting phagocytosis by human neutrophils, and the potential to elicit opsonic antibodies that provide type-specific protection. Several studies conducted mainly in the 1980s provided highly suggestive evidence based on serological cross-reactions with typing sera or monoclonal antibodies raised to M protein from GAS, phagocytosis inhibition, and DNA hybridization analysis that M proteins or M-like proteins are also encoded and expressed by GCS and GGS (69). Subsequent cloning and sequence determination of the corresponding genes as well as analysis of immunoreactivity afforded conclusive evidence in support of this notion (47, 70–76). The first M protein gene to be isolated from a human GGS strain, *emmG1*, encodes a product with structural features characteristic of GAS class I M proteins that are epidemiologically associated with rheumatogenic, opacity factor-negative serotypes (76). As expected, structural similarity between MG1 and the GAS class I M proteins was significantly greater in the C-terminal regions (containing the anchor domains and the C and D repeats) than in the N-terminal portions (containing the A and B repeats) of the mature proteins (86 to 94% versus 31 to 35% amino acid identities), an observation consistent with a difference in antigenicity between MG1 and the GAS M types. An *emmG1* probe encoding the variable region of MG1 detected homologous DNA in seven additional M-positive GGS strains but also revealed at least four distinct *emm* alleles harbored by these strains. The essential aspects of these findings were confirmed and expanded by others (31, 63, 77–79) using a greater and epidemiologically unrelated number of GGS isolates that also included strains recovered from animal sources.

It is noteworthy that none of the DNA specimens isolated from GGS strains originating from animals hybridized with appropriate *emm* gene probes from GAS, whereas all DNAs from human GGS strains (large-colony-forming isolates) did (31, 77). Probes designed to differentiate between GAS M class I and class II genes detected only GGS *emm* genes related to GAS M class I genes (31). The complete sequence of an additional GGS *emm* gene (*emmLG593*), together with the sequences of the 5′ ends of 30 additional GGS *emm* genes, revealed six distinct "genetic types" which in paired comparisons shared <95% sequence identity in the *emm* gene region coding for the mature N termini (31). This observation

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adds further support to the genetic heterogeneity of the M proteins of GGS and, if consistent with future supplementary obsonophagocytotic studies, may be used for the development of GGS M typing schemes for epidemiological purposes. Most interestingly, in the latter studies (31) as well as in those conducted independently by others (78, 79), certain GGS *emm* genes were identified, defined portions of which exhibited exceedingly high levels of sequence identity to the variable regions of well-established GAS class I M protein genes, most notably *emm12* and *emm57*. Inasmuch as the results of total genomic DNA-DNA hybridization, chromosomal restriction endonuclease profiling, and MLEE all indicate that GGS are appreciably divergent from GAS in overall genomic character and, hence, have no recent GAS ancestors, the most likely explanation for the high local homology of certain *emm* genes in the two serogroups is intergroup horizontal genetic exchange followed by homologous recombination events (78, 79). Although several scenarios can be envisaged to suggest possible modes of DNA transfer, the actual transfer mechanisms remain in the dark.

In GCS isolated from horses, several M or M-like proteins have been characterized at the molecular level: SeM (functionally closely related to FgBP) (80) produced by *S. equi* subsp. *equi*, and SzP and SzPSe produced by *S. equi* subsp. *zooepidemicus* (73, 74). SzP and SzPSe are structurally only marginally related to SeM. They are antigenically cross-reactive and are coded by allelic variants of the same hypervariable gene. Functionally, however, all three proteins resemble M proteins of GAS in being capable of inhibiting phagocytosis, eliciting serum opsonic and protective responses, and binding (equine) fibrinogen (74, 80, 81). The complete sequences now available for these proteins are consistent with earlier serological and other observations indicating that the *S. equi* subsp. *equi* proteins are antigenically conserved, whereas those from equine isolates of *S. equi* subsp. *zooepidemicus* exhibit great antigenic variation (52). These observations, together with data derived from comparative MLEE, suggest that *S. equi* subsp. *zooepidemicus* is the ancestral species from which a clone now named *S. equi* subsp. *equi* emanated (51, 52); later, multilocus sequence typing (82) and genome comparisons (83) provided conclusive evidence for this suggestion. Although, unlike the SzP protein family, SeM is highly conserved, sequencing the variable regions from 145 isolates revealed 21 alleles assignable to four phylogenetic clusters (84). This should allow the differentiation of strangle outbreaks. Furthermore, SeM is the stronger opsonizing antigen in small laboratory animals, which together with its proven involvement in pathogenicity (74), has made it the main component of commercial strangles vaccines (8).

Analysis of truncated FgBP/SeM fragments has shown that binding to fibrinogen requires a fairly large stretch of the protein reaching from the vicinity of the N-terminus to about residue 350 of the 534 total amino acids (85). FgBP also uses distinct structural features to bind IgG-Fc (86, 87) and acquires thermal stability owing to an extensive α-helical coiled-coil structure situated in the C-terminal portion of FgPB (88). Of note, in the regions external to the cell wall, the SeM and SzP family proteins share no obvious sequence homology with GAS M proteins (74). However, M antigens closely related to the GAS class I M proteins and to those of the human GGS strains have also been detected on *S. dysgalactiae* subsp. *dysgalactiae* associated with bovine mastitis (75) and GCS isolated from humans (71, 89, 90). It is noteworthy that sequencing the DNA upstream of these M protein genes identified cooriented open reading frames designated *dmgA* (75) and *mgc* (71), respectively, with a high degree of sequence similarity to *mga*, the regulator of *emm* gene expression in GAS. In this connection, the FAI protein found in a GCS isolate from a horse (91) deserves special attention. FAI consists of overlapping functional modules responsible for fibrinogen, IgG, and albumin binding. Its three C repeat copies share 40 to 50% sequence identity with the B repeats of GAS M type 49 and the C repeats of M12 but exhibit no sequence similarity to any portions of SeM or the SzP proteins. Since GAS are usually not distributed in hosts other than humans, it seems doubtful that the strain producing FAI is a genuine horse strain and that the *fai* gene results from intergroup DNA exchange (74).

Given that the virulence mechanisms of *S. canis*, the closest relative of GAS, are incompletely resolved, the M protein of this species, SCM, deserves consideration in this connection. SCM is known to bind mini-plasminogen (mini-PG), the cleavage product of the zymogen PG, with high affinity, thus immobilizing plasmin (PM) onto the cell surface where this protease functions as a major virulence factor by mediating spreading of streptococcal infections. Very recently, SCM turned out to add to the great number of streptococcal proteins that bind to the immunoglobulin G (IgG)-Fc region in a nonimmune fashion (92). The IgG receptor function of SCM involves IgG-Fc from many mammalian sources, including human IgGs. This nonopsonic interaction mediated by the antiphagocytotic action of complement component C1q occurs via a conserved 52-amino acid region in the SCM center, which shows no homology to the IgG-Fc receptor of *S. dysgalactiae* subsp. *equisimilis* (92).

Protein G and Related Ig-Binding Proteins

The most thoroughly studied cell surface protein produced by many human GGS and GCS strains is protein G, which binds the heavy chains of IgG-Fc and Fab with an affinity comparable to that of antigen. Conventional thinking implies a contribution to adherence, inhibition of phagocytosis, and host mimicry in the role that protein G may have in pathogenesis, and evidence in favor of this notion has accumulated in recent years (93). Although the structure of protein G is reminiscent of staphylococcal protein A, the two polypeptides share, except for their C termini, little sequence identity (94–96). The protein G genes (*spg*) of distinct *S. dysgalactiae* strains may differ in their actual nucleotide sequence and size. However, allowing for processes such duplication of internal repeats and recombination and replication slippages involving repeats (94, 97), different *spg* alleles exhibit a very high level of sequence identity, making protein G a unique type among the diverse group of Gram-positive Ig-binding proteins. Compared to protein A, protein G binds not only to human, rabbit, mouse, and guinea pig IgG but also to IgG from cow, horse, sheep, and goat, which protein A does not bind. In addition to showing wider IgG species reactivity, protein G, unlike protein A, also binds human subclass IgG_3. Finally, protein G's reactivity is strictly limited to IgG (protein A also binds IgM.) (95). Because of these properties, protein G has become one of the most versatile tools in immunochemistry. Dissection of the molecule by enzymatic and genetic means followed by functional analysis of defined fragments confirmed early observations indicating that protein G is multifunctional in binding IgG, serum albumin, and α_2-macroglobuline (α_2-M) (85, 95, 98–101). Of particular significance in this context is α_2-M, a component of the innate immune system, which as the major proteinase inhibitor of the plasma, serves as a humoral defense barrier

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against pathogens in blood and tissues. Thus, as schematically shown in Fig. 2, IgG Fc-binding resides in the C-terminal portion of the protein G molecule and is mediated by two or more repeated domains, whereas the albumin-binding activity is a separate function of repeated domains in the N-terminal half. α_2-M binding resides in the N-terminal tip of the mature protein that lacks repeated sequence elements. Functional remnants of protein G have been retained in the protein G-related α_2-M-binding protein GRAB of GAS (102); however, sequence similarity of GRAB to protein G of GCS and GGS is limited to its N- and C-terminal regions.

The three-dimensional structures of the 56-residue B1 and the 57-residue B2 IgG Fc-binding domains of protein G from GGS strain GX7809 (94) have been determined by nuclear magnetic resonance spectroscopy (103) and X-ray diffraction analysis (104), respectively. The two approaches yielded essentially identical overall structures characterized by a four-stranded β sheet and a helix lying diagonally across the sheet and connecting the outer two β strands, β2 and β3. The IgG-binding interface of this structure is formed by negative charges distributed around the helix and β3 (105). The entire folding motif is very compact and shows high thermal stability owing to extensive hydrogen bonding and tight packing of its buried hydrophobic core. Taking advantage of these properties, the folding thermodynamics and mechanism of the B1 segment have been studied (106) and an M13 phage-B1 domain display system developed to pinpoint primary structure requirements for thermodynamic stability of the B1 domain (107). Interestingly, the IgG-binding domains also bind, in a nonimmune manner, heavy-chain constant-region sequences of the Fab portion of IgG but use disparate parts of their structure to bind the heavy chains of Fc and Fab. The crystal structure of the complex between Fab and the third IgG-binding domain of protein G shows that the outer β2 strand of the molecule aligns in an antiparallel fashion with the last β strand of the constant heavy chain domain of Fab, CH1 (108). In this manner, the β sheet structure is extended into protein G. Because the CH1 domain is highly conserved in the IgG Fab fragments from diverse species, these findings neatly explain the broad specificity of the protein G-IgG interaction. Moreover, provided that backbone-backbone hydrogen bonding between the interacting β strands is preserved, this arrangement allows Fab binding in the presence of considerable primary structure variability.

Fragmentation of human serum albumin located its single protein G-binding site to disulfide loops 6 to 8, with a 5.5-kDa pepsin fragment showing complete inhibition of the interaction between protein G and the full-length albumin (109). The crystal structure for a complex between human serum albumin and the albumin-binding domain (the GA module) of the PAB protein from *Finegoldia magna* (formerly *Peptostreptococcus magnus*) has been solved (110). As a result of an intergeneric sequence transfer event, the GA module exhibits high sequence similarity to the albumin-binding domain of protein G. The human serum albumin/GA complex sheds some light on the amino acid residues involved in conferring broader albumin-binding specificity on protein G than on PAB, which preferentially binds only primate albumins, in accord with the human pathogenic lifestyle of *F. magna* (110).

More recently, IgG-binding proteins have been found that show interesting functional differences from the prototype protein G molecules. The *mag* gene cloned from an *S. dysgalactiae* strain codes for a polypeptide with three discrete domains corresponding to those of the trifunctional protein G (111). However, the albumin-binding spectrum of Mag is broader than that of protein G, including albumins from at least nine mammalian species (112). The *mig* gene isolated from a case of bovine mastitis specifies a protein with binding activity to IgG, which resides in five C-terminal repeats, and to the α_2-M-proteinase complex. The latter activity is located in the N-terminal portion of the Mig protein, which exhibits only weak sequence similarity to the α_2-M-binding domain of the Mag protein. Furthermore, the Mig protein has no albumin-binding activity (113). Finally, the Zag protein, isolated from an *S. dysgalactiae* subsp. *zooepidemicus* strain, shows a molecular architecture that is similar to that of the Mag protein, but its N-terminal α_2-M-binding domain represents a third unique type of this module with little sequence similarity to the corresponding regions of either the Mag or Mig proteins. However, inhibition assays showed that all three proteins compete for the same or overlapping sites in the α_2-M molecule (114). The pathogenetic role of these proteins remains to be further established; however, evidence has been published showing that proteinase-complexed α_2-M bound to animal GCS inhibits phagocytosis *in vitro* (115).

One shortcoming of native protein G with regard to its application as a reagent for purification and detection of antibodies is the low affinity of its multispecific C2 domain for binding to the antibody IgG-Fab fragment (K_d [dissociation constant] ≈ 3 μM), in contrast to the high affinity of C2 for the IgG-Fc region (K_d ≈ 10 nM). To overcome this disadvantage for single-step Fab purification, a phagemid expressing engineered C2, designated protein G-A1, was recently constructed (116). This G-A1 variant possesses eight amino acid substitutions in C2, which greatly improves its human IgG1-Fab isotype affinity (K_d ≈ 25 nM). Rational mutagenesis based on three-dimensional structural evidence led to a series of additional variants showing (i) abolished IgG-Fc but preserved high-affinity Fab binding useful for the separation of the two fragments, (ii) pH-dependent affinity advantageous to antibody fragment purification (1,000-fold change from pH 7 to 4), (iii) enhanced stability in basic conditions useful for regenerating affinity columns, and (iv) specific Fab-antigen affinity enhancement by tethering two protein G-A1 molecules to yield a dimer. Taken together, the engineered protein G variants should prove useful for a multitude of applications.

Fibronectin (FN)-Binding Proteins

Studies published in the early 1980s showed that most GCS and GGS were able to bind FN (117, 118). However, it was



FIGURE 2 Schematic representation of the domain organization of protein G, adapted from reference 102. S, signal sequence; E, α_2-macroglobulin binding; A and B, human serum albumin binding; C, IgG binding; W, cell wall-associated region; M, cell membrane-associated region.

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not until the early 1990s that surface proteins of GAS, GCS, or GGS that bind to FN were characterized at the molecular level. The primary function of this adhesive matrix protein, extensively studied over the past 3 decades, is to mediate substrate adhesion and migration of eukaryotic cells via integrin receptors that bind to central parts of the molecule containing the arginyl-glycyl-aspartic acid (RGD) sequence motif. Among the best-studied GCS and GGS proteins responsible for FN binding are those encoded by the *S. dysgalactiae* genes *fnbA*, *fnbB* (cloned from the same strain) (119, 120), and *gfbA* (121); the *S. dysgalactiae* subsp. *equisimilis* gene *fnb* (122); and the *S. equi* subsp. *zooepidemicus* gene *fnz* of the animal ZV strain, which also produces the Zag protein (see above) (123). The mature proteins appear to occur in two size classes of about 60 and 120 kDa, but they all consist of C-terminal repetitive domains that are close to the cell wall-spanning regions and mediate binding to the 29-kDa N-terminal segment of FN. Comparison of the C-terminal repeats reveals a consensus sequence consisting of two adjacent glycines surrounded by highly acidic tripeptides that are required for binding activity, as indicated by the inhibitory effect of synthetic peptides that mimic individual repeats and show a high level of cross-reactivity (124, 125).

In addition to these interactions, the sequence motif LAGESGET separating the first and the second repeat of the Fnz protein has been identified as an important part of an FN-binding domain which does not bind the 29-kDa FN fragment (123). The FN-binding proteins F1 and F2 of GAS also have at least two domains, each responsible for binding. The F1 domain N-terminal to the repetitive domain contains the LAGESGET motif (126). The F2 protein, which is homologous to FnbB and Fnb, contains a nonrepeated domain, designated UFBD, which lies upstream of the repeat region and exhibits FN binding independently of the repeat domain (126). UFBD displays a high degree of sequence similarity to FnbB and Fnb regions N-terminal to their repetitive domains, strongly suggesting that FN-binding proteins generally use multiple domains for ligand binding (127, 128). This also includes Fnz (129) and FnbA, in which a previously unidentified region, Au, upstream of the repeats is capable of independent FN binding (130). Interestingly, a monoclonal antibody raised to FnbA enhances, rather than inhibits, FN binding to protein fragments or synthetic peptides containing Au and recognizes only the Au-fibrinogen complex (130). Circular dichroism analysis suggested that the ligand-binding site containing Au has little secondary structure but on binding FN is induced to form a predominantly β sheet structure that is seen by the antibody (131). The infected host might respond to such ligand-induced conformational changes with antibodies directed against the complex, thus stabilizing, rather than blocking, tissue adherence of the pathogen (130). That FN-binding proteins do contribute to adherence is shown, e.g., by the correlation between the presence of the *gfbA* gene (present in 36% of GGS strains tested) and the ability of such cells to adhere to human skin fibroblasts, as well as by the transformability of a nonadherent strain to the adherence phenotype upon introduction of *gfbA* (121). There is also evidence that GCS and GGS, like their GAS relatives, are capable of invading nonphagocytic host cells (99, 132, 133), a potential that is dependent on FN-binding proteins. A model for the internalization process based on structural studies implies that the originally disordered primary C-terminal FN-binding repeats form an extended tandem β-zipper upon binding to the string of F1 modules contained in the N-terminal 29-kDa FN domain. The binding of multiple FN molecules results in integrin clustering on the host cell membrane and subsequent endocytosis of the streptococcal cells (134).

Apart from the microbial surface components recognizing adhesive matrix molecules characterized above, a novel FN-binding protein, Sfs, displaying partial sequence similarity to collagen, was identified in many strains of *S. equi* subsp. *equi* and subsp. *zooepidemicus* (135). Sfs, which also has a counterpart in GAS, binds to the 30- to 40-kDa collagen-binding domain of FN and, accordingly, inhibits the binding of collagen to FN. Furthermore, the N-terminal fragment of protein Fnz from *S. equi* subsp. *zooepidemicus* binds to murine cells, collagen, and FN, suggesting that this protein is involved in the control of complex adhesive processes in infected tissues that express the αVβ3 integrin (136). The *fnz* gene suffered a one-nucleotide deletion in the middle of the coding sequence, giving rise to a frameshift creating the truncated but still functional *fne* gene encoding the N-terminal half of Fnz that binds to collagen. Interestingly, both Fne and Sfs are secreted into the growth medium due to the lack of a cell wall-anchoring motif, rendering their direct involvement in streptococcal adherence to host cells doubtful. However, both proteins interfere with the binding of collagen/gelatin to FN, reiterating their possible mediation of matrix assembly and cell migration processes (137, 138). Regarding Fne, its crystal structure provides surprising evidence for similarity to the *S. pyogenes* SF370 Spy0125 pilus adhesin and binding of the Fne C-terminus to the collagen/gelatin domain through the β-zipper extension mechanism mentioned above (138). Given the importance of the mutual interaction of FN and collagen in the extracellular matrix for the normal development and properties of many tissues, the above findings significantly add to our understanding the GCS and GGS surface proteins' potential for adherence to epithelial host cells and their invasion, enabling spreading of the pathogen into deeper tissue.

Plasmin(ogen)-Binding Proteins

Under this heading, the focus is on GCS and GGS strains that, in interaction with their specific hosts, recruit plasmin(ogen) on their cell surface. Species that carry such nonactivating PG-binding proteins mainly, but not exclusively, include *S. canis* and *S. dysgalactiae* subsp. *equisimilis*. Essentially, these proteins fall into two groups: (i) M-(like) proteins and (ii) so-called moonlighting proteins synthesized in the cytoplasm and transported to the cell surface (139). Although early studies provided evidence for the presence of plasmin(ogen) on the cell surface, the nature of the receptor proteins responsible for binding remained largely unknown (140–142). Sequence information is available, however, for two related M-like proteins from human GCS and GGS strains capable of binding PG to distinct sites of these proteins (141). Moreover, the GGS protein showed partial sequence identity to the PG-binding M-like protein from GAS termed PAM (141), hinting at the possibility that M-like proteins might be responsible for PG binding. Plasminogen binding and activation is also a common property of bovine mastitis-associated group C strains, with features indicating species specificity regarding the origin of the plasma proteins (142). Subsequent studies reviewed in references 139 and 143 provided ample evidence and specific details of the involvement of M-like proteins as the receptor molecules for plasmin(ogen) binding. They are linked to the streptococcal cell wall via a typical LPxTG motif and, in the case of *S. canis* and *S. dysgalactiae* subsp. *equisimilis*, exploit kringle 5 of mini-PG as their binding site. The two species differ,

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however, in the mode of PG activation: while the former uses host-derived urokinase and tissue-type PG activator, the latter expresses streptokinase (SK) to generate plasmin (PM). Surface-bound PG protects against phagocytosis and promotes adherence to endothelial cells, while PM is involved in the degradation of the extracellular matrix facilitating bacterial invasion.

The surprising discovery in GAS that the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which possesses none of the hallmarks of Gram-positive cell surface proteins, can be localized to the cell wall to function as a major binding protein for a variety of mammalian proteins, including PM (144, 145), prompted the isolation and functional analysis of the corresponding gene (*gap*C) in the human group C strain H46A (146). In this strain, *gap*C is an essential gene which occurs in a single copy and is abundantly transcribed. Its product shares 95% sequence identity with *S. pyogenes* GAPDH. Moreover, the number and position of lysine residues shown to be involved in plasmin(ogen) binding (144, 146) are identical in the two proteins. The binding parameters for human PG and PM of purified GapC protein were determined by real-time biospecific interaction analysis, with results indicating that the protein binds PM and PG with about 10-fold different affinities (K_d values, 25 nM and 220 nM, respectively). Thus, the zymogen and active enzyme appear to possess low-affinity binding sites for the *gap*C gene product, a result that is at variance with that ascribed to GAS GAPDH, whose PM-binding activity was reported to be 3 orders of magnitude higher (147). On reexamination, the GAS strain involved turned out to display other PM-binding substances on the surface in addition to GAPDH (148). A candidate substance could be the glycolytic enzyme α-enolase, which was subsequently identified as a novel ubiquitous streptococcal surface protein with strong plasmin(ogen)-binding capacity (149). Given the 2-μM concentration of PG in plasma, even the high K_d value for the association of this protein with GapC would suggest that, in the vasculature, the great majority of the *S. equisimilis* H46A PG-binding sites are occupied, even if GapC is the only PG-binding protein. An SK-negative mutant of H46A, isolated to eliminate endogenous PG-activating capacity, has been shown to bind PG as indicated by the generation of cell-surface-associated PM activity following exposure to exogenous PG activators (150).

A novel aspect characterizing the interaction of plasmin(ogen) with surface GAPDHs relates to the finding that limited prior treatment with PM greatly increases the subsequent PG-binding capacity of these proteins (146). Presumably, the underlying mechanism involves the exposure of new PG-binding sites on GAPDH by PM, which preferentially catalyzes the hydrolysis of α-amino-substituted lysine (and arginine) esters. In the infected host, streptococci capable of transporting GAPDH (and α-enolase) to the cell wall and secreting SK would thus appear to have evolved a remarkable system to generate and amplify cell-surface protease activity. Host PG bound with low affinity can be converted to the active enzyme directly on the cell surface. The cell-bound PM may generate additional PG-binding sites on GAPDH to start new cycles of PG binding and activation, resulting in amplification of the tissue-invasive potential of the pathogen. Although there exist strains producing proteins unrelated to GAPDH that display high-affinity plasmin (ogen)-binding sites (141), the special role of GAPDH (and possibly α-enolase) is due to its general occurrence on the surface of the pyogenic streptococci (145, 149). A major challenge to what is presently understood of the PG-binding and -activating capacity of pathogenic streptococci relates to the identification of possible factors involved in the modulation of cell surface protease activity that may be necessary to avoid destructive proteolysis of surface structures important for colonization and invasion. It is interesting to note that a screen designed to identify *Escherichia coli* genes expressed preferentially by natural isolates when incubated in nutrient-poor aquatic medium identified a streptococcal *gap*C homolog (85% sequence homology), suggesting acquisition of the gene by horizontal transfer from a Gram-positive organism and possible involvement in the preparation of cells for the colonization of a new host (151).

Targeting Alginate

The exopolysaccharide alginate, a copolymer of D-mannuronic acid and α-L-guluronic acid, is produced by mucoid strains of *Pseudomonas aeruginosa* that are the functionally predominant pathogens in the polymicrobial biofilm associated with devastating chronic pulmonary infections in cystic fibrosis (CF) patients (152). The oral commensal GCS species *S. parasanguinis* and *S. anginosus* have recently been found to be able to become numerically dominant in the biofilm, thus specifically modulating the behavior of *Pseudomonas* in clinically interesting ways (153, 154). In a similar vein, a recent study implicated the biofilm matrix rather than *P. aeruginosa* in the anti-inflammatory response in CF patients, thus directing attention to the possible role of the induction of the innate immunity system in CF (155).

Within a 6-hour dual-species biofilm formed by the mucoid *P. aeruginosa* strain FRD1 and the oral commensal *S. parasanguinis* strain FW213, strain FRD1 promoted the number of FW213 colony-forming units (CFU) by more than 10-fold compared to the single FW213 biofilm (153). Similarly, FRD1 promoted the planktonic cell growth of FW213. In the presence of alginate lyase, which destroys the copolymer by cleaving its β-1,4 linkage, FRD1 was unable to promote the FW213 biofilm. Moreover, FRD1 mutants defective for alginate biosynthesis did not enhance the FW213 biofilm. The importance of alginate in the dual-species biofilm was further supported by immunofluorescence microscopy and confocal laser scanning microscopy, showing that the streptococcal cells are colocalized with alginate, thus exploiting the latter as a biofilm matrix component. In the search for the biofilm-related factors that determine the streptococcal interaction with alginate, sortase A was found by mutational analysis to be responsible for promoting not only the mono-species but also the dual species FW213 biofilm. Since sortase A was previously known to anchor the fimbria-associated BapA1 and Fap1 adhesins to the cell surface of oral streptococci, further mutational analyses showed that BapA1 is required for enhanced biofilm formation by *S. parasanguinis* in the presence of alginate. Finally, these results obtained *in vitro* were expanded by employing the *Drosophila melanogaster in vivo* model in combination with the available set of mutants, showing that the wild-type alginate producer FRD1 promotes *S. parasanguinis* colonization *in vivo* and that, under these conditions, either BapA1 or Fap1 suffice to enhance *S. parasanguinis* colonization (153).

The interaction between the mucoid alginate overproducing CF-associated *P. aeruginosa* strain DWW2 and the oral commensal *S. anginosus* strain 3a stands in striking contrast to the dual-species FRD1-FW213 interaction reviewed above (154). To mimic the atmospheric microenvironments relevant to the CF airway, the responses of DWW2 and 3a to biofilm mono- and coculture were investigated in normoxic, hypercapnic, and anoxic conditions, with the focus put on

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numerical developments of CFU, the measurement of pyocyanin expression by DWW2, the influence of strain 3a on the reversion of DWW2 to a nonmucoidal phenotype, and the virulence *in vivo* of the two species using the *Galleria mellonella* infection model. The results obtained revealed that (i) in agreement with the study reviewed above (153), the streptococcal CFU increased in number in coculture, (ii) the streptococcus was instrumental in furthering the reversion of the DWW2 mucoid phenotype to the nonmucoid variant, exhibiting increased pyocyanin expression, and (iii) the nonmucoid, high-pyocyanin-expressing phenotype of DWW2 was highly virulent *in vivo*, causing 10% larval survival after 24 h incubation in mono-infection and 0% survival for coinfection (154).

The contrast evident in the results of the above studies (153, 154) lies in the different effects the two streptococcal species may have on CF pathogenesis. Whereas *S. parasanguinis* appears to interfere in a favorable fashion with the pathogenic effects of *P. aeruginosa* by restricting its potential for biofilm formation, *S. anginosus* behaves in a detrimental way from a clinical point of view by increasing pyocyanin production that impairs a wide range of cellular components and pathways. To resolve the conflict presented by the two streptococcal species, it should therefore be interesting to investigate the effect of *S. parasanguinis* on pyocyanin expression by *P. aeruginosa* FRD1.

Complement Fragment C5a Peptidase

Complement fragment C5a functions as a chemotaxin that mediates phagocyte recruitment to the site of infection. GAS and human GGS have been found to produce a highly specific cell-wall-anchored serine endopeptidase that cleaves C5a in the polymorphonuclear leucocyte-binding site, thereby limiting the activity of this chemotactic signal. In Southern hybridization experiments, probes specific for the GAS C5a peptidase gene, *scpA*, detected homologous *scpG* sequences in all human GGS strains tested (6, 156) but failed to detect the gene in GGS isolated from animals (156). As far as GCS strains are concerned, the latter observation should be expanded by more recent evidence showing that the ScpZ protein of an *S. equi* subsp. *zooepidemicus* strain, C55138, originally isolated from a pig with septicemia, shares sequence identity, particularly at the highly conserved enzymatically active site, with ScpA (157). The *scpA* and *scpG* genes appear to be very similar, and their products cross-react serologically (156). However, the *scpG* and *scpC* genes in GGS and GCS, respectively, show different linkage relationships from *scpA* because, in either serogroup, they are not located 3′-adjacent to *emm* or *emm*-like genes as is the case for *scpA* in the *mga* regulon of GAS (71, 79, 156) (see also Fig. 3). Whether *scpG*/C are members of the *mgc* regulon (71) is an open question; loss of synteny of the component genes of the *mga* regulon in GGS/GCS may be taken to indicate recent acquisition of individual genes by horizontal gene transfer. In fact, in *S. agalactiae*, *scpB* is localized on a composite transposon flanked by insertion sequences, thus highlighting the possibility of lateral transfer involved in the shuffling of *scp* genes (158).

CYTOPLASMIC MEMBRANE-ASSOCIATED ENZYMES

Hyaluronan Synthase

The hyaluronan (hyaluronic acid) capsule of the Pyogenic streptococci has an important role in resistance to phagocytosis and in virulence (8, 159). In GAS, the three genes required for hyaluronan synthesis, *hasA*, *hasB*, and *hasC*, constitute an operon (160). For efficient hyaluronan export, an ATP-binding cassette transporter system is required, the genes of which are located in opposite orientation adjacent to the *has* operon (161). A *hasA* homolog encoding hyaluronan synthase, which catalyzes the alternate addition of UDP-acetylglucosamine and UDP-glucuronic acid to form the capsule polymer, has been cloned from *S. equi* subsp. *equisimilis* (162). The GCS enzyme (seHAS) sequence is 72% identical to that of its homolog from GAS (spHAS), and accordingly, the two proteins cross-react serologically. Both proteins are processive enzymes, and the size distributions of the hyaluronan chains they synthesize are similar. However, seHAS shows a 2-fold faster chain elongation rate than spHAS, making it the most active hyaluronan synthase described thus far (162). Furthermore, the membrane topology of seHAS is likely to be identical to that experimentally deduced for spHAS. This enzyme exhibits four membrane-spanning domains and two membrane-associated domains, with the termini of the protein and several central regions situated intracellularly (163). The hyaluronan synthases seHAS and spHAS were overexpressed as His_6-tagged membrane proteins in *E. coli*, purified to homogeneity, and extensively characterized kinetically. They represent lipid-dependent enzymes, the activity of which is strongly stimulated by cardiolipin and phosphatidylserine (164–166). In contrast, sulfhydryl-modifying reagents and the lipid-soluble 4-methylumbelliferone proved to be inhibitors of seHAS activity (167, 168). Despite suggestions to the contrary (169), no phosphoryl modification of streptococcal HAS has been detected (167). Although the 6.2-kb restriction fragment from the encapsulated GCS strain that served to clone *hasA* (162) is potentially large enough to also accommodate *hasB* and *hasC* (160), definitive proof for the existence and functioning of this operon in GCS or GGS has not been reported.

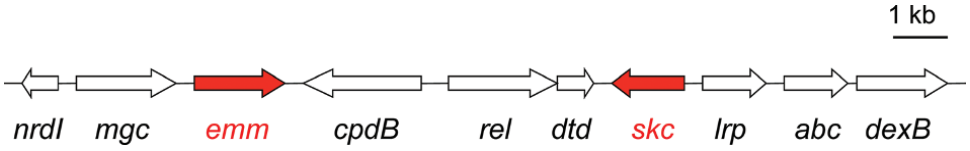


FIGURE 3 Organization of the streptokinase-M protein gene region in the *S. dysgalactiae* subsp. *equisimilis* H46A chromosome based on references 71 and 183. Arrows represent the genes and their orientation: *nrdI*, ribonucleotide reductase; *mgc*, multigene regulator of GCS; *emm*, M protein; *cpdB*, 2′,3′-cyclic nucleotide 2′-phosphodiesterase, *rel*, bifunctional (p)ppGppase and (p)ppGpp synthetase; *dtd*, D-tyrosyl-tRNA^{Tyr} deacylase; *skc*, streptokinase; *lrp*, leucine-rich protein, now recognized as a transcriptional regulator of the PucR family featuring a C-terminal helix-turn-helix domain; *abc*, ATP binding cassette transporter; *dexB*, α-glucosidase.

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Operon organization of the three genes required for capsule synthesis cannot be taken for granted, as evidenced by *S. uberis*, in which *has*C is essential for capsule production but is not situated 3′-adjacent to *hasAB* (64).

Cytoplasmic Membrane Lipoprotein Acid Phosphatase

Attempts to elucidate the linkage relationships of the *S. dysgalactiae* subsp. *equisimilis* H46A *gap*C gene (see above) resulted in the discovery of a novel streptococcal gene 3′-adjacent to, and cooriented with, *gap*C (170). This gene, designated *lpp*C, codes for a 32.4-kDa membrane lipoprotein as indicated by the presence of a lipoprotein-specific signal sequence cleavage and lipidation site (VTG↓C), by globomycin sensitivity of signal sequence processing, and by tight association of the protein with the streptococcal cytoplasmic membrane or the outer membrane of *E. coli* when heterologously expressed in this organism. Southern, Northern, and Western analyses revealed that *lpp*C has homologs in GAS and is transcribed independently of *gap*C as monocistronic 0.9-kb mRNA from a σ^{70}-like consensus promoter (170). Later, lipoproteins cross-reacting serologically with LppC were also detected in disparate strains of two *S. equi* subspecies (171). Currently, database searching shows that LppC homologs are generally distributed in the genus *Streptococcus*, including the known phylogenetic groups harboring GCS and GGS that exhibit 65% (*S. anginosus*) to 93% (*S. canis*) sequence identity to LppC. Moreover, disparate pathogenic genera showing significant sequence identity to LppC include, among others, *Haemophilus*, *Staphylococcus*, *Helicobacter*, *Gemella*, *Sneathia*, *Chlamydia*, *Peptoanaerobacter*, and *Flavobacterium*. Among those, of particular interest is the *hel* gene of *H. influenzae*, which encodes the major outer membrane antigen *e* (P4), exhibiting 58% sequence similarity to LppC.

Biochemical, serological, and genetic evidence has been provided to show that LppC represents a novel class of Gram-positive lipoproteins in functioning as an acid phosphatase (172), now assigned to class C acid phosphatases, which fall into the DDDD superfamily of phosphohydrolases (173). Their enzymatic activity was found in the *Haemophilus e* (P4) antigen as well and led to a re-evaluation of its primary role as an outer membrane protein (173, 174). In a search of a physiological role for LppC, various physiological substrates were tested at an optimum pH of 5.0, and efficient phosphohydrolase activity was found to be restricted to all of the 15-nucleoside and 2′-deoxynucleoside 3′- and 5′-monophosphates examined (175). In contrast, 22 additional mono- or multiphosphorylated *in vivo* substrates tested were not detectably hydrolyzed, among them phosphates involved in signal transduction pathways necessary for oxidative burst activation in neutrophils. In the presence of p-nitrophenyl phosphate and suitable acceptors with free hydroxyl groups, the enzyme also showed transphosphorylating activity, explaining the failure of hydrolyzable phosphomonoesters to support the growth of strain H46A in chemically defined medium lacking free inorganic orthophosphate (P_i); i.e., possible P_i deficiencies cannot be complemented by the hydrolysis of such substrates. Rather, LppC-catalyzed transphosphorylation of *in vivo* acceptors yet to be identified may occur simultaneously, thus constituting a sink that renders growth-sustaining quantities of free P_i unavailable. Growth studies in modified chemically defined medium also showed that strain H46A is capable of *de novo* purine synthesis but depends on salvage pathways for pyrimidine acquisition. However, while UMP could compensate for U deficiency in both the wild type and an *lpp*C mutant, CMP utilization specifically required the functional *lpp*C gene (175).

Although the studies reviewed above provide considerable insight into the physiological role LppC may have in interaction with its hosts, it remains to be investigated whether this protein is additionally involved in specific pathogenetic processes, which might be suggested by its general association with disparate pathogens. Given that its homolog *e* P4 is highly immunogenic, that anti-P4 antibodies exhibit bactericidal activity, and that P4 reduces colonization of *H. influenzae* in mice (176), LppC deserves to be considered a vaccine candidate as well.

PerR-Regulated Iron Efflux Mediated by PmtA

S. pyogenes homeostatically copes *in vivo* with metal sequestration as well as metal overload poisoning by its host. Very recently, the cytoplasmic membrane-associated ATPase PmtA (PerR-regulated metal transporter A) was found by genetic, biochemical, and bacterial growth analyses to act as an iron [Fe(II)] efflux system that is part of the oxidative stress response (177). The broad peroxide-responsive transcription regulator PerR from *S. pyogenes*, which functions to sense and modulate the response of GAS to oxidative stress, is also expressed in *S. uberis*, *S. equi*, and *S. dysgalactiae*, with amino acid sequence identities amounting to 88%, 92%, and 94%, respectively. Regarding PmtA, current databank analyses reveal sequence identity to the *S. pyogenes* protein in *S. phocae* (76%), *S. parauberis* (77%), *S. uberis* (79%), *S. canis* (92%), and *S. dysgalactiae* (94%). Thus, the PerR-PmtA iron efflux system as described in *S. pyogenes* (177) is highly likely also operative in GCS and GGS.

EXTRACELLULAR PROTEINS

Streptokinase

The first SK gene (*skc*) to be cloned (178) and sequenced (179) originates from the most potent and commercially exploited producer of this PG activator, the human *S. dysgalactiae* subsp. *equisimilis* strain H46A. Later, an SK gene (*skg*) from a human GGS strain was also characterized (180), and the two encoded proteins were found to share 98% sequence identity. To date, SK alleles belong to those streptococcal genes that have most frequently been sequenced from diverse strains, and research in this area has appreciably expanded our knowledge of this important streptococcal gene and its product in various areas. Part of this work has been reviewed previously (181, 182); here, I focus on recent studies conducted to elucidate the expression control, structure-function relationships, and aspects of adaptive evolution of SK.

Expression control of the SK gene

Because gene regulation is influenced by the spatial association of genes on the chromosome, it is appropriate to point out that the SK genes show homology of synteny in the genomes of GAS and of human GCS and GGS (71, 183, 184), and numerous subsequent studies based on nucleotide sequence analyses confirmed these early results. Conservation of linkage appears also to be true of an equine group C SK gene that hybridizes with *skc* (184) but encodes an SK that preferentially activates horse PG and antigenically differs from the proteins produced by human strains (185). *skc*, *skg*, and *ska*, collectively designated *skn*, are transcribed as 1.3-kb monocistronic mRNA from a highly conserved chromosomal region in which they are interspersed among unrelated genes transcribed in the opposite direction (Fig. 3).

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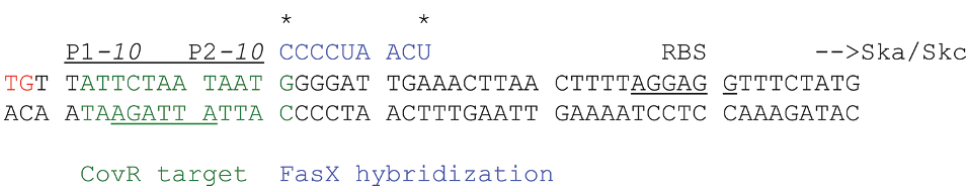


FIGURE 4 Primary structural features of the 5′-untranslated region 47 nucleotides upstream of the *ska/skc* ATG translational start codon. Additionally indicated are the -10 regions of the tandem P1 and P2 promoters (underlined), including (in red) the TG nucleotide extension of P1, the target sequence of CovR (in green) with the consensus binding site underlined, the FasX hybridization site (in blue), the two major transcription initiation sites (stars), and the mRNA ribosome binding sequence (RBS, underlined). These features are based on references 188, 190, 196, and 199.

Transcription is terminated by a hypersymmetrical intrinsic terminator with bidirectional activity shared by the oppositely oriented *skn* and *rel-dtd* transcription units (186, 187). S1 nuclease mapping has identified the core promoter P1 with an extended -10 region that directs transcription initiation predominantly at a G located 32 bases upstream of the *skc* translational start site (188). A second core promoter, P2, with a -10 region immediately 3′-adjacent to that of P1, but distinctly less potent then P1, directs transcription initiation predominantly at an A 24 bases upstream of the Skc start site (Fig. 4). Circular permutation analysis combined with determination of the activity of nested deletions in the DNA upstream of the -35 region identified an intrinsic DNA bending locus that has a pivotal role in *skn* expression (182, 189). Deletions covering the bending locus greatly decrease the core promoter strength in strain H46A but not in *E. coli* (182, 190), consistent with the observation in the latter species that promoters with a TG motif 1 bp upstream of the -10 region, as realized for P1 (Fig. 4), function activator independently, even in the absence of a recognizable -35 region. Region 2.5 of *E. coli* RNA polymerase sigma factor RpoD, i.e., sigma^{70}, implicated in contacting the TG motif (191), is completely conserved in all streptococcal homologs of RpoD (sigma^{42}), including representative strains from three phylogenetic groups of GCS and GGS. In particular, their 2.5 regions do carry the amino acid residues H455 and E458, which are responsible for recognition of the TG extension (Fig. 5).

Nevertheless, as shown by the above findings, the streptococcal system is much more stringent with respect to supplementary structural requirements for full *skn* promoter activity and displays strain-specific *skn* expression levels. Thus, using reporter gene constructs in allele swap experiments between GCS and GAS strains revealed that the host genetic background dictates *skn* expression, suggesting the existence of trans-acting factor(s) with strain-specific activity that modulate *skn* expression. Evidence for the action of such factors was provided after the discovery in GAS of two independent two-component signal transduction systems, *covRS* (192) and *fasCAX* (193), which regulate *skn* expression negatively and positively, respectively (194, 195). Thus, assessing the apparent SK activity of streptococcal strains requires the dissection of the opposing activities of the *cov* and *fas* systems. Specifically, sequence analysis showed that "wild-type" H46A is actually a derepressed mutant for Skc (and streptolysin S) synthesis, carrying a K102amber mutation in *covR* (194). The high-affinity binding site (CB-4; ATTCTAATAATG) of CovR from *S. pyogenes* overlaps the two -10 regions of *ska* (196) and is completely identical to the corresponding *skc* region specified above for P1and P2 (Fig. 4). In general, knowledge of the FasA response regulator is less advanced than that of CovR. The bootstrapped phylogenetic tree published previously (197) places FasA in a widely distributed family of streptococcal response regulators (the FasA-BlpR-ComE family) that is involved in behavioral processes, such as quorum sensing, and that uses the C-terminal LytTR domain for DNA binding (195, 198). The FasA proteins, which are distributed predominantly among the Pyogenic streptococci, form a well-supported cluster within this protein family. Underscoring their distinct position, the *fasX* gene, encoding a nontranslated small regulatory RNA functioning as the terminal effector of the *fasCAX* operon (193, 194), has no homologs in the *comE* and *blpR* clusters. FasX uses its internal 9-ribonucleotide CCCCUAACU sequence to bind 32 nucleotides upstream of the *ska/skc* AUG start codon to the 5′-untranslated region of *ska/skc* mRNA covering the two major transcription initiation sites, thus increasing transcript stability posttranscriptionally (188, 199). Given that, as specified above, the corresponding *skc* mRNA exhibits exactly the same sequence conditions, this mechanism for enhancing SK activity functions beyond reasonable doubt also in GCS. To facilitate comprehension, Fig. 4 illustrates that the binding site of CovR and the hy-



FIGURE 5 Sequence comparison of *E. coli* and *S. pyogenes* σ factors between region 2.4 and the start of region 3. Identical and similar amino acids are marked by asterisks and dots, respectively. The residues identified in *E. coli* (191) as contacting the TG extension of the -10 P1 promoter hexamer are highlighted in red.

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bridization site of FasX share a 1-base overlap; however, because the two opposing regulators target different molecules, i.e., DNA and mRNA, respectively, direct competition between them for binding to the 5′-untranslated *ska*/*skc* regions can be ruled out.

Structure-function relationships of SK

SK produced by *S. dysgalactiae* subsp. *equisimilis* strain H46A was predominantly used in the many structural studies reported in the literature. This protein has at least three functions in the process of PG activation: (i) high-affinity plasminogen binding to form a 1:1 stoichiometric PG:SK complex, (ii) interacting with PG to structure the active site, i.e., forming the virgin enzyme with amidolytic activity (conformational activation; i.e., PG*:SK formation), and (iii) binding and processing of substrate PG molecules, resulting in cleavage of the activating R561-V562 peptide bond to generate the active enzyme, plasmin (PM) (200, 201). Solution structural studies have presented evidence that the 414-amino acid mature SK is an elongated globular molecule that consists of three independent folding domains of roughly equal sizes (202, 203, and references therein). The crystal structure of SK complexed with microplasmin (μPM, i.e., PG residues 542 to 791, containing the catalytic site) illustrates these domains denoted α (residues 1 to 150), β (151 to 287), and γ (288 to 414), interconnected by flexible strands (204). Each of these domains contacts μPM, with the β domain having the fewest interactions and exhibiting the highest degree of residue polymorphism (205). In contrast, the SK termini appear to be unstructured and mobile, explaining early findings showing that SK muteins lacking up to 15 N-terminal or 41 C-terminal residues or that truncated SKs fused to foreign proteins continue to activate PG, albeit with diminished activity (202, 203, 206–208, 263, 264). Studies of binding interactions between SK and PG domains using surface plasmon resonance (209) showed that the β domain, in addition to contacting the PG catalytic domain, interacts with PG kringle 5. Based on these studies, the mechanistic steps involved in PG activation include (i) binding of all SK domains to the PG catalytic domain, (ii) cooperation of the α and γ domains to induce PG*:SK formation, and (iii) recognition of substrate PG by PG*:SK through interactions mediated by the α domain. A unified hypothesis for the mechanism of coupling between conformational and proteolytic PG activation based on parabolic time-course assays implies that PG*:SK initially binds PG in the substrate mode to form a ternary PG*:SK:PG complex, from which free PM is produced. SK affinity for PM is much higher than that for PG, so the catalytic PM:SK complex in the substrate mode readily activates remaining free PG at concentrations of PG>>SK. This ultimately results in full conversion of free PG to PM (200, 201, 210). Taken together, the studies summarized above suggest that, except for the very ends of the polypeptide chain, most of the SK sequence is essential for efficient functionality. Nevertheless, attempts were made preferentially in two fields to improve the therapeutic potential of SK as a cost-effective thrombolytic agent. First, to reduce its immunogenicity, known antigenic epitopes were targeted by directed mutagenesis experiments which, for example, identified an S138K substitution that, after transfer to therapeutic SK, led to a loss of anti-SK antibody binding without detrimentally affecting PG activation activity of the mutein (211). Second, in experiments aimed at increasing the *in vivo* half-life of SK, which is susceptible to cleavage by PM, a K59E mutant was identified to exhibit the desired effect while maintaining PG activation activity (212). Additionally, to reduce the host PG activation activity of SK and hence hinder the acquisition of PM activity on the streptococcal cell surface to counteract invasive processes, SK interaction, particularly between its α-domain and PG, could be a promising target for engineering successful variant SK molecules (213). Finally, taking the gene fusion approach to construct and express hybrid *skc* genes extended at their 5′-ends by DNA segments encoding the fibrin-binding kringle domains of PG led to bifunctional hybrid SKs, the genetic design of which needs to be optimized for ultimately improving the therapeutic potential of SK (207).

Adaptive evolution of SK alleles

It has been known for a long time that SKs exploited by streptococci to infect mammals show species-specific activity, a phenomenon likely due to PG features specific to their respective hosts. More recent studies based on knowledge gained about the mechanism of PG activation have provided molecular insights into this phenomenon (214). Thus, SKs produced by different human streptococcal strains show a strong conservation of amino acid residues that contact sites in the loop residues of human μPm. On the other hand, such residues lack conservation among SKs from animal streptococci that activate the corresponding animal PGs. Conversely, different animal PGs show greater sequence diversity from human μPm in loops that contact SKs produced by human-pathogenic streptococcal strains. Streptococci may thus evolve to produce SKs that match their evolving target PGs, whereas PGs evolve to escape from such interactions. Such coevolutionary patterns in PG activation would thus appear to be advantageous for both sides and make SK a determinant of host species specificity for streptococcal infections (214). In fact, a transgenic mouse line expressing human PG exhibited significantly increased mortality upon infection with GAS (215).

A study designed to uncover molecular evolutionary mechanisms underlying tissue-specific streptococcal infections analyzed the sequences of *skn* genes in skin-tropic strains known to bind host PG via the PAM protein and to depend on both types of genes for virulence at the skin (216). Exploiting the well-known fact that the β domain of SK shows the highest level of sequence heterogeneity, a tight association was found between the presence of PAM and a well-supported subcluster 2b *ska* allele. This observation indicated coinheritance of the corresponding genotypes maintained by coselective pressure due to epistasis rather than a physical genetic linkage of the *ska* and PAM-encoding *emm* loci. Interestingly, in contrast to the β domain-encoding region of the *ska* alleles, the *skn* alleles from GCS and GGS, including the *S. dysgalactiae* subsp. *equisimilis* strain H46A, show a high degree of sequence homogeneity and are most closely related to subcluster 2a alleles of *ska*. This subcluster is predominantly associated with throat-tropic GAS strains, apparently consistent with the lifestyle of GCS and GGS which primarily inhabit the upper respiratory tract. More specifically, the *S. pyogenes* SF370 *ska* allele is much more strongly related to the *skc* alleles than to the subcluster cluster 1 or 2b *ska* alleles, suggestive of relatively recent interspecific gene transfer from GCS or GGS. Although it remains an open question whether subcluster 2a *skn* alleles facilitate colonization of the throat, this finding suggests that an *skn* allele from GCS or GGS was transferred from either group to GAS, where it both stayed as a subcluster 2a allele and evolved into a subcluster 2b allele. Lateral transfer in the opposite direction seems to be less likely because the housekeeping genes of GCS and GGS strains carrying a subcluster 2a allele show great heterogeneity (216).

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Aside from the above considerations, a review focusing on the polymorphism of the SK alleles, including those from GCS and GGS, links this feature with strains associated with acute poststreptococcal glomerulonephritis (205). The basic observation relates to a stretch of about 70 amino acid residues in the SK β-domain in which sequence identity drops to below 50% when the proteins from nephritogenic and nonnephritogenic strains are compared. The sequences unique to the former may be considered the nephrotropic domain with which their SKs, after *in situ* PG activation in the glomerular structures, trigger the complement cascade of proteolytic processes leading to poststreptococcal glomerulonephritis (217, 218).

Streptolysins and Streptodornase

Based on the well-known observation that a positive antistreptolysin O test is not specific for recent GAS disease, successful attempts were made to clone and sequence the streptolysin O (*slo*) genes of GCS (*S. dysgalactiae* subsp. *equisimilis*) and GGS (*S. canis*) (219). The *slo* gene proved to be highly conserved in the three serogroups, with the sequence identity of the protein exceeding 98%. Moreover, in recent comparative studies involving invasive and noninvasive *S. dysgalactiae* subsp. *equisimilis* isolates, *in vivo* evidence was provided that implicated Slo, next to Skc, as a primary virulence factor responsible for necrotizing soft tissue infections (220). Expectedly, the *slo* gene was not detected in two subspecies of *S. equi* (219, 221). However, a streptolysin S-like activity has been characterized in *S. equi* subsp. *equi* and shown to be subject to transposon inactivation at a single locus (221). Conclusive evidence was provided later, showing that the production of this important cytotoxin is encoded in the same nine-gene *sag* operon that was characterized in GAS and *S. dysgalactiae* subsp. *equisimilis* (human GGS) associated with severe necrotizing soft tissue infections (222). Interestingly, a *sag* operon homolog was also detected in all examined strains from the Anginosus group, which in contrast to the strains in the Pyogenic group, exhibited two functional tandem *sagA* genes (*sagA1* and *sagA2*), the primary structures of which are highly conserved (223). Compared to an *S. anginosus* reference strain, *sag* operon sequence identities against the corresponding genes of other streptococcal *sag* operons range from 53 to 75%. The origin of the twin *sagA* genes, which are specific for *S. anginosus*, remains unclear; two possibilities have been suggested: gene duplication in a one-*sagA* gene operon with subsequent (relatively rare) mutations in *sagA2* or, alternatively, previous loss of *sagA2* from a *sag* operon originally carrying two *sagA* genes (223).

Four classes of streptodornases/DNases (A to D) have been described to be produced by the genus *Streptococcus* (224–226). Among them, DNase B appears to be the most common and best characterized. These enzymes, often occurring in multiple forms in the same strains, exhibit different pH ranges for optimal activity and most frequently are phage-encoded. The streptodornase gene *sdc* first cloned from *S. dysgalactiae* subsp. *equisimilis* strain H46A (227) encodes an enzyme exhibiting only 48% overall sequence identity to the *sdaD* gene later isolated from a GAS strain (228). However, sequence identity is much higher (>80%) in the two conserved domains shared by GAS and GCS strains (225).

Although suggestive evidence for the involvement of these enzymes in pathogenetic processes has existed for a long time, conclusive evidence produced more recently in three animal models of infection implicated them in GAS disease progression by enhancing evasion of the innate immune response (229).

CAMP Factor

CAMP (not to be confused with cAMP) refers to a pore-forming cytolysin originally found in *S. agalactiae* (GBS) that acts synergistically with the β-toxin (sphingomyelinase C) of *Staphylococcus aureus* generating a cohemolytic reaction on sheep and bovine erythrocytes previously hydrolyzed by sphingomyelinase (230, 231). In clinical tests, it was long used to differentiate GBS from GAS. However, genome sequencing of GAS strain SF370 revealed a functional secretory CAMP protein (Cfa) exhibiting 59.9% amino acid identity to the equally sized GBS protein (232). Previously, CAMP factor genes were also found in *S. uberis* and *S. canis* with residue identities to the GBS protein amounting to 63% and 67%, respectively (233). Among GCS/GGS, current knowledge restricts the elaboration of the CAMP factor to the above two species, with unclear roles of the protein in pathogenesis. However, after intravenous injection in rabbits, partially purified CAMP protein from GBS (Cfb) was lethal, and intraperitoneal injection together with a sublethal dose of GBS cells led to septicemia and death in mice. In addition, Cfb is capable of binding the Fc sites of immunoglobulins (231). However, compared to the wild type in *in vitro* and *in vivo* assays, deletion of the *cfb* gene in GBS did not cause any changes of the virulence properties of the mutant (234). Possibly resolving this conflicting situation, it has been shown that the expression of β-hemolysin (CylE) and Cfb is intricately coregulated by CovR and the serine/threonine kinase Stk1 in a CovR-dependent manner: CovR represses *cylE* but activates *cfb*; Stk1 alleviates CovR repression of *cylE* as well as activation of *cfb* by phosphorylation of CovR; thus, Stk1 positively regulates *cylE* and negatively regulates *cfb*, and collectively, CovR and Stk1 may fine-tune gene expression in GBS in a beneficial manner for niche adaptation (235). Regarding GCS/GGS, it seems appropriate to point out that both CovR and Stk1 are also elaborated by *S. dysgalactiae* subsp. *equisimilis* and *S. uberis*, making it worthwhile to explore their comparative and functional relationships in these species as well.

Pyrogenic Exotoxins

Pyrogenic exotoxins (Spe) are not only potent superantigenic host T cell mitogens but have also been shown to play a role in the manifestation of acute streptococcal infections (236). GAS are known to produce at least 11 types of superantigens. Although the first reports of such infections involving non-GAS isolates appeared in the 1990s, it was not until the early 2000s that *speA*, *speC*, and *speG* as characterized before in GAS were also detected in human GCS and GGS (237, 238). Similarly, *S. equi* subsp. *equi* strains were found that harbored *speH*, *speI*, *speL*, and *speM* genes (13, 239). While *speH* and *speI* were consistently seen in all isolates, they were absent from *S. equi* subsp. *zooepidemicus* (13). Moreover, these two genes were linked, like their orthologs in GAS, to a bacteriophage genome, strongly suggesting that intergeneric phage-mediated transfer is responsible for their spread. However, more recently, three novel superantigen genes (*szeF*, *szeN*, and *szeP*) were identified in *S. equi* subsp. *zooepidemicus* (240), with their encoded proteins sharing 59%, 49%, and 34% sequence identity with SpeH, SpeM, and SpeL, respectively, previously seen in *S. equi* subsp. *equi* (13, 239). Among numerous *S. equi* subsp. *zooepidemicus* strains screened, about half (7 out of 15) associated with human diseases produced at least one of the novel antigens in question, with *szeN* and *szeP* being located next to a prophage remnant. Overall, these data strongly suggest that veterinary as well as human diseases caused by GCS and GGS

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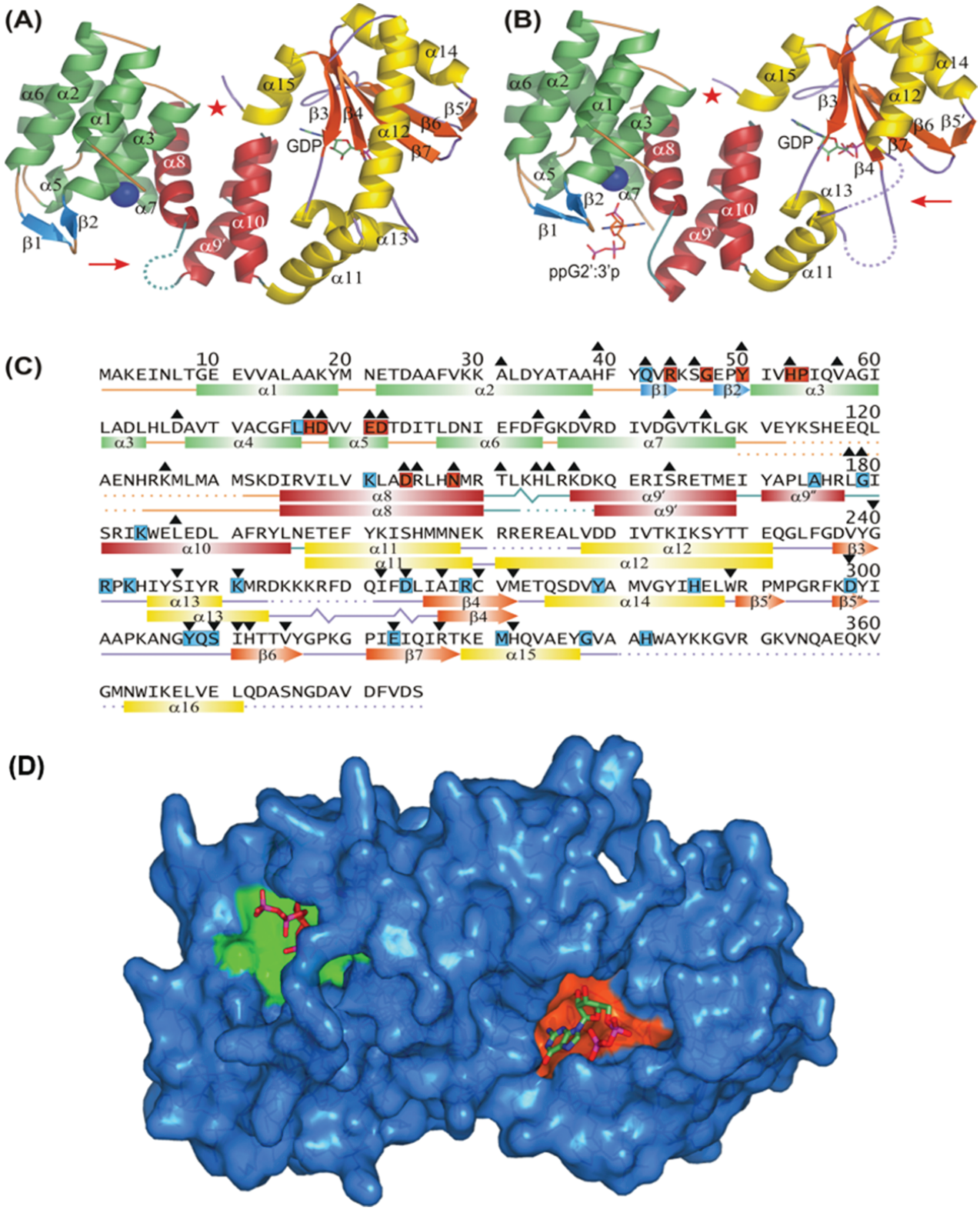


FIGURE 6 Structure of Rel_{Seq}1-385. (A) ppGpp Hydrolase-OFF/synthetase-ON conformation in complex with Mn^{++} (blue sphere) and GDP (stick rendering). The hydrolase domain is highlighted in green α-helices and blue β-strands, the synthetase domain is in yellow α-helices and orange β-strands, and the central 3-helix bundle is red. Part of the substrate binding cleft comprising the hydrolase site (downregulated) is disordered (red arrow). The small synthetase/hydrolase interdomain contact interface involved in signal transmission is labeled with a red star. (B) Hydrolase-ON/synthetase-OFF conformation in complex with Mn^{++}, ppG2′:3′p (which locks the enzyme in the hydrolase-ON/synthetase-OFF conformation) and GDP. The coloring and rendering schemes are the same as

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are afflicted by both intra- and intergeneric exchange of superantigen genes (240).

Although it is not known to represent superantigens, attention may be directed under the above heading to *S. parasanguinis*, which produces members of the LXG polymorphic toxin family, the C-terminal domain of which functions as a lipid phosphatase that degrades lipid II essential for cell wall biosynthesis in this Firmicute species. In a polymicrobial environment, this antibacterial toxin mediates cell contact-dependent interspecies antagonism, thus shaping the composition of such bacterial communities (241).

THE STRINGENT AND RELAXED RESPONSES OF *S. DYSGALACTIAE* SUBSP. *EQUISIMILIS*

Like their closest phylogenetic relatives, GAS, GCS, and GGS are polyauxotrophic, and shortage of an amino acid instantly limits the availability of the corresponding aminoacyl-tRNA. Thus, these organisms have evolved a stimulus response network that involves peptidases, proteinases, and peptide and amino acid transport systems for generating the free amino acids necessary for growth and a dynamic response to the protein-rich environment of their hosts (242).

Sequence analysis of *S. dysgalactiae* subsp. *equisimilis* chromosomal DNA 3′-adjacent to the *skc* gene (183) led to the unexpected discovery of the 739-codon *rel* gene (Fig. 3), the first homolog from a Gram-positive organism of the paralogous *E. coli relA* and *spoT* genes to be cloned, sequenced, and functionally characterized in some detail (243–245). In *E. coli*, these genes are involved in the metabolism of guanosine 5′,3′-polyphosphates [(p)ppGpp] which accumulate in response to starvation for amino acids or glucose exhaustion (246, 247). The accumulated (p)ppGpp mediates a broad pleiotropic response marked by a rapid shutdown of stable RNA (rRNA and tRNA) synthesis (the stringent response) and, concomitantly, by affecting the synthesis of about half of the cellular proteins. The crystal structures of *E. coli* RNA polymerase holoenzyme with (p)ppGpp revealed that both nucleotides bind to a site at the interface between the β′ and ω subunits of RNA polymerase holoenzyme (248).

As revealed by *in vivo* complementation experiments with appropriate *E. coli* mutants and verified by *in vitro* enzyme activity assays, the *rel* gene of *S. dysgalactiae* subsp. *equisimilis*, designated rel_{Seq} here, encodes strong (p)ppGpp 3′-pyrophosphohydrolase activity [(p)ppGppase] and weaker ribosome-independent (p)ppGpp synthetic activity resulting from its ATP:GTP 3′-pyrophosphoryltransferase capacity (243). Homologs of Rel_{Seq} are present in all known GCS and GGS phylogenetic groups, with sequence identities ranging from 91 to 98% in the Pyogenic group and amounting to about 78% in the Anginosus and Mitis groups. *In vivo*, the net effect of the opposing Rel_{Seq} activities favors (p)ppGpp degradation, resulting in the failure of rel_{Seq} to complement the absence of *relA* and, hence, resulting in its functional likening to *spoT*. Structural support for this inference provided the detection of immunological cross-reactivity of Rel_{Seq} with polyclonal antibodies to SpoT but not RelA and, vice versa, of SpoT but not RelA with antibodies to Rel_{Seq}. However, despite these clear observations, disruption of the chromosomal rel_{Seq} gene at codon 216 by insertion mutagenesis abolished the (p)ppGpp accumulation response following amino acid starvation of wild-type *S. dysgalactiae* subsp. *equisimilis*, a phenotype characteristic of *E. coli relA* but not *spoT* mutants. Further functional characterization of rel_{Seq} showed that amino acid deprivation of *S. dysgalactiae* subsp. *equisimilis* triggers a rel_{Seq}-dependent stringent response characterized by rapid (p)ppGpp accumulation at the expense of GTP and abrupt cessation of net RNA accumulation in the wild type but not in the insertion mutant, which displays a relaxed response (244). Thus, one of the most interesting results of this work is the finding that the Rel_{Seq} protein is bifunctional and shows activities that reside separately in the paralogous SpoT and RelA proteins from *E. coli*. This establishes an important variation of the *E. coli* paradigm for regulating (p)ppGpp levels and presumably the expression of genes controlled by the second-messenger properties of (p)ppGpp. In fact, Rel_{Seq} has proved to be of seminal importance to the subsequent establishment of the RelA/SpoT homolog (Rsh) superfamily of bifunctional [(p)ppGpp] synthetases and hydrolases and their distribution and function across all living organisms (249).

Cloning and expression of defined rel_{Seq} fragments followed by catalytic activity assays of the purified protein fragments revealed that the two enzymatic activities reside in the N-terminal half-protein (residues 1 to 385) and that the removal of the C-terminal half-protein has reciprocal regulatory effects on the opposing activities of the N-terminal domain (245). Further dissection of the bifunctional N-terminal domain by a nested deletion analysis revealed nonoverlapping minimal fragments of residues 1 to 198 and 211 to 347 for full hydrolytic and synthetic activities, respectively (250). The 2.1-Å crystal structure of Rel_{Seq}1-385, the first X-ray structure to be reported for an enzyme involved in the metabolism of (p)ppGpp (251), fully explains the solution biochemical studies in trapping the enzyme in two distinct conformations that typify the opposing (p)ppGpp hydrolase-OFF/synthetase-ON and hydrolase-ON/synthetase-OFF states (Fig. 6A to C). The individual active sites are located >30 Å apart (Fig. 6D) and, being mutually exclusive, demonstrate how reciprocal regulation of the two opposing activities can prevent futile cycling in the metabolism of (p)ppGpp. The conclusions derived from the crystal structure are fully supported by extensive point mutagenesis (Fig. 6C) and enzyme inhibition experiments (251). Concomitant with prevention of wasteful macromolecular synthesis by a rapid shutdown

for panel A. The disordered synthetase site (downregulated) is illustrated as dashed lines with a red arrow. (C) Primary and secondary structure of Rel_{Seq}1-385. The secondary structure is color-coded as in panels A and B. Unique secondary structure assignments for panel A are placed immediately below the corresponding assignments for panel B. Residues absolutely conserved throughout the mono- and bifunctional RelA and SpoT homologs are underlaid with blue boxes. Residues conserved in SpoT and the bifunctional enzymes but mutated in the hydrolase-incompetent RelA homologs are underlaid with red boxes. Upright and inverted black triangles above the sequence indicate residues which when substituted experimentally by missense mutations lead to defective hydrolase and synthetase activities, respectively. Reproduced from reference 251, with permission. (D) Semitransparent surface rendering of the bifunctional N-terminal half of Rel_{Seq}1-385 based on its 2.1-Å X-ray structure (251). The hydrolysis site (underlaid in green) binding the ppGpp analog ppG2′-3′p (stick rendering) is separated by the central 3-helix bundle and about 30 Å away from the synthesis site (underlaid in red) binding GDP (stick rendering). The image was generated by Tanis Hogg with the molecular graphics program PyMOL (Warren L. DeLano Scientific, Palo Alto, CA) and kindly provided for publication.

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of futile RNA synthesis following the failure of aminoacyl-tRNA pools to keep up with the demands of protein synthesis, the functional significance of the streptococcal stringent factor consists of strongly supporting cell survival under nutritional stress conditions. After overnight incubation in a chemically defined medium deprived of isoleucine plus valine, the survival fractions of mutants that failed to elicit stringency were 3 orders of magnitude lower than those of the wild type ($N/N_0 \approx 10^{-5}$ versus $\approx 10^{-2}$) (252). The short half-lives of 20 and 60 seconds for pppGpp and ppGpp, respectively, ensure quick recovery from the stasis-like survival state when the nutritional conditions improve (244). Expanding the knowledge of the (p)ppGpp metabolism in the genus *Streptococcus*, the *rel* gene from GAS was included in genetic studies showing that, apart from its primary function of stable RNA synthesis inhibition, (p)ppGpp also negatively affects the transcription of important groups of genes encoding regulators, virulence factors, metabolic enzyme genes, and genes for transport systems (253). Of pivotal interest is the strong negative stringent control of the streptococcal gene, collectively referred to as rsh_{Str}, itself: exposure to isoleucine plus valine starvation results in a 40-fold increase of truncated rsh_{Str} expression in an isogenic rsh_{Str} insertion mutant strain relative to the rsh_{Str} wild type. Such a high degree of negative autoregulation may be indicative of a gene with broad connectivity to sustain cellular homeostasis.

Of further interest and also implicating GCS and GGS are transcriptional analyses of wild type, rsh_{Str}, and *codY* mutant strains exposed to branched-chain amino acid starvation. These experiments showed that in addition to the stringent response, streptococci are capable of mounting an rsh_{Str}-independent response to amino acid deprivation that involves transcriptional modulation of a wide array of housekeeping genes as well as accessory and dedicated virulence genes. For example, among the genes responding with elevated transcript levels to starvation conditions are those encoding aminoacyl-tRNA synthetases, oligopeptide permease, two-component regulators (*cov* and *fas*), and members of the streptolysin S operon. Based on these observations, a regulatory network with feedback mechanisms has been proposed that counteracts the stringent response by the action of the branched-chain amino acid-activated pleiotropic transcriptional repressor CodY. This network links the levels of key rate-limiting enzymes to virulence gene expression and enables streptococci in a dynamic way to take advantage of protein-rich host environments (252, 254).

CONCLUSIONS AND PROSPECTS

It is apparent from the preceding account that molecular biological investigations into GCS and GGS during the past decade have proceeded to generate new information about the lifestyles of these organisms, whether presenting themselves as commensals or opportunistic pathogens causing readily controllable as well as life-threatening invasive infections (255). Molecular taxonomic approaches have solved classification problems, and phylogenomic studies revealed the relationships of GCS and GGS to the phylogenetic species groups of the genus *Streptococcus*. Complete genomic sequences are now available for all species in the Pyogenic group, including *S. phocae* (35), and for most species in the Anginosus and Mitis groups. Through faster, more practical, and less unwieldy techniques for comparative genome characterization in this field, the genomes of GCS and GGS will more comprehensively be shown to be in constant flux, largely determined by intraspecific and interspecific lateral gene transfer.

Concerning virulence gene expression, small noncoding RNAs with regulatory functions at the posttranscriptional level in response to environmental signals have also been detected in GCS and GGS, including *S. dysgalactiae* subsp. *equisimilis*, *S. uberis*, and *S. equi* (256), with FasX involved in SK regulation belonging to those studied best (193) (see also Fig. 4). In GAS, the number of small RNAs considered

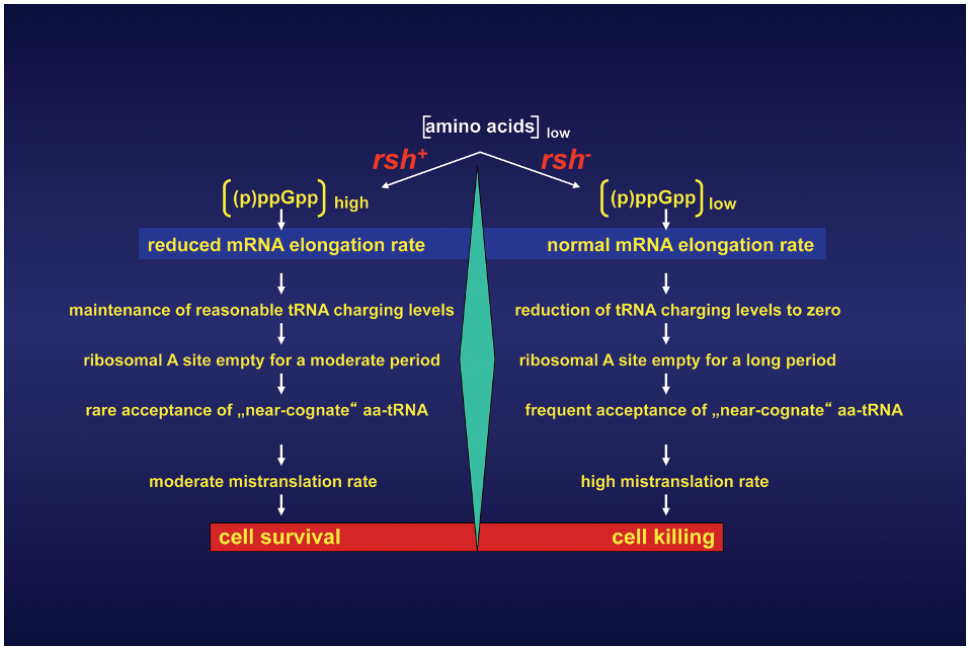


FIGURE 7 Hypothetical metabolic mechanisms explaining differential bacterial survival rates under stringent and relaxed conditions based on data reviewed in reference 246 and references therein.

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candidates for this important gene-regulatory mechanism has been found by a genome-wide analysis to amount to about 75 (257).

On the level of translated proteins with potential pathogenetic significance, this account addresses six cell-surface-associated proteins, three membrane-associated proteins, six extracellular proteins/protein groups, and one intracellular protein. How future basic research and translational medicine might proceed on the basis of current knowledge has been suggested above at the corresponding positions whenever possible. The following considerations single out, from a perspective point of view, the stringent response with its potential for supporting bacterial cell survival under nutritional stress conditions (252, 254, 258). Figure 7 attempts to present a hypothetical mechanism for this potential which covers both the areas of pathogen persistence and treatment approaches. Regarding the former, the stasis-like carrier state requiring survival functions may characterize cells that not only adhere to host cells but also succeed in invading them. However, (p)ppGpp accumulation in these conditions still awaits experimental support, at least as far as the genus *Streptococcus* is concerned (259). Regarding the latter, the now available structural knowledge of the streptococcal stringent factor (251) makes this enzyme a viable target for the design of antibacterial agents. In fact, replacing the 5′- and 3′-pyrophosphate moieties (p-O-p) in ppGpp with nonhydrolyzable bisphosphonate groups (p-C-p) which inhibit the stringent response by competing with GDP/GTP-Rsh binding inhibits ppGpp accumulation with high activity at small concentrations (260). Furthermore, a 2′-desoxyguanosine analog of ppGpp named relacin, in which the 5′- and 3′-pyrophosphate moieties are replaced by glycyl-glycine dipeptides linked to the ring sugar by a carbamate bridge inhibits the stringent response *in vitro* and *in vivo*, thus greatly decreasing cell viability (261). In Gram-positive bacteria, relacin strongly interferes with sporulation and inhibits biofilm formation. Given the ubiquitous occurrence of Rsh proteins in the bacterial kingdom and the absence of homologs in Mammalia, relacin might be a viable candidate for therapeutic application. A lingering issue in this context is whether or not the extracellular and intracellular asymptomatic carrier states feature the stringent response as a prerequisite for relacin to work. Alongside knowledge regarding the structure of Rsh_{Str}, the high-resolution structure of the entire 70S ribosome/RelA/deacyl-tRNA complex from *E. coli* revealed interesting conformational rearrangements of RelA during targeting of the 30S-ribosomal deacetyl-tRNA site. The resolved dynamics at near-atomic resolution may lead to further progress in translational medicine by therapeutic targeting of the stringent response (262).

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Pathogenicity Factors in Group C and G Streptococci

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16

INTRODUCTION

The pyogenic streptococci of Lancefield groups C and G were initially recognized as a cause of animal infections long before they were even considered as agents of human disease, even though they are widely distributed in animals and humans. They comprise a heterogeneous complex of streptococcal species that act as causative agents of a spectrum of diseases ranging from mild pharyngitis to skin infection to life-threatening systemic infections associated with high mortality rates. In this article we provide an overview of the various group C and group G streptococcal species, the diseases they cause, and the major pathogenicity factors that contribute to their virulence (Table 1).

TAXONOMY AND IDENTIFICATION

Taxonomic classification of group C streptococci (GCS) and group G streptococci (GGS) has always proven to be a complex issue. However, extensive taxonomic studies over the past few years have distinguished most of the veterinary pathogens belonging to Lancefield groups C and G from the human pathogens. Previously, GCS and GGS were divided into the following species: *Streptococcus dysgalactiae* subsp. *equisimilis* (only GCS, human pathogen), *Streptococcus dysgalactiae* subsp. *dysgalactiae* (GCS, animal pathogen), *Streptococcus equi* (animal GCS), *Streptococcus zooepidemicus* (animal and human GCS), *Streptococcus canis* (animal GGS), the *Streptococcus anginosus* group, and *Streptococcus phocae* (1). GCS and GGS of human origin are now considered to constitute a single subspecies, *S. dysgalactiae* subsp. *equisimilis*. Therefore, the current taxonomy characterizes the species as follows: *S. dysgalactiae* is divided into the subspecies *S. dysgalactiae* subsp. *equisimilis* and *S. dysgalactiae* subsp. *dysgalactiae* (hereafter referred to in this article as *S. equisimilis* and *S. dysgalactiae*). *S. equi* is divided into the subspecies *S. equi* subsp. *equi*, *S. equi* subsp. *zooepidemicus*, and *S. equi* subsp. *ruminatorum* (hereafter referred to as *S. equi*, *S. zooepidemicus*, *and S. ruminatorum*). However, it has been suggested that *S. equi* may simply be regarded as a subclone of *S. zooepidemicus* (2). On the basis of genetic evidence, *S. dysgalactiae*, *S. equi*, and *S. canis* are more closely related to each other than to the *S. anginosus* group and constitute species with the "large-colony" colony phenotype. *S. phocae* is a new species expressing the group C antigen thus far only isolated from seals (3, 4). Some of these species may also contain strains which express the Lancefield group A or group F antigens (Table 2).

HUMAN DISEASE AND DIAGNOSIS

GCS and GGS can cause a wide range of diseases from mild to severe, among both human and animal populations. GCS and GGS generally colonize the human respiratory, gastrointestinal, and genitourinary tracts, with an estimated <4% asymptomatic pharyngeal carriage rate in adults (5–7). Human invasive infections caused by GCS and GGS have been associated with underlying conditions, such as diabetes, cardiovascular diseases, and chronic skin conditions (8–10). While *S. dysgalactiae* is considered an animal pathogen, *S. equisimilis* is almost exclusively a human pathogen, with increasing prevalence and overlaps in clinical manifestations with group A *Streptococcus* (GAS). *S. equisimilis* infections include acute pharyngitis, pneumonia, endocarditis, cellulitis, peritonitis, septic arthritis, bacteremia, and toxic shock syndrome (11–23). Like GAS, *S. equisimilis* has also been linked to the poststreptococcal sequelae rheumatic heart disease, and in high endemic areas of rheumatic fever, carriage rates of GCS/GGS have been found to be higher than those of GAS (24, 25).

Infections with *S. zooepidemicus* and *S. canis*, normally considered zoonotic species, have also been reported in humans (26–29), including severe infective endocarditis (30).

Generally, person to person transmission of GCS/GGS occurs via respiratory droplets or skin contacts, but other zoonotic vehicles of transmission such as unpasteurized milk products are also possible (26, 27, 31).

S. dysgalactiae predominantly resides in domestic animals such as cattle, sheep, cats, and dogs that are either healthy carriers of the bacterium or go on to develop diseases such as pneumonia, arthritis, septicemia, and abscesses, particularly bovine mastitis. *S. equi*, a pathogen primarily restricted to horses and donkeys, causes strangles, which is a highly contagious disease characterized by purulent discharges from the respiratory tract and the development of abscesses (32). This organism is not considered to be part of the normal flora, because of its close association with disease. In contrast, *S. zooepidemicus* is an opportunistic commensal that colonizes mucosal surfaces and causes rhinopharyngitis, pneumonia (33), endometritis, neonatal septicemia, and wound infections in horses (34) as well as disease in other domestic

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TABLE 1 Pathogenicity factors of group C and G streptococci

Pathogenenicity factors	Organism	Reference(s)
Fibronectin binding proteins		
FnbA	*S. dysgalactiae*	59
FnbB	*S. dysgalactiae*	60
GfbA	*S. dysgalactiae*	64
FNZ/FNE	*S. zooepidemicus*, *S. equi*	61, 65
FNZ2/FNEB	*S. zooepidemicus*, *S. equi*	66, 69
SFS	*S. zooepidemicus*, *S. equi*	70
M-like proteins	*S. dysgalactiae* *S. equisimilis*	91, 105
FOG	*S. dysgalactiae*	93, 94
DemA	*S. dysgalactiae*	105
SzM/SeM	*S. zooepidemicus*, *S. equi*	76, 95–97, 101
SzPSe/SzP	*S. zooepidemicus*, *S. equi*	102
Se18.9	*S. equi*	76, 100
ScM	*S. canis*	104
Others		
C5a peptidase	*S. dysgalactiae*	108
SeCEP/SzoCEP	*S. zooepidemicus*, *S. equi*	112
Immunoglobulin binding proteins		
Protein G	*S. dysgalactiae*	116
MIG	*S. dysgalactiae*	120
MAG	*S. dysgalactiae*	122
ZAG	*S. zooepidemicus*	123
IgG-endopeptidases		
IdeE/IdeE2	*S. equi*, *S. zooepidemicus*	129
IdeE2/IdeZ2	*S. equi*, *S. zooepidemicus*	130
IdeP	*S. phocae*	133
Toxins		
Streptokinase	*S. dysgalactiae*, *S. equisimilis*	134
Streptolysin O	*S. dysgalactiae*, *S. equisimilis*	145
Streptolysin S	*S. dysgalactiae*, *S. equisimilis* *S. equi*, *S. zooepidemicus*	152, 154
Superantigens		
$SpeG^{dys}$/Spegg/SpeG	*S. equisimilis*, *S. canis*	158, 159
SpeH	*S. equi*	167
SpeI	*S. equi*	167
SpeK	*S. equi*, *S. zooepidemicus*, *S. equismilis*	158, 168, 169
SpeL	*S. equi*, *S. zooepidemicus*, *S. equismilis*	158, 168, 169
SpeM	*S. equisimilis*	173
ssa	*S. equisimilis*	173
SDM/SpeM	*S. dysgalactiae*	171
SpeA	*S. equismilis*	170
SpeC	*S. equismilis*	170
SzeN	*S. zooepidemicus*	170
SzeP	*S. zooepidemicus*	170
SzeF	*S. zooepidemicus*	170

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TABLE 2 Species of Lancefield group C and G streptococci[a]

Species	Known sources	Lancefield group
S. dysgalactiae* subsp. *dysgalactiae	Animals	C
S. dygalactiae* subsp. *equisimilis	Humans, animals (rare)	C, G
S. equi* subsp. *equi	Animals	C
S. canis	Animals, humans (rare)	G
S. equi* subsp. *zooepidemicus	Animals, humans	C
S. phocae	Animals (seal)	C
***S. anginosus* group**	Humans	A, C, F, G

[a]From reference 1.

animals such as cattle, sheep, pigs, and chickens. Although uncommon, *S. zooepidemicus* is also responsible for a range of zoonotic infections in humans (26, 27).

The small-colony phenotype of group C and G streptococci is expressed by the *S. anginosus* group, formerly known as *Streptococcus milleri*. The group contains three species, *S. anginosus*, *Streptococcus constellatus*, and *Streptococcus intermedius* (1), and forms a distinct subclade by phylogenomic analysis (35).

Strains belonging to the *S. anginosus* group express group antigens F, C, A, and G or no antigen. These strains are also likely to be non-beta-hemolytic. Members of these species are recognized as common commensal organisms of the human oral cavity, gastrointestinal tract, and genitourinary tract. They are also associated with abscess formation in the mouth and other body sites (36, 37) as well as pharyngitis (38) and endocarditis (39, 40). It has also been reported that *S. anginosus*, along with *Streptococcus mitis* and *Treponema denticola*, can be isolated from esophageal cancer tissue and by initiating inflammation in the cancerous tissue has a role in the development or progression of these cancers (41, 42).

Several assays are available to detect, classify, and genetically describe GCS and GGS. Routine microbiological diagnosis follows the same guidelines that are in use for the identification of the other beta-hemolytic streptococci (43). The beta-hemolytic streptococci isolates are divided into large and small colonies forming groups based on the growth on sheep blood agar: the large-colony-forming group is "pyogenic." The small-colony-forming species make up the anginosus group and are not referred to as GCS or GGS, even though they may cross-react with C or G sera. Lancefield agglutination tests are still used to group beta-hemolytic streptococci into the Lancefield groups. Fermentation tests are used to classify GCS as *S. equisimilis* or *S. zooepidemicus* and are based on a spectrum of biochemical tests which are now available commercially (44). More recently, matrix-assisted laser desorption ionization–time of flight mass spectrometry has been described as a rapid alternative for the identification of streptococci (45). In addition, multilocus sequence analysis of seven housekeeping genes (46) and other sequence-based assays are performed to analyze the streptococcal phylogenies. In particular, sequencing of the *emm* gene, which encodes the hyper-variable surface protein M, and 16s rRNA genes is undertaken for phylogenetic analysis of these streptococci based on sequence similarities (47, 48).

Next-generation sequencing is increasingly being used for genetic characterization, phylogenetic analyses, virulence and pathogenicity profiles, and antibiotic resistance analyses (22, 49). *S. equisimilis* and *Streptococcus pyogenes* (or GAS) share a high genetic similarity (~72%) and similar virulence genes, suggesting a common evolutionary origin and genetic exchange (22, 25, 50).

MECHANISMS FOR ADHERENCE

S. equisimilis causes a spectrum of disease in humans similar to that caused by *S. pyogenes*, and molecular studies have demonstrated virulence determinants that are almost identical. Putative virulence determinants of *S. equisimilis* include adhesins, toxins, and factors that are essential for dissemination in human tissues and for interference with the host immune responses.

Adhesion of microorganisms to host tissues represents a critical phase in the development of infection. It is therefore unsurprising that microorganisms have evolved dedicated mechanisms for attachment and adherence to extracellular matrix components of the host (51, 52). Of these components, the high-molecular-weight glycoprotein fibronectin appears to be the major attachment target of Gram-positive cocci, including GGS and GCS. Fibronectin itself is responsible for substrate adhesion of eukaryotic cells via specific cell surface factors of the integrin family. It also specifically interacts with other matrix components, such as collagen, fibrin, and sulfated glycosaminoglycans, demonstrating that this molecule fulfills multifunctional roles within the extracellular network (53). Being present in the extracellular matrix of most tissues, as well as in plasma and other body fluids in its soluble form, fibronectin represents an exquisite target for bacteria to exploit the cell attachment properties of this molecule by linking the pathogen to specific target cells. Epithelial cells of the human upper respiratory tract are bathed in secretions containing fibronectin in its soluble form. Once bound to the bacterial surface, it enables the pathogen to attach and subsequently colonize the primary site of infection.

Fibronectin binding proteins (FBPs) were first identified in *Staphylococcus aureus* and *S. pyogenes* (54), and approximately 11 have been defined in *S. pyogenes*, highlighting their importance for this pathogen (55). Binding by these bacteria to eukaryotic cells via fibronectin is also an important preliminary event prior to the invasion of these cells (56–58). The FBPs from streptococci and staphylococci share a common architecture, with a putative signal sequence at the N terminus and a wall- and membrane-spanning region. The major fibronectin binding domains are located within the C-terminal part of the proteins and are composed of 3 to 5 repetitive units that consist of 35 to 37 amino acid residues and bind to the 29-kDa N-terminal fragment of fibronectin. Further binding studies of PrtF/SfbI from *S. pyogenes* and FnBPA in *S. aureus* also indicate the presence of a secondary fibronectin binding site upstream of the repeat regions (54).

The first FBPs to be identified in group C and G streptococci were FnbA and FnbB from *S. dysgalactiae* (59). FnbA can opacify serum and bind fibrinogen, like the serum opacity factor of *S. pyogenes* (60). Both FnbA and FnbB bind fibronectin through C-terminal repeat regions (51).

Three FBPs have been defined in *S. zooepidemicus*: FNZ, FNZ2, and SFS. Although the modular organization of FNZ, an *S. zooepidemicus* FBP, is similar to those from other species, homology at the amino acid level is weak (61). Fibronectin

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binding is mediated through a repeat region and a second upstream region containing the amino acid motif LAGESGET. This motif is also present in the secondary binding domain of SfbI (62), where it acts independently of the repeat region in binding to fibronectin (63). This domain is also present in GfbA, the homologue of SfbI in *S. dysgalactiae* (64). *fne* is the homologous gene to *fnz* in *S. equi* (65). However, FNE is unique in that a single nucleotide deletion in the *fne* gene has resulted in the truncation of the protein, so with the loss of the cell-wall binding motif, it is secreted into the surrounding environment. The truncated FNE also lacks the classic fibronectin binding repeats found in the C-terminal region of FNZ but is able to bind fibronectin through the amino-half domain, also found in FNZ. Consequently, fibronectin binding at the bacterial surface is much lower for *S. equi* than *S. zooepidemicus*, although both have an additional cell wall-anchored FBP: FNEB and FNZ2 (66). FNE also binds to collagen, and the protein affects the interstitial fluid pressure *in vivo* (67). The molecular structure of the interaction between FNE and fibronectin has been reported (68). FNZ2 also has both collagen and fibronectin binding properties (69). SFS of both *S. equi* and *S. zooepidemicus* strains contains a signal peptide but no cell wall binding motifs or traditional fibronectin binding motifs. SFS inhibits binding between fibronectin and collagen. If SFS were to bind fibronectin attached to the bacterial surface through FNEB/FNZ/FNZ2, this might inhibit binding of fibronectin to collagen, which could have several physiological consequences (70). The molecular interaction between SFS and fibronectin has been reported (71).

Some of the FBPs and collagen binding proteins identified in *S. pyogenes* are chromosomally located within the fibronectin and collagen binding proteins and T antigen encoding (FCT) locus. There are at least nine types of FCT regions in *S. pyogenes* that vary by gene content and order, but within this region are the genes required to make the pili. The pilus has been shown to contribute to the formation of biofilm and mediate adherence to host cells (72–75). Whole-genome sequence analysis of group C and G streptococci has also identified potential FCT regions. One putative pilin locus, FimI, exists within the genome of *S. equi* strain 4047 (76). Although no pili structures have been identified for *S. equi*, the proteins of FimI are expressed during growth and have the potential to affect adherence *in vivo* (77). The gene encoding the collagen binding protein CNE of *S. equi* (78) is present in this FimI locus. A homologous locus was also found in *S. zooepidemicus* isolates, as well as an additional one or two potential pili loci (76, 79). Two FCT regions were also found in *S. equisimilis*, which share high levels of genetic identity to FCT regions from different *S. pyogenes* genotypes, suggesting multiple horizontal gene transfer events (22, 80).

An alternative way for group C and G streptococci to adhere to host cells is via binding to other extracellular matrix molecules, including fibrinogen, vitronectin, laminin, collagen, and plasminogen (81, 82). The M-protein-like fibrinogen binding protein of G streptococci (FOG) can act as an adhesin by binding collagen IV (83) and has been shown to also bind collagen I fibrils in dermis *in vivo* (84).

Vitronectin is a multifunctional serum protein that affects the humoral immune system by binding to and inhibiting the complement membrane attack complex (85) and is also a major matrix-associated adhesive glycoprotein that regulates blood coagulation. The ability of group C and G streptococci specifically to interact with vitronectin (86) and mediate the adherence to both epithelial and endothelial cells (87, 88) was demonstrated some time ago. However, a specific vitronectin binding protein has never been identified in streptococci.

ANTIPHAGOCYTIC FACTORS

A major requirement of pathogenic streptococci is to be able to resist phagocytosis. The streptococcal M protein, first identified and characterized in *S. pyogenes*, is the major antiphagocytic factor (89). It is a multidomain surface-exposed molecule that forms a coiled-coil secondary structure with significant irregularities that in the B-repeat region are essential for the fibrinogen binding properties (90). Binding of complement factors, fibrinogen, and inhibition of C3b deposition on the bacterial surface are mechanisms by which the M-protein can inhibit opsonization of the organism by the alternative complement pathway, thus evading the host's nonspecific immune defense mechanism.

Several M-like proteins have been identified in group C and G streptococci. Protein MG1, the first group G streptococcal M-like protein characterized on the molecular level (91), exhibits typical structural and biological features of M proteins, such as coiled-coil structure and the ability to generate type-specific opsonizing antibodies. Protein MG1 shares highly homologous sequences with the C-terminal repeat region of class I M proteins, which are frequently associated with rheumatic fever. M proteins of GGS are also responsible for conferring resistance to phagocytosis (92).

In human strains of GGS, FOG is critical for bacterial pathogenesis through its antiphagocytic action of binding fibrinogen (93). It also has the ability to bind IgG subclasses IgG1 and IgG2, although FOG-bound IgG1 can trigger the complement cascade through C1/C1q activation. It is unclear whether this is detrimental to the bacteria, because FOG remains protective against phagocytosis (94).

The antiphagocytic protein SeM (FgBP) appears to be the predominant M-like protein on the surface of *S. equi*, capable of binding fibrinogen and IgG4 and IgG7 subclasses (95–97). SeM confers resistance to phagocytosis, but this also requires the presence of the hyaluronic acid capsule (98). Like the M protein of *S. pyogenes*, SeM is variable at the N-terminal region, suggesting selective pressure (99). Interestingly, this does not appear to be the case for two other M-like proteins expressed by *S. equi*: Se18.9 and SzPSe (99). The antiphagocytic protein Se18.9 is commonly found in strains of *S. equi*, but rarely in strains of *S. zooepidemicus* (76), and acts by binding to fibrinogen and the complement regulatory protein, factor H (100). SzP proteins are also antiphagocytic and are expressed by both *S. equi* and *S. zooepidemicus*. Whole-genome sequence analysis of *S. zooepidemicus* strain H70 identified a potential M-protein homologue in SzM that resembles SeM in its secondary structure, which consists of a predicted C-terminal coiled-coil (76, 101). The N-terminal of SeM is unique to *S. equi* and binds fibrinogen (97, 102). SzM can also bind fibrinogen but cannot bind IgG (103). The M protein of *S. canis* specifically binds the Fc region of IgG in a nonopsonic manner (104).

DemA is an *S. dysgalactiae* protein with homology to FgBP identified by screening of a phagemid expression library (105). The mature DemA protein is 54 kDa in size, contains a signal sequence and a cell wall binding domain, and is predicted to have a coiled-coil secondary structure. DemA shows greatest homology to the FgBP at the C-terminal end in a region that does not participate in fibrinogen binding. The amino acid motif VSKDLADKL present with the repeat units of both DemA and FgBP suggests that the sequence may have an important biological function. DemA is able to bind

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IgG from various animal sources, reminiscent of type IIa Ig-binding proteins of *S. pyogenes* in a domain distinct from the fibrinogen binding domain. Nucleotide sequencing of the *dem*A locus identified an open reading frame upstream of *dem*A that is homologous to *mga*, a positive regulator of M-protein expression in *S. pyogenes* (105).

C5a peptidase of *S. pyogenes* (ScpA) is well known to specifically cleave and inactivate the chemoattractant C5a, but it has also recently been shown to cleave the complement factor C3 and the chemoattractant C3a (106), expanding its impact on immune evasion. ScpA has also been identified as an adhesion factor enabling bacteria to adhere to endothelial and epithelial cells (106). Homologues of ScpA have been identified in group B streptococci (ScpB) and group C and G streptococci isolated from humans but not from GGS from animals (107, 108). Both *S. equi* and *S. zooepidemicus* also carry homologous C5a peptidase genes (*scpE* and *scpZ*, respectively). However, the C5a peptidase gene in *S. equi* is reported to be a pseudogene (76). Unlike in *S. pyogenes*, where *scpA* is located in the *mga* regulon that includes the *emm*-gene, the *scp* genes of group B, C, and G streptococci are associated with a transposon, suggesting that lateral genes transfer between these species (107, 109).

The *S. pyogenes* cell wall envelope proteinase, SpyCEP, is another protease that contributes to the prevention of phagocytosis through the specific cleavage of interleukin-8 and other chemokines to prevent the activation and migration of neutrophils (110, 111). Similar enzymes have been identified in *S. equi* (SeCEP) and *S. zooepidemicus* (SzoCEP) which share 98% identity to each other and 59% identity to SpyCEP (108). SeCEP has been shown to cleave both human and equine IL-8, and vaccination with a recombinant portion of SpyCEP prevented bacterial dissemination in a murine model of *S. equi* invasive infection, suggesting an important contribution to disease (112).

IMMUNOGLOBULIN BINDING AND INACTIVATING PROTEINS

Streptococcal protein G is a surface molecule associated with the majority of group C and G streptococcal isolates of human origin. Protein G interactions with immunoglobulins and other host proteins have been the subject of detailed reviews (113–115). Protein G is defined as a type III IgG Fc receptor and interacts with a wide species range of immunoglobulins, as well as human serum albumin, kininogen, and α_2-macroglobulin. Protein G exhibits a modular structure in which the binding sites for IgG are located in the C-terminal repeat region (114–116). The central A/B-repeat region constitutes the binding domain for serum albumin, and the N-terminal E region is responsible for interacting with the native form of α_2-macroglobulin (82). In contrast to human pathogenic strains of GGS that exclusively bind to the native (slow) form of α_2-macroglobulin via protein G, animal-derived isolates of bovine and equine origin bind the proteinase-complexed (fast) form of the molecule. The B1 domain of protein G involved in immunoglobulin binding consists of an α-helix and four β-strand sheets. This domain has been used as a model structure in numerous biochemical studies examining protein folding, protein interaction, and synthetic protein design (117–119).

Two protein G-related proteins, MIG and MAG, from two mastitis-causing *S. dysgalactiae* strains (120) and MAG (121, 122) and ZAG (123) from *S. zooepidemicus* have been characterized on the molecular level. Like protein G, MAG and ZAG exhibit serum albumin and type III Fc receptor activity, whereas the protein MIG lacks albumin-binding activity. However, MAG is able to bind to immunoglobulins from a greater number of animal sources than protein G. MAG, ZAG, and MIG also bind to the fast form of α_2-macroglobulin. The α_2-macroglobulin binding region in MIG is not homologous to those of protein G, MAG, ZAG, and GRAB. In phagocytosis assays a MIG isogenic mutant strain of *S. dysgalactiae* was not as resistant to opsonization by bovine neutrophils as the parental strain (124). MIG has also been shown to bind to bovine immunoglobulin A (125) and can inhibit bacterial internalization into host cells (126).

As well as IgG binding proteins, group C and G streptococci can express variants of the *S. pyogenes* IdeS and EndoS, which are IgG-degrading enzymes. While IdeS cleaves the hinge region of IgG (127), EndoS removes core IgG glycans (128). Both *S. equi* and *S. zooepidemicus* express two forms of IgG-endopeptidases, IdeE/IdeE2 and IdeZ/IdeZ2, respectively, that are capable of cleaving IgG from several mammalian species (129, 130), although IdeE2/IdeZ2 cleave horse IgG with greater efficiency than IdeE/IdeZ (130). EndoS homologues have also been identified in *S. equi* (EndoSe) and *S. zooepidemicus* (EndoSz), and they share 86 to 89% sequence identity to each other and 70% identity to EndoS of *S. pyogenes* (131). EndoSd has also been found in *S. equisimilis* with IgG hydrolyzing activity (132), and IdeP was identified in the group C *S. phocae* subsp. *phocae* that affects marine animals, although its function has not been confirmed (133).

ENZYMES AND TOXINS

After colonization, adherence, and evasion of host immune responses, the dissemination of pathogenic streptococci in tissues is regarded to be an important step for the onset and development of an invasive disease. One of the factors involved in this process is streptokinase, a protein found in groups C, G, and A streptococci (134). The formation of a streptokinase/plasminogen complex results in the exposure of the plasminogen active site, which then catalyzes the conversion of other plasminogen molecules into plasmin (135). Plasmin is a key serine protease in the fibrinolytic system that is able to break down tissue barriers, thereby enabling the dissemination of streptococci. M proteins coordinately interact with the secreted streptokinase by binding either fibrinogen (136) or plasminogen (137). A high level of variation exists within the streptokinase gene, *ska*, and alleles of *ska* have been associated with tissue tropisms and differing levels of plasminogen activation (138). There is some overlap of alleles between human strains of group C and G streptococci and GAS (139). Streptokinases have been isolated from both human and animal group C and G isolates and have specificity for the plasminogens of their respective hosts (140). Thus, streptokinases from human group A, C, and G streptococci isolates have greater homology to each other than to animal isolates of group C and G streptococci. A study by Caballero et al. (58) found the amino acid homology between streptokinases from human and animal *S. equisimilis* isolates to be only 35%. Homology between the streptokinase from *S. equisimilis* of equine and porcine origins was only 21%. The ability of streptokinases to cleave plasminogen from specific species may therefore be a critical factor in determining the host range of individual streptococcal strains (141). Invasive human strains of *S. equisimilis* have been found to express higher levels of streptokinase, and this can drive virulence in a murine model of invasive necrotizing

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fasciitis, suggesting an important role for streptokinase in disease (142, 143).

Streptolysin O (SLO) is the prototype of a family of thiol-activated cytolysins produced by the genus *Streptococcus* and by other Gram-positive bacteria, including *Bacillus*, *Clostridium*, and *Listeria* species (144). The genes coding for SLO of group C and G streptococci (*S. dysgalactiae*) are almost identical to that of *S. pyogenes* (145). SLO homologues have not been described in *S. equi*. SLO is able to disrupt the cytoplasmic membrane of several eukaryotic cell types that include erythrocytes, leukocytes, macrophages, platelets, and epithelial cells. Separate from its pore-forming activity, SLO also acts to translocate NADase into host cells (146), which contributes to cytotoxicity by depleting energy stores. Other functions of these two streptococcal toxins include limiting neutrophil responsiveness and potentiating bacterial survival, replication, and persistence inside keratinocytes, epithelial cells, and macrophages, which may protect GAS from the immune response and antimicrobial therapy (147–151).

Streptolysin S (SLS) is another cytolysin secreted by groups A, C, and G streptococci, including the animal-pathogenic *S. equi* species (152). SLS belongs to a distinct group of hemolytic toxins that are characterized by their resistance to oxidation and sensitivity to trypan blue. SLS activity results in damage to membranes of various cell types as well as subcellular organelles (153). In contrast to the 57-kDa SLO, SLS is a small, 57-amino acid protein. The gene encoding SLS, *sag*A, is part of a locus containing nine open reading frames which contain significant homology with genes from bacteriocin loci (154, 155). In *S. equisimilis*, SLS expression is under the control of both the *covRS* and *fas*CAX two-component regulatory systems, which in *S. pyogenes* have been shown to be involved in the regulation of multiple virulence factors (156). In a mouse infection model, *S. equisimilis* expressing SLS proliferates and induces necrotic lesions at the site of infection, whereas SLS-negative strains do not, suggesting that SLS is an important factor in the development of necrotizing fasciitis (154).

Streptococcal pyogenic toxins (spes) are superantigen genes capable of cross-linking the major histocompatibility complex class II on antigen-presenting cells to the T cell receptor, leading to proliferation of T cells and substantial release of inflammatory cytokines. Eleven superantigen genes have been identified in *S. pyogenes*, and they are thought to drive the development of scarlet fever and toxic shock syndrome, the latter contributing to high mortality rates following necrotizing fasciitis. Recent work has also identified a role for superantigens in upper respiratory tract infection (157). Homologues of the GAS superantigens have been identified in group C and G streptococci (158). The most commonly found superantigen in *S. equisimilis* is *spe*G (*spegg* or *spe*G^{dys}) (159, 160), and genomic analysis indicates that *S. pyogenes* and *S. equisimilis spe*G genes are orthologues that are direct descendants from a common ancestor gene (161). The gene for *spe*G can also be found in *S. canis* (158). The role of *S. equisimilis spe*G in humans is unclear because the presence of this gene does not correlate with disease severity and it does not confer mitogenic activity toward human mononuclear cells, although it can stimulate bovine T cells (160, 162, 163). The streptococcal superantigens *spe*A, *spe*C, *speJ*, *speK*, *speH*, *speL*, *spe*M, and *ssa* are found infrequently in *S. equisimilis* and other human group C and G streptococci (160, 164–166).

Homologues of *S. pyogenes* superantigens *speH* and *speI* can be carried by strains of *S. equi* (167), and homologues of *speK* and *speL* can be carried by both *S. equi* and *S. zooepidemicus*, although because of confusion with the nomenclature, *speK* homologues were originally termed *speL*$_{se}$/*seeL*/*szeL*, and *speL* homologues were termed *speM*$_{se}$/*seem*/*sze*M (160, 167–169). Three additional superantigen genes, termed *szeN*, *szeP*, and *szeF*, have been identified in *S. zooepidemicus* only and are capable of stimulating equine peripheral blood mononuclear cells (170). *S. dysgalactiae*-derived mitogen shows homology to *spe*M of *S. pyogenes* and can stimulate human mononuclear cells (160, 171). The majority of the superantigens found in *S. pyogenes* and group C and G streptococci are associated with prophages which may drive the lateral transfer of these virulence factors between streptococcal species (76, 172, 173).

CONCLUSIONS

Many recent studies have highlighted the increasing number of systemic infections caused by *S. equisimilis*, particularly among immunocompromised individuals and specific populations, and this therefore suggests that this species will gain even more clinical importance in the future. High nucleotide similarities among virulence genes and their association with mobile genetic elements support the hypothesis of extensive horizontal gene transfer events between streptococcal species of the pyogenic group. A better understanding of the mechanisms of pathogenesis will hopefully be revealed by whole-genome sequencing, and this may result in more effective clinical strategies for the pyogenic group of streptococci in generally.

This chapter is dedicated to the memory of Gursharan Singh Chhatwal, an inspiring "streptococcologist" who leaves us with wonderful memories of Lancefield 2002 held in Goa, India, and many others.

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Infections Caused by Group C and G Streptococcus (*Streptococcus dysgalactiae* subsp. *equisimilis* and Others): Epidemiological and Clinical Aspects

GIO J. BARACCO

17

Conrary to the homogeneity typical of streptococci belonging to Lancefield groups A (*S. pyogenes*) and B (*S. agalactiae*), groups C and G streptococci (GCGS) represent a variety of species that are widely variable in regard to biochemical reactions, hemolytic characteristics, predilection for host species, and clinical illnesses produced in humans and animals. These organisms are found as commensals in the throat, skin, and occasionally the female genitourinary tract, and their epidemiologic patterns and clinical manifestations reflect this distribution.

HISTORY AND TAXONOMY

The classification of GCGS is complex and has been continuously evolving over the past few decades. Most human infections with GCGS are caused either by members of the *S. anginosus* group (SAG) (*S. anginosus*, *S. constellatus*, and *S. intermedius*, also formerly known as the *S. milleri* group), which form small colonies (<5 mm), can be alpha-, beta-, or nonhemolytic, and may also express Lancefield group F (most common) or group A (rare) antigens or no group antigen at all, or by *Streptococcus dysgalactiae* subspecies *equisimilis* (SDSE), which forms large colonies (>5 mm) that resemble those of *S. pyogenes* when cultivated on sheep blood agar plates and is typically beta-hemolytic. SDSE and SAG may express either group C or G antigens. Certain uncommon strains of SDSE may also express Lancefield group L or group A antigens (1–3). In addition, *S. dysgalactiae* subsp. *dysgalactiae* (SDSD), *S. equi* subsp. *equi*, and *S. equi* subsp. *zooepidemicus* express group C antigens, while *S. canis* strains are group G (4).

While well known to veterinarians as a cause of bovine mastitis, *S. dysgalactiae* (from the Greek for "bad milk") was dropped from the Approved List of Bacterial Names in 1980 (5). It is unclear whether this was an oversight or if there was a more concrete reason such as the absence of a reference strain, but in 1983 Garvie sought to correct this error and published an article that included a detailed microbiological description and a reference strain (6). In 1996 Vandamme proposed distinguishing two subspecies of *S. dysgalactiae* based on their host of origin, with animal isolates designated as *S. dysgalactiae* subsp. *dysgalactiae* and human isolates receiving the name *S. dysgalactiae* subspecies *equisimilis* (4). Vieira et al. agreed with the separation into two subspecies but suggested that the distinction be based on the type of hemolysis produced, designating nonhemolytic and alpha-hemolytic GCGS strains as SDSD and beta-hemolytic strains as SDSE (7). This is the currently accepted taxonomic classification, although it has continued to be challenged (8). In 2001 it was proposed that SDSD and SDSE be recognized as distinct species based on clinical and epidemiological grounds, but the motion was rejected by the International Committee on Systematic Bacteriology because taxonomic evidence did not support it (9). Multiple reports of exceptions to the Vieira subdivision have been published, and some authors have found it to be very inaccurate when assessed by DNA fingerprinting (10). It is possible that some of these discrepancies can be explained by continued evolution of the species through horizontal transfer of genes from other beta-hemolytic streptococci, especially *S. pyogenes*, to SDSE (10, 11). In the absence of widespread availability of genomic identification, many clinical laboratories continue to designate animal strains as SDSD and human strains as SDSE or continue to report these bacteria using the Lancefield classification.

In this article we will, to the extent possible, focus on the species-based nomenclature of streptococci. However, many of the studies reviewed, particularly those in the older literature, employed the Lancefield classification, making it impossible to know the causative species.

EPIDEMIOLOGY

The SAG members are normal commensals in the oral cavity. All three species, but especially *S. intermedius*, have been found in gingival crevices, dental plaque, dental root canals, and the naso- and oropharynx. *S. anginosus* isolates are also commonly isolated from urogenital and gastrointestinal sources (1, 12).

SDSE is a common commensal of humans (13). It is thought that the horizontal transfer of virulence factors such as fibronectin-binding protein, M protein, and bacteriocin-like inhibitory substance activity from human streptococci to animal strains of *S. dysgalactiae* enabled them to colonize humans (14). Some studies have suggested that pharyngeal colonization with SDSE may be protective against infection

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with other pathogens, since its presence has been found to have an inverse correlation with the incidence of pharyngitis (15). On the other hand, invasive infections caused by SDSE have been emerging in many parts of the world (16). SDSE is responsible for over 80% of invasive infections caused by beta-hemolytic streptococci of groups other than A and B (2). The incidence of bacteremia caused by SDSE has increased 2- to 3-fold and approaches that of *S. pyogenes* in many countries. In Finland, the incidence of group G streptococcal bacteremia increased from 1.8 cases/100,000 population in 1995 to 4.3 cases/100,000 population in 2004 (17). In Norway, the incidence of invasive GCGS infections increased from 1.4/100,000 inhabitants in 1999 to 6.3/100,000 in 2013 (18). The increased virulence of SDSE seems to be related to clonal expansion of a few clades carrying specific virulence factors, such as *cbp*, *fbp*, *spe*G, *sic*G, *gfb*A, and *bca* (19, 20). Most SDSE strains express a functional homolog of the potent *S. pyogenes* beta-hemolysin/cytolysin streptolysin S that contributes to the pathogenesis of necrotizing soft tissue infection (21). SDSE strains also express M proteins that share structural and functional properties with *S. pyogenes* M proteins (22, 23). Similar to invasive GAS infections, a high percentage of patients with invasive SDSE infections have comorbid illnesses, including diabetes mellitus, malignancies, immunosuppression, intravenous drug use, and breakdown of the skin (1, 16, 24).

Transmission of SDSE, when it occurs, is likely to be from person to person, and most cases are sporadic in nature, rather than associated with common source outbreaks. When outbreaks do occur, they are generally associated with close personal contact or perhaps with environmental contamination. By contrast, *S. equi* subsp. *zooepidemicus* is generally associated with exposure to animals or to common source outbreaks, especially consumption of contaminated dairy products.

ANIMAL INFECTIONS

SDSD is a major cause of mastitis in cows and dromedary camels and various infections in lambs (25–27). *S. equi* subsp. *equi* is occasionally found in the upper respiratory tract of normal horses and is the causative agent of equine strangles. This acute, contagious, and deadly respiratory disease has led to explosive epidemics in horse stables and has serious potential economic consequences for horse fanciers (28). *S. equi* subsp. *zooepidemicus* is a cause of infection in a variety of animal species, including horses, cows, dogs, rabbits, llamas, alpacas, guinea pigs, and swine (25, 26, 29–33).

HUMAN INFECTIONS

The SAG is usually classified, and described, with the "viridans" group of streptococci. These species are characterized for their disposition to form abscesses in various organs, including lung, pleural space, brain, oral and abdominal cavities, skin and soft tissues, and the genitourinary tract with or without associated bacteremia (34–36). Although less common than other viridans streptococci, *S. anginosus* may also cause endocarditis (12, 37, 38). Many invasive SAG strains, however, belong to serogroups other than C or G or are nongroupable (34, 39).

This review will focus on the more common infections caused by SDSE, as well as on the few reported human cases caused by *S. equi* subsp. *zooepidemicus*, *S. equi* subsp. *equi*, and *S. canis*. Strains of these streptococci have been associated with infections of many body sites (14, 26, 40–45).

Pharyngitis and Acute Glomerulonephritis

The role of SDSE in sporadic cases of pharyngitis remains somewhat controversial. Although occasionally isolated from patients with pharyngitis, streptococci of this species are also often cultured from the throats of healthy individuals (12). Marchello and Ebell conducted a meta-analysis of the prevalence of group C streptococci in patients with sore throat. They found a prevalence of 6.1%. However, the absence of a healthy control group did not allow the authors to conclude whether these bacteria were the actual pathogens (46). Several clinical and laboratory-based studies have looked at the clinical manifestations of patients with pharyngitis in whom SDSE was isolated. In general, signs and symptoms of patients with pharyngitis in whom SDSE is isolated are indistinguishable from those in whom *S. pyogenes* is found (47, 48). Turner et al. (49) studied students reporting to a college health service with acute pharyngitis and compared them with controls without infectious problems. Group C streptococci were cultured at a higher rate from students with pharyngitis than from the control group. Patients with positive cultures for group C streptococci were more likely to have features suggestive of a bacterial infection, such as exudative tonsillitis and anterior cervical lymphadenopathy, than were those with negative cultures. Furthermore, these group C strains resisted phagocytosis in human blood and contained genomic DNA encoding an M protein similar in structure to that of group A streptococci, providing further evidence of possible human virulence (50). In a later study, the same group of authors described 265 students with exudative pharyngitis and compared them with 75 patients with rhinovirus infection and 162 students with noninfectious problems. SDSE was isolated significantly more frequently from patients with exudative pharyngitis than from either control group (51). Twenty-two cases of pharyngitis from which group C streptococci were isolated occurred during the fall of 1974 in a school for boys with learning disabilities. Although it is likely in this epidemiologic setting that the infecting strains were SDSE, they were unfortunately not speciated (52). On the more severe side of the spectrum, SDSE has rarely been reported as the cause of severe and even necrotizing tonsillitis (53, 54).

Streptococci expressing group G antigen have clearly been linked to outbreaks of pharyngitis. Many of those outbreaks were related to a common source, usually a food product. In one such outbreak during a single week in 1968, 176 students at a college were evaluated for pharyngitis. The attack rate in the student body was 31%. Signs and symptoms were similar to those characteristic of group A streptococcal pharyngitis, suggesting an etiologic role for the organism. Epidemiologic investigation linked the outbreak to contaminated egg salad (55). In another common source outbreak, 72 people who attended a convention developed pharyngitis, with group G streptococci isolated from most who had cultures performed. All the patients had consumed chicken salad prepared by a single cook whose throat culture was positive for the organism (56). Another epidemic of group G streptococcal pharyngitis involving 68 students occurred over a 1-week period at a North Carolina college. Because no common food source could be identified, the author concluded that the mode of spread was most likely person to person. The very sharp epidemic curve and brief duration of the outbreak suggest, however, that contamination of a common food vehicle is more likely. In support of this conclusion are the facts that all students interviewed had eaten in the campus cafeteria in the week preceding illness and that one student with a positive culture was a food

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handler (57). A community-wide outbreak of group G streptococcal pharyngitis, unrelated to any common source, was documented among private pediatric patients in the winter and spring of 1986 to 1987 in Connecticut (58).

Epidemics and clusters of pharyngitis cases due to *S. equi* subsp. *zooepidemicus* are typically related to a common source, usually consumption of unpasteurized dairy products. A remarkable feature of such outbreaks is their association with poststreptococcal acute glomerulonephritis. From December 1997 to July 1998, 253 cases of acute glomerulonephritis due to *S. equi* subsp. *zooepidemicus* occurred in Nova Serrana, Brazil, among people who had consumed locally produced unpasteurized cheese. Ten patients required dialysis and three died (59). A follow-up report 2 years later of 134 of these patients showed that five of them required continuing dialysis. Of the 69 patients from that cohort who could be found and reevaluated, 42% had hypertension, and there was a high proportion of patients (up to 30%) with persistent renal function abnormalities (60). Duca et al. described 85 patients with pharyngitis due to *S. equi* subsp. *zooepidemicus* following the ingestion of improperly pasteurized milk, 87% of which were adults. Approximately one-third of the patients developed acute glomerulonephritis, generally in the second or third week of illness (61). In a smaller outbreak, five members of a family developed an upper respiratory infection related to *S. equi* subsp. *zooepidemicus* after consuming unpasteurized milk (62). Three of the five family members subsequently developed poststreptococcal glomerulonephritis, which was confirmed in one case by renal biopsy.

Infections of Skin and Soft Tissue

SDSE is a frequent cause of skin and soft tissue infections, and the skin is often the portal of entry for serious invasive disease and bacteremia. These infections can manifest as pyoderma, cellulitis, erysipelas, surgical wound infections, abscesses, necrotizing soft tissue infections, and pyomyositis (63–67). In some series, GCGS have been isolated with as much or greater frequency than *S. pyogenes* in patients with cellulitis and erysipelas (68, 69). Infection caused by these organisms may complicate ulcers associated with diabetes mellitus, immobility, or venous and lymphatic compromise of any cause (14). Recurrent cellulitis may occur, for example, in the limb that underwent saphenous venectomy of patients who have experienced coronary artery bypass grafting or in the extremities of individuals who have had axillary, pelvic, or femoral node dissection for cancer (70). Severe skin and soft tissue infections caused by SDSE include cases of necrotizing fasciitis, Fournier's gangrene, and necrotizing myositis (67, 71). Bruun et al. described a series of 70 cases of necrotizing fasciitis, 9 of which were caused by SDSE. The in-hospital case fatality rate of patients with SDSE was 33%, compared to 11% for those caused by *S. pyogenes* (67). These infections typically occur in patients who are older and have underlying comorbid conditions, such as malignancy, cardiovascular disease, alcoholism, and diabetes mellitus (44, 72). Injectable-drug users seem to be at increased risk for cellulitis and skin abscesses caused by SDSE, and the skin is the usual source of bacteremia in such patients (64). Burn patients are also at risk for skin and skin graft infections with SDSE; such individuals accounted for 8% of cutaneous group G streptococci infections in one series (63, 73).

There are numerous reports of streptococcal toxic shock syndrome (STSS) in patients with severe infections caused by SDSE (74–83). Sachse et al. studied 24 pathogenic isolates of SDSE and found a gene encoding the streptococcal pyrogenic exotoxin G (speG^{dys}), demonstrating that this species has the potential to produce superantigen-like proteins. They failed, however, to show the presence of genes related to the superantigens SPEA, SPEC, SPEZ (SMEZ), SPEH, and SPEI (84). Another study, however, demonstrated that some strains of SDSE do carry the bacteriophage-associated genes *spe*A, *spe*C, *spe*M, *ssa*, or *sme*Z identical to their counterparts in *S. pyogenes*. This suggests that these genes may be transferred from one species to the other, conferring enhanced pathogenicity (85).

In contrast to SDSE, the rare cases of skin and soft tissue infection due to *S. equi* subsp. *zooepidemicus* and *S. equi* subsp. *equi* usually involve exposure to animals. One case of cellulitis with bacteremia due to *S. equi* subsp. *zooepidemicus* was reported in a renal transplant patient who was exposed to horses at a show (86); a case of severe facial cellulitis due to *S. equi* subsp. *equi* was reported in another man who also had equine exposure (87), and a case of necrotizing myositis was reported in a previously healthy Norwegian farmer with two asymptomatic Shetland ponies in his stable (88). Two cases of streptococcal toxic shock syndrome caused by *S. equi* subsp. *zooepidemicus* have also been described (74, 89).

Joint and Bone Infections

While not common, SDSE is a known etiology of infectious arthritis, in both native and prosthetic joints (90–92). A group of reference hospitals in France published their experience with streptococcal bone and joint infections. They found SDSE to be present in 12% of cases and *S. anginosus* in 11%. These species were second only to *S. agalactiae* in frequency. In this series, the isolation of SDSE was associated with an unfavorable clinical outcome (93).

González Terán et al. reviewed 24 patients with group C streptococcal arthritis, two from their experience and the other 22 from the literature. Twelve (50%) cases were caused by SDSE, three by *S. equi* subsp. *zooepidemicus*, and the other nine were not speciated. Nine of the patients had polyarticular involvement. One-third (eight patients) had a preexisting arthropathy, including rheumatoid arthritis, gout, seronegative arthropathy, osteoarthritis, and others. Four of the patients were immunosuppressed, two had HIV infection, and two were on chemotherapy. Only two patients, one infected with *S. equi* subsp. *zooepidemicus*, and the other with SDSE, had a history of animal exposure (horses in both cases) (94).

Numerous cases of infectious arthritis due to group G streptococci have been reported. Serious medical illnesses and previous joint disease were common features in these patients. In five cases of group G streptococcal infectious arthritis from the UCLA hospital system, all patients had prior joint disease and two had infected prostheses (95). In a series of seven patients, only one patient had no underlying systemic or rheumatologic illness. The remaining six patients all had prior trauma, surgery, or inflammation of the affected joint, and four of the six patients also had underlying medical conditions, including diabetes mellitus, alcoholism, and cardiovascular disease (96). In another review of 50 previously reported cases of group G streptococcal arthritis, more than one-third of patients had chronic joint disease, while just under half of the patients had one of four underlying conditions: malignancy, alcoholism, diabetes mellitus, or injectable-drug use (97). Osteomyelitis has also been described with GCGS, but it is reported less frequently than infectious arthritis. In these cases, there is also often a significant underlying disease (44, 63).

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Maternal and Neonatal Infections

Although SDSE can be found as part of the normal female genitourinary flora, its presence in clinical specimens often indicates infection. There have been at least two published outbreaks of puerperal fever caused by SDSE. In the first, 33 confirmed cases in England were caused by a single strain of SDSE. Clinical features included fever and signs of perineal infection. Sources of infection were postulated by the authors to be environmental, because the organism was cultured from toilet seats and bath plug holes. However, the organism was also cultured from the throats of many of the nursing staff (98). In the second outbreak, which occurred 4 years later in England, seven women developed puerperal fever caused by SDSE (99). Interestingly, the isolates shared the same M serotype with the strain responsible for the first outbreak. Though the microorganism was not isolated from the environment, it was speculated that transmission may have occurred through use of a common toilet seat. These epidemiologic and microbiologic data suggesting transmission by fomites must be interpreted with caution. The role of environmental contamination versus health care-related person-to-person transmission in such outbreaks remains to be determined.

Neonatal group C streptococcal infection is rare. In one reported case, meningitis due to SDSE developed in an infant whose mother was being treated for chorioamnionitis at the time of delivery (100). In another case, a preterm infant developed meningitis due to SDSD; the source of infection was not determined, because the mother was not ill and the organism could not be cultured from her (101).

Although clinical infection is rare, colonization of neonates with group G streptococci seems to be a common finding. In one study, cultures were taken from the nose and umbilicus of more than 3,000 neonates over a 1-year period at a New York hospital. The monthly incidence of positive cultures for group G streptococci ranged from 41 to 76%. Seven cases of neonatal sepsis due to these organisms were diagnosed over the same time period. Five of the seven cases occurred in the setting of complications of pregnancy or childbirth (102). In a larger review, which included this series, premature or prolonged rupture of the amniotic membranes was the most common risk factor associated with group G streptococcal infection (103).

Bacteremia, Endocarditis, and Other Serious Invasive Diseases

SDSE, *S. equi* subsp. *zooepidemicus*, and other streptococci with either group C or G antigen have been reported with increasing frequency to cause bacteremia, most commonly secondary to skin and soft tissue infection (16, 24, 42, 104). Many patients affected by these organisms (up to 70% in some series) have serious underlying diseases, especially malignancy, cardiovascular disease, diabetes mellitus, immunosuppression, and alcohol or injectable-drug use (40, 41, 44, 45, 105).

Bacteremia due to SDSE may be primary in approximately 20% of cases or secondary to a focal site of infection, most often from the skin or soft tissues. Auckenthaler et al. reviewed 38 patients who were bacteremic with group G streptococci at the Mayo Clinic-affiliated hospitals, representing 0.25% of all patients with positive blood cultures over a 10-year period. Seventy percent of the patients acquired the infection in the community, and the skin was the portal of entry in approximately three-quarters of the patients. Most of the hospital-acquired bacteremias involved a postoperative wound or a transcutaneous procedure. The patients tended to be older, with most being in the sixth to eighth decades. Many patients had venous insufficiency, lymphedema, or another cause of chronic lower extremity edema (40). Carmeli et al. reported 10 cases of group C streptococcal bacteremia in Israel and reviewed several other case series. In this review, some patients had primary bacteremia, but most cases were secondary to pharyngitis, epiglottitis, pericarditis, pneumonia, skin and soft tissue infection, endocarditis, or an infected aneurysm (41). In a review from Boston University, 29 patients with group G streptococcal bacteremia were identified over a 3-year period. The median age of the affected patients was 68 years, and one-half had a skin infection as the primary source of the bacteremia (45). In another series, six cases of bacteremia were reported in injectable-drug users. The portal of entry for these patients was the skin. All of the infected patients had injected drugs for at least 10 years (64).

Some studies have noted a high rate of relapsing or recurring bacteremias caused specifically by organisms carrying the group G antigen. A series of 84 cases of group G SDSE in Israel included 6 patients (7%) who developed recurrent bacteremia, ranging from two to four episodes per patient. Two of the six patients had SDSE with the same *emm* type (*emm stG*840.0) isolated from their subsequent bacteremias, and the other four had different isolates in each incident (42). Another series, in Singapore, reported a rate of recurrent bacteremia of 5.8% (106). This finding suggests that, although group G streptococci contain M proteins, infections with these organisms may not induce solid protective immunity (22). This assumption is supported by studies in a murine model of group G streptococcal cellulitis (107).

Bacteremia caused by *S. equi* subsp. *zooepidemicus* is generally associated with animal contact and tends to occur in outbreaks associated with exposure to animals or animal products. In 1999, Bradley et al. (108) reviewed 88 cases of bacteremia caused by group C streptococci reported in the literature. Of these patients, 21 reported exposure to animals or animal products and, as expected, most of these had bacteremia due to *S. equi* subsp. *zooepidemicus*. Ten patients had consumed unpasteurized milk, four patients were farmers, one was a butcher, and several had other contact with animals. In the same series, 24 patients with definite or probable endocarditis were described. Of these, five cases were due to *S. equi* subsp. *zooepidemicus*, four were due to SDSE, and the remainder were unspecified. Animal exposure was noted only in patients with infection due to *S. equi* subsp. *zooepidemicus* or unspeciated organisms. Underlying cardiac disease was seen in 60% of the patients for whom adequate information was available. Edwards et al. described an outbreak of 11 cases of bacteremia due to *S. equi* subsp. *zooepidemicus* in West Yorkshire. Presentations included primary septicemia, endocarditis, infected aneurysm, and meningitis. All 11 patients had consumed unpasteurized milk from the same source (43). Yuen et al. (109) reported 11 cases of *S. equi* subsp. *zooepidemicus* bacteremia with sepsis over a 4-year period in Hong Kong. The patients had a variety of presenting syndromes, and 55% had a serious underlying illness. None of the patients reported exposure to animals or animal products. After further investigation, it was believed that the infections were acquired from ingestion of undercooked pork. Furthermore, condemned septicemic pigs were found to be infected with *S. equi* subsp. *zooepidemicus* strains whose DNA fingerprints were identical to the human isolates. More recently, a fatal *S. equi* subsp. *zooepidemicus* bacteremic infection was reported associated with equine exposure (110).

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Endocarditis due to GCGS is also an emerging disease. Like bacteremia from other sources, endocarditis tends to occur in older patients with serious underlying conditions and carries a high risk of severe disease, embolic events, metastatic infections, and death (111, 112). Clinical manifestations are similar to those seen with *S. pyogenes* endocarditis. Oppegaard et al. (113) reported a series of nine cases of definite endocarditis caused by SDSE in Norway between 1999 and 2013. The median age was 64 years, and all but two had underlying comorbid conditions. In general, the patients were acutely and severely ill. The median time of illness to admission was 1 day. Mitral and aortic valves were affected with similar frequency. Four of the nine patients had embolic events, and seven had cardiac complications. Valve replacement was needed in three of the nine patients. Mortality at 30 days was 22%. In a literature review of 40 cases of group G streptococcal endocarditis, the average age was 56 years and the overall mortality was 36%. Underlying disease was present in about one-half of the patients; six patients had a malignancy, six were diabetic, four were alcoholics, and three were injectable-drug users. Also, one-half of the patients had known preexisting valvular disease, with mitral regurgitation being the most common abnormality. Three cases occurred in patients with prosthetic valves (114). In a series of seven cases not included in the above review, the average age of the patients was 72 years, and only one patient was younger than 60 (115). Underlying medical conditions and/or preexisting valvular disease were noted in most cases.

Some patients with SDSE and *S. equi* subsp. *zooepidemicus* bacteremia develop multifocal metastatic infections, including meningitis, peritoneal abscesses, pericarditis, and pneumonia (116–120).

TREATMENT

SDSE and the other large-colony group C and G streptococci are typically susceptible to penicillin, which is considered the drug of choice to treat infections caused by these organisms. Their range of MICs to penicillin G is between 0.03 and 0.06 μg/ml (121). Besides beta-lactams, glycopeptides, daptomycin, and linezolid are also consistently active *in vitro* (122).

Lam and Bayer found that bacterial killing by penicillin is impaired when high concentrations of organisms (>10^8 colony-forming units/ml) are found (123). This phenomenon is well described for *S. pyogenes*, which downregulates the production of penicillin-binding proteins during its stationary phase of growth, resulting in a paucity of targets for penicillin (the "Eagle effect") (124). Another observation has been that in certain patients with endocarditis or septic arthritis, but also in some patients with pharyngitis, there is a poor or delayed response to therapy (125). The reason for this suboptimal response is unclear, and it may be from a combination of microbiological and host factors. The addition of gentamicin to a cell wall active antibiotic has been shown to be synergistic *in vitro*, and some have suggested its use in certain patients with severe and invasive SDSE infections, provided there is no contraindication to use an aminoglycoside antimicrobial agent (126, 127). However, there are no definitive clinical data to support that recommendation at this time. Recently, a penicillin-resistant strain was isolated from the blood of three epidemiologically linked patients in Denmark. The four isolates had a penicillin MIC of 0.5 to 2 mg/liter, belonged to a single clone, and had mutations in multiple penicillin-binding proteins (PBPs), including PBP2x mutations similar to those found in *Streptococcus pneumoniae* and *S. agalactiae*, and mutations in PBP1a and PBP1b (128).

Susceptibility to tetracyclines, macrolides, clindamycin, and fluoroquinolones is variable and cannot be assumed without proper testing (123, 129, 130). Megged (131) reported resistance rates of 38.8% and 27.8% to erythromycin and clindamycin, respectively, in Israel. Mechanisms of resistance are varied, with strains expressing constitutive macrolides-lincosamides-streptogramin B (MLS_B), inducible MLS_B, and M phenotypes. Another study, also from Israel, found resistance to clindamycin, erythromycin, azithromycin, and tetracycline in 11.1%, 18.2%, 21.2%, and 49.5% of SDSE, respectively (132). Similar high rates of resistance have been found in such distant geographical locations as Taiwan, Austria, and Brazil (133–136). Investigators in Portugal reviewed a collection of 314 human isolates of SDSE and found a levofloxacin resistance rate of 12%. Resistance was found in multiple *emm* types and genetic lineages and was associated with mutations in both *gyrA* and *parC*. The data also showed evidence of recombination between SDSE and *S. pyogenes*, but not between SDSE and SDSD (137).

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The Cell Wall of *Streptococcus pneumoniae*

WALDEMAR VOLLMER, ORIETTA MASSIDDA AND ALEXANDER TOMASZ

18

INTRODUCTION

Since the last revision of this article (2006), a number of important publications have appeared in the literature, the inclusion of which made it necessary to limit the topics of this article to information that has bearing on the biochemical and genetic aspects of covalently linked components of the pneumococcal cell wall. Information on proteins noncovalently attached to the cell wall, cell walls and phase variation (Jing Li and Jing-Ren Zhang, in press), and inflammatory activity of cell walls (Allister J. Loughran, Carlos J. Orihuela and Elaine I. Tuomanen, in press) are reviewed separately.

Historically, studies of the pneumococcal cell wall were motivated by such unique features as the presence of choline in the teichoic acids and the pleiomorphic changes that accompany removal or alteration of choline residues and by structural changes in peptidoglycan that are associated with penicillin resistance. Most recent contributions to the field include immunofluorescence (1–7) or fluorescent tagged (8, 9) microscopic localization of cell wall synthetic enzymes at sites of wall synthesis, identification of genetic determinants and enzymes that are involved with the chemical modification of peptidoglycan precursors and peptidoglycan itself, the synthesis of teichoic acid precursors and the assembly of lipoteichoic acid (LTA) and wall teichoic acid (WTA), and the mechanisms and regulation of the cell wall machinery at the central growth zone.

FUNCTIONAL ANATOMY OF THE PNEUMOCOCCAL CELL WALL

The overwhelming majority of natural isolates of pneumococci are wrapped on their outermost surface by one or another of the more than 90 chemically different capsular polysaccharides that this bacterial species is capable of synthesizing (10). Under these diverse structures lies the cell wall, which as far as the resolution of currently used analytical techniques can tell, is much more uniform in its chemistry: it is composed of a peptidoglycan covalently linked to chains of an unusually complex teichoic acid (11–13) which contain as structural components phosphoryl choline residues (14, 15). The phosphoryl choline residues play multiple roles in the physiology and virulence of the pneumococcus (see below). The two polymers, peptidoglycan and teichoic acid, make up the bulk of the cell wall in roughly equal (milligram to milligram) proportions (16). In most but not all cases, chains of the capsular polysaccharide are attached by covalent bonds to the underlying peptidoglycan (17) (Fig. 1).

In electron microscopic thin sections (18) prepared by the method of Kellenberger (osmium tetroxide and glutaraldehyde fixation followed by uranyl acetate and lead citrate staining), the cell wall of *Streptococcus pneumoniae* strain R36A appears as a band of uniform width composed of two electron-dense lines (30 to 40 nm each) enclosing a wider low-density layer (60 to 80 nm). The distribution of teichoic acid chains appears to be uniform within this wall layer (19), and this is presumably also true for the peptidoglycan. Some anatomically differentiated areas may be identified through electron microscopy: (i) The equatorial areas where cell wall growth becomes centripetal (formation of septum or cross wall) represent the "growth zones" of the entire wall. (ii) In dividing cells, a circumferential thickening ("hump") of the cell wall appears at the place of the incipient septa. (iii) Parallel to, or perhaps just prior to the beginning of, the formation of septum, the hump appears to be split at the center, and the two halves begin to move on the cell surface symmetrically to the left and to the right of the ingrowing septum coupled to the growth and eventual division of the cell into two daughter cells. Due to the conservative mode of replication of the pneumococcal cell wall (see below), these two half-humps are morphological age markers: they divide the cell wall of each pneumococcal cell into two hemispheres which differ in age by one cell generation. The functional correlates of these morphological changes began to be identified by the use of immunofluorescence microscopy, which allowed the localization of the various pneumococcal high-molecular-weight penicillin-binding proteins (PBPs) and the FtsZ-ring to equatorial and septal areas of the bacteria in various stages of cell division (2–4, 7). A special role of two hydrolases, the carboxypeptidase PBP3 (1, 20) and the endopeptidase PcsB (21–23), in these processes was also proposed. (iv) The final stage of cell division, the separation of daughter cells, may be inhibited in pneumococci by several means resulting in the formation of long chains of bacteria. Under these conditions, one can observe by electron microscopy a thin bridge of cell wall material connecting

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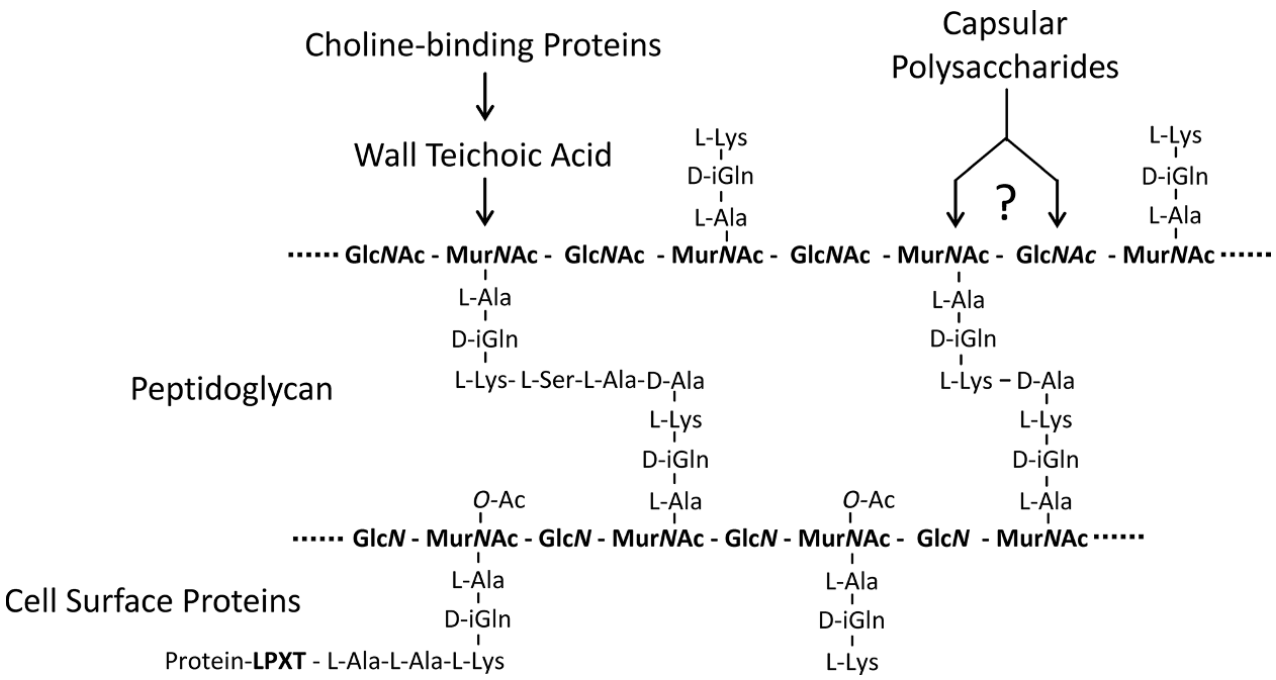


FIGURE 1 Diagram of the cell wall complex of pneumococci. Some of the MurNAc and GlcNAc residues in the glycan chains of peptidoglycan are modified by *O*-acetylation or *N*-deacetylation, respectively. Direct and indirect peptide cross-links are shown. Capsular polysaccharides have been assumed to be connected to MurNAc residues in peptidoglycan, but recent work showed that they might be connected to GlcNAc residues. Surface proteins are covalently linked to peptides in peptidoglycan; choline-binding proteins attach noncovalently to phosphoryl choline residues in WTA.

neighboring cells to one another. The hydrolase, LytB (an endo-β-1,4-*N*-acetylglucosaminidase), has been identified as an enzyme essential for the terminal separation of daughter cells at the end of cell division (24, 25).

STRUCTURE OF PEPTIDOGLYCAN

Purified cell walls of the nonencapsulated strain R6St (streptomycin-resistant) of *S. pneumoniae* were hydrolyzed by the pneumococcal amidase (*N*-acetylmuramic acid L-alanine amidase; the product of the *lytA* gene) under conditions in which this enzyme can quantitatively release the peptide units of cell wall muropeptides. A method that uses muramidase digestion of the pneumococcal cell wall or peptidoglycan followed by high-performance liquid chromatography (HPLC) separation of muropeptides, through an adaptation of the method of Glauner and Schwarz, has been described (26, 27).

In the amidase method, the family of peptides was separated by HPLC and size fractionation and determination of amino acid composition and NH_2 termini. Partial sequencing of the peptides generated by HPLC and analysis using time-of-flight mass spectrometry has allowed the identification of a surprisingly large number of monomeric, dimeric, and trimeric peptides (28) showing a diversity comparable to that seen among the muropeptide species identified in *Escherichia coli*. In the structural assignments, it was assumed that the amino acids within the stem peptides had the usual alternating sequence of L- and D-amino acids beginning with L-alanine in position 1, followed by a D-isoglutamine and then by L-lysine. The carboxyterminus in the stem peptide is occupied by two consecutive D-alanine residues; however, such muropeptides with intact pentapeptide residue are rare in pneumococci cultivated under normal growth conditions. Extension of this analytical technique to cell walls of clinical isolates from a large variety of isolation sites and dates and expressing a variety of different capsules has led to the proposition that the cell wall muropeptide composition of *S. pneumoniae* grown in the commonly used semisynthetic medium and harvested in the late exponential phase is constant and characteristic of the species. The most abundant monomer of the pneumococcal peptidoglycan was a tripeptide, and the most frequent dimer was a directly cross-linked tri-tetrapeptide. Interestingly, the representation of carboxyterminal alanine was extremely rare, suggesting the presence of powerful DD- and LD-carboxypeptidases. These enzymes, PBP3 and LdcB, will be discussed below.

An interesting feature of the peptide network was the presence of both directly and indirectly cross-linked components. In the latter, alanyl-serine or alanyl-alanine dipeptides formed the cross-link. In terms of cross-linking mode, the pneumococcal cell wall may be classified as either A1 (directly cross-linked) or A3 (cross-linked via an interpeptide bridge), depending on which dimer one chooses. A massive distortion of peptidoglycan composition in the direction of preponderance of indirectly cross-linked components was demonstrated in several penicillin-resistant clinical isolates (29).

The amidase also releases the glycan chains, which in *S. pneumoniae* harbor two modifications: *N*-deacetylation of some of the GlcNAc residues, resulting in glucosamine, and *O*-acetylation at some of the MurNAc residues. The latter modification is acid labile and lost during the removal of WTA to purify peptidoglycan, and therefore the glycan chains released by the amidase carry glucosamine residues, the percentage of which depends on the strain and growth conditions. The isolated glycan chains can be chemically reacetylated and analyzed by HPLC with or without partial digestion by muramidase. This analysis revealed that glycan chains of less than 25 disaccharide units are virtually absent

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in pneumococcal peptidoglycan (27), in sharp contrast to that of staphylococci or *E. coli*, in which short glycan chains prevail (30, 31). That pneumococcal peptidoglycan contains relatively long glycan chains was confirmed by size exclusion chromatography (32).

The muramidase method in combination with mass spectrometry allowed detection of ~50 different muropeptides (27). It confirmed the above-mentioned peptide composition and quantified the abundance of modifications in un-cross-linked (monomeric) and cross-linked (dimers, trimers) structures, allowing predictions about the activities of cell wall enzymes. For example, nonamidated D-glutamate residues at position 2 were significantly less abundant in cross-linked muropeptides, suggesting that enzymes performing cross-linking reactions (the PBPs) require the presence of the amidated D-glutamate residue at position 2 (27, 33). The analysis also revealed the presence of cross-linked muropeptides (mainly trimers) that lacked GlcNAc or the GlcNAc-MurNAc disaccharide at one of the peptides, indicating *N*-acetylglucosaminidase and amidase activities, presumably those of LytB and LytA, respectively. Muropeptide analysis has recently been used to study different aspects of pneumococcal cell wall physiology (34–36).

PEPTIDOGLYCAN BIOSYNTHESIS AND REMODELING

Pneumococci contain the essential genes of the general biochemical pathway of peptidoglycan synthesis and cell morphogenesis that were studied extensively in other model species such as *E. coli* and *Bacillus subtilis* (37, 38). Many of the peptidoglycan precursor genes and cell division proteins cluster in three chromosomal regions, instead of one single cluster present in *E. coli* and *B. subtilis*, consistent with the hypothesis that the ovococcal-shaped pneumococci require less coregulation of cell wall and division genes than rod-shaped species (39). In this section we will focus on covalent modifications in the peptidoglycan that are important for the physiology or lifestyle of pneumococci.

Modifications of the Peptidoglycan Precursor

Many Gram-positive species amidate the D-isoglutamate residue at the α-carboxylic group by transfer of an amido group from L-glutamine to the cytosolic precursors UDP-MurNAc-pentapeptide or the bactoprenol-linked precursors lipid I and lipid II. This amidation reaction is catalyzed by a complex of two proteins, MurT and GatD, which were originally identified in *Staphylococcus aureus* (40). MurT and GatD are essential in both *S. aureus* and *S. pneumoniae* (40, 41), consistent with the observation that amidation of the lipid II precursor is required for efficient cross-linking in an *in vitro* assay for peptidoglycan synthesis with purified PBPs (33). Hence, MurT and GatD seem to be essential because they provide a modification needed for the essential peptide cross-linking reaction.

The presence of a branch consisting of 2 to 7 L-amino acids added to the ε-amino group of L-lysine at position 3 of the stem peptide is another widespread modification in Gram-positive bacteria whereby the sequence of the branch varies between species. Virtually all *S. aureus* strains have a pentaglycine branch, but the peptidoglycan is less homogeneous in *S. pneumoniae*, in which strains can significantly differ in the amount of three types of peptides: with no branch, with an L-Ala-L-Ala branch, or with an L-Ser-L-Ala branch (42).

The branch is added to the lipid II precursor by the ligases MurM and MurN. The *in vivo* substrate of MurM is lipid II, and the enzyme catalyzes the addition of a serine or alanine residue (depending on the particular *mur*M allele) to the free amino group of lysine in the stem peptide in a reaction in which the amino acid donor is presumed to be an amino acyl-tRNA (43). This reaction is followed by the MurM catalyzed addition of an alanine to complete the dipeptide branch (see Fig. 2). Although the peptide branches synthesized by MurM and MurN are not essential for pneumococcal growth, they are required in various strains to express high levels of β-lactam resistance. A more detailed description of the connection between the *murMN* operon and β-lactam resistance is provided in "The *murMN* Operon," below.

Penicillin-Binding Proteins

S. pneumoniae has six PBPs, of which three, PBP1a, PBP1b, and PBP2a, are class A bi-functional glycosyltransferase-transpeptidases capable of polymerizing the precursor lipid II to form the glycan chains and performing peptide cross-linking reactions. None of these bi-functional enzymes are essential, but double mutants lacking *pbp1a* and *pbp2a* are not viable (44), and *in vitro* experiments with the purified proteins showed that both require amidated lipid II substrate for efficient transpeptidase activity (33).

S. pneumoniae also has two essential class B monofunctional transpeptidases, PBP2x and PBP2b, involved with septal and peripheral peptidoglycan synthesis, respectively (7, 45). Consistent with their roles, cells depleted of PBP2x become elongated, and cells depleted of PBP2b are shorter. PBP2x has two C-terminal PASTA (PBP and serine/threonine kinase-associated) domains which are required to localize the protein at midcell (46). Genetic evidence points to an important role of branched peptide substrates for these PBPs. The remaining PBP, PBP3, is not a synthase but is a carboxypeptidase for trimming of pentapeptides to tetrapeptides (see below).

Trimming of the peptides by carboxypeptidases

Newly synthesized peptidoglycan contains abundant pentapeptides with a D-alanyl-D-alanine terminus, but these are trimmed in two steps to tripeptides which prevail in the mature pneumococcal peptidoglycan. The DD-carboxypeptidase PBP3 produces tetrapeptides (47), which are the substrate for the LD-carboxypeptidase LdcB (or DacB) to produce the tripeptides. The wall peptide composition in a PBP3-defective mutant (48) and in the laboratory strain R36A growing in the presence of subinhibitory concentrations of clavulanic acid, a selective inhibitor of PBP3 in this bacterium (49), showed accumulation of peptide species that retained carboxyterminal D-alanine residues. Pneumococci grown in the presence of clavulanate also showed abnormal physiological properties: premature induction of stationary phase autolysis, hypersensitivity to lysozyme, and reduced MIC values for deoxycholate and penicillin.

The *ldcB* gene was identified in a search for hypothetical peptidoglycan hydrolase genes, and a deletion mutant has defects in cell shape and septation (50), as has a *dac*A mutant lacking PBP3 (1). However, only *dacB* mutants were significantly attenuated in an intranasal mouse infection model and showed increased uptake by professional macrophages and reduced adherence to lung epithelial cells (51). The *dacB* mutant is rich in tetrapeptides (50, 52), but it is not known why the presence of tetrapeptides instead of tripeptides has these drastic effects on the physiology and pathogenicity of pneumococci. The crystal structure of LdcB shows a catalytic domain with a fold characteristic for the LAS family of Zn-dependent peptidases (51, 52).

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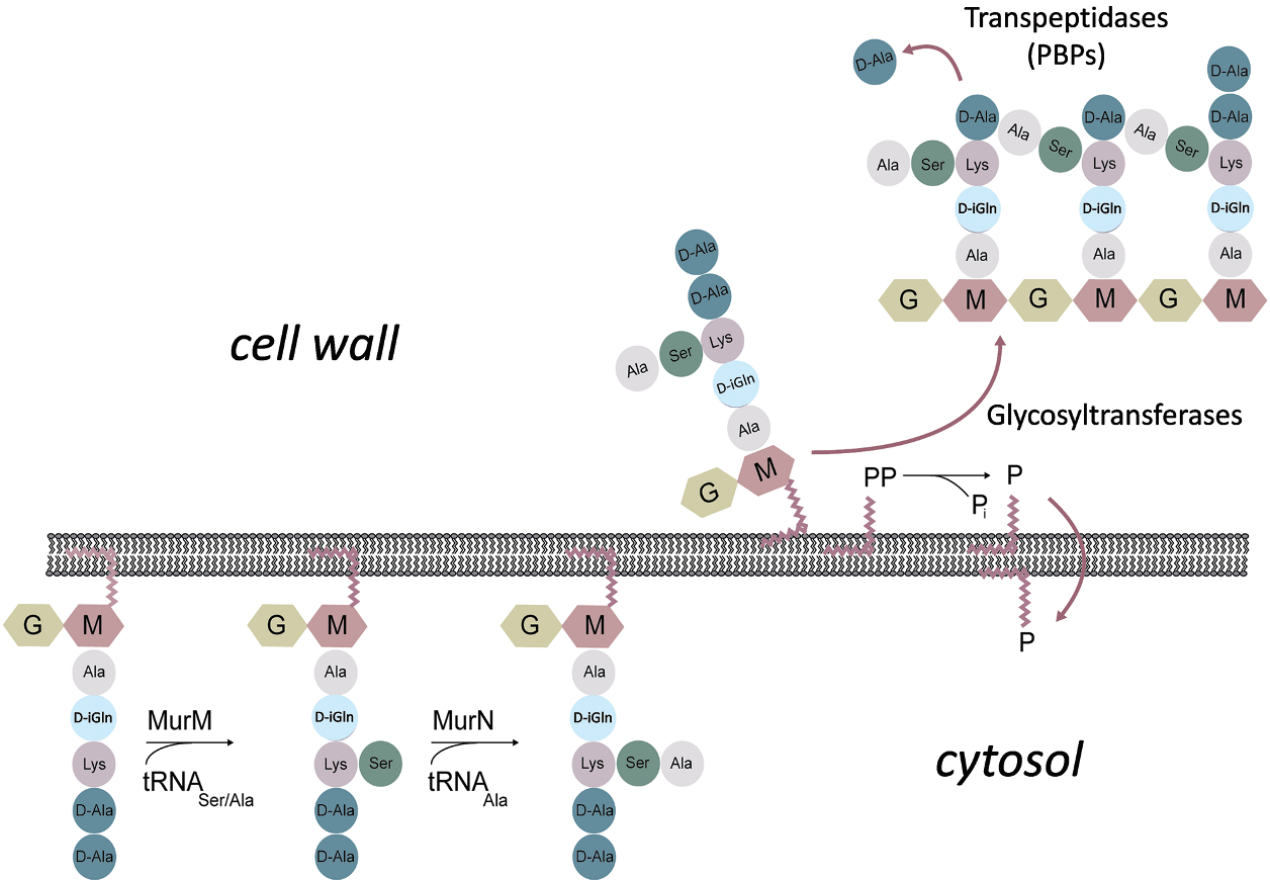


FIGURE 2 Role of MurM and MurN in cell wall branching. The substrate of the MurM- and MurN-catalyzed branching reaction is lipid II, which is composed of *N*-acetylated disaccharide units of glucosamine (hexagon with G) and muramic acid (hexagon with M) with the pentapeptide attached to the M residues. Lipid II is anchored on the plasma membrane through the carrier lipid bactoprenyl phosphate (zig-zag line). Attachment of the completed precursor to the preexisting cell wall occurs on the outer surface of the plasma membrane by the activity of glycosyltransferases and transpeptidases. Reproduced with permission from reference 82.

The Putative Lytic Transglycosylase MltG

The *mltG* (*spd_1346*) gene was recently linked to peripheral peptidoglycan synthesis based on the observation that its inactivation suppresses the requirement for PBP2b, MreCD, RodA, and RodZ, i.e., genes that are all part of the pneumococcal elongation machinery (53). MltG has a YceG-family domain that likely acts as an endolytic transglycosylase since the known MltG endolytic transglycosylase homologue from *E. coli* can replace MltG function in *S. pneumoniae*. A model has been proposed according to which MltG removes nascent glycan strands, produced by PBP1a, from their membrane anchor to facilitate their incorporation into the old cell wall by RodA and PBP2b (53). However, direct biochemical proof of a lytic transglycosylase activity of MltG has yet to be presented, and the lytic transglycosylase products, 1,6-anhydro-*N*-acetylmuramic acid termini in glycan chains and turnover products, have yet to be detected.

Peptidoglycan Hydrolases Involved in Septum Remodeling and Cleavage

Several peptidoglycan hydrolases are important for pneumococcal growth and morphology. The extracellular protein PcsB localizes to the cell division site, where it is required for proper septum formation and cleavage (54). The *pcsB* gene is the only essential gene under the control of the WalRK cell wall stress regulon, and the depletion of *pcsB* results in growth arrest and severe morphological defects. The active site of PcsB resides near the C-terminus and belongs to the CHAP (for cysteine, histidine-dependent amidohydrolase/peptidase) family of peptidoglycan endopeptidases, but such an activity could only be observed for purified PcsB when tested in an in-gel zymogram assay. In the cell, PcsB requires activation by the essential membrane-bound ABC transporter-type FtsEX complex (54, 55). The crystal structure of PcsB shows an inactive homodimer in which the V-shaped coiled-coil domain of each monomer inserts into the active site groove of the other, explaining why this dimer form is inactive (23). It was suggested that FtsEX utilizes ATP hydrolysis to induce conformational changes in the PcsB dimer to unlock this mutual inhibition. However, the precise roles of FtsEX and PcsB in septum formation and cleavage have yet to be established.

LytB is a secreted endo-β-*N*-acetylglucosaminidase with a choline-binding domain consisting of 14 to 18 imperfect repeats, which direct the enzyme to the septum and cell pole regions, followed by an SH3b peptidoglycan-binding domain, a small carbohydrate-binding domain with unknown function, and a C-terminal catalytic domain (56). LytB functions at the final stages of the division process to separate the daughter cells. Mutants lacking LytB grow in deeply constricted chains of unseparated cells (57). Purified LytB is able to disperse chains of cells of a *lytB* mutant. LytB cleaves

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at sites with fully acetylated GlcNAc residues and has preference for monomeric peptides, suggesting a substrate-assisted catalytic mechanism (25).

Apart from their role in cleavage of the septum and autolysis, peptidoglycan hydrolases do not seem to be very active during pneumococcal growth in the laboratory. There is a minimum of peptidoglycan turnover at the hemispherical regions, with the old peptidoglycan remaining virtually inert during growth (58, 59).

Peptidoglycan *N*-Acetylglucosamine Deacetylase PgdA

An unusually high proportion of hexosamine units in the glycan strands of the pneumococcal cell wall are not *N*-acetylated, explaining the resistance of this peptidoglycan to the hydrolytic action of lysozyme, a muramidase that cleaves in the glycan backbone. A gene, *pgdA*, was identified as encoding for the peptidoglycan *N*-acetylglucosamine deacetylase A with amino acid sequence similarity to fungal chitin deacetylases and rhizobial NodB chitooligosaccharide deacetylases (60). PgdA is a metal-dependent enzyme belonging to the family 4 carbohydrate esterases. Its crystal structure shows a His-His-Asp catalytic triad in its active site (61, 62). Pneumococci in which *pgdA* was inactivated produced fully *N*-acetylated glycan and became hypersensitive to exogenous lysozyme in the stationary phase of growth. The *pgdA* gene may contribute to pneumococcal virulence by providing protection against host lysozyme. A pneumococcal strain expressing capsular type II with inactivated *pgdA* showed reduced virulence in the mouse model of intraperitoneal infection (63).

Peptidoglycan *O*-Acetyltransferase

Many bacteria modify the C6-OH group of MurNAc residues in their peptidoglycan by an *O*-acetyl group, and this modification has been implicated in the regulation of autolysins and resistance to lysozyme (64). The pneumococcal *adr* gene encoding the *O*-acetyltransferase has been initially identified in a mariner mutagenesis screen for increased sensitivity to penicillin in the background of the resistant strain Pen6 (65). *Adr* mutant cells also show higher sensitivity to lysozyme, and Adr shares sequence homology with the peptidoglycan *O*-acetyltransferase OatA of *S. aureus* (66). Muropeptide analysis by HPLC and mass spectrometry showed that the *adr* mutant lacked muropeptides with *O*-acetylated MurNAc residues, suggesting that Adr is the pneumococcal peptidoglycan *O*-acetyltransferase. Recent work showed that peptidoglycan *O*-acetylation by Adr occurs at midcell and that this modification protects the cell wall from cleavage by the autolysin LytA (67).

Sortase A-Dependent Covalent Attachment of Proteins to the Pneumococcal Cell Wall

Most virulence-related pneumococcal proteins studied so far were shown to be attached to the cell surface through the choline residues of teichoic acids, and pneumococci have been described as a paradigm for the display of virulence proteins through specific but noncovalent associations with the cell surface (68). Such choline-binding proteins include PspA, PsaA, the enzyme phosphoryl choline esterase (69, 70), and LytA, in which a 20-amino-acid repeat was shown to recognize choline residues in wall or membrane teichoic acids (71). The observations described in this report demonstrate that the mechanism of surface display of proteins does not depend on the choline-binding paradigm alone; pneumococci also use covalent anchoring for some surface proteins. Data available from the genome of *S. pneumoniae* strain R6 (72) indicate that there are at least 23 proteins carrying the LPXTG motif, and 15 of these have this recognition sequence at the C terminus, as expected for proteins that are processed by a typical sortase.

Inactivation of sortase gene *srtA* in *S. pneumoniae* strain R6 caused the release of beta-galactosidase and neuraminidase A (NanA) from the cell wall into the surrounding medium. Both of these surface proteins contain the LPXTG motif in the C-terminal domain. Complementation with plasmid-borne *srtA* reversed protein release. Deletion of *mur*M, a gene involved in the branching of pneumococcal peptidoglycan, also caused partial release of beta-galactosidase, suggesting preferential attachment of the protein to branched muropeptides in the cell wall. Inactivation of *srtA* caused decreased adherence to human pharyngeal cells *in vitro* but had no effect on the virulence of a capsular type III strain of *S. pneumoniae* in the mouse intraperitoneal model. These observations suggest that similar to other Gram-positive bacteria, sortase-dependent display of proteins occurs in *S. pneumoniae* and that some of these proteins may be involved in colonization of the human host (73).

PEPTIDOGLYCAN COMPOSITION AND PENICILLIN RESISTANCE

The first series of highly penicillin-resistant clinical isolates examined using the HPLC method were from South Africa. It was in these isolates that the mechanism of resistance, namely, reduction in the antibiotic "affinity" of PBPs, was identified for the first time (74). It was also in genetic crosses with these isolates used as DNA donors that the stepwise nature of penicillin resistance (i.e., the sequential reduction in the penicillin affinity of several high-molecular-weight PBPs in parallel with the gradually increasing penicillin MIC value) was recognized (74).

Analysis of the penicillin-resistant South African clinical isolates revealed that they produced cell walls of a radically different composition from the one seen in the penicillin-susceptible and nonencapsulated laboratory isolate (75). When the HPLC analysis was extended to the walls of several penicillin-susceptible clinical isolates and several resistant strains (all but one from South Africa), the striking shift toward indirectly cross-linked wall peptide composition in the resistant isolates was fully confirmed. A link between resistance to penicillin and abnormal wall composition was also suggested by the analysis of genetic crosses: a shift toward the distorted wall composition of the resistant DNA donor was observed in genetic transformants above a certain MIC value. It was suggested that the anomalous wall peptide composition reflected the altered substrate preference of the penicillin-resistant PBPs, a shift from the linear to the branched wall peptide precursors (75).

THE *murMN* OPERON

A considerable clarification concerning determinants of cell wall structure and its relationship to penicillin resistance was obtained by the identification of the *murMN* operon, which encodes enzymes involved in the synthesis of branched structured muropeptides in the pneumococcal peptidoglycan (42). The same determinants were also described independently and named *fib* by another group (76). The *murMN* operon shows homology to the *femXAB* genes of *S. aureus*, which are involved with the addition of the pentaglycine

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branches to the epsilon amino group of lysine residues in the staphylococcal peptidoglycan (77).

Inactivation of the *mur*M operon or inactivation of the *mur*M gene alone did not interfere with growth of the bacteria but caused the production of a peptidoglycan composed exclusively of linear muropeptides. Another consequence of inactivation was the complete loss of the penicillin-resistant phenotype. Examination of a large number of *S. pneumoniae* clinical isolates representing different genetic lineages has identified several distinct *mur*M alleles carried by penicillin-resistant strains which also showed different and unique muropeptide composition in their peptidoglycan (78). Analysis of the *mur*M alleles from penicillin-resistant isolates showed that they differed from one another and from the *mur*M carried by penicillin-susceptible strains in regions of considerable sequence diversity that were distributed as heterologous "patches" along the *mur*M gene sequence. Different *mur*M alleles from several penicillin-resistant *S. pneumoniae* strains, each with a characteristic branched peptide composition, were introduced on a plasmid into a common penicillin-susceptible laboratory strain. All transformants remained penicillin susceptible, but their cell wall composition changed in directions which corresponded to the muropeptide pattern of the strain from which the *mur*M allele was derived. This observation suggests that the muropeptide composition of *S. pneumoniae* is determined by the particular *mur*M carried by the strain (43, 78) (Fig. 3A and B; Table 1).

The relationship between *mur*M alleles and the penicillin-resistant phenotype is less clear. In genetic transformation of high-level penicillin resistance, it was shown that successful expression of the resistant phenotype required that the transformants received not only the particular mosaic *pbp* genes of the DNA donor but also the *mur*M allele carried by the donor strain (79). However, in other experiments the linkage between penicillin resistance and abnormal wall composition was lost in transformation experiments in which

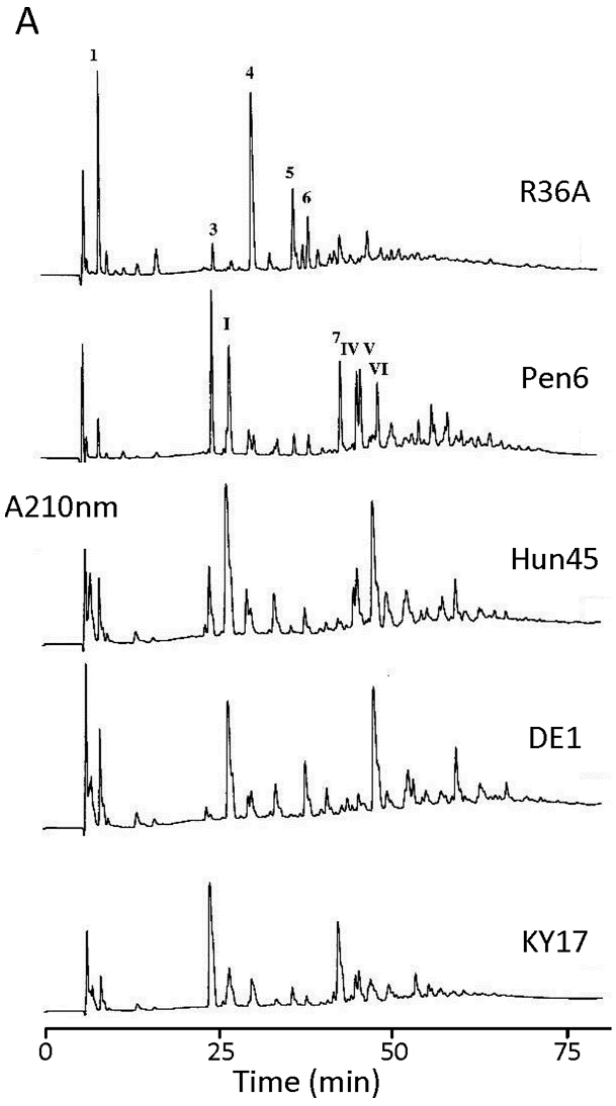


FIGURE 3 (A) HPLC elution profiles of stem peptides of the peptidoglycan from the penicillin-susceptible strain R36A and several penicillin-resistant strains that carry different abnormal *mur*M alleles. (B) Structures of cell wall stem peptides identified in the pneumococcal peptidoglycan of penicillin-susceptible and -resistant strains of pneumococci. Reproduced with permission from reference 42.

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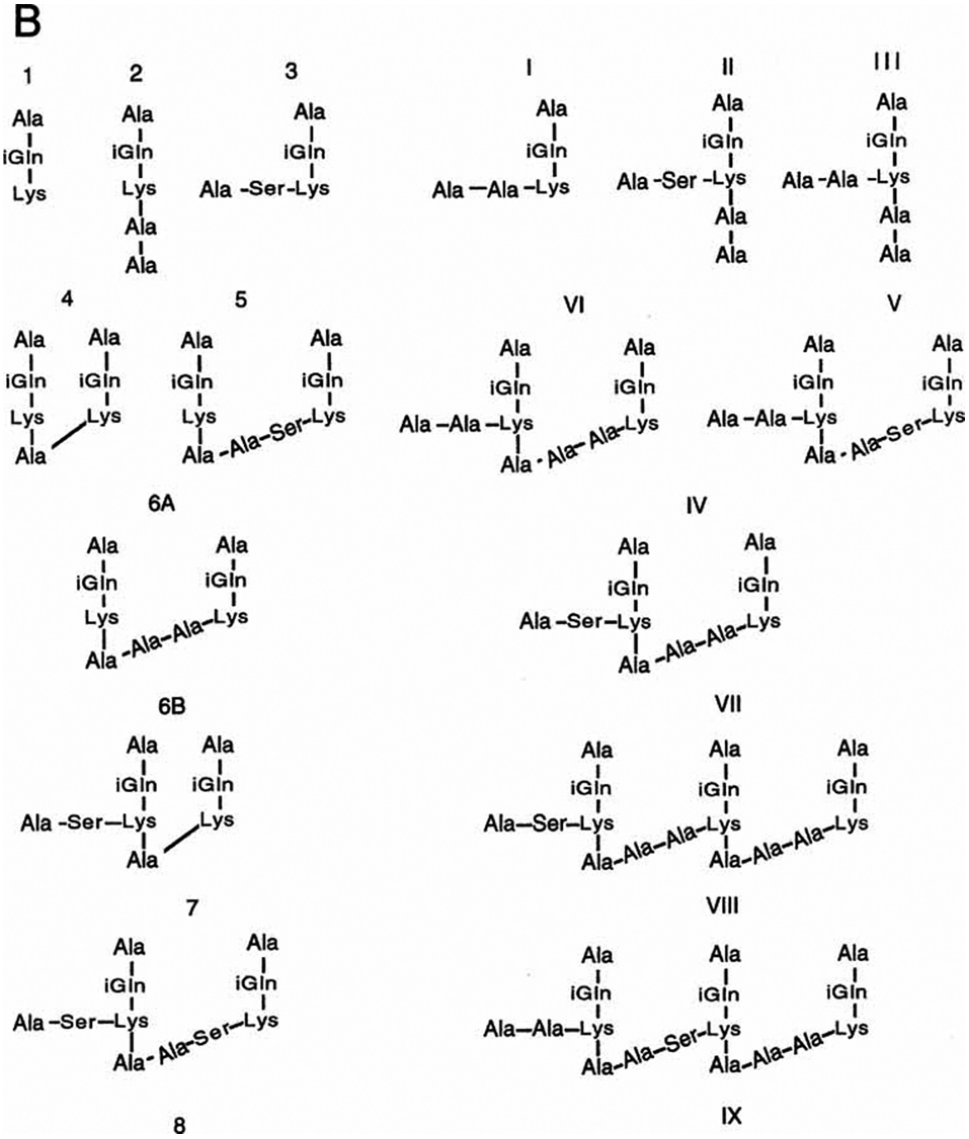


FIGURE 3 *(continued)*

the first round of transformation was followed by a second backcross (80). Interestingly, in this particular experiment the penicillin-resistant secondary transformants began to show "fitness" defects: defective growth and premature autolysis in antibiotic-free medium, similar to the defective physiology observed in laboratory isolates of penicillin-resistant pneumococci (81).

Genetic analysis and molecular modeling of the *mur*M protein has identified amino acid residues that appear to be critical for the specificity of this protein and also pinpointed domains which interact with the aminoacyl tRNA and the bactoprenyl-linked substrate of the *mur*M catalyzed reaction (82).

STRUCTURE OF WTA AND LTA

The Pneumococcal C-Polysaccharide and F-Antigen

In 1930, long before LTAs and teichoic acids were discovered and defined, Tillet and coworkers (83) described pneumococcal teichoic acid as pneumococcal C-polysaccharide by. Thirteen years later, in 1943, Goebel and his colleagues isolated pneumococcal LTA and named it lipocarbohydrate or pneumococcal F-antigen owing to its fatty acid content and immunological properties (84). In these early studies, a structural relationship between C-polysaccharide and lipocarbohydrate was suggested, and in contrast to the various strain-specific capsular polysaccharides, lipocarbohydrate and C-polysaccharide were considered pneumococcal common antigens. This was confirmed by serological methods which showed that all 90 known capsular types of *S. pneumoniae* possess C-polysaccharide and F-antigen (19). The two polymers differ immunologically, because Forssman antigenicity is associated with the LTA (85, 86). As shown by immunoelectron microscopy, C-polysaccharide is uniformly distributed on both the inside and outside of the cell walls, and LTA is located on the surface of the cytoplasmic membrane (12).

Choline, the surface signature of pneumococci, was identified as a component of teichoic acid and LTA (14, 15). The complex structures of WTA and LTA could not be unraveled

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TABLE 1 Cell wall peptide composition of several strains of *S. pneumoniae*[a]

Peptide	% Peptide in				
	R36A (*mur*MA)	Pen6 (*mur*MB2)	Hun45 (*mur*MB1)	DE1 (*mur*MB3)	KY17 (*mur*MB5)
1	13.3	2.6	4.5	10.8	3.4
2	3.4	0.7	0.5	1.4	0.6
3	2.8	14.3	7.2	1.0	28.5
I	1.8	14.4	24.1	23.4	11.2
II	3.4	2.7	5.1	3.1	0.8
4	21.0	3.2	4.4	6.0	7.2
III	1.3	3.5	6.7	6.9	2.1
5	12.7	2.2	0.9	0.5	2.9
6	7.1	2.5	2.8	9.3	1.9
7	7.4	11.1	1.4	0.4	19.7
IV	2.6	6.5	4.7	0.6	4.1
V	2.8	12.3	8.1	3.7	5.2
8	7.8	2.5	1.4	0.6	5.8
VI	6.6	10.1	19.1	27.5	2.0
9	6.0	11.6	9.0	4.9	4.5
Total	100	100	100	100	100
Monomers	26	38	48	47	47
Multimers	74	62	52	53	53
Linear	50	12	14	23	17
Branched	50	88	86	77	83
Branched/linear	1.0	7.4	6.4	3.3	4.9

[a]Reproduced with permission from reference 78.

before modern analytical techniques became available (87). In 1980, Jennings and coworkers published the first complete structure of pneumococcal teichoic acid (11). The structure of pneumococcal LTA was clarified in 1992 (85) and revised in 2013 (88), and subsequent reinvestigation of the WTA, isolated from the same strain from which the LTA had been isolated, revealed that both polymers possess identical chain structures (13, 82). The linkage structure of WTA to peptidoglycan was recently established (13), and all genes required for teichoic acid synthesis were predicted by sequence comparisons (89).

Structure of Teichoic Acid TA and LTA

The chains of LTA and teichoic acid contain 4 to 8 identical pseudo-pentasaccharide repeating units which consist of the rare positively charged amino sugar 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose (AATGal), D-glucose, ribitol 5-phosphate, two *N*-acetyl-D-galactosaminyl (GalNAc) residues, and one or two phosphocholine residues. Hydroxyl groups of ribitol can be substituted by D-alanine. The repeats are joined together by an α-1,4 glycosidic linkage between AATGal and a GalNAc residue of the adjacent repeat. The functionally important phosphocholine residues are phosphodiester-linked to O6 of the *N*-acetyl-D-galactosaminyl residues. The number of phosphocholine residues per repeat is strain specific: in LTA and WTA of strain R6, the majority of repeats carry two phosphocholine residues, whereas most of the repeats in strain Rx1 are substituted with one (Fig. 4). Moreover, the terminal repeating unit of both WTA and LTA can occur with or without the phosphocholine substitution at both GalNAc residues. The chain of LTA is β-1,3 glycosidically linked via the AATGal residue to O3 α-D-Glcp (1–3)-diacylglycerol (Fig. 4).

Microheterogeneity of LTA became apparent by hydrophobic interaction chromatography and mass spectrometry analysis (85, 88). The chain of LTA may vary in length between four and eight repeats, with the range differing from sample to sample. On SDS-polyacrylamide gel electrophoresis, LTA yields a ladder-like pattern of up to six bands, each differing from the next by one repeat. Species with one phosphocholine per repeat are distinguished from species with two by higher mobility of the individual bands.

In addition to the unusual complexity of the chemical structure of teichoic acid, pneumococci are also unique because their WTA and LTA possess identical repeat and chain structures, whereas in other Gram-positive bacteria, WTAs and LTAs are structurally and biosynthetically distinct entities. In LTA and WTA, the AATGal residues are of conformational importance because the positively charged amino groups interact electrostatically or by hydrogen bonding with the negatively charged phosphate groups on the adjacent glucosyl residues (87, 90).

Pneumococcal WTA chains have been isolated with or without attached peptidoglycan fragments and were shown to contain four to eight repeating units (13, 27). WTA is linked to the peptidoglycan by a phosphodiester bond to O6 of some of the MurNAc-residues, which is demonstrated by the release of MurN-6-P on HCl hydrolysis of cell walls (91) and of muropeptides. It is estimated that, dependent on the

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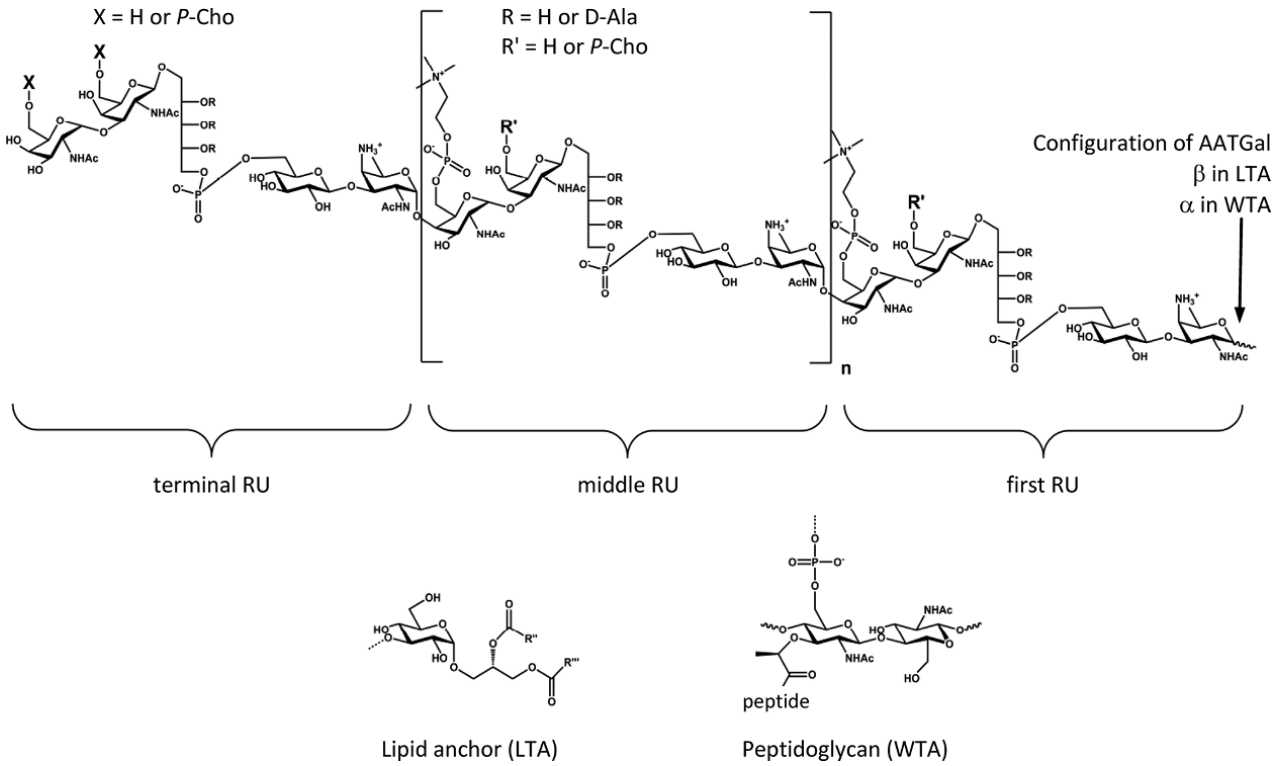


FIGURE 4 Structure of the pneumococcal LTA and WTA. Both types of teichoic acid have identical chains (top) which carry phosphoryl choline and D-alanine residues. In LTA the teichoic acid chains are β-glycosidically linked from AATGal to the lipid anchor (bottom left). In WTA the linkage occurs via an α-linkage from AATGal to MurNAc-phosphate in peptidoglycan. The figure was kindly provided by Nicolas Gisch (Research Centre Borstel, Germany). RU, repeating unit.

particular strain, between 15 and 30% of the muramic acid residues carry WTA chains (16, 92). In other Gram-positive bacteria, connecting the WTA chain and muramic acid by a phosphodiester bond typically has a linkage unit containing glycerophosphate or *N*-acetylmannosamine, but these components could not be detected either in the hydrolysate of pneumococcal cell walls or in WTA-containing muropeptides. Since no other sugar was found either, an acid-degradable sugar like AATGal was suggested as a component of the linkage unit (87). Indeed, peptidoglycan chains with attached WTA could be isolated, showing that the AATGal residue of the first repeating unit has an α-configuration and is directly linked to MurNAc 6-phosphate (13).

SYNTHESIS OF TEICHOIC ACIDS AND MODIFICATIONS

Although only few enzymes of the pneumococcal teichoic acid pathway have been biochemically studied, the complete pathway was deduced from bioinformatics analysis (89). Many teichoic acid genes cluster in three genomic regions, called lic1, lic2, and lic3. The lic1 region contains genes encoding components for the uptake of choline (LicB), the choline kinase LicA, and the cytidyltransferase LicC, which produces CDP-choline. In addition, the lic1 region encodes for TarI and TarJ, which synthesize CDP-ribitol (93). The adjacent lic2 region contains the *tacF* gene, encoding the putative teichoic acid flippase, and the *licD1/licD2* genes, encoding phosphotransferases for decoration of the teichoic acid subunits with phosphoryl choline residues. The lic3 region locates elsewhere on the chromosome and was identified by the presence of a gene, called *licD3*, with sequence similarity to *licD1* and *licD2*, which is surrounded by four other putative teichoic acid genes. LicD3 is most likely the phosphotransferase for the transfer of ribitol phosphate during synthesis of the repeating unit, which involves eight additional enzymes that synthesize the repeating unit linked to the carrier lipid bactoprenol phosphate. Presumably, Spr1222 polymerizes the teichoic acid precursors to the nascent chains, which are still linked to the carrier lipid, before these are flipped across the cytoplasmic membrane by TacF. Interestingly, TacF is specific for choline-loaded teichoic acid precursor chains, ensuring that the mature teichoic acids contain phosphoryl choline residues (94).

After reaching the outer leaflet of the cytoplasmic membrane the teichoic acid chains are transferred to either peptidoglycan to become WTA or to the glycolipid to form the LTA. The transfer to peptidoglycan is catalyzed by members of the LCP (LytR-CpsA-Psr) family of proteins, which have been identified as phosphotransferases involved in cell wall assembly in Gram-positive bacteria (95). The three LCP proteins of *S. pneumoniae* appear to have semiredundant roles in the attachment of capsular polysaccharides and WTA to C6-OH of MurNAc residues in peptidoglycan (96). However, strain D39 and strains with serotypes 8 and 31 link the capsule polymer via a 1,6-glycosidic bond to GlcNAc residues of the peptidoglycan. Such linkage is inconsistent with catalysis by LCP enzymes, indicating that the nature of the capsule attachment enzyme is still elusive (97). In the case of LTA, it is the membrane protein TacL that transfers the teichoic acid precursor chains onto the glycolipid anchor. Interestingly, mutants lacking *tacL* grow normally under laboratory conditions but show attenuated virulence in mouse models of acute and/or systemic infections by *S. pneumoniae* (13).

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Pneumococci contain a *dlt* operon, the products of which are responsible for the decoration of LTA and WTA repeating units with D-alanine residues, which are attached via an ester linkage to OH groups in ribitol (98). This modification introduces positive charges to teichoic acids and thus increases resistance to cationic antimicrobial peptides. While *S. pneumoniae* strain D39 modifies its teichoic acids by alanylation, this modification is not present in the laboratory strain R6 or in strain TIGR4 due to mutations in the *dlt* operon rendering it nonfunctional.

Phosphoryl Choline Esterase Enzymatic Removal of Phosphoryl Choline Residues from the Pneumococcal Cell Wall

The *pce* gene encoding for a teichoic acid phosphoryl choline esterase (Pce), an enzyme capable of removing phosphorylcholine residues from the cell WTA and LTA, was identified independently by two research groups (69, 70). Pce carries an N-terminal signal sequence and contains a C-terminal choline-binding domain with 10 homologous repeating units similar to those found in other pneumococcal surface proteins. The catalytic (phosphorylcholine esterase) activity is localized on the N-terminal part of the protein. The mature protein was overexpressed in *E. coli* and purified in a one-step procedure by choline-affinity chromatography. The product of the enzymatic digestion of ^{3}H-choline-labelled cell walls was shown to be phosphorylcholine. Inactivation of the *pce* gene in *S. pneumoniae* strains by insertion-duplication mutagenesis caused a unique change in colony morphology and a striking increase in the virulence of a capsular type III strain in the intraperitoneal mouse model. Pce may be a regulatory element involved with the interaction of *S. pneumoniae* with its human host. Sequence comparison indicates that Pce, the protein originally identified through its unique enzymatic activity (99), is identical to choline-binding protein E (CbpE), described by Masure and colleagues as a protein implicated in the attachment of pneumococci to nasopharyngeal cells (100). The crystal structure of Pce shows a catalytic site with two zinc ions and an elongated binding domain recognizing the phosphocholine residues of teichoic acid (101).

MULTIPLE FUNCTIONS OF CELL WALL CHOLINE RESIDUES

Nutritional Requirement for Choline

Choline is an essential growth factor for all natural isolates of pneumococci which have to import this nutrient from the growth medium (102). Choline may be replaced by other amino alcohols such as ethanolamine, which can incorporate into LTA and teichoic acid at the same positions as phosphocholine, but it cannot replace phosphocholine functionally. Ethanolamine-grown cells show a number of striking abnormalities, including inhibition of cell separation (growth in long chains), inhibition of DNA uptake in genetic transformation, lack of autolysis during treatment with penicillin and other wall inhibitors and detergents (103), production of an "immature" (enzymatically inactive) form of the autolytic enzyme LytA (104), and production of a cell wall that cannot absorb LytA (105), and is completely resistant to the hydrolytic action of LytA (106). However, the requirement of LytA for built-in phosphocholine residues is no longer seen when solubilized cell wall polymers are degraded to muropeptides (12) or when the teichoic acid is removed from peptidoglycan (48). These observations may be interpreted to indicate that teichoic acid prevents the access of autolysin to its cell wall substrate and that this effect is overcome by binding of the enzyme to the phosphocholine residues.

There are several proteins in pneumococci—including LytA, LytB, and LytC—that specifically recognize and bind to phosphocholine residues. These proteins contain distinct domains which are responsible for their specific biological activities, whereas the choline-binding domains are homologous and contain 6 to 10 choline-recognizing repeats of ~20 amino acids each. The structure of crystallized LytA and LytC and their interaction with choline residues in the cell wall have been described (107, 108).

Choline-Independent Strains

A choline-independent strain, R6Cho^{-}, was recovered from a heterologous cross with DNA from *Streptococcus oralis* (48). *S. oralis* also incorporates phosphocholine into its cell wall teichoic acid but, unlike pneumococci, has no nutritional requirement for choline. Other choline-independent strains, for example, JY2190 and R6Chi, are mutants generated by serial passage of strains in chemically defined medium containing decreasing concentrations of ethanolamine with each passage (94, 109). None of these strains had acquired the capability to synthesize choline or ethanolamine, because phosphorylated amino alcohols could not be detected in TA and LTA. In spite of the absence of phosphocholine, there was no alteration either in the structure of LTA and teichoic acid or in cell wall composition, including the stem peptide profile. Only the phosphate content of cell walls was reduced, consistent with the absence of phosphocholine. *In vivo*, the lack of active autolysins became apparent by impaired cell separation at the end of cell division and by resistance against stationary phase and penicillin-induced lysis. Due to the absence of choline from LTA, PspA was lost into the surrounding medium, whereas in spite of choline-free teichoic acid, the amidase was retained on the cells. Both choline-independent strains retained the capacity to incorporate choline into teichoic acids: when grown in the presence of choline, PspA was retained, cells separated normally and became penicillin sensitive, and phosphocholine was discovered on LTA and teichoic acid.

In the case of strain R6Chi and related strains, the lack of a nutritional requirement for choline was explained by the loss of specificity of the teichoic acid flippase TacF for choline-loaded teichoic acid precursor chains (94). R6Chi contains a single point mutation in the *tacF* gene, rendering the cells choline independent. When growing in the presence of choline, R6Chi still incorporated phosphocholine into its teichoic acids, suggesting that the mutated TacF can transport choline-loaded and -unloaded teichoic acid precursor chains. The presence of the *tacF* mutation also allowed for the deletion of otherwise essential choline utilization genes *licABC*, producing a strain that did not incorporate choline into its cell wall even when growing in the presence of exogenous choline. An encapsulated version of this mutant was used to demonstrate the crucial role of cell wall choline residues in immune evasion (110)

CELL GROWTH REGULATION DURING THE CELL CYCLE

Growth Zone and Cell Wall Segregation

Similar to other streptococci, pneumococci incorporate new cell wall units into the preexisting wall material at a single growth zone located at the cell equator (midcell). Both

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peptidoglycan and teichoic acids units and also capsular polysaccharide (111) enter the pneumococcal surface at this growth zone, which could be visualized by exploiting the unique selectivity of a pneumococcal enzyme, the peptidoglycan hydrolase LytA, for choline-containing segments of the cell wall (58). Pneumococci require choline for growth, and the design of this experiment was based on the observation that the choline component of the WTA can be replaced by structural analogues such as ethanolamine. Pneumococci grown in ethanolamine-containing medium show several striking abnormalities: unlike the choline-grown bacteria, pneumococci utilizing ethanolamine grow in long chains and are completely resistant to the cell wall-degrading activity of LytA (103). Upon addition of trace amounts of radiolabeled choline to a culture grown on ethanolamine, the bacteria immediately shifted to the utilization of choline so that the nascent wall units that began to incorporate into the cell surface contained choline residues in the teichoic acid component of the nascent cell wall and produced regions that were susceptible to hydrolysis by exogenous LytA enzyme added to the medium. It was possible to show by electron microscopy that under these conditions the LytA enzyme performed an enzymatic "microsurgery" on the bacteria: it has selectively removed a thin equatorially located band of cell wall, thus identifying the anatomical site of wall incorporation and growth zone (58).

Another abnormality of the ethanolamine-grown pneumococci, the complete inhibition of cell separation, has allowed the design of experiments to test the mode of inheritance of pneumococcal cell walls. Pneumococci labeled in their wall by titrated choline were shifted to an ethanolamine-containing medium in which the bacteria continued to grow in the form of chains of cells, i.e., "linear clones" in which the distribution of radioactively labeled bacteria could be followed (by autoradiography) as a function of cell generations in the ethanolamine-containing medium. Since the teichoic acid choline does not exhibit turnover during growth, the localization of radioactively labeled cells within the chains of bacteria could provide clues as to the mode of wall segregation. The finding was that the radioactive label remained in large clusters in association with cells that were located either at the tips or at the center of chains. The results demonstrate the conservation of large hemispherical segments of the cell wall which are passed on intact to daughter cells during cell division (112).

Novel methods such as staining nascent peptidoglycan with fluorescent vancomycin (113), incorporating fluorescent D-amino acids (114), and superresolution microscopy (32) confirmed that pneumococci incorporate new cell wall units into the preexisting wall material at a single growth zone located at midcell.

Cell Wall Growth and Cell Division Complexes

The mode of growth and cell wall segregation described above suggests that *S. pneumoniae*, similar to the model rods *E. coli* and *B. subtilis*, grows by alternating cycles of peripheral (side wall) and septal peptidoglycan synthesis, which may overlap for part of the cell cycle in the former (32). Consistent with this, the *S. pneumoniae* chromosome was found to encode proteins that are part of the elongation machinery (elongasome), such as MreC, MreD, MltG, RodZ, RodA, PBP2b, PBP1a, and CozE and others that are part of cell division machinery (divisome), such as FtsZ, FtsA, ZapA, ZapB, EzrA, FtsE, FtsX, FtsK, FtsL, FtsB(DivIC), FtsQ (DivIC), SepF, GpsB, DivIVA, FtsW, PBP1a, PBP2x, PcsB, and LytB, as well as proteins that have a regulatory role in these processes (4, 39, 53, 115–118).

Nevertheless, the characteristic ovococcal shape of pneumococci suggests that there must be major differences in the cell wall growth mechanism compared to those of the rods. One central difference is that pneumococci, despite conservation of all the other components of the elongation complex, lack the rod-shape determinant MreB, i.e., the actin-like protein that in rod-shaped model bacteria provides the dynamic cytoskeletal scaffold to maintain the rod shape (119). The second difference is that, despite previous reports (2), both components of the elongasome and divisome show a clear septal localization, with no substantial variation in timing of recruitment, although they may show differences in localization profile. The latter is best reflected by the specific midcell localization of peptidoglycan synthases PBP2b and PBP2x, which are involved with peripheral and septal cell wall growth, respectively (7). Importantly, these differences from rods have led to the proposal that in *S. pneumoniae* and other oval-shaped cocci, the elongation and septal machineries coexist in a single, intimately interconnected and tightly regulated complex, or supercomplex (Fig. 5) rather than in two separated ones (39, 115). Subsequent studies supported this model and provided insights into the coordination and regulation of the complex during the cell cycle (120–123).

Information regarding gene essentiality among *S. pneumoniae* proteins involved in cell growth and division is available thanks to both global and dedicated studies. However, it was not possible to assess directly the effect of inactivation of these genes until genetic systems allowing the generation of merodiploids and conditional lethal mutants became available. Moreover, recent work employing a clustered regularly interspaced short palindromic repeat interference method (41) provides an additional powerful tool to confirm results obtained with other methods and identify and characterize new essential genes.

Genes encoding pneumococcal elongasome proteins were found to be essential in *S. pneumoniae* strain D39 (53, 123, 124). However, some of these genes were not essential in strain R6, a laboratory derivative of D39, and similar strains (53, 122–124). Presumably, these laboratory strains contain preexisting suppressor mutations that could compensate for the loss of otherwise essential genes. Interestingly, inactivation of *mreC*, *mreD*, *mltG*, and *rodZ* in *S. pneumoniae* D39 is directly suppressed by mutations that inactivate *pbp1a* function or upon *pbp1a* deletion (53, 124). Strain R6 contains an allelic variant of PBP1a (T124A D388E), the functional importance of which has yet to be determined. In agreement, allele swapping experiments showed that the *pbp1a* allele from R6 was sufficient to compensate for *mreCD* loss in the R6 genetic background but less so in the D39 background, suggesting that other mutations present in R6 must be involved in the strong suppression of *mreCD* essentiality (124). This result is also supported by the fact that *pbp2a*, which shows a synthetic lethal relationship with *pbp1a*, can be readily inactivated in strain R6 (44).

A previously uncharacterized gene, *spd_0768* (*spr0777* in R6), encoding the membrane protein named CozE (for coordinator of zonal elongation) was identified in a Tn-seq screen for genes dispensable in D39 with inactivated *pbp1a* (118). Inactivation of *cozE* or *mreCD* in the presence of PBP1a resulted in an aberrant relocalization of PBP1a and peptidoglycan synthesis from midcell to the whole cell periphery, and cells became increasingly spherical before they lysed. Similar to *mreCD*, *cozE* is dispensable in the R6 genetic background. CozE interacts directly with MreCD and PBP1a.

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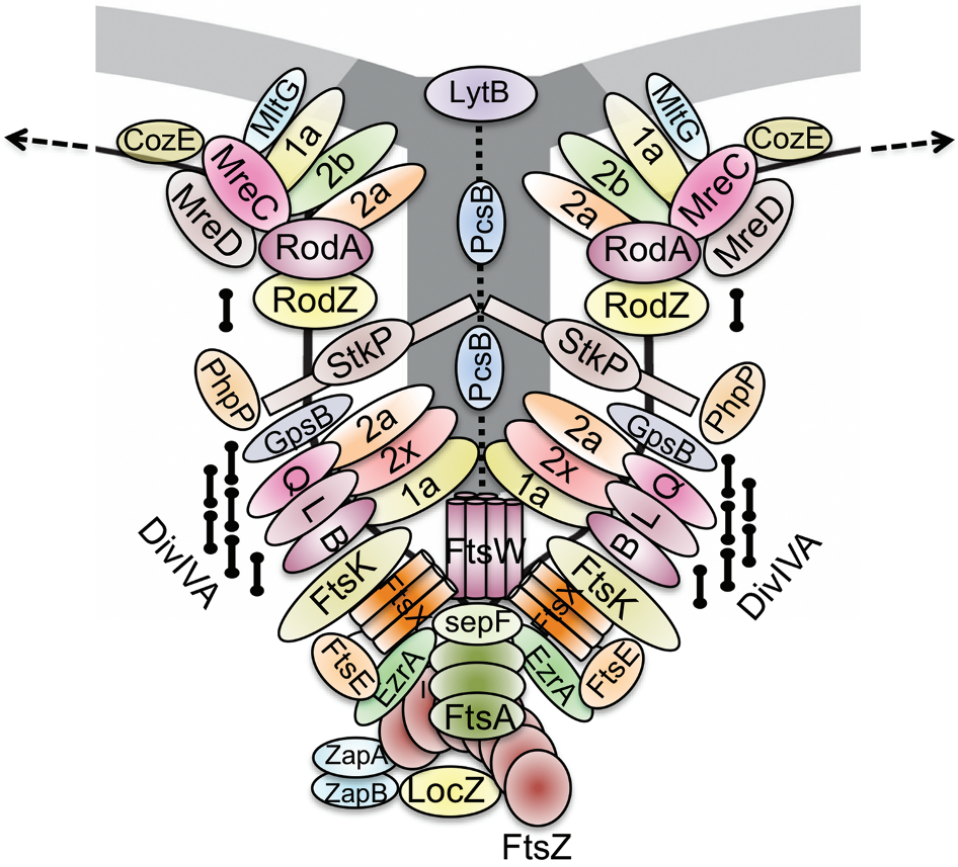


FIGURE 5 Cartoon of a cell wall growth and division complex at midcell showing elongation and cell division proteins and the peptidoglycan hydrolases PcsB and LytB, which cleave the septum for pole formation and cell separation.

Together, the results were interpreted as CozE being part of a coordination mechanism, critical for proper zonal cell wall synthesis, by connecting PBP1a with the other components of the elongation machinery via direct interactions (118).

With the exception of *pcsB* and *gpsB*, which showed strain-dependent essentiality, inactivation of cell division genes was more consistent between strains. Of these, *ftsZ*, *ftsA*, *ezrA*, *ftsEX*, *ftsL*, *ftsB* (*divIC*), and *pbp2x* were demonstrated to be essential in different commonly used genetic backgrounds (7, 45, 46, 54, 121, 125).

As in other bacteria, in *S. pneumoniae*, cell division starts with the localization of the tubulin-like FtsZ and the actin-like FtsA cell division proteins (126) that assemble at midcell to form the Z-ring required for the recruitment of the other components to complete cytokinesis.

Photoactivated localization microscopy showed that as in other bacteria, the Z-ring in *S. pneumoniae* displayed a patchy structure rather than forming a homogenous ring, providing important insight into its *in vivo* organization (127). Following the fluorescent tagged-FtsZ localization and the Z-ring diameter, it was observed that FtsZ molecules assemble at midcell at the beginning of the cell cycle to form a single patchy ring, which thickens and then disassembles during constriction, while new Z-rings assemble at the future division sites (or new equators) of the newborn cells. Notably, unconstricted double Z-rings, suggesting short-lived intermediates, were observed in a small percentage of cells at midcell but not at the future division site (127).

Once the Z-ring is formed, the later pneumococcal cell division proteins localize to midcell, and a few to midcell and cell poles, in exponentially growing pneumococcal cells. A hierarchical order of recruitment to the septum has not been determined, and it may not even be a linear sequence of events, although colocalization studies based on fluorescence microscopy suggest that divisome assembly occurs in at least two steps. In the absence of MreB, it is still unknown how elongation components are targeted to midcell and how lateral and septal growth are coordinated. FtsZ is believed to be the best candidate to act as a scaffold to coordinate both side-wall and septal synthesis (127), although this role has not been experimentally verified, and the effect on *S. pneumoniae* cells of depletion of essential proteins involved in the initial steps of cell division were not available until recently.

A recent study characterized conditional lethal mutants for the cell division protein FtsA, providing insights about how integration of the elongation and septation machineries at a single mid-cell location in *S. pneumoniae* may result in a distinct cell growth pattern characteristic of oval-shaped cocci. Unexpectedly, the complete depletion of FtsA in the pneumococcus resulted in cell ballooning and, ultimately, lysis, in sharp contrast to the cell filamentation phenotype observed in rods (121). FtsZ rings and peptidoglycan synthesis delocalized upon FtsA inactivation, suggesting that the cells could neither elongate nor divide. In contrast, inactivation of genes encoding other cell division components, such as GpsB (5) and SepF (128), which in *B. subtilis* are

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synthetically lethal with *ftsA* (129, 130), resulted in enlarged and elongated cells, with multiple FtsZ rings but not cell ballooning or lysis. Consistent with these results, and in contrast to the model rods where FtsA localizes after FtsZ, in *S. pneumoniae*, FtsZ and FtsA are targeted to midcell at the earliest stages of the process, and both form a ring that remains colocalized throughout the cell cycle, while GpsB and SepF are recruited later, after the Z-ring is assembled (121).

Taken together, these observations led to the hypothesis that in oval-shaped cocci, the actin-like FtsA, and not FtsZ, could play a major role in coordinating peripheral and septal peptidoglycan synthesis, likely carrying out a role similar to the actin-like MreB in preseptal synthesis in *E. coli*. However, since FtsA is needed to tether FtsZ to the membrane (131) and FtsZ may be required for FtsA to be targeted to the septum, additional work is necessary to distinguish any direct role for FtsA in coordinating both modes of peptidoglycan synthesis from that of FtsZ.

While the elucidation of the mechanisms that in *S. pneumoniae* control the intimate coordination between the two peptidoglycan biosynthetic machineries still awaits further study, new insights came from an unanticipated source, the screening for mutations that bypass the need for PBP2b. This approach identified the membrane-bound, likely endolytic transglycosylase, MltG (see above), which is involved in cell elongation (53). More recently, the inactivation of two other genes, *spd_0675* and *spd_1849* (*spr0683* and *spr1851* in R6), was shown to remove the requirement for PBP2b, MreCD, RodA, RodZ, and GpsB in *S. pneumoniae* D39 (123). The corresponding proteins, named KhpA and KhpB, form an RNA-binding complex which regulates cell division posttranscriptionally. Inactivation of *khpA* or *khpB* resulted in cells with a significant reduction in cell volume, but they overall maintained a normal aspect ratio while inducing a strong increase in the expression of the WalRK cell wall stress regulon and modulating the level of FtsA through a posttranscriptional mechanism. Indeed, overproduction of FtsA was found necessary and sufficient to compensate for the loss of PBP2b, MreC, MreD, and RodA but not GpsB, indicating that KhpAB must also regulate other genes in addition to *ftsA* (123).

Two independent studies also identified Spr1851/Spd_1849, either as a novel substrate for phosphorylation by the Ser/Thr protein kinase, StkP, in the laboratory strain *S. pneumoniae* Rx1 (132) or as a suppressor of *pbp2b* in the R704 derivative of laboratory strain R6 (133) and named it Jag (for Jag-domain protein) or EloR (for elongasome-regulating protein), respectively. Jag was found to be phosphorylated at least at T89 (132). Experiments with null or phosphoablative (T89A) alleles of *eloR* in the R704 strain revealed that both *pbp2b* and *rodA* could be deleted, while its phosphomimetic form (T89D or T89E) could only be tolerated in strains that acquired suppressor mutations in *mreC* and *rodZ* (133). In contrast, no phenotype was observed in either KhpB phosphoablative or phosphomimetic derivatives in *S. pneumoniae* D39, indicating that T89~P phosphorylation of KhpB is not necessary for its function in the progenitor strain background (123). Despite discrepancies between strains, these studies highlight the crucial yet complex role of StkP in the regulation of the pneumococcal cell cycle.

Regulators of Cell Wall Growth and Division and Role of Phosphorylation

Similar to other Gram-positive bacteria, *S. pneumoniae* possesses, in addition to histidine kinases of two-component systems, a conserved signaling system consisting of a Ser/Thr protein kinase, StkP, and a cognate PP2C-type phosphatase, PhpP (134, 135). StkP belongs to the subfamily of eukaryotic-type Ser/Thr kinases, ESTKs, and consists of a cytoplasmic kinase domain, a transmembrane region, and a C-terminal part outside the cell made up of four PASTA domains, similar to the two PASTA domains in PBP2x (136).

StkP acts as a dimer and forms a signaling pair with PhpP (137, 138). Several StkP substrates playing a role in cell wall metabolism and cell division were identified to be phosphorylated *in vivo* in a global study of the pneumococcal phosphoproteome (139). However, only some of them have been confirmed to be specifically phosphorylated by StkP *in vitro* and/or *in vivo*. These include the phosphoglucosamine mutase GlmM, the peptidoglycan precursor biosynthesis enzyme MurC, the cell division proteins DivIVA (135, 140–144), the recently discovered substrates MapZ/LocZ (145, 146), and Jag/EloR/KhpB (123, 132, 133, 147). Although StkP is not essential in *S. pneumoniae*, the multiple phenotypes of an *stkP* mutant suggested that it was involved in the regulation of cell growth and cell division (140, 141), virulence, competence (134), and stress resistance (148). It was later shown that both StkP and its cognate PhpP localize to the division site with active peptidoglycan synthesis and that the PASTA domains of StkP were required for its septal localization, indicating that they bind to newly synthesized and still un-cross-linked peptidoglycan chains *in vivo*. Inactivation of *stkP*, or overproduction of PhpP, resulted in elongated cells with multiple and often unconstricted division rings, perturbed in cell wall synthesis. In contrast, *S. pneumoniae* cells overproducing StkP, or lacking functional PhpP, were significantly smaller and rounder (132, 143). These data indicate that StkP and PhpP play an important role in coordinating cell wall synthesis during growth and division to achieve and maintain the characteristic ovococcal shape. StkP was proposed to act as a molecular switch that, through phosphorylation of key division substrates, signals the shift from peripheral to septal cell wall synthesis (143).

A parallel study of the role of StkP in pneumococcal cell division partially confirmed these results and proposed diverse functions for the different StkP domains in the R6-derived strain R800 (142). In this strain, deletion of the *stkP* gene or expression of a truncated protein lacking the kinase domain (StkP-PASTA-TMH) resulted in round and chaining cells rather than in elongated cells, while the elongated morphology was only observed in mutants expressing a truncated protein lacking the PASTA domains (StkP-KD-TMH) or the catalytically inactivated StkP(K42M) (142), in agreement with what was already reported for the *S. pneumoniae* mutant StkP(K42R) and other *stkP* mutants lacking the PASTA domains obtained in different genetic backgrounds (140, 141, 143). The round and chaining phenotype, connected with the *stkP* deletion or truncation (142), was not observed in other studies and was interpreted to be likely due to differences in genetic background, growth conditions, or suppressor mutations in specific *stkP* null strains (39). To date, the molecular mechanisms of StkP's regulatory function on its substrates is still unclear, although recent studies have reported some advances in this direction.

In 2014, Fleurie et al. reported that the DivIVA paralog, GpsB, a putative component of the pneumococcal cell elongation and cell division complex, was required for proper localization and activation of StkP in the *S. pneumoniae* R6 derivative strain R800 (120). R800 cells deleted for *gpsB* were viable but displayed an elongated and twisted-towel phenotype, more severe than that previously reported for *S. pneu-*

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moniae D39 cells depleted of *gpsB*, where this gene is essential (5). Both FtsZ and peptidoglycan synthesis were shown to have a helical pattern of localization in Δ*gpsB* R800 cells, in which phosphorylation of all StkP substrates, including itself, was abolished, consistent with the StkP delocalization observed. The R800 Δ*divIVA* phenotype, instead, was consistent with the Δ*divIVA* phenotype previously reported for *S. pneumoniae* Rx1 strain (3); however, its characteristic rounder and chainy morphology was reinterpreted as a major defect in cell elongation (120) rather than a defect in septum closure and pole maturation as originally proposed (3). In addition, *divIVA* inactivation was found to suppress the elongated phenotype of Δ*gpsB* cells. Together, these observations formed the basis for a model in which DivIVA (required for cell elongation) and GpsB (required for cell division) constituted the mechanism for the molecular switch to coordinate peripheral and septal peptidoglycan synthesis, connecting them to the Z-ring through the cell division protein EzrA: GpsB and DivIVA did not interact directly with FtsZ, but both interacted directly with EzrA, which in turn interacted with FtsZ (120). In this model, GpsB, which has been shown to be phosphorylated in *Streptococcus agalactiae* (149) and *B. subtilis* (150) but not in *S. pneumoniae*, would be needed for StkP localization at midcell and for consequent phosphorylation of itself and its substrates, in particular DivIVA, to activate septum closure. The elongated phenotype of the *S. pneumoniae* R800 cells expressing the phosphoablative DivIVA allele T201A, the only amino acid residue of the protein shown to be phosphorylated (142), further supported this interplay between GpsB, DivIVA, and StkP.

In 2016, Straume et al. reported that *S. pneumoniae* R6 depleted for PBP2b, which grows as long chains of characteristic lentil-shaped cells, has an altered stem peptide composition and is hypersensitive to the peptidoglycan hydrolase CbpD, which is produced during competence (45). They developed a genetic screen to identify genes that, when inactivated, would display a PBP2b-like CbpD-dependent lytic phenotype, encoding proteins functionally related to PBP2b (151). Four out of the twenty proteins tested, RodA, DivIVA, Spr0777 (later identified as Spd_0768/CozE), and MreD (but not of MreC), showed a PBP2b-like competence-induced autolytic response and consequently belong to the cell elongation complex (45). Intriguingly, this study revealed that the *mreD* mutant differed from the expected morphology observed in other studies for *mreD* mutants in the same or similar genetic backgrounds (39, 118, 124), highlighting once more the difficulties in understanding individual effects of gene inactivation in *S. pneumoniae*.

The CbpD-dependent lysis data were then correlated with protein-protein interactions between the identified proteins using the bacterial two-hybrid analysis system (152). PBP2b interacted with RodA, confirming that these proteins have a close functional relationship also in *S. pneumoniae*. Other interactions detected were between DivIVA and PBP2b, between DivIVA and Spr0777(CozE), and the known DivIVA self-interaction (3, 151).

Taken together, these data were interpreted as DivIVA being part of, and required for, proper localization of the pneumococcal elongasome, likely recruited by its interacting partner CozE, supported by the observations that truncations determining the loss of DivIVA localization were also associated with the loss of the elongasome function (151). However, direct experimental proof for the lack of localization of DivIVA in the absence of CozE or known components of the elongasome in the absence of DivIVA, is presently missing. Moreover, Δ*divIVA* cells showed a significantly smaller increase in the amount of branched stem peptide incorporated in the peptidoglycan with respect to that of cells depleted for PBP2b, RodA, or CozE (151). Because DivIVA is dispensable and shows a similar phenotype in all *S. pneumoniae* genetic backgrounds, while other members of the elongation complex are not, a deeper understanding of the localization profile and time of arrival at midcell of the different cell cycle proteins would be needed to reveal their precise functions. The regulatory function in cell wall biosynthesis of its paralog, GpsB, appears to become more clear. Similar to DivIVA, GpsB is also found in Gram-positive bacteria and contains a conserved N-terminal domain, which recognizes the negative membrane curvature at the nascent division septum (130, 153–155).

The *gpsB* gene is essential in *S. pneumoniae* strain D39 but not in its laboratory derivatives, strains R6 and R800 (5, 120). The recent work of Rued et al. (122) showed that GpsB was not strictly required for StkP localization or peptidoglycan synthesis at division septa. GpsB depletion resulted in elongated cells with multiple Z-rings, but no helical localization of FtsZ in twisted-towels cells was observed. However, D39 cells depleted for GpsB showed decreased StkP-mediated phosphorylation that could be suppressed by functional inactivation (mutation or deletion) of its cognate PhpP phosphatase, which also relieved the cells from the need of GpsB. A similar suppressor mutation inactivating PhpP was detected in Δ*gpsB* mutants obtained in the other, commonly used, D39 laboratory derivative, Rx1 (122). Importantly, GpsB forms a complex with EzrA, MreC, StkP, PBP2a, and PBP2x and directly interacts with some of these proteins. Moreover, functional relationships were identified, based on synthetic lethality with Δ*pbp1a* or Δ*pbp2a*, with PBP2a and with PBP2x, in which transpeptidase activity was prevented from localizing at the center of division septa in the absence of GpsB (122). A previous study reported that, although PBP2x is not an obvious StkP substrate, the two proteins formed a complex in the *S. pneumoniae* membrane, and the PASTA domains of StkP interacted directly with the extracellular region of PBP2x, likely allowing correct localization of both proteins at the division site (6).

Taken together, these results highlight a key role for GpsB in regulating the peripheral and septal synthesis through the regulation of PBP activities during the cell cycle, in agreement with what is observed for other Gram-positive bacteria (156, 157). A revised model for GpsB function would then be that, on one hand, GpsB activates PBP2a and StkP-PBP2x to close the septal ring, but on the other hand, it negatively regulates peripheral elongation by inhibiting the activity of PBP2b and MreC (122).

While these aspects of the function of GpsB are relatively well understood, the role of phosphorylation in GpsB function is not. The recent studies of GpsB (120, 122) both agreed that inactivation of GpsB reduces StkP-mediated phosphorylation and, consequently, that GpsB is important for optimal phosphorylation. However, because GpsB does not appear to be a substrate of StkP, the functional relationship between the two proteins remains unclear. Hence, whether StkP phosphorylates DivIVA as part of a molecular switch that together with GpsB and StkP controls peripheral versus septal peptidoglycan synthesis remains controversial. In contrast with what was reported for the *S. pneumoniae* R800 strain (120), Δ*divIVA* mutations were not found to be epistatic to Δ*gpsB* mutations in strains D39 and its derivatives R6 and Rx1 (122), and Δ*divIVA* cells did not show any detectable morphological phenotype when producing the

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phosphoablative DivIVA T201A allele (122, 151), which is also different from what was observed in the *S. pneumoniae* R800 strain (120, 142).

Division Site Selection (MapZ/LocZ) and Origin of Replication

Until recently, no mechanisms for targeting FtsZ and the division complex to the nascent septum were identified, and no counterparts of the widespread or specific systems described in other bacteria, were found to be present in *S. pneumoniae*. Rod-shaped model species use the Min system and nucleoid occlusion to prevent asymmetric cell division and/or prevent the septum from constricting over unsegregated chromosomes, but in contrast to the striking conservation of the proteins that constitute the cell elongation and division complexes, the proteins involved are poorly conserved (reviewed in 158–160).

It came as a surprise when two independent studies reported the characterization of a previously unknown substrate of StkP, annotated as Spr0334 (SPD_0342 in D39), and showed that a specific system for correctly identifying the midcell did indeed exist also in *S. pneumoniae* (145, 146). The protein was named MapZ (for midcell-anchored protein Z) (145) or LocZ (for localizing at the midcell of Z) (146) and was shown to be a membrane protein consisting of a cytoplasmic domain and two extracellular domains separated by a highly entropic serine-rich region. MapZ/LocZ has a rather narrow phylogenetic distribution, being present only in streptococci, enterococci, and lactococci.

Consistent with its proposed function, MapZ/LocZ was found to localize as a ring at midcell at the very early stages of cell division and before FtsZ (145) or FtsZ/FtsA (146). After the equatorial rings of new daughter cells assemble into septal rings, MapZ/LocZ splits into two rings that move away with the so-called wall bands or equatorial rings until they reach the equators of the newly forming daughter cells, marking the future division sites (145, 146).

The gene encoding MapZ/LocZ was found to be dispensable in four genetic backgrounds, R800 (145), D39, R6, and Rx1 (146), and its deletion resulted in misshapen cells often showing misplaced septa (145, 146). Moreover, in the absence of MapZ/LocZ, FtsZ (and FtsA) rings formed but were not localized and/or correctly oriented with respect to the longitudinal axis of the cells. A direct interaction between the MapZ cytoplasmic domain and FtsZ was detected, and MapZ alleles lacking the N-terminal domain were unable to promote correct FtsZ placement (145). Finally, both studies identified that MapZ/LocZ was phosphorylated by StkP at Thr67 and Thr78 and that both phosphomimetic (T67-78E) and phosphoablative (T67-78A) allelic variants localized to midcell, although the phenotypes of the strains differed completely, ranging from severe defects in cell shape and viability with aberrant FtsZ structures and reduced numbers of Z-ring per cell (145) to no evident morphological defects and a wild-type morphology (146).

Thus, although the overall conclusions of the MapZ/LocZ studies agreed on the protein function, the two works differed in important details: (i) the growth and morphological defects are significantly more severe in the R800 background than in the others; (ii) in R800 there is a third MapZ ring forming at midcell after the initial ring splits into two and moves apart from the cell center, but this was not seen for LocZ in other strains; (iii) the relocalization of MapZ at the midcell (third ring) requires that the central FtsZ ring splits into two rings that eventually reach the MapZ rings at the equators, whereas in the other strains, FtsZ disassembles from midcell to assemble *ex novo*, guided by LocZ, at the future division sites, as the FtsZ ring at midcell constricts progressively (145). While these issues need to be clarified, further analysis of MapZ, using both a genetic and a structural approach, demonstrated the importance of the second extracellular domain, which shows a marked flexibility at acid pH, while demonstrating that the first extracellular domain is likely a molecular scaffold or pedestal, required to mark the division site (161).

A more recent study indicated that MapZ/LocZ is not involved in division site selection but, rather, in the selection of the correct division plane and that the replication status and the position of the chromosome have, instead, a crucial impact on spatial regulation of cell division. In particular, the position of the chromosome origin of replication precedes MapZ/LocZ at midcell and promotes the use of the correct division site (162). These conclusions were based on the reasoning that Δ*mapZ*/Δ*locZ* mutants are not elongated but, rather, are shorter and with only minor changes in cell morphology with respect to wild-type cells. Advanced fluorescent microscopy and image analysis showed that FtsZ localization is not affected in cells lacking MapZ, suggesting that it would not be critical for accurate timing of the Z-ring assembly at midcell (162). The localization profile of MapZ/LocZ during the cell cycle was consistent with previous reports; however, two and not three MapZ-rings were observed, in line with what was previously observed for *S. pneumoniae* D39 and related derivatives (146) and not for *S. pneumoniae* R800 (145).

The reported mislocalization of FtsZ in Δ*mapZ*/Δ*locZ* cells was attributed to the FtsZ fusion protein used in the previous studies to detect FtsZ localization in the null mutants (145, 146). This consideration applies only to the MapZ study, where a GFP-FtsZ fusion used was the only source of FtsZ in the cell (145), but not to the LocZ study, where the CFP-FtsZ fusion was ectopically produced, in addition to native FtsZ (146). Nevertheless, the Δ*mapZ*/Δ*locZ* phenotype was reinterpreted in a new light and suggested that MapZ/LocZ could function in cell wall remodeling rather than in site selection, similar to peptidoglycan hydrolases and class C PBPs (162). However, this intriguing hypothesis awaits experimental validation.

In conclusion, the study of the cell cycle of *S. pneumoniae* has benefited greatly from work on other organisms, in particular, the model rods with which the pneumococcus has many similarities as well as many marked differences. The gap in knowledge between *S. pneumoniae* and the model organisms is rapidly closing, and it will be interesting to see how further works develop. Regulation of the cell cycle through phosphorylation is a relatively new and active area of research (163) that will likely provide new insights on how *S. pneumoniae* succeeded to manage peripheral and septal growth that emanates from a single insertion site located at the cell center or midcell.

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Streptococcus pneumoniae Capsular Polysaccharide

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19

BACKGROUND

The presence of what is now recognized as the polysaccharide capsule on the surface of *Streptococcus pneumoniae* was noted by Pasteur in the first published description of the organism in 1880, and since that time it has been the direct or indirect focus of intensive investigation [reviewed by Austrian (1, 2)]. Studies during the first three decades of the 20th century demonstrated the existence of multiple capsular serotypes of *S. pneumoniae* and the fact that antibodies to the capsule conferred type-specific protection against challenge in laboratory animals. The capsular material itself was isolated by Dochez and Avery in 1917 (3), but the fact that it was immunogenic led them to believe that this "soluble substance of the pneumococcus" was proteinaceous in nature. It was not until 1925 that Avery and colleagues (4, 5) demonstrated that the pneumococcal capsule consisted of polysaccharide, the first nonprotein antigen to be recognized.

The capsule forms the outermost layer of encapsulated strains of *S. pneumoniae*. It can be up to approximately 400 nm thick, accounting for more than half of the pneumococcal volume (6), and for the vast majority of cases is covalently attached to the outer surface of the cell wall peptidoglycan (7). A total of 98 structurally and serologically distinct capsular polysaccharide (CPS) types have been recognized to date (8), and the chemical structures of the repeat units for approximately 70% of these have been determined (9). The simplest CPS types are linear polymers with repeat units comprising two or more monosaccharides. The more complicated structural types are branched polysaccharides with repeat unit backbones composed of one to six monosaccharides plus additional side chains. Two nomenclature systems for CPS serotypes have been developed, but the Danish system, which combines antigenically cross-reacting types into groups, is now preferred to the American system, which lists serotypes in chronological order of discovery. Examples of some of these structures are shown in Fig. 1.

The polysaccharide capsule has long been considered to be a *sine qua non* of pneumococcal virulence (2). The vast majority of fresh clinical isolates of *S. pneumoniae* are encapsulated, and spontaneous nonencapsulated derivatives of such strains are almost completely avirulent. Indeed, as early as 1931, Avery and Dubos (10) demonstrated that enzymic depolymerization of the CPS of a type 3 pneumococcus increased its 50% lethal dose more than 10^5-fold. Similar virulence defects were later reported for type 2 and 3 pneumococci with defined mutations in genes essential for CPS synthesis (11–14). However, notwithstanding the critical importance of the capsule for invasive disease, "nontypable" *S. pneumoniae* strains have been associated with outbreaks of conjunctivitis and have been isolated from the nasopharynx and middle ear. While some of these isolates may produce novel CPS serotypes that do not react with existing typing sera, most have genetic defects in the *cps* locus, which encodes CPS biosynthesis, or lack the *cps* locus altogether (8, 15, 16).

The clear morphological distinction between encapsulated (smooth) and nonencapsulated (rough) pneumococci, as well as the massive difference in systemic virulence, facilitated early studies of the phenomenon of capsular transformation. This was first demonstrated by Griffith (17), who found that a proportion of mice injected with a mixture of live rough and killed smooth pneumococci died. Smooth pneumococci expressing the same capsular serotype as the killed smooth strain were isolated from the blood of these mice. The transformation reaction was subsequently performed *in vitro* and led to the seminal discovery that the transforming principle, the carrier of genetic information, was in fact DNA (18).

ROLE IN PATHOGENESIS

The pneumococcal capsule is undoubtedly the most important defense against the host immune system and contributes to pathogenesis in multiple ways. The majority of CPS serotypes are highly charged at physiological pH, and this is thought to promote nasopharyngeal colonization by repelling sialic acid-rich mucopolysaccharides, thereby avoiding entrapment of *S. pneumoniae* in nasal mucus and facilitating access and attachment to the surface of epithelial cells (19). CPS inhibits both the classic and alternative complement pathways by limiting binding of immunoglobulins, complement components, and C-reactive protein to deeper bacterial surface structures. It reduces opsonization with C3b/iC3b and physically impairs interactions between bound C3b/iC3b or Fc regions of immunoglobulins with cognate receptors on phagocytic cells (20, 21). The CPS may also contribute to pathogenesis by inhibiting entrapment of pneumococci in neutrophil extracellular traps (22) and by impeding recognition of Toll-like receptor ligands on the bacterial surface, thereby impairing MyD88-mediated antibacterial defense (23).

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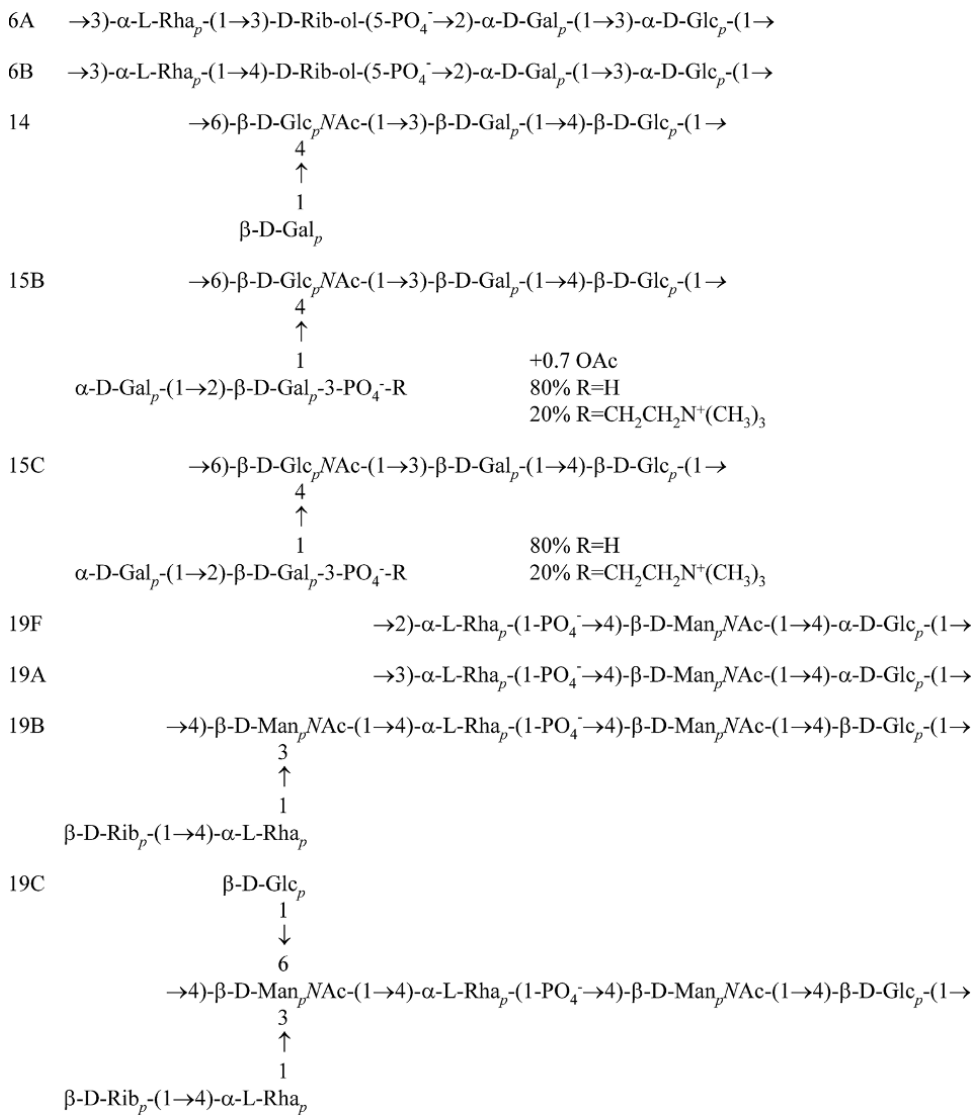


FIGURE 1 Comparison of the CPS biological repeat unit structures of *S. pneumoniae* serotypes 6A, 6B, 14, 15B, 15C 19F, 19A, 19B, and 19C. These are based on published chemical repeat unit structures (9), adjusting for the fact that Glc is the first sugar of the biological repeat unit.

Analysis of a series of mutants producing different amounts of CPS has demonstrated that within a given strain and serotype, the virulence of *S. pneumoniae* is directly related to capsular thickness (24). However, CPS serotypes differ in the efficacy with which they inhibit opsonophagocytosis *in vitro* and in their ability to elicit a humoral immune response (25), which largely correlates with differences between pneumococcal serotypes in their capacity to cause invasive disease (2). Otherwise isogenic pneumococci expressing different CPS serotypes exhibit marked differences in virulence for mice (26, 27), competitiveness in a nasopharyngeal carriage model (28), and efficiency of host-host transmission (29). Capsule-switch mutants expressing CPS serotypes associated with higher rates of invasive disease in humans also bound higher amounts of the alternative complement pathway inhibitor factor H *in vitro* (30). The difference in virulence between pneumococcal serotypes is clearly a function of the biological properties of the CPS itself and is not simply related to the thickness of capsule. For example, type 3 and type 37 pneumococci both produce very thick capsules, but only the former is of high virulence for humans or laboratory animals (2).

CPS-BASED VACCINES

In the immune host, binding of specific antibody to the CPS results in opsonization and rapid clearance of the invading pneumococci. For this reason, a polyvalent pneumococcal CPS vaccine was licensed in the late 1970s. The vaccine provides serotype-specific protection, and the current formulation contains CPS purified from 23 of the 98 recognized serotypes. However, the distribution of *S. pneumoniae* serotypes varies both temporally and geographically. The current vaccine covers approximately 85 to 90% of disease-causing serotypes in the United States and Europe, but in parts of Asia, coverage is <60% (31). Furthermore, serotype prevalence data are scanty for many developing countries, so vaccine coverage is uncertain. The CPS vaccine is protective in healthy adults (against invasive infections caused by those serotypes included in the formulation), but the efficacy is

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much lower in other groups at high risk of pneumococcal infection, such as the elderly; patients with underlying pulmonary, cardiac, or renal disease; and immunocompromised patients. Efficacy is poorest in young children, for whom the CPS vaccine has little or no demonstrable clinical benefit. Polysaccharides are T-cell-independent antigens and thus are poorly immunogenic in young children. This is particularly so for the five pneumococcal CPS types that were the most common causes of invasive disease in children (32). Poor immunogenicity of polysaccharide antigens can be overcome by conjugation to protein carriers, which converts them into T-cell-dependent antigens, resulting in immunoglobulin class switching, affinity maturation, and memory. A highly efficacious 7-valent pneumococcal PS-protein conjugate vaccine (PCV) was licensed in 2000, followed more recently by 10- and 13-valent formulations (9). These PCVs elicit strong, boostable antibody responses to the included serotypes and provide robust, albeit still strictly serotype-dependent, protection. An unexpected benefit of the high levels of serotype-specific immunoglobulin G generated by PCVs has been reduced acquisition of carriage among immunized children that has lowered overall rates of transmission, thereby protecting unimmunized populations against the included serotypes ("herd immunity") (33, 34). However, the clinical benefit of PCVs is being offset by significant increases in both carriage and disease caused by nonvaccine serotypes (35, 36). These occupy the nasopharyngeal niche vacated by the vaccine types as a direct result of immune pressure from widespread use of PCVs (37, 38). Serotype replacement can result from two distinct scenarios. The first of these is by unmasking of strains belonging to nonvaccine serotypes already being carried in the population at low frequency. Elimination of more competitive serotypes covered by the PCV then allows the numbers of the nonvaccine strains to increase in the nasopharynx, increasing the likelihood of both transmission to others in the community and progression to localized or invasive disease. Alternatively, highly competitive strains whose CPS is included in the PCV may acquire the genes encoding synthesis of a nonvaccine serotype CPS by natural transformation with donor DNA from a nonvaccine strain simultaneously present in the nasopharynx. Such "capsule switching" is discussed in more detail later in this chapter. Regardless of the underlying mechanism, widespread use of PCVs is undoubtedly altering the serotype distribution of pneumococcal carriage and disease, which will necessitate ongoing surveillance with a view to periodic adjustment of PCV formulations.

CPS BIOSYNTHESIS GENES

The frequency of transformation of pneumococci from one CPS type to another observed during the classic studies of Griffith, Avery, and others suggested that at least those genes encoding the serotype-specific components of the CPS biosynthetic machinery were closely linked on the chromosome. Additional genetic and biochemical studies by Austrian, Bernheimer, and colleagues (39, 40) demonstrated that during transformation, CPS biosynthesis genes are transferred as a cassette. Advances in gene cloning and DNA sequencing technology in the 1990s resulted in publication of annotated sequences of the complete genetic loci encoding CPS biosynthesis for several *S. pneumoniae* serotypes and functional characterization of some of the genes therein (41–59). Further advances in high-throughput DNA sequencing enabled publication of annotated sequences for the remainder of the known serotypes by the mid 2000s (60). These studies have provided a sound basis for understanding how the pneumococcus acquired the capacity to synthesize such a vast array of CPS serotypes.

The type 3 locus (designated *cps3* [61] or *cap3* [62]) and the type 19F locus (designated *cps19f* [41]) were the first to be completely sequenced and were shown to be located at the same position in the chromosome, between *dexB* and *aliA* (41–44). The *cps* loci from all other serotypes except type 37 have also been localized to the same position on the chromosome. Type 37 has a cryptic copy of the type 33F *cps* locus located between *dexB* and *aliA*; it contains many deletions and point mutations and is not involved in type 37 CPS production. Instead, type 37 CPS biosynthesis is directed by one gene, *tts*, located elsewhere on the chromosome (54). The mechanism of CPS biosynthesis that occurs in type 37 is similar to that in type 3, and this mechanism is distinct from that in all other serotypes analyzed to date, as described below.

CPS Biosynthesis in Types 3 and 37

Type 3 CPS has a simple disaccharide repeat unit comprising glucose (Glc) and glucuronic acid (GlcA). The *cps3*/*cap3* locus contains only three intact genes, which are transcribed as a single unit (42, 43). The first gene (*cps3D* or *cap3A*) encodes the UDP-Glc dehydrogenase required for the synthesis of UDP-GlcA (63). The second gene (*cps3S* or *cap3B*) encodes the type 3 synthase, a processive β-glycosyltransferase that links the alternating Glc and GlcA moieties via distinct glycosidic bonds (41, 42, 64). There is a significant degree of amino acid sequence similarity between Cps3S/Cap3B and other bacterial polysaccharide synthases, including HasA, which synthesizes the hyaluronic acid capsule of group A streptococci (65). These synthases have a common predicted architecture, with four transmembrane domains and a large central cytoplasmic domain. This latter region is believed to contain two distinct catalytic sites capable of forming the two different glycosidic linkages (66). The final complete gene in the *cps3*/*cap3* locus (*cps3U* or *cap3C*) encodes a Glc-1-phosphate uridylyltransferase. However, Cps3U/Cap3C is not essential for CPS biosynthesis in type 3 pneumococci, because inactivation of the gene has no effect on CPS production (42). This enzyme is also encoded by the *galU* gene, which is located elsewhere in the chromosome, and its product (UDP-Glc) is required for CPS biosynthesis in all pneumococci, regardless of serotype (67).

Type 37 CPS is a branched polysaccharide with a linear backbone of Glc with a monosaccharide Glc side chain, and a single gene, *tts*, is responsible for its biosynthesis. Tts is a processive β-glucosyltransferase, which has sequence similarities to a group of plant and bacterial cellulose synthases, especially to the highly conserved motifs thought to be critical for catalysis and/or binding of UDP-Glc (54). The presence of the Cps3S/Cap3B and Tts synthases is sufficient for type 3 and type 37 CPS production, respectively. However, the expression of type 3 CPS also relies on the capacity of the cell to synthesize UDP-GlcA, which is a substrate for Cps3S/Cap3B (68, 69). CPS synthesized by processive transferases does not need a dedicated export system, because the C-terminal hydrophobic domains of these proteins have been predicted to form a pore in the membrane through which the growing polysaccharide chain is extruded as it is synthesized (66).

CPS Biosynthesis in Other Pneumococcal Serotypes

The CPS structures for most other serotypes are more complex than types 3 and 37, with an oligosaccharide repeat

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unit consisting of at least three sugars (9). These repeat units may also contain phosphodiester linkages, pyruvate, glycerol, phosphoryl choline, and/or ribitol. In some serotypes one or more sugars may also be acetylated (9). The respective *cps* loci are also much more complex than those of types 3 and 37, which reflects their more elaborate biosynthetic mechanism. The loci vary from approximately 13 to 30 kb in length and consist of from 10 to more than 20 genes that appear to be arranged as a single transcriptional unit (60). Functions for many of the gene products have been determined experimentally, with the remainder assigned on the basis of amino acid sequence similarities with known proteins and with reference to the structure of their CPS repeat unit. The genes in each *cps* locus are arranged in a common block-wise fashion, as shown for a selection of serotypes in Fig. 2. The first four genes (*cpsABCD* [also referred to as *wzg*, *wzh*, *wzd*, and *wze*, respectively (70)]) comprise the 5′ portion of each *cps* locus; their products have all been implicated in the regulation of CPS production and are discussed later. The central portion of each *cps* locus encodes the glycosyltransferases responsible for assembly of oligosaccharide repeat units, the repeat unit transporter (Wzx), and the polysaccharide polymerase (Wzy). The 3′ regions of the various *cps* loci encode enzymes for synthesis of those activated monosaccharide precursors required for CPS synthesis which are not ubiquitous in *S. pneumoniae*, acetyl transferases and sugar modifying enzymes (60).

Biosynthesis of CPS in these serotypes occurs by a Wzx/Wzy-dependent polymerization pathway, analogous to group 1 CPS biosynthesis in *Escherichia coli* and O-antigen assembly in Gram-negative bacteria (71). The initial step involves transfer of a sugar-1-phosphate moiety to a lipid carrier (undecaprenyl-phosphate) on the cytoplasmic face of the cell membrane. In serotypes containing Glc, this step is carried out by the Glc-1-phosphate (Glc-1-P) transferase CpsE/WchA (60, 72). For those serotypes that lack Glc, the first sugar is linked to the lipid carrier by a *cps* locus transferase of the WciI, WcjG, or WcjH homology groups (60), except for serotype 1, where the function is provided by the glycosyltransferase that initiates teichoic acid biosynthesis

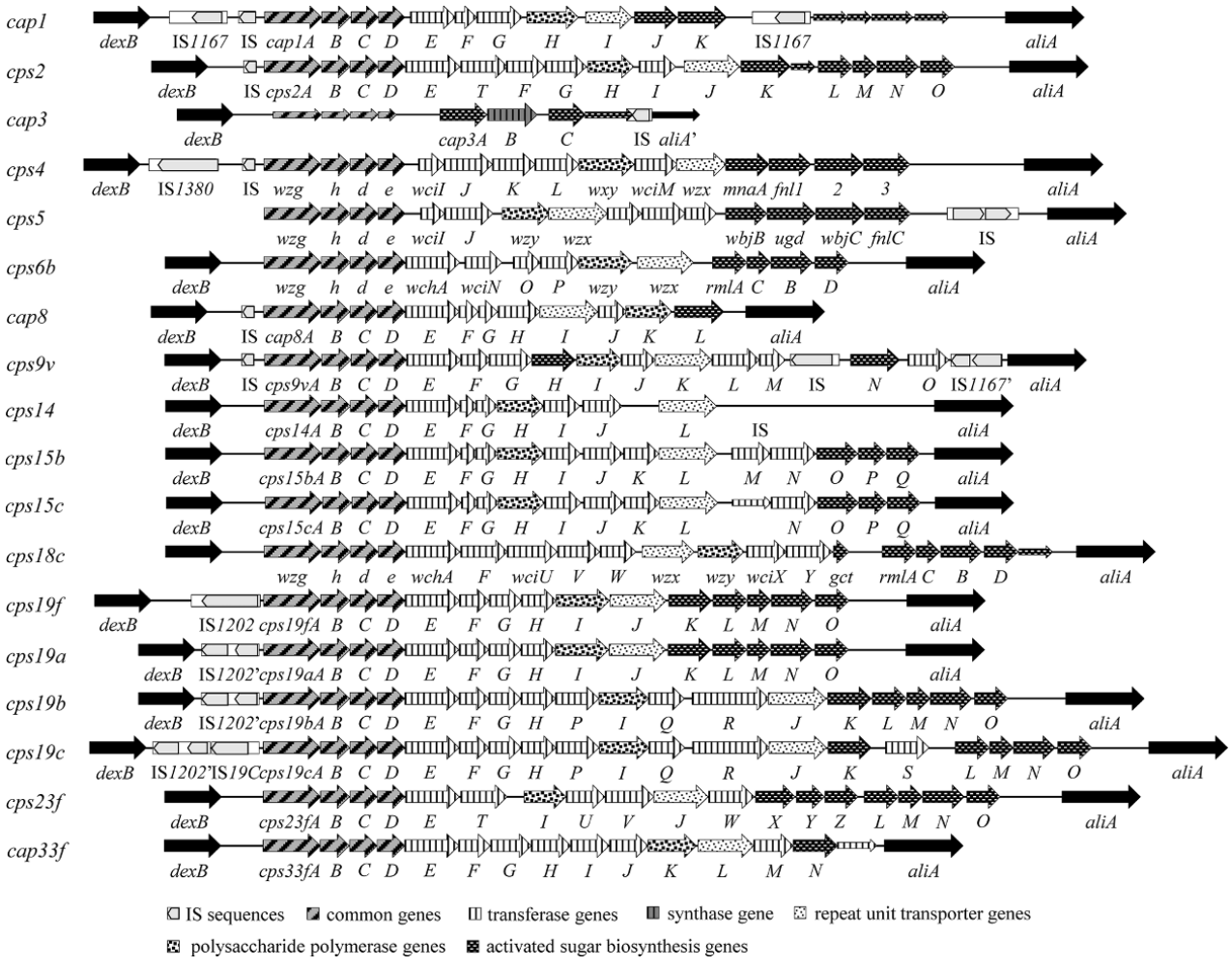


FIGURE 2 Organization of the *cps* loci from selected *S. pneumoniae* serotypes. Gene and locus designations are as published. Open reading frames (ORFs) within the DNA sequence are indicated by large boxed arrows. Highly conserved ORFs, or those encoding proteins belonging to a particular functional group, are identified as shown in the legend at the bottom of the figure. Assignment of an ORF to a given function-related group is based on the published information for each locus as well as on additional database comparisons for some of the ORFs. The short boxed arrows represent cryptic ORFs not required for CPS biosynthesis in the respective serotype.

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(9). These glycosyltransferases are membrane-associated, which facilitates interaction with the lipid carrier (41). Other glycosyltransferases then catalyze the sequential transfer of the other component monosaccharide precursors (synthesized in the cytoplasm by the activated monosaccharide synthesis genes) to form the polysaccharide repeat unit. These lipid-linked repeat units are then translocated from the cytoplasmic to the extracellular side of the cell membrane by the repeat unit transporter ("flippase") Wzx and polymerized in a block-wise fashion by the polysaccharide polymerase Wzy, extending the polysaccharide at the reducing terminus. The final step involves direct covalent linkage of the reducing end sugar (usually Glc) to the GlcNAc residue of the cell wall peptidoglycan via a 1,6 glycosidic bond (73). There is uncertainty regarding which enzyme is responsible for this linkage. CpsA, a LytR-CpsA-Psr protein family member, has been proposed to play a role (74), but CpsA deletion mutants are not defective in CPS attachment (75). A recent proposal is that Wzy may be a bifunctional glycosyltransferase capable of recognizing both peptidoglycan and CPS as acceptor substrates, thereby catalyzing both polymerization of CPS and its linkage to the cell wall (73).

COMPARISON OF *CPS* LOCI FROM DIFFERENT SEROTYPES

The common genomic location and block-wise arrangement of genes in the *cps* loci (see Fig. 2 for examples) has provided insights into the mechanism of generation of capsular diversity. The first five genes, *cpsABCD* and *cpsE/wchA*, are highly conserved in most *cps* loci, except in serotypes that do not contain Glc in their CPS. These serotypes contain *cpsABCD* but not *cpsE/wchA*, which encodes the initiating Glc-1-P transferase (48, 51, 60, 72). The *cpsCDE* genes exist as two distinct classes, suggesting that all pneumococcal serotypes have evolved from two distinct ancestral *cps* types (51, 60). Within one class, the genes share greater than 95% sequence identity but share only 70% identity with those from the other class. Some serotypes, such as type 4, contain a hybrid *cps* locus, where recombination between a class I and a class II locus has occurred. Additionally, the type 3 and type 37 *cps* loci, which do not require the *cpsA-E* gene products for CPS biosynthesis, still retain defective copies of these class I and class II sequences, respectively, suggesting that they also have a common ancestry with other pneumococci of the same class.

The central part of the *cps* locus is serotype-specific, because it encodes glycosyltransferases which exhibit a high degree of substrate specificity and form distinct glycosidic linkages. Accordingly, serotypes only have transferases in common if they have identical glycosidic linkages between identical sugar moieties in their CPS. For example, closely related glycosyltransferases present in the *cps* loci of types 19F, 9N, and 9V are predicted to join Man*N*Ac via a β(1→4) linkage to Glc (44). This central region also encodes the repeat unit transporter/flippase Wzx and the polysaccharide polymerase Wzy. Closely related polymerase and transporter genes only occur between serotypes with near-identical repeat unit backbones, such as types 19F and 19A, types 19B and 19C, and types 14, 15B, and 15C (51, 52, 59). Recognition of the specificity of these genes laid the foundation for definitive determination of serotype by multiplex PCR rather than by classical serological methods (76), and this has been further facilitated by the availability of sequence data for all CPS serotypes.

Activated monosaccharide synthesis genes, located at the 3′ end of the *cps* locus, are more widely distributed among different *S. pneumoniae* serotypes than genes encoding specific glycosyltransferases. Even so, there is substantial deviation among functional homologues. For example, the genes encoding UDP-Glc dehydrogenases from serotypes 1, 2, and 3 exhibit 60 to 90% amino acid sequence identity to each other. Also, the type 19F and type 4 genes encoding UDP-*N*-acetylglucosamine-2-epimerases are only 66% identical. However, the four genes encoding synthesis of dTDP-rhamnose from Glc-1-P have been shown to be closely related with greater than 95% amino acid sequence identity in all serotypes tested that contain rhamnose in their CPS (44). Interestingly, defective copies of these genes have also been identified at the 3′ end of *cps* loci from serotypes not containing rhamnose in their CPS, such as type 1. Their presence suggests that the ancestor of type 1 pneumococci may have been a serotype containing rhamnose (48).

COMPARISON OF *CPS* LOCI FROM CLOSELY RELATED SEROTYPES

Comparison of *cps* loci of serotypes within group 19 enabled one of the earliest reconciliations of genetics with CPS structure. Serogroup 19 comprises serotypes 19F, 19A, 19B, and 19C, and the structures of their CPS repeat units are shown in Fig. 1. Types 19F and 19A have an identical trisaccharide repeat unit and differ only in the nature of the glycosidic linkage formed during polymerization. Both of these types are important causes of human disease, particularly in young children. Types 19B and 19C are only occasionally associated with human disease; they have more complicated CPS repeat units than types 19F and 19A but differ from each other only by one additional Glc side chain in type 19C. The *cps* loci of all four members of group 19 are closely related (41, 44, 45, 52). Thirteen genes (*cps19A-H* and *K-O*) are conserved in all four serogroup members. Nearly all of the common genes from types 19F, 19B, and 19C are >95% identical to each other, whereas those from the type 19A *cps* locus are more divergent. Although the 19A and 19F loci are identical in terms of the number and arrangement of the genes present, sequence homology between individual *cps19a* and *cps19f* genes varies from 70 to 99% (at both the DNA and the deduced amino acid sequence levels). This sequence divergence is surprising given that only an alteration in *cps19fI/wzy*, such that an α (1→3) rather than an α (1→2) linkage is formed during polymerization of the repeat units, is required to change a type 19F pneumococcus into type 19A (52). This suggests either that the type 19F and 19A loci diverged in the distant evolutionary past or that their component genes originated from different sources.

The type 19B and 19C *cps* loci are nearly identical and contain five genes between *cps19H* and *cps19K* unrelated to the genes in the type 19F and 19A *cps* loci (Fig. 2). These genes encode a repeat unit transporter and a polysaccharide polymerase that are specific for type 19B and 19C CPS, as well as two additional putative glycosyltransferases and an unknown protein that might possibly be involved in synthesis of an activated ribose precursor. These five genes encode all of the functions required to convert a type 19F pneumococcus to type 19B (45). The type 19C *cps* locus differs from the type 19B locus only in the insertion of a putative glucosyltransferase gene (*cps19cS*) between *cps19cK* and *cps19cL* (Fig. 2). The presence of this gene accounts for the additional Glc side chain in the otherwise identical repeat unit structures (Fig. 1).

The structures of the type 6A and 6B CPS repeat units differ only in the linkage between the rhamnose and ribitol moieties (Fig. 1), and as expected, the type 6A and 6B *cps* loci

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are very closely related (57, 60). The sequences for the genes encoding the rhamnosyltransferase differ only by a few bases and result in very few amino acid changes. However, these changes are clearly sufficient to alter the serotype-determining linkage. Genetic relationships of *cps* loci within serogroups 9, 12, and 15 and other closely related serotypes have been discussed by Bentley et al. (60).

CAPSULAR TRANSFORMATION *IN VIVO*

There is growing evidence that the phenomenon of capsular transformation first observed by Griffith (17) is a common event *in vivo*, even before the widespread introduction of PCVs which provide additional immune selection. Indeed, the *cps* locus has recently been identified as an evolutionary hot spot with elevated rates of recombination and substitution (77). Even before the advent of high-throughput genome sequencing, the application of molecular typing techniques resulted in the detection of otherwise genetically indistinguishable pneumococci expressing different capsular types. This was particularly evident in clonal groups that are resistant to multiple antibiotics. Indeed, derivatives of a highly successful, multiply resistant type 23F clone (which originated in Spain) expressing types 3, 9N, 14, 19A, and 19F capsules were isolated well before the introduction of PCVs (27, 78–80). Examination of sequence polymorphisms in the conserved regions of the *cps* loci for the parental multiresistant type 23F strain and eight otherwise identical type 19F clinical isolates indicated that in each case, the 5′ recombination occurred upstream of *dexB*. In six of the eight type 19F strains, the 3′ crossover point was downstream of *aliA*. However, in the other two, a recombination crossover point between the introduced type 19F sequences and the type 23F chromosome was identified; this was in *cpsM* in one strain and *cpsN* in the other (81). Thus, capsule switching involves the exchange of very large DNA fragments, ranging from at least 15 kb to over 22.5 kb. The existence of multiple crossover points as well as additional minor polymorphisms within the type 19F-derived *cps* genes also indicated that the eight multiply resistant type 19F strains that were studied arose as a consequence of a minimum of four independent transformation events involving different type 19F donors. It therefore appears that these capsule switching events may be relatively common among pneumococci in nature (81). Multiple serotypes of *S. pneumoniae* are frequently carried concurrently in the human nasopharynx (2), providing ample opportunity for exchange of DNA between types. In addition to enhancing the spread of drug resistance among diverse capsular types, these exchanges provide a mechanism for evasion of serotype-specific host immune defenses, such as those resulting from immunization with PCVs, which provide cover against a limited range of serotypes.

Complete or partial insertion sequences have been located adjacent to many of the *cps* loci, and in some serotypes, the locus is flanked at both the 5′ and 3′ ends by such elements. The *cps*-flanking regions appear to be common targets for insertion elements, and this has led to the suggestion that they may play a role in horizontal transfer of *cps* genes (47, 48). There are precedents for this in other bacteria; the *H. influenzae* type b capsule genes, for example, are located on a 17-kb compound transposon (82). However, the identification of crossover points within the *cps* loci of the type 19F derivatives of the multiresistant type 23F *S. pneumoniae* clone referred to above confirms that at least in these cases, capsular exchange occurred as a consequence of homologous recombination rather than by transposition. Nevertheless, Muñoz et al. (48) demonstrated that IS*1167* sequences flanking the *cap1* locus could direct ectopic integration of these genes into copies of IS*1167* located elsewhere in the pneumococcal chromosome, resulting in genetically binary strains.

The contribution of the CPS itself to *in vivo* recombination is somewhat paradoxical. On one hand, dense genomic sampling of carriage isolates in a refugee camp indicated that the highest frequencies of donation and receipt of recombined DNA fragments occurred in nonencapsulated lineages of *S. pneumoniae*, consistent with the capsule acting as a physical barrier to DNA exchange (83). On the other hand, among encapsulated lineages, recombination was positively associated with serotypes expressing larger capsules and extended nasopharyngeal carriage duration (84). The nasopharyngeal environment is particularly conducive to genetic exchange because not only are multiple *S. pneumoniae* strains carried simultaneously, colocalizing donors and recipients, but transformation frequency itself is enhanced in nasopharyngeal biofilms wherein competence genes are upregulated and capsule thickness is reduced (85). However, the complexity and diversity of pneumococcal *cps* loci can be only partially explained by genetic recombination within the *S. pneumoniae* species. Recent studies have demonstrated the presence of intact *cps* loci genes in a wide range of commensal streptococci. The degree of sequence homology indicates that many pneumococcal CPS serotypes have evolved by import of *cps* fragments from commensal *Streptococcus* species, resulting in a mosaic of genes of different origins (86). Such interspecies recombination is now recognized as having an important role in the evolution of pneumococcal *cps* loci and the emergence of new CPS serotypes (77).

REGULATION OF CPS PRODUCTION

Colonization of the nasopharyngeal mucosa is an essential first step in the pathogenesis of pneumococcal disease and involves direct interaction between pneumococcal adhesins and specific receptors on host epithelial cells. In some cases, asymptomatic carriage progresses to invasive disease, and although the events involved are not fully understood, it is clearly a watershed in the bacteria-host relationship. This transition involves a major switch in the expression of important virulence determinants as the pneumococcus adapts to the altered microenvironment (87, 88). Maximal expression of capsule is clearly important for systemic virulence, but the degree of exposure of other important pneumococcal surface structures, such as phosphoryl choline moieties on cell wall teichoic acid and various surface proteins involved in adherence to host cells, may also be influenced by capsular thickness. Nonencapsulated pneumococci exhibit higher adherence to, and invasion of, human respiratory epithelial cells *in vitro* than otherwise isogenic derivatives expressing either type 3 or type 19F capsules (89). Thus, the very feature (encapsulation) that is most important for systemic virulence of *S. pneumoniae* could be disadvantageous during the colonization or cellular invasion phase. Thus, regulation of CPS production is crucial for survival of the pneumococcus in different host environments.

Role of *cpsABCD*

As mentioned previously, the first four genes of the *cps* locus, *cpsABCD* (*wzg*, *wzh*, *wzd*, and *wze*) are common to all pneumococcal serotypes, except types 3 and 37, and deletion of any one of these genes has been shown to affect the level of CPS expression (90). Homologues of *cpsB-D* are found in most Gram-positive capsule loci, but *cps*A homologues have only been identified in the *cps* loci of other members of

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the genus *Streptococcus*. Interestingly, although CpsA resembles a *Bacillus subtilis* transcriptional attenuator, its homologue in group B streptococci appears to function as a transcriptional enhancer (91). To date, *cpsA* has not been shown to affect *cps* transcription in *S. pneumoniae*. Type 19F and type 2 mutants in which the *cpsA* gene had been deleted produced reduced levels of CPS (approximately two-thirds that of the respective wild-type strains) (14, 90, 92). However, there were no obvious differences in the amounts of CpsB and CpsD proteins detected by Western blotting in any of the strains (90, 93). Nor is there convincing evidence that pneumococcal CpsA catalyzes the linkage of CPS to peptidoglycan, as discussed above. Thus, the precise function of CpsA in pneumococcal CPS biosynthesis or regulation remains uncertain.

CpsB, CpsC, and CpsD collectively constitute a phosphorelay system implicated in modulation of CPS production at a posttranscriptional level. CpsC and CpsD belong to the PCP2b family of polysaccharide copolymerases and were predicted to function together in polymerization and export of CPS in a fashion similar to PCP2a proteins, such as Wzc and ExoP, in the production of CPS and exopolysaccharide in Gram-negative bacteria (41, 71, 94, 95). CpsD has similarities to the C-terminal domain of PCP2a proteins and is an autophosphorylating protein-tyrosine kinase (90, 95). These kinases contain Walker A and B ATP-binding motifs and a tyrosine-rich region at the C-terminus which becomes phosphorylated at multiple tyrosine residues, initially via the ATP-binding domain and also via transphosphorylation which occurs independently of the ATP-binding domain (11, 90, 92). In *S. pneumoniae*, the tyrosine-rich region of CpsD is arranged as an ordered (YGX) motif varying from two to four repeats in different serotypes, with four being the most prevalent. CpsC is a membrane protein with two membrane-spanning hydrophobic domains; the N and C termini are located in the cytoplasm, while the central portion is exposed on the external side of the cell membrane. It has similarities to PCP1/Wzz proteins associated with polymerization of O-antigen in Gram-negative bacteria and to the N-terminal domain of PCP2a proteins (95). CpsC is required for initial CpsD tyrosine-autophosphorylation but is not required for transphosphorylation between CpsD proteins (90). CpsB has been identified as a manganese-dependent phosphotyrosine-protein phosphatase belonging to the PHP (polymerase and histidinol phosphatase) family of phosphoesterases and is required to dephosphorylate CpsD (96). CpsB is also thought to be able to bind CpsD and prevent transphosphorylation between CpsD proteins (11).

Although CpsB, CpsC, and CpsD clearly have a role in modulation of CPS production, the precise manner in which this is achieved remains uncertain, and the situation has been complicated by phenotypic differences between mutants constructed in different strain backgrounds. Indeed, CpsD phosphorylation has been reported to have opposite effects on total CPS production in some studies (90, 93). The capacity of CpsD to cycle between the phosphorylated and dephosphorylated state may facilitate interactions with the polymerization machinery that modulate CPS chain length and cell wall ligation (14, 75, 90, 92, 93). Regardless of its actual role, loss of CPS regulation via phosphorylation of tyrosine in CpsD affects the ability of *S. pneumoniae* to translocate from the lungs into the bloodstream in a murine intranasal challenge model (14). Interestingly, CpsD phosphorylation has been reported to be suppressed by high oxygen concentrations via modulation of CpsB phosphatase activity, which may imply a role in the adjustment of CPS expression between host sites that differ in oxygen availability (97, 98).

Recent advances in cell imaging technologies have provided novel insights into the collective function of the CpsBCD phosphorelay system. CpsC and CpsD have been shown to localize to the division septum in growing pneumococcal cells, and this depends on a functional ATP binding domain in CpsD. In the absence of either CpsC or CpsD, CPS was still produced and linked to the cell surface, but it was absent from the division septum, suggesting that the two proteins are spatial regulators of CPS synthesis (99). Further studies showed that the cytoplasmic C-terminal portion of CpsC required for CpsD autophosphorylation was essential for localization at mid-cell and that the CpsCD complex captures the polymerase Wzy at the division site. Furthermore, dephosphorylation of CpsD impaired both CPS synthesis and cell division itself (100). Collectively, these studies support a model whereby the CpsBCD system ensures that CPS and new cell wall synthesis are colocalized, thereby enabling the newly synthesized cell wall to be promptly masked by CPS to prevent complement deposition at the site (99, 100).

Interestingly, the CpsBCD phosphorelay system has recently been shown to also be capable of phosphorylating and dephosphorylating the major pneumococcal autolysin LytA. LytA amidase activity was stimulated in the phosphorylated state, suggesting a regulatory link between CPS synthesis and LytA-dependent cellular autolysis (101). As mentioned previously, the presence of the pneumococcal capsule significantly impairs both adherence to, and invasion of, epithelial cells *in vitro* (89). However, electron microscopy studies have shown that pneumococci are capable of shedding their capsules at the point of epithelial cell invasion (102). Such capsule shedding is dependent on LytA and protects pneumococci from attack by cationic antimicrobial peptides released in the immediate vicinity by host cells (103). Thus, it may not be a coincidence that the LytA-dependent capsule shedding process is modulated by the CpsBCD phosphorelay (103).

Transcriptional Regulation of the *cps* Locus

The first direct evidence for transcriptional regulation of the *cps* locus in the host environment used quantitative reverse transcriptase PCR (RT-PCR) to show that the level of *cps* mRNA relative to 16S rRNA in pneumococci isolated from the blood of infected mice was approximately 4-fold higher compared to that in pneumococci grown *in vitro* (104). RegM, a homologue of the staphylococcal catabolite control protein CcpA involved in the regulation of sugar metabolism pathways, was also shown to affect transcription of the *cps* locus, suggesting that the carbon source may also influence capsular expression (105).

The *cps* loci are arranged as single transcriptional units in all Wzy-dependent pneumococcal serotypes. The σ^{70} promoter (*cpsp*) is highly conserved, and the transcriptional start site is located about 20 to 30 nucleotides upstream of the initiation codon of the first *cps* gene, *cpsA* (48). The core promoter incorporating the -10 and -35 sequences is essential for *cps* transcription, but sequences immediately upstream also impact the level of transcript (106, 107). The upstream region presumably enables fine-tuning of *cps* transcription, which is necessary for optimal survival of the pneumococcus in diverse host niches (107). Furthermore, sequence polymorphisms in this upstream region result in differences between strains in *cps* transcript levels that correlate with the

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virulence profile *in vivo* (108). A recent study identified a GntR family transcriptional regulator CpsR (*cps* repressor), which is encoded elsewhere on the chromosome but directly binds to a region of DNA approximately 100 nucleotides upstream of the core promoter and represses *cps* transcription (109). A *cpsR* deletion mutant exhibited enhanced CPS production and systemic virulence in mice relative to the wild type but was attenuated in a nasopharyngeal colonization model (109). Interestingly, *in vitro* binding of CpsR to *cpsp* was competitively inhibited by Glc, leading to increased total CPS expression (109). This provides a mechanism whereby CPS expression could be increased *in vivo* when pneumococci enter the bloodstream, where Glc concentrations are maintained at high levels. The competence response regulator ComE has also been shown to be capable of binding to *cpsp* in its phosphorylated form *in vitro*, and a *comE* deletion mutant exhibited increased CPS production (110). Like CpsR, ComE repression of *cps* transcription was alleviated by Glc, but this effect was mediated by repression of *comE* transcription rather than by interfering with the binding of ComE to *cpsp* (110).

Phase Variation and Quorum Sensing

Pneumococci have been shown to undergo bidirectional phase variation between two distinct colonial morphologies, described as "opaque" and "transparent." The transparent phenotype exhibits increased *in vitro* adherence to buccal epithelial cells and cytokine-activated A549 cells relative to opaque variants of the same strain, as well as an enhanced capacity to colonize the nasopharynx of infant rats (111, 112). On the other hand, the opaque form is associated with massively increased virulence in animal models of systemic disease, and this correlates with increased production of CPS relative to cell wall teichoic acid compared with the transparent phenotype. Phase variation also correlated with alteration in the levels of several surface proteins (113). A recent study has shown that the underlying mechanism involves a type I restriction-modification system (SpnIII) with a genetic locus containing inverted repeats that enable spontaneous rearrangement of alternative specificity domain genes. This generates six different SpnIII target specificities, each with distinct genome-wide DNA methylation patterns, gene expression profiles, and virulence phenotypes (114). Moreover, pneumococci were shown to readily switch between SpnIII alleles during progression of disease in a murine model. Differentially expressed genes included the CPS biosynthesis locus *cps*, various sugar transporters, the Mn transporter *psaBCA*, and *luxS* (114). The *luxS* gene is of particular interest because it encodes synthesis of the ubiquitous quorum sensing molecule autoinducer 2 (AI-2), which is known to be an important regulator of biofilm formation and virulence in pneumococci (115). Recent studies showed that AI-2 accumulating in the extracellular compartment is sensed by the pneumococcal fructose-specific phosphotransferase system component FruA, leading to upregulation of the galactose (Gal) ABC transporter and the Leloir pathway, presumably by phosphorylation of the regulator GalR (116). This entire process is repressed in the presence of Glc. However, Gal is an important carbon source *for S. pneumoniae* in the respiratory tract, where Glc is very scarce, and AI-2-mediated quorum sensing is essential for Gal uptake and metabolism. The Leloir pathway converts Gal to Gal-1-P and then to Glc-1-P, the precursor for all the activated nucleotide sugars required for CPS synthesis. In the absence of Glc, Leloir pathway upregulation increases total CPS production and confers a hypervirulent phenotype *in vivo* (116). Two other proteins involved in sugar metabolism have been shown to affect CPS production. PGM is the phosphoglucomutase, which catalyzes the conversion of Glc-6-P (derived from imported Glc when it is available) to Glc-1-P, and GalU is a Glc-1-P uridylyltransferase that catalyzes the formation of UDP-Glc from Glc-1-P. Pneumococcal mutants in which either the *galU* or the *pgm* genes were disrupted produced almost no CPS and exhibited growth defects (12, 67). Glc-1-P is also a precursor for synthesis of other cellular structures, such as teichoic acid, so limiting the supply of this precursor would be expected to impact heavily on CPS production in the pneumococcus. Indirect modulation of CPS production by controlled availability of precursors or cofactors may be one of the regulatory mechanisms used by the pneumococcus.

CONCLUDING REMARKS

In this article we have summarized the current state of knowledge of the CPS of *S. pneumoniae*, with particular reference to the genes encoding biosynthesis of this most important of all pneumococcal surface antigens. Notwithstanding the insights gained from the elegant classical genetic studies of CPS production carried out before the early 1970s, research in the past 2 decades has been revolutionized by the advent of modern methods for gene cloning, DNA amplification, and high-throughput sequence analysis. The complete nucleotide sequences of the *cps* loci of all of the known serotypes are available, and although the functions of many of the individual genes in these loci await confirmation by conventional biochemical and genetic analysis, access to the enormous body of information on sequence databases now available, combined with knowledge of the chemical structures of many of the CPS repeat units, has enabled accurate predictions of function for a significant proportion of these genes. It has also been possible to predict the mechanisms of CPS biosynthesis in pneumococci by analogy with those operating in Gram-negative bacteria. The existence of two distinct mechanisms of CPS biosynthesis in *S. pneumoniae* has already been recognized. However, much remains to be learned about the precise molecular events involved in both of these processes and about how CPS production is regulated in response to the microenvironmental conditions to which pneumococci are exposed *in vivo*. Given the importance of capsules to the virulence of *S. pneumoniae* and several other Gram-positive pathogens, such conserved components of the CPS biosynthesis machinery may prove to be useful targets for novel antimicrobial strategies. Furthermore, the availability of thousands of complete pneumococcal genome sequences in recent years is providing insights into the continuing evolution of diversity of pneumococcal CPS serotypes, particularly in the face of strong immune selection provided by PCVs. A better understanding of these processes will undoubtedly inform ongoing refinement of pneumococcal vaccination strategies.

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Streptococcus pneumoniae: Invasion and Inflammation

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20

INTRODUCTION

Streptococcus pneumoniae (the pneumococcus) is a leading cause of otitis media (OM), community-acquired pneumonia, bacteremia, and meningitis. The pneumococcus is a human-specific pathogen which colonizes the nasopharynx and spreads between hosts through aerosols and potentially through the contamination of objects with mucosal secretions if the bacteria is living within a biofilm (1–3). Rates of carriage vary from 5 to 10% of healthy adults to 20 to 40% of healthy children. However, these numbers can vary widely based on where the samples are collected (4–7). Risk factors associated with higher rates of carriage include race (particularly Australian Aboriginals and Native Americans) (8–12), infancy (13, 14), season, with higher carriage during winter months (13), and crowded areas such as childcare centers, with estimates suggesting that 40 to 60% of children who attend childcare are colonized (15). Duration of colonization decreases with age and varies from 2 weeks to 4 months (14, 16, 17). The introduction of pneumococcal conjugate vaccines has reduced carriage rates for serotypes covered by the vaccine, while nonvaccine serotypes have emerged to occupy this empty niche (18). Nasopharyngeal colonization is usually asymptomatic (19).

Invasive pneumococcal disease (IPD) occurs as a result of the spread of bacteria from the nasopharynx to other parts of the body, including the lungs, blood, and brain. Infants, the elderly, and immunocompromised individuals are at an increased risk for developing IPD (20–22). Pneumococcal models of invasive disease must account for not only the commensal nature of the bacteria, but also the wide spectrum of disease the pneumococcus is capable of causing. Colonization is a prerequisite for IPD, and while the incidence of infection is relatively low, high rates of colonization result in extensive morbidity and mortality that is a global concern. Worldwide, it is estimated that *S. pneumoniae* is responsible for 15 cases of IPD per 100,000 people per year (23) and over a million deaths annually. As of 2004, in the United States, it is estimated that the pneumococcus was responsible for more than 1.5 million cases of OM and 800,000 cases of pneumonia (24). Direct medical costs resulting from infections totaled $3.5 billion (24). The World Health Organization estimates that close to half a million children under the age of 5 years die annually as a result of *S. pneumoniae* infection (https://www.cdc.gov/pneumococcal/global.html). Pneumococcal bacteremia and meningitis are also responsible for significant mortality, particularly in the elderly, for whom rates may be as high as 60% and 80%, respectively (25).

In this article, we review the colonization and spread of *S. pneumoniae* from one anatomical site to another. We also discuss the mechanistic basis of inflammation and cytotoxicity resulting from invasive pneumococcal infection.

TRAFFICKING OF PNEUMOCOCCI THROUGH THE RESPIRATORY TRACT

Interactions with Epithelial Cells of the Nasopharynx

For over a century, *S. pneumoniae* has been categorized by serology, with distinct serotypes identified on the basis of the more than 90 immunologically and chemically distinct polysaccharide capsules that surround and protect the bacteria from phagocytosis (26). The capsular polysaccharide (CPS) is also the basis of the current pneumococcal vaccines. Prior to the introduction of the 13-valent pneumococcal conjugate vaccine in 2010, studies found that only a small subset of the many capsular types was responsible for the majority of IPD isolates (27). The vast majority of pneumococci colonize the nasopharynx for up to 6 weeks and are then cleared with no systemic symptoms in the host (1, 28). IPD is thought to occur most frequently early after the acquisition of a new capsular serotype, as evidenced by shifts in the strains most commonly isolated from IPD patients after vaccine introduction (22, 29–31). Furthermore, attack rates are higher for serotypes that are carried for shorter periods of time versus those that colonize for extended periods (28).

S. pneumoniae undergoes spontaneous phase variation alternating between a transparent and opaque colony phenotype which can be visualized microscopically by oblique transmitted light (32). The various phenotypes occupy different niches based on selection, with the transparent phenotype being the predominant phase in the nasopharynx (32), while the opaque phase is isolated from blood samples (33). The transparent phenotype expresses increased amounts of phosphorylcholine (ChoP) (34) and choline-binding protein A (CbpA) on the surface (35), both of which function as adhesins and contribute to the ability of the bacteria to colonize the nasopharynx. The opaque phenotype expresses increased levels of capsule and pneumococcal surface protein A (PspA), which are important factors for survival in the blood. Phase-variation is one of the mechanisms by which the pneumococcus alternates between an

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adhesive phenotype best suited for the nasopharynx and a phagocytosis-resistant phenotype that can survive in the blood.

ChoP decorates the cell wall (Fig. 1) and serves as a docking group for a set of 15 secreted proteins, termed choline-binding proteins (CBPs) (36). Among the CBPs, CbpA is a major pneumococcal adhesin (37) expressed predominantly in the transparent phenotype (35). Pneumococci lacking CbpA are not only largely unable to bind to the nasopharynx, but also have a diminished capacity to colonize the lower respiratory tract and cause pneumonia (35, 38). *In vitro*, CbpA mutants show decreased binding to both nasopharyngeal epithelial cells and activated type II human lung cell lines (37). Similarly, the CBPs LytB, LytC, CbpD, CbpE, and CbpG also contribute to nasopharyngeal colonization when tested in rat pups. LytB functions as a glucosaminidase (39), whereas LytC is a lysozyme (40) and CbpE is a choline esterase; all three enzymes are active on the bacterial cell wall (37). CbpG is a serine protease that in addition to contributing to nasopharyngeal colonization is required for development of high-grade bacteremia (41).

Other virulence factors affecting the capacity of the pneumococcus to colonize the nasopharynx include sIgA1 proteases (42). IgA antibodies are the predominant type found at mucosal surfaces and serve to aggregate and opsonize pathogens. sIgA1 proteases neutralize the activity of sIgA by cleaving the Fc portion of human IgA1 (43). Hydrolyzed sIgA1 also contributes to pneumococcal adhesion. Fab fragments remaining on the surface of the bacteria after sIgA proteolysis neutralize the negative charge of the capsule and enhance adherence (42).

The two-component system CiaR/H is required for efficient colonization and regulates gene expression in response to oxidative stress (44). Among the many genes regulated by CiaR/H is *htrA*. HtrA, or high-temperature requirement A, is a heat-inducible serine protease that has both proteolytic and chaperone activities. HtrA assists in survival in the presence of environmental factors such as oxidative stress, osmotic

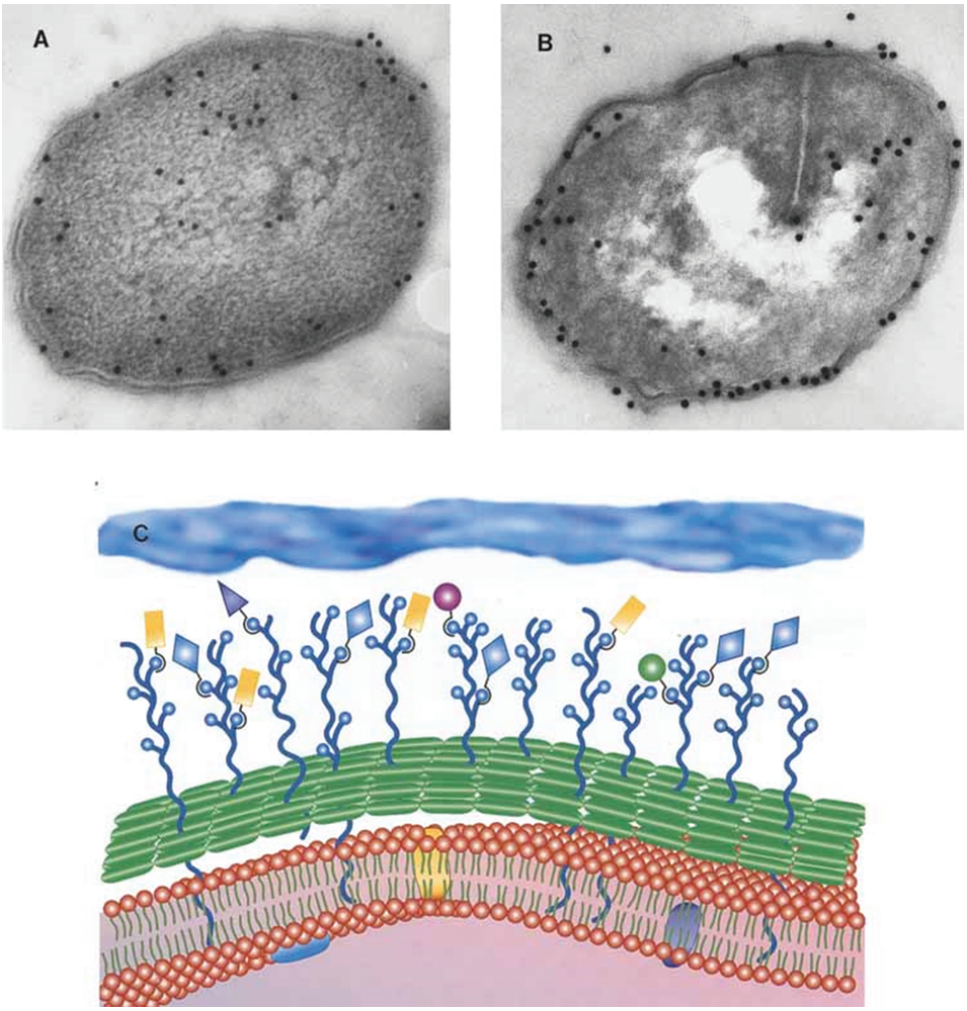


FIGURE 1 Immunohistochemical and schematic depiction of the choline biology of the pneumococcal surface. Immunogold labeling of pneumococci with (A) TEPC-15 antibody recognizing free choline and (B) antiautolysin antibody. These two images contrast free (A) versus CBP-bound (B) choline. (C) Schematic view of the capsule (blue), cell wall (green), and membrane (red). The teichoic and lipoteichoic acids are indicated as dark blue lines bearing choline (circles). A proportion of these are capped by choline-binding proteins. Courtesy of K.G. Murti, St. Jude Electron Microscopy Core Facility.

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stress, and elevated temperatures (45). Deletion of the CiaR/H operon results in a 25-fold decrease in levels of *htrA* expression and a 1,000-fold decrease in the number of bacteria colonizing the nasopharynx. Deletion of *htrA* alone results in a 100-fold decrease in nasopharyngeal colonization, indicating that CiaR/H regulates other factors that contribute to colonization (44). Microarray analysis during bacterial adhesion to epithelial cells *in vitro* has revealed a number of genes with enhanced expression, including CbpA and HtrA (46).

A second regulator important in nasopharyngeal colonization is RlrA, which regulates the transcription of the pilus-1 structural subunit genes *rrg*(A to C) (47, 48). Pili are found in only 20% of *S. pneumoniae* strains but are common in strains belonging to antibiotic-resistant clones (49, 50). Piliated strains show stronger adherence to lung epithelia cells and out-compete nonpiliated strains in mixed infection models (47). Furthermore, introduction of the pilus-1 locus into a nonpiliated strain increased adherence to the lung epithelial cells (47). Pilus-1 expression levels have been shown to change during the different stages of infection, with higher expression during early colonization and lower expression as the disease progresses (51).

Other pneumococcal factors that may aid adherence and colonization of the nasopharynx include PspK, SlrA, and PmpA. PspK, pneumococcal surface protein K, has been suggested to help in the colonization of the nasopharynx in unencapsulated strains by mediating adherence to epithelial cells (52). PspK has also been shown to bind the sIgA but does not appear to be involved in invasion (discussed in further detail below) (53). The surface-associated lipoproteins SlrA and PpmA have both been shown in mutagenesis studies to be important for nasopharynx colonization, but they do not appear to play a significant role in invasive disease (54, 55).

Another strategy employed by the bacteria to colonize and remain in the nasopharynx is formation of a biofilm. While the specific role of biofilm formation in pneumococcal infection is not well understood, it confers increased resistance to antimicrobial peptides and may promote sharing of genetic information between the bacteria as a result of proximity (56). Although bacteria in a biofilm are often metabolically dormant, when they are dispersed, they may be more virulent. Bacterial release from a biofilm can be triggered by changes in the microflora, inflammation, or viral infection (57). These released pneumococci were found to be phenotypically different from planktonic or biofilm bacteria and had an increased ability to disseminate and cause infection (58, 59). These release events represent a potential switch between asymptomatic colonization and invasive disease.

Ascension into the Middle Ear

OM is a highly prevalent pediatric disease and the primary cause of physician visits by small children. As many as 80% of children have presented with at least one case of OM (60). Along with *Moraxella catarrhalis* and *Haemophilus influenzae*, the pneumococcus is a primary cause of OM and is isolated in 30 to 40% of culture positive middle ear infusions (61–63). OM is thought to result when pneumococci in the nasopharynx ascend the eustachian tube and gain access to the middle ear (Fig. 2). Alternatively, it has also been suggested that OM is caused by the blocking of the eustachian tube resulting in a decrease of oxygen and increased dampness on the middle ear surface. While the mechanism is disputed, the transition from colonization of the nasopharynx to OM strongly correlates with accompanying viral infection (64). Typically, OM presents with earache, fever, nasal congestion, a feeling of fullness in the ear, and muffled hearing.

The ability of *S. pneumoniae* to ascend the eustachian tubes involves neuraminidases (61, 65). *S. pneumoniae* encodes two neuraminidases, NanA and NanB, with NanA being the major neuraminidase (66, 67). Neuraminidases cleave *N*-acetylneuraminic acid from glycoproteins and glycolipids on the eukaryotic cell surface. Neuraminidases can also cleave mucin, reducing the viscosity of this barrier and permitting the bacteria to access the epithelium. At the epithelium, neuraminidase cleaves oligosaccharides on the host cell surface, exposing cryptic receptors and enhancing bacterial adherence (66, 67). Support for this mechanism is seen in chinchilla infection models whereby clearance of a neuraminidase-deficient mutant occurs twice as quickly as wild-type *S. pneumoniae*. Furthermore, structural changes in the cell surface carbohydrates of eustachian tube epithelial cells occur following infection with wild-type pneumococci compared to its isogenic *nanA* mutant (68, 69).

Once the pneumococcus is in the middle ear, inflammation is triggered by pneumolysin and cell wall components (70). Pneumolysin is a potent pore-forming toxin that directly kills host cells and activates complement. In addition, cell wall components activate Toll-like receptor (TLR) signaling and the alternative pathway of complement. Pneumolysin strongly contributes to hearing loss and cochlear damage during OM (71, 72). Guinea pigs infected with wild-type *S. pneumoniae* or a mutant deficient in neuraminidase demonstrated significant damage to the reticular lamina, sensory hair cells, and supporting cells of the organ of Corti. Guinea pigs infected with a pneumolysin-deficient mutant had no visible damage. The contribution of pneumolysin and cell wall to inflammation will be discussed in more detail later in this article.

CbpA/pIgR-Mediated Invasion in the Upper Respiratory Tract

Once attached to epithelial cells, the pneumococcus is able to translocate across the mucosal barrier by coopting the polymeric immunoglobulin receptor (pIgR) (73). Mucosal epithelial cells transport IgA and IgM in a vesicle moving from the basolateral to the apical surface by binding to pIgR. On the apical surface, pIgR is cleaved and immunoglobulins are secreted into the lumen. Cleaved pIgR is subsequently shuttled back to the basolateral surface (74). *S. pneumoniae* translocates through epithelial cells by hijacking pIgR on the apical side of the host cell, resulting in transport as the receptor is endocytosed and recycled to the basolateral surface (Fig. 3). The pneumococcus binds to pIgR by ligating the motif YRNYPT of CbpA (75). *In vitro*, CbpA alone is sufficient to mediate translocation across a cell as latex beads coated with CbpA are endocytosed (73). *In vivo*, this interaction manifests as decreased nasopharyngeal colonization in mice lacking pIgR and the reduced capacity for *S. pneumoniae* mutants lacking CbpA to enter the bloodstream (76).

Accessing the Lower Respiratory Tract

Development of pneumonia is contingent on the ability of *S. pneumoniae* to establish a lower-respiratory-tract infection despite host defenses that either kill or clear the aspirated bacteria. The first barrier is the mucociliary escalator, which works mechanically to keep aspirated particles and microorganisms out of the lungs. As is true for many respiratory viruses, neuraminidase plays an important role in initiating bacterial pneumonia. As indicated, neuraminidase-deficient

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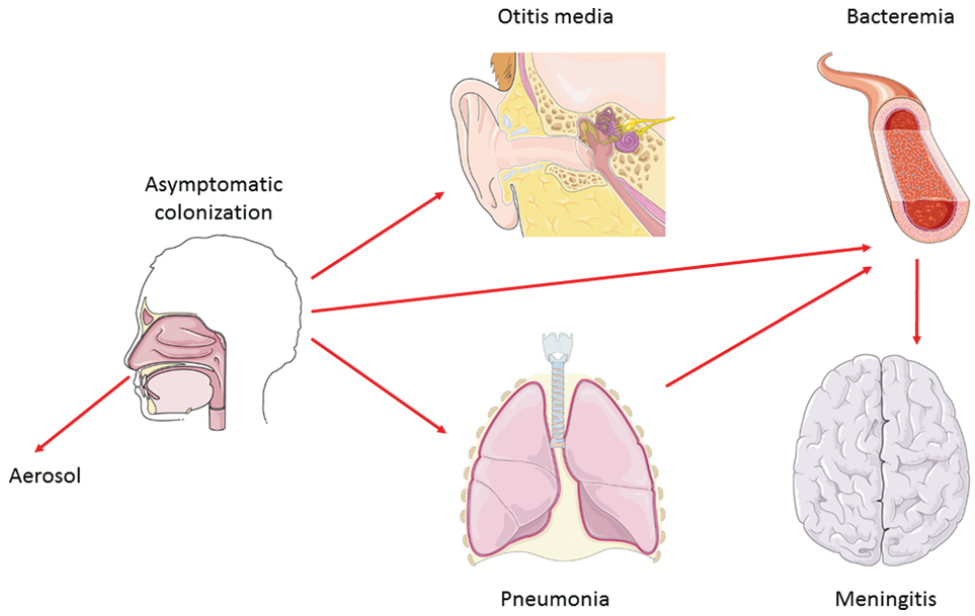


FIGURE 2 Schematic depiction of the spread and progression of *S. pneumonia* infection. Carriage of the pneumococcus occurs in the nasopharynx and is usually asymptomatic in healthy individuals. The bacteria are spread by aerosol from the nasopharynx of carriers. The pneumococcus can spread from the nasopharynx to a number of different tissues. In children the bacteria usually causes otitis media. Invasive diseases generally start in the lungs and spread to the blood, with the most serious complication being meningitis. The switch from asymptomatic colonization to invasive disease in healthy individuals usually occurs when there is a disruption in the innate immune defenses. (This figure contains some artwork produced by Servier Medical Art [http://smart.servier.com/] under Creative Commons license 3.0).

pneumococci do not cleave mucin efficiently and have a diminished capacity to adhere. Mutants deficient in *nanA* have a reduced capacity to bind to chinchilla tracheas *ex vivo* (77) and are attenuated in their ability to cause a lower-respiratory-tract infection following intranasal challenge (38).

The second step in adjusting to the environment of the lung involves changes to the bacterial surface that promote bacterial adherence to epithelium. The respiratory epithelium produces copious antibacterial peptides that kill bacteria on contact. Recent evidence indicates that pneumococci counteract this defense by shedding capsule, which results in relative resistance to the killing by antimicrobial peptides (78). This process is driven by the bacterial autolytic enzyme, LytA. Although LytA has long been known to promote autolysis in response to antibiotics, this new role of the enzyme drives capsule loss without bacterial lysis. Loss of capsule permits a close interaction of the bacteria with host cells, leading to successful initiation of infection.

Pneumococcal adhesion to eukaryotic lung cells is a two-step process that initially entails a loose interaction with host cell surface glycoconjugates followed by a tighter, more secure interaction with host cell protein receptors that promote internalization. During the initial stages of infection, *S. pneumoniae* and other respiratory tract pathogens such as *Pseudomonas aeruginosa* and *H. influenzae*, bind to *N*-acetylgalactosamine β1-3 galactose (79). Neuraminidases cleave the sialic acid and expose *N*-acetylgalactosamine β1-3 galactose and other ligands on the host cell surface (69, 80). The efficient removal of sialic acid by neuraminidases can be reduced by the amount and position of acetylation on sialic acid. It is thought that the major pneumococcal esterase, EstA, deacetylates the sialic acid and increases the release of sialic acid by NanA (81). *In vitro*, pneumococci adhere more efficiently to tissue culture cells treated with neuraminidase (77). The synergism observed in a pneumococcus and influenza coinfection may be the result of enhanced adherence mediated by neuraminidase activity (82). It has long been known that influenza primes the lungs for the development of a secondary bacterial infection. This synergism has been successfully modeled in mice, where pretreatment with influenza readily enhances severe pneumococcal pneumonia, leading to death despite challenge with a dose that is usually insufficient to infect an otherwise healthy mouse (83–85). Mechanistically, superinfection likely occurs as a result of neuraminidase expressed by influenza stripping sialic acid from the mucosa and exposing bacterial receptors. Oseltamivir, a neuraminidase inhibitor, has been shown to prevent pneumococcal superinfection in this postinfluenza pneumococcal challenge model (86).

Interactions in the Alveoli

As pneumonia progresses, the respiratory epithelium is denuded, exposing the underlying extracellular matrix of the bronchioles and alveoli. Surface-bound bacterial proteins such as PepO, PavA, PavB, PfbA, PclA, and PsrP all contribute to attachment through interactions with the extracellular matrix (87–90). Once established in the alveoli, inflammation is particularly intense, resulting in consolidation of the affected lobes. Consolidation progresses through stages of engorgement and red hepatization during which capillaries and epithelial cells become inflamed, and fluid and erythrocytes accumulate in the alveoli in a fibrin mesh (red hepatization). Subsequently, the lungs darken (gray

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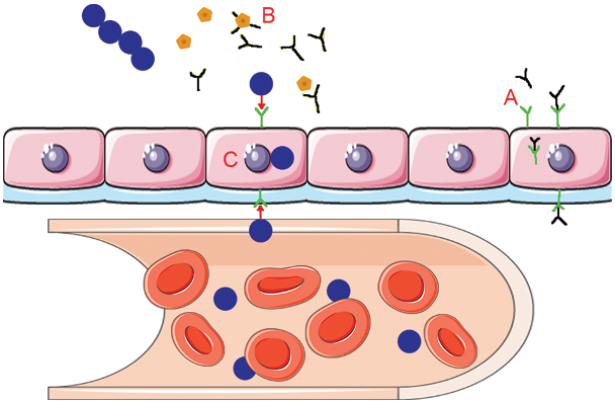


FIGURE 3 Schematic representation of the pneumococcus hijacking the pIgR/IgA system to cross the mucosal epithelia into the blood. (A) Mucosal epithelial cells transport IgA (black) from the basolateral to the apical surface using the receptor pIgR (green). This receptor is then endocytosed and recycled back to the basolateral surface to transport more IgA. (B) To protect itself from IgA, the pneumococcus produces the protease sIgA1 (yellow), which cleaves the host IgA into Fab fragments. (C) The choline-binding protein, CbpA (red), binds to the empty pIgR and shuttles the pneumococcus from the apical side to the basolateral side of the epithelial cells. (This figure contains some artwork produced by Servier Medical Art [http://smart.servier.com/] under Creative Commons license 3.0).

hepatization) as leukocytes enter the lesion and the bacteria are engulfed by macrophages. Resolution continues for several days as capsule-specific antibodies provide efficient opsonization and inflammatory mediators dissipate (91).

Pneumococcal cell wall, pneumolysin, and hydrogen peroxide are the virulence determinants that mediate the greatest inflammation and cytotoxicity observed in the lungs (92–94). Challenge of mice with purified pneumolysin or cell wall TLR ligand products is sufficient to cause edema and influx of neutrophils that recapitulate pneumonia (95, 96). Multiple studies clearly demonstrate that deletion of the genes that encode autolysin, pneumolysin, or the enzyme that produces hydrogen peroxide greatly attenuate the ability of the bacteria to survive and replicate in the lungs (38, 92, 94, 96–98). The contribution of these virulence products is discussed in greater detail below.

INVASION OF PNEUMOCOCCI INTO THE BLOODSTREAM

Access to the Bloodstream

During red hepatization, infected alveoli overflow with bacteria, edema fluid, and erythrocytes wrapped in a fibrin mesh. Fibrin strands pass through interalveolar connections, also known as the pores of Kohn, from one alveolus to the next, and the lymphatics are dilated and filled with cells and fibrin. When infection reaches this stage, a mouse becomes bacteremic. Access to the bloodstream by *S. pneumoniae* may occur through several pathways, including via the lymphatics, via cell damage to the epithelial and endothelial cells, and via direct invasion of endothelial cells. Most likely, all three pathways contribute to bloodstream invasion in an infected animal.

Although the pneumococcus can directly invade cells, this occurs at relatively low efficiency compared to bacterial pathogens that are commonly thought of as invasive, such as *Salmonella* and *Shigella* species (99). Invasion is dependent on activation of the host cell by pneumococcal cell wall components and pneumolysin and results in *de novo* expression of host defenses such as platelet activating factor receptor (PAFr), C3, and other factors. PAFr, a receptor abundant on lung cells, recognizes ChoP on its natural ligand, the chemokine PAF. The surface of the bacterium mimics this by decorating the cell wall with ChoP. CV-1 origin SV40 (COS) cells expressing PAFr have been shown to bind more bacteria than COS cells not expressing PAFr (99). Studies have also colocalized PAFr with adherent bacteria on the surface of human cells (100). PAFr binding is not limited to the pneumococcus; other respiratory pathogens such as *Haemophilus* spp., *Neisseria* spp., and *Pseudomonas* spp. also express ChoP on their surfaces in a phase-variable manner (101, 102). The pneumococcus can also bind the PAFr on other tissues (103, 104). In defense against the widespread expression of ChoP on respiratory pathogens, the host deploys C-reactive protein, an acute-phase reactant that activates complement and opsonizes the bacteria (105). Thus, phase variation of the amount of surface-bound ChoP serves as a mechanism by which the pneumococcus switches from a more adherent form (high ChoP) suited for the mucosa to one that is more resistant to phagocytosis (low ChoP) and well adapted to the bloodstream.

Unlike PAF, the binding of pneumococcus to the PAFr does not result in the activation of a G-protein-mediated signal transduction pathway (100). Rather, pneumococcal uptake requires activation of extracellular signal-regulated kinases consistent with activation by β-arrestin. Uptake of the pneumococcus into a vacuole involves clathrin followed by recruitment of β-arrestin scaffold, Rab 5, and then Rab 7 and Rab 11. Rab 5 is involved in early endocytosis, Rab 7 is found in the late endosome, and Rab 11 is responsible for vacuole recycling (106). Overexpression of arrestin in endothelial cells enhances colocalization of the bacteria with Rab 7 and Rab 11 and increases survival of the pneumococci normally killed by the lysosome. Thus, it is currently thought that association of β-arrestin with the PAFr vacuole complex contributes to the successful translocation of the bacteria away from the lysosome (100).

The pilus-1 system may also be employed in the escape from the lungs and peritoneal cavity into the bloodstream. RrgA, the major adhesive determinant of the pilus, has been shown to facilitate the spread of the bacteria to the bloodstream (107). RrgA binds the complement receptor 3 on macrophages and increases intracellular survival (107). The severity and progression of disease were shown to be affected in mice lacking complement receptor 3 and suggests that phagocytosis by macrophages may facilitate the spread of infection to distal sites (107). This theory is possibly further supported by the fact that ubiquitously expressed pneumococcal protein PepO may lead to an increase in macrophage phagocytosis (108).

Recently, a new macropinocytosis pathway was described that is independent of the PAFr and does not require ChoP (109). This pathway relies on actin interactions with the uptake vesicle independent of dynamin, clathrin, and caveolin (109) and represents a potential alternative pathway for bacterial translocation. However, without the requirement of ChoP it could be used by a wider group of bacterial pathogens. In model systems, roughly half of adherent pneumococci enter epithelial cells via micropinocytosis and half via PAFr. Thus, while the pneumococcus is the prototypical extracellular pathogen, intracellular translocation is an important part of its pathogenesis.

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Survival in the Bloodstream

Once the bacteria escape into the bloodstream, CPS becomes the most important virulence determinant and is responsible for inhibiting phagocytosis. The chemical structure (serotype) and amount of CPS present on the surface of the bacteria contribute to the differential ability of different serotypes to survive in the blood (110–113). Mutants lacking capsule are essentially avirulent (111), requiring 10,000- to 100,000-fold more bacteria to kill a mouse than the encapsulated parent strain following intraperitoneal injection. It is believed that CPS inhibits phagocytosis by preventing phagocytes from physically reaching opsonizing serum components, such as complement, C-reactive protein, mannose-binding proteins, and antibodies that are deposited on the cell wall and by giving the bacteria a negative charge which repels a close association with leukocytes (32, 114, 115). Formation of antibody to the serotype-specific CPS marks the initiation of clearance of the infection, because antibodies to CPS are highly opsonic and are protective against subsequent pneumococcal challenge with the same serotype (116). The fact that capsular antibodies are so effective forms the basis of current effective pneumococcal vaccines.

Pneumococcal proteins also contribute to resistance to defenses in the serum. PspA has been demonstrated to inhibit complement activation mediated via the classical pathway on the bacteria surface (117, 118). Mutants deficient in PspA are cleared more rapidly from the bloodstream of XID mice compared to wild-type mice. XID mice lack the ability to form an antibody response to polysaccharides, and thus, clearance correlates with the amount of complement on the bacterial surface (119). PspA also protects bacteria from the bactericidal peptides of apolactoferrin; blocking PspA with antibodies enhances apolactoferrin killing (120). CbpA also has the ability to inhibit complement deposition by binding to C3 which blocks C3 cleavage and by binding to factor H (121, 122). PspA allows the bacteria to reduce C3 deposition, whereas factor H, a negative regulator of the alternative pathway, leads to interference in the formation of the C3 convertase. Factor H can also be bound by the pneumococcal surface protein Tuf (123). Furthermore, resistance to opsonophagocytic activity of neutrophils is mediated through the action of the exoglycosidases NanA, BgaA, and StrH, which decrease the amount of C3 being deposited onto the pneumococcal surface (124).

TRAFFICKING OF PNEUMOCOCCI INTO THE HEART, BRAIN, AND FETUS

Interactions with the Heart

Once in the bloodstream, pneumococci disseminate widely into many organs by the binding of bacterial CbpA to endothelial laminin receptor and ChoP to the PAFr. For instance, a common complication of severe bacterial pneumonia is cardiac dysfunction. Pneumonia and cardiac events may be closely associated with heart disease predisposing people to pneumonia or, potentially, pneumonia increasing the risk of heart disease; further studies are needed (125). Bacteremia delivers pneumococci to the cardiac vascular endothelium, where CbpA/laminin receptor-mediated translocation into the cardiac tissue occurs. In mice and nonhuman primates, cardiac damage ensues in the form of microscopic lesions in the myocardium (126, 127) caused by the release of pneumolysin and H_2O_2, with added pathology resulting from the influx of immune cells (126, 128). Immunization of mice with the CbpA protects the animals from cardiac damage (126). Importantly, pneumococcal cell wall products are also inhibitory to cardiac contractility (129), as is pneumolysin, which disrupts Ca^{++} signaling due to pore formation even if cells are not immediately killed (130). Once in the heart of the mouse, the bacteria use clathrin-mediated endocytosis to invade cardiomyocytes (131). Interaction between the pneumococcus and the heart is an emerging field.

Interactions at the Blood-Brain Barrier

Pneumococcal meningitis is by far the most devastating complication of IPD. Some estimates suggest that approximately one-third of affected individuals die (132–134) and half of the survivors suffer from permanent neurological sequelae (135–137). Severe damage occurs in the hippocampus, particularly in the dentate gyrus, with survivors suffering from hippocampal atrophy and defects in learning and memory (138). Neuronal damage is, in part, mediated by the response of the host defenses to bacterial products (139); for example, cell wall components are detected by host cells, and the influx of leukocytes leads to extensive inflammation (140, 141). Inflammation in the cerebrospinal fluid (CSF) exacerbates neuronal damage by releasing matrix metalloproteases such as MMP-9 (142), neurotoxic free radicals such as peroxynitrate (143), and proinflammatory cytokines that recruit more leukocytes (141). Ultimately, the overexuberant host response triggers caspase-dependent and -independent apoptosis of neurons (139). Blocking leukocyte entry into the CSF duringmeningitis decreases damage by approximately 50% (144, 145) and as such is the basis for treating individuals with pneumococcal meningitis with dexamethasone prior to antibiotic therapy. The other half of neuronal damage is directly due to cytotoxic compounds such as pneumolysin and hydrogen peroxide that damage the mitochondria and initiate apoptosis (146). These factors are reviewed later in the article. The extent of the host response to damage can be seen by the fact that introduction of purified cell wall alone into the CSF can lead to signs and symptoms of meningitis and ultimately to neuronal damage (147).

Pneumococcal invasion from blood into the CSF is thought to occur either in the choroid plexus or by crossing the blood-brain barrier in the cerebral capillaries that traverse the subarachnoid space. The pneumococcus is thought to initially bind to the blood-brain barrier endothelium through interactions of the NEEK motif of CbpA with the laminin receptor (148). Such binding to the laminin receptor is a common strategy of meningeal pathogens, including *H. influenzae*, meningococcus, prions, and several viruses. Studies with mice have determined that once bound to the endothelium, translocation across the barrier is dependent on the interaction between ChoP and PAFr (148–151). PAFr knockout mice were resistant to development of meningitis (100). Likewise, CbpA mutants were unable to cross the blood-brain barrier despite bacterial titers in the blood of 10^8 CFU/ml (38). Thus, the two-step process of recognition of laminin receptor on the cerebrovascular endothelium by homologs of CbpA followed by translocation across the barrier using surface ChoP binding to PAFr is a conserved process of invasion of the central nervous system that is shared by the most successful meningeal pathogens.

Interactions with the Placenta and Fetus

PAFr is found on a number of tissues, including the placenta during pregnancy. Infection and inflammation during pregnancy were shown to lead to postnatal cognitive deficiencies

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in a number of studies reviewed by Loughran et al. 2016 (103). The pneumococcus itself does not cross the placenta to the fetus. However, fragments of cell wall released during antibiotic treatment cross the placenta in a PAFr-dependent manner and accumulate in the developing fetal cortex in mice. Rather than neuronal death, as seen in postnatal meningitis in mice, the fetal brain responds with an increase in neuroproliferation (104). Neuroproliferation is increased by interfering with the levels of the cytostatic transcription factor FoxG1 as a result of cell wall interaction with TLR2. The abnormal brain architecture is associated with behavioral abnormalities in the postnatal period in mouse models. This leads to the possibility that bacterial products encountered during pregnancy may be associated with cognitive disorders in children.

INFLAMMATION AND CYTOTOXICITY

Intense inflammation is a hallmark of pneumococcal disease, and the pneumococcus serves as a prototype for understanding the molecular mechanisms of inflammation in response to Gram-positive bacteria (152, 153). Inflammatory components released by the pneumococcus include peptidoglycan, teichoic acid, pneumolysin, hydrogen peroxide, and a number of other secreted proteins. Alone, several of these factors have been shown to trigger inflammation and are cytotoxic (see above). In concert, these factors trigger inflammation through multiple inflammatory cascades, including the TLR pathway, the chemokine/cytokine cascade, the complement cascade, and the coagulation cascade. In a naive host lacking serotype-specific antibodies, these cascades hasten the accumulation of leukocytes, which are ineffective in phagocytosing pneumococci coated in capsule. The increased white blood cells at the site of infection lead to an even greater release of proinflammatory mediators without efficient clearance of the bacteria.

Inflammation

The key surface component recognized by the innate immune system is the cell wall (Fig. 4). The pneumococcal cell wall is composed of the peptidoglycan network with teichoic acid attached to roughly every third *N*-acetylmuramic acid residue (154). When tested in animals, peptidoglycan and teichoic acid can elicit inflammation and recapitulate many of the symptoms of pneumonia, otitis media, and meningitis (147, 152, 155). Although CPS is effective in protecting the bacteria, the CPS itself is not inflammatory (156). Components of the bacterial cell wall can mediate inflammation through multiple pathways. At the cellular level, peptidoglycan and teichoic acid bind to pattern recognition molecules such as lipopolysaccharide-binding protein and peptidoglycan recognition proteins. These complexes in turn bind to TLR-2 on the surface of epithelial and endothelial cells, monocytes, and macrophages (157, 158). Cross-linking of TLR-2 triggers intracellular signaling that activates transcriptional regulators such as NF-κB. NF-κB expression then results in production of proinflammatory cytokines such as interleukin-1β (IL-1β), IL-6, IL-8, and tumor necrosis factor (159). Nod receptors in the cytoplasm may also modulate inflammation by binding to intracellular peptidoglycan (160, 161). The importance of Nod receptor activation for resolution of the infection is supported by the need for the expression of the chemokine receptor CCR2 to efficiently recruit macrophages to the site of infection for bacterial clearance (162). Activation of epithelial and endothelial cells results in recruitment of effector cells such as neutrophils and macrophages, altered vascular permeability, and creation of a serous exudate.

Cell wall components not only cause inflammation through interaction with cells but also activate several complement pathways. Antibodies specific to bacterial proteins on the cell surface activate the classical pathway (117). Similarly, CPS and cell walls bind to hydrolyzed C3 and activate the alternative complement pathway (156, 163, 164). The classical and alternative pathways result in release of C3a and C5a, both of which are chemoattractants and potent anaphylactic molecules leading to inflammation. Cell wall also activates complement via the lectin-binding pathway. Mannose-binding lectin, a member of the collectin family, binds to carbohydrates such as *N*-acetyl-glucosamine, a constituent of peptidoglycan (165). The binding of mannose-binding lectin to the bacterial cell results in the formation of a C3 convertase on the surface of the bacteria and deposition of C3b. Mannose-binding lectin deficiency is associated with an increased risk of invasive pneumococcal infection (166). Finally, C-reactive protein bound to cell wall ChoP also activates the classical complement cascade by binding C1q (167). This results in additional opsonization and further release of the C3a and C5a. Pneumococcal PepO can disrupt this process by binding to C1q, increasing bacterial survival (168). However, PepO expression in the lungs has been suggested to trigger release of the chemoattractant IL-8 and IP-10, which leads to neutrophil recruitment and contributes to the host response pathology (169).

Complement activation is not limited to the surface of the bacteria; cell wall fragments released by the bacteria following lysis and during cell wall turnover are also capable of activating complement (170). Likewise, pneumolysin released into the milieu also activates complement (171). Pneumolysin binds to the Fc portion of immunoglobulins and activates the classical pathway. It has been suggested that cell wall components, pneumolysin, and other proteins such as PepO are released by the bacteria to deplete complement (168, 172, 173). This would have the most direct impact during blood stream infections and in the lungs. Release of cell wall components is mediated by the murein hydrolase, LytA. LytA is responsible for pneumococcal lysis in the stationary phase as well as in the presence of antibiotics (174). Furthermore, it has been shown that LytA is important for the release of capsule in response to antimicrobial peptides found on the epithelial surface (78). Autolysin-mediated lysis is responsible for the spike in inflammation observed immediately following antibiotic treatment of meningitis. Autolysin-negative mutants may have reduced virulence compared to the wild type as a result of the inability of the mutant to release cell wall (CW) or the inability to shed the capsule, leaving the bacterium sensitized to the antimicrobial peptides (78, 175).

Although complement is crucial for the opsonization of the bacteria, it also leads to the formation of the membrane attack complex (MAC) formed through the action of C5, C7, and C9, which form a hole in cellular membranes in a fashion similar to pneumolysin. The pneumococcal glycolytic enzyme phosphoglycerate kinase has been shown to inhibit MAC formation by blocking C9 polymerization (176). Gram-positive bacteria, such as *Streptococcus*, are also in general more resistant to MAC killing than their Gram-negative counterparts since they lack an outer membrane, leaving MAC to form on the thick peptidoglycan wall.

Cytotoxicity

In addition to the pathology derived from inflammation, the pneumococcus can directly damage eukaryotic cells. The principal mediators of cytotoxicity are pneumolysin and

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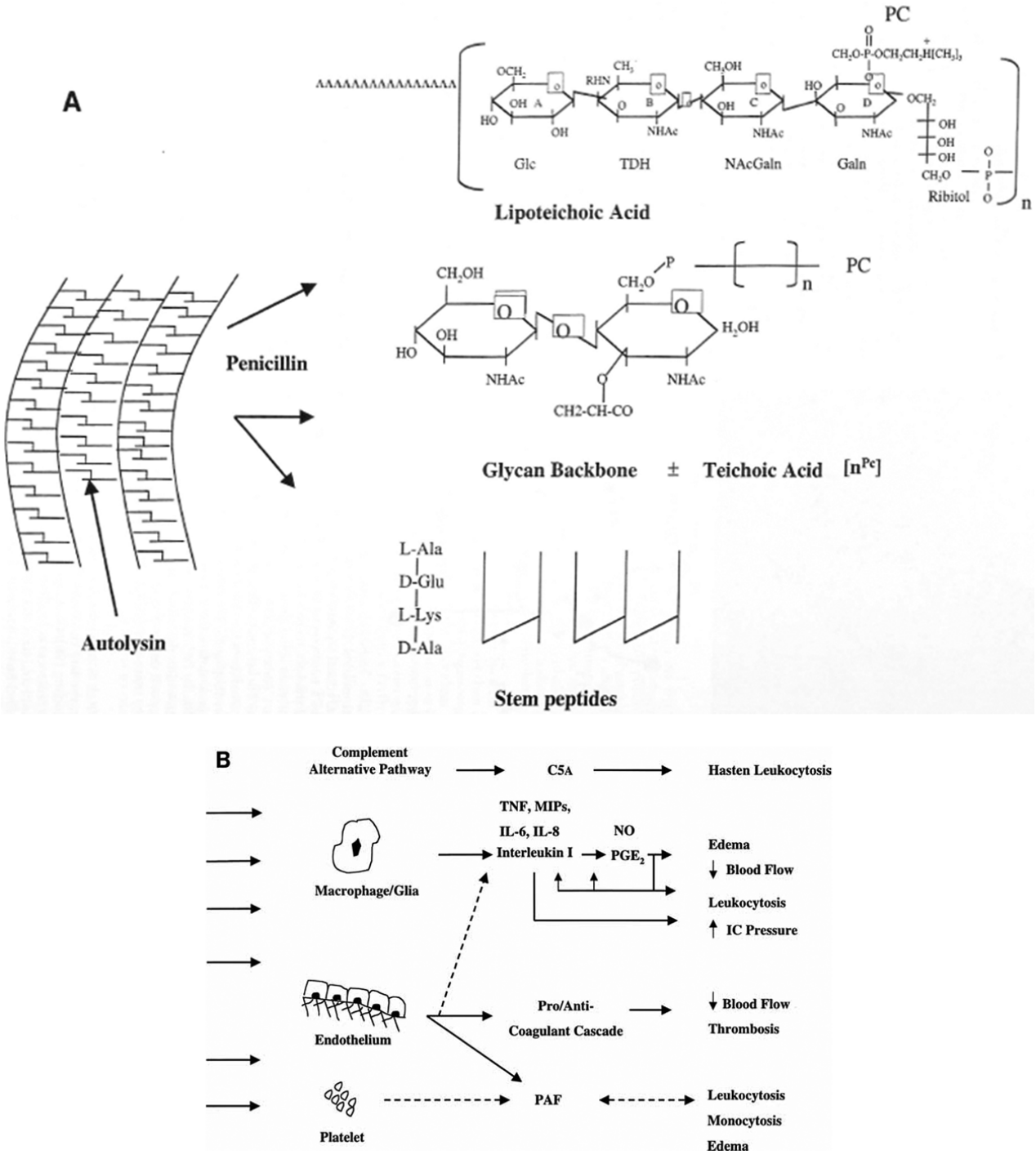


FIGURE 4 Structure of the pneumococcal cell wall and its relationship to inflammation. (A) Penicillin induces cell wall degradation by the autolysin releasing cell wall fragments such as lipoteichoic acid, glycan polymers with and without teichoic acid, and small stem peptides. All teichoicated species contain ChoP, a key component increasing inflammatory activity. (B) All of these components interact with a variety of human cells, which in turn produce inflammatory mediators. Particularly important in this response is the platelet activating factor (PAFr). These mediators combine to produce the symptomatology of pneumococcal infection, including changes in blood flow, fluid balance in the tissue, and leukocytosis. Glc, glucose; TDH, trideoxyhexose; NAcGaln, *N*-acetylgalctosamine; Galn, galactosamine; L-Ala, L-alanine; D-Glu, D-glucose; L-Lys, L-lysine; TNF, tumor necrosis factor; NO, nitric oxide; PGE2, prostaglandin E2; IC pressure, intracranial pressure; MIPS, macrophage inflammatory protein.

hydrogen peroxide (72, 92, 93, 146, 177). Pneumolysin has long been recognized as a principal virulence factor of the pneumococcus (Fig. 5). Studies using a variety of challenge routes and animal models have convincingly demonstrated that pneumolysin-deficient mutants are drastically attenuated (38, 92, 96, 177). The mechanism responsible for pneumolysin secretion from the bacteria is poorly understood. While it was initially thought that pneumolysin was

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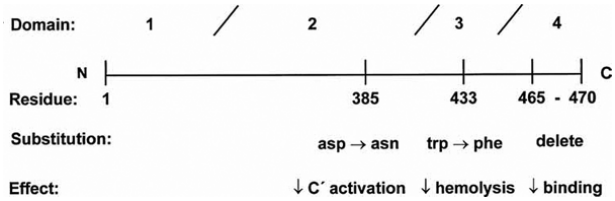


FIGURE 5 Domain structure of pneumolysin. Pneumolysin has three functionally separate domains: one activating complement, one causing hemolysis, and the other binding to cholesterol. Site-specific mutations alter these properties individually (191, 192).

only released as a result of autolysis (178), it has also been shown to have an independent route, because mutants lacking the autolytic enzyme, LytA, show the same pattern of release as the wild-type bacteria (179, 180). Pneumolysin is a pore-forming toxin that kills cells via necroptosis, a programmed mode of necrosis. At high concentrations, pneumolysin, like other pore-forming toxins, triggers necroptosis due to ion dysregulation (181, 182). Critically, pneumolysin-mediated cell death has been shown to be preventable using drugs that block RIPK1, RIPK3, or MLKL, the signaling pathway involved. The toxin binds to cholesterol on the surface of the host cell and oligomerizes to form pores as large as 30 nm in diameter (183). This results in Ca^{++} and K^{+} dysregulation. At lower concentrations, the toxin has a variety of effects on different cell types. Pneumolysin has been demonstrated to slow ciliary beating of epithelial cells (184), disrupt tight junctions (185), and inhibit the capacity of neutrophils and macrophages to kill by inhibiting oxidative burst (186, 187). Disruption of the alveoli-capillary barrier contributes to the leakage that allows serous exudates to enter the lungs and the bacteria to cross into the blood stream (188). In the middle ear, pneumolysin is responsible for damage to the cochlea and hair cells, contributing to hearing loss (72). During meningitis, pneumolysin causes neuronal damage mediated by an influx of extracellular calcium triggering apoptosis (146). Chelating extracellular calcium inhibits the release of apoptosis inducing factor (AIF) and protects cells from pneumolysin-induced apoptosis *in vitro*.

Hydrogen peroxide (H_2O_2) is a major product of pneumococcal metabolism and damages host tissues. H_2O_2 is the result of the activity of the enzyme pyruvate oxidase (SpxB), which decarboxylates pyruvate to produce acetyl phosphate, H_2O_2, and CO_2 (38, 97). Mutation of *spxB* dramatically attenuates virulence in the respiratory tract but not in the blood stream (38). Studies of the cytotoxic effects of H_2O_2 are not as comprehensive as those for pneumolysin. Nonetheless, H_2O_2 also contributes to mitochondrial damage of neurons, resulting in apoptosis (146), and inhibits beating of ciliated ependymal cells lining the ventricular system of the brain and cerebral aqueducts (189, 190). It is also required for cardiac damage (131).

CONCLUDING REMARKS

The ability to invade and cause pathology in such varied organ systems while also avoiding killing by the immune system makes *S. pneumoniae* a highly successful pathogen. Asymptomatic colonization allows the pneumococcus to persist in the population, and extensive serotype diversity complicates the development of effective vaccines. The successful invasion strategy of ChoP/PAFr and CbpA/laminin receptor shared by a number of major respiratory pathogens coupled with cytotoxic pneumolysin makes the pneumococcus a prototypic pathogen for studying host pathogen interactions.

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Phase Variation of *Streptococcus pneumoniae*

JING LI AND JING-REN ZHANG

21

INTRODUCTION OF PHASE VARIATION

Phase variation, a type of phenotypic variation, refers to reversible, ON-and-OFF production of a molecule, structure, or complex characteristic (1). Since Andrewes' description of "diphasic salmonellas" (2), the first report of phase variation in bacteria, phase variation has been discovered in many bacteria, particularly pathogens. Our current understanding of the genetic and molecular basis of phase variation is almost entirely based on the studies of virulence determinants. Examples include variable generation in surface proteins (3–5), fimbriae/pili (6), flagella (7–9), capsule (10, 11), modification of lipopolysaccharides and lipooligosaccharides (12–14), and colony morphology (15–17). This type of phenotypic variation in pathogenic bacteria permits the selection of variants with optimal adaption or fitness in an individual host or distinct host environment. Phase variation is particularly common in Gram-negative pathogens, such as *Escherichia coli*, *Salmonella typhimurium*, *Haemophilus influenzae*, *Campylobacter jejuni*, and pathogenic *Neisseria* (18).

Phase variation is also reported in several Gram-positive bacteria. In *Staphylococcus* spp., previous studies have shown variations in the expression of polysaccharide, intercellular adhesin (19), colony morphology (20), biofilm/biofilm associated protein (21, 22), and antibiotic resistance (23). The bistable expression of pilus has been described in *S. pneumoniae* and *Streptococcus gallolyticus* (24–27). The gene encoding the virulence factor InlA of *Listeria monocytogenes* is also prone to frameshift in its 5′ homopolymeric tract of adenine nucleotide, leading to the ON-and-OFF expression of the protein (28). The variation in colony morphology has been documented in numerous *Streptococcus* spp. (29–31), but the switch in colony opacity of *S. pneumoniae* represents the best-characterized case (see below).

The vast majority of the well-described phase variations are caused by one or combinations of the following four major mechanisms: DNA inversion, homologous recombination, slipped-strand mispairing, and differential DNA methylation (illustrated in Fig. 1).

Variation by DNA Inversion

The Hin recombinase-catalyzed inversion in *S. typhimurium* is the first report of the genetic/molecular mechanisms behind bacterial phase variation. In the context of the "diphasic salmonellas" observation (2), Zieg *et al.* (32, 33) first discovered that the Hin recombinase drives reversible switch of the orientation of the flagellin promoter sequence and alternates the transcription of two flagellin-encoding genes, thereby leading to switch in the biochemical and antigenic characteristics of the flagellum. Similar but more complex DNA-inversions of the promoter-associated sequences have since been identified to drive phase variation in the type 1 fimbriae of *E. coli*. The orientation of the promoter sequence of *fim*A, the structural gene of type I pilin, is capable of DNA inversions, which are catalyzed by two independent tyrosine recombinases, FimB and FimE (6). FimB catalyzes the reactions in both directions, whereas FimE drives the inversion from the "ON" to the "OFF" orientation (34, 35). Promoter sequence inversions are also responsible for phase variation in the polysaccharide antigens of *Bacteroides fragilis* (36–38) and surface proteins of *Mycoplasma* species (4, 39, 40). Lastly, bacteria regulate the frequency of DNA inversion as an adaptive response to environmental conditions. It has been demonstrated that the inversion frequency of the *fim*A promoter is modulated by temperature, media, sialic acid, and nutrient availability (41–43).

Variation by Programmed Homologous Recombination

The phenotypic switch between the piliated and nonpiliated phases of *Neisseria gonorrhoeae* represents one of the best-characterized cases in this category. *N. gonorrhoeae* possesses a transcriptionally active *pilE* locus for producing the pilin of the *N. gonorrhoeae* type IV pilus and homologous *pilS* (transcriptionally silent) sequences located elsewhere in the genome. The RecA-dependent homologous recombinations between the *pilE* and *pilS* sequences sometimes truncate the 5′ region of *pilE*, leading to the production of "nonfunctional" *pilE* alleles, inactive pilin, and ON-to-OFF variation of the pilus structure. However, further recombinations of the nonfunctional *pilE* alleles with the *pilS* sequences may yield a functional *pilE* allele, giving rise to an OFF-to-ON (piliated) variant of the bacterium. Similar programmed homologous recombinations have been described for phenotypic variations of surface-exposed proteins in pathogenic *Borrelia* spp. (44–46).

Variation by Slipped-Strand Mispairing

The vast majority of the well-described phase variation cases are mediated by slipped-strand mispairing (18). This type of phase variation is always associated with the sequences containing homopolymers (stretches of the same nucleotides) or

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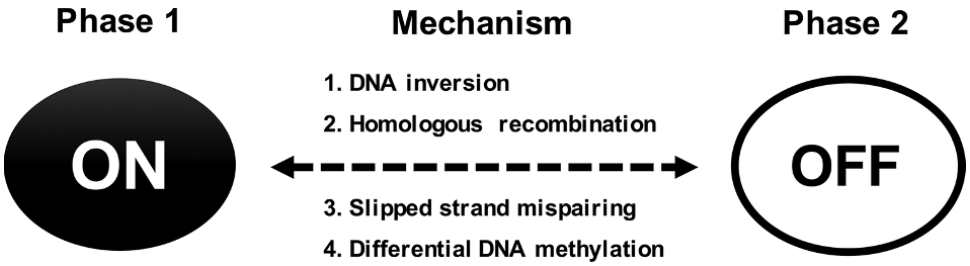


FIGURE 1 Overview of the major mechanisms behind reversible ON-and-OFF phase variation in bacteria.

multimeric repeats. These sequences are prone to transient mispairing during DNA replication, leading to insertion or deletion of one or multiple nucleotides/repeat sequences in the genome of the progeny cells. Mispairing in the promoter and coding sequences may alter transcription of downstream gene(s) and translation (frame shifting) of target genes, respectively. Sequence addition or deletion in the stretch of the cytosine bases adjacent to the promoter region of the *opc* gene is responsible for the ON-and-OFF switch of the Opc outer membrane protein in *N. gonorrhoeae*. While the variants with 12 or 13 bases in the poly(C) tract produce the maximal level of Opc (Opc++ phenotype), those with less than 10 or more than 15 bases fail to produce the protein (Opc– phenotype). Interestingly, the cells with 11 or 14 cytosines in the tract produce an intermediate level of *opc* mRNA and Opc protein (Opc+ phenotype). A similar mechanism of strand slippage also governs the variation in the LKP fimbriae of *H. influenzae*. Tandem repeats of teichoic acid bases in the sequence immediately upstream of the promoter region control the transcription of the *hif* gene cluster, which encodes the LKP fimbriae. The *hif* transcription and fimbriae production reach the maximum when there are 10 teichoic acid repeats. In contrast, the variants with 11 and 9 copies of the di-nucleotides produce reduced and undetectable fimbriae, respectively. Therefore, slipped-strand mispairing not only controls the ON-and-OFF variation, but also regulates the volume of the target molecules.

Disrupting the reading frames of the coding sequences by slipped-strand mispairing causes phase variation in many bacteria. One of the well-characterized examples is the ON-and-OFF variation in the outer membrane protein II of *N. gonorrhoeae* (47). The coding region of its signal peptide contains variable copies of the CTCTT; alteration in the number of tandem repeats leads to frameshifting of the P.II gene. The capsule variation in *Neisseria meningitides* occurs by frameshifting of the *siaD* capsule gene due to expansion and contraction of the poly(C) tract (10). In Gram-positive bacteria, the variable number of the TTTA tetranucleotide repeat appears to be responsible for the phase variation of poly-*N*-acetylglucosamine expression in *Staphylococcus aureus* (19). Reversible expansion and contraction in the number of repeat sequences was also reported in the capsule biosynthetic genes of *S. pneumoniae* (48–50); these events cause an ON-and-OFF switch in the enzymatic activity of the corresponding proteins, leading to phase variation in capsule production.

VARIATION BY DIFFERENTIAL DNA METHYLATION

Methylation status of bacterial genomes represents an epigenetic mechanism that modulates expression of certain genes and associated phenotypes (51–53). Phase variation in P fimbriae of uropathogenic *E. coli* is one of the best-studied examples of this kind (54). *E. coli* cells exhibit reversible phase variation of P fimbriae under certain conditions. This phenotypic switch is determined at the transcriptional level by different methylation patterns of the GATC loci in the promoter region of the fimbriae biogenesis gene (*pap*) locus. Similar methylation-dependent phase variation occurs in the expression of outer membrane protein-antigen 43, which is important for biofilm formation and pathogenesis of *E. coli* (55).

Phase variation also occurs in the DNA methyltransferase (Mod) genes of type III RM systems, also referred to as phasevarions (53). It is controlled by expansion and contraction of nucleotide repeats in the *mod* genes. The ON-and-OFF switch in the production of DNA methyltransferases leads to corresponding alteration in the methylation pattern of bacterial genomes. In *H. influenzae* (56), *Helicobacter pylori* (57), and pathogenic *Neisseria* (58), phase variation in the *mod* genes is associated with changes in stress response and virulence. We and others have recently discovered a complex epigenetic mechanism of phase variation in the type I RM systems of *S. pneumoniae* (52, 59, 60). A series of DNA inversions among homologous *hsdS* genes (encoding the sequence specificity subunit of the RM system) generate HsdS variants, each of which recognizes a unique DNA methylation sequence. Because the HsdS subunit defines the sequence specificity of DNA methylation in the type I RM systems, DNA inversions in the *hsdS* genes generate progeny cells with distinct patterns of genome methylation in an originally clonal population. One of the *hsdS* alleles defines the opaque colony morphology, while the pneumococcal variants with the other alleles produce transparent colonies (52) (see below). This DNA inversion in the *hsdS* genes thus combines the power of DNA rearrangements and differential methylation in phase variation.

PHASE VARIATION OF *S. PNEUMONIAE*

S. pneumoniae has been reported to undergo phase variation in colony opacity, encapsulation, and expression of the type I pilus. As the most important virulence factor of *S. pneumoniae*, polysaccharide capsule protects the organisms from mucosal agglutination and phagocytic killing (61, 62). However, like the immunogens of the current pneumococcal vaccines, the capsular polysaccharides are immunoprotective against pneumococcal disease (63). Besides extensive genetic variation of capsule types by natural genetic transformation, pneumococcus is able to switch between the encapsulated and unencapsulated states. Frequent variation in capsule production was first described in a type 3 strain (48). Reversible sequence

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duplications/deletions in the coding region of the first gene (*cap3A* or *cps3D*) of the type 3 capsule biosynthetic locus is responsible for frameshifting of *cap3A* and the acapsular phenotype. Additional sequence changes in the promoter and coding regions of *cap3A* were later found to cause reversible capsule ON-and-OFF variation (49). Similar capsule variants were later reported in type 8 and 37 strains (50), which are generated by expansions and contractions of repetitive sequences in the capsule biosynthetic genes *cap8E* (type 8) and *tts* (type 37), respectively. Because CapE (the first monosaccharide transferase) is conserved in the strains of other serotypes, this type of variation may occur in other types of pneumococcal capsule.

Independent studies have shown that the pilus of *S. pneumoniae* undergoes bistable expression or phase variation (25, 27). The pilus has been shown as an important contributor to pneumococcal pathogenesis (64, 65), although it is only produced by approximately 25% of strains (66). Immunization with pilus subunits confers protection against lethal pneumococcal challenge (67). The type I pilus consists of a main structural protein (RrgB) and two ancillary proteins (RrgA and RrgC). Transcription of *rrgA*, *rrgB*, *and rrg*C requires activation by the RlrA regulator (26). Basset *et al.* showed that phase variation in the pilus is controlled by an epigenetic mechanism, which is based on autoregulation of *rlrA* by a positive-feedback loop (26). The impact of the pilus phase variation on pneumococcal pathogenesis remains to be defined.

In 1994, Jeffery Weiser's laboratory first reported that single *S. pneumoniae* strains produce both opaque and transparent colonies on translucent agar plates supplemented with exogenous catalase (16). The opaque and transparent variants produce both types of colony when they are restreaked on agar plates. Visualization of phase variation in pneumococcal colony opacity requires careful examination of colony morphology with oblique, transmitted light and magnification on translucent medium (16). Under these conditions, differences in the ability of bacterial cells to pack tightly in a colony affect how light passes through the colony and the level of opacity. The variation appears to exist in all strains, but it is more readily appreciable in some isolates of certain serotypes. While strains of types 6A, 6B, 14, 19F, 23F, and 35B tend to yield easily observable opaque and transparent colonies, isolates of types 2, 3, and 4 do not form appreciable colony variants (52). In addition, different strains require different amounts of time for optimal visualization of colony variants (52, 68). We observed that the polysaccharide capsule of serotypes 2 and 4 blurs the phenotypic difference between the colony variants for an unknown reason (52). However, phase variation in colony opacity does not require the capsule because unencapsulated pneumococci also form both opaque and transparent colonies. Opacity variation is obscured on nontranslucent medium, such as blood agar. It is possible to detect sectored colonies resulting from phase variation during the clonal expansion of a single organism as it forms a colony. The frequency of switching is highly variable from isolate to isolate (range 10^{-3} to 10^{-6}/generation) under the same conditions. Likewise, the fraction of the opaque and transparent variants in a single isolate can differ dramatically, likely due to selection or induction by the environmental conditions. Microscopic analysis of the low-passage clinical isolates reveals that the single isolates from the middle ear produce a remarkably higher proportion of opaque colonies compared with those from the nasopharynx (69). The reasons for these types of site-specific effects remain to be defined.

CHARACTERISTICS OF PNEUMOCOCCAL OPACITY VARIANTS

Variation in Capsular Polysaccharides

The biochemical comparison of pneumococcal variants has focused on two major cell surface structures, the capsular polysaccharide and the C polysaccharide, or cell wall teichoic acid. Relatively small differences in the amount of capsular polysaccharide have been shown to have a major effect on the ability of the pathogen to cause infection (70). The opaque and transparent variants of the same strains are indistinguishable using type-specific antiserum in a Quellung reaction, suggesting that their capsules are antigenically identical. Immunoelectron microscopy demonstrated a larger and denser zone of immunoreactive capsular material in opaque pneumococci (71). Quantification of the capsular polysaccharides with type-specific monoclonal antibodies showed that opaque pneumococci have 1.2- to 22-fold more capsular polysaccharides than the related transparent variants depending on the strains tested (72). Production of capsular polysaccharide in the variants appears to be modulated by environmental conditions because the opaque variants produced significantly more capsule under conditions of reduced oxygen (73).

Variation in Cell Wall Polysaccharides

Opacity variation in unencapsulated pneumococci suggests that differences in the amount or composition of the capsule are not the sole factor responsible for phenotypic variation. The choline-containing cell wall teichoic acid and lipoteichoic acid (pneumococcal Forssman antigen) are the major carbohydrate-containing surface structures on the cell surface (74). Choline, which is imported exclusively from the extracellular environment, is essential for the growth of both transparent and opaque variants. It is estimated that approximately 90% of the choline incorporated into cells is localized to the cell wall teichoic acids, with the remainder in the lipoteichoic acid, which is anchored in the plasma membrane (75). Analysis of choline incorporation revealed that transparent variants incorporated 3- to 8-fold more of the radiolabel choline per cell in the cell wall teichoic acids compared with opaque variants of the same strain (16). This finding was confirmed by multiple immuno-assays (76, 77). Additional evidence that the teichoic acid structure mediates changes in colony opacity comes from mutational analysis of the genes involved in choline incorporation. *S. pneumoniae* contains two copies of *licD*, shown in *H. influenzae* to function as an nucleotide diphosphate-choline transferase, which correlates with the expression of two choline residues/teichoic acid repeat units (78). Loss of *licD2* was associated with decreased uptake of radiolabeled choline and an altered colony phenotype.

These findings suggest that either the structure of the teichoic acid or the amount of teichoic acid per cell is subject to variation, since the only significant reservoir of cellular choline is the teichoic acids. Differences in teichoic acid structure could account for these observations if the amount of choline per teichoic acid chain varied owing to either differences in the average number of repeating units per chain or the number of choline residues per repeating unit. Either of these differences in structure would be expected to affect the size distribution of teichoic acid chains. Western blots using TEPC-15 showed a ladder-like array of doublet bands, which represents chains with differing numbers of repeating units (77). The opaque and transparent variants displayed the same

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pattern in the migration of each chain or in the average chain length. However, the intensity of reactivity for each band was greater for the transparent variant. This observation suggests that the higher amount of choline in transparent pneumococci is a result of increased teichoic acid chain units per cell rather than variation in structure or average number of repeating units per chain. Since the cell wall-associated teichoic acid is covalently attached to the muramic acid in the peptidoglycan and only a fraction of the muramic acids appear to be linked to a teichoic acid chain, the differences in the amount of teichoic acid may reflect more muramic acid residues with teichoic acid chains in transparent pneumococci.

Variations in Proteins

The relationship of several previously identified cell surface proteins to opacity variation has been examined. The role of cell lysis or autolysis in this phenomenon was considered because of several observations: (i) opaque colonies are dome shaped, whereas transparent colonies are umbilicated after equivalent incubation conditions; (ii) electron micrographs of organisms grown under identical conditions revealed that there was breakdown of the cell wall structures only in the transparent cells (16); (iii) opaque organisms undergo spontaneous lysis more slowly and are more resistant to the detergent deoxycholate (79). Autolysis occurs once the bacteria reach stationary phase through the enzymatic degradation of the cell wall primarily by the major murein amidase, LytA (an autolysin). Differences in rates of autolysis could result from variations in the peptidoglycan substrate or the expression of the amidase. The hydrolysis of purified cell walls of opaque and transparent organisms as determined by high-pressure liquid chromatography analysis of stem peptides released by treatment with amidase was indistinguishable (80). This result made it unlikely that differences in autolysis could be attributed wholly to variations in the cell wall. Consistent with relatively faster autolysis in transparent variants, they also showed a higher amount of LytA in immunoblotting detection. Mutants in which the *lytA* gene has been interrupted have altered colony morphology but are still capable of displaying phenotypic variation. This indicated that autolysin is only one of the factors that contribute to phase variation in colony opacity.

Other surface proteins are associated with opacity variation. PspA, a choline-binding protein and one of the major pneumococcal cell wall-anchored proteins (81), is present in greater quantities in opaque variants (72). PspA binds to lactoferrin (82, 83) and contributes to systemic pneumococcal infection in mice by inhibition of complement activation (81, 84). In contrast, CbpA, another choline-binding protein, is more abundant in transparent variants. CbpA, also known as PspC (85) or SpsA (86), binds to an array of host molecules such as secretory immunoglobulin A (86), polyimmunoglobulin receptor (87), complement factor H (88, 89), and complement component C3 (90). It is required for pneumococcal colonization in the nasopharynx of infant rats (91). More recently, analysis of the pneumococcal genome sequence has revealed a number of additional genes whose translation products have sequences related to the choline-binding domain (92, 93). Thus, the family of choline-binding proteins may be more extensive, and differences in their expression on the cell surface seem likely considering the variation in the choline anchor.

Microarray analysis has revealed 24 genes with significantly different expressions between opacity variants of independent strains (94). Among the 21 genes with increased expressions in the transparent variants, 11 are predicted to be involved in sugar utilization, including at least 2 sortase-anchored surface exoglycosidases. Seven of these genes are located in a single locus of pneumococcal genome, including the gene encoding the neuraminidase NanA. NanA is able to desialylate human airway glycoconjugates, such as human lactoferrin, secretory component, and IgA. These host factors bind to the surface of the pneumococcus *in vivo* during pneumococcal pneumonia. The efficiency of desialylation was increased several fold in the transparent variants and enhanced for host proteins binding to the surface of *S. pneumoniae*. Since deglycosylation affects the function of many host proteins, NanA may contribute to a protease-independent mechanism to modify bound targets and facilitate enhanced survival of the bacterium during colonization. Other gene products known to vary with colony opacity did not show differences in mRNA levels in the microarray analysis, suggesting that transcriptional regulation is not the primary mechanism controlling opacity phenotypes.

Using high-resolution two-dimensional protein electrophoresis, Overweg *et al.* showed that compared with transparent variants, the opaque variants overexpress one protein and underexpress two proteins (95). The protein overexpressed in the opaque variants is similar to elongation factor Ts of *H. pylori*. One of the two underexpressed proteins in the opaque variants was homologous to the proteinase maturation protein PrtM of *Lactocobacillus paracasei*, a member of the family of peptidyl-prolyl cis/trans isomerases. A consensus lipoprotein signal sequence suggests that the putative proteinase maturation protein A, designated PpmA, is located at the surface of the pneumococcus and may play a role in the maturation of surface or secreted proteins. The second underexpressed protein was identified as pyruvate oxidase, SpxB. The lower SpxB expression in opaque variants probably explains the reduced production of hydrogen peroxide, a reaction product of SpxB, in this variant (96). Although it was subsequently shown that production of hydrogen peroxide varies consistently with colony morphology, one of the strains analyzed using high-resolution two-dimensional protein electrophoresis was found to have a frameshift mutation in *spxB*, resulting in loss of its function (96, 97). Consistently, mutants in *spxB* and *spxR* (the transcriptional activator of *spxB*), also display the opaque phenotype (98). Since *spxB* is the main producer of hydrogen peroxide and this byproduct inhibits the organism's growth as colonies, some variations in colony morphology may be a consequence of differences in SpxB or its activity (99).

Other Characteristics Associated with Pneumococcal Phase Variation

Membrane property analysis by Aricha *et al.* showed that the opaque and transparent variants carry the same types of fatty acids but differ in their proportions (100). The opaque variants possess a relatively lower degree of unsaturated fatty acids in the cell membrane. Consistently, the opaque variants display higher levels of microviscosity (an increase from 27% to 38%) for the cell membrane of opaque variants.

One of the most thoroughly studied aspects of pneumococcal biology is natural genetic transformation, referring to the ability of the organism to take up and incorporate exogenous DNA. As a variable trait between the two colony variants, the pneumococci derived from transparent colonies are much more competent in natural transformation (101). The transformation frequency of the transparent variants was 9- to 670-fold higher than those of their opaque counterparts. These differences were independent of the DNA incorporated and inversely correlated with the amounts of capsular

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polysaccharide. Genetic transformation therefore tends to select for a less-encapsulated subpopulation with a transparent phenotype.

IMPACT OF PHASE VARIATION IN COLONY OPACITY ON PNEUMOCOCCAL PATHOGENESIS

The biological differences among pneumococcal phase variants are characterized in animal models. In an infant rat colonization model, only the transparent variants were able to establish dense and stable colonization on the mucosal surface in the nasopharynx when the animals were intranasally infected with equal inocula (10^3 CFU) of opaque or transparent variants derived from the same strain (16). After challenge with a large inoculum (10^7 CFU) of an opaque variant, the pups gradually became colonized, but by the end of 7 days the opaque variant had been cleared and there was heavy colonization with transparent forms. This suggested that since such a large inoculum contained a small number of transparent variants, these were selected for from the heterogeneous inoculum. The role of the opaque variant in pneumococcal pathogenesis became evident when the variants were compared in a systemic infection model of adult mice (72). Infant rats are not highly susceptible to invasive pneumococcal infection. After intraperitoneal challenge with opaque or transparent variants derived from the same strain (10^7 CFU), all mice receiving the opaque organisms succumbed to the infection. In contrast, of the few mice that survived the infection with transparent organisms, splenic cultures revealed only organisms that had reverted to the opaque phenotype. This suggests that during invasive infection, there is a strong selection for organisms with the opaque phenotype. Similarly, opaque variants show enhanced survival compared to transparent variants of the same strain during experimental middle ear infection (otitis media) in the setting of inflammation induced by concomitant influenza A virus infection (102).

The animal experiments strongly suggest that the pneumococcus phase varies between at least two forms: one adapted for nasopharyngeal colonization and the other for events following colonization. The animal experiments therefore demonstrated the relevance of opacity variation to the pathogenesis of pneumococcal infection. Furthermore, observations in animals correlate with phenotypic differences in type-matched isolates simultaneously cultured from the nasopharynx (transparent phenotype predominant) and bloodstream (opaque phenotype predominant) of the same individual with pneumococcal infection (73).

CONTRIBUTION OF PHENOTYPIC VARIATION TO HOST CLEARANCE MECHANISMS

The elevated amounts of cell wall choline associated with the transparent phenotype may, in addition, promote clearance of the organism by the host. Human serum contains abundant natural antibody to choline and C-reactive protein (CRP), both of which recognize this structure and promote the *in vivo* clearance of the pneumococcus (103–105). CRP, an acute-phase reactant whose levels may rise 1,000-fold during inflammation, is capable of activating the classical pathway of complement by binding to C1q (106). CRP expression has also been described in leukocytes and in the human respiratory epithelium (107). Only transparent pneumococci appear to bind significant amounts of human CRP (108). Since cell wall choline binds to the receptor for platelet-activating factor on human cells (109), binding of CRP effectively inhibits the receptor for platelet-activating factor-mediated adherence to epithelial cells (110). These factors may lead to a selection of pneumococci with less cell surface choline (of the opaque phenotype) once an organism has bypassed the mucosal barrier.

The contribution of the capsular polysaccharide to the pathogenesis of pneumococcal infection has been ascribed to its antiphagocytic properties. The possibility that the increased virulence of opaque pneumococci could be due to the antiphagocytic effect of the higher amount of capsular polysaccharide associated with this phenotype was addressed in an opsonophagocytosis assay (108). For variants from an individual isolate, the amount of immune serum required for opsonophagocytosis by HL-60 cells in culture was proportional to the quantity of capsular polysaccharides. The average titer of serum required to achieve 50% killing was significantly greater for opaque compared with transparent variants. In addition, natural IgG2 antibody against choline is opsonophagocytic and more effective against transparent variants, which express increased amounts of the cell wall choline antigen and less antiphagocytic capsular polysaccharide (111). This suggests that the ability of opaque orga-

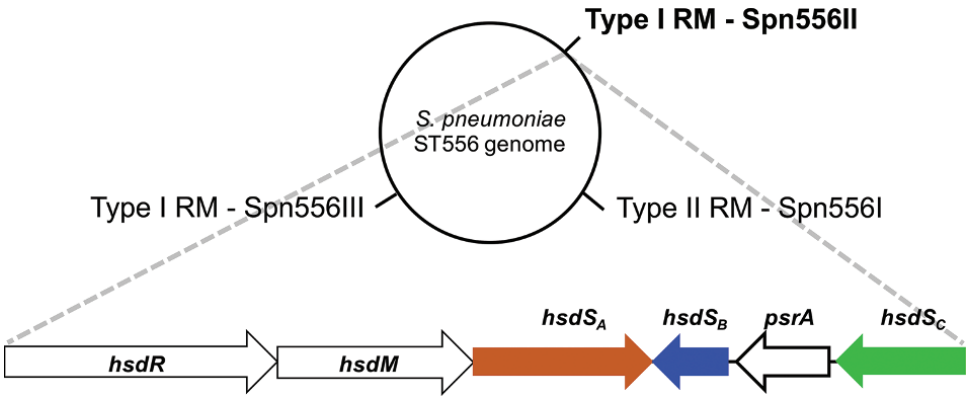


FIGURE 2 Genetic organization of the *cod* locus of *S. pneumoniae* ST556. The positions of three functional RM systems are indicated in the genome of ST556 according to a previous study (59). The gene order and other features in the *cod* locus are based on the published genome of the strain (accession no. CP003357) (122).

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nisms to cause sepsis may be a result of diminished clearance by the combined activity of antibody, CRP, complement, and phagocytes.

MECHANISM(S) OF PNEUMOCOCCAL PHASE VARIATION IN OPACITY

Diversification of Pneumococcal Methylomes by DNA Inversions in a Type I RM Locus

Our laboratory recently identified an epigenetic mechanism that controls a reversible switch between opaque and transparent phenotypes (52). This mechanism depends on DNA inversion events among three *hsdS* genes of a conserved type I RM system in *S. pneumoniae*. Bacterial RM systems are well known for their classical function as defense systems against the invasion of foreign DNA. DNA methylation catalyzed by the DNA methyltransferases in RM systems protects bacterial genomes from self-cleavage by their restriction endonuclease counterparts. As one of the four major RM types, each type I RM system usually possesses three genes: *hsdR*, *hsdM*, and *hsdS* (encoding the restriction enzyme, methyltransferase, and sequence recognition protein, respectively). The three proteins form the functional methyltransferase (M_2S) and restriction enzyme (R_2M_2S) protein complexes (112). The S subunit in both protein complexes is responsible for DNA recognition specificity (112). We (52, 59) and others (60, 113, 114) identified a type I RM system in strains ST556 (Spn556II) and D39 (SpnD39III), which undergoes extensive DNA inversions in the three homologous *hsdS* genes. Because these DNA inversions control the phase variation in opacity, this type I RM locus was named the colony opacity determinant (*cod*) locus (52), while Manso *et al.* named the same locus in strain D39 SpnD39III (60). For the convenience of this discussion, we will follow our nomenclature. As illustrated in Fig. 2, the *cod* locus contains three homologous *hsdS* genes ($hsdS_A$, $hsdS_B$, and $hsdS_C$) and a DNA invertase gene (pneumococcal site-specific recombinase A, *psrA*), in addition to *hsdR* and *hsdM*. Although this locus is highly conserved among the genomes of all *S. pneumoniae* strains available to date, the sequence configurations of the three *hsdS* genes vary in the genomes of different strains (113, 114).

Some details of the DNA inversions in the *cod* locus were recently described in strains ST556 and D39 (52, 60). Since

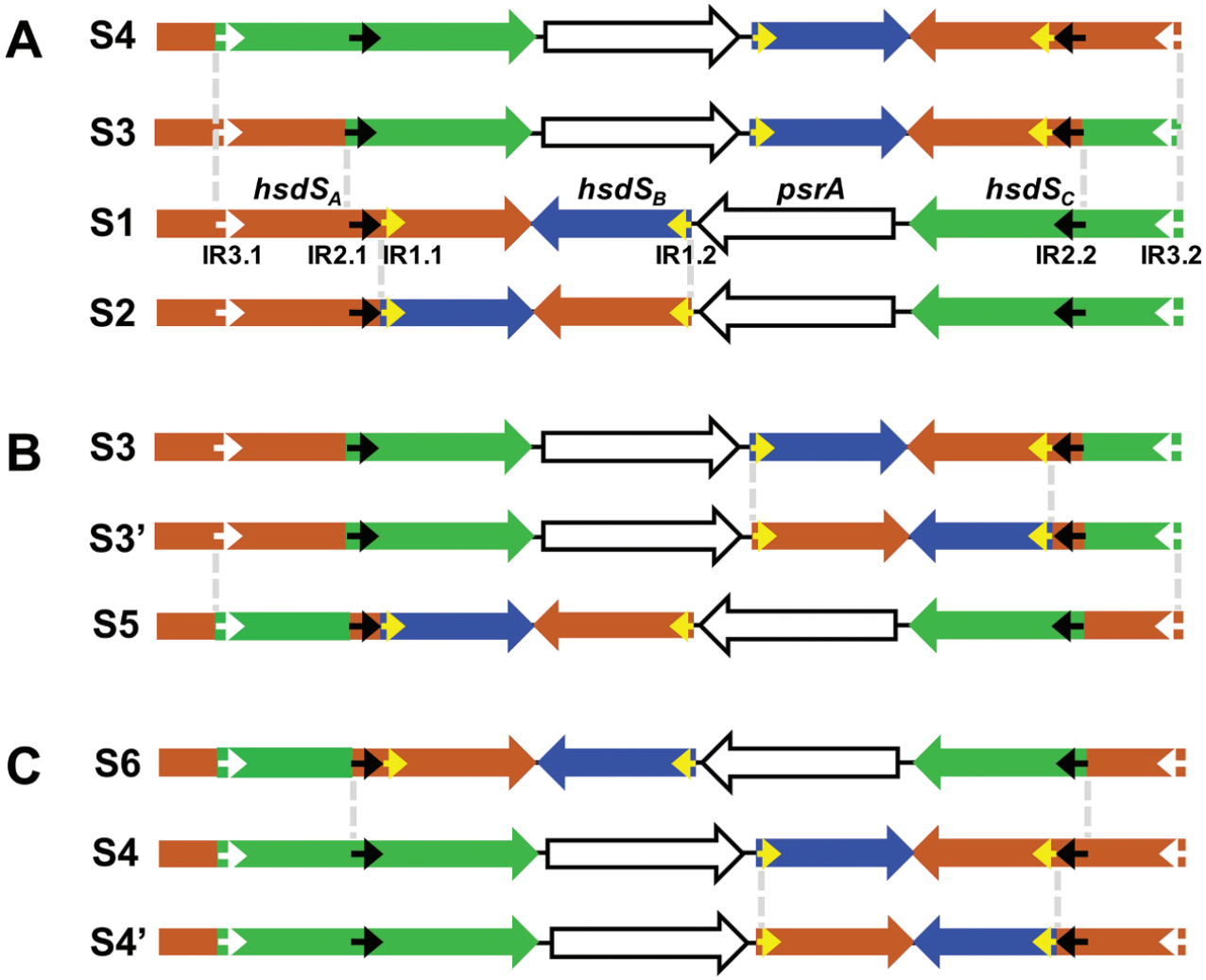


FIGURE 3 Unique genetic configurations generated by DNA inversions in the *cod* locus. The genetic orientations of the *hsdS* and *psrA* genes in the derivatives of forms S1 (A), S3 (B), and S4 (C) are illustrated by arrows. Each DNA configuration is given an S number. The sequence compositions of the *hsdS* genes are indicated by colors. The inverted repeats (IRs) in three *hsdS* genes are represented by yellow (IR1), black (IR2), and white (IR3) arrowheads. The inverted DNA segments are marked by dashed lines.

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the inversion reactions in this locus appear to operate in the same manner among different strains, we only summarize the major findings for ST556 in this article. As illustrated in Fig. 3, the three *hsdS* genes in the *cod* locus undergo different DNA inversions, designated configurations S1 to S6. The inverted sequences are demarcated by three pairs of inverted repeats, which are referred to as IR1 (15 bp), IR2 (298 bp), and IR3 (85 bp). While $hsdS_A$ carries the forward sequences of all three IRs (IR1.1, IR2.1, and IR3.1), the inverted counterparts of these repeat sequences are located in the coding sequences of $hsdS_B$ (IR1.2) and $hsdS_C$ (IR2.2 and IR3.2). The first three forms of DNA sequences in the *hsdS* loci (S2, S3, and S4) can be derived from three independent inversions of S1 (Fig. 3A). S2 is the product of DNA inversion within the coding sequences of $hsdS_A$ and $hsdS_B$. S3 and S4 were generated between $hsdS_A$ and $hsdS_C$. Four additional DNA configurations of *hsdS* loci (S3′, S4′, S5, and S6) can be further derived from DNA inversions in the S3 and S4 configurations. DNA inversions within $hsdS_B$ and $hsdS_C$ mediated by IR1.1 and IR1.2 in S3 lead to the generation of S3′. Similarly, S5 is derived from S3′ by an inversion of the sequence bounded by IR3.1 and IR3.2 (Fig. 3B). S4′ and S6 are generated from S4 by the DNA sequence inversion flanked by the pair of inverted repeat IR1 and IR2, respectively (Fig. 3C). In total, the DNA inversions in this locus generate six unique $hsdS_A$ alleles.

Our recent study reveals that the PsrA invertase encoded by the *cod* locus catalyzes the DNA inversion between $hsdS_A$ and $hsdS_B$ (52). PsrA is homologous to the tyrosine family recombinases. While the deletion mutant of *psrA* does not show detectable inversion between $hsdS_A$ and $hsdS_B$, it is still capable of DNA inversion between $hsdS_A$ and $hsdS_C$. This result indicates that the inversions between $hsdS_A$ and $hsdS_C$ are driven by unknown invertases. It should be mentioned that our preliminary analysis indicated that only the $hsdS_A$ gene of the *cod* locus is cotranscribed from the promoter upstream of *hsdR* (Fig. 2), but $hsdS_B$ and $hsdS_C$ are transcriptionally silent (manuscript in preparation). Consistently, our sequencing analysis showed that $hsdS_B$ and $hsdS_C$ have no detectable impact on the DNA methylation pattern or methylome of *S. pneumoniae* (52). In this context, it appears that the functions of $hsdS_B$ and $hsdS_C$ are solely for donation of their unique sequences to $hsdS_A$.

Subsequent methylation sequencing of the $hsdS_A$ allele-locked strains reveals that each of the six $hsdS_A$ alleles generated by the DNA inversions recognizes a unique DNA sequence and thus determines a distinct pneumococcal methylome (Fig. 4) (52, 60). It is thus conceivable that these DNA inversions in the *cod* locus are able to diversify an originally clonal population into a heterologous pool of at least six epigenetic types. The bacterial cells in each of the epigenetic types possess a unique methylation pattern of genome DNA. Previous modeling analysis suggests that the S subunit (encoded by *hsdS*) in the type I RM system consists of two target recognition domains (TRDs), each of which recognizes one half of the type I RM methylation motif (115). Comparing the coding sequences of $hsdS_{A1}$ to $hsdS_{A6}$ shows alleles A1, A2, and A3 apparently to have the same first TRDs and the first halves of their methylation motifs (5′-CRAA-3′). Similarly, the first TRD shared by A4, A5, and A6 is likely responsible for 5′-CAC-3′ recognition. The second TRD at the terminals of the $hsdS_A$ alleles (indicated by three colors in Fig. 4) correspond to 5′-CTT-3′, 5′-TTC-3′, and 5′-CTG-3′.

Phase Variation in Colony Opacity by Epigenetic Switch

Microscopic analysis of the $hsdS_A$ allele-locked strains reveals that the methylome switch driven by the DNA inversions in the *cod* locus controls pneumococcal phase variation in colony opacity (52). Specifically, the methylome generated by the $hsdS_{A1}$ allele leads to the formation of opaque colonies, whereas the pneumococci carrying the other five $hsdS_A$ alleles or lacking the *cod* locus produce transparent variant (Fig. 5). In addition, the opaque colony associated with $hsdS_{A1}$ requires DNA methylation activity of the type I RM system because the mutants with the loss-of-function mutations in

$hsdS_A$ allele	Allele composite	Methylation motif	Colony opacity
$hsdS_{A1}$		5'-CRAAN$_8$CTT-3' 3'-GYTTN$_8$GAA-5'	Opaque
$hsdS_{A2}$		5'-CRAAN$_9$TTC-3' 3'-GYTTN$_9$AAG-5'	Transparent
$hsdS_{A3}$		5'-CRAAN$_8$CTG-3' 3'-GYTTN$_8$GAC-5'	Transparent
$hsdS_{A4}$		5'-CACN$_7$CTG-3' 3'-GTGN$_7$GAC-5'	Transparent
$hsdS_{A5}$		5'-CACN$_8$TTC-3' 3'-GTGN$_8$AAG-5'	Transparent
$hsdS_{A6}$		5'-CACN$_7$CTT-3' 3'-GTGN$_7$GAA-5'	Transparent

FIGURE 4 The unique methylome specified by each of the HsdS variants in the $hsdS_A$-locked strains. The sequence origins of each $hsdS_A$ allele for the three *hsdS* genes are color-coded as shown in Fig. 3. The methylated adenosine nucleotide in the DNA methylation motif identified by single-molecule real-time sequencing in each $hsdS_A$-locked strain is shown as a red character. The opaque and transparent phenotypes associated with the $hsdS_A$-locked strains in colony opacity are indicated in the right-hand panel. R = A or G, Y = T or C.

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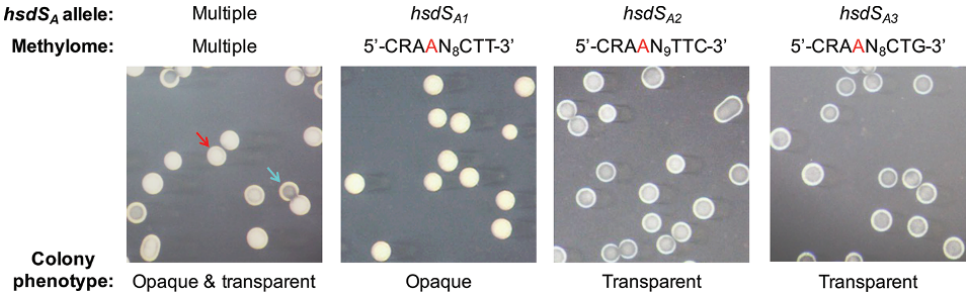


FIGURE 5 Colony phenotypes associated with strain ST556 and its $hsdS_A$-locked derivatives. The colonies were prepared and photographed under a dissection microscope as described (52). The representative opaque and transparent colonies in the wild-type strain are indicated by blue and red arrowheads, respectively.

the *hsdM* gene no longer form opaque colonies even in the presence of the $hsdS_{A1}$ allele. Finally, additional expression of the $hsdS_{A1}$ allele in the strain carrying $hsdS_{A2}$ or $hsdS_{A3}$ reverts the colony phenotype from transparent to opaque. This observation shows that the reversible switch of the $hsdS_A$ alleles by DNA inversion drives spontaneous phase variation between the opaque and transparent colonies. Together with other lines of evidence, this result indicating that DNA methylation of the pneumococcal genome at the 5′-CRAAN8CTT-3′ sequence and cellular conditions defined by the $hsdS_{A1}$ allele is the basis of the opaque colony phenotype; the loss of the $hsdS_{A1}$ allele by DNA inversions switches the pneumococci to the transparent or nonopaque phenotype (Fig. 6). The ON-and-OFF switch of the $hsdS_{A1}$ allele in the *cod* locus driven by DNA inversions results in the pneumococcal methylome variation between the methylated and nonmethylated states of the 5′-CRAAN8CTT-3′ motifs and thereby dictates phase variation between the opaque and transparent phenotypes. There are over 1,000 copies of the 5′-CRAAN8CTT-3′ motifs in the pneumococcal genome, but it remains to be determined which of these sites are involved in dictating the opacity variants. It is worth noting that in our preliminary experiments, the pneumococcal strains carrying the other $hsdS_A$ alleles (A2 to A6) also display unique phenotypes in other characteristics, although they share the transparent colony phenotype (manuscript in preparation). We believe that the DNA inversions in the *hsdS* genes drive additional types of phase variation, in addition to that in colony opacity.

To the best of our knowledge, this DNA inversion-driven epigenetic mechanism of pneumococcal phase variation is the first case in which DNA inversion and differential DNA methylation are combined to generate phenotypic variation. As discussed in the previous secions, DNA inversion or differential DNA methylation alone is able to produce phenotypic variants in bacteria, such as the DNA invertase-driven switch in the *Salmonella* flagellum (32, 33) and Dam methyltransferase-dependent phase variation in *E. coli* fimbriae (54). Both the DNA inversion and differential methylation mechanisms alone can produce limited numbers of variants, but DNA inversion in the *hsdS* methyltransferase genes in the *cod* locus of *S. pneumoniae* appears to take advantage of both the mechanisms, generating multiple types of phase variation in different biological traits. Because of the ubiquitous nature of the *cod*-like locus in archaeal and bacterial species (see below), the switch in colony opacity mediated by DNA inversion-driven epigenetic diversification in *S. pneumoniae* represents a novel mechanism of phase variation in the prokaryotes.

Other Characteristics of Phase-Locked Pneumococci

Because of the previously mysterious nature of the genetic and molecular mechanism behind the generation of pneumococcal opacity variants, the study of the biology of these variants has relied on pneumococcal populations that are prepared with natural opaque or transparent colonies of wild-type strains. The discovery of the epigenetic switch

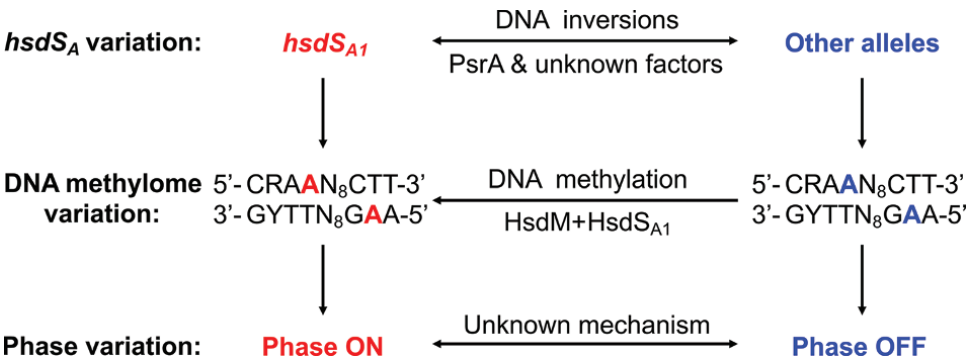


FIGURE 6 Diagrammatic demonstration of the relationship between an epigenetic switch driven by DNA inversion in the *cod* locus and phase variation in colony opacity. The methylated and unmethylated adenine nucleotides in the DNA motif by the $HsdS_{A1}$-associated methyltransferase are highlighted with red and blue characters, respectively. R = A or G, Y = T or C.

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driven by DNA inversions in the *cod* locus has made it possible to generate $hsdS_A$ allele- or phase-locked strains for precise characterization of the biologic and pathogenic implications associated with the phase variants. The following is a summary of what has been learned with the phase-locked strains. First, the site-specific recombinations in the *hsdS* gene of the *cod* locus broaden the defense spectrum against the evasion of foreign DNA. The strains carrying a single $hsdS_A$ allele inhibit transformation with donor DNA molecules that are methylated by other $HsdS_A$ variants (52, 60). Second, in agreement with the previous observation of the natural opacity variants (116), the strains carrying $hsdS_{A1}$ are less adhesive than the transparent counterparts carrying the other $hsdS_A$ alleles in epithelial cell cultures (52). Likewise, opaque colony phenotype, $hsdS_{A1}$-locked pneumococci showed weaker colonization than locked allelic variants with the transparent phase (52). These results indicate that the phase variation driven by reversible ON-and-OFF switches of the $hsdS_{A1}$ allele generate pneumococcal variants with variable capacity in nasopharyngeal carriage (16). Third, the allelic variants expressing $hsdS_{A1}$, $hsdS_{A2}$, and $hsdS_{A6}$ in strain D39 grew more slowly than the other three isogenic variants in the mouse sepsis model (60), suggesting that allelic variation in the *cod* locus maximizes the range of pneumococcal fitness during invasive infection. Finally, unencapsulated pneumococci with deletions within the capsule biosynthesis locus still form colonies with these two phases (52), demonstrating that the capsule is not necessary for the phase variation in colony opacity. The $hsdS_A$ allele-locked strains have also been used to study the transcriptomes of the opacity variants (60, 117). Manso *et al*. found that the genes in the capsule biosynthetic locus are transcriptionally downregulated in the $hsdS_{A2}$ allelic variant of strain D39 compared with those of $hsdS_{A3}$, $hsdS_{A4}$, and $hsdS_{A5}$ (60). However, Oliver *et al*. showed that there is no significant difference in transcription levels of the capsule locus among the six corresponding $hsdS_A$ allele-locked variants of strain TIGR4 (117). This difference may be related to variations in bacterial strain, growth medium, or other conditions used in these studies.

The *cod*-Like Systems in Other Bacteria

The *cod*-like type I RM systems are found in many phylogenetically diverse archaeal and bacterial species, which includes the homologs of the *psrA* invertase gene and multiple copies of *hsdS* in the same locus. These include *Mycoplasma* (e.g., *Mycoplasma pulmonis* and *Mycoplasma bovis*), Spirochetes (e.g., *Treponema medium* and *Treponema denticola*), Gram-negative bacteria (e.g., *B. fragilis* and *Campylobacter upsaliensis*), and Gram-positive bacteria (e.g., *Enterococcus faecalis* and *Streptococcus agalactiae*). *M. pulmonis* has two type I RM systems, each of which undergoes DNA inversions catalyzed by the HvsR tyrosine recombinase between the *hsdS* genes (39, 118–120). Some of the DNA inversions generate HsdS allelic variants, which recognize distinct DNA sequences and thus result in variable restriction activities. DNA inversions of the *hsdS* genes have also been observed in *B. fragilis* (38), although there is no functional information on the bacterial variants. Although it remains to be determined whether these *hsdS* inversions in *M. pulmonis* and *B. fragilis* have any functional impact on bacterial methylomes and other aspects of biology beyond restriction, it is tempting to predict that these type I RM systems likely play important roles in epigenetic and phenotypic diversity among the prokaryotic organisms, based on the DNA inversion-driven pneumococcal phase variation (121).

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Genomics and Genetics of *Streptococcus pneumoniae*

FRANCESCO SANTORO, FRANCESCO IANNELLI AND GIANNI POZZI

22

The first complete genome sequence of the pneumococcal type 4 strain TIGR4 was published in 2001. To date (February 2018), GenBank hosts 37 complete pneumococcal genomes and more than 8,000 draft genome sequences. The genetics of *Streptococcus pneumoniae* began with the discovery of bacterial transformation by Griffith in 1928 and subsequent identification of DNA as the genetic material by Avery, MacLeod, and McCarty in 1944 (1). Many of the *S. pneumoniae* strains commonly used in genetic studies are derivatives of the clinical strains obtained by Avery in 1916 (Table 1) (2–4).

PNEUMOCOCCAL GENOMES

In 2000, the Institute for Genomic Research (TIGR) released the first complete genome of serotype 4 invasive strain TIGR4 (formerly known as JNR.7/87 [5]) for BLAST search and for download. The work describing the genome sequence was subsequently published in 2001 (6). In the same year, two more *S. pneumoniae* genomes were published: (i) the genome of strain G54, an Italian clinical isolate serotype 19F strain (7, 8), first released as a draft genome in 31 annotated DNA contigs (9) and later completed (GenBank accession no. CP001015), and (ii) the complete genome of R6 (10), an unencapsulated derivative of the type 2 Avery's strain D39 (11). Six years later, in 2007, the genome sequence of D39 was completed and released (12).

Of the 37 pneumococcal genomes hosted in GenBank, 9 were obtained by Sanger sequencing and the rest by next-generation sequencing or by combining Sanger with next-generation sequencing technologies (Table 2).

The next-generation sequencing technologies with relatively low costs and high throughput produced more than 8,000 pneumococcal genomes, but only 14 were closed and completed. This is essentially because short sequence reads, such as those obtained by Illumina sequencing, are inherently incapable of covering and closing gaps located in long repeated regions (13). Long read sequencing technologies such as PacBio and Nanopore sequencing could be used to close the draft genome sequences. On the other hand, the database redundancy due to inflation of essentially identical pneumococcal genomes impairs database exploration and data retrieval. In addition, automated gene annotation tends to add redundancy and to amplify annotation errors. To overcome difficulties in pneumococcal database exploration, it is suggested that you perform homology searches using the online tool Microbial Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=MicrobialGenomes), selecting the complete genomes database, and if necessary, extending the search to draft genomes. Alternatively, a stand-alone database containing only genomes of interest can be created and efficiently searched.

Table 2 shows the main characteristics of the 37 complete pneumococcal genomes. Sequenced strains include Avery's classic strains D39 (type 2) and A66 (type 3) and their derivatives (1, 14); the remaining strains are clinical isolates from both invasive and noninvasive diseases or carriage. Four clinical strains belong to lineages that have contributed to the global spread of antibiotic resistance, namely, Spain-23F-1 (ATCC700669), Spain-6B-2 (670-6B, ATCC700670), Hungary19A-6 (ATCC700673), and Taiwan 19F-14 (ATCC700905) (15). Pneumococcal genome analysis showed that the median genome size is 2,085 kb and the average GC content is 39.6%. There is a strong bias in gene orientation, and the large majority of the genes are transcribed in the same direction as the DNA replication (6). The genome of *S. pneumoniae* contains a large number of carbohydrate uptake systems belonging to the ATP binding cassette (ABC) superfamily and to the phosphoenolpyruvate-dependent phosphotransferase system family; notably, these transport systems and the respective operons are components of the variable part of the pneumococcal genome (16). Energy generation relies essentially on substrate-level phosphorylation and glycolysis (17); genomic analysis suggests that *S. pneumoniae* can catabolize a wide array of sugars, as confirmed by fermentation experiments demonstrating that pneumococci were able to metabolize at least 32 carbohydrates, including 6 polysaccharides (16). Comparative genomics of *S. pneumoniae* indicates that there is a core genome formed by about 70 to 80% of sequences common to all strains (18, 19), whereas intraspecies variability is essentially due to (i) allelic variants of genes present in all pneumococcal genomes, as in the case of the capsule genes, *psp*C, and *psp*A (20–23), and (ii) DNA sequences which are "added" to the pneumococcal genome and belong to bacteriophages and other mobile genetic elements (MGEs). This added DNA, known as mobilome, (i) is uniquely present in a strain (or a group of strains), (ii) typically shows a different GC content from the rest of the bacterial genome, (iii) is an insertion of heterologous DNA into the bacterial chromosome, as demonstrated in early work by Guild and collaborators (24), (iv) is not strictly part of the pneumococcal genome but is shared with other bacterial genomes, and (v) contains a higher ratio of open reading frames whose putative gene products are of unknown function. From a genomic perspective, correct definition, careful manual

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TABLE 1 Properties of classic *S. pneumoniae* laboratory strains

Strain	Capsule[a]	Relevant properties[b]
D39	Type 2^+	Clinical isolate (1928); virulent; $pDP1^+$; *comC1*
R36	Type 2^-	D39 passaged 36× in anti-type 2 serum (1944); does not revert to Cps^+ (see R36A, below); Hex^+; $pDP1^+$
R36N	Type 2^-	Colony variant of R36 (1944); nontransformable; $pDP1^+$
R36NC	Type 2^-	Transformable derivative of R36N (1947); *Dpn*I; Hex^+; $pDP1^+$
R36A	Type 2^-	Colony variant of R36 (1944); deletions demonstrated in type 2 capsule locus (45); Hex^+; $pDP1^-$
R6	Type 2^-	Single-colony isolate of R36A (1962); *Dpn*I; Hex^+; $pDP1^-$
R6x	Type 2^-	R6 transformed to the Rx Hex^- phenotype (1973)
Rx	Type 2^-	R36A transformed to type 3 capsule (1949) and subsequently isolated as a spontaneous "unencapsulated"' derivative (1959) (but see Rx1, below); Hex^- (*hexB*$^-$); $pDP1^-$
Rx1	Type 3*	Highly transformable derivative of Rx; contains the type 3 *cps* locus, a 5′ deletion of *ali*A (*plp*A), and a point mutation in *cps3D* responsible for reduced production of capsule (23, 25); virulence is not restored by transformation to type 3 capsule; *Dpn*0; Hex^- (*hexB*$^-$); $pDP1^-$
A66	Type 3^+	Clinical isolate (1928); virulent; donor in 1944 transformation studies of Avery et al.; *Dpn*I; *comC2*

[a]The *cps* genotype is indicated, and the symbols indicate whether the strain is encapsulated (+), unencapsulated (–), or intermediate (*).
[b]Numbers in parentheses indicate the date of original description. The *Dpn*0 phenotype in Rx1 is apparently due to a mutation in the *Dpn*I-encoding locus. Hex phenotypes are described in references 3 and 147. *com*C alleles are described in reference 7.

annotation, and phenotypic characterization of the most common MGEs of a bacterial species are essential for improved performance of comparative genomic studies. For instance, open reading frames of uncharacterized MGEs are automatically annotated as "conserved hypothetical proteins," because they are found in many different bacterial genomes as part of the mobilome. The mobilome includes plasmids, prophages, transposable elements comprising integrative and conjugative elements (ICEs), pathogenicity islands, and insertion sequences (Table 2, Table 3). Pneumococcal genomes also contain three families of small interspersed repeats, namely, BOX, RUP (repeat unit of pneumococcus), and SPRITE (*S. pneumoniae* rho-independent terminator-like element) (25–27). As evidenced from the genome sequences, BOX and RUP are present in more than 100 copies and are found almost exclusively outside of coding regions, and SPRITE elements are also found in intergenic regions but are less represented (about 30 copies/genome). Their functions are unknown, although RUP elements are thought to be mobilizable by the IS*630*-Spn1. The BOX element comprises three subunits of 59, 45, and 50 bp (box A, box B, and box C, respectively), although not all are present in every copy and modulate gene expression (28).

Pneumococcal Population Structure

In the past few years, several studies have investigated the genomes of pneumococcal strain collections from diverse settings. These studies characterized different pneumococcal lineages, i.e., groups of isolates with high genomic similarity. Within the same lineage, ICEs were generally conserved, while sequence variation occurred by recombination of small fragments of DNA and by loss or acquisition of prophages (29). As expected for a naturally competent bacterium, the sequence variation in pneumococcal populations essentially arose from recombination and not by accumulation of base substitutions (30). Recombination events occurring upon horizontal DNA transfer were detected at a relatively small number of loci, such as *psp*A, *psp*C, and the *cps* locus. These loci encode for surface-exposed structures of the bacterial cell, which are under selective pressure imposed by the human host. In particular, *psp*A and *psp*C have a mosaic structure that typically evolves by consecutive recombination events (20, 21). The *cps* locus is located between the conserved *dexB* and *ali*A genes and exhibits high variability, with more than 90 serotypes identified that are encoded by different allelic variants (22). Recombination events, leading to serotype switch, are relatively frequent in pneumococcal populations (191 events were detected in a collection of 3,095 carriage isolates [31]) and can also lead to loss of the whole locus or to its acquisition by unencapsulated pneumococci. Different lineages have been identified even among unencapsulated pneumococcal carriage isolates, which may act as a gene reservoir (32). Other highly recombinogenic loci are those under selective pressure from antibiotics, such as the penicillin binding protein genes *pbp1a*, *pbp2b*, and *pbp2x* encoding cell wall synthesis enzymes or the dihydrofolate reductase gene *fol*A. Allelic variants of *pbp* are responsible for the reduced susceptibility to beta-lactam antibiotics, while variants of *fol*A are responsible for cotrimoxazole resistance.

Genomic variability among isolates belonging to different lineages is mainly due to variation in accessory regions, which contain a wide array of genes, including some important virulence factors (33). Comparison of invasive and noninvasive pneumococcal isolates shows that the invasive potential of a strain is often correlated with the presence or absence of a few specific accessory regions. The pneumococcal pathogenicity island 1 (PPI1) is a highly variable accessory region that is present in about two-thirds of pneumococcal isolates (34). It has a lower GC content than its surrounding regions, and it is composed of (i) an operon coding for an ABC iron transporter (35), (ii) the *pezAT* toxin-antitoxin system (36), (iii) a putative lantibiotic production locus, and (iv) the *nplT* neopullulanase gene. Variants of PPI1 show different levels of virulence in mouse models; in particular, the PPI1 locus from highly virulent isolates contains both *pezAT* and *nplT* and shows enhanced fitness in blood, lungs, and nasopharinx (37). The *pezAT* toxin-antitoxin system is a two-gene operon that codes for a labile PezA antitoxin and a stable PezT toxin (36). The toxin targets the cell wall synthesis pathway, and the antitoxin blocks its activity. The system impairs competence development and makes pneumococcal cells more susceptible to beta-lactams and to lysis in the stationary phase (38).

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TABLE 2 Complete genomes of *S. pneumoniae*

Strain name (serotype)	Size (Mb)	G C %	No. of genes	No. of proteins	MGEs contained[a]	Sequencing Technology	GenBank accession no. (release date)	Reference
R6 (rough type 2)	2.04	39.7	1,967	1,814		Sanger	AE007317.1 (03/10/2001)	10
TIGR4 (4)	2.16	39.7	2,344	2,092		Sanger	AE005672.3 (03/10/2001)	6
D39 (2)	2.05	39.7	2,220	1,996		Sanger	CP000410.1 (12/10/2006)	11
Hungary19A-6 (19A)	2.25	39.6	2,429	2,167	Tn*6002*-like Defective Tn*5253*-like Group 1 phage Group 3 phage	Sanger/454 FLX	CP000936.1 (27/02/2008)	18
CGSP14 (14)	2.21	39.5	2,380	2,112	Tn*2008* (ICE*Sp*23FST81-like/SPnRi3*erm*-like) Tn*3872*-like	Sanger	CP001033.1 (11/04/2008)	171
G54 (19F)	2.08	39.7	2,269	1,986	Defective ICE*Sp*23FST81-like/defective Tn*6002*-like	Sanger	CP001015.1 (22/07/2008)	9
ATCC 700669 (23F)	2.22	39.5	2,403	2,150	ICE*Sp*23FST81/Tn*916*-like MM1 phage (group 3)	Sanger	FM211187.1 (13/01/2009)	38
70585 (5)	2.18	39.7	2,397	2,134	Group 1 phage	Sanger/454 FLX	CP000918.1 (27/03/2009)	18
JJA (14)	2.12	39.7	2,285	2,057		Sanger/454 FLX	CP000919.1 (27/03/2009)	18
P1031 (1)	2.11	39.7	2,336	2,081	Tn*5253*-like/Tn*916*-like Group 1 phage	Sanger/454 FLX	CP000920.1 (27/03/2009)	18
Taiwan19F-14 (19F)	2.11	39.8	2,306	2,052	Tn*2009*-like	Sanger/454 FLX	CP000921.1 (27/03/2009)	18
TCH8431/19A (19A)	2.09	39.8	2,277	2,031	Tn*2010*-like	454	CP001993.1 (14/06/2010)	
INV200 (14)	2.09	39.6	2,264	2,006		Sanger/Illumina	FQ312029.1 (29/07/2010)	
OXC141 (3)	2.04	39.8	2,246	2,005	Group 1 phage	Sanger	FQ312027.1 (29/07/2010)	
INV104 (1)	2.14	39.6	2,347	2,065		Sanger	FQ312030.1 (29/07/2010)	
SPN034156 (3)	2.02	39.9	2,220	1,963	Group 1 phage	Sanger/454 FLX	FQ312045.1 (29/07/2010)	172
SPN034183 (3)	2.04	39.8	2,244	1,986	Group 1 phage	Sanger/454 FLX	FQ312043.1 (29/07/2010)	172
SPN994038 (3)	2.03	39.8	2,232	1,990	Group 1 phage	Sanger/454 FLX	FQ312041.2 (29/07/2010)	172

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SPN994039 (3)	2.03	39.8	2,233	1,992	Group 1 phage	Sanger/454 FLX	FQ312044.2 (29/07/2010)	172
SPN032672 (1)	2.13	39.6	2,362	2,069		Sanger/454 FLX	FQ312039.1 (29/07/2010)	
SPN033038 (1)	2.13	39.6	2,365	2,063		Sanger/454 FLX	FQ312042.1 (29/07/2010)	
AP200 (11A)	2.13	39.5	2,327	2,067	Group 1 phage Tn*1806*	454 FLX	CP002121.1 (07/09/2010)	173
670-6B (6B)	2.24	39.6	2,472	2,199	Tn*5253*-like/Tn*6002*-like Group 1 phage	Sanger	CP002176.1 (09/09/2010)	
ST556 (19F)	2.15	39.7	2,359	2,110	Tn*2009*-like Group 3 phage	Sanger/454 FLX/PacBio	CP003357.2 (06/03/2012)	174
A45 (3)	2.13	39.8	2,394	2,069	Unassigned group phage	Sanger/454 FLX	HE983624.1 (07/09/2012)	18
gamPNI0373 (1)	2.06	39.8	2,277	2,026	Group 1 phage	454/Sanger	CP001845.1 (21/09/2012)	175
110.58 (NT)	2.29	39.8	2,445	2,084	ICE_1*Sp*ST344, ICE_2*Sp*ST344 (ICE*Sp*23FST81-like/Tn*6003*-like) Group 2 phage	PacBio	CP007593.1 (09/01/2015)	31
NCTC7465 (1)	2.11	39.7	2,330	2,064	Group 1 phage	Sanger/454 FLX/Illumina	LN831051.1 (22/03/2015)	18
A66 (3)	1.98	39.7	2,178	1,934		Illumina	LN847353.1 (20/05/2015)	13
SP49 (19A)	2.21	39.9	2,415	2,169	Group 1 phage Group 2 phage Group 3 phage	Illumina/PacBio	CP018136.1 (09/12/2016)	176
SP61 (19A)	2.07	39.9	2,245	2,008	Tn*2010*-like	Illumina/PacBio	CP018137.1 (09/12/2016)	176
SP64 (19A)	2.07	39.9	2,249	2,007	Tn*2010*-like	Illumina/PacBio	CP018138.1 (09/12/2016)	176
SWU02 (19F)	2.09	39.8	2,272	2,019	Tn*2010*-like	Illumina/PacBio	CP018347.1 (13/12/2016)	
KK0981 (3)	2.15	39.6	2,319	2,073	Tn*5253*-like/defective Tn*2017*-like	PacBio	AP017971.1 (25/01/2017)	177
19F (19F)	2.13	39.6	2,276	2,005		PacBio	CP025076.1 (07/12/2017)	
11A (11A)	2.08	39.8	2,317	2,075	Tn*6002*-like Group 2 phage	PacBio	CP018838.1 (07/12/2017)	
Xen35 (4)	2.17	39.7	2,322	2,052		Illumina	CP025256.1 (17/12/2017)	178

[a]MGEs (ICEs and prophages) were searched using Microbe BLAST against *S. pneumoniae* complete genomes. Insertion sites of MGEs and sequences of Tn*5253* and Tn*916* were used as query sequences. When available, publications linked to sequences were used to retrieve the MGE content of genomes. Phages are grouped according to the classification in reference 140.

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Two other important pneumococcal recombination hotspots are the insertion sites for the ICEs Tn*5253* and ICE*Sp*23FST81. These ICEs share homology in the conjugation machinery module and both carry *tet*(M) and *cat* genes but are inserted at different sites since they possess different recombination modules (39–41). The insertion site of Tn*5253* is located downstream of the IgA protease gene *zmpA* (SP_1154, TIGR4 genome), where extensive variability is detected, with the possible insertion of the *zmpA* paralog *zmpD* (42). The insertion site of ICE*Sp*23FST81 is located upstream of the conserved gene *rplL* (SP_1354, TIGR4 genome), which codes for the L7/L12 ribosomal protein. This region contains fragments of homology with ICEs, possibly indicating that it has been a target site for these elements for a long time (29).

It is worth noting that regions of PPI1 are homologous to fragments of ICE Tn*5253*, which also carries two copies of *pezAT*. PPI1 lies in a chromosomal recombination hotspot and is itself a hotspot of recombination, since it can accumulate mobile gene clusters, including the Ω*cat*(pC194) element. Ω*cat*(pC194) is part of Tn*5253* and is able to integrate within PPI1 (40).

HORIZONTAL GENE TRANSFER

Both transformation and conjugation have been described in *S. pneumoniae*. Transformation serves as the primary, and perhaps sole, means of transferring chromosomal genes. Conjugation occurs with plasmids that are capable of self-transfer or mobilization and with ICEs that are integrated into the chromosome. The latter mediate their own transfer but do not cotransfer linked chromosomal markers. Transfer of transformation of ICEs and chromosomal genes are the major mechanisms known to be involved in the spread of antibiotic resistance in *S. pneumoniae*. Generalized transduction has not been observed, although a process termed pseudotransduction, which involves properties of both transduction and transformation, has been described for one pneumococcal bacteriophage (10).

Transformation

Natural transformation in *S. pneumoniae* can be divided into several stages: (i) induction of competence, (ii) binding of double-stranded DNA to the cell surface, (iii) digestion of bound DNA into discrete double-stranded fragments, (iv) uptake of single-stranded linear DNA, (v) binding of the internalized DNA by single-stranded binding proteins, and (vi) recombination of homologous DNA into the chromosome by a single-strand replacement mechanism. Successful transformation of plasmids requires the uptake of two complementary, overlapping DNA strands. Essentially any DNA can be taken up, because no specific recognition sequences are required. Most DNA that is not homologous to the pneumococcal chromosome or that cannot replicate independently is degraded and becomes part of the nucleotide precursor pool. Occasionally, DNA that is not homologous to the chromosome but that is physically linked to DNA that is homologous can be inserted into the chromosome by a process known as homology-assisted illegitimate recombination (43).

Competence development

S. pneumoniae is competent for transformation for only a brief period during the early exponential phase of growth (44). The specific point at which competence is induced is determined by both the cell density and the culturing conditions. Maximal transformation efficiencies are usually observed in a rich medium containing calcium and serum or albumin, at a starting pH of about 7.0 to 7.2. Under optimum culture conditions, transformation of a given marker from a donor chromosomal DNA preparation can be observed in up to 10% of the recipients. Alterations in the starting pH affect both the timing and efficiency of transformation (45). A low pH tends to prevent the development of competence, whereas higher pH values result in multiple waves of competence that occur over a broader range of cell densities and rarely reach the peak efficiency. Under optimum conditions, cotransformation of two unlinked chromosomal markers occurs near the frequency expected for two independent events, indicating that essentially all cells in the culture become competent (46). Cotransformation of unlinked markers during subsequent, postpeak waves of competence occurs more frequently than expected, indicating that only a fraction of these cells are actually capable of being transformed (45). Genomic analysis of cotransformation shows that upon selection of an antibiotic marker, up to two secondary, unselected recombination events can occur within the same genome (47).

The development of competence is mediated by the synthesis and the release of the competence factor (44), a small peptide that induces expression of genes encoding transformation-related proteins. The great majority of *S. pneumoniae* strains contain one of the two main alleles of *com*C, the gene that encodes the competence-stimulating peptide (CSP) (7, 48). The 41-amino-acid translation products from *comC1* and *comC2* each contain an identical 24-amino-acid N-terminal leader sequence that possesses a Gly-Gly processing site common to peptide bacteriocins. The remaining 17 amino acids, which represent the mature, active peptides of CSP-1 and CSP-2, differ at eight positions. Sequence analysis of encapsulated strains representing multiple capsular serotypes has shown that each has either a *comC1* or *comC2* allele, which are both highly conserved. In general, however, transformation of encapsulated isolates is not readily observed under the growth conditions described above. In many cases, the failure to transform is related to the presence of the capsule, which reduces or blocks competence for genetic transformation (49, 50). Noncompetent cultures, as well as many encapsulated strains, can be induced to competence by the addition of CSP-1 or CSP-2 (7).

The identification of the *comC1* and *comC2* alleles and their respective peptides has made possible the transformation of a large number of encapsulated isolates. The efficiencies of transformation can vary widely, but many strains (approximately 50% of those tested) have not been transformed. These observations suggest that other factors are also important in the induction of competence and the ability of recipients to be transformed. Genomic screening shows the presence of other minor *com*C alleles which are present in less than 5% of analyzed pneumococcal strains (51, 52).

During competence development the CSP serves as the peptide pheromone signal. The induction of competence leads to transient restriction of most cellular protein synthesis, with high-level expression of a small set of proteins involved in transformation. Microarray analysis showed that the expression of more than 100 genes is regulated by CSP, and of those only a minor fraction are clearly necessary for transformation (53–56). CSP-responsive genes exhibited four patterns of expression: early, late, and delayed gene induction and gene repression (53, 57). Early genes include those involved in competence induction, whereas late genes include those necessary for DNA processing. Heat shock proteins, proteases, and chaperones occur among the delayed

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TABLE 3 Insertion sequences found in *S. pneumoniae*

Element[a]	Size (bp)	Terminal inverted repeats	Target (bp)	Copies[b]	Reference
IS*1202*	1,747	23 bp, imperfect	27	1–5 (0)[b]	179
IS*1167*	1,435	24 bp, imperfect	8	3–12 (22; 14t)	180
IS*1381*	846	20 bp, imperfect	7	5–7 (12t)	181
IS*1515*	871	12 bp, imperfect	3	2–13 (1)	182
IS*3-Spn*	1,359			(14t)	6
IS*1380-Spn*	1,703	13/15[c] bp, imperfect	4	(12; 1t)	6
IS*630-Spn1*	896	22 bp, imperfect	2	(12t)	6
IS*1239*	1,046	7/15 bp, imperfect		(14t)	6
IS*66*	2,484	15/20 bp, imperfect	8	(2t)	6
IS*200*	747	0 bp		(3; 2t)	6

[a]Each element contains an open reading frame or frames with homology to transposase sequences.
[b]Numbers in parentheses represent the number of copies in TIGR4 and the number of truncated or frameshifted copies (t).
[c]Alternative number of bp, depending on the copy.

genes, possibly reflective of a stress response. Repressed genes include ones encoding ribosomal proteins and carbohydrate/amino acid metabolism enzymes.

The events and genetic loci involved in detecting and responding to the CSP signal are summarized in Fig. 1. Export and maturation of CSP is mediated by an ABC transporter encoded by the *comAB* locus (58). *com*C is the first gene in an operon with *comD* and *comE*, which encode the histidine kinase sensor and response regulator components, respectively, of a two-component signal transduction system (59). ComD has been proposed to be the CSP receptor (58) and has been demonstrated to retain CSP on competent cells (60). Allelic variation at the N-terminal sensor domain of ComD has been reported and is responsible for CSP pherotype specificity (51, 61). Expression of *comCDE* is enhanced approximately 40-fold upon addition of exogenous CSP (59). Maximal expression of *comAB* and *comCDE* occurs within 5 to 10 min after addition of CSP and returns to near basal levels by 15 to 20 min (54, 57). Loss of *comE* abolishes competence induction and *comCDE* expression (59). Binding of CSP to ComD is predicted to activate ComE via phosphorylation, and mutations in ComD that apparently alter its conformation and lead to constitutive phosphorylation also result in constitutive competence (62). The activated ComE binds to conserved target sequences, consisting of two 9-bp direct repeats separated by 12 bp, located upstream of the –10 regions of the *comAB*, *comCDE*, and *comX* promoters (63, 64). The latter gene encodes the alternative sigma factor ComX, which allows core RNA polymerase to recognize competence-specific promoters (63, 65). These promoters contain a com- or cin-box (TACGAATA) located –10 from the transcription start site and a T-rich region at –25 (66). They are located upstream of a number of loci, including *cin-rec*, *cel*, *cfl*, *cgl*, *coi*, and *ssb*, that are involved in the later stages of competence and whose expression is induced in parallel (54, 59, 66). ComX thus serves as the link between the competence induction pathway (*comAB* and *comCDE*) and the DNA processing components (63). It is an unstable protein that is not detected in noncompetent cultures but is apparent by 7.5 min after induction with CSP and about 3 min before the expression of ComX-dependent genes is detected (65). During competence, levels of the major sigma factor, Sigma A (RpoD), are decreased relative to ComX, and the molar amount of ComX exceeds that of Sigma A by approximately 2-fold. ComX may replace Sigma A during competence, thus explaining the preferential expression of competence-related genes during this time (65, 67). An additional factor, ComW, is necessary to maintain adequate levels of ComX, through an effect on either translation or stability, and may also increase the activity of ComX in transcription of the late competence-specific genes (68, 69).

CiaH and CiaR are the sensor and response regulator components, respectively, of a two-component signal transduction system that may be involved in calcium sensing (70, 71). CiaR is necessary to repress competence expression, although it is not clear whether the activated or nonactivated form is involved. Deletion of CiaR results in constitutive *comCDE* expression and competence (56, 72, 73), whereas point mutations in *ciaH* result in no CSP being exported and a failure to respond to exogenously added CSP (71). Enhanced expression of stress response genes occurs in CSP-induced CiaR mutants, along with growth restriction and stationary phase autolysis (56). Mutations that affect stress response proteins can also lead to competence expression (74, 75), and it has been suggested that CSP might be an alarmone that signals environmental stress (72). The Cia system, as well as the oligopeptide permeases encoded by the *ami* and *ali* (also referred to as *plp*) loci, may be involved in sensing the environment. Mutations in the *ami* and *ali* loci, and especially those in more than one locus, affect the spontaneous development of competence but do not alter the response to exogenous CSP induction (57, 76).

Genetic transformability has been proposed to be induced by antibiotic stress. Fluoroquinolones, kanamycin and streptomycin antibiotics, and the DNA-damaging agent mitomycin C induce the expression of the late competence gene encoding a single-stranded DNA binding protein (77). Furthermore, antibiotics, DNA-damaging agents, and other compounds affecting DNA replication have been found to upregulate the expression of genes located near the chromosomal origin of replication, including the *comCDE* operon (78). There has long been evidence for a macromolecular inhibitor that causes the cessation of the competent phase (44). The mechanism by which competence is abruptly shut off remains unknown, but mRNAs of the ComX-dependent loci decay in parallel, and the process requires ComX (54, 63). Other genes or loci necessary for specific steps in transformation have been described and are discussed in the appropriate sections below. Mutant analyses have also identified several additional loci whose precise points of involvement

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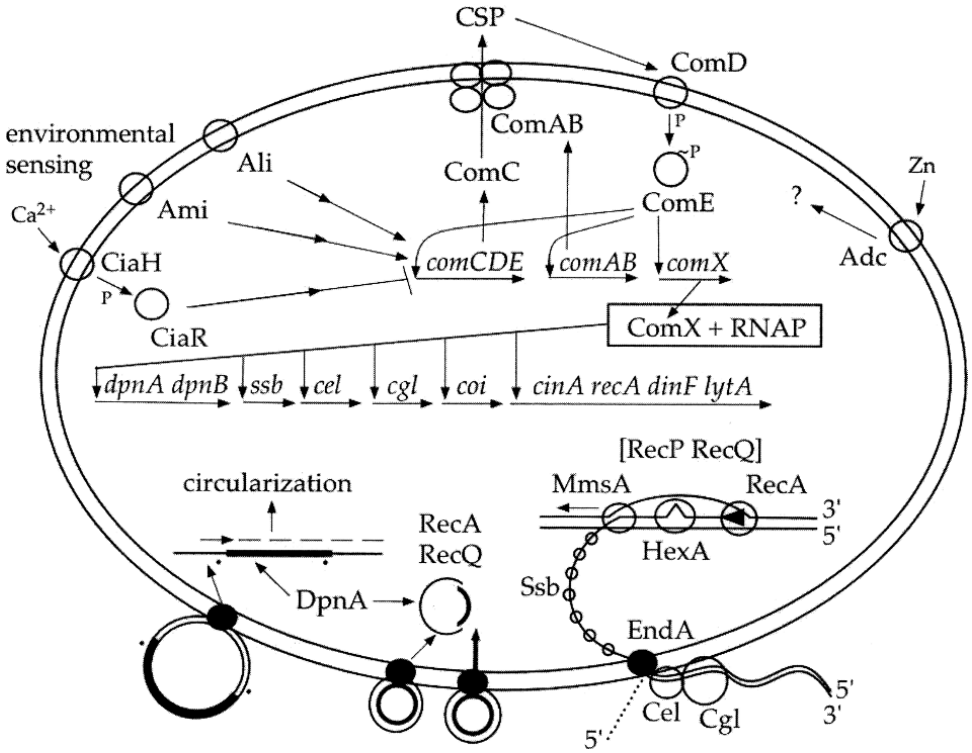


FIGURE 1 Transformation. The model is based on information discussed in the text. In some cases, the putative regulation of gene expression may be either direct or indirect. Phosphorylation of response regulator molecules by their cognate histidine kinase sensors is indicated by P. Uptake and integration of linear DNA is shown in the lower-right portion, with monomer and dimer plasmid uptake shown in the central and lower-left portions, respectively. Plasmid uptake occurs by the same mechanism as linear DNA uptake. For monomers, complementary overlapping strands pair, and DNA synthesis completes the double-stranded circular molecule. For the dimer, a small fragment of the complementary strand can serve as a primer for DNA synthesis, and circularization can occur as described in the text. Dots on the dimer molecule indicate homologous sites.

are not entirely clear. For example, Adc mutants transform poorly unless Zn is added, and the addition of CSP in the absence of Zn does not induce competence. Therefore, the effect is likely downstream of induction and may be related to transmission of the signal from ComD to ComE (79).

DNA release, binding, and uptake

CSP induction results in the release of transformable DNA from competent cells (80–83). The release is dependent on the major autolysin, LytA, and the autolytic lysozyme, LytC. Double-stranded DNA can bind to competent cells at multiple locations (50 binding sites have been estimated) (84), where it is subjected to single-strand nicks. A constitutively expressed membrane-associated endonuclease (EndA) converts the nicks into double-strand cuts, resulting in fragments with a median length of about 7 kb (85–87). Nucleolytic digestion of one strand occurs in the 5′ to 3′ direction, while the other strand is taken into the cell in the 3′ to 5′ direction (88). Entry and degradation occur at equivalent rates of ~100 nucleotides per sec at 30°C (88). Binding and subsequent uptake of a single large molecule may occur at multiple independent sites. Hence, even though digestion results in fragments of relatively small size, linkage of markers hundreds of kilobases apart can be detected using carefully prepared DNA. The average small size also does not preclude the uptake and integration of large DNA fragments or of large heterologous regions flanked by sequences homologous to the recipient chromosome, as evidenced by the exchange of genetic cassettes encoding the capsular polysaccharide biosynthetic enzymes (47).

It has been proposed that EndA is part of a DNA uptake complex (86). The predicted products encoded by several genes induced by CSP and required for efficient transformation are homologous to proteins involved in DNA binding, uptake, and the assembly of DNA transport machinery in *Bacillus subtilis*. These genes are contained in the *S. pneumoniae* loci originally referred to as *cel* or *cilE*, *cgl* or *cilD*, *cilC* or *cclA*, *cilB*, and *cfl* (59, 63, 66). They have since been referred to in a manner consistent with their *B. subtilis* homologs (89). The *B. subtilis* ComG proteins are primarily membrane associated, but some may be processed and translocated to the cell wall. They appear to have a role in allowing the DNA to traverse the peptidoglycan and reach the DNA binding protein, ComEA (90). In *S. pneumoniae*, inactivation of ComGA (*cilD*, *cglA*) eliminated DNA binding and degradation, whereas inactivation of ComEA (*cilE*, *celA/B*) eliminated degradation and internalization of DNA, but low-efficiency binding still occurred. EndA thus cannot access DNA in the absence of these proteins, and they are postulated to be necessary for the surface attachment and deep binding, respectively, of the DNA (89, 91). In these same studies, ComEC (*cilE*, *celA/B*) and ComFA (*cfl*) mutants were found to bind and degrade DNA, but not to internalize it, suggesting a role in membrane transport.

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Chromosomal integration

Once inside the cell, the DNA is contained in an eclipse complex, where it is protected from cellular nucleases by interaction with a 19.5-kDa single-strand binding protein and no longer has transforming activity (92, 93). Homologous recombination into the chromosome proceeds by a single-strand replacement mechanism that involves RecA activity and is affected by the Hex mismatch repair system (see below). Purified RecA mediates single-stranded DNA-dependent nucleoside triphosphate hydrolysis and nucleoside triphosphate-dependent DNA strand exchange (94). CSP induces expression of the *recA* operon, which consists of *cinA-recA-dinF-lytA* (95, 96). In competent cells, all of these genes are induced from a promoter located upstream of *cinA*, whereas in noncompetent cells, lower levels of transcription proceed from other promoters. The induction of *lytA*, which encodes the major pneumococcal autolysin, is consistent with the observation of DNA release and autolytic activity during the competence phase. LytA is not, however, necessary for transformation (96). *dinF* encodes a putative multiantimicrobial extrusion family protein which is involved in quinolone efflux (97), while in *Escherichia coli dinF* is expressed in response to DNA-damaging agents (96). In *S. pneumoniae* CinA (also called CogA) may have a role in facilitating the localization of RecA to the membrane (98), while in *B. subtilis* it is localized in the nucleoid and is not involved in competence (99).

Mutations in at least four other loci are known to affect chromosomal integration. *mmsA* encodes a helicase (RecG homolog) that is proposed to be involved in branch migration of the three-stranded heteroduplex integration intermediate (100). *recP* is required for chromosomal but not plasmid transformation, although it is not clear how its predicted product, a transketolase, is involved. *recQ* is required for both chromosomal and plasmid transformation, but its role has not been further characterized (101, 102). Internalized strands of donor DNA are complexed with SsbB, an about 15-kDa single-stranded DNA binding protein, and *ssb* mutants have a 3- to 5-fold reduction in transformation frequency (66, 93).

Plasmid transformation

Transformation of plasmids proceeds by the same pathway as that for chromosomal DNA. The efficiency (based on donor DNA concentration) is lower, however, because two separate uptake events that involve complementary and overlapping single strands must occur to obtain a circular, partially double-stranded molecule that can then be completed through DNA repair synthesis (Fig. 1). Consequently, plasmid DNA linearized at a unique site cannot be recircularized and established in the recipient cell. The frequency of plasmid transformation is enhanced by the use of dimers as opposed to monomers (103). Indeed, the highest frequencies of plasmid transformations are obtained using $RecA^+$ *E. coli* strains as a source of multimeric plasmids. In these situations, only a small fragment of the complementary strand is necessary to provide a primer for synthesis of a molecule that can be more than one monomeric unit in length when using the dimer/multimer as a template. A recombination event between homologous regions can then restore the circular plasmid. Alternatively, as suggested by Saunders and Guild (103), intramolecular circularization may occur by pairing of a single-strand region of the incoming DNA with a newly synthesized complementary strand and displacement of the template strand. A ligation reaction would restore the circular molecule. The first possibility would be expected to be RecA dependent, whereas the latter likely would not. Whether the transformation of dimers is dependent on RecA has not been determined. Defects in RecA do, however, decrease the efficiency of monomer transformation (84). In addition, RecQ is necessary for efficient plasmid transformation, but RecP is not (101, 102).

The establishment of plasmids can also be facilitated by the presence in the recipient cell of regions homologous to the incoming plasmid (104). Thus, plasmids containing cloned fragments of *S. pneumoniae* DNA are readily transformed, because the homologous chromosomal regions can serve as primers for DNA repair synthesis, if the original double-strand cut occurred within the cloned region of the plasmid. Likewise, plasmids with homology to endogenous plasmids are transformed at high frequency.

Artificial transformation

Replicating plasmids can be introduced into *S. pneumoniae* by electroporation. Unlike plasmids taken up via the natural transformation pathway, they are subject to restriction by the *Dpn* system (see below), indicating that double-stranded DNA is transformed (105). Recombination of markers into the chromosome is not observed, even when competent cells are used as recipients or when DNA is made single-stranded prior to transformation. Electrotransformation of plasmids containing regions homologous to the *S. pneumoniae* chromosome is, however, enhanced in competent cells in a RecA-dependent manner, indicating that pairing between homologous DNAs does occur during this process (106).

Conjugation

S. pneumoniae can serve as both a donor and recipient in the conjugation of plasmid DNA and ICEs (24, 107). Transfers are DNase resistant and require stable cell-to-cell contact. Both capsulated and unencapsulated strains can serve as conjugation donors or recipients, and transfer frequency is strain dependent (40, 108–110). Although no indigenous conjugative plasmids have been identified, several plasmids from other streptococci, including pIP501 of *Streptococcus agalactiae*, have successfully been conjugated into *S. pneumoniae* and then retransferred to other pneumococcal and streptococcal recipients. In addition, nonconjugative plasmids such as pMV158 can be mobilized both to and from *S. pneumoniae* (107).

ICEs that can insert at either multiple sites or only at specific sites have been identified in *S. pneumoniae*. ICEs are transferred in a process that likely involves excision to form a circular intermediate that is nicked and then transferred as single-stranded DNA to recipient cells. Tn*1545*, a 25.3-kb conjugative transposon first detected in *S. pneumoniae*, encodes resistance to tetracycline [*tet*(M)], kanamycin, and related aminoglycosides (*aphA-3*) and to macrolide-lincosamide-streptogramin B type antibiotics (*ermAM*). It is closely related to the *tet*(M)-carrying element Tn*916* from *Enterococcus faecalis* (111, 112). Precise excision of Tn*1545* from the chromosome is mediated by the transposase-encoded proteins Xis-Tn and Int-Tn, which are structurally and functionally similar to the Xis and Int proteins of bacteriophage λ. Excision results in a nonreplicating, circular intermediate that can integrate at another site in the resident chromosome through the action of Int-Tn or can mediate its own transfer to other recipients (113). Numerous other Gram-positive bacteria can serve as both recipients and donors for Tn*1545* transfer (112). Transposition occurs in these organisms, as well as in *E. coli* in which the transposon has been introduced on a plasmid. Integration involves site-specific recombination within a short region of

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nonhomologous DNA and can occur at multiple locations. Specificity is for AT-rich regions and may be related to conformational aspects of the target DNA sequence (110, 114). The family of Tn*916* genetic elements includes many members which share the same general organization but have insertions, deletions, or substitutions of DNA regions relative to Tn*916* (115). Insertions are mainly due to the acquisition of insertion sequences and nonconjugative transposons, such as Tn*917*, the MAS (macrolide-aminoglycoside-streptothricin) element (116), or the MEGA element (macrolide efflux genetic assembly) (117), which carry additional antibiotic resistance genes (118). In *S. pneumoniae*, Tn*916* family elements can be found alone in the chromosome or inserted in larger, composite ICEs such as Tn*5253* and related elements (110, 119).

Tn*5253* [formerly known as Ω(*cat-tet*)], the first ICE identified in *S. pneumoniae*, is a 64,528-bp element in which two distinct genetic elements are integrated, Tn*5251* and Ω*cat*(pC194) (24, 40, 120, 121). Integration of Tn*5253* into the pneumococcal chromosome occurs within a single target site (*attB*) (122). The entire Tn*5253* is capable of conjugal transfer to other streptococci, where it also integrates at a single target site (40). Tn*5251* is 18,033 bp in size, encodes the tetracycline resistance determinant *tet*(M), and is a member of the Tn*1545*/Tn*916* family. Tn*5251* produces circular intermediates, and a deletion in Tn*5253* and is capable of independent conjugal transfer to different bacterial species (110). Ω*cat*(pC194) is a 7,627-bp element and contains a linearized copy of the *cat*-containing staphylococcal plasmid pC194. This element is flanked by two long direct repeats which mediate its excision from Tn*5253* with formation of a circular intermediate. The element is not able to transfer by conjugation but is capable of intracellular mobility, integrating by homologous recombination at an ectopic location in the pneumococcal chromosome corresponding to the PPI1 locus (40).

Tn*1207.1* is a 7,244-bp-long genetic element that carries the *mef*(A)-*msr*(D) macrolide efflux genes and specifically integrates into a specific GA dinucleotide located in *celB*, thus impairing competence (123, 124). Tn*1207.1* is not capable of conjugal transfer but can be transferred by transformation. Three open reading frames, located at the 3′ end of Tn*1207.1*, show homology to the UV resistance genes of Tn*5253* (123). A complete copy of Tn*1207.1* is present at the 5′ end of Φ1207.3 of *Streptococcus pyogenes*, formerly known as Tn*1207.3* (125, 126). Φ1207.3 is 52,491 bp in size; it is integrated into the *comEC* gene of *S. pyogenes* and can be transferred to *S. pneumoniae*, where it inserts into the *comEC* homolog *celB* at the same GA nucleotide of Tn*1207.1* (126).

Pseudotransduction

Although true generalized transduction has not been observed with pneumococcal phages, Porter et al. (10) described a process that involved phage packaging of pneumococcal DNA and subsequent transfer to recipient cells via the transformation pathway. Termed pseudotransduction, the process includes several features consistent with generalized transduction. The phage, PG24, could package different chromosomal regions into DNase-resistant phage particles that adsorbed to recipient bacteria. Gene transfer was inhibited by blocking adsorption with phage-specific antiserum or by trypsin treatment of the phage. Successful gene transfer, however, was dependent on the ability of the recipient cells to become competent and on the presence of the membrane endonuclease (EndA) required for DNA processing and entry via the transformation pathway. Frequency of transfer was affected by the Hex phenotype of the recipient, indicating entry of single-stranded DNA and heteroduplex formation between the donor and recipient DNAs. DNase sensitivity of the process occurred at the time of competence development, further indicating that the bacterial DNA was released from the phage at the outside surface of the cell and not injected into it, as occurs for the phage DNA. Transfer of DNA by pseudotransduction rather than standard transformation permitted the entry of longer fragments, presumably owing to protection of the DNA from excessive cuts by EndA while maintained at the cell surface in the phage head.

Plasmids and Bacteriophages

Plasmids have been detected in a small number of *S. pneumoniae* isolates. Most are either identical or very similar to pDP1, a cryptic 3-kb plasmid first identified in the capsule type 2 strain D39 and a number of its descendants (4, 127). Approximately 3% of over 600 strains examined carry a member of this plasmid family (see reference 128 and references therein). The plasmids belong to a family of rolling-circle plasmids that includes pC194 (4, 128–130). Small plasmids unrelated to pDP1 have occasionally been noted (108), but as with pDP1, no specific functions have been detected (127). The presence of pDP1 or other plasmids is unrelated to the capsular serotype, restriction system, antibiotic resistance profile, clinical infection, or other phenotypes. Plasmids from other Gram-positive bacteria are capable of replicating in *S. pneumoniae* and have been used for both transformation studies and cloning (49, 50, 103, 104).

Bacteriophages that infect *S. pneumoniae* can be readily isolated from clinical samplings (131). The ω, Cp, and Dp groups contain virulent phage that were isolated directly from human throat swabs (132–135). Pneumococcal phages are different in their ability to infect other species of oral streptococci: Cp-1 and Dp-1 are able to infect *Streptococcus mitis* and *Streptococcus oralis* (136, 137), while ω phages do not infect other oral streptococci (135). Temperate phages appear to be present in many pneumococcal isolates and occur in a wide variety of capsular serotypes (138–140). However, as shown using isogenic derivatives of encapsulated and unencapsulated strains, the presence of the capsule prevents infection by virulent ω phages under laboratory conditions (141), suggesting that natural infections may occur when capsule production is reduced, as is expected to occur during colonization of the nasopharyngeal cavity (142, 143).

The properties of some pneumococcal phages are shown in Table 4. Some important similarities between many of the phages have been noted, and the complete nucleotide sequences have been determined for the genomes of many phages, including Dp-1, EJ–1, Cp-1 and its variant SOCP, and MM1 and its variants MM1-1998 and MM1-2008 (132, 137, 140, 144). Comparison of the complete sequences of 10 pneumococcal temperate bacteriophages identified 3 distinct groups of prophages. All prophage genomes are organized in functional modules: (i) the lysogeny module, (ii) the replication module, (iii) the packaging module, (iv) the morphology module, and (v) the lysis module (140). The lysogeny module codes for integrase genes, which determine the site specificity of phage integration. Three main prophage integration sites are present in the pneumococcal chromosome and are located in intergenic regions between (i) SP_0019 and SP_0020 (TIGR4 genome), (ii) SP_0020 and SP_0021 (TIGR4 genome), and (iii) SP_1563 and SP_1564 (TIGR4 genome). Cp-1 and HB-3, as well as their related phages, contain a terminal protein covalently linked to the 5′ end of each DNA strand. In the case of Cp-1, the terminal

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TABLE 4 Properties of some *S. pneumoniae* bacteriophages

Phage	Structure	Genome	GC%	Burst size	N-terminal[a]	C-terminal[b]
ω8 (virulent)	Octahedral hexagonal head (60 nm) Flexible tail (180 × 10 nm) Tail fiber (72–108 nm)			100–200		
Cp-1/SOCP (virulent)	Hexagonal capsid (66 × 42 nm) Noncontractile tail (19 × 7 nm) Neck appendages Head fibers	19.3 kb, double-stranded, linear terminal protein	38.8	94	Lysozyme	Choline
Dp-1 (virulent)	Icosahedral capsid (70 nm) Noncontractile tail (170 nm) Lipid envelope	56.5 kb, double-stranded, linear	40.3	73	Amidase	Choline
MM-1 (temperate)	Icosahedral capsid (60 nm) Noncontractile tail (160 nm)	40.2 kb, double-stranded, linear terminal protein	38.7		Amidase	Choline
EJ-1 (temperate)	Icosahedral capsid (57 nm) Noncontractile tail (130 nm)	42.9 kb, double-stranded, linear	39.6		Amidase	Choline

[a]Activity of the N-terminal domain of the lytic enzyme.
[b]Substrate bound by the C-terminal domain of the lytic enzyme.

protein serves as a primer in initiation of DNA replication for the linear molecule. The mechanism resembles that of adenovirus and *B. subtilis* phage φ29, with which Cp-1 has structural similarity. As in these other viruses, the DNA of Cp-1 and related phages contains inverted terminal repeat structures. A number of the pneumococcal phages are also related by the structural organization of their lytic enzymes. Cp-1, Cp-9, Dp-1, and HB-3 require choline in the pneumococcal cell wall for adsorption and cell lysis. Lytic enzymes have a modular structure composed of a catalytic domain and a specific binding domain, which localizes the catalytic domain of the enzyme on the substrate. Phage lytic enzymes contain a C-terminal choline-binding domain homologous to those of the choline-binding proteins of *S. pneumoniae*, including pneumococcal autolysin. The N-terminal domain of HB-3 has amidase activity that is virtually identical to that of the host autolysin, whereas the N-terminal domains of Cp-1 and Cp-9 lytic enzymes encode a lysozyme. Dp-1 codes for a hydrolase similar to that found in the *Lactococcus lactis* phage BK5-T. The C-terminal region of the Cp-7 lytic enzyme does not encode a choline-binding domain; hence, this phage does not require choline-containing cell walls for infection. Phage lytic enzymes represent examples of modular evolution, in which catalytic domains and binding domains are shuffled and generate new mosaic alleles.

DNA REPAIR

Incoming DNA that contains either transition mutations or small (<6-bp) deletions or insertions is usually corrected to the recipient DNA sequence by the action of the Hex mismatch repair system (145). Incoming markers can thus be observed to transform at either high efficiency (transversions and large deletions/insertions), low efficiency (transitions and small deletions/insertions), or intermediate efficiency (transversions and single-base deletions/insertions). The substrate for repair is the heteroduplex integration intermediate formed between the donor and recipient DNA. Recognition of a single-strand break in the donor DNA, followed by recognition of the mismatch, triggers activity of a 3′ to 5′ exonuclease that creates a gap of up to several kilobases in the donor DNA. The gap is then repaired using the recipient DNA as a template. Thus, a high-efficiency marker located adjacent to a low-efficiency marker may also be repaired. The same system functions to repair errors in DNA replication and is analogous to the Mut system of *E. coli*. Unlike the Mut system, however, the Hex system does not require recognition of unmethylated sequences in hemimethylated DNA. In *S. pneumoniae*, the products of at least two unlinked genes, *hexA* and *hexB*, are essential for the repair system. HexA (homologous to MutS) recognizes the mismatched base pair, but the role of HexB has not been demonstrated. By analogy with its *E. coli* homolog, MutL, it is likely to interact with HexA and other proteins in the mismatch repair complex and stimulate their activities (146, 147). In Hex$^-$ cells, mismatches are not repaired, and high-efficiency transformation of all markers, as well as a high spontaneous mutation rate, are observed. The high transformation efficiency of many of the commonly used *S. pneumoniae* strains is due, in part, to the fact that they are Hex$^-$, as the result of a mutation in *hexB* (147) (Table 1).

Expression of the *hex* genes is not enhanced during competence, and saturation of the system can occur with an excess number of mismatches, leading to the incorporation of low-efficiency markers. In addition, saturation is observed using donor DNA from the related *S. mitis* and *S. oralis*. Thus, the Hex system does not serve as a barrier to interspecies gene exchange, and its role in transformation, unlike that in DNA replication repair, has been proposed to be fortuitous (148).

S. pneumoniae also encodes a MutY-like adenine glycosylase, which repairs A/G mismatches to CG. MutY mutants exhibit a mutator phenotype, where the frequency of AT to CG transversions is enhanced (149).

S. pneumoniae generally lacks a UmuDC-dependent SOS response involved in error-prone repair of UV-damaged DNA (150), although transposon-encoded UmuC and UmuD homologs can mediate this response in cells harboring ICE Tn*5253* (151). Despite the lack of a LexA homolog in the *S. pneumoniae* chromosome, the purified *S. pneumoniae* RecA is able to cleave the *E. coli* LexA (94). Uptake of either homologous or heterologous DNA during transformation results in lysogenic induction of the temperate bacteriophage HB-746 in RecA$^+$ but not RecA$^-$ cells. Similarly, prophage induction by mitomycin C is dependent on a functional

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RecA. Both observations are consistent with an SOS-like repair system that is controlled by RecA (152). The spontaneous mutation frequency is significantly increased in cells undergoing transformation with homologous or closely related DNA but is unaffected when nonhomologous DNA is transformed (153). These observations further suggest the existence of an error-prone SOS-like repair system. The repair of double-strand DNA breaks requires RexAB, the functional homologs of the *E. coli* RecBC (154).

RESTRICTION-MODIFICATION SYSTEMS

Most *S. pneumoniae* isolates contain the genes necessary for expression of either the *Dpn*I or *Dpn*II restriction system. The genes for each of these systems are located at the same site in the respective chromosomes, thus forming genetic cassettes that can be exchanged as units between strains (155). The *Dpn*I endonuclease (DpnC) cleaves double-stranded DNA containing the methylated sequence 5′-GmeATC-3′, whereas the *Dpn*II endonuclease (DpnB) cleaves unmethylated DNA of the same sequence. In addition to their respective endonucleases, the *Dpn*I gene cassette encodes a second protein (DpnD) of unknown function, while the *Dpn*II cassette encodes the adenine methyltransferases, DpnA and DpnM, that are necessary for methylating the host DNA to prevent restriction (156). DpnM acts only on double-stranded DNA, whereas DpnA can methylate both double- and single-stranded DNA (157).

The major function of the *Dpn* system is to protect the cell against entry of phage DNA. Neither of the *Dpn* endonucleases attacks single-stranded DNA or hemimethylated double-stranded DNA. Thus, natural transformation of chromosomal DNA is not affected. In contrast, plasmid transformation is affected to some extent, with an approximately 50% reduction observed for transfers between strains of differing *Dpn* types as compared with transfers between strains of the same type (157). The lack of complete restriction of methylated plasmids by *Dpn*I cells apparently results from the mechanism of plasmid uptake, which can give rise to large segments of hemimethylated DNA following DNA synthesis to restore both strands of the incoming plasmid. Complete restriction of nonmethylated plasmids by the *Dpn*II system is prevented by the action of DpnA, which methylates incoming single-stranded DNA. Expression of *dpnA* is induced by CSP and initiates at a com-box sequence (158). In the absence of such methylation, restriction of the newly restored plasmid that is not methylated on either strand apparently occurs faster than methylation of the double-stranded DNA by DpnM. Single-stranded, unmethylated plasmid DNA introduced by conjugation into *Dpn*II cells is also severely restricted, presumably because synthesis of the complementary strand by enzymes transferred with the DNA occurs more rapidly than protective methylation of the single strand by DpnA (157). The introduction of plasmids by electroporation, or other methods that involve the uptake of double-stranded DNA, is also strongly affected by the *Dpn* system (105).

A third allelic variant of the *Dpn* system has been recently identified in the Spain-23F-1 lineage. This system, named *Dpn*III, is constituted by the genes *m.dpnIII* and *r.dpnIII*, which have opposite orientation on the chromosome and code for an endonuclease and for a C5 cytosine-specific DNA methylase, respectively. The endonuclease recognizes and cleaves the specific 5′-GATC-3 sequence on double-stranded unmethylated or hemimethylated DNA, while the methylase recognizes the same 5′-GATC-3′ tetramer and generates a C5-methylcytosine. Genomic analyses and experimental transformation data suggest that this system limits horizontal transfer of unmethylated DNA (159).

A phase-variable type I restriction-modification system has also been identified in various strains of *S. pneumoniae*. This system contains three cotranscribed genes, *hsdR*, *hsdM*, and *hsdS*, which code for the restriction, methylation, and specificity subunits, respectively. The methylation subunit is an N6 adenine methylase, which is guided by the specificity subunit. The *hsdS* gene contains two variable regions which code for two target recognition domains and are able to recombine with two truncated *hsdS* genes located downstream. In a pneumococcal population, recombination generates six alleles of *hsdS* with different methylation target specificities (29, 160, 161). This system acts as an epigenetic switch, which is able to regulate gene expression, virulence, and phase variation (opaque versus transparent phenotype) in pneumococci (160).

TRANSCRIPTION PROMOTERS

The existence of extended –10 sites in promoter sequences was first noted in the *Dpn*II locus and was subsequently found to be relatively common in *S. pneumoniae*, in contrast to the relatively rare occurrence of such sequences in *E. coli*. The promoter of the *Dpn*II operon contains an extended –10 site (TATGGTATAAT) but does not have a –35 site. Analysis of 35 additional known or putative *S. pneumoniae* promoters revealed that 60% had the complete extended –10 consensus sequence (TNTGNTATAAT) and 71% had at least the TG motif of the extension. None lacked a –35 site, and those lacking a –10 extension tended to have –35 sequences close to the *E. coli* consensus (156). Numerous *S. pneumoniae* sequences exhibit promoter activity in *E. coli* but not in *S. pneumoniae*, apparently as a result of the AT richness of the *S. pneumoniae* DNA and of more stringent sequence recognition requirements of the *S. pneumoniae* RNA polymerase. Such requirements could be fulfilled by the extended –10 sequences. Excessive promoter activity of *S. pneumoniae* DNA in *E. coli* has been used to explain difficulties encountered in cloning pneumococcal sequences. While it is clear that difficulties exist, sequences with promoter activity strong enough to destabilize cloning vectors appear to be rare in the *S. pneumoniae* chromosome (162). The expression of toxic products, the use of large fragments or high-copy-number plasmids, and sequence-specific effects unrelated to promoter activity appear to be more likely causes.

GENETIC MANIPULATION

S. pneumoniae is a human pathogen, naturally competent for genetic transformation, and it can be easily manipulated at the genetic level. *S. pneumoniae* therefore represents a model for the study of bacterial genetics and pathogenesis.

Insertion-duplication mutagenesis has been the classical method to knock out genes in *S. pneumoniae* (163). In this procedure, a fragment of *S. pneumoniae* DNA is cloned into a plasmid that is able to replicate in *E. coli* but not in *S. pneumoniae*. The clone can thus be propagated in *E. coli* and be used to transform *S. pneumoniae*, where homologous recombination into the chromosome at the target site produces insertion by single crossover of the whole vector, with the consequent duplication of the cloned gene fragment and the acquisition of an antibiotic resistance determinant encoded by the plasmid vector. Target sequences of less than 200 bp can be used successfully, but larger fragments increase the frequency of transformation, owing in part to the higher likelihood of digestion of the surface-bound DNA within the

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cloned fragment. Transformation and subsequent integration into the *S. pneumoniae* chromosome result in gene disruption if the cloned fragment is an internal portion of a gene or in restoration of the entire gene if the fragment overlaps one end of the gene (Fig. 2). In the latter case, a mutant phenotype should not be observed unless the target sequence is part of an operon. The mutations produced with this method can display polar effects. Libraries of plasmids containing random fragments of pneumococcal DNA have been used to identify essential genes and mutants impaired in genetic transformation (63).

Transposon mutagenesis using Tn*916* has been employed in *S. pneumoniae* to inactivate the capsule locus (164). *In vitro mariner* transposon mutagenesis has proven to be a useful technique (72, 165). Here, target DNA is mutagenized *in vitro* using the *Himar1* transposase and a minitransposon containing an antibiotic resistance cassette flanked by *Himar1* inverted terminal repeats. Transposase catalyzes the integration of the resistance cassette in the target DNA, then gaps generated at the insertion site are repaired, and the mutagenic DNA is transformed to *S. pneumoniae* with selection for the antibiotic marker. Site specificity is minimal since the *Himar1* target site is any TA dinucleotide, and saturation mutagenesis appears possible. With this method, both site-specific or random mutagenesis can be performed. Site-specific mutagenesis is obtained using a linear PCR fragment as the target DNA, while for random mutagenesis the whole chromosomal DNA is used as a target. The latter technique allows the construction of libraries for the screening of essential genes (165).

Other techniques, employing PCR without the need of cloning, are less time-consuming and minimize the introduction of polar effects. A mutagenic construct containing an antibiotic resistance cassette flanked by DNA fragments located upstream and downstream of the locus to be mutagenized is assembled by splicing by overhang extension PCR and directly used in transformation (166). For the introduction of mutations that do not confer a selectable phenotype, direct transformation of a PCR unmarked mutagenic construct followed by screening using PCR can be used to identify mutants resulting from double crossover events. It is possible to introduce in-frame deletions, insertions, and single nucleotide substitutions in the bacterial chromosome (167). Introduction of unmarked mutations in encapsulated strains with low levels of competence is done using gene replacement techniques involving a negative selection (168). The Janus cassette, which contains a selectable kanamycin-resistance marker and counterselectable *rpsL*, which confers dominant streptomycin sensitivity in a streptomycin-resistant background, is first introduced by homologous recombination at the site of interest. DNA containing the desired mutation is then introduced, and mutants in which recombination has occurred to replace the Janus cassette are identified by their streptomycin-resistant and kanamycin-sensitive phenotypes. The Sweet Janus cassette contains a counterselectable marker that confers sucrose sensitivity and allows a reduction of the false-positive mutants that are isolated (169).

An alternative method for introducing unmarked mutations in the pneumococcal chromosome is the Cheshire cassette, which includes the resistance determinant *ermAM* and the *cre* gene encoding the site-specific recombinase of bacteriophage P1, under the control of the fucose-induced *fcsK* promoter. The cassette is flanked by the recombinase-specific recognition sequence and is introduced by transformation within the target gene. Upon addition of fucose in the growth medium, the recombinase gene is expressed and the cassette is excised (170).

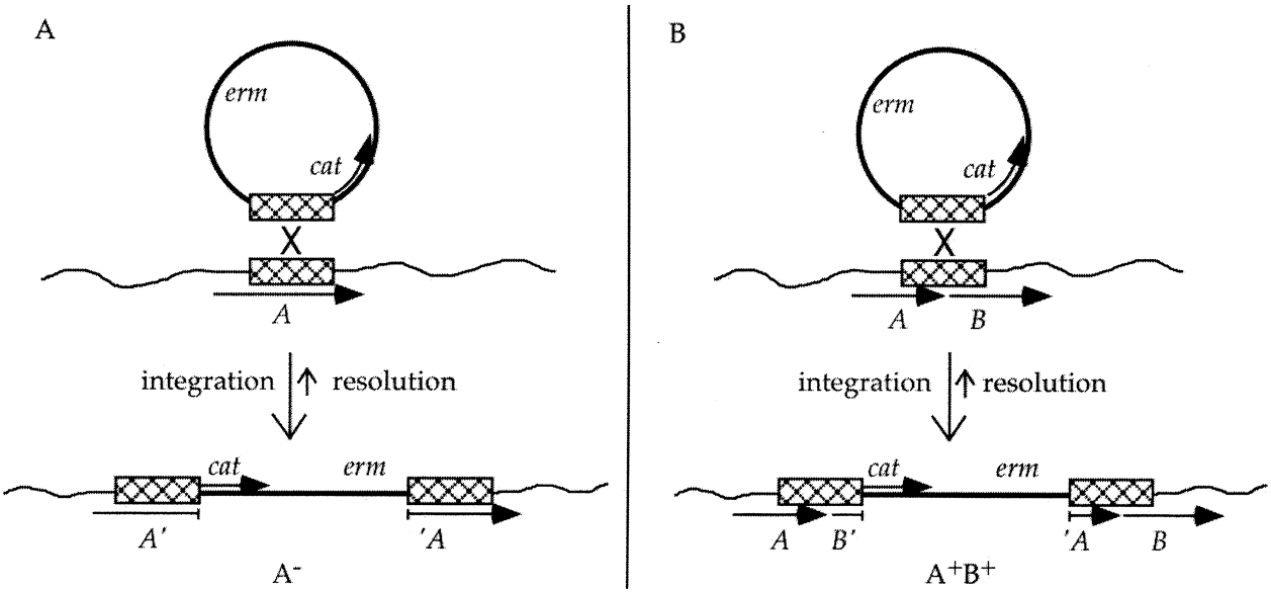


FIGURE 2 Insertion-duplication mutagenesis and restoration. (A) The effect of using an internal gene fragment to direct insertion of a nonreplicating plasmid into the chromosome. Duplication of the target fragment occurs, and the gene is disrupted by the plasmid insertion, resulting in an insertion-duplication mutation. (B) The target fragment overlaps the ends of two genes. Insertion results in duplication of the target fragment, but both genes are completely reconstructed, and the result is an insertion-duplication restoration. Both genes should be functional, unless they form part of an operon, in which case the plasmid insertion would be polar on the downstream gene. The figure shows a selectable erythromycin-resistance gene (*erm*) and a promoterless chloramphenicol-resistance reporter gene (*cat*).

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Pneumococcal Vaccines

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23

With the discovery of the pneumococcus in 1881, it became apparent that this Gram-positive pathogen was a major cause of serious and often fatal pneumonia (1). It is also a major cause of meningitis and otitis media in children. Pneumococci are the largest cause of community-acquired pneumonia in the developed world. According to the Centers for Disease Control and Prevention, in the United States the combined rates of invasive pneumococcal diseases has shown a decline since the introduction of the conjugate vaccines, with rates among the elderly declining from 59/100,000 in 1998 to 23/100,000 in 2015 and rates among children under 5 years of age falling from 95/100,000 in 1998 to 9/100,000 in 2016 (https://www.cdc.gov/pneumococcal/surveillance.html). The rate of meningitis in children in the United States is about 4 cases per 100,000 children, with a fatality rate of about 15% (2). In the developing world pneumococci are an important cause of childhood deaths due to bacterial respiratory infection following viral disease. The World Health Organization suggests that globally, such infections have been reported to have killed an estimated over 500,000 children in 2008 (http://www.who.int/immunization/monitoring_surveillance/burden/estimates/Pneumo_hib/en/). Recently, about one-third to one-half of pneumococci recovered from humans in the United States have been found to be at least partially resistant to penicillin, and penicillin-resistant strains are frequently also resistant to other common antibiotics (3). The rise of antibiotic resistance among pneumococci has already complicated treatment, especially of meningitis (4), and threatens to greatly increase the morbidity and mortality caused by pneumococci unless new control measures are developed.

It has long been recognized that the best management of most infectious disease is prevention. Vaccines offer the prospect of a highly cost-effective means of preventing morbidity and mortality caused by pneumococci. This article provides a concise summary of issues critical to the development and application of pneumococcal vaccines. There are several relatively recent reviews that address this topic in more detail (5–11).

In the preantibiotic era, vaccination attempts utilized whole killed pneumococci injected parenterally. Although such vaccines were sometimes protective in humans, they were also highly reactogenic. These killed vaccines were mainly used to elicit antibody in animals for passive treatment of infected humans (12). In 1933 it was clearly demonstrated that antibody to type-specific capsular polysaccharides (PSs) could be highly protective (13). However it soon became apparent that different strains of *Streptococcus pneumoniae* each expressed one of many different PSs. Most subsequent vaccine attempts focused on the use of mixtures of the isolated PSs to elicit protection (7, 14). However, the inability of young children to make adequate responses to most soluble PSs led to the eventual development and licensing of an immunogenic PS-protein conjugate vaccine for children (6, 15). However, the wide diversity of pneumococcal PS types, the failure of PS vaccines to protect against strains with PSs not in the vaccines, and the complexity and expense of highly valent conjugate vaccines have led to investigations of other much more cross-reactive pneumococcal antigens (primarily proteins) as potential vaccine candidates.

PNEUMOCOCCAL CAPSULAR PS VACCINE

During the 1930s, capsular PS was shown to be able to elicit protective antibodies. At that time it was generally assumed that capsular PS was the target of all, or most, protective antibodies (1, 13). Currently, over 90 distinct serotypes of capsular PS have been identified for pneumococci (16). Each strain can make only one PS, and for a strain to make a different PS requires a genetic transformation with DNA of another strain. Only about one-third of the greater than 90 serotypes occur with a significant frequency in adult infections, and the distribution of common serotypes is even more restricted in children (6, 17–20). Following the identification of capsular PSs as protection-eliciting molecules, vaccines comprising relatively pure PSs were used successfully in nursing homes and among military personnel in the 1940s. However, during this time antibiotics were becoming widely available, and these drugs diminished the perceived impact of pneumococcal infections. The two commercially available pneumococcal vaccines were withdrawn from production for lack of demand (7).

Despite a growing selection of antibiotics and increasingly sophisticated critical care technology, morbidity and mortality from invasive pneumococcal infections have remained high. Antibiotics and supportive care alone are not sufficient to completely eliminate the impact of pneumococcal disease because in many cases deaths occur so quickly that antibiotics are not effective (21). During the 1970s, renewed interest in pneumococcal vaccines (14) led to clinical trials of a PS vaccine in adults at high risk for invasive pneumococcal disease (IPD) in South Africa and New Guinea. Based largely on encouraging results from these trials, a 14-valent PS vaccine was licensed in the United States in 1977. The

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vaccine was expanded to 23 PS serotypes in 1983 and is licensed for use in adults in the United States (6, 7, 22).

Clinical trials examining the efficacy of the PS vaccine continued after licensure, with somewhat mixed, and controversial, results (7). A significant impediment to conducting the most rigorous controlled clinical trials has been the ethical concern of denying at-risk groups an approved and recommended vaccine. A recent systematic review and meta-analysis to examine the efficacy of the 23-valent vaccine (PPSV23) in adults >60 years of age, has shown a pooled vaccine efficacy of 73% (95% confidence interval [CI]: 10 to 92%) against IPD in clinical trials, 45% (95% CI: 15 to 65%) in cohort studies and 59% (95% CI: 34 to 74%) in case control studies (22). The higher efficacy of former trial data, with a 2.5-year follow-up, compared to the lower efficacy seen in the observational cohort and case control studies, with a 5-year follow-up, may reflect a waning protection over time (22, 23). Although the PS vaccine is cost-effective even when considering only bacteremic disease, this vaccine is not generally accepted to reduce the incidence of pneumococcal pneumonia or localized upper respiratory infections such as acute bronchitis, sinusitis, or otitis media (24).

A major drawback to the PS vaccine is the poor immunogenicity of PS antigens in children and elderly adults, primarily due to the vaccine eliciting a T cell-independent immune response (25). The response to PS varies by age and with the presence of certain underlying chronic medical conditions, most notably, infection with human immunodeficiency virus (HIV). Certain capsular PS serotypes in the 23-valent vaccine, such as types 6B, 9V, 19F, and 23F, induce relatively low amounts of specific antibody, which fall to prevaccination levels within 3 years (26). In addition to a reduced quantity of antibody, a significant number of elderly adults have impaired functional antibody responses to PS as defined by *in vitro* avidity and opsonophagocytosis assays (27, 28).

POLYSACCHARIDE-PROTEIN CONJUGATE VACCINES

Children less than 2 years of age are at increased risk for infections caused by encapsulated bacteria; consequently, this group had the highest incidence of invasive pneumococcal infections in the preconjugate era (27). Children in this age group do not consistently respond to PS antigens, which directly cross-link surface immunoglobulin on B cells and do not have an absolute requirement for T cell help (29–31). Whatever the underlying mechanisms, children less than 2 years old respond poorly to many of the common pneumococcal capsular types (32), and alternatives to purified PS antigens are needed to protect young children.

The ability to make antibodies to protein antigens appears quite rapidly after birth, and T cell-dependent antibody production is highly efficient. The development of PS-protein conjugate vaccines for children takes advantage of their ability to elicit T cell-dependent responses to PS covalently attached to immunogenic proteins. This strategy has proven highly successful in preventing both invasive disease and colonization with *Haemophilus influenzae* type b (33). The process of synthesizing conjugate vaccines requires chemical reactions, which covalently attach PS to protein; nonconjugated mixtures of peptides and PS do not induce T cell-dependent PS antibodies (34).

Immunization with PS conjugate vaccines induces effective memory antibody responses in infants and children, and antibody levels can be boosted with repeated immunization (35). A large clinical trial in infants using a four-dose schedule of immunization with a heptavalent conjugate vaccine proved this strategy to be highly effective in preventing invasive (bacteremic) disease (30, 36, 37). However, this conjugate vaccine has shown somewhat more modest efficacy in preventing pneumonia (15) and otitis media (38). The heptavalent pneumococcal conjugate vaccine (PCV7) was licensed in 2000 in the United States for use in all children less than 2 years of age and for high-risk children less than 5 years of age (37). Since the vaccine serotypes that colonize and infect children include most of the serotypes associated with antibiotic resistance, and since children carry pneumococci at a much higher frequency than adults, there are expectations that childhood immunization may reduce the transmission of antibiotic-resistant pneumococci in the entire population (39–42). It has been observed, however, that significant replacement carriage and otitis media occur with capsular types not present in the vaccine (38, 39, 42). Since the introduction of the 7-valent vaccine, a significant increase in invasive disease was seen among nonvaccine strains, which led to the introduction of the 13-valent conjugate vaccine in 2010 (43–45). However, despite the introduction of the new conjugate vaccine, strain replacement still persisted in carriage and invasive disease, with rates of invasive disease due to nonvaccine strains ranging from 57.8% in North America to 71.9% in Europe (46–50). Also of concern are observations that immunization with the conjugate vaccine alters the bacterial flora of the upper respiratory tract in children and has affected the pathogens recovered from children with carriage and acute otitis media (51, 52). A potentially worrisome trend has been noted in increased rates of carriage of *Staphylococcus aureus* in children immunized with the pneumococcal conjugate vaccine (51, 53).

According to 2015 data from the Centers for Disease Control and Prevention, adults over the age of 65 years had the highest risk of IPD in the United States (https://www.cdc.gov/abcs/reports-findings/survreports/spneu15.html). The distribution of pneumococcal serotypes commonly causing invasive disease in adults is much larger than that for children. The Advisory Committee on Immunization Practices recommended the current 13-valent conjugate vaccine for use, in conjunction with the 23-valent PS vaccine, in adults over the age of 65 years (54). This recommendation was based on the results of a large efficacy trial in the Netherlands, which showed 75% and 46% efficacy against vaccine type invasive disease and pneumococcal pneumonia, respectively, among people over 65 years (55). It is recommended that the 13-valent vaccine be administered prior to the 23-valent vaccine, because there is evidence that prior PS vaccine administration reduces the immune response to the conjugate vaccine (56, 57). The CDC is currently evaluating the efficacy of the stand-alone 13-valent conjugate vaccine or the conjugate vaccine in conjunction with the 23-valent PS vaccine in older adults in the United States. The 13-valent conjugate vaccine is also recommended for routine use among people over the age of 19 with immunocompromising conditions, functional or anatomic asplenia, cochlear implants, or cerebrospinal fluid leak (54, 58). Attempts are also underway to develop conjugate vaccines with much higher valence to broaden coverage of different serotypes (59, 60).

Strategies to enhance the immunogenicity of PS have been tested in small animal and human studies, but none have yet reached clinical application. A conjugate of type 6B PS and tetanus toxoid reduces carriage in mice when administered intranasally with the cholera toxin B subunit

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(61). T cell help during response to protein antigens involves binding of CD40 on B cells by CD154 expressed on T cells. Administration of anti-CD40 antibodies along with capsular PS results in predominantly high-affinity IgG antibodies, which is a hallmark of responses to protein antigens (62). Small amounts of interleukin 12 have been shown to be a potent adjuvant for pneumococcal PS in mice (63). Coadministration of immune modulators may prove to be an effective and practical strategy to enhance immune responses to PS. Unless substantial progress is made with protective protein antigens or protein-PS conjugates, novel methods for adjuvant responses to purified capsular PS will remain an important area of investigation into pneumococcal vaccines. One such approach has been the development of a multiple antigen-presenting system (MAPS), which combines various antigens, including PSs and proteins, in a synthetic acellular system (64). Such complexes of antigens in some ways mimic the pneumococcal whole-cell vaccine (WCV) with probably much less risk of undesired reactogenicity. The MAPS complex is highly efficient in generating a broad immune response, including B and T cell (Th1 and Th17) responses, and is particularly effective in the case of *S. pneumoniae*, because it circumvents the issue of covering multiple serotypes to avoid the serious problem of serotype replacement. An approach with multiple antigens that collectively protects against carriage, pneumonia, and sepsis, delivered through a mucosal route, would be expected to be optimally protective.

NONCAPSULAR PS VACCINES

Overview

Polyvalent pneumococcal vaccines based on purified PS have been available for over 3 decades, but their clinical efficacy has been limited by poor immunogenicity in high-risk groups (particularly young children). As discussed above, the problem of poor vaccine immunogenicity in children has been addressed by conjugation of the PS to protein carriers, thereby converting the PS from T cell-independent to T cell-dependent antigens. However, serotype coverage remains limited, and it is unlikely that significantly more than 13 to 15 of the 98 currently known serotypes will be included in future conjugate formulations. Nasopharyngeal colonization with *S. pneumoniae* is a virtual prerequisite for invasive disease. Data from the original conjugate vaccine trials indicated that, although carriage of vaccine types was reduced, the vacated niche was promptly occupied by nonvaccine serotypes known to cause invasive disease in humans (8). Such "replacement" carriage, as well as replacement disease due to nonvaccine serotypes, has occurred to varying extents in virtually all regions where conjugate vaccines have been widely used since the licensure of the original 7-valent formulation in 2000 and continued after introduction of 10- and 13-valent formulations (65–67). In addition, the cost of the conjugate vaccines remains very high; thus, without philanthropic support, their use in developing countries, where the need for effective pediatric vaccines is the greatest, will remain restricted. In view of this, much attention has focused on the possibility of developing vaccines based on pneumococcal protein antigens common to all serotypes (5, 68, 69). Such proteins, being T cell-dependent antigens, are likely to be highly immunogenic in human infants and able to elicit immunological memory. The pneumococcal proteins also have potential as carriers for the PS in the conjugate vaccines. The vaccine potential of various pneumococcal protein antigens are discussed further below.

PspA and PspC

Pneumococcal surface protein A (PspA) is produced by all pneumococci (70). The protein is important for virulence (71, 72); its presence on pneumococci prevents classical pathway complement deposition via C-reactive protein (CRP) and probably anti-PC antibody (72–74). PspA also appears to reduce phagocytosis of pneumococci even in the absence of C′ and antibody (75). Antibodies to PspA, however, strongly enhance complement deposition (73). PspA may also play a role on mucosal surfaces since it blocks killing by apolactoferrin (76). Antibody to PspA reverses this blockage and enhances killing by apolactoferrin (76). Although serologically variable when examined with monoclonal antibody, PspA is highly cross-reactive when examined with polyclonal sera (70, 77). Monoclonal and polyclonal antibodies to PspA can passively protect mice from otherwise fatal bacteremia and otherwise fatal sepsis caused by pneumococci (78, 79). Parenteral immunization with PspA has been able to protect mice against fatal infections, and this protection has been observed to be highly cross-protective regardless of PspA type (79–81). Intranasal and transdermal immunization with PspA has also been shown to protect against nasopharyngeal carriage in an adult mouse carriage model, raising the possibility that vaccines could be developed to prevent carriage and transmission of pneumococci in addition to invasive disease with pneumococci (45, 61, 82–86). A group in Japan has used PspA for the development of a mucosal vaccination protocol which does not use an immunogenic adjuvant or molecules that can follow the nerves from the nose into the brain. Their protocol and the transdermal route could be particularly important in moving PspA and other mucosal immunogens into humans as the vaccines routes able to elicit both mucosal and systemic immune responses (83, 84).

A number of pneumococcal proteins, including PspA, PspC, and LytA, are able to bind choline and share similar, and sometimes indistinguishable, choline-binding domains near the C-terminal ends of the proteins (87–90). The N-terminal end of PspA is composed of a largely coiled-coil α-helical sequence (89, 91) that is responsible for most of the cross-protective immunity elicited by PspA (92, 93). Paradoxically, this region also contains significant structural and serologic variability within the PspA molecule (92, 94). In spite of its variability at the amino acid sequence level, PspAs are very cross-reactive (70, 79) and can be divided into families based on their cross-reactivity and amino acid sequences (77, 94). Ninety five percent or more of clinical isolates belong to families 1 and 2 (77, 94, 95). Immunization of humans with a single recombinant family 1 PspA leads to antibodies that are able to protect mice from otherwise fatal infection with strains expressing either family 1 or family 2 PspAs (80). However, other data indicate that the best vaccine would probably contain PspAs from both family 1 and family 2 (81, 93, 96). In addition, it has been shown that the 80-120 amino acid long proline-rich domain in the middle of PspA, is also able to elicit protective antibody (97). The use of well-chosen alpha-helical and proline-rich domains in immunizing PspA molecules should ensure coverage of diverse PspAs.

The serum of virtually all adults, and most children over 7 months of age contains detectable antibody to PspA (98, 99). The levels of antibody are higher in adults than in children, and it is thus possible that natural antibodies to PspA will contribute more to the immunity to pneumococcal infection in adults than in young children. It is assumed that

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immunization to elicit high levels of antibodies to PspA will be able to enhance protection of young children and adults with waning immunity from infections with pneumococci.

PspC is a protein that has some similarity to PspA in its proline-rich and choline-binding domains (100). The gene for this protein was originally identified through its close similarity to PspA (101). However, the alpha-helical domain of PspC is more complex than that of PspA and is present as several very distinct alleles with distinct combinations of functions (102, 103). This protein has been independently discovered by others based on its ability to bind secretory IgA (SpsA) (104), choline (CbpA) (105), and factor H (Hic) (106). It has potential roles in colonization, adherence, and invasion (103–105, 107, 108). Immunity to PspC is able to protect against pneumococcal infection and carriage (103, 108). Recent studies have shown that the NEEK motif of PspC (CbpA) is able to bind to laminin receptors on the blood-brain barrier and thus is able to elicit protection against fatal pneumococcal infection and is suspected to be particularly important in protection against meningitis (109, 110).

Pneumolysin

All pneumococci produce pneumolysin, a potent 53-kDa thiol-activated pore-forming cytolysin. Pneumolysin is released from pneumococci especially during autolysis. This cell-free pneumolysin can attack any cell that has cholesterol in its plasma membrane (111). In addition to its cytotoxic properties, cell-free pneumolysin is capable of directly activating the classical complement pathway away from the pneumococcal surface and does so in the absence of specific antibody. Classical pathway activation by pneumolysin reduces the local concentration of reactive C3 and other classical pathway complement components. As a result, there is a local decrease in serum opsonic activity (112).

Structure-function analysis of pneumolysin has indicated that a domain toward the C terminus of the toxin (amino acids 427 to 437), which includes a unique cysteine residue, is critical for cytotoxicity (113). This cysteine motif is highly conserved among other members of the thiol-activated cytolysin family. Several single-amino-acid substitutions within this region (and other regions involved in cell binding and pore formation) reduce the cytotoxicity of pneumolysin by up to 99.9%. A separate region, which has a degree of amino acid homology with human CRP, is responsible for IgG binding and complement activation, and a mutation in this domain (Asp385→Asn) interferes with both properties (114).

Pneumolysin has a variety of detrimental effects on cells and tissues *in vitro*, which provide clues to its role in the pathogenesis of pneumococcal disease (111, 115). Complete inactivation of the pneumolysin gene in either a type 2 or type 3 pneumococcus has been shown to reduce virulence for mice challenged by both the intranasal and intraperitoneal routes (116, 117). Compared with the wild-type strain, intranasal challenge with the pneumolysin-negative pneumococci resulted in a less severe inflammatory response, a reduced rate of multiplication within the lung, a reduced capacity to injure the alveolar-capillary barrier, and a delayed onset of bacteremia (118, 119). Pneumolysin also plays a critical role after bacteremia has developed, by modulating the elicited inflammation to reduce its ability to keep bacteremia in check (120). Pneumolysin's ability to modulate inflammation may be related to its ability to stimulate Toll-like receptor 4 (121) and may contribute to chemotaxis of CD4 T cells (122). By using *S. pneumoniae* derivatives, in which the wild-type pneumolysin gene was replaced by mutated genes encoding toxins with point mutations affecting either or both of the cytotoxic and complement activation properties, it has been possible to confirm distinct roles for the two toxin activities in the pathogenesis of pneumococcal pneumonia (123–125).

These studies established the importance of pneumolysin in the pathogenesis of pneumococcal disease and identified it as a target for vaccination. It has been known for many years that immunization with purified pneumolysin protects mice against challenge with highly virulent pneumococci (126). Potential problems of toxicity have been overcome by site-directed mutagenesis of regions of the toxin essential for cytotoxicity and complement activation, as described above. Genes encoding these recombinant pneumolysin toxoids (pneumolysoids) have also been inserted into *Escherichia coli* expression vectors, permitting large-scale production of antigens at low cost (127). Sequence analysis of pneumolysin genes from a wide range of *S. pneumoniae* serotypes has confirmed that there is very little variation in primary amino acid sequences (>99% identity) (128, 129), so a single vaccine antigen should provide coverage against all pneumococci regardless of serotype. Indeed, immunization of mice with a pneumolysoid carrying a Trp_{433}-Phe mutation resulting in a >99% reduction in cytotoxicity (designated PdB) provided a significant degree of protection against all nine serotypes of *S. pneumoniae* that were tested (130). Moreover, mouse monoclonal antibodies to pneumolysin could protect against otherwise fatal infection following intranasal inoculation of *S. pneumoniae* (131). Humans are known to mount an antibody response to pneumolysin as a result of natural exposure to *S. pneumoniae* (132, 133). The expectation that human antibodies to pneumolysin may be protective is supported by evidence that purified human antibody to pneumolysin passively protects mice from challenge with virulent pneumococci (132) and by evidence that a lack of high levels of serum antibody to pneumolysin appear to predispose patients to pneumococcal pneumonia (133). Thus, it is anticipated that immunization of humans with pneumolysoid will increase resistance to pneumococcal infection. The protection elicited by a pneumolysin-containing vaccine may be enhanced by incorporation of other protection-eliciting antigens such as PspA, immunity to which enhances complement deposition and blood clearance (73, 134, 135).

Pneumolysin has also shown promise as a carrier for the otherwise poorly immunogenic PS in experimental conjugate vaccine formulations. Immunization of mice with pneumolysoid conjugated to type 19F PS elicited a strong and boostable antibody response to both protein and PS moieties and provided infant mice with a high degree of protection against challenge with *S. pneumoniae* (127, 136). Similar results have been reported for conjugates of native pneumolysin with type 18C PS (137). A comparison of tetravalent pneumolysoid-PS or tetanus toxoid-PS conjugate vaccines (incorporating PS types 6B, 14, 19F, and 23F) demonstrated that pneumolysoid was at least as good a carrier protein as tetanus toxoid and, in the case of type 23F, superior (138). Clearly, such antigens have the potential to evoke a significant anti-PS response, as well as an anti-virulence-protein response, thereby conferring comprehensive protection against pneumococcal disease in humans.

Other Choline Binding Proteins

Access to the pneumococcal genome sequence facilitated the search for additional vaccine antigens, because it enabled entire families of genes encoding proteins with recognizable

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structural features to be targeted (139). The choline binding proteins are good examples of this approach. Although several members of this family were previously identified by conventional techniques, such as elution from the cell surface with choline, a search of the genome sequence identified a dozen or so functional genes encoding proteins with choline-binding motifs. Site-specific mutagenesis was then used to demonstrate that five of the novel choline binding proteins (CbpD, CbpE, CbpG, LytB, and LytC) were involved in *in vitro* adherence to epithelial cells, nasopharyngeal colonization, or sepsis, thereby identifying them as vaccine candidates (140). LytB and LytC are unusual in that their choline binding domains are located in the N-terminal part of the molecule, while the C-terminal portions have murein hydrolase activity (141). Purified recombinant LytB and LytC were subsequently tested for protective efficacy as part of another large-scale study. Immunization with these proteins conferred significant protection against intraperitoneal challenge in mice, although the degree of protection observed was marginally less than that observed using PspA, which was used as a control antigen (142).

Another choline binding protein with cell wall modification (in this case amidase) activity is the major pneumococcal autolysin LytA. Mutatgenesis of the *lytA* gene prevents the autolysis of pneumococci that occurs spontaneously in stationary-phase cultures, or on addition of deoxycholate and also attenuates virulence in mouse models of sepsis. It might seem paradoxical that inactivation of what is essentially a suicide gene could have such an effect. However, LytA is largely responsible for release of cell-associated pneumolysin, inflammatory cell wall degradation products, and other cell-associated virulence factors, so prevention of autolysis might be of considerable benefit to the host (116, 118, 143). Exogenous antibody to LytA is capable of penetrating the surface layers of the pneumococcus and inhibiting autolysis and release of pneumolysin *in vitro*. Active immunization of mice with purified LytA also elicited a similar degree of protection as pneumolysoid against challenge with fully virulent pneumococci, but it conferred no significant protection against challenge with high doses of a pneumolysin-negative strain. This suggested that the LytA-induced protection is mediated largely through blockade of pneumolysin release (144).

Lipoproteins

The pneumococcal genome includes over 30 putative lipoproteins, with prolipoprotein signal peptidase recognition sequences (LXXC) (145). This so-called lipobox motif directs covalent attachment of a diacyl glycerol moiety to the N-terminal Cys residue of the mature protein, anchoring it to the outer face of the plasma membrane. Thus, they are located beneath the cell wall and the capsule in *S. pneumoniae*. These lipoproteins have diverse functions, the commonest being substrate binding components of ATP binding cassette (ABC) transport systems, and many are important for growth and survival of the pneumococcus *in vitro* and *in vivo*. Their cellular location suggests that they are not exposed on the cell surface to any significant extent, which in turn suggests that they are unlikely to elicit opsonic antibodies. However, this does not necessarily preclude their utility as vaccine targets, since exogenous antibody may diffuse through the capsule and cell wall layers and inhibit the biological function of the lipoprotein. Indeed, several pneumococcal lipoproteins have been shown to have potential as vaccine antigens, as discussed below.

Pneumococcal surface antigen A (PsaA)

PsaA is a highly conserved 37-kDa lipoprotein produced by all pneumococci. It was initially thought to be an adhesin based on sequence homology with putative lipoprotein adhesins of oral streptococci, but it is actually the metal binding component of an Mn^{2+}-specific ABC transport system (146). Defined *psa*A-negative mutants of *S. pneumoniae* are virtually avirulent for mice and exhibit markedly reduced adherence *in vitro* to human type II pneumocytes (147, 148). This is presumed to be a consequence of growth retardation due to an inability to scavenge Mn^{2+} *in vivo*, as well as pleiotropic effects on expression of a range of cellular processes or virulence factors. Intracellular Mn^{2+} appears to play a critical role in the regulation of expression of oxidative stress response enzymes and intracellular redox homeostasis, and *psa*A-negative pneumococci exhibit hypersensitivity to superoxide and hydrogen peroxide (147, 149).

One study has shown that parenteral immunization of mice with purified PsaA in the presence of strong adjuvants elicits significant protection against systemic challenge with *S. pneumoniae* (150). However, in other studies immunization with PsaA elicited only marginal protection and was less efficacious than pneumolysoid in an intraperitoneal challenge model (134, 151). The dimensions of PsaA (approximately 7 nm at its longest axis) (152) are such that if it is indeed anchored to the outer face of the cell membrane via its N-terminal lipid moiety, it is unlikely to be exposed on the outer surface of the pneumococcus. This is consistent with the fact that whereas the known surface-exposed domains of PspA and PspC are variable, the amino acid sequence of PsaA is highly conserved (153). Gor et al. (151) used flow cytometry to compare the surface accessibility of PsaA and PspA to exogenous specific antibodies in 12 *S. pneumoniae* strains. PspA was readily detectable on the surface of all strains, whereas PsaA was not. This directly correlated with the protective efficacy of either active or passive immunization with the respective protein or antibody; significant protection against systemic challenge was achieved using PspA or anti-PspA, but not using PsaA or anti-PsaA. Given the virtual absence of surface exposure, any protection elicited by immunization with PsaA is unlikely to be a consequence of enhanced opsonophagocytic clearance. Rather, it is presumably due to *in vivo* blockade of ion transport, which necessitates diffusion of antibody through the capsule and cell wall layers. Such penetration of antibody is likely to be concentration-dependent, and thus, high anti-PsaA titers may be required for protection. Moreover, accessibility of PsaA to exogenous antibody may be influenced by the thickness of the capsule, which may vary from strain to strain. Expression of pneumococcal capsule biosynthesis genes has also been shown to be upregulated during invasive infection (154). In contrast, pneumococci colonizing the nasopharynx are thought to downregulate capsule expression, thereby facilitating interaction between surface adhesins and the host mucosa. Consistent with this hypothesis, several studies have shown that intranasal immunization of mice with PsaA in the presence of strong mucosal adjuvants such as cholera toxin B subunit significantly reduces the level of nasopharyngeal carriage of *S. pneumoniae* (82, 155). A lesser but still significant reduction in susceptibility to carriage was also achieved by subcutaneous immunization of mice with synthetic lipidated multiantigenic PsaA peptides (156). Thus, at least in the nasopharynx, PsaA may be accessible to antibody. Immunization with a PsaA-cholera toxin B subunit fusion protein also significantly reduced carriage of *S. pneumoniae* in

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mice without significantly disturbing the oropharyngeal microflora (157).

Iron transporter lipoproteins PiuA and PiaA

Two other metal-binding lipoproteins have been proposed as pneumococcal vaccine antigens. These proteins, designated PiuA and PiaA, are components of two separate ABC iron transport systems. At least one of these proteins is required for optimal growth of pneumococci in iron-depleted media, and they are capable of acquiring iron from hemoglobin (158). Indeed, PiuA has been shown to be capable of directly binding both hemin and hemoglobin (159). PiuA and PiaA are produced by all pneumococci, and their genes are highly conserved (160). Mutagenesis studies have shown that both proteins contribute to virulence in mice using both lung and intraperitoneal models of infection (158). They are immunologically cross-reactive, and immunization of mice with either protein conferred a similar degree of protection against intraperitoneal challenge to that elicited by the pneumolysoid PdB. Moreover, immunization with a combination of PiuA and PiaA resulted in additive protection (161). Although a direct comparison has not been conducted, immunization with either PiuA or PiaA provided a higher degree of protection against systemic disease than that previously published for PsaA, using the same mouse model and *S. pneumoniae* challenge strain (134). Like PsaA, PiuA and PiaA are predicted to be attached to the outer face of the plasma membrane (159), and so the superior protective efficacy of the latter proteins ought not to be due to a difference in accessibility to exogenous antibody. However, Jomaa et al. (160) have shown by flow cytometry that both PiaA and PiuA are accessible to exogenous antibody in intact bacteria and that these antibodies stimulate *in vitro* opsonophagocytic activity, particularly in the presence of complement. They also reported that the antibodies did not appear to interfere with iron uptake *in vitro*. Mucosal immunization with PiuA and PiaA has also been shown to elicit antibody responses both in serum and respiratory secretions, which protected mice against intranasal challenge (162). The reason for the apparent difference in surface accessibility between PsaA and the two iron-binding lipoproteins is unclear, given their predicted location. One possibility is that in the latter two cases, at least a proportion of the proteins are released from the membrane and are then able to bind to more exposed domains on the pneumococcal surface, where they can interact with exogenous antibody more freely. Regardless of the underlying mechanism, available data suggest that PiaA and PiuA have more promise than PsaA as vaccine antigens, as least for prevention of systemic disease.

Pneumococcal Histidine Triad (Pht) Proteins

The pneumococcal histidine triad proteins are a recently recognized family of surface proteins that have an unusual polyhistidine motif, HXXHXH, repeated five or six times in their amino acid sequences. The prototype, PhtA, was discovered as part of a genome-wide screen for potential vaccine antigens (142). Over 100 proteins were expressed and tested for efficacy in a mouse model, and PhtA was 1 of only 5 that were protective. The others were the choline binding proteins LytB and LytC (discussed previously), a cell wall-associated serine protease PrtA, and another protein of unknown function, designated PvaA. Further examination of the pneumococcal genome sequence revealed three additional related open reading frames (designated PhtB, PhtD, and PhtE), each with five or six copies of the histidine triad motif. The four proteins range in size from 91 to 114 kDa and are closely related at the amino acid sequence level, exhibiting 32 to 87% identity; this similarity is strongest in the N-terminal regions (163). Although their signal peptides all contain an LXXC motif, this does not appear to function as a true lipobox, because they are not labeled by [^{3}H]-palmitate (164). Flow cytometric analyses have shown that the C-terminal regions are more readily accessible to exogenous antibodies (163, 164), suggesting that they are tethered via their N-termini. Analysis of deletion and point mutants subsequently identified a three-amino-acid region in PhtD (Q27-H28-R29) that is critical for surface attachment (165). The histidine triads are believed to form a novel Zn^{2+} binding motif (166), and the *pht* genes are regulated by the Zn^{2+}-dependent repressor AdcR (167). A major function of Pht proteins is to facilitate Zn^{2+} import via AdcAII, one of two Zn^{2+} binding lipoproteins associated with the AdcBC permease (168). Of the five His triad motifs in PhtD, that closest to the N-terminus appears to have the greatest impact on Zn^{2+} recruitment (169). Pht proteins may also inhibit complement deposition on the pneumococcal surface through binding of factor H (167). They exhibit substantial functional redundancy; significant effects on virulence in mouse models of pneumococcal sepsis, on AdcAII-dependent Zn^{2+} uptake, and on complement deposition *in vitro* were only observed when all four *pht* genes were deleted (167, 168).

There is a high degree of protein sequence conservation among individual Pht proteins from diverse *S. pneumoniae* serotypes (164), which combined with a degree of immunological cross-reactivity between the proteins augers well for broad strain coverage for these candidate vaccine antigens. Immunization with purified PhtA, PhtB, or PhtD has been shown to confer significant protection against intraperitoneal challenge with type 3, 6A, and 6B and one of two type 4 *S. pneumoniae* strains (142, 163). PhtD has also been shown to protect against intranasal challenge with a type 3 strain (170), while immunization with either PhtB or PhtE also protects against type 3 pneumococci in models of sepsis and pneumonia (164). In this latter study, immunization experiments with truncated PhtE fragments localized the protective epitopes to the more surface-exposed C-terminal region of the molecule. However, notwithstanding these promising results, the only direct comparative studies of the protective efficacy of Pht proteins with other well-characterized antigens indicate that the level of protection elicited is no better than that achieved by either PspA or pneumolysoid (142, 171).

Sortase-Dependent Surface Proteins

Sortase-dependent surface proteins of Gram-positive bacteria are identifiable by the presence of a C-terminal anchoring motif, which consists of a conserved LPXTG sequence, followed by a hydrophobic domain and usually a tail of positively charged residues. This motif is recognized by sortase, a membrane-localized cysteine protease which cleaves between the T and G residues and covalently links the processed protein to the peptidoglycan cross-bridges (172). In pneumococci, inactivation of the sortase gene releases known sortase-dependent surface proteins, such as the major pneumococcal neuraminidase NanA, and reduces adherence to pharyngeal cells (173). NanA-deficient mutants of *S. pneumoniae* have been shown to have a reduced capacity to colonize the upper and lower respiratory tracts of mice (174, 175) and the nasopharynx and middle ear cleft of chinchillas (176). An early study indicated that purified NanA had

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modest but significant protective efficacy (weaker than pneumolysin) in a mouse sepsis model (144). It was also shown to be protective against both carriage and otitis media in chinchillas (177, 178).

Significant attention has also been paid to pilus-like structures on the surface of *S. pneumoniae*, which contribute to virulence (179). These are encoded on the *rlrA* pathogenicity islet, which is present in some but not all clinical isolates. The *rlrA* islet encodes a transcriptional regulator, three pilus structural components (RrgA, RrgB, and RrgC), and three sortases (SrtB, SrtC, and SrtD) which are required for pilus assembly (179, 180). RrgB is the major pilin, while RrgA and RrgC are ancillary pilin subunits decorating the shaft and tip (181). Immunization of mice with purified RrgA, RrgB, or the combination of all three pilus proteins elicited significant protection against intraperitoneal challenge with the *S. pneumoniae* strain from which the proteins originated (182). However, the utility of pilus proteins as stand-alone vaccine antigens is limited by the fact that there are three sequence clades of the *rlrA* islet, each associated with distinct *S. pneumoniae* clonal groups. At the amino acid sequence level, RrgB is the most divergent, with only about 50% identity between clades (183), and the extent of cross-protection is unknown. Moreover, *rlrA*-positive pneumococci account for only about 30% of strains, and the majority of these belong to serotypes covered by PCV7 (183, 184). Interestingly, a second pneumococcal pilus locus has recently been described, and this was present in about 16% of strains, again belonging to discrete clonal groups, all but one of which lacked *rlrA* (185). However, the vaccine potential of these distinct pilus proteins is uncertain.

Other sortase-dependent pneumococcal surface proteins have been proposed as vaccine candidates, including hyaluronidase (Hyl) and the IgA1 protease (Iga). The latter is of interest because its sortase motif is located in the N-terminal region of the molecule, but it is nevertheless essential for proper function and surface localization (186). However, although these proteins have been shown to contribute to pathogenesis using either *in vitro* or *in vivo* models, Hyl is at best a weak protective antigen (187), while Iga is yet to be tested individually for protective efficacy.

Other Protein Vaccine Candidates

A number of other apparently surface-associated pneumococcal proteins with at least theoretical vaccine potential have been identified, in most cases using immunoproteomic approaches. This list includes proteins that lack export or anchorage signals and would have been predicted to be cytoplasmic and hence dismissed by previous motif-based searches. Examples include metabolic enzymes such as enolase (which also binds plasminogen) (188), 6-phosphogluconate dehydrogenase (a putative adhesin) (189), fructose-biphosphate aldolase, and glyceraldehyde-3-phosphate dehydrogenase (190), as well as the heat-shock protease ClpP (191). Another candidate is the putative proteinase maturation protein PpmA (192), although its degree of surface exposure and protective efficacy has been questioned (151).

Giefing et al. (193) conducted a comprehensive study of the antibody repertoire induced in humans during natural pneumococcal infection by extensive screening of *E. coli* display libraries representing the entire *S. pneumoniae* proteome with carefully selected panels of convalescent patient sera. Sequence analysis of reactive clone inserts identified not only proteins that reacted consistently with the sera, but also the key epitopes therein. Over 140 immunoreactive proteins were identified, and many of the vaccine candidates referred to above, most notably PspA, PspC, PhtA/B/D/E, NanA, Iga, LytC, and LytA, were detected with high frequency. Interestingly, about 20% of the proteins represented unknown gene products and thus may have been missed in previous motif-based *in silico* screens. A subset of the identified proteins was selected on the basis of further seroreactivity screens of synthetic peptides, surface localization, and opsonophagocytic studies using epitope-specific hyperimmune mouse sera and PCR-based gene distribution analyses. Twenty selected proteins were then purified and tested for protective immunogenicity in mouse models of sepsis and pneumonia. Significantly, two proteins (PcsB and StkP) provided a degree of protection similar to that seen for PspA. Both are highly conserved and appear to play an important role in cell separation and/or formation of division septa. PcsB and StkP both protected against a variety of challenge strains and were superior to PspA when the challenge strain expressed a dissimilar family/clade of PspA (193).

A recent study evaluated the vaccine potential of 52 pneumococcal proteins selected on the basis of genetic conservation across disease-causing serotypes and bioinformatic prediction of antibody binding to the target antigen (194). Seven proteins induced protective responses in a murine infection model, namely, the Pht family proteins PhtB, PhtD, and PhtE and the sortase family proteins PrtA, NanA, PavB, and Eng.

From the above it can be seen that there are a number of pneumococcal proteins that exhibit potential as vaccine antigens. However, assessment of their protective efficacy has generally been carried out in different laboratories using a variety of animal models and challenge strains. Relatively few direct comparative protection studies have been performed to determine which of these proteins provides the strongest protection against the widest variety of *S. pneumoniae* strains.

Combination Protein Vaccines

The vast majority of the pneumococcal proteins under consideration as vaccine antigens are directly or indirectly involved in the pathogenesis of pneumococcal disease. Mutagenesis of some combinations of virulence factor genes, for example, those encoding pneumolysin and either PspA or PspC, PspA, or PspC or all three genes, has been shown to synergistically attenuate pneumococcal virulence in animal models, suggesting that the respective proteins function independently in the pathogenic process (195, 196). This strongly suggests that immunization with combinations of these antigens might provide additive protection. Moreover, there may be differences in the relative protective capacities of the individual antigens against particular *S. pneumoniae* strains, particularly for surface-exposed antigens that exhibit some degree of sequence variation. Thus, a combined pneumococcal protein vaccine may elicit a higher degree of protection against a wider variety of strains than any single antigen.

Immunization of mice with a combination of the pneumolysoid PdB and PspA provided significantly increased protection against intraperitoneal challenge than immunization with either protein alone. However, combining either protein with PsaA did not result in enhanced protection (134). The potential benefits of combination protein vaccines are also well illustrated using a mouse model of nonbacteremic pneumonia, which closely reflects the commonest form of pneumococcal respiratory disease in humans (135). In this system, subcutaneous immunization (using alum adjuvant)

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with either PdB or PspA, but not PsaA, significantly reduced numbers of *S. pneumoniae* in the lungs 7 days after challenge. A significant additional reduction in bacterial load was achieved by immunization with a combination of PdB and PspA, but not when either protein was combined with PsaA (135). These findings contrast with those obtained using mucosal (intranasal) immunization with the same proteins with cholera toxin B subunit as the adjuvant. As discussed previously, immunization with either PspA or PsaA, but not PdB, reduced the level of carriage of *S. pneumoniae* after intranasal challenge, and the combination of PspA and PsaA was more effective than either antigen alone (82). These findings suggest that PsaA is either more important for survival of *S. pneumoniae* in the nasopharynx than in the lung or that it is more accessible to exogenous antibody in the former niche. On the other hand, pneumolysin appears to play only a minor role during the colonization phase but is clearly important once the organism has been aspirated into the lungs. Thus, optimum vaccine formulation will be dependent upon the mode of vaccine delivery and the stage of the pathogenic process being targeted for immunoprophylaxis. In a more recent study, a variety of multivalent protein-based vaccines comprised various combinations of full-length or peptide regions of the immunogens pneumolysin, PspC/CbpA, or PspA. Optimum protection against multiple challenge strains in models of sepsis, pneumonia, and meningitis was obtained using a combination comprising pneumolysin toxoid with an L460D substitution, with two protective peptide epitopes from PspC/CbpA (known to be involved in binding to the host polymeric immunoglobulin receptor and the laminin receptor, respectively) fused to the N- and C-termini, as well as a polypeptide derived from the conserved proline-rich region and SM1 regions of PspA (197).

Only a handful of other pneumococcal protein combinations have been tested for additive protective immunogenicity. Immunization with both the iron transporters PiuA and PiaA was more effective than either antigen alone (161). A combination of pneumolysoid (PdB), PspA, and PspC has also shown stronger protection than single or paired antigens (171). In that study, combinations of PhtB and PhtE were less protective than those involving two or more of PdB, PspA, and PspC, and inclusion of PhtB did not improve the protection imparted by either PdB/PspA or PdB/PspC (171). Additive protection relative to individual components has also been demonstrated for a trivalent formulation comprising another pneumolysin toxoid (PlyD1), PhtD, and pneumococcal choline binding protein A (PcpA) in an infant murine otitis media model (198). Clearly, additional comparative studies of the protective efficacy of the better-characterized proteins, as well as the more recently identified vaccine candidates (both singly and in combination), are desirable to enable informed decisions on the optimum formulation of a protein-based pneumococcal vaccine. However, further development of some potentially efficacious combinations has been frustrated by the intellectual property landscape surrounding individual components, which impacts freedom to operate for commercial partners taking experimental vaccine formulations forward into the clinic.

Of the pneumococcal combination protein vaccines that have reached phase II human trials, the PlyD1/PhtD/PcbA formulation referred to above has been shown to be safe, well-tolerated, and immunogenic in adults, toddlers, and infants (199). A formulation comprising the currently licensed 10-valent PS conjugate vaccine, supplemented with chemically detoxified pneumolysin (dPly) and PhtD was also safe and immunogenic in infants (200, 201), but this vaccine formulation did not reduce nasopharyngeal carriage of non-conjugate vaccine serotypes (202). A cell-free protein extract from a derivative of *S. pneumoniae* TIGR4 expressing a nontoxic pneumolysin gene, enriched for various conserved surface proteins by anion exchange, was also safe and immunogenic in adults at phase I (203).

PNEUMOCOCCAL WHOLE-CELL VACCINES (WCV)

An alternative strategy for eliciting non-serotype-dependent protection is immunization with a simple vaccine comprising killed nonencapsulated pneumococci that display critical protein antigens on their surface. A study looking at historical data from the 1918 influenza pandemic showed that WCVs had the ability to confer cross-protection against different pneumococcal serotypes and that such vaccines were protective against influenza-associated pneumonia (204). A WCV comprising a chemically killed *lytA*-deletion mutant of *S. pneumoniae* Rx1 expressing nontoxic pneumolysin has also been shown to elicit protection against sepsis and nasopharyngeal carriage in mouse models. Protection against sepsis was dependent on antibodies to noncapsular antigens, while prevention of carriage required CD4+ T cell-dependent responses (205, 206). The WCV approach has potential cost advantages over the currently licensed conjugate vaccines and the various multicomponent purified protein formulations currently in clinical development. A chemically killed WCV formulation was safe and immunogenic in a phase I trial and is in further clinical development (207).

Another study has used γ-irradiation to inactivate a similar unencapsulated *S. pneumoniae* strain. Intranasal vaccination with this WCV (without adjuvant) elicited serotype-independent protection in lethal challenge models of pneumococcal pneumonia and sepsis. Vaccine efficacy was shown to be reliant on B-cells and interleukin-17A responses. Interestingly, immunization promoted interleukin-17 production by innate γδ T cells, not T helper 17 (Th17) cells (208). Further enhancement of immunogenicity was observed when this vaccine was coadministered with a γ-irradiated influenza virus vaccine that also provides heterotypic protection against the virus (209).

MUCOSAL IMMUNITY

The development of vaccines that can elicit mucosal immunity could be very important because (i) mucosal immunity is thought to provide the best protection against carriage (61, 210), (ii) nasopharyngeal carriage is the human reservoir for pneumococci, and herd immunity depends on immunity to carriage (39, 82, 210), (iii) at least a short duration of carriage is required prior to almost all invasive disease (211), and (iv) epidemiologic data suggest that meningitis frequently occurs with strains of capsular types which seldom cause sepsis but which are readily carried (50, 212, 213). In the section above about PspA and PspC, mucosal immunity studies are described that used a cationic cholesteryl group-bearing pullulan nanogel, which greatly enhanced mucosal immunity in mice and, like many other recent enhancers of mucosal immunity (214, 215), is not transported to the brain (83, 84).

CONCLUSIONS

The ongoing high global morbidity and mortality associated with pneumococcal disease and the complications caused by increasing rates of resistance to antimicrobials have under-

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pinned extensive efforts in recent years to develop more effective vaccination strategies against *S. pneumoniae*. These efforts have benefited from a better understanding of the mechanisms of pathogenesis of pneumococcal disease and the advances made possible by the advent of recombinant DNA technology and access to genome sequence data. The polyvalent PS vaccines have doubtless prevented a large number of deaths from invasive disease in recipients belonging to those patient groups for whom this vaccine is currently recommended. The newer PS-protein conjugate formulations confer a very high degree of protection in young children against the included serotypes and may also have an impact on the prevalence of drug-resistant strains. However, there is now general acceptance that this vaccination approach is not without its drawbacks, and as explained above, the initially substantial clinical benefits that are expected to be derived from widespread use of conjugate vaccines may diminish with time. It will take many years for the overall impact of conjugate vaccines on disease burden and the population biology of *S. pneumoniae* to become apparent. At the very least, use of the conjugate vaccines will buy time for development of less expensive, largely non-serotype-specific vaccines based on combinations of protein antigens. It must be emphasized, however, that the success of these protein vaccines is not dependent upon real or perceived failure of the conjugates. Rather, the two approaches should be viewed as complementary, each having an important role to play in global prevention of pneumococcal disease. Nor should development of parenteral protein vaccines impede future research on mucosal- or DNA-based delivery systems, which may further improve presentation of protective antigens to the immune system, thereby optimizing host responses and herd immunity.

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Pathogenicity of Enterococci

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24

INTRODUCTION

Enterococci are hardy, Gram-positive cocci that are common residents of the gastrointestinal tracts of nearly all land animals, including humans. While they are a core member of the microbiome, they are also capable of causing a variety of severe infections, most often among antibiotic-treated hospitalized patients with perturbed intestinal microbiota. Here, we present an overview of the pathogenicity of enterococci and discuss the most prominent features of this hospital-associated pathogen.

The origin of the term "enterococcus" dates to the end of the 19th century when Thiercelin described a saprophytic Gram-positive coccus of intestinal origin capable of causing infection (1, 2). In the same year, a very detailed picture of enterococcal pathogenesis was reported by MacCallum and Hastings (3), who isolated and characterized an organism (now believed to be a cytolytic strain of *Enterococcus faecalis*) from a lethal case of acute endocarditis. Termed *Micrococcus zymogenes* by these authors, bacteria recovered from the blood and cardiac tissue of the patient and inoculated into pure culture were able to recapitulate elements of endocarditis in animals, satisfying Koch's postulates and establishing *Enterococcus* as a bona fide pathogen of humans and animals. From these early descriptions, the paradigm of enterococci as a commensal opportunist was established. More than a century later, enterococci are widely recognized as leading hospital pathogens (4, 5).

Infections occurring mainly among hospitalized patients and caused by microbes of the genus *Enterococcus* (most notably, *E. faecalis* and *Enterococcus faecium*) include urinary tract infections, bacteremia, intra-abdominal infections, and endocarditis (6). Enterococci are now the third most common hospital-acquired pathogen, causing 14% of hospital-acquired infections in the United States between 2011 and 2014, an increase from 11% in 2007 (4, 7). In addition to hospital-acquired infections, enterococci are responsible for 5 to 20% of community-acquired endocarditis (8).

This review focuses on the mechanisms by which enterococci cause human disease, with particular attention given to advances in the field since the last edition of this text (9). Additional perspectives and information may be found in the complete volume on enterococcal biology (10).

BIOLOGY AND CHARACTERISTICS

Enterococci can be easily isolated from a wide range of hosts, including invertebrates, insects, and mammals (Fig. 1). This host range highlights the ability of enterococci to survive a diversity of innate host defenses in the gut (11). In humans, enterococci are one of the earliest colonizers of the infant gut, and as a result are core members of the intestinal microbiome (12, 13). They are also common among commensal microbiota of domesticated and wild animals and can cause infections in these animals as well (14–16). Due to their widespread occurrence among humans and animals, they are readily isolated from the environments inhabited by those hosts, often associated with plants, soil, and water (17, 18). Because of their numerous auxotrophies, enterococci persist in the environment but do not likely proliferate to high numbers there.

Enterococci were formerly classified as group D streptococci (19) but were given genus status in 1984 (20) based on nucleic acid hybridization studies that showed a more distant relationship to the streptococci. The genus *Enterococcus* contains over 50 species and appears to have branched from its last common ancestor approximately 425 million years ago (21). Members of this genus are endowed with intrinsic properties that confer the ability to survive host defenses and compete in the intestinal tract and then persist and spread in the natural environment or hospital, leading to colonization of new hosts. Among these traits are the ability to grow over wide temperature and pH ranges, survive desiccation, and grow in the presence of 6.5% NaCl and 40% bile salts (17).

ANTIBIOTIC RESISTANCE

The challenge of disease caused by enterococci is compounded by their limited susceptibility to antibiotics, due to both intrinsic and acquired antibiotic resistances. Enterococci are intrinsically resistant to cephalosporins, aminoglycosides, lincosamides, and streptogramins (6, 22). Intrinsic resistance of enterococci undoubtedly positions them well to acquire additional resistances on mobile genetic elements. In the antibiotic-treated patient, enterococci accumulate to high numbers (23) and coexist in intimate association with other overtly antibiotic-resistant microbes, the precise environment

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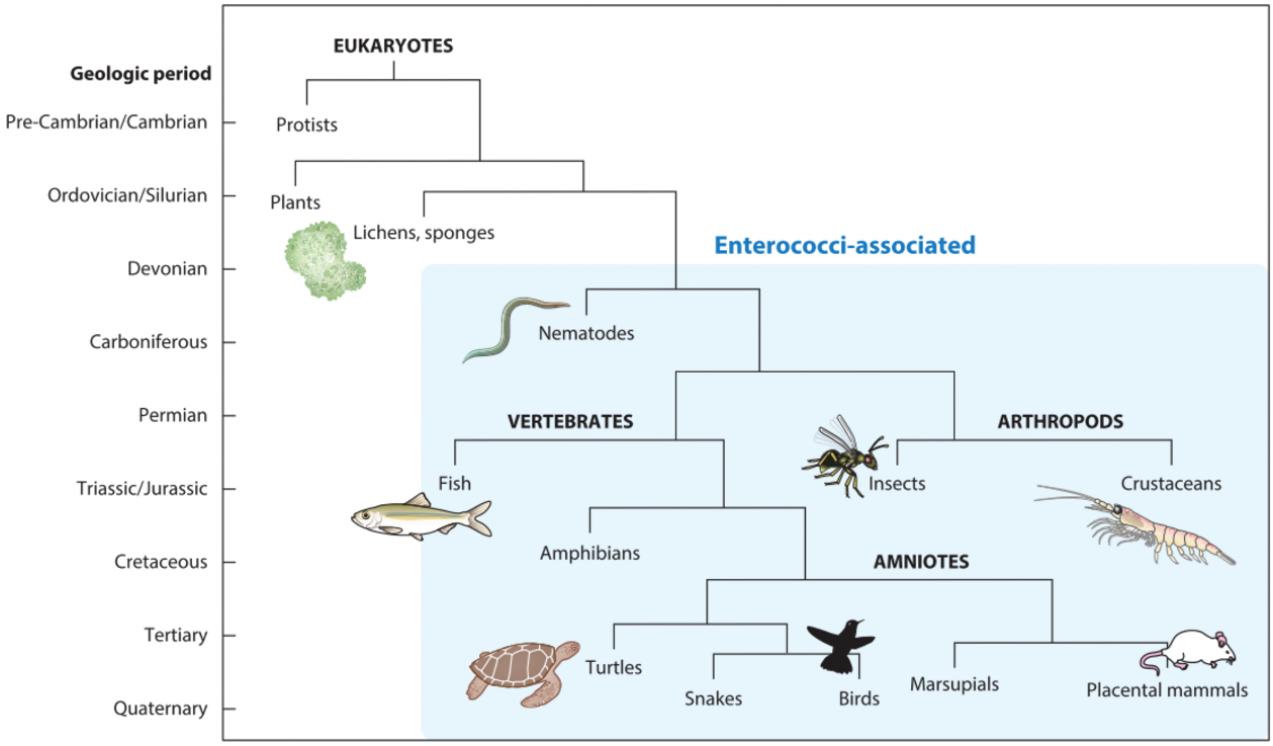


FIGURE 1 Animal hosts that have been associated with enterococcal colonization. A simplified tree of life with blue shading indicating animals from which enterococci have been isolated. Corresponding geologic periods are indicated on the left. Reproduced with permission from reference 11.

to enhance the probability of contact between enterococci and other species harboring new resistances on mobile elements, leading to element acquisition.

Because enterococci are not killed by many β-lactams, new approaches to treatment were sought. The discovery that aminoglycosides were synergistic with β-lactams and could achieve cidality (24) resulted in combination therapy becoming the standard of care. However, only a few years later, aminoglycoside resistance in enterococci was reported (25) and found to be due to the acquisition of a plasmid-borne resistance factor. High-level resistance to aminoglycosides is conferred by aminoglycoside-modifying enzymes, especially a bifunctional enzyme (26), and mobile elements conferring this trait have spread throughout the enterococci (27). Further compromising combination therapy, enterococci that expressed a β-lactamase have been identified (28), but these remain surprisingly rare.

Vancomycin was introduced in the late 1950s (29) and because of its limited spectrum and intravenous dosing received limited application in hospitalized patients with β-lactam allergies and infections due to organisms with other antibiotic resistances (30). Vancomycin was found to be effective against aminoglycoside-resistant enterococci (31) and became a leading alternative therapy in the late 1970s and early 1980s (32). However, vancomycin resistance emerged in the mid-1980s—first in Europe (33, 34) and then in the United States (35). Enterococci resistant to vancomycin are now globally disseminated, with as many as 80% of *E. faecium* isolates being resistant to vancomycin in some hospitals (4, 36).

The rapid rise of vancomycin resistance in enterococci at the beginning of the 21st century raised alarms, because it represented the loss of an important bactericidal, last-line therapy. Daptomycin was shown to be effective against vancomycin-resistant enterococci (VRE) (37), but its value was found to be limited by the rate of daptomycin resistance development during the course of treatment (38–40). Resistant isolates had mutations in several genes that affect cell membrane composition and charge, including the cardiolipin synthase *cls*, the glycerophosphoryl-diester-phosphodiesterase *gdpD*, and the stress-sensing response component *liaF* (41, 42). Confounding a precise understanding of its mechanism of action, *Staphylococcus aureus* was observed to alter different pathways in achieving daptomycin resistance (43). Furthermore, resistance-conferring mutations derived in enterococci *in vitro* only partially overlap with those that arise *in vivo*, potentially because of differences in lipid incorporation from the environment into the enterococcal membrane (44). Lipidomic studies of daptomycin-resistant strains showed differences in lipid profiles compared to sensitive strains, particularly in phosphatidylglycerols, cardiolipins, and glycolipids (45).

Linezolid, the first oxazolidinone antibiotic, was approved by the FDA in 2000, and its introduction into clinical use was quickly followed by the emergence of linezolid-resistant VRE strains in the United States in 2001 (46) and in the United Kingdom in 2002 (47). Sequencing of the resistant isolates revealed a G2576U mutation in the 23S ribosomal RNA subunit (48). This mutation was also seen in resistant organisms isolated from a gnotobiotic mouse model of infection (49), where its occurrence was found to depend on the dose of antibiotic that reached the intestine. A linezolid-resistant isolate from Thailand subsequently was shown to have acquired the *cfr* methyltransferase on a plasmid, leading to methylation of position A2503 in the 23S rRNA and causing high-level resistance (50).

A third antibiotic that has found utility in treating VRE is the glycylcycline tigecycline. It has been used successfully, alone and in combination with other antibiotics, to treat

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VRE infection, but resistance has also emerged. A study of 73 tigecycline-nonsusceptible *E. faecium* and *E. faecalis* isolates collected from 2007 to 2015 found mutations in various efflux pumps that were identified and associated with tigecycline resistance (51). Serially passaged *E. faecium in vitro* in the presence of tigecycline selected for mutations in ribosomal protein subunit *rpsJ*, but this mutation has not yet been functionally validated (51, 52). Nonetheless, enterococci appear able to evolve resistance to antibiotics of all classes that have so far been introduced to practice.

ENVIRONMENTAL PERSISTENCE

As implied by the clonality of infection, epidemiological studies highlight person-to-person transmission of endemic strains within the hospital (53–55), implying that enterococci persist for extended periods in the hospital environment. Transmission occurs through contact with health care personnel and inanimate objects such as bedrails, nursing station keyboards, hospital drapery, and ear-probe thermometers (56). Enterococci are unusually resistant to common antiseptics and disinfectants, as well UV radiation, starvation, and desiccation (21, 57, 58). The ability to survive nutrient-poor environments as well as desiccation has led some to speculate that enterococci may enter a viable but nonculturable state as an adaptation to poor growth conditions (59–61), but details of such a mechanism have yet to be elucidated.

Studies have examined the environmental ruggedness mainly of *E. faecalis* (62–70), which develops tolerance to otherwise lethal levels of bile salts and detergents, such as sodium dodecyl sulfate, if first subcultured in the presence of sublethal levels (62). This phenomenon likely contributes to the ability of enterococci to survive the cleaning regimens employed in most hospitals as part of infection control programs. Regulatory systems govern the adaptive response to environmental insults (71, 72). In a study by Hancock and Perego (71), inactivation of a response regulator, designated RR06, led to an increased sensitivity to heat stress (growth at 46°C), as well as detergent stress (0.003% sodium dodecyl sulfate). Le Breton et al. (72) showed that another response regulator, RR10, negatively regulates the heat shock proteins DnaK and GroEL. Inactivation of the *rr10* gene resulted in acid sensitivity but also enhanced survival at 50°C.

To identify the genetic contributors to this intrinsic ruggedness, a spectrum of enterococcal species, including clinical isolates as well as species never reported to be associated with human infection, were compared for resistances to chemical compounds as well as environmental stresses (21). All enterococci were found to be intrinsically much more resistant to most insults than other related microbes, indicating that many of the underlying traits were acquired as the genus branched from its ancestors. The two enterococcal species that are most pathogenic to humans, *E. faecalis* and *E. faecium*, showed the greatest resistance to desiccation and starvation (21). These two species also show high levels of resistance to the common hospital disinfectants chloroxylenol and chlorhexidine. The molecular mechanisms that contribute to these phenotypes were narrowed to a set of 126 genes that distinguished enterococci from ancestors (21). Additionally, the two-component system ChtRS was recently identified as in important regulator of chlorhexidine resistance in *E. faecium* (73).

PATHOGENIC MECHANISMS

For enterococci to cause disease, several barriers must first be overcome. An initial barrier is the ability to overcome colonization resistance provided by competing microbes, and host defenses such as gastric acid and bile, and colonize the intestinal tract. From this reservoir the bacteria can amplify in number and spread to sites vulnerable to infection. A basic prediction from such a model is that the probability of infection should be a function of the intestinal burden of bacteria in the gut reservoir—the more bacteria, the greater the probability of contamination of a potential infection site in numbers large enough to overcome host defenses. Indeed, colonization of the gastrointestinal tract has been shown to be directly associated with risk of infection (23, 74). Infection occurs when enterococci overwhelm host defenses, when they replicate at rates that exceed clearance, and when pathologic changes result through direct toxin activity, or infection occurs indirectly by bystander damage from the inflammatory response (75, 76).

HUMAN COLONIZATION

Enterococci are core but numerically minor members of the human microbiome (77). The occurrence of two species, *E. faecalis* and *E. faecium*, in similar abundance, suggests that neither evolved in humans but, rather, likely entered the primate lineage early and from organisms consumed from lower in the food chain. Both *E. faecalis* and *E. faecium* are found in relative abundance in human feces (10^5 to 10^7 organisms per gram) (78) but typically represent less than 1% of the total microbial population (17). "Colonization resistance" (79) is a term applied to the challenge an organism faces in colonizing a stable, established community. It is a main mechanism of innate defense of the gastrointestinal tract. Colonization and proliferation of hospital-adapted lineages of enterococci are usually associated with antibiotic-induced disruption of the community structure (80). Therefore, effectively managing the human microbiome in health and disease represents a theoretically promising strategy for preventing hospital infection.

A primary barrier to invading the gut consortium and colonization by orally acquired microbes is gastric pH, which is inhospitable for most microorganisms, including enterococci. Compromise of this barrier enhances the oral acquisition of enterococci from a contaminated environment. In the intensive care unit setting in particular, patients are placed on H_2-receptor antagonists as prophylaxis for treating stress ulcers; the consequence of this action is a pH increase from pH 2 to pH 3.5 to 5.3, depending on the antagonist employed (81). In a study by Başaran et al. (82), H_2-receptor antagonists were shown to promote colonization of the small bowel, as well as translocation of enteric bacteria from the intestinal tract to extraintestinal tissues.

Several studies have examined the ability of enterococci to tolerate acidic pH (64, 83). Flahaut et al. (64) demonstrated that exposure of *E. faecalis* to a sublethal pH level (pH 4.8) for 15 to 30 minutes protected the organism from a normally lethal challenge at pH 3.2, suggesting an adaptive response to changing acid stress. Suzuki and colleagues (83) showed that an *E. faecalis* mutant defective in F_1-F_0 H^+-ATPase activity was unable to grow at a pH of <6. The H^+-ATPase is used to regulate the cytoplasmic pH of *E. faecalis* by proton extrusion, and this enzyme has been shown to be activated at low pH (83). Teng et al. (84) identified a two-component regulatory system, termed EtaRS, which appears to be involved in acid stress response and virulence. Inactivation of the response regulator, EtaR, results in increased acid sensitivity and decreased virulence in a murine peritonitis model.

Colonization resistance is also imposed by the stability of a mature complex community of gut microbes. The use of

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broad-spectrum antibiotics, many of which possess little or no antienterococcal activity, destabilizes this community by removing susceptible members (85–87), opening opportunities for invasion by new organisms. In a study designed to examine the persistence and density of colonization by vancomycin-resistant *E. faecium* in a murine model, Donskey et al. (88) showed that suppression of the anaerobic microflora by the use of antimicrobials that target this microbial population resulted in increased colonization density and prolonged VRE persistence. This observation highlights the importance of the anaerobic flora in suppressing enterococcal growth within the intestinal microenvironment. Several other studies have also examined factors that lead to overgrowth of enterococci, especially VRE, in the gastrointestinal tract of hospitalized patients. Treatment with broad-spectrum antibiotics, especially cephalosporins and metronidazole, to which enterococci are intrinsically resistant, leads to clearance of commensals and overgrowth of enterococci (80, 89). In extreme cases, hospitalized patients treated with these antibiotics develop a near monoculture of VRE in their intestine and are at high risk for enterococcal bacteremia and other enterococcal infections (80). Factors that limit the outgrowth of *E. faecium* in the antibiotic-perturbed gut include reduced expression of the host innate immune factor RegIIIγ, which results indirectly from the loss of Gram-negative members of the gut microflora (90). RegIIIγ production relies on signaling through Toll-like receptor-5 and interleukin-22 (IL-22), and supplementation of Toll-like receptor agonists has been shown to restore RegIIIγ expression and suppress *E. faecium* growth (90). In mice, the IL-22 signaling pathway and, in particular, the IL-22 receptor has been shown to be required for *E. faecalis* colonization resistance. *Il22ra1*$^{-/-}$ mice were observed to be particularly susceptible to *E. faecalis* overgrowth and subsequent systemic dissemination (91). This restriction of *E. faecalis* outgrowth is controlled in part by fucosylated glycans, which are downregulated in *Il22ra1*$^{-/-}$ mice. Oral supplementation of fucosylated glycans was shown to promote diversity of the gut microbiota and restore the growth restriction of *E. faecalis* (91).

In addition to antibiotic and host immune factors, hospital-adapted strains of enterococci are endowed with properties that allow them to colonize microniches that are likely less suitable for colonization by commensal strains. For example, some hospital-associated strains of *E. faecalis* contain an additional bile salt hydrolase (92). *E. faecalis* also produces extracellular superoxide (93), leading to DNA damage in colonic epithelial cells (94) and chromosomal instability (95), potentially relating to local inflammation, changes in the nutritional properties of the mucosa, and development of colorectal cancer. Enterococci also have been associated with inflammatory bowel disease, Crohn's disease and ulcerative colitis. Enterococcal abundance, particularly of *E. faecium*, is higher in fecal samples from Crohn's disease patients compared to healthy controls (96, 97). Increased *E. faecalis* abundance and anti-*E. faecalis* antibodies have also been observed in ulcerative colitis patients (98). Germ-free IL-10 knockout mice colonized with *E. faecalis* develop chronic inflammatory bowel disease-like symptoms (99), highlighting the bacteria's ability to perpetuate gut inflammation. A subset of virulence factors in *E. faecalis*, including the metalloprotease GelE, enterococcal polysaccharide antigen Epa, and cell surface-associated lipoprotein Lgt, were shown to influence induction of inflammatory bowel disease in these mice (Table 1) (99).

Enterococci must compete with other microbes in the gut for space, binding sites, and nutrients. Enterococci have long been known to be prolific producers of bacteriocins, many of which are encoded by mobile elements (100, 101). Plasmid pPD1, harbored by some *E. faecalis* strains, encodes a bacteriocin that inhibits other *E. faecalis* strains, eventually leading under some conditions to strain replacement *in vivo* (102). Importantly, the bacteriocin-expressing strain was able to reduce the intestinal load of VRE in a cocolonization study, highlighting the potential therapeutic value of such agents.

The apparently hasty accretion of mobile elements by enterococci affects their ability to compete in surprising ways. It was recently shown that commensal *E. faecalis* is able to induce self-killing of the vancomycin-resistant strain V583, through the production of the pheromone peptide cOB1 (103). This pheromone induces a cascade of events that involves expression of conjugation-associated functions on a pheromone-responsive plasmid resident within V583, which

TABLE 1 Enterococcal virulence factors

Virulence factor	Description	Reference(s)
Cytolysin[a]	Hemolysin/bacteriocin, lyses a broad range of eukaryotic and Gram-positive cells	128, 126, 129, 218
Gelatinase[a]	Secreted zinc metalloprotease	216, 221, 222
Serine protease[a]	Secreted serine protease	216, 221, 222
Hyaluronidase[b]	Degrades hyaluronic acid	236
Esp[a,b]	Cell wall anchored protein, enhances biofilm formation and colonization of bladder epithelium	92, 237
Aggregation substance[a,b]	Cell wall anchored protein, involved in conjugation and adhesion to eukaryotic cells	128, 132, 214, 267
Ace[a,b]	Cell wall anchored protein, collagen binding protein	135, 136, 138, 139
Epa[a,b]	Enterococcal polysaccharide antigen, anti-phagocytic cell wall polysaccharide	200
Cps[a,b]	Capsular polysaccharide, antiphagocytic cell wall polysaccharide	194, 196
LTA[a,b]	Lipoteichoic acid, enterococcal group antigen, binding substance for conjugation	214, 268
Toxic metabolites[a,b]	Reactive oxygen species: extracellular superoxide, hydrogen peroxide	94, 239

[a]Virulence factors identified in *E. faecalis*.
[b]Virulence factors identified in *E. faecium*.

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then engage in a lethal cross talk with a gene or genes in another mobile genetic element in the chromosome of V583, inducing death of V583 by an as yet unknown mechanism.

The ability to obtain nutrients within the competitive environment of the gut is an important aspect of enterococcal colonization efficiency (104). Within *E. faecium*, the two main clades, A and B, differ in the availability of carbohydrate utilization pathways. Strains in clade A1, which includes most infection-associated strains, express more transporters and metabolic pathways for the utilization of carbohydrates derived from the gut mucosa, as opposed to the diet, such as mucins and epithelial cell glycosidic surface decorations (104). Inactivation of a phosphotransferase system related to this ability reduced gut colonization in an antibiotic-treated mouse model (104, 105). Clade B strains do not possess this pathway (104), highlighting ecological differences between pathogenic and commensal strains. Meanwhile, clade B commensal *E. faecium* strains outcompete clade A strains in the murine intestinal tract in the absence of antibiotics (106).

Antagonisms within the gut community also influence colonization. Among commensals, *Barnesiella* species have been observed to be antagonistic to enterococcal colonization (107). Colonization resistance to VRE could also be artificially effected with a four-species cocktail containing *Bacteroides sartorii*, *Parabacteroides distasonis*, *Clostridium bolteae*, and *Blautia producta* (108). To colonize and establish residence within the gut, then, multidrug-resistant strains of enterococci must successfully navigate the hazards of innate host defenses and enter a destabilized consortium lacking overt antagonists.

TRANSLOCATION FROM THE GUT INTO THE BLOODSTREAM

Enterococal overgrowth in the colon increases the chance, by simple numeric probability, of dissemination into the bloodstream and contamination of other body sites in numbers large enough to establish infection (Fig. 2). Mechanisms that contribute to enterococcal translocation are still being discovered. In some cases, enterococci may be phagocytosed by intestinal epithelial cells, dendritic cells, or other tissue-resident leukocytes and transported across the intestinal wall to the underlying lymph system. Failure to kill the phagocytosed organism could then lead to abscess formation in reticuloendothelial organs and systemic spread (109). Gentry-Weeks et al. (110) showed that *E. faecalis* possesses the ability to survive for up to 72 hours in peritoneal macrophages. Wells et al. (111, 112) induced intestinal *E. faecalis* overgrowth by antibiotic treatment and observed organisms adhering to epithelial surfaces of the ileum, cecum, and colon. They also showed that enterococci possess the ability to translocate from the intestinal lumen into the mesenteric lymph nodes, liver, and spleen (111, 112).

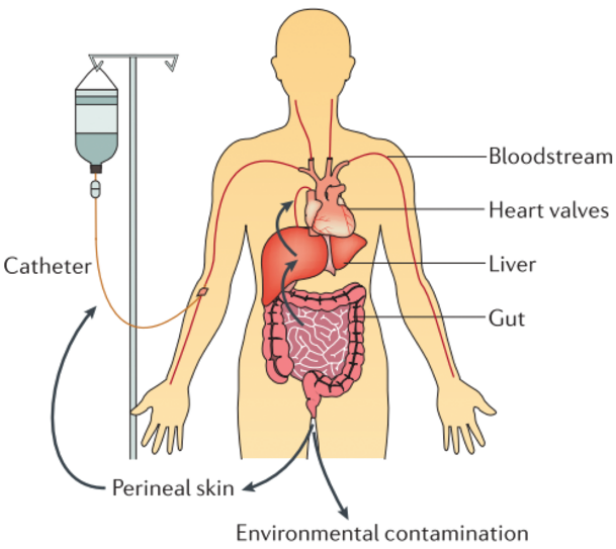


FIGURE 2 Routes of dissemination of enterococci from the intestinal reservoir of a hospitalized patient. Bacteria from the intestine can seed infections throughout the body and contaminate surfaces, leading to patient-to-patient spread. Reproduced with permission from reference 122.

Bacteria also diffuse through the gut barrier at low levels, and enterococci may gain access to the bloodstream that way. Inert particles the size of bacteria, and without any special properties, penetrate the gut mucosal barrier (113); special invasive properties do not appear to be essential for at least some level of translocation into the bloodstream. In a healthy, immune-competent host, innate defenses are well equipped to eliminate such invaders. So long as the microbes leaking into the bloodstream and occurring in the lymphatics are easily killed, such as the predominating anaerobes in the healthy gut community, there is little ill effect. However, because enterococci persist in phagocytic cells, their extraintestinal accumulation may more readily lead to infection, especially in an immunocompromised or disease-stressed patient.

A variety of enterococcal factors have been examined for their role in colonization and translocation out of the gastrointestinal tract. Olmsted et al. (114) found a plasmid-encoded surface protein, aggregation substance (AS), to promote internalization of *E. faecalis* by cultured intestinal epithelial cells (HT-29). To further dissect the functional domains of AS involved in internalization by HT-29 cells, Waters et al. (115, 116) examined the various domains of AS to determine their roles in this process. Surprisingly, the aggregation domain, and not the N-terminal RGD motif of AS, was found to be important for internalization by HT-29 cells (116). An additional finding from this study was the apparent requirement for a cofactor (likely lipoteichoic acid), because expression of AS in the binding substance mutant INY3000 failed to efficiently internalize. In a subsequent study it was shown that the amino-terminal aggregation domain of the protein could bind efficiently to purified lipoteichoic acid (LTA) as well as LTA purified from INY3000, suggesting that another cell component other than LTA was responsible for the cooperative internalization of *E. faecalis* by HT-29 cells (115).

BACTEREMIA

Enterococcal bloodstream infections (BSIs) are associated with a high level of mortality. In a recent large survey study of Canadian hospitals (117), the incidence of enterococcal BSI of hospitalized patients was 6.9 per 100,000, with most cases due to *E. faecalis* (4.5 per 100,000). The incidence of enterococcal BSI increased between 2008 and 2014 (9 to 14 per 100,000) in Switzerland (118), suggesting that enterococcal BSIs are on the rise. Overall, mortality from nosocomial enterococcal BSI is quite high, ranging from 25 to 50% (119, 120).

Most cases of enterococcal BSI are thought to result from translocation of enterococci from the gut into the bloodstream. Other routes of infection include along intravenous lines, endocarditis, urinary tract infections, and abscesses (109, 121, 122). The risk factors for mortality associated with enterococcal bacteremia include severity of illness (based on APACHE II scores), patient age, and use of third-generation

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cephalosporins or metronidazole (123). Huycke et al. (54) found that patients infected with hemolytic, gentamicin-resistant *E. faecalis* strains had a 5-fold increased risk for death within three weeks compared to patients with nonhemolytic, gentamicin-susceptible strains. Moreover, mode of treatment was not associated with outcome, discounting the direct contribution of aminoglycoside resistance. In another study, Caballero-Granado et al. (124) analyzed the clinical outcome, including mortality, for bacteremia caused by *Enterococcus* spp. with and without high-level gentamicin resistance. Mortality associated with high-level gentamicin resistance (29%) was not significantly different from gentamicin-susceptible strains (28%). Taken together, these two studies suggest that high-level aminoglycoside resistance itself does not explain clinical outcome and that the presence of other factors, such as the *E. faecalis* cytolysin (hemolysin), appears to contribute to enhanced lethality. In support of the latter finding, the *E. faecalis* cytolysin has been shown to result in acute toxicity in a number of animal models. Cytolysin significantly lowers the 50% lethal dose of the infecting strain for mice (125–127). As will be discussed later, the cytolysin also contributes to the acute toxicity of lupine endocarditis and endophthalmitis models (128, 129) and contributes to nematode killing in *Caenorhabditis elegans* (130).

URINARY TRACT INFECTION

In a nosocomial population surveyed between 2011 and 2014, enterococci were the most commonly isolated Gram-positive bacteria from catheter-associated urinary tract infections, with over 20,000 cases reported to the CDC National Healthcare Safety Network between 2011 and 2014 (4). Of these cases, just over 50% were caused by *E. faecalis*, followed by "other *Enterococcus* species" (~30%) and *E. faecium* (~20%). Most worrisome was the fact that ~85% of *E. faecium* isolates were vancomycin resistant, with the fraction of resistant isolates increasing each year (4).

A few studies have been aimed at understanding the interaction of enterococci with uroepithelial tissue (131, 132). Kreft et al. (132) showed a potential role for the plasmid-encoded AS in the adhesion of enterococci to renal epithelial cells. *E. faecalis* harboring the pheromone-responsive plasmid pAD1, or various isogenic derivatives, were better able to bind to the LLC-PK cultured pig renal tubular cell line than plasmid-free bacteria. Their findings also showed that a synthetic peptide containing the fibronectin motif, Arg-Gly-Asp-Ser, could inhibit binding. This structural motif mediates the interaction between fibronectin and eukaryotic surface receptors of the integrin family (133). Guzmàn and coworkers (131) analyzed the ability of strains of *E. faecalis* isolated from either urinary tract infections or endocarditis to adhere to urinary tract epithelial cells and to the Girardi heart cell line. Urinary tract infection isolates adhered to urinary tract epithelial cells *in vitro*, whereas strains from endocarditis adhered efficiently to the Girardi heart cell line, suggesting that environmental adaptations can facilitate interactions with host tissues. Shankar et al. (134) showed that the *E. faecalis* Esp surface protein colocalizes in the bladder, but not the kidneys, in an ascending urinary tract infection model, suggesting that the protein has tissue specificity for bladder epithelium. Tomita and Ike (135) showed that highly adherent enterococcal urinary tract infection strains recognize the extracellular matrix proteins fibronectin, laminin, and collagen types I, II, IV, and V. One of these strains, AS14, was subjected to transposon mutagenesis with Tn*916*, and mutants with altered collagen IV and laminin binding were identified. Out of 14 single transposon insertion mutants, 13 mapped to the Ace protein (136–140). The level of adherence of mutants to collagen IV and laminin was 1 to 2 orders of magnitude lower than the wild-type strain, but the level of adherence to fibronectin remained the same as that of the wild-type strain. The collagen binding proteins Ace (*E. faecalis*) and Acm (*E. faecium*) were originally identified because of their homology to the amino terminal end of Cna, the collagen binding protein from *S. aureus* (138, 139). The ability of most *E. faecalis* Ace-positive strains to adhere to collagen and laminin was highly dependent on growth temperature, with binding occurring at 46°C, but not at 37°C (136). No plausible explanation existed for this unusual binding phenotype until observations by Tomita and Ike (135) showed that gelatinase-positive *E. faecalis* strains adhered poorly to collagen and laminin due to the ability of the protease to cleave these proteins. Shiono and Ike (141) showed that some *E. faecalis* clinical isolates adhere efficiently to human bladder carcinoma cells, as well as to human bladder epithelial cells, and that these interactions could be inhibited by pretreating the bacteria with fibronectin or trypsin.

More recently, several studies have identified a pilus in *E. faecalis* (140, 142–145) and have shown that Ebp pili are important for adhesion to the urinary epithelium and urinary catheters through the binding of fibrinogen released into the bladder during catheterization (146–148). In a mouse model of catheter-associated urinary tract infection, *E. faecalis* infection could be blocked by the administration of antibodies raised against the tip subunit of the Ebp pili, EbpA (149). It is hoped that these antibodies could potentially be used to prevent infection in catheterized patients without the use of antibiotics.

ENDOCARDITIS

Of the infections caused by enterococci, infective endocarditis (IE) is one of the most therapeutically challenging (8, 150). Enterococci account for between 10 and 20% of all cases of IE (151, 152), with *E. faecalis* causing a majority of these. In IE, bacterial colonization of the endocardium leads to vegetations within the tissue and a biofilm-like matrix surrounding the bacteria, which is difficult to penetrate either by the immune system or by antibiotics (153–155).

Several virulence factors have been identified as playing a major role in the pathogenesis of enterococcal IE, with adhesion to host tissues by cell surface adhesins among the best characterized. In *E. faecalis*, AS represents a class of large, pheromone-inducible surface proteins involved in mediating cell-cell adhesion as the first step in conjugal plasmid transfer (156). These proteins also appear to be key players in both attachment to heart tissue and the development of biofilm during IE. Three highly related AS proteins have been well studied in this regard, including Asa1, Asc10, and Asp (155, 157, 158). All three proteins appear to aid in cell-cell adhesion and contribute to the exopolymeric matrix that accumulates within the biofilm.

The EfaA antigen is another adhesin that was originally identified as being expressed in serum but not broth culture (159), and database homology searches revealed extensive sequence similarity with several streptococcal adhesins. Shepard and Gilmore (160) showed that of all the suspected *E. faecalis* virulence factors, EfaA resulted in the most dramatic increase in expression when serum-grown and broth-grown *E. faecalis* were compared. It is now known that EfaA forms part of a putative ABC-type transporter specific for manganese, along with EfaB and EfaC. Because manganese is tightly sequestered within the mammalian host, it is not unexpected that *E. faecalis* grown in serum would require the

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induction of a high-affinity manganese transporter, EfaABC. The induction of the *efaCBA* operon occurs at relatively low manganese levels, because under high manganese levels the product of the *efaR* gene, a manganese-responsive transcriptional regulator, represses transcription from the *efaCBA* operon (159). More recently, EfaR inactivation has also been shown to impair the ability of *E. faecalis* to form biofilms, survive inside macrophages, and tolerate oxidative stress (161).

Pili encoded by the Ebp gene cluster in *E. faecalis* have also been shown to be important for both adherence and biofilm formation in IE (162). High titers of antibodies against the EbpABC proteins in patients with enterococcal IE have been demonstrated, indicating that they are expressed at high levels within the host during infection (162). Other important virulence factors in *E. faecalis* IE include the metalloprotease GelE (163), collagen-binding adhesin Ace (164), fibronectin-binding protein EfbA (165), cytolysin (128), and phosphotransferase system permease BepA (Table 1) (166). A review of the many virulence factors that play a role in *E. faecalis* endocarditis was recently published (167).

In *E. faecium*, less is known regarding specific virulence factors and the roles they play in IE. However, the enterococcal surface protein Esp and the collagen-binding protein Acm have been shown to be important for the pathogenesis of *E. faecium* IE in a rat model of infection (168, 169). In infected animals, *E. faecium* strains that lack either Esp or Acm were recovered at lower numbers than their wild-type counterparts. Antibodies against both proteins have also been detected in patients diagnosed with *E. faecium* IE, and pretreatment of bacteria with anti-Acm antibodies derived from an infected patient was shown to reduce the ability of the bacteria to bind to collagen (162, 168). It seems that targeting the adhesive and biofilm development properties of enterococci, in addition to treatment with antibiotics, may be a promising method of treating enterococcal IE in the clinic.

SURGICAL SITE INFECTIONS

Enterococci are common causes of surgical site infection at all anatomical sites (170), including the eye (129, 171). The eye affords a unique opportunity to visualize the evolution of such an infection using readily available equipment. Small numbers of organisms injected into the vitreous of a rabbit (129) or mouse (172), can be directly studied using an ophthalmoscope or slit lamp, and declining organ function can be measured quantitatively by electroretinography (173). Enterococci rapidly proliferate at this site, with a 10-μl injection of enterococci suspended in phosphate-buffered saline remaining as a light-refracting bead. After a few hours, the small bead acquires a coat of fibrin. About 12 hours later, the microvessels in the retina begin to dilate and subsequently turn white as they become coated with adherent neutrophils. After about 24 hours, neutrophils can be seen streaming from vessels in the optic nerve head to the infection nidus. These measurement parameters allow various steps in the pathogenesis of infection to be dissected and the roles of various traits expressed by the bacterium to be defined. In this model, the enterococcal cytolysin makes a profound contribution to organ destruction, which is not salvaged by otherwise effective antimicrobial or anti-inflammatory therapy (173). In contrast, identical infections initiated with isogenic cytolysin knockout strains resolved completely with antimicrobial and anti-inflammatory therapy, with little loss of organ function. Using this model, GelE and SprE proteases, both controlled by the Fsr quorum-regulated system, were observed to make measurable contributions to virulence in the rabbit model of endophthalmitis (174).

ROOT CANAL FAILURE

Due in part to its resistance to many disinfectants and antiseptics, *E. faecalis* is an important cause of root canal failure (175, 176). This is particularly true in cases of chronic and persistent periradicular lesions leading to root canal failure, where *E. faecalis* was isolated in 24 to 77% of cases (176). Genetic analysis of *E. faecalis* strains isolated from infected root canals suggests that these strains, while not clonal, are highly related and may share additional traits that lead to efficient colonization of the oral cavity (177, 178). Calcium hydroxide, a common disinfectant used within the root canal, has been shown to be ineffective at killing *E. faecalis* on its own (179), as have other disinfectants such as EDTA and citric acid (180, 181). It appears that the bacteria can resist starvation within the root canal for long periods of time and are then able to be resuscitated by serum that enters the canal from the alveolar bone and periodontal ligament (59). Provided with an adequate food supply, *E. faecalis* may utilize proteases such as SprE and GelE, and the collagen binding protein Ace, to bind to dentin and form biofilms (182). Once the infection is established, the most efficient treatments include prolonged washing of the canal with sodium hypochlorite or chlorhexidine solution (176). Using chlorhexidine or sodium hypochlorite washes during the initial procedure are thought to be the best options for preventing *E. faecalis* infection of the root canal and preventing failure.

IMMUNE EVASION

For enterococci to maintain an infection, they must successfully evade both specific and nonspecific host defense mechanisms. Other Gram-positive pathogens possess attributes that allow them to survive in the host in spite of powerful nonspecific host defenses mediated primarily by professional phagocytes, i.e., neutrophils, monocytes, and macrophages. These factors include antiphagocytic polysaccharide capsules, antiphagocytic surface proteins, such as the group A streptococcal M protein, and various secreted toxins with direct phagocytic cell toxicity. The most important cell type for clearing bacteria during the early innate immune response to enterococcal infection is neutrophils, with complement and antibody-mediated killing also playing a role (183). Neutropenic mice are less able to clear *E. faecium* from their bloodstream compared to their wild-type counterparts and show higher inflammatory cytokine levels after intraperitoneal infection (184).

Numerous studies have characterized the host response to enterococcal infection (183, 185–187). *E. faecalis* has been found to be able to actively cleave the complement protein C3, mediated by the metalloprotease GelE, leading to reduced phagocytosis and higher rates of bacterial survival (188). In a case report, Bottone and colleagues (189) isolated three highly mucoid encapsulated strains of *E. faecalis* from patients with urinary tract infections. These mucoid isolates appeared to persist longer in the mouse following intraperitoneal injection compared to nonmucoid controls, but crude mortality was not changed, suggesting a role in preventing or delaying phagocytic clearance (190). Once phagocytosed, *E. faecalis* is able to resist intracellular killing in both polymorphonuclear leukocytes and macrophages (110). In polymorphonuclear leukocytes, opsonized bacteria are more readily killed than unopsonized cells, and AS appears to limit opsonization, leading to survival within phagosomes (191). Recent studies examining *E. faecalis* survival in macrophages showed that phagosomes containing *E. faecalis* did not acidify or fuse with autophagosomes, leading to greater bacterial survival (192). *E. faecalis* is also able to survive the oxidative

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burst within macrophages, which is in part mediated by the production of catalase (112, 193). Survival within macrophages may also allow for bacterial translocation across the intestinal epithelium, leading to dissemination of *E. faecalis* throughout the body (112).

Several studies have examined the diversity of cell wall polysaccharides produced by *E. faecalis* (194–197). Based on genetic and serologic data, *E. faecalis* capsular polysaccharides fall into four serogroups (197, 198). Antibodies to *E. faecalis* capsular polysaccharides do, however, cross-protect across the species boundary (196), because antibodies to *E. faecalis* capsular antigens are reactive with some *E. faecium* capsular antigens and mediate opsonophagocytosis. The genetic basis for *E. faecalis* capsular biosynthesis is encoded by the *cpsC-K* operon (194, 199). In addition to capsular antigens, Murray and coworkers identified another cell wall polysaccharide, called the enterococcal polysaccharide antigen, whose synthesis is encoded by the *epa* locus (200–202). This pathway appears to be widespread and conserved within *E. faecalis*, and it has been designated the *E. faecalis* group antigen (194), analogous to the streptococcal group antigens. Antibodies to the Epa polysaccharide have also been shown to promote opsonophagocytic clearance by neutrophils (200), but this prospect remains controversial (196). A recent study showed that inactivation of a predicted wall teichoic acid biosynthetic gene in *E. faecalis* caused differences in wall teichoic acid and Epa composition and led to increased susceptibility to complement-mediated killing by neutrophils (203). Finally, enterococcal cell surface-associated polysaccharides have been found to impact gastrointestinal tract colonization and infection in a variety of settings (99, 195, 204–207). The extent to which enterococcal surface-associated polysaccharides could be used as potential vaccines awaits further study.

TISSUE DAMAGE

Following adhesion to host cell surfaces and evasion of the host immune response, the last step in the pathogenesis of enterococcal infection is the production of pathologic changes in the host. Such changes can be induced by the host inflammatory cascade or by direct damage as a result of secreted toxins or proteases, as well as the production of toxic metabolic byproducts. Each of these mechanisms has been observed in studies of *E. faecalis* pathogenesis.

Indirect Tissue Damage

Enterococcal LTA, also known as the group D streptococcal antigen, has been implicated in a variety of biological processes (204, 208–211). Some properties ascribed to LTA include modulation of the host immune response and mediating the adherence of enterococci to host cells. Bhakdi et al. (212) found LTA from enterococci to be as inflammatory as lipopolysaccharide of Gram-negative bacteria, although more recent findings dispute this claim (213). Strains of *E. faecalis* defective in enterococcal binding substance (EBS) (which is at least partially derived from LTA) and also defective in the protein adhesin AS did not induce clinical signs of illness when injected intraventricularly into rabbits (214). While $EBS^{+}AS^{-}$ or $EBS^{-}AS^{+}$ strains caused signs of illness and pericardiac inflammation, all rabbits injected with the $EBS^{+}AS^{+}$ strain developed illness and died. Surprisingly little inflammation was observed in rabbits injected with the $EBS^{+}AS^{+}$ strain, despite the lethality observed. The authors state that such observations are consistent with the presence of a superantigen. The presence of LTA (EBS) and AS together may mediate effects on the host immune response that differ from those seen when either component acts alone.

Direct Tissue Damage

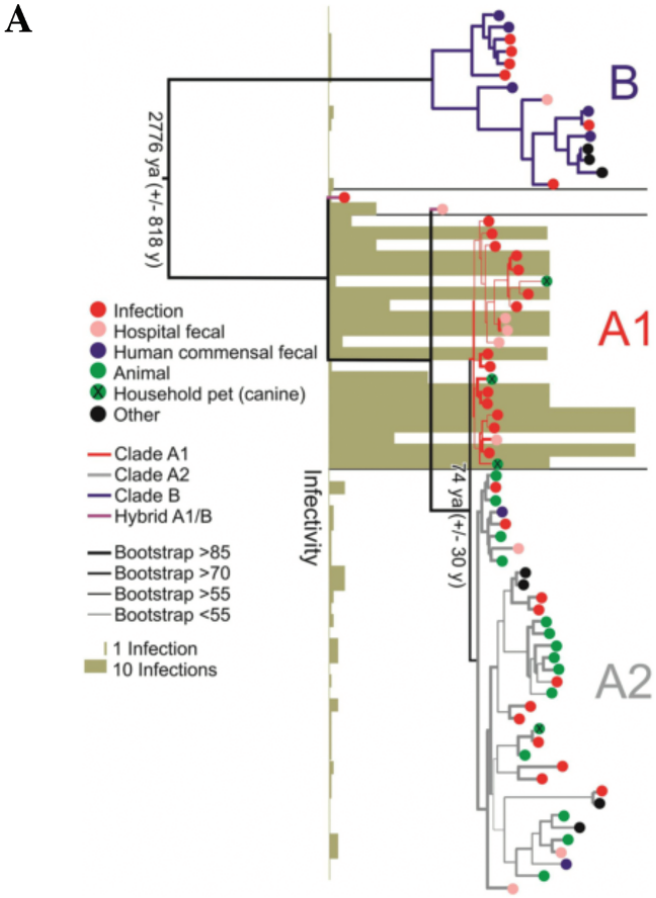
The enterococcal cytolysin and two proteases, a zinc metalloprotease (gelatinase) and a serine protease, are secreted factors well suited to contribute to disease severity (126, 128, 129, 215, 216). The enterococcal cytolysin is well established in several independent labs and animal models (126, 128, 129). The presence of the cytolysin has also been shown to promote the appearance of *E. faecalis* in the bloodstream (217). The genetics and biology of the enterococcal cytolysin have been reviewed elsewhere (218, 219). The toxin is genetically and structurally related to the lantibiotic family of bacteriocins but diverges from this family in that it possesses both bacteriocin activity and toxicity toward a variety of mammalian cell types. The cytolysin operon is typically encoded on large pheromone-responsive plasmids but has also been shown to reside within an *E. faecalis* pathogenicity island (134). The operon consists of six genes, designated $cylL_L$, $cylL_S$, *cylM*, *cylB*, *cylA*, and *cylI*, with each gene product playing an essential role in toxin synthesis, modification, secretion, activation, and immunity. Transcription of the operon is repressed by the products of a divergent operon encoding CylR1 and CylR2, and repression is alleviated by the accumulation of fully processed $CylL_S$, which serves as a quorum sensing molecule to prime the synthesis of more toxin components as cell density increases (220).

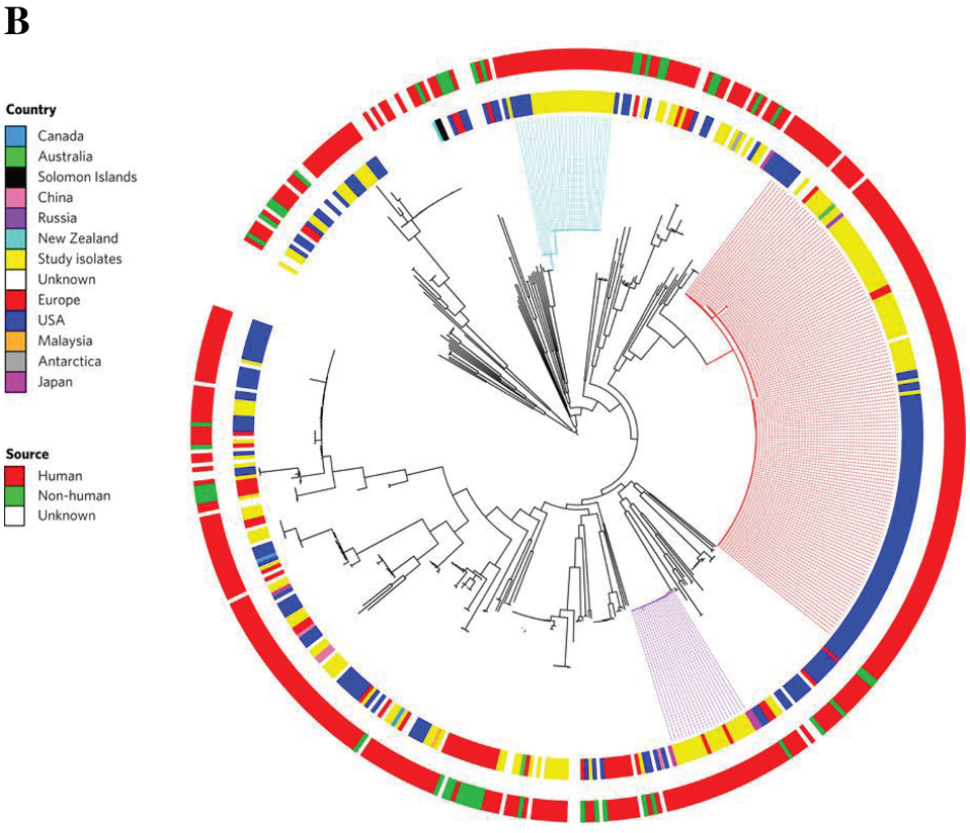
The most direct and quantitative evidence for pathologic damage attributable to the cytolysin was obtained using a rabbit model of endophthalmitis (129). This model was selected because a robust infection can be established with as few as 10 organisms due to the natural aberrations in the intraocular immune response. This limited response provides the offending bacterium an opportunity to adapt to *in vivo* growth conditions and environmental cues. Moreover, highly sensitive and quantitative measurements of the evolution of disease can be made. A role for the cytolysin in tissue pathology was unambiguously demonstrated by both a reduction in β-wave response and complete destruction of retinal architecture 24 hours postinfection (129).

Because of its broad protease specificity, the enterococcal gelatinase may also play a measurable role in systemic disease (127), as well as in a model of dental caries using germ-free rats (215). In the germ-free rat model, Gold et al. (215) showed that a proteolytic (Gel+) strain exhibited cariogenic activity, whereas three nonproteolytic strains exhibited little cariogenicity. Dupont et al. (127) showed a reduced 50% lethal dose for mice injected with gelatinase-producing (Gel+) strains, and these findings were subsequently confirmed using isogenic strains defective in protease production (216). On the same transcript as the *gelE* gene resides the *sprE* gene, which encodes a serine protease. Both proteases have been shown to play important roles in *C. elegans* nematode killing (221), lupine endophthalmitis (222), and murine peritonitis (216). As discussed previously, gelatinase has been shown to be important in *E. faecalis* biofilm formation (223, 224) and also appears to play a role in the degradation of the host extracellular matrix proteins collagen and laminin (135).

Both gelatinase and the SprE serine protease are regulated at the transcriptional level by a quorum sensing signal transduction system termed *fsr* (225). The *fsr* locus encodes a response regulator, FsrA, which is thought to be activated by

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phosphorylation at a conserved aspartyl residue by phosphotransfer from a histidine kinase, FsrC. The kinase is thought to undergo auto-phosphorylation upon sensing the accumulation of a peptide lactone quorum molecule (226), which is encoded at the carboxy-terminus of FsrB. Most if not all of the phenotypic effect of inactivating the *fsr* locus resides in the lack of protease production. While initially the only genes thought to be transcriptionally controlled by activated FsrA were *fsrB*/C and *gelE*/*sprE*, various studies have suggested that additional genes are controlled by the *fsr* locus (221, 222, 227). More recently, a third locus under the control of FsrA was identified (228); this locus encodes the antimicrobial peptide enterocin O16 (229).

There is some indication that clinical isolates of *E. faecalis* may be enriched for the proteolytic trait, because greater than 50% of isolates from both endocarditis and other clinical sources exhibited gelatinase activity, whereas only 27% of community fecal isolates possessed this trait (230). Studies that have explored the presence of *fsr* genes and *gelE* in epidemiology have found widely differing results (231–234). In addition to differences in sample size and setting, this could be because a large percentage of gelatinase-negative clinical isolates contain a 23.9-kb chromosomal deletion that results in the deletion of both *fsrA* and *fsrB* and yields a null protease phenotype (235). Because this represents a defined deletion, it is tempting to speculate why *E. faecalis* would delete these important virulence factors. Analogous to the description of the *E. faecalis* pathogenicity island, which is known to be modulated through the insertion and deletion of genetic information (92), the organism may simply choose to modulate its virulence for fear of overtly offending its host.

While *E. faecium* appears to lack both proteolytic and cytolytic activities, it has been shown to encode a hyaluronidase (236), which appears to be enriched in nonstool isolates. Hyaluronidase activity has yet to be demonstrated in these isolates, but it is conceivable that this trait is emerging within the *E. faecium* population. An *E. faecium* pathogenicity island has also been described, which contains an ortholog of the *E. faecalis esp* gene (237, 238). Apart from the *esp* gene and an *ara*C-like transcriptional regulator, none of the remaining open reading frames resemble those present in the *E. faecalis* pathogenicity island, and the nature of this element remains largely unknown.

TOXIC METABOLITES

In addition to secreted proteins, *E. faecalis* and *E. faecium* have also been shown to produce toxic oxygen metabolites that can cause cell or organ damage (94, 239). Using a worm model, Moy et al. (239) demonstrated that *E. faecium* produces hydrogen peroxide at levels that cause cellular damage. *E. faecium* transposon insertion mutants were identified which altered *C. elegans* killing activity and displayed altered levels of hydrogen peroxide production. Mutation of an NADH oxidase-encoding gene eliminated nearly all NADH oxidase activity and reduced hydrogen peroxide production and killing, whereas mutation of a gene encoding an NADH peroxidase resulted in enhanced levels of hydrogen peroxide and more rapid nematode killing. Depending on the culture conditions, *E. faecium* can produce hydrogen peroxide at varying levels, and this production appears to correlate inversely with nematode survival.

According to one study, the vast majority (87/91) of *E. faecalis* strains tested produce superoxide (O_2^-), whereas *E. faecium* isolates (5/13) did so less frequently (240). The study authors speculated that membrane-damaging effects of oxygen radicals might potentiate cellular damage to nearby intestinal epithelial cells. In proof, it was shown that the production of extracellular superoxide and hydrogen peroxide damages colonic epithelial cell DNA (94). The answer to why *E. faecalis* produces extracellular oxygen radicals stems from the fact that this oxygen radical is a byproduct of an incomplete respiratory chain. *E. faecalis* is capable of reconstituting a cytochrome complex in the presence of exogenous heme (241), enabling it to effectively respire. The fact that enterococci generate a byproduct that damages its host appears to be an unintended consequence of incomplete respiration.

COMPARATIVE GENOMICS OF *E. FAECALIS* AND *E. FAECIUM*: EMERGENCE OF PATHOGENIC, RESISTANT LINEAGES

With the development and widespread use of next-generation sequencing and comparative analysis of bacterial genomes, a great deal has been learned about the genetic makeup of pathogenic *E. faecalis* and *E. faecium* strains. The first enterococcal genome to be completely sequenced was a vancomycin-resistant *E. faecalis* strain isolated from an HIV-infected patient (35, 242). The genome of this isolate, termed V583, made apparent the importance of mobile genetic elements in the development of *E. faecalis* into a hospital-adapted pathogen (35, 242). When compared to a commensal-like *E. faecalis* strain, OG1RF (243), which harbors no known functional mobile elements, V583 was found to possess a genome more than 25% larger as the result of harboring three plasmids, a pathogenicity island (92, 243), and several integrated prophages (242).

First identified in the multiple drug-resistant strain MMH594 (92), the pathogenicity island has been shown to be widely distributed among hospital-adapted, pathogenic strains of *E. faecalis*. It encodes the virulence factors cytolysin and AS, ESP, and a variety of other elements believed to play a role in pathogenesis (92, 244). Overall, the trend of increased mobile genetic element content among multidrug-resistant, hospital-adapted strains holds true within *E. faecalis* and is strongly correlated with the absence of a functional clustered regularly interspaced short palindromic repeat-Cas system (245).

The population structures of *E. faecalis* and *E. faecium* have been widely studied, initially by multilocus sequence type and related techniques (246–249) and more recently by comparing sequences of entire genomes of many isolates of both species (104, 250, 251) (Fig. 3). These studies revealed a

FIGURE 3 Population structure of *E. faecium* and *E. faecalis* based on comparisons of whole genomes. (A) RAxML tree of 1,344 single copy core genes found in 73 *E. faecium* genomes, showing the clade structure of the *E. faecium* population. Reproduced with permission from reference 104. (B) Phylogeny of 515 *E. faecalis* isolates based on 1,293 conserved core genes found in 99% of isolates. The L1, L2, and L3 lineages are highlighted with red, purple, and turquoise lines, respectively. Reproduced with permission from reference 250.

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pronounced clade structure within the *E. faecium* species. The two main branches, clades A and B, are so divergent from one another that they approach the limits for designation as separate species (Fig. 3A) (104, 248). Most hospital-adapted *E. faecium* isolates belong to clade A and closely resemble strains isolated from agricultural and companion animals. Non-hospital associated human fecal isolates of *E. faecium*, on the other hand, mainly belong to clade B and appear to have diverged from clade A strains several thousand years ago (104, 248, 252–254).

In contrast to the pronounced clade structure of *E. faecium*, the population structure of *E. faecalis* is less discrete (Fig. 3B) (250, 251). A study of *E. faecalis* mainly derived from Great Britain revealed three dominant lineages, named L1, L2, and L3 (Fig. 3B) (250). However, these lineages are fairly closely related compared to the A and B clades of *E. faecium*. This finding supports previous work comparing a smaller set of *E. faecalis* genomes (253).

ANIMAL MODELS USED TO STUDY PATHOGENIC MECHANISMS

Several animal models have been developed to study different aspects of enterococcal pathogenesis. Using both invertebrate and vertebrate host organisms, many important discoveries have been made in identifying the roles of specific virulence factors in enterococcal disease (Table 1). Model organisms have also been employed in the discovery of novel antibacterial compounds. Here, we summarize the major advances in the establishment of relevant models of enterococcal disease; a more complete examination of models of enterococcal infection has been presented recently in two excellent reviews (255, 256).

Invertebrates

Invertebrate models of enterococcal infection have been very useful due to the ability to use large numbers of organisms in each experiment and because they are known to be natural hosts of enterococci in the environment. *C. elegans*, *Drosophila melanogaster*, and *Galleria mellonella* have all been used in different infection contexts, largely to examine the effects of virulence factors on lethality of the whole organism (130, 257, 258). It has been observed that there is good correlation between virulence factors in invertebrate hosts and in mammalian infection models (130, 221). Invertebrates, particularly *C. elegans*, have also proven to be very fruitful for high-throughput screening for novel antienterococcal compounds, because they allow for identification of bactericidal compounds with low host toxicity, as well as host immunomodulatory molecules that impact the course of infection (259).

Vertebrates

Several infection models have been established in small mammals for many of the most important enterococcal diseases. Two of the earliest infection models were developed for enterococcal IE, first in rabbits by MacCallum and Hastings in the first description of enterococcal disease (3) and later modified by Garrison and Freedman (260). Santoro and Levison developed a rat model of infection in the 1970s (261). Both the rabbit and rat IE models involve the insertion of a catheter into the heart valve followed by administration of bacteria. The introduction of a foreign body allows for initial adherence of enterococcal cells, resulting in a robust infection of the cardiac tissue. The determination of many important virulence factors for endocarditis have been shown using these animal models (128, 157, 255).

Intestinal colonization by *E. faecalis* and *E. faecium* has been studied in antibiotic-treated mice to model antibiotic treatment in hospitalized human patients. Generally, mice are treated with high concentrations of broad-spectrum antibiotics, particularly spectinomycin or metronidazole, to allow enterococcal invasion into the microbiota. Kommineni and colleagues obtained stable, long-term colonization of an introduced *E. faecalis* strain into an intact mouse microbiota through long-term administration of the bacteria in drinking water (102). Recently, studies examining the localization of *E. faecalis* within the intestinal tract have been performed in germ-free mice, allowing for direct visualization of bacteria in contact with intestinal tissue (262). How closely this localization resembles that of enterococci in a fully colonized animal has yet to be determined.

Mice have also been used with great success to study bacteremia and peritonitis caused by *E. faecalis* and *E. faecium* (126, 263). Urinary tract infection models, both catheter-associated and endogenous, have been established in female mice and were instrumental in identifying virulence factors, particularly adhesins, in enterococcal pathogenesis (134, 147, 264, 265). Finally, to study postoperative infections, Stevens and colleagues established a highly tractable rabbit endophthalmitis model in 1992 (266). This model involves the direct placement of small numbers of bacteria into the vitreous, directly behind the lens, where the evolution of infection can be easily observed by direct ophthalmoscopy as well as slit lamp biomicroscopy. This allows the influence of a number of virulence traits on the host-microbe dynamic to be assessed in real time (129).

From one of the first descriptions of enterococcal infection in 1899 (3) to the understanding of the role of virulence factors in disease and identification of novel antimicrobials, animal models have proven to be extremely important in the study of enterococcal pathogenesis, and their further development will continue to provide insight into diseases caused by enterococci.

CONCLUSIONS AND PERSPECTIVES

Enterococci are unusually well adapted for survival and persistence in a variety of adverse environments, including inanimate surfaces in the hospital environment and at sites of infection. This intrinsic ruggedness undoubtedly plays a role in providing opportunities for enterococci to interact with other overtly drug-resistant microbes and acquire additional resistances on mobile elements. The rapid rise of antimicrobial resistance among hospital-adapted enterococci has rendered hospital-acquired infections a leading therapeutic challenge. Having added about a quarter of a genome of additional DNA conveyed by mobile elements, there are undoubtedly many more properties that have been acquired that help enterococci persist and spread in the hospital setting and cause diseases that have yet to be defined. Much remains to be learned about these ancient and rugged microbes.

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Enterococcal Genetics

KEITH E. WEAVER

25

Interest in enterococcal genetics began with three landmark discoveries: (i) identification of the first conjugative plasmids whose transfer systems are induced by an identifiable signal (1), (ii) identification of the first "transposons" capable of intercellular (conjugative) transposition (2, 3), and (iii) the acquisition of vancomycin resistance (4, 5). Since the most prevalent antibiotic resistance genes are located on plasmids and transposons, early work on enterococcal genetics focused heavily on mobile genetic elements (MGEs). Examination of the complete sequence of a vancomycin-resistant clinical isolate of *Enterococcus faecalis*, V583, reaffirmed the importance of MGEs in the evolution of this species, revealing that over a quarter of the genome consists of mobile and/or exogenously acquired DNA (6). Further genome sequencing has revealed extensive variability in the proportion of horizontally acquired DNA in *E. faecalis* strains, with the presence of clustered regularly interspaced short palindromic repeats (CRISPR) with CRISPR-associated (Cas) protein (CRISPR-Cas) loci playing an important role in regulating MGE acquisition (7, 8). More recent genomic analysis has revealed that MGEs are important drivers of genome diversity and evolution in *Enterococcus faecium* as well (9).

While work on enterococcal MGEs continues apace, other aspects of enterococcal genetics are beginning to come into sharper focus. While the basic mechanisms of chromosome replication, partition, and cell division remain poorly defined, progress on other chain-forming ovococci provides a framework for further detailed analysis. Meanwhile, substantial progress has been made in understanding transcription and posttranscriptional regulation of gene expression, intercellular communication, and global response via second messengers, particularly of genes involved in virulence of the two major pathogens of the genus, *E. faecalis* and *E. faecium* (for a comprehensive review see 10). This article will focus primarily on the most recent advances in this field, with references to detailed reviews where available. Unless otherwise stated, identification of *E. faecalis* genes orthologous to genes in other bacteria utilized the V583 genome for searches (6), and gene annotations where given will use the V583 designation.

CHROMOSOMAL REPLICATION, PARTITION, AND CELL DIVISION

The fundamentals of chromosomal DNA replication in enterococci are probably similar to those in the model organism *Bacillus subtilis* (11). The *E. faecalis* genome includes homologs of all known components of the typical Gram-positive DNA polymerase III (Pol III) holoenzyme, including a replicative DNA helicase (which is named DnaB according to the *Escherichia coli* convention rather than DnaC as in *B. subtilis*), helicase loaders, primase, clamp and clamp loaders, and DnaE- and PolC-type catalytic alpha subunits. Most have also been identified in the *E. faecium* genome. It has been proposed that PolC may be the primary leading strand DNA polymerase, while DnaE plays dual roles as the lagging strand polymerase and as an error-prone polymerase during DNA repair in *B. subtilis* and *Streptococcus pyogenes* (12, 13). The *E. faecalis* PolC and DnaE proteins have been purified and found to have characteristics similar to the *B. subtilis* homologs, even cross-reacting with antibodies generated against *B. subtilis* PolC and DnaE (14), suggesting that *E. faecalis* and *B. subtilis* enzymes are structurally and functionally similar. The putative enterococcal *ori*C has a bipartite organization with DnaA binding sites flanking the *dnaA* gene as in *B. subtilis* and distinct from *E. coli*.

In addition to DNA Pol III, the *E. faecalis* V583 chromosome also encodes a DNA Pol I homolog, two putative phage-encoded DNA polymerases, and one member of the mutagenic Y-family polymerases (15) that have been shown to be involved in lesion bypass in other organisms. Y-family polymerases are commonly found on bacterial plasmids (16), and all three of the plasmids in *E. faecalis* V583 encode at least one member of the family. The previously described *uvrA* gene of *E. faecalis* plasmid pAD1 (17) also belongs to this group and was shown to impart increased resistance to UV exposure.

Other genes associated with DNA repair present in the *E. faecalis* genome include homologs required for transcription-coupled DNA repair (*mfd*), nucleotide excision repair (*uvrA*, *uvrB*, and *uvrC*), mismatch repair (*mutS* and *mutL* homologs annotated as *hexA* and *hexB*, respectively), and numerous other nucleotide repair proteins, including seven potential *mutT* homologs. It should be noted that the previously designated *uvrABC* genes located on plasmid pAD1 (17) are not homologs of the excision repair enzymes that usually bear this name but are instead associated with a lesion bypass polymerase. Multiple homologs of genes involved in recombination repair were also identified, including genes for a LexA-like regulator, RecA, a RexAB DNA processing enzyme, a RuvAB Holliday junction helicase, a PriA replication restart primase, a RecFOR complex, and a variety of other recombination proteins. A recombination-deficient mutant of *E. faecalis*, UV202, has a phenotype similar to *recA*

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mutants in other bacteria (18). Sequencing and complementation analyses confirmed that this phenotype is due to a single point mutation that results in a G265D change in the RecA protein (19). Other putative recombinational repair proteins have not been characterized.

The *E. faecalis* genome includes topoisomerases I to IV, a *Streptococcus pneumoniae* condensin homolog, and a homolog for the nucleoid associated protein HU, suggesting that the chromosomal DNA is organized and compacted as in other bacteria. No replication terminator protein is annotated in the genome, and no identifiable homolog of the *B. subtilis* Rtp terminator could be identified. In fact, Rtp appears to be limited to the *Bacillus* genus, suggesting that proteins involved in termination of replication are poorly conserved. A single XerD homolog, involved in chromosome dimer resolution in other bacteria, is annotated in the *E. faecalis* genome, but several other integrase/recombinases have been identified that could play the role of the XerCD resolution recombinase. The C-terminus of the cell division protein FtsK has been implicated in organizing both the XerCD dimer resolution complex and the topoisomerase IV decatenation complex near the chromosomal replication terminus for efficient separation of daughter chromosomes in *E. coli* (20, 21). The C-terminal domain of *E. faecalis* FtsK is 42% identical to the C-terminus of *E. coli* FtsK, but the N-terminal domains of the two proteins are not similar, suggesting that they may play similar roles at the end of chromosomal replication but different roles in cell division.

The division/cell wall cluster of *E. faecalis* was identified in strain A24836 (22) and appears to be identical in the V583 genome (note that *divIB* and *ftsQ* are orthologs). This cluster includes *ftsZ*, the gene for the bacterial tubulin homolog essential for contraction of the cell membrane during cell division, two other cell division genes, *ftsA* and *ftsQ*, and several genes involved in peptidoglycan synthesis. There are also multiple *ftsW*- and *ftsK*-like genes in the V583 genome that may be involved in cell division. Details of the mechanism of FtsZ ring closure have been examined in *S. pneumoniae* (23–25) and may be applicable to enterococci.

In the model rod-shaped organisms *E. coli* and *B. subtilis* the primary systems responsible for division site selection and coordination with chromosome segregation are the Min and nucleoid occlusion systems (26, 27). In the Min system, the MinCD division inhibitor functions with a topological specificity determinant (MinE in *E. coli* and DivIVA in *B. subtilis*) to prevent division site placement at the poles, while nucleoid occlusion prevents septum closure over the bulk chromosomal DNA. Gram-positive cocci lack recognizable genes for MinCD and known nucleoid occlusion systems, and evidence suggests that nucleoid occlusion does not function in these organisms (28, 29). Recent work in *S. pneumoniae* suggests that proper origin placement and chromosome compaction drive division site selection, and a protein unique to the *Enterococcaceae* and *Streptococcaceae* families, MapZ (30), is required for proper perpendicular orientation of the division plane (31). The mechanism of origin placement remains unclear but may involve as yet unidentified proteins, coupling of transcription to segregation (32), or entropic effects (31).

The *S. pneumoniae* system provides a useful model for understanding enterococcal partition and cell division. Enterococcal division is at least superficially similar to streptococcal cell division, and the enterococcal chromosome includes orthologs for many of the important players, including genes for partition, chromosome compaction, and MapZ. But there are also several potentially significant differences. First, the *S. pneumoniae parB* gene, whose product is required for proper partition and recruitment of the nucleoid organizing protein Smc (33), is located immediately adjacent to the origin region and is not genetically linked to a gene for the ParA protein, which is normally essential for ParB-mediated partition function. The closest *S. pneumoniae parB* homolog on the *E. faecalis* genome (EF3298) is not closely linked to the putative origin region and has a clear *parA* partner (EF3299). Gene order at the *E. faecalis* and *E. faecium* origins conform more closely to that found in *B. subtilis* than in *S. pneumoniae* (34). Further, the homology between *S. pneumoniae* and enterococcal MapZ is primarily in the C-terminal peptidoglycan binding domain and does not extend to the transmembrane and cytoplasmic domains potentially involved in midcell divisome placement. While these observations may simply represent variations on a general theme, further research is required to determine how similar coordination of chromosome partition and cell division are in the enterococci and streptococci.

REGULATION OF GENE EXPRESSION

Like many other aspects of enterococcal genetics, regulation of gene expression was initially investigated in the MGEs of *E. faecalis*. These include the two-component signal transduction system (TCSTS) regulation of transposon-borne vancomycin resistance genes, signal peptide-dependent and antisense RNA-mediated regulation of conjugation, and antisense RNA regulation of plasmid-encoded toxin-antitoxin (TA) genes. The mechanisms of regulation of these systems will be examined in detail in later sections of the article. This section will focus on more recent advances in the understanding of the regulation of enterococcal chromosomal genes.

The *E. faecalis* genome encodes genes for RNA polymerase α, β, β', ω, and δ subnits and four recognizable σ factors. The σ factor genes include a putative housekeeping σ factor (*rpoD* or *sigA*), two members of the extracytoplasmic family (ECF), and a σ^{54}-like sigma factor (*rpoN*). σ^{54} is unique among bacterial sigma factors in that σ^{54} RNA polymerase is unable to form open complexes and initiate transcription without the help of ATP-dependent activator proteins (35). Such "enhancer binding proteins" are highly conserved, and genes encoding four such proteins have been identified in the *E. faecalis* genome (36). All belong to the LevR subfamily of σ^{54} activators, which includes a conserved DEAH helicase motif (37). Immediately downstream of each of these genes is a putative σ^{54} promoter and operons encoding four distinct sugar phosphotransferase systems (PTSs) of the mannose family (36). Mutation of σ^{54}, enhancer binding protein MptR, or PTS component MptD resulted in decreased sensitivity to class IIa bacteriocins, implicating MptD as the bacteriocin receptor and σ^{54} and MptR as its regulators (36, 38). V583 σ^{54} mutants showed increased biofilm formation while decreasing autolysis, cell death, and extracellular DNA release (39). This phenotype was independent of the four known enhancer binding proteins. Because biofilm development is believed to be dependent on extracellular DNA, it was proposed that σ^{54} mutants adapt an alternative matrix for biofilm establishment. The *E. faecium* genome also encodes an *rpoN* and five enhancer binding proteins of the LevR family, but the functions of the genes are so far undefined.

ECF σ factors generally facilitate transcription of genes involved in bacterial stress responses. They are sequestered by membrane-localized anti-σ factors and released by stress-induced anti-σ factor degradation by regulated

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intramembrane proteolysis (40, 41). The SigV ECF of *E. faecalis* was shown to contribute to survival following heat, acid, and ethanol stress (42) and to play a key role in lysozyme resistance and virulence (43). The gene adjacent to *sigV*, *rsiV*, encodes the anti-σ factor of the system (42) and is targeted by the Eep membrane-embedded zinc metalloprotease and at least one other unidentified protease for degradation in response to lysozyme and perhaps other stresses (44). The function of the second *E. faecalis* ECF σ factor is unknown.

TCSTSs have emerged as ubiquitous bacterial signaling systems for regulating gene expression in response to environmental conditions (45). The canonical TCSTSs are composed of a sensor histidine kinase (HK) that modulates a response regulator (RR) via phosphotransfer. The RR is frequently a transcriptional regulator. Analysis of the *E. faecalis* genome revealed the presence of 17 HK/RR pairs and 1 orphan RR (46). The best-characterized enterococcal TCSTSs are the Van system, required for regulation of vancomycin resistance genes (see below), and the Fsr system. The Fsr system regulates production of two proteases, gelatinase and serine protease, that are associated with virulence and biofilm formation (47–51). Fsr is a quorum sensing system that responds to the accumulation of a peptide lactone, designated GBAP (52). GBAP is the product of a small open reading frame, *fsrD*, that is processed and secreted by FsrB (53). GBAP is sensed at high cell density by the FsrC HK, which then activates the FsrB RR by phosphorylation. Phosphorylated FsrB then activates transcription from the *gelE-sprE* target promoter and the *fsrBDC* promoter, completing the autoinduction loop. The *fsr* system is analogous to the well-studied *agr* system of *Staphylococcus aureus* (54) except for the absence of the global small RNA (sRNA) regulator RNA III; FsrA appears to be the terminal regulator of the Fsr system.

The CroRS TCSTS is essential for PBP5-mediated intrinsic cephalosporin resistance in both *E. faecalis* and *E. faecium* (55–57). The CroS HK is a sensor of cell wall homeostasis and is stimulated to phosphorylate the CroR RR in response to a number of cell wall-active antibiotics. The CroR-regulated effector genes responsible for cephalosporin resistance have not been identified, but neither PBP5 itself nor its substrates appear to be the primary target. In some *E. faecalis* strains, cross-regulation is observed with a second TCSTS CisRS (56). The situation is further complicated by the involvement of a transmembrane serine/threonine kinase, IreK, its cognate phosphatase, IreP, and their substrate, IreB, which also modulate intrinsic cephalosporin resistance by an unknown mechanism (58–61).

The GrvRS TCSTS system, also known as EtaRS, is orthologous to the important *S. pyogenes* virulence regulatory TCSTS CovRS (62). In *E. faecalis* it has been demonstrated to be involved in virulence as well as resistance to low pH and high temperature (63). GrvRS has also been implicated in the regulation of the important extracellular matrix adhesin Ace (64), catalase (65), and the heat shock proteins DnaK and GroEL (66). It seems likely that the GrvRS regulon will continue to grow with further investigation.

Disruption of RR 04, 08, and 18 (as defined in 46) each resulted in sensitivity to heat, and transcription of HK-RR 07, 13, and 14 was induced by heat, suggesting that these TCSTSs may also play a role in stress resistance (66).

In *E. faecium* the EntKR TCSTS regulates expression of the bacteriocin enterocin A (67). EntKR is a quorum sensing system that responds to a small peptide produced from the same locus, designated EntF. There appear to be no close homologs to the EntKR system in *E. faecalis*. The ChtRS TCSTS contributes to resistance to both bacitracin and the disinfectant chlorhexidine (68), and CroRS is associated with intrinsic cephalosporin resistance (57). Clear homologs of *E. faecalis* HK-RR 01, 03, 04, 05, 07, 09, and 10 (as defined in 46) are present in the *E. faecium* genome sequence. In addition, a system with linked homologs of the *S. aureus agrA*, *agrB*, and *agr*C genes is present in *E. faecium*, but this system is not closely related to the *E. faecalis fsr* system. The *E. faecium* genome contains 10 more annotated RRs and 9 more HKs that either have no strong homology to counterparts in the *E. faecalis* genome or are no more closely related to *E. faecalis* genes than to genes from any other *Firmicutes*. Therefore, *E. faecalis* and *E. faecium* have about the same number of TCSTSs but not necessarily the same complement of homologs.

The *E. faecalis cyl* cytolysin-producing operon is induced by a quorum sensing mechanism operating through a two-component system, but in this case the sensor and regulator show no homology to HK/RR of typical TCSTSs (69). Active cytolysin is composed of two posttranslationally modified peptides, $CylL_L$ and $CylL_S$, the latter acting as the autoinducer of the system. CylR2 is a DNA binding protein that represses expression of the *cyl* operon encoding the structural genes for the toxin, an enzyme for modification of the toxin peptides, and an ABC transporter for secretion. The structure of the CylR2-promoter interaction has been determined (70). The $CylL_S$ inducing signal is transmitted to CylR2 via the membrane receptor CylR1. The system is sensitive to both bacterial cell density and the presence of target cells. Target cells are detected by preferential binding of $CylL_L$ from the dual toxin complex, releasing $CylL_S$ to induce the operon (71).

The *E. faecalis* genome includes numerous other putative regulatory proteins in a variety of other classes, but few of these regulators have been characterized. The *E. faecalis* version of the catabolite control protein gene, *ccpA*, has been mutationally inactivated and shown to regulate a variety of glucose starvation proteins and was capable of complementing a *B. subtilis ccp*A mutant (72). CcpA/P-Ser-Hpr binding to catabolite responsive elements (cre) in *E. faecalis* has been shown to participate in (i) the repression of citrate utilization operons through transcriptional inhibition of both the structural genes and the gene for the GntR-type citrate-responsive positive regulator CitO (73), (ii) the partial repression of genes for malate utilization, including *maeKR* encoding a malate response TCSTS (74), and (iii) repression of the agmatine deiminase pathway in cooperation with the mannose PTS and in opposition to the AguR-positive regulator (75). CcpA has also been shown to be important for the growth and virulence of *E. faecium* (76).

Metal homeostasis is frequently important in bacterial pathogens, and regulatory responses to copper and manganese have been investigated in the enterococci. Copper uptake in *Enterococcus hirae* is encoded in the four-gene *cop* operon consisting of the *copA* and *copB* genes, which encode proteins for copper uptake and efflux, respectively, *copY*, which encodes a copper-responsive repressor, and *copZ*, which encodes a copper chaperone that delivers copper from CopA to CopY (77). At low copper levels, CopY in complex with Zn(II) binds to DNA sequences overlapping the promoter as a dimer and inhibits transcription. At high copper levels, CopZ delivers Cu(I) to CopY, displacing Zn(II) and causing dissociation from the promoter. CopZ complexed with Cu(I) is subject to proteolysis, preventing excessive accumulation of intracellular copper, which can damage the cell. The Efa adhesin, encoded by the *E. faecalis efaCBA*

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operon, is regulated in response to manganese by the EfaR protein, a member of the DtsR/MntR repressor family (78). The EfaR binding motif was later identified in the promoter regions of 30 genes. *efaR* mutation impaired biofilm formation, survival in macrophages, and oxidative stress tolerance, suggesting that EfaR is an important modulator of virulence (79).

Four regulators, OxyR (80), PerR (81), Spx (82), and HypR (83), have been implicated in the response of *E. faecalis* to oxidative stress. HypR is the best studied of these regulators, acting as a transcriptional activator of at least six genes encoding catalase, glutathione reductase, two subunits of alkyl hydroperoxide reductase, thiol peroxidase, and HypR itself through binding to a "HypR box" (84, 85). HypR mutants are deficient in macrophage survival and virulence.

Transcriptomic analyses have identified a number of regulatory networks and putative regulatory proteins involved in the virulence of *E. faecalis*. These have been recently reviewed (10). Information relevant to stress responses in *E. faecalis* may also be found in a recent review on the stress physiology of lactic acid bacteria (86).

Posttranscriptional regulation by various RNA-mediated mechanisms has recently increased in prominence in the regulation of gene expression in bacterial cells. As in most other bacteria, the first examples of RNA-mediated regulation in enterococci were identified in MGEs (see below). More recently, a number of examples of RNA-mediated regulation were identified on the enterococcal chromosome. The best studied of these is regulation of the *eut* regulon, which encodes genes essential for ethanolamine (EA) utilization (87, 88) (Fig. 1). Regulation of the *eut* regulon requires a response to two regulatory inputs: the presence of EA substrate and the availability of adenosyl cobalamine (AdoCbl), a cofactor in the first enzymatic step of EA catabolism. The presence of EA is sensed by the EutVW TCSTS. Binding of EA by the EutW HK leads to phosphotransfer to and dimerization of the EutV RR. Unlike most other RRs, EutV is an RNA binding protein in the ANTAR family of antiterminator proteins (89). EutV~P dimers bind to and stabilize a dual hairpin structure in nascent RNA that precludes formation of multiple intrinsic terminators in four *eut* polycistronic RNAs, allowing transcriptional read-through into downstream *eut* genes. AdoCbl is sensed via a unique riboswitch located upstream of the *eutG* structural gene, designated EutX. In the absence of AdoCbl, EutX adopts a structure that allows read-through to a EutV~P dimer binding site. The relative abundance of EutX allows sequestration of EutV~P dimers, preventing mRNA binding and antitermination (+EA) (Fig. 1). AdoCbl binding to nascent EutX causes a conformational change that allows formation of an intrinsic terminator that stops transcription before the EutV~P binding site (+AdoCbl) (Fig. 1), allowing EutV~P dimers to perform their antitermination function (+AdoCbl,+EA) (Fig. 1).

Several other examples of posttranscriptional regulation have been identified in the enterococcal core genome. The *pyr* operon of *E. faecalis* appears to be regulated by PyrR via an attenuation mechanism similar to that described for *B. subtilis* (90). Bioinformatic analysis suggests that genes involved in amino acid biosynthesis and transport are subjected to T-box regulation in *E. faecalis* and *E. faecium* (91). A transcription attenuation mechanism for regulation of the *tetM* gene of Tn*916* was proposed to be based on the observation of (i) a short leader peptide encoded between the promoter and the TetM-encoding open reading frame, (ii) the existence of multiple alternative RNA secondary structures surrounding the leader peptide coding sequence, and (iii) the apparent extension of a short transcript upon induction with tetracycline (92). RNase J2 has been implicated in the regulation of

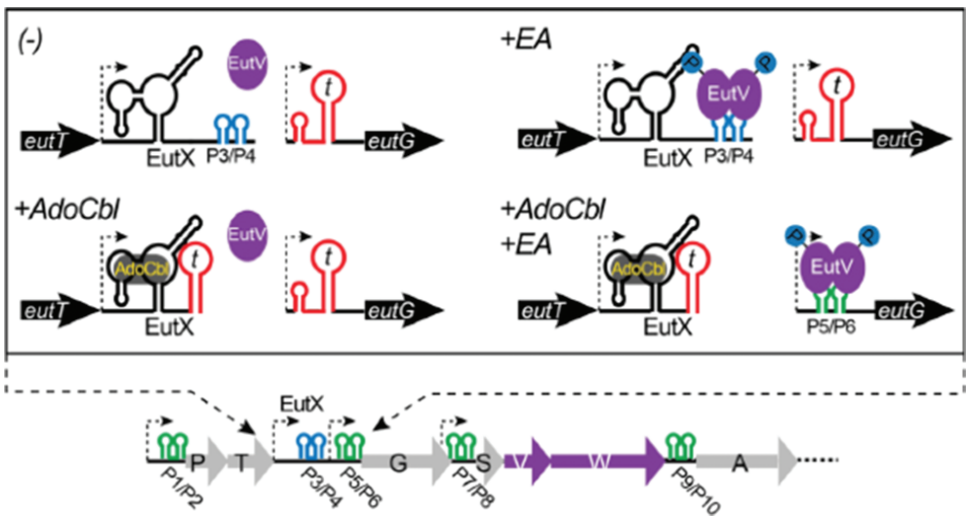


FIGURE 1 Model for EutV- and EutX-mediated regulation of the *eut* regulon. In the absence of either EA or AdoCbl (–EA), EutV~P dimers are not available to disrupt terminators upstream of *eut* genes. In the presence of EA but the absence of AdoCbl (+EA), the EutX riboswitch adopts a conformation that binds EutV~P dimers, titrating them away from the *eut* gene terminators. In the presence of AdoCbl but the absence of EA (+AdoCbl), EutX adopts an alternative conformation that is not competent for binding EutV~P dimers, but such dimers are absent due to the lack of EA. In the presence of both EA and AdoCbl (+AdoCbl, +EA), EutX is unable to bind EutV~P dimers, which then interfere with termination in *eut* transcripts, allowing the genes to be transcribed. (Adapted from reference 87.)

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the *ebp* pilus operon in *E. faecalis* independent of an effect on the level of the mRNA for EbpR, the transcriptional activator of the operon (93). Mutation of the *E. faecalis mjB* gene encoding RNase J2 was further shown to affect the expression of 62 genes and biofilm formation, bile salt resistance, and virulence in a model system (94).

Recently, a comprehensive map of all 5′ RNA ends in *E. faecalis* V583, discriminating processed from primary ends, was obtained by a combination of differential tagging and RNA-seq (95). This study identified 85 novel putative regulatory RNAs, which when added to those identified in previous studies (96, 97), brings the total number to >120. This includes putative *trans*-acting sRNAs and antisense RNAs as well as such functional RNAs as transfer-messenger and 4.5S RNA. The specific functions and targets of these putative sRNAs and antisense RNAs remains a mystery, although four have been implicated in modulating virulence and stress responses (98). *E. faecium* has also been subjected to experiments designed to detect putative regulatory RNAs with the identification of 61 sRNA candidates (99). Several of these RNAs were demonstrated to be induced or repressed during exposure to daptomycin or development of resistance to it. One putative sRNA, sRNA_0160, was demonstrated to be downregulated in the presence of daptomycin and repressed in a daptomycin-resistant mutant. Further work will be required to determine the target of this sRNA and its functional mechanism.

VANCOMYCIN RESISTANCE

Enterococci are intrinsically resistant to a variety of commonly used antibiotics and have acquired resistance to many others either by chromosomal mutation or acquisition of MGEs (100). As such, the enterococci provide a collection and distribution center for the dissemination of antibiotic resistance genes to other, potentially more pathogenic, Gram-positive bacteria. Dramatic evidence of this was provided by the characterization of the first high-level vancomycin-resistant *S. aureus* (VRSA) isolate (101, 102). Examination of the plasmid content of the VRSA isolate and coisolated vancomycin-resistant *E. faecalis* (VRE) and vancomycin-sensitive *S. aureus* (VSSA) strains suggested that a *vanA*-carrying transposon, Tn*1546*, was delivered from the VRE to the VSSA on a conjugative plasmid. The Tn*1546* then transposed to a plasmid resident in the VSSA, and the donating plasmid was subsequently lost. Further studies have implicated Inc18 plasmids, in particular, as the driving force for dissemination of vancomycin resistance to *S. aureus* (103). However, even though the Tn*1546*-bearing plasmids are conjugative in *S. aureus*, vancomycin resistance has not become widespread in *S. aureus* infections. A total of only 14 VRSA were isolated between 2002 and 2015 (104). Whether this is due to some fundamental incompatibility between vancomycin resistance and pathogenesis in this species or some bottleneck in *van*A transmission is unknown.

Vancomycin is a cell wall-active antibiotic that represents the last line of defense against many multiply resistant Gram-positive pathogens. Vancomycin and related glycopeptide antibiotics inhibit cell wall synthesis by binding to the terminal D-Ala-D-Ala dipeptide of peptidoglycan precursors, preventing their transfer from the lipid carrier to the cell wall by transglycosidases (105). Transpeptidases and D,D-carboxypeptidases involved in cell wall synthesis are also inhibited. VRE circumvent vancomycin action by substituting D-Ala-D-Lac or D-Ala-D-Ser for the D-Ala-D-Ala dipeptide, decreasing the affinity of peptidoglycan precursors for glycopeptides. Hydrolysis of pre-existing D-Ala-D-Ala dipeptides is also required for full resistance (106).

Unlike resistance to most other antibiotics, resistance to vancomycin involves the acquisition of a suite of enzymes required to reprogram peptidoglycan synthesis. Nine genotypically and phenotypically distinct systems designated VanA to VanE and VanG (reviewed in 107), VanL (108), VanM (109), and VanN (110) have been identified in VRE. Van gene clusters generally include three components: (i) a TCSTS, VanRS, required for sensing the presence of the antibiotic and inducing the resistance operon; (ii) an essential three-gene core encoding enzymes responsible for producing the D-Lac (VanH) or D-Ser (VanT) precursors, ligating those precursors to D-Ala (VanA to VanN), and eliminating D-Ala-D-Ala dipeptides (VanX, VanY, and/or a VanXY fusion); and (iii) accessory genes performing various and sometimes unknown roles. While the core and accessory genes are generally transcribed as an operon, the TCSTS is transcribed from a separate promoter (Fig. 2).

The VanC, VanE, VanG, VanL, and VanN systems all encode low-level vancomycin resistance by producing peptidoglycan peptides terminating in D-Ala-D-Ser. All encode a VanXY bifunctional dipeptidase/carboxypeptidase for degrading D-Ala-D-Ala, a VanT serine racemase for producing D-Ser, and their respective ligases. The VanC systems are responsible for the intrinsic resistance of motile enterococci, are chromosomally encoded, and do not appear to be on mobile elements. Although VanC systems appear to encode a TCSTS, resistance is either expressed constitutively or only sluggishly induced (111, 112). The VanE system is very similar to VanC in sequence and gene organization but is present in an *E. faecalis* strain (113). Although likely acquired from one of the intrinsically resistant enterococci, it is chromosomally located and could not be transferred to a vancomycin-susceptible strain. VanE is inducible in spite of a nonsense mutation in the gene for the VanS HK, suggesting some level of cross-talk with other cellular TCSTSs. The VanG system is also chromosomally located in an *E. faecalis* strain but resides on a large (~240 kb) mobile element that also encodes an *ermB* erythromycin resistance gene (114). The VanG system is inducible, probably due to the presence of a functional VanRS TCSTS. VanL and VanN were recently described, with VanN being the first D-Ala-D-Ser-producing system in *E. faecium* (108, 110).

The VanA, VanB, VanD, and VanM systems produce peptidoglycan precursors terminating in D-Ala-D-Lac. The VanA and VanB systems are widespread in both *E. faecalis* and *E. faecium*. In general, vancomycin resistance is more prevalent in *E. faecium* than in *E. faecalis* (115). Phenotypically, VanA-type resistance is characterized by high-level resistance to both vancomycin and teicoplanin, while VanB-type resistance provides variable levels of resistance to vancomycin only. Susceptibility to teicoplanin in the VanB system is due to a failure to induce the resistance genes in response to this antibiotic; VanB cells induced with vancomycin are resistant to both antibiotics. Genotypically, the VanA and VanB systems are quite similar (Fig. 2). Each encodes a VanRS TCSTS, a VanH pyruvate dehydrogenase for production of D-Lac, their respective ligase VanA or VanB, and separate VanX dipeptidase and VanY carboxypeptidase enzymes. Only the *vanH*, *vanA/B*, and *vanX* genes are essential for resistance. In addition, the VanA system encodes an additional gene, *vanZ*, which confers low-level resistance to teicoplanin by an unknown mechanism, and the VanB system encodes a gene, *vanW* (also present in VanG), of unknown function.

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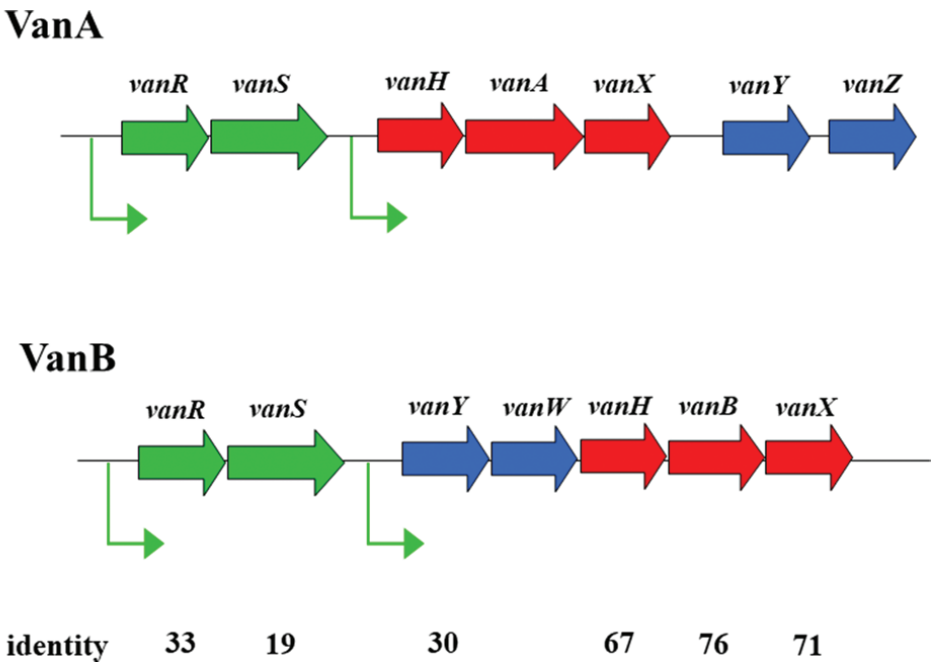


FIGURE 2 Organization of the VanA and VanB vancomycin resistance systems. Gene functions are color coded: green, sensory and regulatory; red, essential resistance genes; blue, auxiliary resistance genes. Detailed functions of each gene are described in the text. Green arrows show the position of the VanR inducible promoters. The percentage identity of the VanB genes to their VanA homologs is given below the individual VanB genes. Homologs have identical names in both systems except for the *vanA* and *vanB* ligases.

The VanRS TCSTS of the VanA and VanB systems have been extensively investigated, and reference 116 provides an excellent review. *In vitro* the VanRS system functions much like other TCSTSs. The HK, VanS, autophosphorylates its conserved histidine residue and then transfers the phosphate to the VanR RR (117). Phosphorylated VanR (VanR~P) produces a larger footprint and binds with higher affinity to its target promoters than unphosphorylated VanR, consistent with its *in vivo* role as a transcriptional activator (118). However, in the absence of VanS, dephosphorylation of VanR~P is unusually slow compared to related RR, and VanS stimulates dephosphorylation (117). In addition, VanR is capable of stimulating its own phosphorylation in the absence of VanS, using acetyl phosphate. These *in vitro* results are consistent with the observation *in vivo* that inactivation of VanS results in constitutive activation of vancomycin resistance rather than failure to induce expression. Apparently, VanR~P is the default condition *in vivo*, with phosphorylation occurring either by acetyl phosphate or by cross-talk with other cellular HK. The primary role of VanS, then, is to dephosphorylate VanR in the absence of the inducing signal (119, 120). The nature of the inducing signal is beginning to emerge. Given the differences in induction patterns and the poor conservation of VanS, the signal may be different for the VanA and VanB systems. Interestingly, single amino acid substitutions in the signaling domain of $VanS_B$ allow induction by teicoplanin (121). A recent report examining structural and activity effects on $VanS_A$ of a range of potential ligands including vancomycin, teicoplanin, and a variety of peptidoglycan precursers identified the likely inducers as the glycopeptides themselves (122).

Both the VanA and VanB gene clusters are frequently, if not always, associated with MGE. VanA is located on a Tn*3*-type transposon, Tn*1546*, and is frequently found on conjugative plasmids. In one *E. faecium* outbreak, a unique mechanism of facilitating horizontal transfer of VanA was observed (123). In this case, a nonconjugative Tn*1546*-bearing plasmid was observed to integrate into a pheromone-responsive plasmid via a mechanism involving a series of insertion sequences located outside of Tn*1546*. Conjugative transfer was enhanced by the fact that plasmid fusion occurred within a homolog of *prgX*, the central negative regulator of the pheromone response in pCF10-like plasmids (see below). The resulting insertional inactivation of *prgX* derepressed conjugation functions, and transfer occurred in the absence of pheromone. Tn*1546*-like elements have also been identified on plasmids related to the conjugative non-pheromone-responsive plasmid pMG1 (124) (see below). VanB has been localized on a variety of MGEs. In some strains, conjugative transfer of VanB has been associated with the movement of large (90 to 250 kb), chromosomally located elements that may be complex conjugative transposons (125). VanB was further localized on one of these elements to a 64-kb composite transposon, Tn*1547*, capable of transposition to plasmids under laboratory conditions. In other cases, VanB resistance was associated with conjugative plasmids (126), but the identification of similar genes on a variety of plasmids also suggested a transposon location. A VanB gene cluster was localized on a Tn*916*-like element designated Tn*5382* (127). Although independent mobility of this element was not demonstrated, it appears to be associated with a larger, 130- to 160-kb chromosomally located conjugative element. A similar element, designated Tn*1549*, was identified on a pAD1-like pheromone-responsive plasmid (128). Mobility of chromosomally located vancomycin resistance genes may also be accomplished by Hfr-like transfer

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mechanisms as described below and in references 129 and 130.

Unlike VanA and VanB, VanD systems provide constitutive vancomycin resistance due to various lesions in the *vanS* gene (131–133). In spite of the presence of an apparently intact *vanX* gene, VanD systems produce very little VanX D-Ala-D-Ala dipeptidase activity. Nevertheless, peptidoglycan precursors in VanD strains contain very little or no D-Ala-D-Ala terminal peptides due to mutations in the chromosomally encoded *ddl* gene responsible for producing the D-Ala-D-Ala precursors (131, 133, 134). Thus, a combination of constitutive expression of VanD and mutation of the chromosomal *ddl* ensure vancomycin resistance. VanD systems have been found only in *E. faecium* and do not appear to be mobile.

The complex nature of the vancomycin resistance systems argues against their acquisition by accumulation of mutations or piecemeal acquisition of individual genes. More than likely, they were acquired *en masse* from a single source. In this context it is interesting to note that the gene organization and sequence of the VanA and VanB systems are highly similar to self-protection systems present in glycopeptide-producing species (135, 136).

Resistance to Other Antibiotics

In addition to vancomycin, enterococci express intrinsic and/or acquired resistance to a wide variety of other antibiotics, including daptomycin, aminoglycosides, rifampicin, quinolones, macrolide/lincosamide/streptogramin B, β-lactams, and linezolid. For information on the genetics of these resistance mechanisms and the MGEs with which they are associated the reader is referred to a recent extensive review (100).

PHEROMONE-RESPONSIVE PLASMIDS

The enterococci host a multitude of plasmids, including both theta and rolling circle replicating replicons (RCRs) (137). The majority of these plasmids fall into six classes based on conserved domains in their putative initiator proteins: three RCR classes (Rep_trans, Rep_2, and Rep_1) and three theta classes (Rep_3, inc18, and RepA_N). These plasmids have been further subdivided into 10 families (of 19 present in the *Firmicutes*) based on initiator sequence homology (138). RCR and inc18 plasmids generally have a broad host range and are widely disseminated in Gram-positive and even some Gram-negative bacteria. They have been extensively studied in bacteria other than the enterococci and have been the subject of several excellent reviews (137, 139, 140). Rep_3 replicons are ubiquitous in bacteria and include some of the best-studied bacterial plasmids, including F, P1, and pSC101 (141, 142). The RepA_N plasmids are a class of replicons broadly distributed among the *Firmicutes* but whose individual members have a narrow host range (143). They include both conjugative and nonconjugative plasmids ranging in size from 3.3 to 281 kb. RepA_N proteins have also been detected on streptococcal bacteriophages and integrated conjugative elements (ICEs). Of particular relevance to enterococcal genetics are the RepA_N replicons that encode conjugation systems that are induced by small peptide pheromones secreted by potential recipients, the pheromone-responsive plasmids. These plasmids replicate primarily in *E. faecalis* and remain one of only two plasmid systems in which a specific signal for induction of conjugation has been identified (the quorum sensing system of *Agrobacterium* Ti plasmids is the other [144]). These plasmids will be discussed below, but the reader is referred to detailed reviews that discuss their replication, stable inheritance, and conjugation (137, 145–150).

Pheromone-responsive plasmids range in size from 37 to 92 kb and are maintained at a low copy number in their native host. In general, the pheromone-responsive conjugation system works as follows. Conjugation in cells containing pheromone-responsive plasmids is normally repressed in the absence of an appropriate recipient. When cocultivated with or exposed to culture filtrates of plasmid-free cells, plasmid-containing cells respond by producing a plasmid-encoded surface adhesin called aggregation substance (AS). AS binds to a ligand called enterococcal binding substance that is present on the surface of both donor and recipient cells, leading to the formation of macroscopic cellular aggregates that provide the contact required for plasmid transfer. Once transfer is complete, production of the pheromone specific for the transferred plasmid is shut down in recipient cells by the combined action of two plasmid-encoded components: a peptide that competitively inhibits pheromone activity and a membrane protein that reduces pheromone secretion. These inhibitor mechanisms are specific for the cognate pheromone of the particular plasmid acquired; production of pheromone(s) specific for other plasmids continues, allowing the recipient to induce a mating response in other potential donors and continue to collect plasmids. A cartoon showing the relevant players involved in the pheromone response, how they interact, and the organization of the plasmid-encoded regulatory genes is shown in Fig. 3.

Regulation of the Pheromone Signal Transduction Circuit

All sex pheromones and inhibitors characterized to date are linear, hydrophobic peptides of seven or eight amino acids. The genetic determinants responsible for the production of five pheromones were identified in the *E. faecalis* V583 genome and found to be encoded within signal peptide coding sequences of different lipoprotein genes (151). The involvement of these genes in the production of pheromones specific for pAD1 (cAD1), pCF10 (cCF10), and pAM373 (cAM373) was confirmed by cloning and mutagenesis experiments (152–154). Mutations of the mature lipoprotein-encoding portion of the genes for cAD1 and cAM373, *cad* and *camE*, respectively, had no effect on pheromone production or mating potential, suggesting that the lipoproteins themselves are not involved in conjugation. Pheromone sequences are generally found at the C-terminus of the lipoprotein signal peptide, indicating that signal peptidase II is required for the initial processing step. Maturation of the pheromone is then completed by the site 2 protease Eep in most cases (155). As described above, Eep belongs to a family of intramembrane processing proteins involved in regulated intramembrane proteolysis in organisms as diverse as bacteria and humans (156). The plasmid-encoded genes for the inhibitor peptides (iAD1, iCF10, iAM373, etc.) encode prepeptides resembling unattached lipoprotein signal peptides (157) and therefore require only Eep for processing. Some variability is observed in the processing of specific pheromones, suggesting further complications and potential alternative pathways. For example, Eep-processed cCF10 is further processed by removal of three C-terminal amino acids by an unknown mechanism (153, 158), and Eep is not required for cOB1, cAM373, or iAM373 processing (155, 158). Interestingly, both cAD1 and cAM373 activities can be detected in some *S. aureus* strains. In both cases the responsible peptides are produced from the signal sequences of lipoproteins unrelated to those that produce the same pep-

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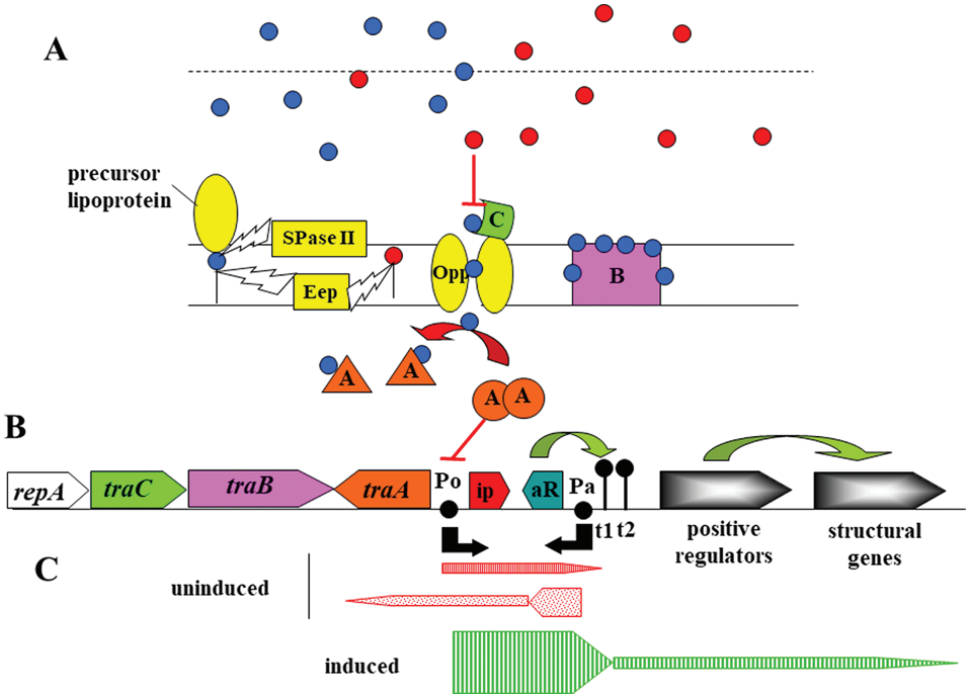


FIGURE 3 Regulatory circuitry of the pheromone response. (A) Events occurring at the cell surface. Important chromosomally encoded determinants are colored yellow with the exception of the pheromone itself, which is shown as blue circles. Plasmid-encoded determinants are color coordinated with their genes shown in panel B. The inhibitor peptide (ip) is shown as red circles and competitively inhibits pheromone binding to TraC. TraB inhibition of pheromone secretion is depicted here as sequestration, but may also involve peptide degradation. (B) Events occurring at the DNA level. Binding of pheromone by TraA links events at the cell surface with events at the DNA level. A conformational change in TraA due to pheromone binding is indicated by the change in shape of the molecule and the change in dimerization state, although more subtle conformational changes are likely. Pheromone-free TraA binds Po and inhibits transcription. Direction of transcription from Po and Pa is indicated by the arrows. The antisense RNA, generalized to aR in this figure, stimulates termination at t1, indicated by the green arrow. Positive regulatory elements vary between different pheromone-responsive plasmids, and their mechanisms of inducing downstream transcription of the conjugation structural genes may also vary. For simplicity, the more common gene names and order are used. It should be noted that the gene order of the *traC* and *traB* genes are reversed in pAD1. The RepA gene is shown to orient the reader relative to Fig. 4. (C) Events at the RNA level. Relative levels of RNA produced from the Po and Pa promoters under uninduced (red) and induced (green) conditions are depicted by the sizes of the arrows.

tides in *E. faecalis* (154, 159). Linear synthetic peptides of the appropriate sequence function as effective inducing signals for the cognate plasmids, indicating that no further processing is necessary. Hydrophobic septa- and octapeptide pheromones are pumped out of the membrane by a membrane ABC pump (160). Release of lipoprotein signal sequence fragments appears common among the *Firmicutes*, and perhaps pheromone-responsive plasmids evolved to take advantage of this process. It is interesting and perhaps surprising that so far, this efficient cell-cell communication system is mainly found in the species *E. faecalis* and is not more widespread.

Pheromones are imported into plasmid-containing cells by the chromosomally encoded oligopeptide permease (Opp) system in combination with a plasmid-encoded OppA homolog, TraC/PrgZ, that provides increased sensitivity and specificity for the cognate pheromone (161–164). The chromosomal Opp system transports sufficient pheromone to induce conjugation at high peptide concentrations, but TraC/PrgZ allows responses to pheromone levels of 10^{-10} to 10^{-11} M (161). Evidence indicates that inhibitor peptides compete with their cognate pheromones for TraC/PrgZ binding and that this at least partially accounts for their function (but see below) (102, 165).

Once inside the cell, pheromone is bound by a key negative regulatory protein, TraA/PrgX (161, 166). These proteins belong to the RRNPP (Rap, Rgg, NprG, PlcR, and PrgX) family of linear peptide pheromone-responsive regulators that are widespread in *Firmicutes* (167). Results with three TraA/PrgX proteins, those from pAD1, pPD1, and pCF10, show that the repressors bind both the stimulatory pheromone peptides and their cognate inhibitor peptides (166, 168, 169). In pPD1 and pCF10, both inhibitor- and pheromone-bound repressors bind the promoter required for expression of the conjugative apparatus (Po in Fig. 3) but with subtly different configurations that make the difference between preventing or allowing RNA polymerase binding (168, 170). In pPD1 this involves the binding of TraA to alternate sites within the promoter region (168). In pCF10 it involves subtle conformational changes in the peptide-bound PrgX tetramers that allow greater competition with

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RNA polymerase promoter binding in the inhibitor-bound than in the pheromone-bound state (170). Thus, competition between pheromone and inhibitor peptides occurs at two levels, import via TraC/PrgZ and interaction with repressor protein TraA/PrgX. The complex dual peptide regulation of TraA/PrgX systems is unique among RRNPP family members, giving rise to several interesting evolutionary questions that have been addressed elsewhere (148).

As shown in Fig. 3, even in the absence of pheromone, transcription from Po is not completely repressed. In fact, basal-level expression is required to maintain the uninduced state since the gene for the inhibitor peptide, ip in Fig. 3, is under Po control. In fact, results in pCF10 suggest that pheromone induction results in only a 5-fold or less increase in transcription from Po (147, 171). Thus, the pheromone response must rely on regulatory mechanisms beyond simple derepression of transcription from Po. Multiple lines of evidence suggest that this added layer of regulation occurs at the t1 terminator (Fig. 3), which separates the Po promoter from the rest of the conjugation genes. First, pPD1 TraA has been shown to bind to both t1 and t2 either alone or when iPD1-bound but not when cPD1-bound (168). It is proposed that this facilitates termination in the uninduced state, but precisely how this occurs is not known. Second, transcription from the Pa promoter results in the production of an antisense regulatory RNA (aR in Fig. 3) that binds to the nascent Po transcript, stabilizing a conformation including an intrinsic terminator. mD and Qa antisense RNAs have been identified and described in pAD1 and pCF10, respectively (172, 173), and their sequences and location are highly conserved in other pheromone plasmids. Details of this countertranscript-driven attenuation mechanism have been described for the pCF10 system (174, 175). In pCF10, PrgX and Qa are transcribed from Pa, and PrgX is required both for positive autoregulation of Pa and proper processing of Qa (171). In both the pAD1 and pCF10 systems, pheromone induction results in a significant decrease in antisense RNA production (176–178). Based on these observations, it was proposed that, under noninducing conditions, the level of antisense RNA is precisely poised to ensure that all Po transcripts adopt a termination-competent structure. Even a moderate increase in transcription from Po tips the balance in favor of antitermination by three amplification mechanisms: (i) titration of the Pa transcript by the excess Po transcript, (ii) transcriptional interference favoring the Po transcript and further reducing Pa transcript levels (179), and (iii) RNase III-mediated degradation of the complex between the Po and Pa transcripts, which reduces translation of TraA/PrgX (180). These overlapping regulatory mechanisms allow the cell to precisely set the pheromone:inhibitor ratio high enough to prevent fruitless induction of the costly conjugation apparatus but low enough to allow efficient transfer of the plasmid DNA when sufficient numbers of recipients are present. Tight regulation at t1 is most likely required to prevent transcription of strong downstream positive regulators. In the case of pAD1, this regulator is TraE1, which positively autoregulates its own transcription and is essential for expression of the downstream structural genes required for conjugation (177, 181). The pCF10 plasmid lacks a TraE1 homolog but produces several RNAs in the region immediately downstream of t1 and t2 that positively regulate downstream structural genes (182–184). The mechanisms of action of these regulators are unknown but may involve both stimulation of transcriptional read-through and translation.

The inhibitor peptide is essential for modulating the plasmid-containing cell's response to exogenous pheromone, but it is not sufficient to prevent self-induction by endogenously produced pheromone. A second plasmid-encoded protein, TraB/PrgY, is required to specifically reduce secretion of the cognate pheromone (185–187). The pCF10-encoded PrgY protein has been studied in detail (158, 188). It consists of four transmembrane domains with the N-terminal two-thirds of the protein located on the outer surface of the cytoplasmic membrane. The cCF10 pheromone binding region has been mapped to an approximately 120-amino acid region in the extracytoplasmic domain adjacent to the first transmembrane segment. PrgY has been proposed to function by specifically binding to cognate pheromone as it emerges from the membrane, either following or concomitant with cleavage by Eep. The protein then either degrades the peptide directly or chaperones it to a cell wall bound protease for degradation (147). Recent data favor direct degradation (G. Dunny, personal communication). TraB/PrgY proteins appear to be part of an orthologous family of proteins found in all domains of life but only spottily distributed (188). For instance, there are representatives in humans, plants, and Gram-negative bacteria but not in any other *Firmicutes* species outside of the enterococci. This phylogenetic distribution suggests that horizontal gene transfer has been critical for their distribution. No function has been assigned for any of the TraB/PrgY orthologs, but homology suggests that they are involved in some form of peptide signaling or processing. Interestingly, pAM373 appears to control extracellular pheromone levels without a clear TraB/PrgY homolog (189).

Altogether then, pheromone plasmids encode three proteins, TraC/PrgZ, TraA/PrgX, and TraB/PrgY, that bind their cognate pheromone yet are divergent enough to be able to selectively respond only to the pheromone specific for their encoding plasmid. Structural analyses of these proteins suggest that the pheromone binding pockets did not diverge from a single ancestral domain but rather evolved independently and convergently (148). Assuming that all pheromone plasmids descended from a single ancestor, divergence to respond to new pheromone and inhibitor peptides requires coordinated evolution of the binding pockets of all three proteins. How these specificity switches occur is an intriguing evolutionary question.

The Conjugation Apparatus

Downstream of the positive regulatory elements of pAD1, transcription of a contiguous region of ~20 kb is inducible by pheromone (190). In most pheromone plasmids, the first two genes of this region encode a surface exclusion protein and AS (191). Both proteins encode signal sequences and LPXTG cell wall-anchoring motifs and have been demonstrated to be present on the surface of only pheromone-induced plasmid-bearing cells (192). The surface exclusion proteins limit conjugation between strains harboring the same pheromone plasmid (193, 194). AS is essential for the cellular aggregation response to pheromone and efficient mating in broth but is not required for plasmid transfer on solid surfaces, suggesting that it is not an integral part of the mating pore (195). AS is a cell wall-anchored protein and is highly conserved in all pheromone plasmids except pAM373. pAM373 does not encode an entry exclusion function (196) and encodes an AS that is half the size of other AS proteins and shows only localized homology (189). AS facilitates intercellular aggregation by binding to an enterococcal binding substance which is present on the surface of both donor and recipient cells. Lipoteichoic acid

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(LTA) appears to be at least one component of enterococcal binding substance, since free LTA inhibits aggregation (197) and cells containing mutations affecting LTA are poor recipients (198). Mutagenesis and *in vitro* binding analysis of the AS from pCF10 has identified two distinct aggregation domains, one of which binds tightly to LTA *in vitro* (199, 200). However, pCF10 AS also binds LTA from *E. hirae* which does not aggregate with AS-expressing *E. faecalis* cells, suggesting that LTA binding may not be sufficient for aggregation. AS has also been implicated as a virulence factor in several pathogenicity models (201–205) and is induced by plasma by means of the pheromone-sensing machinery (206). A recent study demonstrated that the pCF10 surface exclusion protein, AS, and the product of the *prg*C gene immediately downstream all play roles in conjugation, biofilm formation, and virulence (207). Another gene located between the AS gene and *prg*C, designated *prgU*, encodes a small protein with an RNA binding fold that mitigates the toxicity associated with overproduction of AS and other related adhesins in multiple Gram-positive bacteria (208). Its mechanism of action remains to be determined.

Comparison of the sequences downstream of the AS gene of five completely sequenced pheromone-responsive plasmids, pAD1 (209), pAM373 (189), pCF10 (210), pTEF1, and pTEF2 (6), show a high degree of conservation in the presumed structural genes required for conjugation. Comparison of these sequences to those of conjugative systems of both Gram-negative and Gram-positive bacteria suggests that they are orthologous to the family of protein and DNA secretion machineries collectively referred to as type 4 secretion systems, or T4SSs (211). Functional studies of the products of these genes from pheromone-responsive plasmids are limited, but work on pCF10 has identified PcfC as the coupling protein responsible for bringing the nicked DNA substrate to the secretion apparatus (212, 213), PrgJ as a VirB4-like ATPase in the T4SS apparatus (214), and PrgK as a peptidoglycan hydrolase required to generate localized lesions in the cell wall for assembly of the T4SS (215).

Within the conserved region encoding the T4SS is a short segment of localized reduced homology. In pAD1, this region has been shown to encode a functional origin of transfer (*oriT2*) located between a relaxase gene, *traX*, and *traW*, the pAD1 coupling protein homolog (209). A similarly located *oriT* was identified in pAM373, and the two *oriT*s were found to be mobilized only by their cognate plasmids. Plasmid-specific single-strand nicking by the putative relaxase was demonstrated within a large, conserved inverted repeat in *oriT*. Specificity was imparted by nonconserved direct repeats, presumed to be the relaxase binding site, adjacent to the conserved inverted repeat (216). The relaxase of these plasmids is unique in that it lacks the "three-histidine motif" common to most other plasmid relaxases (217) and instead relies on a catalytic domain related to the PD-(D/E)XK family of restriction endonucleases for nicking activity. Thus, the pAD1 TraX relaxase is the prototype for a distinct family of relaxases designated MOB_C (218). pAD1 also contains a second *oriT* located within the *rep*A initiator protein gene that is similar to IncP-type *oriT*s (209), but this *oriT* is utilized with 1,000-fold lower efficiency than *oriT2* and its biological relevance is unclear. The pCF10 relaxase, PcfG, is unrelated to pAD1 TraX and instead is closely related to the pheromone-independent *Lactococcus lactis* plasmid pRS01 relaxase (210, 219, 220). A comparative study of the pCF10 and pRS01 systems identified related accessory proteins important for assembly of the relaxosome. In spite of clear sequence homology between the pCF10 and pRS01 relaxases and their target *oriT*s, each system effectively mobilized only its cognate target (221).

Replication and Stable Inheritance

On the opposite side of the pheromone-response regulatory module from the conjugation structural genes are the genes required for replication and stable inheritance (189, 222–224). The genes of the basic replicon include a replication initiator protein RepA/PrgW, a partition system RepBC/PrgPO, and an antisense RNA-regulated type I toxin-antitoxin (TA-1) module designated *par* (Fig. 4). The *repA*/*prgW* genes belong to the RepA_N family of replication initiator proteins, which along with their associated partition modules, have been recently reviewed (143). RepA_N initiator proteins are not restricted to pheromone plasmids and are widespread among conjugative and nonconjugative plasmids in the *Firmicutes*. The genes encoding RepA_N initiators encode a centrally located series of repeats whose organization and sequence vary between plasmids and, in some cases, have been demonstrated to function as an origin of replication (*oriV*) (225–228). Work on pAD1 has shown that (i) *oriV* is sufficient to support plasmid replication if its RepA_N initiator, RepA, is supplied *in trans*, (ii) the *rep*A gene itself is sufficient to support plasmid replication if RepA is overexpressed from an artificial promoter, and (iii) RepA binds specifically to *ori*V in double-stranded DNA and nonspecifically to single-stranded DNA (225). The pCF10 initiator protein, PrgW, binds cCF10 pheromone, and provision of cCF10 in a host not normally permissive for plasmid replication, *L. lactis*, allows plasmid establishment (229). The role of cCF10 in plasmid replication and whether this observation can be extended to other pheromone-responsive plasmids has not been determined. The mechanisms of strand opening, initiation of replication, and regulation of initiation frequency have yet to be determined for the pheromone-responsive plasmids. However, work with the RepA_N proteins of staphylococcal plasmids pSK41 and pTZ2162 may be instructive (230). Both domains flanking the central repeat region of these proteins adopt a structural fold similar to the DnaD primosome. However, the N-terminal domain has evolved to bind the *oriV* repeats bending the DNA and presumably facilitating separation of the DNA strands. The C-terminal domain retains primosome function and recruits DnaG primase to initiate replication. Furthermore, the N-terminal domain was also demonstrated to be capable of bridging two plasmid origins, suggesting that it could function in replication control by handcuffing (231). It had been previously proposed that the pattern of conservation of RepA_N proteins, with a highly conserved N-terminal domain and genus-specific conservation of the C-terminal domain, suggested a universally conserved function (e.g., origin binding) for the former and a genus-specific function (e.g., interaction with host replication proteins) for the latter (143). These results support that prediction. A second method of copy number control involving an antisense RNA that regulates expression of the initiator protein is superimposed on handcuffing in the staphylococcal plasmids (232). No analogous antisense RNA has yet been detected, either physically or bioinformatically, on the pheromone plasmids.

The pheromone-responsive plasmid partition systems are variants of the previously described type Ib class (233). Type I systems encode a well-conserved Walker-type ATPase designated ParA, a poorly conserved DNA binding protein designated ParB, and a centromere-like site to which ParB binds. In type Ib systems, the ParA component lacks a

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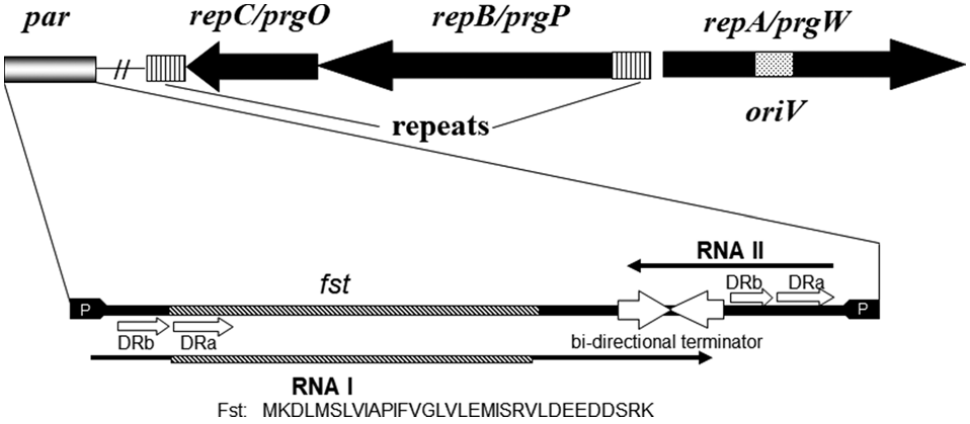


FIGURE 4 Genetic organization of a general pheromone-responsive plasmid replicon. Gene names are given as pAD1/pCF10. The stipled box within *repA/prgW* represents the origin of replication (*oriV*) to which the *repA/prgW* product binds. The lined boxes at each end of the *repB/prgP-repC/prgO* operon represent the likely centromere-like sites to which the *repC/prgO* products bind and, with the *repB/prgP* product, direct plasmid partition. The organization of the *par* region is blown up below the replicon. The arrowheads at each end of the locus marked P represent the promoters for the RNAI and RNAII transcripts. The extent and direction of transcription of the transcripts are shown above and below the genes for RNAII and RNAI, respectively. The *fst* gene is designated by the diagonally lined box, and the sequence of the peptide is shown at the bottom. DRa and DRb are direct repeats in the DNA sequence that provide complementarity between RNAI and RNAII when transcribed in opposite directions. Overlapping transcription at the bidirectional transcriptional terminator also provides complementarity.

DNA-binding domain, and the centromere-like site is located upstream of the *parA-parB* operon where binding of the ParB component can autoregulate transcription as well as direct partition. The RepB/PrgP and RepC/PrgO proteins correspond to type Ib ParA and ParB proteins, respectively. In all four pheromone-responsive plasmids examined, including pAD1, pCF10, pPD1, and pAM373, a series of repeats is present upstream of the *repB/prgP* gene (Fig. 4), with at least one repeat overlapping a putative promoter sequence, suggesting that protein binding to the repeats could suppress transcription. A series of similar repeats is also located downstream of the *repC/prgO* gene (143, 150). Each plasmid has repeats with a distinct DNA sequence and organization. For example, pAD1 contains 26 TAGTARRR (R = purine) repeats upstream of *repB* in two clusters of 13 and 12 tandem repeats with a single isolated repeat overlapping the promoter. Three more tandem repeats are located downstream of *repC*. pCF10 contains six tandem ATATATNNN (N = any nucleotide) repeats upstream of *prgP* with a single isolated repeat overlapping the promoter and two sets of four tandem repeats downstream of *prgO*. Work with the pAD1 RepBC system indicates that it functions similarly to other partition systems (234). Thus, *RepBC*, with the flanking repeats, is capable of stabilizing heterologous plasmids, the upstream repeats act as a strong incompatibility determinant, RepC binds specifically and with high affinity to the repeats, RepB binds the repeats only in the presence of RepC and ATP, and RepC downregulates its own expression. Plasmids bearing either the full complement of 25 upstream repeats or the half site containing 12 repeats but not the downstream 3 repeats can be stabilized by *repBC* supplied *in trans*. Spontaneous expansion of the pAD1 *repB* upstream repeats has also been associated with loss of regulation of the conjugative response and constitutive aggregation (235). The molecular basis of this phenomenon is unknown but may not be directly related to the normal function of the repeats. RepB proteins are broadly distributed in the *Firmicutes* but are not phylogenetically congruent with the associated replication initiator proteins, suggesting mosaicism of partition and replication modules (143).

TA modules are ubiquitous on bacterial plasmids and function as stability determinants by programming plasmid-free segregants for death (236). To accomplish this, TA modules encode a stable toxin and an unstable antitoxin; as long as the plasmid is retained, antitoxin is continuously replenished and the cell is protected, but if the plasmid is lost, the antitoxin is degraded and the toxin kills the cell. There are now five classes of TA modules (237), but only two are currently relevant to enterococci. In TA-1 the antitoxin is an unstable small regulatory RNA that binds to the toxin message and suppresses its translation. In TA-2 the antitoxin is an unstable protein that binds to the toxin protein and inhibits its activity. A general review of these systems is available (238), and enterococcal TA systems have been reviewed (137). Only one TA system has been characterized on pheromone-responsive plasmids, the TA-1 *par* locus (see 137, 239–241 for extensive reviews). *par* encodes two convergently transcribed RNAs, RNAI and RNAII, initiating from each end of *par* and terminating at a common bidirectional intrinsic terminator (242, 243) (Fig. 4). RNAI encodes the toxic component of the system, a 33-amino-acid peptide designated Fst. RNAII is a 65-nucleotide small regulatory RNA that is the *par* antitoxin. Although the RNAI and RNAII genes overlap only at the terminator, each reads in opposite directions across a pair of direct repeats (DRa and DRb in Fig. 4). Therefore, RNAII contains regions of complementarity with both the 3′ and 5′ ends of RNAI. Furthermore, the RNAI repeats are positioned such that interaction with RNAII sequesters the translation initiation region of *fst*, preventing ribosome binding and translation (244, 245). Formation of the RNAI-RNAII complex proceeds in at least two steps, with the interaction initiating at a

U-turn motif in the RNAI terminator loop which stimulates interaction with the complementary sequences in the RNAII terminator loop. Interaction then extends to the repeats at the 5′ end, suppressing translation (246) (Fig. 5). An intramolecular stem-loop within RNAI that partially sequesters the *fst* ribosome binding site is required to inhibit translation until RNAII binding can be completed (244, 247). A long distance interaction between sequences near the 3′ end of RNAI and its 5′ nucleotides is responsible for the stability of RNAI relative to RNAII (248). Unlike most antisense RNA systems, binding of RNAI by RNAII results in formation of a highly stable complex rather than specifically targeting the complex for degradation (248, 249). Formation of such a stable complex allows the toxin-encoding RNAI to persist in plasmid-containing cells. Over time, however, RNAII is slowly removed from the complex to allow translation of *fst*. The mechanism of this preferential degradation of RNAII from the complex is unknown.

The Fst toxin sequence is shown in Fig. 4. It consists of a central hydrophobic region flanked by charged N- and C-terminal tails. Overexpression of Fst has been shown to be toxic in *E. faecalis*, *S. aureus*, *B. subtilis*, and *E. coli* (247, 250–252). In all four species overexpression results in condensation and mislocalization of the nucleoid and interference with proper partition and cell division. Membrane permeabilization is only a secondary effect. Internal expression of Fst and external addition of the lantibiotic nisin synergistically effect cell killing, suggesting that the two peptides have different but complementary effects on the cell envelope (252). Saturation mutagenesis experiments revealed that the central hydrophobic region and charged residues in the N-terminus were important for overexpression-mediated toxicity but that the C-terminal charged tail was nonessential (251). An atomic resolution structure determined in a membrane mimetic by nuclear magnetic resonance spectroscopy revealed that the essential hydrophobic region forms an α-helix that spans the membrane with the charged N- and C-termini protruding (253). The charged C-terminal seven amino acids were disordered and predicted to extend from the cytoplasmic side of the membrane. The authors suggested that the primary function of membrane insertion was to facilitate interactions with a specific target, rather than being directed against the membrane itself. They also predicted that the disordered C-terminus might become structured upon recognition of the target. This would appear to contradict results indicating that the C-terminal amino acids are nonessential for toxicity, but it should be noted that the expression system used in reference 251 could not distinguish levels of toxicity. Therefore, it is possible that the toxicity is substantially decreased in the truncated mutants and could even be an artifact of overexpression. This possibility is under further investigation.

TA-1 type toxins in general are small proteins, less than 60 amino acids, that have hydrophobic regions typical of transmembrane domains (254). These toxins have been divided into eight superfamilies, and Fst is the founding member of the Fst/Ldr family (255). The Fst members of this family are widespread among the *Firmicutes* and appear to be

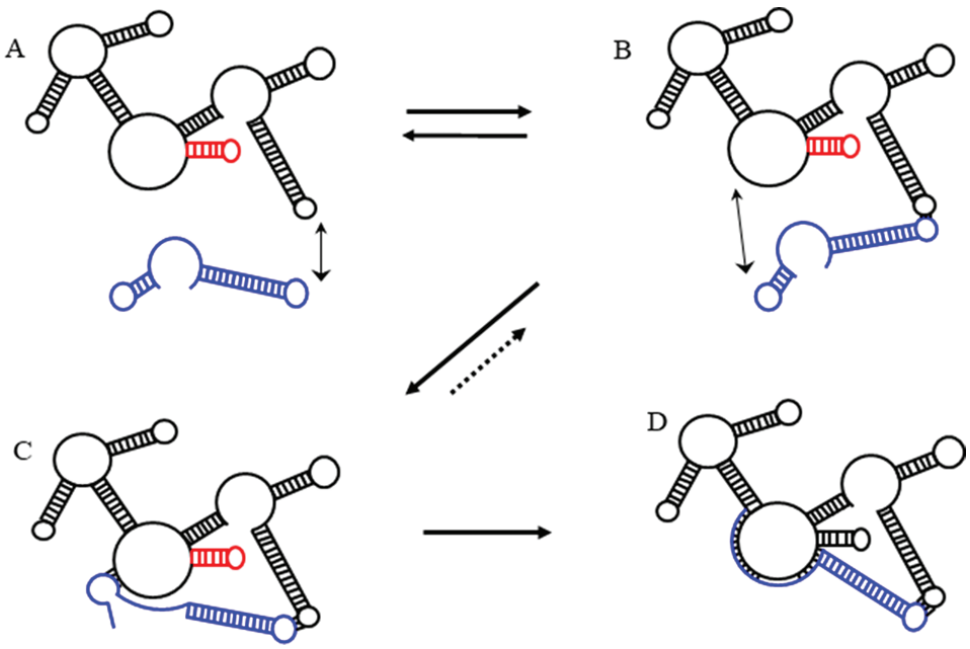


FIGURE 5 Model of RNAI-RNAII interaction. RNAI is the larger, black structure and RNAII is the smaller, blue structure. Stems, loops, and bulges are depicted in their approximate locations and sizes as determined by experimentation. The red stem-loop structure within RNAI sequesters the ribosome binding site and prevents translation until a complex is formed. The initial interaction occurs at a U-turn motif in the terminator loop of RNAI (A), followed by interaction between the complementary repeats in the 5′ end of each RNA (B and C). Since RNAII-mediated protection from the RNAI-encoded toxin occurs *in vivo* even when one of the repeats is mutated, the structure shown in panel C is apparently sufficient to prevent translation *in vivo*. Once complex formation is complete (D), the structure is extremely stable *in vivo* and *in vitro* perhaps due to the gap between the interacting repeats.

regulated and organized similarly to the pAD1 *par* determinant (251, 255, 256). The Ldr members are present in the γ-proteobacteria and are regulated in a manner more similar to *hok-sok* family members. In spite of their phylogenetic distance, Ldr overexpression has effects on nucleoid condensation and cell division similar to those Fst has in *E. coli* (247, 257). Like most other TA systems, the *par*-like Fst-encoding systems have both MGE and chromosomal members, and like other TA systems, the function of the chromosomal members is obscure. One of these chromosomal members, the toxin of which is annotated as EF0409, is conserved in *E. faecalis* genomes (251). For purposes of nomenclature, we have established a convention of using the locus or MGE name as a subscript for the relevant element. While the par_{EF0409} and par_{pAD1} systems show clear genetic similarity, the predicted RNAI-RNAII interaction sites are divergent, and it has been demonstrated that the antitoxins do not cross-protect (258). Par_{EF0409} is located between two paralogous mannitol-type phosphotransferase sugar transport systems, suggesting that it may be involved in regulating carbon flux. Transcriptomic analysis of cells overexpressing either Fst_{pAD1} or Fst_{EF0409} revealed that effects on the expression of a variety of transporters were predominant (258, 259), including effects on several genes for PTS components other than those to which par_{EF0409} is genetically linked. It was also observed that interference with the function of ABC transporters rescued cells from Fst_{pAD1} overexpression (259). Utilizing a novel expression vector, pCIE, that allows tight regulation and a large dynamic range of induction, the effects of low-level expression of Fst_{pAD1} and Fst_{EF0409} were compared. It was found that, while there was some overlap, overexpression of the two toxins had distinct transcriptomic effects (258). This suggests that the toxins have different targets or have distinct effects on the same target. Finally, overexpression of the Fst proteins is approximately 10-fold more toxic to mannitol-grown than to glucose-grown cells (K. Weaver, unpublished observation), strengthening the circumstantial link between *par* function and carbon flux. Somewhat surprisingly a mutant deleted for the par_{EF0409} antitoxin was isolated in *E. faecalis* strain EryS (98). The mutant strain showed increased virulence and improved survival in macrophages under several stress conditions. Proteomic analysis performed on this strain showed significant differences from the wild type, but these differences did not correlate with the transcriptome differences observed in strain OG1RF (258). These differences could be strain-related, due to differences between transcription and translation effects, or due to the accumulation of compensatory mutations in the deletion strain. Further investigation of the enterococcal *par* systems should elucidate the function of the chromosomal par_{EF0409} locus, determine the mechanism of action of the Fst toxins, and provide insight into the evolution of the *par*-like systems.

CONJUGATIVE TRANSPOSONS (ICES)

The enterococci carry the full range of transposon varieties found in other bacteria. Two Tn*3*-type transposons, Tn*917* encoding erythromycin resistance and Tn*1546* encoding vancomycin resistance, have been extensively studied (260, 261), and Tn*917* and its derivatives have been used for transposon mutagenesis in a number of Gram-positive organisms. The genome sequence of *E. faecalis* strain V583 revealed the presence of 38 insertion sequence elements, with IS*Ef1*, IS*256*, and IS*1216* being the most frequent (6). Both IS*256* and IS*1216* have been associated with simple composite transposons (123, 262) and large, complex, mosaic elements (263, 264). Many of these elements contain antibiotic resistance genes, and some are conjugative by means that have yet to be fully explained (see below). For a more detailed description of these elements see references 137 and 150.

The *E. faecalis* transposon Tn*916* was the first well-characterized self-conjugative transposon (2, 3) although large, conjugative chromosomal elements had been previously identified in other streptococci (265). Conjugative transposons were later found to be widespread in the lactic acid bacteria, and mechanistically similar but unrelated elements were identified in *Bacteroides* species (266). Characterization of new elements from both Gram-positive and Gram-negative organisms and the reinterpretation of data on older elements formerly classified as integrative plasmids have revealed that MGEs with characteristics similar to conjugative transposons (i.e., that excise from their chromosomal locus, circularize, transfer to a new host cell by conjugation, and insert in the recipient cell's chromosome without extensive replication) are probably as ubiquitous in bacteria as plasmids. Since the mobility of most of these elements is dependent on a tyrosine or serine recombinase rather than a DDE-type transposase and since many of them have relatively site-specific integration preferences, the appropriateness of the term "conjugative transposons" has been questioned and alternative names have been suggested, the most inclusive of which is ICE, for integrative and conjugative element (267). The full spectrum of ICEs has been described in detail in numerous reviews (268–271). This section will focus primarily on Tn*916* and related elements and other ICE-related issues specific to enterococci. A map of Tn*916* is shown in Fig. 6. For more detail on the Tn*916*-family of ICEs see references 272 and 273. For a comprehensive list of Tn*916*-like elements and other ICEs in enterococci see reference 137.

Unlike transposons using a DDE-type transposase, Tn*916*-like transposons are not flanked by direct repeats of target sequence. Preferred insertion sites tend to be AT-rich and possess intrinsic curvature, but the DNA sequence is not conserved and flanking sequences on each end are heterologous (274, 275). Unlike many other ICEs, Tn*916* has relatively little insertion site preference. Two transposon-encoded proteins, Int and Xis, are required for excision (276, 277). Int is a tyrosine recombinase (278) with N-terminal and C-terminal DNA binding domains (279). The N-terminal domain (Int-N) binds via a three-stranded β sheet to direct repeat sequences near each end of Tn*916* (280, 281). The C-terminal domain (Int-C) binds to the transposon termini as well as to target sequences. Int-C contains the conserved active site residues involved in nicking-closing. Like other tyrosine recombinases, Int produces staggered cuts at each end of the integrated transposon. One cut is made flush with the end of the transposon, producing a recessed 3′ end covalently jointed to Int via a phosphotyrosine linkage. The second cut is made 6 nucleotides into the host sequence, leaving a free 5′ OH that attacks the phosphotyrosine linkage at the other end of the transposon, resulting in circularization (282). Since the joint is derived from chromosomal DNA at each end that is not complementary, a 6-bp heteroduplex is formed in the circular intermediate, called the coupling sequence (283). The fact that Int must bind and cleave at the coupling sequences, which vary from insert to insert, may explain the wide range of conjugation frequencies (from $<10^{-9}$ to $>10^{-4}$ per donor cell) observed using different donor strains (284). How the coupling sequences affect conjugation frequency is not known, but it

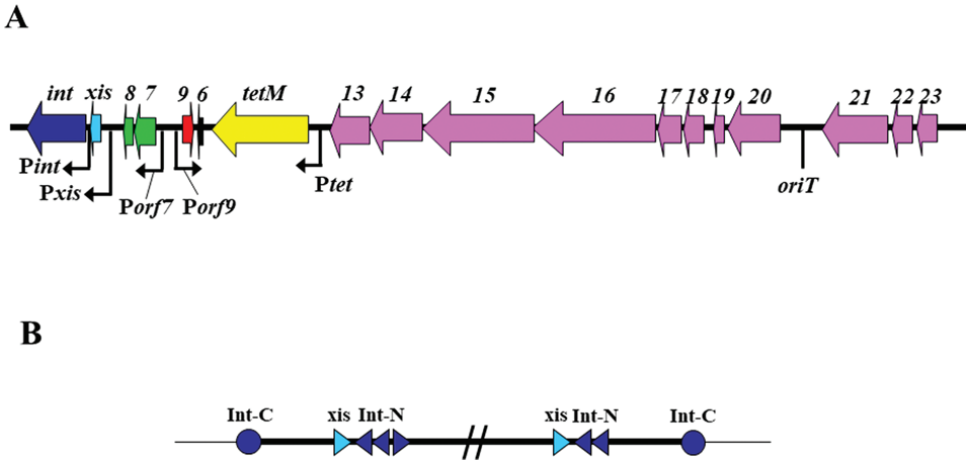


FIGURE 6 Genetic organization of Tn*916* and the relative positions of Int and Xis binding sites. (A) Functions of Tn*916* genes are color coded: blue, recombination; green and red, positive and negative regulation, respectively; yellow, tetracycline resistance; magenta, conjugation. Gene names and open reading frame numbers are shown above the individual genes. The origin of transfer is designated by the line between *orfs 20* and *21*, labelled *oriT*. Promoters and the direction of transcription are designated by labeled arrows below the gene line. (B) Binding sites for the Int-C and Int-N DNA binding domains and Xis are designated by marked circles and triangles. Int and Xis binding sites are color coordinated with the genes shown in panel A.

does not appear to be due to effects on integrase affinity (285) or on excision frequency (286).

Xis binds adjacent to the Int-N binding sites on both ends of the transposon (287) and stimulates Int-mediated excision of Tn*916* in *E. coli* (276, 288), *L. lactis* (289), *B. subtilis* (277), and *in vitro* (290). In *E. coli*, Xis binding to the left end is implicated in playing an architectural role similar to λ-Xis in excision of bacteriophage λ (291). HU protein plays a significant role in excision in *E. coli*, but no host factors affecting excision have been identified in Gram-positive hosts. Interestingly, Xis binding to the right end of Tn*916* inhibits excision, perhaps by competing with Int-N for binding, suggesting that Xis has dual roles in excision, both stimulating and inhibiting the process (292).

Once the circular intermediate is formed, the transposon is transferred to the recipient cell by conjugation. DNA strand transfer is initiated by nicking at *oriT* directed by the Orf20 relaxase and the Int protein (293, 294). While the Orf20 protein is responsible for strand nicking, Int functions as a specificity factor, conferring both strand and sequence specificity on its endonucleolytic activity (294). Although Tn*916* *oriT* bears similarities to IncP-type origins of transfer (293), Orf20 does not show similarity to any of the identified families of plasmid-encoded relaxase proteins (217). Instead, it appears to have similarity to the Rep_trans family of RCR initiator proteins and may represent a novel solution to the problem of conjugative nicking (217, 294). On the opposite side of the *oriT* from *orf20* is *orf21*, which encodes a product in the FtsK/SpoIIIE/coupling protein superfamily of DNA translocases. It is likely that Orf21 connects the *oriT*/Orf20 complex to the T4SS proteins encoded in *orfs14* to *16* (137). A combination of genetic analyses and DNA sequence determination have identified a number of genes involved in Tn*916* conjugation (295, 296), but the precise roles of none of these genes have been experimentally determined.

Tn*916* does not mobilize plasmids into which it has integrated, nor does it mobilize chromosomal genes adjacent to its integration site during intercellular transposition. This suggests that some mechanism suppresses utilization of the *oriT* site until after the transposon has excised from its host genome and circularized. Supporting this suggestion, induced expression of the transfer genes requires a functional *int* gene, and transcription is initiated from the *orf7* promoter, which since it is located at the opposite end of the transposon, is linked to the transfer genes only in the circular intermediate (297). Activation of the *orf7* promoter requires *orf7* and *orf8* and is repressed by *orf9*, but the mechanisms of regulation are unknown. Conjugation is also induced by the presence of tetracycline, which induces transcription of the *tetM* gene via ribosomal override of a transcriptional attenuator at its 5′ end (92). Transcription then proceeds through *tetM*, increasing transcription of the positive regulators *orf7* and *orf8* and blocking the *orf9* regulator presumably by antisense RNA interactions and/or transcriptional interference. This in turn increases transcription through *int* and *xis*, stimulating excision and circularization, leading to read through into the structural conjugation genes (272). Recent evidence suggests that subinhibitory concentrations of other ribosome-targeting antibiotics also stimulate Tn*916* conjugation and may contribute to its spread (298).

Once a copy of the transposon is transferred, integration into the recipient chromosome requires Int but not Xis (277). Since the known tyrosine recombinases function only on double-stranded DNA, replacement synthesis of the transferred strand presumably takes place prior to integration. Experiments with *int-* versions of Tn*916* where Int was provided *in trans* from a plasmid in the donor revealed that *int* gene expression was not required in the recipient for integration, suggesting that the Int protein might be transported with the transposon DNA (299). Recipients frequently acquire multiple copies of the transposon, and there is no evidence that Tn*916* expresses either an entry exclusion function or transposon immunity (145, 300). Interestingly, in cells harboring more than one copy of the

transposon, excision of one element transactivates excision and mobility of other elements located elsewhere in the genome (301).

Sequence analysis of known and putative ICEs in sequenced genomes suggests that these mobile elements are modular and that the modules may have different evolutionary histories (267, 302, 303). For example, Tn*5397* from *Clostridium difficile* is greater than 84% identical at the DNA sequence level to Tn*916* across all of the conjugation-related genes and the *tetM* gene, but Tn*916* Int is a tyrosine recombinase, while the recombinase protein of Tn*5397* is a serine recombinase. Conversely, the recombination module of the VanB transposon Tn*1549* is closely related to the Tn*916 int/xis* genes, but most of the conjugation genes are unrelated (128). Putative ICEs identified in the *E. faecalis* V583 genome sequence, EfaC2 and EfaD2, include conjugation-related genes apparently acquired from pAD1 and pAM373 (267). A more global analysis including plasmids, bacteriophages, and genomic islands as well as ICEs indicates that the degree of exchange between these elements is very high, suggesting that, rather than representing distinct groups, they represent a continuum of structures that maximize mobility under various circumstances (304).

OTHER FEATURES OF THE ENTEROCOCCAL MOBILOME

The above discussion focused on MGEs that are unique to the enterococci and/or were originally described in the enterococci, but the enterococcal "mobilome" is extraordinarily diverse and includes plasmids, insertion sequences, and transposons in a variety of families, group II introns, integrons, pathogenicity islands (PAIs), and bacteriophages. A recent review provides tabular lists of many of these elements (137). For a comprehensive overview of MGEs associated with the mobilome of clinically important *E. faecium* and *E. faecalis* lineages, the reader is referred to a recent report (305). Enterococcal phages have also been recently reviewed (306). Addressed below are some issues of particular interest related to the enterococcal mobilome.

Important Non-Pheromone-Responding Plasmids

pAMα1 is an ~10-kb multicopy tetracycline resistance plasmid that is a composite of two replicons (307, 308), an RCR replicon similar to *Bacillus cereus* plasmid pBC16 that carries the tetracycline resistance gene but does not function in *E. faecalis* and a putative theta-replicon similar to pS86 that supports replication in *E. faecalis*. Each component plasmid encodes a relaxase gene, and the junction sites contain *oriT* sequences recognized by them. The *oriT*/relaxase complex allows mobilization of the plasmid in the presence of another conjugative plasmid. The presence of the *oriT* sites at the plasmid junctions suggests that the fusion giving rise to pAMα1 occurred via a site-specific recombination event catalyzed by the relaxases as was previously observed in other plasmids (309, 310). Under tetracycline selection, cells accumulate pAMα1 derivatives containing tandem repeats of the pBC16 replicon, amplifying the tetracycline resistance gene. Amplification is initiated by a RecA-independent recombination event requiring at least one of the relaxase genes and both *oriT* sequences (311) followed by more extensive RecA-dependent amplification between redundant sequences in the duplicated region (312). Thus, the relaxases of pBC16 and pS86 perform three functions: (i) stimulate cointegration to form pAMα1, (ii) facilitate mobilization of the cointegrate, and (iii) trigger amplification of the tetracycline resistance gene in the presence of the antibiotic.

pMG1 is an ~65-kb conjugative plasmid originally isolated from *E. faecium* and encoding high-level gentamicin resistance (313). pMG1-like plasmids bearing Tn*1546* encoding the *van*A determinant have been associated with the spread of vancomycin resistance in enterococci (124, 314). Like the pheromone-responsive plasmids, pMG1 is capable of high-frequency transfer in broth. Unlike pheromone plasmids, pMG1 transfer is not induced by exposure to culture filtrates from potential recipient cells, conjugating broth cultures do not form macroscopic cellular aggregates, and transfer occurs freely between *E. faecium* and *E. faecalis* cells. Furthermore, no homology between pMG1 and the conjugative systems of pheromone-responsive plasmids or Inc18 plasmids was observed (313). Rather, sequencing results indicated significant homology of suspected conjugation genes to those from pXO2, the capsule-encoding plasmid of *Bacillus anthracis* (315, 316). Microscopic aggregates were observed during mating, and this phenomenon is related to a region of the plasmid encoding five open reading frames. One open reading frame encodes a large protein with similarity to a pXO2-encoded surface protein that could represent an "aggregation substance" unrelated to the pheromone plasmid AS (316). Genes potentially encoding the coupling protein, a VirB11-family protein, a relaxase, and the *oriT* were also identified. A gene designated *traA* appears to encode a key positive regulatory protein (317).

TA Systems

In addition to par_{pAD1} and par_{EF0409}, several other TA systems have been identified on enterococcal plasmids and chromosomes. *Par* homologs are present on several other enterococcal plasmids, although no chromosomal loci analogous to par_{EF0409} have been described outside of *E. faecalis* (215, 256). The *E. faecium* multiresistance plasmid pRUM (318) and the related vancomycin resistance plasmid pS177 (319) encode a TA-II locus designated Axe-Txe. Axe-Txe stabilizes heterologous plasmids in *Bacillus thuringiensis*, *E. coli*, and its native host. The Txe toxin belongs to the RelE superfamily of RNA interferases and was shown to cleave mRNA one base downstream of the AUG initiation codon (319, 320). Interestingly, Axe is more closely related to Doc, the antitoxin for the Phd toxin, than it is to RelB, the antitoxin most commonly associated with RelE toxins, supporting the hypothesis that TA-II systems have undergone extensive recombinatorial mixing and matching during their evolution (321). The ωεζ TA-II system is commonly present on the broad-host-range Inc18 plasmids (139), many representatives of which are present in enterococci (322), including the VanA-encoding *E. faecium* plasmid pVEF3 (323), pAMβ1 (308), and the pheromone plasmid pSL1 (324). Unlike most TA-II systems, the ε antitoxin does not autoregulate the locus. Instead, it is regulated by an independent regulatory component, ω, which also regulates the plasmid active partition locus and the copy control factor CopS (325). ζ-family toxins have been proposed to cause cell growth arrest by depleting ATP and GTP pools in the cell and may be associated with persistence to antibiotics (326). The ubiquitous *higBA* and *mazEF* chromosomally encoded TA-II systems are prevalent in *E. faecium* and *E. faecalis* clinical isolates, and *mazEF* has been suggested as a target for novel antibacterials (327).

In *E. faecalis* V583 a *mazEF* TA-II homolog is closely linked to a TA-I locus distantly related to *B. subtilis txpA-ratA* (328). Upstream of the *mazEF* locus is a direct repeat motif

that binds MazE and MazEF, suggesting that the locus is negatively autoregulated as is observed in other TA-II modules (329). It was also observed that ectopic expression of MazE or MazEF stimulated transcription of the *ratA* antitoxin RNA for the *txp-ratA* locus, and a sequence with similarity to the direct repeat motif was found upstream of the *ratA* gene. Thus, the MazE toxin suppresses the toxicity of both *mazEF*, by reducing overall transcription, and the *txpA-ratA* locus, by increasing synthesis of the *ratA* antitoxin, which then targets the *txpA* mRNA for degradation. Conversely, one would expect triggered degradation of MazE, by an as yet unknown mechanism, to activate toxicity of both systems. Thus far, this is the only example of coordinated regulation of TA-I and TA-II loci, and the complete system has been dubbed a TAI-II locus.

PAIs and Intercellular Transfer by Hfr Formation

PAIs are chromosomal regions containing large clusters of genes associated with bacterial virulence (330). Frequently, PAIs include genes associated with known MGEs, such as phage- and ICE-related integration/excision modules and plasmid-related conjugation genes, and some have been shown to be either independently mobile (331) or mobilizable by other MGEs (332). The first enterococcal PAI was identified in *E. faecalis* strain MMH594, and related islands were described in strains V586 and V583 (333). This PAI is ~150 kb long, encodes several pathogenicity factors, including the enterococcal surface protein (*esp*), cytolysin, AS, a bile acid hydrolase, various surface proteins, and general stress proteins, and contains numerous transposons. The PAI also contains phage-related integration/excision proteins, homologs of plasmid conjugation functions, and is flanked by 10-bp terminal direct repeats, suggesting that it could be independently mobile. Genomic analysis of clinical *E. faecalis* isolates revealed that PAI genetic composition is highly variable, suggesting the involvement of modular accretion during its evolution and dissemination (334, 335). Intercellular transfer of either portions of the PAI (336, 337) or the complete PAI (130, 338) has been demonstrated between *E. faecalis* strains and between *E. faecalis* and *E. faecium*. Complete transfer from V583 was shown *in vitro* to be independent of PAI-encoded mobility genes and to frequently include chromosomal genes outside of the element. This *in vitro* transfer was dependent on the conjugation functions of pTEF1 and pTEF2, two pheromone-responsive conjugative elements present in the same strain, suggesting that the plasmids mobilized the PAI via chromosomal integration, perhaps at IS*256* elements, followed by mobilization either by an Hfr- or F′-like mechanism (130). In contrast, precise excision, circularization, and recipient insertion of the PAI was observed in *E. faecalis* strain UW3114, suggesting that the PAI is capable of independent mobility (338), perhaps at a rate substantially lower than that mediated by mobilization. Interestingly, the PAI circular intermediate was not detected in V583, suggesting that an important component for self-mobilization may be absent in that strain or not expressed under the conditions tested. However, even in UW3114, a coresident pheromone-responsive plasmid, pLG2, was transferred in parallel with the PAI and may have facilitated its transfer, and the authors did not determine whether or which PAI genes might be required for mobility. So whether the *E. faecalis* PAI is fully self-mobile is still an open question, although genes associated with excision, integration, and transfer appear to be intact.

Similar *esp*-associated PAIs have also been identified in *E. faecium* that may be subject to conjugative transfer (337, 339, 340). Plasmid-mediated transfer of chromosomal determinants, including determinants involved in β-lactam and vancomycin resistance, has been demonstrated in *E. faecium* strain C68. Transfer in this case was mediated by pLRM23 and a second plasmid and appeared to proceed via cointegration of the plasmid with the donor chromosome, excision of the plasmid with neighboring chromosomal DNA, circularization in the donor prior to transfer, integration of the chromosomal segment into the recipient chromosome by homologous recombination, and subsequent loss of the unstable plasmid remnant (129). Whether this mechanism can also facilitate PAI transfer has not been determined.

Enterococcal Bacteriophage and Genome Defense Systems

Bacteriophages provide a sizable fraction of the mobilome of both *E. faecalis* and *E. faecium*, amounting to close to 10% of the genome of *E. faecalis* strain V583 (6) and 3 to 5% of seven sequenced *E. faecium* strains (340). V583 contains seven putative prophages, five of which have sufficient gene content to generate functional virions. The phage02 region was subsequently determined to be part of the core genome of *E. faecalis* and is likely no longer capable of mobilization (335). The phage07 region is capable of producing infectious virions but requires phage01 as a helper phage to provide packaging and structural elements (341, 242). Sequencing of multiple *E. faecalis* genomes has confirmed the variable presence of these prophage elements except for phage02 (335, 343, 344), and the OG1RF genome contains only phage02 (345).

Comparative genomic analysis of *E. faecalis* phages suggests that they have a modular structure much like bacteriophages infecting other bacteria (306). The modules consist of three transcriptional units, one encoding the lysogeny control functions, one encoding genes required for the lytic pathway, and a variable "cargo" region encoding potential lysogenic conversion genes, some of which could encode important virulence factors. Such cargo genes include a set of putative membrane proteins in the φEF11 phage (346) and a ferrochelatase gene and cold shock protein gene in phage04 (6). However, no systematic analyses have been performed to unequivocally link phage determinants to enterococcal pathogenicity (306). Laboratory studies have demonstrated a capacity for transduction by enterococcal phages, including interspecies transfer of antibiotic resistance, so the potential for phage effects on virulence does exist (347, 348).

CRISPR-Cas function as adaptive immune systems for bacteria, generally targeting the DNA of invading MGEs for degradation (see 349 for a recent review). Three distinct type II-A CRISPR loci have been identified in *E. faecalis*: CRISPR1 and 3 are complete functional loci with associated Cas genes, while the CRISPR2 locus is an "orphan" locus harboring repeats and spacers of the same family as CRISPR1 but lacking Cas genes (8, 345). CRISPR1-Cas and CRISPR3-Cas are variably present in *E. faecalis* genomes and never cooccur in the same strain, while CRISPR2 is ubiquitous and can thus be considered part of the core genome of this species. CRISPR1-Cas-related systems have also been detected in several environmental and nonclinical enterococcal species (350). Spacer sequences from all three CRISPR loci show identities to phage and plasmid sequences, suggesting that all have acquired MGE segments for genome defense (8). However, CRISPR2 only functions when the Cas genes of CRISPR1 are provided *in trans* (351), suggesting that strains containing only CRISPR2 should be more sensitive to MGE invasion. Indeed, the absence of complete CRISPR-Cas loci

is associated with larger genomes, higher percentages of horizontally acquired genes, greater antibiotic resistance, the presence of certain virulence factors, and high-risk multilocus sequence type (7, 8). Therefore, *E. faecalis* CRISPR-Cas loci, in cooperation with restriction-modification systems (351), are effective in limiting invasion by MGEs, but loss of CRISPR-Cas facilitates the evolution of pathogenicity by allowing greater horizontal gene transfer. Recently, a method for activating CRISPR2 and using it to modify the *E. faecalis* population structure was proposed (352).

A type II-A CRISPR-Cas locus has also been identified in *E. faecium*, related to the *E. faecalis* CRISPR1-Cas locus (8). Like in *E. faecalis*, the locus is not present in all *E. faecium* strains and is absent in most multiple antibiotic-resistant strains and in the major hospital-adapted clade of this species (353).

CONCLUDING REMARKS

From the beginning, enterococcal genetics has been focused on MGEs. Pioneering studies of the enterococci gave us one of the first examples of intercellular communication in the bacterial world in the pheromone-inducible conjugative plasmids and the first chromosomally located conjugative elements in the conjugative transposons, later to be named ICEs after their distribution was found to be ubiquitous in bacterial cells. Studies of the complex regulatory circuits controlling the pheromone response and the conjugative mechanisms of ICEs continue to inform those interested in the molecular mechanisms of gene regulation in prokaryotes in general. The importance of MGEs in enterococci became even more apparent with the emergence of vancomycin resistance and the multitude of ways the *van* gene clusters could be shared among enterococci and with other genera. Further studies identified the enterococci as a promiscuous hub for the dissemination of a wide variety of antibiotic resistance genes. The accumulation of genomic information on the enterococci has further revealed just how important MGEs are to enterococcal pathogenesis and evolution, particularly in the way that the CRISPR MGE defense system impacts the structure of *E. faecalis* and *E. faecium* populations.

Complicated regulatory schemes and important antibiotic resistance phenotypes are not limited to enterococcal MGEs, and progress is now being made on important systems in the core genomes, particularly those of of *E. faecalis* and *E. faecium*. Work is proceeding on the intrinsic mechanisms of antibiotic resistance in these organisms, and the complex transcriptional and posttranscriptional mechanisms of regulation of the EA regulon presage more interesting regulatory phenomena to be discovered. While roles for enterococcal TCSTSs are being defined, genomic analyses have revealed a plethora of potential sRNA regulators about which we know very little. To this point, most detailed genetic analyses have been performed *in vitro* or have focused on the effects of the various systems during pathogenic processes, but the enterococci are common intestinal bacteria, and it is likely that events occurring within the gut in the presence of the microbiota are more relevant to normal enterococcal physiology. Here again, studies with MGEs are likely to lead the way. Clearly, much remains to be discovered about the genetics of these interesting and clinically important Gram-positive bacteria.

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Biology of Oral Streptococci

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26

THE ORAL ENVIRONMENT

Warm, moist, and rich in nutrients, the oral cavity provides an ideal environment for colonization by a community of bacteria, fungi, protozoa, archaea, and viruses, often in the form of a complex structure called biofilm or plaque (1). In addition, several microbial niches exist within the oral cavity (e.g., cheek, gingiva, teeth, tongue, palate) that vary in nutrient content, pH, oxygen tension, and shear force due to salivary flow and mastication. These physico-chemical characteristics select for suitable microorganisms for each oral niche such that the microbial compositions can differ greatly from one site to another. Saliva is the biological fluid of the oral cavity, and its microbial composition is a collection of bacteria that have shed from various oral niches. As an easily accessible body fluid, saliva can be used in assessment of general oral health, as the disappearance or emergence of certain taxa can indicate health or disease (2, 3). While biofilms from oral soft tissues, such as the tongue, cheeks, and palate, and the supragingival biofilm (plaque on tooth surfaces above the gum line) are constantly bathed in saliva and subjected to an array of environmental fluctuations, the subgingival plaque (plaque on tooth surfaces below the gum line) is a unique niche with less abrupt environmental fluctuations. In particular, with the gum serving as a physical barrier, the oxygen tension and shear forces are reduced, and gingival crevicular fluid (serum) serves as a major nutrient source (4).

TAXONOMY OF ORAL STREPTOCOCCI

Streptococci are found in almost every location in the human body and are the dominant species in the human oral cavity and upper respiratory tract. Species within the genus *Streptococcus* were initially differentiated based on hemolysis patterns on blood agar plates with β-hemolysis (complete lysis), α-hemolysis (partial lysis), and γ-hemolysis (nonhemolytic) (5). Many β-hemolytic species can be further differentiated by carbohydrate group antigens (Lancefield groups) (6), such as the classic human pathogens *S. pyogenes* (group A *Streptococcus*) and *S. agalactiae* (group B *Streptococcus*), which are the subjects of other articles. Historically, the oral streptococci were referred to as viridans streptococci (*Streptococcus viridans*) due to their high propensity to display partial hemolysis when cultured on blood agar plates, resulting in a green coloration surrounding the colonies. While the viridans streptococci or α-hemolytic designations are still used today to classify the oral streptococci, this term is not fully accurate, because some isolates can display β-hemolysis or completely lack any hemolytic activity (γ-hemolysis). As more streptococcal species were identified, it became apparent that additional criteria were required to separate the growing number of species, prompting the development of biochemical and physiologic tests to differentiate streptococcal groups (7, 8). Initially, there were seven named groups of streptococci based on carbohydrate fermentation tests, chain length, and growth on gelatin: *Streptococcus salivarius*, *Streptococcus mitis*, *Streptococcus anginosus*, *Streptococcus equinus*, *Streptococcus pyogenes*, *Streptococcus faecalis*, and *Streptococcus pneumoniae* (pneumococcus) (reviewed in reference 5). Eventually, *Streptococcus faecalis* was reclassified, and it is now a member of the genus *Enterococcus* (9, 10).

The advent of DNA sequencing introduced a new and more accurate approach to the identification of bacteria at the species level, and sequencing of the 16S rRNA region could be used to determine phylogenetic relationships (11). Initially, the oral streptococci were separated into four groups: anginosus, mitis, mutans, and salivarius, while the remaining streptococci isolated outside of the oral cavity were classified into either the bovis or pyogenic groups (12). Recently, using a more robust phylogenetic approach, Richards et al. separated the streptococci into eight distinct groups: mitis, sanguinis, anginosus, salivarius, downei, mutans, pyogenic, and bovis (Fig. 1) (13). Presently, oral streptococci are found in all groups except the pyogenic and bovis groups. The mitis group is the largest of the groups found in the oral cavity, currently with 20 species. Species within the mitis group have been challenging to differentiate based on 16S RNA sequence alone, particularly *Streptococcus oralis* and *S. mitis*. Recent efforts to reclassify many of the species within this group indicate that there are many strains that have been misclassified, and new species descriptions may soon emerge (14).

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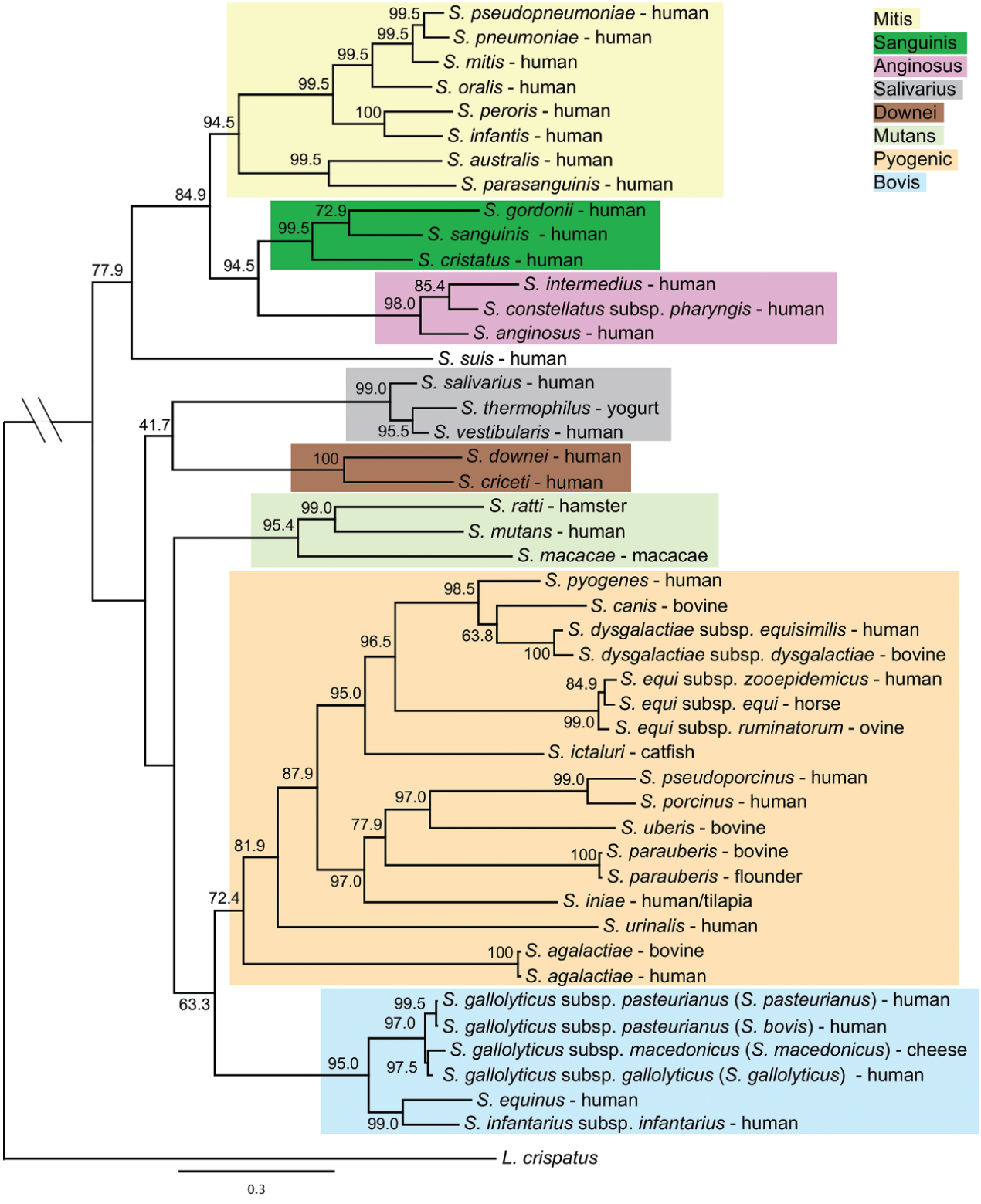


FIGURE 1 Phylogeny of the indicated streptococcal species derived from a core set of 136 concatenated genes. Numbers on branches show bootstrap support for each relationship. The color shading indicates the eight major groups.

MICROBIOME AND METAGENOMICS STUDIES: THE ORAL MICROBIOME

While initial attempts to define the oral microbiome relied on culture-based techniques, 16S rRNA sequencing has identified more than 700 microbial species residing within the oral cavity. It is estimated that roughly half of these species are nonculturable by current methods. The Human Microbiome Project (http://commonfund.nih.gov/hmp/), launched in 2012, aimed to define the core microbiome of 18 locations in the human body, including the oral cavity, and provided the first comprehensive analysis of bacterial diversity and abundance in humans (15, 16). Nearly all microbiome studies today use next-generation sequencing technologies based on 16S rRNA sequences to determine the community composition (17), and large manually curated databases, such as www.homd.org, are now available to aid in the identification of bacterial species unique to the oral cavity. Based on these studies, we now can clearly appreciate that there are distinct communities of organisms living on the different surfaces of the mouth, which vary significantly depending on the site of sample collection, health status, and behavior of the host (18–20). Thus, as indicated in the beginning of the article, slight variations in the local environment within the mouth can have profound effects on the composition of the resident organisms (18, 19). However, regardless of the individual sampled, streptococci are universally present in all sites of the oral cavity and are the dominant genus found in saliva and on soft tissues of the mouth (16, 18, 19). Critically, streptococci, especially members of the mitis group, are the first organisms detected in the mouth of newborn infants and are considered pioneer species (primary colonizer) that allow for the assembly of a complex microbiota.

Dental plaque is readily accessible on the surface of the teeth and therefore is one of the most well-characterized microbial communities in the human body (21, 22). Two of the most common human diseases, dental caries and periodontitis, are directly related to the presence and metabolic activities of dental plaque bacteria. A recent analysis that compared supragingival plaque microbiomes from carious lesions to healthy sites found substantial differences among children with different caries status (23). In particular, communities collected from dentin carious lesions contained notorious acidogenic (acid-producing) and aciduric (acid-tolerant) species, including *S. mutans*, *Scardovia wiggsiae*, *Parascardovia denticolens*, and *Lactobacillus salivarius* (23). In contrast, *Streptococcus sanguinis*, *Neisseria* species, and *Leptotrichia* species were found associated with samples collected from healthy sites. However, a decrease in species diversity was associated with dental caries progression, but an increased overall abundance of *S. mutans* has been demonstrated (24). Moreover, an association of *S. mutans* with other acidogenic species, such as *S. wiggsiae* and bifidobacteria, in severe early childhood caries has been shown (25). One study that focused on the microbiota that thrive at different pH ranges also found a decrease in microbial diversity in communities from acidic pH environments but found *Lactobacillus* spp., *Prevotella* spp., *Atopobium* spp., *Olsenella* spp., and *Actinomyces* spp. to be the most abundant taxa in dentinal caries (26). Microbiome studies of subgingival plaque identified high abundance of *Streptococcus parasanguinis*, *S. sanguinis*, *S. mitis*, *Streptococcus intermedius and Streptococcus infantis* associated with health (27–31). In diseased sites (gingivitis and periodontitis), a decrease in the proportions of health-associated *Streptococcus* (32) and an increase in anaerobic proteolytic bacteria such as *Porphyromonas gingivalis* is observed. However, *Streptococcus constellatus* and *S. intermedius* are often reported to be associated with periodontitis (33, 34).

Most microbiome studies are initially conducted with the intent of correlating the presence of disease with a particular signature microbiome. However, confounding factors such as genetic heterogeneity and phenotypic diversity within a species contribute to a more complex relationship between the presence of a particular bacterial species and the establishment of disease. Studies comparing the phenotypic diversity within streptococcal strains from the same species have identified major phenotypic variations among isolates that are directly related to virulence or properties associated with healthy biofilms (35, 36). For this reason, the combination of 16S rRNA community profiling with other "-omics" technologies (i.e., transcriptomics, proteomics, and metabolomics) is essential for a global understanding of the specific contributions of oral bacteria to health and disease (17), providing a better understanding of how complex communities function and interact (37).

SUGAR METABOLISM OF ORAL STREPTOCOCCI

Streptococci are well known for their ability to assimilate a large array of carbohydrates by glycolysis and for their enhanced tolerance to acidic pH. Employing the Embden-Meyerhof-Parnas pathway, streptococci convert one molecule of glucose or other equivalent carbohydrates (e.g., fructose) into two molecules of pyruvate, with the concomitant generation of two molecules of ATP and two molecules of NADH. Under the conditions of excess carbohydrates and oxygen limitation, streptococci tend to perform homolactic fermentation, reducing pyruvate into lactic acid and regenerating NAD from NADH. The production of lactic acid results in rapid acidification of the environment and allows streptococcal species to outcompete acid-sensitive microorganisms. When faced with carbohydrate limitation or increased oxygen tension, streptococci produce alternative fermentation products such as formate, acetate, and ethanol (38) (Fig. 2). In fact, streptococci lack the capacity for oxidative phosphorylation and electron chain transport systems and depend exclusively on substrate-level phosphorylation for energy production. Therefore, most streptococci are considered facultative anaerobes and susceptible to growth inhibition by oxygen.

Resulting from a reductive evolution as a consequence of adaptation to survival within human and animal hosts,

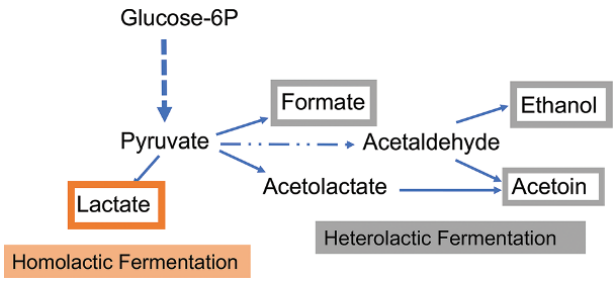


FIGURE 2 Simplified schematic of homolactic fermentation and heterolactic fermentation. While homolactic fermentation generates almost exclusively lactate, heterolactic fermentation generates formate, acetoin, ethanol, and reduced amounts of lactate.

streptococci possess an incomplete tricarboxylic acid cycle, or Krebs cycle, with some enzymes of the pathway being preserved in certain species, such as *S. mutans* and *S. gordonii*, and none present in species such as *S. pyogenes* or *S. pneumoniae*. Because metabolites of the tricarboxylic acid cycle are often precursors required for amino acid biosynthesis, it follows that varying degrees of amino acid auxotrophy exist for different streptococcal species. Another outcome of this reductive evolution is the fact that streptococci did not maintain a functional Entner-Doudoroff pathway, an alternative pathway that utilizes glucose-6-phosphate (glucose-6-P) to generate pyruvate through a series of enzymatic reactions that are distinct from glycolysis. Also, many species lack a complete pentose phosphate pathway (38), which represents a parallel process to glycolysis as glucose-6-P is oxidized and then decarboxylated into a five-carbon product, ribulose-5-phosphate (ribulose-5-P), yielding the important reducing equivalent, NADPH. Further metabolism of ribulose-5-P by the nonoxidative phase creates precursors for biosynthesis of nucleic acids, histidine, and aromatic amino acids. In bacteria lacking the oxidative phase of the pentose phosphate pathway (e.g., *S. mutans*), alternative reactions and enzymes (e.g., $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenase or GapN) exist to compensate for the generation of both nucleotides and NADPH (39).

In contrast to these reductive evolutions, streptococci have maintained a significant arsenal of carbohydrate-uptake mechanisms, proteins, and enzymes of the sugar: phosphotransferase system (PTS) (40, 41) and the ATP-binding cassette transporter family (42). The PTS consists of two general enzymes, enzyme I and a phospho-carrier protein, HPr, and a series of multidomain, membrane-associated, substrate-specific sugar transporters called enzyme II. Using phosphoenolpyruvate as an energy source, enzyme I and HPr initiate a cascade of phospho-relay reactions that funnels phosphoryl groups, via various enzyme IIs, onto incoming carbohydrates (40) (Fig. 3). Recent sequencing projects of streptococci have uncovered large portions of these genomes as predicted carbohydrate transporters, many of which are apparent products of horizontal gene transfer and have origins

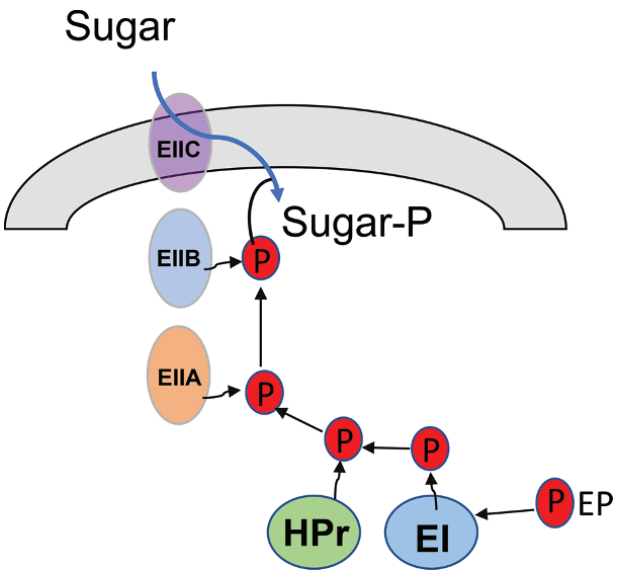


FIGURE 3 Simplified schematic of the phosphoenolpyruvate sugar:phosphotransferase system. EI, enzyme I; EII, enzyme II P, phosphate; PEP, phosphoenolpyruvate.

in related bacterial species (43–45). Together, these transporters allow metabolic flexibility when glucose, the preferred energy source for most streptococci, is not available. A major coordinator of these processes is the global regulator CcpA (catabolite control protein A). CcpA oversees carbon catabolite repression, a regulatory mechanism by which enzymes associated with catabolism of nonpreferred energy sources are repressed until glucose is no longer available (46, 47). When glucose is abundant, the cofactor HPr becomes phosphorylated at a serine residue. In association with this phosphorylated cofactor HPr(Ser-P), CcpA binds to the catabolite response element (*cre*) within the promoter region of a target gene to influence transcription (46). In addition to overseeing carbohydrate metabolism, CcpA has been associated with expression of virulence factors in streptococci (48–50). Notably, it has been demonstrated in some streptococci, e.g., *S. mutans*, *S. pyogenes*, and *S. pneumoniae*, that substrate-specific, CcpA-independent carbon catabolite repression plays profound roles in controlling carbohydrate prioritization and virulence expression (see references 51–55). Therefore, it appears that interactions among streptococci and with their host environment have shaped these bacteria for a successful lifestyle that specializes in carbohydrate catabolism and environment acidification. As such, infectious diseases and virulence associated with streptococci are often heavily dependent on their metabolic activities and, consequently, their ecological relationships with commensal bacteria in each particular biofilm environment (45, 50, 56).

MECHANISMS OF COLONIZATION

As important primary colonizers of the mouth, oral streptococci possess multiple high-affinity adhesins that mediate initial binding to tooth surfaces via interactions with substrates in the acquired salivary pellicle, including albumin, proline-rich proteins, glycoproteins, mucins, and sialic acid. Bacterial extracellular polysaccharides and extracellular DNA are major constituents of the plaque biofilm matrices whose production is triggered by bacterial adherence (57, 58). These macromolecules further facilitate bacterial colonization and accumulation of multicellular clusters. Salivary amylase also appears to play a role in bacterial colonization of mammalian oral surfaces since amylase-binding bacteria (such as *S. mitis*, *S. gordonii*, *S. salivarius*, and *Streptococcus cristatus*) were found to colonize only hosts with salivary amylase activity (59). Late colonizers, both Gram-positive and Gram-negative species, build upon the initial streptococcal foundation through a series of coaggregation and coadhesion events until a mature plaque is achieved within any given individual (60). Once established, the plaque community is relatively stable, and under healthy conditions, the different species in the community exist in homeostasis.

Colonization, as well as accumulation of oral streptococci on hard and soft tissue surfaces, is guided by a series of adhesin-receptor interactions and regulated in response to environmental cues, including the presence of other microbial species (61). The antigen I/II family of adhesins (SpaP, P1, PAc, SspA, SspB) is shared by most oral as well as several of the pyogenic streptococci (62). These proteins mediate binding to multiple host proteins, with the salivary glycoprotein gp340 (DMBT1) being the most widely studied. *S. gordonii* possesses two paralogs of antigen I/II, SspA and SspB, although the purpose for this duplication is not known (63). Notably, *S. gordonii* is a master of adherence and possesses numerous adhesive structures. Among them, GspB and Hsa are serine-rich repeat (Srr) glycoproteins that bind to sialylated glycans present on the soluble human salivary mucin MG2/MUC7, a

component of the mucosal pellicle (64), and the platelet glycoprotein GPIb (65, 66). Srr proteins are important for biofilm formation and have been identified in many other oral streptococci, including Fap1 of *S. parasanguinis* (67) and *S. oralis* (68), SrpA of *S. sanguinis* (69) and *S. cristatus* (70), and SrpA, B, and C of *S. salivarius* (71). Interestingly, Srr homologs are not present in the caries pathogen *S. mutans*. Genes encoding Srr family proteins are located within tightly coordinated genetic loci that also encode proteins that mediate their glycosylation, acetylation, and export (72–74). Notably, instead of the general secretion machinery utilized for other surface proteins, an alternative secretion system including SecA2 and SecY2 is dedicated to the translocation of this family of serine-rich proteins (67, 75). In addition, CshA of *S. gordonii* and CshA-like proteins of *S. oralis* and *S. sanguinis* are multifunctional fibrillar adhesins that bind to multiple host proteins, including fibronectin, and to other microorganisms (76, 77).

As detailed, production of extracellular glucans via the glucosyltransferases GtfB, GtfC, and GtfD is well known to facilitate adhesion of *S. mutans* to saliva-coated tooth surfaces (78). Secreted *S. mutans* Gtfs bind to the salivary pellicle and to other organisms, facilitating the recruitment of oral microorganisms to the dental biofilm. Although some other oral streptococci also express Gtfs, these enzymes have not been extensively studied like the Gtfs of *S. mutans*. A Gtf-negative mutant of *S. gordonii* showed diminished persistence on the teeth of sucrose-fed rats, although initial colonization was not affected (79). In addition, deletion of the single *gtf* gene of *S. gordonii*, *gtfG*, impacted its ability to form mixed biofilms with *Candida albicans* (80). *S. sanguinis* also produces water-soluble glucans from sucrose through a Gtf enzyme called GtfP. Its biofilm formation in monoculture in the presence of sucrose was significantly decreased upon inactivation of *gtfP* in *S. sanguinis*, although little effect on biofilm production was observed when the mutant strain was cocultured with other oral streptococcal species (81). Additional studies are necessary to firmly establish the contribution of Gtfs from non-*S. mutans* oral streptococci to oral colonization and biofilm assembly *in vivo*.

BETWEEN GOOD AND EVIL: WHEN THINGS GO WRONG

Dental caries, or simply cavities, is a multifactorial disease in which a carbohydrate-rich diet and the oral microbial composition play key roles in the development and progression of the disease. The common main culprits associated with dental caries are two species from the mutans streptococccci group, *S. mutans* and *Streptococcus sobrinus*, but other organisms can also be involved in the process, because caries-active subjects have been identified that do not harbor *S. mutans* or *S. sobrinus*. In healthy subjects, there is a balance between the numbers of cariogenic bacteria and non-cariogenic commensal species, with the latter keeping the cariogenic population in check through the production of alkali, hydrogen peroxide, or other inhibitory substances. However, frequent exposure to dietary carbohydrates creates a dysbiotic environment, in which the generation of organic acids such as lactic acid as fermentation by-products allows *S. mutans* and other aciduric species to outcompete less acid-tolerant commensal bacteria present in the dental plaque. Eventually, the repetitive acidification of environmental pH below the critical point (pH 5.5) leads to enamel demineralization and consequent cavitation of the tooth surface.

The distribution of oral streptococcal species differs within various ecological niches of the oral cavity (82). The mitis and sanguinis groups, including *S. oralis*, *S. mitis*, *S. gordonii*, and *S. sanguinis*, are the primary colonizers of the tooth surface and are commonly considered commensals, although all have been implicated in cases of infective endocarditis. Oral bacteria can reach the bloodstream not only during invasive procedures such as dental extractions and oral surgery but also after simple daily activities such as mastication, brushing, and flossing. Fortunately, our immune surveillance readily clears these bacteria from the bloodstream in a process that can take just a few seconds. However, in the rare occasions when these organisms successfully avoid the immune surveillance and cause systemic disease, interactions with platelets are especially important during the pathogenic process of infective endocarditis. Platelet binding by *S. mitis* was reported to be higher in strains that produce fewer exopolysaccharides, whereas high exopolysaccharide producers demonstrated less platelet aggregation (83). Another primary colonizer of the tooth surface that can cause transient bacteremia and endocarditis is *S. parasanguinis*. Its dual-function lipoprotein FimA participates in both metal transport (84) and adherence to fibrin, critical for vegetation formation on damaged heart tissue (85).

THE GOOD STREPTOCOCCI: COMMENSAL ORAL FLORA

In the human mouth, most microorganisms are embedded within a complex extracellular matrix known as biofilm or plaque (86). In a healthy individual, the oral biofilm is dominated by bacteria that are commensal in nature, engaging the host immunity in an intricate dialog that maintains a homeostasis integral to human health. Significantly, the commensal communities afford the host additional benefits by fending off pathogenic species. Dental health is often associated with a greater proportion of commensals with beneficial properties that include *S. gordonii*, *S. sanguinis*, and *S. parasanguinis*, some of which can cause opportunistic diseases at different body sites as discussed above, but in the mouth, their presence is generally associated with lower proportions of cariogenic *S. mutans* and oral health. In the case of dental caries, repetitive exposures to acidic pH values, reduced salivary flow, and a diet rich in fermentable carbohydrates are the known triggers of dysbiosis in dental plaque, tilting the balance toward acidogenic and aciduric populations (87). Here, we discuss a few mechanisms that are critical to understanding the antagonism between the commensal streptococci and dental pathogens: (i) production and neutralization of acids, (ii) generation of hydrogen peroxide (H_2O_2) that inhibits the persistence of *S. mutans*, and (iii) production and secretion of antimicrobial compounds.

Acid is the driving force behind dental caries, but pH in dental plaque is modulated in a complex way by the buffering capacities of salivary secretions, bacterial cells, certain foods, and the metabolic products of the microbiota. As previously mentioned, commensal streptococci are early colonizers of the oral cavity and play pivotal roles in moderating biofilm acidification by, among other things, generating large amounts of alkali from salivary and dietary substrates. A primary route for alkali generation by oral streptococci is the arginine deiminase system (ADS), which consists minimally of three enzymes: arginine deiminase, a catabolic ornithine transcarbamylase (cOTC), and a catabolic carbamate kinase (cCK). The ADS acts on free arginine to generate one molecule of ornithine and CO_2 plus two molecules of ammonia, with the concomitant production of ATP (88). Although free arginine is found in micromolar concentrations in ductal saliva, it is abundant in salivary

proteins and peptides (89). The pH-modulating effects of ammonia production benefit the commensals and other species that are generally less aciduric, or overtly acid sensitive. Another well-characterized alkali-generating mechanism is the urease system identified in *S. salivarius*, a three-part enzyme complex and a set of accessory proteins that break down urea to release ammonia and CO_2. The urease system could have considerable impact on oral bacterial ecology, given the millimolar concentration of urea present in saliva and gingival crevicular fluids. Of note, subjects with chronic renal failure, who secrete 10-fold more urea in saliva than normal individuals, rarely developed dental caries despite being on a carbohydrate-rich diet (90, 91).

Importantly, there are multiple reasons to believe that the ADS and arginine both have a potent influence on the promotion of a microbiota that is less cariogenic and that prevents caries pathogens from emerging in substantial numbers. In a mixed-species biofilm model containing arginine in the growth medium that included *S. mutans* and *S. gordonii*, activities of ADS provided a two-fold beneficial impact: they promoted the dominance of commensals and disrupted the biofilm matrix of *S. mutans* by interfering with exopolysaccharide production (92). Multiple clinical studies involving both children and adults support that arginine and elevated ADS levels are correlated with dental health, whereas low ADS activity appears to be strongly associated with active disease (35, 93–95). As a result, arginine has been incorporated into oral health products around the globe to promote oral health.

A second strategy employed by oral commensal streptococci to achieve a competitive advantage over oral pathogens is the generation of hydrogen peroxide (H_2O_2), which inhibits the growth of *S. mutans*, the periodontal pathogen *P. gingivalis* (96), and other oral pathogens. Among the known peroxigenic commensals, which include *S. oralis*, *S. mitis*, *S. sanguinis*, *S. gordonii*, and *Streptococcus oligofermentans*, there are multiple enzymatic pathways for the generation of H_2O_2. A dominant mechanism in dental biofilms appears to involve the enzyme pyruvate oxidase (Pox, encoded by *spxB*), which is present in many abundant commensal streptococci. Pox catalyzes the conversion of pyruvate and inorganic phosphate to H_2O_2, CO_2, and acetyl phosphate in the presence of oxygen. Thus, not only does the commensal gain a competitive advantage over H_2O_2-sensitive pathogens, but also, the acetyl phosphate provides a bioenergetics benefit as it is further metabolized, via acetate kinase, to acetate with the production of ATP. Moreover, the production of CO_2 contributes to some unknown degree the overall buffering capacity of the biofilm (97–99).

The H_2O_2-generating enzymes lactate oxidase (100, 101), L-amino acid oxidase (102, 103), and NADH oxidase are also present in some commensal oral streptococci. A lactate oxidase enzyme has been identified in *S. oligofermentans* that is capable of utilizing the lactic acid generated by the glycolytic activities of lactic acid bacteria to yield pyruvate and H_2O_2, which has the added benefit of removing a strong acid (lactate) from the environment. *S. oligofermentans* also encodes a Pox enzyme, but there is evidence to suggest that these enzymes may not work synergistically because of the way the genes are regulated (100). Notably, in *S. gordonii* and *S. sanguinis*, expression of *spxB* is sensitive to carbon catabolite repression by preferred carbohydrates such as glucose (104). Thus, it remains to be determined to what degree H_2O_2 production by Pox and other enzyme systems is active under conditions that are conducive to the formation of caries, i.e., when the host is consuming large quantities of readily metabolizable sugars. Likewise, since mature dental biofilms tend to be depleted for oxygen and have a low redox potential, Pox and other systems may exert their benefit primarily in the early phases of dental plaque development and have a more potent influence on the establishment of caries pathogens, whereas other pathways for competition become essential in more mature biofilms.

More recently, another mechanism whereby commensals may specifically affect the fitness of *S. mutans* has come to light. Most isolates of *S. mutans* carry the ComDE two-component system, which is required for the activation of transcription of a number of genes encoding lantibiotic and nonlantibiotic bacteriocins, small peptides that exhibit antimicrobial activity against other species. ComDE depends on a quorum sensing peptide, competence-stimulating peptide (CSP), for activity (105). *S. gordonii* DL-1 produces a protease, designated as challisin, which is able to degrade CSP and block the activation of bacteriocin gene expression by *S. mutans* (106). Interestingly, *Streptococcus* A12, a recently identified commensal with multiple strategies to interfere with *S. mutans* (105), also produces a challisin-like protease with similar activities against CSP and the ability to interfere with exopolysaccharide production by *S. mutans*. Notably, A12 can also block a second peptide-dependent signaling pathway (XIP-ComRS) that is highly conserved in *S. mutans* (105).

CONCLUDING REMARKS

As our understanding of oral infectious diseases evolves toward a multimicrobial, dysbiosis-based perspective, the field of oral microbiology has embraced a more holistic approach, exemplified by significant enhancements in our knowledge of molecular mechanisms underlying the physiological and ecological shifts that control the composition and activities of the microbiome in health and disease. Nevertheless, the metabolic plasticity, the ability to colonize multiple oral surfaces, and the production of inhibitory substances make oral streptococci key players in oral homeostasis and disease.

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The Biology of *Streptococcus mutans*

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27

In 1924, J. Clarke isolated an organism from carious lesions and called it *Streptococcus mutans*, because he thought the oval-shaped cells observed were mutant forms of streptococci (1). However, it was in the late 1950s when *S. mutans* gained widespread attention within the scientific community, and by the mid-1960s, clinical and animal-based laboratory studies depicted *S. mutans* as an important etiologic agent in dental caries (2). The natural habitat of *S. mutans* is the human oral cavity, more specifically, the dental plaque, a multispecies biofilm formed on hard surfaces of the tooth. It has been largely accepted that the cariogenic potential of *S. mutans* resides in three core attributes: (i) the ability to synthesize large quantities of extracellular polymers of glucan from sucrose that aid in the permanent colonization of hard surfaces and in the development of the extracellular polymeric matrix *in situ*, (ii) the ability to transport and metabolize a wide range of carbohydrates into organic acids (acidogenicity), and (iii) the ability to thrive under environmental stress conditions, particularly low pH (aciduricity) (3). While *S. mutans* does not act alone in the development of dental caries, studies from several laboratories have convincingly demonstrated that *S. mutans* can alter the local environment by forming an extracellular polysaccharide (EPS)-rich and low-pH milieu, thereby creating a favorable niche for other acidogenic and aciduric species to thrive. As a human pathogen, *S. mutans* is also implicated in subacute bacterial endocarditis, a life-threatening inflammation of heart valves, while a subset of strains has been linked to other extraoral pathologies such as cerebral microbleeds, IgA nephropathy, and atherosclerosis.

Strains of *S. mutans* can be classified into four serological groups (*c*, *e*, *f*, and *k*) based on the composition of cell-surface rhamnose-glucose polysaccharide, with ~75% of strains isolated from dental plaque belonging to serotype *c*, ~20% to serotype *e*, and the remaining 5% classified as serotypes *f* or *k* (4). While biochemical and genetic approaches to dissect the biology of *S. mutans* have been used for at least the past 4 decades, the publication of the complete genome sequence of the *S. mutans* strain UA159 in 2001 (5) dramatically changed the landscape, and today, *S. mutans* is one of the best-characterized Gram-positive pathogens. In this article, we highlight some of the key studies that have led to our current understanding of *S. mutans* genetics, physiology, and virulence. For a historical perspective and complete survey of the field, we direct the reader to the review articles in references 2, 3, and 6–9.

GENETIC AND PHENOTYPIC HETEROGENEITY

The first *S. mutans* genome sequenced (serotype *c* strain UA159) was found to contain ~2.0 Mb of DNA and to encode approximately 2,000 genes (5). As the cost of next-generation sequencing technologies has gone down, genomes from dozens of *S. mutans* strains have been sequenced and assembled and are now available on public databases such as the NCBI (www.ncbi.nlm.nih.gov/assembly/?term=streptococcus+mutans). This influx of genome sequences has led to an increase in comparative genomic studies focused on *S. mutans* (10–13). One of the first such studies was based on the shotgun genome sequence from 57 geographically diverse *S. mutans* clinical isolates. This study concluded that the *S. mutans* pan-genome contains a minimum of ~3,300 possible genes and has a core genome (genes that are common to all strains) of 1,490 genes (14). This means that in any one *S. mutans* isolate, ~500 genes could be distinct from any other strain, perhaps significantly influencing virulence potential or fitness. The same group, using population demographic analysis based on single nucleotide polymorphisms of the core genes, determined that a large expansion took place in the *S. mutans* population between 3,000 and 10,000 years ago, which coincided with the advent of human agriculture and increased consumption of carbohydrates in the human host diet (14). This study also identified 73 unique core genes that are found only in *S. mutans* and not in its closest relatives, many of which are involved in carbohydrate metabolism and acid resistance (14). In a follow-up study, Palmer et al. (15) characterized 15 of the most genetically diverse isolates of the 57 strains sequenced by Cornejo et al. (14) and found great variation in the phenotypes directly related to virulence, including the ability to form biofilm in the presence of sucrose and the ability to tolerate low pH and oxidative stresses, suggesting that not all strains of *S. mutans* are equally virulent and providing a rational explanation for why attempts to correlate the carriage of certain genotypes of *S. mutans* with the incidence of dental caries has proven so difficult (10, 16–18).

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Work to characterize the unique core and noncore genes is ongoing and will likely lead to a greater understanding of the physiology and diversity of *S. mutans* as a species. For example, one of the unique core hypothetical genes, SMu.1147, which encodes a small peptide, was found to regulate genetic competence and other traits of general importance to *S. mutans* virulence (19). In contrast, efforts to characterize the noncore genes indicate that they likely provide a competitive advantage under particular circumstances. Such is the case for a galactose-specific phosphotransferase system (PTS) transporter found in several strains (20). Among the many noncore genes of *S. mutans* identified in recent years, those encoding the collagen-binding proteins (CBPs) Cnm and Cbm were shown to confer adhesion to collagen and laminin, invasion of endothelial and epithelial cells, and virulence in the *Galleria mellonella* invertebrate model (21, 22) as well as in rabbit and rat models of infective endocarditis (23, 24). Epidemiological studies indicate that *cnm* is present in approximately 15% of *S. mutans* isolates, whereas *cbm* is rarely found (~2%) (21, 22). Genes encoding CBPs have an uneven distribution among the different serotypes and are found at higher frequency among serotype *e*, *f*, and *k* strains but are rarely present in serotype *c* strains (25). Notably, CBP^+ *S. mutans* strains are more frequently isolated from dental plaque of individuals with bacteremia and infective endocarditis, suggesting a correlation between the production of these adhesins and systemic infections (26). Interestingly, the presence of *cnm* in *S. mutans* strains isolated from saliva has been linked to IgA nephropathy, cerebral microbleeds, and cognitive impairment (27, 28). Finally, animal and clinical studies have suggested an association of CBPs in caries risk and severity (27, 29, 30).

CARBOHYDRATE METABOLISM

As a lactic acid bacterium, *S. mutans* relies exclusively on glycolysis for energy production (Fig. 1). A signature characteristic of this organism is its ability to metabolize a large variety of carbohydrates. The genome of the UA159 type strain encodes 14 phosphoenolpyruvate-dependent sugar: PTSs with specificities for various mono- and disaccharides, as well as two ATP-binding cassette (ABC) transporters involved primarily in internalizing oligosaccharides (5). Sucrose is a β2,1-linked disaccharide composed of glucose and fructose that has proven, for a number of reasons, to be the most cariogenic of all carbohydrates. *S. mutans* has evolved multiple pathways to catabolize sucrose for acid production (31), and several glycosyltransferase enzymes (Gtfs) convert sucrose into a glue-like extracellular polymer glucan that promotes the buildup of biofilms by way of cellular attachment to dental surfaces and other oral microorganisms (7). As discussed in more detail below, recent multispecies modeling studies have confirmed the unique roles of Gtfs in the formation of a heterogeneous, diffusion-limiting, low-pH matrix that is conducive to both dental demineralization and the eventual dominance by acid-tolerant species (7, 32).

Carbon Catabolite Repression

Most bacteria have evolved regulatory capacities that enable them to respond efficiently to changes in carbon source and thereby shift gene regulation, a phenomenon called carbon catabolite repression (33, 34). The majority of low-GC Gram-positive bacteria depend on a LacI-type transcriptional regulator called CcpA to ensure that catabolic genes for less preferred carbohydrates are suppressed when a preferred carbohydrate (normally glucose) is present. Interestingly, *S. mutans* deviates from this paradigm in that CcpA plays a less direct role. Instead, the glucose/mannose-PTS (EII^{Man}) system regulates the expression of catabolic operons required for assimilating secondary carbohydrates such as oligo- and disaccharides (35). Inactivation of *manL*, which encodes the A and B domains of the EII^{Man} permease, results in enhanced expression of the *fruAB* and *levDEFG* (EII^{Lev}) operons. These operons contain genes which encode two different fructanases, FruAB for hydrolyzing fructose polymers (36), and a fructose/mannose-PTS (37), respectively. As a result, a *manL* mutant demonstrates improved growth on media supplemented with fructose polymers such as inulin or levan (35). CcpA and its cofactor HPr-Ser-P are also involved in this regulation (38, 39). Whereas CcpA interacts directly with cognate *cis*-elements called *cre* (catabolite response element) near the *fruA* promoter, ManL influences *fruAB* and *levDEFG* expression independently of CcpA (39). It is now believed that EII^{Man}-dependent glucose transport influences carbohydrate metabolism via substrate-specific signaling, which occurs primarily at submillimolar concentrations of carbohydrate (38). However, substrate-level regulation is also influenced indirectly by CcpA under excess-carbohydrate conditions, since CcpA appears to regulate the transcription of *manL* (38, 39). A transcriptomic study of a *manL* deletion strain validated the key roles of EII^{Man} in global gene regulation (40), and transcriptional studies of a *ccpA* deletion strain revealed both a central role for CcpA in controlling carbon flux and the existence of a substantial network of CcpA-independent genes (41, 42).

Catch and Release

To further explore substrate-dependent carbon catabolite repression in *S. mutans*, Zeng et al. assessed the relative contributions of various sucrolytic enzymes (31). The majority (>95%) of sucrose encountered by *S. mutans* is believed to be internalized via the EII^{Scr} PTS (43, 44), while the rest is likely metabolized extracellularly by the glucosyltransferases (GTFs) and fructosyltransferase. Enhanced transcription of the *fruAB* and EII^{Lev} operons was observed in the presence of sucrose, with such activation requiring both the sucrose PTS (ScrA) and the LevQRST regulatory pathways. LevQRST is an unconventional four-component system that activates transcription of *fruAB* and EII^{Lev} in response to extracellular fructose or mannose (37, 45). Since free fructose was detected in the culture media of an *S. mutans* strain deficient in all extracellular sucrolytic enzymes after treatment with sucrose (46), it was hypothesized that following sucrose internalization and subsequent cleavage of sucrose-6-P into glucose-6-P and unphosphorylated fructose by the ScrB hydrolase, some of the fructose is released into the medium and can activate the LevQRST circuit but is insufficient to trigger CcpA-dependent carbon catabolite repression. Such a strategy of carbohydrate metabolism would be energetically efficient and avoid perturbing intracellular ATP levels, which is particularly important to a bacterium such as *S. mutans* that actively pumps protons out of the cell at the expense of ATP to maintain pH homeostasis. Interestingly, fructose may not be the only carbohydrate to be released and reinternalized by *S. mutans*. Recent studies have characterized maltose and maltooligosaccharide metabolism and demonstrated the presence of free glucose in the culture medium that is apparently reinternalized by way of the glucose-PTS EII^{Man} (47, 48). Transient expulsion of glucose was also observed when *S. mutans* was grown on a mixture of glucose and lactose (49). Considering diffusion limitations within biofilm matrices (50), hexose release may likely serve as temporary energy

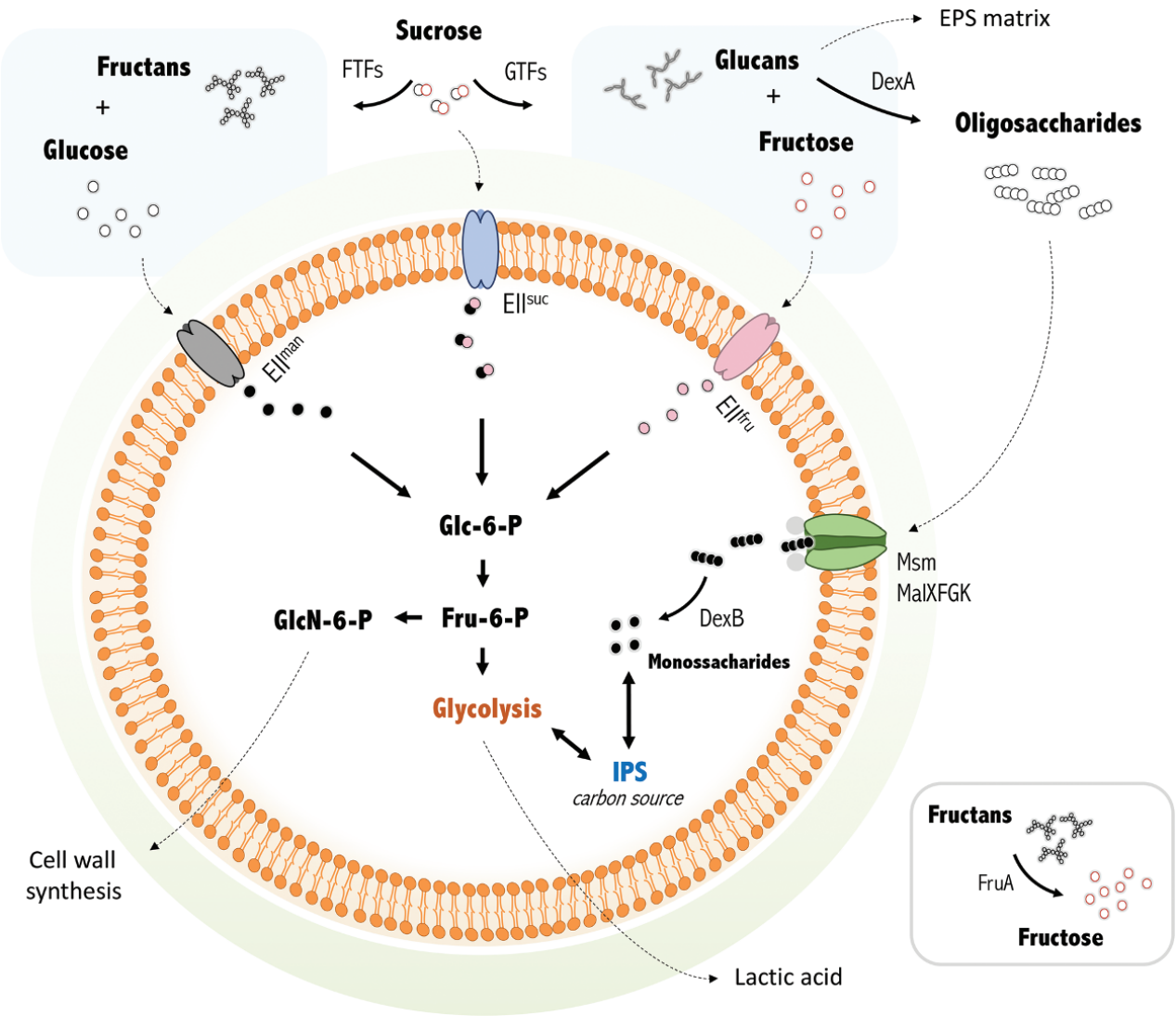


FIGURE 1 Carbohydrate metabolism in *S. mutans*. While *S. mutans* can metabolize a large variety of carbohydrates, the figure shows the metabolism of most common dietary sugars (fructose, glucose, and sucrose). Sucrose is a β2,1-linked disaccharide composed of glucose and fructose that has proven to be the most cariogenic of all carbohydrates. In the extracellular environment, sucrose is a substrate of glucosyltransferase (GTF) and fructosyltransferase (FTF) enzymes, which catalyze the production of glucans and fructans, respectively. The formation of glucans plays a key role in virulence, because they contribute to biofilm buildup by forming a glue-like polysaccharide matrix. Fructans serve as short-term extracellular carbohydrate sources and are degraded by the fructanase enzyme FruA, yielding fructose, which can be internalized for energy production. Glucans are susceptible to the action of an extracellular dextranase, DexA, which breaks down the α1,6-linkages, thereby yielding oligosaccharides (e.g., maltodextrans). After being transported into the cell, oligosaccharides are degraded into monosaccharides by the action of the DexB glucosidase. Oligosaccharides are primarily transported into the cells by ATP-binding cassette (ABC) transporters (e.g., Msm and MalXFGK transport systems), whereas monosaccharides (e.g., glucose and fructose) and disaccharides (e.g., sucrose) are predominantly taken up by the phospho-enolpyruvate:sugar PTS. In *S. mutans*, multiple PTSs can transport the same carbohydrate, with at least three PTSs being involved in fructose transport and several PTSs and permeases being involved in glucose transport. In the intracellular environment, carbohydrates are phosphorylated and processed to fructose-6-phosphate (Fru-6-P) and fermented by glycolysis with production of organic acids, mainly lactic acid. In addition, glucosamine-6-phosphate (GlcN-6-P) is synthesized from Fru-6-P and serves as an initial precursor for cell wall biosynthesis. Cells can synthesize an intracellular polysaccharide (IPS), a polymer of the glycogen-amylopectin type, when carbohydrates are in excess that can be stored as intracellular granules and are used as an energy source reserve during starvation.

storage and/or as a carbohydrate source for neighboring cells that may not be actively invested in metabolizing these disaccharides. This would thereby represent a bet-hedging strategy within the population and also contribute to a mutualistic relationship with other microorganisms that are closely associated with *S. mutans* (51, 52).

BIOFILM FORMATION

As a major etiological agent of human dental caries, *S. mutans* lives primarily in biofilms on the tooth surfaces, the so-called dental plaque. Strains of *S. mutans* produce up to three GTFs, GtfB, -C and, -D, that utilize the glucose moiety of sucrose as the substrate to synthesize glucose polymers of

glucans (also known as mutans) (Fig. 1) (7). GtfB synthesizes water-insoluble glucans rich in α(1-3)-linkages, GtfC produces a mixture of soluble glucans rich in α(1-6)-linkages and insoluble glucans, and GtfD makes primarily soluble glucans (often called dextran). These polymers, especially the α3,1-linked water-insoluble glucans, are major constituents of plaque biofilm matrices. Gtfs also bind to other oral microbes, even those that do not naturally express Gtfs, thereby converting them into *de facto* glucan producers (7). In addition, *S. mutans* encodes several surface-associated glucan-binding proteins, GbpA, -B, -C, and -D. Together, the Gtfs, Gbps, and adhesive glucans serve as an integrated scaffold for sucrose-dependent biofilm formation central to this organism's cariogenicity by promoting the local accumulation of microbial cells while forming a diffusion-limiting polymeric matrix that protects the embedded bacteria (Fig. 2).

S. mutans also possesses multiple high-affinity surface adhesins that enable colonization even in the absence of sucrose. One of the most widely studied adhesins is the dual antigen I/II, also known as P1, SpaP, or PAc. This structurally complex multifunctional adhesin mediates bacterial attachment to the tooth's salivary pellicle via interactions with the host scavenger receptor glycoprotein GP340 or DMBT-1 (53–55). The AgI/II family adhesins also interact with other

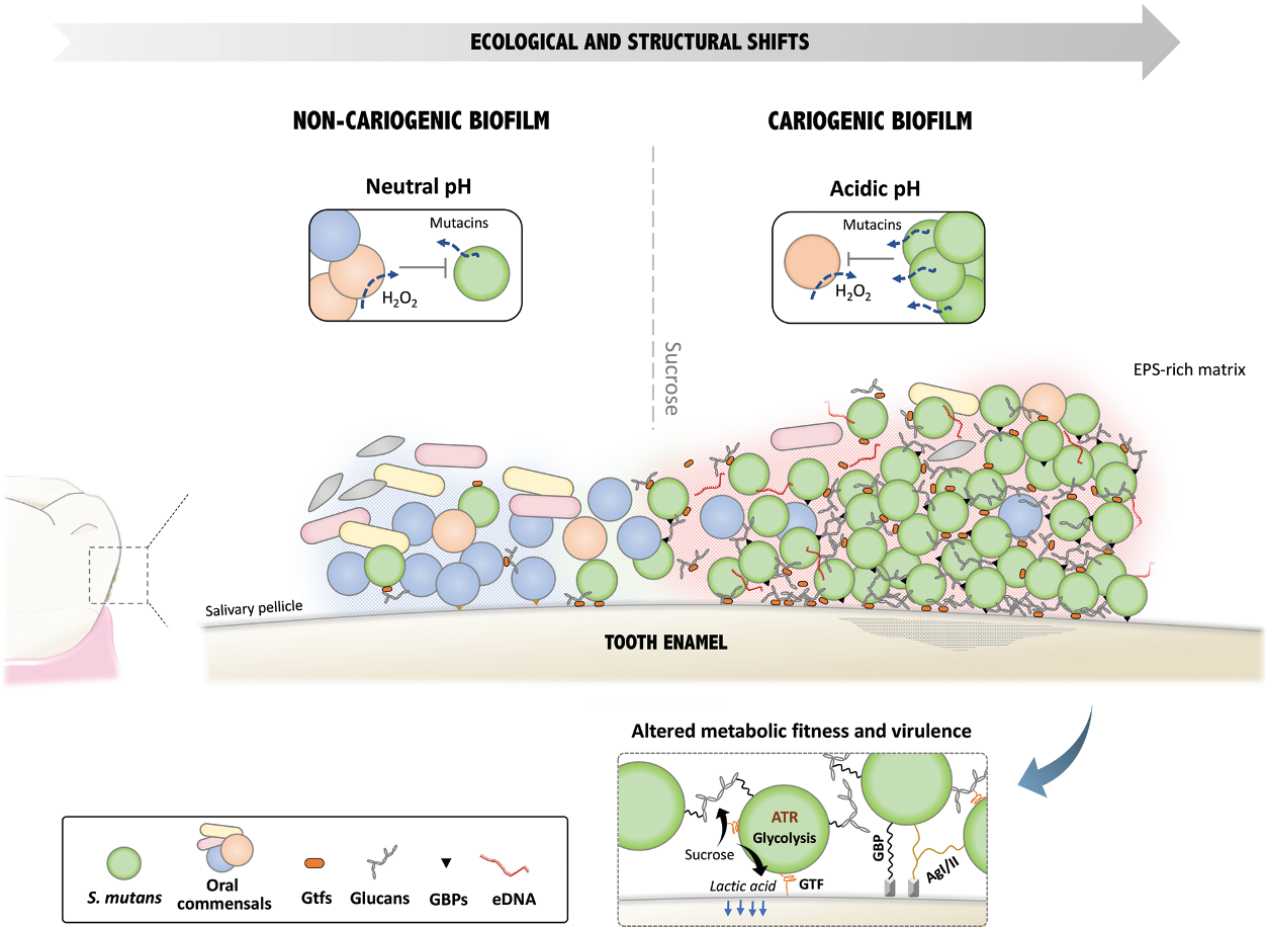


FIGURE 2 Biofilm formation and host-pathogen interactions in *S. mutans*. Early colonizers (e.g., *S. gordonii*, *Streptococcus mitis*, and *Actinomyces* spp. among others) attach to the tooth enamel via salivary proteins and start to form three-dimensional biofilms under noncariogenic conditions. At pH levels close to neutrality, production of H_2O_2 by peroxigenic bacteria and other antimicrobial products produced by oral commensals prevents the overgrowth of specific pathogens (e.g., *S. mutans*) on dental biofilms. Glycosyltransferases (GTFs) secreted by *S. mutans* adsorb onto the enamel pellicle or bacterial surfaces. In the presence of sucrose, GTFs catabolize sucrose to produce large amounts of insoluble and soluble glucans, which contribute to the buildup of a robust extracellular polysaccharide matrix (EPS), particularly insoluble components. The EPS matrix serves as an architectural scaffold for the biofilm structure, mediating tight adherence to the tooth enamel and bacteria, as glucans provide binding sites for *S. mutans* glucan-binding proteins (GBP) and other organisms. Extracellular DNA (eDNA) is another functional constituent of the oral biofilm matrix, forming nanofibers that connect cell to cell and cell to substratum and that contribute to biofilm structural integrity and stability. Continuous intake of sucrose by the host leads to a series of ecological and structural shifts that favor the growth of aciduric and highly acidogenic bacteria, such as *S. mutans*. These changes alter the oral biofilm metabolism so that copious amounts of organic acids are produced, contributing to a decrease in environmental pH. Once *S. mutans* becomes dominant, the secretion of large amounts of mutacins kills nearby competitors such as peroxigenic streptococci. The constant low-pH milieu surrounding the hydroxyapatite structure of the enamel leads to demineralization and initiates the carious process.

bacteria and host proteins such as fibronectin and collagen (53, 56). Relative to the wild type, a P1-deficient mutant demonstrates reduced binding to saliva or GP340-coated surfaces, aberrant biofilm formation, and reduced cariogenicity in a rat caries model (57, 58). In addition, P1 and WapA, another surface-localized adhesin, have been recently shown to form fibrillar amyloid aggregates (59). Amyloids are increasingly recognized as integral bacterial biofilm matrix components that interact with extracellular DNA (eDNA) and confer stability to the exopolysaccharide matrix (60). An *srtA* mutant lacking the only surface-anchoring sortase enzyme found in *S. mutans* is severely defective in biofilm development and does not produce amyloid, suggesting that fibril nucleation occurs at the cell surface (61). Treatment of *S. mutans* with known amyloid inhibitors inhibits biofilm formation via P1 and WapA-dependent mechanisms (59). In addition to P1 and WapA, a third amyloidogenic protein (SMU_63c) was recently identified. This secretory protein serves as an apparent negative regulator of biofilm cell density and genetic competence (59). Its production is associated with K^+ availability and influenced by growth phase (62).

Several cell envelope-associated proteins also contribute to *S. mutans* biofilm formation, including AtlA, RgpG, BrpA, and Psr. AtlA is an autolysin whose deficiency results in decreased autolysis and longer chain length and drastically reduces biofilm formation regardless of the carbohydrate used for growth (63). RgpG is the first enzyme of the biosynthetic pathway for rhamnose-containing glucose polymers, a major surface antigen of oral streptococci responsible for the different serotypes (64, 65). RgpG deficiency does not impair growth but leads to a pronounced reduction of cell surface antigens and major defects in cell morphology and cell division (65). RgpG-deficient mutants display long chains of swollen "squarish" dividing cells and form fewer biofilms irrespective of carbohydrate source. The BrpA and Psr proteins are members of the LytR-CpsA-Psr family of proteins that are widespread in Gram-positive bacteria (66–68). Deletion of *brpA* has little effect on growth but causes major impairment in biofilm formation. The *brpA* mutant is also less tolerant to acid and oxidative stresses and more susceptible to cell envelope antimicrobial agents, all of which likely contribute to its reduced biofilm phenotype. Psr also strongly influences *S. mutans* biofilm formation and acid tolerance, but unlike BrpA, Psr deficiency does not diminish oxidative stress tolerance or resistance to cell envelope antimicrobial agents. Both BrpA and Psr exhibit ligase functions for cell wall antigens, such as the rhamnose-containing glucose polymers, and thereby globally impact cell envelope composition. Deficiency of either BrpA or Psr results in a reduction of cell wall antigens and their concurrent accumulation in cell-free culture medium (65). Finally, eDNA is another functional constituent of the *S. mutans* biofilm matrix; eDNA forms nanofibers that connect cells to one another and to the substratum contributing to bacterial adherence and biofilm structural integrity and stability (69).

STRESS TOLERANCE

The ability of *S. mutans* to adapt to sudden and substantial environmental changes within the dental plaque is a key attribute that contributes to its status as the major etiologic agent of dental caries. Fermentable carbohydrates consumed by the host provide a substrate for *S. mutans* and other lactic acid bacteria that ultimately results in the production of acidic end products that accumulate within the biofilm. To thrive at low pH values, *S. mutans* mounts the so-called acid tolerance response, a robust transcriptional and physiologic adaptation mechanism that encompasses the induction of pathways that contribute to cytoplasm buffering and changes in membrane fatty acid composition, ultimately protecting the cellular machinery from acid damage and contributing to the survival of the bacteria during stress (Fig. 3) (3, 6, 70). Collectively, the different cellular processes that constitute the acid tolerance response contribute to the ability of *S. mutans* to maintain an intracellular pH that is more alkaline than the surrounding environment (ΔpH) by about 0.5 to 1 pH unit (71). Environmental acidification also triggers *S. mutans* to modify the composition of the plasma membrane in a way that may alter proton permeability. These modifications are accomplished by increasing the proportion of monounsaturated fatty acids (both by incorporating exogenous fatty acids and by *de novo* synthesis) and by increasing the length of the carbon chains composing these membrane fatty acids (72). Inactivation of the gene responsible for biosynthesis of monounsaturated fatty acids in *S. mutans*, *fabM*, results in extreme sensitivity to low pH, inability to maintain ΔpH, and reduced virulence in a rat caries model (73, 74). In addition to changes in fatty acid composition, the phospholipid cardiolipin has also emerged as an important contributor to acid tolerance, because deletion of the cardiolipin synthase (*cls*) increased acid sensitivity (75).

Alkalinization of the cytoplasm occurs either by pumping protons out of the cell or through the generation of neutralizing molecules. In *S. mutans*, the membrane-bound F_1F_0-ATPase (F-ATPase) is the primary mechanism by which protons are extruded to maintain pH homeostasis. The *S. mutans* F-ATPase is transcriptionally induced by low pH values and has an optimal pH of 6.0, which is lower than that of most streptococci associated with oral health (76, 77). Some oral streptococci utilize the urease enzyme (e.g., *Streptococcus salivarius*) or the arginine deiminase system (e.g., *Streptococcus gordonii*) to produce the neutralizing molecules ammonia and CO_2 in order to cope with acid stress. Although these pathways are absent in *S. mutans*, the agmatine deiminase system (AgDS), analogous to the arginine deiminase system, is present in this species (78, 79). The AgDS converts agmatine, a decarboxylated derivative of arginine found in dental plaque, to ammonia, CO_2, putrescine, and ATP. Though the AgDS does not appear to have a considerable impact on environmental alkalinization, the ammonia generated internally may contribute to neutralization of the cytoplasmic pH, while the ATP generated can be used to fuel proton extrusion via the F-ATPase (80). Malolactic fermentation converts malate, an acid commonly found in wine and in fruits such as apples, to the less acidic lactate and to CO_2. The CO_2 product can then be used for cytoplasmic neutralization by conversion to bicarbonate via carbonic anhydrase. In *S. mutans*, transcription of the genes encoding the malolactic enzyme and permease is acid-inducible, and malolactic fermentation activity was found to be optimal at an extracellular pH of 4.0 (81). Most importantly, malate was shown to protect *S. mutans* against acid killing and was associated with maintenance of ATP pools during starvation (82).

While the dental plaque environment was initially thought to be anaerobic, it is now known that the oral microbial community has a high capacity to reduce oxygen, resulting in the generation of toxic reactive oxygen species (83). Most *S. mutans* strains are susceptible to exposure to H_2O_2, which can be generated by metabolism of other species

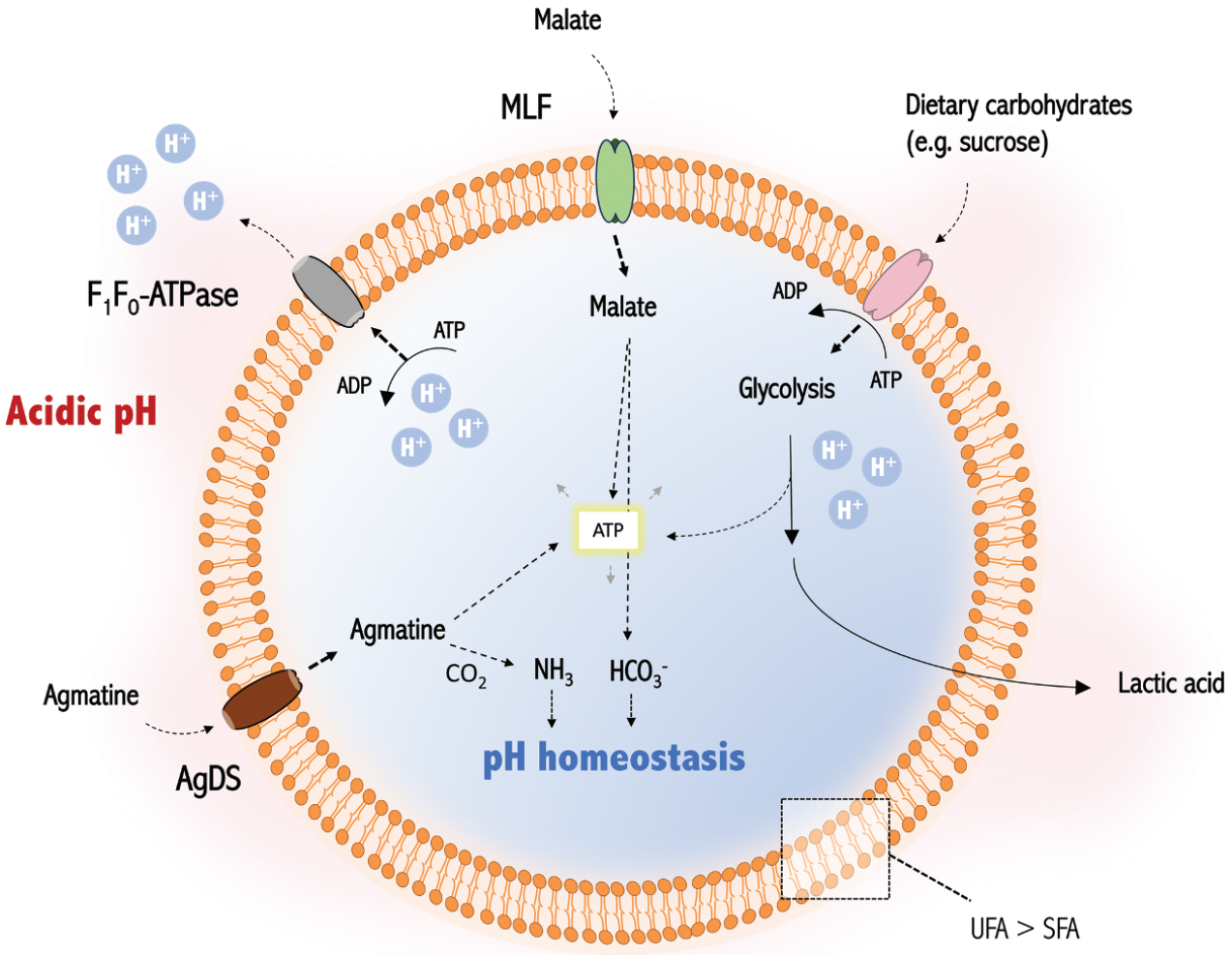


FIGURE 3 Acid stress tolerance mechanisms of *S. mutans*. The ability of *S. mutans* to catalyze fermentable dietary carbohydrates into organic acids can promptly drop the environmental pH. Exposure to sublethal low pH triggers an acid-adaptive response known as ATR (acid tolerance response), which is a robust transcriptional and physiologic adaptation mechanism for pH homeostasis through alteration of proton permeability, generation of neutralizing molecules, and changes in membrane fatty acid composition. The membrane F_1F_0-ATPase (F-ATPase) is transcriptionally induced by low pH and serves as the primary mechanism by which protons are extruded to maintain pH homeostasis. The modifications in membrane composition refer to an increase in the proportion of monounsaturated fatty acids (UFA) over saturated fatty acids (SFA) and in the length of the carbon chains composing the membrane fatty acids. Production of neutralizing molecules, such as ammonia and CO_2, is also an important way to cope with acid stress. In *S. mutans*, the agmatine deiminase system (AgDS) converts agmatine, a decarboxylated derivative of arginine found in dental plaque, to ammonia, CO_2, putrescine, and ATP. The ammonia generated internally may contribute to cytoplasmic buffering, while the ATP generated can be used to fuel proton extrusion via the F-ATPase. A decrease in environmental pH also triggers activation of malolactic fermentation (MLF), which converts malate to the less acidic lactate and to CO_2. The CO_2 product can then be used for cytoplasmic neutralization by conversion to bicarbonate (HCO_3^-) via carbonic anhydrase.

within the dental plaque, such as peroxigenic streptococci, or is contained as a component of oral hygiene and tooth bleaching products. Exposure to high levels of H_2O_2, and its more damaging breakdown products hydroxyl radical and superoxide anion, can rapidly cause irreversible cellular damage by triggering mismetallation of enzymes, by damaging proteins through oxidation of sulfurous amino acids and metal-binding sites, and by disturbing DNA integrity (84). Although oral streptococci lack catalase, *S. mutans* utilizes a number of scavenging and protective systems to prevent the accumulation of toxic reactive oxygen species. Among these are a manganese-dependent superoxide dismutase, an alkyl hydroperoxidase reductase, a thioredoxin reductase, and a glutaredoxin system (GshA/B/R) (85). Tight control of free iron in the cell is an important aspect of minimizing exposure to reactive oxygen species, because free radical formation is a direct result of the Fenton chemistry, when H_2O_2 comes into contact with ferrous iron. For this reason, the iron binding protein Dpr is a critical mediator of oxidative stress exposure in *S. mutans* (86, 87). Finally, the Spx regulators, SpxA1 and SpxA2, are responsible for the transcriptional activation of virtually every major oxidative stress response gene in *S. mutans* (85, 88). Not surprisingly, *spx* deletion strains were ill-equipped to tolerate oxidative challenges and caused fewer sulcal caries lesions in rats fed a highly cariogenic diet (85, 89, 90).

SIGNALING PATHWAYS

Two-Component Signal Transduction Systems (TCSTSs)

TCSTSs play important roles in bacterial adaptation, survival, and virulence by sensing changes in the environment and altering the expression of specific sets of genes to mount coordinated responses to environmental stimuli. In TCSTSs, a signal is sent by phosphotransfer between histidine senor kinases and their cognate response regulators, triggering the response regulators to dimerize and bind to conserved DNA motifs, ultimately tuning cellular functions such as biofilm formation, stress tolerance, and nutrient uptake (91). The genome of the type strain UA159 encodes 14 TCSTSs and one orphan response regulator with no genetic link to a histidine kinase (92). Among those, ComDE, LevRS, VicRK, and the orphan CovR regulator have been studied in some detail and shown to coordinate the expression of a number of virulence attributes. The LevRS system, which is important for management of carbohydrate metabolism, was introduced in the "Carbohydrate Metabolism" session, whereas the ComDE system, which is involved in the regulation of bacteriocin production and competence, is discussed in the next paragraph. VicRK influences acid and oxidative stress responses and competence and is the only TCSTS essential in *S. mutans* (93, 94). Several studies have proposed phosphotransfer cross talk between VicRK and the LiaFSR TCSTS, which contributes to surface adhesion, mutacin production, and the ability to tolerate environmental stresses, including cell envelope damage and heat shock (95–98). In addition, VicRK and CovR directly regulate genes implicated in the synthesis of and interaction with extracellular polysaccharides (7, 99). For example, transcription of *gbpB* is positively regulated by VicR (100, 101), while *gtfB*, *gtfC*, and *gbpC* are repressed by CovR (99).

Quorum Sensing (QS)

QS systems are communication networks that enable microbes to sense and respond to environmental conditions such as nutrient availability and population density, because signaling molecules naturally accumulate along with bacterial density. In Gram-positive bacteria, QS is coordinated by peptide pheromones, which serve as extracellular signaling molecules that trigger changes in gene expression and, ultimately, activation of a coordinated response by the population (102–104). In *S. mutans*, QS systems have evolved to regulate production of bacteriocins (known in *S. mutans* as mutacins), which are peptide antibiotics used in defense against other oral microbes (105–107) and in competence (natural transformation), a transient state in which the organism is primed to take up foreign DNA (Fig. 4). The competence-stimulating peptide signaling molecule (CSP) (also called the mutacin-inducing peptide) is encoded by *com*C. Once CSP has been secreted and processed, it is recognized by the TCSTS ComDE (104, 108, 109). Activated (phosphorylated) ComE regulates mutacin production by recognizing a conserved sequence in the promoter region of its target genes (95, 110, 111). Also sharing regulatory links with ComDE in *S. mutans* is another TCSTS, CiaRH, which contributes to biofilm formation, acid tolerance, and entry into competence, possibly by interacting with CSP (112). Among the consequences of ComE activation is the stimulation of *comRS*, the QS pathway directly responsible for competence activation via the alternative sigma factor ComX (also called SigX) (113, 114). Once exported to the extracellular space, the prepeptide ComS is presumably processed into the peptide pheromone XIP (*comX*- or *sigX*-inducing peptide) (114, 115). When imported back into the cell via the oligopeptide permease system, XIP is sensed by ComR, which binds to conserved inverted repeat sequences upstream of both *comX* and *comS*, thereby causing autoinduction of the ComRS pathway and activation of ComX-regulated genes. It is worth noting that while all *S. mutans* isolates sequenced to date possess a functional ComRS-ComX system, not all strains harbor a complete ComCDE pathway, nor are all isolates equally competent (15).

The mutacins produced by *S. mutans* are categorized as either lantibiotics (broadly active against Gram-positive bacteria) or nonlantibiotics (most active against closely related species), and production of these molecules is strain specific (116). Mutacins provide a competitive advantage to *S. mutans* by inhibiting the growth of neighboring bacterial species (117, 118). A close relationship exists between competence induction and mutacin production, because a threshold concentration of CSP activates ComDE to result in increased mutacin production (95, 111). Adding to the layers of regulation of mutacin production are two LytTR regulatory systems, HdrRM and BrsRM, which include a membrane-bound inhibitor protein that prompts the activity of the transcriptional regulator (119, 120). In this case, an overlap in the regulatory systems that govern mutacin production and competence is again observed because LytTR systems also induce competence through ComX (120, 121). A complete understanding of the environmental conditions that trigger these regulators and hence influence mutacin production is yet to be achieved.

Another *S. mutans* QS mechanism employs the autoinducer-2 (AI-2), the collective name for a group of furanones formed as a by-product of LuxS-mediated methyl metabolism (122). The lack of species specificity of AI-2 signaling molecules, which are produced by both Gram-positive and Gram-negative organisms, facilitates interspecies communication as population growth contributes to accumulation of AI-2. Deficiency of LuxS reduces the ability of *S. mutans* to tolerate acid and oxidative stress and to form biofilms, especially during growth in sucrose (123, 124). In agreement with those phenotypes, LuxS deficiency causes alterations in expression of a large number of genes, including those with known roles in stress tolerance response and biofilm formation (123–125). Addition of synthetic AI-2 to the growth medium can restore the phenotypes of LuxS mutants with regard to the stress tolerance response, biofilm formation, and the expression of selected genes (124, 125). To date, however, a specific receptor for AI-2 has yet to be identified (125).

Regulatory Nucleotides

The regulatory nucleotides (p)ppGpp and cyclic-di-AMP have also been shown to modulate biofilm formation and stress responses in *S. mutans* (126–128). Guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp), collectively called (p)ppGpp, are the effector molecules of a conserved stress response referred to as the stringent response (129). Three (p)ppGpp-metabolizing enzymes have been identified in *S. mutans*: RelA is a bifunctional synthetase/hydrolase contributing to metabolism of (p)ppGpp, while two small alarmone synthetase enzymes, RelP and RelQ, harbor only the synthetase domain (130). In *S. mutans*, changes in (p)ppGpp pools affect biofilm formation, carbohydrate metabolism, and stress tolerance, suggesting an overlap between circuits that govern general stress tolerance and biofilm formation. While the mechanism is not completely understood,

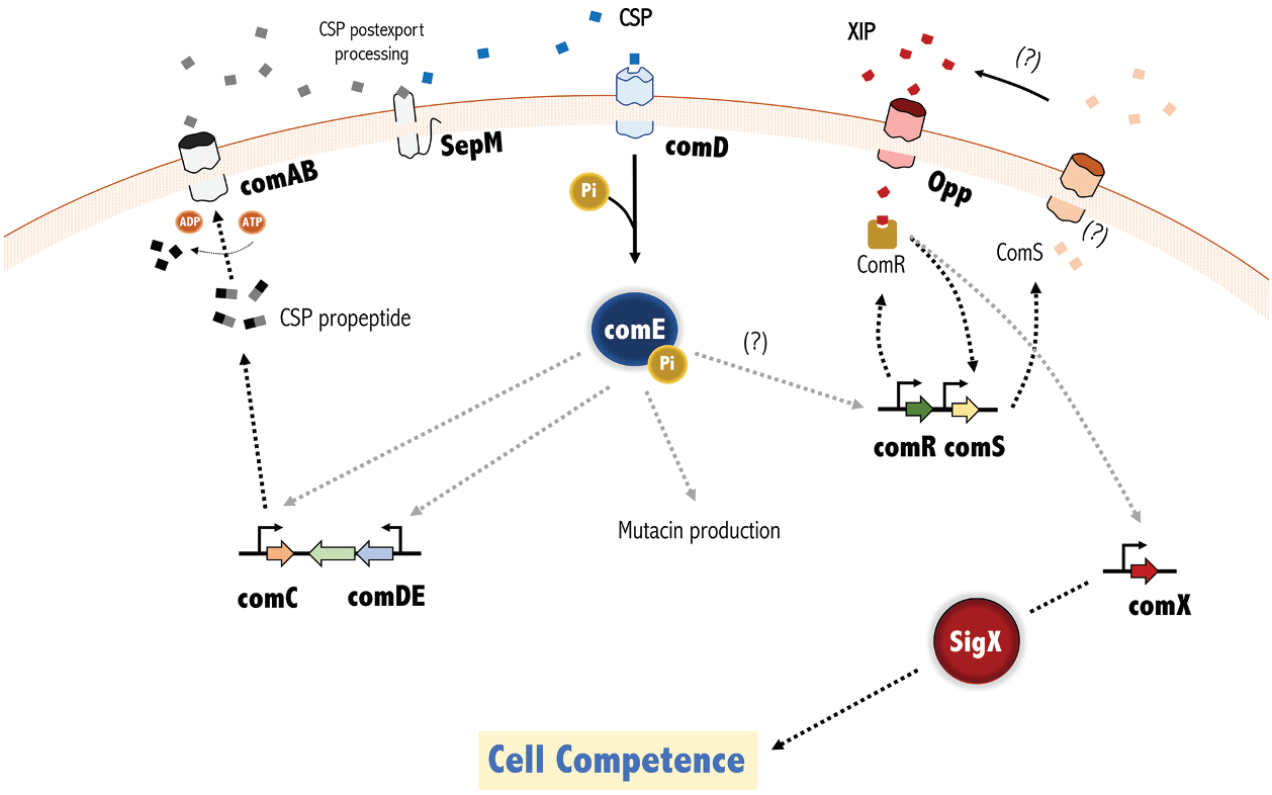


FIGURE 4 Quorum-sensing systems involved in the regulation of bacteriocin production and competence in *S. mutans*. Cell density, nutrient availability, and other environmental conditions induce the expression of *comC*, a gene encoding the competence-stimulating peptide (CSP) precursor. The CSP propeptide is cleaved off inside the cell and exported through a specific ABC transporter encoded by *comAB*. In the extracellular environment, CSP then undergoes a final postexport processing mediated by the SepM protease. Upon reaching a certain threshold, mature CSP is recognized by the two-component system ComDE, triggering a phosphorylation cascade. Activated (phosphorylated) ComE activates transcription of (i) *comC* and *comDE*, creating a positive feedback loop, (ii) genes involved in mutacin production, and (iii) by a yet-to-be-determined mechanism, *comRS*, the quorum-sensing pathway directly responsible for competence activation via the alternative sigma factor ComX. Once exported to the extracellular milieu, the prepeptide ComS is processed into the peptide pheromone XIP (*comX*- or *sigX*-inducing peptide). XIP is transported back into the cell via the oligopeptide permease system (Opp) and sensed by the Rgg-type regulator ComR. Activated ComR binds to both *comX* and *comS* promoters, thereby causing auto-induction of ComRS and activation of late (ComX-regulated) competence genes.

(p)ppGpp pools also modulate competence signaling through interactions with an ABC-type transporter, RcrRPQ, and at least one peptide encoded within *rcrQ* (126, 131). Cyclic di-AMP is an emerging second messenger in bacteria that has been shown to play important roles in bacterial fitness and virulence (132). In *S. mutans*, increased cyclic-di-AMP levels promoted biofilm formation through interactions with the VicRK-*gtfB* network that ultimately resulted in in the transcriptional activation of *gtfB* (128).

INTERSPECIES AND CROSS-KINGDOM INTERACTIONS

Interactions between *S. mutans* and certain members of the dental plaque community have been documented and have recently been shown to exert a major influence on the development and pathogenicity of plaque. These interactions can be synergistic, i.e., promote *S. mutans* growth, or antagonistic, i.e., inhibitory to *S. mutans*. A classic example of an antagonistic interaction occurs between *S. mutans* and members of the mitis streptococci group such as *Streptococcus sanguinis* and *S. gordonii*. Specifically, several members of the mitis group secrete millimolar concentrations of H_2O_2, which can be highly inhibitory to *S. mutans* (118). On the other hand, strains of *S. mutans* not only produce mutacins that are specific against mitis streptococci, but most are also generally more aciduric than species from the mitis group. This antagonistic relationship becomes more evident in dental plaque, whereby there is an inverse association between the abundance of oral commensal streptococci such as *S. sanguinis* and *S. gordonii* (abundant in healthy plaque) and that of *S. mutans* (abundant in caries lesions) (133) (Fig. 2).

While production of lactic acid by *S. mutans* is inhibitory to the growth of many acid-sensitive oral commensals, lactic acid serves as a carbon source for *Veillonella* spp., a prevalent genus in the oral cavity (134). Thus, it is thought that *Veillonella* may act as an "acid sink," preventing the dental biofilm from reaching extremely low pH values that even aciduric organisms such as *S. mutans* may not be able to survive. This nutritional mutualism may explain the close association of *Veillonella* with streptococci in the oral cavity, which excrete lactate as a waste product of carbohydrate fermentation. In dual-species biofilms, *S. mutans* and *Veillonella parvula* form distinctive structures and display increased resistance to chlorhexidine and other antimicrobials when compared to single-species biofilms (135, 136). Using a

three-species mixed culture, Liu et al. showed that the presence of *V. parvula* negated the growth inhibition of *S. mutans* caused by peroxigenic *S. gordonii* (137).

In addition to bacteria, the oral cavity is also colonized by yeasts. *Candida albicans* in particular is frequently found in the oral mucosa. Interestingly, *C. albicans* is often associated with *S. mutans* and detected in high numbers in cases of early childhood caries (138). Recent studies have shown that the *S. mutans* GtfB enzyme binds to mannan receptors on the *C. albicans* surface, leading to enhanced adherence and biofilm accumulation by *C. albicans* and thereby enhancing biofilm formation and cariogenicity (139, 140). At the same time, *C. albicans* synthesizes farnesol, which enhances extracellular polysaccharide production by *S. mutans* (52). In a rat caries model, coinfection with *S. mutans* and *C. albicans* led to higher levels of microbial carriage in plaque biofilms and significantly increased the cariogenic potential of dental plaque, resulting in aggressive disease onset and rampant caries lesions (140).

S. MUTANS AS A MODEL ORGANISM: SHIFTING LONG-STANDING PARADIGMS

Key advances in the field of bacteriology have come from studies with *Escherichia coli* and *Bacillus subtilis*, which represent the Gram-negative and Gram-positive bacterial paradigms, respectively. Despite the important contributions of these two species to bacteriology research, no model organism is a perfect representative of other species. In the case of Gram-positive organisms, *B. subtilis* is a free-living sporulating organism found in soil and plants but not in humans. Conversely, *S. mutans* is an obligate human pathogen with a biofilm-dependent lifestyle. It does not come as a surprise that a greater overlap in the mechanisms of gene regulation and metabolic pathways are observed between *S. mutans* and related Gram-positive pathogens that share a host-associated lifestyle. Such organisms, like *S. mutans*, generally have compact genomes with low GC contents. Like *E. coli* and *B. subtilis*, *S. mutans* is highly amenable to genetic manipulation and is therefore also easy to study and control in the laboratory setting. Among closely related streptococci, it has the most complete and sophisticated set of genetic tools that can be used in combination with simple and efficient *in vitro* and *in vivo* models to enhance our current understanding of *S. mutans* biology and beyond (141). As a result, a number of *S. mutans* investigations have challenged long-standing bacterial dogmas that were established based on work conducted with *E. coli* and *B. subtilis*. One such example is the signal recognition particle (SRP) pathway involved in cotranslational protein translocation (142). The first clue that protein transport in *S. mutans* differs from the established *E. coli* model came from the identification of an acid-sensitive transposon mutant in which *ffh* encoding the 54-kilodalton homolog of the eukaryotic SRP was insertionally inactivated. Subsequently, all three conserved bacterial elements of the cotranslational translocation SRP pathway, including the particle components Ffh and small 4.5S cytoplasmic RNA, along with the membrane-associated SRP receptor FtsY, were proven dispensable in *S. mutans*. This was noteworthy because the SRP pathway was long believed to be essential for viability in all living cells. An explanation for this surprise lay in the presence in *S. mutans* of two paralogs of the YidC/Oxa/Alb family of membrane-localized chaperone insertases found in bacteria, mitochondria, and chloroplasts. Unlike Gram-negative bacteria, most Gram-positive organisms possess two, if not more, YidC homologs. Another almost universal distinguishing feature between Gram-positive and Gram-negative bacteria, also discovered in *S. mutans*, is the SRP accessory factor YlxM, which interacts with both Ffh and scRNA and influences the GTPase activity of the Ffh/FtsY heterodimer, which is necessary for pathway component recycling once ribosome/nascent protein chain complexes are delivered to the membrane-localized SecYEG translocon (143). Another example occurred during the initial characterization of the enzyme responsible for the synthesis of (p)ppGpp in *S. mutans*, the effector molecule of the bacterial stringent response (see "Signaling Pathways" section). Before the work with *S. mutans*, the bifunctional RelA enzyme (also known as Rsh or Rel) was considered the sole enzymatic source of (p)ppGpp synthesis and degradation in all *Firmicutes*. The serendipitous finding that deletion of *relA* did not abolish (p)ppGpp production in *S. mutans* led to the discovery of two additional enzymes, RelP and RelQ, capable of synthesizing (p)ppGpp (130). Subsequent bioinformatics and genetic analysis revealed that these enzymes are ubiquitous in *Firmicutes*, and they were eventually found in other bacterial species.

S. MUTANS AS A THERAPEUTIC TARGET IN CARIES PREVENTION

While caries is a polymicrobial disease, selective targeting of *S. mutans* in dental biofilms is viewed as a suitable approach for its prevention. This is mainly because the synthesis of insoluble glucans from sucrose by *S. mutans* is central for the formation of a stable biofilm matrix that facilitates bacterial colonization of the tooth surface and, at the same time, serves as a diffusion barrier helping to maintain the acidic milieu within which cariogenic bacteria thrive (144). One of the current lines of thought in dental caries prevention is that it may be possible to halt the development of cariogenic biofilms by selectively targeting *S. mutans* such that the oral microbiome associated with health is not disturbed. Most of the early research conducted in the late 1960s into the 1980s focused on the development of anti-*S. mutans* vaccines. Despite some recent successes in animal models with vaccines containing or encoding single or a combination of antigens (145–148), emphasis on an immunoprophylactic approach has slowed somewhat due to the many challenges in obtaining long-term salivary IgA responses, safety concerns with antistreptococci antibody cross-reactivity, and providing species-specific protection without disrupting related beneficial commensal organisms. Over the past 2 decades or more, a number of natural products, such as propolis (149), curcumin (150), cranberry (151), and green tea extracts (152) among many others, have been shown to be effective against *S. mutans* biofilms and to prevent dental caries, although to date, none of these products has proven to be selective toward *S. mutans*. More recently, antimicrobial peptides (AMPs), small molecules, and probiotics have emerged as promising new approaches for the development of novel anticaries strategies that specifically target *S. mutans*. While AMPs kill bacteria indiscriminately, the selective killing of *S. mutans* by AMPs has been achieved by creating a synthetic peptide that combines a large-spectrum AMP with the *S. mutans*-specific CSP (153, 154). Phase 2 clinical trials recently showed that a single varnish application of one such peptide achieved significant reductions of *S. mutans* in the oral cavity (155). More recently, a small molecule derived from 2-aminotriazole (designated 3F1) was also shown to selectively disperse *S. mutans* biofilms (156). Most importantly, 3F1 was shown to reduce caries without disturbing the oral microbiome in a rat caries model. An alternative approach to combat *S. mutans* and therefore prevent dental caries may be

the use of alkalinogenic bacteria as a probiotic or the use of substrates for such alkalinogenic activities, e.g., arginine, as a prebiotic. This is because some oral bacteria can neutralize plaque pH by producing large quantities of ammonia by metabolizing arginine or urea, thereby creating an environment favorable to health-associated bacteria (157). A promising candidate is a highly arginolytic streptococcal strain, designated A12, isolated from supragingival plaque of a caries-free individual (158). Not only did the A12 strain neutralize acid by metabolizing arginine, but it was also shown to kill *S. mutans* by producing H_2O_2 and to interfere with signaling pathways that control bacteriocin production.

FUTURE PERSPECTIVES

Evidence accumulated over many decades has clearly shown that *S. mutans* is a major agent in dental caries vis-à-vis its capacity to orchestrate changes in the plaque microbiome via EPS and acid production. Thus, continued efforts to elucidate how *S. mutans* senses and responds to environmental cues through interconnected circuits that govern stress tolerance and biofilm formation can facilitate the identification of new targets for caries treatment and prevention. As the biology of *S. mutans* continues to be unraveled, a better understanding of the consequences of genomic and phenotypic heterogeneity among strains is an important area for development. Specifically, how *S. mutans* isolates with different properties contribute to the different stages of disease through synergistic or antagonistic interactions with the microbiome is a largely unexplored area. Future investigations should also consider the role of certain strains of *S. mutans* in systemic infections.

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Genetics of *sanguinis*-Group Streptococci in Health and Disease

ANGELA NOBBS AND JENS KRETH

28

INTRODUCTION

Our view of oral streptococci has largely been influenced by the approach taken in the last century to identify etiologic agents of disease. As a consequence, beneficial aspects of streptococcal colonization of the oral cavity were initially overlooked. The first comprehensive analysis of the resident oral microbiota was accomplished in 2005 (1), and with this, a new picture began to emerge. With the availability of high-throughput sequencing techniques and an increased sensitivity in analysis methods, the presence of a defined microbiome associated with oral health has been shown (2). Alongside this, "omics" techniques have revealed that prevalent oral diseases such as caries and periodontal disease are polymicrobial in nature and the result of microbial dysbiosis (3, 4). Even more striking, the metabolic output of these mixed microbial communities seems to be more relevant than their precise microbial composition (4). This is also reflected by the fact that the severity of caries and periodontal disease is heavily influenced by the synergistic interactions of the individual members of the polymicrobial consortium, including metabolic cross-feeding and interspecies signaling with transcriptional adjustment to the metabolic output. Thus, the ecological context of the microbial community seems to be of importance to understanding oral health and disease development. As a consequence, polymicrobial diseases cannot be explained by the behavior of one bacterial species and certainly cannot be treated like diseases that follow Koch's postulates (5–7). Novel approaches to combat oral polymicrobial diseases should therefore focus on the bacterial community that is present in the healthy oral cavity. Since oral streptococci are abundant during initial colonization of the tooth (8, 9), their function is to provide a favorable environment for incorporation of later species and to support accretion of the mature oral biofilm, which in general has a health-protecting function (10, 11).

One of the oral *Streptococcus* species that is repeatedly isolated in great abundance as part of the health-associated microbiome is the initial colonizer *Streptococcus sanguinis* (12–14). Because of the strong association of *S. sanguinis* with oral health, this commensal can serve as a model to understand how an individual species is able to interact with other members of the bacterial community to shape the composition of a benign oral biofilm. *S. sanguinis* is in general classified as a non-spore-forming, catalase-negative, chain-forming coccus. *S. sanguinis* is non-beta-hemolytic but is able to produce a green coloration on blood agar plates, referred to as alpha-hemolysis, which is a consequence of hydrogen peroxide (H_2O_2) production (15). *S. sanguinis* has been placed in the *mitis* group of streptococci based on 16S rRNA sequence analysis; however, it has also been classified in its own group together with *S. gordonii* and *S. parasanguinis* (15). A more recent analysis using the housekeeping genes *rpoB*, *sodA*, *ddl*, and *gdh* showed a more distant relationship with *S. parasanguinis* but confirmed the phylogenetic relationship with *S. gordonii* (16). Here, we present the *sanguinis*-group streptococci in the context of molecular commensalism, highlighting those aspects of their biology that are important for health-associated biofilm development, including polymicrobial interactions and regulatory and mechanistic events (Fig. 1).

MOLECULAR DETERMINANTS OF *S. SANGUINIS* AS A COMMENSAL PIONEER COLONIZER

Initial colonization is intimately linked to the adhesion capabilities of oral streptococci. *S. sanguinis*, together with *S. gordonii*, *S. oralis*, and *S. mitis*, are well adapted for initial colonization. These streptococci express a multitude of bacterial surface proteins, named adhesins, which are able to interact with salivary proteins covering the tooth surface (17–19). *S. sanguinis* seems to be particularly well equipped with adhesins that recognize this salivary pellicle (20). Overall, initial colonization has two important consequences. First, the ecological niche is occupied, which plays a significant role in the process of colonization resistance and the exclusion of potential incoming pathogens. Second, initial colonization shapes the overall composition of the biofilm, since the pioneer colonizer can produce metabolic products or provide a substratum for compatible partner species. Initial biofilm development requires the formation of macromolecular complexes. Complex formation is facilitated through electrostatic interactions of salivary proteins with the tooth surface to form the acquired enamel pellicle, a process that occurs within seconds after a clean enamel surface is exposed to saliva (21). Microbial attachment to the acquired enamel pellicle is then mediated via protein-protein

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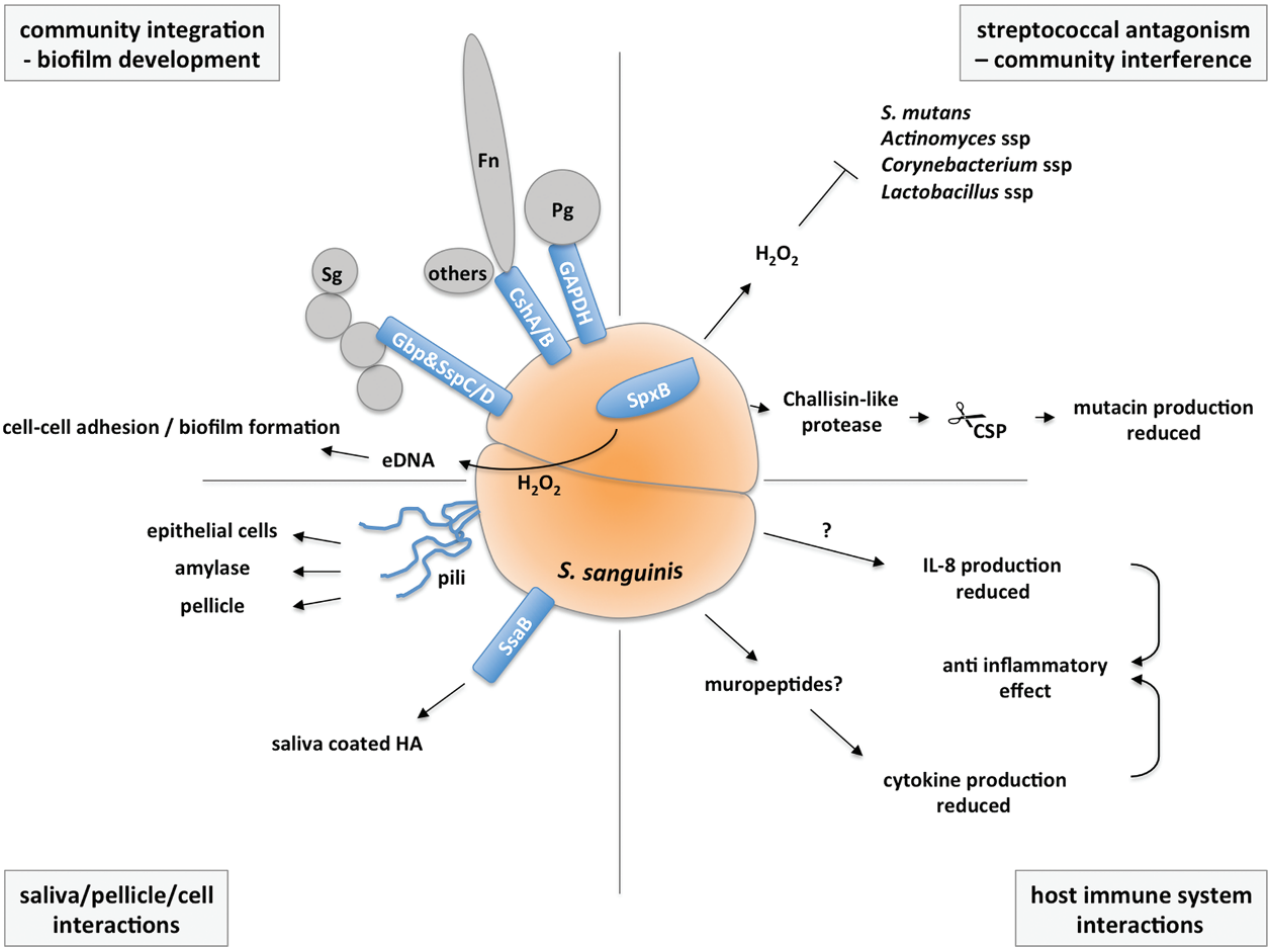


FIGURE 1 Summary of key *S. sanguinis* components important in commensalism. The schematic shows important components for the role of *S. sanguinis* as a commensal organism, including community integration and biofilm development, community interference and streptococcal antagonism, and interactions with salivary proteins, host cells, and the immune system. Pg, *Porphyromonas gingivalis*; Fn, *Fusobacterium nucleatum*; Sg, *Streptococcus gordonii*; eDNA, extracellular DNA; CSP, competence stimulating peptide. Reprinted with permission from reference 144.

and lectin-like interactions. The protein content of the acquired enamel pellicle is thus a major determinant of colonization sequence, dictating the strength of microbial adhesion as well as localization, since pellicle proteins seem to differ according to anatomical site (22).

A prominent protein in saliva and the acquired enamel pellicle is α-amylase, which is responsible for the catalytic hydrolysis of starch (23, 24). Amylase-binding proteins have been identified in several oral streptococcal species (24). The best studied is the amylase-binding protein A (AbpA) in *S. gordonii* (25). Mutation of AbpA results in deficient biofilm formation and bacterial adhesion *in vitro* (26). Although the sequenced reference strain *S. sanguinis* SK36 seems to encode an *abpA* homolog in a similar chromosomal context with its accessory sortase, *srtB* (27), its function is currently unknown. Interestingly, *S. sanguinis* is able to bind directly to surface-bound amylase and *vice versa* (24). This function has been shown to be mediated by long filamentous pili organized in a four-gene operon (ssa1631 to ssa1634) that encodes three pilin subunits (PilA-C) and a dedicated sortase (SrtC) for cell-surface anchoring of the pilin structural proteins (28). Pili are relatively long and thin appendages, and the pili of *S. sanguinis* can be as long as 1 μm, as shown by immune-gold staining of PilA (28). This poses a potentially interesting dynamic with AbpA, which is strictly confined to the outer cell surface, as shown for *S. parasanguinis*, also with immune-gold labeling (27). Taking into account that pili are flexible, one could hypothesize that binding to amylase in acquired enamel pellicle is possible even when the molecule is scarce, since the pili could serve as a flexible "arm," latching onto free amylase within saliva. A recent publication demonstrating that amylase in acquired enamel pellicle is actually less abundant than that in saliva (29) is in agreement with this hypothesis, suggesting an advantage of a flexible arm over a rigid arrangement on the bacterial surface. The pili also showed binding to other salivary proteins, and their deletion diminished biofilm formation on saliva-coated surfaces (28). However, the mutant was still able to bind amylase, albeit with lower efficiency (28), suggesting that other surface proteins are also able to bind amylase, possibly the aforementioned AbpA homolog (27). A pilus-bound amylase also offers the advantage of retaining about 50% of its enzymatic function (30). Gaining access to the amylase substrate starch through a flexible pilus would increase the chance for

hydrolysis of the alpha-1,4-glycosidic linkage into glucose, maltose, and maltodextrins, promoting sugar uptake and subsequent metabolism by simply increasing the accessible radius of the cell.

Two major mucins are found in saliva, MUC7 (low molecular weight) and MUC5B (high molecular weight) (31, 32). The majority of mucins are synthesized and secreted by the submandibular and sublingual glands, as well as minor glands located in the palatal, buccal, and labial mucosae. Mucins are heavily glycosylated glycoproteins and form a lubricating, viscoelastic coating on all oral surfaces. They are abundant proteins in saliva and the acquired enamel pellicle (31, 32). Both MUC7 and MUC5B contain sialic acid as a glycoconjugate, and this can be targeted by *S. sanguinis* SK36 sialic-acid-binding adhesin SrpA (33). SrpA contains a subdomain in its binding region that is similar to the V-set Ig-like fold adopted by mammalian Siglecs (sialic acid-binding immunoglobulin-like lectins) (34, 35). Indeed, Siglec-like domains have been identified in potential adhesins of several *S. sanguinis* isolates and other oral streptococci (34). Glycoarray dot blots with human salivary samples and naturally occurring glycoconjugates have demonstrated a high specificity of *S. sanguinis* SK36 SrpA for MUC7 but no binding to MUC5B. This is in contrast to *S. gordonii*, which showed, in general, better binding to several glycoconjugates, including MUC5B and amylase (34), which are known to form a heterotypic complex (36). As mentioned above, amylase and MUC5B seem to be depleted in the acquired enamel pellicle compared to saliva (29), but this was not seen for MUC7. Thus, the specificity of binding to MUC7 within the acquired enamel pellicle may go some way to explaining why *S. sanguinis* seems to be one of the first oral colonizers and is found in greater abundance than *S. gordonii*. The glycoarray also revealed an interesting role for divalent cations Ca^{2+} and Mg^{2+} in the binding of *S. sanguinis* SK36 to MUC7, as well as to other glycoconjugates (34). Chelation of Ca^{2+} and Mg^{2+} decreased binding to several of the tested components. This was also observed with two *S. gordonii* strains (34), suggesting a dominant role for divalent cations in the binding process of not only *S. sanguinis*, but of other oral streptococci to salivary components and the acquired enamel pellicle. This is further supported by the increased abundance of proteins known to bind divalent cations such as Ca^{2+} in the acquired enamel pellicle compared to saliva (29). Interestingly, *S. sanguinis* encodes a surface-associated, dual-function protein that bridges substratum attachment and interactions with divalent cations. SsaB is able to bind to saliva-coated hydroxyapatite through an unknown mechanism (37), but its principal function seems to be the transport of divalent cations (38). While this transport was demonstrated to be specific for Mn^{2+} and Fe^{2+}, playing a pivotal role in oxidative stress defense (38), other divalent cations such as Ca^{2+} and Mg^{2+} might still be able to interact with SsaB to facilitate binding to the acquired enamel pellicle.

Recently, the ability of *S. sanguinis* to be motile on surfaces such as solidified agar has been reported for several strains. Spreading zones after prolonged incubation can be observed around colonies, indicating active movement (39). Whether or not this so-called twitching motility has any function or is used as a means to disseminate within the oral biofilm is not yet clear. However, the *pil* locus that encodes the type IV pilus involved in twitching motility is conserved in most of the sequenced *S. sanguinis* genomes (39), suggesting biological importance.

Overall, *S. sanguinis* does not rely on a single mechanism to bind and establish itself within the acquired enamel pellicle. Rather, the process is elaborate and ensures the role of *S. sanguinis* as a pioneer colonizer. This correlates with the observation that signal peptidase I is required for biofilm development (40). In general, signal peptidases are membrane-bound endo-proteases that cleave the signal peptide portion from the majority of secreted proteins (41). *S. sanguinis* encodes two signal peptidases, SSA_0849 and SSA_0351, which are crucial for biofilm formation. Deletion of SSA_0351 abolishes biofilm formation but does not affect planktonic growth. Although the substrates for signal peptidase processing are not experimentally verified, *in silico* prediction identified 168 potential candidates, including several adhesins (40). Further characterization of signal peptidase-processed surface proteins will most likely identify new proteins involved in the adhesion process.

S. SANGUINIS IN BIOFILM FORMATION

The initial step in biofilm development is attachment, followed by microcolony formation of newly attached cells through growth expansion. This process then leads to a series of ordered and temporal events, in which coadhesion predominates, ultimately resulting in formation of a mature biofilm. The production of extracellular matrix material is a defining step in biofilm development and maturation. Matrix materials include polymers such as carbohydrates, proteins, extracellular DNA (eDNA), and lipids and are collectively referred to as extracellular polymeric substance (EPS) (42). Since EPS is produced by the biofilm inhabitants themselves, specific enzymatic functions and cellular processes are associated with its formation.

Exploiting the intake of dietary sugars, many oral *Streptococcus* species have evolved glucosyltransferases (Gtfs) that hydrolyze sucrose and polymerize the glucose into glucans. These, in turn, promote biofilm development. A single Gtf, GtfP, is carried on the *S. sanguinis* genome, which synthesizes mainly water-soluble α-1,6-linked glucans that branch at α-3,6-linked glucose residues (43). The TetR family regulator BrpT was recently identified as a repressor of *gtfP* expression. While a *gtfP* mutant formed only a fragile biofilm, the biofilm formed by the *brpT* mutant was thicker, more robust, and had a higher glucan content (44). By modulating the levels of glucan synthesis, BrpT may control the switching of *S. sanguinis* biofilms from an adherent to a dissemination state. *S. gordonii* also carries a single *gtf* gene, *gtf*G, encoding an enzyme that synthesizes both α-1,3- and α-1,6-linked glucans. This activity was found to promote mixed biofilm formation with *Candida albicans* (45), and a *gtf*G mutant was unable to persist on the tooth surface in rats (46). Expression of GtfG is under the positive regulation of Rgg (47), a homolog of which is present in *S. sanguinis*. As for other oral streptococci, it is likely that expression of Gtfs and thus the glucan content of biofilms is a tightly controlled process, affected by multiple factors and transcriptional regulators.

Compared to carbohydrates, the presence of eDNA within EPS is a relatively recent discovery. Nonetheless, growing evidence suggests that eDNA is a critical contributor to cell-to-cell adherence and overall biofilm stability (48). Courtesy of its negative charge, eDNA may facilitate association with the acquired salivary pellicle (49), and cell aggregation was shown to be promoted by eDNA for *S. sanguinis*, although not for *S. gordonii* (50). Visualization of "yarn" and "sweater" structures of eDNA that wrap around cells within *Enterococcus faecalis* biofilms (51) provides compelling evidence for how eDNA may contribute to the

structural integrity of biofilms, and similar structures have been seen for biofilms of *S. gordonii* (A.H. Nobbs, unpublished data). Moreover, eDNA regulates the viscoelastic properties of biofilms that allow them to withstand mechanical stress (52), a property that is particularly pertinent to the oral cavity environment. In line with this, the presence of DNABII proteins within the EPS of *S. gordonii* and other oral bacterial biofilms was recently found to be essential for eDNA integrity and biofilm structure (53). The regulation of eDNA release is not fully understood, and both lytic and active mechanisms have been reported. For *S. sanguinis* and *S. gordonii*, the autolysins LytF and AtlS play significant roles via mechanisms closely linked to H_2O_2 production and competence development. These are discussed in more detail below.

Multicellular entities such as biofilms require a high level of coordination, and quorum sensing (QS) circuits and two-component signaling systems (TCS) are intimately involved in these processes. One QS system found across several bacteria is LuxS/AI-2. LuxS is an integral component of the activated methyl cycle for correct methylation of nucleic acids and proteins but as a by-product of this cycle generates autoinducer 2 (AI-2), an interspecies chemical signal. LuxS mutants in *S. gordonii* and *S. sanguinis* were altered in their ability to form biofilms, and the lack of AI-2 resulted in altered *S. gordonii* microcolony architecture (54). However, a definitive role for AI-2 has not been identified, and for *S. sanguinis*, a disrupted activated methyl cycle rather than an absence of AI-2 was found to underpin the *luxS* mutant biofilm phenotype (55). A TCS associated with regulation of biofilm formation for both *S. sanguinis* and *S. gordonii* is BfrAB (56). This TCS modulates expression of two ABC transporters (BfrCD and BfrEFG) and a putative membrane-bound metalloprotease (BfrH). Given such functions, it is postulated that this system transports and processes proteins or peptides across the cell membrane that promote biofilm development, although the precise targets have yet to be identified. More recently, defects in the TCS SptRS have been found to promote biofilm formation by *S. sanguinis*, which is associated with elevated levels of H_2O_2 and eDNA (57), while stand-alone ArcR was identified as a key regulator of *S. gordonii* biofilm development, perhaps via modulation of the phosphotransferase system (58).

A final aspect of streptococcal biology that is closely associated with biofilm formation is competence development. This is a QS system that controls transformation, i.e., the capacity for bacteria to actively take up exogenous DNA, and in species such as *S. sanguinis* and *S. gordonii*, this is regulated via the *comCDE* operon (59, 60). The gene *comC* encodes a precursor molecule that is cleaved and exported via the ABC transporter ComAB, releasing the mature competence-stimulating peptide (CSP) into the local environment. For *S. gordonii* this is a 19-amino acid peptide, while the CSP of *S. sanguinis* has 17-amino acid residues, thereby minimizing interspecies cross-talk. The CSP is sensed by the TCS ComDE. Once the CSP pheromone exceeds a threshold concentration, ComD phosphorylates ComE, which upregulates expression of early competence genes, including *comCDE* and *comAB*, establishing a positive feedback loop, and *comX*. ComX then drives expression of the late competence genes required for DNA binding, uptake, and recombination. The coordination of competence and biofilm development contributes to the adaptability of bacteria such as *S. sanguinis* to changing environmental conditions via horizontal gene transfer. This is explored in more detail below.

S. SANGUINIS IN COMMUNITY DEVELOPMENT

S. sanguinis and *S. gordonii* can form monospecies biofilms, but within the host the biofilm communities are typically polymicrobial in nature. As pioneer colonizers, these streptococci can have profound consequences for niche occupation and subsequent colonization by incoming species and thus significantly influence whether a community is predisposed to health or disease. Such interactions do not occur at random but, rather, are directed in an ordered and temporal manner as a consequence of direct physical engagement (coadhesion), metabolic relationships, and interspecies communication.

Alongside streptococci, *Actinomyces* species constitute the predominant, health-associated early colonizers of the oral cavity, and both *S. sanguinis* and *S. gordonii* are able to coadhere with *Actinomyces oris*. This is mediated by recognition of the streptococcal receptor polysaccharide (RPS)-containing linkages GalNAcβ1-3Gal or Galβ1-3GalNAc by the FimA subunit of *A. oris* type 2 fimbriae (61, 62), with variations in the genetic loci for synthesis of RPS (*rps*) and RPS precursors (*rml*, *galE1*, *galE2*) subtly altering the coadhesion profile with different streptococcal species (63). In addition, the *S. gordonii* antigen I/II family protein SspB targets an extracellular polysaccharide produced by *A. oris*, although the precise composition and structure of this polysaccharide has yet to be determined (64). Once bound, *A. oris* may then promote *S. gordonii* survival under low arginine conditions by stabilization of arginine biosynthesis (65). Similarly, *S. gordonii* adhesin Hsa binds surface receptor Hag1 of early colonizer *Veillonella* species (66), but this interaction is also underpinned by a strong metabolic dependency. Lacking a fully functional glycolytic pathway, veillonellae must utilize hydroxyl acids for growth. These are provided by streptococci as the excreted metabolic waste product lactate, and utilization of lactate, in turn, protects streptococci from low pH (67). Once established, this community of pioneer colonizers then supports the incorporation of secondary or late colonizers, with *Fusobacterium nucleatum* serving as an important "bridging" organism due to its promiscuous coadhesion capabilities. *S. sanguinis* supports this engagement via interaction with the arginine-inhibitable adhesin RadD of *F. nucleatum* (68), while a second fusobacterial outer membrane protein, coaggregation-mediating protein A (CmpA), has recently been shown to promote biofilm formation with *S. gordonii* (69).

These community interactions with compatible species illustrate how *sanguinis*-group bacteria are able to promote development of a health-associated microbiota. Nonetheless, the social life of these bacteria is not exclusively beneficial, and other microbial partnerships may facilitate a more disease-prone state. Such examples have been described largely for *S. gordonii* rather than for *S. sanguinis*, leading to the designation of *S. gordonii* as an "accessory pathogen." Some of the best-characterized interactions are with the periodontopathogens *Porphyromonas gingivalis* (70, 71) and *Aggregatibacter actinomycetemcomitans* (72) and with the fungal pathogen *C. albicans* (73). Evidence from *in vitro* studies and animal models shows that these relationships can enhance both the persistence and virulence potentials of the microbes involved (74).

Chemical communication in modulation of the oral microbial community is exemplified by the complex effects of the interspecies signaling molecule AI-2. This molecule promotes dual-species biofilm formation between *S. gordonii* and *S. oralis* but can also modulate the relative proportions of these species in a concentration-dependent manner (75).

Likewise, while AI-2 from *F. nucleatum* promotes biofilm development with *S. gordonii*, it has the opposite effect on *S. oralis* (76). AI-2 from *S. gordonii* is essential for mutualistic biofilm growth with *A. oris* (77) but may also promote biofilm formation with *P. gingivalis* (78) and *C. albicans* (79). Ultimately, QS molecules and peptide pheromones work together with the molecular mechanisms described above to exquisitely coordinate biofilm development. The result is a community optimized to survive and persist under the prevailing environmental conditions in a manner that exceeds the capabilities of the individual component species.

COMPETITIVE BEHAVIOR: INHIBITION BY *S. SANGUINIS* H_2O_2 AND BACTERIOCIN PRODUCTION

Addressed above are examples of synergistic interactions with *sanguinis*-group streptococci that promote incorporation and retention of the partner microbes within the biofilm community. Nonetheless, within a defined ecological niche, resources are limited, and thus, competitive forces also work to shape the developing biofilm. One of the best-investigated competitive measures of *S. sanguinis* (and the vast majority of oral streptococci) is the production of H_2O_2 (80). The enzyme responsible for the production of H_2O_2 is pyruvate oxidase, SpxB (81, 82). SpxB is encoded by the majority of commensal oral streptococci, with an unusually high degree of conservation of over 96% amino acid identity compared to SpxB of *S. sanguinis* strain SK36. SpxB catalyzes the conversion of pyruvate to acetyl phosphate, CO_2, H_2O_2, and ATP. Therefore, besides generating inhibitory amounts of H_2O_2, SpxB confers a growth advantage on the producer via ATP generation during biofilm development (81, 82).

The overall importance of H_2O_2 production is further indicated by the fact that neither *S. sanguinis* nor *spxB*-encoding oral streptococci seem to encode for the H_2O_2 detoxifying enzyme catalase. Consequently, these bacteria are able to produce considerable amounts of H_2O_2 that can influence the surrounding environment and inhibit susceptible species. H_2O_2-dependent competitive behavior faces two challenges. First, SpxB requires oxygen for its activity (83, 84). Its production therefore declines once biofilm formation reaches a certain density resulting in an anaerobic environment. Second, released H_2O_2 is a substrate for detoxifying enzymes such as salivary lacto-peroxidase (85). Therefore, the effect of H_2O_2 production on biofilm development is confined to the immediate vicinity of the producer and most likely does not result in active killing of competitors. Rather, H_2O_2 affects susceptible species just enough to gain a growth advantage. For *S. sanguinis*, the oxygen-dependent production of H_2O_2 makes perfect sense from an ecological point of view. As the initial colonizer, *S. sanguinis* finds a sparsely inhabited environment with enough salivary oxygen tension to promote H_2O_2 production (86). However, once biofilm growth through proliferation and integration of compatible species is sufficient to result in a decline in oxygen tension, *S. sanguinis* is already an established member of the community, so H_2O_2 production is no longer required (80). The inhibitory spectrum of H_2O_2 as evaluated *in vitro* with deferred antagonism plate assays includes cariogenic *S. mutans*, as well as the periodontopathogens *A. actinomycetemcomitans*, *Prevotella intermedia*, and *P. gingivalis* (83, 87).

The genetic regulation of *spxB* expression holds a mystery. A clear repressive function of the carbon catabolite repressor CcpA has been shown since deletion of CcpA in *S. sanguinis* strain SK36 lifts *spxB* repression (88). Further, two *cre* (carbon responsive element) sites for the binding of CcpA have been verified experimentally (89), yet no glucose effect on *spxB* expression and H_2O_2 production can be observed. This is in contrast to other species such as *S. gordonii*, which follows classic carbon catabolite repression in the presence of glucose (90). Why CcpA in *S. sanguinis* does not respond to the presence of glucose, and if any environmental factor influences *spxB* expression, has yet to be determined. Compared to *S. gordonii* and other oral streptococci, *S. sanguinis* H_2O_2 production in general seems to be lower (89, 91), and a clue to the consequences of low H_2O_2 production and tight control by CcpA might be the increased susceptibility of a *S. sanguinis* CcpA knockout mutant toward its own H_2O_2 production (88). However, whether or not this is a direct effect of H_2O_2 or the result of altered gene expression from other CcpA-controlled genes is not known. From an ecological standpoint, the numerical abundance of *S. sanguinis* over other species might not require high production of H_2O_2, while other species such as *S. gordonii*, which is known to be a less prominent member of the oral biofilm, might require more aggressive H_2O_2 production to establish themselves within the oral community. Taking into account that oral streptococci intermingle during biofilm formation and can be found at the same location (92), the sum of H_2O_2 production could be more important than the quantity produced by individual species. Nevertheless, competitive H_2O_2 production and its role in community development is important and can be used to study biofilm dynamics.

Interestingly, *S. sanguinis* is also able to produce antimicrobial activity via bacteriocins. Several reports describe the antimicrobial activity of sanguicins (93, 94). Initial characterization showed bacteriostatic activity against a number of oral species, including *Actinomyces naeslundii*, but not against the periodontopathogens *P. gingivalis*, *P. intermedia*, and *F. nucleatum* (53). However, a more recent study using purified sanguicin demonstrated antimicrobial activity against such pathogens (93). Since both studies used a different strain of *S. sanguinis* and reported different molecular weights (65,000 Da versus 280,000 Da) for the purified peptide, it is most likely that different sanguicins were characterized. It is also worth mentioning that none of the studies determining the effect of H_2O_2 on periodontopathogens or *S. mutans* reported an inhibitory effect when catalase was added to the deferred antagonism assay (87, 95). Therefore, either strain-specific antagonistic activity exists or the culture conditions used in the studies were selective for specific production of bacteriocins or H_2O_2.

S. sanguinis bacteriocins are also reported to exhibit antifungal activity (96, 97). As part of the normal microbiota, *C. albicans* is commonly isolated from subjects but can also cause problems such as oral candidiasis (98). *S. sanguinis* produces a bacteriocin that can cause changes in the cell surface hydrophobicity of several *Candida* spp., a factor that influences the initial adhesion of *Candida* to oral epithelium. Furthermore, the bacteriocin can impair fungal cell membrane permeability and general cell structure (96, 97). How this ultimately affects survival of the fungal cell *in vivo* is not known, but analogous to the effect of H_2O_2 production, it might provide a competitive advantage to *S. sanguinis* by impairing the growth of competing oral fungi.

HORIZONTAL GENE TRANSFER AND THE ANTIBIOTIC RESISTANCE RESERVOIR

An important feature of oral streptococci is the ability to take up eDNA when developing competence, as discussed above.

While the process of competence development is well characterized at the molecular level (99), the origin of DNA in the environment and the molecular mechanisms of DNA release from bacterial cells are less well understood. As a vital part of the oral biofilm matrix, the eDNA available for uptake by competent bacteria is quite abundant (100, 101). In general, cell lysis can contribute to the release of DNA from bacterial cells. This process can be an active autolysis process involving murein hydrolases that weaken or completely lyse the cell wall, consequently expelling DNA into the environment (102). Interestingly, both *S. sanguinis* and *S. gordonii* release DNA in an H_2O_2-dependent manner under aerobic conditions (83). The eDNA is largely intact, high-molecular-weight DNA of chromosomal origin. Release of eDNA peaks when *S. sanguinis* grows aerobically under optimal H_2O_2-producing conditions, and release declines when cells are grown under oxygen-limiting conditions, when no H_2O_2 is produced. Furthermore, deletion of *spxB* severely impacts the release (83). However, eDNA release can be induced even under anaerobic conditions with the addition of H_2O_2, as shown for *S. gordonii*, but only when cells are metabolically active (103). The addition of H_2O_2 to cells suspended in buffer does not release DNA, indicating the requirement for active metabolism, gene expression, or protein synthesis. This is also consistent with the observation of a time delay between the production of H_2O_2 and the appearance of the released DNA and with the evidence that the addition of chloramphenicol, known to block synthesis of new proteins, also blocks release of DNA (103). This is relevant in the context of the oral biofilm, where SpxB-positive streptococci that have access to oxygen can produce H_2O_2 that might then diffuse through the oral biofilm with limited or no oxygen. Remarkably, the H_2O_2-dependent release of DNA is not the result of obvious cell lysis (50), as reported for other streptococci such as *S. pneumoniae* (104). Although the exact molecular mechanism involved in the release is not known at this moment, *S. sanguinis* might encode a dedicated system for eDNA release. This could involve partial lysis and/or active transport across the membrane. In a recent study, the transport of eDNA in *Haemophilus influenzae* was found to be dependent on the competence-related protein ComE (105), the transporter involved in the uptake of DNA. *S. sanguinis* encodes for ComE, and intriguingly, expression of competence genes seems to increase under aerobic conditions ideal for H_2O_2 production in streptococci (83, 103). However, experimental evidence that this mechanism is conserved in *S. sanguinis* is not available. Under anaerobic conditions, eDNA release is not completely absent, suggesting another mechanism involved in the release (50). One autolytic enzyme involved in this process in *S. sanguinis* is LytF, which is also under the control of the competence system as one of the early competence genes (106). LytF is a secreted autolysin and can be recovered from the environment in its active form. LytF is a fratricin but also acts on neighboring cells, causing release of eDNA not only from *S. sanguinis* but also from other streptococci (106). In general, streptococci seem to control or connect competence development with the release of eDNA (107), exemplifying their role as masters of efficacy. Utilizing this approach increases the chance that DNA is available for uptake and transformation.

Uptake of eDNA has two pronounced functions; first, it serves in horizontal gene transfer during bacterial transformation and therefore plays a pivotal role in the promotion of diversity among oral bacterial species, and is an important mechanism of evolution allowing the acquisition of new genetic traits stored in the genomic information contained in the multispecies biofilm (108). Second, eDNA in the oral cavity can serve as a reservoir for diverse antibiotic resistance mechanisms (109). A recent comparison of *S. sanguinis* and *S. gordonii* genomes revealed the presence of several genes potentially associated with antibiotic resistance determinants, including GCN5-related *N*-acetyltransferases, *parE*, and TetR family regulators (110). Combined, they potentially provide resistance to diverse antibiotics such as aminoglycosides, fluoroquinolones, and many more. Additionally, several genes were identified that encoded anion channels in *S. sanguinis* that confer resistance to fluoride (111), which is particularly important since fluoride is successfully used to prevent caries. Although the direct transfer of antibiotic resistance determinants via eDNA from other species to *S. sanguinis* has not been confirmed, evidence exists that, in general, the acquisition of new genetic traits occurs. For example, the pathway for vitamin B_{12} biosynthesis as well as the degradation of ethanolamine and propanediol, which is encoded on a large genomic region, has been acquired through horizontal gene transfer (112), although it is unknown if this was mediated through conjugation, transduction, or transformation. The best evidence for the potential of H_2O_2-induced transfer of antibiotic resistance was shown with *S. gordonii* using engineered strains carrying antibiotic resistance cassettes. Coincubation of strains encoding distinct antibiotic cassettes under ideal H_2O_2-producing conditions increased the occurrence of intraspecies genetic exchange by 300-fold compared to nonpermissive conditions (103). Overall, antibiotic resistance in the oral biofilm seems to be mainly acquired through horizontal gene transfer as suggested by a recent review (113). The dental biofilm is an ideal environment for the development and transfer of antibiotic resistance, even under conditions where no external pressure through antibiotic administration is present. This is supported by the finding that biofilm evolution and selective pressure through competitive bacterial interactions mediated by bacteriocins alone gave rise to antibiotic resistance (114). The oral microbiome is able to elicit this evolutionary pressure due to the abundance of bacteriocins (115).

S. SANGUINIS AS A MODULATOR OF THE HOST

While the predominant ecological niche of *S. sanguinis* is the tooth surface, dental plaque formed at the gingival margin brings the oral biofilm into contact with the oral epithelium and associated immune defenses. The host-microbe interplay that occurs at these sites represents a critical step in determining progression of the biofilm community below the gum line and potential transition from oral health to the onset of gingivitis, periodontitis, or other disease manifestations. Another facet of *S. sanguinis* as a coordinator of the microbial community is therefore its interactions with host tissues.

Under conditions of oral health, an equilibrium exists between microbiota and host, representing a delicate balance of antimicrobial factors from immune cells, together with pro- and anti-inflammatory molecules released from the host in response to the sustained microbial challenge. In contrast, disruption of this homeostasis is the hallmark of chronic inflammatory periodontal disease and resultant tissue damage. In keeping with this, *S. sanguinis* biofilms were found to be a poor stimulant of the proinflammatory cytokines interleukin-1α (IL-1α), IL-6, and IL-8 from OKF4 oral epithelial cells compared to biofilms of *F. nucleatum* (116). Likewise, in contrast to cell wall extracts of *F. nucleatum* or

P. gingivalis, those of *S. sanguinis* failed to induce significant upregulation by gingival keratinocytes of genes encoding human β defensin peptides, proinflammatory cytokines (e.g., IL-8), or matrix metalloproteinase-9. These keratinocyte responses were mediated by Toll-like receptor 2, with differences in acylation patterns of bacterial lipopeptides purported to underpin the differential outcomes of Toll-like receptor 2 stimulation by the bacterial species (117). Moreover, while only a weak stimulant of host responses itself, *S. sanguinis* is also able to suppress the effects of other microbes. In mixed culture, *S. sanguinis* impaired induction of IL-8 release from gingival HOK-18A epithelial cells by *A. actinomycetemcomitans*. Such effects were also seen with *S. sanguinis* spent culture medium alone, suggesting a secreted molecule as the mediator (118). Similarly, through blocking lipopolysaccharide engagement with the monocyte receptors lipopolysaccharide-binding protein and CD14, peptidoglycan from *S. sanguinis* was able to inhibit induction of genes encoding tumor necrosis factor-α, IL-6, and IL-8 by the periodontopathogens *P. gingivalis*, *A. actinomycetemcomitans*, and *Tannerella forsythia* (119). This mechanism correlates well with the observation that Gram-positive bacteria release large quantities of peptidoglycan fragments, muropeptides, during cell division (120). Such coordination of both host responses and the oral microbiota is likely critical to the role of *S. sanguinis* as a health-associated member of the oral biofilm community.

S. SANGUINIS AS AN ETIOLOGICAL AGENT OF EXTRAORAL DISEASES

Despite its main role as a benign oral commensal, the name *S. sanguinis* derives from its role in cardiovascular disease infective endocarditis (IE). In a note to the *Journal of Bacteriology*, Niven and White described a new species isolated from approximately 100 cases of subacute bacterial endocarditis (121). About one-third of the isolates failed characterization as previously described streptococci and were referred to as *Streptococcus* s.b.e. (for subacute bacterial endocarditis) (121). Overall, the group was quite homogeneous in its physiological characteristics (122). Further serological characterization confirmed the isolation of a new species since no cross-reactivity was observed between rabbit serum from *Streptococcus* s.b.e. and other identified streptococci belonging to various Lancefield groups (123). Interestingly, isolation of *Streptococcus* s.b.e. from the human throat was unsuccessful at that time, despite testing over 800 streptococcal isolates. The only other positive culture came from an extracted tooth, which we now know coincides with the preferred colonization site. However, in the original publication, the natural habitat was not identified, and the only source was from the blood of endocarditis patients; hence the species name *sanguis*, Latin for "blood" (122, 124). This has been changed fairly recently to the grammatically correct version, *S. sanguinis* (125).

IE is a relatively rare but potentially fatal disease and can affect the heart valves or endocardium. The annual incidence ranges from 3 to 7 cases per 100,000 people and has been relatively constant, whereas the etiology of IE has changed over time (126, 127). A major concern with this disease is that mortality rates remain high; the in-hospital mortality rate ranges from 15 to 22%, with a 5-year mortality rate of around 40%. Currently, *Staphylococcus aureus* is the most common pathogen associated with IE. However, oral streptococci are responsible for an estimated 35 to 40% of cases, with *sanguinis*-group species being the most common isolates (128).

Our understanding of the molecular mechanisms that might contribute to the capacity for *S. sanguinis* to cause IE has benefited from the availability of an excellent animal model for IE. New Zealand white rabbits can be catheterized at specific sites of the heart, causing injury-induced IE after bacterial blood inoculation (129). The injury provides exposed extracellular matrix components, fibrin, and platelets for attachment of the bacterial cells and subsequent aberrant clot formation, which leads to infective vegetations forming on the heart valves. Todd Kitten's group at Virginia Commonwealth University has used this model to decipher molecular determinants expressed by *S. sanguinis* during the infection process. Using a random signature-tagged mutagenesis approach, six chromosomal loci were identified from an initial screen of 800 mutants. The mutants carried transposons in an intergenic region and in genes encoding undecaprenol kinase, homoserine kinase, anaerobic ribonucleotide reductase, adenylosuccinate lyase, and a hypothetical protein (130). The screening method is elegant but has some technical limitations, since important determinants such as surface-exposed proteins, which have been shown to contribute to the infection process (131), were missed. Nonetheless, the identification of determinants regarded as housekeeping genes involved in cell wall (undecaprenol kinase), amino acid (homoserine kinase), and nucleic acid (ribonucleotide reductase) synthesis suggested a potential role for these genes in pathogenesis that had not previously been appreciated. Furthermore, since these genes are not found in humans, they may represent potential targets for drug development. However, a functional analysis of the clonal structure of *S. sanguinis* strains isolated from the oral cavity and from subjects with IE demonstrated that housekeeping and virulence genes are subject to considerable intraspecies recombination events. Thus, potential drug targets in such genes may correlate with a high potential for the selection of resistant mutants (132). Interestingly, the same study also concluded that the endocarditis strains did not form a distinct subcluster. This supports the notion that *S. sanguinis* strains are human pathobionts, and thus all strains have the potential to cause IE (132).

Following from this study, a targeted signature-tagged mutagenesis approach was employed to identify surface proteins important in IE. A total of 33 proteins were classified as cell wall associated, and of these, mutants in 31 were tested in the aforementioned rabbit model of IE, alongside mutants in 3 sortase genes (133). Interestingly, no single cell wall-associated protein was found to be essential for the development of IE (133). It may be concluded from this that *S. sanguinis* does not require any specific virulence determinants to cause IE. Rather, what is expressed and present during colonization of the oral cavity may be sufficient to colonize the endocardium. In support of this, the surface adhesins Hsa, PadA, and SspA/B from *S. gordonii* have been implicated in IE through their engagement with platelets but also contribute to the capacity for *S. gordonii* to bind salivary pellicle and other oral microbes (134–137). Such highly optimized genomes might explain the success of these bacteria as early colonizers, with their ability to cause IE on rare occasions coincident in their ubiquitous presence and abundance within the oral cavity.

Mutations in the genes encoding lipoprotein SsaB (a putative manganese transport protein) (38), *spxA1* (encoding a global regulator involved in H_2O_2 production) (138), and *nox* (encoding an NADH oxidase that also influences H_2O_2 production) (139), together with the anaerobic ribonucleotide reductase already mentioned, have all been

shown to impair the capacity of *S. sanguinis* to cause IE. This suggests that the ability of *S. sanguinis* to adapt to differences in oxygen tension and the production of and resistance to reactive oxygen species are important in the development of IE, although molecular details have yet to be explained. A surface-bound nuclease designated SWAN (streptococcal wall-anchored nuclease) has also been proposed to enable *S. sanguinis* to evade killing by neutrophil extracellular traps and thus to promote survival both in the bloodstream and within infective vegetations (140).

Surviving within the bloodstream to access extraoral sites and inducing thrombosis are key steps in IE, but these capabilities also associate *S. sanguinis* with other systemic diseases. These include meningitis, following infection of the lining of the spinal cord or brain, and disseminated intravascular coagulation, in which aberrant activation of the coagulation cascade leads to the formation of small clots that can occlude blood flow to major organs and tissues (141, 142). It is also widely recognized that oral streptococci such as *S. sanguinis* exist in biofilm communities with *Pseudomonas aeruginosa* in the lungs of cystic fibrosis patients. However, with evidence that H_2O_2 production by these streptococci can impair *P. aeruginosa* growth (143), the impact of *S. sanguinis* on cystic fibrosis disease progression remains to be fully understood.

CONCLUSION AND OUTLOOK

Considering the impact on the individual, it is easy to understand why disease outcomes have historically dominated microbiological research. Nonetheless, technological advances are providing a growing appreciation for the importance of our resident microbiota. Within the oral cavity, bacteria such as *S. sanguinis* are ubiquitous and abundant, reflecting their evolution to be exquisitely adapted to colonization of their ecological niche. As a consequence, *S. sanguinis* is able to orchestrate accretion of the dental plaque biofilm, promoting the acquisition of beneficial microbes while serving as an imposing competitor to others and ultimately providing the foundation of a health-associated biofilm community. Complex molecular mechanisms regulating physical interactions and communication networks underpin these capabilities. If these can be understood at both the molecular and ecological levels, they offer immense potential for exploitation in the development of novel strategies to combat infections from a point of health as opposed to disease.

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Genetics of Lactococci

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29

INTRODUCTION

Lactococci have been used for centuries in dairy fermentation. These Gram-positive, generally nonpathogenic, nonmotile, and nonsporulating bacteria are members of the *Streptococcaceae* family, which includes food, commensal, and virulent species (Fig. 1). *Lactococcus lactis* is a relatively simple bacterium, with a 2.4-Mbp genome. Many of its functions of interest are nonredundant, which facilitates functional genetic studies of nonessential genes. Lactococci are presumed to be devoid of virulence factors (although isolated cases of *L. lactis* as the infectious agent in human and bovine infections have been reported [1, 2]). The goal of this article is to confront previous and current information in different areas of lactococcal genetics, keeping in mind the relevance of findings to related bacteria, especially pathogens. Work on pathogens has long focused on surface and secreted virulence factors, while work on lactococci has gone deeper in characterizing basic metabolic properties, nutrient uptake, and survival. Genes in basic metabolic pathways (e.g., respiration, metal homeostasis, amino acid metabolism) are now known to be essential not only for fitness but also for virulence. Numerous *Lactococcus* researchers who shifted their focus to pathogens have contributed to this understanding. The overall nonvirulence of lactococci has also been useful in determining how metabolic and virulence factors participate in bacterial "everyday life" outside the animal host. Our deep knowledge of *L. lactis* physiology has led to new concepts and general findings, for example by (i) establishing the bases for dialog between *Firmicutes*, (ii) providing concrete *in vivo* data on the biomedical or probiotic potential of recombinant and wild-type lactic acid bacteria (LAB) (3, 4), and (iii) establishing the existence of an inverse correlation between bacterial mRNA concentration and stability (5).

This article, organized in five sections, highlights major work in lactococci, including metabolic capacities, physiology, stress response, interbacterial effects, and studies leading to novel uses of lactococci for protein delivery or as probiotics. Prophages have a primordial role in fermentation processes, genetic diversity, expression, and cell lysis; this important area of research, including abortive infection mechanisms, is not a focus of this review (see 6–12). Comparative *L. lactis* genome organization analyses are considered in references 13 and 14 and references therein.

The first section, "Basic Features of Lactococci," presents a primary description of *L. lactis* and its relationship to other *Streptococcaceae*. The next section is "Metabolic Options for Lactococci." Because lactococci are industrial bacteria, the study of them has focused on optimizing growth and flavor production during fermentation. Lactococci shift to a respiration metabolism when provided with an exogenous heme source. Metabolic flexibility provides a valuable prototype for the lifestyle of other LAB and certain streptococcal pathogens, which respire when supplied with heme and menaquinones. Nitrogen metabolism is extensively studied in *L. lactis*, because it determines the capacity to grow in dairy medium for food fermentation. Remarkably, nucleotide metabolism coordinates intracellular pools with the envelope state. The third section is "Lactococcal Stress Responses." Lactococci, though largely consumed through fermented foods, have been considered noncolonizers of the animal host. Lactococci express numerous and diverse factors that allow bacteria to respond and survive in a crowded or hostile environment and were identified by simple selective systems. Unique structures discovered on the lactococcal envelope (pellicles and pili) may extend survival in harsh environments, notably the gut, and narrow the divide between food bacteria and pathogens. The fourth section, "*L. lactis* and Interbacterial Dialogue" discusses how lactococci impact expression and/or development of other bacteria in their biotopes, including pathogens. Several findings may prove valuable when considering the roles of cognate functions in pathogens. The last section is "Applications and Tools of Lactococci." Lactococci are useful bacterial vectors for biomolecule delivery. Protein expression systems were developed in lactococci for applications in biotechnology and have been used for expression in related low-GC-content Gram-positive bacteria, including pathogens; remarkably, some of them have stayed in use for over 25 years. As a simple nonpathogen, *L. lactis* is a useful host for separating potential virulence factors from pathogenic bacteria and analyzing their roles. While medical applications have not been marketed, numerous proof of concept studies show the feasibility

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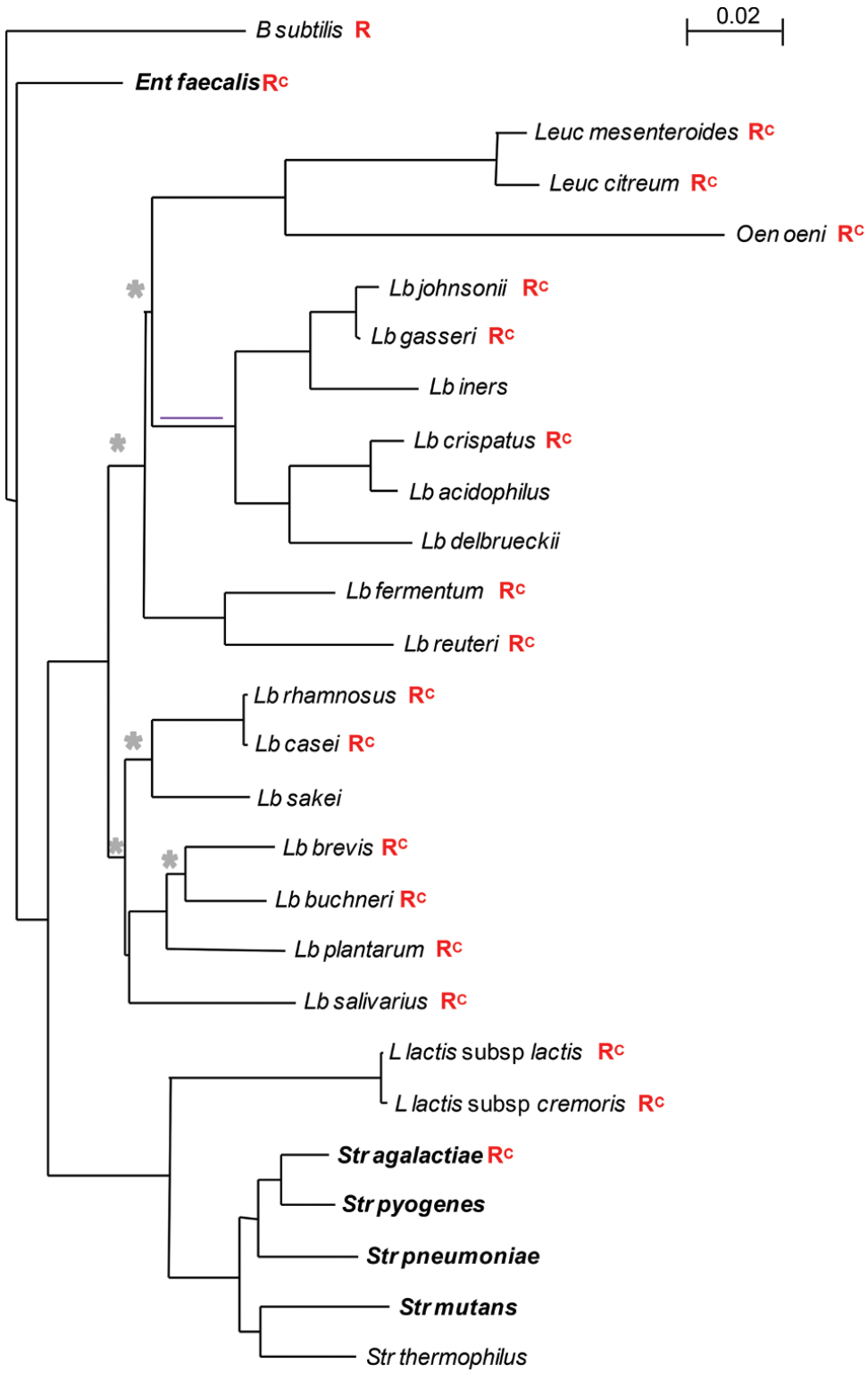


FIGURE 1 The phylogenetic tree reveals similarities between lactococci and streptococcal pathogens. A phylogenetic tree built on 16S sequences was constructed directly on the Ribosomal Database interface (210). Branches with a bootstrap value below 60% are indicated with an asterisk. Respiration capacity (see text) is indicated by a red R. R^c indicates conditional respiration: for *L. lactis*, *E. faecalis*, and *Leuconostoc* spp., aerobic respiration is activated by exogenous heme. For all *Lactobacillus* spp. and *S. agalactiae*, respiration is activated by exogenous heme and menaquinone. Opportunist pathogens are indicated in bold. *B subtilis*, *Bacillus subtilis*; *Ent*, *Enterococcus*; *Leuc*, *Leuconostoc*; *Oen*, *Oenococcus*; *Lb*, *Lactobacillus*; *Str*, *Streptococcus*. This figure is based on reference 155.

of using *L. lactis* or other LAB as biomolecule delivery vectors (see 15 and 16 for reviews).

BASIC FEATURES OF LACTOCOCCI

What Is *L. lactis*?

LAB are named for their ability to produce lactic acid via fermentation metabolism. *L. lactis* is a mesophilic LAB with an optimal growth temperature of ~30°C. It is the most extensively characterized of the LAB, which comprise a highly diverse group (Table 1, Fig. 1), including various cocci and bacilli. However, the term "LAB" is misleading, because although "LAB" generally refers to bacteria used in food fermentation, lactic acid producers also include opportunists and pathogens, including streptococci and enterococci; of note, *Enterococcus faecalis*, despite its reported beneficial effects for fermentation, has now clearly emerged as an important clinical antibiotic-resistant opportunist pathogen that may lead to intestinal dysbiosis after antibiotic treatments (17–19). Among sequenced relatives, *L. lactis* is related to *Streptococcus mutans* (Fig. 1). The 2.4-Mb genome of *L. lactis* strain IL1403 is intermediate in size between streptococcal pathogens such as *Streptococcus pneumoniae* and *Streptococcus pyogenes* (reported as 2.1 and 1.9 Mb, respectively) and *E. faecalis* (3.4 Mb). Although described as "cocci," *L. lactis* subpopulations may differentiate to a rod shape, possibly reflecting a change in the proportions of wall and division enzymes (20).

L. lactis is classified within the *Streptococcaceae* family, and it appears that *L. lactis* and the pathogenic streptococci may have a common origin. In addition to high genetic relatedness, DNA motifs called Chi, which are required for chromosomal integrity and are overrepresented in *L. lactis*, are identical to Chi sites present in both pathogenic and food-derived *Streptococcaceae*, providing evidence that a common core genome organization preceded divergence of streptococcal pathogens and food bacteria (21). Relatedness between *L. lactis* and streptococci surpasses by far that with many other LAB (including *Lactobacillus* species; Fig. 1). Streptococci also produce lactic acid and thus may be considered a branch of LAB. The *Streptococcaceae* family diverged as the result of unknown selective pressures to generate groups of pathogens, colonizers, and food bacteria, which seem to have diversified to adapt to their preferred biotopes.

Varied Lifestyle of LAB

L. lactis and LAB in general seem to have a varied lifestyle. Lactococci are isolated from plants and are likely to be ingested by grazing animals, together with milk in the case of calves. Coingestion could explain how lactococci ended up in milk. The need for several plasmid- or transposon-encoded characteristics for growth in milk (e.g., enzymes for sugar and protein metabolism, DNA restriction and abortive phage infection functions, and bacteriocins) supports the hypothesis that milk is not the original habitat of LAB.

Genome Plasticity

Studies of lactococci have focused on two organisms, IL1403, an *L. lactis* subsp. *lactis* strain, and MG1363, an *L. lactis* subsp. *cremoris* strain. Despite ~80% sequence identity between these genomes, they differ by a large chromosomal inversion (22). Even closely related isolates of MG1363 show considerable polymorphism, corresponding to large rearrangements (23) that might be mediated by mobile elements. Studies of artificial chromosomal rearrangements suggest that lactococci tolerate certain large genomic inversions if the origin and terminus regions are not disturbed (24).

Genome transfer and rearrangements may occur in lactococci via conjugation, transposition or insertion sequences, and phage transduction, as suggested by the identification of the required elements in different species, or by experimental systems in which natural DNA transfer occurs (see 25–28 for examples). DNA transfer occurs in streptococci by natural competence, as first discovered and then characterized in *S. pneumoniae* (29). Since then, competence among LABs was shown for *Leuconostoc mesenteroides* and *Streptococcus thermophilus* (30–33). *L. lactis* strains encode homologs of all late competence genes that are regulated by ComX; *com*X overexpression led to the induction of several genes linked to late competence (e.g., *com*GA, *GB*, *GC*, *GD*, *EA*, *EC*, *FA*, *FC*, *C*, *dprA*, and *coiA*), but these bacteria seemed to be defective for competence (34). Nevertheless, overexpression of ComX did lead to competence in a subgroup of lactococci (35, 36) but not in *L. lactis* model strains IL1403 and MG1363. In *L. lactis* strains KW2 and KF147, *com*X overexpression led to a transformation rate of 10^{-5} to 10^{-7} transformants/total cell number/μg of plasmid. These rates are similar to those obtained by electroporation in glycine-treated cells (37).

TABLE 1 Characteristics of *L. lactis*

Characteristic	Description
Classification	Gram-positive 38% GC content 2.4 Mb Nonpathogenic food microorganism (referred to as a lactic acid bacterium)
Close neighbors	Streptococci (food, commensal, pathogen)
Optimal growth temperature	30°C
Growth media	Plants, milk, food silage, farm animals, and human gut constituents
Environmental contacts	Plants (environmental niche) Farm animals Milk and other foods (environmental niche may rely on plasmid-encoded factors) Gastrointestinal tract
Metabolism	Fermentation Respiration in aerobic, heme-containing medium
Survival	Usually poor after fermentation growth Good long-term survival after growth by respiration

L. lactis IL1403 contains 40 putative insertion elements, 14 of which correspond to an insertion sequence element similar to IS*1070* from *Leuconostoc lactis*, and at least 4 prophage elements (38, 39). *L. lactis* and *S. thermophilus* share common integrative conjugative elements, as well as highly conserved regions coding, for example, exopolysaccharide synthesis enzymes (26). The existence of functional genes that have been transferred to *L. lactis* is suggested by the presence of atypical regions, i.e., those containing DNA that differs structurally from its context, or unexpected open reading frames (Orfs), e.g., a hemolysin-like protein. Similarly, an *S. thermophilus* isolate was shown to encode a pigment that was suspected to be transferred from pathogens and usually lost in milk bacteria (40).

Close interactions between microorganisms, e.g., in the gastrointestinal or vaginal mucosa of animals, or in industrial milk fermentation processes, could lead to horizontal genetic exchange. Contact between these bacteria is also suggested by the existence of nearly identical genes, e.g., in lactococci and *S. thermophilus* (26). Close physical contact, visualized microscopically between *S. thermophilus* and *Lactobacillus bulgaricus*, may facilitate genetic exchange (41).

METABOLIC OPTIONS FOR LACTOCOCCI

Lactococcal metabolism has been intensively studied because of its industrial importance in fermentation processes, with a focus on metabolic pathways and their engineering (42). Importantly, basic metabolic functions may have far-reaching effects, and as described below, metabolic shifts can result in dramatic changes in *L. lactis* growth characteristics and survival.

A remarkable metabolic process in lactococci, respiration, was essentially overlooked with the focus on dairy fermentation (43). Researchers confirmed and developed a 1970 study showing that lactococci not only ferment sugars but are also capable of forming an active electron transport chain to generate respiration metabolism (44–47). *L. lactis* respiration requires an external heme source due to an incomplete biosynthetic pathway (48). The respiration process and relevance for certain pathogenic streptococci and numerous lactobacilli is discussed below.

The two energy metabolism options, fermentation and aerobic/anaerobic respiration, are presented in the following sections. Pathways for nucleotide metabolism are also described, including a link established between nucleotide homeostasis and cell wall regulation.

Energy Option 1: Fermentation in *L. lactis*

Simplistically, lactococci use sugars to provide energy and amino acids to synthesize proteins (Fig. 2). Dairy lactococci have multiple nutritional requirements for amino acids and vitamins, probably resulting from their adaptation to a life in milk. Lactose is the major sugar source in milk, and through its uptake and degradation, lactococci generate energy in glycolysis. Casein, the major protein component in milk, is degraded to provide the major carbon source for anabolism. The flow of carbon for energy production is therefore almost separable from the flow of carbon for anabolism in these bacteria, making them ideally suited for metabolic studies.

All species belonging to the genus *Lactococcus* produce acid from glucose, fructose, mannose, and *N*-acetylglucosamine. *L. lactis* species used for dairy fermentation undergo mainly homolactic fermentation of lactose and other sugars. *L. lactis* subsp. *lactis* strains are more versatile than *L. lactis* subsp. *cremoris* in their use of diverse sugar sources, including maltose, ribose, and trehalose (reviewed in 49). Sugars may be transported by a plasmid-encoded phosphotransferase system in dairy strains or at a slower rate by a permease in nondairy strains (50).

Carbohydrate fermentation may be shifted from homolactic (lactate production) to mixed acid fermentation (acetic acid, formic acid, CO_2, acetoin, and ethanol produced in addition to lactate; Fig. 2). Two sets of conditions accompany this change, namely, (i) an altered redox state created by increased aeration during growth or (ii) reduced entry of sugar that is used for energy production.

Under the first set of conditions, oxygen appears to be involved in maintaining the NADH/NAD ratio, which itself seems to regulate the switch between homolactic and mixed acid fermentation (50); aerobic conditions result in oxidation of NADH to NAD^+ (catalyzed by NADH oxidases), thereby reducing the NADH/NAD^+ ratio in the cell. Lactate dehydrogenase (Ldh) is active at high NADH/NAD^+ ratios (i.e., low oxygen), while glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is inhibited. Increasing the amounts of water-forming NADH oxidase from a nisin-inducible promoter was shown to decrease the NADH/NAD ratio and to decrease *in vivo* activity of Ldh. The increased pyruvate pool was directed to acetoin and the flavor compound diacetyl. Other types of engineering (e.g., through mutations which block specific pathways) can also result in altered flavor properties in lactococcal fermentation (reviewed in 42, with descriptions of applications in 51–54). Note that Ldh is essential in *S. mutans* but not in *L. lactis*, possibly because *S. mutans* lacks alternative pathways for reoxidation of NADH that are present in *L. lactis*. Among the three Ldh-like Orfs potentially present on the *L. lactis* IL1403 genome, studies suggest that at least two genes encoding functional Ldh are potentially expressed (55).

Under the first set of conditions, sugar entry may be decreased if galactose is used as an energy source or when lactose is transported by a permease (56). All sugar-carbon utilization produces pyruvate regardless of the growth conditions and the pathway; the activity of enzymes using pyruvate as a substrate determines if fermentation is homolactic or mixed acid. Decreased sugar flow thus favors the activity of enzymes giving rise to mixed fermentation products. One hypothesis is that when carbon fluxes are high, GAPDH is a bottleneck in glycolysis, resulting in high pools of upstream intermediates. These pools would then inhibit pyruvate formate-lyase in one of the fermentation pathways from pyruvate, resulting in homolactic fermentation under anaerobic conditions. The extent to which GAPDH is a bottleneck in lactococci was examined by constructing strains with GAPDH activities ranging from 13 to 210% of the normal activity and measuring metabolic fluxes (57, 58). Surprisingly, GAPDH was in large excess even when carbon fluxes were high; in terms of flux control, GAPDH would thus not be the controlling factor. Moreover, the fermentation pattern remains homolactic even after 4-fold reduction of GAPDH activity, which shows that GAPDH has no control over mixed acid flux either. Similar studies were carried out to determine the importance of other glycolytic enzymes on the fermentation pattern. Phosphofructokinase had no control over glycolytic flux or on mixed acid flux (59), despite the presence of this enzyme in excess (60). Ldh had no control over glycolytic flux either but did exert a strong negative control over mixed acid flux (61). An intriguing possibility is that the excess glycolytic enzymes are somehow shuttled to the surface, where they could play other roles in bacterial dis-

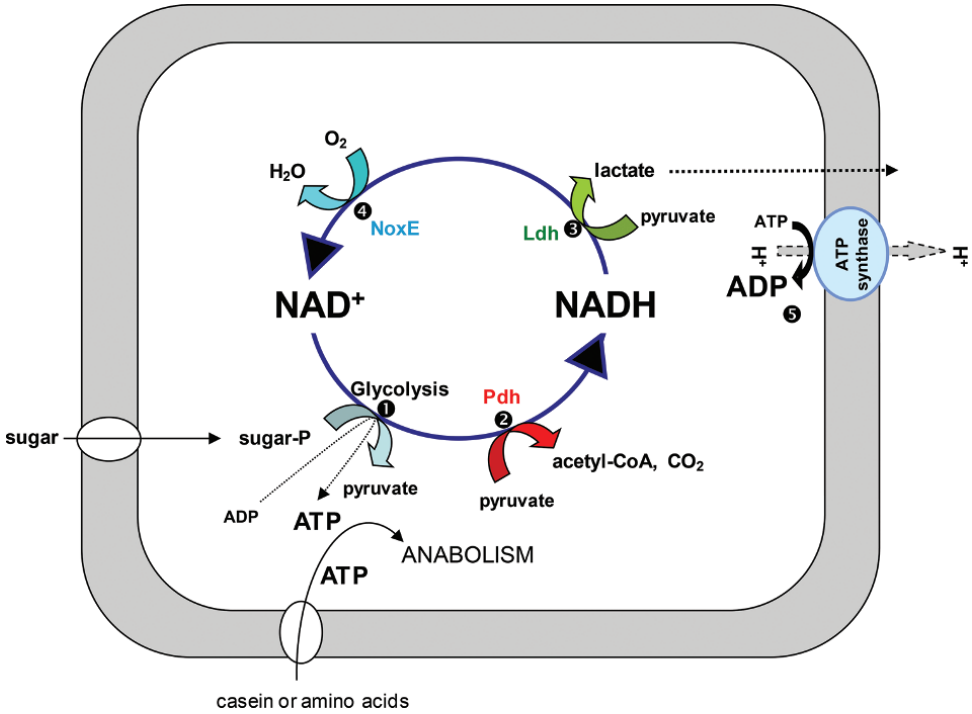


FIGURE 2 Basics of *L. lactis* fermentation. The NADH/NAD^+ ratio is placed as a central determinant of carbon metabolic choice in *L. lactis* (56). (1) Sugar fermentation generates ATP, which is used for amino acid anabolism. In anaerobic conditions and rapid sugar flux, essentially all sugar is converted to lactate (homolactic fermentation) from pyruvate (glycolysis). When sugar flux is slower, in the presence of sugars other than glucose or lactose or in aerobic growth, mixed acid fermentation may occur. The latter conditions are characterized by lower NADH/NAD^+ ratios than those found during homolactic fermentations. Besides NADH, glycolysis generates ATP and pyruvate from sugar degradation. (2) Pyruvate dehydrogenase (Pdh) provides extra NADH from pyruvate when oxygen is present. (3) Lactate dehydrogenase (Ldh) oxidizes NADH into NAD^+ by conversion of pyruvate into lactate, thus maintaining glycolytic activity during fermentation. (4) When oxygen is present, NADH can be oxidized by the cytoplasmic H_2O-forming NADH oxidase (NoxE), generating NAD^+. (5) The ATPase expulses H^+ at the expense of ATP to avoid acidification due to glycolysis. Pyruvate build-up leads to synthesis of acetate or the neutral acetoin and diacetyl (also see Fig. 4). This figure is modified from reference 155.

semination; glycolytic enzymes, like GAPDH, have been reported to be present on the surface of numerous *Streptococcaceae* (62–64).

The genetic organization of enzymes involved in sugar utilization may reveal regulation at the transcriptional level. *L. lactis* appears to coordinate the expression of three genes, each involved in key but distinct steps in fermentation, *pfk*, *pyk* (encoding phosphofructokinase and pyruvate kinase, respectively) and *ldh* genes, by having them present in one operon (called *las*). The operon organization may prevent unwanted accumulation of glycolytic intermediates. In *S. pyogenes* and *S. pneumoniae*, the *ldh* gene is not within this operon, suggesting that a common regulation of the three genes may be unique to lactococci. The *las* operon is induced in the presence of glucose via the catabolite control protein CcpA (65). Expression of the entire *las* operon was modulated to determine its importance for metabolic flux; flux was highest when expression was at its normal level and decreased rapidly when expression was reduced or increased (59).

In *Escherichia coli*, glycolytic metabolic flux is controlled almost exclusively by the demand for ATP. In *L. lactis*, the ATP demand also controls flux in slow- or nongrowing cells but not in fast-growing cells (59, 66–68).

The importance of individual enzymes for system properties such as fluxes, metabolite concentrations, frequencies of infection, survival rates, etc., can be accessed by modulating the activities of the respective components. Using synthetic promoters has proven useful for obtaining accurate tuning of gene expression and optimizing metabolic fluxes (57, 58). Moreover, this technique allows numerous genes to be modulated simultaneously and at differential levels in a cell; in one example an *L. lactis* strain was constructed in which all 10 genes encoding the enzymes of the glycolytic pathways were upregulated using synthetic promoter libraries (69).

Energy Option 2: Respiration in *L. lactis*

Components of the electron transport chain

An active respiration chain comprises three elements: (i) an electron donor, supplied by NADH dehydrogenases, (ii) menaquinones (including demethylmenaquinones or DMK), which are non-protein components of the respiration chain that deliver electrons to the terminal oxidoreductases, and (iii) terminal oxidoreductases, which contain heme as an essential cofactor and use oxygen as the final electron

acceptor (70, 71). Although *L. lactis* undergoes fermentation in a rich medium, addition of a heme source to an aerated medium activated respiration metabolism in *L. lactis* (44, 46). Demonstration of the existence of an electron transport chain and heme-dependent membrane NADH oxidase activity, and identification of factors implicated in respiration, all point to the major impact of this metabolic mode on bacterial growth and survival (Fig. 3) (45–47, 72–75). Heme-stimulated growth leads to an approximate doubling of cell biomass (in keeping with greater energy production by respiration activity), increased pH, and massive production of acetoin rather than lactic acid (46, 76). In combination with sugar-limiting conditions, as occurs during growth on maltose, the biomass yield increased further and indicated a reversal of the function of the H^+-ATPase toward the direction of ATP synthesis (77). An important feature of respiration metabolism is decreased oxidation in the cell, due to respiration-dependent oxygen elimination. This leads to a more reduced intracellular environment and consequently less oxygen-induced damage. This feature, together with increased pH, may explain the extraordinary increase in bacterial survival during respiration growth (46, 72).

Like other Gram-positive bacteria that grow via respiratory metabolism, *L. lactis* utilizes only menaquinones as electron carriers to the terminal oxidoreductase. Inactivation of *menB*, which encodes a menaquinone biosynthesis enzyme (dihydroxy naphthonic acid synthase), totally abolished both quinone production and respiration in *L. lactis* (78). However, while other respiring bacteria have several oxidoreductases that ensure respiration under different conditions, *L. lactis* uses a single enzyme, the cytochrome *bd* quinol oxidase (encoded by *cydAB*) (45, 46). This kind of oxidase has a high affinity for oxygen (79). Moreover, as shown for *Streptococcus agalactiae*, cytochrome *bd* quinol oxidase contributes to both virulence and colonization (80, 81; Y. Yamamoto, A. Gruss, and P. Gaudu, unpublished data).

Respiration in *L. lactis* requires uptake of heme (iron is insufficient), possibly suggesting the presence of a heme transporter. Genetic studies suggested that the *fhu* operon mediates heme uptake (75). However, more recent studies suggest that heme might diffuse into *L. lactis* membranes and then be recovered by cytoplasmic heme-binding factors. Heme reduction by menaquinones appears to favor membrane incorporation (82). Other factors that facilitate heme entry via diffusion are being investigated.

Based on the dramatic differences in growth, survival, and metabolite production, we had anticipated that respiration growth would induce massive changes in gene expression compared to fermentation. Surprisingly, the expression of very few genes was specifically altered by respiration as evaluated by proteomic (21 proteins) and transcriptomic (11 transcripts) analyses of late exponential phase cells (73, 83). These studies led to the hypothesis that NADH/NAD balance, which is shifted toward NAD by respiration chain activity, is responsible for modulating enzyme activities that accompany respiration growth (Fig. 3). Significant changes in expression were observed for a single operon, as discussed below (see section on heme homeostasis) (73).

Role of CcpA in respiration

The regulator CcpA, which controls catabolite carbon repression, imposes a hierarchical use of carbohydrates (84). CcpA controls carbohydrate catabolism (glycolysis) and positively controls production of Ldh (65), which converts pyruvate to lactate. CcpA also seems to coordinate respiration metabolism at different levels. The first control concerns sugar uptake, because *L. lactis* does not use amino acids or glycerol as a carbon source (85). CcpA also controls the transporter *fhu*, which appears to contribute to heme uptake (75). Third, CcpA regulates expression of genes encoding protein components of the respiratory chain.

In *L. lactis*, *ndh* (encoding NADH dehydrogenase [Ndh]) and *cydAB* are present on distinct operons (85, 86), while in other LAB they appear to be organized in a single operon (80, 87). The latter organization suggests coordinated expression of *ndh* and *cydAB* and may indicate that the respiratory chain is fueled by just one NADH dehydrogenase. In *L. lactis*, two putative NADH dehydrogenase genes (*noxA llmg_1735* and *noxB*, *llmg_1734*) are present in the genome, and both enzymes could drive electrons to menaquinones. Though *noxA* is adjacent to *noxB*, transcriptome analyses indicated that they are not in an operon (86). In the case of *noxB*, direct control by CcpA is suggested by the presence of a CcpA binding motif *cre* (catabolite response element) in the promoter region. Altogether, *noxB* seems to be repressed by CcpA, while *noxA* and *cydAB* are induced (88). Deletion of *noxA* abolished respiration activity on glucose growth, while the role of *noxB* is unknown (89). Because *noxB* is likely induced late in growth (when CcpA is less active), it may drive electrons to menaquinone, as does NoxA, but in late growth.

Interestingly, CcpA does not control respiration-induced acetoin production, because gene expression of this pathway was not affected by *ccpA* deletion (88). A challenging question concerns regulation of the pyruvate-acetoin pathway, which is functional late in respiration growth, as evidenced by acetoin accumulation in stationary phase cells; ~70% of the pyruvate pool is converted to acetoin (46, 73, 76) (Fig. 3). Interestingly, this pathway produces not only acetoin, but also carbon dioxide, which may contribute to lowering medium acidification in respiration growth (pH 6, compared to pH 4.5 in fermentation; both media contain 1% glucose as an energy source) (212). The search for regulators and/or cofactors of the pyruvate-acetoin pathway and for regulatory checkpoints of *L. lactis* respiration is ongoing.

Although *L. lactis* is only equipped for aerobic respiration, other LAB can also perform anaerobic respiration. In *Lactobacillus plantarum*, nitrate can be used as an electron acceptor instead of oxygen. Reduction of nitrate into nitrite is performed by a quinone-nitrate reductase (NarG). Nitrate respiration is repressed by glucose, suggesting potential participation of CcpA in anaerobic metabolism in this organism (89).

Heme homeostasis is a key to *L. lactis* respiration

Transcriptome studies of *L. lactis* in respiration versus fermentation conditions during exponential growth revealed strong upregulation of components of a single operon, *ygfCBA*, encoding a putative transcriptional regulator (YgfC; also detected by proteomics [83]), a predicted permease (YgfB), and an ATPase (YgfA) (73). YgfB and YgfA are HrtB and HrtA orthologs (*hrtBA* for heme-regulated transporter") encoding HrtBA, an ABC transporter that was found in parallel in *Staphylococcus aureus* (90). Its function was subsequently clarified as a dedicated heme-efflux pump, which was necessary and sufficient to maintain intracellular heme concentrations at subtoxic levels while allowing sufficient intracellular amounts for functional respiration (Fig. 4). The predicted TetR family repressor YgfC, renamed HrtR, regulates expression of *hrtBA* in *L. lactis*. HrtR shows high affinity for heme through a noncovalent hexacoordinated interaction with heme iron (74). HrtR binds a 15-nucleotide palindromic sequence in the *hrtRBA* promoter region, which is needed for repression (74). Heme binding to HrtR modifies its conformation, releasing it from its target promoter and alleviating transcriptional repression of *hrtRBA* (74, 91).

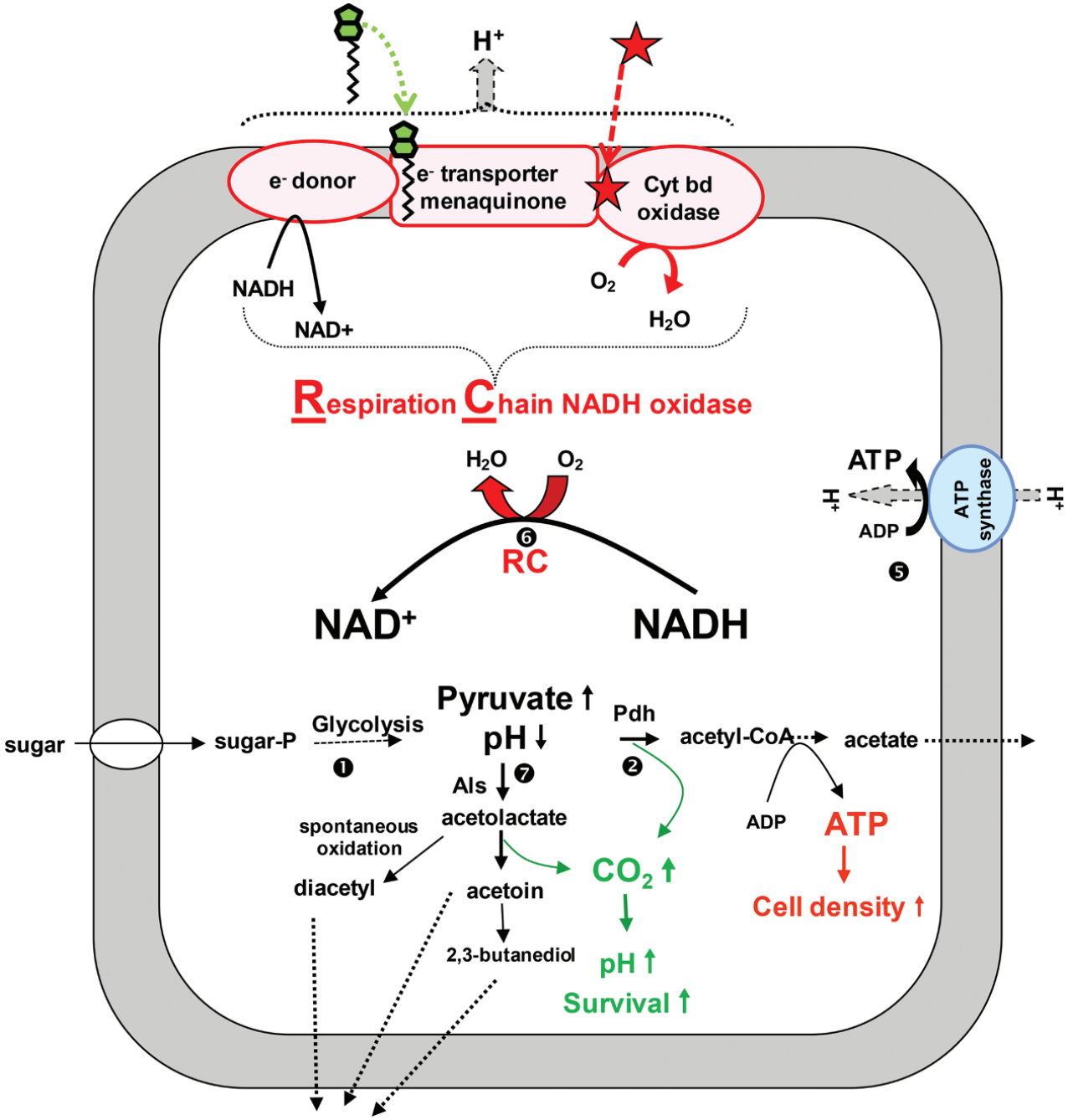


FIGURE 3 Basics of *L. lactis* respiration. Refer to Fig. 2 for reactions 1, 2, and 5, which are common to fermentation and respiration (numbering is the same). (6) The membrane respiration chain (RC) comprises an electron donor (putatively encoded by *noxAnoxB* [45, 89]), menaquinones (encoded by *men* operon genes or provided exogenously [80]), and a terminal electron acceptor (the cytochrome oxidase encoded by *cydAcydB* [46, 211]). Heme (red star) must be added exogenously (red arrow) to activate cytochrome oxidase. *S. agalactiae* and lactobacilli with respiration capacity (see Fig. 1 legend) require menaquinones (schematic molecule with green center) and heme to activate respiration. Respiration chain activity results in H^+ expulsion. (5) The ATPase might import H^+, which generates ATP but with low efficiency (47, 77). *L. lactis* lacks a complete Krebs cycle. Thus, NADH, which is needed for the respiratory chain, is produced by carbon catabolism. (1) Once phosphorylated, sugar is catabolized to pyruvate via glycolysis with production of ATP and NADH. As the respiration chain consumes NADH, Ldh activity decreases and pyruvic acid accumulates. Pyruvic acid dissociates to pyruvate and a proton, decreasing the internal pH. (7) To avoid acidification, pyruvate/pyruvic acid is converted to acetolactate via acetolactate synthase (Als) and then to the neutral compound acetoin with production of CO_2. Diacetyl is produced by spontaneous oxidation of acetolactate. This pathway raises the pH and improves cell survival. Some LAB convert acetoin to 2,3-butanediol. (2) Pyruvate may also be converted to acetyl-CoA via pyruvate dehydrogenase (Pdh), providing extra NADH and CO_2. Acetyl-CoA is further converted to acetate with production of ATP, promoting higher cell density. Acetate, acetoin, diacetyl, and 2,3-butanediol diffuse or are secreted into the medium. This figure is modified from reference 155.

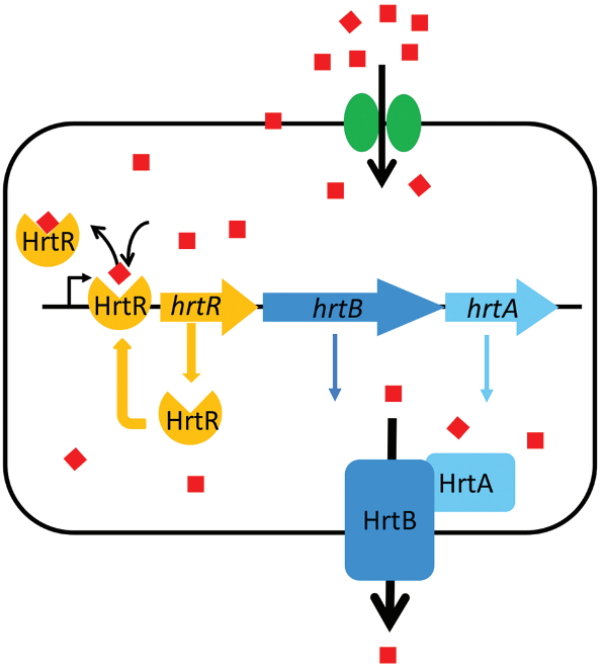


FIGURE 4 Schematic representation of *L. lactis* heme-sensing and HrtBA-mediated efflux, which regulate heme homeostasis. In *L. lactis* and numerous commensal bacteria, heme is suggested to be taken up by *fhuDBA* gene products (green ovals) and/or by diffusion through membranes (75, 82). Internalized heme binds to the HrtR repressor, which releases binding to the *hrtRBA* operon. Consequent activation of *hrtBA* results in heme efflux (74). Red squares, heme.

The use of HrtR as an intracellular heme sensor and *hrtBA* regulator appears to be conserved among commensal bacteria. In contrast, numerous Gram-positive pathogens use an extracellular two-component system, *hssRS*, to regulate *hrtBA* (92). These findings point to an essential role of efflux for heme homeostasis in *L. lactis*. In contrast, heme import mechanisms remain elusive, despite a partial role for *fhu* (75). Membrane-associated menaquinones were shown to favor the accumulation of reduced heme in membranes (82). An oxidative environment, provided by oxygen, prevents and reverses hemin reduction by menaquinone and thus limits heme accumulation in membranes (82). HrtBA counteracts menaquinone-dependent membrane retention of excess heme in membrane, suggesting direct efflux from this compartment. Moreover, both HrtBA and menaquinone-mediated reduction have a strong impact on heme intracellular pools, detected as induction of an HrtR heme sensor. This indicates that intracellular heme acquisition is controlled at the membrane level without the need for dedicated import systems, at least at high heme concentrations (82). While pathogens may have access to blood heme during infection, the existence of heme-responsive genes in lactococci and other commensal bacteria raises questions concerning the nature of heme sources in their natural ecosystems (48).

Roles of respiratory chain components in nonrespiration processes

Milk, one of the main industrial media for food fermentation, lacks heme and thus cannot support *L. lactis* respiration metabolism. However, respiration chain activity does affect fermentation by decreasing the milk redox potential of *L. lactis*, which is one of most reducing LAB, providing a very negative redox potential value in milk. To establish a low redox potential, oxygen must be removed and oxidized compounds should be reduced. During fermentation, water-forming NADH oxidase (NoxE) eliminates oxygen, and respiration chain components (NoxAB and MenC) provide a lower redox potential by reducing oxidants in milk. The redox potential of milk is known to affect the microbiota and sensorial quality of fermented dairy products. Thus, although the *L. lactis* respiration chain is incomplete in milk due to the absence of a heme source, its reducing ability plays a significant role during fermentation (93).

L. lactis is a respiration prototype

Lactococci, unlike *E. coli* and *Bacillus subtilis*, respire using a restricted electron transport chain, and only if heme is provided. Comparative studies revealed that certain, but not all, *Streptococcaceae* and certain other *Firmicutes* have the capacity to respire when provided with heme, or heme plus a menaquinone source (45) (Fig. 1). They include *S. agalactiae* (80, 94), *E. faecalis* (95, 96), and *Lc. plantarum* (89). Since the first characterization of *L. lactis*, a far wider group of LAB have been revealed to adopt respiration metabolism when provided with heme, or heme plus a menaquinone source, is added (see 48 for review).

Nitrogen Metabolism

In a milk medium, lactococci derive amino acids from casein via hydrolysis by the extracellular protease PrtP, transport of the generated peptides, and further degradation by a multitude of intracellular and envelope proteases and peptidases (97, 98). Amino acids readily available in milk are used both directly as amino acid building blocks and as a general carbon supply for other forms of anabolism in lactococci. Extracellular proteases plus at least 14 intracellular peptidases are of key importance for amino acid utilization. Lactococci grow poorly or die in milk fermentation conditions in mutants that are devoid of different combinations of these peptidases (99).

Dairy lactococci differ from plant lactococci in that they require several amino acids for growth. Surprisingly, strains of both origins appear to have the necessary genes for biosynthesis. Nevertheless, dairy lactococci require Ile, Leu, Val, and His, and sometimes Arg, Met, Pro, and/or Glu (100). These amino acid requirements in dairy strains appear to result from multiple mutations rather than deletions in the structural genes (39). This may suggest that mutations accumulated as an economic measure in strains maintained in a dairy environment. Similar results have been reported for *S. thermophilus*, a dairy bacterium related to *S. pyogenes*.

Nucleotide Metabolism

Nucleotides are not only substrates for DNA and RNA polymerases but are also substrates or allosteric effectors for many enzymes, and furthermore they constitute parts of different coenzymes. Thus, mutants in nucleotide metabolism may display numerous phenotypes. Cell nucleotide pools are also influenced by the presence of exogenous nucleobases or nucleosides in the medium. Pathways of uptake and utilization of these compounds (the so-called salvage pathways, which vary among organisms) are key contributors to bacterial responses to changes in the medium and to increased intracellular degradation of nucleic acids as illustrated below.

As seen above for operons involved in carbon metabolism, gene organization has unique characteristics in

lactococci, which may impact regulation. For example, genes encoding the pyrimidine (thymine, cytosine, and uracil) biosynthesis pathway leading to the formation of UMP are organized in five different operons in *L. lactis* and in a single operon in most investigated Gram-positive bacteria. Similarly, purine (adenosine, guanine, and xanthine) biosynthesis genes involved in the first 10 steps leading to IMP production are located in 5 separate operons in *L. lactis* and in a single operon in *B. subtilis*.

Pyrimidine biosynthesis is regulated by attenuation and antitermination by PyrR. The PyrR protein binds to PyrR "boxes" (5′-UCCAGAGAGGCUNGCAAG-3′) present on the 5′ ends of the untranslated mRNAs on four of the identified pyrimidine biosynthetic operons (101, 102). Mutation of the *pyrR* gene results in constitutively increased levels of the pyrimidine biosynthetic enzymes (101). The active RNA binding form of PyrR in *Bacillus* is a dimer which is formed when UMP, UTP, or PRPP (5-phosphoribosyl-1-pyrophosphate) is bound to PyrR; GTP counteracts UTP binding and thus promotes transcriptional read-through (103). The regulatory mechanisms seem to be the same in *L. lactis* (104). Interestingly, several *pyr* operons in *Mycobacterium smegmatis* have been found to be regulated by PyrR by translational repression (105).

Purine biosynthesis in *L. lactis* is positively controlled by PurR, and *purR* mutants are purine auxotrophs. PurR binds to a Pur box consensus sequence (5′-ANNNCCGAACAAT-3′) (106–108) to activate transcription of the *pur*C and *purD* operons. Data show that the PurR activating effector is PRPP. Because PRPP synthetase is inhibited by ADP and the addition of purine leads to increased purine pools (104), the addition of purine may indirectly decrease gene expression (108, 109). Interestingly, riboswitch control of the PurR-regulated *xpt* operon is modulated by purine pools (104). In operons activated by PurR, the distance between the Pur box and the −10 region was 57 to 58 bp. Mutational studies of pur boxes supported the importance of the central CCGAAC sequence. Interestingly, while *L. lactis* PurR often acts as an activator, the homologous PurR in *B. subtilis* usually works as a repressor. In addition to the importance of PurR binding sites, the two PurR proteins also differ in their PRPP binding properties. The two types of PurR proteins are related, and the *Bacillus* type seems to have evolved early from the activator type of PurR (109).

The PurR regulon includes purine biosynthetic genes but also genes involved in purine uptake and conversion into purine monophosphates. Genes involved in C1 carbon metabolism, guanosine tetraphosphate (ppGpp) metabolism, phosphonate transport, and pyrophosphatase activity were also identified. Interestingly, a Pur box was also identified upstream of two ribosomal RNA operons, which could imply an interconnection between the purine pathway and translation, but this link needs to be confirmed (104, 106).

L. lactis can utilize the exogenous nucleobases or nucleosides present in the medium or formed from intracellular degradation of nucleic acids via the salvage pathways. The nucleobases uracil, guanine, and adenine, as well as hypoxanthine and xanthine, are taken up and converted to nucleotide monophosphates, while cytosine is not utilized by lactococci (110). Orotate may be incorporated in plasmid-bearing *L. lactis* strains that express an orotate transporter and may be used for UMP biosynthesis, since orotidine.5′-monophosphate (OMP) is a normal intermediate (111, 112). Thymine may be incorporated via pyrimidine phosphorylase to thymidine and further phosphorylated by thymidine kinase (*tdk*) to the corresponding monophosphate (110). Thus, all common nucleobases except cytosine can be transported and incorporated into nucleotides. The ability of *L. lactis* to scavenge nucleobases or nucleosides can confer a survival advantage in stress conditions.

In *L. lactis*, all nucleosides except cytidine can be degraded to the corresponding nucleobase. However, cytidine may first be deaminated to uridine and then further to uracil. Pyrimidine nucleosides may also be directly converted to nucleotides since the corresponding nucleoside kinases (*udk* and *tdk*) are functional in lactococci. Two high-affinity nucleoside transport systems were identified: UriP is specific for uridine and deoxyuridine, while BmpA-NupABC takes up cytidine and purine nucleosides and the corresponding deoxyribonucleosides (113).

LACTOCOCCAL STRESS RESPONSES

Stress response is the adaptation to homeostatic changes due to the environment and is generally modulated by sensors and regulators that sense the state and respond to signals. Major signals for *L. lactis* include metabolites such as nucleotides, acid (self-induced), oxygen, salt, metals such as heme (natural or industrially provoked), and toxic products (e.g., disinfectants). We refer readers to recent reviews on LAB stress responses (114) and on regulatory signals in *L. lactis* (115). Here, we focus on specific effectors and responses that were not previously considered, i.e., nucleotides, Spx, cyclic-di-AMP, and heme and respiration stress responses.

Regulation by Nucleotide Pools

Low nucleotide pool sizes may serve as internal stress signals that provoke expression of stress response genes in *L. lactis* (116, 117). An important link between nucleotide pools and cell wall synthesis was uncovered: as part of the uridine synthesis pathway, PyrB converts L-aspartate (L-Asp) to N-carbamoyl-L-Asp. PyrB competes with cell wall enzymes for L-Asp; thus, L-Asp consumption during rapid growth favors cell wall flexibility, while its accumulation in the stationary phase may lead to a more rigid cell envelope (118). Additionally, high UTP pools in the human pathogen *S. pneumoniae* correlated with more capsule formation and lower biomass yield (119). These examples suggest that nucleotide pools connect nucleotide metabolism to the cell envelope structure.

The Spx Family of Regulators

A hot spot for *L. lactis* mutants that compensated for the stress sensitivity of a *rec*A mutant, thermosensitivity of a *clpP* mutant, metal toxicity, and oxidative stress mapped to the *spx* gene (117, 120, 121). Spx is conserved in numerous *Firmicutes* and was shown in *B. subtilis* to be involved in oxidative stress regulation via thioredoxin gene expression (122). Seven *spx* homologues exist in *L. lactis* MG1363, five of which contain redox-reactive CXXC regions (121). Exploration of other Spx proteins revealed that SpxB, a non-redox-sensitive Spx, is a key regulator of cell wall resistance to lysozyme by activating *O*-acetylation of peptidoglycan (PG) (123). The Spx family is intrinsic to cell responses to oxidative stress and cell wall damage conditions.

Cyclic-di-AMP as a Second Messenger in Bacterial Adaptation and Its Potential Role in Heme Sensitivity

Nucleotide pools are required for DNA synthesis but are also involved in numerous stress responses, including amino acid

starvation and acid stress (e.g., via ppGpp) (124). More recently, cyclic-di-AMP was identified as a second messenger in stress adaptation (125). In *L. lactis*, cyclic-di-AMP homeostasis is a balance between synthesis via the adenylate cyclase enzyme CdaA (Llmg_0448) and degradation by the cyclic-di-AMP phosphodiesterase enzyme (GdpP, Llmg_1816). Increasing the cyclic-di-AMP pool by deletion of *gdpP* increased heat resistance and salt hypersensitivity in *L. lactis* (126). These phenotypes might be due to a change in PG synthesis (127), because (i) salt sensibility in the *gdpP* mutant is suppressed by deletion of *cdaA* or *glmM* encoding a phosphoglutaminase enzyme involved in PG integrity, (ii) *cdaA* is in an operon with *glmM*, (iii) GlmM interacts with CdaA to modulate its activity, and (iv) *L. lactis* cells spontaneously lyse when cyclic-di-AMP pools are decreased. The exact role of cyclic-di-AMP in PG synthesis in *L. lactis*, though unknown, may be to modulate enzymatic activity to control cell wall plasticity. In contrast to CdaA, small molecules might interact with GdpP to modulate its activity. In *B. subtilis*, the GdpP homolog (YybT) harbors a PAS domain, which contains a heme binding site (128). Interestingly, *L. lactis* GdpP also harbors this domain, and deletion of the *L. lactis gdpP* gene increased the sensitivity of cells to heme (128). These observations suggest a dialogue/link between heme homeostasis and the cyclic-di-AMP pool enabling bacteria to adapt their physiology in response to exogenous heme.

In addition to its involvement in PG synthesis, cyclic-di-AMP changed pyruvate carboxylase (*pycA*) activity in *L. lactis* (129). PycA is required for aspartate synthesis. Milk acidification is delayed in a *pycA* mutant compared to the wild-type strain, indicating that cyclic-di-AMP might modulate the fermentation process in some conditions.

Strategies for Survival in Stress Conditions

L. lactis is equipped to deal with several stress conditions confronted in nature or in industrial settings, as summarized below. Salt and acid induce expression of GadB and GadC, which are putatively involved in glutamate transport by an antiporter; glutamate transport presumably involves efflux of H^+, thereby maintaining intracellular pH (130, 131).

Toxic products such as bile, quaternary compounds, and antibiotics may be actively pumped out of the cell by specialized transport functions. Among the numerous transport systems that shuttle metabolites in and out of the cell, some mediate drug expulsion and, consequentially, can confer drug tolerance (132). In *L. lactis*, LmrA is a multidrug pump that effluxes a wide range of amphiphilic, cationic drugs (including potentially toxic antibiotics, quaternary ammonium compounds, aromatic dyes, and phosphonium ions) in exchange for H^+ influx. LmrA (590 amino acids) is similar to the human multidrug resistance P-glycoprotein, thus raising questions about the origins of the pump. Judging from sequence analyses, an LmrA dimer would be the functional equivalent of the P-glycoprotein (LmrA is 32% identical to half of the P-glycoprotein, particularly within known functional domains). Remarkably, LmrA is functional in eukaryotic cells and is able to replace P-glycoprotein defects, thus making *L. lactis* an excellent model to study drug extrusion (132). Note that sequence comparisons predict an LmrA homologue in *S. pneumoniae* (an Orf with ~30% identity over 539 amino acids is present). The CmbT transporter (Llmg_1104), described initially as a cysteine and methionine biosynthesis transporter, was subsequently identified as a multidrug efflux pump that effluxes a wide range of antibiotics and toxic drugs (133).

Other efflux systems protect *L. lactis* from toxic metabolites: the dedicated heme-specific HrtBA efflux pump (see above) protects cells from heme toxicity. *L. lactis* homologs of heme and protoporphyrin IX efflux pumps identified in *S. agalactiae* may suggest that backup systems are available (134).

A series of exciting reports reveal that there is more to the envelope than was previously thought: first, pili discovered in *L. lactis* led to the idea that these bacteria might persist in the gut upon ingestion of fermented food products (135, 136). Second, a pellicle-like carbohydrate structure was visualized on the *L. lactis* MG1363 surface and characterized biochemically (137). A third structure, described as trapped within the PG, corresponds to another pellicle-like carbohydrate thin outer layer surrounding *L. lactis* strain MG1363 (138).

Finally, basic cell metabolism can determine how well the bacterium copes with oxidative conditions. Respiration metabolism presents a clear advantage to lactococci in an aerobic environment for both growth and long-term survival (Fig. 5) (46, 72).

Selections Leading to Improved Adaptation to Environmental Stress Situations

Oxygen is a ubiquitous stress. If not eliminated, reactive oxygen derivatives provoke cell damage that can be lethal. One means of reducing oxygen-related damage is by removing oxygen. In fermenting cells, H_2O-forming NADH oxidases do eliminate oxygen during growth (139), although cells are sensitive to oxygen-related damage in the stationary phase (140). Overproduction of H_2O-forming NADH oxidase could not only change metabolic end products (139) but also might improve survival in an oxidizing environment due to oxygen removal. An alternative means of creating a more reducing environment is by adding glutathione, a redox peptide (141), or dithiothreitol, a reducing agent (75), to the medium. Lactococci lack catalase, which eliminates hydrogen peroxide in many aerobic bacteria. Hydrogen peroxide has been effectively removed by cloning catalase in *L. lactis* (142). Respiration metabolism in lactococci is a "natural" and efficient means of eliminating oxygen compared to fermentation, leading to good survival in the stationary phase (46, 72).

L. lactis generates an acidic environment during fermentation growth, which might compromise its survival (143). Significantly, acidification may be more severe if cells are immobilized, because acid diffusion may be slower. This situation may provide a natural selection for strains to escape from a constrained environment, and it was shown experimentally to generate multistress-resistant mutants (116). Interestingly, acid-resistant mutants evoked changes linking nucleotide pools (ppGpp) and cell wall alterations (116, 118). Studies using a semiliquid medium have been used to impose a natural selection for mutants that can more readily escape a constraining environment (144). In one case, bacteria that make chains diffused more slowly than single cells (Fig. 6). "Dechaining" mutants, affected in penicillin binding protein and cell wall-synthesis enzyme PBP1A were isolated; the mutants no longer formed chains and were able to disperse more readily in the semiliquid medium; these mutants display greater permeability, probably due to increased cell wall breaks (144). In *S. agalactiae*, interruption of *ponA* results in reduced virulence that was linked to host antimicrobial peptide susceptibility (145, 146). In view of the identified role of PBP1A in lactococci, it is tempting to speculate that the *ponA S. agalactiae* mutant may also be defective in cell wall permeability and its chain-forming ability, thus

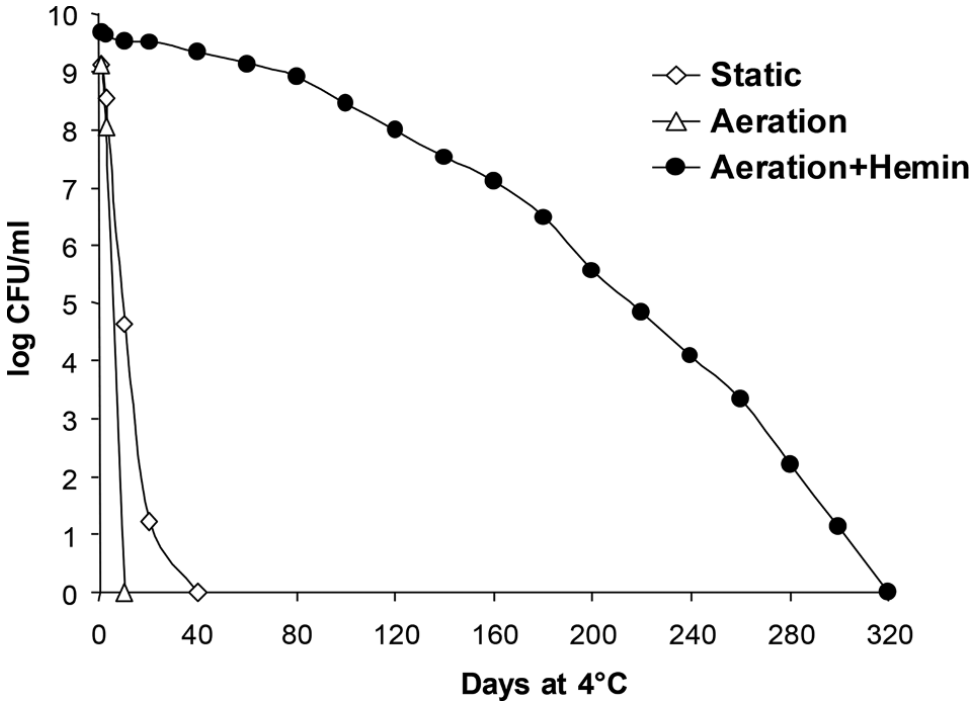


FIGURE 5 Respiration metabolism increases the survival capacity of lactococci. When supplemented with hemin, aerobically grown lactococci can undergo respiration metabolism. As a result, cells stored at 4°C show markedly better survival compared to cells grown aerobically in the absence of heme or in static conditions. Improved survival was also observed when cells were maintained at 30°C. The experiment shown was performed by Karin Vido in the authors' laboratory.

impacting its *in vivo* localization. Use of semiliquid medium for selections might prove effective in examining factors that are at work when bacteria are immobilized in their host and has also proven useful in other types of selections in which surface properties are altered (137).

Transposon insertional mutagenesis was also used to select for stress-resistant strains of lactococci. Three examples are given. (i) A combination of stress conditions is lethal for lactococci (and possibly for other organisms), although each condition alone may be nonlethal. Simultaneous high temperature (37°C), oxygen, and either low pH (i.e., similar to conditions in the stomach) or a *recA* background give rise to mutants, many of which seem to affect intracellular metabolic pools of guanosine-phosphate and phosphate in stress response (116, 117). Low intracellular levels of these metabolite pools in the mutants may constitute a starvation signal to induce a stress response. These mutant strains show better long-term survival than their nonmutated parents. It thus appears that a general stress response is induced in *L. lactis* when intracellular guanosine-phosphate and phosphate pools are low. In accordance with this, *L. lactis* showed resistance to acid and heat stress when grown in milk or a synthetic medium without purines (147). (ii) Hydrogen peroxide is toxic to lactococci. An H_2O_2-resistant mutant was isolated at high temperature (37°C), and although its resistance was 1,000-fold greater than the parental strain, it displayed no other stress resistance phenotypes (148). H_2O_2-resistant mutants might have a survival advantage in coculture with strains producing mM amounts of H_2O_2, e.g., some lactobacilli (149) and streptococci (150). In the former case, use of H_2O_2-resistant lactococci could lead to development of new fermented products; in the latter, more efficient growth of lactococci could improve the hygiene of food products. (iii) Mutants were selected for increased resistance to dithiothreitol (DTT), a reducing agent, at elevated temperature. By preventing the formation of disulfide bonds, DTT disables a part of the oxidative stress response pathway (numerous lactococcal stress-response proteins contain one or more CXXC motifs). All 18 DTT-resistant mutants mapped within a single operon, *pst*, involved in phosphate transport. Greater oxidative stress tolerance of *pst* was linked to its effects on copper and zinc homeostasis (151).

Stress-resistant lactococci have several potential uses. First, such strains are potentially valuable in dairy fermentation. Their greater resistance to stress may overcome survival variability as seen in conventional strains. The specifically acid-resistant strains may provide resistance to extreme acid pH conditions or may be better at maintaining a neutral internal pH. Stress-resistant strains may survive longer in fermentation and may also be more resistant to harsh storage conditions (such as freezing and lyophilization). Second, because such strains may survive the harsh environments in the gut better, they may be attractive for probiotic uses. A proof of concept mouse inflammatory gut model provided evidence that antioxidant superoxide dismutase produced by wild-type *L. lactis* may improve gut integrity (4, 152, 153). Third, LAB are potentially valuable candidates for the production of molecules with medical or biotechnological uses; Lactococci are nontoxic and have documented potential for expressing and exporting proteins or other molecules of interest, either for industrial production or in the gut.

L. LACTIS AND INTERBACTERIAL DIALOGUE

Lactococci have long been known to inhibit the growth of coexisting bacteria, including pathogens, via acid production

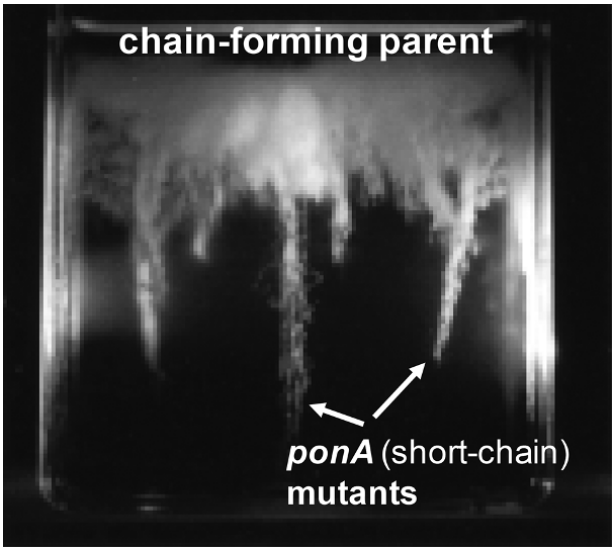


FIGURE 6 Bacterial root formation in semiliquid medium. Bacterial chains (here, an *acmA* mutant of *L. lactis*; parental strain) diffuse slowly in a semiliquid (0.035% agar) medium. Bacterial dechained mutants diffuse more quickly to form roots. In this experiment, all the roots corresponded to independent mutants in the same gene, *ponA*, encoding PBP1A (reproduced from Kulakauskas and coworkers [144]). Note that a similar strategy of semiliquid medium selection was used to uncover the existence of a cell-surface carbohydrate pellicle in *L. lactis*; the system is readily applied to other bacteria (137).

and are an important feature of fermented foods. This property has led to broader potential applications in the probiotic field, e.g., with respect to cholera infections (154). Recent studies also uncovered novel *in trans* effects of *L. lactis* and led to the identification of similar "public contributions" by other bacteria. Some examples are described in the following subsections.

Interbacterial Cross-Feeding

Via menaquinones

Other than *Lactococcus*, *Leuconostoc*, and *Enterococcus* species, most known LAB (pathogenic or not) lack a complete menaquinone biosynthesis pathway and require exogenous menaquinone or a precursor, in addition to heme, for respiration growth (48, 89, 155, 156). Unexpectedly, menaquinone-deficient strains can overcome this deficiency via contacts with menaquinone producer species. For example, respiration of *S. agalactiae*, an opportunistic pathogen, is activated in the presence of *L. lactis via* menaquinone donation and a heme source (78) (Fig. 7). Remarkably, *E. coli* expulses dihydroxynapthoate acid (DHNA), which is used by *S. agalactiae* to synthesize its own menaquinone (in this case a DMK) (156). DHNA is coupled to an isoprenyl chain by menaquinone prenyltransferase, encoded by *menA*. In *Lb. plantarum*, a *menA* ortholog is adjacent to the *cydAB* locus, suggesting that this organism can also use DHNA to synthesize menaquinones. Free menaquinones and DHNA are likely present in host ecosystems and can be used to activate metabolic pathways in commensal and pathogenic bacteria as well. Thus, environmental nonpathogens might contribute to the fitness, and potentially virulence, of neighboring pathogens.

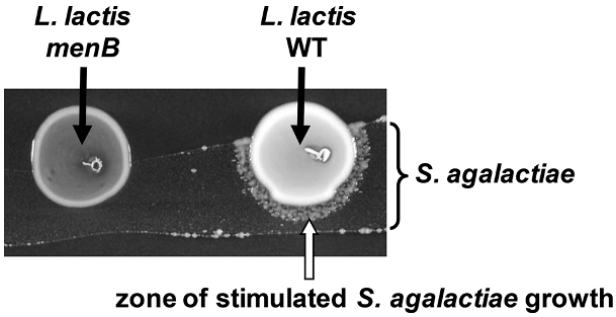


FIGURE 7 *L. lactis* produces menaquinones that cross-feed the opportunist pathogen *S. agalactiae*. Heme is present in the solid medium. A broad horizontal streak of an *S. agalactiae* strain (NEM316) is shown. Spots of cultures of *L. lactis* wild type (left) or *menB* that is defective for menaquinone synthesis (right) are deposited directly over the streaks. A stimulated growth zone is observed directly surrounding the wild-type *L. lactis* strain but not the *menB* mutant. From *Molecular Microbiology* (78).

Via respiration

Respiration growth leads to higher pH and efficient oxygen consumption. The presence of a respiration-positive *L. lactis* strain (grown aerobically with heme) rescued growth and survival of a respiration-negative mutant strain (tested with *cydA*) (Fig. 8).

Interbacterial Cross-Inhibition

Via secreted products

Lactococci, like other *Streptococcaceae*, produce toxic hydrogen peroxide under aerobic fermentation conditions, which may inhibit growth of peroxide-sensitive bacteria (72). Numerous LAB produce bacteriocins, which have widely different host spectra (see 157 for review); lysis of heterologous bacteria by bacteriocins may provide needed

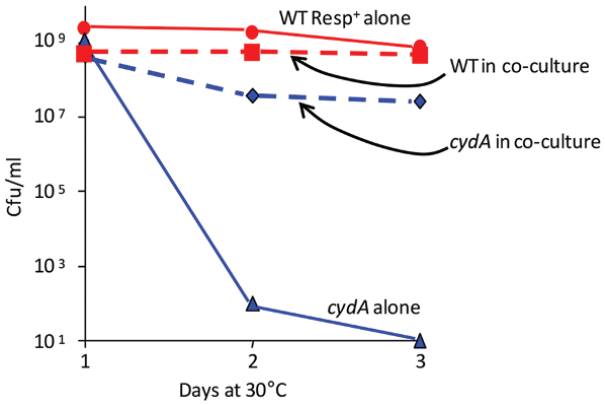


FIGURE 8 Respiring *L. lactis* can improve survival of nonrespiring bacteria in coculture. Differentially marked wild-type and *cydA* (nonrespiring mutant) *L. lactis* strains were grown separately or together in coculture. Nonrespiring *cydA* grew less well and showed poor survival when maintained in an aerobic medium with heme over a 3-day period. However, the respiring wild-type strain thrived. In contrast, the *cydA* strain fared much better when grown in coculture with the wild-type strain, as determined by cell count determinations. From *Molecular Microbiology* (72).

nutrients for the producer cells. Production of lysins may act to regulate bacterial cell wall properties *in trans* (158).

Via regulation of expression *in trans*

Studies of mixed cultures provide evidence for inhibitory effects of *L. lactis* on *S. aureus* growth and/or virulence gene expression. Tests conducted in either food or mammary gland cell lines indicate that (i) *L. lactis* reducing activity contributes to inhibition of a major virulence gene regulator (*agr*) and (ii) *L. lactis* at high bacterial concentrations inhibits *S. aureus* internalization in a mammary epithelial cell line (159, 160). These findings open prospects for antibiotic alternatives for prevention of infection, although their applicability to food industry and *in vivo* situations remains to be confirmed.

APPLICATIONS AND TOOLS OF LACTOCOCCI

The use of lactococci in "bioprotein" delivery, i.e., for antigen or enzyme delivery *in vivo*, is based on the idea that *L. lactis* can act as an effective nonpathogenic carrier, which can be administered orally or nasally without provoking a reaction other than that induced by the bioprotein of interest. Prior to this update, numerous reports described encouraging results of using lactococci for (i) prophylaxis to prevent bacterial infection (161), (ii) treatment of inflammatory bowel disease (3), (iii) prophylaxis and/or treatment of virally induced tumors (162), and (iv) allergy prevention (163, 164). Other properties of lactococci, including the expression of pili that might mediate bacterial adhesion *in situ* (135, 136), and novel applications have since been reported (165) which support their uses in biomolecule delivery. Despite a strong start, pharmaceutical drug development using *L. lactis* has not been reported, possibly due to the cost of development and the existence of industrially backed alternatives. Reported success with bioactive molecules might change this picture in the future.

Numerous applications using lactococci resulted from the development of genetic tools. Interestingly, a thermosensitive plasmid developed in *L. lactis* over 20 years ago, pG^+host, is still the gene replacement and transposition tool of choice in numerous *Firmicutes*, including pathogenic bacteria (166, 167). pG^+host is a derivative of the broad-host-range plasmid pWV01 and is nonreplicative in *L. lactis*, streptococci, staphylococci, and some lactobacilli at temperatures of 37.5°C or above. The thermosensitive replication protein has been retooled to produce numerous derivatives. Furthermore, pG^+host is replicative in *E. coli*. The existence of an *E. coli* strain bearing a chromosomal copy of the nontemperature-sensitive *rep* gene (168) facilitates gene cloning, as well as characterization of pG^+host insertions.

Another tool that has withstood time is the nisin-inducible expression system. Nisin is a bacteriocin encoded by a conjugative transposon (169–171). The promoter for the nisin biosynthesis gene, *nisA*, is regulated by *nisR* and *nisK* gene products. In the absence of nisin, or of *nisR* and *nisK* genes, promoter activity is very low. Addition of sublethal amounts of nisin results in strong induction of promoter activity, as demonstrated in innumerable applications of this system. This system has been shown to be functional in other *Firmicutes* (see 172 for review). Expression systems have also been developed for wider use among *Firmicutes* and in some cases may be advantageous over the nisin system. Controlled expression systems have arisen from studies of pH, salt, metal, heme, chloride, and sugar-regulated promoters (see 115 for review); some systems may overcome two potentially important limitations of the nisin system: (i) the requirement for either a specific host strain or a second plasmid (to provide *nisR* and *nisK* genes) and (ii) effects of nisin on the membrane, which may be particularly undesirable in studies of membrane proteins.

Highly regulated promoters can also be turned around for use as specific sensors. An interesting example concerns the HrtR repressor, which regulates heme efflux (Fig. 4). This and other heme-responsive promoters have been developed as sensitive heme sensors, with proof of activity *in vivo* (81; D. Lechardeur, unpublished data).

Constitutive expression of promoters at fixed levels can be valuable for quantitative physiological studies or for fine-tuning of gene expression in biotechnology. The *L. lactis* promoters P45 and P32 are commonly used, and strong constitutive promoters have been described (173). A set of synthetic promoters that differ by the sequence and length of spacers between the consensus sequences allows a broad range of constitutive activities (57, 174). A high-expression promoter based on the phosphotransferase system is further enhanced by cellobiose and was shown to be active in *B. subtilis* (175).

Site-Specific Single-Copy Integration

Lactococcal bacteriophages were initially studied with the goal of controlling starter culture lysis during fermentation. These phage studies led to the characterization of numerous bacterial strategies to abort phage activity. Phage studies have also been exploited to develop a site-specific integration system. Integration of temperate phages uses a phage-specified integrase that catalyzes insertion of the phage at a specific bacterial target, which is often localized at or near a tRNA gene. Using elements of lactococcal bacteriophage TP901-1, a site-specific integrative vector was designed to obtain chromosomal single-copy integration (176). This system should allow stable insertion and expression of foreign genes and can also be used to study the expression of genes in single copies under different growth conditions. The TP901-1 integration system has also been used in human cells (177). Another integration system based on a lactococcal intron led to efficient and stable insertion of genes without the need for selection (178).

Immune System against Phages: the CRISPR-Cas System

Chromosomal manipulation was upstaged by a mechanistic breakthrough made in *S. thermophilus* on phage resistance via the clustered regularly interspaced short palindromic repeat (CRISPR)-CRISPR-associated (Cas) system, which makes up a natural immune system against phage infections (179). During phage DNA infection, a bacteriophage DNA fragment is integrated in a specific chromosomal CRISPR locus. During a second attack with the same bacteriophage, small RNA expressed from the CRISPR system directs adjacently expressed Cas nuclease to cleave phage DNA, which prevents new infection. CRISPR-Cas is present in various LAB, including *S. thermophilus* and lactobacilli, but is uncommon in lactococci (180), although applications of the system are seen as ubiquitous (181).

Protein Export and Display Systems

Protein export reporters were developed to probe the membrane protein structure and identify export signals (182–184). One such reporter, the nuclease of *S. aureus*, is a stable,

well-characterized protein which is active when present as an amino- or carboxy-terminal fusion to other peptides and is faithful in reporting export events and in determining membrane protein topology. It is used in *L. lactis* and other *Firmicutes* to follow expression of exported proteins under different environmental conditions. The major advantages of using the nuclease over previously described export reporters are that it rapidly assumes its conformation and thus avoids degradation, and as few as ~300 nuclease molecules per cell can be detected in colony assays (183–186).

The use of *L. lactis* as a bacterial factory has been developed to deliver proteins of interest in the extracellular medium or on the bacterial surface. The export signal of Usp45, a secreted *L. lactis* protein, or signals from identified secreted native or heterologous proteins, is routinely used. Secretion efficiency can be improved by introducing (if necessary) an overall negative charge at the N-terminal end of the mature, translocated secreted protein (185). Advantages of using LAB other than *L. lactis* concern transit time in the intestinal tract; systems are thus being transposed to lactobacilli, such as *L. plantarum*, to improve efficacy (see 165 for review).

Anchoring of exported proteins via C-terminal LPXTG motifs is widely documented, particularly with respect to virulence factors such as *S. pyogenes* M-protein (187–189). *L. lactis* encodes several anchored proteins and at least two sortase-like proteins (190). Expression of the anchoring motif of the *S. pyogenes* M6 protein has been adopted to express recombinant proteins on the lactococcal surface (191). The AcmA autolysin is also cell wall associated, due to the presence of a three-times-repeated LysM motif (192). Anchoring via the AcmA binding motif was found to effectively present antigens at the lactococcal surface; a spacer between the anchoring domain and the protein of interest (a fimbrial protein) facilitates its access to target epithelial cells (193).

Expression Strains

Lactococci are remarkable for their simple genomes compared to complex bacterial models such as *B. subtilis* or *E. coli*. An interesting example is HtrA, which is the only surface protease in *L. lactis*. In contrast, *B. subtilis* and *E. coli* both encode numerous exported proteases (194). An *htrA* mutation leads to temperature sensitivity; at 30°C, exported proteins showed increased stability, although activity was not necessarily improved (194).

Cell Lysis Systems

Controlled cell lysis is a potentially powerful means of arresting cellular and metabolic activity; in fermentation, it may additionally result in a coordinated release of enzymes which could accelerate product maturation. The host autolysin, AcmA (195), or bacteriophage-encoded lysins and holins (which allow lysin release) are good candidates for this purpose. This application is potentially useful in controlling cell growth in fermented dairy products, as well as for enzyme release (196, 197). Expression of lysin and holin by a nisin-induced promoter does appear to accelerate cheese ripening (198). Lysins, derived from bacteriophage specific to several pathogens, effectively prevented or treated infections by streptococci, bacilli, and staphylococci in mice (199–201).

The cell envelope is an important barrier protecting the cell from stress situations. Cell wall damage via autolytic enzymes can render cells more sensitive to environmental conditions. Bacteria that have undergone even partial cell wall damage are permeable to small labeled probes used in standard *in situ* hybridization methods, while undamaged cells are not (202). As mentioned above, the SpxB protein mediates regulation of cell wall integrity (123). Rather than affect autolysin activity, SpxB is induced by lysozyme and/or PG digestion products, leading to PG *O*-acetylation, which renders it lysozyme resistant. Such changes could have profound effects on bacterial state, fitness, and resistance to autolysins, with applications for probiotic uses.

Containment and Food-Grade Strains

Inactivation of the *L. lactis* thymidylate synthase *thyA* gene results in a requirement for thymine or thymidine (66, 203). In *L. lactis*, a *thyA* mutant was exploited as a means of strain containment; i.e., strains can grow in a thymine-containing environment, such as the gut, but not in more limiting environments (203).

Nonsense suppressor strains are used in *E. coli* genetics to analyze phenotypes of point mutations. Plasmids carrying the suppressor genes could suppress an otherwise lethal nonsense mutation in the cell. This property of suppressors was exploited in *L. lactis* to construct and establish a food-grade plasmid (i.e., no foreign DNA) containing the suppressor genes in a suppressible purine auxotroph; this plasmid is stable in a milk media which cannot sustain growth of a purine auxotroph (204).

Higher Antigenicity

Studies have compared the effectiveness of presentation of different antigens as bacterial cytoplasmic, surface-anchored, or secreted proteins. Cell wall-associated antigens seem to induce a greater immune response than secreted or cytoplasmically expressed proteins (205, 206). Furthermore, immune response was reportedly enhanced in mutant strains with cell wall defects due to mutation of the alanine racemase (207).

CONCLUSIONS

L. lactis is likely the microorganism most eaten by humans. It belongs to a family comprised of pathogens (e.g., *S. pneumoniae*, *S. pyogenes*), commensal and opportunist microorganisms (e.g., *Streptococcus gordonii*, *S. mutans*), and food microorganisms (e.g., *S. thermophilus* and *L. lactis*). Studies of *L. lactis* reveal that differences between pathogens and nonpathogens are limited. As a bacterium that acidifies its own medium, *L. lactis* may have a high capacity for stress resistance when preadapted; stress-resistant mutants with constitutive stress resistance can be selected. Its metabolic flexibility by shifting to respiration metabolism can dispense with most acid production. As a food microorganism, *L. lactis* comes into close contact with, and may cross-feed, other bacteria in both the food environment and in the gut; as such, it may impact the behavior of other bacteria in complex ecosystems. As a nontoxic bacterium that secretes relatively few proteins in quantity, *L. lactis* may also be an organism of choice for oral vaccine or protein delivery design and biotechnological uses (see 165, 208, 209 for reviews). As a simple organism with diverse biotopes, including plants, milk products, and the gut, *L. lactis* may be a good choice for studies of the influence of environmental stress on evolution.

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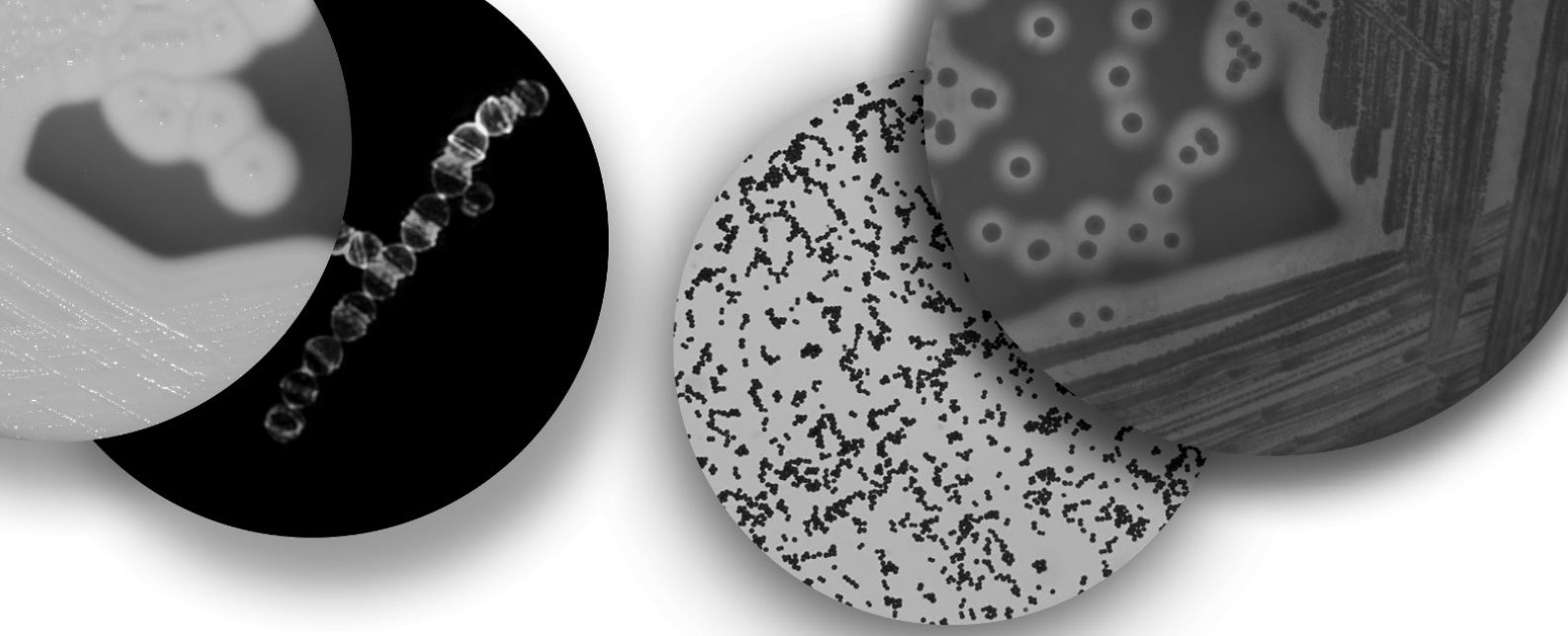
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SECTION 3

The Staphylococcus

Section Editor: **Richard P. Novick**

During the 12 years since the second edition of Gram positive Pathogens, the staphylococci have joined the best understood, best characterized, and most productively studied of the bacteria, driven, not least, by the revolution in genome sequencing. According to Lindsay, in chapter 30, there are now at least 275 fully sequenced *S. aureus* genomes and nearly 10,000 shotgun compilations in GenBank, plus a substantial number of non-aureus genomes, many of them of species associated with animals. This information has revealed the extent and importance of horizontal gene transfer and the nature, genetic contents, and mobility of the mobile elements, which is arguably responsible for the diversification and spread of pathotypes. Regulatory molecules and networks have been deciphered, not only those that govern metabolism, but also those that govern virulence and the production of toxic exoproteins and virulence-enhancing surface proteins, all of which has greatly enhanced our understanding of how and why these bugs cause disease. We now have better understanding of the relation between colonization and disease, and of the fascinating and remarkable battle between the staphylococci and the immune system, which leads one to

envision the host animal as a walled city surrounded and besieged by an invading army with a vast armamentarium of weapons designed to infiltrate and subvert or to breach or undermine the ramparts and kill the populace and its defenders. And the city in turn with its lookout towers and solid walls surmounted by an army of archers with arrows directed against the invaders and each of the invaders weapons, plus soldiers that venture forth to attack enemy individuals and a populace ready to mobilize and fight to the last. And the invaders with shields and armor to ward off the arrows and disarm the soldiers plus the ability to tunnel under the walls and construct their own defensive ramparts. How is the outcome of this siege decided? When the city is strong and well-armed, its ramparts well-maintained, and its soldiers healthy, its populace determined, it usually (but not always) prevails — but sometimes the invaders have secret weapons against which the besieged populace is defenseless. When the city is old and its ramparts and defenses weakened by desuetude or inattention and its populace indifferent, the siege is successful unless an outside force comes to the rescue with re-enforcements in the form of its own secret weapons — weapons that the invaders are learning all too rapidly to counter.

Staphylococci: Evolving Genomes

JODIE A. LINDSAY

30

INTRODUCTION

The genome of a bacterial cell encodes all of the potential functions of that cell and how they respond to environments (1). The first staphylococcal genomes to be sequenced in full were landmark achievements in 2001 (2), allowing the first overview and predictions of the metabolic, regulatory, and virulence potential of *Staphylococcus aureus*. As more genomes were sequenced in the next few years, different regions of the genomes were discovered that were core and conserved, often including essential genes, as well as regions that were variable, nonessential, or mobile and allow the cell to adapt to new environments (3–7). Now that thousands of genomes are available, we understand the constant flux that staphylococcal genomes are under. Change occurs in small or single nucleotide variations (SNVs) or by horizontal gene transfer (HGT), and evolution occurs when these changes provide a selective advantage to the bacterial cell and become fixed in subpopulations. Particular variants adapt to evolving habitats, new environments, and stresses, leading to the spread and expansion of successful clones that continue to evolve.

Staphylococci are colonizers and common opportunistic pathogens of mammals and birds. The pathogenic importance of staphylococci ensures that they are one of the most frequently sequenced genera. *S. aureus* is the species most studied due to its virulence and antimicrobial resistance (AMR) (8, 9). *S. aureus* is a colonizer of humans, predominantly the nose, throat, axillae, and groin of healthy humans. The colonization rate of populations is approximately 25% but is dependent on geography, age, and risk factors (10). *S. aureus* colonization is a risk factor for subsequent infection (11), and most infection is caused by the colonizing isolate (12, 13). *S. aureus* is a common cause of minor skin infections that do not require antibiotic treatment due to a healthy innate immune response driven by neutrophils. In hospitalized or immunocompromised patients, particularly those with breaches in the protective skin barrier, infection risk is higher and treatment may require antibiotic therapy. *S. aureus* is one of the most common causes of hospital-associated infection (8).

Acquisition of AMR genes ensures that *S. aureus* it is a formidable pathogen in health care. Penicillin resistance due to penicillinase enzymes that digest the β-lactamase ring is almost ubiquitous in human isolates (14). The widespread introduction of second-generation β-lactamase antibiotics such as methicillin selected for a new type of penicillin-binding protein-mediated resistance encoded by the *mecA* gene. Methicillin-resistant *S. aureus* (MRSA) emerged in the 1960s and spread globally and dramatically in the 1980s to 1990s (15); it is prevalent in health care clones of *S. aureus* at varying frequency across the world (16) and increases the overall burden of *S. aureus* infection. MRSA strains are often endemic in hospitals and resistant to a range of additional antimicrobials, drastically reducing the options available for prophylaxis and empirical treatment of *S. aureus* infection, leading to more infections, economic costs, and increased morbidity and mortality (8, 17). More recently, MRSA spreading among healthy community populations (CA-MRSA) has dramatically impacted health care provision (9), and livestock-associated MRSA clones have caused concern in high-risk groups with exposure to animals (18).

Humans are also colonized by many coagulase-negative staphylococci species (19), so called because they are not *S. aureus*, which is easily differentiated in the laboratory by its ability to clot plasma via coagulase production. Coagulase-negative staphylococci are generally considered less pathogenic than *S. aureus* yet are a common cause of infection in immunocompromised patients, particularly those with indwelling medical devices such as catheters and artificial joints and valves. The ability to produce extracellular polysaccharide biofilm and toxins and to carry multiple antimicrobial resistances contributes to their pathogenesis. The most prevalent species is *S. epidermidis*, and other important pathogens include *S. hominis*, *S. capitis*, *S. haemolyticus*, *S. simulans*, *S. xylosus*, *S. warneri*, *S. cohnii*, and *S. carnosus*. Uniquely, *S. saprophyticus* is a common cause of urinary tract infection and encodes proteins for binding to human urinary tract epithelial cells (20). Recently, several new coagulase-positive or -intermediate staphylococci have been identified that are capable of causing disease equivalent in humans to *S. aureus* but are much less prevalent, including *S. lugdunensis* (21) and *S. argenteus* (22).

S. aureus is also a common cause of infection in companion and livestock animals, in particular, bovine mastitis in the dairy industry (14) and invasive infections in chickens (23). *S. pseudintermedius* is considered to be a major canine pathogen (24), while many other staphylococci have unique associations with particular hosts (25).

Adaptation of successful clones to evolving habitats, such as host and antimicrobial pressure, is increasingly being studied using genomic tools. Analyses of whole genomes, genomic variability, epidemiology, and evolution of staphylococci shed light on staphylococcal colonization of and

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interaction with hosts, pathogenic pathways, and adaptation and spread in response to selective pressures. Ultimately, these studies identified key features of genetic, microbiological and biological phenomena as well as being applicable to preventing and treating infection in humans and animals.

GENOMES

A typical staphylococcal genome consists of a circular piece of DNA of between 2.5 and 3.0 million base pairs, which may be supplemented by additional plasmids. This equates to around 2,500 to 3,000 putative open reading frames or predicted protein coding sequences, as well as some tRNAs, rRNAs, noncoding RNAs, and pseudogenes. An example genome is shown in Fig. 1.

Signatures in the DNA sequence allow automated prediction of the start and stop of each coding sequence, and the coding sequences can be compared to genes of known function in other bacteria to predict protein function. Signaling sequences and conserved domains can identify the active sites of proteins and their physical location in the cell. Promotors can be predicted, and estimates can made of how genes are expressed and which other genes are expressed at the same time. A holistic picture of the potential of the cell emerges (1).

The first staphylococcal genomes sequenced to completion belonged to the colonizing and pathogenic species *S. aureus* (7). These early sequencing projects set the groundwork, identifying the key regions of the genome responsible for essential processes in growth, replication, and survival and identifying novel genes and pathways.

Over the next few years, another 10 *S. aureus* genomes were sequenced to completion, predominantly methicillin-resistant isolates (1) and a laboratory reference strain (5). These projects began to identify the regions of the chromosome that were conserved among strains and those that were variable. Most of the variability was due to variation in genes associated with surface proteins and regulators, as well as the presence or absence of mobile genetic elements (MGEs) and variations in their genetic content.

At the time of writing, the public database GenBank holds 272 completed *S. aureus* genomes and whole-genome shotgun genomes of another 9,065 *S. aureus* isolates (https://www.ncbi.nlm.nih.gov/genome/154). Including those genomes in the NCBI Sequence Read Archive and the European Nucleotide Archive, this number is almost 50,000 (26). This enormous increase in the amount of sequencing data is driven by increasingly inexpensive technology, particularly the Illumina sequencing platforms, which generates shotgun genomes of short reads of sequence that are unable to be resolved into a single contig of DNA but can be aligned to a reference genome of the same lineage. Similarly, other species of staphylococci are increasingly being sequenced. GenBank lists the whole genomes of at least 31 staphylococcal species, including colonizing and pathogenic coagulase-negative staphylococci, animal colonizers and pathogens, and food organisms. More than 200 staphylococci have been deposited without a designated species, indicating that the taxonomy is not completely resolved, although identifying and classifying species is increasingly accurate using whole genomes (27).

These databases are somewhat skewed by the choice of isolates for genome sequencing (26) and tend to favor those with clinical or industrial significance. Nevertheless, the extraordinary volume of data available ensures an

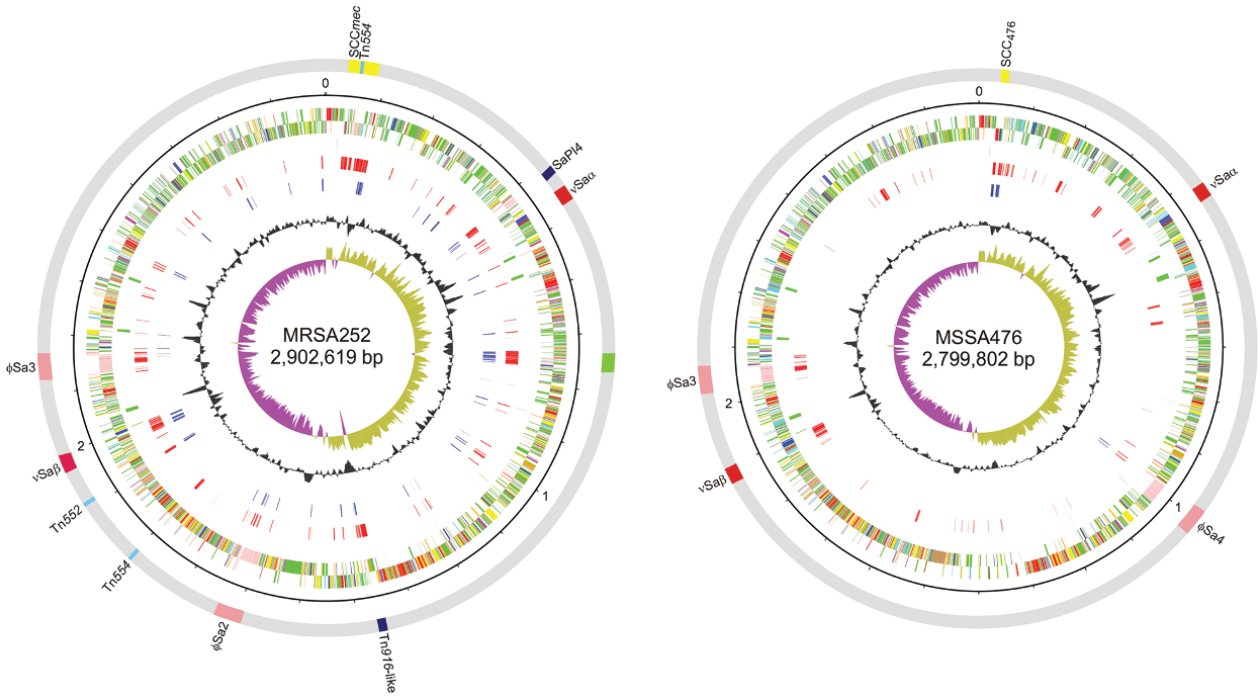


FIGURE 1 Example of *S. aureus* genomes illustrating variation in the core genome and mobile genetic elements. MRSA252, an example of a hospital-associated EMRSA16 ST36 CC30 isolate (left) and MSSA476, an example of a CC1 isolate (right). The core genome is highly conserved, with each lineage (CC) associated with specific variation. The outer gray ring indicates the location and distribution of the mobile genetic elements in these individual isolates, which can be highly variable. From reference 4 (https://www.pnas.org/content/101/26/9786/tab-figures-data).

unprecedented resource for the study of trends in the evolution, selection, and epidemiology of staphylococcal isolates.

The volume of information can be overwhelming to the nonspecialist. There are increasing numbers of tools to assist with the interpretation of genome information. For example, AureoWiki (aureowiki.med.uni-greifswald.de) is useful for searching for common genes across multiple *S. aureus* reference genomes with links to gene annotation and expression data (28). Staphopia assembles, maps, and annotates raw genome sequences (26). The Artemis Comparison Tool (ACT) is useful for aligning genomes and comparing where they vary (29). PanX can identify the distribution of accessory genes across multiple isolates (30). Regulatory RNAs can be identified using the SRD database (31). The Mykrobe predictor reads an *S. aureus* rapid shotgun sequence result and identifies up to 12 AMR markers in only 3 min (32). The Microreact project compares whole-genome sequences (WGSs) of isolates over time and geography to identify clones of public health importance (33). For laboratory researchers, an extremely useful tool is the Nebraska Transposon Library, identifying essential genes and actively distributing mutants deleted in specific genes in the reference strain JE2 (34) and tools to manipulate these strains (35).

The next generation of long-read sequence hardware technologies includes single-molecule real-time sequencers such as PacBio (155), and nanopore sequencing such as MinIon (156). Long-read technologies remove the need for alignment to reference genomes and are particularly useful for studying MGEs. Additional advantages include increased information about the epigenomes (methylation maps) of the DNA, and enhanced portability and low cost will ensure new insights into staphylococcal genomes in the future.

CORE GENOME VARIATION

The core genome is highly conserved among all isolates of a species and typically has a conserved gene order. The core genome encodes all the essential genes as well as many nonessential genes, including those involved in metabolic, housekeeping, replication, structural, regulatory, and some virulence functions (1). We might consider the genome that is not composed of MGEs as the core genome.

Multiple colonies from the same host are likely to have identical core genomes or vary in only a few single nucleotide variants (SNVs) or single nucleotide polymorphisms compared to each other (36). This type of SNV variation may be due to a swap in a base pair or an addition or deletion of a base pair or a very short sequence. From studying multiple colonies from the same host, it has been suggested that isolates are closely related if their core genomes contain 40 or fewer SNVs (37, 38). This level of variation within a colonizing population in a host is called a "cloud of diversity." Bacteria that spread between carriers or infected patients can have few SNV differences and therefore high levels of similarity, suggesting that the transmission event was recent (39).

SNVs arise as errors in replication catalyzed by the DNA polymerase enzyme as it rapidly unwinds and replicates the 3 million base pairs of staphylococcal genome in minutes during cell replication. Errors include incorporation of an incorrect base, deletion of a base, or insertion of an extra base. Bacteria have DNA error repair mechanisms, and in staphylococci these include the mismatch repair system. Occasionally, a mutation in a gene such as *mutL* or *mutS* leads to a "mutator" phenotype, where mutations accumulate at a higher frequency due to the lack of ability to repair them (40, 41).

From population studies of MRSA clonal types, the "molecular clock" of *S. aureus* is estimated to result in one SNV per genome every 5 to 10 weeks (36, 38, 42). Based on this concept, it is presumed that patients with larger clouds of diversity have been colonized for longer periods of time (43). As bacteria spread to new hosts and across nations and continents, this molecular clock of variation can reveal the transmission pathway. This was first seen in a large-scale epidemiological study of MRSA ST239 isolates from across the globe (36), which confirmed expected transmission pathways and identified unexpected ones.

It is important to note that the Illumina WGS methodology used to compare bacterial genomes and identify SNVs for transmission studies restricts analysis to the conserved regions of the genome in any given data set. This is typically the core genome only and will vary between experiments. The analysis will be most discriminatory when related isolates are compared, such as those belonging to the same lineage. Variable regions of the chromosome, such as the MGEs, are usually excluded from analysis. Nevertheless, it is an extremely powerful method for analysis of the evolutionary relationship of isolates and for predicting how recently they shared a common ancestor.

S. AUREUS LINEAGES

The *S. aureus* species has a population structure that divides into lineages, which are often referred to as clonal complexes (CCs) (44). Some of these lineages are dominant (common), while most others are rare (45). Isolates from the same lineage share a core genome that is extremely conserved and unique compared to other lineages. Each lineage is sufficiently different that WGS is rarely used to compare isolates from different lineages because the variation is too great to infer epidemiological relationships. Lineage-specific variation includes thousands of SNVs throughout the genome, as well as insertions, deletions, and variable regions. Isolates from the same lineage have extremely high levels of conservation in their core genomes across these SNVs and variable regions, indicating a close evolutionary relationship.

The naming of lineages is based on the original CCs assigned by a multilocus sequence type (MLST) system, where the sequence of seven housekeeping genes was compared. Isolates with seven identical sequences are grouped together into the same sequence type, and those with five or six identical sequences are assigned to the same CC (45). In contrast, two isolates that have four or fewer identical sequences typically belong to different CCs. Subsequent analysis of the highly conserved core genome across the species by WGS analysis confirms the lineage structure and that assignment of isolates to CCs by MLSTor to lineages by WGS correlate extremely tightly (26).

Microarray analysis across thousands of *S. aureus* genes and pan-genomes identified a broad range of genes that were present, absent, or highly variable in *S. aureus* populations but were conserved within lineages. These included many genes encoding surface-bound virulence factors and their regulators and immune evasion mechanisms (46, 47) that were predicted to play a role in host-adaptation or virulence. In some cases, the variation represents the acquisition or loss of an individual gene, such as collagen-binding protein. In other cases, one or more genes within an operon have been swapped with the corresponding genes from strains or other lineages, such as the four genes in the capsule locus that differentiate capsule type 5 from capsule type 8 or those determining the type VII secretion system. In some cases, a specific region within a gene varied, such as active sites

within the fibronectin-binding protein or the region within the *agr* regulatory locus determining the different *agr* classes (1, 46).

S. aureus genomes typically contain two "genomic island" regions of 20 to 30 kb called alpha and beta, which contain strings of duplicated but variable genes (44, 48). These islands are highly variable between unrelated lineages but very conserved within lineages. GIα typically contains 7 to 11 variant copies of staphylococcal enterotoxin-like genes that have superantigen-like (*ssl*) like properties involved in immune evasion, as well as multiple copies of related lipoprotein genes. GIβ carries multiple copies of serine proteins and different types of superantigens, as well as additional leukocidins or lantibiotic synthesis genes. Both GIα and GIβ also carry a restriction modification system with a modification and restriction specificity that is lineage specific. Such a system allows DNA to be transferred more efficiently between isolates of the same lineage than between isolates of different lineages (49, 50). The Sau1 restriction-modification (RM) variant genes (*hsdS*) are potent markers of lineage (49, 51) and likely contribute to the formation and independent evolution of the lineages.

Despite these major differences in genomes between lineages, isolates from most lineages of *S. aureus* appear to cause the same types and incidence of disease, and lineages generally have similar pathogenic potential. (The exceptions are some specific toxin-mediated diseases such as toxic shock syndrome, scalded skin syndrome, and food poisoning, where pathogenesis is dependent on the carriage of MGEs encoding relevant toxins.) Variation in the incidence of disease caused by each lineage correlates well with variation in the incidence of carriage, suggesting opportunistic infection (44, 45). Some lineages are more successfully carried in some hosts (for example, animals or humans) compared to others (14), and carriage appears to be the main risk factor for subsequent infection (12, 13). The importance of lineage-specific genomic markers in colonization and infection is still unclear, but redundancy of gene function is a common feature of staphylococcal genomes, suggesting that when one gene is missing or variable, others can compensate.

All *S. aureus* lineages share a common ancestor, but each lineage contains a unique combination of common variant genes, indicating extensive recombination. Only some of these lineages have become prevalent, including CC1, CC5, CC8, CC15, CC22, CC25, CC30, and CC45 (44, 45). This evolutionary pattern of repeated recombination of surface and host-interacting genes with expansion of only a small number of successful combinations has led to some very stable lineages. There is a surprisingly close relationship between isolates from the same lineage that can have highly conserved lineage-specific features despite very different epidemiological behavior and geography. An example is the USA400 MRSA lineage CA-MRSA represented by the MW2 isolate from the USA in 1998, which caused severe skin and soft tissue infection in children compared to the methicillin-sensitive isolate MSSA476 identified in a nasal carrier in Oxford, United Kingdom, in 1999 (4). The two isolates both belong to CC1 and have only 285 SNV differences in their core genomes within functional coding regions. In contrast, they have many differences in MGEs (4). Similarly, CC8 isolates, such as the laboratory strain 8325 (5) and MRSA USA300 FPR3757 (52), both belong to CC8 and have highly conserved core genomes despite extensive variation in MGEs and pathogenic and epidemiologic behavior. Therefore, the success of some of these lineages appears to be due to the emergence of specific clones that have acquired MGEs and resistances, allowing rapid adaptation to new habitats and environments.

MOBILE GENETIC ELEMENTS

MGEs are discrete pieces of DNA that replicate on their own or integrate into the host chromosome. They are not essential for the host but have specific mechanisms ensuring their own ability to transfer between bacterial cells and survive. They include bacteriophages (53), *S. aureus* pathogenicity islands (54) (also known as phage-inducible chromosomal islands [55]), plasmids (56), transposons, and the staphylococcal cassette chromosome (SCC) elements. MGEs are important because they carry many virulence, host-adaptation, and AMR genes, so their carriage and transferability can have a substantial impact on the success and spread of staphylococci.

MGEs are widely distributed in *S. aureus* and can make up 10 to 20% of the genome (7). There is enormous variation in MGE gene carriage and distribution, even among related bacterial isolates. Variation and recombination between MGEs appears to be common (53, 56). Having multiple MGEs per cell ensures a flexible bacterial genome that can acquire and lose a variety of virulence and resistance genes.

Bacteriophages

Virtually all natural isolates of *S. aureus* harbor one or more intact bacteriophage (or phage) genomes inserted into the chromosome (53). *S. aureus* phages are typically around 45 kb, temperate, reside quiescently within the chromosomal DNA (where they are termed prophages), and can be induced by DNA damage or stressful conditions to excise, replicate their DNA, and produce phage proteins, including those that assemble into phage particles. Phage DNA is packaged into new phage particles and released on bacterial cell lysis, and the particles bind to and inject their DNA into new host bacteria. This results in either the lytic pathway or the lysogenic pathway. The lytic life cycle induces further replication of phage DNA and protein production, lysis of the bacterial host, and release of infectious particles. The lysogenic life cycle results in the phage DNA integrating into the bacterial chromosome at the phage-specific conserved integration site. Some virulence factors, such as Panton-Valentine leucocidin, enterotoxin A, and exfoliative toxin A, are encoded on lysogenic prophages, where they may provide a benefit to the host bacterium during infection. Host-specific immune evasion pathways such as *chips*, *scin*, and *sak* are also found on the φ3 family of bacteriophages associated with human carriage and disease (14, 53).

Pathogenicity Islands and SaPIs

S. aureus pathogenicity islands (SaPIs) are related to prophages but do not carry the genes for phage particles (54). They excise, replicate, and package their DNA into phage particles, but the phage particles are supplied by a helper phage (57, 58) which also regulates induction of the SaPI and directs lysis of the bacterial cell. SaPIs carry toxic shock syndrome toxins and can also carry biofilm-associated genes, iron uptake pathways, and antibiotic resistance genes.

SCC*mec*

In terms of clinical significance, the most important MGE may be the SCCs that carry the *mecA* gene encoding methicillin resistance. SCC*mec* elements have been classified into

at least 11 types, each with different combinations of *ccr* genes that catalyze excision and integration of the element into the chromosome (http://www.sccmec.org) (59). Integration only occurs at one chromosomal site, *orfX*, and each bacterial cell has either zero or one copy, ranging in size from 3 to 70 kb (60). Early SCC*mec* types tended to be larger and carry multiple resistance genes, while the smaller and streamlined SCC*mec* type IV is probably now the most successful and widely distributed type. While SCC*mec* regions can transfer between isolates, they appear to be more stable than other MGEs in clonal MRSA. The acquisition of an SCC*mec* by a sensitive *S. aureus* strain appears to be the first step in the evolution of MRSAs with unique epidemiology (36, 61–63) and the ability of MRSA clones to subsequently spread in specific environments, particularly health care, community, and livestock, and will be discussed below in the section on AMR. Successful MRSA clones tend to have stable SCC*mec* elements (36, 61, 64).

Plasmids

Staphylococcal plasmids are circular supercoiled DNA molecules ranging in size from 1 to >60 kb. They replicate autonomously and regulate their own copy numbers. Smaller plasmids replicate by the unidirectional rolling circle mechanism (65), while larger ones utilize the bidirectional theta mechanism (66). Plasmids are common, with most isolates harboring one or more, and strains with up to four have been found. Plasmids carry a large proportion of AMR genes in *S. aureus* (56, 67).

Transposons

Transposons are MGEs that encode transposases, which are capable of catalyzing replication and/or excision of the transposon, followed by integration into a new insertion site on the genome. Insertions can be site-specific or random and can be located on the chromosome, plasmid, or another MGE. In staphylococci, transposons are particularly associated with antimicrobial gene carriage (67), especially penicillin, macrolides, tetracycline, and vancomycin.

Insertion Sequences

Insertion sequences (ISs) encode only a transposase, but two ISs can form a composite transposon and transfer the DNA between them, including resistance genes (68). ISs such as IS*256* can insert into coding or regulatory regions of the genome, altering gene function and expression. An example is IS*256* that affects biofilm expression in *S. epidermidis* (69).

HORIZONTAL GENE TRANSFER

Horizontal gene transfer (HGT) requires several steps for success. First, DNA must transfer from one cell to another, and it must find a way to replicate or integrate, evade host defense mechanisms, and not cause a fitness cost to the new cell (70). DNA can transfer between bacterial cells by transformation, conjugation, or transduction. In *S. aureus*, transformation is inefficient (71) and probably rare. While conjugation functions effectively in staphylococci, the distribution of conjugative genes necessary for transfer is relatively constrained, suggesting that conjugation is not the major driver of HGT in *S. aureus*. In contrast, bacteriophages are widespread in *S. aureus* genomes, and HGT via transduction is efficient and widespread, probably the most common mechanism (70).

Transduction involves DNA transfer via bacteriophage packaging and delivery of DNA, such as transfer of phage DNA between cells. Generalized transduction occurs when host bacterial DNA is packaged into the phage particles, which are released by lysed cells, and the noninfectious particles inject DNA into new bacterial hosts. Packaging can be dependent on *pac* sites or *cos* sites scattered through the genome (72, 73), and packaging of host DNA is not accidental but dependent on environmental conditions such as antibiotic exposure (74). Some host DNA is packaged at a higher frequency, particularly plasmids, which may be a reflection of copy number or concatemer formation (75). SaPIs are preferentially packaged into phage particles by targeting the terminase packaging proteins of the phage directly (58). Phage heads typically hold a maximum of 45 kb of DNA, yet some larger MGEs appear to have been transferred by generalized transduction, perhaps by multiple transfer events (76).

Once DNA is delivered into the recipient cell, it needs to find a way to replicate in order to survive the next round of bacterial cell division. Plasmids replicate autonomously as circular DNA. Most other MGEs have a specific mechanism for integrating into the chromosome or a plasmid, using integrases or transposases. These may be site-specific or random integration events. In contrast, a chromosomal piece of DNA that enters a recipient cell without a mechanism of replication or integration relies on homologous recombination with the chromosome (77). In *S. aureus* this is an inefficient process.

Transduction of MGEs is highly efficient in colonizing populations of bacteria. In a controlled evolution experiment in four gnotobiotic piglets, plasmids were shown to transfer between coinoculated isolates within 4 hours (78). Multiple phage and plasmid transfers occurred continuously during 16 days of colonization, resulting in heterogeneous colonizing populations that varied enormously in MGE content (78). Gene transfer appeared to be localized to the bacterial populations within each of the four piglets, despite the close physical contact of the animals and the opportunity for strains to spread (78). Similarly, in human MRSA carriers, free generalized transducing phages are detectable, and clonal colonizing populations in individual hosts often vary in MGE and AMR gene content, indicating frequent gene transfer (79). These studies also suggest that loss of MGEs occurs, although the mechanisms are not understood.

Since high-level MGE transfer occurs within the host, and MGEs are also lost within the host, it is expected that variation in MGE carriage is extensive in clonal population isolates from multiple hosts. Since epidemiological studies typically analyze only one colony or isolate from each host, only a sampling of this variability is detected in studies investigating MGE distribution. This limits our ability to make predictions about the distribution and importance of virulence and AMR genes. However, genomic trees generated to investigate the epidemiology of successful MRSA clones typically identify frequent acquisition and loss of the same or similar MGEs (44, 61, 80), suggesting that they are widespread among the population and undergo their own evolutionary adaptation.

There are several barriers to HGT that protect bacteria from phage DNA that otherwise might lyse and destroy populations. First, prophages produce regulators that prevent phage excision and replication of incoming related phages and therefore protect the cell from lysis (53). The second barrier is RM. The type I Sau1 system is widespread and lineage specific, ensuring that DNA transferred from one

bacterial cell to another of the same lineage is recognized as self due to specific modification and protection of target site sequences (49), whereas DNA transferred from one bacterium to another of a different lineage is recognized as foreign by its unprotected DNA target sites and digested with restriction enzymes (49, 53). Additional RM systems are also found, such as the type II, III, and IV systems (70), and many of these genes are encoded on MGEs themselves, protecting host bacteria as well as protecting themselves from other MGEs. Clustered regularly interspaced short palindromic repeat (CRISPR) systems were first described in *S. epidermidis* (81) but are very rare in *S. aureus* (70, 82). CRISPRs are an adaptive immunity system for bacteria, because they incorporate segments of invading DNA, which they recognize and target for digestion by CRISPR-associated (Cas) endonucleases on the next encounter (83).

Staphylococci coevolve with some MGEs, with each providing advantages and disadvantages to each other. For example, resistance to phage infection by host receptor modification prevents lysis by the phage (an advantage to the cell) but also HGT by that staphylococcal phage (a disadvantage to the cell) (84). The phage itself requires a host to replicate, driving adaptation to respond to altered host receptors. Plasmids and phages can also evolve to expand their host range. Plasmid immunity (incompatibility) occurs when two plasmids share the same replication mechanism, ensuring that after several rounds of cell division, one plasmid tends to be lost (85). Plasmids have therefore recombined extensively so that related genes are carried on plasmids with different replication genes (56). Interestingly, some large conjugative plasmids have overcome type I RM barriers by removing restriction target sites from their genomes, freeing their transfer between certain lineages (50).

The high efficiency of MGE HGT, the inefficiency of transformation and homologous recombination, and the distribution of barriers to gene transfer markedly shape the genome of *S. aureus* and the population structure compared to other species. As a result of these mechanisms, the *S. aureus* lineages are distinct and stable, while the MGEs encode most of the adaptive traits and can be highly mobile and unstable, and those MGEs that maintain stability in successful clones are likely to be important and continuously selected.

SELECTION AND ADAPTATION

Evolution occurs when changes in the genome are selected for and variants outcompete established populations and may move into new niches. MGEs that encode host-adaptive or AMR markers are particularly involved in the evolution of successful clones that can then expand and spread. Considering how rapidly MGEs can be transferred between isolates and lost, the stable presence of an MGE is taken as an indicator that it plays an important role in adaptation.

Virulence and Pathogenicity

S. aureus produces dozens of known and putative virulence factors, including toxins, immune evasion proteins, and adhesins. Regulation of these virulence processes is controlled by a multitude of gene regulators that respond to environmental conditions and ensure efficient expression at the appropriate time and place. Despite many virulence factors being encoded on MGEs, there are few cases where the presence of a single virulence factor has been associated with evolution and adaptation. Since the normal niche of staphylococci is not the diseased host but the normal skin and mucous membranes, we can imagine that the ability to cause disease may not necessarily provide the bacteria with a selective advantage during colonization. Early studies suggested that the presence or absence of virulence factor genes on MGEs was similar in both colonizing and infecting isolates (44). For example, the role of the immune evasion cluster genes on φ3 phages appears to be associated with successful human clones but is also common in colonizing isolates rather than being specifically selected in infection-causing isolates. Another interesting example is the Panton-Valentine leukocidin, which is associated with severe skin and soft tissue infection, where the toxin may play a role in disease but is also relatively stable in colonizing populations (86), where perhaps it reduces clearance by neutrophils in the nose. A third example is the *sasX* gene which is carried on the φSPb-like prophage in the successful ST239 health care-associated MRSA (HA-MRSA) clone and encodes a surface protein for adherence to nasal epithelial cells (36, 87).

More recently, single nucleotide polymorphisms in genes associated with virulence were detected with a 3.1-fold enrichment in infecting versus nose-colonizing bacteria from the same host (88). This suggests that these mutations arose and were beneficial to the bacteria during the pathogenic process. In particular, genes associated with the regulation of virulence were variable, such as the accessory gene regulator, *agr* (88, 89). While these regulators control virulence in mouse models of infection (90, 91), they are lacking in some human clinical isolates, and in others they may play a role in adaptation of colonizing isolates as the infection develops (92, 93). In contrast, no differences were detected between isolates causing bacteremia and those causing endocarditis (94). Bacteremia isolates were shown to be less toxic to eukaryotic cells *in vitro* than nonbacteremia isolates, and genome-wide association studies suggested that multiple loci could be involved (95).

Surprisingly, successful clones can maintain mutations in common virulence factors. The successful CA-MRSA USA300 does not produce capsular polysaccharide, due to multiple mutations in the capsular operon (96). The HA-MRSA strains have mutations in the alpha hemolysin gene and *agr* (157). Extensive redundancy in the staphylococcal genomes, where multiple genes can encode for similar functions, may account for the lack of a virulence Achille's heel. Unfortunately, this reduces options for therapeutics targeting a single virulence factor (97).

Antimicrobial Resistance

Genomic analysis demonstrates that AMR is the most important evolutionary adaptation of staphylococcal populations. In particular, MRSA strains carry SCC*mec* elements which encode the genes necessary to resist nearly all types of β-lactam antibiotics. MRSA strains do not just replace sensitive *S. aureus* populations, but cause an additional burden of disease (98).

Since β-lactam antibiotics are the most successful antibiotic class—effective, inexpensive, easy to tolerate, and with limited side effects—pharmaceutical companies have continually developed new β-lactam antibiotic variants to evade bacterial resistance mechanisms (9). MRSA is only susceptible to ceftaroline and ceftobiprole, which dramatically reduces the types of antibiotics that can be effectively used for prophylaxis (prevention) and empirical therapy (treatment prior to diagnosis). The *mecA* gene carried on the SCC*mec* encodes an alternative penicillin-binding protein, PBP2A, allowing synthesis of cell wall peptidoglycan in the presence of

β-lactams that block a crucial enzyme, PBP2 (99). β-lactams are widely used in humans and in agriculture, and hosts exposed to β-lactams provide an advantageous niche for MRSA over other methicillin-susceptible colonizing microbes. Once colonizing successfully, MRSA acts as an opportunistic pathogen. Since there are only a relatively small number of successful MRSA clones and they cause significant concern in health care, they are often screened for and reported and their epidemiology and spread investigated. Therefore, we investigate the evolution of these clones more than any other staphylococci (47).

In epidemiological terms, there are three main types of MRSA, each evolving on multiple occasions and each with unique features of their genome (100). They are health care associated (HA-MRSA), community associated (CA-MRSA), and livestock associated. Each type has emerged in multiple lineages, demonstrating that lineage is not the major factor responsible for the unique epidemiology. Instead, there are common MGEs associated with each MRSA type, and these MGEs are relatively stable in the clonal population, indicating repeated selection for the MGEs for survival in the respective epidemiological niche (47). In each geographical location and niche, only one or two MRSA clones tend to dominate.

HA-MRSA has emerged in lineages CC5, CC8, CC22, CC30, CC45, CC59, CC80, and ST239. Each lineage carries an SCC*mec* type, usually type II, III, or IV. Interestingly, ST239 evolved by a large recombination event between CC8 and CC30, where a large region of the genome containing the SCC*mec* region of HA-MRSA CC30 swapped into a CC8 lineage (101, 102). HA-MRSA clones also carry the ϕ3 human host-adapted bacteriophage, thought to be important for human colonization. Successful HA-MRSA strains are often multidrug resistant, and a particular association between HA-MRSA and fluoroquinolones has been identified. In the CC22 HA-MRSA clone, mutations in the *gyrA* and *par*C genes seem to have been essential for successful dissemination (61). In the United Kingdom, usage of fluoroquinolones appeared to select for HA-MRSA, and a decrease in usage correlated with a decrease in hospital infection incidence with HA-MRSA (98). This is consistent with exposure to fluoroquinolones being associated with increased HA-MRSA colonization in Europe, Asia, and the United States (103–106). Resistance to aminoglycosides, macrolides, and others is also associated with HA-MRSA, but resistance rates are variable within and between geographical locations (100), possibly due to different selective pressures.

CA-MRSA strains have evolved in lineages CC1, CC8, CC30, and CC59. CA-MRSA causes severe skin and soft tissue infection in healthy patients, often children, military members, athletes, prisoners, and those in close contact with others. Successful clones carry both an SCC*mec* and the φ3 human-adapted bacteriophage with genes for evading the host immune response. Uniquely, they also carry the φ2 bacteriophage encoding Panton-Valentine leucocidin. While the importance of this toxin was initially controversial in models of skin infection, it is now clear that Panton-Valentine leucocidin is host specific and targets human and rabbit neutrophils (107). Successful clones include the USA400 (CC1) clone, initially identified in the United States but now less prevalent, the successful USA300 (CC8) clone in the United States, which is spreading globally, the original Southwest-Pacific clone (CC30), the Asian clone (CC59) (108), and the Australian clone (CC93) (42). Antimicrobial resistance levels appear to be increasing (109), and fluoroquinolone resistance may play a role in the spread (86).

Livestock-associated MRSA strains have evolved in lineages CC398 and CC9 and are associated with pig and veal-calf agriculture. The isolates do not cause significant disease in these animals but can colonize the humans that come into contact with the farms, and the organisms can cause subsequent opportunistic infection. Interestingly, the isolates do not spread in hospitals as effectively as HA-MRSA (110), and colonization of farmers requires repeated exposure (111). CC398 isolates were originally considered untypable due to a modification of the SmaI restriction site that is lineage specific (51, 112). These successful clones are prevalent in mainland Europe and are spreading to the United States and South America and have acquired SCC*mec* elements but not φ3 with human host-specific immune evasion genes. While the reasons for their adaptation to pigs is unclear, they typically encode resistance to tetracycline antibiotics (113). Tetracyclines are widely used in agriculture but are used much less frequently in hospitals, and tetracycline resistance is relatively uncommon in hospital-acquired *S. aureus*. CC398 tetracycline resistance is due to genes carried on plasmids and/or transposons, and up to three distinct mechanisms in a single isolate have been identified (113). CC9 isolates in Asia have been reported to be multidrug resistant (114, 115). A recent increase in CC398 MRSA infection in humans with no contact with livestock has been attributed to higher carriage of this clone in the community and the acquisition of φ3 host-adaptation genes (116).

Genomic analysis has also revealed that other resistances can be selective. For example, in New Zealand, high usage of fusidic acid in the community has driven the evolution and selection of three MRSA clones that carry the *fus*C resistance gene (117). However, not all resistances are selective. Vancomycin and other glycopeptides are widely used to prevent and treat MRSA infection, yet resistance is still rare. Only 14 cases of fully vancomycin-resistant *S. aureus* (VRSA) have been described in the United States. They have evolved independently of each other due to the acquisition of the *van*A operon on a transposon from vancomycin-resistant enterococci (118). VRSA is unfit compared to its MRSA parents and is susceptible to both antibiotics in combination (119). VRSA has been reported in India, Brazil, Portugal, Turkey, and Iran (120–124) but is still rare, and there is little evidence of patient to patient spread. In contrast, intermediate-level vancomycin resistance in MRSA (vancomycin-intermediate *S. aureus* [VISA]) is more widely reported, and these bacteria have thickened cell walls that absorb excess antibiotic, slightly reducing susceptibility. Populations are often hetero-resistant with only a small subpopulation showing the VISA phenotype (125). These isolates probably have limited ability to resist vancomycin in the clinic, and associations between laboratory susceptibility and response to therapy are unclear (126). VISA does not appear to be a clonal trait associated with successfully spreading clones. Instead, VISA organisms are generally isolated from patients with chronic infection who have been exposed to long-term antibiotic therapy, and they arise due to SNV variation (125, 126). It can be concluded that despite substantial selective pressure, *S. aureus* has not yet been able to develop resistance to vancomycin that allows maintenance of fitness in its normal habitats and spread to new hosts.

Host

Genomic analyses of isolates from different hosts, particularly animal versus human, have identified key pathways that are

associated with adaptation to these hosts. Evasion of host innate immune responses is central to the survival of *S. aureus*, particularly escape from complement and neutrophil killing (48). Particular lineages are often associated with specific hosts, with each lineage carrying different combinations or variants of immune evasion genes, particularly on the genomic islands, such as inhibitors of complement cascade or antibodies, neutrophil signaling, migration and recognition, and cytolytic toxins (48).

In addition, host specificity can be strongly associated with MGEs. Human isolates typically carry the φ3 prophage with at least two or three genes at one end that are associated with evasion of the human immune response, while animal isolates are usually missing the phage (14, 127). These genes are the staphylococcal complement inhibitor (*scin*), which blocks activation of the human complement system (128), the chemotaxis inhibitory protein (*chips*), which prevents human neutrophil recruitment (129), and staphylokinase (*sak*), which activates human plasminogen and local spreading (130). Interestingly, the enterotoxin A (*sea*) gene, encoding the most common cause of staphylococcal food poisoning, is also carried on the φ3 phage adjacent to the immune evasion genes but is missing in many human isolates, suggesting that there is frequent recombination of this phage region and that *sea* is not required for host adaptation (14). The specific role of the φ3 phage in selection is most likely to be a requirement for survival in the normal colonizing habitat of the human nose, suggesting an active neutrophil response targeting *S. aureus* during colonization. It is unclear why only about 25% of humans are continuously colonized with *S. aureus* while others are colonized intermittently or not at all.

Leukocidins are two-component pore-forming toxins and function by binding to host-specific receptors on the surface of target cells (131, 132). One of these, Panton-Valentine leucocidin, targets human neutrophils (107) and is carried on a φ2 phage. While only a small proportion of human *S. aureus* bacteria carry Panton-Valentine leucocidin toxin, it has been implicated in rare lethal necrotizing pneumonia (133), as well as severe skin and soft tissue infection and the success of CA-MRSA clones (107).

S. aureus is a common cause of bovine mastitis, causing economic losses in the dairy industry. Unique lineages are associated with bovine isolates (14). A novel SaPI found in bovine isolates coagulates ruminant plasma only (23). The leukocidin toxin LukMF is carried on a φ1 phage in ruminant isolates only (48) and targets bovine neutrophils and immune cells (131).

In horses, a specific phage, φSaeq1, encodes a novel inhibitor of complement from horses, humans, and pigs, eqSCIN (134). Isolates from chickens often carry avian-adapted phages and plasmids, putatively involved in lysing chicken heterophils (135). In both wild mice and laboratory mice, *S. aureus* colonizing isolates have lost φ3 and immune evasion cluster genes (136).

A unique situation occurs in rabbit *S. aureus*, because host specificity can be conferred by a single naturally occurring nucleotide mutation in the *dltB* gene altering teichoic acid and lipoteichoic acid charge (137), a receptor commonly used by bacteriophages and for HGT (138).

Microbiome and Competing Bacteria

The normal habitat of staphylococci is the skin and mucous membranes of animals and birds, and this is the reservoir of infecting isolates. During colonization, a variety of adapted bacteria compete for space and nutrients (139). Competition and coevolution of these bacteria can result in direct inhibition, synergistic cooperation, and/or gene exchange. Environments can also change in nutrient availability, humidity, immune responses, infection with viruses, or antibiotic exposure (139). The microbiome is constantly adapting, and the ability of staphylococci to adapt to these environments is vital for their survival.

In the absence of a microbiome, such as a gnotobiotic animal model, *S. aureus* inoculated onto a single skin site can colonize the whole host within hours (78), presumably due to the lack of competing bacteria. However, once colonized, hosts in close contact exchange strains infrequently (78). Antimicrobials can also deplete microbiomes. In a mouse model of colonization, those treated with streptomycin were more likely to be colonized and colonized with greater numbers of bacteria (140). Humans exposed to antimicrobials that *S. aureus* is resistant to are more likely to be colonized (141).

Nutrients are also important. *S. aureus* survives better on the nutrients available in the human nose than coagulase-negative staphylococci (142), accounting for their different niches.

During evolution, a staphylococcal cell that has altered its genome by SNV or MGE acquisition must compete with not only the microbiome, but with its peers that have not altered their genome. The relative fitness of an evolving isolate can be compared to its peers, but its ability to survive depends on environmental factors.

Competition between microbiome species can be for nutrients and space or can be targeted. *S. lugdunensis* colonizing the human nose produces lugdunin, a peptide antibiotic that inhibits *S. aureus* colonization (143). Some *S. aureus* isolates also produce lantibiotics that target other *S. aureus* isolates, staphylococcal species such as *S. epidermidis*, and lactococci (144, 145), and staphylococcal species and lactococci, in turn, are capable of producing inhibitory lantibiotics against *S. aureus* (146, 147). *Corynebacterium* spp., *Haemophilus* spp., and streptococci also inhibit *S. aureus* colonization (148–150).

In the cystic fibrosis lung, *S. aureus* has a negative correlation with *Pseudomonas aeruginosa*, which eventually displaces it. *P. aeruginosa* produces a range of antistaphylococcal factors, but most interestingly, may also activate the host to clear *S. aureus* specifically by inducing a phospholipase (151, 152). Within a *Caenorhabditis elegans* worm model, coinfection with *Enterococcus faecalis* caused *S. aureus* to evolve to produce less siderophore for iron scavenging (153), while *E. faecalis* evolved to produce more superoxide antimicrobial to combat *S. aureus* (154).

SUMMARY

Staphylococci live on the skin and mucous membranes of mammals and birds and have adapted to this environment. They are formidable opportunistic pathogens, carrying multiple colonization and virulence factors that are often variable, redundant, and highly mobile. This genetic variability contributes to their ability to adapt to changing hosts, antimicrobial exposure, and bacterial competitors, expanding their niches and ability to cause opportunistic infection. New and successful clones with unique epidemiology teach us about genetic adaptability and the selective pressures in different environments. As we improve our understanding, we improve our ability to predict the next wave of multidrug-resistant staphylococci in new host populations and identify opportunities to reduce their impact on health, wealth, and welfare.

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Staphylococcal Plasmids, Transposable and Integrative Elements

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31

As has been the case for other bacterial genera, studies of the genetic basis of staphylococcal pathogenicity and antimicrobial resistance highlighted the presence of determinants not uniformly represented in the genomes of all staphylococcal strains. Although not a fundamental requirement for survival *per se*, such accessory DNA usually encodes functions that are advantageous in a particular environmental niche. The extent of the accessory component of the genome has been revealed by comparative analysis of whole *Staphylococcus aureus* genome sequences, which indicate that it can constitute in excess of 20% of a strain's genetic makeup (1–5). It is clear that the acquisition, maintenance, and dissemination of accessory functions have been central to the ongoing success of staphylococci as pathogens. These processes are underpinned by interactions between mobile genetic elements, such as insertion sequences and transposons that mediate intracellular movement of DNA, and plasmids, bacteriophage and integrative and conjugative elements (ICEs) that facilitate intercellular DNA mobility.

MECHANISMS OF GENETIC EXCHANGE

DNA can be introduced into staphylococci by each of the three classical bacterial gene transfer mechanisms of transformation, transduction, and conjugation. Transformation is thought to be limited by extracellular nucleases and is usually very inefficient (6, 7). However, homologs of competence genes from other Gram-positive species are evident in the *S. aureus* chromosome, leading to the suggestion that natural competence might arise under suitable growth conditions and/or in subpopulations of cells (8). Staphylococci usually possess one or more prophages integrated within their genomes, and generalized transduction is likely to be a frequent mechanism of genetic exchange between strains (9, 10). Staphylococcal conjugative plasmids not only mediate their own self-transfer, but also facilitate the exchange of other coresident nonconjugative plasmids, either by mobilization if the other plasmid carries a relaxation system (11) or more generally via cointegrate formation, and potentially subsequent resolution, in a process termed "conduction" (12, 13). Conjugation is also thought to represent the most likely basis for the apparent transmission of large DNA segments that correspond to approximately 10 to 20% of the chromosome (14). In addition to their own transfer, chromosomally located ICEs in staphylococcal strains (15) might also mediate transmission of plasmid and chromosomal DNA (16). A novel but poorly understood mechanism of staphylococcal genetic exchange, termed mixed-culture transfer or phage-mediated conjugation, was described in the 1980s (6, 17, 18). Although phage, or perhaps components of phage, play a role, the process has been shown to be mechanistically distinct from transduction. More recently, another phage-mediated mechanism, termed autotransduction, has been defined (19), which like mixed-culture transfer, requires the DNA recipient to be lysogenic for specific prophage. It is possible that mixed-culture transfer and autotransduction are actually different perspectives on the same phenomenon. There have been several reports that describe enhanced DNA transfer between staphylococci, by conjugation and transduction, in response to subinhibitory levels of some antibiotics; however, in most cases the basis of this stimulation is yet to be delineated (10, 20–22). Notably, genetic exchange has also been observed to occur more efficiently *in vivo* than *in vitro* (23).

Identical or nearly identical accessory genes, elements, and plasmids have been detected in different staphylococcal species and other bacterial genera, such as enterococci and streptococci (24–28). Such observations suggest that, directly and/or indirectly, the gene transfer mechanisms operating in staphylococci facilitate not only intraspecific transfer, but also interspecific and intergeneric exchange and hence access to an extended and shared reservoir of determinants.

STAPHYLOCOCCAL PLASMIDS

One or more plasmids are usually found in clinical isolates of *S. aureus* and coagulase-negative staphylococci (7, 29). Historically, staphylococcal plasmids can be categorized into one of three main classes based on physical/genetic organization and functional characteristics (24, 25, 30). These correspond to small plasmids that utilize a rolling circle (RC) replication mechanism and usually carry a single resistance gene or are cryptic, larger multiresistance plasmids that often carry several resistance genes and conjugative plasmids that usually also contain multiple resistance determinants and are typically larger, again due to the conjugation-associated genes they contain; theta-mode replication is used by multiresistance and conjugative plasmids. However, categorization

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based on resistance genes is parochial and does not necessarily reflect relatedness between plasmids, since resistance determinants can be readily lost from, or acquired by, plasmid backbones. For example, there are plasmids closely related to multiresistance and conjugative plasmids that lack any recognized resistance genes. Toxins are also encoded by some plasmids (29, 31–34), and the functions of many genes carried by larger plasmids have not been investigated, let alone elucidated.

Analysis of fully sequenced *S. aureus* plasmids in the databases has illustrated that plasmid size correlates strongly with the mode of replication used and hence with the replication initiation gene possessed. Plasmids smaller than 10 kb use the RC mechanism, whereas those larger than 14.5 kb utilize theta-mode replication, with plasmids in the intermediate 10 to 14.5-kb size range using either of these mechanisms (35). Fifteen plasmid incompatibility groups (Inc1 to Inc15) were established for staphylococcal plasmids, incorporating both RC replicating (RCR) and theta replicating plasmids (24, 36). However, the rate of plasmid discovery through genome sequencing has rendered incompatibility testing impractical. A plasmid typing system for plasmids from Gram-positive bacteria (including staphylococci) based on replication region DNA sequence has been established (37) and incorporated into the PlasmidFinder database (38). Unfortunately, coincident identification of additional replicon types (39, 40) has subsequently resulted in discordant classification of some staphylococcal plasmids.

Small RCR Plasmids

The most thoroughly characterized staphylococcal plasmids are those that utilize asymmetric RC replication via a single-stranded DNA intermediate (for detailed reviews of RC plasmid replication, see Khan [41] and Ruiz-Maso et al. [44]). Probably reflecting constraints imposed by this replication strategy, RCR (sometimes alternatively called just RC or single-stranded DNA) plasmids are usually less than 5 kb in size and are rarely found to contain transposable elements (24). This restriction in RCR plasmid size probably reflects elevated production of high-molecular-weight DNA with increasing plasmid size, which impairs cell growth, leading to counterselection against host cells and hence the plasmid itself (42). RCR plasmids are frequently found cointegrated within larger theta replicating plasmids, commonly flanked by insertion sequences such as IS*257*; in such cases the replication system of the integrated RCR plasmid is often inactivated in some way (28, 43).

Staphylococcal RCR plasmids can be subdivided into four families, exemplified by pT181, pC194, pE194, and pSN2, based on replication region sequence similarity (24); representative plasmids are shown in Fig. 1. These families contain plasmids found in different staphylococcal species and are related to plasmids of other bacterial genera, such as *Bacillus*, *Lactobacillus*, and *Streptococcus*, attesting to the horizontal transmission of RCR plasmids (42). Plasmids belonging to each of these four families encode evolutionarily distinct replication initiator (Rep) proteins that possess the conserved domains Rep_trans, Rep_1, Rep_2, and RepL, respectively (35). However, despite the Rep proteins having unique evolutionary origins, they perform a remarkably similar role in recognizing, binding, and nicking specific DNA sequences in the double-strand origin (*dso*). In the RCR plasmids examined so far, the Rep proteins also perform cleavage and ligation reactions at the termination of leading strand synthesis (44). pT181 family Rep proteins recruit the host-encoded helicase, PcrA, which is essential for synthesis of the new, and displacement of the parental, leading strands (45, 46). The leading strand contains a single-strand origin of replication (*sso*) that, upon displacement, is recognized and primed for replication by host-encoded RNA polymerase (47). The inability of host RNA polymerase to recognize and prime the *sso* limits the host range of RCR plasmids. For example, the *sso*A carried by pT181 only functions efficiently in staphylococci, whereas the *sso*U carried by pE194 family plasmids allows stable replication in a broader range of Gram-positive hosts (48).

RCR plasmids are usually maintained at 10 to 60 copies per cell (7), and copy number control is achieved via an antisense RNA that is transcribed divergently to the replication initiation gene (35). In the case of pT181, binding of the antisense RNA causes transcriptional attenuation of *rep*C mRNA (49), whereas for pC194 family plasmids antisense binding is believed to block translation of the Rep protein (50, 51). Antisense inhibition of Rep translation is also thought to occur in pE194 family plasmids, but transcriptional regulation mediated by a repressor protein may also play a role (35), as has been shown for the streptococcal RCR plasmid pMV158 (52, 53).

Some staphylococcal RCR plasmids contain mobilization systems that facilitate their horizontal transmission when coresident with a conjugative plasmid. Plasmids such as pC221 possess a multigene mobilization system (*mob*CAB and origin of transfer [*oriT*]) (Fig. 1) (11). The pC221 MobA relaxase and MobC relaxase accessory proteins are both required for nicking at *oriT* (54, 55). Other RCR plasmids possess a different mobilization locus that is homologous to the *mob*M relaxase gene and *oriT* of pMV158 (56, 57). This locus was originally identified as a site-specific recombination function on staphylococcal plasmids such as pT181 and pE194 (Fig. 1) (24), where the constituent sequences were named *pre* and RS_A (corresponding to mob_{pre} and $oriT_{RSa}$, respectively) (Fig. 1). Additionally, the replication initiation protein and *dso* of several RCR plasmids have been shown to "moonlight" as a relaxase and *oriT* for mobilization mediated by the ICE Tn*916* (see below) (58).

RCR plasmids, including those in different families, often share highly similar DNA segments, such that they appear to be mosaic structures consisting of discrete functional cassettes encoding replication, resistance, and sometimes recombination/mobilization functions (24, 59). Exchange of these segments between plasmids is thought to be promoted by the single-stranded intermediates generated by the RC replication strategy, which enhance the frequency of homologous recombination events between regions of flanking sequence similarity (60). DNA processing at replication and transfer origins also promotes the swapping of such cassettes (24, 61).

Theta Replicating Plasmids

Most staphylococcal plasmids larger than 10 kb employ theta-mode replication and are maintained at low copy numbers (7, 35, 62–65). The majority encode a replication initiation protein containing an N-terminal conserved RepA_N domain (29, 35, 66), which corresponds to a winged helix-turn-helix DNA binding domain that binds to origin Rep box repeat sequences characteristically located centrally within the *rep* gene coding sequence (67–69); the C-terminal domain of the Rep protein from the *S. aureus* conjugative plasmid pSK41 (see below) has been shown to interact with the host encoded DnaG primase (70). An interesting variation is seen in the plasmid pTZ2162, where the RepA_N-type initiation protein has been split into two smaller distinct polypeptides, expressed from two partially overlapping *rep*

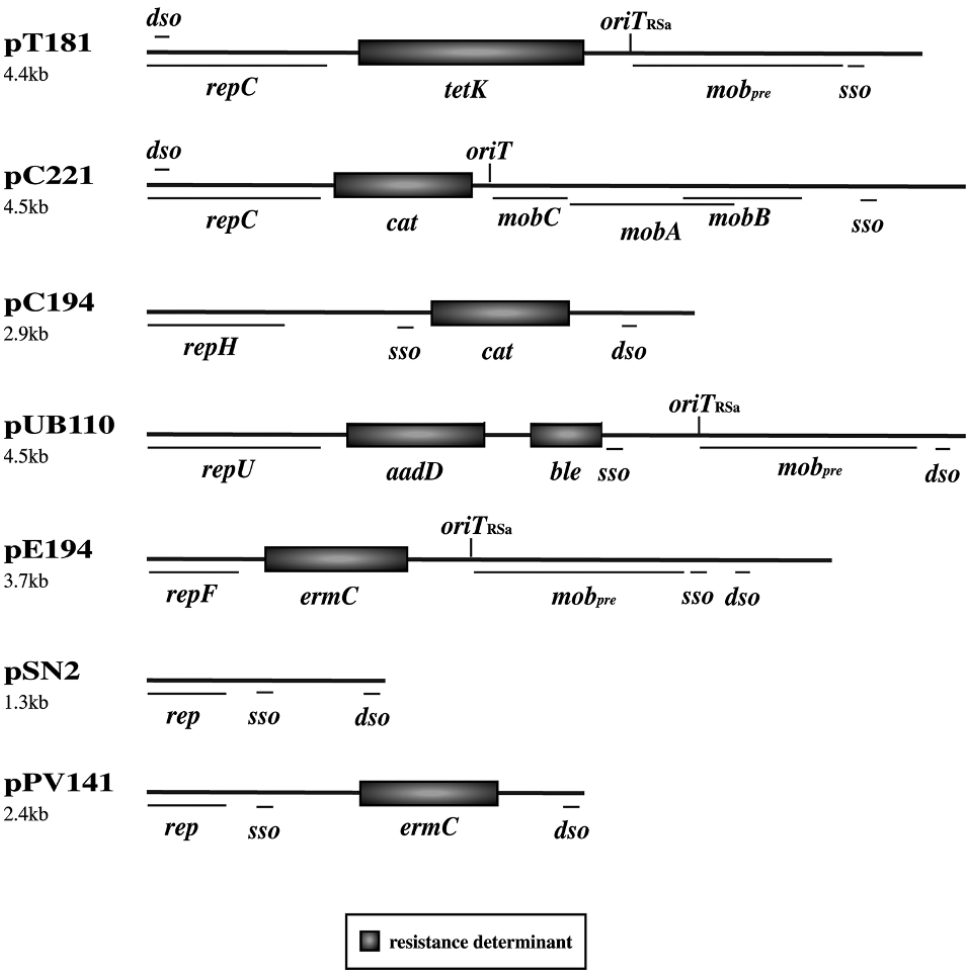


FIGURE 1 Maps of representative RCR plasmids: pT181, pC221, pC194, pUB110, pE194, pSN2, and pPV141 (25, 231); see text for additional references. Plasmid sizes are shown on the left. Resistance and plasmid maintenance genes/loci are shown: *rep*/*repC*/*repF*/*repH*/*repU*, initiation of replication; *dso*, double-stranded origin of DNA replication; *sso*, single-stranded origin of DNA replication; *mobA*/*mobB*/*mobC*/mob_{pre}, mobilization; *oriT*/$oriT_{RSa}$, origin of DNA transfer; *cat*, chloramphenicol resistance. Refer to Tables 1 and 2 for the antimicrobial resistance(s) conferred by other resistance determinants.

genes that have likely arisen from a frameshift (71). In staphylococcal plasmids encoding RepA_N type initiators, copy number is thought to be controlled by a small antisense RNA that binds to the leader region of the *rep* mRNA to block its translation (68, 72, 73).

Two groups of nonconjugative multiresistance plasmids were described previously in staphylococci: the β-lactamase/heavy-metal resistance plasmids and the pSK1 family plasmids; both utilize RepA_N initiation proteins (74). *S. aureus* strains from the 1960s and 1970s commonly contained plasmids that conferred resistance to β-lactam antibiotics and heavy metals or other inorganic ions. These plasmids characteristically carried a Tn*552*-like β-lactamase-encoding transposon or a derivative thereof (75, 76) and frequently possessed a composite structure designated Tn*4004* conferring resistance to mercuric ions and operons encoding resistance to arsenical and/or cadmium ions (Fig. 2) (6, 77). Additionally, some β-lactamase/heavy-metal resistance plasmids carry the transposon Tn*4001* or Tn*551* conferring resistance to aminoglycosides (6, 78) and macrolide-lincosamide-streptogramin type B (MLS) antibiotics (79), respectively, and/or a *qacA* or *qacB* gene mediating multidrug resistance to antiseptics and disinfectants (Fig. 2) (6).

Plasmids related to the prototype, pSK1 (Fig. 2), were first identified in epidemic *S. aureus* and coagulase-negative staphylococcal strains isolated in Australia during the 1980s and later in isolates from Europe (25). In addition to a *qacA* gene mediating multidrug resistance to antiseptics/disinfectants carried ubiquitously by pSK1 family plasmids (80, 81), members of this family variously encode Tn*4001*, which confers resistance to gentamicin and other aminoglycosides (82), a Tn*552*-like β-lactamase transposon, Tn*4002* (83), and/or a composite structure designated Tn*4003* that mediates trimethoprim resistance (84). Tn*4003* represents a vestige of a cointegrated pSK639-like plasmid (see below (26, 85).

As more plasmid sequences have accumulated through genome sequencing it has become clear that there is a broad diversity of plasmids with a *repA_N*-type initiation gene, including plasmids lacking resistance genes and/or containing

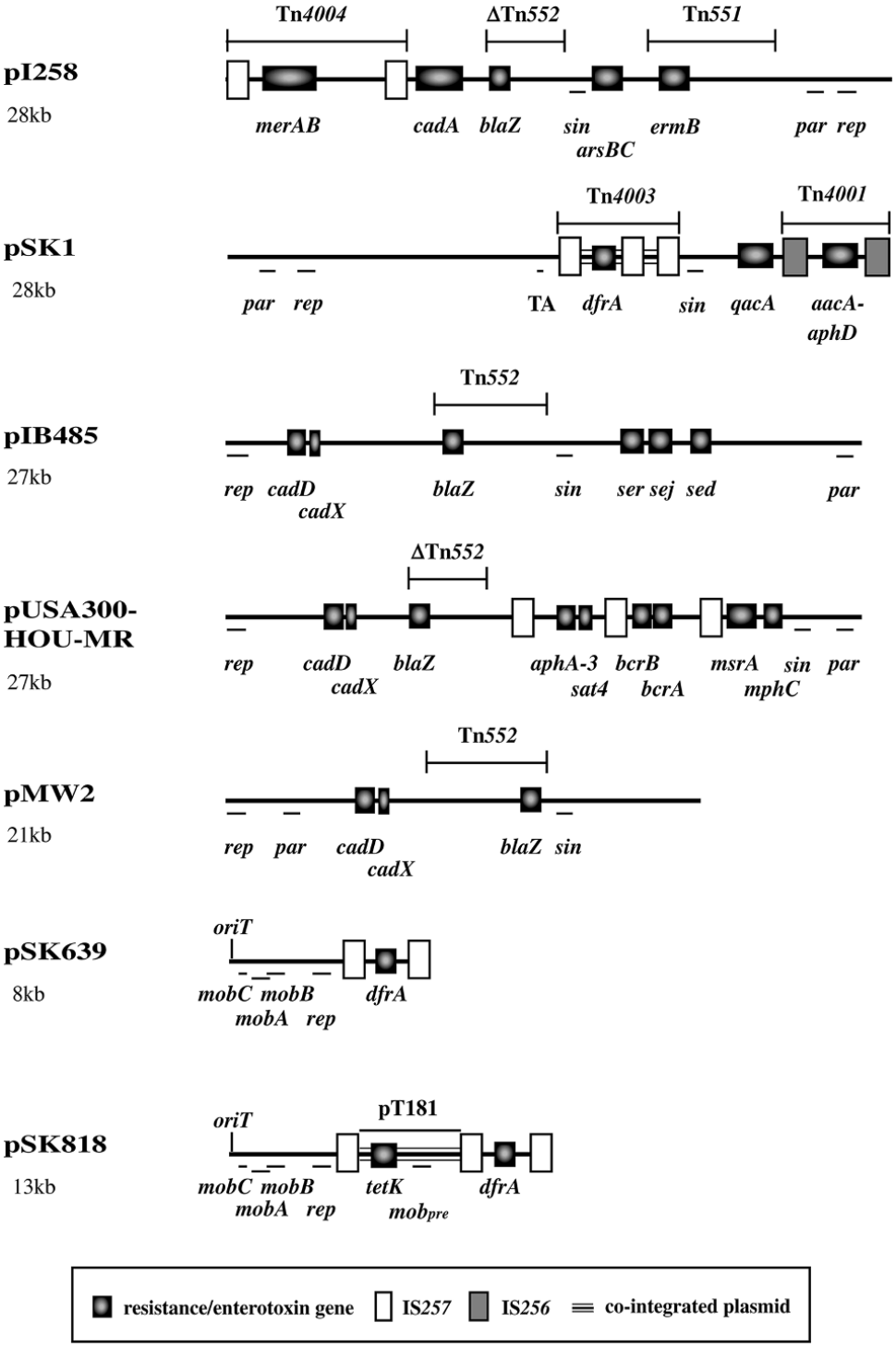


FIGURE 2 Maps of representative nonconjugative theta-replicating plasmids: pI258, pSK1, pIB485, pUSA300-HOU-MR, pMW2, pSK639, and pSK818 (3, 25, 29, 65, 85, 89, 232); see text for additional references. Resistance/enterotoxin genes, transposons, insertion sequences, and cointegrated plasmids are shown: *ars*BC, arsenic resistance; *cad*A, cadmium resistance; *mph*C, macrolide resistance; *msr*A, macrolide/streptogramin B resistance; *qac*A, antiseptic/disinfectant resistance; *ser*, *sej*, and *sed*, enterotoxins. Refer to Tables 1 and 2 for the antimicrobial resistance(s) conferred by other resistance determinants. Plasmid maintenance genes/loci are also shown: *par*, novel partitioning system; *rep*, initiation of replication; *sin*, multimer resolution; TA, Fst-like toxin-antitoxin system; *mobA*/*mobB*/*mobC*/mob_{pre}, mobilization.

toxin genes, such as pIB485 (Fig. 2) and related plasmids that have been isolated from strains of wide geographic distribution (29). Two other loci are nearly ubiquitous on RepA_N-encoding plasmids, suggesting that they are important for plasmid survival (29). The first is a multimer resolution system that includes a gene coding for a serine recombinase, which is usually annotated as *sin* or *bin3* (86, 87). The second is homologous to the pSK1 *par* (formerly *orf245*) gene, which is usually located upstream of, and transcribed divergently from, the *rep* gene. This locus increases plasmid segregational stability and is thought to constitute a novel single-protein partitioning system (64, 88). In contrast, type I and II partitioning systems, comprising two protein-encoding genes, are found only occasionally on these plasmids (29). Finally, an Fst-like type I toxin-antitoxin system, which might contribute to plasmid segregational stability, is carried by pSK1 (89), and related loci have been identified on numerous other theta replicating staphylococcal plasmids (90, 91).

A minority of theta replicating staphylococcal plasmids code for a Rep protein containing a Rep_3 conserved domain (35); initiation proteins of this type from Gram-negative bacteria, encoded by iteron plasmids such as F, pPS10, and R6K, contain tandem winged helix-turn-helix domains (92). This type of replication system was first detected in the genus in plasmids from *Staphylococcus epidermidis* related to the 8-kb trimethoprim resistance plasmid pSK639 (Fig. 2), which also contains a mobilization system related to that of the RCR plasmid pC221 (65). More recently, *rep_3*-type genes have been found in addition to the usual *repA_N*-type gene on larger β-lactamase/heavy-metal resistance plasmids such as pN315 (1), whereas in the case of pMW2 (3) (Fig. 2), only a remnant of the *repA_N* gene remains. Finally, plasmids employing an initiation protein containing a PriCT_1 conserved domain, such as pETB, which encodes exfoliative toxin B (32), have also been found in staphylococci but appear to be quite rare; some also encode a RepA_N or Rep_3 type initiator (35).

Conjugative Plasmids

The largest staphylococcal plasmids (>30 kb) are those that encode their own conjugative transfer (7, 25). Three distinct families of conjugative plasmids have been identified in staphylococci: the well-known pSK41/pGO1-like plasmids and the recently characterized pWBG4 and pWBG749 families.

S. aureus plasmids such as pSK41 (43, 93, 94) (Fig. 3), pGO1 (95, 96), and pJE1 (20) were first identified in association with gentamicin resistance in the mid 1970s (13, 25). Structurally related conjugative plasmids were found in coagulase-negative staphylococci (97, 98), and interspecific transfer has been demonstrated (99, 100). In pSK41 and many relatives, the plasmid backbone consists of two IS*257*-flanked segments, termed the transfer (*tra*) region and region I (Fig. 3) (94). However, in other family members (e.g., pPR9) these segments are contiguous, and this likely represents the ancestral configuration prior to the incorporation of additional DNA segments (43, 101, 102).

Multiple copies of IS*257*, mostly in the same orientation, are a feature of many pSK41-like plasmids (Fig. 3). These usually delimit segments that carry resistance genes and often correspond to cointegrated copies of smaller plasmids (43, 103). Such determinants include the aminoglycoside and bleomycin resistance genes *aadD* and *ble*, respectively, on a copy of pUB110 and the small multidrug resistance determinant *qac*C on another RCR plasmid (Fig. 3). Plasmids such as pJE1 and pGO1 also contain a Tn*4003*-like structure derived from a pSK639-like trimethoprim-resistance plasmid (see above). The *aacA-aphD* gene encoded by Tn*4001*-IS*257* hybrid structures is responsible for resistance to gentamicin and other aminoglycosides (104). Members of the family have also been found that contain IS*257*-flanked genes encoding resistance to tetracycline (*tetK*) (29), MLS antibiotics (*erm*C) (105), mupirocin (*mupA*/*ileS2*) (102, 106), and most recently, multidrug resistance to phenicols, lincosamides, oxazolidinones (e.g., linezolid), pleuromutilins, and streptogramin A compounds ($PhLOPS_A$ phenotype; *cfr*) (107). Plasmids of this type can also carry unit transposons, such as Tn*552* (*blaZ*) (43) and the *vanA* glycopeptide resistance transposon Tn*1546* (27). The pSK41-like plasmid pLW1043 (Fig. 3) was responsible for the first reported high-level vancomycin-resistant *S. aureus* strain, isolated in Michigan in 2002 (27), and resulted from the transposition of Tn*1546* into a copy of IS*257* within the pSK41-like plasmid pAM829 (27, 108). A Tn*1546*-containing Inc18 broad-host-range conjugative plasmid, pAM830, carried by a coisolated *Enterococcus faecalis* strain, is thought to have mediated the intergeneric transfer event (27, 109). Such enterococcal Inc18 *vanA* plasmids themselves have subsequently been detected in some vancomycin-resistant *S. aureus* strains, but their scarcity in *S. aureus* suggests some limitation on their establishment in this species, possibly inefficient replication, an incompatibility with staphylococcal cell wall biosynthesis, a restriction barrier, and/or high metabolic burden associated with *vanA* carriage (108). Cointegrates composed of Inc18 and pSK41-like plasmids are also thought to occur (110), and it has been suggested that carriage of pSK41-like plasmids might enhance the recipient ability of *S. aureus* cells for enterococcal donors containing Inc18 plasmids, but the mechanism has not been determined (111).

Conjugative transfer of pSK41 family plasmids occurs only on solid surfaces and does so at comparatively low frequencies, in the range of 10^{-5} to 10^{-7} transconjugants per donor cell (13). The majority of genes thought to be responsible for conjugative transfer are located in the *tra* region (Fig. 3) (93, 95). The *tra* genes are most similar to the transfer genes of the *Lactococcus lactis* plasmid pMRC01 (112) and the broad host range plasmid pIP501 (43), originally identified in *Streptococcus agalactiae*, exhibiting both synteny and product amino acid sequence similarity (43, 113, 114). Several deduced products show homology to proteins from the type IV secretion system superfamily, including to coupling proteins, peptidoglycan hydrolases, and several other core mating pair formation-type proteins (115, 116). Thus, the basic conjugative process is likely to resemble that of more extensively characterized Gram-negative counterparts, *viz.*, transfer of a single strand of plasmid DNA via direct cell-cell contact. However, no pilus-like structure seems to be associated with Gram-positive conjugation (114), perhaps reflecting the distinction in the cell envelope organizations and accounting for the solid surface requirement for conjugative transfer. The origin of transfer, *oriT*, and the relaxase, Nes, that acts upon it, are encoded in region I (117, 118), as are a number of genes of unknown function that might also play a role in conjugative transfer (20, 43, 113).

As mentioned above, pSK41-like conjugative plasmids encode a RepA_N type replication initiation protein, whose translation is regulated by an antisense RNA called RNAI (67, 70, 72, 73). However, several members of the family, such as pPR9 and pUSA03, possess a novel chimeric *rep* gene where the sequences that usually encode the N-terminal RepA_N DNA-binding domain, and the origin Rep box

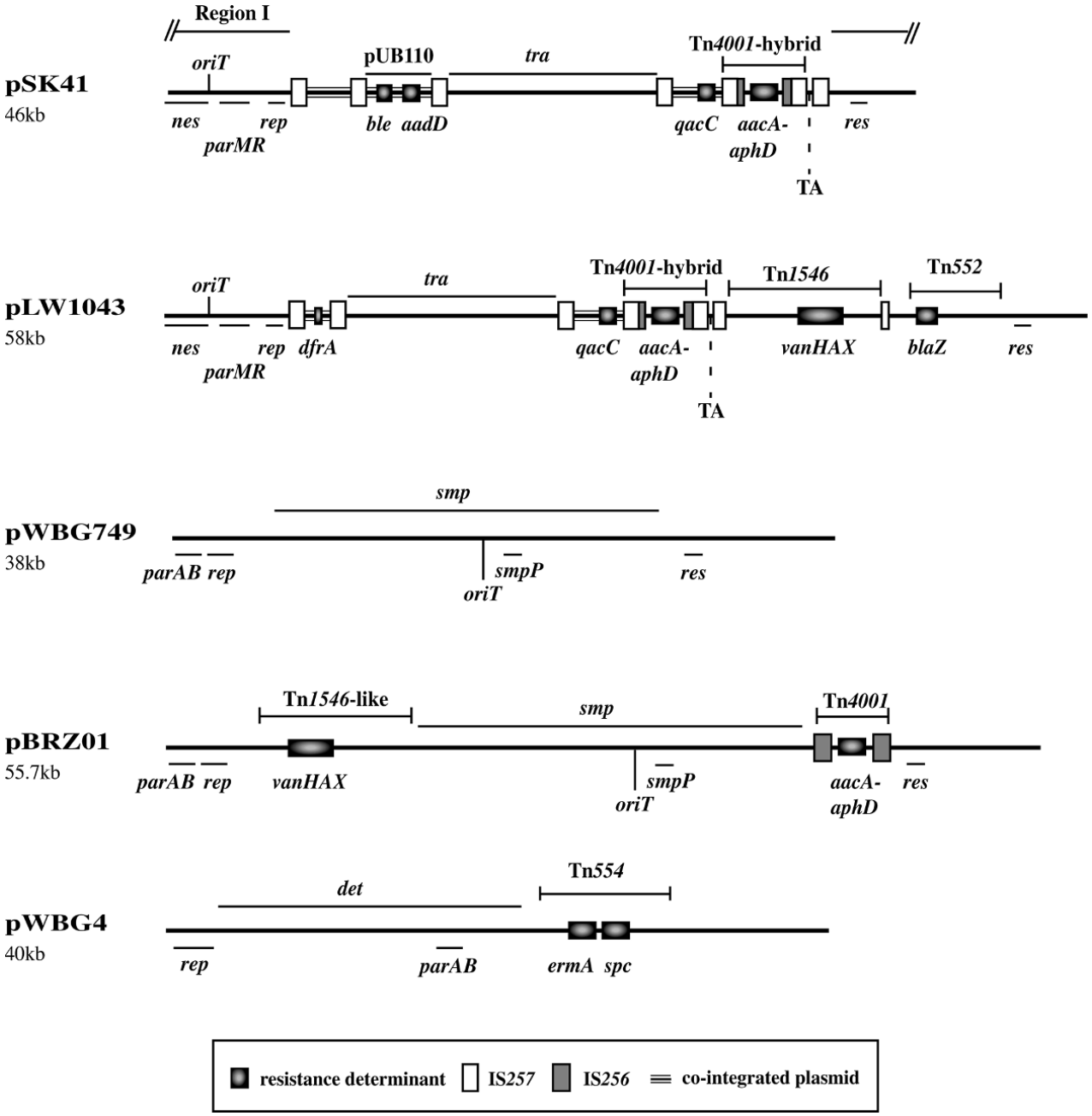


FIGURE 3 Maps of representative conjugative theta-replicating plasmids: pSK41, pLW1043, pWBG749, pBRZ01, and pWBG4 (27, 43, 128, 136, 137); see text for additional references. Resistance genes, transposons, insertion sequences, and cointegrated plasmids are shown: refer to Tables 1 and 2 for the antimicrobial resistance(s) conferred by the resistance determinants. Plasmid maintenance genes/loci (if known) are also shown: *parAB*, type I partitioning system; *par*MR, type II partitioning system; *rep*, initiation of replication; *res*, multimer resolution; TA, Fst-like toxin-antitoxin system; *tra*/*smp*/*det* genes, conjugative transfer; *nes*/*smpP* genes, relaxases; *oriT*, origin of transfer. Note that pLW1043 and pBRZ01 are members of the pSK41 and pWBG749 families, respectively.

repeats to which it binds, appear to have been replaced by sequences encoding a helix-turn-helix protein sequence related to phage replication proteins and distinct DNA sequence repeats (35, 102). The usual upstream antisense RNA control region and downstream in-frame end of the *rep* gene, encoding the conserved C-terminal end of the Rep protein that interacts with host DnaG primase (70), remain intact in these plasmids, but the variation is sufficient to allow compatibility with the pSK41 replication system (102).

All known pSK41 family plasmids encode a serine recombinase and a type II active partitioning system, the latter representing a significant difference from *rep*A_*N* replicating nonconjugative staphylococcal plasmids, which usually contain an unusual *par* gene or occasionally a type I partitioning system (see above). The pSK41 *res* locus is functional, facilitating the conversion of plasmid multimers into monomers, with the Res protein shown to bind to a resolution site, comprising three subsites, located in the intergenic

region upstream of the *res* gene; Res binding also autoregulates transcription of its own promoter (119). Functionality of the pSK41 partitioning system has also been demonstrated (120). It comprises a centromere-like site, *par*C, and two genes in an operon, *par*M and *par*R. The ParM protein is an actin-like motor protein that forms single-stranded helical filaments (121). ParC is a ribbon-helix-helix protein that binds to DNA repeat sequences within *par*C (120) and interacts with ParM to modulate its activity (122). ParR also autoregulates transcription from the partitioning operon promoter, which is also controlled by the plasmid-encoded ArtA protein (see below). Some pSK41-like plasmids encode an additional potential plasmid maintenance determinant in the form of an Fst-like toxin-antitoxin system, which is located on a small IS*257*-flanked DNA segment adjacent to Tn*4001*-hybrid elements in plasmids conferring gentamicin resistance (94).

In pSK41, transcription of the majority (21 out of 30) of the plasmid backbone genes is controlled by the product of the conserved *art*A gene (corresponding to *trsN* on pGO1) (101, 123). The ArtA protein is a ribbon-helix-helix DNA-binding protein that represses five promoters in addition to its own—including two operon promoters in the *tra* region and two more operon promoters in region 1 (Fig. 3). ArtA is therefore thought to coordinate basal-level transcription of most of the plasmid's backbone genes (101).

The pWBG4 family of conjugative plasmids, also called diffusible pigment (DiP) plasmids, were first identified as a distinct family of *S. aureus* conjugative plasmids in the 1980s by the Grubb laboratory (124–127), but the complete sequence of pWBG4 (Fig. 3) was only reported in 2016 (128). The first example of this family, pWBG14, was isolated in Western Australia in 1968 and conferred resistance to MLS antibiotics (*erm*A) and spectinomycin (*spc*) and production of a diffusible orange pigment. Related plasmids were subsequently identified carrying resistance to aminoglycosides and trimethoprim (*dfrD*), and more recently several pWBG4-related plasmids were implicated in the conjugative transfer of linezolid and phenicol resistance (*cfr* and *fex*A) (128, 129) in America, China, and Germany (128, 129). pWBG4 family plasmids carry an ~18-kb conjugation-gene cluster *detA-detV* which is distinct from that of pSK41/pGO1 family plasmids and uniquely includes a MobC-like conjugative relaxase (DetB) and T4CP (DetA) related to those of the mobilizable *Enterobacter cloacae* plasmid CloDF13 (128, 129). pWBG4 plasmids also encode a PriCT_1-domain replication-initiation protein common to enterococcal Inc18 plasmids (35). pWBG4 family plasmids have been isolated from both *S. aureus* and *S. epidermidis*, and transfer between and within these species has been demonstrated (125).

The pWBG749 family was also only recently sequenced and identified as being a distinct family of *S. aureus* conjugative plasmids. pWBG749 (Fig. 3) was originally isolated in 1995 (29, 125, 130, 131) from colonizing *S. aureus* present in remote indigenous communities in Western Australia, but more recent sequencing has identified variants isolated as early as 1985 (36, 128, 132–135). pWBG749 encodes a RepA_N initiation protein, possesses a type IB partitioning system (29), and carries an ~23-kb conjugation gene cluster *smpA-X* (136) (Fig. 3). While most identified pWBG749 family plasmids lack antimicrobial-resistance determinants, members carrying resistance to penicillin (*blaZ*), aminoglycosides (*aacA-aphD*), and vancomycin (*vanA*) have been isolated (130–133, 137). Despite their frequent cryptic nature, pWBG749 family plasmids likely have an extensive impact on antimicrobial resistance dissemination through their so-far unique ability to horizontally mobilize large multiresistance plasmids in addition to smaller plasmids (131).

The mechanism by which pWBG749 mobilizes nonconjugative plasmids was only recently recognized as a naturally occurring phenomenon, potentially explaining why it was previously overlooked (128, 136). Most characterized plasmid mobilization systems described require a mobilizable plasmid to carry its own relaxase gene (138). Plasmids capable of being mobilized by pWBG749 need only carry a short "mimic" of the pWBG749 origin-of-transfer sequence (136). The conjugative relaxase of pWBG749, SmpP, along with an *oriT* specificity factor, SmpO, acts *in trans* on the *oriT* to pilot it to the mating apparatus. Approximately 50% of unique nonconjugative staphylococcal plasmids carry a pWBG749 family *oriT* mimic (136). Most common mimics are near-identical to those on pWBG749 or the closely related conjugative plasmid pWBG745 and its vancomycin resistance-encoding derivative pBRZ01 (Fig. 3) (137). Bioinformatic inspection of 397 sequenced clinical *S. aureus* isolates from the MRSA Orange County initiative of the Broad Institute (broadinstitute.org) identified just 24 contigs matching pWBG749 or pWBG745, but astonishingly, 736 *oriT* mimics were identified among the sequence contigs. This abundance is likely explained by the presence of up to three *oriT* mimic variants per plasmid on common large multidrug resistance plasmids such as pIB485, pMW2, and pUSA300-HOU-MR (Fig. 2) and on small RCR plasmids (128, 136, 138).

Analogous to pWBG749-like *oriT* mimics, multiple mimic types that resemble the pSK41 *oriT* sequence were also detected recently on numerous staphylococcal plasmids (139). Although pSK41 could not mobilize the variants tested, it would seem likely that other pSK41 family plasmids encoding relaxation systems with appropriate specificity might also mediate relaxase-*in trans* mobilization. Recent bioinformatic analyses have revealed that nearly all theta replicating *S. aureus* plasmids carry at least one *oriT* mimic sequence, with many possessing both pWBG749 and pSK41 types, suggesting that the capacity for conjugative mobilization via the relaxase *in trans* mechanism is widespread (138).

TRANSPOSABLE ELEMENTS

Insertion sequences and composite transposons detected in staphylococci are listed in Table 1, and unit transposons are presented in Table 2. For many, designation as a transposable element is based on possession of diagnostic characteristics, such as terminal inverted repeats, flanking insertion sequences and/or target duplications, presence of an open reading frame encoding a transposase homolog, and/or detection in varied genetic contexts, rather than formal demonstration of mobility in a *rec*A recombination-defective host. As outlined for specific elements below, in addition to facilitating the translocation of genes between replicons, insertion sequence elements are increasingly being shown to play more subtle roles in phenotypic expression and genome evolution.

Inverted copies of IS*256* flank the composite transposon Tn*4001* (Figs. 2 and 3; Table 1) (82). Tn*4001* and elements related to it are responsible for the emergence of linked resistance to the aminoglycosides, gentamicin, tobramycin, and kanamycin in staphylococci, having been found on the chromosome and theta replicating plasmids (Figs. 2 and 3) in clinical *S. aureus* and coagulase-negative staphylococcal isolates from around the world (6, 25). Indeed, some of the earliest reported gentamicin-resistant strains, dating from 1975, have been shown to carry a Tn*4001*-like structure on

TABLE 1 Insertion sequences and composite transposons[a]

IS[b]	Tn[c]	Associated resistance(s)/phenotype(s)	Determinant(s)	TD[d] (bp)
IS*256*	Tn*4001*[e]	Gentamicin/kanamycin/tobramycin	*aacA-aphD*	8
		Phenicols/lincosamides/oxazolidinones/pleuromutilins/streptogramin A	*cfr*	
IS*257*[f]		Kanamycin/neomycin/paromomycin/tobramycin	*aadD*	8
		Kanamycin/neomycin	*aphA-3*	
		Bacitracin	*bcrAB*	
		Bleomycin	*ble*	
		Cadmium	*cadDX*	
		Trimethoprim	*dfrK*	
		MLS	*ermC*	
		Fosfomycin	*fosB5*	
		Fusidic acid	*fusB*	
		Mupirocin	*ileS2 (mupA)*	
		Lysostaphin immunity factor	*lif*	
		Preprolysostaphin	*lss*	
		Antiseptics/disinfectants	*qacC*	
		Streptothricin	*sat4*	
		Tetracycline	*tetK*	
		Tetracycline	*tetL*	
		Streptogramin A	*vat*(A)	
		Streptogramin A/pleuromutilins/lincosamides	*vga*(A)	
		Streptogramin B	*vgb*(A)	
	Tn*4003*	Trimethoprim	*dfrA*	
	Tn*4004*	Mercury	*merAB*	
	Tn*6072*	Gentamicin/kanamycin/tobramycin	*aacA-aphD*	
	Tn*6072*	Spectinomycin	*spc*	
IS*1181*				8
IS*1182*	Tn*5405*[g]	Streptomycin	*aadE*	8
	Tn*5405*	Kanamycin/neomycin	*aphA-3*	
	Tn*5405*	Streptothricin	*sat4*	
IS*1272*	Tn*Sha1*	Triclosan	*fabI*	Absent
	Tn*Sha2*	Triclosan	*fabI*	
IS*1293*		Lysostaphin immunity factor	*lif*	Unknown
		Preprolysostaphin	*lss*	
IS*21-558*[f]		Phenicols/lincosamides/oxazolidinones/pleuromutilins/streptogramin A	*cfr*	6
		Lincosamides	*lsa*(B)	
IS*Enfa4*		Phenicols/lincosamides/oxazolidinones/pleuromutilins/streptogramin A	*cfr*	8
IS*Sau10*		Kanamycin/neomycin/paromomycin/tobramycin	*aadD*	8
		Trimethoprim	*dfrK*	
		MLS	*ermC*	
		MLS	*ermT*	
		Tetracycline	*tetL*	
IS*Sha1*				8

[a]See Paulsen et al. (25), Firth and Skurray (26), Fu et al. (237), Allignet and El Solh (238), Chen et al. (239), Kadlec and Schwarz (240), Highlander et al. (232), O'Neill and Chopra (241), de Vries et al. (242), and the text for references. Additional elements detected in genome sequences but not described here (e.g., IS*Sau1* through IS*Sau8* and IS*Sau11*) are listed in ISfinder (http://www-is.biotoul.fr).
[b]In the case of IS, association is based on probable involvement in the acquisition and dissemination of the resistance.
[c]Associated composite transposons are shown.
[d]TD, target duplication.
[e]Tn*3851* and Tn*4031* are likely to be similar or identical to Tn*4001*.
[f]IS*257* is also known as IS*431*, and IS*21-558* is also known as IS*Sau9*.
[g]Tn*3854* may be similar to Tn*5405*.

TABLE 2 Unit transposons[a]

Transposon	Family	Associated resistance(s)	Determinant(s)	TD[b] (bp)
Tn*551*	Tn*3*	MLS	*ermB*	5
Tn*1546*		Vancomycin/teicoplanin	*vanHAX*	5
Tn*552*[c]	Tn*7*	Penicillins	*blaZ*	6/7
Tn*5404*		Streptomycin	*aadE*	6
		Kanamycin/neomycin	*aphA-3*	
		Streptothricin	*sat4*	
Tn*554*[d]	Tn*554*	MLS	*ermA*	Absent
		Spectinomycin	*spc*	
ψTn*554*		Cadmium	*cadA*	Absent
Tn*558*		Chloramphenicol/florfenicol	*fexA*	Absent
Tn*559*		Trimethoprim	*dfrK*	Absent
Tn*5406*		Streptogramin A/pleuromutilins/lincosamides	*vga*(A)*v*	Absent
Tn*6133*		MLS	*ermA*	Absent
		Spectinomycin	*spc*	
		Streptogramin A/pleuromutilins/lincosamides	*vga*(E)	

[a]See Paulsen et al. (25), Firth and Skurray (26), and the text for references.
[b]TD, target duplication.
[c]Tn*3852*, Tn*4002*, and Tn*4201* are likely to be similar or identical to Tn*552*.
[d]Tn*3853* is likely to be similar or identical to Tn*554*.

the chromosome (140). Resistance is mediated by the gene *aacA-aphD*, which encodes a bifunctional enzyme possessing both aminoglycoside acetyltransferase and phosphotransferase activities (75). Transposons closely related to Tn*4001* are also evident in enterococci (141) and streptococci (142). In enterococci, IS*256* and related insertion sequences are also associated with elements encoding resistance to vancomycin (143) and erythromycin (144). IS*256* also appears to be contributing to the spread of the *cfr* multiresistance gene, forming a composite-like structure with a copy of the related enterococcal element IS*Enfa4* (145, 146).

Roles in gene expression and regulation have been attributed to IS*256* via both transcriptional promotion and insertional inactivation. Transcription of the *aacA-aphD* resistance gene of Tn*4001* is directed by a complete promoter located within the end of the upstream copy of IS*256*, and it has also been found that a small flanking deletion can result in a −35-like sequence at the terminus of that element forming part of a hybrid promoter which mediates a higher level of aminoglycoside resistance (147). Additionally, roles for IS*256* hybrid promoters have been demonstrated in the expression of methicillin resistance (148, 149). Insertional inactivation of genes by IS*256* has been shown to modulate expression of several phenotypes, including virulence and glycopeptide resistance. Alternating IS*256* insertions and excisions in the *ica* operon responsible for the synthesis of the polysaccharide intercellular adhesin mediate phase-variable biofilm formation in *S. epidermidis* strains responsible for indwelling device infections (150). Notably, biofilm phase variation has also been attributed to IS*256* insertions that affect global regulatory circuits, including the *sarA* global regulator, the quorum-sensing *agr* system, and *rsbU* which positively regulates the σ^B stress response factor (151, 152). IS*256* inactivation of the *tcaA* locus was found to increase the teicoplanin resistance of a glycopeptide intermediate-resistant *S. aureus* strain (153), and IS*256* insertion into the promoter region of the *walKR* two-component regulator system similarly resulted in decreased glycopeptide susceptibility (154, 155). Insertion into the promoter repressor of toxins gene, *rot*, was also found to enhance the virulence of a USA500 strain (156).

IS*256* utilizes a "copy-out-paste-in" transposition mechanism via an excised circular double-stranded DNA intermediate (157–159). Its transposase contains an N-terminal helix-turn-helix domain that mediates binding to the element's terminal inverted repeats (160). The capacity of IS*256* to mediate phase variability has been linked to a capacity for precise excision of the element and the target duplication generated during insertion (150). Interestingly, this excision behavior occurs independently of the element's transposase and is instead thought to involve host-mediated illegitimate recombination acting on the flanking target duplications (161).

IS*257* (also known as IS*431*) has been found in diverse genetic contexts in staphylococci, variously associated with a number of determinants (Table 1). The prevalence of resistance genes flanked by copies of this element resulted in the designation of several such composite structures as transposons, *viz.*, Tn*4003* and Tn*4004*. Although the possibility that these structures can behave as traditional composite transposons cannot be excluded, such an organization is probably a consequence of the transposition mechanism of IS*257*. This insertion sequence element is thought to undergo nonresolved replicative transposition (26, 103). The expected outcome of such an event involving two replicons is cointegration, so that the replicons become fused with a directly repeated copy of IS*257* at each junction, as has been experimentally demonstrated by Needham et al. (162). The IS*257*-like element IS*Sau10* is also found in such configurations (163, 164). Many IS*257*-flanked segments represent plasmids cointegrated into larger plasmids (Figs. 2 and 3) or the chromosome (26, 103). Frequently observed deletion events adjacent to IS*257* may result from intramolecular transposition of IS*257*. However, replicon fusions and

sequence deletions have also been shown to have resulted from homologous recombination between pre-existing copies of IS*257* (43).

IS*257* is a member of the IS6 family of insertion sequences (159). Another member of this family, IS*26*, which is found flanking resistance genes with increasing prevalence in Gram-negative bacteria, has recently been shown to undergo a second "conservative" mode of transpositional movement (165, 166); the process was IS*26* transposase dependent and *recA* independent in *Escherichia coli*. It involves the insertion of a single copy of the element and a flanking DNA fragment (often containing a resistance gene), termed a translocatable unit (TU), adjacent to an existing copy of IS*26* in another replicon. The resulting composite structure can be equivalent to an IS*26*-mediated cointegration event into an "IS*26*-less" replicon, but importantly, movement of an IS*26* TU was found to occur at a much higher frequency than cointegrate formation mediated by transposition of just an IS*26* copy. As a consequence, the presence of IS*26* in a replicon predisposes it to the acquisition of IS*26* TUs. The capacity of IS*257* to promote movement via comparable TUs is yet to be investigated, but the similar properties of these elements in their distinct hosts, particularly their prevalence in gene arrays punctuated by directly repeated insertion sequence copies, suggests it is a distinct possibility (165, 166).

The activities of IS*257* are illustrated by the pSK41 family plasmids (Fig. 3). It would seem that IS*257*-mediated cointegration has enabled these conjugative plasmids to collect functions, in the form of smaller plasmids, as they move horizontally through bacterial populations. Insertions and flanking deletions mediated by this element provide a mechanism for the inactivation or removal of deleterious sequences, such as redundant replication function.

Interestingly, short (148-149 bp) elements, named ISLE39 and ISLE49, that lack any transposase coding sequence but possess terminal inverted repeats similar to those of IS*257*, have been identified in several staphylococcal species (167). These presumably represent non-autonomous miniature inverted repeat transposable elements (MITEs) that could be rendered mobile by transposase encoded by IS*257* copies present in the same cell.

A central role for IS*257* has been suggested in the evolution of staphylococcal trimethoprim resistance, including the probable capture of the resistance gene, *dfrA*, from the chromosome of an *S. epidermidis* strain (26, 103). Furthermore, an IS*257*-hybrid promoter is responsible for transcription of this determinant (103, 168). In some plasmids, the high-level trimethoprim resistance typically conferred by this determinant has been moderated as a consequence of IS*257*-associated flanking deletions. An analogous IS*257*-hybrid promoter transcribes the *tetA*(K) tetracycline resistance gene of a cointegrated copy of pT181 within type III SCC*mec* elements (see below), and several other resistance genes are likely to be similarly promoted (102, 169).

One or more copies of IS*257* are carried by most types of staphylococcal cassette chromosome *mec* (SCC*mec*) elements (where it is commonly denoted IS*431*; see below). As well as flanking various resistance determinants within these elements, in several *SCCmec* types an IS*257* copy has mediated deletion of the *mecI* and part of the *mecR1* regulatory genes, resulting in derepressed transcription of the *mecA* methicillin resistance gene (170). Equivalent *mecI-mecR1* deletions are associated with another insertion sequence in some other SCC*mec* types, which instead contain a remnant of IS*1272*, ψIS*1272* (see below) (171–173). Multiple intact copies of IS*1272* have been found on the chromosomes of *S. aureus* and coagulase-negative staphylococcal strains, and it is particularly prevalent in *Staphylococcus haemolyticus* (4, 172, 174). IS*1272* is also associated with the elements Tn*Sha1* and Tn*Sha2*, which encode the *fabI* determinant responsible for resistance to the biocide triclosan (175); these elements are believed to target palindromic sequences and not generate target duplications during transposition.

IS*21-558* (also called IS*Sau9*) and derivatives thereof have been linked with the *cfr* multiresistance gene in a range of contexts and hosts (145). Its name reflects the fact that it is a member of the IS*21* family of insertion sequence elements and that it was initially detected within a variant of Tn*558* (176), which itself carries a *fexA* determinant that mediates chloramphenicol and florfenicol efflux (177). Within the Tn*558* variant, two directly repeated IS*21-558* copies formed a composite configuration flanking both *cfr* and the *lsa*(B) lincosamide resistance gene (176).

In addition to the high-level glycopeptide resistance VanA element Tn*1546* of enterococcal origin (27) described above, one other Tn*3*-type transposon, Tn*551*, has been detected in staphylococci. This MLS (*ermB*) transposon is closely related to Tn*917* from *E. faecalis* but confers constitutive rather than inducible MLS resistance (7). Tn*551* was originally identified on the β-lactamase/heavy-metal resistance plasmid, pI258 (Fig. 2) but has subsequently been shown to transpose to numerous sites in other large plasmids and the chromosome. Despite a tendency to "hot-spot," it has served as a valuable tool for mapping and mutagenesis studies (7, 178).

Tn*552* and Tn*5404* are Tn*7* family unit transposons. Tn*552* appears to be restricted to a very limited set of insertion sites on the chromosome and in plasmids (25). In theta replicating plasmids (Figs. 2 and 3), the insertion sequence sites of Tn*552* and Tn*552*-like elements are normally located within resolution sites upstream of resolvase (recombinase) genes encoded by the plasmids (43, 86, 87, 179). An invertible segment can be formed between resolution sites that are present in both the plasmid and transposon (180). In some ICE*6013* elements (see below) Tn*552* is also found proximal to the probable recombinase genes (181). This property is shared with Tn*5035*-like elements from Gram-negative bacteria, which are termed "*res* site hunters" (182). Despite such insertional specificity, Tn*552*-type transposons are thought to represent the source of all staphylococcal β-lactamase genes (76). However, in many plasmids, only remnants of such transposons remain, presumably as a consequence of multiple insertion events and/or rearrangements mediated by the recombinase systems present on both the transposon and plasmids.

Tn*5404* is thought to have resulted from the transposition of a chromosomal element to a plasmid in a clinical *S. aureus* strain (183). This transposon shares an invertible segment (between resolution sites) with an adjacent copy of Tn*552*, such that an entire copy of one or the other of these elements is generated depending on the orientation of the segment (183). Another invertible segment within Tn*5404* represents a composite structure designated Tn*5405* (183), which encodes resistance to aminoglycosides (*aadE*, *aph*A) and streptothricin (*sat4*) (184). Tn*5405* is bounded by copies of IS*1182*, an element related to IS*1272* (see above) (185). Moreover, one of the IS*1182* elements flanking Tn*5405* is interrupted by a copy of IS*1181*, which is both prevalent and active in *S. aureus* (1, 185, 186).

An incomplete vestige of an element related to IS*1181* has been detected on the plasmid pACK1 from *Staphylococcus simulans* biovar *staphylolyticus* ATCC1362. This element,

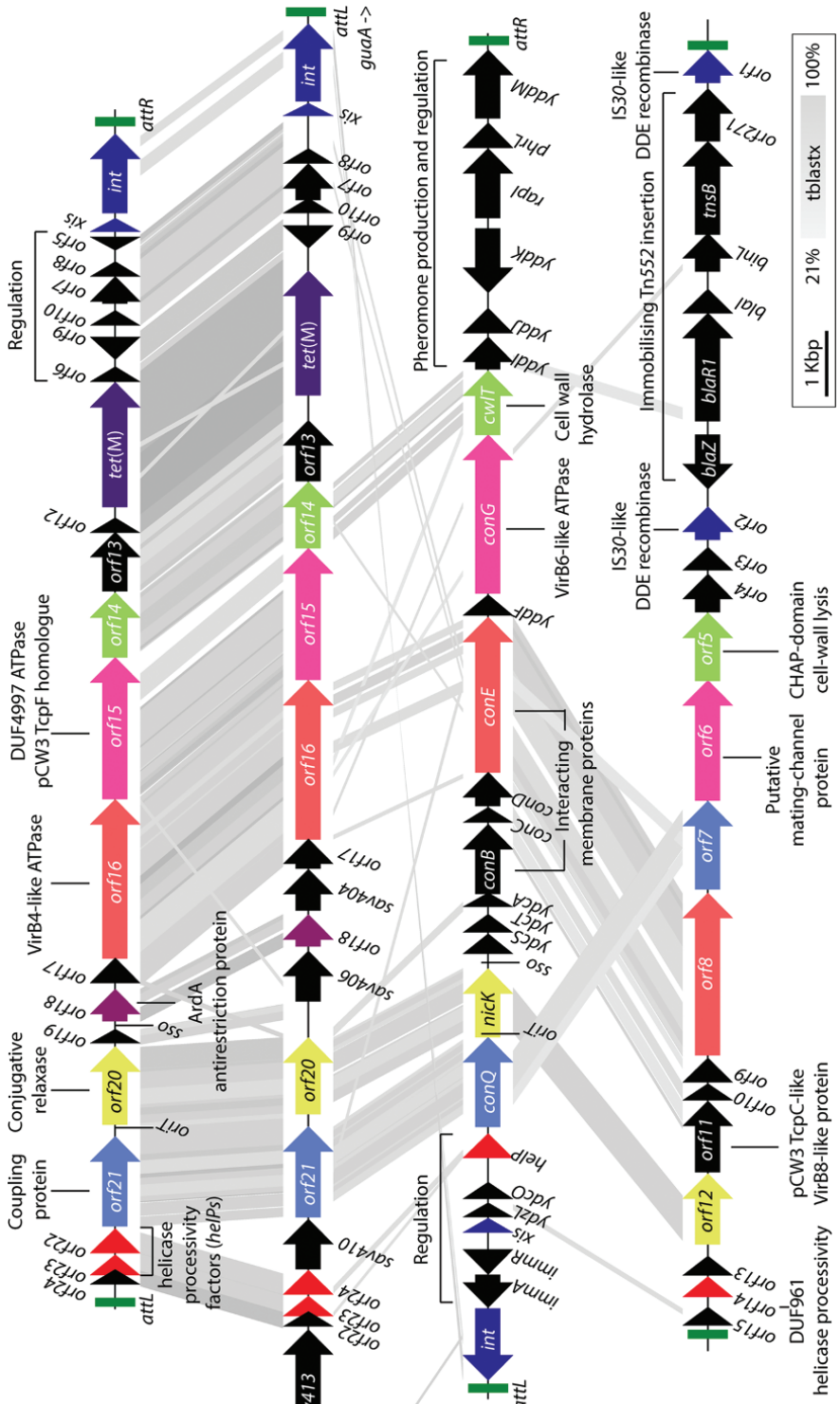
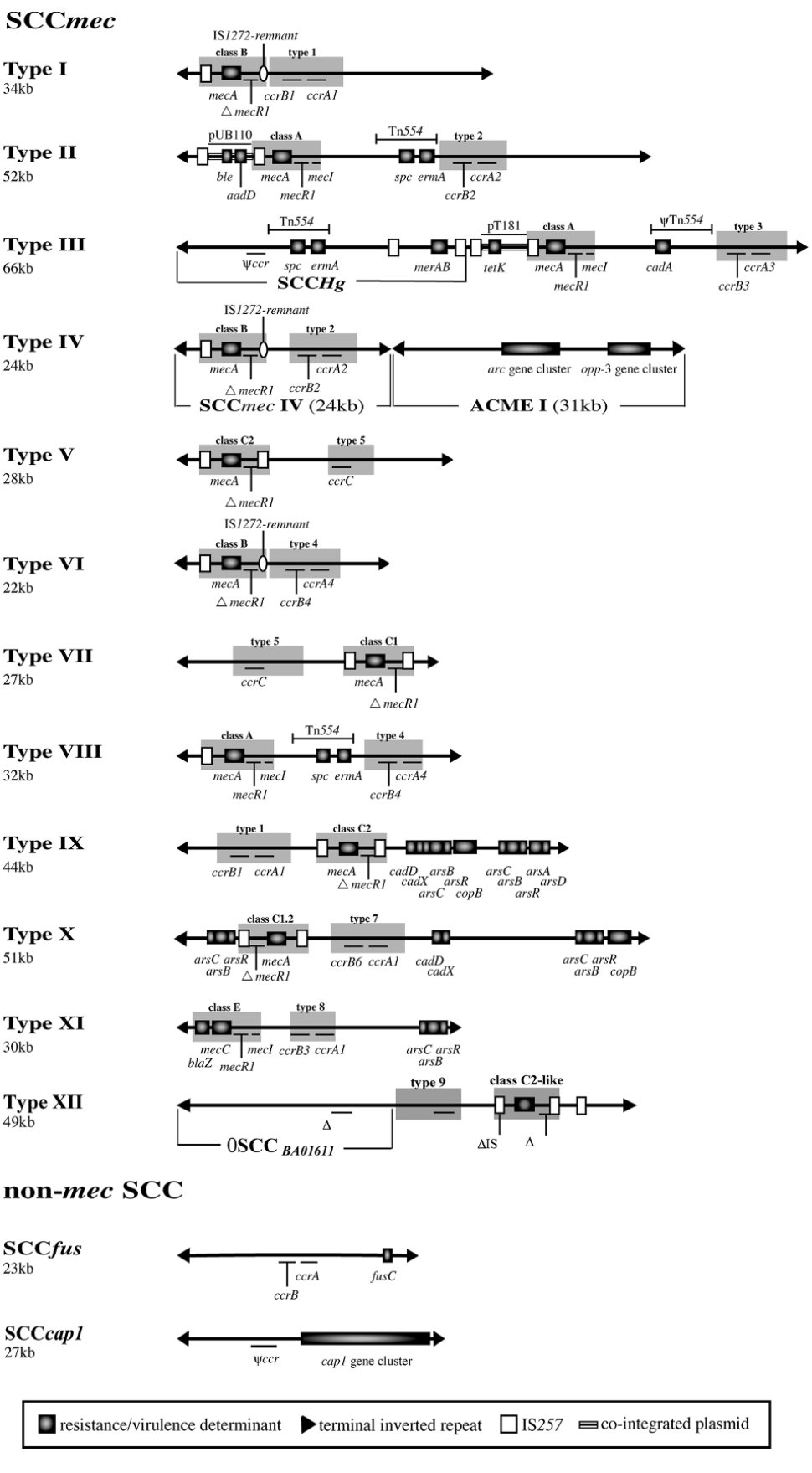


FIGURE 4 Genetic organization of Tn*916*, Tn*5801*, ICE*Bs1*, and ICE*6013*. A comparison of Tn*916*, Tn*5801*, ICE*Bs1*, and ICE*6013* constructed using EasyFig (233). Amino acid similarity comparisons (20% lower cutoff) using tblastx and are shown as gray shading between regions on each ICE. Genes with likely common functions—regardless of sequence similarity—are similarly colored. Positions of the conjugative origin of transfer (*oriT*) and single-strand origin of replication (*sso*) are indicated if known. Recombinase attachment/target sites are indicated by green rectangles. Annotations were collated from previously published figures for each ICE (15, 181, 197, 204, 206, 234, 235).


SCCmec
Type I
34kb
IS1272-remnant
class B
type 1
mecA
ccrB1
ccrA1
△mecR1
Type II
52kb
pUB110
class A
Tn554
type 2
ble
aadD
mecA
mecR1
mecI
spc
ermA
ccrA2
ccrB2
Type III
66kb
Tn554
pT181
class A
ψTn554
type 3
ψccr
spc
ermA
merAB
tetK
mecA
mecR1
mecI
cadA
ccrA3
ccrB3
SCCHg
Type IV
24kb
IS1272-remnant
class B
type 2
mecA
△mecR1
ccrA2
ccrB2
arc gene cluster
opp-3 gene cluster
SCCmec IV (24kb)
ACME I (31kb)
Type V
28kb
class C2
type 5
mecA
△mecR1
ccrC
Type VI
22kb
IS1272-remnant
class B
type 4
mecA
△mecR1
ccrA4
ccrB4
Type VII
27kb
type 5
class C1
ccrC
mecA
△mecR1
Type VIII
32kb
class A
Tn554
type 4
mecA
mecR1
mecI
spc
ermA
ccrA4
ccrB4
Type IX
44kb
type 1
class C2
ccrB1
ccrA1
mecA
△mecR1
cadD
cadX
arsB
arsR
arsC
copB
arsC
arsB
arsA
arsD
arsR
Type X
51kb
class C1.2
type 7
arsC
arsR
arsB
mecA
△mecR1
ccrB6
ccrA1
cadD
cadX
arsC
arsR
arsB
copB
Type XI
30kb
class E
type 8
mecC
blaZ
mecR1
mecI
ccrB3
ccrA1
arsC
arsR
arsB
Type XII
49kb
type 9
class C2-like
Δ
ΔIS
Δ
0SCC BA01611
non-mec SCC
SCCfus
23kb
ccrA
ccrB
fusC
SCCcap1
27kb
ψccr
cap1 gene cluster
resistance/virulence determinant
terminal inverted repeat
IS257
co-integrated plasmid

designated IS*1293*, is located at one end of a segment that contains the genes encoding preprolysostaphin (*lss*) and lysostaphin immunity factor (*lif*) (187). It is likely that the *lss-lif* segment of this plasmid, which possesses a truncated remnant of IS*257* at its other end, has been acquired through horizontal transfer (187). Numerous copies of another IS*1181*-like element, IS*Sha1*, have been found in *S. haemolyticus* strains (188).

The MLS- and spectinomycin-resistance element Tn*554* is unusual in that it lacks terminal inverted repeats and generates no target sequence duplications upon insertion (189). It inserts at an efficiency approaching 100% into a unique site in the *S. aureus* and *S. epidermidis* chromosomes, termed *att554* (189), within the *radC* gene (190), with a preference for one orientation. Secondary insertions can be generated in the chromosome or plasmids, albeit at much lower frequency, if *att554* is absent or occupied by a preexisting copy of Tn*554*. However, natural isolates have been identified that carry this or related elements, such as ψTn*554* encoding cadmium resistance, at different secondary sites, including some located in SCC*mec* regions (see below) (1, 179). Insertion of Tn*554* is dependent on two tyrosine-based site-specific recombinases encoded by the *tnpA* and *tnpB* genes and the product of a third, *tnpC*, that influences efficiency and orientation specificity (191, 192). The recombinases of Tn*554* are related to integrases of bacteriophage and ICEs such as Tn*916* (see below). The Tn*554*-like elements, Tn*558* (177), Tn*559* (190), Tn*5406* (193), and Tn*6133* (194) exhibit equivalent insertion site specificity but encode additional or alternative resistance determinants.

INTEGRATIVE AND CONJUGATIVE ELEMENTS

ICEs are mobile genetic elements that share characteristics of both conjugative plasmids and chromosomally integrating elements such as transposons and prophage. ICEs primarily exist within the host chromosome but can excise, circularize, and transfer via conjugation. ICEs encode all genes necessary for mating-pair formation and DNA transfer. The broad impact of ICEs on an organism's capacity for horizontal gene transfer and dissemination of antimicrobial resistance and virulence factors is significant. However, perhaps due to the often-changing classification of ICEs as genomic islands, transposons, or conjugative transposons and the evolving differences in naming conventions (195, 196), the roles of ICEs in *S. aureus* gene transfer are not yet as widely recognized as those of their plasmid and bacteriophage counterparts.

Identified *S. aureus* ICEs can be grouped into two families: those identical or closely related to the Tn*916* ICE and those related to the distinct ICE*6013* family (16). The conjugation gene clusters of Tn*916* and ICE*6013* family elements both carry conjugation genes related to those of *Bacillus subtilis* ICE*Bs1* (Fig. 4) and more distantly, plasmids such as *Clostridium perfringens* pCW3 and *Enterococcus* spp. pIP501 (197). *S. aureus* Tn*916* and ICE*6013* family elements vary in their integration mechanism, insertion site preference, and antimicrobial-resistance gene cargo.

Tn*916* elements are extremely promiscuous in Gram-positive organisms, and those identified in *S. aureus* are often identical to those in other Gram-positive genera. Tn*916* transfer from *E. faecalis* to *S. aureus* and transfer between *S. aureus* strains has been experimentally demonstrated (15, 198). The 18-kb Tn*916* element carries genes for conjugation, integration, excision, and the tetracycline-minocycline-resistance gene *tetM*. Tn*916* integration is mediated by the tyrosine recombinase Int, and excision is stimulated by Int and the excisionase Xis. Int preferentially targets pairs of poly-A and poly-T tracts separated by a 6-bp spacer for Tn*916* insertion (199). Tn*916* excision is stimulated in the presence of tetracycline and other ribosome-targeting antibiotics through an antiattenuation mechanism resulting in the derepression of *xis*; therefore, antibiotic exposure directly drives the spread of Tn*916*-mediated resistance (200–202).

Various distinct Tn*916*-like elements have been described in *S. aureus*, but the most well-documented example is the Tn*5801* subfamily. Tn*5801* (Fig. 4) was originally identified in *S. aureus* strain Mu50 (1) and carries conjugation and resistance genes (*tetM*) closely related to those of Tn*916* in both sequence and organization. Tn*5801* is larger (25 kb) than Tn*916* and harbors several Tn*5801*-specific genes within the conjugation-gene cluster. *int* and *xis* genes of Tn*5801* are distinct from those of Tn*916*, as is the integration preference for the 3′ end of the conserved GMP-synthase gene, *guaA*. Tn*5801* transfer between *S. aureus* strains has also been demonstrated (15). Like Tn*916* elements, Tn*5801* elements have been identified in human and animal-associated *S. aureus* and are particularly prevalent in *S. aureus* clonal complex CC398 (15, 203, 204). Both elements are also abundant in other animal-associated *Staphylococcus* spp. (204, 205), suggesting that agricultural and companion animals may be reservoirs of Tn*916* and Tn*5801*-carrying staphylococci.

ICE*6013* is the most recently identified family of *S. aureus* ICEs, and although it is also related to Tn*916* and Tn*5801*, it is more closely related to *B. subtillis* ICE*Bs1* than any other *S. aureus* element (206) (Fig. 4). Unlike Tn*916* and Tn*5801*, ICE*6013* family members generally lack antimicrobial-resistance determinants, although the first discovered ICE*6013* element in *S. aureus* HDG2 carries a Tn*552* insertion conferring penicillin resistance, and the insertion has immobilized this copy of ICE*6013* (181, 206) (Fig. 4). As well as being one of the smallest known families of ICE, with a minimum size of 12.7 kb, ICE*6013* is the only known ICE to utilize a DDE family transposase for its integration and excision and has an insertion preference for the sequence 5′-TTTTTANNNTAAAAA-3′ (181). ICE*6013* was originally identified in the ST239 *S. aureus* strain HDG2, and related elements have been identified in 44% of distinct *S. aureus* sequence types, including the dominant American community-associated lineage USA300. Conjugation frequencies of ICE*6013* approach rates similar to that of the conjugative

FIGURE 5 Maps of representative SCC elements (4, 216); see text for additional references. Resistance/virulence genes, transposons, insertion sequences, and cointegrated plasmids are shown: *arsBC*, arsenic resistance; *cadA*, cadmium resistance; *cap1* genes, capsular polysaccharide (220); *fusC* (previously known as *far*), fusidic acid resistance (236); *mecA*/*mecC*, β-lactam resistance. Refer to Table 1 for the antimicrobial resistance(s) conferred by other resistance determinants. Cassette recombinase genes (*ccrA*, *ccrB*, and *ccrC*), *mecA*/*mecC* regulatory genes (*mecI* and *mecR1*), and an arginine catabolic mobile element (ACME I [227]) are also shown; *mec* classes and *ccr* types are denoted by gray shading. Note that *cch* genes, *polA*, and SAUGI are not shown and that IS*257* is also known as IS*431*.

plasmid pSK41/pG01. Some strains harbor various combinations of up to four unique ICE*6013*, Tn*916*, and Tn*5801* family ICEs simultaneously (23, 181). The Tn*916*/Tn*5801* *orf18* gene encodes the ArdA antirestriction protein (Fig. 4), which inhibits type I restriction modification systems ubiquitous in *S. aureus*, so capture of Tn*916*/Tn*5801* ICE may increase the propensity for horizontal acquisition of additional mobile elements (207–209).

In addition to their own conjugative transfer, ICEs can mobilize the transfer of compatible nonconjugative plasmids or other integrative elements (16, 210). ICE*Bs1* is able to mobilize the transfer of RCR plasmids encoding RepU replication initiators through an interaction dependent on RepU and the ICE*Bs1* T4CP, ConQ (58, 197). ICE*6013*, Tn*916*, and Tn*5801*-family ICEs share closely related ConQ homologs (Fig. 4), so it is plausible that *S. aureus* ICEs may facilitate mobilization of RCR plasmids. Indeed, ICE*Bs1*- and Tn*916*-mediated mobilization of *S. aureus* antimicrobial-resistance plasmids pC194 and pUB110 has already been demonstrated, albeit in a *B. subtilis* host (58, 211). The impact of *S. aureus* ICEs on the capacity to facilitate horizontal gene transfer of other mobile genetic elements warrants further investigation (16, 138).

STAPHYLOCOCCAL CASSETTE CHROMOSOME

Most methicillin-resistant *S. aureus* isolates contain a 20- to 70-kb DNA segment, generically termed the *mec* region, that is not present in methicillin-sensitive strains. These segments are a type of genomic island (i.e., are bounded by terminal inverted repeats and subject to horizontal transfer) called staphylococcal cassette chromosome *mec* (SCC*mec*) (179). Methicillin resistance is mediated, at least in part, by the *mecA* gene or the more recently identified *mecC* gene (212, 213), both of which encode a related low-affinity penicillin-binding protein, termed PBP2a. Two regulatory loci, *mecI* and *mecR1*, are commonly found upstream of, and transcribed divergently to, *mecA*/*mecC* (however, see discussion of IS*1272*/IS*257*, above). In combination, the *mecA*/*mecC*, *mecI*, and *mecR1* genes and associated insertion sequence(s) are referred to as the *mec* gene complex, of which several classes have been defined (A, B, C1, C2, D, and E) on the basis of structural diversity (http://www.sccmec.org).

Additionally, SCC*mec* contains the *ccr* gene complex, which comprises either a combination of *ccrA* and *ccrB* genes (one of each) or a single *ccrC* gene; different allotypes for these genes also exist, and several types of the *ccr* gene complex have been defined (http://www.sccmec.org) (214). Each *ccr* gene encodes a recombinase that mediates the excision and circularization of the cassette and its site- and orientation-specific insertion into the $attB_{SCC}$ site of the *S. aureus* chromosome and homologous sites in other staphylococcal species (179). Recently, a conserved gene located upstream of the *ccr* gene(s) has been shown to encode a DNA helicase (215) referred to as *cch* or *cch2*, and the latter is also preceded by a putative primase (*pol*A). This implies that SCC elements might be able to replicate (postexcision), and multiple copies would likely facilitate the horizontal transfer process. Moreover, a gene located downstream of the *ccr* gene(s) encodes a uracil-DNA glycosylase inhibitor (SAUGI). *S. aureus* pathogenicity islands, which are specifically induced to excise and be packaged by invading bacteriophage, carry homologs of *cch*, and regulate their excision in response to phage-induced changes in uracil metabolism. These commonalities suggest that phage may also have a role in stimulating and/or facilitating SCC*mec* transfer (128, 215).

At present, SCC*mec* has been classified into 12 allotypes based on the organization of the *mec* and *ccr* gene complexes (Fig. 5) (216); subtypes also exist and are defined by the structure of the three joining (J) regions (previously called junkyard regions) located between these gene complexes and the chromosomal regions flanking SCC*mec*. The J regions also act as a chromosomal hot-spot for the insertion of additional antimicrobial resistance determinants, often in association with transposable elements (173). These include Tn*554* or related elements encoding resistance to erythromycin and spectinomycin, or cadmium, and IS*257*-flanked segments conferring resistance to mercury, tetracycline, and/or aminoglycosides and bleomycin; the latter two segments are known to be cointegrated copies of the plasmids pT181 and pUB110, respectively (Fig. 5) (26, 103). Based on this classification system, an online tool, SCC*mec*Finder, has recently been developed for rapid whole-genome sequence-based SCC*mec* typing (217).

It is important to note that SCC*mec* elements are not exclusive to *S. aureus* and are frequently carried by coagulase-negative staphylococci. Moreover, the ubiquitous carriage of a *mecA* homolog by *Staphylococcus sciuri* has led to the suggestion that this or closely related species represent the origin of the *mec* determinants found in other species (218). In this regard, a recent study by Rolo et al. (219) indicates that *Staphylococcus vitulinus* and *Staphylococcus fleurettii* contributed to the evolution of the *mec* complex and that *S. sciuri* likely provided the mobile element into which this complex was later incorporated. In any case, it is clear that coagulase-negative staphylococci act as a reservoir from which methicillin-sensitive *S. aureus* can acquire SCC*mec*, contributing to the emergence of new methicillin-resistant *S. aureus* clones.

However, not all SCC elements encode resistance to methicillin, because related structures have also been identified (179, 220, 221). These include SCC*cap1*, which encodes a type I capsular polysaccharide (220), and SCC*fur* (also known as SCC_{476}) and SCC*Hg* (also known as SCC*mercury*), which encode resistance to fusidic acid and mercuric chloride, respectively (4, 222) (Fig. 5). Additionally, the arginine catabolic mobile element, which is broadly associated with certain coagulase-negative staphylococci (223–225), has also been identified in association with SCC*mec* allotypes, largely type IV (226). This element is a virulence/colonization determinant that utilizes SCC*mec*-encoded cassette recombinases and integrates into the $attB_{SCC}$ site (223, 227, 228) (Fig. 5). Arginine catabolic mobile element allotypes are defined by the presence or absence of the *opp*-3 and *arc* gene clusters, the latter of which encode a complete arginine deiminase pathway, and carriage of this element has been shown to enhance fitness and skin colonization (228–230).

PERSPECTIVES

Mobile genetic elements and the mechanisms of genetic exchange they facilitate/exploit act in concert to catalyze rapid microbial adaptation by providing access to an extended reservoir of niche-adaptive functions. Clinical staphylococci represent a salient illustration of the evolutionary potential this affords. Genomic technologies have greatly expanded our understanding of variability between strains (clinical and nonclinical) and the relationships between them and revealed a growing repertoire of mobile genetic elements, thereby illustrating the significance of horizontal genetic exchange. There is little doubt that the ongoing acceleration of genomics will yield further insights in the future.

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Temperate Phages of *Staphylococcus aureus*

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32

INTRODUCTION

The diversity of the *Staphylococcus aureus* species is mainly determined by mobile genetic elements, many of which are prophages or phage-related genomic islands. Strain evolution as a result of short- and long-term adaptation to diverse environments is tightly linked to phages. Many phages carry accessory genes coding for staphylococcal virulence factors, which are important for the success of certain *S. aureus* clonal complexes (CCs). Second, phages support the induction, packaging, and transfer of genomic islands (1, 2). This topic is reviewed elsewhere. Third, phage-mediated transduction is an efficient way to transfer not only extrachromosomal mobile elements, such as plasmids, but also chromosomal markers (albeit with lower efficiency). *S. aureus* is thought not to be naturally competent, so that recombination and horizontal gene transfer are mostly phage mediated and, to a lesser extent, conjugative. Here, we will first give a brief overview of previously used methods to classify *S. aureus* phages. Then we will mainly focus on the impact of temperate phages on the evolution of the bacterial host.

CLASSIFICATION OF *S. AUREUS* PHAGES

All known *S. aureus* phages belong to the order *Caudovirales* (tailed phages) and are composed of an icosahedral capsid filled with double-stranded DNA and a thin filamentous tail. Based on the complete genomes of 27 phages (3), *S. aureus* phages were assigned to three size classes: siphoviruses, with a genome size of 39 to 43 kb, podoviruses, with a smaller genome size of 16 to 18 kb, and myoviruses, with a genome size of 120 to 140 kb. This capsid and genome size-based classification correlates with the tail morphology as observed by electron microscopy: *Podoviridae* have a very short tail, *Siphoviridae*, a long noncontractile tail, and *Myoviridae*, a long contractile, double-sheathed tail (for recent reviews, see 4–6). All of the known temperate staphylococcal phages belong to the *Siphoviridae*, which are the primary focus of this chapter.

The siphovirus genomes are usually organized into six functional modules: lysogeny, DNA replication, packaging, head, tail, and lysis (7–9). The evolution of phage lineages was driven by the lateral gene transfer of interchangeable genetic elements (modules), which consist of functionally related genes. A functional module found in one phage can be replaced in another phage by a sequence-unrelated module that fulfils the same or related functions; often, genes within such modules travel together (10). Thus, multiple alignments of *S. aureus* phage genomes reveal a chimeric and mosaic structure resulting from horizontal transfer and recombination (3, 5, 9, 11–13).

Due to this modular structure, phage nomenclature and classification of the *Siphoviridae* are challenging and are a matter of debate. In Table 1 the different designations and properties of a representative set of *S. aureus Siphoviridae* are illustrated. In one approach phages were classified based on protein repertoire relatedness (5, 7). Thereby, *Siphoviridae* from *S. aureus* were clustered into six major clades. Clades 4 to 6 (Sfi21-like phages) share characteristic features with the capsid region of *Escherichia coli* HK97 phage and use the cos-site strategy for DNA packaging. Clades 1 to 3 (Sfi11-like pac-type phages) are related to the *Bacillus subtilis* SPP1 phage (7). The Sfi11/Sfi21-like grouping of *Siphoviridae* is based on genome analysis of lactococcal phages. Gene clusters extending from the DNA packaging genes to the tail genes were found to be represented by two unrelated configurations: one is characteristic of cos-site phages (prototype: phage Sfi21), and the other is characteristic of pac-site phages (prototype: phage Sfi11).

In another approach, *S. aureus Siphoviridae* were classified according to polymorphism of the *int* gene and the modular composition of lysogeny, regulation, replication, structural, and lytic modules (9, 12) (Table 1). The modules can be defined by multiplex PCR or sequence analyses of selected genes within the modules. *S. aureus* prophages were primarily classified on the basis of *int* gene homology (9, 12, 13). By including information on the allelic variation of other modules, a further meaningful subdivision can be achieved. A similar approach to the general classification of bacteriophages has been proposed previously (14, 15). Kahánková *et al.* (9) established a mulitplex PCR assay to distinguish between different types of integrase (10 types), antirepressor (five types), replication proteins (four types), dUTPase (four types), portal protein (eight types), tail appendices (four types), and endolysin (four types). The proposed extended typing scheme covering seven major genomic modules enables not only the differentiation of phages but also the design of a classification system. For the basic description and rapid differentiation of a phage strain, the serological group type, the *int* type, and the endolysin type are the most relevant (located at the left side, the middle, and the right side of the prophage genomes). Using a simplified scheme, e.g., phage φ11 can be classified as Sa5int-Ba-ami1 or phage φ13 as Sa3int-Fb-ami3 (9). Despite the multiple mosaic variants, there are close links between particular modules; e.g., all phages using *polA* for replication are serogroup A.

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TABLE 1 Classification and properties of selected *S. aureus Siphoviridae*[e]

Phage name	Sfi type[a]	Clade[a]	Int[b]	Sero[b]	Holin[c]	Virulence[d]	cos/pac	attB	Phage or attB in 8325[c]
Φ55	Sfi11	II	Sa1	B	438				SAOUHSC_00851: 823130-823148
ΦMu50B	Sfi11		Sa1	B	438		pac	TTCGAAATGGAAGGTAGTA	
ΦETA	Sfi11	2	Sa1	B	276	*eta*			
ΦSa1JH1			Sa1	B	276				
ΦETA2			Sa1	B	276	*eta*			
Φ252A			Sa2	A	303				SAOUHSC_01583: Φ12, 1463618-1508581
ΦSa2mw	Sfi21		Sa2	A	303	*lukFS-PV*	cos: CGGCGGGGGC	ACCATCACATTATGATGA TATGTTTATTT	
ΦPVL108	Sfi21	5	Sa2	Fb	303	*lukFS-PV*			
Φ2958PVL	Sfi21	4	Sa2	A	303	*lukFS-PV*			
ΦPVL	Sfi21	5	Sa2	Fb	303	*lukFS-PV*			
ΦSa2USA300	Sfi21		Sa2	A	303	*lukFS-PV*			
ΦSLT	Sfi21	4	Sa2	A	303	*lukFS-PV*			
Φ12	Sfi11	4	Sa2	A	303				
Φ13	Sfi21	5	Sa3	Fb	255a	*sak*, *chp*, *scn*	cos: CGGAGCAGA	TGTATCCAAACTGG	*hlb*: Φ13, 2031923-2074632-
ΦN315	Sfi21	6	Sa3	Fa	255a	*sep*, *sak*, *chp*, *scn*			
ΦSa3mw	Sfi21		Sa3	Fb	255a	*sek*, *seq*, *sea*, *sak*, *scn*			
ΦNM3		6	Sa3	Fa	255a	*sea*, *sak*, *chp*, *scn*			
ΦMu50A	Sfi21	ND	Sa3	Fa	255a	*sea*, *sak*, *scn*			
ΦSa3USA300			Sa3	Fa	255b	*sak*, *chp*, *scn*			
ΦSa3JH9			Sa3	Fa	255b	*sak*, *chp*, *scn*			
Φ42E	Sfi21	IV	Sa3	A	255a				
ΦSa4JH1			Sa4	A	438			CATGTAATTCC	SAOUHSC_00958 (*htrA*): 933127-933137
ΦSa4ms			Sa4	A	303				
Φ11	Sfi11	1	Sa5	B	438		pac	CTTCCCATGG	SAOUHSC_02090: Φ11, 1923398- 1967013

ΦPV83			Sa5	Fb	255a	*lukM*, *lukF-PV*			
Φ187	Sfi11	I	Sa5	L	255a				
Φ29	Sfi11	II	Sa5	B	438				
ΦNM1	ND	1	Sa5	B	303				
Φ88	Sfi11	II	Sa5	B	438				
Φ52A	Sfi11	II	Sa6	B	438				SAOUHSC_00300 (*geh*): 316250-316257
Φ80	Sfi11	2	Sa6	B	438				
ΦNM4		2	Sa6	B	303			ATCATACAAGGATGGGAT	
ΦSa6JH9			Sa6	B	438				
ΦCOL			Sa6	A	303				
L54a			Sa6	A	?				
Φtp310-2			Sa6	A	303				
Φ53	Sfi11	I	Sa7	B	303				Intergenic, downstream SAOUHSC_01079 (*isdB*): 1042159-1042167
Φ80alpha	Sfi11	1	Sa7	B	303		pac	AGGTATCTG	
Φ85	Sfi11	I	Sa7	B	303				
ΦNM2			Sa7	B	438			AGGTATCTG	
Φ6390			Sa7	B	255a	*sak*			
Φ92	Sfi11	II	Sa7	B	438				
φSaov2			Sa7	F					
ΦRF122			Sa8	B	438		pac	CGGAAGGTAAGGGA	SAOUHSC_T00050 (*tmaS*): 1864312-1864325
Φ96	Sfi11	2	Sa9	B	303		pac	ND	tmRNA, 788659
ΦEW	Sfi11	3	Sa11	ND	435		pac	ND	SAOUHSC_00581: 584240

[a]Classification based on reference 5.
[b]Classification based on reference 12.
[c]Location of the native phage or the attB site in the genome of reference strain 8325 (GenBank accession number NC_007795.1).
[d]virulence genes
[e]Abbreviations: ND, Not determined; tmRNA, transfer messenger RNA.

There are several reasons to classify *S. aureus* prophages primarily based on *int* polymorphism. First, nucleotide sequences are well conserved within *int* groups, making the gene an ideal target for PCR amplification. The *int* grouping has good discriminatory power, reflecting the diversity of the *S. aureus* phage population. Recombination and exchange of certain modules seem to occur more often between phages of the same *Sa-int* group than between phages of different *Sa-int* groups (16). Second, the *int* typing allows for prediction of the chromosomal location of the prophage. Last, the *int* type is closely linked to the virulence gene content of the prophage and can therefore convey information about the pathogenic potential of the bacterial lysogen (12). Most of the *S. aureus* phages can be assigned to one of the major *Sa-int* types 1 to 8.

PHAGE INTEGRATION/EXCISION

All prophages integrate to position the *int* gene in close proximity to the bidirectional origin of chromosome replication. Based on amino acid sequence homology and catalytic residues, most integrases belong to the tyrosine recombinase type family. Only the integrases of Sa7int phages were found to belong to the serine recombinase type family (12). Despite the usually strong association of the *int* type with the integration site, there are also events where a phage may integrate in an illegitimate attachment site. This phenomenon was described to occur for Sa3int phages during chronic lung infections of cystic fibrosis patients (17). Under these conditions, the reconstitution of the phage-interrupted *hlb* gene may be an advantage. When these mislocated phages were induced and used to reinfect *S. aureus in vitro*, the phages reintegrated at their dedicated attachment site within *hlb*. Sa3 phages are only rarely found in livestock-associated *S. aureus* strains of CC398. In these strains the canonical attB site within *hlb* is altered, and therefore Sa3int phages integrate at diverse alternative attB sites (18, 19). The mechanism for excision of *S. aureus Siphoviridae* is much less well understood. Excision followed by replication may result not only in bacterial lysis and phage release but also in a process termed "active lysogeny." This term was recently introduced to describe instances where prophages temporally and reversibly excise from the chromosome without lysing the bacterial host (20). This might be seen as a form of bacterial gene regulation that possibly improves bacterial fitness. Evidence for active lysogeny in *S. aureus* comes from a recent sequencing approach to detect extrachromosomal phages in *S. aureus* (21). Furthermore, it could be demonstrated that phages are readily induced under infectious conditions (17, 22–25). Analysis of isolates from cystic fibrosis patients revealed that translocation of the Sa3int phages often leads to a splitting of the bacterial population (17) into Hlb-positive (phage-cured) and phage-positive fractions. Both the phage-encoded virulence factors and Hlb are secreted factors; thus, functional complementation can be assumed.

PHAGE-BACTERIAL RECOGNITION

Adsorption to the bacterial host is the first critical step within the phage life cycle. Interaction of the receptor-binding protein (RBP) of the phage and its receptor initiates the infection cycle and, importantly, determines the host range and specificity (26). Phage receptors have to satisfy the following distinct requirements to be suitable for viral attachment. (i) Accessibility: phage receptors have to be accessible to the phage by random Brownian motion, flow, or diffusion. (ii) Abundance: with attachment being a stochastic process, the abundance of the receptor must be high enough to permit a sufficient probability of RBP-receptor contact. (iii) Constancy: the chemical composition of the receptor must be stable enough to allow evolutionary adaption of the phage to possible changes in the receptor appearance. Wall teichoic acid (WTA) of *S. aureus* fulfills all three requirements imposed on a proper phage receptor and was shown to act as the primary phage receptor. It is a major component of the cell wall and is expressed by all *S. aureus* strains. Most *S. aureus* strains produce WTA comprised of ribitol-phosphate (RboP) repeats. The complex biosynthesis of WTA is reviewed elsewhere (27, 28). WTA is covalently attached to the peptidoglycan of the cell wall (29, 30). Further derivatization of WTA is achieved in two ways. Attachment of *N*-acetyl-glucosamine residues (GlcNAc) occurs at the C4-position of the RboP unit by two glycosyltransferases, TarS and TarM, which attach GlcNAc in the β-position and α-position, respectively (31, 32). Recently, a third prophage-encoded glycosyltransferase, named TarP, was identified (33). Unlike TarS, TarP catalyzes the attachment of GlcNAc in the β-1,3-position.

The second modification of WTA is attachment of D-alanine residues at the C2-position by the Dlt-machinery (34). In contrast, a completely different type of WTA, composed of a glycerol-phosphate (GroP) repeating unit modified with α-*N*-acetyl-galactosamine (GalNAc) residues is found in *S. aureus* strains belonging to CC395 (35). This GroP WTA resembles the WTA structure found in many coagulase-negative staphylococci (36). Early studies identified *B. subtilis* mutants lacking WTA or WTA glycosylation. Those mutants appeared to be resistant against certain *B. subtilis* bacteriophages (37, 38). It was subsequently shown that the sugar moiety of WTA is also important for *S. aureus* phage interaction. Treatment of *S. aureus* with exo-β-acetylglucosaminidase or use of WTA glycosylation mutants leads to a decrease in phage binding (39, 40).

Although a majority of publications describe only carbohydrate-based phage interactions (WTA in combination with peptidoglycan structures), work by Nordström *et al.* (41) suggested that additional proteinaceous factors, prominent among them, surface protein A, interfere with phage adsorption. To our knowledge, this is the only account of surface protein phage interactions reported for *S. aureus*. Interestingly, a secondary protein receptor besides the primary receptor WTA has been described for the Gram-positive model organism *B. subtilis*. The lytic phage Spp1 adsorbs first in a reversible manner to WTA, which accelerates an irreversible interaction with the membrane receptor YueB and injection of viral DNA (42). A similar phenomenon was described for the membrane protein PIP, which serves in lactococcal species as a secondary phage receptor (43). However, PIP homologues in *S. aureus* have not been shown to be necessary for phage adsorption (44). Thus, so far, there is no clear evidence for a secondary proteinaceous receptor in *S. aureus*.

The advent of bacterial genetics allowed further insights into the molecular basis of bacteria-phage interactions. An early study suggested that besides WTA, a different anionic polymer of the *S. aureus* cell envelope, lipoteichoic acid, might serve as a receptor for the tail protein ORF636 of *S. aureus* phage ΦSLT (45). Lipoteichoic acids (LTA) consist of D-alanylated glycerol phosphate repeating units attached to the cell membrane of *S. aureus* (46). However, using an LTA-deficient mutant strain of RN4220, no difference in the infectivity of phages expressing homologues of

the tail protein of ΦSLT could be observed (47). In contrast to the LTA-deficient mutant, gene deletion of *tag*O, the first gene of the WTA biosynthesis pathway, leads to a phage-resistant phenotype (47, 48). By creating a transposon mutant library, Xia *et al.* (32) were able to isolate the RN4220 mutant K6, which is deficient in WTA glycosylation. K6 carries a transposon interrupting the gene coding for the α-glycosyltransferase TarM. K6 additionally carries a premature stop codon in *tar*S coding for the β-glycosyltransferase (31). K6 was resistant to a wide range of siphoviruses (47). Interestingly, as reviewed later, lytic myoviruses (Φ812 and φK) were able to infect K6 despite the lack of WTA glycosylation.

By using defined knockouts of the two identified ribitol-phosphate glycosyltransferases, *tar*M and *tar*S, it could be demonstrated that siphoviruses of serogroup B do not seem to differentiate between α- or β-GlcNAcylation at the ribitol C4 position (31, 44) (Fig. 1A). The receptor promiscuity of siphoviruses might reflect their role as the main vectors of horizontal gene transfer. Of note, Winstel *et al.* (49) demonstrated that *S. aureus* pathogenicity islands (SaPIs) can be transferred between strains expressing α- or β-GlcNAcylated WTA. Notably, β-1,3-GlcNAcylation by TarP seemed to reduce the infection and SaPI-transfer capacity of serogroup B siphoviruses (33) (Fig. 1A). More importantly, the Winstel and coworkers publication established the compatibility of phage and host receptors as a key driver of cross-species horizontal gene transfer (49). Even distantly related bacterial species such as *Listeria monocytogenes* can engage in horizontal gene transfer with *S. aureus* (49, 50), as long as they express a compatible phage receptor. In contrast, lack of RboP-GlcNAc WTA excludes bacteria from the exchange of genetic information. For instance, *S. aureus* PS187 (CC395), equipped with GroP-type WTA, is cut off from horizontal gene transfer with RboP-WTA-expressing *S. aureus*, but is able to engage in exchange with coagulase-negative species that share a similar GroP-type WTA (35, 49). Interestingly, ectopic expression of RboP-type biosynthesis genes in coagulase-negative staphylococci with GroP-WTA rendered them susceptible to *S. aureus* phages (49). Hence, it can be speculated that RBP-receptor incompatibility is a major hindrance for horizontal gene transfer between *S. aureus* and coagulase-negative staphylococci. The other two described classes of morphological groups, namely *Myoviridae* and *Podoviridae*, show more diverse receptor specificities, that are discussed later in the article.

Returning to the postulated requirements for a phage receptor (accessibility, abundance, and constancy), one can audit whether they hold true for WTA as the phage receptor of *S. aureus*. WTA is an exposed cell surface molecule attached to the peptidoglycan (51), which would allow easy access to phages. The negative charge of WTA, conferred by phosphate residues, might allow polar or ionic interaction

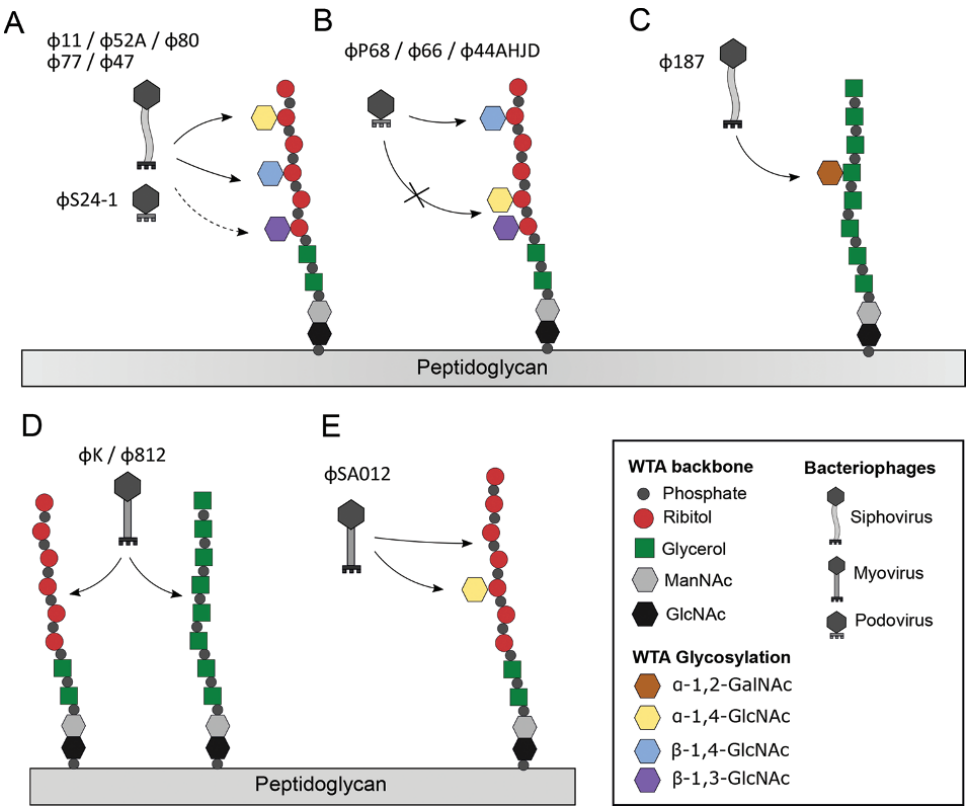


FIGURE 1 Receptor specificity of *S. aureus* phages. (A) Siphoviruses Φ11, Φ80, Φ52A, Φ47, and Φ77 and podovirus SA24-1 recognize α-or β-1,4-GlcNAc-RboP WTA. β-1,3-GlcNAc-WTA is adsorbed to less strongly by Φ80, Φ52A, and Φ11. (B) Podoviruses ΦP68, Φ44AHJD, and Φ66 bind to β-1,4-GlcNAc-RboP WTA and are blocked by β-1,3-GlcNAc or α-1,4-GlcNAc modifications. (C) Siphovirus Φ187 binds to α-GalNAC-GroP. (D) Myovirus ΦK, Φ812, attache to the backbone of GroP and/or RboP. (E) ΦSA012 recognizes both the RboP WTA backbone and α-1,4-GlcNAc-RboP by two different RBPs.

with RBPs. LTA, due to its presence in the more internal cell membrane of *S. aureus*, might therefore be of less utility as a receptor for bacteriophages. Additionally, WTA is a highly abundant glycopolymer that constitutes up to 60% of the cell wall mass in many Gram-positive bacteria. It is a key component of the cell wall, and no natural isolates of *S. aureus* without WTA have been reported so far. The indispensability and abundance of WTA ensure a high probability of phage-receptor contact, which is crucial for the adsorption process. Coagulase-negative bacteria, especially *Staphylococcus epidermidis*, show an increased diversity of WTA glycosylation patterns (36, 52). *S. aureus* appears to encode only one housekeeping glycosyltransferase, TarS. The *tarS* gene is found in almost all *S. aureus* genomes (53). The second glycosyltransferase, TarM, appears to have been acquired very early in *S. aureus* evolution (54). However, certain clonal lineages, such as CC5 and CC398, seem to have deleted *tarM* during their emergence or may have acquired phage-encoded *tarP*. The introduction of more sophisticated WTA glycosylation patterns might lead to less phage interaction and therefore to a decrease in the ability to participate in horizontal gene transfer. The described receptor constancy of WTA together with the widespread absence of clustered regularly interspaced short palindromic repeat (CRISPR)/Cas systems allows rapid acquisition and exchange of genetic elements by phages or phage-like particles (SaPIs), which ensures the role of *S. aureus* as a major human pathogen.

OCCURRENCE AND ROLE OF ACCESSORY PHAGE GENES

Although phages may be regarded as selfish elements, bacteria have learned to use them for their own purposes, and lysogeny can be regarded as a motor for short-term evolution. In many pathogens, phages provide the bacteria with additional genes that enable them to establish a new lifestyle. In *S. aureus*, several such phage-encoded virulence factors have been described, an observation originally described as phage conversion. Genes coding for Panton-Valentine leukocidin (*lukSF*) exfoliative toxin A (*eta*) (55), the cell-wall anchored protein SasX (56), and the immune evasion cluster (IEC) composed of enterotoxin S (*sea*), staphylokinase (*sak*), the chemotaxis inhibitory protein (*chp*), and the staphylococcal complement inhibitor (*scn*) (57) are the best-characterized phage-encoded virulence factors in *S. aureus*. A transposon mutant library was screened for virulence genes, and phage-encoded virulence genes were detected on all four prophages from strain Newman (58). Moreover, small RNAs involved in gene regulation (SprD = teg14) (59) or coding for the type I toxin-antitoxin system (SprF1/SprG1) (60) are encoded on Sa3int phages. With the availability of new phage and *S. aureus* genomes, new putative phage-encoded virulence genes (e.g., those coding for putative Clp protease or phospholipase) are being discovered and are awaiting functional analysis (18, 61–63).

Interestingly, accessory genes are strongly associated with phages of certain *int* groups and are localized at the left or (more frequently) right ends of the phage. There is a link between the encoded virulence factors, the *int* module, and the lytic module (holin and amidase genes), which are localized at the opposite end of the prophages (9). One may assume that it is evolutionarily beneficial to interchange this whole unit. Of note, these modules are in close proximity after phage excision in the circular and/or concatamer form of the phage. The close link of the lytic module and the inserted virulence factors is perhaps favored to optimize the phage control of the expression of the virulence genes (64). For instance, the expression of the virulence genes becomes cotranscribed with the late phage genes upon phage induction (64–66).

Sa1int Phages Carrying *eta*

The exfoliative toxins are virulence factors of *S. aureus* that cause bullous impetigo and its disseminated form, staphylococcal scalded-skin syndrome. The clinical symptoms vary from blisters anywhere on the body to multiple lesions complicated by conjunctivitis and staphylococcal scalded-skin syndrome (67). The exfoliative toxin A gene (*eta*) is carried in the genomes of Sa1int phages (9, 12, 13). An *eta* homolog is also carried by SaPI2 but is not known to be functional (1). However, these phages can be differentiated into at least six types due to variation in different modules (68). These *eta*-phages were associated with outbreaks of methicillin-resistant *S. aureus* (MRSA) and methicillin-susceptible *S. aureus* strains of various CCs in Japan and the Czech Republic (67, 69, 70) and are also present in a subpopulation of CC121 strains associated with superficial infections (25, 71).

Sa2int Phages Carrying *lukSF*

lukSF encodes the bi-component leucotoxin PVL, which targets human phagocytes through interaction with the complement receptors C5aR1 and C5aR2 (72). *lukSF*-encoding phages are strongly associated with skin and soft tissue infection and necrotizing pneumonia, which can also affect young, immunocompetent people (73–76). The majority of *lukSF*-carrying phages are Sa2int phages (66, 77–82). These phages are integrated within a conserved ORF which is surrounded by a cluster of tandemly repeated genes. In CC80 strains, phage induction led to the acquisition of host DNA into the phage genome probably due to a homologous recombination event between direct repeats of the two paralogous genes adjacent to the phage integration site (83). Phage excision was accompanied by an additional chromosomal deletion in this region. PCR-based typing schemes were established and modified to further subtype the *lukSF*-encoding phages (78, 84, 85). These studies confirm the high mosaicism of phage genomes and show that different *S. aureus* lineages have acquired a diverse set of *lukSF* phages. The combination of *lukSF* phages with methicillin resistance is characteristic of community-associated MRSA strains that are spreading in various continents. Strains of the most prominent USA300 lineage (CC8 isolates) are mainly spreading in North America, whereas in Europe CC80 strains are spreading, in Asia, CC59 strains, in the Asia Pacific region, CC30 strains, and in Australia, CC93 strains are more prevalent. The global spread of different *S. aureus* clones containing different *lukSF*-carrying phages supports the idea that PVL provides some fitness advantage to the bacterial host. The most common feature of all these strains is the strong association of *lukSF* phages and superficial, recurrent skin infections (75).

Sa3int Phages Carrying the IEC

Sa3int phages are by far the most prevalent *S. aureus* phages. Up to 96% of human nasal isolates were found to carry Sa3int phages integrated into the *hlb* gene (17, 86). Hlb can modify endothelial cells and platelets by both toxin sphingomyelinase and biofilm ligase activities, thereby increasing infection severity (87). The *hlb*-converting phages carry genes representing the IEC (*sea*, *sak*, *chp*, and *scn*) (57), coding for highly human-specific virulence genes. SEA has been

described to modulate the function of chemokine receptors such as CCR1, CCR2, and CCR5 (76). CHIPS blocks two G-protein coupled receptors involved in chemotaxis and phagocyte activation (C5a and the formylated peptide receptor) (88). SAK is a potent plasminogen activator with pleiotropic function including fibrinolysis and antiopsonic activity. The latter occurs through degradation of immunoglobulin G and C3b/C3bi on the surface of staphylococci (89). SCIN is a specific inhibitor of the C3 convertase of the complement system (90). Seven IEC variants were discovered, carrying different combinations *of sea* (or *sep*), *sak*, *chp*, and *scn*. The genes' order was conserved and always in the same 5′ to 3′ orientation. *Scn* was the only gene present in all IECs analyzed, whereas, e.g., *sea* was detectable in only 27% of the IECs (57). Interestingly, genes coding for a type I toxin-antitoxin system (SprF1/SprG1) are also localized within the IEC gene cluster (60).

Sa7int Phages Carrying *sak*

As an exception to the rule, *sak* could be detected not only on Sa3int phages but also occasionally on Sa7int phages. Curiously, such a *sak*-encoding Sa7int phage is found in derivatives of the laboratory strain 8325-4 (17). The widely used phage-cured strain 8325-4 was somehow lysogenized with such a phage and has since been distributed to laboratories worldwide (designated strain RN6390 or ISP479C). A similar phage was detected in most of the prevalent hospital-associated MRSA clones (belonging to the ST5 lineage) isolated in southern Germany (91). These strains were highly successful over the years and were quickly diversifying, as illustrated by differences in pulsed-field gel electrophoresis patterns and antibiotic susceptibility. Notably, these isolates can be clearly discriminated from other CC5 isolates (ST225, a single-locus variant of ST5) circulating in Germany, which are characterized by the high prevalence of a typical Sa1int phage (91, 92).

SasX-Carrying Phage in MRSA

The assumption that mobile genetic elements promote the spread of bacterial clones was emphasized by the emergence of highly epidemic MRSA strains carrying a phage harboring a newly identified cell wall-anchored virulence factor, SasX (56). SasX promotes nasal colonization, bacterial aggregation, and virulence. *sasX*, similar to other phage-carried virulence genes, is located as an accessory gene at the right end of a phiSPβ-like prophage. The genome size of 127 kb is significantly larger than that of a typical *S. aureus* siphovirus and highly similar to a prophage found in *S. epidermidis* strain RP62A (93), indicating that the phage and thus a new virulence trait was acquired from *S. epidermidis*. This phage is spreading between *S. aureus* strains and is also found in MRSA strains of the CC5 lineage (56). It is unclear whether this phage and other genetic elements managed to cross the species barrier and, if so, how.

TarP-Carrying Phages

Recently, the phage-associated WTA-glycosyltransferase TarP was found to glycosylate the WTA in an alternative way (33). The *tarP* gene is located in the lysogenic module of three different *S. aureus* prophages with integrase groups, Sa1int, Sa3int, and Sa9int. The *tarP*-Sa3int phage, ΦN315, additionally encodes the IEC discussed above. *tarP*-encoding phages can be found in clonal lineages CC5 (hospital-associated clones with Sa3int TarP-phages) and CC398 (livestock-associated clones with Sa1int and Sa9int TarP-phages). Modification of WTA by TarP lead to reduced opsonization by IgG in human sera. The immune evasion capabilities of TarP were reflected by lower immunogenicity of TarP-modified WTA in comparison with TarS-modified WTA.

ROLE OF PHAGES IN HUMAN ADAPTATION OF ANIMAL ISOLATES

S. aureus colonizes a variety of animal species and adapts to particular species through changes in the core genome as well as potential phage-encoded virulence genes (94). However, transmission of strains between human and animal reservoirs also occurs. Since the early 2000s MRSA strains with the sequence type ST398 were described as colonizing pigs and also causing infections in humans living in close contact with livestock (95, 96). Strains belonging to the CC398 lineage are commonly found in livestock and are resistant to multiple antibiotics but lack several important virulence factors (97–99). More recently, human to human transmission of CC398 strains was reported. Critical in this jump are Sa3int phages that generally are absent in CC398 strains but are present in most livestock strains infecting humans without livestock contact (61, 100). The importance of Sa3int for virulence of CC398 strains to humans has also been demonstrated more directly, because the presence of Sa3int decreased phagocytosis by human but not by pig polymorphonuclear neutrophils, while β-hemolysin production was abolished due to integration of the phage in the *hlb* gene (101).

Interestingly, the livestock-associated MRSA CC398 strains originated in humans as a methicillin-susceptible *S. aureus* strain, and upon introduction into livestock they lost Sa3int and acquired methicillin resistance and resistance to tetracycline, which is commonly used in livestock production (102, 103). The recent reintroduction of Sa3int into the livestock-associated MRSA strain population appears to be restricted by mutations in the 14-base pair phage attachment site of many CC398 strains (104) that leave the *hlb* reading frame intact but reduce integration of Sa3int (18, 19, 105). In these strains Sa3int integrates elsewhere in the chromosome, and the majority of those integration sites contain a 4-nucleotide sequence (5′-CTGG-3′) that is shared with the bona fide integration site in *hlb* (19). Importantly, the location of the integration appears to influence the stability of the Sa3int prophage in the livestock strains (18, 19), indicating that the success of the livestock MRSA strains in humans may depend on the location of the prophage. Interestingly, Sa3int also can promote animal adaptation, because some of the avian isolates carry a Sa3int-like phage with two putative avian-niche-specific genes (102).

The loss of Sa3int in the original jump of methicillin-susceptible *S. aureus* strains to livestock and the observation that the *hlb* gene of livestock-associated CC398 strains is mutated such that the phage integration site is eliminated but the *hlb* gene is kept intact underscores the role of the phages in lysogenic conversion. It also underscores the probability that Hlb is important for livestock but not for human pathogenesis. Also, the human to bovine jump of CC8 strains has been associated with the loss of Sa3int, similar to the human to livestock jump of CC398 (102, 106).

Phages other than Sa3int have also been associated with the human adaptation of CC398. Several studies document the presence of a phiMR11-like phage in these strains, and further analysis suggested that it is a defective phage that may act as a helper phage which interacts with a coresiding Sa3int phage to promote the expression of phage gene products (61, 107, 108). In another subset of CC398 strains associated with

human to human transmission, Sa7int phages were detected (103). Little is known about this phage, but its association with strains being transmitted between humans suggests that it may be important for human adaptation.

DUAL CONTROL OF PHAGE-ENCODED VIRULENCE GENES: LINK TO HOST REGULATORY SYSTEMS AND PHAGE LIFE CYCLE

Phage-encoded virulence genes are integrated into the regulatory mechanism of the bacterial host and modulated in a manner surprisingly similar to bacterial chromosome-encoded virulence factors. The alternative sigma factor B seems to inhibit the expression of most, if not all, of the currently analyzed phage-encoded virulence factors (109–111). Furthermore, the two-component regulatory system *saeRS* and, to a lesser extent, the quorum-sensing system *agr* (66, 109, 110, 112) are required for the activation of most of the phage-encoded virulence factors, such as *eta*, *pvl*, *scn*, and *chp*. Interestingly, as an exception to this observation, *sak* was not, or was only marginally, influenced by *sae* and/or *agr* (66, 110). Both the *sae* and *agr* regulatory systems are essential for the coordinated expression of many bacterial chromosome-encoded virulence factors, and mutants deficient in these factors are clearly less virulent as shown in different animal models of infection. Thus, the phage-encoded virulence factors are integrated into different regulatory circuits employed by the bacteria. It is likely that the prophages acquired these virulence genes along with their pre-existing chromosomally determined regulatory features. Moreover, the expression of phage-encoded virulence genes is also influenced by subinhibitory concentrations of certain antibiotics. β-Lactam antibiotics, for instance, enhance PVL production presumably via the transcriptional factors SarA and Rot (112).

The expression of these virulence factors is also tightly linked to the phage life cycle. Prophages are induced by environmental conditions that lead to DNA damage, including exposure to reactive oxygen species generated by leukocytes or exposure to exogenous agents such as antibiotics (65, 113, 114). It has been demonstrated that under such phage-inducing conditions, the transcription of the virulence factors that are localized in close proximity to the lysis module of the phage genome is increased (64–66). This phenomenon is partially due to a multicopy effect caused by phage replication. However, it has also been shown that transcription becomes intimately linked to the phage genes through cotranscription with the now derepressed lysis genes (65, 66). In this regard, the use of antibiotics that induce the SOS response, such as quinolones or β-lactam antibiotics, is a special concern. Antibiotic-induced expression of phage-encoded toxin genes is well documented for *E. coli* prophages harboring Shiga-toxin-encoding genes (*stx*). Quinolones enhance *stx* transcription, Stx production, and toxin release from the bacterial cells via phage-mediated lysis and death in mice (115).

MYOIRIDAE AND *PODOVIRIDAE*

Although more is known about the siphoviruses, the phages belonging to the *Myoviridae* and *Podoviridae* are receiving increasing attention because of their therapeutic potential in combating *S. aureus* infections. In this context, their nontemperate and lytic nature does not carry the risk of enabling the spread of virulence or antibiotic-resistance genes upon phage treatment (116).

The receptor interaction of *Myoviridae* and *Podoviridae* demonstrates more diversity in comparison with the discussed *Siphoviridae*. The well-described Myovirus ΦK and Φ812 only require either a GroP or RboP backbone of WTA (Fig. 1D) (35, 47). Structural elucidation of the RBP of Myovirus φ812 demonstrates a sophisticated conformational change of the double-layered baseplate upon binding of the bacterial cell wall (116). Hence, their high receptor promiscuity allows infection of many coagulase-negative staphylococci expressing GroP-WTA (49, 117). Alternatively, Twortlikevirus ΦSA012 appears to distinguish between α- and β-GlcNAcylation (118). At least two RBPs are encoded by ΦSA012: one interacting with the RboP backbone and the other, with α-1,4-GlcNAc residues (Fig. 1E). Interestingly, Podovirus ΦP68, Φ44AHJD, and Φ66, requiring β-1,4-GlcNAcylated WTA, fail to adsorb and infect strains carrying WTA modified with α-GlcNAc or β-1,3-GlcNAc residues in a dominant manner (33, 54). This finding demonstrates that certain WTA glycosylation patterns are able to protect *S. aureus* from viral predation by preventing proper phage adsorption. Furthermore, it shows that the alternative WTA glycosyltransferase, TarM and TarP, play an antiviral role. Obtaining phage resistance by blocking the host receptor is an often-observed feature (119, 120). However, TarM-mediated glycosylation does not block all *S. aureus* podoviruses from infection. Certain podoviruses, such as S24-1, appear to have evolved an RBP that allows adsorption to α- or β-GlcNAcylated WTA (121). Noticeably, this RBP shares key amino acids with the RBP of siphovirus φ11, GP45, also shown to facilitate binding to α- or β-GlcNAcylated WTAs (44).

The application of phages to limit *S. aureus* both *in vitro* and *in vivo* has been tested extensively, and there seems to be some therapeutic potential (122, 123). In the following, we mention just a few examples of how phages may be used to combat *S. aureus*. Additionally, the therapeutic use of phage lytic proteins in *S. aureus* is yielding promising results, showing good efficacy without apparent side effects (124). During dairy production a phage cocktail consisting of *Myoviridae* and *Podoviridae* as well as lytic *Siphoviridae* eradiated a 10^6 CFU/g *S. aureus* population after 14 days in Cheddar cheese curd during ripening at 4°C (125). Also, in biofilms, phages appear to reduce *S. aureus* numbers as exemplified in two studies using members of the *Myoviridae* family (126, 127). However, if present in sublethal doses, the action of the lytic phages may promote DNA release and collectively enhance biofilm formation, suggesting that caution should be used when considering phage therapy for eradication of *S. aureus* biofilms (128). The potency of phage therapy *in vivo* has also been evaluated. In a mouse model of bovine mastitis a phage cocktail significantly reduced infection with a clinical bovine mastitis when applied 4 h post-inoculation as demonstrated by improved pathology and decreased bacterial counts. Importantly, phage quantification indicated that the phage cocktail maintained high intramammary phage titers without spreading systemically (129). Future studies are likely to further address the therapeutic potential of targeting *S. aureus* with phages.

PHAGE DYNAMICS: MOVEMENT OF THE PHAGE WITHIN AND BETWEEN STAPHYLOCOCCAL SPECIES

Whereas most *S. aureus* isolates harbor multiple phages, less is known about the prevalence and nature of phages in coagulase-negative staphylococci. Analyses of available

phage genome sequences of coagulase-negative staphylococci revealed a modular structure similar to that of *S. aureus* phages. The transfer of phages between different staphylococci is also supported by cluster analyses of phages from different staphylococcal species (130). CRISPR/Cas loci are present in some *S. epidermidis* strains but are lacking in most *S. aureus* isolates. These loci are involved in the recognition and cleavage of foreign DNA. Therefore, it was postulated that the gene flow is uni-directional (131), as indicated by several instances in which genetic material was presumably transferred from *S. epidermidis* to the more pathogenic *S. aureus* species. For example, the staphylococcal cassette chromosome expressing methicillin resistance (SCC*mec*) genomic islands that carry the *mec*A gene conferring resistance to methicillin at least occasionally originate from *S. epidermidis* (132). Genetic exchange might be possible between different staphylococcal species because they live in similar environments, such as on the skin or in the nose. Additionally, phages might persist in a specific environment even though the bacterial host is already eliminated through the action of the immune system or antibiotics. Such phages or transducing particles may then infect coinhabitants, providing them with new properties.

PHAGES MEDIATE HORIZONTAL GENE TRANSFER

Horizontal gene transfer is commonly observed in *S. aureus*, and phages are believed to be major contributors (6, 16). In 1959, transduction was described for a staphylococcal phage (133), and soon thereafter, several phages were shown to be transducing (134), particularly those belonging to serological group B (135), such as Φ11, Φ80a, and Φ80 (136, 137). Transduction is the process by which bacterial DNA, during lytic replication of the phage, is mis-packaged into phage capsids forming transducing particles and upon release can be taken up by bacterial cells. In generalized transduction, the phage machinery recognizes pseudo *pac*-sites that mimic the sequence from where phage DNA packaging is initiated during lytic growth, and thus, in principle, any bacterial DNA can be transferred by this process. Normally, it is thought that the recipient strain should be susceptible to the transducing phage, but this appears not to be the case, because plasmid DNA is effectively transduced into recipient *S. aureus* strains by φ29, φ52A, and φ80α as well as by prophage φ53 in spite of their insensitivity to the lytic action of the transducing phage (138). In *S. aureus*, the transfer of antibiotic resistance genes has mostly been studied, but metabolic traits have also been transduced, as have been various mobile genetic elements (139, 140). The size of the DNA transferred by a transducing particle is limited to that of the *S. aureus* siphophage genome (approximately 43 kbp) (8, 141), and molecules of smaller sizes such as plasmids are transferred as linear multimers (142). The size limitation of the phage capsid prevents transfer of larger chromosomal elements such as the SCC*mec*. The SCC*mec* cassettes vary in size from 20 kbp to 60 kbp, so only the smaller SCC*mec*s are expected to transfer through generalized transduction, as demonstrated for SCC*mec* type IV and SCC*mec* type I (143, 144). Even then, transfer of methicillin resistance is a rare event, and other factors are likely required, such as lysogeny prior to transduction and the presence of a penicillinase in the recipient strain (145). The need for penicillinase activity was confirmed and explained by the transcriptional regulation of the *mec* gene by the plasmid-borne *blaR1-blaI* regulatory genes (143). Thus, transduction is possibly responsible for the transfer of SCC*mec* elements, although additional factors are required.

In the laboratory, the phages Φ11 and Φ80a in particular have been used extensively for genetic manipulations of *S. aureus* (146). However, outside of the lab a diverse range of transducing phages are being reported (49, 147, 148). One example is Φ187, which only binds and transduces ST395 strains expressing an unusual WTA (Fig. 1C) that, surprisingly, is also present in *S. epidermidis* and *L. monocytogenes*, which are also transducible (49). Another phage, S6, isolated from sewage, proved to be a giant myophage that is able to transduce plasmid and methicillin resistance between *S. aureus* and non-*aureus* staphylococcal species, including *S. epidermidis*, *S. felis*, *S. sciuri*, and *S. pseudintermedius* (148).

Although some phages are able to readily transduce between staphylococcal species, horizontal gene transfer appears generally limited within the species through restriction-modification (R-M) systems. The principle function of these R-M systems is to protect the cell by degrading foreign DNA. If the phage is derived from a host with the same R-M system, the phage DNA becomes methylated at the cognate restriction site and thus is protected. Strains of the major CCs were shown to differ in their R-M specificity genes (149). Thus, mobile genetic elements present in one strain move to a strain of the same lineage at a higher frequency than to strains of other lineages. Consequently, *S. aureus* lineages carry a unique combination of core variable genes, suggesting only vertical transmission of these genes (149). It has also been shown that prophage prevalence is associated with the clonal background of *S. aureus*, indicating that the spread of the phages in the bacterial population is at least partially restricted (12, 13). In certain CCs, some phage groups are completely absent, whereas others are significantly less or significantly more frequent. The most prominent disequilibrium was the finding that CC15 strains do not carry Sa3int phages, although this is the most common phage group found in *S. aureus*, with a prevalence of up to 90% (17, 57, 150). In addition, many isolates from the CC15 complex carried none of the seven prophage groups, suggesting that this lineage is particularly restrictive to the uptake of foreign DNA. From these studies, it is clear that restriction barriers are important in limiting phage transmission and likely also transduction. Other barriers may also be present, such as the CRISPR/Cas system, but their biological importance is unknown because the system has only been reported for a few *S. aureus* strains (151).

Although it is well accepted that transduction is central for horizontal gene transfer in staphylococci, surprisingly little is known of the process under biologically relevant conditions. Recently, we observed that phages spontaneously released from a subpopulation of lysogenic cells can infect, lyse, and efficiently transfer DNA from a phage-susceptible bacterial population back to the intact lysogenic population, which itself survives because of immunity to phage killing (152). The process, which we termed "auto-transduction," is driven by the spontaneous release of phages from the lysogenic population. Upon infection of susceptible cells, phages are formed together with transducing particles containing bacterial DNA and mobile genetic elements such as plasmids or SaPIs. These elements are effectively introduced in the original population, which due to lysogeny resists phage killing (152). Even though the phenomenon of transducing particles entering lysogens was observed in the first transduction experiments (153), we still do not know the extent to which it has biological impact. What, for example, remains to be studied are the conditions that promote the spontaneous

release of prophages and those that influence the formation of transducing particles. A recent study showed that the ratio of transducing particles to phages is affected by antibiotics (154), thus stressing the need for *in vivo* studies that assess the potential impact of antimicrobial therapy on transduction.

Recently, another study investigated transduction and found that packaging of bacterial DNA in transducing particles differs depending on whether they arise from an induced, temperate phage or from an infection (155). Interestingly, while infection with a transducing phage leads even to packaging of the bacterial chromosome in transducing particles, the induction of a temperate transducing phage leads to preferential packaging of bacterial DNA downstream of the integration site in a process termed "lateral transduction." By lateral transduction, several hundred kilobases of *S. aureus* DNA is packaged with high frequency, leading to hypermobility of this chromosomal region (155). With these findings in mind, we are beginning to understand how bacteriophages can be the main vehicle of staphylococcal horizontal gene transfer.

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Pathogenicity Islands and Their Role in Staphylococcal Biology

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33

INTRODUCTION

Once upon a time, bacteria, like other organisms, were considered to have stable genomes with essentially constant overall composition that unequivocally defined each species. The bacterial genome was initially seen to consist of a single very large circular DNA molecule (chromosome) containing all of the organism's genes (1). In retrospect, the proof that prophages were physically integrated into the chromosomal DNA provided the first inkling that this view was incorrect: discrete, variable, and mobile genetic units could exist within the bacterial chromosome. The profundity of this observation, however, was not immediately realized, since phages were seen as parasitic invaders that had found integration as a way to achieve stable intracellular inheritance. It was soon realized, however, that not only temperate phages but also the F plasmid and, as it was then thought, the ColE1 plasmid could reversibly integrate into the host cell's chromosome and that there were also short DNA segments (insertion sequences) that could be found in various locations (2). These observations led to the idea of discrete elements of genomic variability superimposed on the overall constancy of the chromosomal genophore; indeed, by 1996 the intergeneric transfer of mobile genetic elements had been documented so widely that David Summers was led to suggest, a bit whimsically, that all bacteria are but a single megaspecies, cohered by a vast marketplace of exchangeable genetic units (3).

Within this overarching firmament of genetic mobility are the genomic islands (GIs)—elements that were first realized to be members of this universe when several reports described DNA segments that carried genes involved in pathogenicity, lacked genes belonging to the core chromosome, and were present in some strains of a species but absent from others (4). Termed "pathogenicity islands," they launched the concept of the GI, which is now the generic term for all inserted DNA segments in bacteria (5). However, despite the clarity of this concept, it has often been misapplied and therefore deserves a very clear and exclusive definition: "a genomic island (GI) is a discrete segment of DNA with defined ends, that has a limited phylogenetic distribution" (6); because a GI lacks genes belonging to the core chromosome, it is dispensable. Membership in the island family involves acquisition by horizontal transfer; although extant islands may not be or may no longer be mobile, most possess stigmata of their horizontal acquisition, including flanking direct repeats, mobility genes such as *int* and *xis*, transfer origins, *tra* genes, replication-related genes, and transposition genes. It is generally possible to distinguish a GI from an indel by these criteria. Included in the island family are insertion elements and other transposons, integrating conjugative elements, pathogenicity islands, resistance islands, symbiosis islands, integrating plasmids, and prophages (see Table 1). With the exception of symbiosis islands, all of these occur in staphylococci, where they have a major role in the biology of the organism.

This article focuses on a critically important group of staphylococcal GIs, the *Staphylococcus aureus* pathogenicity islands (SaPIs). They are a coherent family of phage-inducible, highly mobile GIs of 12 to 18 kb that are widely distributed among *S. aureus* strains. Although the SaPIs have a modular organization, reminiscent of prophages, and encode several prophage-like functions, they are not defective phages but distinct genetic elements with a characteristic life cycle, novel functions, and unique cargo. They play a significant role in the pathogenesis, evolution, and biology of the organism.

SaPI Encapsulated

Like prophages, the SaPIs reside quiescently in the *S. aureus* chromosome under the control of a SaPI-coded master repressor (Stl) and replicate along with the chromosomal DNA. However, unlike most prophage repressors, the SaPI repressors are not SOS-inducible but are derepressed by specific (helper) phage-coded proteins. When helper phages infect the cell or undergo induction as prophages, they produce antirepressors that counteract Stl, initiating expression of the overall SaPI genome, which leads to excision and replication. Postreplicative SaPI DNA is packaged in phage-like particles composed of phage virion proteins, which are released by helper phage-induced cellular lysis. SaPI particle titers are commensurate with helper phage titers, resulting in extremely high transfer frequencies. The SaPIs frequently carry and disseminate staphylococcal signature toxin genes and therefore play an important role in pathogenesis. Like *pac* phages, most SaPIs are capable of mediating generalized transduction (GT) and therefore play a role in the adaptability and evolution of the organism. However, the SaPI's actual *raison d'etre* may, ironically, be to protect the staph cell from its most formidable foe, the

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TABLE 1 GIs in staphylococci[a]

Island	Size (kb)	Accessory genes	GI stigmata
Insertion sequences	1–2	None	Flanking DRs, transposase
Transposons	>5	Ab resistance genes	Flanking DRs, transposase
SCC elements	20–60	*mec*A, other Ab resistances	Flanking DRs, *ccr* genes
Prophages	40–50	Toxin and other converting genes	Flanking DRs, *int*/*xis*, replicon, virion genes, autonomous replication, mobility, etc.
SaPIs	12–27	Sag genes, other virulence and resistance genes	Flanking DRs, *int*/*xis*, replicon, *terS*, autonomous replication, mobility
Integrated plasmids	4–40	Ab and heavy metal resistance	Typical plasmid genes, replicon
Conjugative transposons	15–50	Ab resistance	Flanking DRs, *tra* genes, oriTs, *int*/*xis*, mobility
ICE elements	15–50	Ab resistance	Flanking DRs, *tra* genes, oriTs *int*/*xis*, mobility

[a]Abbreviations: Ab, antibiotic (not antibody); DR, direct repeats; ccr, cassette chromosome recombinae; Sag, superantigen; SCC, staphylococcal cassette chromosome; tra, transfer; oriT, transfer origin; ICE, integrative and conjugtive element.

bacteriophage. Although the SaPIs are derived from and dependent on phages, all SaPIs code multiple mechanisms for interfering with phage reproduction.

A Brief History of SaPIs

Some 20 years ago, the Novick lab cloned *tst*, the gene for toxic shock toxin-1 (TSST-1) and confirmed that it was a variable gene, present in only about 10% of clinical *S. aureus* isolates. Strains lacking the gene were also missing about 14 kb of flanking sequences, and when the gene was transferred by GT to a new strain, using an inserted tetracycline resistance gene to enable genetic selection, the flanking 14 kb were also transferred, and the transfer occurred at an extremely high frequency. Examination of different TSS strains revealed that the gene was in more than one location, suggesting that the 15-kb unit was a transposon. Analysis of a *tst* element in a different location revealed that the flanking sequences were different, ruling out a transposon and prompting us to consider it a pathogenicity island (7). We named the first one SaPI1 and the second, SaPI2. Though it was assumed that pathogenicity islands were mobile, this was the first demonstration. Several others were soon characterized, including one from a bovine isolate (SaPIbov1) (8). Later, when large numbers of *S. aureus* genome sequences became available, we observed that SaPIs made up a huge family, with an average of one per strain, with those having two, three, or more balanced by those with none. At least two major families were discerned, of which the prototypes are SaPI1 (7) and SaPIbov5 (9) (Fig. 1). Five SaPI attachment (*att*) sites were identified in *S. aureus*, in which only SaPIs but no other mobile genetic elements were located, nor were SaPIs found at any other location. These sites are utilized by members of both SaPI subsets.

SaPI-like elements are also found in non-*aureus* staphylococci, but much less frequently. Although the SaPIs were initially designated pathogenicity islands and are the only known source of TSST-1 (7), many of them do not carry identifiable virulence genes, so the "SaPI" designation is something of a historical accident. Although the term "SaPI" is too well established to consider modifying it, similar elements, with which other Gram-positive cocci are replete and which have yet to be found carrying virulence genes, are called phage-inducible chromosomal islands (PICIs) (10, 11).

The SaPI Genome

The SaPIs and their relatives in other Gram-positive bacteria were, almost certainly, derived from a bacteriophage or protophage in the very distant past by a deletion eliminating the phage morphogenesis and lysis modules, as illustrated in Fig. 1. The resulting SaPI genome contains three types of genes: life-cycle genes, interference genes, and accessory genes. It has, however, retained its prophage-like modular organization in which the life cycle genes are organized as follows: there is an integrase gene (*int*) at one end followed by a key site of transcriptional divergence flanked by two key regulatory genes, a *c1*-like master repressor (*stl*) and a *cro*-like activator (*str*) (see reference 12 for a review). In some SaPIs, the region between *int* and *stl* contains accessory genes. The integrase is always accompanied by an *xis* function; a *pri-rep/ori* complex defines the SaPI replicon, which is organized similarly to typical prophage replicons in which the replication origin (*ori*) is directly downstream of the replication initiator (*rep*) gene; upstream of *rep* is a primase homolog (*pri*), which is fused to *rep* in some SaPIs; the *ori* consists of two sets of GC-rich short repeats flanking an ~80-bp AT-rich region. Downstream of the replicon is a prophage packaging inhibitor (*ppi*), one of the phage interference genes (see below). Although a detailed transcription map is not available, three main transcripts have been defined (the green arrows in Fig. 1). The SaPI genome can be readily identified by genome scanning; for example, a typical SaPI at the *groEL* (SAV2029) site in mu50, from genes SAV2008(*sec3*) to SAV2028(*int*) is shown in Fig. 2.

The SaPI1 Subset

In this group of SaPIs, *ppi* is followed by operon 1, which contains several other phage interference genes and ends at *terS* (Fig. 1). The TerS (terminase small subunit) is highly conserved among these SaPIs and is specific for the packaging of SaPI DNA; unlike in prophages, the *pac* site is separated from the *terS* gene, which enables transcomplementation of *terS* mutants and is a crossover suppressor of heterologous recombination in that region. Unlike the typical prophage *pac* sites, which are embedded within the *terS* coding sequence (13), the SaPI *pac* site is typically separate from and upstream of the SaPI $terS_S$ coding sequence (14). Beyond *terS* is a region occupied by accessory genes.

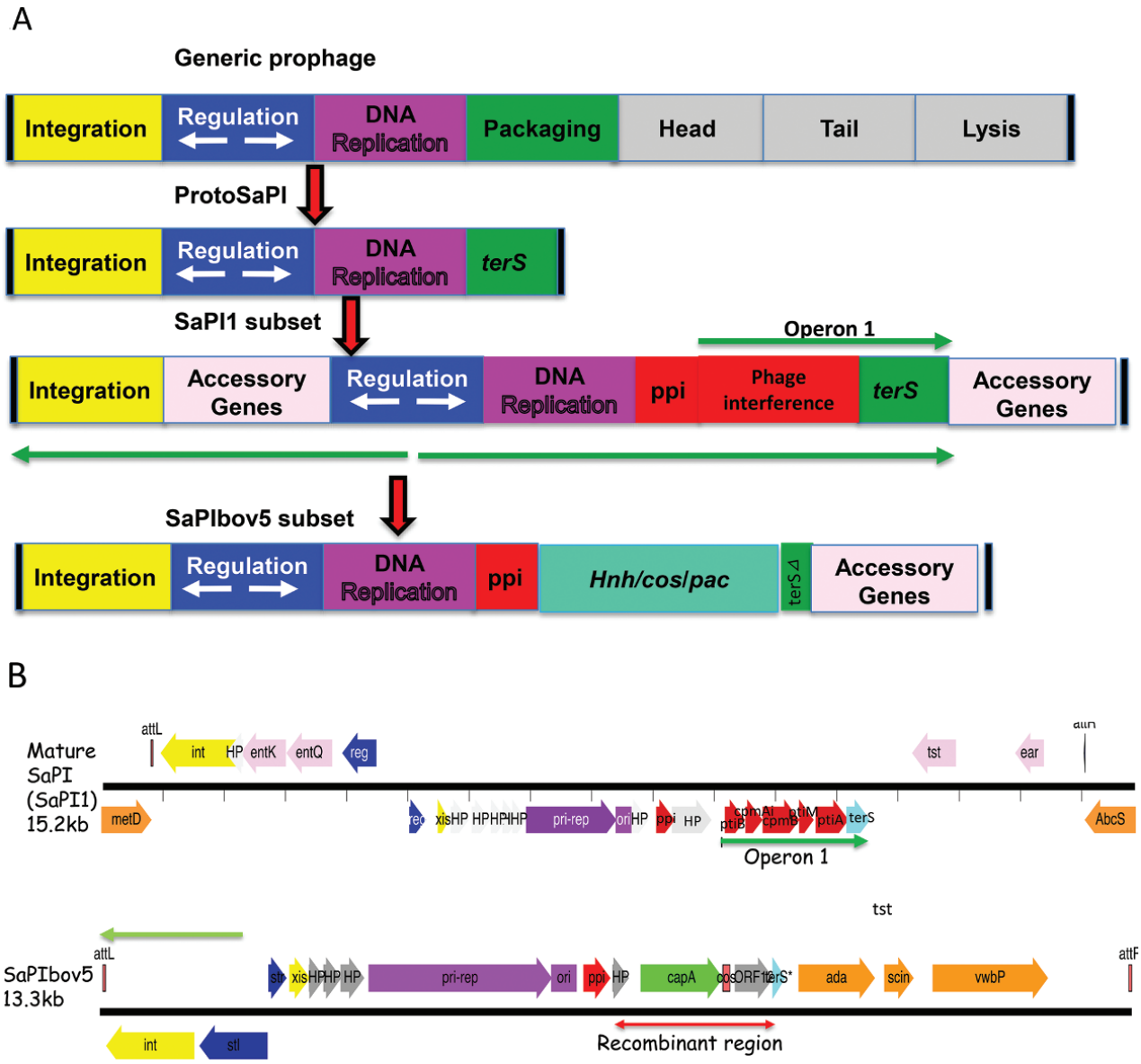


FIGURE 1 The SaPI genome and its putative origin. (A) Block diagrams. At the top is a typical prophage genome, and below that is the putative SaPI precursor, presumably the result of a major deletion. Next are two extant SaPI genomes. (B) Genetic maps of SaPI1 and SaPIbov5. Colors: red, interference genes; aqua, *terS*; orange, accessory genes; blue, regulatory genes; yellow, *int*/*xis*; gray, hypotheticals; purple, replication.

The SaPIbov5 Subset

Many *S. aureus* strains of domestic animals harbor variant SaPIs of which the prototype is SaPIbov5 (9, 15). These lack a functional *terS* but have a C-terminal remnant of a typical SaPI *terS* gene. They were probably derived from a typical SaPI by recombination with a *cos* phage. This recombination event replaced most of operon 1 by a prophage DNA segment containing a *cos* site, an *hnh* protease gene, and *ccm*, a gene encoding a homolog of the phage major capsid protein. The *terS* remnant marks one end of the putative recombinational event. It is possible that this putative recombination event occurred several times, since SaPIs of this type are found in several SaPI *att* sites (see Table 2).

THE SAPI LIFE CYCLE (FIG. 3)

Regulation

As noted above, SaPI DNA expression is controlled by a c1-like master repressor, Stl, which is countered by helper phage-encoded derepressor proteins, all of which have significant roles in the phage life cycle and are therefore moonlighting proteins (16, 17). The Stl amino acid sequence is highly variable, and different Stls are countered by different derepressors. Thus far, four derepressors have been analyzed in *S. aureus* and one in *Enterococcus faecalis*. Table 3 lists the SaPIs that encode these Stls and their respective phage-specified derepressors. Remarkably, 80α encodes four

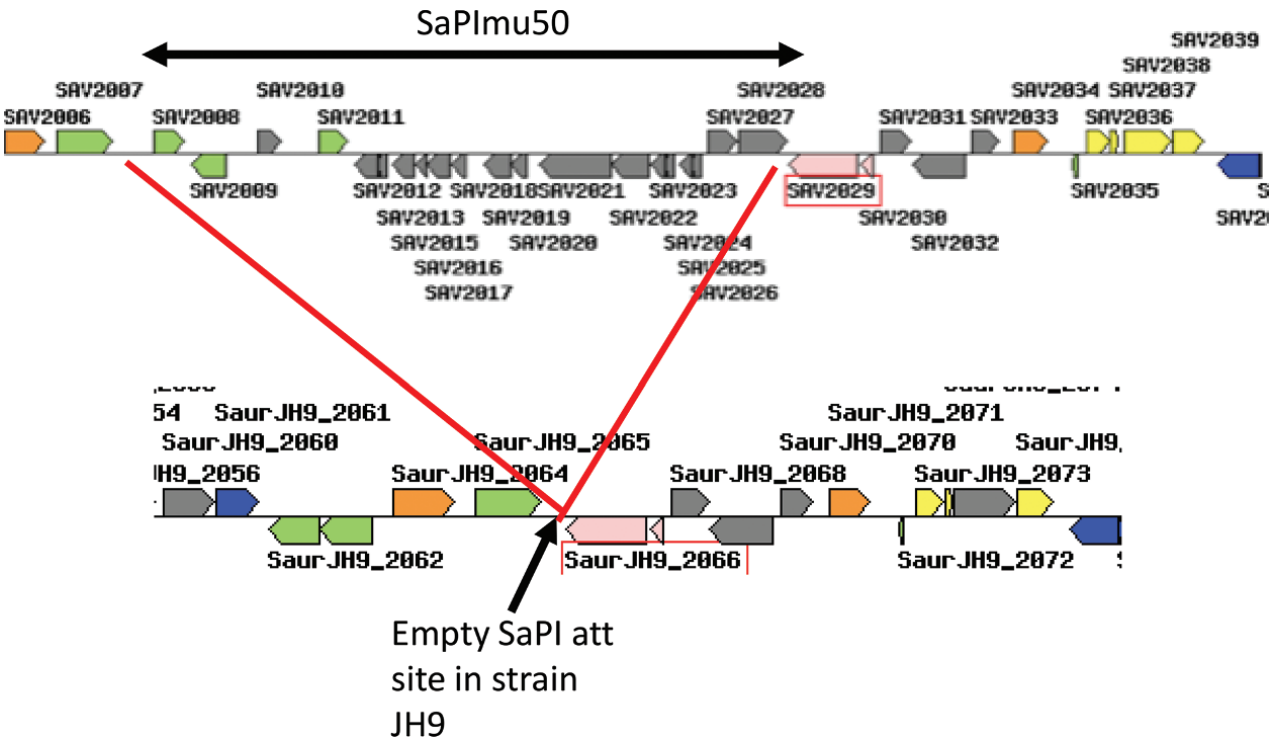


FIGURE 2 SaPI genomic pattern. At the top is the *groEL* region of *S. aureus* strain mu50, with a classical SaPI inserted at the 3′ end of *groEL*. The basic SaPI genes are in gray, and accessory genes at the left end are in green. Note the typical transcriptional divergence. Below is shown the corresponding region of strain JH9, in which the SaPI *att* site at *groEL* is empty.

independent and unrelated SaPI derepressors, and there could easily be additional ones that are as yet unrecognized. It is also highly likely that 80α is not unique among temperate staphylococcal phages in its possession of multiple SaPI derepressors.

The fascinating question arises of how these remarkable relationships could have evolved. Perhaps the SaPIs were derived from a prophage induced by a *trans*-acting derepressor protein (18) (although in the known cases, the derepressors are SOS induced). Alternatively, the SaPI followed an evolutionary pathway in which a classical SOS-induced repressor evolved to one that is bound and inactivated by an important phage-coded protein. The Stls are remarkably diverse, and the evolutionary forces driving their diversity are far from obvious. Moreover, even those that interact with a given derepressor do not seem to have any common motif that could be identified as a binding target. The most interesting of the derepressor proteins is dUTPase, which is universally conserved among temperate phages and has a significant (but nonessential) role in the phage life cycle. dUTPase is a bifunctional protein whose enzymatic and derepressor activities are genetically separable but functionally linked, in that binding of dUTP causes an allosteric shift which enables the protein to bind the Stl (19). dUTPase occurs in dimeric and trimeric forms, and both have the same derepressor specificity. In other species, the repressor-binding region has an important role in signaling and in immune modulation (20). Among the other known derepressor proteins, Sri inhibits loading of DnaB helicase and thus blocks chromosomal replication (21); Sak is a single-strand DNA binding protein that functions in recombination (22); Xis is the classical excision protein, and gp15 is a small protein of unknown function.

The SaPI-Phage War

As detailed below, all known SaPIs and similar elements from other organisms devote considerable genetic energy to interfering with the reproduction of their helper phages. The biological rationale for this is probably that the SaPIs are engaged in supporting their host cells, and one of their most potent weapons is the blocking of phage predation while at the same time commandeering phage proteins for their own life cycle. This generates a phage versus SaPI war, against which the phage's defense is for its derepressor to mutate. This idea is supported by a study demonstrating the frequent occurrence of SaPI-resistant mutations in the derepressor genes during passages of helper phages on SaPI-containing strains (23). Although this might seem to win the war for the phages, in actuality it does not. The derepressor proteins have important roles in the phage life cycle, and one of them, Sak, is evidently essential (22). The experimentally observed mutations are always detrimental to the phage and would not persist in the absence of continuous selection by the presence of a SaPI. Nevertheless, genome data suggest that many phages have actually won this war; the derepressor genes all have homologs in nonhelper phages, and these always differ considerably at the amino acid level from their derepressor cousins (23). If these proteins were once derepressors, this suggests that the development of SaPI resistance must have been a slow and arduous process. Not to be thus outdone, the SaPIs have "learned" to utilize related phage proteins as derepressors. A striking example is the utilization by SaPI2's Stl of distantly related proteins in four different phages as derepressors (22). Remarkably, the SaPI2 Stl appears to have different domains that interact with the different derepressors (22), indicating that the SaPI2 Stl is structurally rather accommodating and not so easily defeated. While this

TABLE 2 Features of two SaPI types[a,b]

Strain	Size	Orientation	Location	*int*	tx	tx	tx	*stl*	str	*xis*	*rep*	*ppi*	*pac*	*cpm*	*ptiA*	*ptiB*	*terS*	*ccm*	HNH	*cos*	tx	tx	tx	tx	*ada*	*scin*	vwbP	Comment
Group 1 - classical SaPIs																												
6850 (MSSA)	16	3 to 5	*groEL*	1	0	0	0	1	1	1	1	1	1	1	1	1	1	0	0	0	bcinIII	HP	HP	HP	0	0	0	
RF122 (SaPIbov1)	15.5	5 to 3	*guaA*	1	0	0	0	1	1	1	1	1	1	1	1	1	1	0	0	0	tst	bcnIIc	sec	sel	0	0	0	Bovine
RF122 (SapI122)	16.5	3 to 5	*groEL*	1	0	0	0	1	1	1	1	1	1	1	1	1	1	0	0	0	mdr	0	0	0	0	0	0	Bovine
BMB9393	14.8	5 to 3	*metQ*	1	0	0	0	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	
55/2053	12.1	5 to 3	*metQ*	1	0	0	0	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	
TCH60	12.1	3 to 5	*metQ*	1	0	0	0	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	
SA957 (CA-MRSA)	14.4	5 to 3	*metQ*	1	sek	seq	0	1	1	1	1	1	1	1	1	1	1	0	0	0	seb	ear	0	0	0	0	0	
n315 (SaPIn1)	15.2	3 to 5	*groEL*	1	0	0	0	1	1	1	1	1	1	1	1	1	1	0	0	0	tst	ear	sec	sel	0	0	0	
RN4282 (SaPI1	15.2	5 to 3	*metQ*	1	sek	seq	0	1	1	1	1	1	1	1	1	1	1	0	0	0	tst	ear	0	0	0	0	0	
RN3984 (SaPI2)	15.3	3 to 5	*groEL*	1	0	0	0	1	1	1	1	1	1	1	1	1	1	0	0	0	tst	eta	0	0	0	0	0	
COL (SaPI3)		5 to 3	*metQ*	1	1	1	0	1	1	1	1	1	1	1	1	1	1	0	0	0	seb	0	0	0	0	0	0	
FDARGOS_5		3 to 5	*metQ*	1	1	0	0	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	0	0	0	0	0	
MS4	15	5 to 3	*metQ*	1	1	0	0	1	1	1	1	1	1	1	1	1	1	0	0	0	seb	0	0	0	0	0	0	
Z172 (MRSA/ VISA)	14.9	5 to 3	*metQ*	1	sek	sel	0	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	
TO131/00383	10.6	5 to 3	*rpsR*	1	0	0	0	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	
TO131/00870	16.1	5 to 3	*rnr*	1	0	0	0	1	1	1	1	1	1	1	1	1	1	0	0	0	eta	0	0	0	0	0	0	
TO131/00934	16.4	5 to 3	*metQ*	1	1	1	0	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	
TO131/02151 SaPI6D	6	5 to 3	*groEL*	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	
TO131/02158	12.8	3 to 5	*groEL*	1	0	0	0	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	

Group 2 - ccm-cos																											
JP5338 (SaPIbov5)	13.5			1	0	0	0	1	1	1	1	1	0	0	0	0	3′rem	1	1	1	0	0	0	0	1	1	1	Bovine
BA4 (SaPIbov4)	13.9			1	0	0	0	1	1	1	1	1	0	0	0	0	3′rem	1	1	1	0	0	0	0	0	1	1	Bovine
UTSW MRSA55	11.1	5 to 3	*metQ*	1	1	1	1	1	1	1	1	1	0	0	0	0	3′rem	1	1	1	0	0	0	0	0	0	0	
JE2	12.3	5 to 3	*metQ*	1	1	1	1	1	1	1	1	1	0	0	0	0	3′rem	1	1	1	0	0	0	0	0	0	0	
CZ406	12.3	5 to 3	*metQ*	1	1	1	1	1	1	1	1	1	0	0	0	0	3′rem	1	1	1	0	0	0	0	0	0	0	
M6	12	5 to 3	*guaA*	1	0	0	0	1	1	1	1	1	0	0	0	0		1	1	1	0	0	0	0	1	?	1	Cow? to pig
NX-T55	9	3 to 5	*guaA*	1	0	0	0	1	1	1	1	1	0	0	0	0	3′rem	1	1	1	0	0	0	0	1	1	1	Animal defective?
USA300_TCH1516	12.9	5 to 3	*metQ*	1	1	1	0	1	1	1	1	1	0	0	0	0	3′rem	1	1	1	ear	0	0	0	0	0	0	
08BA02176	13	5 to 3	*guaA*	1	0	0	0	1	1	1	1	1	0	0	0	0	3′rem	1	1	1	0	0	0	0	1	1	1	
MW2	13.8	5 to 3	*rnr*	1	0	0	0	1	1	1	1	1	0	0	0	0	3′rem	1	1	1	ear	sec	sel	0	0	0	0	
ST398(MRSA)	12.3	5 to 3	guaA	1	0	0	0	1	1	1	1	1	0	0	0	0	3′rem	1	1	1	0	0	0	0	1	1	1	Animal strain causing human endocarditis
ED133	14.1	5 to 3	*guaA*	1	0	0	0	1	1	1	1	1	0	0	0	0	3′rem	1	1	1	tst	sec	sel	0	0	0	0	Ovine
ED133	10.1	3 to 5	*groEL*	1	0	0	0	1	1	1	1	1	0	0	0	0	3′rem	1	1	1	0	0	0	0	0	1	1	Ovine
AR0223	11.1	3 to 5	*metQ*	1	1	1	0	1	1	1	1	1	0	0	0	0	3′rem	1	1	1	0	0	0	0	0	0	0	

[a]Abbreviations: MSSA, methicillin-susceptible *S. aureus*; MRSA, methicillin-resistant *S. aureus*; CA-MRSA, community-acquired, MSSA; VISA, vancomycin-intermediate *S. aureus*; vWBP, von Willebrand factor binding protein

[b]1, the presence of a gene; 0, the absence of a gene.

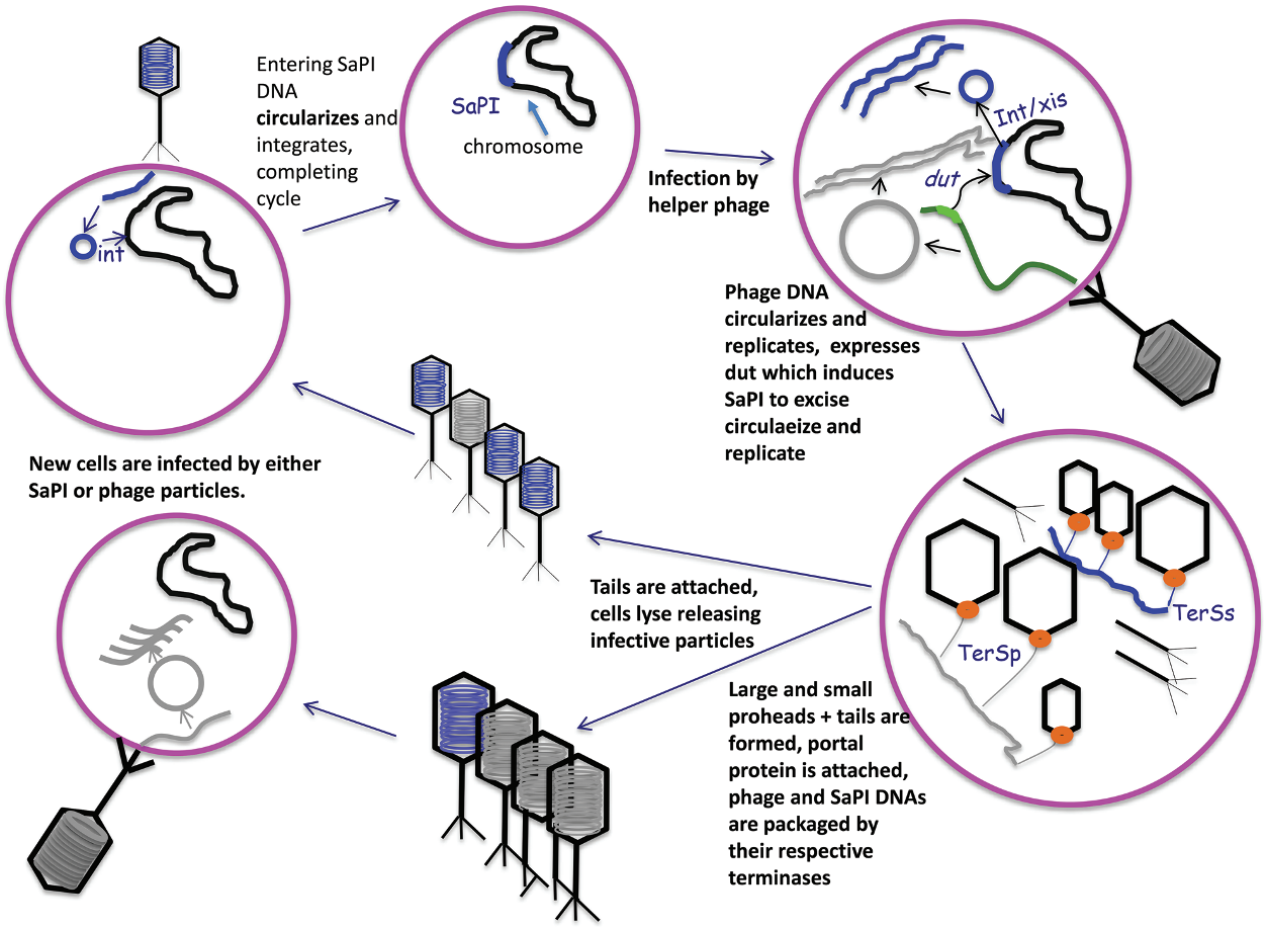


FIGURE 3 The SaPI life cycle.

capability does not enable the SaPI to utilize a phage whose derepressor has evolved away from SaPI sensitivity, it means that the range of phages that a given SaPI can utilize as helpers is immeasurably broad and extends *trans*-specifically and even *trans*-generically (22, 24).

Integration-Excision

SaPI integrases are of the tyrosine type, with *att* site cores of 15- to 22-bp direct repeats; the long flanking sequences generally required by tyrosine integrases have not been mapped. Early results suggested that following the entry of SaPI DNA, integration is delayed for more than an hour because the recombination event required for circularization is very slow (25). Integration is inhibited by 10- to 100-fold if the recipient's *att* site is occupied by a resident SaPI; this inhibition is not caused by the resident SaPI's repressor, but rather by the occupation of the *att* site (26), presumably because the hybrid *att* sites flanking the resident SaPI are poor substrates for the integrase. Nevertheless, integration eventually occurs at either of these hybrid *att* sites, generating a tandem double. The tandem double is highly unstable, ran-

TABLE 3 De-repressor proteins and their targets

	SaPI/PICI					
Phage	SaPI1	SaPI2	SaPIbov1[a]	SaPIbov2	SaPIbov5[a]	PICIef583
80α	Sri	Sak	dUTPase[3b]	gp15	dUTPase[3]	
80/52A		Sak4				
φ11			dUTPase[3]		dUTPase[3]	
φNM1			dUTPase[2]		dUTPase[2]	
φSLT		Erf				
φn315		Redβ				
φ1						Xis

[a]SaPIbov1 and SaPIbov5 have identical StIs.
[b]Superscripts 2 and 3 indicate the dimeric and trimeric forms of dUTPase.

domly losing either integrant by excision. Since the excised SaPI cannot replicate, owing to the presence of the SaPI repressor protein, it is immediately lost (26). A SaPI entering a recipient lacking its normal *att* site integrates with remarkably high efficiency (perhaps as high as 10% of the frequency of its integration into its primary site) into a secondary *att* site, of which there are at least 25 for SaPI1 (24). These secondary *att* sites generally match the primary site rather poorly, indicating that the integrases have rather loose site specificity. A remarkable unsolved mystery is that a SaPI in a secondary site is not induced by SOS induction of a resident helper prophage, although it is induced normally following superinfection of a nonlysogen by the same helper phage (24).

Replication

Replication is initiated by the Rep (or *pri-rep*) protein, which is SaPI specific; specificity is determined by a short C-terminal region of the protein, as shown by an experiment in which this region was switched between SaPIs of different specificities (27). The Rep protein is a helicase which binds to the two sets of flanking repeats that define the *ori*, causing the intervening AT-rich region to form a loop. Superhelix-driven melting of this loop exposes single-stranded regions which serve as the sites for Rep-initiated bidirectional replication (27). Following initiation, the host DNA polymerization system, modified to accommodate phage replication, takes over, and replication is completed by this system, resulting in concatenated multimers, the substrate for packaging (27).

Capsid Morphogenesis and Packaging

Most SaPIs package their DNA in small capsids commensurate with their small genomes. The precedent for phage satellite capsid modification is the *Escherichia coli* P2-P4 system, in which P4 modifies the P2 capsid to fit its 12-kb genome (28). This is accomplished by a P4-encoded scaffold protein, Sid, which forms an external shell within which the P2 capsid proteins are assembled to form the small P4 capsids (29). Many SaPIs also direct the helper phage capsid proteins to form small capsids, commensurate with their 15-kb genomes. For members of the SaPI1 subset, SaPI capsid size is determined by SaPI-coded CpmA and CpmB proteins (5), which act rather differently than the P4 Sid protein. CpmA and CpmB are both required for efficient small-capsid production (5), with CpmB modifying the internal scaffold to switch the capsid from T = 7 to T = 4 icosahedral geometry and CpmA ensuring the efficiency of size determination (5). For members of the SaPIbov5 subset, the Ccm protein (a homolog of the major phage capsid protein) is responsible for capsid size determination. In this case, the resulting small capsids have a morphology entirely different from that of the helper phage (30). In either case, deletion of the capsid morphogenesis gene(s) results in packaging of SaPI DNA in full-size phage capsids (30, 31).

Following replication, for members of the SaPI1 subset, headful packaging of SaPI DNA is initiated by a SaPI-coded terminase small subunit ($TerS_S$), which is distinct from the helper phage counterpart ($TerS_P$) and recognizes the SaPI-specific *pac* site (16, 32). $TerS_S$ complexes with the phage TerL to direct the SaPI DNA into preformed capsids composed of phage virion proteins (5, 33, 34). Packaging specificity is determined by TerS recognition specificity and is independent of procapsid size (31). Upon phage-induced lysis, SaPI particles are released in large numbers to infect other cells. Deletion of $terS_P$ eliminates phage DNA packaging but does not affect lysis, so that lysates are produced with particles of both sizes containing only SaPI DNA (21). For members of the SaPIbov5 subset, packaging is initiated by the helper phage terminase and occurs by the headful mechanism if the helper phage is a *pac* phage or by the *cos* packaging mechanism if the helper phage is a *cos* phage.

SaPIs of the bov5 type also reside quiescently under the control of a master repressor, which is countered by helper phages. Because they lack a functional *ter*S, they rely on helper phage terminases for packaging, and they use two independent packaging pathways. They can be packaged by a typical helper phage, φ11, using the φ11 terminase and by a φ11*pac* site (which has not been mapped). Alternatively, they can be packaged by φ12, which is not a helper phage for the SaPI1 family, using the φ12 terminase/HNH complex and a *cos* site derived from φ12 (or a related cos phage). The putative φ12 derepressor has yet to be identified.

Interference Mechanisms

Bacteria have developed a vast array of phage resistance mechanisms; indeed, phage-resistance mutations have been isolated affecting every stage of the phage life cycle. As noted above, a critical and highly evolved feature of SaPI biology is the presence of phage interference systems, which do not resemble known bacterial phage resistance mechanisms and utilize SaPI-specific genes, some of which have no counterparts among the phages or elsewhere. Among the well-characterized SaPIs of the SaPI1 subset, four such systems have been described (25, 35, 36) (see Table 4). The first of these to be identified (25, 35) is the capsid morphogenesis system, which catalyzes small capsid formation. Since SaPI DNA can be packaged equally well in capsids of either size, the selective value for the SaPI of small capsid formation must therefore be attributed to interference with helper phage reproduction, which amounts to over 90% in some cases (25). This system causes interference not only by diverting the phage virion proteins, but also by causing the phage to waste its DNA by packaging in small capsids, which can accommodate only one-third of the phage genome. The second interference mechanism discovered consists of a SaPI-encoded protein, Ppi, that binds to and inhibits the function of the phage small terminase ($TerS_P$) but does not affect the SaPI counterpart ($TerS_S$), sharply reducing the packaging of phage DNA (35). The third interference system involves a protein, PtiA, that blocks transcription of the late phage genes, including the virion protein and lysis modules, by binding to and inhibiting the late gene activator, LtrC (36). Unchecked, PtiA would block SaPI as well as phage particle production and would be suicidal for both; consequently, there is a second component of this inhibition system, PtiM, which binds to and moderates the inhibitory activity of PtiA (36). There is also a second *ltr*C-blocking protein, PtiB, which thus represents a fourth interference mechanism (36). Three of the phage interference systems are encoded in operon 1; the fourth, *ppi*, is not. Whether this is biologically significant or is an evolutionary "accident" is unclear. The multiplicity of unrelated interference systems, necessarily the products of convergent evolution, underlines their key role in SaPI biology. Since the interference systems are expressed only following SaPI induction, they were first identified as acting on helper phages; however, they also act with undiminished efficiency on other phages, so long as the SaPI is induced by some other agency.

As far as is known, the PICIs of other Gram-positive bacteria also interfere with phage reproduction (11). For members of the SaPIbov5 subset, the primary interference

TABLE 4 Mechanisms of SaPI-mediated phage interference

Action	Gene(s)	SaPI1	SaPI2	SaPIbov1	SaPIbov2	SaPIbov5
Diverts phage virion proteins for the formation of small capsids	*cpmAB*	++	+++	+	–	–
Blocks procapsid formation by binding to the phage capsid protein	*ccm*	–	–	–	–	+++
Binds to and blocks function of phage TerS	*ppi*	–	+++	++	+++	ND
PtiA binds to LtrC and blocks activation of late phage gene transcription; modulated by PtiM	*ptiAM*	++	++	–	ND	Absent
Blocks activation of late phage gene transcription by an unknown mechanism	*ptiB*	ND	++	ND	ND	Absent

+, Relative strength; –, inactive; ND, no data.

mechanism involves Ccm, which interferes with prohead formation by interacting with the major capsid protein (30). Here, the interference mechanism is much more stringent than that seen with members of the SaPI1 subset, resulting in a 10^6- to 10^7-fold reduction in phage titer (30). Since the SaPI capsids also utilize the major phage capsid protein (30), this interference necessarily results in lower SaPI titers than those seen with the SaPI1 subset.

There is an interesting contrast between SaPI-mediated phage interference and the powerful clustered regularly interspaced short palindromic repeat (CRISPR) antiphage system: the SaPIs not only do not totally block phage reproduction, but they also use their interference systems to aid in their own transfer and that of unlinked bacterial genes, whereas the CRISPR system destroys the phage genome and thus precludes phage-mediated gene transfer. Perhaps the high frequency of SaPIs and the rarity of CRISPRs in *S. aureus* is related to the effects of both on gene transfer. This is especially noteworthy since phage- and SaPI-mediated gene transfer are by far the most import genetic exchange mechanisms in this species.

SaPI OPERON 1, A FEATURE OF THE SaPI1 SUBSET, AND ITS KEY ROLES IN HOST CELL BIOLOGY

Host Cell Protection by Operon 1

Virtually all naturally occurring *S. aureus* organisms are lysogenic, many carrying several prophages, most of which are SOS-inducible. Although SOS induction is a stress response which counters DNA damage (37), the presence of an SOS-inducible prophage defeats this because its induction is lethal for the cell. (One must imagine that the SOS response evolved before prophages came on the scene.) SaPIs, however, counter this lethality in a remarkable way through their unique possession of an SOS-inducible operon, operon 1. The discovery of operon 1 and its SOS induction, early in the study of the SaPIs (38), was highly puzzling. Why should this element, which was not itself SOS inducible but required a phage-coded antirepressor instead, contain an SOS-inducible operon? Part of the answer, as noted below, is that SOS induction of operon 1 promotes SaPI-mediated GT. A more important rationale is that operon 1 encodes three of the four known phage interference systems (see below). Thus, DNA damage, resulting in the SOS response, induces operon 1 and is thus predicted to counter the lethal SOS induction of resident prophages, rescuing the cell and enabling the stress response. Perhaps the presence of several different interference mechanisms could be related to differential phage sensitivities. It is perhaps highly relevant in this context that most prophages are not helpers for SaPIs that happen to be coresident; instead, the presence of a SaPI would protect the cell from lethal phage induction during the SOS response. The story is slightly different if an SOS-inducible prophage happens to be a helper for a coresident SaPI. In this case, both SaPI and phage are induced, and the interference dynamics are more complicated; although the induced SaPI interferes with phage reproduction by one or more mechanisms, it blocks phage reproduction only partially because it needs the phage packaging and lysis systems for the production of infectious SaPI particles.

SaPI-Mediated Gene Transfer

In addition to their own high-frequency transfer, SaPIs mediate GT based on $TerS_S$-directed mis-packaging of genomic DNA (39), entirely analogous to phage-mediated GT. In both cases, $TerS_S$ and $TerS_P$ recognize pseudo-*pac* sites homologous to the respective SaPI and phage *pac* sites. Since $TerS_S$ is encoded in operon 1, which is SOS-induced independently of the SaPI life cycle, SOS-induced DNA damage promotes SaPI-mediated GT independently of SaPI transfer. This transfer, like phage-mediated GT, occurs with widely varying frequencies, depending on the similarity of the pseudo-*pac* site to the true SaPI *pac* site. Interestingly, SaPI-mediated GT has a strong preference for genes involved in iron metabolism and therefore impacts pathogenesis.

ACCESSORY GENES

The third class of SaPI genes is the accessory genes, mostly virulence genes—genes that have important beneficial roles for the host bacteria, aiding in their survival in the animal host, often by directly causing diseases. Unlike converting phages, which rarely carry more than one virulence gene, many SaPIs carry four or more, chiefly superantigens, though resistance genes and other accessory genes are also encountered, some of which have no obvious role in virulence. The accessory genes are located at either of two sites: between *int* and *stl* and downstream of *terS*. They are transcribed by promoters that are not under Stl control and therefore are usually expressed in the quiescent state. It is assumed that these genes were acquired from some foreign source by the intact SaPI. As is generally the case with converting phages, neither the source nor the mechanism of acquisition of most of the accessory genes by the SaPIs is known. An exception is the presence of the immune evasion gene, the staphylococcal complement interference gene, on some members of the SaPIbov5 family, a gene that also occurs on the converting prophage φSa3 (40). The observation that *tst* in SaPI1 and *seb* in SaPI3 are in the same location but in opposite orientations suggests that nonhomologous end joining may be the

mechanism of their acquisition, even though nonhomologous end joining is very hard to demonstrate and is perhaps nonexistent in most bacteria. Indeed, one of the great unsolved mysteries of bacterial genetics is how certain genes have moved between genomes, movements that cannot be accounted for by known mechanisms of recombination.

A SaPI-Carried Determinant of Animal Adaptation

Staphylococci have evolved along with their animal hosts, and particular genotypes have come to be associated with particular animals (see chapter 46), implying that there are animal-specific bacterial genes or homologs. One such gene is carried by many members of the SaPIbov5 subset. This gene encodes a homolog of the von Willebrand factor binding protein, which is a coagulase and occurs in different allelic forms associated with SaPIs carried by staphylococci that are specific for different livestock; each allele specifically coagulates the plasma of the animal with which its SaPI is associated (41), suggesting that it is an animal-specific virulence factor. A nonspecific allele is also encoded by a chromosomal gene in most staphylococcal strains (41). Many of these SaPIs also carry an adenosine deaminase and the phage-derived staphylococcal complement interference protein (42). Others carry superantigen genes including TSST-O, a nontoxic variant of TSST-1, typical of ovine strains (43). An animal ST398 strain causing human endocarditis carried a SaPI of this type and had probably jumped from animal to human. Table 2 lists examples of the two known SaPI subsets.

Virulence Gene Clusters: Pretenders to the GI Throne

S. aureus strains encode several clusters of virulence-related genes: a cluster encoding serine protease-like (*spl*) proteins, one encoding exotoxin-like (*set*) proteins, a third encoding enterotoxin-like (*egc*) proteins, and a fourth encoding lipase-like (*lpl*) proteins. These sets of gene clusters are widely regarded as pathogenicity islands (40, 44) and are considered an important feature of the staphylococcal pathogenicity mobilome. However, because they are universally conserved among the available *S. aureus* genomes, they do not satisfy the primary island criterion, limited phylogenetic distribution (6), and moreover, they lack most island stigmata. It is suggested instead that despite their genomic organization and their potential role in pathogenesis, these clusters are not GIs and are not mobile but, rather, are sets of paralogous genes that evolved *in situ* by successive gene duplications, and though transferrable like other genes, this occurs by GT only.

SaPI EVOLUTION

We have proposed (see Fig. 1) that a proto-SaPI arose from a protoprophage by deletion; this was followed by evolutionary divergence resulting in several critical modifications that led to the currently extant SaPI genome as exemplified by SaPIs 1 and 2. SaPI evolution has resulted in deep divergence of all of the phage-related genes. Although these all retain a low degree of similarity to ancestral phage genes, they are much more closely related to cognate SaPI genes (10). Some of the extant SaPI genes, however, have no counterpart among prophages and are clearly products of the independent evolution of the SaPIs following their original split. The most important of these is operon 1, which is widely conserved and, as noted above, unlike the overall SaPI life cycle, is regulated by the SOS response (38). It contains three sets of genes that are responsible for phage interference and is responsible for gene transfer (39), all unique features of the SaPI life cycle. The similarity among SaPI genes also extends dramatically to genes of unknown function, encoding hypothetical proteins (HPs) that may or may not be translated. Most of these HP genes are well conserved among the SaPIs but have no detectable similarity to phage genes (10); phages have their own HPs that are not related to those of the SaPIs. This suggests that the HPs evolved *in situ* or were acquired following SaPI-phage divergence. Most important for the SaPI lifestyle is the possession of a master repressor that is

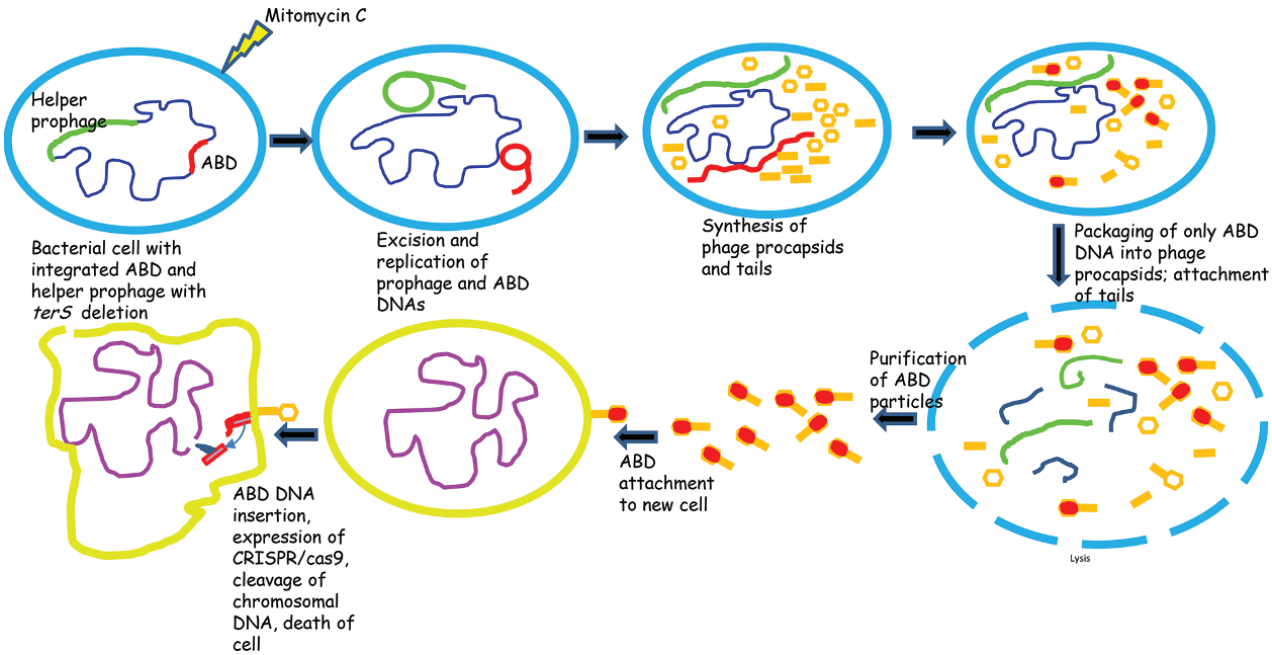


FIGURE 4 Induction, maturation, release, and cell killing by a CRISPR/cas9 ABD.

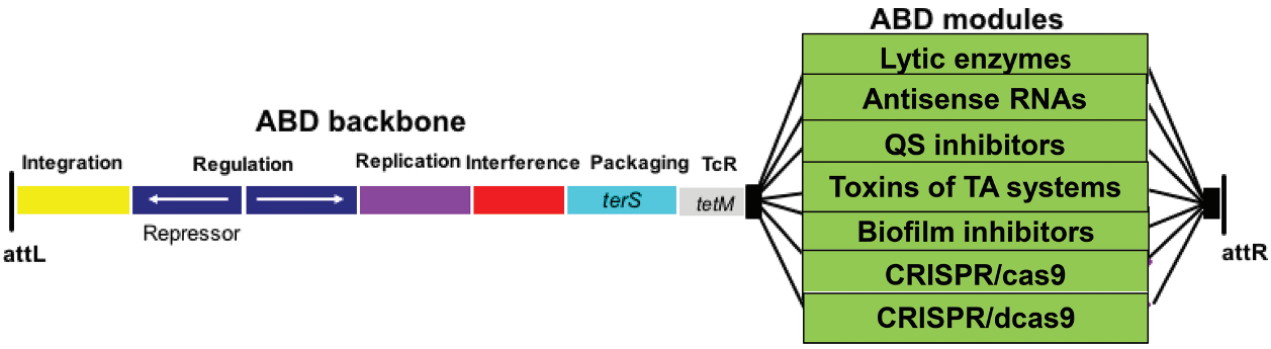


FIGURE 5 Diagrammatic representation of the ABD backbone.

counteracted by a phage-coded derepressor (17) rather than by the SOS response.

CONVERSION FROM WORKHORSE TO TROJAN HORSE: THE ABD STORY

Many years ago, Hope Ross, a research scientist in the Novick lab, realized that we could exploit the high-frequency transfer and great packaging capability of the SaPIs to create an effective delivery system and that if we added antistaphylococcal cargo to this system, we could convert the SaPIs into therapeutic islands and convert the workhorse into a Trojan horse. Accordingly, the genome of a typical SaPI1 family member, SaPI2, was modified by replacing the superantigen and other virulence and resistance genes with genes that, when expressed within a bacterial cell, would either kill the cell or block its virulence and thus cure an infection (Fig. 4). Several antibacterial cargo modules were added to this backbone vehicle (Fig. 5 and Table 5), including CRISPR-Cas9 with spacers matching either *agrA* or *fnbB*, CRISPR-dCas9 with a spacer matching the *agr* promoter region, and *lsp*, the gene for lysostaphin. This versatility was achieved by removing the capsid morphogenesis genes, causing the island to be packaged exclusively in full-sized phage particles, providing >30 kb of cloning space. Packaging in full-sized phage particles had no adverse effect on SaPI reproduction and actually increased SaPI transfer frequency (35). The *terS* gene of the helper prophage was also deleted, resulting in the production of mitomycin C-induced lysates that contained only SaPI particles, typically at titers of $\sim 10^{10}$/ml. These islands, known as antibacterial drones (ABDs), functioned as expected *in vitro* and cured mice infected subcutaneously or

TABLE 5 Existing and proposed ABDs based on SaPI2[a]

SaPIs and ABDs	Size (kb)	Deletions	Insertions	Function
Existing				
SaPI2 (WT)	15.7			
ABD2001	12.5	*cpmA&B tst eta*	*tetM*	ABD backbone
ABD2002	17.6	*cpmA&B tst eta*	CRISPR/cas9/nontargeting	Generic CRISPR/cas9
ABD2003	17.6	*cpmA&B tst eta*	CRISPR/cas9/*agrA*	Causes lethal DSB in *agrA*
ABD2004	17.6	*cpmA&B tst eta*	CRISPR/cas9/*hly*	Causes lethal DSB in *hly* (listeriolysin O)
ABD2005	17.6	*cpmA&B tst eta*	CRISPR/dcas9/nontargeting	Generic CRISPR/dcas9
ABD2006	17.6	*cpmA&B tst eta*	CRISPR/dcas9/$agrP_2P_3$	Blocks virulence by inhibiting expression of *agr* locus
ABD2007	18.8	*cpmA&B tst eta*	CRISPR/cas9/*fnbpB*	Causes lethal DSB in *fnbpB*
ABD2008	18.8	*cpmA&B tst eta*	CRISPR/cas9/*agrA*/*fnbpB*	Causes lethal DSB in *agrA* and *fnbpB*
ABD2009	14.0	*cpmA&B tst eta*	*Isp*	Secretes lysostaphin, lyses ABD-infected and surrounding uninfected cells
Proposed				
ABD2010	19.0	*cpmA&B tst eta*	CRISPR/cas9/*agrA*/*Isp*	Causes lethal DSB in *agrA*
ABD2011	14.5	*cpmA&B tst eta*	*dut*	Replicates autonomously
ABD2012	18.3	*cpmA&B tst eta*	*dut*, hybrid *agr-com*, anti-*spa* RNAIII, anti-*agrA*, anti-*secY*	Replicates autonomously; blocks *agr*, *spa*, PSMs, *secY* expression
ABD2013	14.9	*cpmA&B tst eta*	*dut*, *rsbW*, *icaR*	Replicates autonomously, clocks biofilm formation
ABD2014	16.2	*cpmA&B tst eta*	*pezT*, *ghoT*	Kills ABD-infected staphylococci

[a]Abbreviations: *agrA*, *agr* response regulator; fnbA, fibronectin binding protein A; DSB, double-strand break; *dut*, dUTPase gene; *com*, competence; *spa*, staphylococcal protein A; *secY*, gene for secretory protein Y; PSM, phenol-soluble modulin; *rsbW*, inhibitor of *sigB* activation; *icaR*, regulatory gene for biofilm-promoting *ica* operon; pezT and ghoT – toxins of TA systems; WT, wild type.

intraperitoneally with virulent staphylococci (45). It is planned to expand this system to other bacteria; it may help to counter the problem of antibiotic resistance.

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Respiration and Small Colony Variants of *Staphylococcus aureus*

RICHARD PROCTOR

34

STAPHYLOCOCCAL METABOLISM AND RESPIRATION

The end point of bacterial respiration is the production of ATP as it uses oxygen, or other molecules, to accept electrons while oxidizing dinucleotides (Fig. 1). Because multiple cell wall biosynthetic enzymes require ATP (1), lowering ATP levels reduces the rate of growth of *Staphylococcus aureus*, resulting in small colonies. Some small colony variants (SCVs) have been found to be defective in respiration (2). Under glucose-rich conditions, ATP is produced via glycolysis without using oxygen, wherein glucose metabolism ends with the production of lactate (3). Under aerobic conditions, once glucose and other simple sugars are exhausted, pyruvate is further metabolized to acetate/acetyl-CoA, which enters the tricarboxylic acid (TCA) cycle (also called the Krebs cycle), and the chemical energy is fed into dinucleotides such as NADH and reduced flavin adenine dinucleotide ($FADH_2$). Under anaerobic conditions, pyruvate is mainly metabolized to lactate (3, 4). In addition to the oxidation of carbohydrates, the metabolism of amino acids and lipids also results in the production of acetyl-CoA (3). As acetyl-CoA is oxidized to CO_2 and H_2O, there is a simultaneous reduction of NAD^+ and FAD^+ to NADH and $FADH_2$, respectively. Ultimately, these reduced dinucleotides must be reoxidized because many reactions in the cell, e.g., dehydrogenases, require $NAD(P)^+$ for their activity.

Oxidation of dinucleotides can occur through multiple reactions, but the largest amount of oxidation is accomplished via the electron transport chain, wherein electrons flow from NADH and $FADH_2$ to quinones and then onto the cytochromes as terminal oxidases (5, 6). In *S. aureus*, there is only one quinone, specifically, menaquinone, which is formed from menadione. An isoprenoid tail attaches to menadione and facilitates its entry into the membrane (7) and the transfer of electrons to the cytochromes. There are three types of cytochromes in *S. aureus* (5). Cytochrome types aa_3 and bo_3 are most active when oxygen is abundant. These cytochromes are oxidized by oxygen with the formation of water. In contrast, under reduced oxygen conditions, cytochrome type *bd* is most active during and linked to the nitrate reductase complex (8, 9). The cytochromes and nitrate reductase complex both contain heme that is used in the oxidoreductase reactions. As the dinucleotides are oxidized, the protons are translocated across the membrane, creating a membrane potential.

Staphylococci use nitrate as an alternative electron acceptor during anaerobic respiration. Activation of the genes in the nitrate reductase complex involve an oxygen-sensitive two-component regulator, NreBC, wherein NreB is the sensor with an oxygen-labile iron-sulfur cluster (10). NreBC then activates the genes involved in nitrate reduction to ammonia as NADH is oxidized. The nitrate reductase complex contains both a cytochrome and menaquinone in the oxidoreductase reactions. In summary, the three major complexes that oxidize dinucleotides and create a membrane potential via transport of protons across the membrane in *S. aureus* are Ndh2 (NADH dehydrogenase), cytochromes, and the nitrate reductase complex.

The reentry of the protons through the F_0F_1 ATPase produces ATP in bacteria (11). The F_0F_1 ATPase is a large transmembrane protein complex that uses the proton gradient to release the ATP into the cytoplasm. The net amount of ATP produced by glycolysis is 2 ATPs, whereas the net quantity of ATP production per glucose molecule under aerobic conditions via the F_0F_1 ATPase is 30 to 32 ATPs. Uptake of glucose, mannitol, mannose, lactose, sucrose, maltose, and glycerol requires ATP (12), which is calculated into the net amount of ATP produced via glycolysis (3).

RESPIRATION AND TCA CYCLE ACTIVITY

ATP Levels and Control of the TCA Cycle

Low levels of ATP stimulate a stress response in *S. aureus* (13). ATP levels can be sensed by ClpC, which is a chaperone that binds stress regulators, and by a proteolytic partner, ClpP, in *S. aureus* (14). When ATP levels are high, ATP binds ClpC, and then it associates with ClpP, a protease that digests multiple proteins in the stress regulon. However, ClpC can play a dual role not only by proteolytic removal of proteins (13) but also as a chaperone to enhance the activities of other proteins (14). While this ClpC regulon spans the production of virulence factors to controlling metabolism, one particular link to respiration comes in thymidine auxotrophic *S. aureus* SCVs. ClpC is required for *citB* activation, and *thyA* mutant strains of *S. aureus* have reduced levels of

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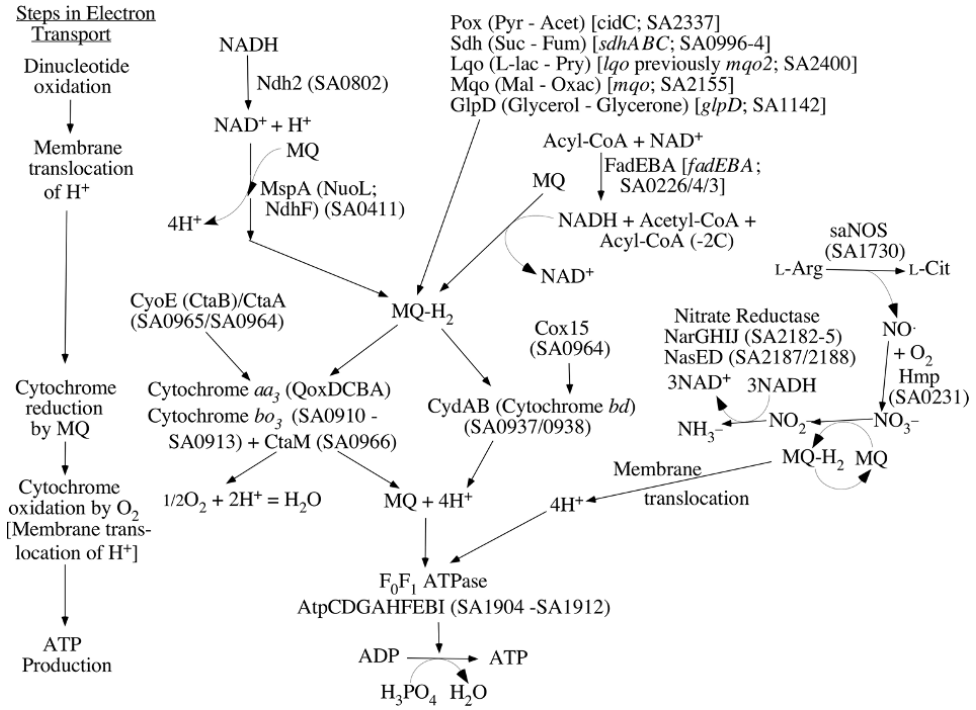


FIGURE 1 Electron Transport in *Staphylococcus aureus*.

ClpC (15–18). The exact pathway involved in *citB* activation through ClpC in *S. aureus* is not known, but it is clear that *thyA* mutants have a reduced TCA cycle because of reduced levels of aconitase. When the TCA cycle is inactive, the electron transport chain also shows reduced activity. Therefore, *thyA* mutants are respiratory deficient, giving them a very similar phenotype to menadione and hemin auxotrophic SCVs (19).

NADH Levels and Control of the TCA Cycle

Redox balance must also be maintained, as is reflected in the NADH/NAD^+ ratio. High NADH levels slow growth of respiratory-deficient bacteria because many dehydrogenases require NAD^+, thereby having a widespread impact on cellular metabolism. The TCA cycle uses the largest quantity of NAD^+ to release NADH. When NADH levels are high, several Krebs enzymes are inactivated by NADH through negative allosteric regulation of the enzymes, and TCA cycle genes are downregulated via redox-responsive regulators (3, 20). Thus, when electron transport is interrupted, levels of NADH rise, which downregulates the Krebs cycle.

Another example of the importance of redox balance comes from our observation that mannose, but not mannitol, stimulates growth of electron transport-deficient *S. aureus* (21). Both mannose and mannitol are metabolized to fructose-6-phosphate, and this leads to ATP production via glycolysis. Consequently, the difference between these compounds is due not to differences in net ATP production but, rather, to the first metabolic step in mannitol metabolism, wherein NAD^+ is reduced to NADH as mannose is produced from mannitol, thereby increasing NADH levels. This small difference makes respiratory-deficient SCVs unable to grow on mannitol salt agar, which is routinely used to identify *S. aureus* (22).

Host Nitric Oxide Redirects Metabolism, Including TCA Activity, in *S. aureus*

A fascinating interplay exists between nitric oxide (NO·) generated by the host and *S. aureus* metabolism, wherein NO· interferes with the TCA cycle, aerobic respiration, fatty acid metabolism, pyruvate metabolism, and nucleic acid synthesis. NO· causes widespread compensatory responses in *S. aureus* (9, 23–28). Overall, this disruption of respiratory metabolism results in rising levels of NADH that inactivate the transcriptional repressor, Rex, which allows derepression of lactate dehyrdogenases, *ddh*, and *ldh1* (29). Moreover, NO· stimulates SrrAB, which increases *hmp* and *qoxABCD* (Cyto aa_3) as well as other regulators such as Fur, SarA, CodY, and Rot (23–25, 29, 30). Finally, *S. aureus* makes its own NO· via nitric oxide synthase that modulates the respiratory chain (31). Taken together, these factors allow *S. aureus* to respond to a normally toxic host defense system by utilizing its regulation of its nitrogen respiratory pathways to its survival advantage.

GENES INVOLVED IN ELECTRON TRANSPORT AND SCVs

Mutations in genes that express the enzymes involved in electron transport (Fig. 1) result in the formation of respiratory-deficient SCVs (Table 1). As would be expected, mutations in genes whose protein products are involved in electron transport result in slower growth, thereby producing SCVs.

Mutation of the genes active in the first step in electron transport, dinucleotide oxidation, e.g., *ndh2* (type 1 NADH: quinone oxidoreductase) or *sdhCAB* (succinate dehydrogenase complex), results in *S. aureus* with an SCV phenotype (32, 33). While a second Ndh2 has been described in *S. aureus*, it is probably not a major Ndh in *S. aureus* (34). Deletion of *sdhCAB* in *S. aureus* produced an SCV with

TABLE 1 Metabolic pathways and mutations found in SCVs with reduced ATP[a]

Stage in electron transport	Biochemical reaction	Genes involved in electron transport pathways	Mutated genes that produce SCVs	Mutations discovered in clinical SCVs
Dinucleotide oxidation	NADH oxidation	*ndh2* (major pathway)	*ndh2*	No
		nasED	No	
		lqo, *mqo*, *glpD*	No	
	FADH oxidation	*sdh*, *fadD*	*sdhCAB*	No
Membrane translocation of protons	Makes a proton gradient	*mpsABC*	*mpsA*	No
		qoxABCD (Cyt aa_3 oxidase)		
		cydAB (Cyt *bd* oxidase)	*cydAB*	No
Transfer of electrons to cytochromes	Menaquinone transfers electrons to cytochromes	Menadione biosynthesis	*menB*, *menC*, *menE*, *menF*	Yes, yes, yes, yes
			menG, *aroD*	Yes, no
			menA, *menD*	Yes, no
		Isoprenoid tail	*ispA*, *mvaS*	No, no
Oxidation of cytochromes	Electrons move from cytochrome to oxygen (nitrate)	Heme biosynthesis	*hemB*, *hemG*	Yes, yes
			hemC, *hemD*, *hemE*, *hemH*, *hemQ*	No, no, no, no, no
		Cytochrome protein biosynthesis	*ctaA*, *ctaB*	No, no
			qoxB + *cydAB*	No, no
ATP production	Proton reentry	*atpCDGAHFEBI*	No	No
Production of NADH	Tricarboxylic acid cycle is major producer of NADH	*citB* (reduced aconitase activity turns off TCA)	*thyA* mutation suppresses *citB* activation	Yes
		odhAB (α-ketoglutarate dehydrogenase complex)	*odhA*, *odhB*	No, no

[a]Gene abbreviations: *atpA-H* (SA1904-SA1912), F_0F_1 ATP synthase; *citB* (SA1184), aconitase; *ctaA* (cox15, SA0965), heme synthase; *ctaBM* (SA0965-6), cytochrome c_{aa3} assembly factor; *cydAB* (SA0937-8), cytochrome *bd* oxidase; *fadD* (SA0265), glutaryl-CoA dehydrogenase electron transfer flavoprotein; *glpD* (SA1142), aerobic glycerol-3-phosphate dehydrogenase; *ispA* (SA1352), geranyltranstransferase; *lqo* (SA2400), lactate:quinone oxidoreductase; *menAFDHB* (SA0894-SA0899), *menCE* (SA1614, SA1615), menadione biosynthesis; *mpsABC* (SA0411-3), membrane potential-generating system; *mqo* (SA2155), malate:quinone oxidoreductase; *mvaS* (SA2334), 3-hydroxy-3-methylglutaryl (HMG) CoA synthase; *nasED* (SA2187-8), nitrite reductase; *ndh2* (SA0411), NADH deydrogenase; *odhAB* (1244-1245), α-ketoglutarate dehydrogenase complex; *qoxABCD* (SA0910-0903), cytochrome aa_3 menaquinol oxidase; *sdhCAB* (SA0944-0946), succinate dehydrogenase complex; *thyA* (SA1260), thymidylate synthase.

reduced pigmentation and increased aminoglycoside resistance whose phenotype is identical to clinical SCVs (2). These SCVs have been found in the laboratory but not yet in clinical isolates. Similarly, decreased translocation of protons across the cell membrane also results in SCVs. Mutations is *mps*A (33) and in the *cydAB* also interrupt electron transport and produce SCVs (5, 6). Again, no clinical isolates with these mutations have been reported. While the nitrate reductase complex also transports protons across the membrane, and mutations in the *nar*G operon slow the growth of *S. aureus* under anaerobic conditions, identifying these mutants as having an SCV phenotype is difficult because *S. aureus* grown anaerobically already demonstrates many of the features of respiratory-defective SCVs (2).

Moving down the electron transport chain, one finds that mutations in genes controlling menaquinone and cytochrome biosynthesis produce SCVs that are auxotrophic for menadione and heme, respectively (2) (Table 1). Mutations in genes for the biosynthesis of the menadione core or for the biosynthesis of the isoprenoid tail produce SCVs that are deficient in menaquinone. Point mutations in *menB*, *menC*, *menD*, and *menF* have been found in clinical isolates of *S. aureus* (35, 36). Mutations in other genes involved in (i) the menadione biosynthetic pathway, such as *men*A, *menD*, and *aroD*, and (ii) the biosynthesis of the isoprenoid tail of menaquinone, such as *isp*A (geranyltransferase) and *mva* (3-hydroxy-3-methylglutaryl CoA synthase), also produce SCVs (7, 37–42). Further support for a role of reduced menaquinone in the formation of SCVs comes from studies wherein inhibition of menaquinone reduction by diarylurea compounds also produced typical SCVs in *S. aureus* (43). Thus, the biochemical and genetic basis is well established for menaquinone-defective SCVs.

Oxidation of the reduced menaquinone is accomplished by the cytochromes (Fig. 1), which are membrane proteins that contain heme, an iron-containing porphyrin. SCVs have been identified with mutations in biosynthetic genes for the porphyrin and protein portions of the cytochrome. Clinical SCV isolates with mutations in *hemB* and *hemG* wherein these mutants reproduce the phenotype have been seen in laboratory-generated and clinical hemin auxotrophs (44, 45). Mutations in *hemE*, *hemH*, and *hemQ* produce SCVs, but these have not yet been reported in clinical specimens (37, 46, 47). In addition, mutations in genes involved in the biosynthesis of the protein portion of cytochrome aa_3 [*ctaB*(*cyoE*), *qoxDCBA*] or cytochrome *bd* (*cox15*, *cydAB*) also produced SCVs (5, 48), but again, these genes have yet to be described in clinical SCV isolates. Genes for both terminal oxidases in *S. aureus* (*cydB qoxB*) must be mutated to produce an SCV in *S. aureus*, suggesting that there is overlapping activity of these cytochromes (49).

While mutations in *atp*A, *atp*B, *atp*G, and *atpH* produce slow-growing *S. aureus*, they do not show typical SCV characteristics (50). Indeed, in an effort to generate ATP, these mutants have increased respiratory activity, thereby creating an enhanced membrane potential, which makes them more susceptible to gentamicin, whereas menadione and hemin auxotrophic SCVs are often more resistant to aminoglycosides (2, 51). Also, by reducing AtpE via *atpE* mRNA antisense, small colonies were also obtained (52). Inhibition of AtpE with diarylquinoline compounds also produces SCVs (52). Thus, while mutations in the biosynthetic genes for the F_0F_1 ATPase produce slower growth and smaller colonies, they do not produce a phenotype found in typical clinical SCVs.

Finally, as noted above, reduced activity of the TCA cycle results in decreased electron transport activity. Thus, it is not surprising to see that mutations in genes controlling the expression of TCA cycle enzymes also produce SCVs typical of the electron transport-defective phenotype. Mutations in *citB* (aconitase), *odh*A (a-ketoglutarate dehydrogenase), and *ohdB* (dihydrolipoamide succinyltransferase) have been reported to form SCVs (32).

COMPENSATORY METABOLIC PATHWAYS IN *S. AUREUS* SCVs

The loss of respiration in SCVs results in the upregulation of many compensatory pathways and the decrease in other pathways, which are summarized in Table 2. It is apparent that these variants are optimizing reactions that produce ATP and oxidize NADH by non-oxygen dependent mechanisms. In addition, because of decreased Krebs cycle activity in SCVs, large amounts of lactic acid cannot be metabolized (53); therefore, reactions that alkalinize the medium are favored, e.g., the arginine deiminase pathway. This pathway has the added benefit of producing ATP. Data that support the findings summarized in Table 2 are based upon transcriptional analysis of genes, protein expression, and isotopologue studies wherein the data using these multiple techniques strongly support one another (18, 53–57). There is upregulation of the genes involved in uptake of sugars and the enzymes in the glycolytic pathway, as this supplies ATP for *S. aureus*, which cannot produce ATP via electron transport. The metabolism of glycerol, pyruvate, butanoate/acetoin, arginine, and nitrate are all increased, because these can oxidize NADH and produce ATP. Reactions that are unfavorable produce NADH, and these are downregulated. As noted above, mannose, but not mannitol, can support growth of *menD*-defective *S. aureus* because mannitol use requires the reduction of NAD^+ to NADH (21, 58). Of note, this profile of genes and pathways up- and downregulated in *hemB* and *menD* mutant *S. aureus* is strikingly similar anaerobically that of grown *S. aureus* (18, 54–56, 59, 60). Finally, of the several reactions shown in Fig. 1 that lead to the reduction of menaquinone, the only gene showing increased expression was *glpD*, whereas the fatty acid oxidation pathway (*fadEBA*) was reduced and *ndh2*, *sdh*, *lqo*, and *mqo* were unchanged. Perhaps this is not surprising, because Ndh2 might be already maximal at baseline since it is the major pathway for oxidation of NADH, but MpsABC increases to ensure a membrane potential in the absence of the cytochrome oxidases.

RESPIRATION AND VIRULENCE FACTOR PRODUCTION IN SCVs

A connection between respiration and toxin production was established decades ago when *S. aureus* grown anaerobically on blood agar failed to cause hemolysis. This was found to be due to reduced production of α-hemolysin (Hla) (61). Similarly, the production of toxic shock syndrome toxin-1 (TSST-1) was found to be reduced under strict anaerobic conditions (62). It is now known that both Hla and TSST-1 are regulated by the *agr* operon via RNAIII (63). RNAIII levels are reduced under anaerobic conditions (64). Hence, a strong connection between respiration and expression of virulence factors has been noted for decades.

Respiration and virulence factor production have been studied in detail in staphylococcal SCVs wherein defects in electron transport and reduced production of toxins are found (2, 22). While multiple pathways can produce slow growth, hence SCVs, in *S. aureus*, only respiratory-defective

SCVs, including thymidine-auxotrophic ones, have consistently shown reduced levels of RNAIII (19). In many cases, low RNAIII leads to reduced toxin, protease, and phenol-soluble modulin (PSM) production but increased expression of adhesins and ligands for uptake by host cells (2, 22, 65). Because SCVs are often unstable, this allows the organisms to shift from a virulent to a quiescent form that can hide within host cells (2, 66). This instability can in some cases be attributed to point mutations in *men*, *hem*, and *thyA* genes (22). Thus, *S. aureus* alteration of respiration allows for populations of virulent organisms during acute infection and quiescent bacteria during chronic infections (discussed below).

REGULATORS, SCVs, AND PERSISTENCE WITHIN THE HOST

SCVs have been associated with unusually persistent, recurrent, and antibiotic-resistant infections (2, 22). Thus, it is not surprising that the reduced activity of *agr* seen in respiratory-defective SCVs is associated with persistent infections, because loss of *agr* activity has been associated with persistent infections even when there are no SCVs (67, 68). Early studies with *hla* mutants revealed that they persist within cultured endothelial cells (69) and that *S. aureus* SCVs behaved similarly because they were not producing Hla (70). Thus, the reduced *agr* activity in SCVs results in persistence because of low levels of Hla production. Of interest, during prolonged infections of cultured cells, the intracellular milieu is able to select for *S. aureus* with reduced Hla production and the SCV phenotype (66, 71, 72). Similarly, *S. aureus* strains harvested from cows with chronic bovine mastitis show lower expression of *agr* and less Hla production than strains harvested from acute mastitis (73).

In addition to Hla, PSMs are highly lytic to mammalian cells (74). The *psm* genes are strongly upregulated by RNAIII. Thus, mutations in *agr*CA or in *agrD* result in low levels of PSMs (75), which might allow persistence. Persistence of *agr*CA or *agrD* mutants requires the presence of *nanK* (ManNAc kinase gene) in the sialic acid operon (75). Of note, *S. aureus* can grow on sialic acid as an energy source (76), which may have relevance to mucosal surfaces where sialic acid is found. Hence, the low RNAIII levels in SCVs result in low levels of PSMs, which may help with intracellular persistence. Naturally, an intracellular location would facilitate persistence by protecting organisms from host defenses, and the ability to switch from rapidly growing organisms that produce large amounts of toxins to SCVs, which express fewer toxins but more adhesins, is an excellent survival strategy for *S. aureus* (2, 19, 66).

Also, suppressing Agr activity with diflunisal, which blocks AgrA, reduced the production of Hla and Psmα (77, 78) and strongly reduced osteoblast cytotoxicity caused by *S. aureus*-secreted toxins independently of effects on bacterial growth (77). This resulted in reduced damage to bone. However, isolates from patients with chronic osteomyelitis showed elevated expression of *agr* and *sarA*, yet these were more invasive into cells (high FnbA and Emp expression), had less cytotoxicity and strong hemolysis in isolates from chronic osteomyelitis, and formed a higher percentage of SCVs (79). The expression of PSM was not found to be different in the groups of isolates. In summary, *agr* plays a role in the persistence seen with SCVs, but changes in Arg activity are not the whole story.

SigB is required for formation of SCVs and establishment of chronic infection in bone (80, 81). A prominent role for SigB in persistence was provided by a study using respiratory *S. aureus* isolates from patients with cystic fibrosis (82), wherein the genes under the positive control of the alternate sigma factor SigB, were specifically upregulated and differed in that way from those seen in prototype *S. aureus* and the *hemB* mutant. These clinical SCVs persisted much more efficiently in cultured cells than the *hemB* and prototype strains, and a *sigB* mutant was a poor persister. Further support for SigB playing an important role in persistence comes from studies with 4-hydroxy-2-heptylquinoline-*N*-oxide (HQNO). Exposure of *S. aureus* to the *Pseudomonas aeruginosa* exoproduct HQNO activates SigB, inhibits cytochromes, suppresses growth, provokes the emergence of SCVs, and stimulates biofilm formation (24, 46, 86). SigB activation also increases the expression of the fibronectin-binding protein A and the biofilm-associated *sarA* genes. All of these changes enhance the persistence of *S. aureus* in the airways of patients with cystic fibrosis (83). Thus, it appears that the *agr* locus plays a minor role in the regulation of the virulon of SCVs, unlike SigB, which may have a key role in intracellular persistence.

Respiratory-defective SCVs have another mechanism for persistence. The decreased use of oxygen by these variants means they would not activate HIF-1, an oxygen-sensing protein that notifies the host of intracellular invasion. In fact, *S. aureus* SCV failed to activate HIF-1 (84). This provides a further mechanism to help them hide from host defenses.

The lower membrane potential of respiratory-defective SCVs is likely to be another factor in the intracellularly persistent SCVs (85). The intracellular milieu contains cationic molecules that kill *S. aureus*, but their association with the membrane and bactericidal action requires that the membrane has a normal membrane potential. *S. aureus* SCVs are more resistant to cationic molecules such as tPMP, protamine, spermine, lactoferrin B, hBd-2, hBD-3, LL-37, and daptomycin (which behaves as a cationic peptide when loaded with calcium). This is due in part to reduced membrane potential (86–94). Resistance to spermine has some unique features in that it is menadione auxotrophic but not hemin auxotrophic. *S. aureus* SCVs are more resistant, so the resistance is not strictly related to oxygen or nitrate respiration (88). Moreover, USA300 is different from many strains of *S. aureus* that are susceptible to spermine in that it contains SpeG, which allows this strain to metabolize (detoxify) spermine (88). However, exposure of *S. aureus* to spermine also results in reduced expression of the F_0F_1 ATPase (93). Therefore, respiration and membrane potential seem to be involved in *S. aureus* resistance to many host cationic compounds.

Some experimental compounds have been reported that treat SCVs. Persistence of SCVs within host cells can be altered by tomatidine, which is the first representative of a new group of compounds that target respiratory-defective *S. aureus* (95, 96). This unique activity against respiratory-defective *S. aureus* SCVs decreases persistence (95). Moreover, tomatidine prevents virulence gene expression (97). Of further interest is the finding that tomatidine acts in synergy with aminoglycoside and β-lactam antibiotics against multiresistant *S. aureus* (97, 98). Thus, tomatidine and its derivatives may have strong relevance in the treatment of patients with cystic fibrosis, who frequently have combined *P. aeruginosa-S. aureus* infections (22, 99). In addition, iron chelators, such as deferiprone, have activity against *S. aureus* SCVs (100). They have been tested in the *Caenorhabditis elegans* and wound models and shown to enhance the activity of antibiotics against *S. aureus* SCVs (100). Finally, NP108, a lysine polymer, is a bactericidal compound that kills

TABLE 2 Compensatory changes in redox and ATP-producing reactions in SCVs[a]

Reaction	Enzyme	Genes (N315 genome numbers)
Favorable reactions produce ATP and NAD^+ increased in SCVs		
Glycolysis (glucose or fructose) + ADP = 3 carbon + ATP		Multiple glycolytic enzymes
Membrane potential formation	MspA(NuoL)	*mspABC*(SA0411-3)
Glycerol pathways		
Glycerone~P + NADH + MQ = Glycerol-3-P + MQ-H_2 + NAD^+	GlpD	*glpD* (SA1442)
Glyceraldehyde-3-P + ADP = glycerate + ATP	GlxK	*glxK* (SA2220)
Glycerate + NADH = glyceraldehyde + NAD^+	AldH	*aldH* (SA1736)
Glyceraldehyde + NADH = glycerol + NAD^+	AdhE	*adhE* (SA0143)
Pyruvate pathways		
Pyruvate + NADH = lactate + NAD^+ (linked to Ndh)	LctE(Ldh1)	*ldh1* (SA0232)
2 Pyruvate + CoA = 2 acetyl-CoA + formate	PlfAB	*plfAB* (SA0219/8)
Acetyl-CoA + NADH = ethanol + NAD^+	AdhE	*adhE* (SA0143)
Acetaldehyde + H_2O + NADH = ethanol + NAD^+	AdhH	*adhH* (SA1736)
Pyruvate + NO + NADH = NH_4 + NAD^+ + lactate	Hmp	*hmp* (SA0231)
Pyruvate = acetolactate + CO_2	AlsS (IlvB)	*alsS* (SA2008)
Pyruvate + Pi + O_2 = acetyl~P + CO_2 + H_2O_2 (MQ/Cyto)	CidC(Pox)	*cidC*(SA2327)
Acetyl~P + ADP = acetate + ATP	LctE	*lctE* (SA0232)
Phospho-enolpyruvate + CO_2 + ADP = ATP + oxaloacetate	AdhE	*adhE* (SA0143)
Butanoate/acetoin pathways		
Acetoin		
Pyruvate = acetolactate + CO_2	AlsS	alsS (SA2008)
	IlvB	*ilvB* (SA1859)
Acetolactate = acetoin + CO_2	AlsD	*alsD* (SA2007)
Acetoin + NADH = butane-2,3-diol + NAD^+	ButA	*butA* (SA0126)
Butanal/propanoate		
Butanoyl-CoA + NADH = butanal + NAD^+	AdhE	*adhE* (SA0143)
Acetoacetyl-CoA + NADH = 3-OH-butanoyl-CoA + NAD^+	FadB	*fadB* (SA0224)
α-ketobutyrate + NADH = 2-hydroxybutanoate + NAD^+	LctE	*lctE* (SA0232)
Arginine deiminase pathway	ArcR	*acrR* (SA2424)
Arginine = citruline + NH_3	ArcA	*acrA* (SA2428)
Citruline + P = ornithine + carbamoyl-P	ArcB	arcB (SA2427)
Carbamoyl-P + ADP = ATP + CO_2 + NH_3	ArcC	arcC (SA2425)
Nitrate reductase		
Nitrate + MQ → Nitrite + MQ-H_2	NarGHIJ	*narGHIJ* (SA2182-5)
Nitrite + NADH → NAD^+ + NH_3	NasED	*nasED* (SA2187-8)
L-Arg → L-Cit → NO	saNOS	*nos* (SA1730)
NO + O_2 → NO_3-	Hmp	*hmp* (SA0231)
Nitrite uptake	NirBC	*nirC* (SA0293)
Unfavorable reactions produce NADH—decreased in SCVs		
Mannitol-1-P + NAD^+ = mannose-6-P + NADH	MtlD	*mltD* (SA1963)
Entry into TCA cycle		
Oxoaloacetate + Acetyl-CoA → Citrate	CitZ 0.4	*citZ* (SA1518)
Citrate → Isocitrate	CitB 0.3	*citB* (SA1184)
Glutamate → isocitrate entry into TCA	GltBD	*gltBD* (SA0430/1)
Aspartate → oxaloacetate entry into TCA	HisC	*hisC* (SA0902)
β-oxidation of fatty acid = 1 NADH/acetyl group	FadEBA	*fadEBA*(SA02243/4/6)
Formate + NAD^+ → CO_2 + NADH	Fdh	*fdh* (SA0171)

(*Continued on next page*)

TABLE 2 (*Continued*)

Reaction	Enzyme	Genes (N315 genome numbers)
Pyruvate + NAD^+ → Acetyl-CoA + NADH	PdhABCD 0.3–0.7	*pdhABCD* (SA0943-6)
α-ketobutyrate + NAD^+ → Succinyl-CoA + NADH	OdhBA	0.3 *odhBA* (SA1224-5)
Not found increased in SCVs—unchanged in SCVs		
NADH + MQ = NAD^+ + MQ-H_2	Ndh2	*ndh2* (SA0802)
Oxaloacetate + NADH + CO_2 = malate + NAD^+	PycA	*pycA* (SA0963)
Oxaloacetate + ADP = pyruvate + ATP	PycA	*pycA* (SA0963)
Pyruvate = 2 acetaldehyde + $2CO_2$	Pdc	*pdc* (SA0182)
Pyruvate + NADH = lactate + NAD^+ (aerobic LDH)	Ldh2	*ldh2* (SA2395)
Pyruvate + NADH = lactate + NAD^+ (linked to quinol oxidase/NAD^+ independent)	Lqo	*lqo* (SA2400)
Malate + MQ = oxaloacetate + MQ-H_2	Mqo1	*mqo1* (SA2155)

[a]ADH, alcohol dehydrogenase; ArcA, arginine deiminase; ArcB, ornithine transcarbamolyase; ArcC, carbamoylate kinase; CitB, aconitase; FadB, 3-hydroxyacyl-CoA dehydrogenase; HMP, hydroxmonocarboylic acid phosphatase (flavohemoprotein or pyruvate/nitrite reductase); LDH, lactate dehydrogenase; MltD, mannitol-1-phosphate 5-dehydrogenase; MQ, menaquinone; Mqo1, malate:quinone oxidoreductase; PycA, pyruvate carboxylase; PdhB, pyruvate dehydrogenase E1 component beta subunit; PflB, pyruvate formate lyase; SA0182 and SA0224, gene numbers in the *S. aureus* N315 genome; saNOS, nitric oxide synthase.

S. aureus, including SCVs, by membrane disruption and cell lysis through mechanisms found in lysine-rich cationic peptides (101).

The rationale for examining intracellular persistence comes from clinical observations. *S. aureus* SCVs produce persistent, recurrent, and antibiotic-resistant infections (2, 22). In the past 20 years, there have been over 40 studies that involve over 350 patients where persistent infections have been reported (2, 22). When referring to these clinical studies, the term "persistent" means that the organisms are found in the patient for years, which is different from the term "persister" within a broth culture. Thus, this is different from clinical practice, where the term "persistent infection" applies to the failure of apparently active antibiotics and host immunity to clear infection, and this may not be related to the definition of persistence used when defining organisms *in vitro* (102). Thus, when SCVs are found in infections that are not cleared by active antibiotics, these have strongly correlated with unusually persistent infections, and the organisms recovered from patients often do have defined genetic mutations (2, 22, 103). These studies show that patients infected with SCVs often have a history of long-term treatment with antibiotics. They may have prolonged disease-free intervals (as long as 53 years), but during recurrences, they show unusual resistance to apparently active antibiotic therapy (103).

P. AERUGINOSA-S. AUREUS SCV COOPERATION

Several studies have found that respiratory-deficient *S. aureus* and *P. aeruginosa* may actually work synergistically to make infections worse. As noted above, HQNO, which targets cytochrome *bd* (6), selects for respiratory-defective *S. aureus* SCVs (104, 105). Similarly, another exoproduct of *P. aeruginosa*, pyocyanin, selects for menadione auxotrophic SCV (39). Both menadione auxotrophic and *hemB* mutant SCVs are resistant to pyocyanin (39, 106). Genome sequencing of the isolates selected with pyocyanin identified mutations in four genes: *saeS*, *menD*, NWMN_0006, and *qsrR* (39). Mutations in *menD* and *qsrR* allow for pyocyanin resistance. QsrR is a quinone-sensing repressor of quinone detoxification genes. Inactivation of *qsrR* resulted in significant pyocyanin resistance, and additional pyocyanin resistance was achieved through combined inactivation of *qsrR* and menadione biosynthesis (39). *P. aeruginosa* selection of *S. aureus* SCVs increases *S. aureus* resistance to aminoglycosides and other antibiotics (107). Of note, not all *P. aeruginosa-S. aureus* strain pairs produce this devastating synergistic effect (108), which is welcome information, because *S. aureus* SCVs are associated with worse lung disease in children with cystic fibrosis (109). Finally, *S. aureus* SCVs can serve as a source of iron to increase the virulence of *P. aeruginosa* (110). Therefore, there are several ways that combined infections with *P. aeruginosa-S. aureus* SCVs can produce synergic virulence.

CLINICAL LABORATORY IDENTIFICATION OF *S. AUREUS* SCVS

The clinical microbiologist must be alerted to look for SCVs, because their slow growth means that they may either be missed entirely or may be overgrown by more rapidly dividing strains or revertants. Furthermore, they are easily misidentified because of their unusual colony morphology and biochemical profile. Because *S. aureus* SCVs are often very slowly catalase positive and produce little pigment and hemolysins, they may be misidentified as viridans streptococci or coagulase-negative staphylococci (2, 22). In addition, these SCVs often fail to ferment mannitol and are resistant to aminoglycoside antibiotics. All aspects of this unique phenotype can be attributed to the loss of one or another of the enzymes critical to the biosynthesis of electron transport chain components (Fig. 2). At 24 hours, the colonies can be very small and easily missed if there is a mixed culture of rapidly and slowly growing colonies (22). Slow growth may occur owing to several factors when electron transport is inoperative. One direct effect of interrupting electron transport is reduced synthesis of teichoic acid, because the biosynthesis of phosphatidyl glycerol, a teichoic acid precursor, requires electron transport (111–114). Also, ATP is required for many cellular functions and supports electron transport in the synthesis of menaquinone, such as cell wall biosynthesis and uptake of amino acids and carbohydrates; hence, organisms interrupted in electron transport have a lower capacity to produce ATP and show slower

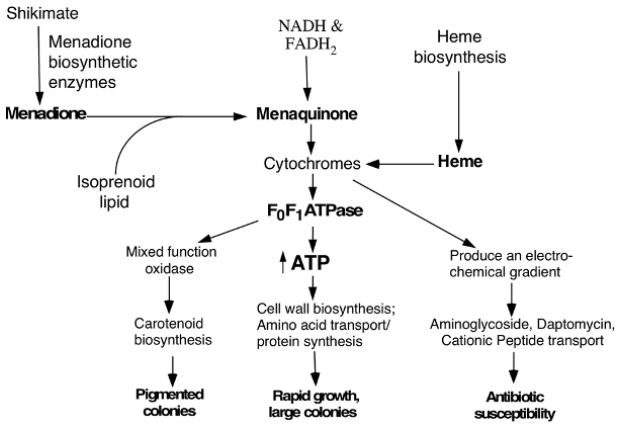


FIGURE 2 Relationship between electron transport and the small colony variant phenotype in *Staphylococcus aureus*.

growth, resulting in small colonies. We found that the interdivision time for *S. aureus* 6850, a clinical isolate (parent strain), was 20 min, compared to 180 min for *S. aureus* JB-1, a menadione auxotrophic SCV, when grown in tryptic soy broth (70). Hale (115) found similar growth rates with an *S. aureus* parent and SCV at 20 min and 120 min, respectively, in nutrient broth, and Massey and Peacock found division times of five independent strains to be 20 min, compared to 210 min in SCVs selected by gentamicin exposure (116, 117). In contrast, a hemin auxotrophic SCV had a 131- to 173-min generation time, compared to its parent's time of 53 min (113). While there are clearly differences in the growth rates of several respiratory-defective strains of *S. aureus*, all of these variants produce small colonies and fulfill the definition of an SCV, i.e., an organism that forms colonies that are 10-fold smaller than those of its parent.

The failure of SCVs to ferment carbohydrates, such as mannitol, can also be related to defects in electron transport (3). As noted above, interruption of electron transport results in the accumulation of NADH, which downregulates the citric acid cycle enzymes, thereby making the fermentation of mannitol and other such carbohydrates unfavorable.

Electron transport is also required for carotenoid biosynthesis. Electrons are shuttled through the electron transport system to the mixed function oxidase system, which is linked to pigment formation (118, 119). Hence, SCVs that are defective in electron transport form nonpigmented colonies.

Thus, *S. aureus* SCVs do not show features that are typical for wild-type *S. aureus* and can easily be missed due to the small sizes of colonies and misidentified because of atypical characteristic (22). Nevertheless, most of the phenotypic features reported for clinical and laboratory-generated *S. aureus* SCVs can be explained by the model shown in Fig. 2.

ANTIBIOTICS AND SCVs

Ever since Bigger first described the "persistence" of staphylococci in broth cultures exposed to penicillin (120), there has been interest in understanding this perplexing observation as organisms survived in broth cultures despite apparently active antibiotics. "By definition, persisters are not mutants but are regarded as phenotypic variants that are isogenic to normally growing cells within a common culture" (121). Slow growth and low ATP have been found among many types of persisters (122, 123). This provides a link to respiratory-deficient SCVs, which have low levels of ATP.

With respiratory-deficient *S. aureus*, we are able to define a number of mechanisms for *S. aureus* failing to respond to antibiotics. First, slow division of the bacterial cell wall is associated with resistance to penicillin and other β-lactams (124, 125). Second, the decreased membrane potential in respiratory-deficient *S. aureus* SCVs results in decreased uptake of aminoglycosides, cationic peptides, and daptomycin (7, 33, 86, 87, 89–92, 94, 126). These are all cationic antimicrobials, because the active form of daptomycin carries calcium. Third, alterations in membrane fluidity caused changes in the fatty acids of the membrane as seen in *fabF* mutant SCVs (127) or due to decreased carotenoid content of the membrane (128) result in daptomycin resistance. Fourth, respiratory-deficient *S. aureus* SCVs can have a hypermutator phenotype (47). This may relate to the lower levels of ATP. SOS DNA repair led by RecA only binds single-stranded DNA in the presence of ATP, and hence, mutations leading to resistance are more frequent in *S. aureus* SCVs (129). While ciprofloxacin induces the SOS response in *S. aureus* (130), development of ciprofloxacin resistance in *S. aureus* was found to occur when the organisms passed through a transient SCV phase, which was then followed by mutations that altered ciprofloxacin targets (131). Paradoxically, the SOS response may have a dual effect. Antimicrobials that reduce replication fidelity increase the formation of SCVs through activation of the SOS response and thereby potentially promote persistent infections that are difficult to treat (132). Finally, intracellular location of *S. aureus* SCVs (66, 72) provides a location where there is reduced penetration of some antibiotics (133–135). However, the intracellular location is not a benefit in all cell types, because the acidic environment of and presence of oxidant species within the phagosome greatly enhance the activity of β-lactams against *S. aureus* SCVs (133–135). In addition, SCVs release large quantities of lactic acid (53), which decreases the activity of aminoglycosides (136).

Comparing the activity of a large number of antibiotics in broth culture, within tissue-cultured cells, and *in vivo* reveals that some antibiotics may have an advantage in treating SCVs (31, 53, 54). Finafloxacin has greater activity in acidic environments where *S. aureus* SCVs may hide (137). In addition, oritavancin is especially good at killing stationary phase organisms, which also translates to the slower-growing SCVs (99, 138). Based upon tissue culture and animal studies, adding rifampin may add efficacy for killing *S. aureus* SCVs (133, 139, 141). Of the different types of respiratory-deficient *S. aureus*, *menD* was found to be the most resistant (133). Finally, a *fabF* mutant of *S. aureus* formed an SCV that was less susceptible to vancomycin and daptomycin, but this variant does not appear to be respiratory defective (127).

One solution to avoiding these antibiotic-resistant variants may be prevention of *S. aureus* SCV infection through a vaccine. This is being attempted for bovine intramammary infections (140). Also, as noted above, drugs used in combination with antibiotics may help to treat SCVs.

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Noncoding RNA

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35

GENERAL INTRODUCTION

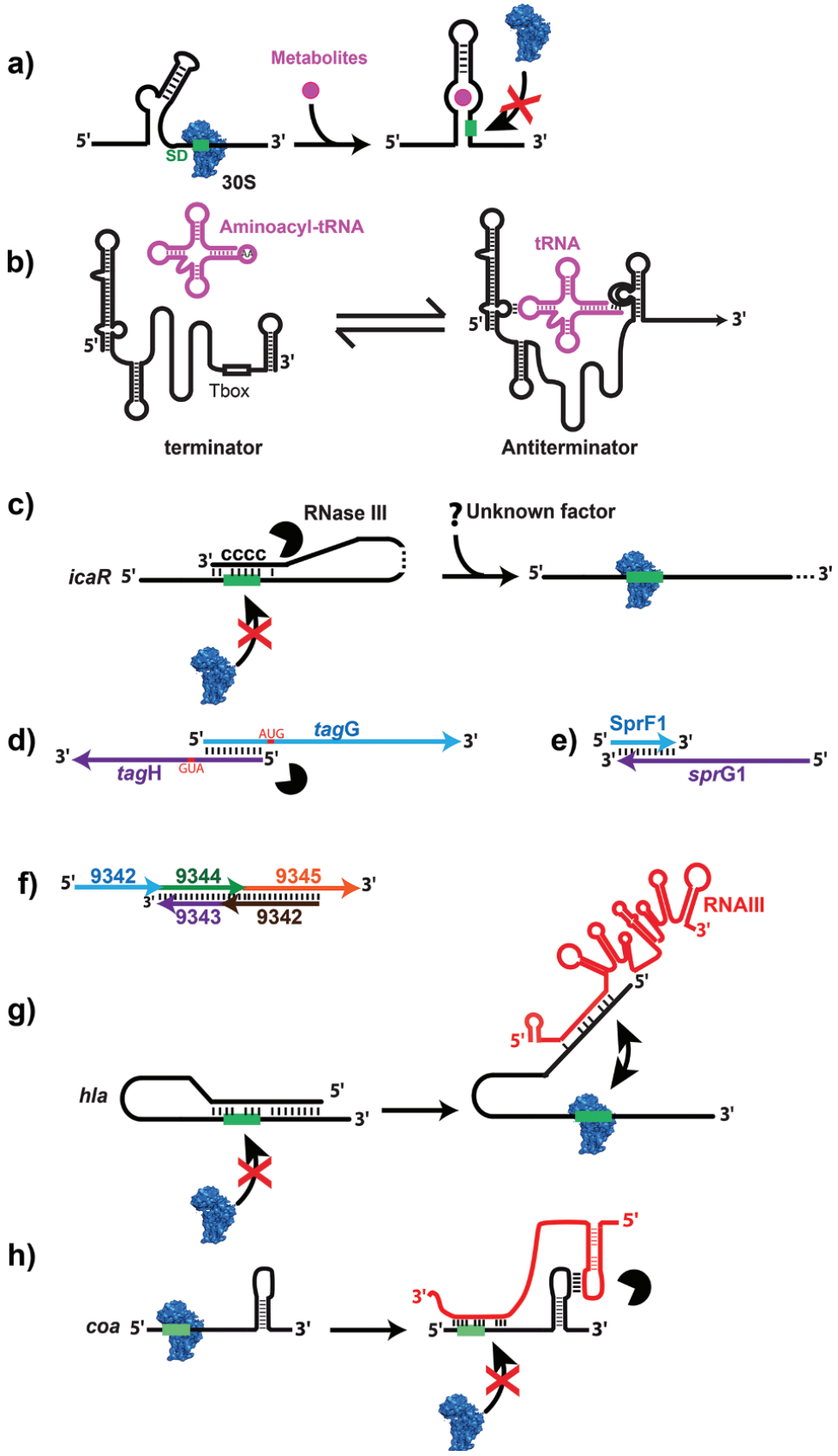
Regulatory RNAs have been identified in many bacteria and in pathogenic bacteria such as *Staphylococcus aureus*, where they play major roles in the regulation of virulence or the synthesis of metabolic proteins, besides transcriptional factors and two-component systems (1–4). Most of them are noncoding RNAs (sRNAs), but some of them express small peptides. Certain sRNAs, acting in *cis*, are situated at the 5′ untranslated regions (UTRs) of mRNAs and act as sensors of metabolites, tRNA, or environmental stimuli (e.g., temperature, pH) or are situated at the 3′ UTR. In contrast, the genes encoding sRNAs, which act *in trans*, sit on the opposite strand of the regulated mRNA or at genomic locations distant from the mRNAs they regulate. *Cis*-encoded sRNAs, also called antisense RNAs (asRNAs), are fully complementary to their targets. In contrast, *trans*-encoded sRNAs share only partial complementarity, and as a consequence, they can regulate many mRNAs. Most of them are encoded mainly in the core genome, while a few of them are localized within mobile elements, pathogenic islands, or plasmids. In this review, we will focus on the most recent mechanisms of RNA regulation discovered in *S. aureus* and how regulatory RNAs are part of sophisticated networks that allow the bacteria to adapt quickly to their environment or survive in their host.

mRNA 5′ UTRs: RIBOSWITCHES, T-BOXES, AND THERMOSENSORS

5′ UTRs of mRNAs contain riboswitches, T-boxes, or thermosensors with potential impacts for novel antibiotherapy. Riboswitches and T-boxes are found in the 5′ UTR of some mRNAs and contain highly structured domains, which recognize metabolites such as cofactors, vitamins, amino-acids, nucleotides, second messenger cyclic di-GMP, Mg^{2+}, or nonaminoacylated tRNAs (5). Binding of these metabolites induces structural changes that modify the expression of the downstream mRNA, for example, by inducing premature transcription arrest, repression/activation of translation, or cleavage (Fig. 1a). A T-box senses the aminoacylation status of tRNAs and mainly controls transcription of downstream genes that encode proteins involved in biosynthesis, transport of amino acids, or aminoacylation of tRNAs (Fig. 1b). Based on sequence and structure conservation, most of the T-boxes and riboswitches were predicted in *S. aureus* genomes (4). A large proportion of riboswitches control the expression of genes involved in metabolic pathways. Because these genes are often essential for growth, they represent interesting targets for the development of alternative antimicrobial drugs in the battle against multidrug-resistant *S. aureus*. This strategy was used with the guanine and glucosamine-6-phosphate (GlcN6P) riboswitches. Mulhbacher et al. (6) identified a pyrimidine derivative that binds to the guanine riboswitch and represses the expression of *guaA*. This compound significantly attenuated *S. aureus* infections in a mouse model. In Gram-positive bacteria, the *glmS* mRNA is both a ribozyme, which catalyzes its own cleavage, and a riboswitch responding to GlcN6P. Its product encodes an essential enzyme, which converts fructose-6-phosphate into GlcN6P, a building block of bacterial peptidoglycan. Tight regulation of *glmS* mRNA is crucial to maintain a homeostatic level of GlcN6P in the cell. At high concentrations of GlcN6P, its binding to the 5′ UTR of *glmS* leads to site-specific self-cleavage, which generates a 5′ hydroxylated end molecule rapidly degraded by the RNase J1 (7, 8). A recent study led to the design, synthesis, and characterization of a GlcN6P analogue, carba-GlcN6P, which constitutively activates the *glmS* ribozyme of vancomycin-resistant *S. aureus* and destabilizes its mRNA (7). This compound was experimentally shown to induce the efficient self-cleavage of the *glmS* mRNA in a similar fashion as the natural metabolite and thus represented an important step in the development of antibiotics with a new mode of action. Very recently, a new approach called "Term-seq" revealed that several antibiotic resistance genes are under the control of riboswitches responding to antibiotics commonly used against Gram-positive pathogenic bacteria such as *Listeria* spp. and *Enterococcus faecalis* (9). These results suggest that the same phenomenon could exist in *S. aureus* and that RNA-mediated regulation could play a broader role in antibiotic resistance mechanisms than has been envisioned.

Not surprisingly, the presence of antibiotics can also modulate the regulatory activity of T-boxes. In *S. aureus*, an unusual *glyS* T-box regulates transcription antitermination (Fig. 1b) of the unique glycyl-tRNA synthetase (glyRS) gene responsible for catalyzing the aminoacylation of the five $tRNA^{Gly}$ isoacceptors, independently of their anticodon (GCC or UCC) and with different binding affinities (10). Thereafter, the T-box senses the availability of glycine not only for its incorporation into nascent polypeptide chains during translation but also for the formation of pentaglycine bridges into the peptidoglycan molecule, linking two essential pathways. Antibiotics targeting the small ribosomal

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a)
Metabolites
5'
3'
SD
30S
b)
Aminoacyl-tRNA
tRNA
Tbox
terminator
Antiterminator
c)
RNase III
? Unknown factor
icaR
cccc
d)
tagG
AUG
tagH
GUA
e)
SprF1
sprG1
f)
9342
9344
9345
9343
RNAIII
g)
hla
h)
coa

subunit stabilized the T-box/tRNA complex and induced a read-through of transcription, while chloramphenicol and linezolid attenuated *gly*S transcription (11). The outcome depended on the binding sites of the protein synthesis inhibitors (11). Although T-boxes can be direct targets for antibiotics against *S. aureus*, it was also reported that a high concentration of the antibiotic tigecycline might induce possible off-target inhibition of the RNA polymerase (11).

RNA thermosensors are regulatory elements often localized at the 5′ UTR of mRNAs encoding heat or cold shock proteins and virulence factors (for review see 12). Briefly, at low temperatures, the mRNA cannot be translated, since the Shine and Dalgarno (SD) sequence is trapped in a hairpin structure, which melts gradually when the temperature increases. The best-studied example in Gram-positive bacteria is the thermosensor regulating the expression of the transcriptional factor *prf*A, which activates the expression of most of the virulence genes in *Listeria monocytogenes* at high temperatures (13, 14). No such example has yet been demonstrated for other Gram-positive bacteria, including *S. aureus*.

3′ UTRs OF mRNAs ACT IN *CIS* OR ARE RESERVOIRS OF sRNAs

Transcriptome analysis of the human pathogen *S. aureus* revealed that at least one-third of mRNAs carry long 3′ UTRs and thus might display multiple regulatory functions (15). Some of them have direct action on the expression of their own mRNA (15, 16). Long 3′ UTRs (>100 nucleotides) can end at an intrinsic Rho-independent terminator of transcription (TT) and can be generated from a specific RNase cleavage or from a termination read through of the RNA polymerase. Remarkably, several 3′ UTRs contain riboswitches functioning in metabolite-sensing regulation (16). Indeed, the TT of the riboswitch in an OFF conformation also serves as the TT of the gene encoded upstream of the riboswitch.

A singular example of a newly identified posttranscriptional regulatory mechanism was shown for the *icaR* mRNA (Fig. 1c), which possesses an unusually long 3′ UTR (390 bp). In this case, the expression of the mRNA is modulated through a long-distance interaction between 3′ UTR and 5′ UTR (16). Because *icaR* codes for a transcriptional repressor of the *icaADBC* operon, encoding enzymes involved in the synthesis of PIA-PNAG, the main polysaccharides of the biofilm matrix, this regulation has a direct impact on biofilm formation. Base-pairing interactions between the long 3′ UTR of *icaR* mRNA and the SD sequence of the same mRNA hindered efficient translation initiation (16). This long-range RNA duplex generated a specific site for the double-stranded endoribonuclease III (RNase III) for cleavage. As a consequence of this cleavage, PIA-PNAG synthesis increased. However, the mechanism allowing IcaR translation is not known to prevent the action of the 3′ UTR as a *cis*-acting antisense RNA (Fig. 1c).

It is also postulated that the 3′ UTR length provides other types of transcript-specific regulation. Indeed, in *Salmonella* spp., the 3′ UTRs are also reservoirs for sRNAs, which originate either by transcription from an internal promoter or by processing. In both cases, the sRNA generated from the 3′ UTR regulates trans-encoded mRNA targets. For instance, the sRNA CpxQ is generated by RNase E cleavage of the 3′ UTR of *cpxP* and represses the translation of mRNAs encoding a family of envelope proteins, whereas the sRNA DapZ is transcribed from an internal promoter within the *dapB* gene and inhibits translation of the major ABC transporters, DppA and OppA (17, 18; for review see 19). Recent work has shown that such 3′ UTR-derived sRNAs also exist in *S. aureus*, although their functions remain to be addressed (E. Desgranges, S. Marzi, P. Romby and I. Caldelari, unpublished data).

asRNAs IN PERVASIVE TRANSCRIPTION AND ACTING AS ANTITOXINS

asRNAs are transcribed from the opposite strand of the mRNAs they regulate, so that they display perfect complementarities with their targets. Short asRNAs are often encoded on mobile elements such as plasmids, transposons, and phage-like elements. Such elements can potentially be transferred horizontally to other bacterial species or be duplicated (20). In *S. aureus*, they were first described to control plasmid conjugation and replication (21). The size of asRNAs can vary from 10 to thousands of nucleotides, because these RNAs can overlap with part of a gene (3′ or 5′ ends), the entire gene, or a group of genes (Fig. 1d to f). This phenomenon is called pervasive transcription. Initially, pervasive transcription was considered a nonfunctional transcriptional noise. However, considering the large number of asRNAs expressed from the entire genome and in several bacterial species, these RNAs might play an important role in

FIGURE 1 Several mechanisms of RNA regulation in *S. aureus*. (a) Schematic drawing of the flavin mononucleotide riboswitch. The 5′ UTR adopts a particular structure recognized by the flavin mononucleotide, which in turn leads to the stabilization of a stem-loop structure sequestrating the SD sequence to inhibit translation. 30S is for the small ribosomal subunit. (b) An example of a T-box motif as found in the 5′ UTR of many mRNAs encoding aminoacyl-tRNA synthetases. Nonaminoacylated tRNA binds to the leader region at two sites and stabilizes an antiterminator structure, allowing transcription of the downstream gene. The drawing is adapted from reference 4. (c) The 3′ UTR of the biofilm repressor IcaR possesses a cytosine-rich motif, which binds to the SD sequence and hinders ribosomes from its binding site on the mRNA (see text for details). (d) Overlapping 5′ UTRs of *tag*G and *tag*H mRNAs are processed by the endoribonuclease III (Rnase III). Shorter 5′ ends might facilitate ribosome recruitment. (e) The antitoxin RNA SprF1 interacts at the 3′ end of the toxin encoded by sprG1 and triggers its degradation. (f) A cluster of five sRNAs was sequenced in the *S. aureus* Newman strain that encodes a putative toxin-antitoxin system (see text for details). (g,h) sRNAs act by an antisense mechanism. Binding of the 5′ UTR of RNAIII to the 5′ UTR of *hla* mRNA liberates its SD and activated translation (g), whereas the 3′ domain of RNAIII acts as a repressor domain, which contains C-rich motifs for base-pairing with the SD sequence of mRNA as *coa* mRNA depicted in the figure (h). Green bar, SD sequence; black circle, RNase III (for references and more details, see text).

the regulation of gene expression. Genome-wide analysis of *S. aureus* highlighted that the expression of a significant proportion (75%) of antisense transcripts to annotated open reading frames are synthesized from the complementary strand and that these sense/antisense duplexes are digested by RNase III, generating short fragments all along the genome (22). Another study using the RIP-Seq approach confirmed the involvement of RNase III in the regulation of sense/antisense transcripts and overlapping UTRs (23). The situation might be even more complex, because recent results suggested that the termination factor Rho plays a major role in preventing pervasive transcription in *Bacillus subtilis*, but also in *S. aureus* (24, 25). Although the biological outcome of pervasive transcription is not clearly understood, some of the asRNAs produced are functional and control several biological processes (15).

Type I toxin-antitoxin (TA) systems are particular cases of short asRNAs, in which the antitoxin is an asRNA regulating the translation or the fate of the toxin-encoding mRNA, whereas in the type III system, the antitoxin sequesters the toxin (reviewed in 26, 27). In *S. aureus*, several type I TA module systems have been described (reviewed in 28). One of them, called SprF1/SprG1, expresses SprF1 as the asRNA and *spr*G1 mRNA (Fig. 1e), which encodes two short secreted peptides with hemolytic and antibacterial activity (29). The antitoxin SprF1 binds to the 3′ end of *spr*G1 mRNA, which leads to mRNA degradation and inhibition of peptide synthesis to protect cells against lethality (29). The SprA1/asSprA1 pair is another intriguing and unconventional system. The asSprA1 is transcribed from the opposite strand of the *spr*A1 mRNA, producing a cytolytic peptide. Their 3′ ends overlapped by 35 nucleotides, but experimental data indicated that the functional domain of asSprA1 is outside the complementary sequence with *spr*A1 mRNA. Both RNAs were expressed concomitantly, and asSprA1 5′ base-paired with the ribosome-binding site (RBS) of *spr*A1, impairing its translation (30). Thus, asSprA1 acts as a *trans* regulator with its complementary target, suggesting that it can potentially interact with other RNA targets. Finally, a cluster of five genes encoding sRNAs specific to the *S. aureus* Newman strain contained a putative TA system (31) (Fig. 1f). Three genes were transcribed from the positive strand and two from the negative strand. Moreover, one small open reading frame was detected within one of the genes from the minus strand and coded for a secreted peptide with similarity to the RelE toxin (32). Whether these two overlapping genes corresponded to a novel TA system remains to be addressed. Interestingly, this locus was expressed in a growth-phase-dependent manner, in nutriment starvation, and in oxidative stress. Type I TA systems have been involved in many functions (e.g., membrane depolarization, plasmid maintenance), mainly in *Escherichia coli*, including the persistence phenomenon (for review see 33), but not in *S. aureus* until now (28).

TRANSCRIPTIONAL FACTORS AND sRNAs BUILD COMPLEX REGULATORY NETWORKS

The sRNAs belong to intricate networks of regulation, and their synthesis is often dependent on transcription factors or on two-component systems. In addition, sRNAs can also control transcription factors at the posttranscriptional level (Fig. 2). Typical examples will be described below. In addition, *trans*-acting sRNAs regulate mRNAs by imperfect base-pairings, which signifies that one sRNA can modulate several targets and one target can be controlled by several sRNAs. In *S. aureus*, the annealing region between sRNA and mRNAs are often longer than in *E. coli* and mostly targets the RBS of mRNAs affecting translation. In several cases, a second distinct site of interaction occurs in the coding region. Unlike Gram-negative bacteria, in which Hfq and ProQ proteins participate in sRNA regulation by stabilizing and facilitating their pairings with mRNA targets (see reviews in 34, 35), no RNA chaperones have yet been identified in *S. aureus*. Indeed, there is no identifiable *proQ* homolog, and the role of Hfq is still unclear. Recent work has shown that the rim domain of Hfq has an amino acid composition (low in arginine) incompatible with RNA annealing activity compared to *E. coli* Hfq (36).

AgrA, the response regulator of the *agr* quorum sensing system, activates the transcription of RNAIII. The bifunctional RNAIII codes for δ-hemolysin and regulates the expression of virulence genes at the posttranscriptional level (see below). It interacts with various mRNAs either to activate or to repress translation (Fig. 1g,h). The RBS of *hla* mRNA encoding α-hemolysin is embedded in a hairpin, which prevents ribosome binding and blocks the start of translation. The 5′ UTR of RNAIII possesses complementary sequences to the leader region of *hla*. Interaction between these RNAs enables the recruitment of ribosomes to initiate Hla translation. In its 3′ UTR, RNAIII carries conserved UCCC motifs that are used as the seed sequences to bind with the RBS of the target mRNAs coding for protein A, coagulase, Sbi protein, the transcription factor Rot (a repressor of exotoxins), and the endopeptidase LytM (for review see 37). Moreover, AgrA represses the sRNA ArtR, which inhibits translation of the SarA homolog SarT (38), and activates RsaE (see 39) (Fig. 2).

The staphylococcal accessory regulator SarA is synthesized from three distinct promoters (P1, P2, P3) and binds DNA or RNA (1). As a transcription factor, it regulates many genes involved in virulence, autolysis, biofilm formation, stress response, antibiotic resistance, and metabolism, but also two sRNAs, SprC and Srn_9340, located on the same pathogenicity island (40). SprC prevents ribosome binding to the SD of the *atl* mRNA coding for an autolysin. Deletion of the *spr*C gene causes enhanced phagocytosis of *S. aureus* by monocytes and macrophages, and this effect was found to be partly due to the deregulation of *atl* expression (41). SarA represses both *spr*C and *srn_9340* transcription and requires an ATTTTAT sequence in its binding site (40). However, while the SarA level remains relatively constant during bacterial growth, the expression of SprC fluctuates, which suggests that additional factors might control its synthesis and that a mechanism of derepression should coexist under specific conditions. These are the first examples of two sRNAs regulated by the same transcription factor.

In the following, we will describe an sRNA whose transcription is controlled by three independent transcription factors. The sRNA RsaE possesses two consensus sequence motifs UCCC as found in RNAIII, which interact with the RBS of several mRNAs involved in central metabolism to repress their translation (39, 42). RsaE is highly conserved between the *Staphylococcaceae* and *Bacillaceae* families. Not only is the sequence of RsaE conserved between *B. subtilis* and *S. aureus* species, but so are its regulation and functions. Recent studies have shown that its transcription is activated by AgrA (see above), the two-component system SrrAB (staphylococcal respiratory response) in response to NO, and a binding site for the redox sensing repressor Rex has been predicted (39, 43, 44). In *B. subtilis*, the Rex-repression of RsaE (also called RoxS) has been proposed to readjust

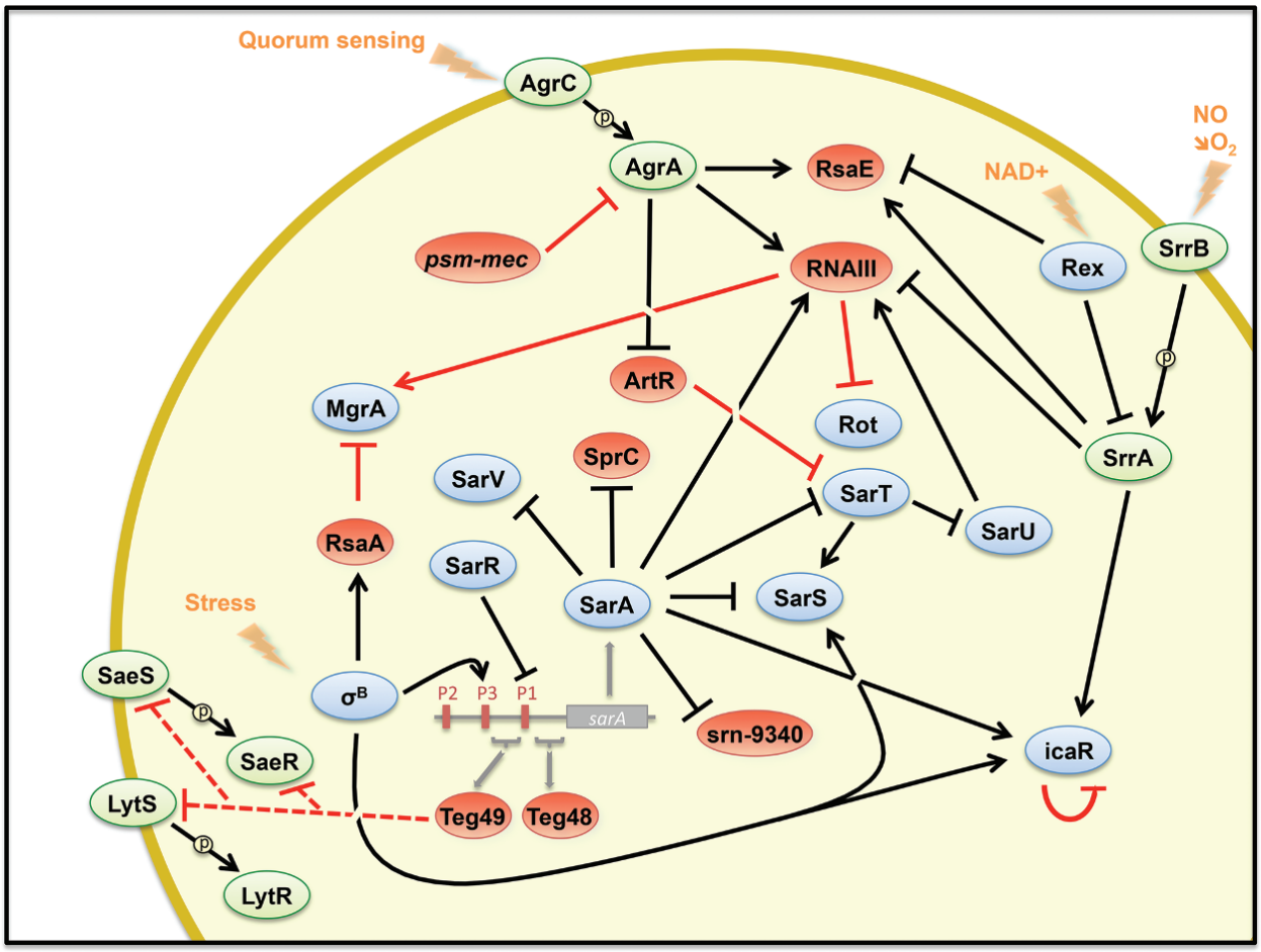


FIGURE 2 Examples of the complex network between sRNAs and transcriptional factors in *S. aureus* in response to stress. Arrows show activation and bars show repression. Blue, transcriptional regulators; green, two-component systems; red, regulatory sRNAs. Red lines corresponded to posttranscriptional regulation, and black lines, to transcriptional regulation. Dotted lines are for the target mRNAs that were not experimentally validated. Only sRNA-dependent mRNA targets encoding transcriptional factors are depicted in the figure.

the cellular balance of NAD^+/NADH on various signals (44).

The alternative sigma B factor (σ^B) together with RNA polymerase guides transcription of genes mainly in the stationary phase of growth and under stress conditions. Its regulon comprises more than 200 genes, including several virulence factors, transcription factors, and sRNAs (Fig. 2). Among the sRNAs induced by σ^B are SbrA, B, and C activated by KOH (45), and RsaA (39). The stability of RsaA depends on RNase III and the endoribonuclease RNase Y (23, 46). RsaA acts as an acute virulence attenuator in *S. aureus* (see below) by inhibiting translation of the MgrA transcription factor (Fig. 2) (47), which in turn causes the activation of the synthesis of several surface proteins (48). RsaA possesses two UCCC motifs, which in the case of *mgrA* mRNA, bind to two distant regions, involving an imperfect duplex masking the SD sequence of the mRNA and a loop-loop interaction occurring downstream in the coding region. These two distant binding sites are required for efficient repression and RNase III-dependent degradation of the repressed mRNA (47). Finally, the sRNA Teg49 is transcribed from the σ^B- dependent P3 promoter of *sar*A and is probably processed by RNase III and RNase Y with the help of the helicase CshA (49–51). Transcriptomic analyses revealed that besides genes involved in virulence and autolysis, Teg49 might posttranscriptionally affect the SaeRS and LytRS two-component systems, yet the exact mechanism is not known (51). In addition, another sRNA, Teg48, whose role is not known, is transcribed from the P1 promoter of *sar*A (Fig. 2). Even if the maturation process and the function of Teg48 and Teg49 are not clearly established, the long 5′ UTR of *sar*A mRNA encoding a master regulator of virulence in *S. aureus* represents a putative reservoir for novel sRNAs.

crRNA, tracrRNA, AND THE CRISPR-Cas ADAPTIVE IMMUNITY SYSTEMS IN *S. AUREUS*

Phages are the most abundant forms of life on earth and the natural killers of bacteria because in most cases their lytic life cycle ends with the death of the bacterial cell. Outnumbering their microbial hosts, phages impose selective pressure for the diversification of microbial defense systems. These include

various innate phage-resistance mechanisms such as restriction/modification enzymes, receptor masking, blocking DNA injection, abortive infection (52, 53), and the adaptive defense mechanism based on clustered, regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated (*cas*) genes (54, 55). The latter RNA-interference-like mechanism relies on small noncoding RNAs, CRISPR RNA (crRNA), and in some cases trans-activating crRNA (tracrRNA), through which prokaryotic hosts (bacteria and archaea) can acquire heritable resistance to genetic parasites such as phages, but also plasmids and transposons (for reviews see 53, 56). To date, CRISPR-Cas systems have been found in about 50% of bacterial genomes and 95% of archaeal genomes (57, 58). The CRISPR and the *cas* loci are often located next to each other in the genomes, sometimes organized into operons, but a significant number of genomes have also isolated *cas* loci and/or CRISPRs (59).

Despite the large diversity of the CRISPR-cas systems, they share common features. Briefly, the CRISPR loci are characterized by an array of short and palindromic repetitive sequences interspaced by sequences called "spacers" that are derived from plasmid and viral DNAs (in some cases, also RNAs). During the initial infection with a virus or plasmid, these spacers are first integrated into the CRISPR array in the host genome to provide the host with immunity (acquisition step [60, 61]). During a second infection event they are transcribed and used as guides to inactivate the viral or plasmid genome. This two-step pathway involves a variety of Cas proteins, leading to several major types of CRISPR-cas systems (reviewed in 58, 62). However, the acquisition step involves two highly conserved Cas1 and Cas2 proteins (58, 63). The Cas1-Cas2 integrase is a heterohexameric complex of four Cas1s and two Cas2s which preferentially incorporates foreign DNA at the first CRISPR repeat and participates in the discrimination against self-DNA and in the minimization of off-targeting insertions (64–66). Both proteins have been found in several *S. aureus* strains (57). During the second step, activation of transcription from a promoter located in an AT-rich leader sequence preceding the first CRISPR repeat (67, 68) leads to expression of the whole array into precursor CRISPR transcripts (pre-crRNA). The pre-crRNAs are then processed into mature crRNAs consisting of partial repeat(s) and a single spacer sequence, each complementary to a unique invader sequence (69). Different endonucleases participate in the maturation step, which might vary in different bacteria. Type I and III systems perform the function by a multisubunit Cas protein complex and are characterized by Cas6 processing (69). Type II uses another sRNA, tracrRNA, to direct RNase III-dependent maturation of the pre-crRNA in the presence of Cas9, the hallmark protein of the type II system (70). Cas9 endonuclease remains associated with the dual-tracrRNA:crRNA structure, which during the interference phase, guides the cleavage of site-specific cognate target DNA (71). Type I and II CRISPR-Cas systems target DNA (72, 73), whereas type III systems provide immunity against DNA and RNA (74).

With the completion of the genome sequences of several *S. aureus* strains, it appears that the CRISPR-Cas systems are not highly prevalent, and only a few of them have been experimentally demonstrated. A CRISPR Finder analysis (57) of the 115 sequenced *S. aureus* genomes present in the CRISPRdb (http://crispr.i2bc.paris-saclay.fr) showed that the majority of CRISPR-like loci contain only a few spacers (1 or 2), a few have between 3 and 10 spacers, and only 1 genome (from methicillin-resistant *S. aureus* [MRSA] 08BA02176, isolated from a patient) has 15 spacers. Using CRISPRone (http://omics.informatics.indiana.edu/CRISPRone), several genes belonging to the type III-A CRISPR system (including Csm2, Cas1, Cas2, and Cas6) (Fig. 3a) have been recently predicted (72) in different isolates (MSHR1132, JS395, CIG290, and 21252). Multilocus sequence typing performed on one of these isolates (MSHR1132) has shown that it belongs to a divergent clonal complex, which appears to be closely related to *Staphylococcus epidermidis* and *Staphylococcus lugdunensis* (75), and which has been renamed *Staphylococcus argenteus* (76). It has thus been hypothesized that CRISPR/cas was present in a common ancestor of *S. epidermidis*, *S. lugdunensis*, and *S. aureus* and was later lost in most conventional *S. aureus* strains. It is noteworthy that numerous repeat-spacer-like structures resemble CRISPR elements but lack spacer diversity and have been classified as false-CRISPR (72). *S. aureus* repeat-like elements (GC-rich direct repeats) belong to this class of RNAs, for which the functions remain to be addressed (77, 78).

More recently, a type II-C CRISPR-Cas system was found in an MRSA strain isolated from an Irish patient (Fig. 3b). This CRISPR element is located on a pseudo staphylococcal cassette chromosome *mec* (SCC*mec*) composite island, which was probably horizontally acquired from an *S. epidermidis* strain (79). The peculiarity of this system (SaCas9) is that it contains a shorter version of Cas9 protein. The crystal structure of a complex containing SaCas9, the sgRNA (single-guide RNA; an artificial fusion product of a crRNA and a tracrRNA [71]) and its target DNA provided a model to understand how crRNA and tracrRNA guide Cas9 on the target DNA and prepare it for double-stranded DNA cleavages (80). In this structure, the DNA duplex of the protospacer-adjacent region contains the signals recognized by the Cas9 protospacer-adjacent region-interacting (PI) domain, which discriminates the invader DNA against self-DNA and facilitates unwinding of the target DNA, leading to the formation of a heteroduplex. The beginning of the RNA-DNA heteroduplex ("seed" region) adopts a distorted structure critical for Cas9-catalyzed DNA cleavage. This conformation results from the RNA-DNA helix structure, the interactions with protein residues, and the RNA helix formed by the repeat/antirepeat regions mimicking the tracrRNA-crRNA interactions (Fig. 3c). The discovery of the smaller Cas9 protein led to recent improvements in genome editing (81). The seminal works of Jennifer Doudna and Emmanuelle Charpentier on the use of CRISPR-Cas systems for genome editing have inspired many studies showing the incredible potency of the system (82–84). Thousands of publications have reported the use of *Streptococcus pyogenes* Cas9 (85) directed by the sequence of a sgRNA for site-specific genome modifications, gene knockouts or replacements, gene expression control, and functional genome screenings in over 40 species. Interestingly, SaCas9 has been successfully used for eukaryotic genome editing since its smaller size makes it easier to be delivered via adeno-associated virus vectors to somatic tissues (81).

The fact that CRISPR-Cas has been mapped on mobile elements in MRSA confirms the importance of horizontal gene transfer from other cocolonizing bacteria in the acquisition of novel functions and in the evolution of *S. aureus* strains. Interestingly, transcription of the CRISPR-Cas genes can be highly regulated and induced upon infection (86–88) by membrane stress (89) and, in Gram-negative bacteria, by quorum sensing signaling (90, 91). Recent work has developed genetic engineering tools to apply the CRISPR/Cas9 system as an antimicrobial strategy against *S. aureus* (92).

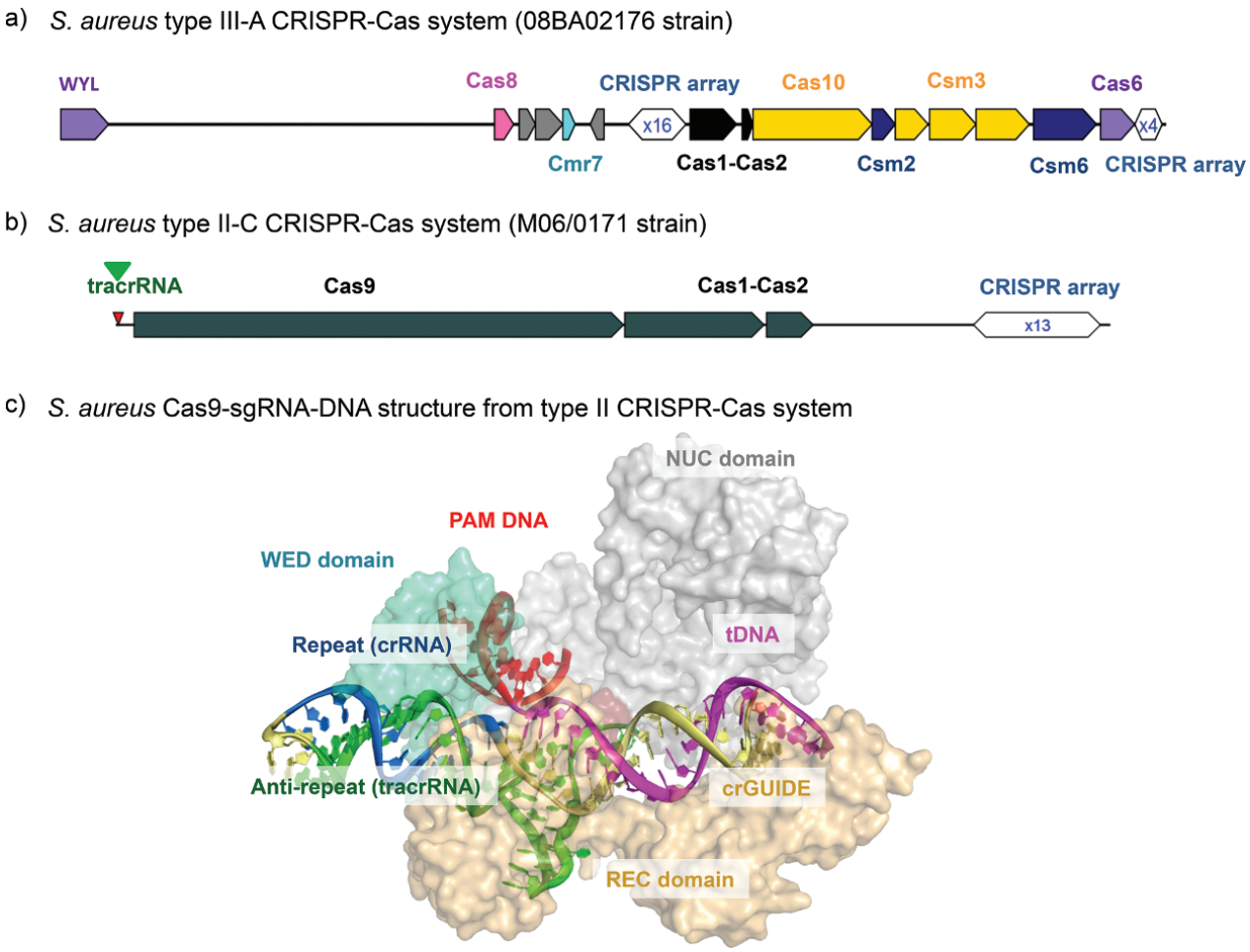


FIGURE 3 (a) Genomic organization of the loci for the type III-A CRISPR system of *S. aureus* strain 08BA02176. Type III is the typical *S. aureus* CRISPR organization. The scheme was obtained using CRISPRone (72), and the genome sequence was deposited in GenBank (accession number 08BA02176; RefSeq accession number GCF_000296595.1). (b) Genomic organization of the loci for the type II-C CRISPR system of *S. aureus* strain M06/0171. The CRISPR-Cas genes were found on an SCC*mec* inserted into the 3′ end of the chromosomally located *orfX* gene. The scheme was obtained using CRISPRone (72), and the SCCmec sequence was deposited in GenBank (GenBank accession number HE980450.1). (c) Cartoon (RNA and DNA) and surface (Cas9) representations of the SaCas9-sgRNA-target DNA complex (pdb file 5AXW) (80). The SaCas9 sgRNA consists of the crRNA guide region (crGUIDE represented in pale yellow) forming a heteroduplex with the target DNA strand (tDNA in magenta) and the repeat/antirepeat helix (blue, the repeat crRNA-derived strand, green, the antirepeat trascrRNA-derived strand). The protospacer adjacent region-containing DNA duplex is red. Cas9 domains are colored as follows: cyan, WED domain; pale orange, REC domain; gray, NUC domain. Molecular graphics images were prepared using PyMol.

Clearly, the CRISPR/Cas9 system offers an alternative therapy to conventional antibiotics.

ROLE OF sRNAs IN PHYSIOPATHOLOGY

S. aureus pathogenesis can take different forms depending on the infected tissue and the invading bacterial strain. This is often accompanied by the expression of various virulence factors involved in the colonization and the alteration of the tissue but also by the capacity to escape from the host immune response. Among key regulators of virulence, several sRNAs have been shown to modulate the synthesis of virulence factors in a dynamic manner, and some of them contribute to specific aspects of bacterial virulence in animal models of infection (1).

The most studied RNA in *S. aureus*, *agr*-RNAIII, is the main intracellular effector of the quorum sensing system *agr*. As described above, RNAIII regulates expression of virulence factors known to be associated with infectious diseases. For instance, it represses the synthesis of protein A, which triggers inflammatory signaling pathways and contributes to evasion of the immune response. Conversely, RNAIII induces the synthesis of a battery of toxins, which contribute to the degradation of tissues and subversion of host defenses, such as the pore-forming toxins, and peptides with proinflammatory and lytic activities. Recent modeling of the quorum sensing system and of its regulators has illustrated the importance of the *agr* system in promoting dissemination of the bacteria from biofilms or dense populations (93, 94). Nevertheless, despite the fact that many clinical isolates from

acute infections express RNAIII, its steady state level varies considerably among them (95, 96). In particular, a higher level of RNAIII has been observed in the community-acquired MRSA strains with increased virulence compared to other *S. aureus* lineages (97, 98). Interestingly, a recent study showed that the level of RNAIII is lower in strains isolated from patients with sepsis than in those from commensal carrier patients (99). Perhaps more surprisingly, heterogeneity has been reported in patients in which *agr*-positive and *agr*-negative strains coexisted. This has been proposed as a factor that might modulate the outcome of the infections (100, 101).

S. aureus is also frequently exposed to other microbes during colonization and infection, providing opportunities to acquire mobile genetic elements that contribute to the evolution of the genome. Some of these genomic islands play key roles in pathogenesis through their possession of new virulence factors (pathogenicity islands) or through the synthesis of novel regulators modulating the expression of genes of the core genome. As an example, SprD is an important small regulatory sRNA (142 nucleotides) expressed from a pathogenicity island, which significantly promoted *S. aureus* diseases in a mouse sepsis model of infection (102, 103). SprD interacts through base-pairings with the *sbi* mRNA, which encodes an immune evasion molecule protecting the bacteria from the host immune responses (104, 105). However, the phenotype of the Δ*sprD* mutant strain was not linked to the SprD-dependent regulation of *sbi* since the Δ*sbi* mutant strain behaved like the wild-type strain in the mouse sepsis model (102). Therefore, these data strongly suggest that SprD might regulate the expression of other proteins important for infection.

SSR42 (for small stable RNAs) is an 891-nucleotide-long sRNA whose stability is greatly enhanced in the stationary phase of growth (106, 107). It regulated the expression of approximately 80 mRNAs in 2 genetically different *S. aureus* strain backgrounds. While it increased the expression of capsule Cap5a, SSR42 downregulated the expression of protein A, α and γ hemolysin, and Panton-Valentin leukocidin (107). Because no direct binding was evidenced between SSR42 and mRNAs encoding virulence determinants, the effect was predicted to be indirect through the modulation of the expression of a transcriptional regulator. Phenotypically, the deletion of the SSR42 gene affected erythrocyte lysis, resistance to opsonization killing, and pathogenesis in a murine model of skin and soft tissue infections (107). More recently, SSR42 was identified as an important effector of intracellular virulence by screening of a transposon mutant library pool. After internalization in epithelial cells, the ΔSSR42 mutant strain was significantly enriched in the intracellular fraction, most likely due to an attenuated cytotoxicity (108).

In contrast to RNAIII, SprD, and SSR42, which contribute to enhanced virulence of *S. aureus*, other regulatory RNAs behave as attenuators of virulence. This is, for instance, the case of another encoded pathogenicity island sRNA, the so-called SprC (103). Indeed, the virulence of the isogenic strain lacking SprC was significantly and reproducibly enhanced in a mouse systemic model. Furthermore, SprC reduces *S. aureus* susceptibility to phagocytosis by human monocytes and macrophages (41). Another example is the *psm-mec* RNA, which is a bifunctional RNA located in the SCC*mec* mobile genetic element. It encodes a phenol-soluble modulin (PSMmec) cytolytic toxin and acts as a translational repressor through direct binding with *agr*A mRNA (109). The transcription of all *psm* genes is positively regulated by AgrA. This activation is linked to a specific binding of the phosphorylated form of AgrA to the promoter sequences upstream of the *psm* genes, except that this binding was not yet demonstrated for the *psm-mec* gene (reviewed in 110). The deletion of the *psm-mec* RNA increased the expression of AgrA, which resulted in an increase of toxin and PSMα production and enhanced virulence in mice (109). Interestingly, community-acquired MRSA that does not carry the *psm-mec* gene has been shown to be more virulent than the hospital-associated MRSA that harbors the gene (109, 111). Finally, a mutant strain that did not express the sRNA, RsaA, was attenuated in the severity of acute systemic infection in a mouse model (47). This deletion is linked to the deregulation of MgrA, a master regulator of capsule synthesis and clumping (112). This phenotype in pathogenesis is probably linked to the high sensitivity of the mutant strain to opsonophagocytosis by host polymorphonuclear leukocytes. Because the expression of these three sRNAs is detrimental for bacterial spreading within colonized host organisms, one may suggest that during evolution they have favored commensalism with the host.

To evaluate the impact of sRNA expression in the context of host infection, two studies explored the possible relationships between infection severity and RNA expression levels. In the first study, the expression levels of five sRNAs (RNAIII, RsaA, RsaE, RsaG, and RsaH) were analyzed in samples from acute cutaneous infection, cystic fibrosis sputum, or nasal colonization. The expression profiles did not correlate with the type of infection, but the authors noticed that the expression of these five RNAs was more homogeneous in the nasal colonization isolates than in those responsible for infection (96). More recently, the expression levels of RNAIII and SprD were measured in 40 strains cultivated from patients with sepsis or septic shock and compared to 21 strains isolated from asymptomatic colonized carriers. It appeared that strains from septic shock had significantly lower levels of RNAIII and to a lesser extent for SprD (99). It is important to note that this analysis was performed on clinical isolates cultured *in vitro* and does not necessarily reflect the expression of these RNAs during infection within the host. In fact, it is very difficult to obtain reliable data from *in vivo* studies to assess the role and importance of RNAs in the establishment or evolution of infection. The great variability of *S. aureus* strains, the difficulty in obtaining highly controlled cohorts of patients, and the reliability of sampling protocols, sample processing, and RNA expression analysis are all obstacles to be overcome. Moreover, biological variables may influence the analysis since the relationships between host immune systems and microbes seem to be particularly individualized and can influence the disease outcome (113, 114). Furthermore, interspecies interactions between bacterial pathogens and the commensal microbiota, as well as limited nutrients, play major roles in promoting or preventing *S. aureus* colonization (113). Interestingly, it was shown that the *agr* system is repressed by high concentrations of hemoglobin in the nasal fluids, leading to the expression of several cell surface proteins and favoring nasal colonization (115). Similar data were obtained when *S. aureus* was cocultivated with the nasal strain of *Corynebacterium striatum* (116). A recent study also demonstrated that the commensal *S. epidermidis* can influence the expression of one ncRNA of *S. aureus* (117).

Clearly, we are just beginning to better appreciate the roles of regulatory sRNAs during colonization and in the pathophysiology of *S. aureus* infections.

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The Staphylococcal Cell Wall

RITA SOBRAL AND ALEXANDER TOMASZ

36

HISTORICAL OVERVIEW

Ever since the recognition of the cell wall in the late 1940s to early 1950s as a unique anatomical component of all eubacterial cells, *Staphylococcus aureus* has often served as the Gram-positive model in wall-related studies. One of the first demonstrations that bacterial cell walls can be isolated as physical entities with the size and shape of the whole bacterium was with *S. aureus*. It was in penicillin-treated *S. aureus* that the UDP-linked amino sugar-containing wall precursor peptides were discovered, providing the first insights into the unique building blocks of cell wall biosynthesis. The history of interest in the staphylococcal cell wall also reflects the history of success and failure of the antibiotic era. The clue that eventually led to the discovery of penicillin (and later to the autolytic enzymes) was provided by the lysis of staphylococcal colonies in the vicinity of a mold contaminant on an agar plate in Fleming's laboratory. Elucidation of the mode of action of several important antibiotics in the 1960s and 1970s has been intimately linked to studies of the biosynthesis of staphylococcal cell walls. This included studies of the mode of action of penicillin and other β-lactam antibiotics as specific inhibitors (acylating agents) directed against the active site of penicillin binding proteins (PBPs)—transpeptidases with or without an additional transglycosylase function—which catalyze terminal stages in the assembly of the bacterial cell wall. It was mainly from studies of *S. aureus* and from parallel studies of *Escherichia coli* that by the early 1980s a coherent picture emerged of the biosynthetic pathway that leads to the formation of the lipid-linked disaccharide pentapeptide, which with some structural variations, is the universal building block of cell wall peptidoglycan in both Gram-positive and Gram-negative bacteria. Reviews and references summarizing various aspects of studies of the cell walls of staphylococci up to the late 1980s are available (1, 2).

Changing Image of Cell Walls

During the early antibiotic era, biochemists and microbiologists working with cell walls viewed these as more or less inert exoskeletons essential to withstand the turgor pressure of cytoplasm. However, these views are changing rapidly with the recognition of the complexity of chemical structure and biosynthetic pathways and the large number of genetic determinants involved with the synthesis of the cell wall. Cell walls are also intimately involved with host-related functions of staphylococci (see "Complex Functions of Cell Walls" below). The new image of cell walls emerging is that of a dynamic and very live structure. The assembly and replication of cell walls in dividing cells pose some of the most challenging questions of microbial cell biology, surpassing in complexity the questions of chromosome replication. How is the flow of precursor molecules and their polymerization on the outer surface of the plasma membrane controlled? What controls the unique species-specific chemical composition of peptidoglycan? What principles govern the organization of wall polymers into supermolecular sacculi which have the same size and shape as the particular bacterium? How and by what mechanism does the nascent innermost layer of this envelope mature while moving outward toward the cell surface? How and why and through what signals and by what catalysts are the outermost layers of cell wall shed into the medium during wall turnover? And how is the spatial and temporal accuracy of wall synthesis coordinated with cell division? Although large and successful efforts have been made in the past decade to provide answers to these questions, the scope of these key issues remains the roots of priority research lines.

ABOUT THIS REVIEW

In addition to the reemergence of interest in cell walls in the context of modern microbial cell biology, some approaches have been making great impact on discoveries in this field: in addition to high-resolution analytical techniques (high-performance liquid chromatography [HPLC] and mass spectrometry) and the application of molecular genetic approaches, more recently, cryo-electron microscopy, confocal fluorescence microscopy, superresolution microscopy, and nuclear magnetic resonance spectroscopy are contributing to a clearer picture of the ultrastructure of the Gram-positive cell envelope. Comprehensive coverage of all the data in a period of such rapid expansion of a field would be difficult. The purpose of an updated review may be better served by putting the interested reader "on track" of some of the most recent findings and emerging trends by quoting the relevant literature. The structure of the article will be as follows. After a brief reminder of the anatomy of staphylococcal cell walls, new information will be reviewed under four headings: –"High-Resolution Analysis of the Staphylococcal Peptidoglycan," "Variation in Peptidoglycan Composition," "Genetic Determinants and Enzymes in Cell Wall Synthesis," and "Complex Functions of Cell Walls."

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ANATOMY OF THE *S. AUREUS* CELL WALL

Most clinical isolates of *S. aureus* express on their outermost surface one of the 13 chemically different capsular polysaccharides that have been identified so far (3, 4). The chemical subunit structures of these important and often antiphagocytic carbohydrate polymers have been elucidated, and rapid advances are being made in the identification of genetic determinants and the organization of capsular loci (5). Underneath these somewhat varied surface layers is the staphylococcal cell wall. In electron-microscopic thin sections followed by heavy metal staining, the cell wall appears as a triple layer: a diffusely staining middle layer sandwiched between two electron-dense lines (Fig. 1). The electromicrograph in Fig. 1 also shows the effect of inhibited cell wall turnover (Fig. 1B) resulting in the accumulation of large amounts of unstructured cell wall material on the cell surface of a vancomycin-resistant clinical isolate of *S. aureus* (6). The photograph of the isogenic vancomycin-susceptible parental cell recovered from the same clinical source is shown on the left side of the figure. Cell wall thickening is often the phenotype of *S. aureus* with vancomycin-intermediate *S. aureus* (VISA)-type vancomycin resistance (7).

Earlier studies established basic compositional/structural features of the *S. aureus* cell wall. It is composed of a highly cross-linked A3α-type peptidoglycan with pentaglycine oligopeptide units connecting the ε-amino group of the lysine component of one muropeptide to the penultimate D-alanine of another. This peptidoglycan, together with ribitol-type teichoic acid chains [which are attached to the 6-hydroxyl groups of some of the *N*-acetylmuramic acid residues of the glycan chain through linkage units, (glycerol phosphate)3-*N*-acetylmannosaminyl beta(1→4)N-acetylglucosamine] (8, 9), surrounds the *S. aureus* cell in the form of a multilayered envelope (Fig. 2). The peptidoglycan of *S. aureus* is typically 20 to 30 nm thick and is characterized by short glycan strands (5 to 25 disaccharide units) (10) and a high degree of cross-linking (~90%) (11), with striking specific structural characteristics that contrast not only with the peptidoglycan of Gram-negative bacteria but also with that of other Gram-positive model organisms (Fig. 3) (12). This review article only presents information on the peptidoglycan component and does not address teichoic acids.

HIGH-RESOLUTION ANALYSIS OF THE *S. AUREUS* PEPTIDOGLYCAN

Progress in the high-resolution chemistry of the *S. aureus* cell wall came from the introduction of HPLC and mass spectrometric methods (13) for the analysis of the primary structure of peptidoglycan. First, studies with gel permeation HPLC established the presence of muropeptide oligomers in lengths extending to nanometers and beyond (14). This was followed by the adaptation of the reverse-phase HPLC system in combination with mass spectrometry for the analysis of strains of *S. aureus* (11, 15). Analysis of the peptidoglycan of methicillin-resistant *S. aureus* (MRSA) provided the first high-resolution view of the complexity of cell wall structures in Gram-positive bacteria.

Enzymatic hydrolysis (with the M1 muramidase) of the staphylococcal peptidoglycan was followed by reduction and separation of the muropeptide components on a reverse-phase HPLC column. This method resolved the hydrolysate to more than 21 distinct UV-absorbing peaks plus a "hump" of unresolved material of longer retention times which made up more than 25% of the muropeptides (Fig. 4A).

Analysis of muropeptide components obtained after digestion with muramidase, or with the combination of muramidase plus lysostaphin (a bacteriolytic endopeptidase that attacks the pentaglycine bridge), revealed that the major monomeric building block is a disaccharide pentapeptide carrying D-isoglutamine in position 2, an intact D-alanyl-D-alanine carboxyterminal, most frequently with a pentaglycine substituent attached to the ε-amino group of the lysine residue (muropeptide 5) or, occasionally, without it (muropeptide 1). These monomeric units make up about 6% of all muropeptides. Another 20% consist of dimers in which

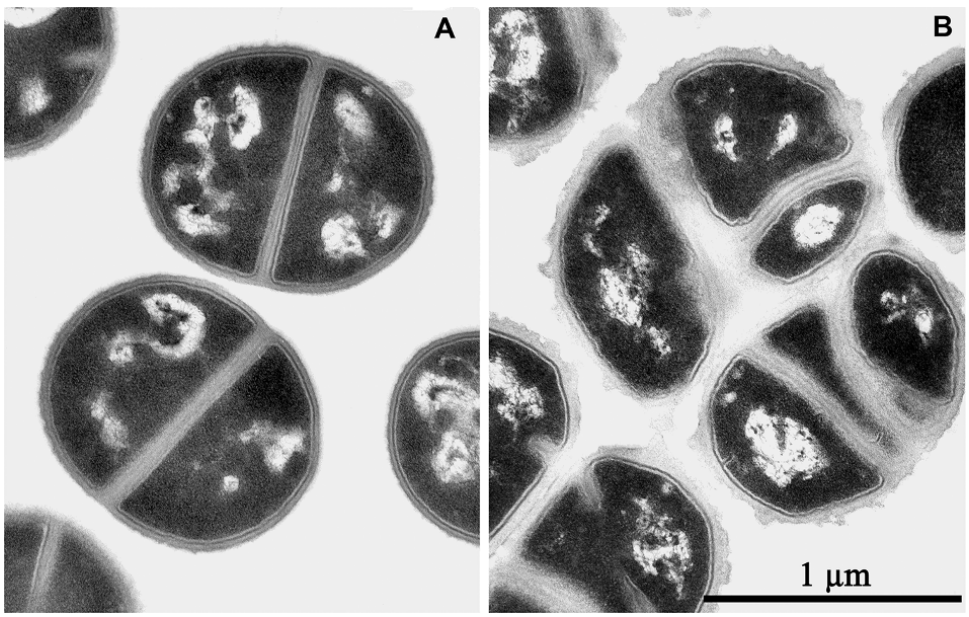


FIGURE 1 The anatomy of cell walls in normal (A) and vancomycin-resistant (B) *S. aureus*. Reproduced with permission from reference 32.

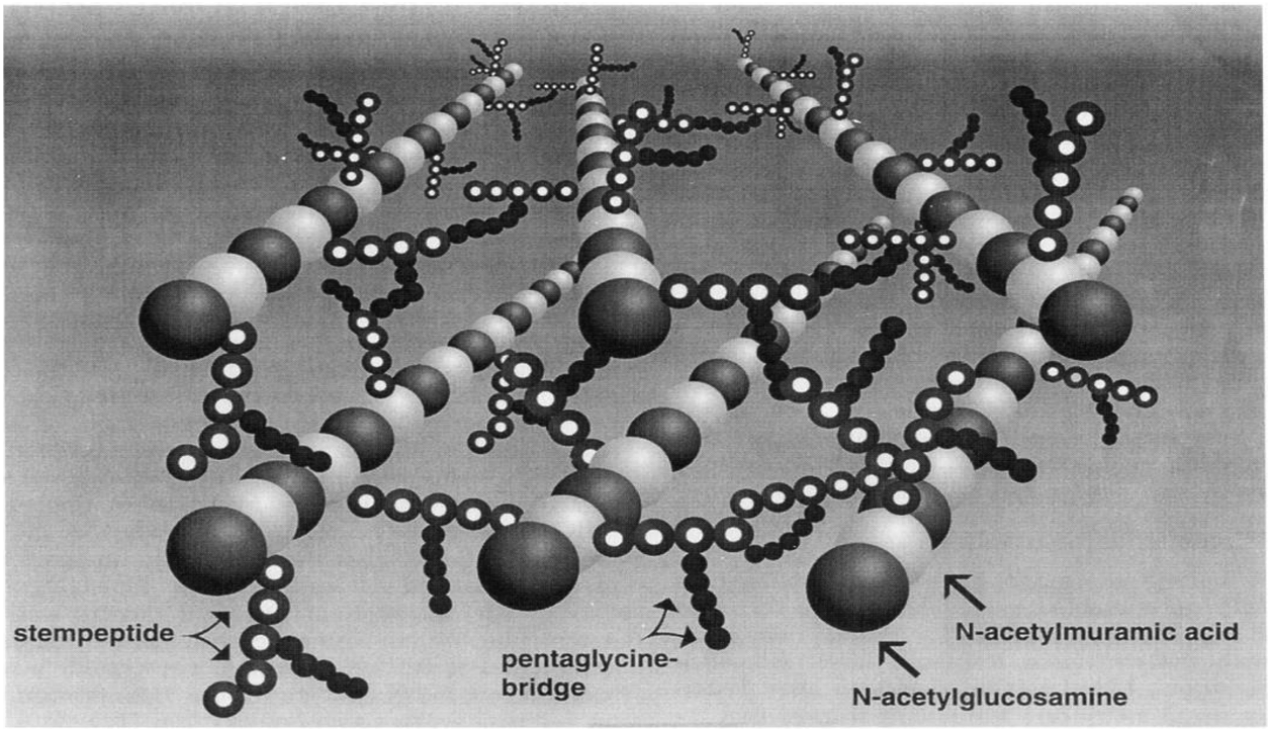


FIGURE 2 Three-dimensional structure of staphylococcal peptidoglycan. Straight lines of large globes represent sugar moieties of the peptidoglycan. Each globe in these lines symbolizes an amino sugar, *N*-acetylglucosamine (black globe), or *N*-acetylmuramic acid (white globe). Stem peptides, branching from *N*-acetylmuramic acid, are characterized by small dark globes with a white center. The connecting interpeptide bridges (pentaglycines) between the stempeptides are shown as small black globes. Schematic drawing by Peter Giesbrecht, Thomas Kersten, Heiner Maidhof, and Jorg Wecke; Robert-Koch Institute, Berlin, Germany. Reproduced with permission.

the pentaglycine substituent of one muropeptide unit is cross-linked to the penultimate D-alanine of a neighboring one (muropeptide 11). About 40% consist of higher oligomers containing 3 (muropeptide 15) to 9 muropeptide units generated by the same cross-linking principle. Still higher oligomers account for an additional 15 to 25% of the muropeptide units (the hump of unresolved components eluting with retention times longer than 110 min.).

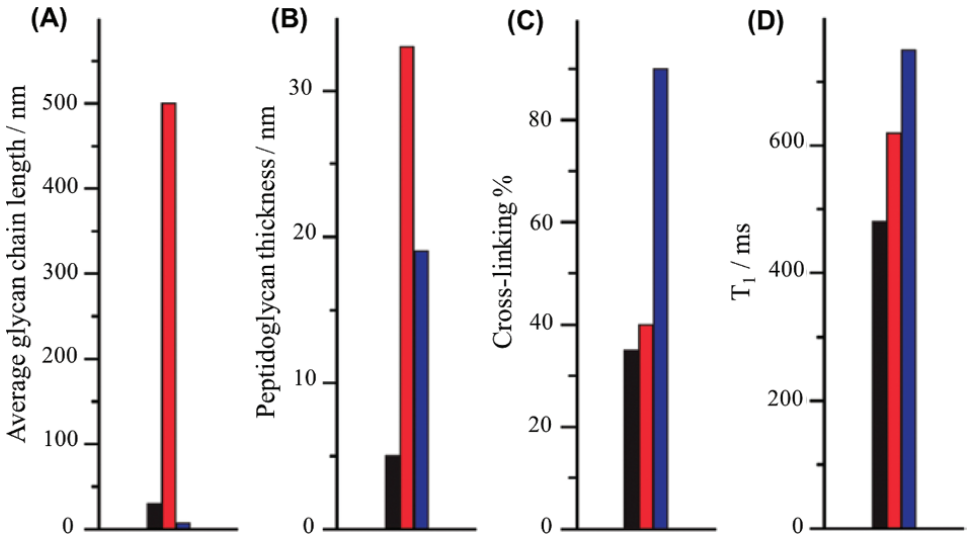


FIGURE 3 Variation of peptidoglycan global structural parameters between *S. aureus* (blue), *B. subtilis* (red), and *E. coli* (black) (164). (A) Chain length, (B) peptidoglycan thickness, (C) percentage of peptidoglycan cross-linking, and (D) relaxation time-constants, with shorter values indicating a higher peptidoglycan flexibility. Hence, the peptidoglycan of *S. aureus* is the most rigid of the three bacterial species. Reproduced with permission from reference 12.

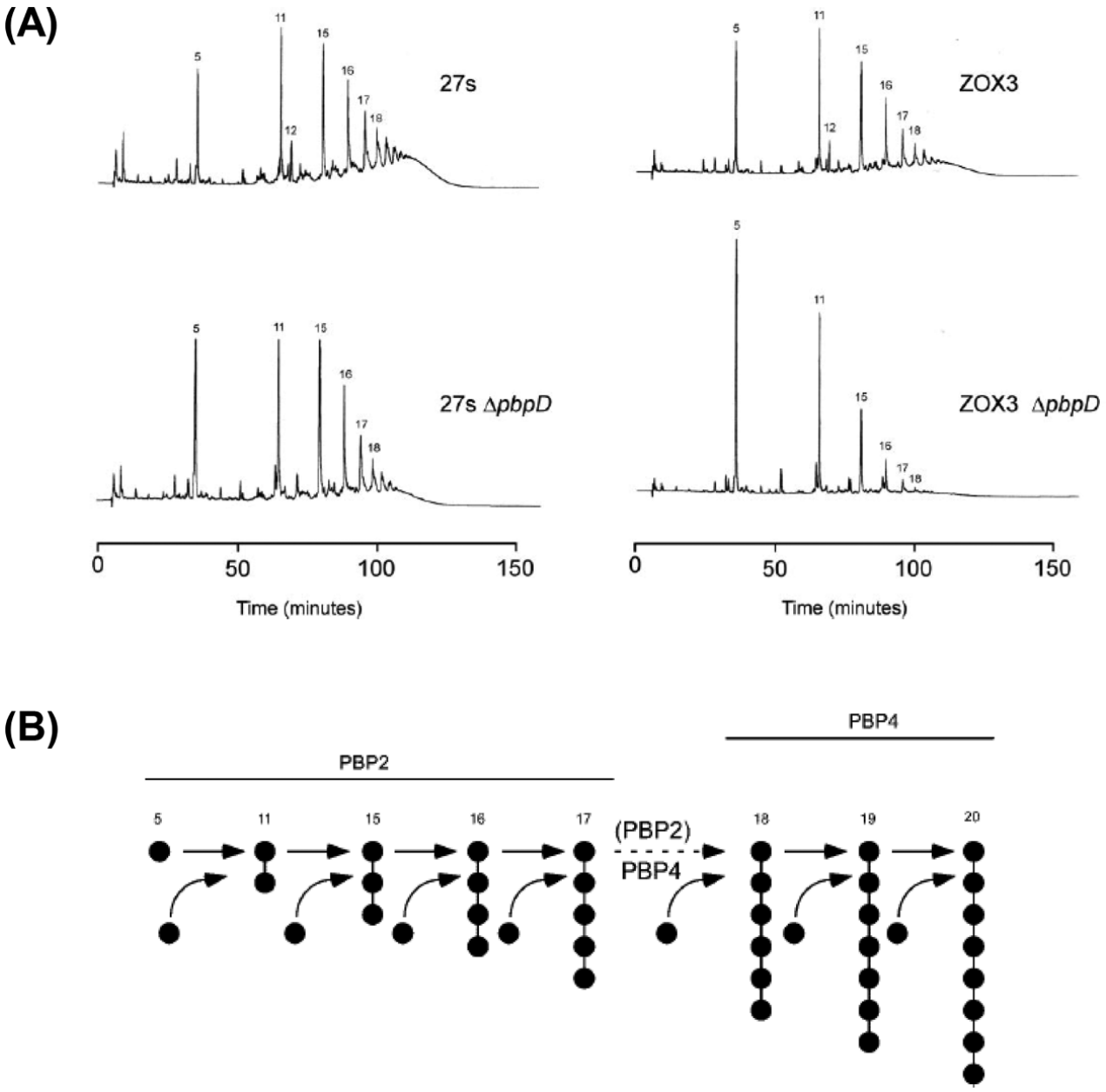


FIGURE 4 (A) Analysis of the peptidoglycan of methicillin-susceptible *S. aureus* strain 27s and its isogenic derivatives ZOX3 (PBP2 TPase point mutant), 27s*ΔpbpD* (PBP4 deletion mutant), and ZOX3 *ΔpbpD* double mutant. Numbers above the HPLC peaks identify the structure of the respective muropeptides. Peaks 5, 11, 15, 16, and 17 represent the monomeric muropeptide and its di-, tri-, tetra-, and pentameric derivatives, respectively. The poorly resolved part ("hump") of the HPLC profile eluting after peak 18 contains highly cross-linked oligomeric components. (B) Model for the cooperative functioning of PBP2 and PBP4 for the cross-linking of the *S. aureus* peptidoglycan. Muropeptides are identified by the respective peak numbers in the above HPLC chromatograms. Monomers are indicated by solid circles, and the bead diagram symbolizes the number of monomeric units cross-linked. The primary TPase activity of PBP2 is suggested to involve the cross-linking of muropeptides of lower oligomerization degree (up to pentamers), and PBP4 is suggested to catalyze the cross-linking of higher multimers. Reproduced with permission from reference 55.

A variation in the preparative technique also allows determination of the structure of the glycan chains. In this case, peptidoglycan is first hydrolyzed with lysostaphin to disrupt the pentaglycine bridges connecting neighboring muropeptides, followed by removal of stempeptides by the pneumococcal amidase (*N*-acetylmuramyl-L-alanine amidase), which hydrolyzes the covalent bond connecting the L-alanine residue to the acetyl muramic acid residue of the glycan chain. After removal of the stempeptides, the size distribution and composition of the glycan chains is determined by reverse-phase HPLC. Application of this method has resolved the glycan strands of *S. aureus* to a family of major peaks which represent oligosaccharides composed of repeating dissacharide units (*N*-acetylglucosamine beta-1,4-*N*-acetylmuramic acid) with different degrees of polymerization and terminating with *N*-acetylmuramic acid residues at the reducing ends. The method allowed separation of strands up to 23 to 26 disaccharide units. Minor satellite peaks were

also present throughout the HPLC elution profile, most likely representing products of *N*-acetylglucosaminidase activity (10).

Stability of Muropeptide Composition

Analyses of peptidoglycan prepared from a large number of *S. aureus* isolates of different clonal types, either susceptible or resistant to methicillin and grown without antibiotic challenge, all showed virtually identical HPLC muropeptide patterns (A. Ornelas-Soares, H. de Lencastre, and A. Tomasz, unpublished observations). Selective Tn*551* inactivation of the *mec*A gene did not cause any detectable change in muropeptide composition (15). These data suggest that similar to other bacteria, the muropeptide composition of *S. aureus* cell walls is specific for the species.

Ultrastructural Analysis of the *S. aureus* Peptidoglycan

Knowledge of the primary structure of peptidoglycan and other cell wall components was more recently complemented by structural data provided by high-resolution approaches. Cryo-electron microscopy has allowed a change of the previous simple concept of a thick and undifferentiated cell wall. The substitution of chemical approaches of fixation by rapid freezing as a physical method with simultaneous chemical fixation allowed observation of the cell envelope organization, namely, the identification of a low-density inner wall zone limited by the membrane and a high-density outer wall zone. The low-density region, proposed to be a periplasmic space, lacks cross-linked polymeric structures and harbors soluble components, while the high-density region consists of an intricate net of peptidoglycan and teichoic acids. Furthermore, the cell wall structure is differentiated at the septum, with two high-density zones corresponding to nascent cross walls separated by a wide low-density mid-zone, suggested to result from the activity of autolysins, separating daughter cells during cell division (16, 17).

Another step forward on the definition of the structure of the peptidoglycan of *S. aureus* was possible due to solid-state nuclear magnetic resonance (NMR) spectroscopy of fully hydrated cell wall material. First, this method showed that the peptidoglycan structure is not altered by the removal of teichoic acids, validating the established HPLC-based peptidoglycan analysis (12). Also, it corroborated the longtime notion that glycan strands are more rigid than peptide stems and that the peptidoglycan of *S. aureus* is more rigid than that of *E. coli* or even *Bacillus subtilis*. This decrease in dynamics is directly related to the higher degree of cross-linking of *S. aureus* peptidoglycan, which compensates for the shorter glycan strands and the presence of interpeptide bridges (12), that should enhance peptidoglycan flexibility. Furthermore, rotational-echo double-resonance NMR, allowed measurement of bridge-glycan distances and determined that the pentaglycyl bridge is in close proximity (5 Å) of the neighboring glycan chain, suggesting a three-dimensional model in which the peptide stems are in a plane perpendicular to the glycan chain and parallel to each other. The study was widened to include *fem* cell wall mutants affected in the glycine interpeptide bridge and VISA strain Mu50 (18). More recently, the same approach was used to study the effects of glycopeptides on the biosynthesis of peptidoglycan and teichoic acids (19).

Fluorescence microscopy and, more recently, superresolution microscopy have driven important breakthroughs on cell morphology and division patterns. In fact, *S. aureus* cells were revealed not to be spherical throughout the cell cycle and to include cell elongation steps that depend on peptidoglycan synthesis and remodeling, allowing for new models of *S. aureus* cell division to be proposed (20–23).

VARIATION IN PEPTIDOGLYCAN COMPOSITION

Effect of Antibiotics

Growth of the highly methicillin-resistant *S. aureus* strain COL in a wide range of subinhibitory concentrations of methicillin caused striking changes in muropeptide composition: the representation of trimeric plus higher oligomeric components was drastically reduced (from a combined representation of close to 50% to less than 10%), while the proportion of monomeric and dimeric components increased (from about 15% to about 50%). It was suggested that the abruptness of this change in peptidoglycan composition may represent the switching over from the normal wall biosynthetic system (the four native PBPs) to another one (PBP2A, encoded by the methicillin resistance gene *mec*A) capable of functioning in the presence of high concentrations of methicillin (11). The anomalous composition of the peptidoglycan produced under these conditions would then reflect the limited capacity of PBP2A for cross-linking more than single monomeric muropeptides. Nevertheless, similar albeit much less abrupt compositional changes were also observed when methicillin-susceptible staphylococci or an isogenic derivative of strain COL (with inactivated *mec*A) were grown in sub-MIC concentrations of methicillin.

Effect of Vancomycin and Teicoplanin Resistance

Laboratory mutants with increased MIC values for glycopeptide antibiotics have been isolated (24–26). The cell wall composition was determined in a series of isogenic laboratory mutants of the highly methicillin-resistant *S. aureus* strain COL (27). The peptidoglycan of the mutants showed distorted muropeptide composition which paralleled the increasing vancomycin MIC values, and it was suggested that some of the unusual properties of these mutants and the mechanism of resistance are related to the enrichment of the cell walls in muropeptide monomers. Muropeptides terminating in the D-alanyl-D-alanine residues are known to form the binding sites for glycopeptide antibiotics. The mechanism of resistance may then be related to trapping the glycopeptides in the mature layer of the peptidoglycan enriched for muropeptide monomers, thus preventing the antibiotic molecules from reaching sites of wall biosynthesis at the plasma membrane (26, 28). Another structural change, increased glycan chain length, was also detected in a vancomycin-resistant mutant (29). Recently, the effects of glycopeptides were analyzed by rotational-echo double-resonance NMR (19). Vancomycin subinhibitory concentrations resulted in a strong reduction of D-alanine incorporation into wall teichoic acids but no changes in the peptidoglycan stem composition, suggesting that, upon glycopeptide challenge, the cell drives the available D-alanine to peptidoglycan biosynthesis at the expense of the wall teichoic acids.

VISA Isolates

Clinical isolates of *S. aureus* with reduced susceptibility to vancomycin have been repeatedly described in the literature, and in at least some of these so-called VISA isolates,

alterations in peptidoglycan composition have been observed, including increased monomeric and decreased oligomeric components, excess of cell wall material, aberrant separation of daughter cells, and altered autolysis (6, 30–32). The genomic analysis of clinical VISA isolates, together with laboratory mutants, has contributed to the understanding of the genetic and biochemical mechanisms of intermediate-level vancomycin resistance in *S. aureus*. Isogenic groups of susceptible and resistant isolates recovered from patients with a history of vancomycin therapy or laboratory strains submitted to vancomycin selection have been studied using high-throughput whole-genome sequencing to identify the altered genes (33–36). The mutated genes were commonly identified in VISA isolates, with the most frequent mutations being in the *vraRS* (37), *graRS* (38, 39), and *walKR* (35, 40) two-component regulatory systems (detailed below). The most relevant mutated genes seem to be somehow related to cell wall metabolism and autolysis (35, 37, 38), but mutations in the RNA polymerase gene *rpoB* were also described (41). Nevertheless, the genetic alterations identified in the *in vivo* VISA isolates were not the same as the ones found in laboratory-derived VISA strains (42). For comprehensive reviews, see references 43 and 44.

Vancomycin-Resistant *S. aureus* (VRSA)

In 2002, highly vancomycin-resistant strains of *S. aureus* were recovered from clinical specimens. These bacteria acquired the vancomycin resistance gene complex through the enterococcal transposon Tn*1546*. Expression of Tn*1546* produced drastic changes in the cell wall composition: all pentapeptides were replaced by tetrapeptides, and the peptidoglycan contained at least 22 novel muropeptide species that frequently showed a deficit or complete absence of pentaglycine branches. The UDP-Mur-NAc pentapeptide, the major component of the cell wall precursor pool in vancomycin-sensitive cells, was replaced by the UPD-MurNAc depsipeptide in the resistant bacteria, and PBP2A, the product of the methicillin resistance gene *mecA*, was unable to utilize such precursors for wall synthesis. The MRSA strain COL carrying *mecA* and the *vanA* genes had extremely high MICs both for β-lactam antibiotics and for vancomycin (45). The major PBP responsible for the assembly of the abnormally structured peptidoglycan produced in the vancomycin-resistant cells was PBP2 (46).

Since 2002, the number of VRSA isolates in the United States has increased to 14 (47). Thirteen of these Tn*1546*-containing *S. aureus* isolates belong to the clonal complex 5 (CC5), an MRSA lineage that has been repeatedly isolated from clinical specimens (43, 44, 47), in contrast to VISA strains that have been associated with different genetic backgrounds, including CC5, CC8, CC30, and CC45 (48, 49). The fact that VRSA strains have not become more frequent is probably related to the specificities of the clinical scenario in which they emerge. Most VRSA isolates were retrieved from diabetic wounds of patients that carried both vancomycin-resistant enterococci and VRSA (45).

GENETIC DETERMINANTS AND ENZYMES IN CELL WALL SYNTHESIS

Penicillin Binding Proteins

Tentative roles for the four native staphylococcal PBPs in wall synthesis were originally assigned on the basis of morphological/biochemical effects of β-lactams that showed more or less selective binding to individual PBPs (50). More recent studies used genetic techniques and *in vitro* transpeptidation assays with lipid II and labeled substrates to clarify the functions of PBPs; the minimal protein composition of peptidoglycan synthesis machinery was addressed by generating a mutant strain in which seven of the nine genes encoding peptidoglycan synthesis enzymes (the five PBPs, four native and one acquired; the two MTGases, MGT and SgtA; and FmtA and FmtB, which have homology to transpeptidase [TPase] domains) were deleted. *S. aureus* was found to be able to grow and divide *in vitro* with only the PBP2 and PBP1 enzymes, without morphological changes, suggesting that the other enzymes have redundant functions. Transcriptomic analysis showed that, as could be expected, the *vraSRF* operon is upregulated and several murein hydrolases are inhibited in this mutant. However, cells with this minimal set of peptidoglycan synthetic enzymes were impaired in pathogenesis and antibiotic resistance, indicating that a complete synthetic pathway is important in natural conditions (51).

Allelic replacement approaches had already established the essential nature of PBP1 (52), and later the contribution of this PBP (a high-molecular-weight monofunctional transpeptidase) to cell division and separation was explored (53, 54). PBP1 localizes to the division septum and does not depend on other peptidoglycan synthetic enzymes for correct localization (51). A PBP1 depletion mutant rapidly loses viability but maintains a normal peptidoglycan composition (53). However, analysis of a PBP1 mutant with impaired TPase activity suggested that PBP1 plays a dual essential role, because its presence, independent of the TPase activity, is required for the formation of septa, while the TPase activity—being necessary for the mechanism of cell separation (54)—can act in a timely fashion at the end of the septal growth.

Of the four native PBPs, PBP2 is the only one composed of a TPase and a transglycosylase (TGase) domain, the former being essential for growth of the bacteria. Biochemical evidence for TPase activity was obtained through the analysis of the peptidoglycan muropeptide profile of a spontaneous ceftizoxime-resistant mutant with a point mutation in the TPase domain (55). The decreased level of highly cross-linked oligomers in the mutant suggests that the primary TPase function of PBP2 is in producing muropeptide dimers (Fig. 4A, peak 11), trimers, tetramers, and pentamers (peaks 15 through 17).

Studies of conditional mutants of *pbpB* demonstrated that this essential function of PBP2 may be replaced by PBP2A, the product of the acquired drug resistance gene *mecA* (56). The TGase domain of PBP2 was shown to cooperate functionally with PBP2A when the bacteria were growing in the presence of antibiotics (57). Critically located point mutations in *pbpB* were associated with methicillin resistance both in laboratory mutants and among some clinical isolates that did not carry the *mecA* gene but showed low-level β-lactam resistance (58). Recently, the substrate preferences of PBP2A, PBP2, and PBP4 were compared regarding the completeness of the glycine bridge. PBP2 was found to accept penta-, as well as tri- and monoglycine-associated muropeptides as substrates of transpeptidase activity, while PBP4 exhibits different enzymatic activities according to each of the three substrates, and PBP2A does not accept monoglycine muropeptides (59).

PBP3 is a class B high-molecular-weight protein with a C-terminal penicillin binding domain showing the

conserved motifs characteristic of transpeptidases and an N-terminal nonbinding domain of unknown function. Unlike in the cases of *pbpA* and *pbpB*, inactivation of *pbpC* in either methicillin-susceptible or -resistant backgrounds allowed growth of the bacteria, and there was no change in the muropeptide composition. The only observable alteration of such mutants was a reduction in autolysis rates. However, when mutants were grown in the presence of sub-MIC concentrations of methicillin, the bacteria frequently showed aberrant morphologies (60). The possible function for this PBP in cell wall synthesis and cell division is not yet described.

PBP4 is a nonessential low-molecular-weight PBP. Bacteria with inactivated PBP4 grew normally but produced a peptidoglycan in which the highly oligomeric components were substantially reduced. An extensive deletion in the promoter region was identified in β-lactam-resistant laboratory isolates which showed increased peptidoglycan cross-linking (61). Inactivation of *pbpD* was demonstrated in a highly vancomycin-resistant lab mutant which produced peptidoglycan with greatly reduced cross-linking (28). These findings confirm earlier proposals that PBP4 can act as a secondary transpeptidase. It was recently demonstrated that PBP4 not only acts preferentially as a transpeptidase, accepting both nascent peptidoglycan and lipid II as substrates but also is the main agent responsible for the incorporation of exogeneous D-amino acids into peptidoglycan. It was proposed that this PBP may be responsible for adding cross-links to partially cross-linked peptidoglycan, primarily to pentamers and higher oligomeric muropeptides (55) and to repair peptidoglycan damage (62). Furthermore, regarding the cross-linking preferences, PBP4 is much more selective than PBP2 and shows a wider range of activities; PBP4 acts exclusively as a transpeptidase for pentaglycine muropeptides and shows 90% of hydrolytic activity with triglycine muropeptides and carboxypeptidase activity for monoglycyl muropeptides (59). Initially, PBP4 was thought not to be required for β-lactam resistance as tested in hospital-acquired MRSA strains. However, in the background of community-acquired strains it was deemed to be involved in the optimal expression of resistance (63). Using atomic-force microscopy to determine the elasticity of the cell wall, the analysis of PBP4 mutants showed that *S. aureus* cells suffer an overall enhancement in elasticity upon decrease of the cross-linking level and that this effect was stronger in the community-acquired MRSA background (64).

PBP2A—the protein product of the resistance gene *mecA*–was crystallized, allowing the identification of structural features responsible for the extremely low affinity of this protein for most β-lactam antibiotics (65). The basis of the PBP2A preference for a peptidoglycan substrate is an allosteric site (distal by 60 Å from the active site) that regulates the conformation of a structural loop guarding the active site. When the allosteric site is bound to a neighboring peptidoglycan strand, the active site becomes available to its ligand, peptidoglycan. Furthermore, the catalytic serine residue changes conformation to maintain the substrate preference toward the β-lactam molecule (66–68). Such structural insights are crucial for mechanistic studies aimed to understand the mode of action of the different β-lactam molecules and the emerging PBP2A mutations. Regarding the transpeptidase activity, it was earlier proposed that PBP2A would only cross-link stem peptides with a pentaglycine branch; however, PBP2A was recently found to also accept as a substrate triglycine-containing strands but not monoglycine ones (59).

Methicillin-Conditional Mutants

The central genetic determinant of MRSA strains is the heterologous gene *mecA*. Several lines of evidence suggest that *mecA* evolved from a housekeeping gene of *Staphylococcus sciuri* (69–72). The evolution toward a resistance determinant, promoted by antibiotic use in humans and animals, involved alterations in the promoter region, structural changes in one protein domain, and the adaptation of the genetic background of bacteria (73).

Inactivation of *mecA* results in complete loss of resistance. Nevertheless, a reduction in the resistance level from a modest decrease to a virtually complete loss of resistance can also be the consequence of inactivation of a surprisingly large number of domestic genes, over 30, which have been referred to as *fem* or auxiliary genes and are not directly involved in the expression of the *mecA* gene (74–77). The transposon mutants of methicillin-resistant *S. aureus* strain COL, which led to the identification of auxiliary genes, were originally isolated to clarify some complex genetic features of staphylococcal methicillin resistance (75, 78). Biochemical analysis indicated that in several of these mutants the inactivated genes were involved with staphylococcal cell wall synthesis. This finding suggested that a reduction in the methicillin resistance level may provide a relatively easily selectable phenotype for the identification of genes in cell wall synthesis and metabolism (75). As a working model, it was proposed that the methicillin MIC value in such "methicillin-conditional" mutants may primarily reflect the success of the *mecA* gene product (PBP2A), positioned close to the end of a long and complex metabolic pathway, to continue catalyzing peptidoglycan incorporation even when this metabolic pathway is disrupted by an inhibited auxiliary gene.

Tn*551* MRSA mutants selected for reduced MIC (such as *femXA/B*, *gluM*, *murE*, *llm*, *fmtA* to C, etc.) or specifically constructed mutants (*murF*, *murT-gatD*, etc.) have led to insights into hitherto unknown or not fully characterized steps in staphylococcal cell wall precursor biosynthesis (74–76, 79–85). More recently, the list of auxiliary genes was widened by the large-scale identification, using antisense interference conditional mutants, of essential genes required for β-lactam optimal resistance (86). This study allowed the identification of essential cell division (*ftsZ* and *ftsA*) and teichoic acid biosynthesis genes (*tarL*), among others.

The Role of the Stringent Response in the β-Lactam Resistance Mechanism

Recently, the above-described model has been challenged by a sequence of studies that culminated in the identification of the stringent response as a key player in a *mecA*-dependent mechanism of homogeneous and high-level β-lactam resistance (87–89). The stringent response is a bacterial reaction to a nutrient shortage, with the aim to economize metabolic resources. Such a response involves the transcriptional regulation by RNA polymerase and the modulation of the activity of certain proteins and RNAs through the cellular level of guanosine pentaphosphate (p)ppGpp (90). A comparative study based on whole-genome sequencing of highly resistant subpopulations of early MRSA strains with different degrees of oxacillin resistance identified mutations in 27 genes of diverse functional categories. These mutations were shown to induce high-level β-lactam resistance by triggering the stringent stress response, causing increased production of PBP2A (91). Nevertheless, the mechanism of high-level resistance induced by the stringent response corroborates the

previously established model of β-lactam resistance, which considers the essentiality of the transglycosylase activity of PBP2 (87). Moreover, auxiliary mutants were shown to regain the homogeneous resistance level of the parental strain COL when challenged with mupirocin, an isoleucyl-tRNA homologue that induces stringent stress (92).

FemAX/B

The *femX* (Fmhb), *femA*, and *femB* genes are part of an operon involved with the synthesis and attachment of the pentaglycine branches to the epsilon amino group of the lysine residue of the *S. aureus* muropeptides. The synthesis of pentaglycine bridges occurs in three sequential steps catalyzed by the FemABX nonribosomal peptidyl transferases, which cannot substitute for one another. The protein product of *Fmhb* catalyzes the addition of the first glycine residue to the muropeptides. This gene is essential for bacterial survival and growth (93). The protein product of *femA* is involved with the addition of the first and second, and the protein product of *femB*, with the addition of the third and fourth, glycine residues to the cross-bridge (83, 94). In an elegant series of studies, the entire sequence of pentaglycine branch synthesis and attachment has been determined and reproduced *in vitro* using bactoprenol-linked disaccharide pentapeptide as the acceptor and t-RNA-linked glycine as the source of the amino acid residues (95, 96). Structural NMR studies of *femAB*-deletion mutants with shortened pentaglycine interpeptides showed that the pentaglycine bridge is required to reduce the average distance between the nearest-neighbor bridge and glycan and that a poorly cross-linked cell wall may not be able to resist the cell's internal turgor. To counteract the internal osmotic stress in the absence of *femAB* genes, transcriptomic studies suggested that the cell resorts to metabolic alterations, namely, of the arginine-deiminase pathway and nitrogen metabolism (18, 97). Nevertheless, viability in the absence of *femA* and *femB* suggests that one or more of the native PBPs can cross-link stem peptides with monoglycyl or triglycyl bridges.

Secondary Modifications

Secondary modifications of the glycan strands of the staphylococcal peptidoglycan, namely, *O*-acetylation and *N*-deacetylation of *N*-acetylglucosamine, have been mostly associated with evasion of the human host. *O*-acetylation occurs at the C-6 position of muramic acid residues and is catalyzed by the integral membrane *O*-acetyltransferase OatA; this modification confers resistance to lysozyme, a muramidase present in most human biological fluids and secreted by cells of the immune system (98–100). The proposed basis of resistance is that the *O*-acetyl group sterically prevents the binding of lysozyme to peptidoglycan (101), and only pathogenic species of *Staphylococcus* have *O*-acetylated peptidoglycan (98). The stem peptide of *S. aureus* also suffers modifications to the primary structure, including the addition of the pentaglycine bridge (see previous section) and amidation of the second residue D-glutamate to D-iso-glutamine. The genetic determinants of the amidation reaction, the *murT* and *gatD* genes, were described in 2012, and the protein products form a bi-enzymatic complex, with MurT being a member of the mur ligase family and GatD a glutamine amino transferase that uses glutamine as the source of the amino group (79, 102). As for *O*-acetylation, peptidoglycan amidation is involved in lysozyme resistance, but in contrast, it is not restricted to pathogenic species but, rather, is widely distributed among Gram-positive bacteria. The MurT-GatD catalyzed reaction is essential for viability and for resistance to β-lactams, showing different levels of involvement in the resistance mechanism according to the genetic background of the strain (79, 102, 103).

The pentaglycine bridge is a modification of *S. aureus* peptidoglycan and the specific target of lysostaphin, a bacteriolytic enzyme secreted by *Staphylococcus simulans*. This species is resistant to the activity of lysostaphin since it contains serine/glycine cross-bridges, a modification catalyzed by Epr (lysostaphin endopeptidase resistance) of the FemAB protein family (104).

Insights into Regulation and Contribution of Transcriptomic Studies

Upregulation of the transcription of *pbpB*, the structural gene of PBP2, by exposure of *S. aureus* cells to oxacillin, vancomycin, and other cell wall inhibitors has been reported (105), suggesting transcriptional regulatory pathways linking the cell wall structure and metabolic functions. This first observation was corroborated and widened by a large number of transcriptomic studies using state of the art DNA microarrays to study cell wall exogenous stress conditions (37, 106–110) or cell wall mutations (37, 97, 107, 111–114). Utaida et al. were the first to describe a large set of genes, the so-called cell wall stimulon (CWS), the expression of which was activated in response to cell wall-targeting antibiotics of diverse classes, suggesting the sensing of cell wall damage (110, 115). The CWS included cell wall-associated genes such as *pbpB*, *murZ*, and *sgtB*, encoding a TGase, and *fmtA*, encoding an enzyme with TPase activity. The vancomycin resistance-associated (VraSR) two-component system, identified for being activated by vancomycin challenge, was found to induce all members of the CWS (37, 116). Upregulation of the CWS is suggested to provide resistance to VraSR-inducing agents, including β-lactams.

S. aureus contains 16 sets of 2-component regulatory systems that monitor cell wall damage and activate an appropriate physiological response; among these is not only VraSR, but also GraRS and WalKR, which interestingly were all found to be implicated in the development of VISA strains (40, 117, 118).

The VraSR system autoactivates its own expression and most CWS genes, which are suggested to contribute to the repair of the sensed cell wall damage (113, 119). VraS is an intramembrane sensing kinase that undergoes autophosphorylation upon challenge with a cell wall-targeting antibiotic and subsequently phosphorylates the VraR response regulator (120), which binds to its own promoter and activates or represses the genes of the regulon (121). The molecular signal detected is not yet defined but is suggested to be a by-product of cell wall damage since a diverse class of unrelated antibiotics was shown to induce the system. The Vra system effects methicillin resistance by a still unclear mechanism, independently of *mecA* gene (116).

The glycopeptide resistance-associated (GraSR) system confers resistance to cationic antimicrobial peptides by increasing the positive charge of the cell surface, contributing to electrostatic repulsion through the modulation of the D-alanylation of teichoic acids and of lysinylation of phosphatidyl glycerol (122, 123). A transcriptomic study showed that the GraSR system is also involved in pathogenesis, stress response, and cell wall signal transduction, and its regulon overlaps the WalKR regulon (112). This system is proposed to be a five-component system composed of GraS, an

intramembrane sensing kinase, GraR, the response regulator, GraX, a regulatory cofactor, and the VraFG ABC transporter that senses the presence of cationic antimicrobial peptides and is involved in signaling transduction (112, 124).

The two-component system, WalKR, is essential for *S. aureus* viability and is primarily involved in the regulation of cell wall metabolism by positively modulating autolytic activity through the control of the expression of most of the cell wall hydrolase genes, including the direct binding of WalR to the *atl* promoter (125). LytM autolysin seems to play a particularly important role in this mechanism by reducing the degree of cross-linking in the peptidoglycan through its endopeptidase activity toward the pentaglycine bridge (126). Mutants for this system showed enhanced biofilm formation, decreased peptidoglycan biosynthesis and autolytic activity, thickened cell walls, and reduced vancomycin susceptibility (125, 126). A role in virulence was also described for this system, because WalKR was found to trigger the host inflammatory response through the release of peptidoglycan fragments as a consequence of increased autolysis (111). A mutation in WalK sensor kinase that was also found in a clinical VISA strain was responsible for deficient autophosphorylation and concomitant reduced phosphorylation of WalR, which binds less efficiently to the *atl* promoter, impairing the control of the autolytic system (118).

Two monofunctional glycosyl transferases were identified and characterized in *S. aureus* (127, 128), and genomic analysis suggested that besides PBP2, only MGT and SgtA contain TGase motifs. MGT enzyme was shown *in vitro* to catalyze glycan chain polymerization, using lipid II as a substrate, with an efficiency of 5,800 $M^{-1}s^{-1}$ (129). All three enzymes have the capacity to elongate glycan chains, and although the monofunctional glycosyl transferases are not essential, *S. aureus* cannot survive without the TGase activity of PBP2 or MGT, indicating that the activities/specificities of MGT and SgtA are different. Both MGT and SgtA were found to interact with PBP2, suggesting the existence of a cell-wall synthetic complex in *S. aureus* (127). Expression of the *mgt* gene was found to be upregulated in the presence of cell wall antibiotics (110). However, although MGT can compensate for the lack of PBP2 TGase activity in methicillin-susceptible *S. aureus* and MRSA strains, it does not replace PBP2 upon challenge with high concentrations of oxacillin (127).

Although never fully described, several pieces of evidence support the existence of a coordinated multienzyme complex responsible for cell wall assembly. One piece of evidence was provided by the analysis of the peptidoglycan of a PBP2 TPase mutant and of its corresponding double PBP4 deletion mutant (Fig. 4A). This study allowed the description of the cooperative functioning of PBP2 and PBP4 in which the TPase activity of PBP2 is suggested to produce the substrates for PBP4, dimers, trimers, and tetrameric muropeptides, which are subsequently transformed into higher oligomers by PBP4 activity (Fig. 4B). In the same study, PBP2A was proposed to directly provide "assistance" to this PBP2-PBP4 transpeptidation system through a still undescribed mechanism (55).

COMPLEX FUNCTIONS OF CELL WALLS

The peptidoglycan is a protective barrier against the host and also a scaffold for the attachment of surface proteins and polysaccharides, which are essential for cell division and pathogenesis. The carboxyterminal end of staphylococcal surface proteins (such as protein A) are covalently bound to the oligoglycine cross-bridge of peptidoglycan muropeptide units through a threonine residue in the protein (generated after cleaving of the wall sorting signal LPXTG), which is then linked to the amino terminal glycine residue of the muropeptide. Sortase-anchored surface proteins were shown to be released into the external environment together with peptidoglycan immunostimulatory fragments linked to their C-terminus by the action of murein hydrolases (130). The structure of these cross-bridges is under the control of the *fem* gene complex. Therefore, the already pleiomorphic *femA/B* mutant (showing defective wall structure, reduced methicillin resistance, and slow wall turnover) may also be affected in virulence.

The active involvement of the cell wall in complex functions is also being recognized in studies of the transport (release and uptake) of large molecules (131). Increased susceptibility of *femA/B* null mutants to some antibacterial agents other than β-lactams may be related to increased porosity of the peptidoglycan. Increased extractability of proteins (wall porosity?) was noted in a highly teicoplanin-resistant staphylococcal mutant with greatly decreased peptidoglycan cross-linking (132).

Murein Hydrolases in Cell Division and Antibiotic Resistance

Peptidoglycan is a dynamic structure, being constantly synthesized and hydrolyzed. The cell biological functions of staphylococcal murein hydrolases and their regulation have been intensively investigated, and these studies benefited greatly from zymography for profiling bacteriolytic enzymes and from genetic work. The major autolysin gene *atl* was found to encode a bifunctional protein that undergoes proteolytic processing at the cell surface into the independent *N*-acetylmuramyl-L-alanine amidase and endo-β-*N*-acetyl glucosaminidase domains (133, 134). The still "double-headed" *atl* gene product containing the fused amidase and glucosaminidase proteins was found to be localized on the plasma membrane as a circumferential ring at the future cell divisional site, and this localization precedes the appearance of centripetally growing cell wall (135) (Fig. 5). The molecular signature that directs the autolysin gene to its site of action at the cellular equator was shown to reside within the three repeat elements present in each domain (136, 137). The presence of wall teichoic acids at the septum was described to prevent the binding of Atl domains to the septal region through an avoidance strategy (138). Atl is primarily involved in the separation of daughter cells during cell division but also contributes to cell wall turnover and antibiotic-induced lysis (139, 140).

In zymograph gels, a Δ*atl* mutant showed a single lytic band that was determined to correspond to the amidase Sle1 (141), also involved in cell separation. Besides the *atl*, *sle1*, and *lytM* genes, *S. aureus* is thought to contain 10 additional genes encoding peptidoglycan hydrolases, although their products are not biochemically characterized.

Alteration of autolytic properties has been consistently seen in staphylococcal mutants isolated for changed β-lactam resistance. Increases in autolytic rates (and cell wall turnover) in parallel with the increase in resistance level were demonstrated in cefotaxime-resistant mutants (142), while reduced rates were observed in *femA*, *femB*, and *femC* mutants. Also, the downregulation of PBP2 and PBP1 expression resulted in an associated reduction in the transcription of *atl* and *sle1*, indicating a coupling of cell wall synthesis to the autolytic enzymes at the transcriptional level (54, 143).

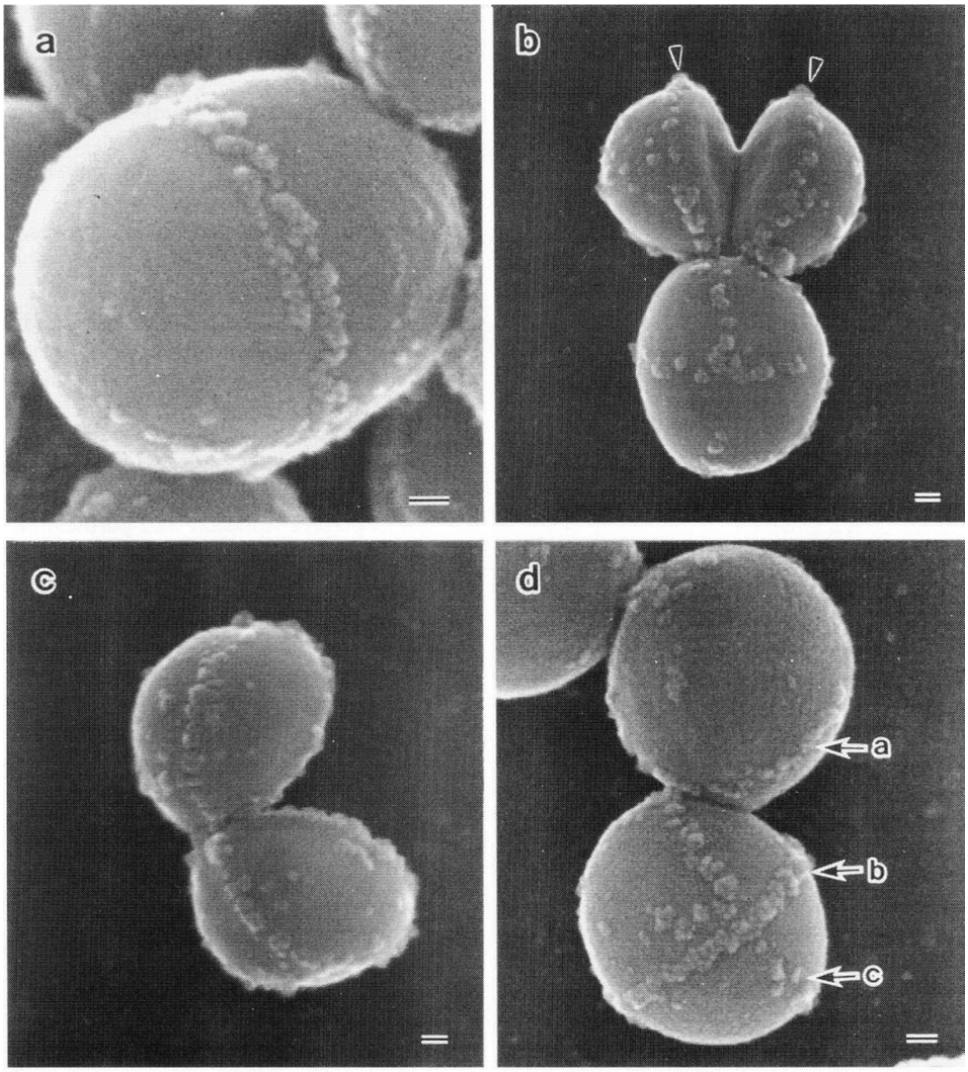


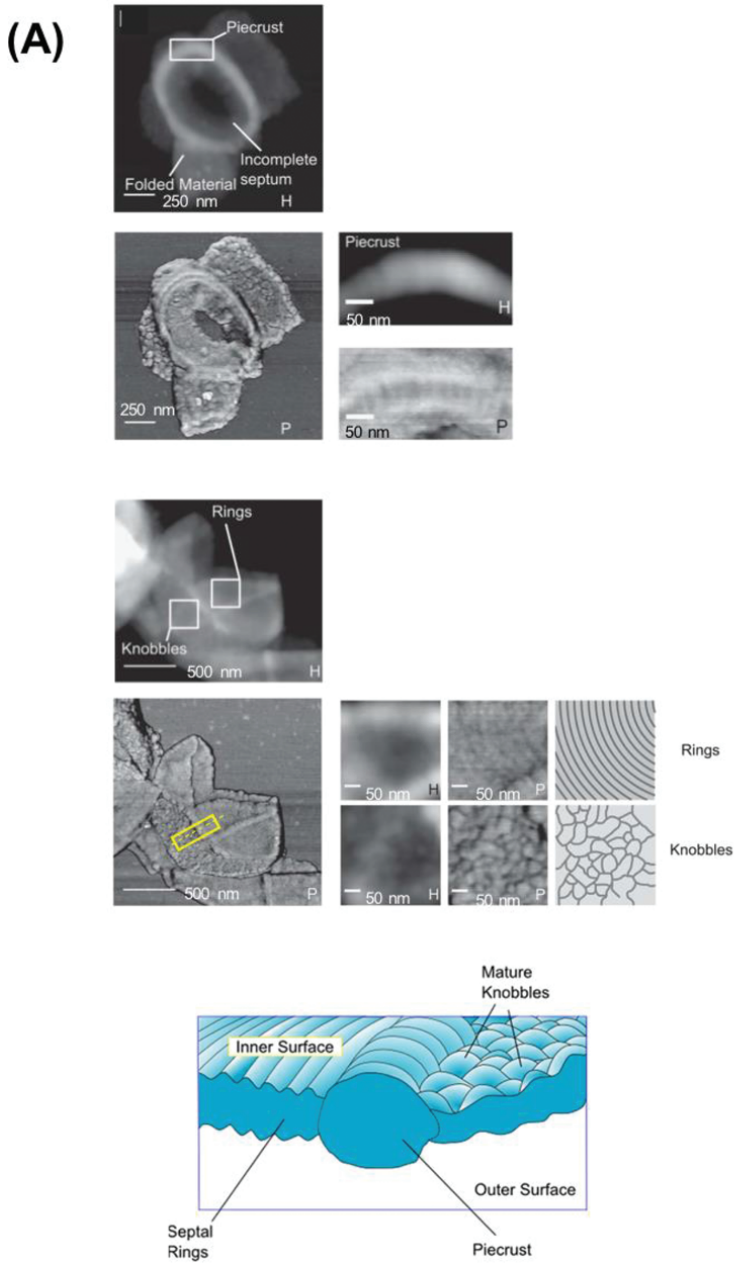
FIGURE 5 Localization of *atl* gene products on the cell surface of *S. aureus* during the division cycle as determined by scanning electron microscopy. (a to d) The gold labeling patterns on cells at different stages of the cell cycle. Bar, 100 nm. Reproduced with permission from reference 135.

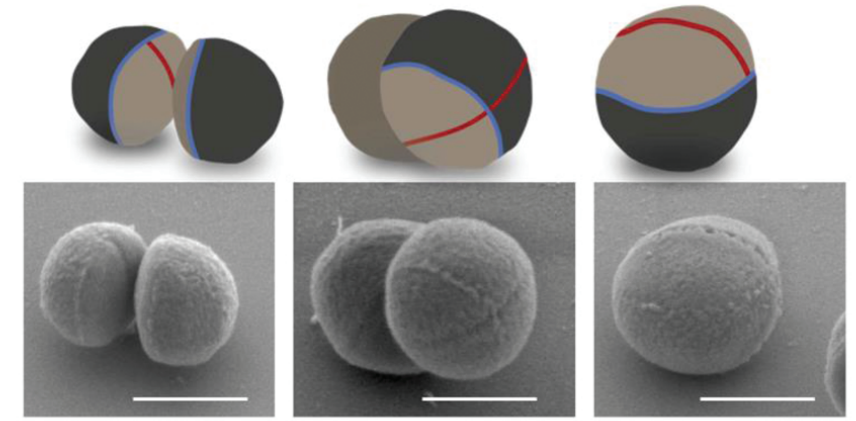
Cell Division

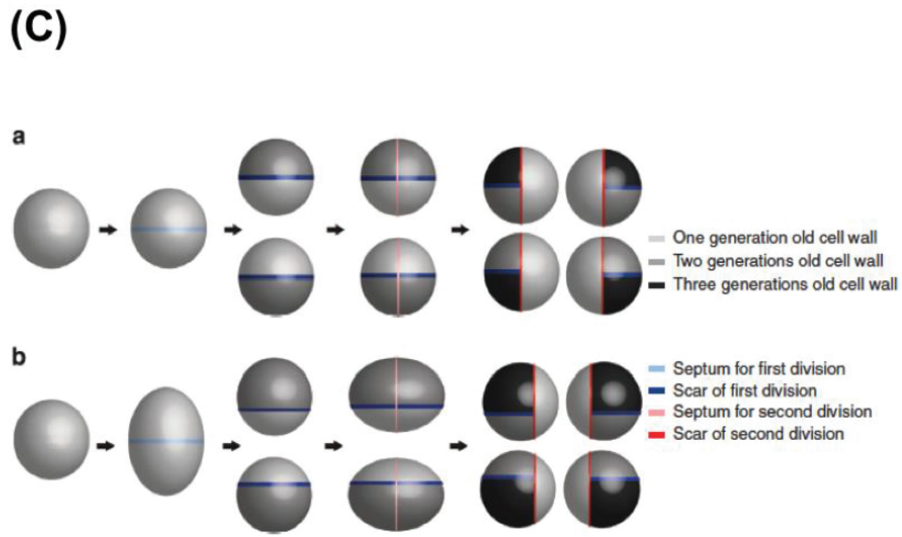
Recent methods allowed the identification of functional links between the peptidoglycan structure and synthesis pathway and the cell division process. Fluorescence microscopy localization studies demonstrated the essential role of FtsZ and of lipid II peptidoglycan precursor in the correct localization of PBP2 to the septum (144, 145). On the other hand, wall teichoic acids were shown to indirectly control PBP4 localization to the septum (146).

The process of cell division in *S. aureus* occurs equatorially and on consecutive orthogonal planes in three dimensions in accordance with the previous suggestion that peptidoglycan insertion only occurs at the septum (145, 147). It was possible to correlate the architecture and dynamics of peptidoglycan with the cell cycle using atomic force microscopy and fluorescence microscopy by observing a thick band of peptidoglycan, the so-called piecrust, at the division plane before the formation of the septum. After division, these structures remained as orthogonal ribs, marking the location of the previous division event, possibly to enable the cell to maintain the division plane localization sequentially orthogonal over the generations. Upon cell expansion after separation, the peptidoglycan mesh must undergo rearrangements since the area of the flat septal disc is much less than the area of half of the new cell (Fig. 6A) (21, 148). Some peptidoglycan hydrolases (Atl, SagA, ScaH, and SagB) with glucosaminidase activity have recently been shown to be required for cell enlargement, presumably by altering the mechanical properties of peptidoglycan that becomes more flexible for stretching (23).

The model of functional association between peptidoglycan synthesis and cell division still needs further work in light of new observations obtained by super-resolution microscopy. In contrast to original beliefs, *S. aureus* cells were shown to elongate before dividing, and the septum forms in a slightly unsymmetrical plane, suggesting that the peptidoglycan "piecrust" does not mark quadrants of the cell (20) (Fig. 6B and C).


(A)
Piecrust
Incomplete septum
Folded Material
250 nm
H
Piecrust
50 nm
H
250 nm
P
50 nm
P
Rings
Knobbles
500 nm
H
500 nm
P
50 nm
H
50 nm
P
50 nm
H
50 nm
P
Rings
Knobbles
Mature Knobbles
Inner Surface
Outer Surface
Septal Rings
Piecrust


(B)


(C)
a
One generation old cell wall
Two generations old cell wall
Three generations old cell wall
b
Septum for first division
Scar of first division
Septum for second division
Scar of second division

Interaction with the Host

Purified insoluble peptidoglycan of *S. aureus* was shown to induce arthritic symptoms in mice and interleukin-1b expression in macrophages, indicating that live bacterial cells are not necessary to elicit an inflammatory response (149, 150). The fact that this heterogeneous polymer is ubiquitous, essential, and unique to bacteria makes it the perfect target for the innate immune system through the action of lysozyme, a muramidase that is well distributed among the animal kingdom (151).

S. aureus peptidoglycan undergoes several secondary modifications (see above), such as *O*-acetylation of MurNAc and amidation of the second position of the stem peptide, contributing to lysozyme resistance, with a Δ*oat*A mutant showing impairment in the capacity to mediate septic arthritis (152). Other surface alterations that add to such resistance in *S. aureus* by modification of the net charge include the incorporation of D-alanine in teichoic acids and L-lysine in the membrane phosphatidylglycerol (153, 154).

Peptidoglycan fragments, released by cell wall turnover, have strong proinflammatory capacity, since they are recognized by host immune receptors, including nucleotide oligomerization domain protein-2 (NOD2), which detects L-lysine-containing muropeptides and the muramyl dipeptide as the minimal recognition structure (155). Peptidoglycan is also recognized by the extracellular Toll-like receptor-2 (TLR2) (156) and by peptidoglycan recognition proteins (PGRPs) that are present in leucocytes, liver, and epithelial cells and also in insects (157). Another clever host concealment strategy of *S. aureus* was described to rely on the activity of autolysins to trim the peptidoglycan fragments that stand out from the cell wall surface and become exposed to PGRP (158). Recognition of peptidoglycan by all these receptors, and perhaps others that remain to be identified, leads to the activation of multiple host defense responses (159).

Interaction with the External Environment and Recycling

Bacteria build and remodel their peptidoglycan scaffold not only through the primary biosynthetic pathway but also by interaction with the external environment. One form of interaction is peptidoglycan recycling, which although well described in Gram-negative bacteria for which turnover products are retained in the periplasm, has been a subject of debate for Gram-positive organisms. Recently, recycling of *N*-acetylmuramic acid was described for *S. aureus*, *B. subtilis*, and *Streptomyces coelicolor*, mediated by MurNAc-6P etherase, which converts per generation about 5 to 10% of MurNac-6P to *N*-acetylglucosamine-6-phosphate and lactate intracellularly. Deletion mutants showed no growth defects during exponential phase, because recycling was shown to occur mainly upon transition to stationary phase, suggesting that this process contributes to survival in the late phase of growth (160). Another form of interaction with the environment is the peptidoglycan chemical plasticity in challenging conditions. Noncanonical D-amino acids are known to be released to the environment by a wide range of bacterial species and to be exogenously incorporated into the mature peptidoglycan structure, through the action of surface associated enzymes, as a mechanism of peptidoglycan editing (161). In fact, the effect of exogenous glycine on the structure of staphylococcal peptidoglycan was described in early studies. Growth of *S. aureus* in the presence of high concentrations of glycine, D-serine, or other D-amino acids caused dramatic distortions of the peptidoglycan scaffold with reduction in cross-linkage and replacement of the muropeptide components by abnormal structures in which the carboxyterminal D-alanine residues were replaced by glycine or the other amino acids added to the medium (11, 162). One of the best-documented roles for such peptidoglycan editing capacities is to confer resistance to glycopeptides, but other functions related to adaptation to sudden environmental stresses, presence of the host, or an aging cell population have been suggested (161, 163). Most recently, incorporation of exogenous amino acids into *S. aureus* peptidoglycan was described to occur inside the infected live organism *Caenorhabditis elegans*, revealing a process of *in vivo* cell surface remodeling, eventually as a communication platform between pathogen and host (163).

SUMMARY

Through these studies, the staphylococcal cell wall is beginning to emerge not only as a critically important exoskeleton responsible for the structural integrity of the bacterium, but also as an organelle of multiple functions: it is intimately involved with interactions with the system of innate immunity of the host; it is a dynamic scaffold undergoing permanent remodeling upon external changes; it provides covalent attachment sites for a number of host-related surface proteins; and it occupies center stage in resistance to

FIGURE 6 (A) AFM height (H) and phase (P) images of purified sacculi from exponential phase *S. aureus* and key architectural details of the piecrust, division rings, and knobbles (21). (Top panel) Sacculus showing apparent incomplete septum parallel to the plane of the image, encircled by a piecrust feature associated with the initiation of septum formation; inset shows detail of piecrust features. (Middle panel) Sacculus showing ring texture associated with nascent peptidoglycan and knobble texture associated with older peptidoglycan; insets show details of rings and knobbles. (Bottom panel) Interpretive diagram drawn from the yellow rectangle in the middle panel. Reproduced with permission from reference 148. (B) SEM images of *S. aureus* strain COL showing an asymmetric scar (blue line) corresponding to the previous division site and a fissure located in the middle of the cell (red line), presumably corresponding to the next division site. The new cell wall (light brown), resulting from septal material from the mother cell, which has a smooth surface immediately after division (first panel), occupies less than half of the total surface. Scale bars, 600 nm. (C) Comparison of two models for *S. aureus* growth and division. (a) An earlier model which assumed that *S. aureus* cells remained approximately spherical over the cell cycle and that, on division, the cell wall material from the septum of the mother cell increased in cell surface area to constitute half (one hemisphere made of new cell wall) of the cell surface of each daughter cell. As a consequence, "scars" of previous divisions were proposed to encode epigenetic information that could be used to determine orthogonal placement of division septum. (b) In the other model proposed, *S. aureus* cells are approximately spherical at the beginning of the cell cycle and elongate as the cell cycle progresses. On division, there is no increase in the surface area of the previous septum, which becomes ~33% of the surface area of each daughter cell. This asymmetry in the regions composed of new and old cell wall results in scars of previous divisions that do not divide cells in quadrants. Consequently, T-junctions of scars of two previous divisions are not located at the cell poles. Two consecutive divisions in orthogonal planes are depicted in panels a and b. Reproduced with permission from reference 20.

β-lactam and glycopeptide antibiotics. Some of these observations have also begun to throw light on how the control of degradative and synthetic activities and morphogenetic principles come together in the assembly and replication of cell walls.

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Staphylococcal Protein Secretion and Envelope Assembly

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37

The cell wall envelope of *Staphylococcus aureus* is a surface organelle with anchored proteins, teichoic acids, and polysaccharides that enable bacteria to promote specific interactions with their environment (1). During colonization and invasion of their mammalian hosts, secreted and cell wall-anchored surface proteins fulfill a wide spectrum of functions, including bacterial adhesion to host cells or tissues, evasion of innate host defenses, and diversions of host adaptive immune responses (2). Secreted proteins trafficking in and out of the cell wall envelope promote both the persistent colonization of mammalian hosts and the pathogenesis of specific disease states that are characteristically associated with *S. aureus* (2).

CELL WALL SYNTHESIS AND ENVELOPE ASSEMBLY

When analyzed by electron microscopy of thin-sectioned bacteria, staphylococci are spherical cells whose envelope is composed of a plasma membrane and surrounding cell wall layer (20 to 40 nm in diameter) (3). Cell walls are isolated by physical disruption of staphylococci and chemical treatments that remove secondary polymers, including wall teichoic acids (WTAs), polysaccharides, and proteins (4). The remaining structures, designated murein sacculi, retain the spherical shape of the bacteria and are composed of peptidoglycan, including glycan strands with a repeating disaccharide, →4 (*N*-acetylmuramoyl-β-(1→4)-*N*-acetylglucosaminyl-β-(1→), abbreviated $(MurNAc\text{-}GlcNAc)_n$, and cross-linked wall peptides, L-alanyl-D-iso-glutaminyl-(pentaglycyl)L-lysyl-D-alanyl$_n$, abbreviated $[Ala\text{-}iGln\text{-}(Gly_5)Lys\text{-}Ala]_n$ (5, 6). Wall peptides are tethered to glycan chains via amide bonds formed by the amino-group of L-alanyl and the carboxyl-group of MurNAc (6, 7). Wall peptides of neighboring peptidoglycan strands are cross-linked by amide bonds formed from the amino group of pentaglycyl and the carboxyl-group of D-alanyl (8). During peptidoglycan synthesis, the bactoprenol-linked intermediate, lipid II $[C_{55}\text{-}(PO_4)_2\text{-}MurNac(\text{-}Ala\text{-}iGln\text{-}(NH_2\text{-}Gly_5)Lys\text{-}Ala\text{-}Ala\text{-}COOH)\text{-}GlcNAc]$, is translocated across the staphylococcal plasma membrane (9, 10). Penicillin binding protein 2 polymerizes lipid II substrate to generate glycan strands and cross-linked wall peptides via hydrolysis of its phosphodiester and Ala-Ala bonds (11–13).

WTAs and capsular polysaccharide are also synthesized as bactoprenol-linked intermediates (14, 15). Once translocated across the membrane, WTAs and capsular polysaccharides are anchored to the peptidoglycan scaffold (16, 17). Staphylococci replicate by forming cell division septa at midcell and by synthesizing peptidoglycan in the cross-wall compartment, which is bounded by the septal membranes of newly dividing staphylococci (3, 18). Once completed, cross-wall peptidoglycan is split to separate the dividing cells (3). Staphylococcal murein sacculi are not permeable for proteins and other large macromolecules. Cell division, assembly of secondary polymers, and the secretion of proteins across the bacterial cell wall requires specific murein hydrolases that modify murein sacculi, thereby enabling protein secretion across the cell wall envelope and the anchoring of secondary cell wall polymers to peptidoglycan (19, 20).

TEICHOIC ACID SYNTHESIS AND ENVELOPE ASSEMBLY

The envelope of *S. aureus* encompasses both polyribitol-phosphate and polyglycerol-phosphate polymers (21, 22). Polyribitol-phosphate $[(Rib\text{-}PO_4)_n]$, also designated WTA, is synthesized in the bacterial cytoplasm from CDP-ribitol and tethered to its bactoprenol-linked carrier, thereby generating lipid III $[C_{55}\text{-}(PO_4)_2\text{-}GlcNAc\text{-}ManNAc\text{-}(Gro\text{-}PO_4)_2\text{-}(Rib\text{-}PO_4)_{11\text{–}40}]$ (23, 24). Rib-PO_4 is polymerized via phosphodiester bonds engaging ribitol hydroxyls at *O*1 and *O*5; the remaining hydroxyl moieties (*O*2, *O*3, and *O*4) are variably substituted with α- or β-linked GlcNAc (25–27). Following translocation of lipid III across the membrane, LCP enzymes tether WTA to peptidoglycan, generating phosphodiester bonds between the *O*1 of GlcNAc and the *O*6 of MurNAc (28). The Rib-PO_4 polymer of anchored WTA is further modified via esterification with D-alanyl (29, 30). Peptidoglycan synthesized at the cross-wall is initially devoid of WTA (31) (Fig. 1). Upon cross-wall splitting and cell separation, cross-wall peptidoglycan is modified with WTA (31). LtaS polymerizes polyglycerol-phosphate $[(Gro\text{-}PO_4)_n]$, also designated lipoteichoic acid (LTA), from phosphatidylglycerol, transferring the assembled Gro-PO_4 polymer onto its glycolipid anchor gentiobiosyldiacylglycerol: Glc-β-(1→6)-Glc-β-(1→3)-diacylglycerol $[Glc_2\text{-}DAG]$ (32–34). The *O*2 hydroxyl of Gro-PO_4 is variably substituted with esterified D-alanyl (30, 35). LtaS and LTA synthesis are restricted to septal membranes and are required for staphylococcal cell division and growth (36) (Fig. 1). Thus, assembly sites of WTA and LTA synthesis are unevenly distributed in the envelope of dividing staphylococci.

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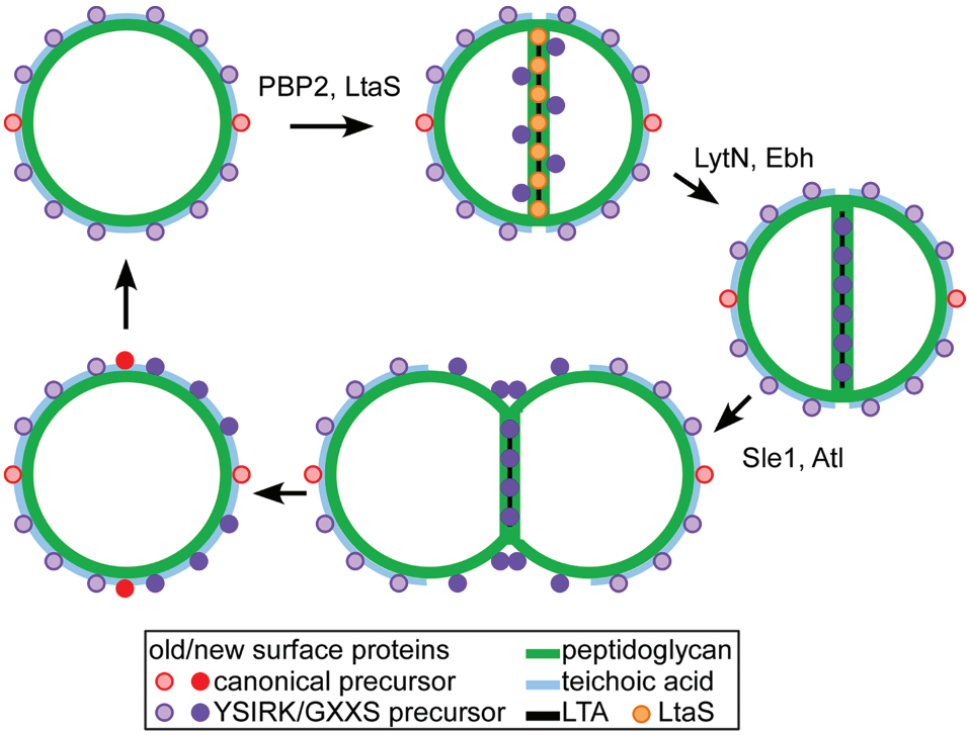


FIGURE 1 Schematic illustrating *S. aureus* cell division, PBP2-mediated synthesis of cross-wall peptidoglycan, LtaS-mediated synthesis of lipoteichoic acid (LTA) in septal membranes, and the trafficking of surface protein precursors with YSIRK-GXXS motif signal peptides (blue circles) to septal membranes. Following translocation, sortase A-mediated cell wall sorting, and incorporation into the cross-wall, the peptidoglycan layer is split and divided cells are separated, exposing cross-wall-incorporated proteins on the bacterial surface. Surface proteins with canonical signal peptides (red circles) are deposited into polar segments of the peptidoglycan layer.

PROTEIN SECRETION

The genome of *S. aureus* carries more than 1,300 genes with products encompassing predicted N-terminal signal peptides (37). When subjected to proteomic analysis, most of these gene products can be identified in the filtered supernatant of centrifuged *S. aureus* cultures (38). Genetic and biochemical experiments characterized the Sec secretion pathway in the model organism *Escherichia coli* (39–41). Elements of this pathway are conserved among all bacteria, including staphylococci (42). Some of the *sec* genes have also been characterized in *S. aureus*. Signal peptide-bearing precursors are translocated across the plasma membrane through the Sec translocon, a complex formed from three membrane proteins, SecY, SecE, and SecG (43). Protein translocation requires SecA, a cytoplasmic ATPase that pushes unfolded precursors through the translocon (44, 45). SecDF, a chaperone whose activity is sustained by the proton motive force, assists in the folding of translocated proteins (46, 47). Similar to *E. coli*, *S. aureus secA* is absolutely essential for bacterial growth and protein secretion, whereas *secDF* is not essential, providing auxiliary functions to protein secretion (48–50). Translocated precursors are cleaved by signal peptidase I, LepB in *E. coli* (51). *S. aureus* carries two signal peptidase I genes, *spsA* and *spsB* (52). SpsB, but not SpsA (it lacks a critical catalytic residue), can substitute for *E. coli* LepB, and expression of *spsB* is required for staphylococcal growth and the cleavage of secreted precursors in *S. aureus* (52). Suppressor mutations that induce expression of a putative ABC transporter (ABC1-3) can restore both growth and precursor secretion in *S. aureus spsB* mutants (53, 54). A physiological role has not yet been assigned to the putative ABC1-3 transporter. SagB glucosaminidase cleaves staphylococcal peptidoglycan to generate short glycan strands with variable lengths $(MurNAc\text{-}GlcNAc)_{3–10}$. *S. aureus sagB* mutants exhibit diminished growth, cross-wall separation defects during cell division, and reduced secretion of proteins into the extracellular medium (19). Thus, the generation of short glycan strands is a prerequisite for the transport of protein across the cell wall peptidoglycan. Three other murein hydrolases—autolysin (Atl amidase and glucosaminidase), Sle1, and LytN (CHAP domain amidases and endopeptidases)—contribute to cell division, cross-wall separation, and likely also to protein secretion across the staphylococcal cell wall envelope (55–59).

PROTEIN ANCHORING TO THE CELL WALL

Surface proteins of staphylococci are covalently linked to peptidoglycan (60). Following translocation of signal peptide-bearing precursors across the plasma membrane, the C-terminal end of surface proteins is cleaved between the threonyl (T) and the glycyl (G) of their LPXTG motif sorting signal by sortase A, which generates a thioester-linked acyl-enzyme intermediate (61–64) (Fig. 2). Nucleophilic attack of the peptidoglycan biosynthetic intermediate, i.e., of the amino-group of pentaglycyl within lipid II, resolves the sortase A acyl-enzyme, synthesizing an amide linkage between the carboxyl group of the C-terminal threonyl of surface proteins and the amino-group of pentaglycyl (65, 66). The surface protein-lipid II intermediate is subsequently incorporated into the peptidoglycan via penicillin binding protein

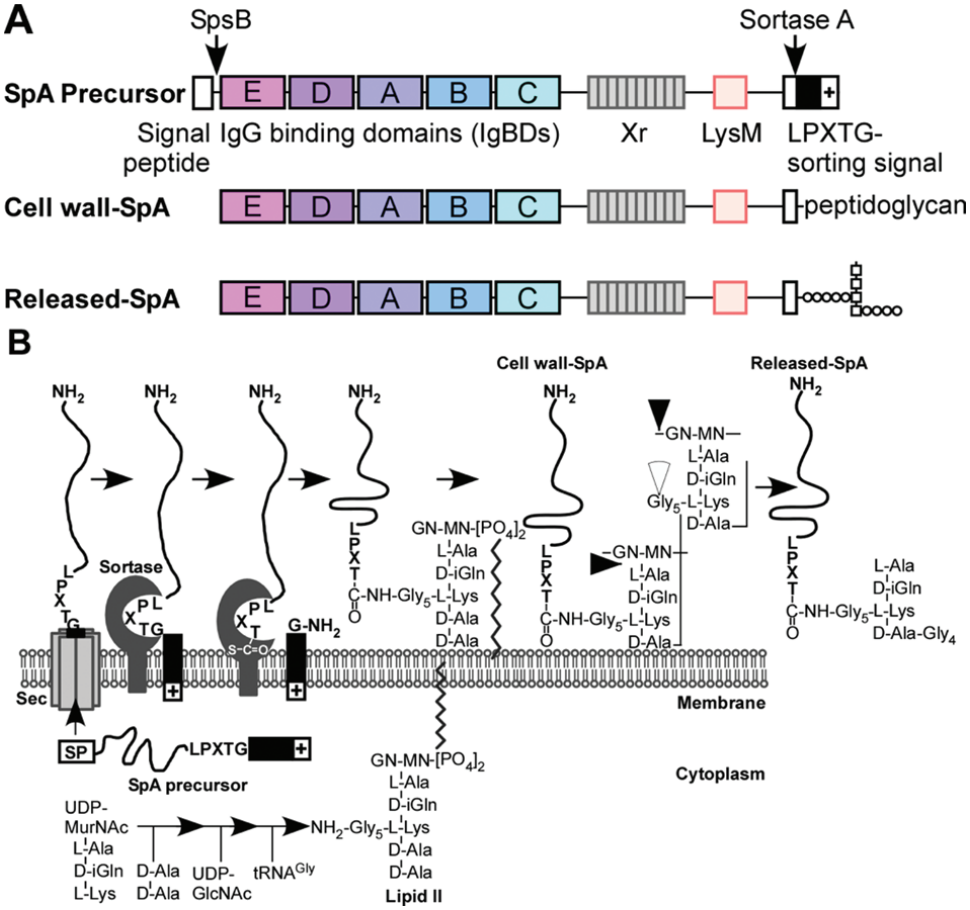


FIGURE 2 Surface protein anchoring to the cell wall of *S. aureus*. **(A)** Primary structure of the precursor for staphylococcal protein A (SpA) with an N-terminal signal peptide, signal peptidase (SpsB) cleavage site, five immunoglobulin binding domains (IgBDs), region X (Xr), LysM domain, and C-terminal LPXTG-motif sorting signal. Cell wall-anchored SpA is tethered to the envelope via an amide bond between the carboxyl-group of its C-terminal threonyl (T in the LPXTG motif) and the amino group of the pentaglycine cross-bridge within peptidoglycan. Released nSpA encompasses murein tetrapeptide-tetraglycyl [L-Ala-D-iGln-(SpA-Gly_5)L-Lys-D-Ala-Gly_4] linked to the C-terminal threonyl of the surface protein. **(B)** Cell wall sorting pathway of surface proteins in *S. aureus*. Following Sec-mediated translocation of surface protein precursors across the membrane, sortase A cleaves the LPXTG-motif of the C-terminal sorting signal between the threonyl (T) and the glycyl (G) residues and forms a thioester bond between its active site cysteinyl and the carboxyl-group of the C-terminal threonyl. The sortase acyl-enzyme intermediate is relieved by the nucleophilic attack of lipid II, thereby generating surface protein linked to peptidoglycan precursor, which is subsequently incorporated by penicillin binding protein 2 into the cell wall peptidoglycan. Through the action of murein hydrolases, surface protein is released from the cell wall into the extracellular milieu.

2-catalyzed transglycosylation and transpeptidation reactions (67) (Fig. 2). Sortase A and surface protein anchoring to the cell wall envelope occur both within the cross-wall compartment and within polar peptidoglycan (68, 69) (Fig. 1). Genome sequences of thousands of isolates reveal between 18 and 23 genes for sortase A-anchored surface proteins in *S. aureus* (70).

PROTEIN SECRETION INTO THE CROSS-WALL COMPARTMENT

Thirteen *S. aureus* surface protein genes encode precursors with YSIRK-GXXS signal peptides, and their products are secreted across septal membranes into the cross-wall compartment (69, 71). Septal secretion promotes abundant deposition and broad distribution of these proteins over the staphylococcal surface (68). In contrast, surface proteins secreted via canonical signal peptides are deposited with low abundance into polar peptidoglycan (69). In addition to sortase A-anchored surface proteins, four other staphylococcal precursors carry YSIRK-GXXS signal peptides: the LytN murein hydrolase (55), the giant protein Ebh (72), and two glycerol-ester hydrolases, GehA and GehB (73). Septal secretion of LytN into the cross-wall promotes cleavage of peptidoglycan and the separation of cells that have completed the division process (55). The 10,421-residue Ebh precursor comprises an N-terminal YSIRK-GXXS signal peptide, a hyperosmolarity resistance domain (residues 179 to 2530), 7 repeats of the 54-residue FIVAR domain (possibly associated with polysaccharide binding), 50 repeats of the

123-residue FIVAR-GA domain, 7 repeats of DUF1542 (a 72-residue domain of unknown function), a putative SMC domain (structural maintenance of chromosomes; residues 8976 to 9576), a transmembrane domain (residues 10227 to 10247), and a positively charged cytoplasmic domain (72). Mutations that abolish *ebh* expression increase staphylococcal cell size and perturb the integrity of the cell wall envelope, suggesting that Ebh is a size determinant for the staphylococcal murein sacculus (72). GehA and GehB are secreted as 72-kDa pro-proteins with 250-residue N-terminal domains that are thought to assist in the folding of C-terminal glycerol-ester hydrolase domains (74). GehA cleaves the ester bonds of short-chain fatty acids within acyl-glycerol, whereas GehB hydrolyzes preferentially long-chain fatty acid esters (75–77). Following cell division, GehB pro-protein is released into the extracellular medium and cleaved by aureolysin, a cysteine protease that is secreted via the canonical Sec pathway (75). A specific role for GehA and GehB during staphylococcal cell division has thus far not been revealed.

During secretion, the precursor of SpA (staphylococcal protein A) is cleaved at Gly^{13}, i.e., between the conserved (underlined) elements of its YSIRKLG^{13}VGIAS motif (50). After translocation across the plasma membrane, SpsB signal peptidase cleaves SpA at Ala^{37}, thereby removing the N-terminal hydrophobic signal peptide (78). Translocated SpA is retained within the secretory pathway by its C-terminal sorting signal and is subsequently anchored to the cell wall envelope (79). Amino acid substitutions in the YSIRK-GXXS motif perturb both the proteolysis and the secretion of SpA precursors, suggesting that cleavage of the YSIRK-GXXS motif is a prerequisite for septal secretion (50). SecA ATPase and SecDF foldase promote the secretion of precursor proteins at septal and polar membranes (50). In contrast, LtaS and LTA synthesis are required for septal, but not polar, secretion in *S. aureus* (50). Thus, synthesis of LTA within septal membranes represents a unique trait of staphylococcal biology that directs precursors with YSIRK-GXXS signal peptides into the cross-wall compartment (Fig. 1). It is not yet clear, however, whether the abundance of LTA, the depletion of phosphatidylglycerol, i.e., the lipid substrate of LtaS, or the accumulation of di-acylglycerol, i.e., the byproduct of LTA synthesis, make specific contributions toward septal secretion. It is also conceivable that septal secretion of GehA and GehB may contribute to the specific features of septal membranes that support trafficking of YSIRK-GXXS precursors into the cross-wall compartment.

RELEASE OF SURFACE PROTEINS FROM THE STAPHYLOCOCCAL ENVELOPE

During staphylococcal growth, cell wall-anchored surface proteins are released from the staphylococcal envelope into the extracellular milieu, where they fulfill specific functions (80). For example, release of SpA requires LytN CHAP amidase and LytM endopeptidase, releasing surface protein with murein tetrapeptide-tetraglycyl [L-Ala-D-iGln-(SpA-Gly_5)L-Lys-D-Ala-Gly_4] linked to the C-terminal threonyl (81) (Fig. 2). Surface proteins of staphylococci fulfill complex biological functions both on the bacterial surface and following their release from the peptidoglycan scaffold by murein hydrolases (82).

STAPHYLOCOCCAL PROTEIN A

All clinical isolates of *S. aureus* harbor the *spa* gene for staphylococcal protein A (SpA), which generates a precursor product composed of an N-terminal YSIRK/GXXS signal peptide followed by 4 or 5 immunoglobulin-binding domains (IgBDs), region X repeats (Xr), an LysM domain, and an LPXTG sorting signal (70, 83, 84). Following septal secretion and anchoring to cross-wall peptidoglycan, SpA is displayed on the bacterial surface (68). The IgBDs of SpA bind to the Fcγ domain of human and animal IgG, effectively blocking effector functions of antibodies, i.e., the binding of C1q complement or the engagement of Fc receptors, thereby inhibiting opsonophagocytic killing of staphylococci (85–87). SpA released from the bacterial envelope crosslinks V_H3 idiotype IgM B cell receptors and promotes the nonproductive proliferation of B cells and secretion of V_H3 idiotype antibodies that do not recognize staphylococcal antigens (88, 89). This B cell superantigen activity requires endocytosis of SpA crosslinked to B cell receptors, signaling via RIPK2 kinase, and $CD4^+$ T helper cell functions (90). The B cell superantigen activity of SpA effectively blocks host adaptive immune responses and the establishment of protective immunity against *S. aureus* (85).

SURFACE PROTEINS AND HEME-IRON TRANSPORT

S. aureus encodes a second sortase gene, designated sortase B (*srtB*) (91). Sortase B cleaves the NPQTN-motif sorting signal of IsdC (iron-regulated surface determinant C) (92). Unlike sortase A, the sortase B acyl-enzyme intermediate is resolved by nucleophilic attack of the amino group of pentaglycyl within polymerized peptidoglycan, thereby anchoring of IsdC to peptidoglycan in the vicinity of the staphylococcal membrane (92). The sortase B anchoring mechanism enables *S. aureus* surface proteins to promote heme-iron scavenging from host hemoglobin and haptoglobin (93, 94). Initially, sortase A-anchored IsdB and IsdH retrieve heme-iron from host hemoproteins (95, 96). IsdA, another sortase A-anchored surface protein, captures the heme-iron from IsdB and IsdH for passage of the nutrient across the staphylococcal cell wall envelope and transfer to IsdC (97). IsdE lipoprotein and IsdF transporter are thought to capture heme-iron from IsdC for import into the bacterial cytoplasm (97, 98). IsdG and IsdI cleave the tetrapyrrol ring of heme to generate staphylobilin and iron for the assembly of iron-sulfur cluster proteins (99, 100). NEAT domains (near-iron transporter domains about 120 residues in length) promote the heme-iron binding and transfer reactions and are found in variable numbers within IsdA, IsdB, IsdC, IsdE, and IsdH (93, 101, 102).

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Surface Proteins of *Staphylococcus aureus*

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38

INTRODUCTION

Cell wall-anchored (CWA) proteins are characterized by the presence of a sorting signal at the C-terminus which is responsible for coupling the protein covalently to peptidoglycan. The surface of *Staphylococcus aureus* is decorated with up to 24 CWA proteins. The precise number depends on the strain and the growth conditions. The repertoire of CWA proteins expressed by *S. aureus* is limited and many have evolved to perform important interactions with the host. They can be categorized into distinct structural and functional groups (Fig. 1, Table 1). Several are multifunctional due to the proteins comprising distinct domains which recognize different ligands, while for others a single domain is capable of binding different ligands by different mechanisms.

The multifunctionality of CWA proteins has resulted in some confusion over nomenclature, with the original names often referring to the first-assigned function, while in other cases proteins were named *S. aureus* surface protein (Sas) prior to a function being ascribed and were then assigned a new name when a function or structural feature was discovered; e.g., SasH is now called AdsA (extracellular adenosine synthase), and SasA is now SraP (serine-rich surface protein).

This review will describe our current understanding of the structure and functions of CWA proteins of *S. aureus* in adhesion to the extracellular matrix (ECM), invasion of host cells, evasion of immune responses, biofilm formation, and acquisition of iron. The review will include a discussion of posttranslational modifications that contribute to function and will describe the contribution of CWA proteins to colonization of the host and to pathogenesis.

CWA PROTEINS: STRUCTURE AND FUNCTION

Several distinct structural and/or functional classes of CWA protein occur in *S. aureus* (Fig. 1, Table 1). N-terminal domains are projected away from the cell surface and are the part of the protein that is usually engaged in interactions with the host. An important exception is the fibronectin binding repeats of fibronectin binding proteins (FnBPs), which are located toward the C-terminus.

The MSCRAMM Family

"Microbial surface components recognizing adhesive matrix molecules" (MSCRAMMs) is a term that was originally applied to CWA proteins that bind to host proteins from the ECM, such as fibrinogen and fibronectin (1). It has become widely used to encompass all CWA proteins whether or not they bind ECM molecules. It was recently proposed that the term "MSCRAMM" should be reserved for proteins with a common structure motif, *viz.*, two adjacent IgG-like folds and similar mechanisms of ligand binding exemplified by dock lock latch (DLL) and the collagen hug (2).

Ligand binding by DLL: the basic mechanism

Proteins in the Clf-Sdr subfamily of MSCRAMMs have an N-terminal A domain comprising three subdomains called N1, N2, and N3, with N2 and N3 being the minimum required for ligand binding by DLL. The N2 and N3 subdomains each comprise two β-sheets that are arranged in a variation of the IgG fold called DEv-IgG (3). The DLL mechanism was originally defined by analyzing the structure of the *Staphylococcus epidermidis* protein SdrG in the apo form and in complex with the β-chain peptide of fibrinogen (4). The same mechanism was found to apply to clumping factor A (ClfA) and the A domains of FnBPA and FnBPB binding to the γ-chain peptide of fibrinogen and to ClfB binding to cytokeratin 10, loricrin, and the α-chain of fibrinogen (5–7).

In essence, an unfolded peptide ligand binds to the open form of the MSCRAMM in a hydrophobic trench located between the N2 and N3 subdomains (Fig. 2). This stimulates a conformational change which results in the unfolded extension of N3 covering the peptide to lock it in place and creating the latch by forming an extra β-strand in one of the β-sheets of N2 by β-strand complementation (2, 4). The peptide ligand binds β-strand G′ in N3 through backbone interactions while side chains of residues in the peptide and residues in the binding trench of the MSCRAMM determine binding specificity.

Recent studies using atomic force microscopy showed that the rupture force of SdrG in complex with fibrinogen is very high, being equivalent to that of a covalent bond (8). Similar forces were required to separate cells expressing Cna binding to collagen (9) and ClfB expressing cells binding to loricrin (10). In the latter the strength of the interaction between ClfB and loricrin increased when mechanical forces were applied, suggesting that shear forces might induce the conformational changes involved in DLL. When the MSCRAMM is attached to the surface of a staphylococcal cell, these changes must result in considerable reorientation of the protein (Fig. 3) with implications for binding to other ligands (see section FnBPs bind plasminogen).

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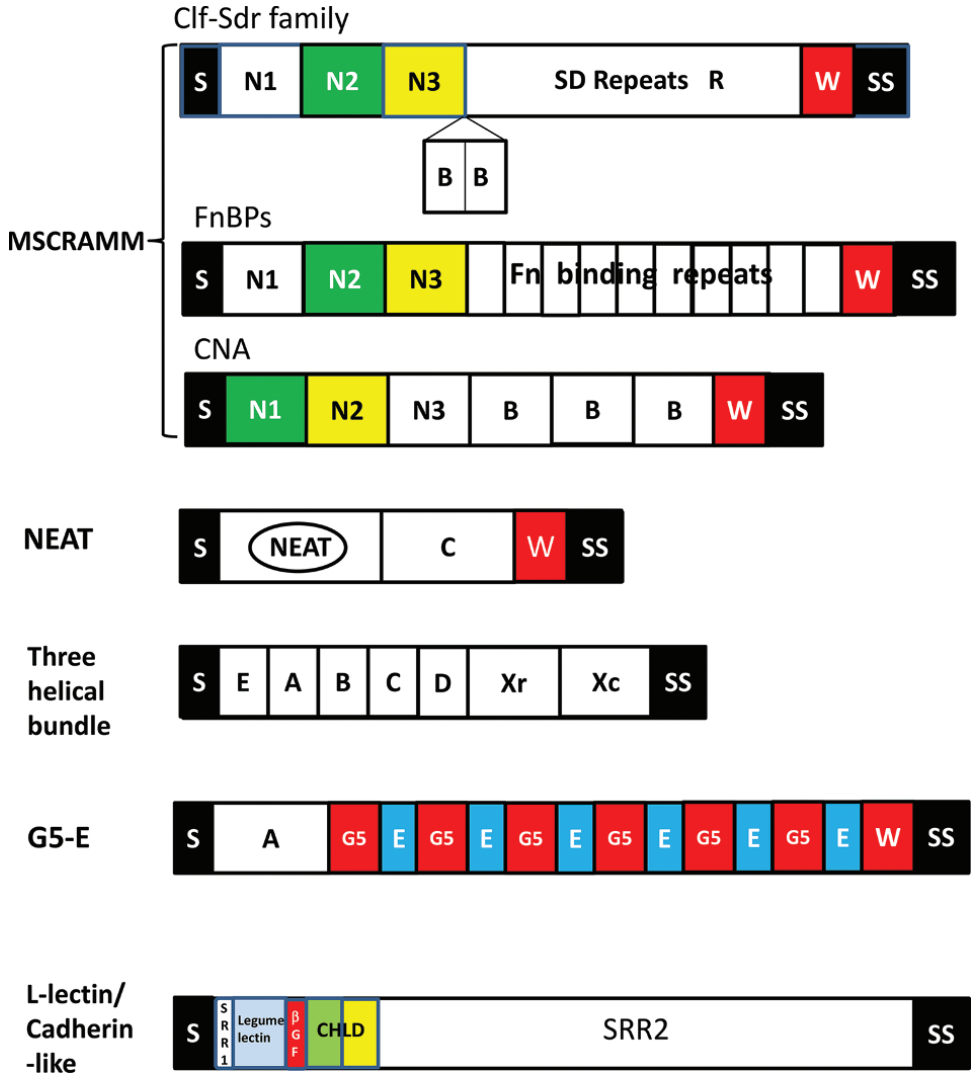


FIGURE 1 CWA surface proteins classified based on structural motifs. The primary translation products of all CWA proteins contain a signal sequence (S) at the amino terminus and a wall-spanning region (W, Xc) and sorting signal (SS) at the carboxyl terminus. The CWA proteins that are depicted are those for which structural analysis has facilitated classification into five distinct groups. (A) Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). The clumping factor (Clf)-serine aspartate repeat (Sdr) group comprises proteins that are closely related to ClfA. ClfA and ClfB have a similar domain organization, whereas SdrC, SdrD, and SdrE contain additional B_{SDR} repeats that are located between the A domain and the serine-aspartate SD repeat R region. The N-terminal A region contains three separately folded domains, called N1, N2, and N3. Structurally, N2 and N3 form IgG-like folds that bind ligands by the DLL mechanism. Fibronectin-binding protein A (FnBPA) and FnBPB have A domains that are structurally and functionally similar to the A domain of the Clf-Sdr group. Located in place of the serine-aspartate repeat region are tandemly repeated fibronectin-binding domains (11 in FnBPA, 10 in FnBPB). The A region of the collagen adhesin (Cna) protein is organized differently than other MSCRAMMs, with N1 and N2 comprising IgG-like folds that bind to ligands using the collagen hug mechanism. The A region is linked to the wall-spanning and anchorage domains by variable numbers of B_{CNA} repeats. (B) Near iron transporter (NEAT) motif protein family. The iron-regulated surface determinant (Isd) proteins have one (for IsdA), two (for IsdB), or three (for IsdH) NEAT motifs that bind to hemoglobin or heme. The figure depicts IsdA, which has a C-terminal hydrophilic stretch that reduces cell surface hydrophobicity and contributes to resistance to bactericidal lipids and antimicrobial peptides. (C) Three-helical bundle motif protein A. The five N-terminal tandemly linked triple-helical bundle domains (known as EABCD) that bind to IgG and other ligands are followed by the repeat-containing Xr region and the nonrepetitive Xc region. (D) G5-E repeat family. The alternating repeats of the G5 and E domains of *S. aureus* surface protein G (SasG) (and the accumulation-associated protein [Aap] from *S. epidermidis*) link the N-terminal A region to the wall-spanning and anchorage domains. If the A domain is removed, the G5-E region can promote cell aggregation. (E) Legume-lectin, cadherin-like domain protein. The BR region of the serine rich adhesin of platelets (SraP) protein is flanked by serine-rich repeat domains. The BR region comprises three distinct structural domains: the legume lectin-like, the β-grasp fold (β-GF), and the cadherin-like (CHLD) domains.

Ligand binding by DLL: variations on a theme

Several subtle variations on the DLL theme have been observed. Both parallel and antiparallel orientations of ligand peptide binding to the trench occur (SdrG parallel; ClfA and ClfB antiparallel), which results in the ligands binding in opposite orientations with respect to the MSCRAMM (3, 4, 7).

The ligand residues bound by SdrG and ClfB have extensive flanking residues located both C-terminally and N-terminally and thus can only bind to the open apo form of the MSCRAMM. In contrast, the ClfA ligand binding residues occur at the extreme C-terminus of the γ-chain of fibrinogen and are able to penetrate the MSCRAMM in the locked position (11).

ClfA has a second binding site on fibrinogen in addition to the γ-chain peptide (12). The epitope for the monoclonal antibody tefibazumab that blocks binding of ClfA to fibrinogen was mapped to a location on the "top" of subdomain N3 well away from the ligand binding trench involved in DLL. Molecular modeling predicted that tefibazumab partially blocks a second fibrinogen binding site involving the fibrinogen γ-globule and that this is required for high-affinity binding to the ligand (Fig. 3). Indeed, tefibazumab has a high affinity for ClfA but has only a half maximal inhibitory concentration for blocking ClfA binding to fibrinogen. Studies with ClfA variants showed that high-affinity binding requires the two sites to act cooperatively in a complex multicontact binding mechanism. It is possible that other MSCRAMMs bind ligands by similar multistep mechanisms, particularly where the affinity of a peptide is lower than the full-length protein. Ultimately, proof of two binding sites requires x-ray crystal structures of the proteins in complex.

SdrE binds to the host recognition C-terminal unit 20 of the complement control protein factor H by a variation of DLL called "closed dock lock latch" (13). In SdrE the ligand binding trench is occluded by loop A-B that protrudes from N2 forming four main chain H bonds with β-strand G′ in N3. For the factor H unit 20 to bind by DLL, it must first displace the AB loop, which is flipped out of the way (Fig. 4).

ClfB binds several ligands by DLL

ClfB binds to cytokeratin 10, loricrin, and the α-chain of fibrinogen by DLL (5, 7, 14). Thus, several different ligands can be accommodated by the same binding trench. This has allowed the definition of a consensus ligand binding sequence (7). Another example is the A region of FnBPA and FnBPB, which can bind to elastin as well as the γ-chain peptide of fibrinogen like ClfA (15, 16). However, this conclusion resulted from the study of FnBP variants with substitutions in residues predicted to be involved in DLL and not from x-ray crystal structures. It is possible that other MSCRAMMs bind several different ligands by DLL.

Collagen binding

The collagen binding protein Cna binds collagen by a variation of the DLL mechanism called the collagen hug (17). The N1 and N2 subdomains of the A region of Cna are composed of IgG-like folds and perform roles corresponding to the N2 and N3 subdomains of other MSCRAMMs, with the C-terminal extension of N2 forming the latch (Fig. 5).

The linker separating N1 and N2 is long and is able to accommodate the thick rod of the collagen triple helix. The ligand binds to a shallow trench in N2. This stimulates a conformational change, which enables the linker to wrap around collagen. Latching then takes place by β-strand complementation between the C-terminal extension of N2 and a β sheet in subdomain N1 (17).

Cna also binds to complement factor C1q

The complement pathway is initiated when complement protein C1q binds to antibodies attached to the surface of a pathogen. C1q forms a bouquet-like structure that is composed of six identical heterotrimers, each comprising a globular head that forms the antibody recognition domain and a long collagenous triple helix (Fig. 6) (18). These coalesce to form a stalk. The C1r and C1s proteins are associated with the C1q complex and are necessary for initiating the complement cascade. Cna can bind to the C1q triple helices and displace C1s-C1r, resulting in inhibition of complement fixation (19). The collagen hug mechanism is most likely to be involved because Cna variants with reduced collagen binding also bind C1q with lower affinity. Thus, Cna and other members of the Cna family can act both as adhesins to collagenous tissue and as immune evasion factors by inhibiting complement fixation.

SdrC and FnBPs engage in homophilic interactions and promote biofilm formation

The MSCRAMMs SdrC, FnBPA, and FnBPB can promote cell-cell attachment and biofilm formation (20–22). In both cases the A domains on adjacent cells undergo homophilic interactions (Fig. 7). SdrC subdomain N2 has two interaction sites that were identified by screening phage display libraries for inhibitory peptides (21). Molecular modeling showed that a β-neurexin peptide which binds SdrC occludes one of the interaction sites and can prevent biofilm formation (23, 24). Studies with bacterial cells expressing FnBPs and variants thereof confirmed that the A domains are required for biofilm formation and that in the case of FnBPA, the ability to engage in DLL is not involved (20).

Atomic force microscopy has been employed to study forces involved in interactions between MSCRAMMs either with the atomic force microscopy cantilever tip conditioned with the purified A domain protein or with a single bacterial cell. It was concluded that cell-cell binding results from multiple weak interactions between proteins occurring in parallel between molecules on two individual cells (23, 25). The rupture force required to separate a single CWA protein-protein interaction was very low compared to that required to separate an MSCRAMM from a ligand bound by DLL or the collagen hug. Weak interactions might facilitate detachment of bacterial cells from the mature biofilm and their dissemination.

In the case of SdrC, the A domain can also promote attachment to hydrophobic surfaces and could thus be involved both in attachment to an indwelling medical device and in biofilm accumulation (23).

FnBPs bind plasminogen

Several CWA proteins contribute to the ability of *S. aureus* cells to capture plasminogen from serum (26). Mutants lacking FnBPs are only partially defective in plasminogen binding, whereas a sortase A defective mutant lacking all CWA proteins binds very little. Thus, the full repertoire of CWA proteins that bind plasminogen is not known, with the two studies published so far focusing only on FnBPs (26, 27). The A domain of all seven isoforms of FnBPB bind plasminogen with similar affinity (26). Binding of FnBPB was inhibited by ε-amino caproic acid and lysine, which implies that binding occurs between lysine residues on the MSCRAMM and negatively charged residues on plasminogen. Indeed, alanine substitutions of the two conserved patches of lysines in FnBPB N2 resulted in reduced plasminogen binding. The ability of whole cells expressing FnBPs to bind to plasminogen is greatly enhanced by fibrinogen

TABLE 1 Properties of CSA surface proteins[a]

Protein structural and functional group	Ligand and binding mechanism	Function	References
MSCRAMM family			
Clumping factor A (ClfA)	Fibrinogen γ chain C-terminus (DLL)	Adhesion to immobilized fibrinogen, immune evasion by binding soluble fibrinogen	3, 11
	Complement factor I Mechanism unknown	Immune evasion, degradation of C3b	130, 131
Clumping factor B (ClfB)	Fibrinogen α-chain repeat 5, keratin 10 and loricrin (DLL)	Adhesion to desquamated cells from nares and from skin of AD patients; nasal colonization and colonization of AD skin	5, 7, 14, 118, 150
Serine aspartate repeat protein C (SdrC)	β-neurexin (DLL)	Unknown	23, 24
	Unknown	Adhesion to desquamated epithelial cells Nasal colonization?	93
	SdrC	Homophilic SdrC-SdrC interaction; biofilm formation	21, 23
Serine aspartate repeat protein D (SdrD)	Desmoglein-1 Mechanism unknown	Adhesion to desquamated epithelial cells Nasal colonization?	93, 120
Serine aspartate repeat protein E (SdrE)	Complement factor H	Immune evasion, degradation of C3b	132
	Close DLL		13
Bone sialoprotein binding protein (isoform of SdrE)	Fibrinogen α-chain (DLL)	Adhesion to immobilized fibrinogen	151
Fibronectin binding protein A and B	FnBPA A domain binds fibrinogen γ-chain C-terminus and elastin (FnBPA A domain, DLL)	Adhesion to ECM	6, 16, 152
A domains			
	FnBPB A domain also binds fibronectin but not by DLL		152
	FnBPA, FnBPB	FnBPA-FnBPA A domain homophilic interaction; biofilm formation	20, 25
Fibronectin binding repeats	Fibronectin, tandem β-zipper	Adhesion to ECM Invasion of mammalian cells	73–75, 86, 153
Collagen binding protein (Cna)	Collagen triple helix (CH)	Adhesion to collagen-rich tissue	17
	Complement protein C1q (CH)	Prevent classical pathway of complement activation	19
	Laminin	Adhesion to basement membrane	154
NEAT motif family			
Iron-regulated surface protein A (IsdA)	Heme, fibrinogen, fibronectin, cytokeratin 10, loricrin (N-terminal NEAT motif region)	Heme uptake and iron acquisition	64
		Adhesion to desquamated epithelial cells	65, 119, 155
	Unknown ligand(s); C-terminal domain NEAT motif region	Resistance to lactoferrin	156
		Resistance to bactericidal lipids and antimicrobial peptides Survival in neutrophils	66
IsdB	Hemoglobin, heme (N-terminal NEAT motif region)	Heme uptake and iron acquisition	64, 157
	β3 integrins (NEAT motif regions)	Invasion of nonphagocytic cells	68
IsdH	Heme, hemoglobin (N-terminal and/or C-terminal NEAT motif region)	Heme uptake and iron acquisition	69

(*Continued on next page*)

TABLE 1 (*Continued*)

Protein structural and functional group	Ligand and binding mechanism	Function	References
	Unknown ligand(s); N-terminal NEAT motif region)	Accelerated degradation of C3b	69
Three-helical bundle			
Protein A	IgG Fc	Inhibits opsonphagocytosis	53
	IgM Fab V_H3 subclass	B cell superantigen	55
	TNFR-1	Inflammation	54
	von Willebrand factor	Endovascular infection; endocarditis	56
	Unknown ligand (region Xr)	Inflammation	58
G5-E domain family			
S. aureus surface protein G (SasG) and plasmin-sensitive surface protein (Pls, SasG homolog in MRSA)	A domain; no known ligand	A domain; adhesion to desquamated epithelial cells; primary attachment to unconditioned biomaterial	43, 45, 93
	G5E repeats of SasG; homophilic Zn^{2+}-dependent homophilic interaction	Biofilm formation	48, 49, 51, 52
Legume lectin-like, cadherin-like			
SraP N-terminal legume-lectin domain	*N*-acetyl neuraminic acid (Neu5AC) Salivary agglutinin gp340	Adhesion to and invasion of mammalian cells	71, 72
SraP CHDL	SraP CHDL	Homophilic CHDL-CHDL interaction; biofilm formation Projection of L-lectin domain	71
5′-nucleotidase			
Adenosine synthase A	Converts ATP to adenosine	Survival in neutrophils; inhibits oxidative burst	87, 88
	2′-deoxyadenosine 3′-monophosphate; degradation product of DNA converted to 2′-deoxyadenosine	Macrophage and monocye apoptosis	90
EF-hand motif (calcium structural domain)			
Biofilm-associated protein Bovine/ovine mastitis isolates only	Biofilm Ca^{2+} dependent	Promotes persistence in ovine mastitis	158
	Binds GP96	Promotes adhesion to epithelial cells, reduces invasion	159
Other proteins No known functional motifs			
S. aureus surface protein X (SasX)	Ligands unknown	Biofilm, cell aggregation, squamous cell adhesion, neutrophil evasion	115
SasC N-terminal FIVAR-containing domain	Ligands unknown	Promotes primary attachment and accumulation phases of biofilm formation	160
SasB, SasD, SasF, SasJ, SasK, SasL	Unknown	SasF implicated in resistance to bactericidal effects of long chain unsaturated fatty acids	161
		Putative LPXTG proteins identified from genome sequences; no known structure or function	162

[a]AD, atopic dermatitis; CH, collagen hug; CHDL, cadherin-like domain; C1q and C3a, complement components; DLL, dock lock latch; ECM, extracellular matrix; FIVAR, found in various architectures; NEAT, near iron transporter; MRSA, methicillin-resistant *S. aureus*; *S. aureus* surface protein, Sas; Pls, plasmin-sensitive; SraP, serine-rich repeat protein.

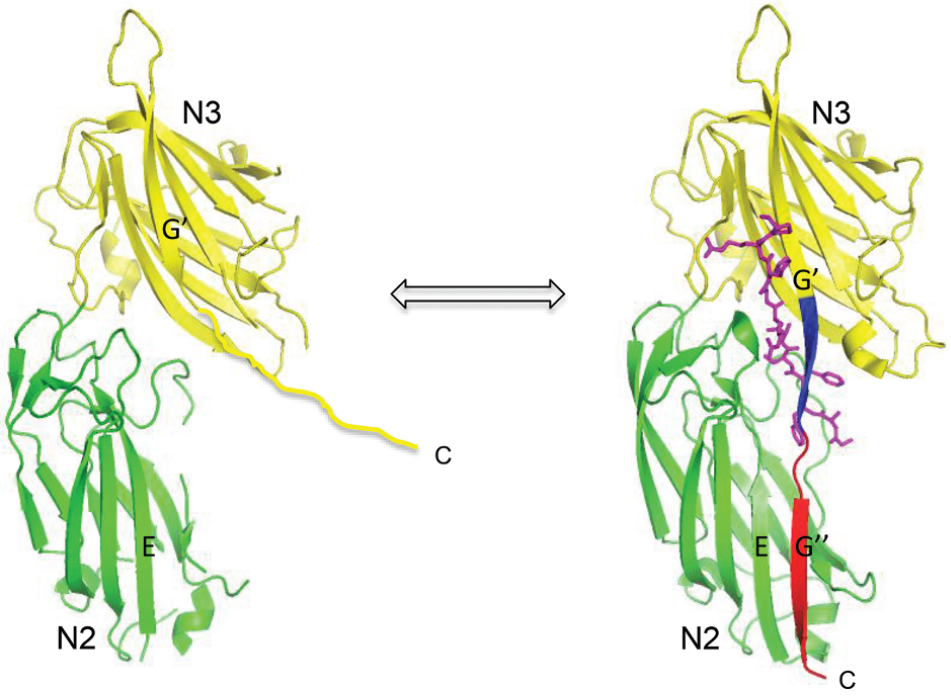


FIGURE 2 MSCRAMM binding to ligand by DLL. Ribbon diagram showing the structure of the N2 (green) and N3 (yellow) subdomains of SdrG in the apo form and in complex with a peptide from the β-chain (purple) of fibrinogen following ligand binding by the DLL mechanism. The C-terminal extension of N3 in the apo form undergoes a conformational change following ligand binding, resulting in an additional β-strand in a β-sheet in subdomain N2 forming the latch (red) and lock (blue). The letters refer to β-strands.

(27). This implies that the conformational changes that occur when fibrinogen binds FnBPA by DLL reorients the cell-bound MSCRAMM and exposes the plasminogen binding domain (Fig. 3).

It is likely that MSCRAMMs and, indeed, other CWA proteins are also able to bind several ligands. Indeed, the A domain of FnBPB can also bind to fibronectin by a mechanism that does not involve DLL (15).

Functions of other regions of MSCRAMMs

The SdrC, SdrD, and SdrE proteins have two or more repeated domains of 110 to 113 residues (B_{SDR}) located between the A region and the SD region (Fig. 1) (28). The B_{SDR} repeats are folded separately and form rigid rod-like structures that depend on Ca^{2+} for their structural integrity. They have no known ligand binding activity (29), although the related B_{SDR} region of SdrF from *S. epidermidis* can bind weakly to collagen (30).

It has been suggested that the B1 domain of SdrD makes contact with the adjacent N3 subdomain of region A, resulting in the ligand binding groove between N2 and N3 opening further than seen in the structure of the A domain alone, which possibly influences binding activity (31).

Cna proteins have 2 to 4 repeats of the B_{Cna} domain (Fig. 1) that are not related to the B repeats of Sdr proteins. Atomic force microscopy studies of bacteria expressing Cna proteins on their surface indicated that the B repeats act as nanosprings that project the A domain from the cell surface to facilitate the conformational changes required for the A domain to engage in ligand binding by the collagen hug (9). The B_{SDR} repeats might have a similar function.

Flexible stalks

MSCRAMMs from the Clf-Sdr family are attached to the cell wall via long flexible unfolded peptides comprising either serine-aspartate dipeptide repeats (ClfA, ClfB, SdrC, SdrD, SdrE) or 10/11 tandem repeats of ~38 residues that bind fibronectin by the tandem β-zipper mechanism (see below). The SD repeats of ClfA act like a stalk that projects the N-terminal ligand binding A domain away from the cell surface (32). Bacteria expressing ClfA truncates lacking SD repeats bound fibrinogen less avidly, perhaps because the A region was impaired by the proximity of the cell surface and could not undergo the conformational changes required for DLL.

Posttranslational modifications

The Sdr proteins are glycosylated by the action of the glysosyltransferases SdgA and SdgB that are encoded by genes located near the *sdrCDE* locus (33). They attach two *N*-acetylglucosamine molecules to serines in the SD repeat domain in a stepwise fashion (Fig. 3). Glycosylation creates a dominant epitope for human antibodies, which can represent up to 1% of IgG. Glycosylation protects the MSCRAMMs from degradation by host proteases such as the neutrophil cathepsin G and could thus contribute to virulence (33). Indeed, Sdg mutants have reduced virulence in a mouse sepsis infection model (34).

The Pls protein encoded by the type I staphylococcal cassette chromosome *mec* (SCC*mec*) element has a short C-terminal SD repeat region that is glycosylated by a combination of glycosyltransferases encoded by the genes *gtfC* and *gtfD*, which that are closely linked to the *pls* gene in SCC*mecI* along with SdgA and SdgB (35). Proteases can

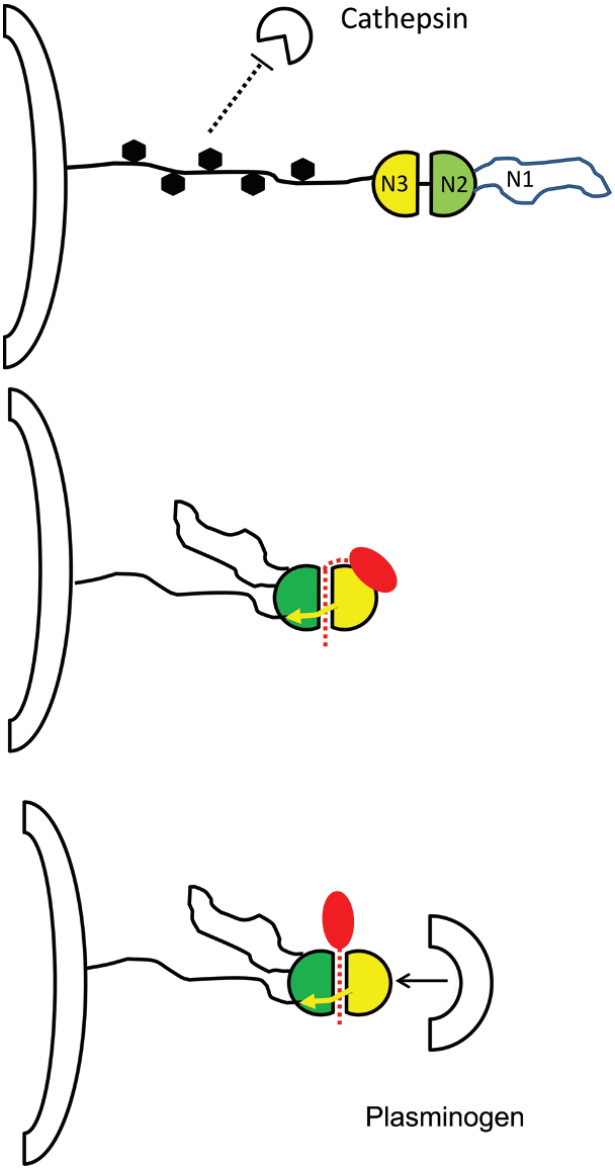


FIGURE 3 Schematic diagrams of MSCRAMMs before and after ligand binding. The top figure depicts an MSCRAMM in the apo form with the N2 (green) and N3 (yellow) subdomains shown as semicircles and the unstructured N1 subdomain. Serine residues in the flexible stalk are glycosylated, which prevents degradation by cathepsin. The middle diagram shows the conformational change in an MSCRAMM with respect to the bacterial cell that occurs following ligand binding by DLL. The C-terminal γ-chain peptide of fibrinogen is depicted by the red dashed line, and the gamma globule domain is in contact with the second ligand binding site in ClfA subdomain N3. It is not known if other MSCRAMMs have two binding sites on their ligands. The bottom diagram indicates that binding to fibrinogen by FnBPs exposes the N3 subdomain to plasminogen, which binds the MSCRAMM more efficiently in the presence of fibrinogen.

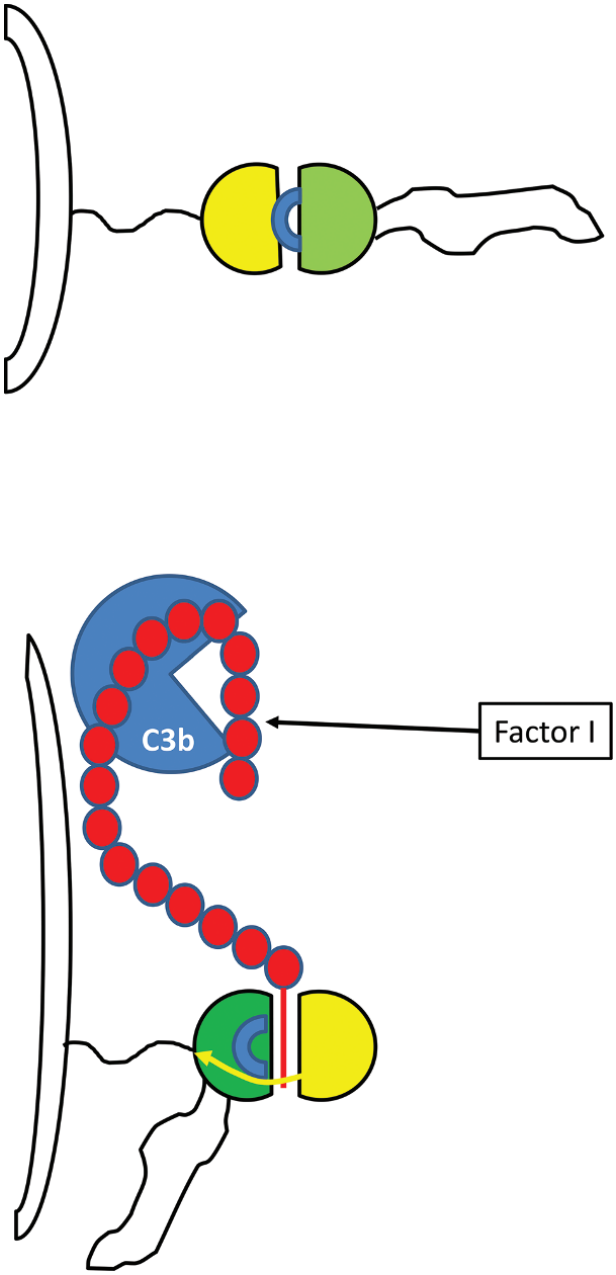


FIGURE 4 SdrE binding to complement factor H. The top figure shows SdrE in the apo form with the unstructured N1 subdomain and the N2 and N3 subdomains (yellow and green semicircles, respectively). The loop that occludes the ligand binding trench is shown in blue. The bottom figure shows the conformational changes that occur when complement factor H binds by closed DLL. Factor H (red) can then engage nearby C3b molecules (blue) and facilitate binding and activation of the protease factor I, which cleaves C3b.

remove subdomain N1 from MSCRAMMs. The staphylococcal protease aureolysin cleaves close to the boundary between subdomains N1 and N2 of ClfA and ClfB, while the host protease thrombin removes the N1 subdomain of FnBPA. Following removal of N1, ClfB can no longer bind to fibrinogen (36). It is possible that removal of subdomain N1 also reduces the capacity of *S. aureus* to adhere to loricrin and cytokeratin 10 since these ligands share a common binding site in ClfB. The molecular basis for the loss of ligand binding is not known (7). The biological significance of the removal of subdomain N1 from ClfA and FnBPA is unclear since it does not reduce the ability of FnBPA to promote biofilm accumulation or the ability of ClfA and FnBPA to mediate

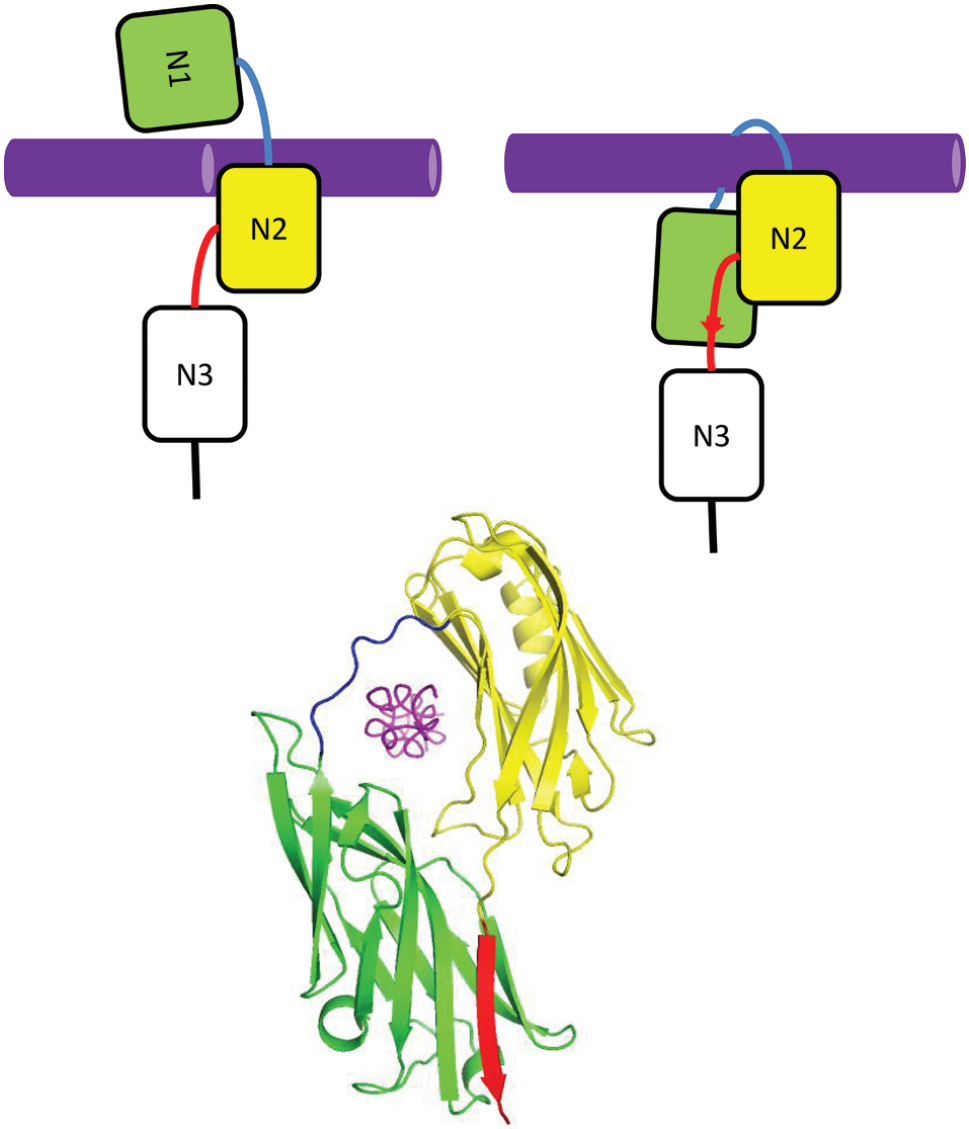


FIGURE 5 Collagen binding protein and the collagen hug. The upper part shows a schematic diagram of the Cna protein in the apo form on the left and following binding by the collagen hug to the collagen triple helix on the right. Below is a ribbon diagram of the Cna protein in complex with the collagen triple helix. The N1 subdomain (green) and N2 subdomain (yellow) are separated by a long unfolded region (blue) that forms the lock around collagen (purple). β-strand complementation by the red strand completes the hug.

adherence to fibrinogen (20, 37). Under certain conditions, FnBPs are degraded by the *S. aureus* protease V8 (38).

G5-E Repeat Proteins

Three staphylococcal proteins are characterized by the presence of G5-E repeats: SasG and Pls of *S. aureus* and Aap of *S. epidermidis*. Pls is encoded within the type I SCC*mec* cassette and is therefore only expressed by hospital-associated methicillin-resistant *S. aureus* strains carrying that element (39).

The proteins form highly extended fibrillar structures due to the unusual properties of the G5-E repeats and are able to mask the ability of shorter adhesins such as ClfA and FnBPs to promote adhesion to the ECM or invasion of mammalian cells (40–43). The G5-E repeats can also engage in homophilic interactions and promote biofilm formation (see below).

The N-terminal A domains of these proteins bind to cornified squamous epithelial cells by recognizing as yet unknown ligands and thus might contribute to nasal colonization (41, 43, 44). The A domain of Aap also promotes adhesion to hydrophobic surfaces in the initiation of biofilm formation by *S. epidermidis* (45, 46).

The ability to form very strong and stable (long and strong) extended structures is an unusual property of the intrinsically unstable repeated region (47–49). Both the G5 and E domains comprise two triple-stranded β-sheets with a central collagen-like triple helical region (Fig. 8). When an E domain is expressed alone or combined with a single G5 domain, the proteins are intrinsically disordered and

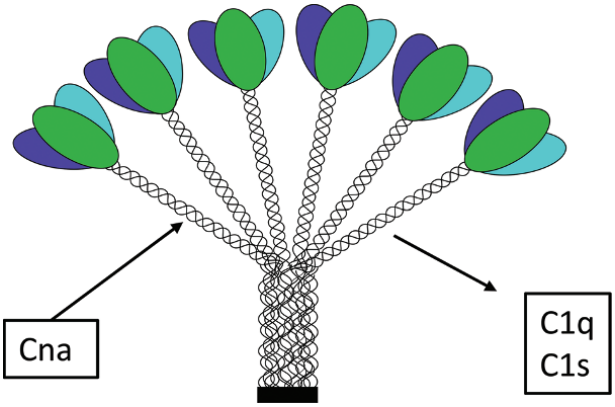


FIGURE 6 Complement protein C1q. C1q is a complex of six identical heterotrimers that form a bouquet-like structure. The globular domains (blue, green, and cyan ovals) make up the six IgG binding sites. Each heterotrimer forms an extended collagen-like triple-helix stalk which coalesces into a complex stem. C1r and C1s bind to the triple-helix region and are displaced when Cna binds. The figure was kindly provided by Nicole Thielens, CNRA-CFE-Université Joseph Fournier, Grenoble, France.

unfolded (47). However, a $G5_1$-E-$G5_2$ moiety folds to form an elongated structure. When $G5_2$ folds, it triggers folding of the central E domain, which in turn promotes folding of $G5_1$. Thus, the entire repeat region can be considered to comprise overlapping G5-E-G5 cooperative folding units.

Cell-cell adhesion and biofilm formation can be promoted by G5-E repeats. First, the N-terminal A domain must be removed by proteolysis (49–51). Only then can the folded G5-E repeats engage in the homophilic interactions involving multiple contacts along the lengths of the two molecules. Molecular modeling suggested that the G5-E domains twist around each other in a twisted rope-like structure (Fig. 7). Atomic force microscopy allowed measurement of the forces involved in cell-cell interactions, and in cases where full engagement was likely to have occurred, separation required

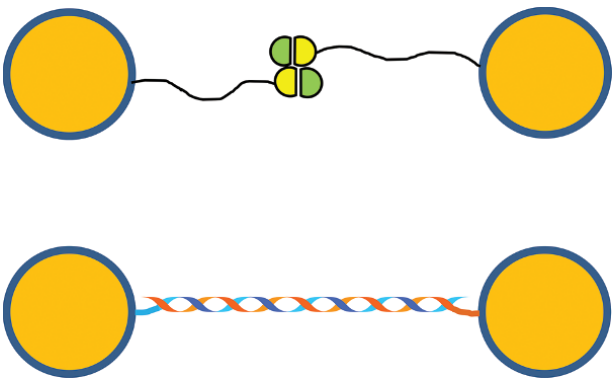


FIGURE 7 Homophilic interactions and biofilm formation. The upper part shows a schematic diagram of the model for homophilic interactions between the A domains of the MSCRAMMs FnBPA, FnBPB, and SdrC, which promote cell-cell accumulation of staphylococcal cells (yellow spheres) during biofilm formation. See Fig. 2 and 3 for the key. The lower part shows the extended fibrillar region of SdrG and Aap (orange and blue strands), which form extended zinc-dependent zipper interactions predicted to form a twisted rope-like structure.

the two proteins to become unwound by successively unfolding the E and G5 subdomains (52).

Protein A, a Protein Comprising Three-Helical Bundle Repeats

The N-terminal region of protein A (Spa) comprises a tandem array of five separately folded three-helical bundles, each of which can bind to several different ligands (Fig. 1, Table 1). The A1 domain of von Willebrand factor, tumor necrosis factor receptor 1, and the Fc region of IgG each bind to the interface between helices 1 and 2, while the Fab region of IgM binds to the interface of helices 2 and 3 (53–56). The three-helical bundles of Spa can exist in slightly different conformations, a conformational plasticity that facilitates binding to structurally diverse ligands (57). Upon binding to the Fc region of IgG, there is considerable loss of conformational heterogeneity combined with structural rearrangements at the interface.

Located between the helical bundle domains and the cell wall-spanning region Xc is a an octapeptide repeat region Xr, which is highly variable in number. Region Xr is proinflammatory by activating interferon-β expression (58).

The *S. aureus* binder of Ig Sbi (59) contains four tandemly arrayed three-helical bundles, two of which can bind to IgG in a way similar to Spa. Sbi associates with the cell envelope by binding noncovalently to lipoteichoic acid (60). This allows the most N-terminal repeats to be exposed on the cell surface and to contribute to immune evasion. The C-terminal repeats are buried but are biologically active in promoting fruitless consumption of complement protein C3 when released into the medium (61).

The NEAT Motif Family

The near iron transporter (NEAT) domain proteins capture heme from hemoglobin and contribute to the survival of bacteria in the human host, where iron is restricted. Heme is transported by several CWA iron-regulated surface (Isd) proteins to the membrane transporter. Once in the cytoplasm, heme oxygenases release free iron (62, 63). The defining feature of Isd proteins is the presence of one or more NEAT motifs, which bind either hemoglobin or heme. The structures of NEAT domains have been solved and the molecular mechanisms of ligand binding elucidated (64).

Isd proteins have additional functions other than transport of heme. IsdA promotes adhesion to squamous epithelial cells and promotes nasal colonization (65). The C-terminal domain of IsdA also confers resistance to bactericidal lipids (66). IsdB binds directly to β3-containing integrins and promotes platelet activation and invasion of mammalian cells (67, 68). IsdH helps *S. aureus* to avoid phagocytosis by promoting degradation of complement opsonin C3b by an as yet unknown mechanism (69).

The Legume Lectin Domain of SraP

The serine-rich adhesin of platelets (SraP) is a member of a family of glycoproteins in Gram-positive cocci (70). The structural gene *sraP* is included in a locus comprising genes that encode glycosyltransferases (GtfA and GtfB) and accessory secretion factors (SecY2 and SecA2). A short serine-rich repeat region (SSR1) is located at the N-terminus of the CWA protein followed by the ligand binding BR domain (Fig. 1). The BR domain is composed of four separately folded subdomains that form a rigid bent rod (71). Two cadherin-like domains and a β-grasp fold domain act to project the legume-lectin domain. This has structural similarity to

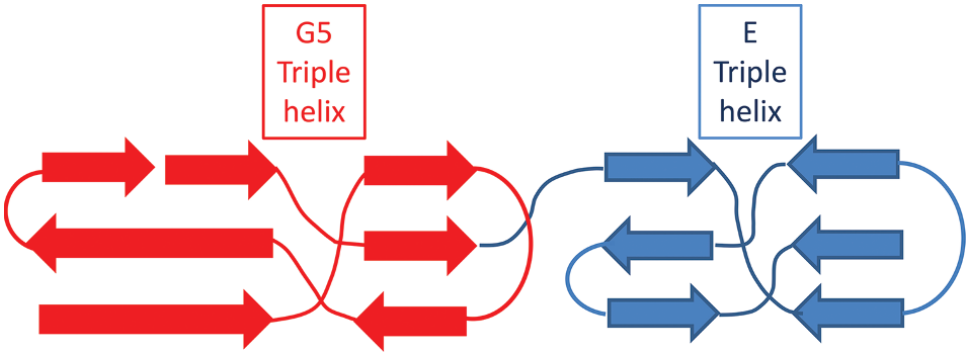


FIGURE 8 G5-E domains of SasG and Aap. The G5 (red) and E (blue) domains each form two triple-stranded β-helices separated by a short collagen-like triple helix.

legume lectins and binds *N*-acetyl neuraminic acid (Neu5Ac) containing glycoproteins that are attached to the surface of mammalian cells. It also binds to salivary glycoprotein gp340, which is rich in a 5NeuAc containing trisaccharide (72). SraP promotes bacterial adhesion to and invasion of mammalian cells (71). This was apparent using an experimental system where there was little or no fibronectin present and employing a strain that expressed low levels of FnBPs. Thus, there was minimal involvement of the highly efficient FnBP-mediated uptake (see below). SraP, like IsdB, might act as an accessory invasin. The cadherin-like domains dimerize in solution and promote cell-cell accumulation and biofilm formation (71).

The Tandem β-Zipper Binding to Fibronectin Promotes Invasion of Mammalian Cells

S. aureus can invade many types of mammalian cells that do not normally engage in phagocytosis, such as various epithelial cells, endothelial cells, fibroblasts, osteoblasts, and keratinocytes (73–80). This allows bacteria to evade host immune defenses and antibiotics. Bacteria can escape into the cytosol by lysing the phagosomal membrane and then multiply before eventually destroying the integrity of the cell and being released. In some cases staphylococci enter a semidormant state called the small colony variant (81). Small colony variants do not express cytolytic toxins, so the invaded cell stays intact. Small colony variants express FnBPs at high levels, which facilitates efficient uptake into nearby cells when they are released (82).

Fibronectin is a dimeric glycoprotein comprising three distinct domains: FI, FII, and FIII (83). In plasma fibronectin exists in a compact state which is held together by intramolecular interactions between F1 modules in the N-terminal domain and FIII modules in the C-terminal cell binding domain. The binding of FnBP to FI modules in the N-terminal domain of fibronectin by the tandem β-zipper mechanism (Fig. 9) forces a conformational change in fibronectin (84). This exposes a cryptic RGD integrin binding site in the 10th FIII module. This RGD motif is recognized by α5β1 integrins on the surface of mammalian cells. In this way, fibronectin acts as a bridge between *S. aureus* and host cells. Clustering of integrins triggers intracellular signaling by the focal adhesion kinase and Src kinase and subsequently endocytosis (77, 85, 86).

Nucleotidase Motif

A nucleotidase motif was discovered in a previously uncharacterized CWA protein called SasH. It was shown to be enzymatically active and, importantly, to contribute to the ability of *S. aureus* to evade innate immune responses in the infected host (87, 88). The ability of SasH (now called adenosine synthase [AdsA]) to convert intracellular ATP to the potent immunoregulatory molecule adenosine inhibited the oxidative burst and promoted survival within neutrophils.

Neutrophils also contribute to the host defenses by releasing DNA, which forms neutrophil extracellular traps (89). These can entrap bacteria and facilitate killing by their attached granule contents and histones. *S. aureus* expresses an extracellular deoxyribonuclease which degrades neutrophil extracellular trap DNA producing phosphomononucleotides, including 2′ deoxyadenosine 3′ monophosphate (dAMP) (90, 91). AdsA converts dAMP to 2′ deoxyadenosine, which triggers caspase 3-promoted apoptosis in macrophages and monocytes (90). By excluding macrophages from a developing abscess, AdsA helps to promote persistent infection.

CWA PROTEINS AS COLONIZATION AND VIRULENCE FACTORS

Investigating the Contribution of CWA Proteins to the Virulence of *S. aureus*

To investigate if a CWA protein can act as a virulence factor, a mutant defective in the protein can be isolated and its virulence compared to the wild type in an appropriate animal infection model. However, functional redundancy sometimes makes it difficult to show conclusively that a mutant lacking a single factor has reduced virulence. For example, *S. aureus* expresses several CWA proteins that bind fibrinogen, and most strains elaborate two fibronectin binding proteins. Another approach is to express the CWA protein in a surrogate host such as *Lactococcus lactis* (14, 92–94) or *Staphylococcus carnosus* (95). Until recently, it was difficult to isolate mutations in most clinically relevant strains, so earlier studies of the role of CWA proteins were performed with laboratory strains such as Newman and derivatives of NCTC8325. However, it is now possible to circumvent the restriction barriers that prevent transfer of plasmid DNA from *Escherichia coli* cloning hosts into *S. aureus*, rendering feasible genetic manipulation of diverse clinical isolates (96–98).

Another important consideration is differences in animals used for infection models compared to humans. Results of experimental infection studies must be interpreted with caution if the surface protein being analyzed has a lower affinity for ligands in the animal compared to humans. One way to circumvent such difficulties is to engineer a humanized mouse expressing the human version of the ligand (99). For

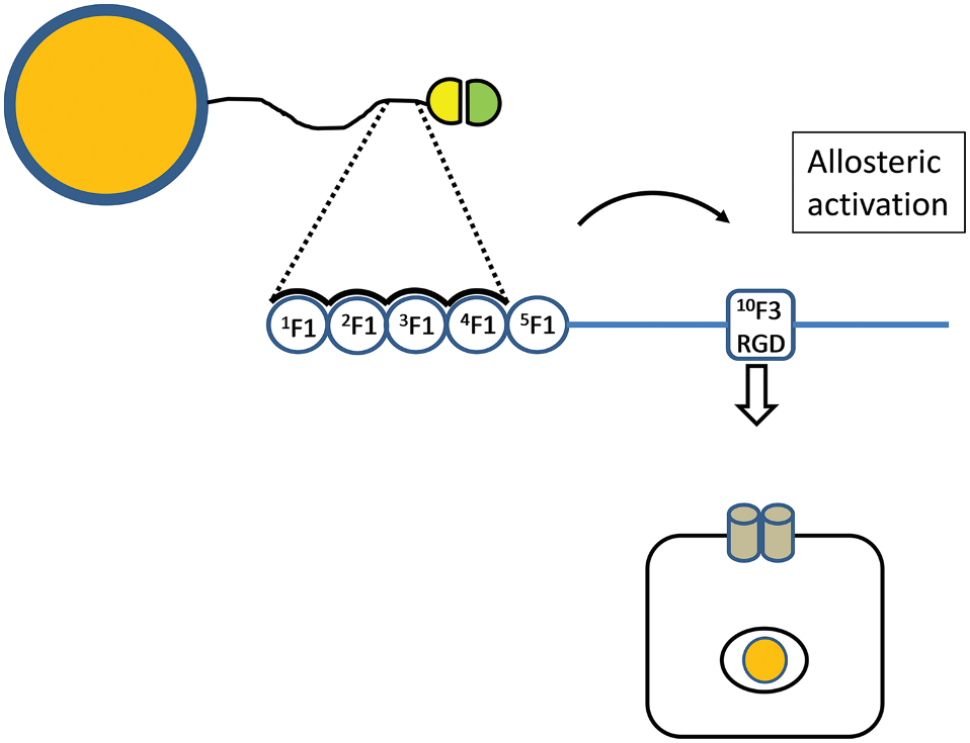


FIGURE 9 Fibronectin binding by FnBPs. The figure shows how one fibronectin binding repeat of the unstructured fibronectin binding region of FnBP binds to N-terminal type I modules of fibronectin by the tandem β zipper mechanism. Potentially up to 10 such interactions can occur per molecule of FnBP. Intramolecular interactions between the N-terminal type I modules and C-terminal type III modules result in allosteric activation of the 10th type III module, exposing an RGD motif which engages an $\alpha_5\beta_1$ integrin on the surface of a mammalian cell to promote invasion by endocytosis.

example, IsdB does not bind mouse hemoglobin, so a transgenic mouse expressing the human version of the protein has been employed in infection studies (100). Another option is to murinize the pathogen by engineering the virulence factor to bind to the murine version of the ligand as has been achieved with internalin A of *Listeria monocytogenes* (101).

A summary of studies demonstrating that bacterial mutants defective in CWA proteins have reduced virulence in different infection and colonization models is shown in Table 2. A systematic analysis of mutants defective in individual surface proteins was performed with mutants of strain Newman using a mouse model of bacteremia, dissemination, and abscess formation (102). Mice were injected intravenously with bacteria, and the bacterial load in the kidneys and the number of abscesses were measured. This model measured the ability of bacteria to survive in the bloodstream and to invade kidney tissue and establish an abscess. Several mutants had a statistically significant >1 log reduction in viable counts in kidney tissue, indicating the importance of CWA proteins, although the precise role of each protein is not known (103). The study was limited by the fact that strain Newman does not express Cna and lacks the ability to display FnBPs on its surface due to mutations in the *fnbA* and *fnbB* genes, which prevent anchorage of the proteins to the cell wall (104).

The collagen binding protein promotes virulence in infections where adhesion to collagen-rich tissue is important, such as ocular keratitis and septic arthritis. The strength of adhesion of the recombinant protein to immobilized collagen *in vitro* correlated with the severity of the disease (105). Cna also interferes with complement fixation and most likely contributes to innate immune evasion during infection (19).

Studies of *S. aureus* strains that colonized cardiac devices in human patients compared to those isolated from cases of uncomplicated bacteremia identified polymorphisms in the *fnbA* gene encoding FnBPA associated with complicated bacteremia that increased the affinity of the protein for fibronectin *in vitro* (106). This implies that selection occurred *in vivo* for strains that had a high affinity for fibronectin that coated the device implanted in the patient.

Rather than isolate null mutants that lack the ability to express a surface protein bacteria expressing variants, the protein on the cell surface with amino acid substitutions that cause loss of function can be studied. For example, mutants expressing mutants of ClfA (107) and Cna (105) with reduced ligand binding were shown to lack virulence in a model of septic arthritis. A mutant expressing AdsA that was defective in adenosine synthase survived less well than the wild type in blood stream infection (87). An advantage of this approach is that possible pleiotropic effects of the missing surface protein on the elaboration of other CWA proteins are avoided.

Infection studies with the surrogate host *L. lactis* expressing CWA proteins have been used to study aspects of the infection process. The ability of *L. lactis* to colonize a sterile thrombus on damaged heart valves in a rat model of endocarditis was dependent on bacteria being able to adhere to fibrinogen/fibrin on the damaged tissue. This could be mediated either by ClfA or the A domain of FnBPA (94). Subsequent bacterial proliferation and expansion of the lesion required FnBP-promoted invasion of cells of the surrounding endothelium.

TABLE 2 CWA proteins as colonization and virulence factors studied using animal models

Role in colonization or infection	CWA protein	Mechanism	References
Nasal or skin colonization	ClfB	Adhesion to loricrin on squames	14
	IsdA	Adhesion to squames	119
	SasX	Adhesion to squames	115
Endocarditis	ClfA	Adhesion to thrombus	163
	FnBPA	Adhesion to thrombus; invasion of adjacent endothelium	94
	ClfB	Adhesion to thrombus	164
	SraP	Adhesion to platelets; colonization of thrombus	165
Mastitis	FnBP	Invasion of epithelial cells in mammary gland	(166)
Pneumonia	Protein A	Enhanced inflammation of lung epithelium	167
Foreign body infection	FnBP	MRSA[a] biofilm-promoted infection	168
		Adhesion to intra-aortic patch	169
Ocular keratitis	Cna	Enhanced colonization and infection	170
Septic death	ClfA	Reduced opsonophagocytosis	102, 107, 141
Survival in blood	Protein A		171, 172
	IsdH		69
	AdsA		87
	SasX		115
Kidney abscess	AdsA, ClfA, ClfB Spa, IsdC. IsdA, IsdB, SdrD SdrE	Increased survival in blood stream prior to kidney infection	87, 102
Septic arthritis	ClfA, protein A	Enhanced survival in bloodstream prior to invasion of joint	141, 172
	Cna	Enhanced survival in blood stream; adhesion to cartilage within joint	173
Joint infection	Fibrinogen binding MSCRAMMs	Biofilm formation in synovial fluid	174
Subcutaneous abscess	Spa	Abscess development, bacterial load	171
	FnBPs, ClfA		175
	SasF		161

[a]MRSA, methicillin-resistant *S. aureus*

CWA Proteins Promote Colonization of the Host

Approximately 20 to 30% of the population are colonized persistently by *S. aureus* in the nares, with the remainder being intermittently colonized (108, 109). The factors that determine whether an individual is a persistent carrier are not completely understood (110). *S. aureus* colonizes the nares by adhering both to ciliated epithelial cells in the posterior region of the nares and to squamous epithelial cells in the anterior nares (111).

Adhesion to ciliated epithelial cells is mediated in part by the zwitterionic bacterial wall teichoic acid (WTA) binding to the scavenger receptor SREC-1 (112). This occurs through interactions involving positively charged D-alanine residues that decorate WTA binding to negatively charged residues on the host protein (112). Glycosylation of WTA contributes to the ability of *S. aureus* to colonize the nares of cotton rats, although the receptor for the *N*-acetyl glucosamine residues is not known (113).

Several CWA proteins promote adhesion of *S. aureus* to the cornified envelope of squamous cells (corneocytes). ClfB and IsdA have been shown to promote nasal colonization in rodent models and, in the case of ClfB, humans (14, 65, 114). SdrC, SdrD, SasG, Pls, and SasX contribute to bacterial adhesion to corneocytes *in vitro*, and SasX can also promote colonization of the nares of mice (43, 93, 115). Pls and SasX are encoded by genes located within mobile genetic elements and are thus only expressed by a subset of methicillin-resistant *S. aureus* strains carrying the relevant element. Pls is encoded by a gene carried within the type I SCC*mec* cassette (116), while the *sasX* gene is carried by a lysogenic phage found only in ST239 methicillin-resistant *S. aureus* strains that are prevalent in the Far East (115). The *sasG* gene is carried by 50% of the most prevalent lineages (clonal complex 1 [CC1], CC5, CC8, CC15, and CC22) but is absent from CC12, CC25, CC51, CC45, and CC30 strains (117).

It is well established that ClfB can bind to cytokeratin 10 and loricrin, proteins that only occur in corneocytes (5, 7, 118). Loricrin is probably the more important ligand *in vivo*; loricrin knockout mice are defective in colonization (14). ClfB binds to loricrin by the high-affinity DLL mechanism (14). IsdA can also bind to loricin, cytokeratin 10, and involucrin, but the mechanistic basis of these relatively weak interactions is not known (119). SdrD binds to desmoglein 1, a component of both the desmosomes that hold keratinocytes together and the corneodesmosones that regulate desquamation in the stratum corneum (120, 121).

S. aureus is regarded as a minor or transitory resident of healthy skin. However, in eczema (atopic dermatitis [AD]) patients suffering from a flare-up, the diversity of normal skin microbiota is reduced and *S. aureus* proliferates (122). The bacterium releases several proteins that can exacerbate inflammation in AD skin (123). Colonization of AD skin is at least in part promoted by the ability of bacteria to adhere to corneocytes in a ClfB-dependent fashion (124). Adhesion to loricrin and/or to ligands that become exposed on the surface of the deformed corneocytes where corneodesmosome proteins are present on villous-like protrusions is an important first step in colonization (125).

IMMUNE EVASION PROMOTED BY CWA PROTEINS

Evasion of Innate Immune Responses: Inhibition of Complement Activation

Protein A is a multifunctional CWA protein of *S. aureus* that promotes evasion of innate responses by its ability to capture IgG from serum. Bacterial cells become coated with IgG that is bound incorrectly so that the Fc region of the antibody cannot be recognized by the Fc receptor on neutrophils (126, 127). To initiate the classical pathway of complement fixation, the complement protein C1q must be able to bind to the Fc region of bound IgG (128, 129). Thus, opsonophagocytsosis is comprehensively inhibited by preventing recognition of the opsonins IgG and C3b by both the Fc receptor and the complement receptor on neutrophils.

The collagen binding protein also blocks initiation of the classical pathway of complement fixation by binding to the collagen triple-helix structures of C1q, displacing C1r-C1s and preventing complement activation (19).

Evasion of Innate Immune Responses: Enhanced Destruction of C3b

Complement activation results in the complement protein C3b being deposited on the bacterial cell surface, allowing it to be recognized by the complement receptor on phagocytic cells. Accumulation of C3b begins the amplification process of the alternative activation pathways and also triggers activation of C5 convertase (129). Three CWA proteins, SdrE, ClfA, and IsdH, contribute to enhanced complement degradation (69, 130–132). Only in the case of SdrE is the mechanistic basis understood. SdrE binds the host complement regulatory protein factor H, which in turn recruits and activates the specific C3b-degrading protease factor I (13). It is possible that ClfA can bind and activate factor I directly without the requirement of factor H, but evidence for this is incomplete and further work is required.

Interference with Adaptive Immune Responses

A hallmark of *S. aureus* infections is the elaboration of superantigens which interfere with adaptive immunity. Most strains have the ability to secrete one or more small proteins (enterotoxins and toxic shock syndrome toxin 1) that have the ability to bind the MHC-II receptor of antigen-presenting cells and promote binding to T cell receptors (133). This triggers activation and proliferation of a plethora of T cells without appropriate guidance and recognition by bound antigen fragments. The results is uncontrolled expression of cytokines, which in the worst case scenario can cause toxic shock syndrome. Depletion of T cells also results in failure to mount an appropriate adaptive immune response.

Recently, it has been recognized that protein A is a crucially important factor in the interaction of *S. aureus* with its host. Protein A is expressed by all clinical strains of *S. aureus* and is important in persistent nasal carriage and in disrupting adaptive immune responses during infection (134, 135). Protein A is released from the cell surface by the action of autolysins, resulting in soluble molecules with fragments of peptidoglycan bound to the C-terminus (136, 137). The Ig binding repeats of Spa bind to and cross-link the variant V_H3 chain of receptors on B cells in lymphoid tissue (55). This results in B cell proliferation. During infection there is a rise in the level of V_H3 clonal IgM and IgG in serum, but these antibodies lack the ability to bind to *S. aureus* antigens (137, 138). This inappropriate expression of Ig requires the cooperation of $CD4^+$ T cells (137), although it is not clear if antigen presentation is involved. The C-terminal peptidoglycan fragment is implicated in activation of (nucleotide-binding oligomerization domain) NOD1/NOD2 signaling, which in turn triggers maturation of the B cells into antibody-secreting plasma cells (55, 137, 139). Depletion of B cells from lymphoid tissues causes anergy, and the lack of immunological memory results in *S. aureus* being able to reinfect and is probably responsible for bacteria being able to proliferate in the nares of persistent carriers.

CONCLUDING REMARKS

CWA proteins provide opportunities for *S. aureus* to interact with the host, both in the commensal state and during infection. Given that the repertoire of CWA proteins is restricted, it is perhaps not surprising that several of these proteins have been found to be multifunctional.

Recently, it was proposed that CWA proteins should be categorized based on structural motifs and functional attributes (2, 140). There has been a tendency to use the acronym MSCRAMM inappropriately for all CWA proteins regardless of function and whether or not they bind to components of the ECM.

The functions of some CWA proteins have yet to be established, and it is likely that new ligands will be discovered for those with known functions. Structural analysis will provide clues about function as well as the application of high-throughput techniques for discovering binding partners. Far Western blotting, phage display, and two-hybrid analysis have identified potential ligands which must then be validated by detailed analysis to show biological relevance (21, 24, 26, 120).

Until recently, most studies were performed with laboratory strains. It is now possible to genetically manipulate strains from all genetic lineages so that clinically relevant bacteria can more easily be studied (96–98). This is important because since variations in the repertoire of surface proteins occur in different lineages, sequence variations in ligand binding domains might lead to different affinities for ligands, and the timing of and regulation of gene expression can result in different levels of protein abundance on the cell surface.

Several CWA proteins have been investigated as potential antigens for vaccination to protect against *S. aureus* infections and have been shown to offer protection in murine models of infection (65, 141–143). Furthermore, recombinant truncates that have been engineered so as not to bind host ligands offer the potential for improved antigenicity (107, 144). Several combinations of antigens including fragments of CWA proteins and other surface components have been formulated (145–147). However, the only phase III clinical trial performed to date was with a single-antigen

IsdB and resulted in failure for reasons that are not clear (148, 149). Any future clinical trials will require the use of an adjuvant that triggers a cellular response involving T_H1 cells and/or T_H17 cells as well as interleukin-17 and interferon-γ-mediated recruitment of neutrophils and must avoid the immune dysregulation caused by expression of superantigens (146, 149).

In conclusion, CWA proteins carry out a range of important functions and are essential for successful colonization of the host and for infection. Structural and functional analysis has provided a rational framework for classification of CWA proteins and has helped to define the mechanistic basis of host-pathogen interactions.

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Immune Evasion by *Staphylococcus aureus*

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39

INTRODUCTION

The Gram-positive bacterium *Staphylococcus aureus* is considered a commensal bacterium because roughly one-third of the human population is colonized by it without developing disease (1). Colonization occurs in the human nose, whereby the host nasal microbiota plays a major role in promoting or inhibiting *S. aureus* colonization (2). Despite the fact that *S. aureus* is considered a commensal, nasal carriage of *S. aureus* is linked to bacteremia (3). The bacterium may cause a range of infections, from cellulitis and superficial skin disease to abscesses, bacteremia, sepsis, endocarditis, and pneumonia (4). Moreover, *S. aureus* has been shown to adapt in its interaction with humans by increasing resistance against methicillin and is currently a leading cause of human bacterial disease worldwide. Methicillin-resistant *S. aureus* (MRSA) was identified in the 1960s as a nosocomial pathogen, when hospitalized patients showed distinct risk factors for acquisition (5). The prevalence of methicillin resistance among nosocomial *S. aureus* isolates increased from 2.1% in 1975 to 35% in 1991 (6). MRSA epidemiology changed in the 1990s when infections of healthy individuals outside hospitals were reported. These strains, with increased virulence, were the first reports of community-acquired MRSA (7, 8). Now, community-acquired MRSA has been reported as the leading cause of bacterial infections in the bloodstream, skin, soft tissue, and lower respiratory tract in developed countries (9). As a consequence, research interest in the pathophysiology of *S. aureus* has increased.

This extensive research during the past decades resulted in the identification and characterization of around 40 proteins which are able to evade various processes of the innate and adaptive immune system (10), the so-called immune evasion proteins. A list of these evasion molecules with their abbreviation, what they evade and location on which MGE is listed in Table 1. Proteomics has shown that around 100 to 200 *S. aureus* proteins are secreted, many with a still unknown function (11). These unknown secreted proteins are potential evasion molecules, demonstrating that the identification and characterization of new evasion proteins is not yet complete and will most likely expand in the future.

Many evasion proteins are targeted against neutrophils. This is not a surprise, because neutrophils are the most prominent leukocytes in blood, covering 60% of the leukocyte population, and play a prominent role in fighting *S. aureus* infection. They are equipped with various granules with specific content to kill both Gram-negative and Gram-positive bacteria (12, 13). Neutrophils originate and mature in the bone marrow and are then released into the bloodstream. There, these end-stage cells are induced to circulate and migrate toward the site of infection by chemotactic signals produced both by the bacteria themselves and by host cells (13). Upon arrival at the infection site, neutrophils are able to "eat" microorganisms that are opsonized (labeled) by the complement system or immunoglobulins (antibodies). The complement system is especially important in clearing staphylococcal infections. Mice deficient in complement C5 showed decreased clearance of *S. aureus* after pulmonary and bloodstream infections (14, 15). In addition, antibodies play an important role in fighting staphylococcal infections. Opsonization of bacteria subsequently leads to phagocytosis and, ultimately, killing of microbes, because neutrophils release the content of their antimicrobial granules and produce reactive oxygen species (ROS) (13).

Impaired neutrophil function is linked to staphylococcal infections, as shown in multiple studies. For instance, patients suffering from congenital neutropenia often have severe infections, including staphylococcal infections, which can be fatal (16). Patients with chronic granulomatous disease have defects in their NADPH oxidase, which leads to impaired formation of ROS. Catalase-positive microorganisms in particular, such as *S. aureus*, cause recurrent infections in these patients because catalase breaks down the bacterial hydrogen peroxide and thereby prevents the generation of ROS (17). Another example where *S. aureus* infections play a major role is in burn patients. The burn wound is especially susceptible to bacterial colonization and infection, because neutrophils are known to be decreased in burn patients (18, 19). From these observations we can conclude that neutrophil-mediated killing in healthy individuals is crucial in the defense against *S. aureus*.

After the first response, the acquired immune system comes into the picture. However, this part of the immune system has an insignificant role in acute infection. Nonetheless, antibodies are important in the long term with recurrent staphylococcal infections, but antibodies against staphylococcal proteins do not show effective protection; even though most adult humans have high levels of circulating antibodies against different *S. aureus* proteins, these are not immunologically protective (20).

In this article we discuss the different facets of the antistaphylococcal innate immune system. First, we describe the mechanisms of neutrophil extravasation through the endo-

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TABLE 1 Abbreviations of staphylococcal immune evasion proteins, what they evade, and on which MGE or paralogous gene cluster they are located[a]

Abbreviation	Full name	Evades process[b]	MGE[c]
Aur	Aureolysin	III, V	
CHIPS	Chemotaxis inhibitory protein of *Staphylococcus*	II	IEC-1/φSa3
Can	*S. aureus* collagen adhesion	III	
Eap and EapH	Extracellular adherence protein and extracellular adherence protein homologue	I, V	
Ecb	Extracellular complement-binding protein	III	IEC-2
Efb	Extracellular fibrinogen-binding protein	III	IEC-2
FLIPr	FPR2 inhibitory protein	II	IEC-2
Hla	Hemolysin-alpha	V	
Hlg	Hemolysin-gamma	V	
LukS-PV	Panton Valentine leukocidin	V	φSa2
Luk-AB	Leukocidin AB	V	
Luk-DE	Leukocidin DE	V	
Nuc	Nuclease	IV	
PSMs	Phenol-soluble modulins	V	
Sags	Superantigens	V	IEC1, SaPIn1, vSaβ[d]
SAK	Staphylokinase	III, V	IEC-1/φSa3
Sbi	Staphylococcal binding of IgG	III	
SCIN	Staphylococcal complement inhibitor	III	IEC-1/φSa3
ScpA	Staphopain A	II	
SdrE	Surface-associated serine-aspartate repeat protein E	III	
SelX	Staphylococcal enterotoxin-like X	I	
SpA	Staphylococcal protein A	III	
SPIN	Staphylococcal peroxidase inhibitor	V	vSaα[d]
SSL	Staphylococcal superantigen-like 1-11	I, II, III	vSaα[d]
SSL	Staphylococcal superantigen-like 12, 13, 14	I, II, III	vSaβ[d]

[a]MGE, mobile genetic element.
[b]The innate immune mechanisms that are inhibited: I, neutrophil extravasation; II, priming, chemotaxis, and activation of neutrophils; III, opsonization and phagocytosis; IV, NET formation; V, bacterial killing by neutrophils.
[c]IEC, immune evasion cluster; SaPI, staphylococcal pathogenicity island.
[d]vSaα and vSaβ are clusters of paralogous genes that have evolved in situ, are nearly universally conserved in *S. aureus*, and are not MGEs.

thelium. Second, we give an overview of the proteins and receptors involved in neutrophil priming, chemotaxis, and activation. Third, we describe the processes involved in bacterial opsonization and phagocytosis by neutrophils. Fourth, we provide an overview of the processes involved in bacterial killing by neutrophils. After each section we describe the various evasion molecules that interrupt that specific process. Finally, we discuss why *S. aureus* has evolved so many evasion proteins and the therapeutic implications of these proteins with respect to staphylococcal infections. Thus, we give an overview of what we know so far about *S. aureus* immune evasion and what lessons we can draw from this knowledge.

NEUTROPHIL EXTRAVASATION THROUGH THE ENDOTHELIUM

Once pathogens cause infections in tissues, neutrophils leave the bloodstream and migrate toward the site of infection. This multistep process is called extravasation and includes neutrophil rolling, crawling and firm adhesion to the endothelial cells, extravasation through the endothelium (diapedesis), and migration to the infection site over a chemokine gradient (21). An overview of this process is shown in Fig. 1.

First, the neutrophils in the bloodstream slow down near the site of infection. This is initiated by changes on the endothelial surface by inflammatory mediators (e.g., histamine, cytokines, and leukotrienes) (22). The endothelial cells are activated and start to express P-selectin and E-selectin, which interact with P-selectin glycoprotein ligand-1 (PSGL-1) on the surface of neutrophils (23). This leads to the tethering (capturing) of freely moving neutrophils to the endothelial surface as they start to roll along the vessel in the direction of the blood flow. In the second step the neutrophils adhere and "crawl" along the vessel wall by the interaction between intercellular adhesion molecule 1 (ICAM-1) on endothelial cells and β2 integrins (such as LFA-1 and Mac-1) on the surface of phagocytes (24–26). Neutrophils express constitutively high levels of β2 integrins, which undergo conformational change upon either intracellular activation ligand binding (outside-in signaling) or integrin adhesiveness

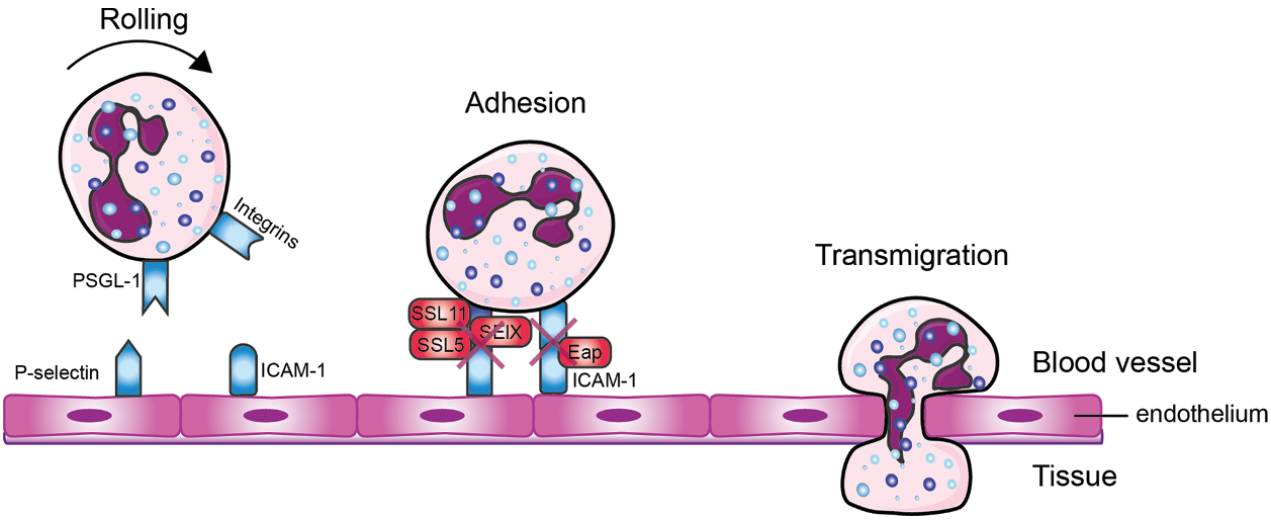


FIGURE 1 Evading neutrophil extravasation to the infection site. Mechanisms by which *S. aureus* evades the steps in neutrophil extravasation. Neutrophils start to roll on the activated endothelium, which leads to firm adhesion and subsequently to transmigration through the endothelium. Red boxes indicate staphylococcal proteins, and blue boxes indicate host proteins. Abbreviations: PSGL-1, P-selectin glycoprotein 1; SSL, staphylococcal superantigen-like protein; ICAM-1, intracellular adhesion molecule 1; Eap, extracellular adherence protein; SElX, staphylococcal enterotoxin-like X. The figure was adapted from Servier Medical Art.

(inside-out signaling). β2 integrins are relocalized to the cell surface upon activation by chemokines (27, 28). The neutrophils enter the arrested state after expressing high levels of ICAM-1. This leads to the initiation of transendothelial cell migration through a paracellular (passing through the endothelium between the cells) or a transcellular (movement through the endothelial cell) pathway. Leukocytes mainly migrate via the paracellular route but follow the transcellular route in the central nervous system and in various inflammatory settings (29). After extravasation, the neutrophils continue their journey toward the infection site via chemotaxis.

EVADING NEUTROPHIL EXTRAVASATION

An overview of the molecules interfering with the extravasation of neutrophils is shown in Fig. 1. *S. aureus* is able to modulate the first step in neutrophil extravasation, rolling of neutrophils on endothelial cells, by secreting staphylococcal superantigen-like 5 (SSL5). SSL5 directly binds PSGL-1 on leukocytes and human HL-60 leukemia cells. This prevents the interaction of PSGL-1 with its natural ligand, P-selectin, in a sulfation- and sialylation-dependent manner (30, 31). Cocrystallization data revealed SSL5 in complex with tetrasaccharide sialyl Lewis X, a key posttranslational modification of PSGL-1 binding to P-selectin (32). SSL5 is part of a larger group of 14 structurally related proteins (SSL1 to SSL14), which are involved in innate immune evasion. SSL11, of all the SSLs, shares the highest amino acid sequence identity to SSL5 and also shows binding to sialyl Lewis X, as seen in the cocrystal structure of the complex. SSL11 also binds other sialic acid-containing glycoproteins, such as FcαRI, the Fc receptor for IgA (33). *S. aureus* secretes another immune evasion molecule, named SElX, which also interacts with PSGL-1 in a glycosylation-dependent manner (34). Before the molecular mechanism was identified, SElX was thought to possess superantigenic activity and was associated with a virulence factor in MRSA necrotizing pneumonia (35). Recently, SElX was also found to have the sialylated-glycan-dependent active site, similar to a subfamily in SSLs (SSL2 to SSL4 and SSL6) (36). This leads to binding of neutrophils and monocytes via multiple glycosylated neutrophil surface receptors, thereby disrupting IgG-mediated phagocytosis and contributing to pathogenesis, as revealed in a necrotizing pneumonia rabbit infection model (37, 38).

The second step of extravasation, the adhesion of neutrophils to endothelial cells, is also targeted by *S. aureus*. The pathogen secretes extracellular adherence protein (Eap) that directly binds ICAM-1 and thereby inhibits neutrophil recruitment to the infection site (39).

PRIMING, CHEMOTAXIS, AND ACTIVATION OF NEUTROPHILS

When neutrophils have migrated through the endothelial barrier, they are primed, recruited toward the site of infection by various chemoattractants, and activated by multiple inflammatory stimulants. Here, we will discuss various sets of proteins and receptors involved in this process. An overview is shown in Fig. 2.

Priming occurs when a neutrophil is exposed to a primary priming stimulus which enhances the neutrophil's functional response (for example, adhesion, phagocytosis, degranulation, and superoxide production [40]), although the priming stimulus does not give the functional response itself (41). Examples of known priming molecules for neutrophils are complement components C3a and C5a (42), interleukin-8 (IL-8) (43), granulocyte colony-stimulating factor (43), tumor-necrosis factor-α (44), and interferon-γ (45).

Chemoattractants are chemokines and bacteria- and complement-derived products that activate phagocytes by interacting with receptors at the surface of neutrophils which belong to the superfamily of G protein-coupled receptors (GPCRs) (46). These GPCRs also belong to the rhodopsin subfamily of GPCRs. Rhodopsin is a seven-transmembrane receptor with seven helical membrane-spanning regions connected by six extramembrane loops, as shown by crystallization (47). The mechanism of stimulation of the GPCR depends on the type of ligand. For example, small peptides (e.g., *N*-formyl-methionyl-leucyl-phenylalanine [fMLP]) and

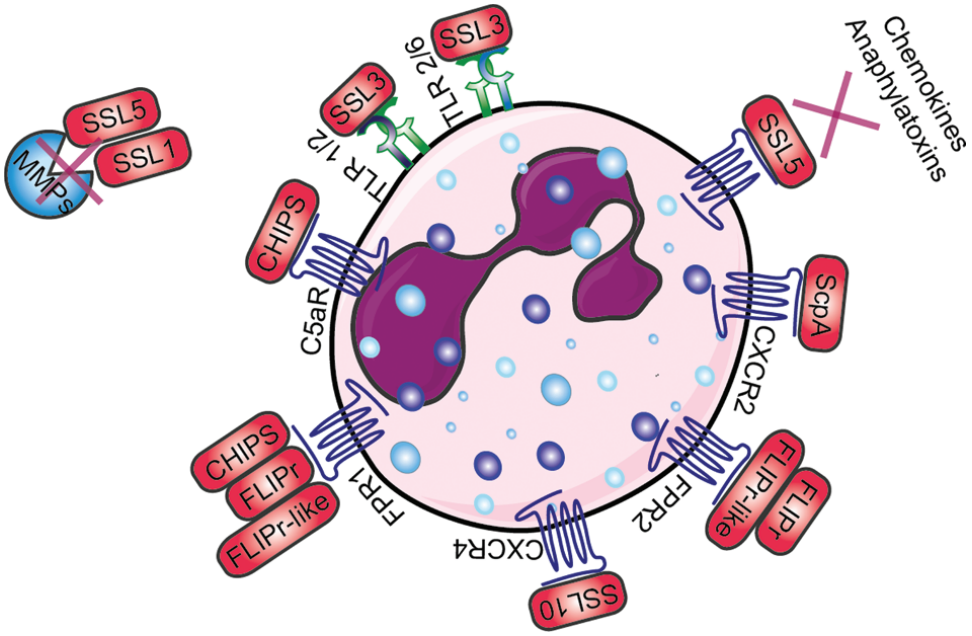


FIGURE 2 Schematic overview of how *S. aureus* evades priming, chemotaxis, and activation of neutrophils. Red boxes indicate staphylococcal proteins, and proteins shown in blue indicate host proteins. Abbreviations: TLR, Toll-like receptor; CXCR, chemokine receptor; ScpA, staphopain A; SSL, staphylococcal superantigen-like protein; FPR, formyl peptide receptor; FLIPr, FPR2 inhibitory protein; C5aR, C5a receptor; CHIPS, chemotaxis inhibitory protein of *Staphylococcus*; MMP, matrix metalloproteinase. The figure was adapted from Servier Medical Art.

lipid-derived stimuli (e.g., platelet-activating factor and leukotriene B4) primarily activate their GPCRs through the transmembrane regions. Larger stimulatory molecules (e.g., chemokines and anaphylatoxins) activate GPCRs via a two-step process whereby the stimulus binds the N-terminus of the specific GPCR, whereupon conformational change of the receptor leads to activation by the interaction with the pocket formed by the transmembrane domains (48). CXC chemokine receptors expressed on the surface of various immune cells also belong to the family of GPCRs, and to date, seven receptors have been described (named CXCR1 through CXCR7). They interact with chemokines, a large family of 8- to 12-kDa proteins. The CXC chemokines are one of the most prominent groups and are mostly chemotactic for neutrophils, such as CXCL8 (also known as IL-8) (49).

Newly synthesized bacterial proteins contain formylated methionine, so bacteria secrete a lot of N-formylated proteins and peptides, which were identified as chemoattractants in 1975 (50). These formylated peptides interact with formyl peptide receptors 1 (FPR1) and 2 (FPR2) (FPR2 is also known as FPRL1) on neutrophils (51), both belonging to the GPCR family. The prototype *N*-formyl-peptide, fMLP, binds with higher affinity to FPR1 than its homologue, FPR2 (52). Neutrophils and monocytes express FPR1 and FPR2, whereas the third homologue, FPR3 (also known as FPRL2), is only expressed in monocytes (52). Staphylococci produce phenol-soluble modulins (PSMs) in addition to formyl peptides. PSMs are not only important secreted staphylococcal toxins, but they are also sensed by FPR2, which leads to activation and attraction of leukocytes (53).

Two complement receptors (CRs) on neutrophils also belong to the family of GPCRs, namely, the C3a receptor (C3aR) and the C5a receptor (C5aR). Their ligands are the small complement components C3a and C5a, formed during complement activation. C5a and C3a are commonly also called anaphylatoxins and are both chemoattractants, acting by attracting phagocytes to the site of infection (54).

Neutrophils also express another class of innate immune receptors that are involved in pathogen recognition, the Toll-like receptors (TLRs). The discovery of these proteins in the mid-1990s revealed that pathogen recognition by the innate immune system is specific, because it recognizes different components of foreign pathogens, which are called pathogen-associated molecular patterns. Pathogen-associated molecular patterns that are important for staphylococcal infections are bacterial lipoproteins (recognized by TLR1, TLR2, and TLR6) and unmethylated CpG sequences in DNA molecules (recognized by TLR9) (55). Ligand binding to the extracellular domains of TLRs causes dimerization of the receptor complexes and triggers recruitment of MyD88 to the intracellular TLR domains, which ultimately leads to the activation of transcription factor NF-κB (56). Most TLRs appear to function as homodimers, although TLR2 forms heterodimers with TLR1 or TLR6. TLR1/2 recognizes triacylated lipoproteins, whereas TLR2/6 specifically responds to diacylated lipoproteins (57). TLRs are not directly involved in chemotaxis, but they contribute to phagocytosis.

Another class of proteins important for controlling inflammation are the matrix metalloproteinases (MMPs). Members of this family of 23 endopeptidases are secreted by numerous cells and are important in the recruitment and migration of neutrophils during bacterial infections. They break down extracellular matrix components so neutrophils can migrate toward the infection, but they also stimulate proinflammatory signals and cleave chemokines. This leads to enhanced inflammation and improved bacterial clearance. The two main neutrophil MMPs are MMP8 (also known as neutrophil collagenase) and MMP9 (also known as neutrophil gelatinase B) (58).

EVADING PRIMING, CHEMOTAXIS, AND ACTIVATION OF NEUTROPHILS

Studies thus far have not shown whether *S. aureus* can directly modulate neutrophil priming to influence the function of neutrophils. Additional studies are necessary to understand the influence of *S. aureus* on neutrophil priming (59). However, more knowledge has been gained about the impact of *S. aureus* on neutrophil chemotaxis and activation. Here, we describe the function of the evasion proteins SSL5, SSL10, SSL3, and SSL4; staphopain A; CHIPS; FPR2 inhibitory protein (FLIPr); and FLIPr-like in their role in evading neutrophil chemotaxis and activation, as shown in Fig. 2.

SSL5 not only binds to PSGL-1 to block neutrophil extravasation, but it also binds glycosylated N-termini GPCRs, thereby inhibiting the ligands that require the N-terminus of the receptor for activation. Therefore, it binds but does not interfere with the activation of FPR1 and FPR2, leukotriene B4 receptor, platelet-activating factor receptor, and nucleotide receptor P2Y2, since ligands of these receptors are small and signal via the transmembrane domains of the GPCRs (60). Pretreating neutrophils with SSL5 blocks the activation induced by C3a and C5a, because chemokines and anaphylatoxins bind the N-termini of their receptors. SSL5 also inhibits activation and neutrophil migration induced by IL-8, which interacts with CXCR1 and CXCR2, but it also targets the response induced by chemokine ligand 1 (CXCL1), which acts only on CXCR2 (60). SSL5 together with SSL1 inhibits neutrophil migration through breakdown of collagen and potentiation of IL-8 by inhibiting cleavage of the neutrophil-specific MMP8 (neutrophil collagenase) and MMP9 (neutrophil gelatinase B) as well as other members of the MMP family (MMP1, 2, 7, 12, 13, and 14) (61, 62). Additionally, SSL5 activates and aggregates platelets by interacting with platelet membrane receptor GPIbα and collagen receptor GPVI, leading to a beneficial environment for staphylococcal colonization (63, 64).

SSL10 targets another chemokine receptor, CXCR4, expressed on neutrophils and cancer cells. It binds to human T-cell acute lymphoblastic leukemia, lymphoma, and cervical carcinoma cell lines and inhibits calcium mobilization and cell migration induced by the ligand of CXCR4, CXCL12 (also known as stromal cell-derived factor-1α) (65).

SSL3 binds the extracellular domain of TLR2 and inhibits its activity on human and murine neutrophils and monocytes (66, 67). The cocrystal structure of SSL3 in complex with TLR2 reveals that SSL3 blocks ligand binding and prevents TLR heterodimerization of TLR1 and TLR6 and downstream signaling by interacting with an already formed TLR2-lipopeptide complex (68). SSL4 shares the highest homology with SSL3 and also blocks TLR2 activation, albeit less potently compared to SSL3. Therefore, it remains unknown whether this is the primary function of SSL4, because SSL4 was also shown to bind myeloid cells in a glycan-dependent manner (66, 69). Recently, antibodies against SSL3 and SSL4 were detected in human serum, showing that both evasion proteins are secreted *in vivo*. SSL3 is able to bind both murine and human TLR2, so SSL3 was investigated in an intravenous murine infection model, where it was shown that the presence of the gene correlates with high virulence. To circumvent low expression rates of SSL3 in mice, an SSL3-overproducing strain was used (70).

S. aureus also secretes a protease that interferes with chemokine signaling. Staphopain A cleaves the N-terminus of CXCR2 and thereby causes unresponsive neutrophils to undergo activation by CXCR2. The protease also hampers neutrophil migration toward CXCR2 chemokines (71). Mature staphopain A has a broader substrate specificity than CXCR2 alone because it is also able to degrade elastin fibers. However, it is also prone to autolytic degradation, which could explain the loss of activity of staphopain A over time (71, 72).

S. aureus supernatant showed chemotaxis-inhibiting properties by targeting the fMLP and C5a response of neutrophils (73). Later, the protein responsible in the supernatant was identified, and this 14.1-kDa evasion protein was named chemotaxis inhibitory protein of *Staphylococcus* (CHIPS). CHIPS binds and inhibits the GPCRs FPR1 and C5aR on neutrophils and monocytes. Thus, it blocks the binding of these receptors by their ligands, namely, C5a and fMLP, and thereby impairs the activation and chemotaxis of phagocytes (74, 75). N-terminal peptides of CHIPS inhibited FPR1, while the activity of C5aR was unaffected (76). This showed that the N-terminus of CHIPS is specific for its activity toward FPR1 and that CHIPS probably had another binding site for C5aR. Indeed, CHIPS specifically targets via its C-terminal domain the N-terminus of C5aR through binding to amino acids 10 to 18 and thereby prevents the binding of C5a to its receptor (77, 78). Arginine 44 and lysine 95 of CHIPS in particular appear to be highly important in the antagonism of C5aR (78).

The importance of CHIPS as a potential virulence factor led to the search for homologous excreted proteins in the genome of *S. aureus*. This resulted in the discovery of the gene encoding FLIPr, which showed 49% similarity to the gene for CHIPS (*chp*). Similar to CHIPS, FLIPr is able to inhibit FPR1 but can also inhibit FPR2, whereas binding with FPR3 was not observed (79). Later, the evasion protein FLIPr-like was discovered by a BLAST search through sequenced *S. aureus* genomes with FLIPr as the reference sequence. FLIPr and FLIPr-like are two allelic variants of the same gene, with 73% similarity on the amino acid level. The function of FLIPr-like is also similar to that of FLIPr, because it is an antagonist of both FPR1 and FPR2 (80). Moreover, FLIPr and FLIPr-like also target FcγR, the receptor for IgG, where FLIPr is almost exclusively restricted to class II receptors, with a preference for FcγRIIa, while FLIPr-like binds to most FcγR isoforms (81).

OPSONIZATION AND PHAGOCYTOSIS

Opsonization

The next step in clearing a bacterial infection, after neutrophils have been attracted to the site of infection, is uptake of the pathogens by phagocytes. For this phagocytosis to be effective, bacteria have to be opsonized: coated with components of the complement system, immunoglobulins, or other innate immune components. We will first describe opsonization by the complement system, followed by opsonization by immunoglobulins.

Opsonization by the complement system

Complement is a proteolytic cascade comprising more than 30 proteins in plasma that can (i) opsonize bacteria by depositing complement activation products on the bacterial surface, (ii) attract and activate other immune cells by formation of chemoattractants, and (iii) lyse and kill Gram-negative bacteria directly by the formation of the membrane attack complex (54). The complement system can be activated via three separate pathways that differ in their method of recognition but come together at one central step: formation of C3 convertase, which cleaves C3. One pathway, the lectin pathway, is activated upon recognition of conserved microbial sugars such as ficolins and mannan-binding lectin. The second pathway, the classical pathway, is primarily ini-

tiated by the interaction of C1q with antigen-antibody complexes (54). The third pathway, the alternative pathway, is initiated on the surfaces of neutral or positively charged pathogens that do not contain complement inhibitors. Its activation is due to the spontaneous "tick-over" reaction of C3 with water to form hydrolyzed C3, and it exists in an activated state at all times. Importantly, this pathway serves as an amplification loop after C3b is formed on bacterial cells via the lectin and classical pathways (54). C3 convertases are enzyme complexes with proteolytic activity that cleave C3. The classical and lectin C3 convertase is formed by cleavage of C4 by C1s to C4b, after which C4b covalently attaches to the bacterial surface and binds C2. C1s is then able to cleave C2 as well, thereby forming the C3 convertase C4b2a. The alternative pathway C3 convertase is different in that it contains a surface-bound C3b molecule attached to protease Bb, a subunit of factor B. Factor H controls complement activation by stimulating the decay of Bb from the alternative pathway convertase (C3bBb) and is a cofactor for factor I-mediated cleavage and inactivation of C3b (82).

Cleavage of C3 results in the formation of chemoattractant C3a and the deposition of C3b at the bacterial surface. C3b can be cleaved to the proteolytically inactive product iC3b by factor I on the surface of bacteria. iC3b is still able to opsonize bacteria, but it cannot associate with Bb (83). Both C3 convertases can bind an additional C3b molecule to form C5 convertases, and this convertase can cleave C5 into C5a and C5b. C5a is a chemoattractant and has been shown to have a protective role during staphylococcal bloodstream infections in mice (15). C5b together with C6, C7, C8, and multiple copies of C9 form the membrane attack complex (54). The membrane attack complex is able to directly lyse Gram-negative bacteria, whereas Gram-positive bacteria are unaffected due to their thick peptidoglycan layer (84). However, the complement system is important in the opsonization process because it deposits C3b on the surface of Gram-positive bacteria, such as *S. aureus*. Together, this sequence of events results in rapid and efficient detection and elimination of bacterial invaders.

Opsonization by immunoglobulins

Immunoglobulins (Igs) are a class of proteins that not only enable highly efficient opsonization, but are also involved in agglutination, neutralization of toxins and other virulence factors, and inhibition of adhesion. After binding to antigens on pathogens via their Fab segments, they are recognized by Fc receptors on the surface of phagocytes via their Fc region. The four isotypes of Igs vary in complement activation and are recognized by their own Fc receptor (FcγR, FcαR, FcεR, and FcμR). IgG, IgA, and IgM play roles in controlling infections, whereas IgE is more important in immunity to parasites. IgM is especially effective at opsonization through complement activation due to its polymeric structure (85). IgG consists of four subclasses (IgG1, IgG2, IgG3, and IgG4), and the differences in effector functions between the four IgGs are caused by differences in structure, especially the length and flexibility between the variable Fab segments and the stable Fc segment (86). Therefore the different subclasses of IgG bind differently to C1q, which is at the start of the classical pathway cascade. IgG3 binds the most strongly, whereas IgG1 and IgG2 bind more weakly (IgG1 > IgG2). IgG4 completely lacks the ability to activate the complement system (87).

Phagocytosis

Phagocytosis is a process by which other cells, cell fragments, and microorganisms are engulfed by white blood cells and end up in an internal compartment called the phagosome. As described above, opsonized microorganisms bind to specific receptors on the phagocyte surface. Subsequently, invagination of the cell membrane causes envelopment of the bacterium. This uptake is aided and highly enhanced by factors such as C5a, TLR ligands on the surface of the bacterium, and ligands for C-type lectins (such as DC-SIGN, dectin-1, and the mannose receptor on the surface of neutrophils) (88).

Cross-linking of FcγR by ligand binding on the surface of neutrophils activates several effector functions directed toward killing of pathogens and an inflammatory response (16). FcγRs are members of the immunoglobulin superfamily, and they are capable of binding the Fc region of IgG antibodies. There are several activating receptors (FcγRI/CD64, FcγRIIa/CD32a, FcγRIIc/CD32c, and FcγRIIIa/CD16a), one inhibitory receptor (FcγRIIb/CD32b), and one glycosylphosphatidylinositol-anchored receptor that contains no signal motif (FcγRIIIb/CD16b) (89). Human neutrophils express only two FcγRs, FcγRIIa and FcγRIIIb, and FcγRIIa induces mainly phagocytosis (89).

CR1 (CD35) is found on circulating monocytes, neutrophils, and B-lymphocytes. This receptor binds C4b, C3b, and iC3b and induces phagocytosis (90). CR3 (CD11b/CD18, Mac-1) and CR4 (CD11c/CD18, p150/95) are heterodimeric glycoproteins of the integrin family with a shared β-chain (CD18). They bind the iC3b fragment and to a lesser extent also C3b (91). Stimulation of neutrophils and monocytes via CR3 and CR4 results in induction and enhancement of phagocytosis, degranulation, and generation of ROS (91).

EVADING OPSONIZATION AND PHAGOCYTOSIS

S. aureus has evolved numerous molecules that enable it to evade the different parts of the complement cascade, immunoglobulins, and elements of the phagocytic process. These molecules are illustrated in Fig. 3. Evasion of these components results in a highly effective delay or reduction of the immune response and creates a beneficial situation for the bacterium to survive and multiply within its host.

Capsule Production

The best way for the bacterium to prevent phagocytosis is to hide the antigenic or immunogenic proteins at the surface of the bacterial cell wall with a polysaccharide capsule. Up to ~75% of all clinical *S. aureus* isolates have a capsule or microcapsule whereby most of them contain either capsular polysaccharide 5 (CP5) or capsular polysaccharide 8 (CP8). These bacteria grown under optimal capsule-production conditions showed resistance to opsonophagocytosis and thus killing (92). However, the capsule of *S. aureus* does not completely block deposition of complement components at the bacterial surface or binding of specific antibodies (93). Other bacteria, such as *Streptococcus pneumoniae*, produce a very thick capsule (94).

Proteins Targeting Immunoglobulins

The first evasion molecule described to have antiopsonic properties is staphylococcal protein A (SpA). SpA is linked to the staphylococcal cell wall via its LPXTG anchor but can be released upon hydrolysis during growth (95, 96). This protein contains four or five immunoglobulin-binding domains capable of binding the Fc part of IgG, which leads to blockage of FcR-mediated phagocytosis (97). SpA also binds to the Fab domain of variable heavy 3 type B cell receptors of

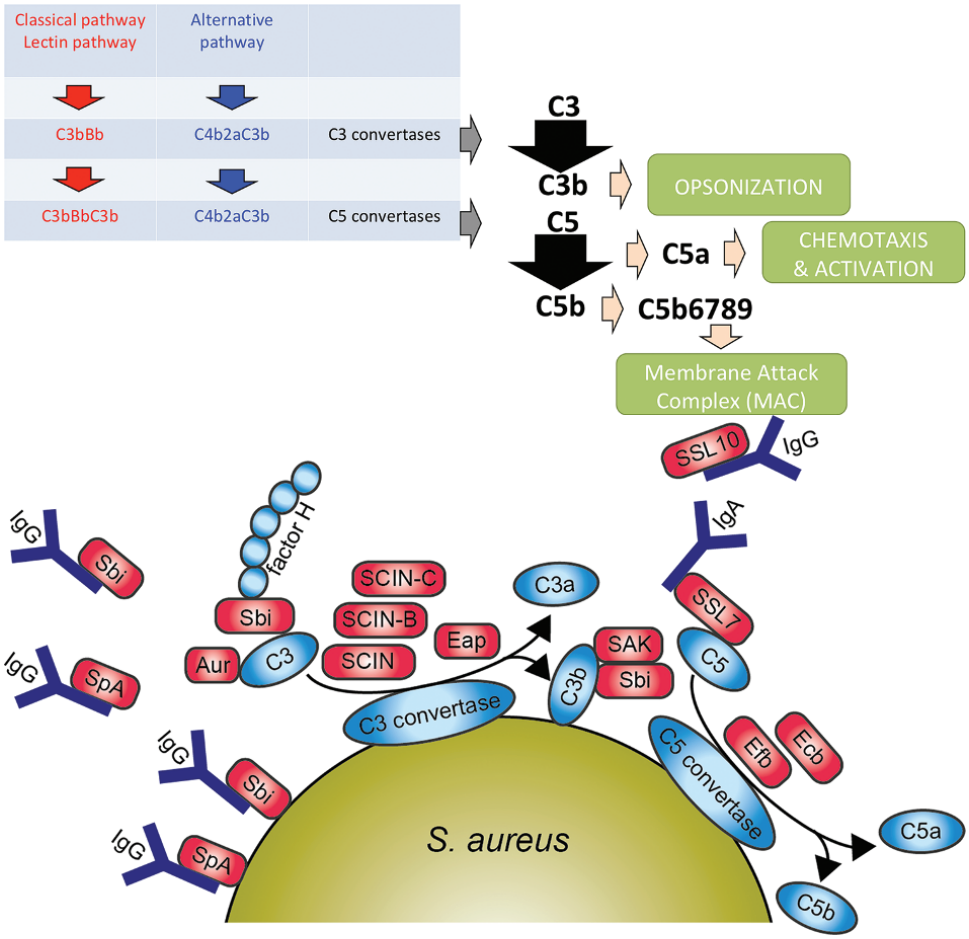


FIGURE 3 Diagram of the main pathways and components of the human complement system 3a and a schematic representation of *S. aureus* evading opsonization and phagocytic uptake by neutrophil 3b. Red boxes indicate staphylococcal proteins, and blue boxes indicate host proteins. Abbreviations: IgG, immunoglobulin G; SpA, staphylococcal protein A; Sbi, staphylococcal binding of IgG; SCIN, staphylococcal complement inhibitor; SAK, staphylokinase; Aur, aureolysin; SSL, staphylococcal superantigen-like protein; Efb, extracellular fibrinogen-binding protein; Ecb, extracellular complement-binding protein. The figure was adapted from Servier Medical Art.

IgM and thus serves as a B cell antigen by stimulating B cell activation (98–100). Consequently, this shows that SpA effectively interferes with the adaptive immune response. Recently, guinea pigs were shown to be a good model to test this therapeutic approach *in vivo*, which will be important for vaccination strategies in the future (101).

A second IgG-binding protein, *S. aureus* binder of IgG (Sbi), was identified through a phage display screen against immobilized human IgG (102). Like SpA, Sbi is also expressed at the staphylococcal cell surface and is secreted during bacterial growth (103, 104). Sbi also has two binding targets because it is able to bind to another serum component, β_2-glycoprotein I, also known as apolipoprotein H. This was similarly identified by phage display, and the binding site was clearly different from the IgG-binding domain (103). However, in contrast to SpA, Sbi can only interact with the Fc domain of IgG (105). Furthermore, the extracellular region of Sbi consists of four globular domains, where domains I and II are immunoglobulin-binding domains and domains III and IV are able to bind complement component C3 and factor H, forming a stable tripartite complex (Sbi:C3:factor H) (106, 107). Thus, Sbi is a versatile evasion protein, interfering with the innate immune system by binding IgG and apolipoprotein H, as well as the complement components factor H and C3.

SSL10, a multifunctional evasion protein, is yet another protein able to bind IgG1 (108). This leads to the inhibition of complement activation via the classical pathway, and this is the second function of SSL10, since it is also a CXCR4 antagonist. The N-terminus of SSL10 binds to the Fc domain of IgG1, preventing association with C1q and FcγRs and leading to inhibition of FcγR-mediated phagocytosis (109). Furthermore, SSL10 also targets prothrombin and factor Xa to inhibit blood coagulation (110), binds to phosphatidylserine, and recognizes apoptotic cells (111). Finally, *S. aureus* secretes a serine protease, V8, which is able to cleave immunoglobulins (112).

Proteins Targeting the Complement System

Not all known evasion proteins interfering with opsonization target immunoglobulins. Some of them are specialized in evading the complement system. For example, the secreted metalloprotease aureolysin cleaves the central complement

component C3 in a zinc-dependent manner. The cleavage site on C3 of aureolysin differs by two amino acids from the C3 convertase cleavage site generating active C3a′ and C3b′. This suggests that aureolysin mimics C3 convertases, but it also degrades the cleaved C3b′ as well as factors I and H. This cleavage is more effective *in vivo* than *in vitro* because it is more effective in serum than without serum. Aureolysin is a secreted protease, and thus it can cleave C3, removing it from the bacterial surface and creating a C3-free microenvironment and thus preventing C3b′ from covalently attaching via its thioester to bacteria (113).

Staphylococcal complement inhibitor (SCIN), initially identified as a prophage-coded protein, inhibits all three complement pathways: the alternative, classical, and lectin pathways. SCIN stabilizes and inhibits surface-bound C3 convertases, and this results in a decline of C3b deposition and release of chemoattractant C5a, which results in the inhibition of phagocytosis (114). Later, SCIN-B, SCIN-C, and open reading frame D were identified in the *S. aureus* genome. Characterization of these homologues shows that SCIN-B and SCIN-C also inhibit complement, whereas open reading frame D shows no inhibitory activity (115). Cocrystallization studies of SCIN with C3 convertase reveals that SCIN inhibits convertase activity in three ways: (i) SCIN prevents Bb mobility, which prevents Bb from accessing substrate C3 (116); (ii) SCIN may dimerize two C3 convertases and thereby prevent substrate binding (116); (iii) by binding to a single C3b molecule, SCIN slows the rate of factor B loading onto C3b and thus lowers the amount of C3 convertase formation (117). This all leads to inhibition of phagocytosis, C3b deposition, and C5a generation (114). Furthermore, a new SCIN variant was recently identified in equid *S. aureus* isolates. This equine SCIN has adapted to horses by inhibiting the equine complement system.

Extracellular fibrinogen-binding protein (Efb) and extracellular complement-binding protein (Ecb) are homologous proteins (118). The first characteristic described for Efb was that it binds via its N-terminus to fibrinogen—hence its name (119). Efb can also bind to platelets, as identified by phage display, interfering with platelet aggregation, contributing to virulence in wound infections, and delaying the healing process in rats (120–122). Additionally, the C-terminus of Efb is able to bind C3 and its cleavage products containing the thioester domain (C3d) (123, 124). Efb blocks phagocytosis *in vitro* of *S. aureus* by neutrophils in plasma and human whole blood as well as *in vivo* in a mouse peritonitis model. This evasion protein forms a unique bridge between complement and coagulation systems, since it can bind both C3 and fibrinogen. Efb covers bacteria with a thick layer of fibrinogen, which leads to shielding of surface-bound C3b and antibodies from recognition by phagocytic receptors (125). This mechanism to shield the bacterial surface from phagocytosis together with the formation of a capsule enhances the evasion of *S. aureus* against phagocytosis (126). It also causes clumping of staphylococcal cells *in vivo*, thus enhancing *agr* quorum-sensing (127).

Ecb (also known as Ehp) compared to Efb lacks fibrinogen binding activity, but like Efb, it does inhibit the complement system by binding the C3d domain of C3 (118, 128). This results in blocking of C3 convertases of the alternative pathway and C5 convertases of all three complement pathways (129). The binding of Ecb to C3d is stronger than Efb to C3d, because Ecb contains a second, lower-affinity, C3 binding site. This results in enhanced complement inhibitory effect of Ecb compared to Efb (128). The function of many evasion molecules is restricted to the human host; however, Ecb and Efb efficiently inhibit the complement system in both humans and mice. Therefore, the importance of Ecb and Efb could be examined *in vivo* in a murine infection model. Mice experienced higher mortality rates in an intravenous model with wild-type bacteria (79%) compared to an isogenic ΔEcbΔEfb mutant (21%). In addition, Ecb and Efb promoted bacterial survival and blocked neutrophil influx in the lungs. These results indicate that Ecb and Efb are essential to *S. aureus* virulence *in vivo* and could be attractive targets for vaccine development (130).

S. aureus recruits factor H to its surface with the surface-associated serine-aspartate repeat protein E (SdrE), and this leads to inhibition of the alternative pathway of the complement system (131). Recombinant SdrE recognizes its ligand via a unique "close, dock, lock, and latch" mechanism as determined by crystallographic studies (132). Moreover, factor H bound to SdrE retains its activity for factor I-mediated cleavage of C3b to iC3b (131). Factor I is directly targeted by the full-length cell wall component clumping factor A as well as a secreted moiety of around 50 kDa. Binding of cell wall component clumping factor A to factor I promotes cleavage of C3b to iC3b, which results in disruption of opsonophagocytosis (133, 134).

Extracellular adherence protein (Eap) has a variety of functions in evading the immune system. We already discussed the binding of Eap to ICAM-1 causing impaired function in neutrophil extravasation (39), but Eap is also able to inhibit the complement system. C4b is targeted by Eap, and this leads to inhibition of C2 binding to C4b and thus blocks formation of the active C3 convertase of the lectin and classical pathway (135).

Staphylokinase (SAK) also acts as an antiopsonic immune evasion protein by converting human plasminogen into the active bacterium-bound serine protease plasmin. Plasmin, from its location on the bacterial surface, degrades IgG and C3bf, resulting in decreased phagocytosis by human neutrophils (136).

SSL7 inhibits opsonization in two ways: it selectively binds both IgA and complement C5 (137). The binding of SSL7 to the Fc region of IgA, mediated via its N-terminus, causes interference with antibody recognition (138), whereas its C-terminus binds to C5 and thereby inhibits terminal complement activation and cleavage of C5 by interfering with the binding of C5 to C5 convertases (139). SSL7 was also found to be important *in vivo* by inhibiting complement-induced neutrophil influx in a murine inflammatory model (139).

S. aureus has also evolved a protein attached to the cell wall that specifically inhibits the classical pathway of the complement system. This protein, *S. aureus* collagen adhesin (Cna), belongs to the microbial surface component recognizing the adhesive matrix molecule (MSCRAMM) family of adhesins. Cna binds C1q and thus inhibits classical pathway activation. Cna belongs to the structurally related Cna-like family, members of which are found in many other Gram-positive bacterial species. Thus, the function of Cna-like MSCRAMMs as inhibitors of the classical pathway could serve as an immune evasion strategy for numerous Gram-positive pathogens (140).

NEUTROPHIL EXTRACELLULAR TRAP FORMATION

Neutrophils have recently been shown to have an antibacterial defense mechanism whereby they release their DNA, in association with antimicrobial peptides, histones, and proteases, to form a network of extracellular fibers which entrap

and kill various microbes. These networks are known as neutrophil extracellular traps (NETs) (141). There are current studies to address the molecular mechanism behind NET formation, but more research is needed to fully understand this phenomenon (142). The formation of NETs *in vitro* is activated via different proinflammatory stimuli, including hydrogen peroxide, phorbol myristate acetate, lipopolysaccharide, IL-8, and various bacteria, including *S. aureus* (143). The formation of NETs has also been shown *in vivo*, where intravascular NETs were observed in the mouse liver during sepsis induced by *S. aureus* (144).

EVADING NET FORMATION

A secreted nuclease of *S. aureus*, Nuc, is important in the breakdown of NETs. This has been shown both *in vitro* and *in vivo*, whereby an isogenic nuclease knock-out of *S. aureus* shows impaired degradation of NETs compared to the wild-type strain. This leads to the linkage of nuclease production to delayed bacterial clearance in the lung and increased mortality after intranasal infection *in vivo* (145). Thus, Nuc helps staphylococci to escape from the extracellular fibers and to avoid getting killed by antimicrobial peptides and proteases. Nuc was also shown to play a role in immune cell death together with secreted adenosine synthase. These two enzymes are able to convert NETs to deoxyadenosine, which triggers the caspase-3-mediated death of immune cells (146).

BACTERIAL KILLING BY NEUTROPHILS

Neutrophils are end-stage cells with a high concentration of antimicrobial proteins safely stored within different granules. They kill pathogens by releasing their granules containing antimicrobial proteins into the phagosome (also called degranulation) and generating ROS (also known as the oxidative burst) upon phagocytosis of bacteria. Phagocytes, especially macrophages, also form reactive nitrogen species via the oxidation of nitric oxide (NO•), which is produced by an inducible nitric oxide synthase (147). The granules of neutrophils can be divided into three groups: the primary or peroxidase-positive granules, the secondary or specific granules, and the tertiary or gelatinase granules. The peroxidase-positive granules are also called azurophilic granules due to their affinity for the basic dye azure A (12). Secretory vesicles contain plasma proteins and are probably formed by endocytosis (12). Azurophilic granules contain a variety of antimicrobial proteins, such as pore-forming peptides (α-defensins), neutrophil serine proteases (NSPs; proteinase 3 and 4, cathepsin G, and elastase), myeloperoxidase (MPO), the bactericidal/permeability-increasing protein, and lysozyme. Secondary granules contain hCAP-18, lactoferrin, neutrophil gelatinase-associated lipocalin, and lysozyme. Tertiary granules contain a number of metalloproteases, such as MMP8 (collagenase) and MMP9 (gelatinase). Secretory vesicles contain plasma proteins and membrane-associated receptors essential at the earliest phase of neutrophil-mediated inflammatory response, such as the extremely rapid upregulation of β_2-integrin CD11b/CD18, CR1, and FPR1 and FPR2 (148). However, it should be mentioned that heterogeneity exists between the different granules and that their granule contents overlap, because they are sequentially formed during myeloid cell differentiation (149).

Oxygen-Independent Killing by Neutrophils

Oxygen-independent killing mechanisms by neutrophils greatly contribute to microbial killing (150). Agents that contribute to oxygen-independent microbicidal activity include antimicrobial peptides and proteins such as lactoferrin, lysozyme, α-defensins, cathelicidins such as hCAP-18, azurocidin, proteinase 3, cathepsin G, and neutrophil elastase (12). The C-terminal part of hCAP-18, named LL-37, forms an amphipathic α-helix and has antimicrobial properties against Gram-negative bacteria and Gram-positive bacteria, such as *S. aureus* (151). LL-37 is effective against both intra- and extracellular *S. aureus* (152). Other antimicrobial peptides include α-defensins (also known as human neutrophil peptide-1). These peptides are antimicrobial, acting by the formation of multimeric pores and applying this on various microorganisms (153). The structurally related serine proteases (cathepsin G, elastase, and proteinase 3 and 4) have various functions, including the cleavage of bacterial virulence factors and regulation of immune responses by cleaving receptors and chemokines, and they are able to kill bacteria in the extracellular milieu after being secreted (154). The serine proteases also proved to be important *in vivo*, since mice lacking cathepsin G showed impaired clearance of *S. aureus* during infection (155). Lactoferrin and calprotectin are chelating proteins; lactoferrin sequesters iron needed for microbial growth, and calprotectin chelates zinc and manganese and thereby inhibits staphylococcal growth (156). Calprotectin is located in the cytoplasm of neutrophils and makes up more than 60% of the proteins in the cytosol (157). Lysozyme is a cationic antimicrobial peptide and is present in all granule subsets, with a peak concentration in secondary granules. Lysozyme cleaves bacterial cell wall peptidoglycan polymers by breaking the β-1,4 glycosidic bonds between *N*-acetylmuramic acid and *N*-acetylglucosamine (158). This results in lysis of various bacteria, such as *Bacillus subtilis*, in human plasma, but has only limited action against staphylococci because of their modified peptidoglycan (159).

Oxygen-Dependent Killing by Neutrophils

Upon phagocytosis of bacteria, the azurophilic granules are the first to fuse with the phagosome, and this leads to assembly of the five essential parts of the NADPH oxidase complex and activation of the complex (160). Active NADPH oxidase transfers electrons across the phagosomal membrane from cytosolic NADPH to intraphagosomal molecular oxygen to produce superoxide, which is compensated for by an influx of cations or protons. Although superoxide has limited direct microbicidal capacity, the molecule is involved in generating secondary derived ROS such as hypochlorous acid, chloramines, hydroxyl radicals, and singlet oxygen, which directly contribute to polymorphonuclear microbicidal activity. The superoxide anion is converted to hydrogen peroxide spontaneously or with the help of superoxide dismutase. MPO catalyzes the reaction of hydrogen peroxide with chloride to form the highly bactericidal agent hypochlorous acid (161, 162). Chlorine, chloramines (e.g., monochloramines, dichloramines, and taurine chloramine), singlet oxygen, hydroxyl radicals, and ozone are subsequently formed in secondary reactions and are also potent antimicrobial compounds (163).

The role of MPO in intracellular bacterial killing is controversial. In the early 1970s it was thought that MPO was the main factor in killing of *S. aureus* by hypochlorous acid production, because MPO-deficient patients showed a 10-fold decrease in killing bacteria after phagocytosis (164). However, this process is incompletely understood, and reproducible results are lacking. Later, MPO was shown to contribute poorly to the pulmonary defense when comparing wild-type mice with MPO-deficient mice infected with

S. aureus. In contrast, MPO-deficient mice showed a significant decrease in clearing other microorganisms (e.g., *Candida albicans*, *Candida tropicalis*, *Pseudomonas aeruginosa*) compared to wild-type mice (165). Back then it was believed that the pH inside the phagosome dropped to 4.0 (166). Later, the pH inside the phagosome was believed to be 7.8 and to drop to 6.9 after 15 minutes of phagocytosis (167) or even to 5.7 after 60 minutes (168). Recently, data revealed that the pH becomes more alkaline due to K^+ influx, which is optimal for granule proteases (155). This brings into question the role of MPO in bacterial killing because an alkaline pH results in a virtual absence of the peroxidase and chlorinating activities of MPO (169). It is possible that MPO has two functions *in vivo*: (i) peroxidase activity at pH ~6 when the neutrophil is unable to fully engulf an organism and (ii) superoxide dismutase (161) or catalase (170) activity in an alkaline milieu in a fully enclosed vacuole with a pH of ~9 to favor conditions for the granule proteases (e.g., proteinase 3, cathepsin G, and elastase) (171). MPO could also protect the microbicidal enzymes against oxidative damage, so both oxygen-dependent and oxygen-independent machineries inside the phagosome synergize for most effective killing of microbes. In our own experiments we have demonstrated that the MPO-dependent part of killing of *S. aureus* in neutrophils is 26% in the first 60 minutes (172).

EVADING KILLING

In this section we give an overview of the evasion proteins and pigments that are involved in staphylococcal survival of oxygen-dependent and -independent killing. These proteins are summarized in Fig. 4. Then the various staphylococcal toxins will be discussed. An overview of these toxins is shown in Fig. 5.

Evading Oxygen-Dependent Killing by Neutrophils

S. aureus's species name is derived from the fact that this bacterium has "a jacket" of the golden pigment staphyloxanthin. This staphyloxanthin serves as an antioxidant and is protective in killing by hydrogen peroxide and singlet oxygen. Bacteria lacking staphyloxanthin showed impaired survival *in vitro* and *in vivo* (173). The genes *crtM* (dehydrosqualene synthase) and *crtN* (dehydrosqualene desaturase) are essential in the biosynthetic pathway to produce staphyloxanthin. Therefore, synthetic inhibitors of CrtM are interesting new virulence factor-based therapies that act by targeting the biosynthesis of staphyloxanthin (174, 175).

S. aureus has developed other proteins that contribute to the resistance against ROS. The bacterium produces two superoxide dismutases, *sodA* and *sodM*, that convert the harmful superoxide radicals into hydrogen peroxide and oxygen (176). Isogenic *sodA* and/or *sodM* knockout bacteria had reduced virulence in a mouse abscess model, showing that both *sodA* and *sodM* contribute to the virulence of *S. aureus* (176). Probably, *sodA* has the major SOD activity in *S. aureus* throughout all growth stages, whereas *sodM* becomes active under oxidative stress during the late exponential and stationary growth phases (177). The hydrogen peroxide generated by the neutrophil is degraded into water and oxygen by KatA, a staphylococcal catalase, which is described to be a major virulence factor (178). However, studies conducted with a catalase mutant strain showed no difference in virulence in a murine abscess infection model (179). Another

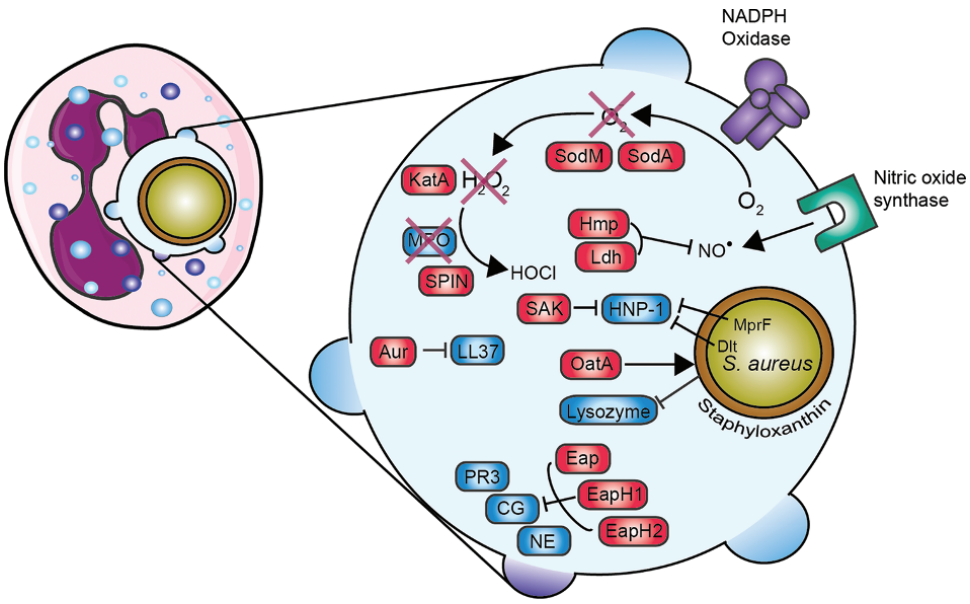


FIGURE 4 Overview of evasion proteins that are involved in evading neutrophil killing. Enlargement of the phagosome is shown on the right. Red boxes indicate staphylococcal proteins, and blue boxes indicate host proteins. Staphyloxanthin provides a protective shield, KatA neutralizes hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2), and SPIN inhibits MPO activity. MprF and the Dlt operon lead to an increase in positive charge of the bacterial surface. Abbreviations: SOD, superoxide dismutase; SAK, staphylokinase; KatA, catalase; MPO, myeloperoxidase; SPIN, staphylococcal peroxidase inhibitor; Aur, aureolysin; Hmp, flavohemoglobin; Ldh, L-lactate dehydrogenase; Eap, extracellular adherence protein; EapH, extracellular adherence protein homologue; PR3, proteinase 3; CG, cathepsin G; NE, neutrophil elastase. The figure was adapted from Servier Medical Art.

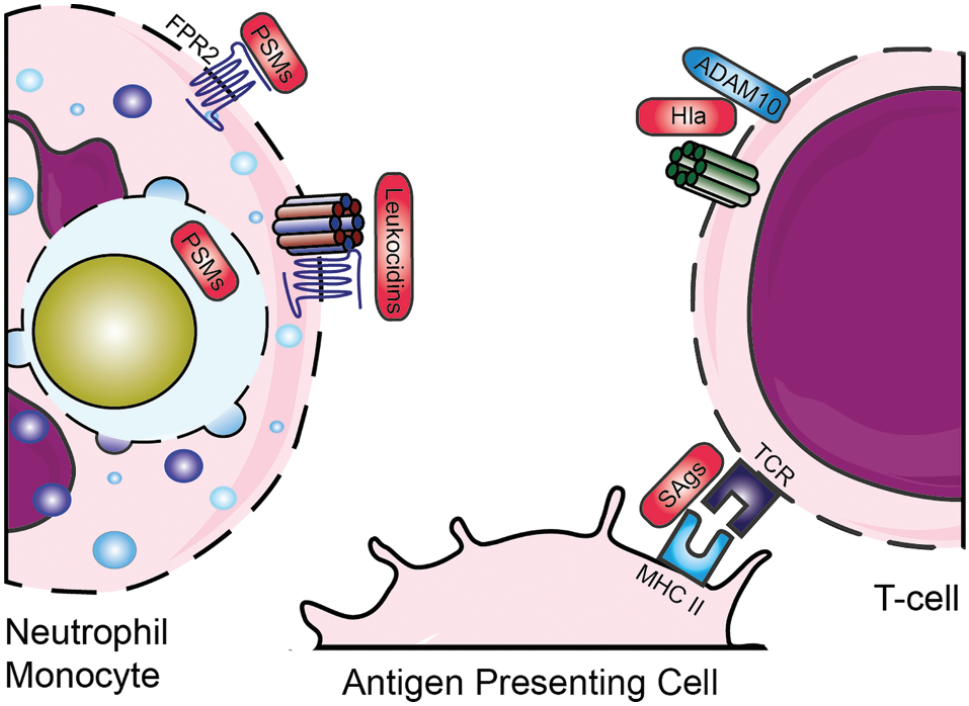


FIGURE 5 Evasion by staphylococcal toxins. Various leukocidins bind specific GPCRs, after which they form a pore and lyse host cells. PSMs are released inside the phagosome and can bind via FPR2. SAgs cross-link major histocompatibility complex class II and T-cell receptors. Abbreviations: GPCR, G-protein-coupled receptor; FPR, formyl protein receptor; PSMs, phenol-soluble modulins; Hla, hemolysin-alpha; SAgs, superantigens; MHC II, major histocompatibility complex II; TCR, T-cell receptor. The figure was adapted from Servier Medical Art.

catalase-like protein produced by *S. aureus* is alkyl hydroperoxide reductase (AhpC). KatA catalase appears to be important for protection against external oxidative stress, whereas AhpC clears endogenously produced hydrogen peroxide. In that study, AhpC and KatA were not required for virulence of *S. aureus*, but AhpC and/or KatA are important for nasal colonization (180). Another virulence factor, at first unidentified, regulated via the SaeR/S two-component system was suspected to play a role in the decrease of hydrogen peroxide and hypochlorous acid production. This factor was independent of SOD and catalase, since their expression is not regulated by SaeR/S (181). Later, this factor was identified as staphylococcal peroxidase inhibitor (SPIN), a secreted protein of 8.4 kDa which is able to bind and inhibit MPO. From crystallographic studies, we know that SPIN acts as a molecular plug that prevents access of substrate hydrogen peroxide to the MPO active site. Despite the controversy concerning the importance of MPO in bacterial clearance, SPIN protects *S. aureus* from MPO-mediated killing (172).

S. aureus has also evolved two proteins to resist the stress of nitric oxide produced in activated phagocytes. Flavohemoglobin scavenges host-derived NO•, and the NO•–inducible L-lactate dehydrogenase catalyzes the production of L-lactate, which maintains redox homeostasis during nitrosative stress (182, 183).

Evading Oxygen-Independent Killing by Neutrophils

S. aureus contains two independent loci (*dltABCD* and *mprF*) that affect susceptibility to defensins and other cationic pore-forming peptides by modifying the net charge of the cell wall envelope. The *dlt* operon is responsible for the incorporation of D-alanine into teichoic acids, and this leads to a decrease in negative charge on the bacterial surface and tolerance of high concentrations of positively charged antimicrobial peptides (184). A *dlt* knockout mutant was more susceptible to killing by defensin peptides and human neutrophils, even in the absence of a functional respiratory burst. As a control, there was no difference in killing between wild-type and *dlt* knockout bacteria by monocytes, which do not produce defensins (185). The *mprF* (multiple peptide resistance factor) gene encodes a lysylphosphatidylglycerol synthetase which is involved in modification of membrane phosphatidylglycerol with L-lysine, which also results in a reduction of the negatively charged membrane surface (186, 187). In contrast to most defensins, cathelicidin-derived bactericidal peptides, such as LL-37, have substantial activity against staphylococci. However, *S. aureus* has also evolved a protein to evade the antimicrobial effects of LL-37 by degrading and thereby inactivating LL-37 by the staphylococcal metalloproteinase aureolysin (which is also involved in the opsonization process by cleaving C3) (188). As described above, lysozyme degrades the cell wall peptidoglycan layer. Nonetheless, *S. aureus* resists lysozyme by expressing the enzyme *O*-acetyltransferase A (OatA). OatA causes *O*-acetylation of the peptidoglycan, and this leads to resistance to the muramidase activity of lysozyme. OatA is regarded as a virulence factor since it is expressed only in pathogenic, lysozyme-resistant staphylococci (for example *S. aureus*, *S. epidermidis*, and *S. lugdunensis*) (189, 190).

Eap inhibits the activity of NSPs (neutrophil elastase, cathepsin G, and proteinase 3). Its homologues, EapH1 and EapH2, each contain one EAP domain, whereas the earlier described Eap usually contains four or five EAP repeats.

A single EAP domain impairs the function of NSPs; therefore, EapH1 and EapH2 also inhibit NSP activity (191). Recently, the interaction between EAP and NSPs was investigated, and despite the homology between EapH1 and EapH2, they form different complexes with NSPs (192). *S. aureus* protects its own immune evasion arsenal from degradation and cleavage by inhibiting these NSPs (193). For example, the N-terminus of SPIN is susceptible to proteolysis by NSPs, which results in a loss of function to inhibit MPO, but it is protected again by Eap. SAK is not only involved in evading opsonization by cleaving plasminogen to active plasmin (136), but it also binds and inhibits α-defensin (human neutrophil peptide 1). Human neutrophil peptide 1 is an important antibacterial peptide in neutrophil granules. This inhibition affects intracellular killing (194).

Escape by Toxins

S. aureus also protects itself by the secretion of toxins (Fig. 5). Toxins can directly lyse and thereby kill (immune) cells by disrupting the cell membrane and protecting the pathogen both before and after engulfment by neutrophils. These lytic toxins can be divided into two groups based on their structure: the β-barrel pore-forming toxins such as α-hemolysin and leukocidins and the small α-helical peptides such as the PSMs. A different group of toxins, the superantigens, activate T-cells by cross-linking major histocompatibility complex class II and T-cell receptors, resulting in massive nonspecific activation of T-cells. This temporary overactivation of a large population of T-cells causes secondary inhibition of the adaptive immune system and thereby contributes to immune escape.

β-Barrel pore-forming toxins target eukaryotic cells expressing specific factors. Hemolysin-alpha (Hla, also known as α-toxin) is secreted as a monomer, which forms a homo-heptameric pore upon binding to its receptor, the zinc-dependent metalloprotease ADAM-10 (195). Hla also upregulates ADAM10 on endothelial and epithelial cells, causing disruption of the endothelial and epithelial barrier (196, 197). Hla does not lyse granulocytes (e.g., neutrophils), but it does lyse other blood cells, such as erythrocytes, macrophages, and subpopulations of lymphocytes (198). Other β-barrel pore-forming toxins are leukocidins. These toxins consist of two independent secreted monomers known as F (fast) and S (slow) and form octamer pores of four F and four S subunits in membranes of host cells (199). Currently, seven *S. aureus* leukocidins have been described, including five in human-associated strains: LukAB (also known as LukGH), LukSF (also known as PVL), LukED, and two γ-hemolysins (HlgAB and HlgCB). Two animal-associated leukocidins (LukPQ [200] and LukMF′ [201]) were recently added to the arsenal (202). The receptors for these leukocidins have recently been identified, and this explains the cellular tropism and biological consequences of these pore-forming toxins during infection (203). Their receptors, all GPCRs and almost exclusively chemoattractant receptors, are located on various immune cells, but mainly on phagocytes. This indicates that the major role of these toxins is in immune evasion. They are designed to kill those cells that are crucial in eliminating staphylococci. HlgAB and LukED, however, also target the Duffy antigen receptor for chemokines expressed on erythrocytes, which leads to release of hemoglobin and promotes bacterial growth (204). Furthermore, LukAB has been shown to play a role in intracellular lysis of neutrophils, thereby promoting bacterial replication and outgrowth (205). Interestingly, the presence of these leukocidins in the genome of *S. aureus* is highly diverse. For example, the *HlgACB* and *lukAB* genes are located in the core genome and are present in more than 95.5% of human *S. aureus* isolates, whereas PVL is located on a phage and is found in fewer than 2% of all clinical isolates, but it is found in the majority of community-acquired MRSA strains in the United States. The presence of the PVL genes (lukS-PV and LukF-PV) in staphylococci correlates with the occurrence of necrotizing pneumonitis, a severe and devastating form of bacterial pneumonia (206).

The other class of toxins is PSMs, which are small α-helical peptides. These peptides are associated with enhanced virulence in community-acquired MRSA and are produced at high concentrations. PSMs contain a common amphipathic α-helical region which is responsible for disrupting the cell membrane; nonetheless, they are categorized in two groups: the β-type PSMs (around 44 amino acids long) and the shorter α-type PSMs (20 to 25 amino acids long), which have more enhanced toxic characteristics (207). The function of PSMs is inhibited by serum lipoproteins, which are present in large quantities in human serum (208). Therefore, their function is mainly intracellular, where they are able to lyse phagocytes from the inside out. The critical concentration of PSM is easily reached inside the condensed phagosome (209). Through the interaction between PSMs and FPR2 (see "Opsonization," above), PSMs can not only lyse neutrophils, but can also attract and activate leukocytes via FPR2 (53).

S. aureus also encodes a variety of toxins (the superantigens) that modulate the adaptive immune system. Around 20 serologically distinct staphylococcal superantigens are known, which are divided into enterotoxins, enterotoxin-like, and toxic shock syndrome toxin-1. Their nomenclature is differentiated on the basis of proven emetic activity when orally ingested (enterotoxins SEA-E, G-J, and S-T) or unconfirmed activity (enterotoxin-like SEIK-R, U and U2, V, and X-Y) (210). Superantigens cross-link major histocompatibility complex class II and T-cell receptors. This causes massive nonspecific activation of T-cells and an increase of many cytokines to toxic levels, which leads to damage of tissue and organs. This is called the toxic shock syndrome, and it is often lethal (211). The SSLs were first annotated as SET proteins for "staphylococcal enterotoxin-like" but were later renamed staphylococcal superantigen-like (SSL) since these proteins lack enterotoxic activity (210). Even though their enterotoxic activity is lacking, the SSLs have distinct functions in evading the innate immune system.

DISCUSSION AND CONCLUSION

Staphylococcal Innate Immune Evasion

Bacteria adapt to their environment. Pathogenic bacteria adapt to survival in their host. In the human host this means that they have evolved various virulence strategies to overcome innate and adaptive immunity. These virulence strategies are (i) camouflage of the microbial surface to avoid immune recognition by the production of an inert capsule, often composed of sugars, (ii) hiding from the immune system by adapting to an intracellular lifestyle in immune cells or other cells, and (iii) secretion of small proteins that inhibit specific elements of the immune system (212). These approaches all have advantages and disadvantages. Production of a capsule requires a considerable amount of energy. In addition, at some stages during infection, the bacteria need to remove their capsule, for example, for adherence to host cells, thereby exposing themselves to the immune system. For example, pneumococcal capsules are 60 nm thick, covering

all surface structures on the bacterium. Hiding from the immune system through survival inside cells is also a challenge owing to the presence of multiple microbicidal enzymes and proteins inside these cells. Moreover, discovery of intracellular TLRs, NOD-like receptors, and inflammasomes revealed that bacteria can also be recognized inside cells (55). Therefore, the production of small secreted inhibitory proteins is, by itself or in combination with the other strategies, a strong evasion strategy to survive within the host and works extracellularly as well as intracellularly. *S. aureus* has extensively evolved proteins to evade all facets of the initial attack by the host immune system.

S. aureus remains a leading cause of bacterial infections and has an increasing risk for human health with the rise of antibiotic resistance in community-acquired strains. Also, the financial burden is growing due to increasing pressure on the health care system. Research into immune evasion molecules is very important, since these proteins determine staphylococcal virulence and pathogenesis. Understanding the mode of action of these evasion molecules will help us to find new therapeutics for prophylaxis or will improve treatment against *S. aureus*, since a functional vaccine is still lacking.

So Many Evasion Molecules: Luxury or Necessity?

To date, the functions of around 40 immune evasion proteins have been identified, and this number is still growing. These evasion molecules are present in clinical isolates and are expressed in the human host, since healthy carriers and noncarriers in adults and even young children have antibodies against these proteins (20, 213). Some of these evasion molecules were detected by high-resolution mass spectrometry from nasal polyp tissue from patients with chronic rhinosinusitis (214). One question arising from this observation is Why is *S. aureus* expressing so many evasion molecules? It seems a waste of time and energy for the bacterium to produce and secrete all of these proteins. Most of them target different specific parts of the immune system. We think that this demonstrates the strength, the significance, and the redundancy of our innate immune system. A very efficient and redundant immune system can only be overcome by a redundant counterattack. Moreover, other microbes have different methods to avoid recognition and killing by the immune system. As said before, a thick capsule is an efficient method to hide and sterically hinder access to the highly immunogenic surface, for example, masking the binding of C3 fragments to the complement receptor. This leads to decreased opsonization, phagocytosis, and subsequently, killing of the microorganisms by neutrophils (94). Gram-negative bacteria such as *Neisseria meningitides* and Gram-positive bacteria such as group A *Streptococcus* and *S. pneumoniae* are known to produce this thick capsule (94, 215, 216). A typical capsule is encoded by at least 15 large glycosyltranferases that are encoded by 3 to 4% of all base pairs in the genome, because the average size of a glycosyltransferase is between 200 and 300 kDa. Evasion proteins are much smaller, typically between 10 and 30 kDa, and encoding 40 of these therefore amounts to less than 1% of the whole *S. aureus* genome. Also, the production and maintenance of a thick polysaccharide capsule consumes a large amount of energy. Therefore, the production of 40 small proteins that directly attack the immune system is cheaper for the bacterium than the strategy of making a thick capsule.

Another explanation for the enormous number of different immune evasion proteins is variability in the human's defense. The arsenal of immune evasion molecules makes it easier to fight the immune system of different individuals with variable antigens and genomic variation in their immune response. In addition, the expression of the various evasion molecules is tightly regulated via multiple regulation systems, and this results in differential expression during different growth stages. Interestingly, some of these relatively small molecules have multiple functions. The total number of secreted proteins in *S. aureus* is 100 to 200, and many of these proteins still have no known function (11). Thus, it is likely not only that new evasion molecules will be identified in the future, but also that new functions will be associated with already known proteins (11).

Despite the fact that *S. aureus* produces so many evasion molecules, the majority of colonized individuals will not suffer a staphylococcal infection. This tells us how complex and efficient our own immune system is. Invading microorganisms can be cleared within minutes by our innate immune system, possibly before some immune evasion proteins are produced. The pathogens are recognized by pattern recognition receptors and opsonized by components of the complement system and immunoglobulins. These immunoglobulins from the adaptive immune system are also able to neutralize bacterial components, such as superantigens, toxins, and other immune evasion proteins. The opsonized bacteria are efficiently ingested by neutrophils and killed by oxidative and nonoxidative proteins, which are safely stored inside their granules.

In conclusion, it seems that there is a delicate balance between the immune system and *S. aureus* in their ongoing battle to fight each other. However, *S. aureus* is gaining ground due to the rise of antibiotic resistance and community-acquired and highly virulent strains.

Evasion Proteins and Therapeutic Strategies

Over the past 2 decades we have gained in-depth knowledge about the existence, the importance, and the molecular mode of action of many immune evasion proteins. Now, what can we do with this knowledge? As illustrated below, one crucial goal is to find a treatment specifically against staphylococcal infections, but these immune evasion proteins can also be used to dampen the immune response in inflammatory diseases.

Combating *S. aureus* with (new) antibiotics is a challenge, since the bacterium has the extraordinary ability to develop resistance against antibiotics and the discovery of new antibiotics is declining (7). Vaccination is a strategy that could solve many of the problems that we encounter with antibiotic resistance. A few vaccine candidates have been tested in clinical trials, but none of them successfully passed phase III studies (217). For example, the vaccine candidates against the relatively small capsule of *S. aureus*, capsular polysaccharides types 5 and 8, have not passed clinical trials yet (218). This is possibly due to the presence of natural nonopsonic antibodies to another *S. aureus* cell surface polysaccharide, poly-*N*-acetylglucosamine, in human serum, and this interferes with the vaccine (219). Other vaccines targeting a single cell surface-associated antigen have failed clinical trials, for example, the iron surface determinant B (IsdB). Antibody against IsdB showed good tolerance in healthy volunteers, but in phase III studies patients administered with this vaccine were more susceptible to developing *S. aureus* infections and were five times more likely to die than unvaccinated patients with *S. aureus* infections (220). A recent overview of vaccine candidates and the status of their clinical trials was described, and this shows that

although there is still ongoing research in this field, no functional vaccine will be available in the near future (217).

Another way to neutralize evasion molecules is by the generation of small inhibitory peptides or neutralizing antibodies via passive immunization, which is short-term immunity provided by antibodies obtained outside the body. For example, a CCR5 antagonist often used in HIV treatment, maraviroc, also protects T-cells and myeloid cells from LukED-mediated toxicity (221). Thus, these types of drugs could also be used as therapeutic alternatives against staphylococcal infections. Also, it might be a good approach to target one of the toxins produced by *S. aureus*. Currently, there are three antistaphylococcal monoclonal antibodies in clinical trials. Two of them target the secreted virulence factor alpha-toxin, which has recently been shown to protect against *S. aureus*-induced pneumonia (222). Unfortunately, so far, antibody-based therapy has not passed clinical trial phase II. Earlier research focused on single antigens displayed at the bacterial surface, but recently, this has moved toward agents targeting multiple *S. aureus* proteins, for example, immune evasion molecules and intracellular reservoirs of the bacterium (223). For example, the monoclonal antibody 6D4 interferes with the activity of SCIN, but it is likely that this will not be the only target in antistaphylococcal therapy since SCIN-deficient *S. aureus* strains still cause infections (224). Nevertheless, despite the lack of a functional monoclonal antibody/antibodies at this moment, antibody-based therapy is a promising therapeutic agent to use against staphylococcal infections in the future (225).

The fact that, so far, the development of an antistaphylococcal vaccine has failed can be attributed to several factors. Vaccines targeting single antigens without adjuvants were insufficient to give protection. This is likely due to the secretion of the manifold evasion molecules. Therefore, research focus has switched to developing a vaccine against a combination of proteins. Also, the lack of a highly reliable animal model is another factor and is partially caused by immune evasion molecules since many of the evasion proteins have high-affinity binding capacity to only human targets. These high-affinity protein-protein interactions between virulence factor and target cause a lack of affinity in targets in other, less related hosts. Because multiple immune evasion proteins are restricted to the human host, it leads to difficulties in studying *S. aureus* infections *in vivo*, such as in mice. Therefore, *in vivo* studies of SCIN, CHIPS, SAK, and PVL are difficult. Some isolated virulence factors are, however, not human specific and do allow studies in other species, for example, Ecb and Efb in a murine infection model (130). The genes located in the core variable genome are immobile and generally have a broad species specificity, whereas mobile genetic element-encoded immune evasion proteins (e.g., pathogenicity islands and prophages) are highly species specific (226). The third reason for the failure of previous vaccination attempts is the existence of immune evasion by itself, which leads to lower local immunity. Even if the vaccine raises sufficient opsonizing antibodies during this lowered local immunity, the induced antibodies cannot confer protection by the lack of an effector system (such as complement and neutrophils). A way to overcome this is to incorporate evasion molecules into the vaccine and raise neutralizing antibodies against these evasion molecules. Inhibiting the inhibitor and restoring local immunity could be a solution to solve vaccination issues in the future. Nevertheless, redundancy of immune evasion molecules and a good animal model remain obstacles to be overcome.

The evasion molecules can also be used as anti-inflammatory therapy in diseases where there is a deflecting immune activation. This is a growing area of research due to the increased prevalence of staphylococcal infections in Western countries. There are numerous inflammatory diseases, for example, allergy, asthma, autoimmune diseases, vasculitis, and inflammatory bowel disease. Many evasion molecules inhibit the central players in human inflammatory diseases, such as some GPCRs, complement components, and TLRSs, and are therefore interesting therapeutic targets (227–229). This approach has already been used in several studies; for example, SCIN-derived peptides were screened for complement inhibition through phage display to develop new complement-directed therapies (230). However, the evasion molecules are immunogenic by themselves. Thus, we need something else that is based on these evasion proteins, such as small peptides or derivatives which are no longer immunogenic. The (co)crystal structure is known from many evasion molecules, and this shows us where the exact binding interface at the amino acid level is between the staphylococcal protein and its binding partner of the immune system (226). This is very helpful in designing (structured) peptides.

Another way to use peptides derived from evasion molecules is in anticancer strategies. Some of these immune receptors promote cancer growth, such as FPR1 in brain tumors. Thus, the staphylococcal inhibitor of FPR1, CHIPS, is a potential anticancer drug. Recent research with mice expressing human FPR1 on astrocytoma cells treated with CHIPS showed increased survival of the mice and reduced tumor growth (231).

Immune Evasion Cluster 1/φSa3: a Mobile Element on a Quintuple Converting Bacteriophage

CHIPS, SCIN, and SAK (136) are small, secreted proteins that play a crucial role in the staphylococcal defense against the human innate immune system (complement and neutrophils) (46). The genes for SAK (*sak*), CHIPS (*chp*), and SCIN (*scn*), and also for the superantigen SEA (*sea*), are clustered on an innate immune evasion cluster (ϕSa3) in *S. aureus*, an 8-kb region at the 3′ end of β-hemolysin-converting bacteriophages. When this phage inserts into the β-hemolysin gene, it inactivates the gene itself, and the bacterium can no longer produce β-hemolysin. In addition, the bacterium now can produce four virulence/evasion factors, which have roles in different aspects of the pathogenetic strategy of the organism, as described above. Therefore, these phages are known as quintuple converting phages (226). When studying the occurrence of this mobile element in different *S. aureus* strains, it becomes immediately clear that it is strictly limited to human strains and is absent in, for example, bovine strains (226). Further, when studying the functional interaction between the four evasion molecules present on this element, it becomes clear that all four proteins act only on human targets, never on animal receptors or molecules. This is true for CHIPS in its interaction with C5aR and FPR, is true for SCIN in its interaction with the complement components C3b and Bb, and is true for SAK and even for the superantigen SEA. CHIPS does not work on animal neutrophils, and SEA does not work on animal T-cells. SCIN does not work in animal sera (116). From the molecular data from crystallography and nuclear magnetic resonance studies between evasion molecules and targets, we can now deduce the action on the precise amino acids in the host (78, 115). Taken together, the conclusions from epidemiological studies of various animals and humans, the functional studies conducted with sera and isolated cells versus recombinant bacterial proteins, and the structural data

generated over the years are very clear: this mobile element and all of the components located on it are strictly human specific. It can transform a staphylococcus into a human pathogen. This also illustrates the importance of these virulence factors in the pathophysiology of infections in humans.

Concluding Remarks on Biological Warfare between Staphylococci and their Hosts

Our immune system can rapidly clear bacteria from our body after infection. However, infecting bacteria do not idly stand by when confronted by the host immune response. The following bacterial immune evasion strategies are often employed by pathogenic bacteria to resist our immune system.

1. Form a capsule.
2. Invade host cells and survive within them (intracellular lifestyle).
3. Change surface/change recognition molecules.
4. Secrete modulators/inhibitors/immune evasion molecules.

Hiding within an inert capsule is the hallmark of pneumococci. Mycobacteria are renowned for their capacity to hide within host cells. Staphylococci are the masters of surface modulation and immune evasion by secreted proteins. On the one hand, in the past 15 years, these phenomena have been studied extensively in *S. aureus*; on the other hand, in the next decades, it is likely to become clear how far this understanding can be extended to other bacteria. For any pathogenic bacterium, we know that there are still hundreds of nonannotated secreted proteins.

Staphylococcal immune evasion is very important for the bacterium, especially to fight the immune system during acute infection. Therefore, most evasion proteins are directed against this initial attack by neutrophils. This review gave an overview of the various evasion proteins and their function(s) during infection. Interestingly, the repertoire is still expanding with the recent discovery of novel proteins and new functions of existing evasion molecules. It is an important field of research, since functional therapeutics against staphylococcal infections are still lacking, while the economic burden increases. However, with the current speed of experiments, our knowledge is growing every year, and most likely it will only be a matter of time before we find the appropriate therapeutics for prophylaxis or treatment of this extremely clever bacterium.

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Staphylococcus aureus Secreted Toxins and Extracellular Enzymes

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40

EXOTOXINS

Introduction

Staphylococcus aureus is a highly successful pathogen that colonizes ~30% of the population asymptomatically, but it is also capable of causing infections ranging from mild skin and soft tissue infections to invasive infections, such as sepsis and pneumonia (1). When *S. aureus* infects the host, it produces many virulence factors that promote the manipulation of the host's immune responses while ensuring bacterial survival. These virulence factors include secreted toxins (exotoxins), which represent approximately 10% of the total secretome (2). While there are over 40 known exotoxins produced by these bacteria, many of them have similar functions and have high structural similarities. Closer examination of these seemingly redundant exotoxins revealed that each has unique properties. Exotoxins fall into three broad groups based on their known functions: cytotoxins, superantigens (SAgs), and cytotoxic enzymes (Table 1). Cytotoxins act on the host cell membranes, resulting in lysis of target cells and inflammation. Superantigens mediate massive cytokine production and trigger T and B cell proliferation. Secreted cytotoxic enzymes damage mammalian cells. Collectively, these exotoxins modulate the host immune system and are critical for *S. aureus* infections.

Cytotoxins

β-barrel pore-forming toxins

α-Toxin: the prototypic pore forming toxin (PFT)

α-Toxin (also known as α-hemolysin or Hla) is encoded by the gene *hla* as part of a monocistronic operon in the core genome of *S. aureus*. Like all conventionally secreted proteins, α-toxin is synthesized with an N-terminal signal peptide. Water-soluble, α-toxin monomers form heptameric β-barrel pores in target cell membranes, resulting in cell lysis (Fig. 1A) (3). The α-toxin heptamer resembles a mushroom and has three major domains: an extracellular cap domain, a stem domain that forms the β-barrel pore, and a rim domain that confers receptor specificity (Fig. 2A to C) (4). The β-barrel pore is formed from a prepore by a conformational change in a toxin substructure known as the amino latch (5). The critical role of the amino latch in the β-barrel pore formation is exemplified by a single amino acid mutation at His-35, which disrupts interprotomer stabilization, thus preventing pore formation and inactivating the toxin (6–8).

The role of α-toxin in disease has been studied extensively. α-Toxin causes lysis of many cell types: erythrocytes, platelets, endothelial cells, epithelial cells, and certain leukocytes (9–12). For many years, α-toxin was thought to mediate cytolysis through nonspecific binding to the lipid bilayer of cells. However, this model did not explain the species specificity exhibited by the toxin (i.e., lysis of rabbit but not human erythrocytes). In 2010, Wilke et al. identified the protein ADAM-10 (a disintegrin and metalloprotease 10), as the cellular receptor for α-toxin, thus providing an explanation for the observed species and cell type specificities (Fig. 3) (13). Recently, the mammalian junction protein, PLEKHA7 (plekstrin-homology domain-containing protein A7), was also demonstrated to be involved in α-toxin cytotoxicity and is thought to contribute to α-toxin-mediated tissue injuries in murine skin infection and pneumonia models (14).

α-Toxin is not only lethal, but can also modulate cellular responses at sublytic concentrations, including the release of nitric oxide from endothelial and epithelial cells, extracellular Ca^{2+} influx, production of proinflammatory cytokines, and pyroptosis of monocytes through the activation of caspase-1 and the nucleotide binding oligomerization domain-like receptor protein 3 (NLRP3) inflammasome (10, 15–19). Additionally, sublytic levels of α-toxin upregulate the expression of ADAM10 and activate the ADAM10 protease to cleave the junction protein E-cadherin, resulting in disruption of the epithelial barrier (11). Nanogram to microgram amounts of α-toxin can cause severe dermonecrosis when administered subcutaneously in rabbits and mice (20, 21). Moreover, intravenous administration of this toxin also results in rapid lethality of the animals (20, 21). *S. aureus* Δ*hla* strains are severely attenuated in several infection models, resulting in enhanced host survival, decreased bacterial burden, inflammation, and tissue injuries (22–27).

The bicomponent PFTs

The bicomponent PFTs are relatives of α-toxin (Fig. 4), share structural homology with α-toxin, and have a similar pore formation mechanism (Fig. 1 and 2). However, in contrast to α-toxin, the bicomponent PFTs require two subunits: the fast-eluting subunit, F-subunit, and the

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TABLE 1 Major exotoxins produced by *S. aureus*

Exotoxin(s)	Gene(s)	Function(s)
α-Toxin	*hla*	Pore-forming toxin
PVL (LukSF-PV)	*lukS*, *lukF*	Pore-forming toxin
HlgAB	*hlgA*, *hlgB*	Pore-forming toxin
HlgCB	*hlgC*, *hlgB*	Pore-forming toxin
LukED	*lukE*, *lukD*	Pore-forming toxin
LukAB/HG	*lukA/H*, *lukB/G*	Pore-forming toxin
LukMF′	*lukM*, *lukF′*	Pore-forming toxin
LukPQ	*lukP*, *lukQ*	Pore-forming toxin
PSMα1 to PSMα4	*psmα1* to *psmα4*	Phenol soluble modulins
PSMβ1, PSMβ2	*psmβ1*, *psmβ2*	Phenol soluble modulins
δ-Toxin	*hld*	Phenol soluble modulins
PSM-mec	*psm-mec*	Phenol soluble modulins
ε-Toxin	*cytE*	Cytotoxin
SEA to SEE, SEG	*sea* to *see*, *seg*	Enterotoxins, T cell superantigens
SE-l H to SE-l Y	*selh* to *sely*	T cell superantigens
TSST-1	*tst*	T cell superantigen
SpA	*spa*	B cell superantigen
β-Toxin	*hlb*	Sphingomyelinase, biofilm ligase
Exfoliative toxin A	*eta*	Serine protease
Exfoliative toxin B	*etb*	Serine protease

slow-eluting subunit, S-subunit, named on the basis of their liquid chromatography behavior (28, 29). The current model for leukocidin pore formation suggests that the S-subunit recognizes and binds to a surface receptor on the target cell and then recruits the F-subunit for dimerization (30–32). This is followed by oligomerization with three additional dimers to form an octameric prepore on the target cell membrane (33). Next, the stem domains of the prepore extend in the center of the structure, forming a β-barrel pore that inserts into the target cell membrane, resulting in cell lysis (Fig. 1B) (33, 34). Similar to the α-toxin heptamer, the bicomponent PFT octamer also resembles a mushroom, consisting of the cap, the rim, and the stem domains (Fig. 2).

The bicomponent PFTs primarily target leukocytes, and thus they are known as leukocidins (Luk). Currently, five of the leukocidins are known to be associated with human infections: LukSF-PV (originally known as the Panton-Valentine leukocidin [PVL]), γ-hemolysins AB and CB (HlgAB, HlgCB), LukED, and LukAB (also known as LukHG) (30–32). Two other bicomponent PFTs, LukMF′ and LukPQ, are associated with animal infections (35–37).

All the leukocidins share structural homology and sequence identity, ranging from 40 to 90% within each S-subunit and F-subunit family (Fig. 4) (38). The only exception is LukAB, which shares only ~30% sequence similarity with the others (38). LukA has an ~33-amino acid sequence at the N-terminus and a 10-amino acid C-terminal tail that are absent from other S-subunits, contributing to its divergence. Nevertheless, the structure of LukAB remains homologous to the other bicomponent PFTs (39).

In additional to mediating cell lysis, many of the leukocidins have sublytic effects, causing extracellular Ca^{2+} influx on host cells (40–42) and production of proinflammatory cytokines (40, 43–47). Several of the leukocidins, PVL, HlgAB, and LukAB, stimulate K^+ efflux, which is involved in the activation of the NLRP3 inflammasomes and caspase-1, resulting in a form of inflammatory cell death known as pyroptosis (43, 45–47).

Although the bicomponent PFTs exhibit many similarities, there are subtle differences that confer unique properties on each of them, which are briefly summarized below (see references 30–32 for in-depth summaries).

LukSF-PV (PVL). The *pvl* locus is encoded within the genomes of at least six prophages (48–51). Although less than 40% of clinical isolates from the United States carry a *pvl*-encoding prophage, over 90% of strains associated with severe necrotizing pneumonia and community-acquired infections carry *pvl* (1, 52). The relationship between *pvl*-encoding *S. aureus* and severe infections in humans is supported by strong epidemiological data and recent data using animal models of infection (53–57).

PVL exhibits species specificity, killing only rabbit and human leukocytes. The species specificity is due to the targeting of the human and rabbit G-protein-coupled receptors, C5aR1 and C5aR2, but not the murine counterparts (Fig. 3) (58, 59). Consequently, regular mice are inappropriate for the study of this toxin; the availability of rabbit models and human *ex vivo* models has provided insight into the complexity of PVL-mediated pathology. PVL is a critical factor in invasive diseases such as osteomyelitis and pneumonia in rabbits. Deletion of *pvl* results in less inflammation, reduces tissue injuries and bacterial burden, and promotes host survival (57, 60). Remarkably, sublytic levels of PVL can enhance phagocytosis and killing of the bacteria by primary human neutrophils (61). In contrast, the role of PVL during skin and soft tissue infections is unclear. While one study demonstrated that PVL did not contribute to lesion sizes and bacterial burden (62), another study showed that infections caused by Δ*pvl* strains have smaller lesions compared to the wild type and complemented controls (63). These studies seem to suggest that the pathogenic effect of PVL could be dependent on the site of infection, but more work must be done to test this hypothesis.

γ-Hemolysin (HlgAB, HlgCB). The γ-hemolysin locus is part of the core *S. aureus* genome, present in ~99% of sequenced *S. aureus* genomes (64, 65). This locus comprises three genes: the *hlgA* gene, transcribed by its own promoter, followed by an operon containing *hlgC* and *hlgB*, transcribed by a different promoter (66). HlgA and HlgC are S-subunits that share the same F-subunit, HlgB, to form two leukocidins, HlgAB and HlgCB, each possessing its own unique properties.

HlgAB binds to the human receptors CXCR1, CXCR2, CCR2, and DARC (the Duffy antigen receptor for chemokines), lysing human erythrocytes, neutrophils, monocytes, and macrophages (Fig. 3) (44, 67). HlgAB can also target murine monocytes and macrophages but cannot target murine neutrophils, because it cannot bind to murine CXCR2 (44). In contrast, HlgCB is a human-specific toxin that targets cells expressing the receptors C5aR1 and C5aR2 (the same receptors targeted by PVL) (Fig. 3) (44).

γ-Hemolysins cause acute tissue injury and inflammation and contribute to *S. aureus* disease in various animal models. Retroorbital administration of microgram amounts of HlgAB is lethal to mice (68). Intravitreal injection of γ-hemolysins in rabbits is highly toxic, resulting in destruction of the eye and tissue injury in surrounding areas (69). The tissue damage could be the result of a combination of toxin-mediated cell lysis and pyroptosis caused by sublytic concentration of the

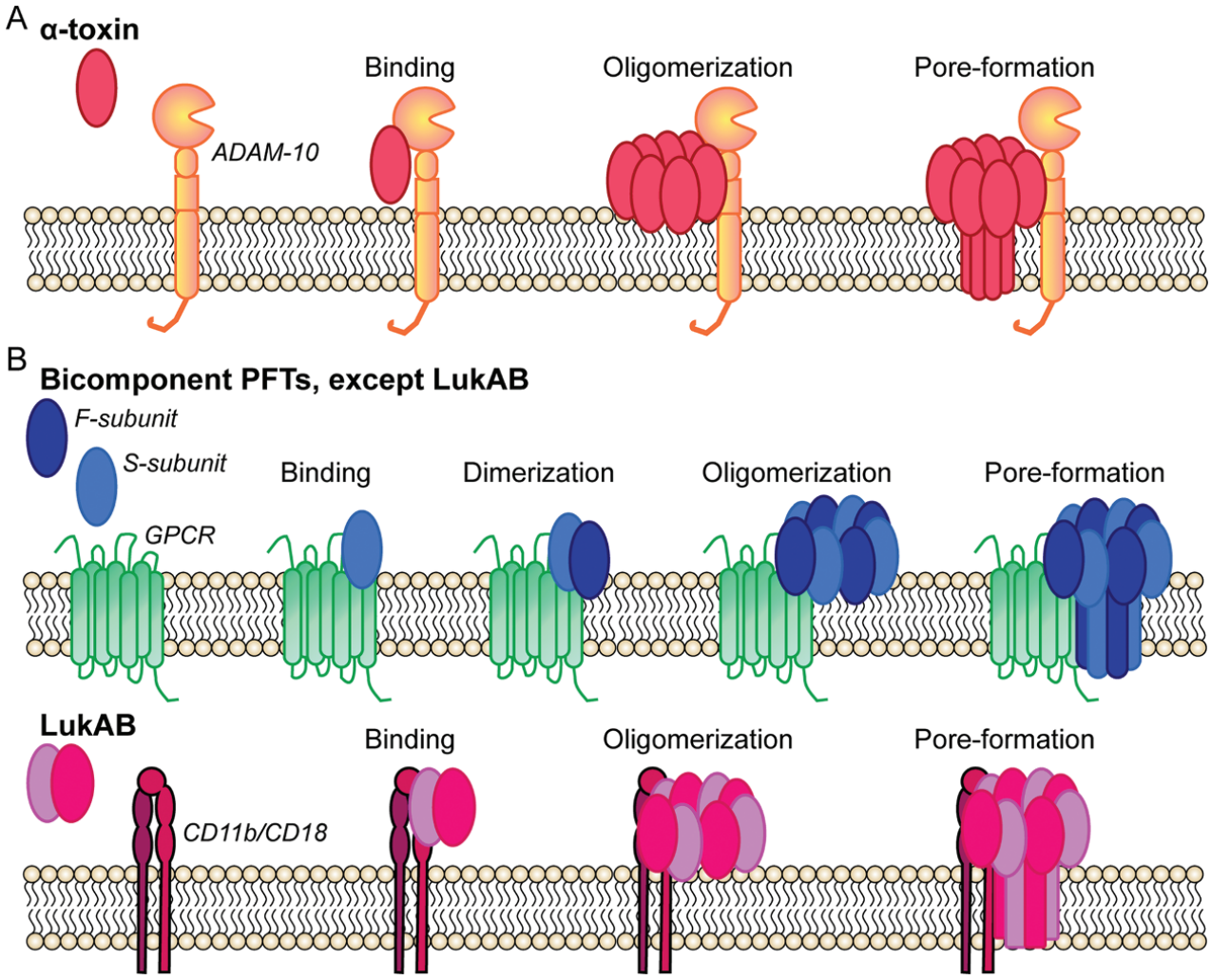


FIGURE 1 Current models for PFT pore formation for (A) α-toxin and (B) the bicomponent PFTs. (A) α-toxin is secreted as a monomer. Upon binding to the host receptor, ADAM-10, the toxin monomers oligomerize to form a heptameric prepore on the target cell surface. The prestem domains of the prepore then extend to form a β-barrel pore that punctures the target cell membrane. (B) The bicomponent PFTs are also secreted as monomers (except LukAB, which is secreted as dimers). The S-subunit recognizes the target cell by binding to cell surface receptors (LukPQ is an exception; the F-subunit LukQ is the receptor recognition subunit). These receptors are typically G-protein-coupled receptors (except for LukAB, which binds to the integrin CD11b). Upon receptor binding, the S-subunit dimerizes with the F-subunit, followed by oligomerization of three additional leukocidin dimers, resulting in an octameric prepore. Similar to the α-toxin pore formation model, the prestem domains of the prepore extend to form a β-barrel pore, thus disrupting the target cell membrane.

toxins (47). The contribution of HlgAB to disease has been further demonstrated in several infection models with strains that do not produce HlgAB. With such strains, there is reduced neutrophil lysis, less inflammation, reduced bacterial burden, and enhanced host survival (44, 70–72).

LukED. The *lukED* locus is in the νSaβ gene cluster. The *lukED* locus is present in ~70% of *S. aureus* isolates and is conserved in a lineage-specific manner (64, 65). The two genes in the locus, *lukE* and *lukD*, are cotranscribed during the late exponential phase (73).

Early studies of LukED demonstrated the lytic activity of the toxin in rabbit and human erythrocytes and neutrophils (74). Subsequently, LukED was demonstrated to also mediate lysis of many different human and murine bone-marrow-derived cells (75, 76). LukED targets the G-protein-coupled receptors (CXCR1, CXCR2, CCR5, and DARC) on neutrophils, monocytes, macrophages, dendritic cells, NK cells, T-cells, and red blood cells, conferring on the toxin broad leukocidal activity (Fig. 3) (67, 76, 77). Moreover, the pathogenic effects of LukED are receptor-dependent (75–77).

LukED is an important contributor to the virulence of *S. aureus*. Initial studies of LukED demonstrated toxin-induced dermonecrosis of rabbit skin (73). Retroorbital administration of microgram amounts of the toxin leads to acute lethality in mice (68). Corroborating the intoxication studies, Δ*lukED* strains are severely attenuated, resulting in lowered inflammatory responses, reduced bacterial burden, and enhanced host survival in murine systemic infections (75–77).

LukAB (also known as LukHG). The *lukAB* locus is part of the core *S. aureus* genome found in 99% of *S. aureus*.

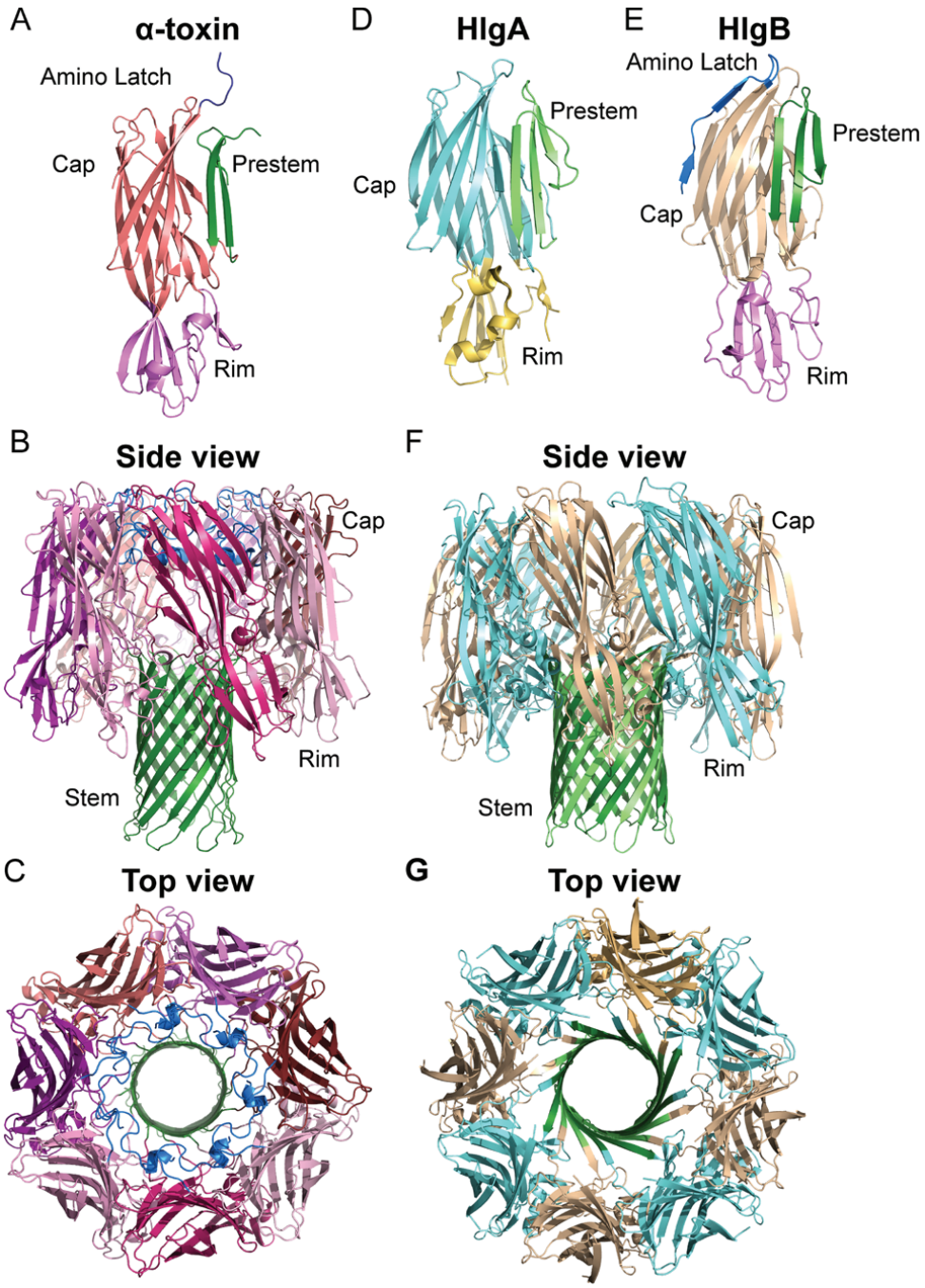


FIGURE 2 Structures of (A to C) α-toxin and (D to G) the bicomponent PFT, HlgAB. (A) The α-toxin monomer (PDB:4U6V) (351). The amino latch is colored blue, the cap domain red, the rim domain pink, and the prestem domain green. (B, C) The α-toxin heptamer (7AHL) (4). Each α-toxin is colored a different shade of pink to denote individual protomers. The amino latches are highlighted in blue, and the β-barrel pore is green. The monomers of (D) HlgA (2QK7) (352) and (E) HlgB (1LKF) (353). The amino latch of HlgB is blue; the cap domain for HlgA is cyan and HlgB is beige; the rim domains are yellow for HlgA and pink for HlgB; and the prestem domains are green. (F, G) The HlgAB octamer (3B07) (33). The HlgA protomers are cyan, the HlgB protomers are beige, and the β-barrel pore is green.

The toxin is found in abundance in the secreted proteome during the late exponential growth phase, which led to its discovery (78, 79). The C-terminal region of LukA is critical for toxin activity, because its deletion or mutation within this region (i.e., the E323A mutation) renders the toxin inactive (80). Unlike the other leukocidins, which are secreted as monomers, LukAB is secreted as heterodimers (80).

LukAB exhibits sharp species specificity, with its greatest potency being for human and primate cells, followed by

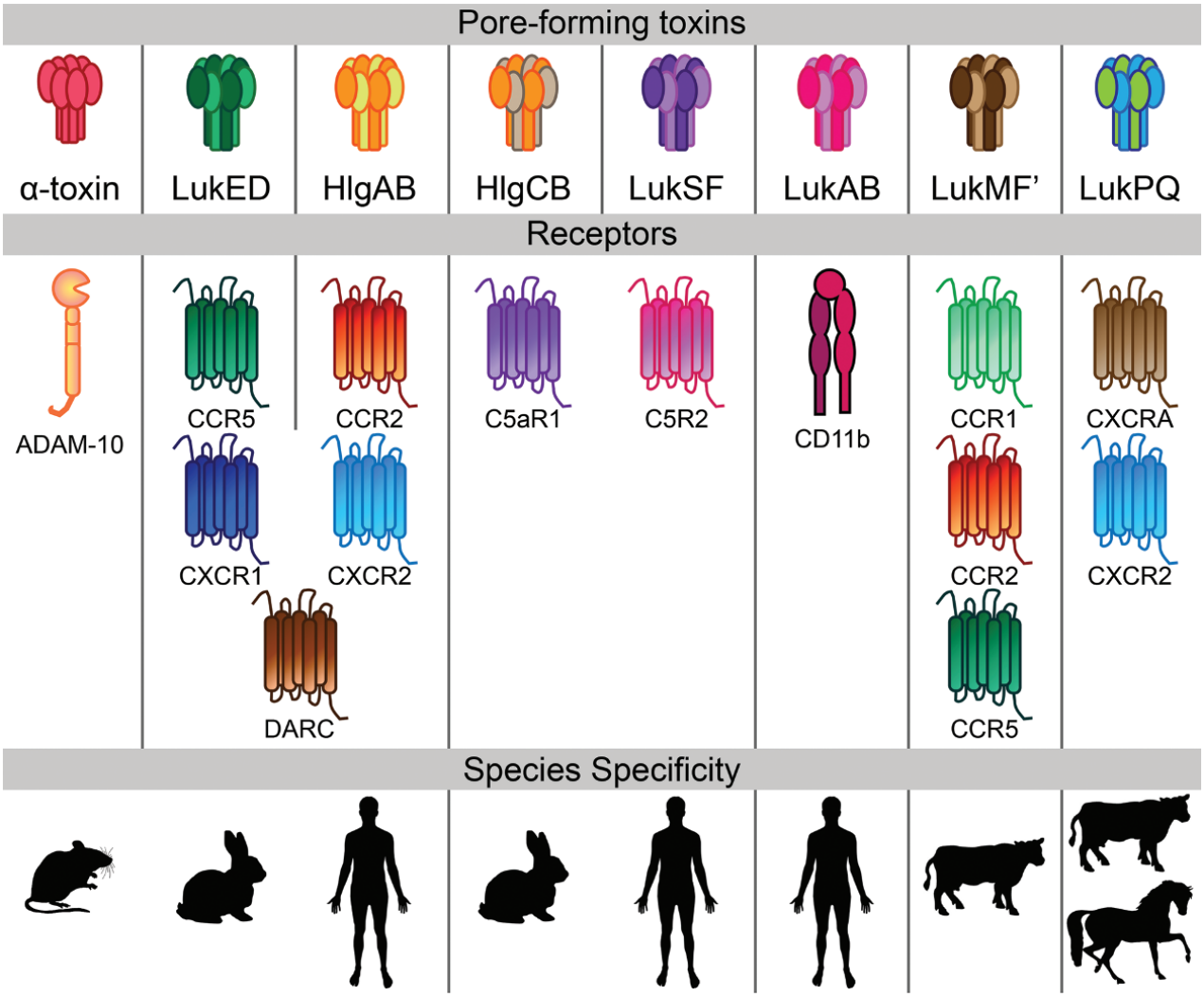


FIGURE 3 *S. aureus* PFTs and their receptor, species, and cell type specificities. Currently, *S. aureus* is known to produce eight β-barrel PFTs. Each of these PFTs targets different cell surface receptors. While some PFTs share the same receptors, they can differ in their species specificity. Collectively, the PFTs exert their sublytic and lytic effects on a variety of cells, including erythrocytes, endothelial cells, epithelial cells, neutrophils, monocytes, macrophages, dendritic cells, and T cells.

rabbit, and it is ~1,000-fold less active in mice (81). The selectivity of LukAB was explained when it was discovered that it mediates cytotoxicity through targeting the I-domain of the CD11b receptor present on leukocytes, including neutrophils, monocytes, macrophages, dendritic cells, and NK cells (Fig. 3) (80, 82).

Since LukAB is only weakly active toward murine leukocytes and mildly active with rabbit cells, the role of LukAB during infection remains to be fully elucidated. Although the field currently lacks robust *in vivo* models to directly assess the pathogenicity of the toxin, *in vitro* and *ex vivo* studies have provided insights into the role of LukAB in disease, revealing that *lukAB*-defective mutants are greatly attenuated for virulence. For example, primary human neutrophils infected with Δ*lukAB* strains exhibit enhanced survival compared to wild type (78). Additionally, LukAB promotes the escape of phagocytosed *S. aureus* from neutrophils and monocytes (46, 83). Anti-LukAB antibodies were observed in the serum of patients with invasive *S. aureus* disease, thus demonstrating production of the toxin during infection (84, 85).

LukMF′. The *lukMF′* locus is encoded by the temperate phage ΦSa1 (37). While *lukMF′* is infrequently found among human isolates, it is commonly found among animal isolates (86). LukMF′ exhibits high cytolytic activity toward bovine neutrophils and macrophages through targeting the surface receptor bovine CCR1, CCR2, and CCR5 (Fig. 3) (87). LukMF′ can be isolated from bovine mastitis tissue samples, indicating a role of this toxin in disease progression (36). However, unlike the other leukocidins, purified LukMF′ does not elicit a strong proinflammatory response when incubated with primary bovine macrophages (88).

LukPQ. LukPQ is encoded by the temperate phage ΦSaeq1 (35). Like other bicomponent PFTs, LukPQ demonstrates species and cell type specificities. Equine neutrophils are most sensitive to the lytic effect of LukPQ, followed by bovine neutrophils; human neutrophils are relatively insensitive to the toxin (Fig. 3) (35). LukPQ targets the equine G-protein-coupled receptors, CXCRA and CXCR2, to initiate cytolysis (35). At a high concentration, the toxin also targets equine CCR5. However, toxin binding can be

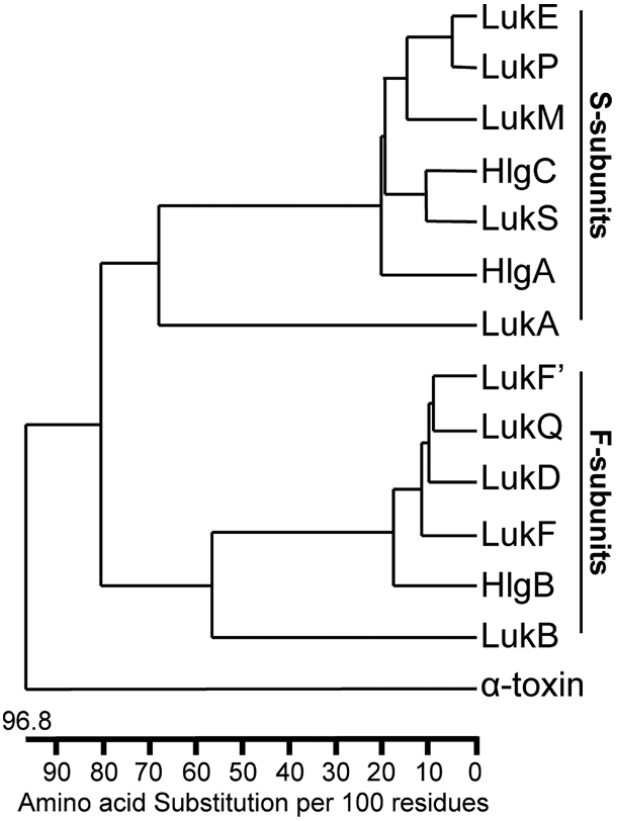


FIGURE 4 Phylogenic tree of *S. aureus* PFTs. The tree is constructed based on the mature protein sequences using the DNASTAR MegAlign ClustalW method for multiple sequence alignment.

inhibited by cytokines binding to these receptors, suggesting that LukP and the receptor ligands may share a common binding site (35). Uniquely, the F-subunit, LukQ, is responsible for conferring species specificity of LukPQ, whereas species specificity is conferred by the S-subunit in the other leukocidins (35).

Other cytotoxins

Phenol soluble modulins

Phenol soluble modulins (PSMs) belong to a family of amphipathic peptides uniquely found in staphylococci. In *S. aureus*, PSMs are encoded in three loci in the core genome: (i) the *psmα* operon, encoding PSMα1-4, (ii) the *psmβ* operon, encoding PSMβ1-2, and (iii) *hld*, encoding δ-toxin (89, 90). *hld* is also part of the coding sequence of RNA III, the master regulatory RNA in staphylococci. Additionally, certain methicillin-resistant staphylococci carry PSM-mec, encoded by *psm-mec* in the staphylococcal cassette chromosome *mec* (SCC*mec*) (91). Like *hld*, *psm-mec* is encoded within a regulatory RNA (92).

Unlike the other cytotoxins described thus far, PSM peptides are often secreted without a signal peptide (90). Therefore, most PSM peptides isolated from staphylococci contain an N-terminal formylmethionine. However, some PSM peptides lack this N-formylmethionine due to the cytoplasmic enzyme N-deformylase (93). PSMs are secreted by the ABC transporter phenol-soluble modulin transporter (Pmt). The absence of Pmt causes accumulation of PSM in the cytosol, resulting in cell death (94).

PSMs are classified based on length (95). The α-type PSMs are typically 20 to 25 amino acids long, most having a neutral or positive net charge. PSMα and δ-toxin are α-type PSMs, the entire protein forming one α-helix (96, 97). In contrast, the β-type PSMs are longer, typically 43 to 45 amino acids in length, most having a negative net charge. The structure of the β-type PSM, PSMβ2, consists of three α-helices that fold to a "v"-like shape (97).

PSMs have multiple roles in *S. aureus* pathogenesis, including cell lysis, biofilm formation, and immune modulation. α-type PSM peptides have high potency in lysing eukaryotic cells in a receptor-independent manner through targeting the cell membranes (90, 98–100). However, lipoproteins present in the serum can inhibit the cytolytic activity of PSMs (101). Therefore, the role of PSMs in extracellular cytolysis *in vivo* is unclear. In contrast, phagocytosed *S. aureus* produces PSMs to lyse neutrophils and osteoblasts intracellularly (99, 100). As such, the role of PSMs could be to mediate intracellular escape of *S. aureus*.

Sublytic concentrations of PSMs have immune modulatory effects on host cells. In humans, PSMs are detected by the pattern recognition receptor formyl peptide receptor 2 (FPR2) (102). FPR2 is a member of the G-protein-coupled receptor family that specializes in recognizing pathogen-associated molecular patterns produced by bacteria. FPR2 is predominately expressed on innate immune cell types, including neutrophils, monocytes, macrophages, and immature dendritic cells. Upon activation by PSMs, FPR2 induces a series of proinflammatory responses, including cytokine production, neutrophil chemotaxis, and leukocyte activation (102).

PSMs can shape biofilms by forming channels needed for nutrient delivery and dissemination (103). Additionally, the α-type can cause a leaky *S. aureus* membrane, resulting in the release of cytoplasmic proteins (104).

ε-toxin

Merriman et al. identified ε-toxin in 2015. The gene encoding ε-toxin, *cytE*, is conserved in the core genome of *S. aureus* (105). Since this is a recently identified toxin, the regulation of ε-toxin expression and the mode of action of the toxin are unknown. However, ε-toxin lyses rabbit erythrocytes and human keratinocytes. Lytic concentration of ε-toxin in keratinocytes promotes the secretion proinflammatory cytokine, interleukin 8 (105). In contrast, sublytic concentration of ε-toxin slows the rate of keratinocyte proliferation, suggesting a role for the toxin in impairing normal wound healing (105). A microgram amount of ε-toxin can result in neutrophil recruitment to the injection site when administered subcutaneously in rabbits (105). Moreover, the same dosage of ε-toxin can cause rabbits to develop fever after intravenous administration of the toxin (105). The role of this toxin in *S. aureus* infections remains to be fully elucidated.

Staphylococcal Superantigens

T cell Superantigens

T cell superantigens (SAgs) represent the largest family of exotoxins produced by *S. aureus*. Their molecular weights range from 19 to 30 kDa. SAgs are unique because they are resistant to heat, proteolysis, and desiccation (106). Due to their extreme stability and high toxicity in humans, some of them are classified as select agents for bioterrorism (i.e., staphylococcal enterotoxin B).

The genes encoding SAgs are found in various components of the *S. aureus* genome. The recently discovered *selX*

and *selW* are part of the core genome (107, 354). The other SAgs are encoded in different mobile genetic elements, such as bacteriophages, plasmids, and pathogenicity islands (108–111). However, the distribution of SAgs is highly variable in the same mobile genetic element found in different strains (112).

SAgs share structural homology with another family of closely related proteins, the SSLs (staphylococcal superantigen-like proteins). The *ssl* genes are encoded in the gene cluster νSaα (113). This family of proteins was originally called SETs for staphylococcal enterotoxin-like proteins (113). However, the International Nomenclature Committee for Staphylococcal Superantigens renamed them in 2004 to reflect their lack of emetic and mitogenic properties (114). The primary role of these proteins seems to be immune evasion (see reference 111 for a review on the SAg-like proteins).

SAgs exhibit tremendous sequence diversity (Fig. 5), but their overall structures are similar. SAgs have two primary domains: an N-terminal oligosaccharide/oligonucleotide binding fold that is shaped like a β-barrel and a C-terminal β-grasp domain composed of antiparallel β-sheets. The two domains are connected by an α-helix (115). Additionally, all SAgs have a dodecapeptide binding site, a Vβ T cell receptor (TCR) binding site, and up to two major histocompatibility complex (MHC) binding sites (106).

An SAg exerts its mitogenic property by cross-linking the Vβ TCR on a T cell with the MHC class II molecule (MHCII) on an antigen presenting cell, resulting in polyclonal T cell proliferation (Fig. 6) (116). SAgs are highly effective T cell mitogens that can stimulate up to 50% of T cells (106). SAg-induced T cell proliferation is followed by a state of T cell anergy, when activated T cells failed to proliferate and/or undergo apoptosis. SAgs are one of the many ways *S. aureus* manipulates the host immune system to prevent the generation of functional adaptive immunity.

SAgs can be broadly divided into three groups: staphylococcal enterotoxins (SEs), staphylococcal enterotoxin-like (SE-*l*) SAgs, and toxic shock syndrome toxin-1 (TSST-1). Each group of SAgs is briefly summarized below (see references 106, 355).

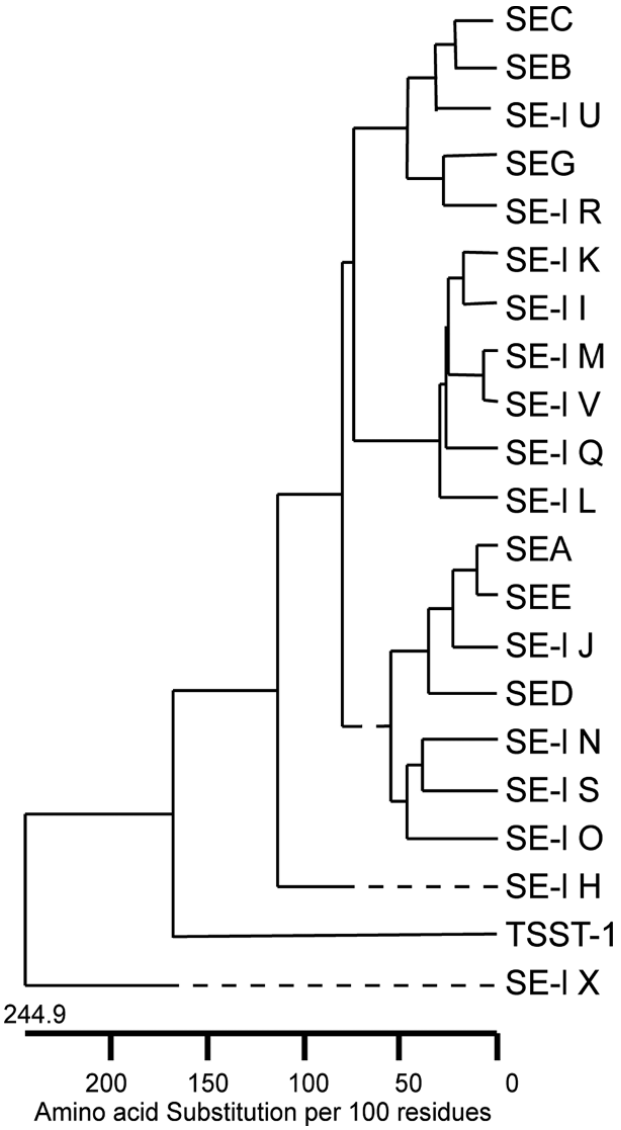


FIGURE 5 Phylogenic tree of *S. aureus* SAgs. The tree is constructed based on the mature protein sequences using the DNASTAR MegAlign ClustalW method for multiple sequence alignment.

Staphylococcal enterotoxins

There are six distinct SEs: SEA to SEE and SEG. Additionally, several variants of SEB and SEC have been identified. They were originally named for their ability to induce emesis, a key characteristic of staphylococcal food poisoning (114). Ingestion of SEs causes vomiting and diarrhea. However, the disease is usually self-limiting. The emetic activity of SEs is correlated with the presence of a 9- to 19-amino acid-long disulfide loop in the protein.

SEs can have up to two MHC binding sites. SEBs and SECs contain only one MHC binding site, while the other SEs have two MHC binding sites (106, 117, 118). The low-affinity binding site targeting the MHCII α-chain is common to all SAgs. The second site is a Zn^{2+}-dependent high-affinity binding site that targets the β-chain of MHCII (119). SAgs that have two MHC binding sites are 10- to 1,000-fold more potent than SAgs that have only one. However, they are produced at a much lower abundance compared to some other SAgs that contain one MHC binding site, such as TSST-1 and SEB (106).

Staphylococcal enterotoxin-like SAgs. The other SAgs are SE-*l* H to SE-*l* Y (106, 114, 355). SE-*ls* include all newly identified SAgs that are T cell mitogens but have unproven emetic activity (114). However, this group also contains SE-*ls* that lack the emesis-associated disulfide loop and are proven to not induce emesis (106). SE-*ls* can have up to two MHC binding sites and a 15-amino acid extension for specific TCR interactions (106, 120).

Toxic shock syndrome toxin-1. The gene *tst* is encoded in several pathogenicity islands, including SaPI1, SaPI2, and SaPIbov1 (121). TSST-1 has only one low-affinity MHC binding site that targets the MHCII α-chain, a Vβ TCR binding site, and a dodecapeptide binding site (122). This dodecapeptide binding site is proposed to be important for the interaction of TSST-1 with epithelial cells and the immune stimulatory molecules, CD40 and CD28 (123–125).

TSST-1 originally was known as SEF. In 1984, it was renamed to reflect the lack of emetic activity and to emphasize its association to toxic shock syndrome (TSS). TSS is an acute systemic illness characterized by hypotension, fever, rash, and desquamation 1 to 2 weeks after onset. As

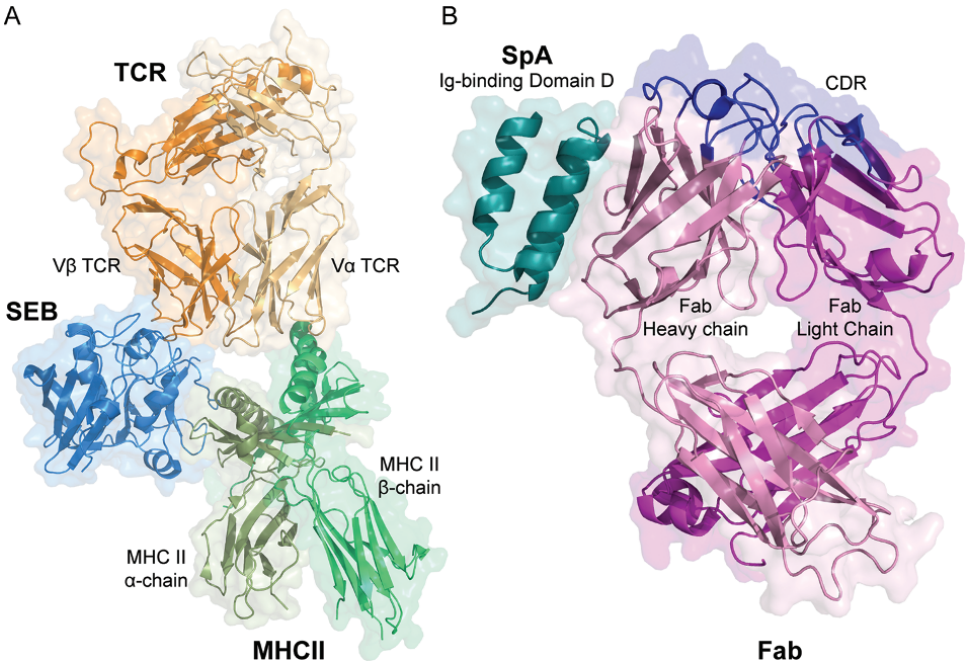


FIGURE 6 Crystal structures of *S. aureus* superantigens in complex with their cellular targets. (A) The T cell superantigen, SEB, in complex with the TCR and MHC II molecule (4C56) (116). SEB (blue) cross-links the α-chain of MHC (dark green) to the Vβ TCR (orange) to induce T cell proliferation that results in T cell anergy and/or apoptosis. (B) The B cell superantigen, SpA (teal), in complex with the Fab fragment (pink/magenta) (1DEE) (138). Conventional antigens bind to B cell receptors at the complementarity-determining region (blue), a hypervariable region that confers antigen specificities. SpA binds at a constant region of the receptor to activate B cells for supraclonal expansion, which leads to clonal deletion of SpA-activated B cells.

defined by the Centers for Disease Control, TSS involves at least three of the following organ systems—gastrointestinal, muscular, mucous membrane, renal, hepatic, hematologic, or central nervous system (126). TSS can be further classified as menstrual and nonmenstrual TSS. Menstrual TSS is usually associated with vaginal/cervical mucosae colonization of TSST-1-producing *S. aureus* and tampon use (127, 128). Approximately 50% of nonmenstrual TSS cases are caused by TSST-1-producing strains, and the remaining are caused by strains that produce SEB or SEC (129).

B cell Superantigen

Staphylococcal protein A (SpA) is the only known B cell superantigen produced by *S. aureus*. A majority of clinical isolates contain *spa* in the core genome (130). The SpA precursor has an N-terminal signal peptide that is cleaved prior to the secretion of the mature protein. Mature SpA has four to five highly conserved immunoglobulin (Ig) binding domains connected by short linkers at the N-terminus (131). These are followed by a hypervariable region called region X, which comprises subregions Xr and Xc (132). The highly variable and repetitive octapeptide in Xr is the basis of SpA-typing, a high-throughput method of grouping *S. aureus* isolates (130). Region X is followed by the C-terminal LPXTG motif for covalent anchoring of the protein to the cell wall (131). However, SpA proteins can be released from the cell wall by the cell wall hydrolase, LytM (133).

The Ig binding domains confer upon SpA the ability to bind the Fcγ portion of Igs to prevent opsonization (134). These Ig binding domains also mediate SpA binding to B cells by cross-linking V_H3-expresssing B cell receptor, which results in B cell activation; however, activation without costimulatory signals results in death and subsequent clonal deletion of B cells (135–137).

Conventional antigen recognition by a B cell receptor requires antigen recognition at the complementarity-determining region. In contrast, SpA exerts its mitogenic activity by binding to the variable region of the heavy chain, away from the complementarity-determining region, thus bypassing the antigen specificity requirement for B cell activation (Fig. 6) (138, 139). SpA-mediated clonal deletion of V_H3-expressing B cells can lead to the impairment of the B cell repertoire that is important for mounting effective antimicrobial defenses against the pathogen (140, 141).

During intravenous infection, SpA prevents opsonophagocytosis of the bacteria by sequestering Igs and impedes the development of specific anti-*S. aureus* antibodies (142). In contrast, isogenic strains that lack *spa* or express variants that cannot bind to Ig exhibited reduced kidney abscess formation and elicited specific anti-*S. aureus* antibodies (142). Mice immunized with the Ig binding-deficient SpA variant, SpA_{KKAA}, acquired protective immunity and could mount a more effective humoral response against *S. aureus* antigens (143).

Cytotoxic Enzymes

β-toxin (also known as β-hemolysin)

The β-toxin encoding gene, *hlb*, is part of the core *S. aureus* genome. However, due to the presence of the *hlb*-converting prophage (i.e., ΦSa3, Φ13), which disrupts the gene, only a

limited number of human clinical isolates produce β-toxin (144, 145). The prophage carries the immune evasion gene cluster encoding for immune evasion factors, such as the staphylococcal complement inhibitor proteins, chemotaxis-inhibitory proteins, and staphylokinase (145). These virulence factors are thought to be involved in *S. aureus* immune evasion and survival in the human host. The *hlb*-converting prophage is prevalent in strains associated with human infections (~90%), but it is less frequently found in animal isolates (~30%) (146). However, chronic infections or environmental pressures (i.e., oxidative stress, antibiotics, temperature) can promote the excision of the prophage and the production of β-toxin (147–150). Thus, the contribution of β-toxin during human infection is unclear.

β-toxin is a Mg^{2+}-dependent neutral sphingomyelinase (SMase), a phospholipase that specifically cleaves sphingomyelin to produce ceramide and phosphocholine (151). This toxin was first identified in 1935 by Glenny and Stevens based on several unique observations: hemolysis of erythrocytes in the presence of α-toxin neutralizing serum, lysis of sheep but not rabbit erythrocytes, and enhanced hemolysis caused by the temperature shifting from 37°C to a lower temperature (152). As such, β-toxin is also known as a hot-cold hemolysin. This unique phenomenon is the result of ceramide hydrolysis products at 37°C being held together by cohesive forces in the membrane. When the temperature decreases (i.e., to 4°C), this causes a phase separation that condenses ceramide into pools and collapses the lipid bilayer, resulting in the invaginations observed on erythrocyte membranes by electron microscopy (153).

The crystal structure of β-toxin reveals structural homology to members of the DNase I superfamily (154). β-toxin is a single domain protein consisting of four layers: two layers of β-sheets at the center and two outer layers that comprise α-helices and β-strands (154). Based on the structural homology to the DNase I superfamily, a secondary function for β-toxin was hypothesized. Later, β-toxin was shown to enhance biofilm formation through catalyzing the formation of nucleoprotein matrix in biofilms; therefore, β-toxin is also a biofilm ligase (155).

β-toxin exhibits species-dependent hemolytic activity that correlates with the amount of sphingomyelin content in erythrocytes: sheep, cow, and goat erythrocytes are highly sensitive to the toxin, rabbit and human erythrocytes exhibit intermediate sensitivity, and murine and canine erythrocytes are resistant (156). The SMase activity of β-toxin also causes the lysis of human keratinocytes, monocytes, T cells, and bovine epithelial cells (154, 157–159). β-toxin stimulates the production of proinflammatory cytokines in human monocytes (158) but suppresses IL8 production and cell adhesion molecule expression in human endothelial cells, and therefore the toxin can prevent leukocyte migration across the endothelium (160).

Infection with β-toxin-producing *S. aureus* results in larger lesions in the organs without affecting the overall bacterial burden in the rabbit endocarditis and pneumonia models (147). The presence of β-toxin enhances *S. aureus* colonization of the skin (159) and induces injuries to the scleral epithelial cells during ocular keratitis in mice (27). In an infective endocarditis model, rabbits infected with *S. aureus* that produce β-toxin mutants that lack SMase activity have enhanced survival and smaller lesions in the heart, but there were no differences in bacterial burden compared to the isogenic strain that produces active β-toxin (161). Intranasal administration of β-toxin induces the shedding of syndecan-1, a major heparan sulfate proteoglycan molecule on lung epithelial cells, and causes neutrophil infiltration into the lungs in mice (162). The shedding of sydecan-1 is caused by the SMase activity of β-toxin, because intranasal intoxication with SMase mutants reduced shedding of this protein *in vivo*, resulting in reduced lung pathology (162).

Exfoliative toxins

Exfoliative toxins (ETs) are also known as epidermolytic toxins. There are four antigenically distinct forms found in *S. aureus*: ETA, ETB, ETC, and ETD. Each ET is encoded on a different mobile genetic element: *eta* is encoded in the genome of a temperate phage that has been shown to convert *eta*-negative strains to toxin producers (163, 164). *etb* is found on the plasmid pETB (165), and *etd* is encoded as part of a pathogenicity island (166). ETC was purified from an *S. aureus* isolate from equine infection; however, the genetic locus of ETC has not been described (167).

Most of what is currently known about the ETs is based on ETA and ETB. ETs are the causative agents for staphylococcal scalded skin syndrome (SSSS), including Ritter's disease, toxic epidermal necrosis, bullous impetigo, and certain erythema cases. SSSS predominantly affects neonates, infants, and immunocompromised adult patients (168). Symptoms of SSSS are characterized by formation of blisters and superficial desquamation, involving only the skin layer (168). Although SSSS was initially described in 1878, its association with *S. aureus* infections was first suggested in 1967, and the contribution of ETs to the blistering symptoms was not identified until in the 1970s (163, 169–171). This delay was in part contributed by the ETs' unique mode of action. The lesions characteristic of SSSS are often sterile, because the ETs can be distributed through the bloodstream from a distant site to cause symptoms (171).

ETs are glutamate-specific serine proteases of the chymotrypsin family. The catalytic triad (histidine, aspartate, serine) is conserved in all ETs (168). ETA and ETB are similar in structure and share homology with other staphylococcal serine proteases: SspA and the serine protease-like proteins (Fig. 7) (172–174). The N-terminal α-helical extension is required for enzyme activity (172, 174). The crystal structures of both ETA and ETB show that the key residues in loop D occupy the oxyanion hole required for enzymatic activity of all serine proteases, and thus the crystal structures represent the inactive forms of the enzymes (172, 174). These findings suggest that the protease activity of ET may require a specific cellular target and occur under specific conditions.

In the early 2000s, ETs were shown to interact with human and mouse desmoglein 1 (Dsg1), causing blistering of the superficial skin (175, 176). ET recognition of Dsg1 is conformational, requiring the presence of Ca^{2+}. Lack of Ca^{2+} results in the unfolding of Dsg1 and thus the inability of ETs to cleave the protein (177). Through domain swapping and site mutagenesis studies, it was determined that five amino acids (Q271, Y274, T275, I276, E277) belonging to the extracellular domain 3 of Dsg1 are critical for ETA to exert its protease activity (178).

With the identification of Dsg1 as the substrate for ETs, the pathophysiology of the superficial skin blistering in SSSS was explained. In humans, there are four isoforms of desmogleins, Dsg1 to Dsg4 (179). Desmogleins are cadherins that are required for desmosome cell-to-cell adhesion to maintain the integrity of the epidermis. ETs target only Dsg1, which is expressed throughout the human epidermis. Cleavage of Dsg1 disrupts the cell-to-cell adhesion of the epidermis, resulting in blistering and desquamation of the superficial skin. The other strata of the epidermis are unaffected because of the presence

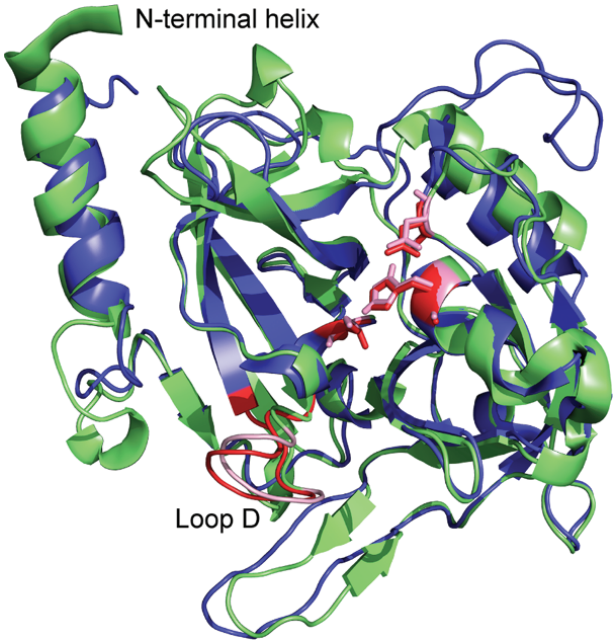


FIGURE 7 Overlay of the crystal structures of ETA and ETB. ETA (1EXF, green) (174) and ETB (1QTF, blue) (173) share high structural identity. ETs cause SSSS by cleaving Dsg1 at the epithelial cell junctions. Both ETs are serine proteases. Loop D and the catalytic triad are highlighted in pink for ETA and red for ETB.

of Dsg2 to Dsg4, which are not targeted by ETs and thus compensate for the destruction of Dsg1 (175, 180).

EXOENZYMES

Introduction

S. aureus devotes a significant amount of its resources to produce virulence factors to evade the host immune system and to acquire nutrients for its survival. The previous section discussed the mechanisms of toxin-mediated host immune evasion and their roles in *S. aureus* virulence. In addition to the toxins, *S. aureus* also produces a large number of virulence factors that have enzymatic properties. They can be broadly categorized into two groups: cofactors that activate host zymogens and enzymes for degradation of tissue components (Table 2). While these cofactors and secreted enzymes (exoenzymes) have different substrates and mechanisms of action, they function to break down bacterial and host molecules for nutrient acquisition, bacterial survival, and dissemination.

Cofactors for Host Enzyme Activation

Coagulase (Coa), von Willebrand factor binding protein (vWbp), and staphylokinase (Sak) are cofactors produced by *S. aureus* that have no enzymatic activities by themselves, but they can activate host zymogens. These three proteins hijack different aspects of the host coagulation system, thereby manipulating the host innate defenses to promote bacterial survival and dissemination.

Staphylococcal coagulases: Coa and vWbp

The ability to induce coagulation is one of the key criteria used in modern medical microbiology for species classification in the genus *Staphylococcus*—separating coagulase-positive and coagulase-negative species. A majority of staphylococci are coagulase negative, but a few are coagulase positive species, including *S. aureus* and *S. intermedius*; however, *S. schleiferi* has both coagulase-positive and coagulase-negative subspecies (181).

S. aureus-induced coagulation of human plasma was initially documented in 1903 (182). The causative agents, Coa and vWbp, are highly active in coagulating human and rabbit plasma (183).

Both *coa* and *vwb* are chromosomally encoded. There are 12 isoforms of *coa* that have been identified thus far; the majority of the variability is attributed to the high sequence variability (>50%) of the N-terminus coding region among different strains (184, 185). In contrast, *vwb*, which encodes vWbp, is relatively conserved, with only two known alleles (184). However, a recent report identified several *vwb* paralogues carried by SaPIs that produce vWbps that coagulate ruminant and equine plasma (186).

Coa and vWbp share ~30% protein sequence homology at the N-terminus (187). They both have a D1D2 domain for prothrombin binding (188, 189). However, they differ significantly at the C-terminus. The C-terminus of Coa has a 188-residue linker region followed by a repeat region composed of tandem repeats of 27 residues responsible for fibrinogen binding (188, 189). In contrast, the C-terminus of vWbp has a von Willebrand factor binding domain and a fibrinogen binding domain (188, 190).

TABLE 2 Major secreted cofactors and enzymes produced by *S. aureus*

Cofactor/enzyme	Gene	Function(s)
Coagulase	*coa*	Cofactor, activates prothrombin
vWbp	*vwb*	Cofactor, activates prothrombin
Staphylokinase	*sak*	Cofactor, activates plasminogen
Nuc (thermonuclease)	*nuc*	Nuclease
Aureolysin	*aur*	Metalloprotease
ScpA (V8 protease)	*sspA*	Serine protease
SplA	*splA*	Serine protease
SplB	*splB*	Serine protease
SplC	*splC*	Serine protease
SplD	*splD*	Serine protease
SplE	*splE*	Serine protease
SplF	*splF*	Serine protease
Exfoliative toxin A	*eta*	Serine protease
Exfoliative toxin B	*etb*	Serine protease
Staphopain A	*scpA*	Cysteine protease
Staphopain B	*sspB*	Cysteine protease
Hyaluronidase	*hysA*	Lyase
β-Toxin	*hlb*	Sphingomyelinase, biofilm ligase
PI-PLC	*plc*	Phospholipase
SAL1	*lip1*	Lipase
SAL2	*geh*	Lipase
FAME	Unknown	Detoxify free fatty acids

Coa or vWbp bind to prothrombin at a ratio of 1:1 to form staphylothrombin. Insertion of the N-terminus of Coa into the Ile16 pocket of prothrombin causes a conformational shift resulting in the activation of the zymogen (189, 191). Staphylothrombin is highly efficient in converting fibrinogen to fibrin (Fig. 8).

The activity of staphylothrombin cannot be inhibited by common anticoagulants (i.e., EDTA, heparin) or thrombin inhibitors, such as hirudin and bivalirudin (189, 191–193). However, two recently discovered small molecules, argatroband and dabigatran, can inhibit the activity of staphylothrombin (194, 195).

The Δ*coa*Δ*vwb* strain is less virulent than its wild-type parent, demonstrating a role of the coagulases during infection (187, 196, 197). However, coagulases must be present concurrently with the infecting strain to promote virulence. Ekstedt and Yotis demonstrated that while intracerebral coinjection of purified coagulase with coagulase-negative *S. aureus* enhances the virulence of coagulase-negative *S. aureus*; preinjection of purified coagulase before infection has no effect (198). Additionally, Coa is suggested to have a role in the formation of device-associated biofilm formation (199).

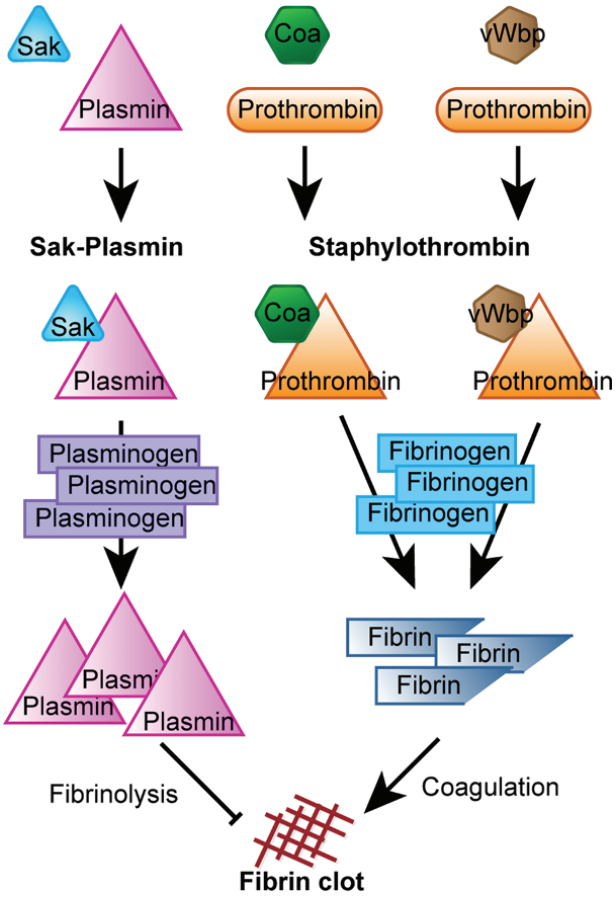


FIGURE 8 *S. aureus* produces cofactors that hijack the host's coagulation system. Coa and vWbp bind to prothrombin and alter the conformation of the protein to form the complex, staphylothrombin. This complex is highly active and cleaves fibrinogens to fibrins, promoting the formation of fibrinous clots. Sak binds to plasmin to form the Sak-plasmin complex. Sak stabilizes plasmin to enhance enzymatic activity. Sak-plasmin cleaves plasminogen to form plasmin, which breaks down fibrin clots.

In an abscess, the coagulases generate a fibrin shield to protect *S. aureus* from immune cell infiltration. Coa is required for the formation of pseudocapsule immediately surrounding the abscess, and both vWbp and Coa are required for fibrin formation around the pseudocapsule (187, 200).

Staphylokinase

Staphylokinase (Sak) is a cofactor that hijacks host plasmin to activate plasminogen for the breakdown of fibrin clots and promotes bacterial dissemination (Fig. 8). Sak is produced by lysogenic strains of staphylococci; the prophage encoding Sak typically carries other genes that encode virulence factors such as enterotoxin A and chemotaxis inhibitory proteins (201, 202). There are three groups of phages that carry the *sak* gene (203). Serotype B phages (i.e., ΦC) cause positive conversion of Sak without disrupting other genes (204, 205). Positive conversion of *sak* can also be mediated by some serotype F phages (i.e., ΦSa3, Φ42D), but the phage integration disrupts the *hlb* gene (144, 202, 206). The phage carrying *sak* has also been reported to disrupt the coding sequences of *N*-acetylmuramyl-L-alanine amidase and peptidoglycan hydrolase (207, 208).

Sak is a single-domain protein that consists of a central α-helix, a five-strand β-sheet, and two shorter β-strands (Fig. 9) (209). Sak forms a 1:1 complex with plasmin in the serum to form Sak-plasmin (210, 211). This complex is highly efficient in converting plasminogen to plasmin. Sak can also bind to plasminogen, but this complex is inactive and must be converted to Sak-plasmin to have enzymatic activity (212). In an active Sak-plasmin complex, the first 10 residues at the N-terminus of mature Sak are removed to expose the charged residue, Lys11 (213). Deletion of Lys11

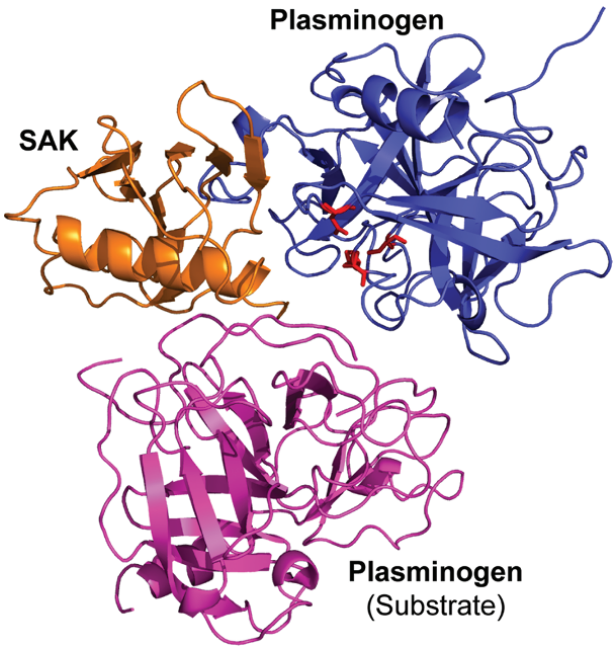


FIGURE 9 The crystal structure of Sak in complex with two plasminogen molecules (1BUI) (215). While Sak binding to plasminogen does not have enzymatic activity, the trimeric complex captures how Sak may bind to plasmin to cleave plasminogen. Sak (orange) is in complex with plasminogen (blue), exposing the catalytic site (red). Sak facilitates the docking of the substrate plasminogen (pink) to promote cleavage by plasmin.

inactivates Sak (214). The binding of Sak to plasmin directs the active site of plasmin to favor cleavage of the activation loop in plasminogen and promotes the conversion of plasminogen to plasmin by enhancing substrate presentation to plasmin. (Fig. 9) (215).

Circulating Sak-plasmin complexes are sensitive to dissociation by α_2-antiplasmin, but fibrin-bound complexes are protected from inactivation (216). The fibrin-bound complexes cleave IgG and human C3b, thus preventing opsonization of the bacteria by the complement system (217). Additionally, Sak-plasmin complexes can activate the matrix metalloprotease 1, which is important for leukocyte migration and activation (218). Importantly, Sak neutralizes the bactericidal activities of α-defensins and LL-37, two major human antimicrobial peptides (219, 220).

Sak is highly species specific. It is active for human, dog, goat, rabbit, and sheep plasminogen but is inactive for mouse, pig, cow, and buffalo plasminogen (221). Using transgenic mice that produce human plasminogen, studies demonstrated that Sak facilitates *S. aureus* invasion of the skin barrier to generate large and open lesions (222, 223). However, plasmin activation is known to promote wound healing and to reduce inflammation. Thus, during skin infection, Sak may function as a vanguard to establish the primary infection, but after the infection is established, Sak limits the severity of infections to promote dissemination (223).

Furthermore, Sak reduces biofilm formation and facilitates the detachment of mature biofilm by activating plasminogen (221). Corroborating these observations, high Sak-producing strains are often associated with less biofilm formation *in vitro* and noninvasive infections in humans (221, 223).

Enzymes that Degrade Host Tissue Components

Nucleases

Staphylococcal nuclease, originally known as micrococcal DNase, was identified in the culture supernatants of *S. aureus* by Cunningham et al. in 1956 (224). Nuclease requires Ca^{2+} ions for activity, but not other divalent cations (224, 225). Staphylococcal nuclease is also known as thermonuclease, named after its resistance to heat inactivation (224, 225). Staphylococcal nuclease functions as both an endo- and exo-nuclease that breaks down DNA and RNA substrates through the cleavage of the 5′-phosphoryl ester bond (224, 225).

With the availability of whole-genome sequencing in the late 1990s, the sequence of the *S. aureus* genome became available, which led to the identification of two staphylococcal nuclease genes, *nuc* (SA0746) and *nuc2* (SA1160) (226, 227). The two genes are located at disparate regions in the genome, under the control of separate promoters. The two nucleases share 34% amino acid similarity overall and 42% similarity within the catalytic domain (228). Both nucleases are Ca^{2+} dependent and heat resistant and are able to use DNA and RNA as substrates (226, 228). A major difference between Nuc and Nuc2 is their cellular localization. Nuc is a secreted enzyme with two isoforms, NucB and NucA (229, 230). In contrast, Nuc2 is surface bound (228).

Much of what is currently known about nucleases is gathered from studies performed on Nuc. During infections, Nuc regulates biofilm formation and mediates bacterial escape from neutrophil extracellular traps (NETs).

Nuc disperses biofilm by breaking down extracellular DNA. Biofilm formation is enhanced in strains that do not produce Nuc (230, 231). Expression of *nuc* is repressed during biofilm formation, providing evidence that *S. aureus* controls nuclease expression to regulate biofilm formation (230, 232). Furthermore, the *nuc* mutant has decreased fitness during intraperitoneal infection *in vivo* (232).

The second role of Nuc is to mediate bacterial escape from NETs. NET is an innate immune defense mechanism by which DNA released from dying neutrophils immobilizes and facilitates killing of invading pathogens (233). Nuc degrades NETs to allow *S. aureus* to escape (234).

Moreover, when Nuc degrades DNA in the abscess or NETs, the degradation products, monophosphate nucleotides, become a substrate for another enzyme, adenosine synthase A (235). Adenosine synthase A converts the degraded DNA to deoxyadenosine, which induces caspase-3 activation, leading to apoptosis of macrophages surrounding the abscess or the NET, thus promoting *S. aureus* survival (235).

The contribution of Nuc2 to *S. aureus* virulence is less clear due to its low expression level compared to Nuc (228). Purified Nuc2 has been demonstrated to disperse biofilms *in vitro* (228). A mutant expressing only Nuc2 but not Nuc showed that the nuclease is produced during intramuscular infections in mice, albeit at a much lower level (228). The identification of Nuc2 *in vivo* suggests that it may have a role in *S. aureus* virulence, possibly performing functions similar to those of the secreted Nuc but on the bacterial surface.

Proteases

Staphylococci encode three families of secreted proteases: metalloproteases, cysteine proteases, and serine proteases. Collectively, these proteases have roles in nutrient acquisition, bacterial dissemination, and immune evasion. Currently, *S. aureus* is known to produce 12 proteases: one metalloprotease (aureolysin [Aur]), two cysteine proteases (staphopain A [ScpA] and staphopain B [SspB]), and nine serine proteases. These serine proteases include V8 protease (SspA), serine protease-like proteins A to F (SplA to SplF), and ETA and ETB. Although Spls, ETA, and ETB are secreted as active enzymes, all the other proteases are secreted as zymogens, requiring proteolytic cleavage of the propeptide for activation (Fig. 10). The roles of ETA and ETB in *S. aureus* virulence are described in the exotoxin section, above.

In the following sections, we will discuss the mode of action of each protease family and its proposed role in *S. aureus* virulence.

Metalloprotease: Aureolysin

The *S. aureus* metalloprotease, aureolysin (Aur), also known as protease III, was identified in the culture supernatant of strain V8 by Arvidson et al. in 1972 (236, 237). The structure of Aur comprises two conserved domains common to bacterial metalloproteases of the thermolysin family: the N-terminal β-pleated domain and the C-terminal α-helical domain (238). The mechanisms of substrate binding and protein catalysis are also common among the proteases in this family. However, unlike other bacterial metalloproteases in the thermolysin family, Aur does not have elastase activity (238, 239).

Aur self-activates by autoproteolysis through the cleavage of the N-terminal propeptide (Fig. 10) (240). The active enzyme prefers to cleave peptide bonds at the N-terminal side of bulky hydrophobic residues, such as alanine, isoleucine, and tyrosine (241). The presence of Zn^{2+} is required for enzyme activity, but Co^{2+} can act as a substitute and increases enzyme activity (242). Additionally, binding to Ca^{2+} ions stabilizes Aur, whereas chelating agents, such as EDTA, irreversibly denature the protein (236, 238).

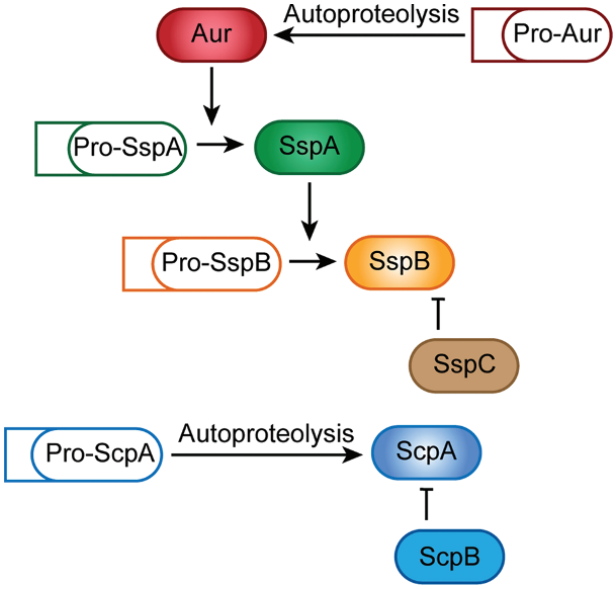


FIGURE 10 Staphylococcal protease cascade. The metalloprotease, Aur, is activated by autoproteolysis after protein secretion. Aur is required to activate the serine protease, SspA. SspA processes one of the staphopains, SspB, from zymogen to active enzyme. The other staphopain, ScpA, is activated by autoproteolysis. Both staphopains are inhibited by staphostatins prior to secretion. SspB is inhibited by SspC, and ScpA is inhibited by ScpB.

The broad substrate specificity of Aur allows the metalloprotease to target a variety of substrates, including other *S. aureus* proteins that are important for virulence and host proteins that are important for immune defense. Aur activates SspA, the second protease in the staphylococcal protease activation cascade (Fig. 10) (243). Additionally, Aur can degrade clumping factor B (ClfB) and the PSMα peptides (244, 245). Collectively, Aur can shape the secreted and surface proteome of *S. aureus* (246).

Aur contributes directly to *S. aureus* immune evasion and dissemination through the cleavage and inactivation of the antimicrobial peptide LL-37, thus promoting *S. aureus* survival (247). Aur can also degrade the human plasma protease inhibitors—α_1-proteinase inhibitor and α_1-antichymotrypsin present in the serum—albeit not as efficiently as SspA (248, 249). As such, Aur and SspA are proposed to work synergistically to achieve immune evasion.

Aur can affect complement activation by cleaving the complement proteins C3 to C3a and C3b in serum (250). The anaphylatoxin, C3a, is further degraded by Aur, preventing leukocyte activation (250). The soluble C3b fragment is inhibited and degraded by factor H and factor I in the serum (250, 251). Degradation of C3 by Aur results in the depletion of C3 proteins, thus preventing the formation of the membrane attack complex on the bacteria, and promotes bacterial survival. Furthermore, Aur activates prothrombin and prourokinases and inactivates plasminogen inhibitors, thereby manipulating the host coagulation system (252, 253). The various roles of Aur in modulating *S. aureus* proteome and host innate defense molecules suggest that Aur has an important role in promoting survival and dissemination of the bacteria *in vivo*. This is corroborated by the detection of Aur in phagocytosed *S. aureus*, suggesting that the protease may have a role during intracellular infection (254).

Serine proteases: SspA

The serine protease, SspA is also known as the V8 protease or GluV8. The gene encoding SspA (*sspA*) is part of the staphylococcal serine protease operon (*ssp*), which consists of three genes: *sspA*, *sspB*, and *sspC* (255). The functions of SspB and SspC are discussed later in this section.

SspA was identified in the culture supernatants of strain V8 by Drapeau et al. in 1972 (256). Around the same time, Arvidson et al. identified protease I, which exhibited properties similar to those of the SspA identified by Drapeau et al.; however, whether these two reports describe the same enzyme was difficult to decipher because there were differences in molecular weight and protease inhibitor sensitivity between the two reports (241, 257). Based on the report by Drapeau et al., the enzymatic activity of SspA can be inhibited by the serine protease inhibitor disopropyl flurophosphate (256).

SspA is a glutamyl endopeptidase, part of a small group of serine proteases that preferentially cleave substrates at the C-terminal side of glutamate and aspartate (256). The preference for negatively charged residues as substrates at neutral pH is due to the protein's positively charged N-terminus (258). The crystal structure of SspA showed that the protein lacks the disulfide bonds commonly found in other proteins of this family (258). However, SspA shares high structural homology to the serine proteases—staphylococcal ETs and bovine trypsin—despite having limited protein sequence similarity (258). The conserved trypsin-like serine protease catalytic triad, consisting of histidine, asparagine, and serine, is found in SspA. The C-terminal repeat domain consists of tandem repeats of Pro-Asp or Asn-Asn that range from 9 to 19 repeats (259–261); however, because this C-terminal repeat domain is not required for activity (262), its role in the function of the protein is unclear.

SspA is secreted by *S. aureus* as a zymogen. However, pro-SspA can undergo autoproteolysis to generate a shorter version of pro-SspA (243). Aur processing is required for both forms of pro-SspA to become active enzymes (Fig. 10) (243).

SspA contributes to *S. aureus* immune evasion and dissemination by breaking down self and host proteins. SspA cleaves fibrinogen binding factors on the *S. aureus* cell surface and thus reduces bacterial adhesion and enhances bacterial dissemination and ultimately results in the breakdown of biofilms (263, 264). SspA can also degrade host proteins such as α_1-proteinase inhibitor (248), the interleukin 6 cytokine (265), and immunoglobulins (266, 267); thus, SspA directly modulates immune activation and opsonization. SspA can cleave LL-37, but the cleavage does not inactivate the antimicrobial peptide (247).

SspA is produced upon *S. aureus* phagocytosis by neutrophils, suggesting its role in facilitating *S. aureus* intracellular escape, potentially through activating the cysteine protease, staphopain B (254). SspA can disrupt epithelial barriers, compromising cell junction integrity (265, 268). Skin infection models of *sspA* mutants suggest a slight decrease in bacterial fitness *in vivo* (269). However, since the activity of SspA can be inhibited by an α_2-macrogobulin present in the serum, its role as a soluble virulence factor in serum during *S. aureus* pathogenesis remains unclear (263).

Cysteine proteases: Staphopains

Staphopain A (ScpA) was the first cysteine protease identified in *S. aureus* by Arvidson et al. (described as protease II) in 1973 (257). Subsequently, staphopain B (SspB) was identified as ORFX in 1998 by Chan et al. (270). A third staphopain, staphopain, C was described in avian-associated

S. aureus isolates (271, 272). Most of what is known about staphopains is derived from studies of staphopains A and B.

ScpA and its intracellular inhibitor staphostatin A are encoded in the staphylococcal cysteine protease operon (*scp*) by the genes *scpA* and *scpB*, respectively (273). Staphopain B (SspB) and it intracellular inhibitor staphostatin B (SspC) are encoded in the staphylcoccal serine protease (*ssp*) operon by the genes *sspB* and *sspC*, respectively (255).

ScpA and SspB are secreted as zymogens. Pro-SspB is processed by SspA as the last step of the proteolytic cascade that begins with Aur (Fig. 10) (255, 274). In contrast, processing of pro-ScpA is not mediated by Aur, SspA, or SspB (269). Instead, pro-ScpA undergoes rapid autoproteolysis, but this process also leads to rapid degradation of the protease (275).

Despite limited primary sequence identity, crystal structures of both staphopains demonstrated structural similarity to papain. Classical papain-like proteases contain two domains: the helical L-domain is composed of the N-terminal part of the protein, containing the catalytic cysteine, and the R-domain is composed of the C-terminal part of the protein, which folds into antiparallel β-sheets, forming a β-barrel-like structure that contains the catalytic histidine and aspartate (Fig. 11) (276–278). The location of the catalytic triad is conserved in both staphopains (276, 277).

Although ScpA and SspB share high structural similarity, subtle differences between the proteases confer different substrate specificities. ScpA cleaves elastins found in connective tissues, pulmonary surfactant protein A in the lungs, and the chemokine receptor CXCR2 on leukocytes (239, 279–281). Additionally, ScpA promotes vascular leakage by activating the plasma kallikerin/kinin system, resulting in hypotension (281). The activity of ScpA in mediating vascular leakage is enhanced by SspB; however, SspB alone does not induce vascular leakage, demonstrating substrate specificity of the two proteases (281).

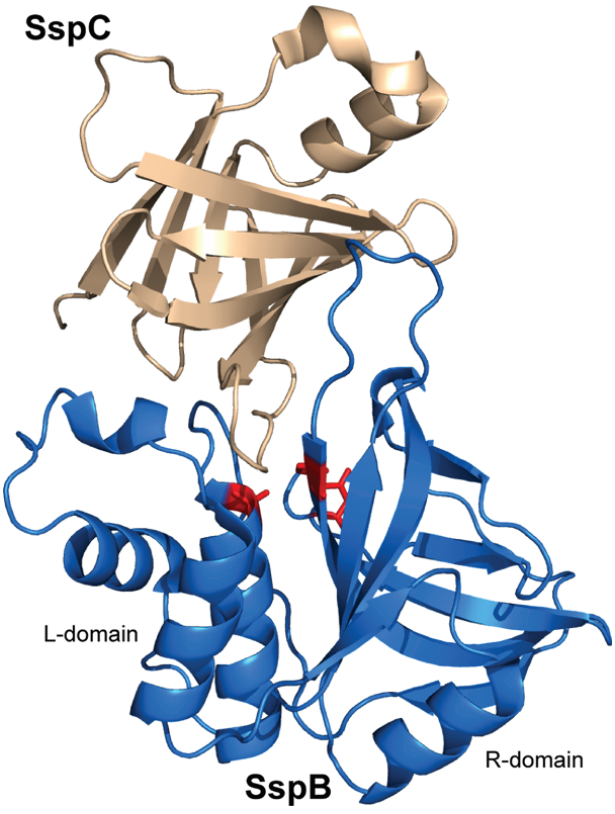


FIGURE 11 Staphopain-staphostatin complex (1PXV) (293). Staphopain SspB (blue) has two domains: the L-domain is helical, and the R-domain consists of β-strands that fold into a β-barrel-like structure. The catalytic site of SspB is highlighted in red. Staphopain SspC (beige) is a single domain protein composed of eight β-strands forming a single mixed β-barrel domain. SspC is a competitive inhibitor of SspB, directly blocking substrate access to the active site.

In contrast, SspB degrades the antimicrobial peptide LL-37, thereby promoting bacterial survival (282). SspB also cleaves CD11b and CD31, surface proteins that are important for the activation and survival, respectively, of phagocytes (283, 284). Thus, SspB prevents *S. aureus* from phagocytosis while diminishing the leukocytes' abilities to detect pathogens. Paradoxically, SspB is also a potent activator of chimerin, a chemoattractant for dendritic cells and macrophages (285). *S. aureus* thrives intracellularly in macrophages and dendritic cells (286, 287). Therefore, SspB may function to promote the intracellular lifestyle of *S. aureus* for persistent infections. In fact, *S. aureus* has been demonstrated to produce SspB, SspA, and Aur after neutrophil phagocytosis (254).

Both ScpA and SspB are implicated in modulating biofilm formation (288, 289). The expression of both staphopains is repressed during biofilm formation, and the production of staphopain results in the dispersal of biofilms (289).

In addition to promoting biofilm dispersal, staphopains have a direct effect on the host's connective tissues and coagulation system. Staphopains inactivate a number of host proteins, including α_1-proteinase inhibitor, collagen, and fibrinogens; however, SspB has higher activity in cleaving fibrinogen and collagen compared to ScpA (248, 290).

The activities of staphopains are inhibited by the cysteine protease inhibitor E-64; by heavy metals, such as Co^{2+}, Ag^{2+}, Hg^{+}, and Zn^{2+}; and by host-derived proteins, including α_2-macroglobulin in human plasma and the epithelial serpin, SCCA1 (239, 257, 291). Additionally, *S. aureus* produces inhibitors against the enzymes, known as staphostatins. Staphostatins are specific reversible inhibitors of staphopains. Staphostatin A can inhibit only staphopain A but not staphopain B (273). Similarly, staphostatin B inhibits only staphopain B (273). Both staphostatins are similar in size and structure. These small proteins (~13 kDa) each comprise eight β-strands that form a single mixed β-barrel domain (292). Staphostatin occupies the same binding site as substrate, and thus they are competitive inhibitors (Fig. 11) (276, 293). Staphostatins lack signal peptides, and thus they are proposed to inhibit intracellular staphopain activities prior to secretion of the proenzyme (273, 294).

Serine proteases: the serine protease-like proteins

Serine protease-like proteins A to F (SplA to SplF) are the newest group of secreted staphylococcal serine proteases identified. SplC (named ORF-2 in the study) was the first Spl identified from a screen of *S. aureus* antigens reactive to serum antibodies from endocarditis patients (295). Soon after, *splC* was discovered as part of the *spl* operon, encoding *splA to SplF* (296). This operon is located in the gene cluster νSaβ, which is present in over 60% of *S. aureus* genomes (296, 297).

Spls share 40 to 60% protein sequence identity, except for SplD and SplF, which have 95% sequence similarity with each other (Fig. 12) (296). The Spls are similar in size, ranging from 21 to 22 kDa. SplA to SplD have been characterized, and their structures were determined by X-ray crystallography (298–301). The crystal structures of the four Spls showed structural homology to the other staphylococcal

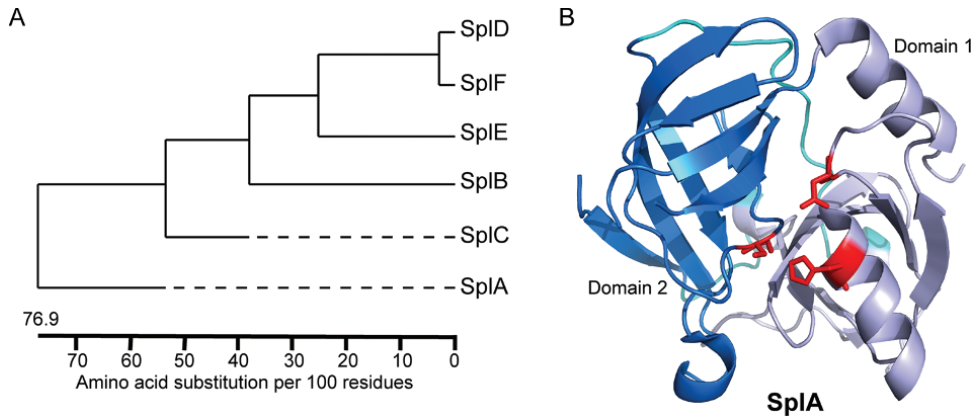


FIGURE 12 (A) Phylogenic tree of *S. aureus* Spls. The tree is constructed based on the mature protein sequences using the DNASTAR MegAlign ClustalW method for multiple sequence alignment. (B) Crystal structure of SplA (2W7S) (299). SplA has two domains connected by a linker (cyan). Domain 1 (light purple) consists of α-helices and β-strands, and domain 2 (blue) consists of of β-strands. Both domains fold into a β-barrel structure. The catalytic triad (red) is located at the center between the two domains.

serine proteases. Spls have a chymotrypsin-like fold, consisting of two β-barrel domains (Fig. 12) (298–301). The catalytic triad typical of serine proteases (His, Asp, Ser) is conserved and is present in the center between the two domains (296, 298–301).

Based on functional studies of the Spls, the precise cleavage of the signal peptide is critical for protease activity. An additional two amino acids (such as those resulting from a thrombin cleavage) in the N-terminus of SplA, SplB, and SplC are enough to render the enzymes inactive (296, 298, 299). Therefore, the signal peptides of Spls serve dual functions: (i) to direct the protein secretion and (ii) to serve as propeptides to prevent enzyme activation prior to secretion.

SplA, SplB, and SplD have extremely narrow substrate specificities, requiring the recognition of substrate consensus sequences that are 4 to 5 amino acids in length (Table 3) (298–300). Mucin-16, an *O*-glycosylated transmembrane protein present in the ocular epithelia, is a substrate for SplA (302). Additionally, searches based on the substrate consensus sequences identified many olfactory receptors as potential Spl substrates, but they remain to be verified experimentally (298–300). Nevertheless, these searches suggest that Spls may be important for nasal colonization. SplD contributes to airway inflammation and asthma by promoting IgE production and Th2 responses (303, 304). The role of SplD and SplF in asthma is supported by the identification of these proteins in nasal polyp samples from asthma patients who were also *S. aureus* nasal carriers (303).

However, the role of Spls in *S. aureus* pathogenesis remains unclear. While murine pneumonia and intraperitoneal infection models using Δ*spl* mutants had no effect on host survival or bacterial burden, the absence of *spl* limited the dissemination of bacteria *in vivo* in a pneumonia model (296, 303). Proteomic analysis of wild-type *S. aureus* and Δ*spl* mutant demonstrated significant changes in many virulence factors important for adhesion and immune evasion; thus, Spls have a role in shaping the *S. aureus* proteome (302).

TABLE 3 Consensus cleavage sequence of Spls

SplA	Trp/Tyr – Leu – Tyr – Tyr – Ser
SplB	Trp – Glu – Leu – Gln
SplC	To be determined
SplD	Arg – Trp/Tyr – Pro/Leu – The/Leu/Ile/Val
SplE	To be determined
SplF	To be determined

Hyaluronidase

Hyaluronic acid (HA) is a linear polysaccharide composed of repeating units of *N*-acetylglucosamine and glucuronic acid linked by alternating β-1,3 and β-1,4 glycosidic bonds (305). HA is a critical component of extracellular matrices in vertebrates, providing homeostasis and structural integrity to cells and tissues; it is also important for immune regulation (306, 307). The enzymes that break down HA are collectively known as hyaluronate lyase or hyaluronidase.

In nature, hyaluronidases can be found in vertebrates, invertebrates, and bacteria. Hyaluronidases found in vertebrates and invertebrates break down HA to tetrasaccharides (308). In contrast, bacterial hyaluronidases act as endo-*N*-acetylhexoaminidases and cleave the β-1,4 linkage in a process known as β-elimination, breaking down HA to unsaturated disaccharides (308).

S. aureus and *S. hyicus* are the only staphylococci known to produce hyaluronidase (309, 310). The activity of staphylococcal hyaluronidase was initially reported by Duran-Reynals in 1933 as a "spreading factor" that increased lesion sizes in a rabbit skin infection model (311). Subsequently, this spreading factor was identified by Chain and Duthie in 1940 as hyaluronidase (312). In 1995, the gene encoding for staphylococcal hyaluronidase, *hysA*, was eventually cloned and the corresponding protein purified (313).

As a spreading factor, hyaluronidase is implicated in the dissemination of bacteria through breaking down HA in extracellular matrices and biofilms. The skin and the lungs are two locations where extracellular matrices are abundant. Deletion of *hysA* resulted in reduced skin and lung pathology and lowered bacterial burden in skin and lung infection models, respectively (314, 315). Deletion of *hysA* was also demonstrated to cause increased biofilm formation and reduced bacterial dissemination (316).

Lipases

Phospholipases

S. aureus can produce two phospholipases: β-toxin and phosphatidylinositol-specific phospholipase C (PI-PLC). β-toxin is a neutral sphingomyelinase with hemolytic and cytolytic activities, discussed in the exotoxin section, above. The other staphylococcal phospholipase, PI-PLC was discovered in the 1960s in *S. aureus* culture supernatants where PI-PLC hydrolyzed phosphatidylinositol (PI) to diglyceride and inositol phosphate (317, 318). Today, *S. aureus* remains the only staphylococcus known to produce PI-PLC (319). *S. aureus* membrane does not contain PI, and thus *S. aureus* is thought to have acquired PI-PLC to adapt to the host environment (320).

Like other bacterial PI-PLCs, the staphylococcal PI-PLC has an imperfect $(\beta\alpha)_8$-barrel structure (also known as the TIM barrel) (321). The active site of PI-PLC is conserved and is located at the C-terminal end of the β-strands that form the β-barrel (321). The elucidation of the staphylococcal PI-PLC crystal structure provided explanations for many of PI-PLC's biochemical properties (321). PI-PLC is reported to have an optimum pH between 5.5 and 6.0 (319). This property can be explained by the unrestricted substrate access to the active site under acidic conditions. In contrast, accessibility of the substrate is restricted under basic conditions (321). PI-PLC is inactivated by NaCl, $HgCl_2$, and Cu_2SO_4 (319). The salt sensitivity of PI-PLC can be explained by the high electropositivity of the barrel rim region and the active site (321). The presence of phosphocholine enhances the activity of PI-PLC (321). Structural analysis of PI-PLC suggests that the presence of phosphocholine enables transient dimerization of two PI-PLC monomers, resulting in the enhancement of enzyme activity (322).

Bacterial PI-PLC hydrolyses PI in two steps: first, PI is hydrolyzed to diacylglycerol and the intermediate product myo-inositol 1,2-cyclic phosphate (cIP). This is followed by a second slower hydrolysis of cIP to myo-inositol 1-phosphate (323, 324). Diacylglycerol is an important secondary messenger for activating intracellular pathways in mammalian cells for growth and survival (325). PI-PLC can also release glycosyl-phosphatidylinositol-anchored proteins on the cell membrane (319). Two such proteins are C8 binding protein and the decay-accelerating factor (326, 327). Both proteins are complement regulators that are normally present on host cells to restrict complement activation on self (328, 329). Recently, PI-PLC was demonstrated to promote survival of *S. aureus* in human blood and neutrophils (330).

Glycerol ester hydrolases (lipases)

S. aureus has two lipases: *S. aureus* lipases 1 and 2 (SAL1 and SAL2). SAL1- and SAL2-encoding genes are sometimes annotated as *gehA and gehB*, respectively, for glycerol ester hydrolase (331–333). SAL1 has also been annotated as *lip1* in the literature (331). The two genes are encoded in disparate regions in the *S. aureus* genome, but they share protein sequence similarity with each other and with other lipases found in other staphylococcal species (332, 333).

The lipases are produced as pre-pro-enzymes (334). The pre-pro-enzyme is processed by signal peptidase I, which cleaves the signal peptide for secretion. The secreted pro-enzyme is cleaved by aureolysin to yield the mature lipase (331). However, cleavage of the propeptide is not required and has no effect on the enzymatic activity (331, 335). Utilizing chimeric lipases of *S. hyicus* expressed in *S. carnosus*, the lipase propeptides were found to be important for the translocation of the lipases to the extracellular milieu and for stabilizing the proteins to prevent degradation (336, 337).

Enzymatic activities of the lipases are conferred by the conserved catalytic triad, formed by serine, aspartate, and histidine (331, 332). Although sharing a similar catalytic mechanism, SAL1 and SAL2 differ biochemically and have different substrate preferences. SAL1 functions optimally at pH 6.0 and is stable under acidic conditions, but it is inactivated when the pH is above 10 (338). Biochemical and molecular analyses showed that Ca^{2+} stabilizes the structure of SAL1 and increases its activity (334, 338). Accordingly, chelators, such as EDTA and EGTA, inhibit SAL1 activity (338). SAL1 has a strong preference for short-chain triglycerides but cannot hydrolyze long-chain triglycerides (338).

In contrast, SAL2 functions optimally around pH 8.0 and is inactive under acidic conditions (339). The presence of Ca^{2+} does not enhance the activity of SAL2 (339). As such, chelators have minimal effects on activity. SAL2 prefers long-chain triglycerides as substrates (331). However, SAL2 has also been shown to hydrolyze short-chain triglycerides, monoglycerides, and diglycerides with lower efficiency and with no apparent positional specificity (331).

The conservation of lipases in staphylococcal species implies their evolutionary importance. However, the contribution of lipases during disease is unclear. *S. aureus* clinical isolates from deep tissue infections produce more lipases than isolates from superficial infections (340). Purified lipases cause aggregation of granulocytes and decrease phagocytosis at high concentrations (341, 342). During infection, SAL2 was shown to be important for biofilm formation and contributed to the virulence of *S. aureus* strain RN4220 in a murine intraperitoneal infection model (343). These observations suggest that lipases are involved in the overall virulence of *S. aureus* and promote bacterial survival in biofilms and abscesses. Paradoxically, lipase-mediated triglyceride hydrolysis liberates bactericidal free fatty acids, which can interfere with pathogenicity (344). For most lipase-producing strains, these bactericidal fatty acids can be detoxified by fatty acid-modifying enzymes (FAMEs).

Fatty acid-modifying enzyme

The FAME was first described in 1992 by Mortensen et al., who observed that *S. aureus* culture filtrates inhibited the bactericidal activities of host lipids in abscesses (345). Since the initial discovery, FAME activity has been well documented in many staphylococcal species (346, 347). Approximately 80% of *S. aureus* and *S. epidermidis* organisms produce this enzyme (347, 348). Despite its prevalence, the corresponding gene for FAME is not known and the protein has not yet been identified.

FAME promotes staphylococcal survival by esterifying the bactericidal free lipids with an alcohol substrate to form alcohol esters. Although FAME can esterify free lipids with methanol, ethanol, 1-propanol, 2-propanol, and 1-butanol, it prefers cholesterol, which is highly abundant in abscesses (345). Saturated and unsaturated fatty acids with 15 to 19 carbons are efficiently esterified by FAME; however, esterification is also observed for fatty acid chains of between 11 and 24 carbons (349). The optimal pH of the enzyme ranges between 5.0 and 5.5 and has an optimal temperature of about 40°C (345). Enzyme activity is inhibited by di- and triglycerides with unsaturated fatty acid side chains (349).

In abscesses, lipases and FAME are thought to complement each other to enhance staphylococcal survival (350). While lipases break down triglycerides that inhibit FAME activities, FAME processes the free fatty acids liberated

by lipases to protect the staphylococci. This hypothesis is corroborated by the observation that most *S. aureus* strains that carry genes encoding lipases have FAME activities and that they are correlated with the invasiveness of the bacteria *in vivo* (345, 348).

CONCLUSION

S. aureus devotes a significant amount of energy to the production of virulence factors to protect the bacteria from host immune surveillance and to promote bacterial survival in hostile environments (Fig. 13). The importance of these virulence factors during infection has been demonstrated extensively in various *ex vivo* and *in vivo* infection models. Pathogenic *S. aureus* is usually present in hostile host environments with limited resources; thus, it follows that the production of many virulence factors that serve the same purpose can be a waste of limited resources and be disadvantageous for survival. In contrast, this redundancy can ensure protection of the bacterium if one of the virulence factors is rendered ineffective. Alternatively, the bacterium may have acquired these seemingly redundant virulence factors during its evolution to better adapt to different types of infections or colonization sites.

Many of the exotoxins and secreted enzymes discussed in this article share structural and functional similarities. However, closer examination of these proteins reveals subtle differences that have functional significance. Minor differences in the PFTs lead to the cytolysis of various cell types that are critical for immune defense. Each of the SAgs target different Vβ TCRs, resulting in broad suppression of the T cell repertoire. Proteases, such as the serine proteases, which have structural homology have disparate substrate specificities. Other exotoxins and enzymes have similar functions but differ in when and where they are produced during growth and pathogenesis, suggesting that the complex and seemingly redundant virulence factor repertoire is critical for the success of *S. aureus* as a versatile pathogen. With the rise in antibiotic resistance in microbes, including *S. aureus*, there is an urgent need to develop novel therapeutics and vaccines to combat this deadly pathogen. Understanding the roles these important virulence factors have during diseases can provide the knowledge necessary for designing better therapeutics and identifying vaccine targets.

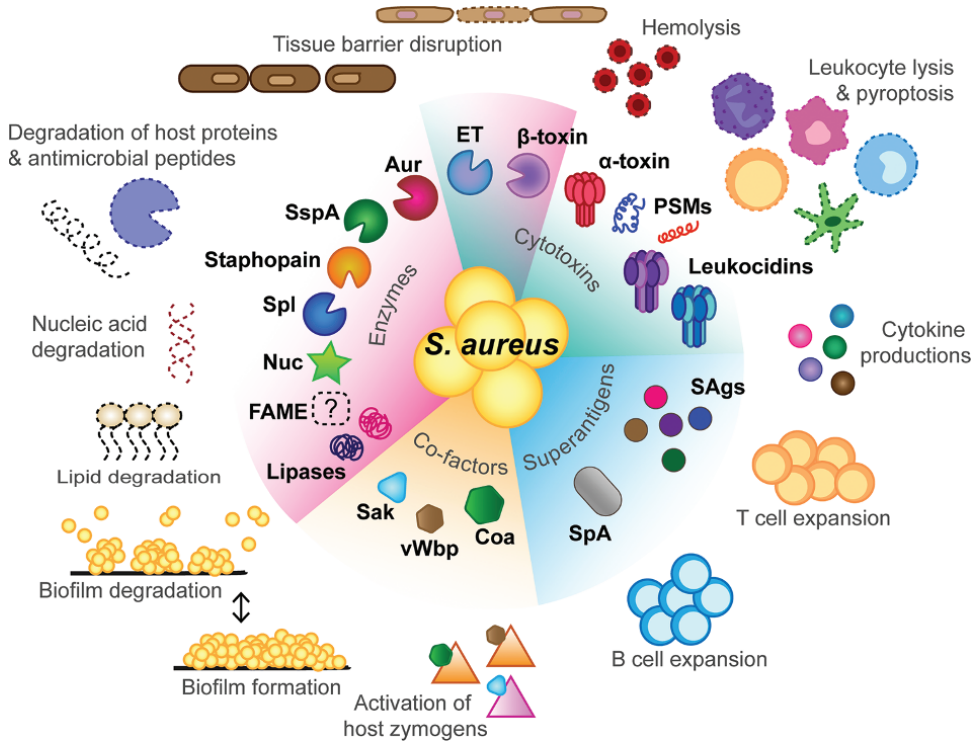


FIGURE 13 *S. aureus* secretes many different toxins and enzymes. Superantigens are proteins that have high mitogenic properties, causing T and B cell expansions that result in clonal deletion and massive cytokine production. Cytotoxins, such as α-toxin and the leukocidins, cause cytokine production, hemolysis, and leukocyte cell death through targeting specific cell surface receptors. The amphiphilic PSM peptides mediate cytolysis by inserting into the lipid bilayer of cell membranes. Enzymes, such as β-toxin and the ETs, cause cytotoxicity on mammalian cells, resulting in cell death, inflammation, and tissue barrier disruptions. Other enzymes, including various proteases and nucleases, mediate host protein degradations, thwarting many important host immune surveillance and defense molecules. These enzymes can also act on self-proteins to degrade biofilms for bacterial dissemination. Lipases and FAME work synergistically to degrade lipids in the environment for nutrients. Cofactors, including Coa, vWbp, and Sak, bind and activate host zymogens in the coagulation system to mediate clot formation and dissolution. Altogether, these toxins and enzymes provide critical nutrients (i.e., iron and carbon) that are important for the growth and survival of the bacteria. Importantly, they target various aspects of host immune defenses, thus contributing to the overall virulence of *S. aureus* during infections.

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Regulation of *Staphylococcus aureus* Virulence

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41

INTRODUCTION

Staphylococcus aureus is a Gram-positive commensal bacterium and opportunistic pathogen. The main sites of colonization are the skin and mucous membranes, and approximately 30% of the healthy adult population are colonized by *S. aureus* (1). Although *S. aureus* is primarily a commensal microbe, it has the potential to cause a wide range of diseases that can vary considerably in severity. The most common problems are skin infections, and some of the most severe are bloodstream infections, endocarditis, osteomyelitis, and necrotizing fasciitis (2). To survive and adapt to different environmental niches, *S. aureus* has evolved an intricate regulatory network to control virulence factor production in both a temporal and host location manner (3). The regulatory machinery and virulence factors are known as accessory genes, since they are not essential for normal growth. These accessory factors are used to establish dominance in the host and contribute to the pathogenicity of *S. aureus*, and they include cell surface components and proteins directly released into the extracellular environment. The functions of these molecules include adherence to host cells, evasion of host defenses, nutrient degradation, and acquisition. These accessory genetic elements are encoded directly on the chromosome and on mobile elements that include phages, plasmids, and pathogenicity islands.

S. aureus is a bacterial species with a conserved core genome (4), and its evolution is mainly driven through mutation and horizontal gene transfer. Mobile genetic elements, such as integrated bacteriophages (prophages), are one of the most common contributors to *S. aureus* strain-to-strain variation. Due to its low level of natural competence, bacteriophage transduction is a frequent mode of DNA transfer between *S. aureus* strains (5). Panton-Valentine leucocidin and the immune evasion cluster are two examples of virulence factors found on prophages (6, 7), with the latter being on a bacteriophage integrated into the *hlb* gene (8). Other important bacteriophage-encoded virulence factors include exfoliative toxin A (9, 10), cell wall-anchored virulence factor SasX (11), staphylococcal inhibitor of complement (*scn*) (12), staphylokinase (*sak*), chemotaxis inhibitory protein (*chp*) (13), and the enterotoxins encoded by *sea* (14), *selk2*, and *selp* (15). Staphylococcal pathogenicity islands are another mobile genetic element that can encode pyrogenic toxins called superantigens. The superantigen genes encoded on staphylococcal pathogenicity islands include toxic shock toxin (*tsst*) (16), enterotoxin B (*seb*), and enterotoxin-like protein Q (*selq*) (15). Plasmids play an important role in the acquisition of antibiotic resistance of *S. aureus*, but there are also a few examples documenting their importance for the acquisition of virulence factors. To date, it has been shown that several toxins can be plasmid encoded, including exfoliative toxin B (17–19), enterotoxin D (20), the enterotoxin-like protein SER (21), and enterotoxin-like protein J (22). The focus of this article is the regulation of these virulence factors in *S. aureus*.

REGULATION OF *S. AUREUS* VIRULENCE FACTORS

Within *S. aureus*, the regulation of the virulence factors is subject to a complex network that integrates host- and environment-derived cues into a coordinated response. Two-component systems (TCSs) are one mechanism to perceive environmental changes and transform them into a regulatory program. For a typical TCS, an external signal activates the membrane-associated histidine kinase, leading to its autophosphorylation and subsequent phosphorylation of the response regulator. Once phosphorylated, the response regulator can bind to a specific DNA sequence motif, resulting in the alteration of target gene expression. Most strains of *S. aureus* encode 16 different TCSs (23, 24); one of these is essential (WalKR), and the other 15 have been inactivated in multiple strains (25, 26). Some TCSs, such as *agrAC*, *saeRS*, and *arlRS*, are linked to *S. aureus* virulence and regulate a large number of host-impacting secreted proteins. The best studied of these regulatory systems is the accessory gene regulator (*agr*), which was first described in 1986 and encodes a quorum-sensing system that acts as a master virulence regulator (27). Coupled with these TCSs, *S. aureus* survives in the host environment using a suite of important cytoplasmic regulators (23). Most important among them are the SarA protein family of transcriptional regulators (SarA, Rot, MgrA, etc.) and the alternative sigma factors (SigB and SigH). The goal of this article is to provide an overview of these regulatory systems (summarized in Table 1), and the interplay and critical features of each system will be covered.

THE AGR QUORUM-SENSING SYSTEM

Architecture of the *agr* Quorum-Sensing System

The gene cluster that encodes the peptide quorum-sensing system in *S. aureus* is called the accessory gene regulator (*agr*)

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TABLE 1 Major virulence regulatory systems of *S. aureus*

Regulator system	Role	*In vivo*	References
agr	Cell-to-cell communication (quorum sensing) with AIPs as signal; *agr* activation leads to expression of exo-toxins and exo-enzymes	Required for virulence in animal models of skin infection, pneumonia, and endocarditis	27, 30, 50–54, 56, 59, 62, 87, 89–92, 94
SaeRS	Induction of exo-protein production, including many virulence factors	Required for virulence in animal models of skin infection and pneumonia	97, 98, 105–108
SrrAB	Oxygen-responsive TCS; induction of *plc* and *ica* expression; repression of *agr*, TSST-1, and *spa*	Required for defense against neutrophils	71, 80, 113, 118
ArlRS	Autolysis and cell surface TCS; induction of MgrA expression and repression of *agr* and autolysis	Required for virulence in animal models of skin infection and endocarditis	121–123, 125, 202
SarA	Cytoplasmic regulator; induction of exo-proteins and repression of *spa*	Required for virulence in animal models of biofilm infection	135, 137, 139, 161
Rot	Cytoplasmic regulator of toxins and extracellular proteases; *agr* activation prevents Rot translation	Mutation of *rot* restores virulence in *agr*-null background in rabbit endocarditis model	61, 155–160
MgrA	Cytoplasmic regulator; induction of efflux pumps and capsule expression; repression of surface proteins	Required for virulence in animal models of skin infection and endocarditis	84, 163, 164
SigB	Stationary phase sigma factor; inhibits *agr* activity	Important for the establishment of chronic infection in rat lung model	134, 183–188, 190, 203

(Fig. 1). The signal sensed by the *agr* system is an autoinducing peptide (AIP), which can be 7 to 9 amino acids in length and contains a five-membered thiolactone ring between the C-terminal end and a conserved cysteine residue (3, 28, 29). The AIP signal accumulates in the extracellular environment, and once it reaches a critical concentration, usually at a "quorum" of cells in the population, the system is activated. *S. aureus* employs the *agr* system to adapt to changing environmental conditions during growth and to regulate virulence (27, 29, 30). The *agr* system consists of two adjacent transcripts, called RNAII and RNAIII, whose expressions are driven by the P2 and P3 promoters, respectively (31). The RNAII transcript is an operon of four genes, *agrBDCA*, that encode the machinery of the quorum-sensing system, and the RNAIII transcript is the major effector and regulates the expression of most *agr*-dependent target genes. As shown in Fig. 1, AgrD is the ribosomal peptide precursor of AIP (32) and is proteolytically processed by AgrB, an integral membrane-bound peptidase (33, 34). AgrB-mediated cleavage of AgrD results in the formation of an enzyme-bound thiolactone intermediate (33–35), and through an unclear mechanism this structure is transported across the membrane. The type I signal peptidase SpsB performs the final processing step to release the mature AIP into the extracellular environment (36). Once outside the cell, AIP is then sensed by AgrC, the membrane-bound histidine sensor kinase of the AgrCA TCS. The binding of AIP to the AgrC receptor leads to histidine autophosphorylation (37), and this signal is relayed to the aspartate receiver on the response regulator AgrA (38). The phosphorylated AgrA can then bind to the P2 and P3 promoters to drive expression of RNAII and RNAIII, respectively (39). Expression of RNAII, which encodes for all components of the *agr* system, effectively leads to a positive feedback loop. This autocatalytic regulation is a hallmark of quorum-sensing systems and enables *S. aureus* to readily produce exoproteins, even though growth is slowed down (38).

AIP Types

To date, four classes of AIP structures are known to exist in *S. aureus*. Their length ranges from 7 amino acids (type III) to 8 amino acids (type I and IV) to 9 amino acids (type II) (Fig. 2). AIP types I and IV are the most conserved, differing in only one amino acid, and as a result, they function interchangeably (40). Early studies of *agr* revealed that the AIPs can cross-inhibit the function of an AgrC receptor from another *S. aureus* strain in a mechanism termed "*agr* interference" (41). This cross-talk gave rise to three functional AIP groups, each of which can effectively cross-inhibit other *agr* systems within *S. aureus* (41), resulting in an intriguing mechanism of intraspecies signaling (Fig. 2). The divergence among the four AIP types stems from a highly variable region that spans the C-terminus of *agrB*, the whole *agrD* gene, and the N-terminal region of *agrC* (42). These differences also explain the variability of the AgrC protein and its specificity for its cognate AIP (41, 42). As mentioned above, the hypervariable region responsible for the four AIP types also reaches into the coding sequence of *agrB*, which suggests that AgrD processing might also be type specific. Indeed, it was demonstrated that AgrB of the AIP type II system can only process its cognate AgrD, while AgrB of the AIP type I and type III systems is not able to process the type II AgrD (41, 43).

Agr Regulon

AgrA directly regulates the expression of RNAII and RNAIII from the P2 and P3 promoters, respectively (Fig. 1). This was long thought to be the only relevant regulatory role of AgrA, until a study compared gene regulation between the wild-type, an *agrA* mutant, and an RNAIII mutant (44). It was discovered that AgrA also regulated the phenol-soluble modulin (PSM) genes, which have received considerable attention in recent years for their involvement in *S. aureus* virulence (45–47). Electromobility shift assays demonstrated that AgrA is capable of directly binding the promoters of the alpha- and beta-PSM-encoding operons (44). The main effector of the *agr* quorum-sensing system is RNAIII, a regulatory RNA that also encodes the delta-hemolysin gene (*hld*) (48), also known as delta-toxin. Interestingly, the translation of *hld* is delayed by 1 hour after RNAIII transcription by an unknown mechanism (49). Among the genes upregulated by RNAIII are many well-characterized

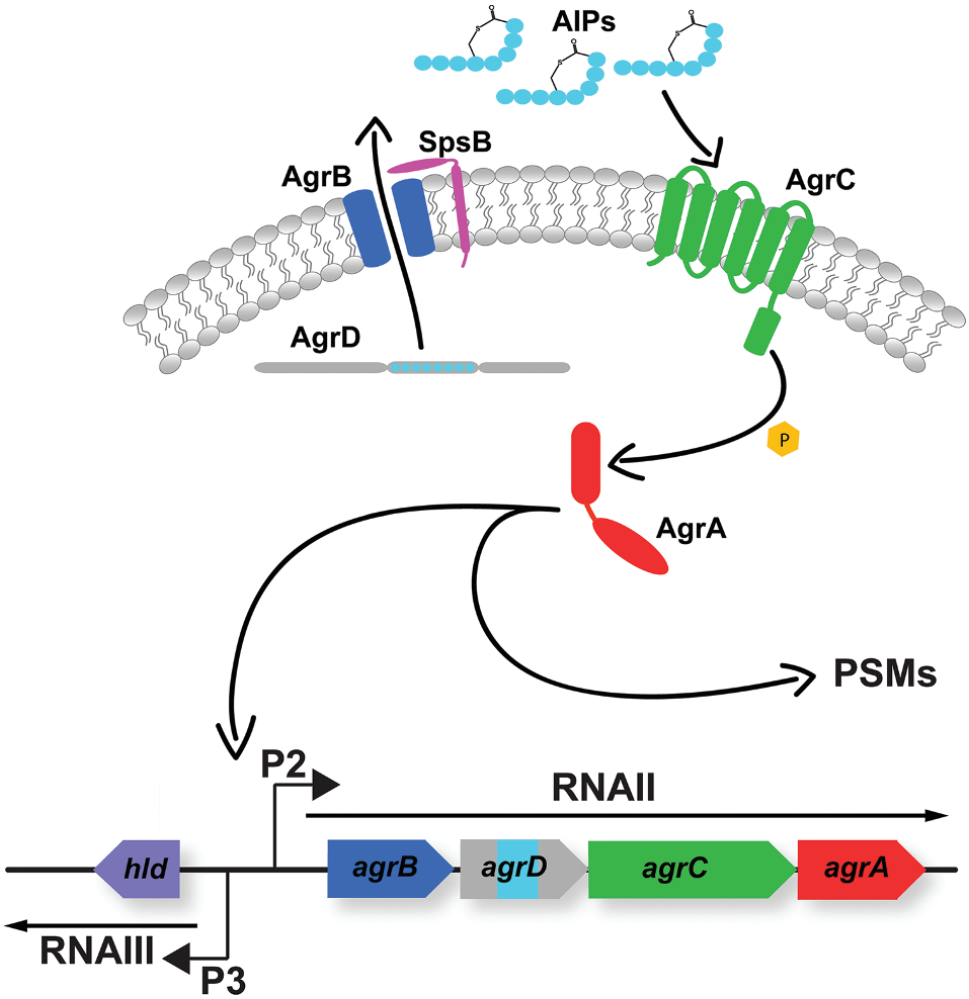


FIGURE 1 Schematic of the molecular organization, signal biosynthesis, and transduction cascade of the *agr* quorum-sensing system. The autoinducing peptide (AIP) signal is encoded within the AgrD peptide. AgrD is processed and transported into the environment by AgrB with the aid of signal peptidase SpsB. When the extracellular AIP concentration reaches a critical level, the signal is sensed by the histidine kinase AgrC, which undergoes autophosphorylation. Then the phosphate is relayed to AgrA, which in turn can bind the P2 and P3 promoters, driving expression of the RNAII and RNAIII transcripts, respectively. The RNAII transcript harbors the *agrBDCA* operon, encoding the primary machinery for AIP biosynthesis and detection. RNAIII is the main effector molecule of the *agr* system and drives expression of downstream target genes. Phosphorylated AgrA also binds the promoters for the phenol-soluble modulin (PSM) genes, leading to their expression.

virulence factors, including alpha-toxin (Hla), serine proteases (SplA-F, SspA), cysteine proteases (ScpA, SspB), gamma-hemolysin (Hlg), and lipase (Geh) (30, 50, 51). Alpha-toxin is one of the most prominent regulated toxins and is an important virulence determinant in skin and soft tissue infections (52, 53), pneumonia (54), and endovascular infections (55). In contrast, some surface proteins, including protein A (Spa), cell wall secretory protein (IsaA), and surface receptors (MnhA, MnhF, and MnhG), are downregulated by RNAIII (30, 50, 51). Among these surface proteins, protein A stands out due to its dominant role in pathogenicity in several types of *S. aureus* infections, including pneumonia (54, 56), bloodstream infections (57), and septic arthritis (58).

RNAIII enacts its regulatory role either directly on the transcriptional level by modulating transcription initiation or at the posttranscriptional level by interacting with the target gene transcript (48). RNAIII is a large regulatory RNA that has a complex secondary structure with several C-rich hairpins, which are important for the interaction of RNAIII and its target mRNAs (59). One well-studied example is the positive regulation of alpha-toxin (*hla*) by RNAIII. The *hla* mRNA normally forms a hairpin loop that prevents the ribosome from accessing the ribosome-binding site. RNAIII can bind to the *hla* mRNA, relieving the hairpin loop structure and allowing the ribosome to recognize the binding site for translation initiation (51). On some occasions, posttranscriptional control by RNAIII results in translational inhibition, rather than promotion. For example, RNAIII exerts its regulatory activity on *spa* (protein A) gene expression at the posttranscriptional level by two distinct mechanisms. The first mechanism is direct RNA-RNA interaction with the *spa* mRNA, which inhibits access to the ribosome-binding site, as well as the *spa* start codon, pre-

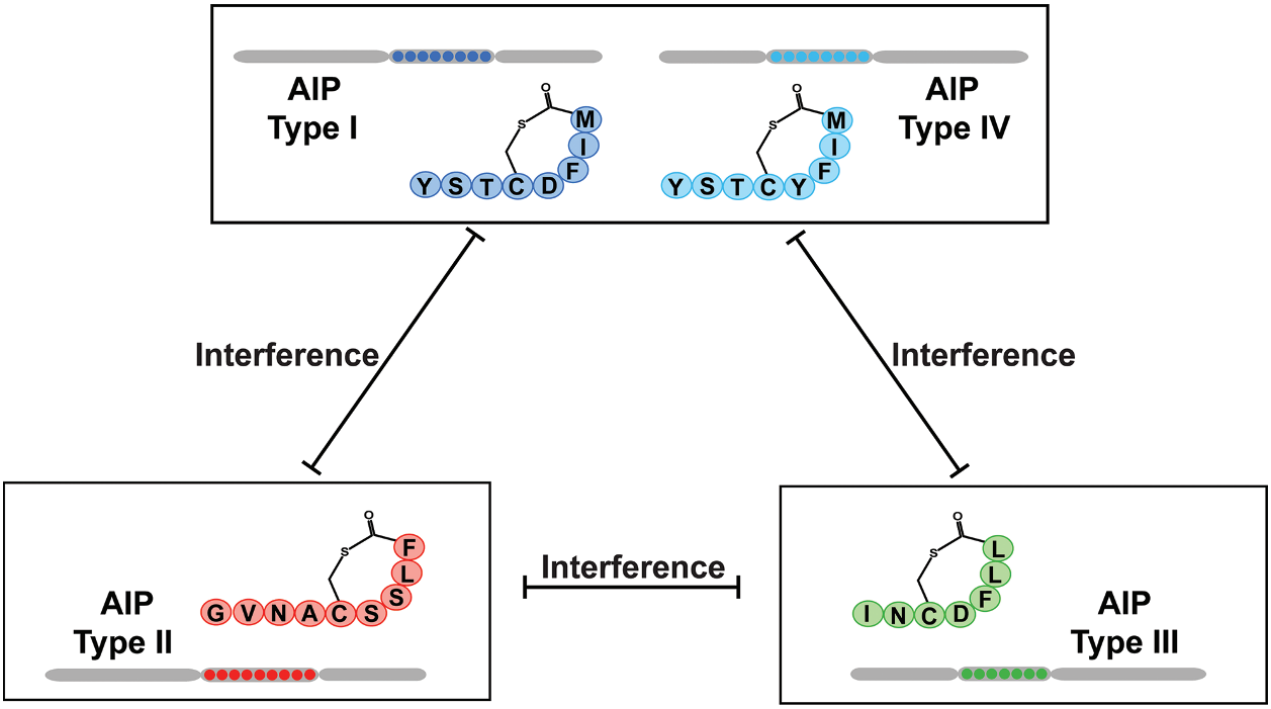


FIGURE 2 The autoinducing peptides (AIPs) and *agr* interference within *S. aureus* strains. Every *S. aureus* strain has a single *agr* system that can produce one of four different AIP signal structures. Each of the AIPs has a five-residue cyclic thiolactone ring, but the amino acids within the ring and the N-terminal extension are variable. The type I and IV AIPs differ by only one amino acid and can function interchangeably, while the type II and III AIPs are more divergent. Interference is observed between the three groups of AIPs as shown. In each case, the cognate AIP signal from a producing strain cross-inhibits the AgrC receptor, and in turn inhibits *agr* function, on an *S. aureus* strain representing a different *agr* group.

venting the initiation of translation (60). The complex formed between RNAIII and *spa* mRNA is also a substrate for RNAseIII. Thus, RNAIII can also inhibit protein A production by enhancing the degradation of *spa* mRNA (60). Although RNAIII is considered the major effector of the *agr* system, regulation of many genes is achieved indirectly through the global regulator Rot (61). Rot is part of the SarA family of cytoplasmic regulators, and Rot's regulatory mechanism is detailed later in this article. Similar to the protein A mechanism, RNAIII controls Rot expression by blocking the ribosome-binding site (59, 62, 63), facilitating *rot* mRNA cleavage by RNAseIII directly at this site (59, 63). When the *agr* system activates, RNAIII levels are high, which prevents the translation of Rot and alters the expression of downstream target genes (64).

Impact of Environmental and Host Stimuli on *agr* Function

Because the *agr* system plays a central role in pathogenesis, *S. aureus* has evolved many strategies to fine-tune the expression of *agr*, allowing it to adapt quickly to changing conditions. These *agr*-modulating factors include environmental cues, other *S. aureus* regulatory proteins, and host-derived stimuli. The following sections outline some of these factors and the mechanisms through which they influence the *agr* quorum-sensing system.

Environmental cues

One of the first abiotic factors described to influence the *agr* system is pH. It was shown that acidic pH inhibits *agr*, which can be achieved either through the catabolism of glucose (65) or by directly lowering the pH of the growth medium in the absence of glucose (66). Interestingly, alkaline pH also represses RNAIII transcription, and it is evident that maximum *agr* activity is observed near neutral pH (67). Reactive oxygen species can also influence the *agr* activity. Neutrophils are some of the first responders to *S. aureus* infection, and they bombard the bacteria with oxidative killing mechanisms. Myeloperoxidase transforms H_2O_2 and chloride to HOCl (bleach), which modulates the *agr* system by oxidizing the methionine side chain of AIP-I and -IV (Fig. 2), rendering the AIPs ineffective (68). Oxidative stress caused by reactive oxygen species can also induce disulfide bond formation in AgrA, leaving it incapable of binding its target promoters (69). In addition, indirect regulation of *agr* activity by reactive oxygen species is achieved by altering the activity of the AirSR (70) and the SrrAB TCS (71), which is discussed further elsewhere in this article.

Host-derived factors

Host serum was one of the first identified inhibitors of the *S. aureus agr* system (72). In follow-up studies, it was found that apolipoprotein B within serum is primarily responsible for this phenotype (73, 74). Apolipoprotein B sequesters AIP signal and prevents *agr* activation. In the host, *S. aureus* obtains iron from hemoglobin (75), but hemoglobin in turn can impact *agr* function. Initially, this was observed using the hemoglobin α and β chains, which inhibited exotoxin production and increased protein A levels, suggesting that the *agr* system was repressed (76). Later studies demonstrated that, indeed, hemoglobin downregulates the *agr* P3 promoter activity and that this can happen during colonization or infection (77).

Regulatory proteins

Many studies have defined how *S. aureus* regulators modulate *agr* activity, and a comprehensive list of these regulators impacting *agr* can be found in Table 2. Of these regulators, CodY, MgrA, and SarA are covered in more detail here. CodY is a transcription factor present in several low-G+C Gram-positive bacteria, and it senses the intracellular concentration of branched-chain amino acids and GTP. It regulates the cell's response to nutrient limitation and metabolic stress (78). In a *codY* mutant, expression of the *agr* locus is upregulated (79). The *agr* locus is overexpressed in the exponential growth phase in a *codY* mutant, which correlates with accumulation of AIP and the dependency on phosphorylated AgrC and AgrA. CodY only weakly binds to the P2 and P3 promoter regions, and instead binds to a region within *agr*C that contains the P1 promoter (79, 80). Although the P1 promoter is weak and only drives *agr*A expression, the binding of CodY to this region could explain the negative regulation of *agr* function. In contrast to CodY, MgrA is a positive regulator of *agr* expression and belongs to the SarA-family of regulators. Two independent studies have shown that an *mgr*A mutation results in a decrease of RNAII and RNAIII transcripts (81) or even complete loss of RNAIII production and P3 promoter activity (82). However, a subsequent microarray study did not observe an impact of MgrA on RNAIII production (83), and a more recent RNA sequencing study had the same observation (84). These studies used different strains, and therefore the regulation of *agr* by MgrA seems to be highly strain specific. Finally, SarA is a positive regulator of *agr* activity and one of the best-studied virulence regulators in *S. aureus*. SarA exerts its positive regulation over *agr* by binding to the P2 and P3 promoter regions and subsequently activating transcription of RNAII and RNAIII (85, 86). A comprehensive description of SarA's role in *S. aureus* virulence regulation is given in a later section of this article.

Importance of *agr* During Infection

Numerous studies have shown that the *agr* system is required for different animal models of infection. Strains with *agr* mutations show smaller lesion sizes and reduced bacterial load in murine skin infection models compared to wild-type strains (28, 87–90). These *agr* mutants are also attenuated in acute murine pneumonia models (54, 56, 89, 91), and they were shown to have decreased rates of infective endocarditis in rabbit models (92). Additionally, *agr* mutants display a decreased ability to establish osteomyelitis in a rabbit model (93). Using a P3-*lux* bioluminescence reporter strain, the dynamics of *agr* activation have been monitored in real time during infection (90, 94, 95), and there is evidence that *agr* activation occurs in repeating waves *in vivo* (94).

TWO-COMPONENT SYSTEMS

The SaeRS TCS

First described in 1994, the *sae* (*S. aureus* exoprotein expression) locus was initially characterized as a regulator of exoprotein production (96, 97). The SaeRS TCS consists of the histidine kinase SaeS and the SaeR response regulator (96–98). The operon encoding the SaeRS system contains four genes: *saeP*, *saeQ*, *saeR*, and *saeS* (Fig. 3). Expression of *saeR* and *saeS* is driven by the constitutive P3 promoter, which is located within the coding region of *saeQ* (99, 100). The P1 promoter is autoinduced by SaeRS and drives the expression of all four genes (101). Although expression of the SaeRS system from the P1 promoter is much stronger than expression driven by the P3 promoter, the overall SaeRS activity is not elevated. This is explained by the fact that SaePQ forms a complex with SaeS, leading to enhanced phosphatase activity (102) and resulting in reduced signal transduction between SaeS and SaeR. Topological studies of the SaeS sensor kinase revealed a 9-amino acid extracellular loop that plays an important role in signal transduction. Three amino acids in the extracellular loop (M31, W32, and F33) were found to be important for SaeS activity. Interestingly, site-directed mutation in M31 and F33 abolished the ability of SaeS to respond to α-defensins (HNP-1) (103), which activates the SaeRS system at subinhibitory concentrations (104). Interestingly, a mutation of SaeS W32 still allowed a response to HNP-1. Exposure to human neutrophils showed that changing the aromatic amino acids (W32 and F33) in the anchor region of SaeRS did not inhibit its activity, while mutating M31 rendered SaeS nonresponsive to neutrophils (103). In general, *S. aureus* wild-type strains survive better after neutrophil uptake compared with an *saeRS* mutant strain (105), indicating a crucial role for SaeRS in the sensing of and defense against neutrophils.

TABLE 2 Regulators impacting *agr* system function

Regulator	Impact on *agr*	Function	Reference(s)
ArlRS	Negative	Represses *agr* P2 and P3	121
CcpA	Positive	Activates *agr* P3	204
CodY	Negative	Repress *agr* P1 promoter in *agr*C	79, 80
MgrA	Positive	Activation of *agr* P3	82
Rsr	Negative	Represses *agr* P3	205
SarA	Positive	Activates *agr* P2 expression	136, 206
SarR	Negative	Represses *agr* P2 expression	136
SarT	Negative	Represses *agr* P3	147
SarU	Positive	Activation of *agr* P2 and P3	148
SarX	Negative	Represses *agr* P2 and P3	207
SarZ	Positive	Activates *agr* promoter	208
SrrAB	Negative	Represses *agr* P2 and P3	71, 117
σ^B	Negative	Represses *agr* P3	185, 209

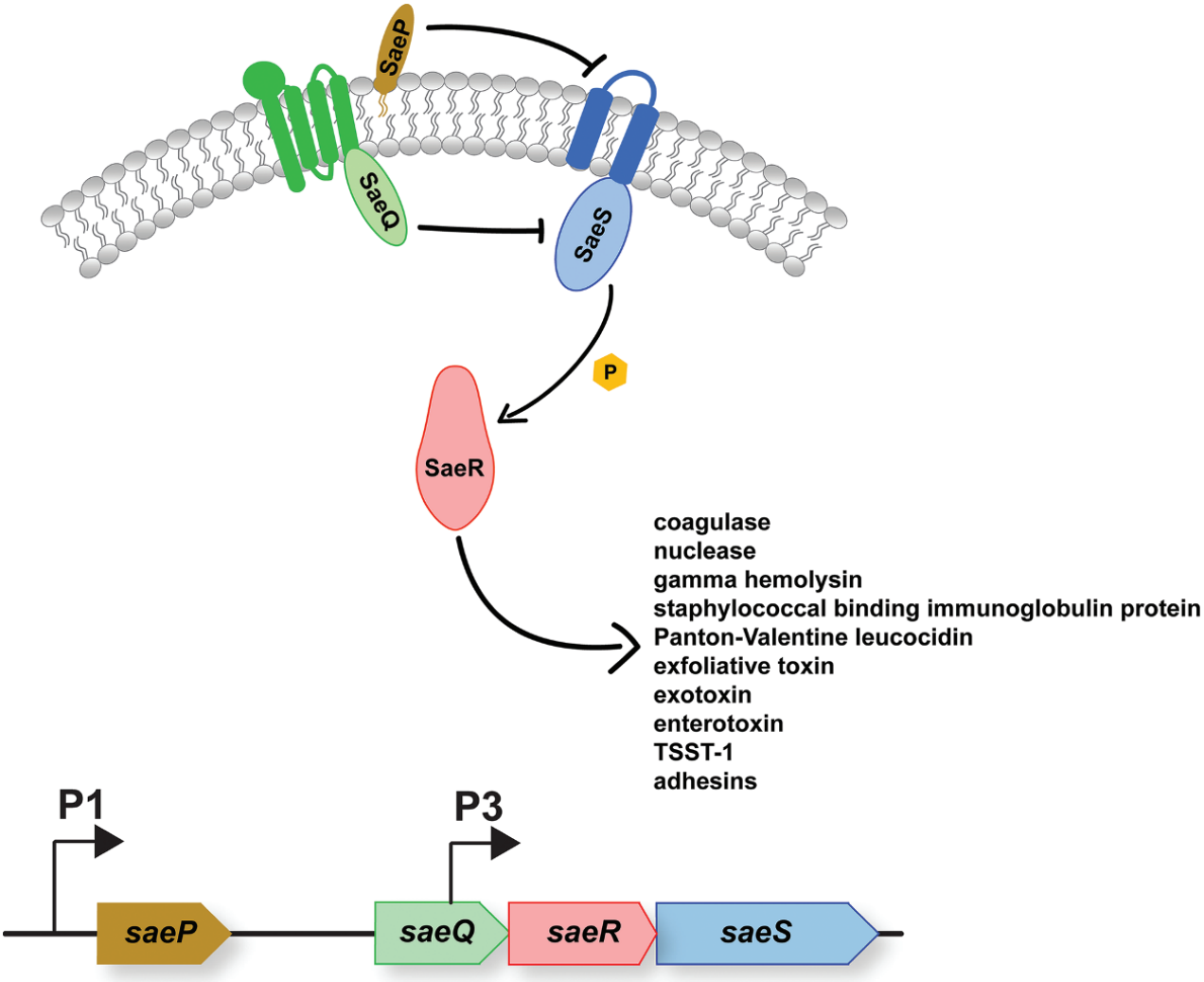


FIGURE 3 Schematic of the molecular organization and signal transduction of the SaeRS TCS. (Top) The histidine-kinase SaeS phosphorylates its cognate response regulator SaeR. Phosphorylated SaeR can then bind to the promoter region of target genes and induce expression of numerous virulence factors (listed). The phosphorelay from SaeS to SaeR is inhibited by the combined action of SaeP and SaeQ. (Bottom) The *sae* gene cluster consists of four genes: *saeP*, *saeQ*, *saeR*, and *saeS*. All four genes are transcribed from the P1 promoter. In addition, transcription of *saeS* and *saeR* is enhanced via the P3 promoter, which is located within the coding region of *saeQ*.

The SaeRS TCS drives the expression of several important exoproteins (Fig. 3), including coagulase, alpha-toxin, beta- and gamma-hemolysins, staphylococcal immunoglobulin-binding protein, Panton-Valentine leucocidin (LukGH), toxic shock syndrome toxin 1 (TSST-1), exfoliative toxin, and nuclease (97, 98, 105–107). The latter two were also shown to be SaeRS regulated *in vivo* during infection (107, 108). Across *S. aureus* strains, the sensor kinase SaeS has polymorphisms, and the three known variants are designated $SaeS^{P}$, $SaeS^{SK}$, and $SaeS^{SKT}$. The $SaeS^{P}$ variant, which is present in the *S. aureus* strain Newman, leads to SaeRS hyperactivity and higher transcription rates of numerous output genes, including *fnbA*, *coa*, *sib*, *efb*, *fib*, *eap*, and *sae*, while other target genes, such as *hla*, *hlb*, and *cap*, are not sensitive to the $SaeS^{P}$ variant (109). Besides its autoinducing regulation, the SaeRS system is regulated by environmental cues and host-specific signals. Low pH (5.5) and high salt concentration (1M NaCl) inhibit *sae* P1 promoter activity (66, 104). The inhibitory effect is relieved in an *saeP* mutant (110), suggesting that the SaeP lipoprotein contributes to this regulatory mechanism. Metals, specifically copper and zinc, interfere with the signal transduction from SaeS to SaeR by inhibiting the SaeS kinase activity. Interestingly, zinc-bound calprotectin, a neutrophil intracellular protein, protects the Sae system from metal-mediated inhibition through an indirect mechanism (111). In addition, the human skin-derived fatty acid *cis*-6-hexadecanoic acid was reported to inhibit the SaeRS system by an unknown mechanism (112).

The SrrAB TCS

The SrrAB (staphylococcal respiratory response) TCS, also known as SrhSR, was first described as a global regulator of virulence factor production under low-oxygen conditions (71, 113). The *srrAB*/*srhSR* locus encodes a histidine kinase SrrB (SrhS) and the paired response regulator SrrA (SrhR). The SrrAB TCS was shown to be important for resistance to

nitrosative stress (114). More recent studies have shown that SrrAB also confers H_2O_2 resistance during high dioxygen-dependent respiration, which is achieved by the positive regulation of the H_2O_2 resistance genes *kat*, *ahpC*, and *dps* (115). *S. aureus* Δ*srrAB* mutants are also attenuated in their fermentative biofilm-forming capacity. This phenotype is in part due to the decreased abundance of autolysin (*atlA*) transcript in a Δ*srrAB* mutant strain, resulting in impaired autolysis and extracellular DNA accumulation under fermentative conditions (116).

In regard to other global regulatory effects, the *agr* system, protein A (*spa*), and TSST-1 are repressed by SrrAB, especially under low-oxygen conditions (71). The observed repression is a result of a direct interaction of SrrA with the respective promoters (117). Besides the aforementioned virulence factors, the SrrAB TCS is also a major driver of *ica* operon expression under low-oxygen conditions, and this operon encodes the polysaccharide intercellular adhesin. An *srrAB* mutant is impaired in a neutrophil killing assay, suggesting that SrrAB-dependent polysaccharide intercellular adhesin production may protect *S. aureus* against nonoxidative killing by neutrophils (118). Transposon sequencing (TnSeq) analysis revealed SrrAB to be essential for *S. aureus* survival in an osteomyelitis model, which could be rescued by neutrophil depletion (119). SrrAB was also found to be a positive regulator of the phosphatidylinositol-specific phospholipase C (PI-PLC) enzyme. PI-PLC is important for *S. aureus* survival in human blood and in human neutrophils (25), which could in part explain the importance of SrrAB for defense in these environments.

The ArlRS TCS

Bacteria can undergo autolysis to remodel their cell walls by the action of murein hydrolases, which hydrolyze various components of peptidoglycan (120). The autolysis regulated locus (ArlRS) TCS was first described as a negative regulator of the autolytic process (121, 122), and it acts as a repressor of *agr* by downregulating RNAII and RNAIII expression (121). More recently, it was observed that ArlRS is a repressor of autolysis in methicillin-sensitive *S. aureus* strains (122, 123) but not in methicillin-resistant strains, including hospital- and community-acquired methicillin-resistant strains (123). In terms of virulence factors, ArlRS was reported to be a positive regulator of extracellular proteolytic activity, specifically serine protease activity (122). Serine proteases are involved in *S. aureus* virulence due to their ability to cleave antibodies and block their activity. This was, for example, demonstrated for the V8 serine protease of *S. aureus*, which cleaves immunoglobulin G *in vitro* (124). Although early reports demonstrated differential toxin regulation between wild-type and *arlRS* mutant strains, this was not observed in other studies using clinical isolates (125), supporting the notion that some of this reported regulation is highly strain specific.

In addition to *S. aureus* virulence, a microarray study showed that ArlRS represses sugar uptake systems and activates genes involved in amino acid utilization as a carbon source (126). These early results were corroborated by a recent study that showed positive regulation of genes involved in amino acid catabolism by ArlRS in the presence of calprotectin, an antimicrobial protein that starves bacteria for manganese and zinc (127, 128). A functional ArlRS system was necessary to facilitate resistance against calprotectin-mediated manganese starvation. Interestingly, manganese sequestration by calprotectin inhibited growth of *S. aureus* when glucose was utilized as the main energy source but not when amino acids served as the primary energy source. This implies that glucose utilization results in a higher demand for manganese compared to amino acid utilization. Therefore, *S. aureus* protects itself from calprotectin-mediated manganese starvation by switching its carbon source, and this process seems to be regulated by the ArlRS TCS (127).

Recently, it was demonstrated that the ArlRS TCS is required for *S. aureus* clumping in the presence of fibrinogen (125). *S. aureus* uses surface proteins, such as ClfA and ClfB, to interact with the abundant dimeric fibrinogen molecule, bringing cells together as a "clump" to survive in the host (129). The inactivation of *arlRS* results in the dramatic upregulation of the large surface protein Ebh, which is thought to inhibit clump formation. Indeed, the *arlRS* mutant strain also showed lower virulence in a rabbit model of sepsis and endocarditis, and this phenotype was partially restored by mutation of *ebh* (125). A subsequent study revealed that ArlRS directly regulates MgrA (84), which is the primary controller of the clumping mechanism. Through RNA transcriptome sequencing analysis, a total of eight large surface proteins (Ebh, SraP, Spa, FnbB, SasG, SasC, FmtB, and SdrD) were found to be repressed by MgrA (more on this regulator below). Depending on the strain, a deletion of SraP, SasG, or Ebh (in combination) within an *mgrA* mutant background restores the clumping phenotype to wild-type levels (84). The fibrinogen-mediated clumping phenotype of *S. aureus* was shown to have a major influence on *agr* signaling. Clumping creates a microenvironment in which the AIP concentration is increased and thus quorum sensing is uncoupled from the actual bacterial density. This results in a boost in *agr* activation and subsequent production of virulence factors, leading to increased bacterial burden as well as host morbidity and mortality (130).

THE SARA PROTEIN FAMILY

SarA

SarA is the prototypical member of the SarA protein family in *S. aureus*. As outlined in Fig. 4, SarA is expressed from three different promoters, leading to expression of three overlapping transcripts (131). SarA binds to its own promoter and auto-upregulates its own expression (132). It is negatively regulated by the SarA-like protein SarR (133) and positively regulated by the alternative sigma factor σ^B (133, 134). One of the ways SarA exerts its global regulatory function is via the upregulation of *agr* (Fig. 4), resulting in enhanced expression of toxins and the repression of protein A (*spa*) (135). More specifically, SarA significantly boosts the expression of the *agr* system from the P2 promoter (136). Besides indirect regulation through the *agr* system, SarA also exerts posttranscriptional regulation of targets (e.g., *spa*) by binding and altering the mRNA turnover (137).

Several studies have shown that SarA is a strong repressor of protease production, which effects several phenotypes, such as biofilm development (138), and the accumulation of proteins important for *S. aureus* virulence (139, 143). An *in vivo* study showed that a *sarA* mutant of the USA300 strain LAC was attenuated in a murine infection model. The *sarA* mutant displayed increased extracellular protease production, which facilitated the degradation of extracellular virulence factors. Subsequently, this phenotype could be reversed by eliminating extracellular protease activity (139). Another interesting aspect of SarA-mediated regulation is the decreased accumulation of alpha-toxin and PSMs in a USA300 strain. The altered virulence factor levels could be correlated with the increased extracellular degradative protease activity of the strain, rather than a direct regulatory effect of SarA on either

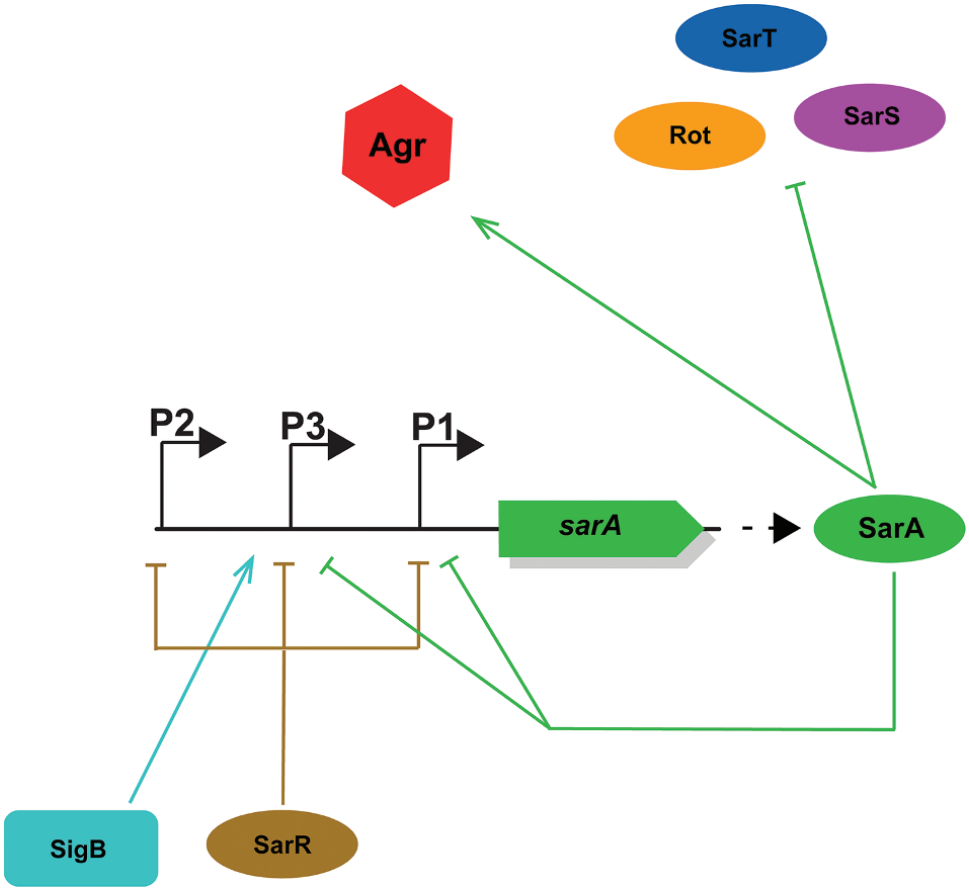


FIGURE 4 The *sarA* promoter region and SarA regulatory network. *sarA* gene expression is driven by three promoters (P1, P2, and P3) as shown. The alternative sigma factor σ^B (SigB) drives expression of *sarA* by binding to the P3 promoter. The binding of SarR to all three promoters inhibits expression and impedes autoregulation by SarA. Finally, SarA is an activator of the *agr* system, and it can also function as a negative regulator of the three SarA-like proteins SarH1, SarT, and Rot.

agr or *hla*. In contrast, mutation of *sarA* in strain Newman did not impact PSM or alpha-toxin accumulation (140), which might be explained by the fact that strain Newman has the $SaeS^P$ variant, leading to constitutive activity of the SaeRS system. A knockout mutation of *sarA* also leads to significant reduction of cytotoxicity against osteoblasts and osteoclasts and to reduced osteomyelitis in a murine model. This phenotype is thought to be mediated by the SarA-regulated production of alpha-PSMs (141).

Besides these regulatory functions, SarA also directly upregulates other virulence factors, including TSST-1, through direct promoter binding (142). Interestingly, the same research group identified SarA as a negative regulator of TSST-1 expression in another *S. aureus* strain background (134), suggesting that TSST-1 regulation by SarA is strain specific. Exfoliative toxin A and B, the causative agents of staphylococcal scalded-skin syndrome, are both downregulated by SarA *in vitro*. Negative regulation of exfoliative toxin B (ETB), but not exfoliative toxin A (ETA), by SarA was also observed in a neonatal mouse model (107). Another facet of SarA-mediated regulation is the recently discovered repression of the regulatory small RNAs *srn_3610* (*Spr*C), known to attenuate *S. aureus* virulence in an animal infection model (144), and *srn_9340* (145). Repression is achieved by direct binding of SarA to the respective promoters of the two small RNAs, which consequently blocks RNA polymerase loading (145).

SarR

SarR is a 13.6-kDa homolog of the SarA regulator protein and is a negative regulator of *sarA* expression (133) (Fig. 4). SarR accomplishes this regulation by binding to all three promoters of the *sarA* locus (146). SarR also acts as a negative regulator of *agr* expression from the P2 promoter, and SarR binds better to the *agr* promoter than SarA does. The result is that SarR can displace SarA and maintain negative regulation over *agr* function (136).

SarT and SarU

Two other members of the SarA protein family are encoded by the adjacent and divergently transcribed genes *sarT* and *sarU* (147, 148). Transcription of the *sarT* gene is regulated by *agr* and *sarA*, both of which repress *sarT* expression. While SarT has only a minimal effect on SarA and SarR expression, the mRNA levels of the *agr* effector RNAIII are increased in a *sarT* mutant (147). Therefore, SarT and the *agr* system seem to interact in a negative feedback loop. Besides this regulation, SarT also represses *hla* (147). In addition, SarT is also a

negative regulator of the *sarU* gene through binding to the promoter region. In contrast to a *sarT* mutation, the activities of the RNAII and RNAIII promoters are decreased in a *sarU* mutant when measured with a green fluorescent protein (GFP) reporter plasmid (148). In terms of the *agr* system, SarT and SarU play opposite roles in the regulatory mechanism. SarT production is also regulated by a small noncoding RNA called ArtR. The ArtR transcript binds to the 5′ untranslated region of the *sarT* mRNA, promoting its degradation (149).

SarH1

SarH1, also known as SarS (150), is a 29-kDa protein from the SarA family. Like other SarA family proteins, its regulation circuit is interwoven with other SarA family proteins and the *agr* system. Expression of SarH1 is strongly repressed by SarA as well as *agr* (150, 151), while it is upregulated by SarT (152). On the posttranscriptional level, SarH1 is regulated by GdpS. The mRNA product of the *gdpS* gene directly interacts with the 5′ untranslated region of *sarH1*, resulting in a more stable *sarH1* mRNA (153). SarH1 itself acts as a repressor of *hla*, and it also acts as a positive regulator of *spa*, which is located directly downstream the *sarH1* locus (151). The regulation of *spa* seems to be organized in a cascade. The *agr* system regulates SarT, which upregulates SarH1 and in turn positively regulates *spa* production (152). Another virulence factor regulated by SarH1 is exfoliative toxin. Two exfoliative toxins, ETA (*eta*) and ETB (*etb*), are expressed by *S. aureus* strains. Of these, SarH1 downregulates production of ETA, but it does not affect ETB production. ETB and ETA production is also negatively regulated by SigB and SarA, while *saeRS*, *arlRS*, and the *agr* system act as positive regulators (107). A recent study showed that SarH1 might also be involved in the spread of antibiotic resistance via the staphylococcal cassette chromosome *mec* element (SCC*mec*). SarH1 can upregulate the chromosome cassette recombinases A and B, which are responsible for the excision of SCC*mec* (154), which could possibly lead to its horizontal transfer.

Rot

The repressor of toxins (Rot) regulatory protein is a 15.6-kDa member of the SarA-like family. The name is derived from the fact that mutation of the *rot* locus in an *agr*-null background restores toxin production and protease activity and consequently restored virulence in a rabbit model of endocarditis (61, 155). In terms of toxin regulation, Rot acts as a repressor of enterotoxin B (*seb*), alpha-toxin (*hla*), proteases encoded by the *spl* and *ssp* operons, and lipase (*geh*). Enterotoxin B is directly repressed by Rot through binding to the *seb* promoter (156). The repression of *hla*, on the other hand, is exerted indirectly via the SaeRS TCS. Rot represses *sae* transcription from the P3 promoter, which then leads to reduced *hla* expression (157).

Rot also acts as a positive regulator of multiple virulence factors. Protein A (*spa*) and the SarA-family protein SarH1 (158) are upregulated by Rot, and by directly binding to its promoter, Rot can positively regulate superantigen-like proteins (Ssl) (159). A subsequent study demonstrated that Ssl production is also dependent on the SaeRS TCS, which synergizes with Rot to activate the Ssl promoter (160). The Rot regulon overlaps with the *agr* system, and Rot acts as an intermediate regulator of *agr* function. As described above, RNAIII, the main effector of the *agr* system, blocks the translation of the *rot* gene (59, 62). Besides RNAIII, Rot is also repressed by SarA, through direct binding to the Rot promoter (161), and by the sigma factor B (σ^B) during stationary growth (63). On the posttranscriptional level, Rot production is also dependent on the presence of the ClpX chaperone (162).

MgrA

MgrA is an important member of the MarR (multiple antibiotic resistance regulator)/SarA protein family. MgrA is a positive regulator of 175 genes and a negative regulator of 180 genes in *S. aureus* strain Newman, as shown in a microarray study (83). Among these regulated genes are several encoding for virulence factors, including alpha-toxin, coagulase, protein A, nuclease, extracellular serine proteases, and capsule production (163, 164). The regulation of genes responsible for capsule production (*cap5*) has also been implicated in infective endocarditis, while alpha-toxin production was not MgrA-dependent in the same model (163). MgrA also regulates the expression of SarZ, which itself controls exoprotein production (165). Looking at the regulatory pattern of MgrA, it is evident that exoproteins are positively regulated and surface proteins are negatively regulated by MgrA (83), with some parallels to the *agr* system. Related to this point, the *mgrA* gene is transcribed from two promoters (P1 and P2), and the mRNA transcript derived from the P2 promoter is stabilized by RNAIII (166).

In addition to RNAIII, MgrA production is regulated by another small RNA-mediated mechanism. The noncoding RNA RsaA represses MgrA production by interacting with its ribosome-binding site, making a loop-loop interaction with the *mgrA* mRNA (167, 168). The activity of MgrA is also regulated on the posttranslational level via the phosphorylation of a conserved cysteine residue. This phosphorylation is mediated by the Stk1-Stp1 proteins, a eukaryote-like kinase-phosphatase pair. Deletion of Stp1 results in elevated protein phosphorylation on cysteine residues and significantly reduces virulence in mouse infection models (169). The phosphorylation status of MgrA also plays a key role in the regulation of the NorA and NorB efflux pumps, which confer resistance to several antibiotics. Phosphorylated MgrA is able to bind to the NorB promoter, acting as a repressor, and unphosphorylated MgrA binds to the NorA promoter, acting as an activator (170–173). Further studies on MgrA suggest that it has the ability to regulate the *lytRS*, *lrgAB*, and *arlRS* TCSs, which are all involved in *S. aureus* autolysis (81). MgrA was called Rat (regulator of autolysis, in answer to "Rot") in early studies due to its role in autolysis regulation.

Several studies have shown that MgrA plays an important but conflicting role in *S. aureus* biofilm formation (Fig. 5). A mutation of *mgrA* has been reported to both promote (84, 174, 175) and repress biofilm formation (176). The disparity is most likely explained by the use of different *S. aureus* strains in these studies. The downregulation of surface proteins by MgrA has been corroborated in a recent study, which established a link between MgrA and the clumping activity of *S. aureus* in the presence of fibrinogen (84). As described above (in the ArlRS section), MgrA represses eight large surface proteins, and of these Ebh, SraP, and SasG are important in preventing clumping with fibrinogen. Fibrinogen-mediated clumping facilitates increased *agr*-mediated virulence factor production and decouples quorum sensing from the actual cell density (130). This can potentially be detrimental for *S. aureus*, because virulence factor production is often a trigger for host defense. Thus, MgrA might act as a fail-safe switch for premature virulence factor production. Relating to biofilm development, the derepression of SasG in an *mgrA* mutant strain promotes biofilm formation (84).

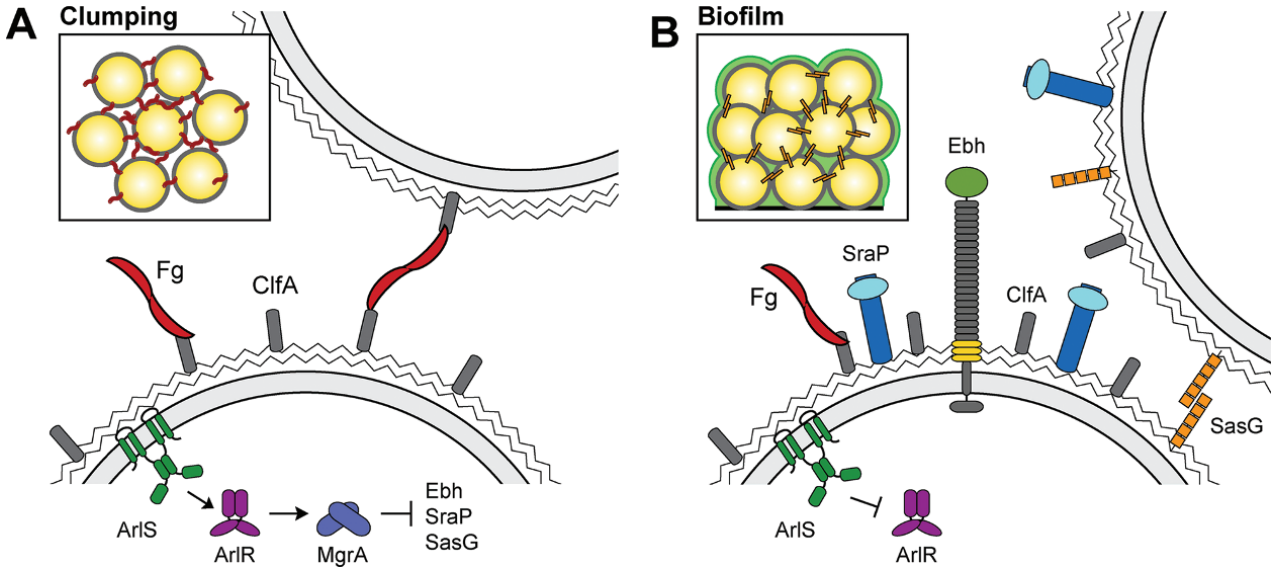


FIGURE 5 Regulatory pathway of ArlRS TCS and MgrA in biofilm formation and clumping. (A) The ArlRS TCS is activated by an unknown signal and subsequently activates MgrA, which in turn represses the production of large surface proteins (Ebh, SraP, and SasG), allowing ClfA/ClfB to interact with fibrinogen (Fg). Neighboring cells binding to the dimeric Fg molecule leads to clumping. (B) When ArlRS is inhibited, MgrA is not expressed and the repression of the large surface proteins (Ebh, SraP, and SasG) is relieved, preventing proper interactions of ClfA/ClfB with Fg. At the same time, SasG overproduction leads to homodimeric interactions with other cells expressing SasG, resulting in enhanced biofilm formation. This figure is a reproduction from one published in reference 84.

ALTERNATIVE SIGMA FACTORS

SigB

Sigma factors confer promoter specificity to the RNA polymerase holoenzyme and are required for transcription initiation. While the primary sigma factor controls housekeeping functions (177), alternative sigma factors regulate the response to changing conditions and allow *S. aureus* to adapt to different environments. Sigma factor B (σ^B) is known to respond to different stresses and accordingly regulates stress-response proteins. In addition, it counterbalances the activity of the *agr* system on virulence factor expression and thus overlaps with the *agr* regulon (178). In *S. aureus*, the *sigB* gene is part of an operon, formed with *rsbU*, *rsbV*, and *rsbW*. Expression *of sigB* is driven by SigA, which leads to the transcription of the whole operon, as well as through autoregulation by SigB itself, leading to transcription of *rsbV* and *rsbW* (179). SigB activity is regulated on the posttranslational level by the Rsb (regulation of sigma B) proteins. SigB is bound and kept inactive by the anti-sigma factor RsbW during exponential growth. Binding of the anti-anti-sigma factor RsbV leads to the release of SigB from RsbW and subsequently allows its binding to the RNA-polymerase holoenzyme (180). The first gene in the SigB operon, *rsbU*, was shown to be necessary for the activation of SigB (181) and is the major activator of SigB during acidic stress (182).

In *S. aureus*, SigB directly and indirectly controls approximately 200 genes, including genes with functions in virulence, biofilm formation, persistence, cell internalization, membrane transport, and antibiotic resistance (180). Several of these regulatory functions are executed by the regulation of known virulence regulators, such as *agr* and SarA (29). An early study of the phenotypic variation of wild-type *S. aureus* strains, and their corresponding *sigB* mutants, suggested a role of SigB in staphyloxanthin pigment production and sensitivity to hydrogen peroxide (183), demonstrating its role in stress response. Strain 8325-4 is a natural *rsbU* mutant and produces high levels of protease and alpha-toxin activity, and this phenotype is dramatically reduced when *rsbU* is repaired (184, 185). Similar results were obtained using the clinical isolate KS26, a prototype V8 protease producer, and strain Wood46, a hyper-alpha-toxin producer, and both of these strains were shown to have mutations in the *sigB* operon (186). Another natural *rsbU* mutant strain is BB255, rendering it effectively a SigB-deficient strain. Complementation of *rsbU*, as well as SigB overproduction in this strain background, increased the strain attachment to fibrinogen- and fibronectin-coated surfaces, as well as adherence to platelet-fibrin clots (187). These phenotypes are thought to derive from the positive regulation of the cell wall-attached adhesins ClfA and FnbA by SigB (187, 188). SigB also regulates TSST-1 through an indirect mechanism involving the two virulence regulators *agr* and SarA (134, 189). In addition, the enterotoxin Seh and the enterotoxin gene cluster *egc* are activated by SigB, while transcription of enterotoxin Seb is negatively regulated by SigB (189). SigB also plays an important role during conditions present in the pulmonary environment. When colonizing the lung, bacteria come into contact with pulmonary surfactant that damages the bacterial cell membrane. In response to the surfactant, *S. aureus* induces the expression of the gamma-hemolysin subunit B, a component of the type-VII secretion system, and the PsiA damage repair protein. The upregulation of these genes is dependent on SigB, indicating that SigB plays an important role in *S. aureus* adaption to the lung environment (190).

SigH

Besides SigB, *S. aureus* harbors a second alternative sigma factor, known as SigH. It was recently shown that overexpression of SigH leads to natural competence of *S. aureus*, although with very low efficiency (191). Indeed, some genes

known to be involved in natural competence of other bacterial species are regulated by SigH (192, 193), but several other competence genes are expressed poorly in *S. aureus* and are not effected by SigH (193). SigH also stabilizes the lysogenic state of *S. aureus* prophages (194). Because prophages often carry genes for virulence factor production, SigH might play an underappreciated role in *S. aureus* virulence.

CONCLUSION

As outlined in this article, the regulation of *S. aureus* virulence factors is subject to a complex and intricate network of factors that integrate physiological processes in response to environmental cues and host signals. This complex network allows *S. aureus* to readily adapt to changing conditions and establish itself in the available niches through its commensal and pathogenic life cycle. Some of the regulatory schemes outlined in this article are highly specialized and fine-tune gene expression in a unique environment, such as in a specific host site. On the other hand, *S. aureus* has evolved several master regulators of virulence that have more global effects, with the most prominent being the *agr* quorum-sensing system, SaeRS, SarA, Rot, ArlRS, and MgrA as summarized herein. An overview of the interconnections between these global regulators is depicted in Fig. 6.

Going forward, there is growing interest in targeting regulatory pathways as a means of preventing infection. As antibiotic resistance in *S. aureus* continues to spread (195), new and unconventional approaches to fight bacterial infections are needed to maintain antibiotic stewardship (196). Due to the importance of the *agr* system in virulence, researchers have extensively targeted this system as a potential means of therapy (197–199). Efforts have been made to target the other global regulators, but in some cases these inhibitors have had broader effects on numerous systems (200). In regard to the *agr* inhibitors, some of the best and most effective candidates are the AIPs themselves, and numerous synthetic studies have been performed to identify improved and more global AIP-based inhibitors (28, 198). More recently, it has been realized that commensal microbes sharing the same niche as *S. aureus* may compete through *agr* interference (90, 201), which ultimately may dictate the ability of *S. aureus* to colonize, or not colonize, the host. An improved understanding of these natural competition

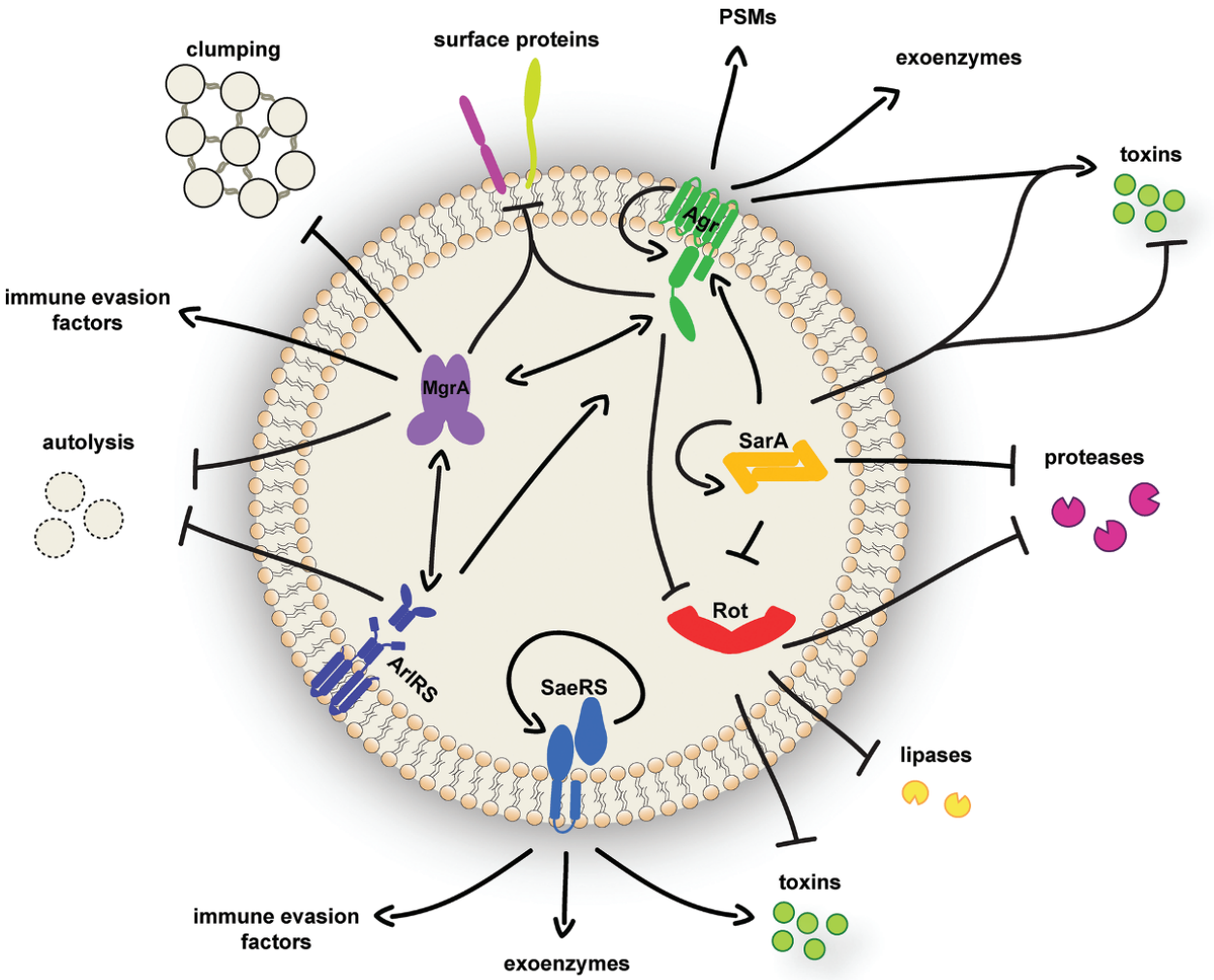


FIGURE 6 Interaction network of the major *S. aureus* global regulators. The schematic depicts a comprehensive overview of five regulatory systems, including the *agr* quorum-sensing system, the ArlRS and SaeRS TCS, and three members of the SarA-protein family (SarA, Rot, and MgrA). The virulence-associated traits controlled by each regulator are also shown.

mechanisms may shape future treatment approaches for certain types of infections.

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Virulence and Metabolism

ANTHONY R. RICHARDSON

42

INTRODUCTION

Of the many species of staphylococci, *Staphylococcus aureus* has evolved to be far and away the most virulent. This stems largely from the horizontal acquisition of virulence determinants that are all coordinately regulated by several transcription factors covered elsewhere (e.g., AgrA, SaeR, Rot, and SarA) (117). In parallel, a separate set of transcription factors act in concert to control the expression of enzymes in various metabolic pathways (e.g., CcpA, CcpE, CodY, RpiRc, SrrA, and Rex). Of note, recent findings have demonstrated that many of these metabolic transcriptional regulators both directly and indirectly influence the functions of the virulence gene regulators. Moreover, many of the virulence gene regulators also affect the expression of key metabolic pathways. Thus, *S. aureus* has intertwined transcriptional control of virulence and metabolism. In addition, several groups have published findings that shed light on the direct contributions of *S. aureus* metabolism to disease outcomes. Different sets of metabolites are available at different infection/colonization sites, thereby necessitating unique metabolic pathways. For instance, the skin surface has abundant lactate, urea, and certain amino acids, but carbohydrates are relatively scarce. This is in contrast to deep tissue sites where *S. aureus* would have access to serum glucose. In addition to metabolite availability, other environmental variables differ among infection sites, such as pH, oxygen tension, and the presence of robust inflammation. For instance, the skin surface is oxygenated and acidic (pH ~5.5) with minimal inflammation, whereas deep tissue infection sites become hypoxic, are relatively well buffered (pH ~6.5), and are almost always accompanied by an immune cell infiltrate. These contrasting environmental factors constrain and alter the metabolic landscape in *S. aureus*, requiring significant adaptation to cause infection/colonization. This chapter highlights recent findings in this area and outlines current knowledge about the linking of virulence with metabolism in this important human pathogen.

CARBOHYDRATE CARBON/ENERGY SOURCES

S. aureus is capable of utilizing at least 15 carbohydrates as primary carbon/energy sources (glucose, glucose-6-phosphate, mannose, fructose, galactose, mannitol, GlcNAc, ManNAc, ribose, maltose, sucrose, trehalose, lactose, turanose, and maltotriose) (1–7). All but four (glucose, glucose-6-phosphate, ribose, and maltotriose) are solely dependent on the phosphoenolpyruvate transport system (PTS) for import (1). PTS transport is coupled directly to phosphorylation of the sugar without the need for cytosolic glucokinase (*glk*, SACOL1604). Briefly, a high-energy phosphate is transferred from phosphoenolpyruvate to the enzyme I (EI) component (*ptsI*, SACOL1092) of the PTS shared by all transporters. This phosphate is then shuttled to His15 on the shared HPr protein (*ptsH*, SACOL1091), which then passes the phosphate onto the sugar-specific enzyme II (EII) component. These EII transporters are made up of three domains in *S. aureus* (EIIA, EIIB, and EIIC) and can be encoded by individual genes or together as fused polypeptides. The phosphate is shuttled from the EIIA subunit to the EIIB subunit, which finally phosphorylates the sugar as it passes through the EIIC pore. *S. aureus* encodes ~29 putative sugar transporter genes, 10 of which are unique to this pathogenic species (1). The substrates for many have been identified and independently validated in multiple strain backgrounds. For instance, *lacE* and *lacF* (SACOL2181-82) encode an EIIBC fusion protein and an EIIA subunit, respectively, that confer growth on lactose and galactose (8). Similarly, *mtlA* and *mtlF* (SACOL2146 and SACOL2148) confer growth on mannitol (9, 10). In addition, *fruA* encodes an EIIABC fusion polypeptide that allows growth on fructose. However, this locus is frameshifted in *S. aureus* COL, which consequently has trouble growing on fructose. Additionally, inactivation of SACOL2316 reduced growth on maltose and maltotriose, insertions in SACOL2376 reduced growth on sucrose, inactivation of SACOL0517 eliminated growth on trehalose and reduced growth on maltotriose, and loss of SACOL2663 limited growth on mannose (1).

Given the redundancy of substrate specificity for many PTS transporters, full identification of the substrates for each putative locus has proven difficult. For instance, glucose transport is highly redundant in *S. aureus*, far more than in coagulase-negative staphylococci (CoNS). While there is an EIIABC fusion peptide encoded by *glcB* (SACOL2552) and a non-PTS proton symporter encoded by *glcU* (SACOL2246) that are specific for glucose and shared by many species of staphylococci, there are additional glucose transporters that are specific to *S. aureus*. Specifically, *glcA* (SACOL0175) encodes an EIIABC fused polypeptide that is specific for glucose and GlcNAc and, to a lesser extent, mannose (1). This gene is not typically found in CoNS. Similarly, *glcC* (SACOL0224) encodes an EIIBC fusion peptide that transports glucose as well as ManNAc and is also absent from the genomes of many CoNS (1). Finally, *S. aureus* can also

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use glucose-6-phosphate, which is imported via a transporter encoded by *uhpT* (SACOL0200) that is rarely found in CoNS other than *Staphylococcus epidermidis* (11, 12). This expansion of glucose transport capability in *S. aureus* is noteworthy because glucose is in low abundance on the skin surface. Likely, *S. aureus* encounters higher glucose levels in deeper tissue replete with serum glucose, a habitat less frequented by CoNS. In fact, it has been shown that the two PTS-dependent glucose transporters unique to *S. aureus* (GlcA and GlcC) contribute more to glucose uptake in this species than do the common shared glucose transporters (GlcB and GlcU) (1). Additionally, deletion of all four glucose transporters in *S. aureus* resulted in a highly attenuated mutant strain. This is significant, because this mutant was still capable of using all other carbohydrates yet was still avirulent. Conversely, mutating His15 of HPr eliminates PTS transport and the use of virtually all carbohydrates except glucose (glucose can still be imported via the proton symporter), and yet this mutant retained virulence in a murine skin abscess model (1). Thus, during skin infection, *S. aureus* requires host glucose to thrive, and consequently, it has evolved to maximize glucose uptake by acquiring additional glucose-specific transporters that are uncommon among CoNS.

Each compatible carbohydrate enters the Embden-Meyerhof-Parnas glycolytic pathway and/or the pentose phosphate pathway and is eventually converted to pyruvate, generating ATP and reducing equivalents in the form of NADH/NADPH. The fate of pyruvate depends on the redox state of the cell (see below). However, when grown aerobically on carbohydrates, very little pyruvate enters the tricarboxylic acid (TCA) cycle, because the genes that encode TCA-cycle enzymes are transcriptionally repressed by CcpA when glucose is present (see below). Rather, to yield additional ATP, most of the pyruvate is converted to acetate and then excreted, which can significantly impact pH in unbuffered media. However, once glucose in the media is exhausted, CcpA repression of the TCA cycle is relieved, and the excreted acetate can be reassimilated, provided the strain has an intact TCA cycle (13).

NONCARBOHYDRATE CARBON/ ENERGY SOURCES

S. aureus can utilize a number of noncarbohydrate nutrients, including peptides, free amino acids, fatty acids, and small organic acids. Peptides ranging from 3 to 8 amino acids in length are imported by the ATP-dependent Opp3BCDFA transporter (SACOL0991-95), whereas di- and tripeptides are imported by DtpT (SACOL0788) (14). *S. aureus* can also presumably utilize the cysteine-containing tripeptide glutathione as a nutrient/sulfur source by the ABC transporter SACOL0184-86, which is upstream of *ggt* (SACOL0188) encoding the first step in glutathione breakdown. *S. aureus* also encodes several transporters predicted to import single free amino acids, some of which have been implicated in virulence (15). Some amino acids can be directly catabolized as carbon/energy sources and are therefore rapidly consumed in culture medium, including alanine, arginine, aspartate, cysteine, glutamate, glycine, proline, serine, and to a lesser extent, histidine. Others are consumed much more slowly because they are used primarily for protein synthesis rather than energy sources (e.g., isoleucine, leucine, lysine, methionine, phenylalanine, tyrosine, and valine) (16). Most *S. aureus* strains are conditionally auxotrophic for arginine and proline in that CcpA represses *de novo* synthesis of these pathways in the presence of a preferred carbohydrate (17, 18). However, *de novo* arginine synthesis is required for virulence in a murine model of sepsis, likely due to a limitation of host arginine in the context of inflammation (18). In addition, cysteine and methionine serve as the primary sulfur sources during infection because *S. aureus* cannot assimilate sulfur-oxides and is sensitive to excess sulfide (19).

While *S. aureus* cannot grow using fatty acids as a sole carbon/energy source since it lacks a glyoxylate shunt, it has a full complement of genes encoding enzymes for fatty acid beta oxidation (SACOL0215-0211). However, cells cultivated *in vitro* appear to incorporate exogenous fatty acids into membrane phospholipid, and they are not oxidized for energy (20). The role for fatty acid oxidation in *S. aureus* metabolism and virulence has not been studied in detail, but it likely provides the pathogen with a source of energy. In contrast, the use of short 2- hydroxyacids such as lactate in *S. aureus* has been thoroughly studied. Reports describing NAD-dependent and -independent lactate dehydrogenase activities span the last 50 years (21, 22). Only recently, however, have the genes encoding some of these systems been characterized. *S. aureus* can utilize both D- and L-lactate with NAD-dependent Ddh (D-lactate, SACOL2535) or Ldh1/2 (L-lactate, SACOL0222/2618) (23). However, these enzymes generally operate in the lactate-producing reaction to maintain redox balance during respiratory stress (see below). In addition, *S. aureus* encodes an NAD-independent lactate dehydrogenase, lactate-quinone oxidoreductase (Lqo, SACOL2623), that is largely responsible for respiration-dependent lactate utilization (24). Lactate generation/utilization is critical to *S. aureus* virulence because strains with mutations affecting these enzymes exhibit attenuated phenotypes in a variety of murine infection models (25, 26).

Beyond short chain 2-hydroxyacids, *S. aureus* has also been demonstrated to use sialic acid as a carbon/energy source (27). Furthermore, the catabolic gene segment for sialic acid utilization has been identified and characterized. Interestingly, while present in many CoNS, it was absent from those more closely related to *S. aureus*, including *S. epidermidis*, potentially indicating a patchy phylogenetic history for this metabolic pathway (27). While sialic acid adducts are abundant modifications of host proteins and lipids, *S. aureus* apparently lacks a cognate glycosidase to liberate free sialic acid. Accordingly, it must rely on cohabiting commensal species known to possess sialic acid-specific glycosidases or neurominidases (e.g., *Streptococcus* spp.). Thus, sialic acid utilization likely contributes to *S. aureus* colonization, whereas a role in invasive infection has not been demonstrated directly and is less likely since deep infections are generally mono-associated.

MICRONUTRIENTS

In addition to carbon/energy sources, *S. aureus* must acquire a variety of micronutrients during infection to cause disease. For instance, detailed work has outlined the mechanisms by which this pathogen acquires transition metals. Iron acquisition is critical because this element serves in the active sites in enzymes that detoxify immune radicals such as superoxide dismutase (SodM, SACOL0118), catalase (SACOL1368), and flavohemoprotein (Hmp, SACOL0220) as well as enzymes involved in the TCA cycle and respiration. Similarly, manganese is critical for radical-detoxifying enzymes (SodA, SACOL1610) and key glycolytic and gluconeogenic reactions. Zinc is critical for bacterial DNA replication/repair as well as serving in the active sites of key glycolytic enzymes.

Thus, for pathogens to successfully cause disease, they must be able to acquire all of these metal micronutrients.

Given the above, the host exerts significant resources to preclude would-be bacterial invaders from accessing these key micronutrient metals. For instance, free iron is systemically limited by host scavenging proteins such as albumin, transferrin, and lactoferrin. Similarly, iron atoms coordinated in heme, either free or associated with hemoglobin, are another rich iron source and are actually preferred by *S. aureus* (28). Accordingly, free heme is scavenged by the host hemopexin, and free hemoglobin is protected by host haptoglobin. *S. aureus* has acquired the means to obtain iron from all of these sources save hemopexin. High-affinity nonprotein iron scavengers known as siderophores are secreted by numerous bacterial pathogens and act to tightly bind free iron or strip it from iron scavenging proteins such as transferrin and lactoferrin. The iron-bound siderophore is then taken up by bacterial siderophore receptor/transporters replenishing intracellular iron pools. *S. aureus* synthesizes two independent citrate-based siderophores, staphyloferrin A and B (29). Staphyloferrin A is synthesized by enzymes encoded by *sfaABCD* (SACOL2168-71), and iron-bound staphyloferrin A is transported back into *S. aureus* by the ABC transporter HtsABC (SACOL2165-67) under the control of the FhuC ATPase (SACOL0704) (30, 31). Likewise, staphyloferrin B is synthesized by enzymes encoded in the *sbnABCDEFGHI* operon (SACOL0100-08), and iron-loaded staphyloferrin B is brought into the cell by the SirABC transporter (SACOL0096-98) (32, 33). In addition to endogenously produced siderophores, *S. aureus* is capable of utilizing siderophores from other species (xenosiderophores) including hydroxamate- and catecholate-based siderophores via the FhuCBGD1/2 (SACOL0704-06, SACOL2010/2227) and SstABCD (SACOL0796-99) ABC transport systems, respectively (34–36). While siderophores and xenosiderophores are capable of stripping iron from many iron-binding host proteins, they cannot obtain iron coordinated in heme. However, *S. aureus* can still use free heme, hemoglobin, and haptoglobin-bound hemoglobin as heme/iron sources via the iron-regulated surface determinant (Isd) system encoded by *isdBACDEFGHI* (SACOL1139-44, SACOL1146, SACOL1781, and SACOL0152) (37). Hemoglobin and haptoglobin-hemoglobin are recognized by IsdB and IsdH, respectively, and upon heme removal, the iron source is passed to IsdA, then to IsdC, and finally through the ABC-transporter IsdDEF (38–42). Once cytosolic, the iron can be liberated by one of two independent heme oxygenases encoded by *isdG* and *isdI* (43). Thus, *S. aureus* can acquire heme-iron from most host sources except the heme-scavenging protein, hemopexin.

In addition to the systemic scavenging of iron, manganese and zinc are scavenged away from pathogens locally by neutrophils, which express high levels of calprotectin (44, 45). This highly abundant protein binds both manganese and zinc at two distinct sites with high affinity (46, 47). Moreover, *S. aureus* residing within acidic phagosomes is faced with divalent metal deficiencies (including iron, manganese, and zinc), owing to the presence of eukaryotic NRAMP1, a proton/divalent metal symporter (48, 49). NRAMP1 has been shown to effectively remove metals from the phagosomes and transport them into the cytoplasm out of reach of engulfed bacteria (48). To combat these host nutritional immunities, *S. aureus* expresses two independent manganese transporters: the ABC transporter MntABC (SACOL0690-688) and the bacterial NRAMP-family transporter MntH (SACOL1114). Inactivation of *mntABC* when *S. aureus* is cultivated under manganese-depleted conditions results in a significant growth defect, while inactivation of *mntH* does not (50). However, both transporters must be inactivated to cause significant reductions in organism burden in a murine sepsis model (51). The two transporters are regulated by MntR (SACOL0691), the gene for which is positioned upstream of *mntABC*. MntR represses *mntABC* in a manganese-dependent fashion, while it seems to activate *mntH* in response to manganese in the environment (50).

In addition to manganese acquisition, recent advances have revealed how *S. aureus* competes with the host for available zinc. This pathogen expresses two zinc transporters, the ABC transporter AdcABC (SACOL2403, SACOL1612-13) and an Opp/NikA-family transporter, CntABCDF (SACOL2476-72) (52, 53). The two transport systems are partially redundant in that both have to be inactivated to cause growth defects in Zn-depleted culture conditions. In contrast, the two serve two distinct roles during infection. While AdcABC imports free zinc, CntABCDF imports a broad-spectrum metallophore known as staphylopine (54). This "siderophore-like" metal chelator is synthesized by CntKLM (SACOL2479-77) and is presumably exported via the MFS-family transporter CntE (SACOL2471). While the Cnt system can bind and transport a variety of metals, the Adc/Cnt double mutant only failed to accumulate zinc, prompting staphylopine to be designated as a "zincophore" (52). Given the high affinity of calprotectin toward zinc, the Adc system contributes little to zinc acquisition in the presence of this host protein. However, the high affinity of staphylopine toward zinc allows this metallophore to "steal" zinc from calprotectin. Both of these transporters contribute to virulence in that the double mutant was highly attenuated in murine sepsis models, though the Cnt system alone measurably contributes to virulence, presumably due to its ability to scavenge zinc from host calprotectin (52). In total, *S. aureus* has amassed an impressive arsenal of metal transporters to compete with host nutritional immunity.

Apart from metal micronutrients, recent advances have highlighted the role of sulfur in *S. aureus* virulence. In addition to being required for cysteine and methionine synthesis, sulfur is a key component of proteins that employ iron-sulfur clusters or enzymes that utilize the lipoic acid cofactor. Sulfur is also a key component of several vitamins, including biotin, thiamin, pantothenate, and consequently coenzyme A (CoA). Finally, *S. aureus* synthesizes the small-molecular-weight sulfur-containing bacillithiol (Fig. 1), which is critical to the intracellular detoxification of a variety of molecules (55). Sulfur sources for *S. aureus* during infection include cysteine or its oxidized-dimer form, cystine, via the sodium symporter TcyP (SACOL0454) or the ABC transporter TcyABC (SACOL2412-10) (19). Additionally, sulfur can be obtained from methionine via the ABC transporters MetNPQ1 (SACOL0882-84) and MetNPQ2 (SACOL0504-06) or the Na^+/H^+-symporter MetT (SACOL2319) (19). *S. aureus* can also potentially utilize host glutathione as a sulfur source (see above). While *S. aureus* cannot assimilate sulfur from sulfate or sulfite, it can assimilate hydrogen sulfide, thiosulfate, or taurine, though the abundance of these molecules during infection may be well below that of the sulfur-containing amino acids (56, 57). Assimilated cysteine sulfur can be used to synthesize iron sulfur clusters in TCA cycle enzymes such as aconitase and succinate dehydrogenase. *S. aureus* utilizes a Suf system for Fe-S cluster assembly, encoded by the *sufCDSUB* operon (SACOL0914-18) (58). SufS is the cysteine desulfurase that removes S^0 from cysteine and transfers it as persulfide to SufU

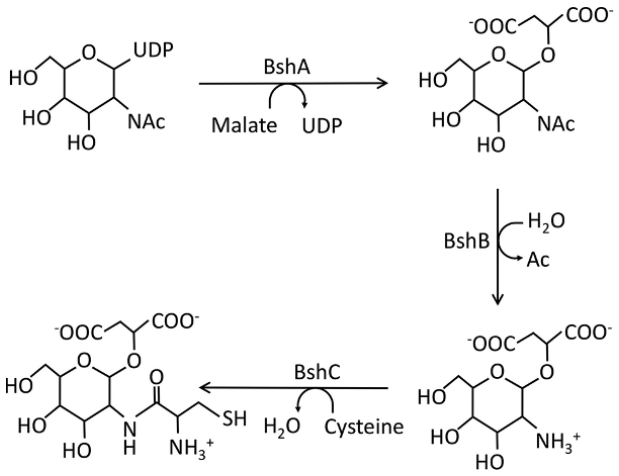


FIGURE 1 Biosynthetic pathway for bacillithiol in *S. aureus*. The major low-molecular-weight thiol in *Firmicutes* is bacillithiol, which is synthesized from UDP-GlcNAc, malate, and cysteine by BshABC. In *S. aureus*, the genes encoding these biosynthetic pathways are not linked in a discreet operon but are located individually throughout the genome.

for incorporation into the iron sulfur cluster on the SufB component of the SufBCD scaffold. The Fe-S cluster can then be transferred to target apoproteins by carriers such as SufA (SACOL0943) and Nfu (SACOL0939) (59). Fe-S assembly (but not repair) is also dependent on a newly characterized SufT protein (SACOL0977), which becomes essential in environments heavily dependent on Fe-S cluster enzymes or lipoic acid-containing enzymes (e.g., growth on peptides or amino acids) (60, 61). Loss of Suf function results in cells with significant fitness defects as well as hypersensitivity to immune radicals and killing by human neutrophils (58). Another important use of assimilated sulfur is in the synthesis of bacillithiol. This molecule is synthesized from UDP-GlcNAc, malate, and cysteine sulfur by the enzymes BshA, -B, and -C (SACOL1498, SACOL0614, and SACOL1190, respectively) (62, 63). Bacillithiol is used in critical detoxification schemes that limit damage caused by immune radicals, toxic electrophiles, certain antibiotics (e.g., fosfomycin), and excessive buildup of metals (55, 64). Furthermore, bacillithiol has a role in Fe-S cluster homeostasis, and mutants lacking the capability of synthesizing this molecule show decreased survival in whole human blood (65).

Respiration versus Fermentation

S. aureus is a facultative anaerobe, meaning it respires if oxygen is present but is capable of either fermentation or anaerobic respiration if oxygen is absent. However, aside from the mere presence or absence of oxygen, the facultative anaerobe designation has implications for virulence in that infection sites are often replete with immune radicals that are highly damaging to respiratory enzymes. Thus, *S. aureus* has evolved to ferment, even in the presence of oxygen, if it senses a drop in respiratory capacity due to the presence of host immune radicals. This likely contributed to the evolution of *S. aureus* pathogenesis. During aerobic respiration, cellular reducing equivalents (e.g., NADH) are recycled via one of the type 2 NADH-dehydrogenases encoded by *S. aureus* (NdhF and NdhC; SACOL0941 and SACOL0944, respectively) (66). Alternatively, electrons can enter the pathway via complex II (succinate dehydrogenase) as well as a host of quinone-dependent oxidoreductases (Fig. 2). Electrons are passed to the sole quinone species in *S. aureus*, menaquinone, and from there can be passed to oxygen-dependent terminal quinol-oxidases. *S. aureus* lacks cytochrome c because it lost the biosynthetic genes early on phylogenetically (Fig. 3). Instead, this species produces three cytochromes (aa_3, bd, and bo_3), which are the products of two independent gene clusters (67). Cyanide-sensitive cytochrome aa_3 is encoded by the *qoxBACD* (SACOL1070-67) gene cluster, as is cytochrome bo_3 when heme a becomes limiting (68). Cyanide-resistant cytochrome bd is encoded by the *cydAB* operon (SACOL1094-95), which is induced during periods of respiratory stress. While these quinol-oxidases are likely necessary for efficient skin colonization given the skin's inherent aeration, each is also required for the colonization of internal organs. Specifically, staphylococcal growth in the heart and liver requires CydAB and QoxABCD, respectively (67). In the absence of oxygen, *S. aureus* is capable of respiring on nitrate via the NarGHJI (SACOL2395-92) nitrate reductase. This molybdenum-dependent enzyme complex transfers electrons from quinol to reduce nitrate to nitrite. The nitrite is excreted but, upon nitrate exhaustion, can be further reduced to ammonia by the siroheme-dependent nitrite reductase, NirBD (SACOL2398-97) (69).

If no external electron acceptor is available, either due to the lack of oxygen/nitrate or if host immune radicals preclude efficient respiration, *S. aureus* enters into fermentative metabolism. The major fermentative end products produced are L-lactate, D-lactate, formate, ethanol, and 2,3-butanediol (70, 71). L-lactate is the dominant fermentative product and is generated by Ldh2 and Ldh1, the latter of which is unique to *S. aureus* (26). D-lactate is generated via Ddh, and all three of these enzymes serve to recycle NAD^+ by reducing pyruvate to lactic acid. Ethanol and 2,3-butanediol are produced from acetyl-CoA and pyruvate by AdhE (SACOL0135) and BudAB (SACOL2199-98), respectively. These are generally produced at much lower levels compared to the organic acids, but they do serve important functions in cells undergoing respiratory stress (72). Formate is generated from pyruvate by PflAB (SACOL204-05). This reaction circumvents the reduction of NAD^+ or quinone by pyruvate dehydrogenase or pyruvate oxidase, respectively, and the formate can be used for generation of formylated peptides (73). Given the inherent redundancy of fermentation pathways, individual loss of function mutations in fermentation genes have little or no phenotype during infection, with Ldh1 being the only exception (26). However, multiple mutations in fermentative pathways lead to severe virulence defects, suggesting that aerobic respiration is not always available to *S. aureus* during infection.

Fermentation inherently comes with metabolic limitations. For instance, fermenting carbohydrates is much less efficient with respect to energy production, and thus, *S. aureus* requires ~4-fold more carbohydrate per cell than during respiration (1). Furthermore, growing independently of respiration limits the compatibility of many carbon sources. With the exception of pyruvate, only glycolytic carbon/energy sources support fermentative growth (74). In fact, eliminating merely cytochrome aa_3 but not bd or bo_3 also necessitates glycolytic carbon/energy sources (68). This has important implications for *S. aureus* virulence. Most of the enzymes in the Embden-Meyerhof-Parnas pathway are reversible and therefore essential since they are required for both glycolysis and gluconeogenesis. However, certain

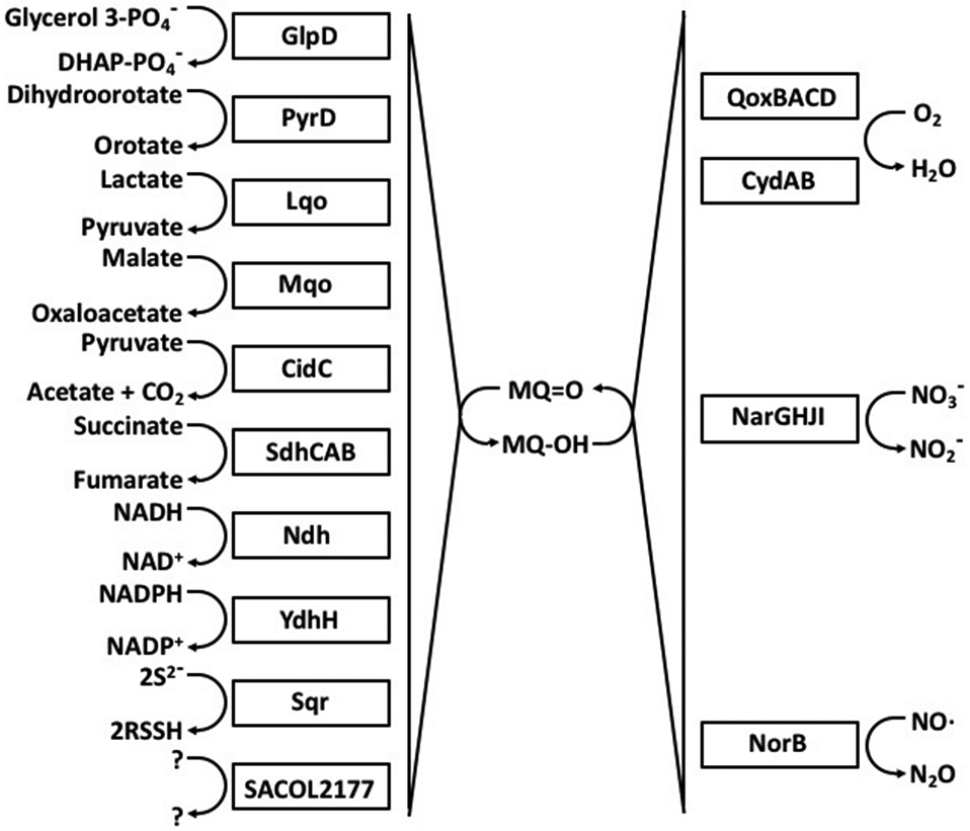


FIGURE 2 Branched respiratory scheme in *S. aureus*. The left side lists all of the known/putative quinone-oxidoreductases in *S. aureus* along with their substrates. Electrons are passed via these enzymes to the menaquinone pool and then to one of many terminal oxidases (right side with substrates). While most *S. aureus* organisms possess QoxBACD, CydAB, and NarGHJI, NorB seems to be limited to strains in clonal complex 30 (e.g., UAMS-1 and MRSA252).

reactions are not readily reversed, such as those of phosphofructokinase (*pfkA*, SACOL1746) and pyruvate kinase (*pyk*, SACOL1745). These genes encode enzymes required only for glycolysis, and therefore, mutants grow like wild type on gluconeogenic substrates (e.g., peptides) but cannot grow in the presence of glucose. These mutants are particularly attenuated for survival within activated macrophages (74). In contrast, gluconeogenic mutants (e.g., Δ*pckA*) were not. Moreover, eliminating macrophage nitric oxide (NO·) production enhanced survival of glycolytic mutants to wild-type

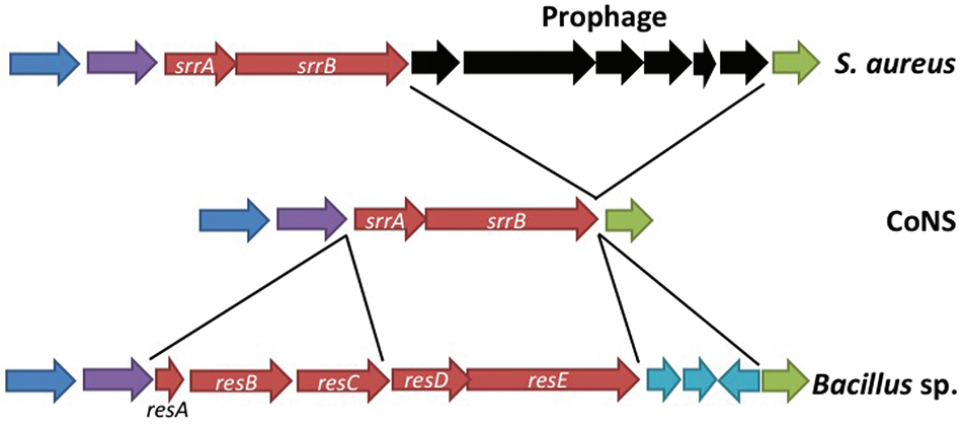


FIGURE 3 Loss of cytochrome C synthesis while retaining SrrAB. In other *Firmicutes*, SrrAB homologues (termed ResDE) are encoded in the same operon as ResABC, which are involved in cytochrome c biosynthesis. Across most staphylococci, the *resABC* genes are missing, while *resDE* (*srrAB*) the two-component system was retained.

levels, suggesting that *S. aureus* has access to a variety of carbon/energy sources within the phagosome, but host NO· blocks respiration, thereby necessitating the use of glycolytic carbohydrates. Also, given the importance of host glucose to infection (see above), it is not surprising that these glycolytic mutants were highly attenuated in murine infection models (74).

Wild-type *S. aureus* can switch between fermentative and respiratory modes of energy production via the coordination of metabolic gene expression by a set of transcriptional regulators that monitor the redox status of the cell (see below). However, many chronic or recurrent *S. aureus* infections are caused by persistent antibiotic-tolerant small colony variants (SCVs) (75). These arise commonly through mutations that eliminate the cell's ability to respire (e.g., heme or menadione biosynthetic mutations). These slow-growing, nonrespiring SCVs exhibit reduced membrane potential and consequently are more resistant to many antibiotics. However, it has been proposed that mixed populations of SCVs with different genetic etiologies (e.g., a mixed infection with heme- and menadione-deficient subpopulations) can "cross-feed" each other any deficient metabolites. This restores growth and virulence but does not return the population to full antibiotic susceptibility (76). Whether this phenomenon occurs clinically has yet to be described. Furthermore, over time, nonrespiring SCVs evolve faster growth rates, in some cases through mutations that enhance fermentative capacity (77). This also improves growth without restoring antibiotic susceptibility. Additional studies of SCVs have uncovered a number of causes beyond the direct loss of respiratory capacity, including mutations in *thy*A that result in loss of thymidine synthesis, thereby reducing trimethoprim/sulfonamide susceptibility (78). However, due to effects of *thy*A mutations on TCA cycle enzyme expression, these SCVs also exhibit reduced respiratory flux. Other means of achieving the SCV phenotype involve mutations in components of the RNA degradasome, changes in small noncoding RNA expression including toxin-antitoxin systems, and persistent stringent response mutations (75). In addition to decreased antibiotic sensitivity, SCVs also exhibit reduced virulence factor production and increased uptake and survival within host cells, accounting for their association with chronic and recurrent infections (79).

Metabolic Gene Regulation

While it would be highly cumbersome to outline all of the metabolic gene regulation in *S. aureus*, here, I focus on regulators that intersect between metabolism and virulence. Specifically, the sensing of carbon sources and redox homeostasis is discussed, because these pathways have been outlined above. Compatible carbohydrates are sensed and responded to by CcpA, while a close homologue, CcpE, responds to citrate levels in the cell. In parallel, the availability of amino acids and nucleotides is sensed by CodY. Finally, *S. aureus* senses respiratory capacity via a two-component system, SrrAB, and the cytosolic redox state via Rex. Nearly all of these regulators are conserved among *Firmicutes*, and most have direct and indirect intersections with virulence factor expression and pathogenesis. Of note, compared with *Bacillus subtilis* and *Escherichia coli*, *S. aureus* lacks an FNR homologue. This Fe-S cluster-containing regulator is conserved in many bacterial species and is required for the maximal expression of anaerobic respiration and fermentative pathways (80). The Fe-S cluster is oxygen labile; therefore, in the presence of oxygen, *B. subtilis* and *E. coli* do not efficiently express fermentative enzymes (81). In contrast, *S. aureus* can fully express fermentative pathways in the presence of oxygen since it does not utilize this oxygen-sensing scheme. Thus, in the presence of host immune radicals, *S. aureus* can ferment available carbohydrates regardless of the aeration of the local environment. The following subsections describe a few of the key metabolic regulators employed by *S. aureus* to coordinately regulate metabolic genes with virulence factor production.

CcpA

In general, CcpA (SACOL1786) mediates carbon catabolite regulation in Gram-positive *Firmicutes*. It binds along with its coregulator (HPr~P) to weakly conserved catabolite-responsive elements (CRE sites; TGTAAA/TTTACA) in the genome (82). While HPr can accept a phosphate from EI at His15 during PTS phosphotransfer (see above), it can also be phosphorylated at Ser46 by the serine kinase HprK (SACOL0825) (83). HprK becomes active once it senses the build-up of the glycolytic intermediate fructose bisphosphate, signifying the presence of a compatible carbohydrate (84). HprK-mediated Ser46 phosphorylation of HPr allows this coregulator to associate with CcpA, rendering it capable of high-affinity CRE binding. Activation of CcpA alters the expression of many genes in *S. aureus*. It leads to the activation of glycolytic genes as well as the repression of TCA cycle genes, genes encoding amino acid catabolism, and in some cases, biosynthetic pathways (16, 85). Another complicating feature is that the conserved serine/threonine kinase 1 (Stk1, SACOL1232) can phosphorylate CcpA at residues Thr18 and Thr33 within the DNA-binding domain, rendering CcpA inactive (86). This mode of regulation is not evident in *Bacillus* spp. and thus may be unique to staphylococci. CcpA is known to be responsible for the glucose stimulation of biofilm formation and expression of the regulatory RNA III as well as toxic-shock syndrome toxin 1 (82, 87, 88). However, caution should be used when interpreting gene expression associated with CcpA, specifically glucose-dependent gene regulation that is absent in a Δ*ccp*A mutant. This pattern of regulation was observed for the *S. aureus*-specific *ldh*1 gene, yet it was shown to be indirectly regulated by CcpA (89). Rather, Δ*ccp*A mutants do not efficiently catabolize glucose in the medium; instead, these mutants preferentially break down amino acids and other TCA cycle/gluconeogenic substrates. It appears that the utilization of glucose, not merely its presence, stimulated *ldh*1 transcription by an unknown mechanism despite the presence of a putative CRE site upstream of the *ldh*1 promoter (89). Whether a similar mechanism mediates glucose-dependent toxic-shock syndrome toxin 1, RNA III, or biofilm production has yet to be determined. Indeed, glucose can stimulate Agr activity, leading to increased virulence factor production so long as the medium is buffered (82). Otherwise, glucose in the medium leads to acetate production and concomitant acidification that seems to repress Agr activity (90). Given the buffering capacity of human blood, but the acidity of human sweat, it is hard to say whether glucose stimulates Agr activity in the host. However, recent work has demonstrated that hyperglycemia in nonobese diabetic mice can affect pathogenicity and virulence factor production in a CcpA-dependent fashion (91).

CcpE and RpiRc

Fructose bisphosphate is not the only metabolite signal that can alter virulence factor production in *S. aureus*. CcpE is a citrate-sensing LysR-type CcpA-homologue that directly binds to and activates the aconitase (*citB*, SACOL1385) promoter (92, 93). Two conserved arginine residues (Arg145

and Arg256) assist in coordinating the citrate molecule. In addition to *citB*, CcpE directly influences the expression of genes involved in iron acquisition, toxin production, pigmentation, and capsule biosynthesis (94). Additionally, several secreted and surface exposed virulence determinants were reduced in the Δ*ccpE* mutant (95). Still, mutants lacking CcpE appear to be more virulent in several murine infection models and survive better in whole human blood. Metabolomic profiling of the Δ*ccpE* mutant shows that carbon becomes rerouted away from the TCA cycle, resulting in a buildup of citrate, lactate, acetate, and alanine (93). Beyond CcpE-mediated citrate sensing, *S. aureus* encodes three homologues of ribose-responsive regulators: RpiRa, RpiRb, and RpiRc (96). Recently, RpiRc has been implicated in dampening expression of RNA III, which has impacts on virulence factor production (97). As such, Δ*rpiRc* mutants exhibit enhanced exotoxin production and are hypervirulent in murine sepsis and skin infection models (97, 98). Additionally, loss of RpiRc limits expression of TCA cycle genes and induces central glycolytic and amino acid metabolic pathways. Taken together, *S. aureus* has integrated virulence factor regulation with the sensing of key metabolic intermediates, specifically, fructose bisphosphate, citrate, and potentially, ribose.

CodY

This repressor senses both branched-chain amino acids (BCAAs) and cytosolic GTP to control many genes involved in amino acid biosynthesis and import as well as virulence factor production (99). Abundant BCAAs bind to CodY and afford it the ability to recognize its cognate DNA sequence (AATTTTCWGAAAATT) (100). GTP also binds CodY and, in combination with BCAAs, improves the DNA-binding capacity of this repressor. BCAAs (Iso, Leu, and Val) are often the most common codons in many genomes, including that of *S. aureus*. Thus, as amino acids become depleted, these BCAAs may become scarce relatively quickly. As BCAAs become depleted, CodY loses affinity for DNA. Furthermore, as general amino acid depletion ensues, the stringent response is activated and a bulk conversion of GTP to the alarmone ppGpp(p) occurs (99). This reduction in the cytosolic GTP pool further reduces the binding capacity of CodY, resulting in derepression of global amino acid synthesis/acquisition. Additionally, CodY negatively affects the expression of extracellular proteases and many secreted virulence factors. In fact, CodY seems to negatively impact Agr signaling, though likely in an indirect fashion (101), and this could be responsible for downregulation of virulence factor production by CodY. It was suggested that in Δ*codY* mutants that constitutively express amino acid synthesis/import functions, intracellular amino acid pools would be high, maximizing the amount of secreted Agr autoinducer. The effects are reduced biofilm formation and hypervirulence in murine skin models for Δ*codY* mutants (102). However, while the impact of CodY on biofilm production has been well described (103), in a murine sepsis model of infection, the Δ*codY* mutant was recently shown to be attenuated (104). Thus, loss of CodY hinders the pathogen's ability to balance virulence factor and extracellular protease production, leading to a variety of outcomes in different infection settings.

Rex

This redox-sensing regulator is also highly conserved among *Firmicutes* and generally represses genes encoding fermentation enzymes that harbor well-conserved Rex sites ($TGTGAN_6TCACA$) near their promoters. Specifically, three lactate dehydrogenases, a lactate permease, two alcohol dehydrogenases, an alanine dehydrogenase, and pyruvate-formate lyase are all directly repressed by Rex (Table 1) (105). However, as redox imbalance ensues, the ratio of

TABLE 1 Dehydrogenases of *S. aureus*

Gene name[b]	Locus name	Putative function	Rex-Reg[a]
*ald*1	SACOL1478	Alanine dehydrogenase	Yes
*ald*2	SACOL1758	Alanine dehydrogenase	
*ldh*1	SACOL0222	L-lactate dehydrogenase	Yes
*ldh*2	SACOL2618	L-lactate dehydrogenase	
adhE	SACOL0135	Bifunctional acetylaldhyde/EtOH dehydrogenase	Yes
adhP	SACOL0660	Zn-dependent alcohol dehydrogenase	Yes
aldA	SACOL0154	Aldehyde dehydrogenase—NAD(P)H-dependent	
ddh	SACOL2535	D-lactate dehydrogenase	Yes
budA 1	SACOL2198	Acetolactate decarboxylase	
budA 2	SACOL2617	Acetolactate decarboxylase	
budB	SACOL2199	Acetolactate synthase	
–	SACOL0111	Acetoin reductase	
–	SACOL2114	Aldehyde dehydrogenase—NAD(P)H-dependent	
–	SACOL2574	D-isomer-specific 2-OH acid dehydrogenase	
–	SACOL1543	Aldol/keto oxidoreductase—NAD(P)H-dependent	
–	SACOL1545	Short-chain alcohol dehydrogenase	
–	SACOL2594	Short-chain alcohol dehydrogenase	
–	SACOL2293	Octopine dehydrogenase	
–	SACOL2488	Short-chain alcohol dehydrogenase	

[a]Rex-Reg: Indicates Rex binding to confirmed Rex sites directly demonstrated from reference 103.
[b]–, Dashes indicate genes that have not had a gene name assigned.

NAD^+:NADH diminishes. While Rex binds both NAD^+ and NADH, it binds the latter with higher affinity (K_M < 100 nM) (105). Furthermore, while NAD^+-bound Rex can effectively bind DNA and repress gene expression, NADH-bound Rex cannot. So as NADH accumulates, Rex loses affinity for DNA, and expression of dehydrogenases specifically designed to restore NAD^+:NADH ratios become highly expressed. In addition, Rex also represses transcriptional regulators, including *srrAB* and *nirR*, thereby indirectly affecting the expression of genes involved in aerobic and anaerobic respiration (105). However, Rex does not appear to directly affect virulence factor expression, and consequently, the effect of Rex on virulence remains unclear.

SrrAB

This two-component system is thought to sense the redox status of the menaquinone pool analogous to the ArcAB system in *E. coli* (106). While this has not been directly demonstrated, many indirect observations support this hypothesis. For instance, genes known to be directly regulated by SrrA can be induced in a Δ*hemB* mutant but not in a Δ*menD* mutant (107). Loss of HemB results in cells that cannot synthesize heme and therefore are unable to respire, since no terminal oxidases function without heme. Consequently, the menaquinone pool becomes completely reduced, presumably activating SrrB. In contrast, a Δ*menD* mutant, while also being devoid of respiration, has no menaquinone, and therefore, SrrB is not stimulated. Similarly, SrrAB affects biofilm formation because it affects the expression of the murein hydrolase Atl as well as *cidABC* and the *ica*-dependent extracellular polysaccharide (108–110). As such, a Δ*hemB* mutant exhibits SrrAB-dependent hyperbiofilm formation, while a menaquinone mutant does not (110). By sensing a reduced menaquinone pool, SrrB can detect drops in respiratory flux. In response, the SrrAB regulon stimulates the production of respiratory cytochromes to increase respiratory capacity (111). It also stimulates pyruvate-formate lyase and alcohol dehydrogenase analogously to Rex. If the drop in respiratory flux is due to the lack of oxygen, SrrA induces the expression of the anaerobic ribonucleotide reductase (NrdDG, SACOL2635-34) (112). If respiration is being inhibited by host immune radicals, SrrA stimulates NO·-detoxifying *hmp* as well as *scdA*, which is involved in Fe-S repair (111, 113). In general, SrrAB senses respiratory flux and responds to a drop in respiration, whether the drop stems from hypoxia or respiration inhibition by host immunity. However, this two-component system also affects virulence factor production (113). Hypoxia was shown to induce the Agr system in an SrrAB-dependent fashion (110). SrrAB has also been implicated in driving toxic-shock syndrome toxin 1 and Spa expression. SrrAB mutants are sensitive to NO· and H_2O_2 as well as to neutrophil killing, and they are attenuated in murine sepsis and osteomyelitis models of infection (112, 114–116). Whether the attenuation stems from the inability to restore respiratory capacity during infection or from the inability to fully express key virulence factors remains unknown.

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Staphylococcal Biofilms

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43

Over the last decades, biofilms have gained more and more recognition as the common mode of growth that microorganisms adapt in nature (1). Furthermore, many types of human infection have been found to progress with the involvement of biofilms or originate from biofilm-associated primary infections (2). Second only to the Gram-negative *Pseudomonas aeruginosa*, staphylococci have been the focus of biofilm researchers. As common colonizers of the human skin, staphylococci are the most frequent sources of biofilm infections on surgically implanted indwelling medical devices. These include serious infections, including endocarditis and prosthetic joint infections (PJIs), and may lead to life-threatening conditions, such as sepsis (3–5).

For the longest time, biofilm research even in pathogenic bacteria has been characterized predominantly by *in vitro* investigation, from which—despite the obvious limitations—researchers attempted to extrapolate to biofilm-associated infection. More recently, biofilm research has begun to integrate *in vivo* infection models to obtain a better understanding of the mechanisms governing biofilm-associated infection. Infection models for *P. aeruginosa*, the biofilm-forming pathogen most intensely studied with *in vitro* research, are extremely challenging to establish and perform (6). Thus, due to the possibility of performing biofilm-associated infection models that reflect human biofilm infection, staphylococcal biofilm research has gained considerably in relative importance. As an important further prerequisite to perform mechanistic biofilm research, methods to genetically manipulate staphylococci are continuously being improved.

This article will discuss the role of staphylococcal biofilms in the commensal state and during infection, our current knowledge of the molecular basis of staphylococcal biofilm formation and its regulation, and the role of biofilm formation *in vivo* as an important means of immune evasion. Finally, potential strategies to develop therapeutics to treat biofilm-associated infections by staphylococci will be presented. There will be a focus on *Staphylococcus aureus* and *Staphylococcus epidermidis*, because we know the most about biofilms in these two species.

STAPHYLOCOCCAL BIOFILMS IN THEIR NATURAL HABITAT AND DURING INFECTION

Many definitions have been used for biofilms. Most define a biofilm as a microbial community of cells that is embedded in a matrix of extracellular polymeric substances that they have produced. Some researchers restrict the term "biofilm" to aggregates attached to a surface, which can be biotic or abiotic. For others, free-floating bacterial aggregates also represent biofilms. Cells in a biofilm characteristically show a phenotype, with respect to metabolism, gene transcription, and protein production, that is different from that during planktonic growth. This has been shown for both *S. aureus* and *S. epidermidis* (7, 8).

Microbial aggregates on infected catheters or other indwelling medical devices obtained from patients resemble biofilms grown in *in vitro* microtiter plates or biofilm reactors, and there is no doubt that these fit the definition of a biofilm (9). Macro- or microscopic evaluation from wound infections also indicates that those infections, which often contain staphylococci among other pathogenic microbes, can be described as biofilm associated (10). The situation becomes less clear when looking at the commensal colonization of the skin, nares, or intestines or at disease-associated colonization, such as during atopic dermatitis. Some researchers would argue that the physiological situation of bacterial aggregates in those habitats may resemble that commonly found in biofilms, while others have raised doubt (11–13). Currently preventing a more detailed analysis of those potential "colonizing" biofilms is the lack of animal models that closely resemble human colonization. Therefore, in the following, only biofilms on devices and wound infections will be discussed.

Medical devices particularly prone to infection include contact lenses, peritoneal dialysis catheters, urinary and central venous catheters (CVCs), endotracheal tubes, mechanical heart valves, pacemakers, and prosthetic joints (9). Staphylococci generally are the most frequent causes of infections on indwelling medical devices, with *S. aureus* and *S. epidermidis* representing the leading species (14). This is mostly due to their predominance in causing intravascular and prosthetic device-associated infections. However, on some devices, such as urinary catheters, other bacteria may dominate (15). Most commonly, indwelling medical devices are contaminated with the biofilm-forming bacteria during surgery. The source can be the skin or other colonized body sites of the patient or the health care personnel (9). In that regard, it is important that only ~20% of individuals are colonized by *S. aureus*—in contrast to many coagulase-negative staphylococci, including *S. epidermidis*, which are ubiquitous skin colonizers in humans (16, 17). Thus, the *S. aureus* carrier state of the patient is a risk factor for device

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infection, as it is for other types of *S. aureus* infection (18). Alternatively, already implanted devices can be infected via hematogenous seeding, although this is considered to be less frequent (19). Not surprisingly, immune- or otherwise compromised patients, such as AIDS patients, patients receiving immunosuppressive therapy, or premature newborns, are at increased risk of developing biofilm-associated infections on indwelling devices (9). These populations are also at increased risk regarding serious complications that may arise from infected devices. The most notable among those is blood infection (bacteremia), which may result in life-threatening sepsis. Central line (CVC)-associated bloodstream infections are among the most frequent and serious complications of medical device infection and cause thousands of deaths annually. In a 2004 study including 24,179 cases of health care-associated bloodstream infections (most of which stem from contaminated devices) in 49 U.S. hospitals over a 7-year period from March 1995 through September 2002, coagulase-negative staphylococci were responsible for 31% and *S. aureus* for 20% of cases, far ahead any other microorganism (20).

Chronic wounds, such as chronic skin ulcers in diabetes patients and acute burn wounds, are highly susceptible for infection by many pathogenic microorganisms. According to clinical observation, the infecting organisms, which usually include more than one type, present as a biofilm (21). The species most often found in wound infections is *S. aureus* (22, 23). From a microbiological point of view, the multispecies biofilms in wounds are much more difficult to understand than infections of indwelling medical devices, which usually present as a monospecies infections. Several studies claim that bacterial interactions exist in wounds between *S. aureus* and, for example, *P. aeruginosa*, but there is no commonly accepted view of their exact nature and role (24).

THE MOLECULAR BASIS OF BIOFILM FORMATION IN STAPHYLOCOCCI

Attachment Phase

Biofilm development has been extensively studied *in vitro* using many different setups and microorganisms. Commonly, it is described as a process of three main stages: attachment, proliferation/maturation, and detachment (dispersal) (25) (Fig. 1).

Attachment to a surface is considered the first stage of biofilm development, although in "free-floating" biofilms, this stage is not relevant. In contrast to motile organisms, which may reach a surface in an active fashion, staphylococci gain contact with a surface passively. Staphylococci may directly attach to the abiotic surface of indwelling medical devices or other abiotic surfaces in the environment (26). The hydrophobic character is the main factor enabling attachment to the usually hydrophobic material surface. However, specific staphylococcal molecules also have been implicated in surface attachment. These include the *S. epidermidis* major autolysin AtlE and its *S. aureus* homologue Atl (27, 28) and wall teichoic acids (WTAs) in *S. aureus* (29). These two may be linked mechanistically, because WTAs impact binding of Atl to the cell surface, controlling cell separation (30). Of note, primary attachment is often measured merely as a short-term variation of the frequently used simple microtiter-based biofilm assay. Whether results represent true primary attachment or initial phases of proliferation cannot be determined clearly using that assay.

In vivo, direct interaction of staphylococcal cells with the abiotic surface is not considered relevant, because indwelling devices rapidly become covered by host matrix material, including fibronectin, fibrinogen, vitronectin, and other matrix molecules (26). Under those conditions, attachment to the device is facilitated by a series of specific staphylococcal surface proteins that interact with those human matrix proteins. The most important family of such surface-expressed staphylococcal binding proteins is the MSCRAMMs (microbial surface components recognizing adhesive matrix molecules). MSCRAMMs consist, from the N- to C-terminus, of a signal peptide, a ligand binding domain with characteristic repeat sequences, a cell wall-anchoring region, a membrane-spanning region, and a positively charged tail (31). At the C-terminus, the MSCRAMM contains a conserved LPXTG motif, which is covalently linked by the enzyme sortase to peptidoglycan (32). According to genome analysis, *S. aureus* has about 20 and *S. epidermidis* about 12 MSCRAMMs (33). Prominent members are the fibrinogen- and fibronectin proteins, which include clumping factors A and B (ClfA, ClfB), the serine/aspartate-rich (Sdr) protein family, and fibronectin-binding proteins A and B (FnBPA, FnBPB) (31). Non-covalently bound surface proteins may also mediate attachment. Among them are predominantly enzymes that have a primary catalytic role, such as the Atl-type autolysins. Furthermore, there is a loosely defined group of non-covalently surface-attached proteins collectively called SERAMs (secretable expanded repertoire adhesive molecules), which bind to the surface by not yet characterized mechanisms and usually show broad specificity in human matrix protein binding (34). The extracellular adherence protein Eap (also called MHC class II analogous protein [Map]) and the extracellular matrix and plasma binding protein Emp of *S. aureus* belong to this group. Finally, the giant 1.1-MDa Embp protein of *S. epidermidis* and its *S. aureus* homologue Ebh (extracellular matrix binding protein homologues) are non-covalently surface-bound fibronectin-binding proteins (35, 36).

Proliferation and Matrix Formation

During the second stage of biofilm development, the microcolonies that have formed after attachment grow by proliferation. Additionally, cells secrete polymeric molecules to form the biofilm matrix. Among all aspects of biofilm development, the polymeric molecules that form the biofilm matrix have received the most attention. Biofilm matrix polymers have a divergent chemical nature and include polysaccharides, proteins, and teichoic acids. In addition to those actively secreted molecules, polymeric substances from dead cells are also believed to contribute to the biofilm matrix. The most notable among those is DNA, which after release from dying cells is known as extracellular DNA (eDNA).

In many biofilm-forming bacteria, exopolysaccharides represent the matrix components that are most specifically implicated with biofilm formation. Staphylococci produce one main biofilm exopolysaccharide, which is called polysaccharide intercellular adhesin (PIA), or according to its chemical composition, poly-*N*-acetylglucosamine (37) (Fig. 2). In contrast to chitin, another important *N*-acetylglucosamine homopolymer found in nature, the *N*-acetylglucosaminyl moieties in PIA are β-1-6 linked. One characteristic feature that distinguishes PIA from most other staphylococcal surface molecules, and also from many other bacterial biofilm exopolysaccharides, is its cationic character, which is due to the enzyme-catalyzed removal of ~15 to 20% of *N*-acetyl groups after secretion. This feature may be important for electrostatic interaction with other surface polymers, and thus the

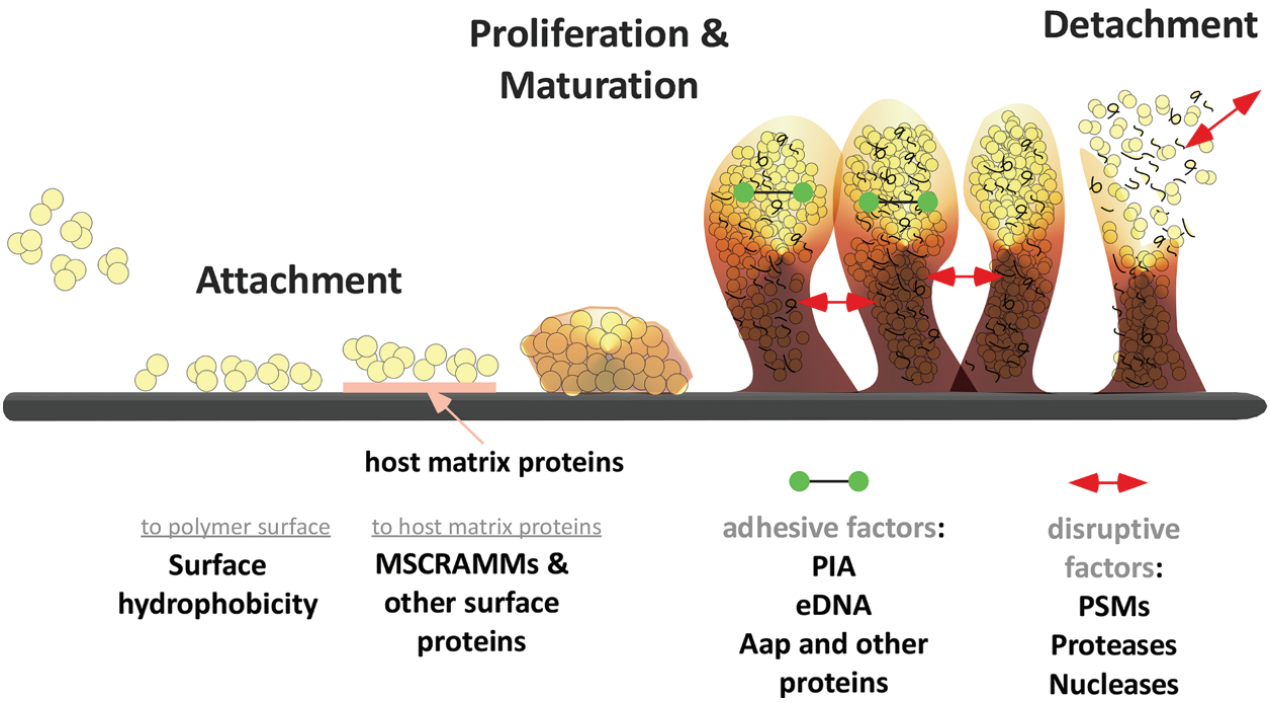


FIGURE 1 Staphylococcal biofilm development. Attachment of cells to a surface (in the case of surface-attached biofilms) occurs via hydrophobic interactions to an abiotic surface or via surface proteins that bind in a specific fashion to host matrix proteins covering an indwelling medical device. Growth of the biofilm in the proliferation/maturation stage is accompanied by the production of cell-cell adhesive matrix components (such as PIA, eDNA, and proteins), as well as disruptive factors (such as PSMs and degradative secreted enzymes). Those disruptive factors can also cause detachment, a process of great importance for the initiation of complications of biofilm-associated infection, such as bacteremia.

formation of the sticky extracellular biofilm matrix (37, 38). There appears to be no specific "anchor" for PIA on the cell surface, and it has been shown directly that this function is not accomplished by wall teichoic acids (39).

PIA biosynthesis is accomplished by the products of the *ica* (intercellular adhesion) gene locus, which comprises the *icaA*, *icaD*, *icaB*, and *icaC* genes (40). IcaA is the *N*-acteylglucosamine transferase, which together with IcaD has been shown to produce an *N*-acetylglucosamine oligomer (41). Chain growth is dependent on the presence of IcaC, a membrane protein assumed to be the PIA exporter. IcaB, which is located on the bacterial surface, is the PIA deacetylase (38). Deacetylation is crucial for the retention of PIA on the surface and the various functions PIA fulfills in staphylococcal physiology, which in addition to biofilm formation comprise increased resistance to antimicrobial peptides (AMPs) and neutrophil phagocytosis (38). Upstream of the *ica* operon is the *icaR* gene, which is transcribed in the opposite direction of the *icaADBC* operon. IcaR works as a repressor of the *ica* operon (42).

Several studies have shown that biofilm formation *in vitro* or *in vivo* can be accomplished by *S. epidermidis* or *S. aureus* isolates that do not harbor the *ica* locus (43–45), although the extension, structuring, and robustness of PIA-negative biofilms appears to be lower than those formed by PIA, at least in *S. epidermidis* (46, 47). In *S. aureus* isolates, biofilm formation appears to be predominantly protein-dependent (48). Earlier studies reported differences between methicillin-resistant and -sensitive isolates in that regard, while a more recent study indicates that both methicillin-resistant and -sensitive *S. aureus* isolates form almost exclusively protein-dependent biofilms (48, 49). In contrast, *S. epidermidis* more frequently relies on PIA to form biofilms, with an additional protein-mediated contribution (45). Replacing PIA in *ica*-negative isolates, or adding to its function in *ica*-positive isolates, are a series of macromolecules that include polymeric proteins and nonprotein polymers. Among the many surface-located proteins implicated in staphylococcal biofilm formation, the *S. epidermidis* accumulation-associated protein (Aap) and its *S. aureus* homologue, SasG (50), stand out due to their capacity to self-polymerize and form fibrils that interconnect cells (51–53) (Fig. 2). Aap is a 220-kD protein that is cleaved by the SepA protease to an active, mature form (54–56). At the N-terminus, the A domain of Aap contains repeats that may initiate biofilm formation in some strains and also bind to human corneocytes (57). It is followed by a lectin domain. These two domains are not present in mature Aap, which contains a large stretch of B repeats in front of a C-terminal proline/glycine-rich region and the cell wall-anchoring domain. The B repeats are responsible for self- and intercellular adhesion in a Zn^{2+}-dependent manner, assisted by cooperative folding (52, 58–60). Recently, the proline/glycine-rich region was shown to form an extended stalk that pushes the B repeat binding domain away from the cell surface, likely to enable better interaction with Aap from other cells, promoting biofilm formation (61). Notably, it has been shown that the homologous proteins SasG and Aap can interact, enabling interspecies biofilm connections (62). Several other surface proteins have also been implicated in intercellular adhesion, but in many of those cases it is difficult to distinguish between a surface/tissue attachment and genuine intercellular adhesion function. These proteins contain many surface binding proteins of the MSCRAMM family in addition to the biofilm-associated protein (Bap),

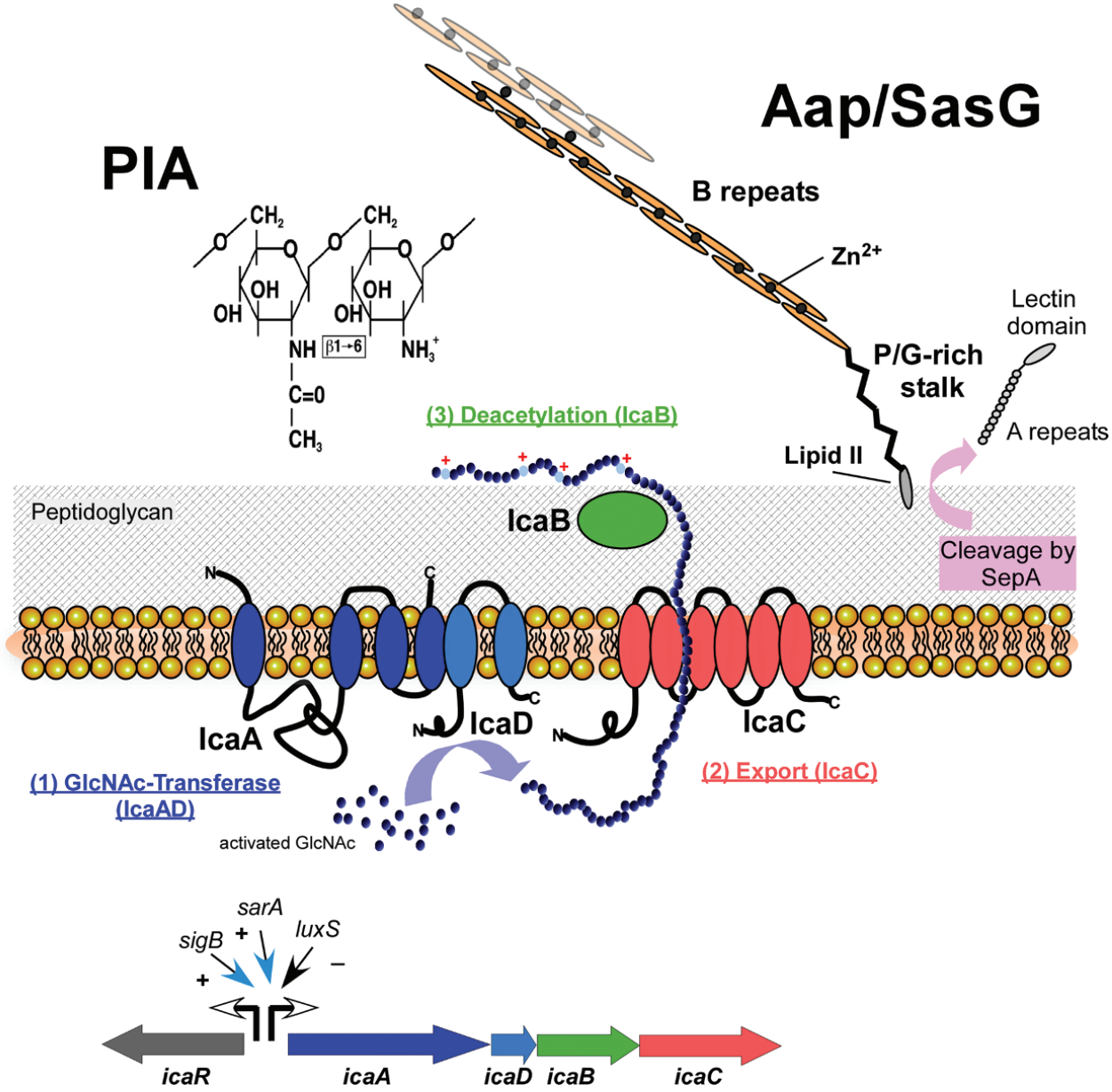


FIGURE 2 Predominant staphylococcal biofilm matrix components. The exopolysaccharide PIA (left), produced by many *S. aureus* and *S. epidermidis* isolates, is a β1-6-linked homopolymer of *N*-acetylglucosamine. It is synthesized in the cell by the combined activity of the membrane enzymes IcaA and IcaD and likely exported by IcaC. The extracellular surface-bound enzyme IcaB removes a certain percentage (~15 to 20%) of *N*-acetyl moieties, which gives the otherwise neutral PIA molecule a positive net charge, anchoring PIA to the negatively charged cell surface. In addition to the *ica* biosynthetic genes *icaADBC*, the PIA biosynthesis locus also contains a regulatory gene, *icaR*. Several global regulators impact transcription from the *icaR* and *icaADBC* promoters. The accumulation-associated protein (Aap), which is present in *S. epidermidis* and has a homologue in *S. aureus* called SasG, is produced as a 220-kD precursor protein, from which the secreted protease SepA cleaves off the N-terminal A-repeat and lectin domains. It is anchored to the cell wall via sortase-catalyzed covalent linkage to lipid II. Mature Aap forms extended fibrils out of B repeat domains, whose polymerization is dependent on Zn^{2+} ions. Zn^{2+} is also required for the interconnection of Aap/SasG proteins from different cells, which can happen in an interspecies manner.

found in *S. aureus* mastitis isolates, of which a homologue (Bhp) is present in *S. epidermidis* (63, 64). In support of a specific involvement in matrix formation, Bap has recently been reported to assemble into amyloid-like matrices to promote biofilm assembly (65), reminiscent of the function of Aap.

Nonprotein extracellular polymers implicated in biofilm formation include teichoic acids and eDNA. Teichoic acids occur in two forms: WTA, which is covalently linked to the cell wall, and lipoteichoic acid, which is surface-anchored via a lipid moiety that intercalates in the cytoplasmic membrane lipid bilayer (66). A specific contribution of the teichoic acid surface polymers to the biofilm matrix is likely but has only been shown for WTA of *S. epidermidis* (67). Teichoic acids devoid of D-alanylation, which alters their net charge, show diminished biofilm formation in *S. aureus* (29). Teichoic

acids are also implicated in receptor-mediated adhesion to the nasal epithelium (68). Finally, eDNA contributes to biofilm formation *in vitro* (69). Likely due to the highly polymeric and anionic character of DNA, it is extremely "sticky" and thus, eDNA is prone to interact with many other surface molecules, contributing to the formation of the extracellular biofilm matrix network.

Structuring and Detachment

Biofilms do not grow as undifferentiated "bricks" but have a characteristic three-dimensional structure, which contains towers, often described as "mushroom"-like, as well as fluid-filled channels between those towers (25). The second (maturation) stage of biofilm development comprises disruptive forces to create those structures in addition to the adhesive mechanisms described above. These are mediated by enzymes that degrade biofilm polymers, such as nucleases and proteases, and surfactant-like molecules, such as the staphylococcal phenol-soluble modulins (PSMs), which disrupt noncovalent interactions (70, 71). The same forces can also ultimately cause biofilm detachment, which facilitates systemic dissemination and subsequent complications during device-associated infection. Both biofilm structuring and detachment/dispersal effects have been described for the PSMs, while research on degradative enzymes has been mostly limited to detachment/dispersal.

PSMs are a family of alpha-helical peptides with pronounced amphipathy, thus exhibiting characteristics that give them surfactant-like properties (72). Members of the PSM family are produced in *S. aureus*, *S. epidermidis*, and most likely virtually all staphylococci (72, 73). PSMs can be grouped in α-type peptides (~20 to 25 amino acids in length), which in *S. aureus* comprise the PSMα and δ-toxin peptides, and the longer β-type PSMs (~44 to 45 amino acids) (72). Confocal laser scanning microscopy, a method often used to visualize biofilm structure, revealed that the characteristic channel-containing biofilm structure is not present in *psm* deletion mutants, which grow in an undifferentiated and extended fashion (47, 74). In contrast to biofilm polymer-degrading enzymes, PSMs structure PIA-dependent and -independent biofilms (47). PSMs work on growing biofilms; when biofilms are formed, external addition of PSMs has only limited biofilm-dispersing activity. Somewhat contrastingly, PSMs have also been described to form amyloid structures that prevent the enzymatic degradation of preformed biofilms (75). A double mutant in the *psm*α and *psm*β loci was impaired in preventing degradation, while the δ-toxin was not specifically addressed in that study. While there appears to be agreement about the *in vitro* capacity of PSMs to form amyloid-like structures, the role of PSM amyloids in biofilms remains controversial (76).

Among biofilm-degrading enzymes, proteases are the most important (71), while enzymes degrading PIA are not produced by staphylococci. *S. aureus* and *S. epidermidis* produce a series of exoproteases. *S. aureus* secretes ten proteases, including seven serine proteases (SspA and SplA-F), two cysteine proteases (SspB and ScpA), and one metalloprotease (Aur) (77). *S. epidermidis* produces at least three major proteases: a homologue of the staphopain cysteine protease SspB, a metalloprotease called SepA, and a homologue of the *S. aureus* SspA (V8) serine protease (16). Mutations in specific *S. aureus* protease genes, or addition of protease inhibitors, impair biofilm formation or lead to biofilm degradation, respectively, in biofilms that are protein dependent (48, 78). While specific matrix protein targets of several of the *S. aureus* proteases have been identified (79, 80), they overall show relatively low target sequence specificity (81). The *S. epidermidis* protease SspA (Esp) has been shown to be able to degrade protein-dependent *S. aureus* biofilms (82). Because biofilm formation in *S. epidermidis* is often strongly PIA-dependent, research on the role of *S. epidermidis* proteases in biofilm development has been limited.

The biofilm matrix component eDNA is subject to degradation by nucleases, which originally has been shown using human DNase I (69), which is abundant in human serum. Both *S. aureus* and *S. epidermidis* secrete nuclease activity. *S. aureus* is known to produce two secreted nucleases, Nuc1 and Nuc2 (83). Expression of Nuc1 reduces biofilms, and a *nuc1* mutation in strain USA300 shows increased *in vitro* biofilm formation (84), as would be expected from abolished eDNA degradation. However, isogenic *nuc1* or *nuc2*, or combined *nuc12* mutants in a different strain (UAMS-1), did not exhibit enhanced *in vitro* or, notably, *in vivo* biofilm formation (85).

REGULATION OF STAPHYLOCOCCAL BIOFILM FORMATION

Biofilms have been recognized as a common form of microbial growth. It is thus not surprising that many regulators that adapt staphylococcal gene expression to changing environmental conditions have an impact on biofilm formation. This section will focus on regulatory effects on the main biofilm-forming factors and on the biofilm effects of the major global regulators, the quorum-sensing system Agr, the Sar family of regulators, and the alternative sigma factor, SigB.

In 1998, Davies et al. reported that a quorum-sensing regulator in *P. aeruginosa* positively impacted biofilm formation (86), a result soon generalized regarding the relationship of quorum-sensing control and biofilm formation in bacteria. Soon after, Vuong et al. reported increased biofilm formation in isogenic mutants of the Agr quorum-sensing system in *S. aureus* (87), in clear contrast to the generalized *P. aeruginosa* finding. Since then, mechanistic investigation has shed more light on the impact quorum-sensing systems have on biofilm formation in several bacteria. As a common theme, quorum-sensing systems appear to frequently control biofilm structuring processes, which may result in more or less extended biofilms, depending on the specific conditions (70). Agr in staphylococci controls the two main biofilm structuring and dispersal factors described above, PSMs and proteases, with the control of *psm* genes being exceptionally direct and tight (8, 88, 89). As a result, biofilms of *agr* mutants grow in an unstructured and extended fashion, as shown for both *S. aureus* and *S. epidermidis* (74, 90), and applying the extracellular autoinducing peptide signal of the Agr quorum-sensing system results in biofilm dispersal (78). However, it remains poorly understood which mechanisms lead to the spatial and temporal heterogeneity of Agr expression that is observed in biofilms (74) and which are a theoretical prerequisite for the Agr-mediated biofilm structuring effect. Finally, the autoinducer-2 quorum-sensing system, which remains less well investigated in staphylococci, represses biofilm formation in an *ica*-dependent manner (91, 92). Both quorum-sensing systems thus affect biofilm formation negatively but using different mechanisms.

The prototype of the Sar paralogue family of regulators, SarA, impacts biofilm formation via regulation of Agr, as well as in an Agr-independent manner (93–96). The strong regulatory impact of SarA on protease production appears to play a preeminent role in this effect (97), and this, historically, was how the biofilm effect of *S. aureus* proteases was discovered. Furthermore, SarA positively controls *ica*

transcription in both *S. aureus* and *S. epidermidis* (94, 96), while its paralogue SarZ appears to have opposite effects in the two species—positive in *S. epidermidis* and negative in *S. aureus* (98, 99). Generally, which environmental signals stimulate Sar expression is not known, and therefore the biological context of biofilm regulation by Sar paralogues remains poorly defined.

Sigma factors are transcription initiation factors that provide promoter recognition specificity to RNA polymerase (100). The "alternative" sigma factor SigB is expressed during the stationary growth phase and environmental stress and is involved in controlling manifold aspects of staphylococcal physiology and virulence. SigB regulation of biofilm formation in *S. epidermidis* biofilms was reported to occur by an impact on *ica* transcription via SigB dependence of the *icaR* promoter (101), and in *S. aureus* possibly via SarA-dependent protease regulation (102, 103). However, other studies found no impact of SigB on biofilm formation in *S. aureus* and *S. epidermidis* (96, 104).

IN VIVO ROLES OF STAPHYLOCOCCAL BIOFILM FACTORS

The recognition that microbes grow in biofilms in nature as well as during many types of infection revolutionized microbiology in the second half of the previous century. However, in comparison to the wealth of *in vitro* biofilm studies, biofilm microbiology has not yet widely included *in vivo* experimentation to understand mechanisms of biofilm-associated infection and determine whether general principles established *in vitro* have *in vivo* relevance. In part, this is due to the problem that animal models for biofilm-associated lung infection by most biofilm researchers' favorite model organism, *P. aeruginosa*, are extremely challenging. The situation is somewhat better for staphylococcal biofilm-associated infection, because catheter infections can quite easily be mimicked by placing a plastic device under the skin of test animals, such as mice or rabbits. Furthermore, more sophisticated models of biofilm-associated staphylococcal endocarditis are available.

Staphylococcal biofilm infection was originally associated mainly with *S. epidermidis*. Biochemical characterization of PIA and genetic approaches to find biofilm-related factors using transposon mutagenesis of *S. epidermidis* then resulted in the identification of the *ica* and *atlE* loci as important for matrix formation or initial adherence, respectively, during *in vitro* biofilm formation (28, 40). For that reason, these were the factors first assayed in animal models of biofilm-associated infection. An isogenic transposon mutant in the *ica* genes (*S. epidermidis* M10) was tested for the impact of PIA production on infection outcome in a mouse model of device-associated infection, using a subcutaneously implanted catheter, and a rat CVC-associated infection model (105, 106). In the mouse subcutaneous catheter model, the wild type produced significantly more biofilm on the catheter and a more pronounced abscess (105). In the rat model, more animals challenged with the wild-type strain developed CVC-associated infection than did those challenged with the *ica*-negative M10 strain (106). The impact of PIA and AtlE were also assayed in a different strain background in the rat CVC-associated model. Both PIA and AtlE had a significant impact on biofilm formation on the device as well as on associated bacteremia (107). Interestingly, later it was also shown that the deacetylation of PIA by IcaB is crucial for the development of biofilm-associated infection using a mouse subcutaneous catheter model (38). As for *S. aureus*, there is no direct evidence from animal infection models that would suggest a key role of PIA in biofilm-associated infection. Furthermore, one study showed that the *S. aureus* homologue of AtlE, the major autolysin Atl, does not impact biofilm-associated infection (108). These results reflect *in vitro* findings indicating that biofilm formation in *S. aureus* is mainly dependent on other factors.

Biofilm formation in *S. aureus* is believed to depend on surface proteins in a majority of strains. However, only for very few surface proteins has an impact on biofilm-associated infection been directly demonstrated using biofilm-associated infection models and deletion mutants. These include *S. aureus* Bap and protein A (Spa), for which a significant impact on pathogenesis in mouse catheter implant infection models was found (63, 109). For some other surface proteins, there is some circumstantial evidence for roles in *in vivo* biofilm formation that is derived from using antibodies. For example, human IgG recognizing ClfA was protective in a rabbit endocarditis model (110). As for *S. epidermidis*, the difficulty in producing isogenic deletion mutants has hampered research along those lines, but circumstantial evidence obtained by heterologous expression has been obtained, indicating a role of the surface protein SdrF, which is produced by a subset of *S. epidermidis* strains (111), in the initiation of experimental device driveline-related infection in mice (112). In general, the paucity of experimental *in vivo* evidence underlining the importance of surface proteins in biofilm-associated infection may be due to their pronounced functional redundancy. More recently, an *ex vivo* approach was taken to investigate biofilm formation during PJI. Dastgheyb et al. used synovial fluid obtained during surgery from uninfected human joints. Using the Nebraska transposon bank, which contains a copy of every nonessential gene in *S. aureus*, they identified ClfA, ClfB, FnBPA, and FnBPB, as well as characteristically low Agr activity and PSM concentration in synovial fluid as driving forces of the extensive formation of antibiotic-resistant biofilms seen in PJI (113–115).

In vivo information on biofilm structuring factors is available for the PSMβ peptides of *S. epidermidis*, which have been shown to drive dispersal and systemic dissemination from an implanted device in a mouse model (47). Mutants in the *psmα*, *psmβ*, or *hld* (encoding δ-toxin, now classified as a PSM) loci of *S. aureus* show the same effect, with that of a triple *psmαβhld* mutant being comparable to that of an *agr* mutant (74). However, at least in the case of α-type PSMs (PSMα peptides, δ-toxin), these outcomes may be influenced also by the cytolysis-mediated role of PSMs in immune evasion (116). Which role proteases play in biofilm-associated infection remains largely undefined. With proteases presumably having multiple roles in infection, their specific role in *in vivo* biofilms is difficult to define. In a recent study using the USA300 strain LAC, a deletion mutant in all ten protease genes did not reveal a significantly changed bacterial load on the device in experimental implanted device infection in mice (117). Similarly, *S. aureus nuc1* and *nuc2* mutants do not have a significant *in vivo* effect during biofilm-associated infection (85), casting doubt also on the role of eDNA in *in vivo* biofilms.

STAPHYLOCOCCAL BIOFILM FORMATION AS AN IMMUNE EVASION AND ANTIBIOTIC RESISTANCE MECHANISM

In vivo biofilm formation is subject to several factors that are specifically due to the *in vivo* environment, such as, most notably, interaction with innate host defenses, in addition to interaction with antibiotics during antibiotic therapy. Innate

host defenses consist primarily of phagocytes, such as neutrophils and macrophages, and AMPs, which are a part of the intracellular killing mechanisms phagocytes employ (118). AMPs are also produced in the skin, where they are secreted to control skin-colonizing or -infecting bacteria (119). Secreted AMPs may thus also form part of the host's defenses on subcutaneously placed catheters (Fig. 3).

Biofilm formation, *per se*, has long been known to provide resistance to attacks by phagocytes, which are impaired in penetrating through the biofilm matrix (120, 121). Similarly, the biofilm matrix may be impermeable to certain AMPs and antibiotics. On the other hand, some can readily penetrate, which is likely dependent on the AMP's or antibiotic's chemical features (122). It has been estimated that biofilms provide 10 to 1,000 times increased tolerance to antibiotic effects (123). One antibiotic that has been shown to be exceptionally active against biofilms and is often proposed to treat biofilm infections is rifampin (124). However, resistance to rifampin can develop quickly during treatment (125). Interestingly, recent *in vivo* research indicates that specific other antibiotics, such as ceftaroline, are more active against *S. aureus* biofilms than those commonly used in the clinical setting (126). Decreased antibiofilm efficacy of antibiotics whose penetration is not decreased is due to the specific physiology of cells in a biofilm. For example, the reduced proliferative and metabolic activity of biofilm cells makes them more tolerant to antibiotics that target those processes, such as cell wall-targeting antibiotics (e.g., beta-lactams) or antibiotics that target DNA or protein synthesis (127). Finally, biofilms have been shown to contain more so-called persisters, dormant cells which are indifferent to antibiotics, compared to the majority of the bacterial population, an effect that enables this subset of cells to withstand antibiotic concentrations far above the MIC (128).

In addition to those general effects, staphylococcal biofilms show specific alterations in physiology compared to planktonic cells, which dampen inflammation and the innate immune response. First, Agr as a regulator of many toxins, which have proinflammatory effects, shows limited expression in biofilms (74, 90). However, whether this effect has *in vivo* consequences remains to be shown directly. In support of that notion, many *S. aureus* and *S. epidermidis* isolates obtained from chronic and device-associated infections are functionally Agr-negative (90, 129). It appears as if phenotypically or genotypically Agr-negative isolates have selective advantages during chronic infection, of which the mechanistic underpinnings still need to be elucidated.

Second, *S. aureus* biofilms have been shown to reduce inflammatory effects, such as phagocyte influx. Specifically, *S. aureus* biofilms in murine biofilm-associated infection appear to skew the immune response away from a microbicidal phenotype by circumventing locus-specific pathogen recognition pathways (130)—a finding in line with reduced Agr activity. Furthermore, during experimental PJI, *S. aureus* biofilms stimulate the influx of myeloid-derived suppressor cells, resulting in reduced phagocyte influx (131), and human PJIs are associated with myeloid-derived suppressor cells (132). Finally, cyclic di-AMP, released by cell lysis, has been shown to stimulate a type I interferon-mediated anti-inflammatory effect in biofilms (133). However, it is not clear whether these immune evasion mechanisms are specific to a biofilm infection as opposed to other *S. aureus* infection

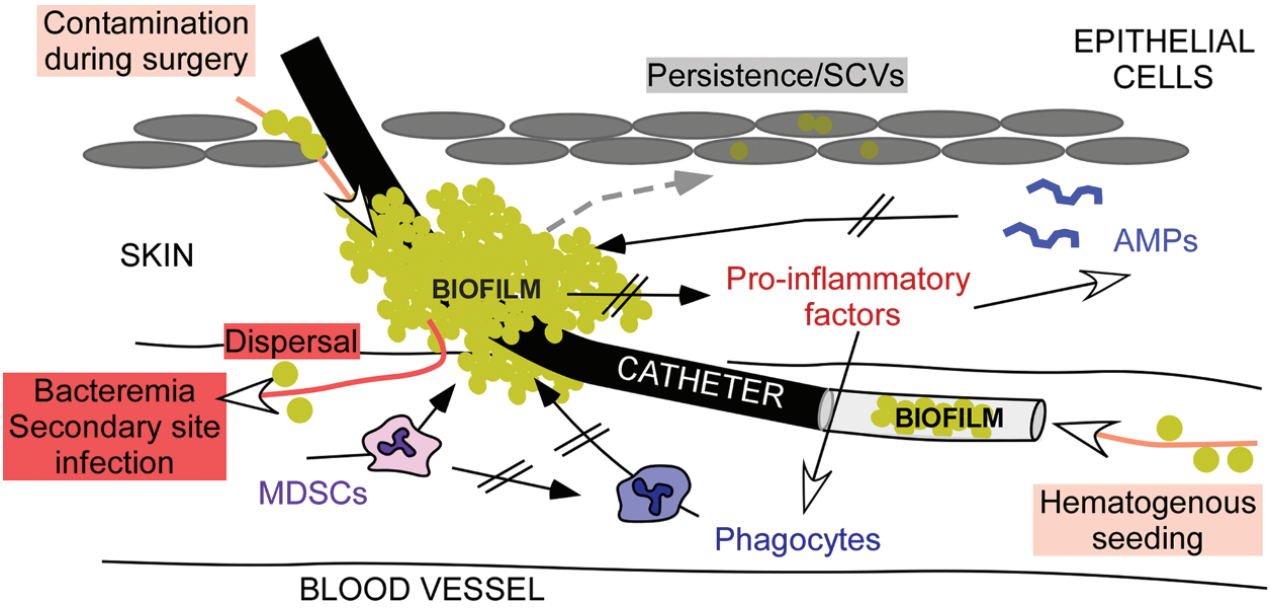


FIGURE 3 Staphylococcal biofilm-associated infection on medical devices. Mechanisms underlying staphylococcal device-associated infection are depicted in an exemplary fashion for an intravascular catheter-associated biofilm. Many of those are still hypothetical. Biofilm formation on a catheter may originate from bacteria introduced as a contamination during surgery/catheter insertion; those are believed to initiate biofilms on the catheter outside. Alternatively, biofilms in the lumen of the catheter can originate from bacteremia and other infection sites due to hematogenous seeding from those sites. Compared to planktonic growth, biofilms secrete fewer proinflammatory factors, which normally cause considerable responses by innate host defenses, such as phagocyte influx and AMP production. In addition to eliciting fewer such responses, the biofilm matrix provides a shelter from AMPs and phagocyte intrusion. *S. aureus* biofilms also attract myeloid-derived suppressor cells (MDSCs), which add to decreasing inflammatory responses, particularly phagocyte influx. Finally, internalization of bacteria, for example, by epithelial cells, can produce SCVs, which are prone to persist and cause recurrent infections.

types, or in the case of cyclic di-AMP release, occur *in vivo*. Somewhat in contrast, it has also been shown that in a long-lasting murine biofilm infection, downregulated responses in regulatory T cells (Tregs) during later stages of the infection provide for an unrestrained inflammatory response (134).

Some prominent biofilm matrix molecules have been described to promote immune evasion mechanisms independently of their role in matrix formation. PIA in its deacetylated form provides resistance to neutrophil phagocytosis and killing by AMPs (38). Possibly, this activity is responsible for the effects PIA has shown in non-biofilm-associated experimental infection (135). The *S. aureus* Bap protein, in addition to promoting adhesion, prevents *S. aureus* internalization by epithelial cells (136). This contrasts with fibronectin-binding proteins, which have long been known to facilitate such internalization (137). Which role internalization and potential intercellular persistence in nonprofessional phagocytic cells have in biofilm-associated infection still needs to be investigated in more depth. There is evidence indicating that the formation of small-colony variants, cells with severely reduced metabolism, contribute considerably to such persistence (138).

THERAPEUTIC STRATEGIES FOR STAPHYLOCOCCAL BIOFILM-ASSOCIATED INFECTIONS

Biofilm infections remain extremely resistant to antibiotic therapy. Currently, there are no alternatives, and excision and replacement of an infected device are often the only remedy. In the following, some strategies to develop alternative biofilm therapeutics will be presented. Most of them are still in the preclinical stage.

Antimicrobial Peptides

With most antibiotics not being efficacious against biofilm cells due to their mode of action that only targets active cell processes, one strategy focuses on developing bactericidal antibacterial compounds. In the center of this approach are AMPs, most of which work by forming pores in the bacterial membrane (139). However, AMPs are subject to a series of resistance mechanisms that are based on the barrier function of the biofilm matrix in addition to more specific mechanisms, such as efflux pumps and AMP repulsion. Furthermore, staphylococci have learned to sense the presence of AMPs during their co-evolution with humans and react with efficient countermeasures (140, 141).

Material Alterations

Many attempts have been made to deal with the adhesion of bacteria to medical devices by altering the surface to lower adhesive features or by coating the device surface with antibacterial compounds (142). The latter are mostly antibacterial metals, such as silver or copper, in addition to antibiotics or AMPs. Such optimized devices, mainly catheters, are in clinical use and have had some success; however, biofilm formation still happens, and it appears impossible to completely inhibit adhesion by altering device surface composition. Unfortunately, once adhesion is accomplished, altered device surfaces do not impact further biofilm growth. One reason for the limited success of altering device surfaces is the abovementioned fact that they are prone to being covered by human matrix proteins, to which bacteria attach independently of the device surface.

Vaccines

So far, there is no working vaccine for *S. aureus*, even for non-biofilm-associated infections; many have argued that development of an *S. aureus* vaccine is inherently difficult (143). It has been proposed that vaccine approaches for biofilm infections be designed depending on which antigens are present in the biofilm mode of growth (144), but no such attempts have been actively pursued. There appears to have been some preclinical success with anti-PIA antibodies, as shown in an *S. aureus* periprosthetic osteomyelitis rat model (145). As for *S. epidermidis*, there has been success using immunization with the surface protein SesC in a subcutaneous foreign body in a rat model (146). However, vaccination against *S. epidermidis* as a beneficial part of the skin microbiota is generally debatable. Furthermore, the many positive results from antistaphylococcal vaccine experiments in animals have so far never resulted in a vaccine that works in humans.

Bacteriophages and Lysins

Bacteriophage therapy is controversial for several reasons, but it has the advantage of having a bactericidal mechanism and thus not being subject to the efficacy-lowering bacterial physiology of biofilms or persister cells (147). Then again, cell surface-located bacteriophage receptor molecules may not be accessible in the biofilm matrix. However, in several cases, bacteriophages have proven efficacious against *in vitro* staphylococcal biofilms. For example, the bacteriophages φIPLA-RODI and φIPLA-C1C reduced biofilms of *S. aureus* and *S. epidermidis in vitro* (148). Bacteriophage K is also often proposed as an antistaphylococcal biofilm agent (149, 150). Furthermore, bacteriophage lysins may represent a means to enzymatically disrupt biofilms, such as bacteriophage lysin CF-301 (151, 152). *In vivo* efficacy of bacteriophages or bacteriophage lysins against *Staphylococcus* biofilm-associated infection still needs to be demonstrated.

Biofilm-Degrading Enzymes

The idea to enzymatically disrupt staphylococci in biofilms for treatment is not limited to bacteriophage-derived lysins. Lysostaphin, a lysin produced by *Staphylococcus staphylolyticus*, is used in the laboratory to digest staphylococcal cells and has been investigated intensely as an antistaphylococcal agent (153). It has also been shown to work against *in vitro S. aureus* biofilms, an effect that could be enhanced when used together with antibiotics (154–156). Notably, lysostaphin eradicated *S. aureus* in a catheter-associated infection model in mice when applied through the catheters (157). *S. epidermidis* shows increased resistance to lysostaphin due to the different composition of the pentapeptide bridge in peptidoglycan, the target of lysostaphin (158). Another enzyme-based potential therapeutic strategy consists of applying matrix-degrading enzymes. Dispersin B is a PIAse produced by *Actinobacillus actinomycetemcomitans* (159). It has been shown to degrade staphylococcal biofilms *in vitro* that are dependent on PIA, thus, predominantly *S. epidermidis* biofilms (160, 161). Other matrix-degrading enzymes, such as proteases, have also been proposed to treat biofilm infections (78, 154), a strategy obviously also dependent on the mode of biofilm matrix formation. The *in vivo* efficacy of biofilm matrix-degrading enzymes still needs to be shown.

Quorum-Sensing Blockers

Finally, quorum-sensing blockers are often proposed for biofilm infections; however, due to the biofilm-structuring

and -dispersing effect of the Agr quorum-sensing system, this approach appears counterproductive in staphylococci. Nevertheless, one needs to consider that severe and life-threatening conditions, such as bloodstream infections, often only arise after dispersal from a biofilm. For those, quorum-sensing blockers or other approaches targeting aggressive virulence factors, such as toxins, may be invaluable. Anti-toxin antibodies show great promise in that regard (162).

CONCLUDING REMARKS

Biofilm formation in staphylococci remains a serious clinical problem due to the enormous and continued difficulties in treating staphylococcal biofilm-associated infections. Therefore, mechanisms of staphylococcal biofilm formation have received much attention over the last decades, and many important results, for example on staphylococcal biofilm genetics and regulation, have been achieved using *in vitro* setups of biofilm development. However, it has become clear that biofilm-related *in vitro* observations are often not transferable to the *in vivo* level, with the *in vivo* situation differing in substantial aspects, such as the interaction of host matrix proteins and immune defenses. With the interest in staphylococcal biofilm formation clearly stemming from the involvement of staphylococci in infections, there should be a more pronounced focus on the in-depth evaluation of biofilm formation in an *in vivo* context in the future. This is equally imperative in preclinical research on potential antibiofilm therapeutics, which currently often lacks this crucial aspect. Such approaches should contain more animal models, which can be optimized to better reflect human biofilm-associated infection, and *ex vivo* research using human cells, particularly immune cells. The increasing recent interest in the genetics and immunology underlying the asymptomatic colonization of human skin and mucous surfaces, which can be seen as proceeding in a biofilm-like fashion, will also be of great value to gain a comprehensive understanding of staphylococcal biofilm physiology in the commensal and infectious states.

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Fulminant Staphylococcal Infections

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44

INTRODUCTION

Fulminant infections are not clearly defined in the field of staphylococcal infections. According to Wikipedia, "fulminant" is a descriptor, especially used in the field of medicine, for any event or process that occurs suddenly and escalates quickly and is intense and severe to the point of lethality, i.e., it has an explosive character. The word comes from Latin *fulminare*, to strike with lightning. For staphylococcal infections in which, despite the fact that *Staphylococcus aureus* is considered a commensal, progression may be devastating in many circumstances, it appears pertinent to add to this definition the notion of an unexpected event leading to a "thunderstorm in a quiet blue sky." Hence, fulminant staphylococcal infection indicates an explosive, intense, and very severe infection occurring in a patient whose previous condition and antecedent would never have suggested any anticipation of life-threatening development. Considering this definition, fulminant is an adjective that could be associated with several staphylococcal infections, including necrotizing pneumonia, necrotizing fasciitis, and to some extent toxic shock syndrome and infective endocarditis (IE). In the three former diseases, toxin production plays a major role, whereas in the latter (fulminant presentation of IE), association with any particular toxinic profile has never been demonstrated.

STAPHYLOCOCCAL NECROTIZING PNEUMONIA

The role of *S. aureus* in pneumonia has been known for decades, but staphylococcal pneumonias are rare, representing only 2% of community-acquired pneumonia in adults (1). Until the end of the 20th century, *S. aureus* was thought to be responsible for community-acquired pneumonia in two main circumstances: first, in respiratory diseases of adults, *S. aureus* pneumonia is observed mainly in the elderly and/or associated with underlying conditions such as immunodepression, cancer, diabetes, or chronic respiratory disease. *S. aureus* is also a major cause of exacerbation in cystic fibrosis. Moreover, *S. aureus* is responsible for 20 to 30% of health care-associated respiratory infections, particularly ventilator-associated pneumonias (2). In such situations, no specific staphylococcal virulence factors are clearly associated with staphylococcal pneumonia, and the main issue for the clinician is to manage methicillin-resistance and multidrug resistance, which are common in hospital-acquired cases. These staphylococcal pneumonias are often severe, but it is always difficult to determine the role of the intrinsic virulence of *S. aureus* or the patient's underlying condition in the potentially unfavorable evolution. The second presentation of severe staphylococcal pneumonia used to be observed only in very young children, aged less than 1 year (3). This disease was commonly described as a bilateral multifocal round-shaped pneumonia with bullous formations in lung parenchyma, leading sometimes to the appellation "bullous pneumonia," considered in children as pathognomonic of *S. aureus* (4). Pleural effusion was common, as was combined pleural empyema and pneumothorax secondary to rupture of bullous lesions in the pleural cavity (5). This severe presentation was associated with a high case-fatality rate, from 10 to 23% (3, 6). Although most cases in the years 1960 to 1970 were associated with the phage type 80-81, now known to encompass strains encoding the Panton-Valentine leukocidin (PVL) (7), no specific staphylococcal virulence factor was identified in staphylococcal pleuro-pneumonia of young children (7). For partially unknown reasons, staphylococcal pneumonia almost disappeared in children in developed countries at the end of the 20th century, whereas it was still commonly described in low-income areas. Nevertheless, despite the undeniable severity of staphylococcal pneumonia, fulminant presentations as defined above were considered very rare, and evolution toward a fatal outcome was usually attributed to underlying conditions rather than to staphylococcal virulence. However, case reports and even small series of fulminant staphylococcal pneumonia were sporadically reported in older children and young adults, but the basis of the severity in these cases has remained unknown.

The first step in understanding how virulence factors impact the severity of staphylococcal pneumonia was reached in 1999, when Lina et al., studying a collection of 172 strains referred to the French National Reference Center for Staphylococci (*Centre National de Référence des Staphylocoques*), showed that 85% of the strains isolated from severe community-acquired pneumonia harbored *luk*S-PV and *luk*F-PV genes, together encoding a rare (less than 5% of strains in Europe) (8) staphylococcal exotoxin: the PVL (9). The clinical presentation of these pneumonias was very unusual in view of their severity (lethality, >75%) but also in view of their epidemiology and symptoms: the cases were observed in young immunocompetent patients but not in very young ones, and most of the patients showed signs of pulmonary necrosis with massive airway bleeding. The complete clinical and biological characterization of this particular form of staphylococcal pneumonia in humans and

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its link to the presence of *pvl* genes was achieved by a prospective comparison between PVL-positive and PVL-negative strains causing community-acquired pneumonia (10). As prospective surveillance continued in France, features associated with severity and mortality were identified (11). Hence, a rare but severe presentation of staphylococcal pneumonia was described and named "staphylococcal necrotizing pneumonia" (SNP). The main clinical characteristics of this disease can be summarized as:

- Occurrence in most cases in young immunocompetent patients, without an antecedent or underlying condition.
- Preceding flu-like illness, with documentation of influenza virus infection in some cases.
- Rapidly progressive bilateral pneumonia, without bullae, evolving toward severe hypoxemia and pulmonary edema and leading to acute respiratory distress syndrome.
- High frequency of airway hemorrhages, massive in some cases.
- Initial leukopenia.
- High case-fatality rate despite young age and absence of underlying preconditions. For the fatal cases, death occurs promptly after the onset of the disease (median survival time, 4 days).
- Unusual pathologic findings on lung necropsy: necrotic destruction of the entire respiratory epithelium, from larynx to alveoli in some cases, without polymorphonuclear infiltrate. Diffuse alveolar damage with hemorrhagic infiltration.

In the comparative study of community-acquired staphylococcal pneumonia based on toxin profiling (12), all the cases matching the above description of necrotizing pneumonia were caused by PVL-positive strains, whereas bilateral pneumonia caused by PVL-negative strains was commonly seen in the elderly and was associated with underlying preconditions. In PVL-negative cases, fulminant evolution was not observed, and leukopenia, airway bleeding, and signs of lung necrosis were absent. These differences suggest that PVL and likely other pore-forming toxins play a central role in the pathophysiology of necrotizing pneumonia.

Since its first description in 2002, (SNP) has been reported worldwide (13–17), and in all cases, the causal strains, despite their genetic diversity and lack of clonality, harbored the *pvl* genes on various prophages (reviewed in reference 18), which is presumably responsible for their dissemination among unrelated strains. Moreover, the finding that previously published cases of fulminant staphylococcal pneumonia had clinical presentations very similar to the "typical" features of SNP (19, 20) may indicate that this disease, although not formally recognized, had been encountered for many years and should not be considered an emerging entity. A particular situation concerning SNP is observed in the United States, where the description of SNP coincided with the emergence of the USA300 community-associated methicillin-resistant *S. aureus* (CA-MRSA) lineage; indeed, almost all the reported cases in the United States were due to USA300 (17, 21, 22). This particular lineage restriction in the United States led to confusion in the understanding of the determinant of severity associated with SNP. First, methicillin resistance was thought to be a major determinant of severity, presumably by inducing a delay in initiation of appropriate antibiotics. However, it has now been demonstrated that methicillin resistance *per se* is not associated with higher mortality in SNP (23) and, despite probable overreporting of cases due to MRSA, more than 70% of the cases of SNP in Europe are due to methicillin-susceptible *S. aureus* strains belonging to distinct lineages. Second, the restriction to USA300 leads to an overestimation of the role of this clone's specific virulence factors. An enhanced production of alpha-hemolysin (24, 25) or phenol-soluble modulin (25, 26) and the presence of Arginine Catabolic Mobile Element (ACME), which is strongly associated with USA300 (27), were therefore suspected to be major determinants of severity in SNP, minimizing the potential role of other virulence factors such as PVL (28). However, the low prevalence of *pvl* genes in strains circulating in Europe (8) and the absence of clonality of infection strains presented a unique opportunity to determine whether or not PVL was associated with severity and poor outcome of staphylococcal pneumonia (29). Apart from the epidemiological association of PVL with the severity of community-acquired pneumonia (10, 13), other features suggest that PVL contributes to the severity of SNP: interestingly, the most typical clinical and biological features associated with SNP, i.e., leucopenia and airway hemorrhages, although they are not fully specific, are strongly associated both with its severity and with the known biological effect of PVL. Hence, leucopenia is probably the result of the lytic effect of leukotoxins on polymorphonuclear (PMN) cells. Leucopenia is associated with mortality in various studies (11, 16, 17), and we have shown that the association of leukopenia with death is "dose-dependent": when the total leucocyte count was above 3 giga elements/liter at hospital admission, survival was observed in >75% of cases, whereas less than 20% of the patients survived when their leucocyte counts were <3 giga elements/liter (11). Airway hemorrhage is associated with poor prognosis too and is a direct consequence of the massive necrosis of the respiratory epithelium, likely due to the direct and indirect leukotoxic properties of PVL and not observed in other staphylococcal pneumonias.

Animal models are required to fully understand the complex interactions between *S. aureus*' virulence factors, host susceptibility, and severity. The model should reproduce the phenotype observed in humans with necrotic hemorrhagic lesions and pulmonary edema. The first model of necrotizing pneumonia was developed in BALB/c mice with nasal instillation of high inoculum (30). Necrotic lesions were observed, but survival of the infected mice was impaired only when overexpressing *pvl* on multicopy plasmids. Other studies in mice failed to demonstrate any role for PVL in severity and even to induce necrotic lesions (24). It is now widely accepted that PVL is poorly active toward murine leucocytes while being highly cytotoxic toward human and rabbit leucocytes (31). The identification of human C5aR and CD45 as the receptors for LukS-PV and LukF-PV, respectively, at the surface of phagocytes offered a molecular explanation for the lack of relevance of mouse models for the study of PVL-associated pathogenesis (31–33). Indeed, due to polymorphisms between mice and humans, neither LukS-PV nor LukF-PV bind to murine C5aR1 and murine CD45, resulting in PVL being inactive in mice. In contrast to murine C5aR1, rabbit C5aR1 is compatible with LukS-PV, and rabbit PMN cells are highly susceptible to PVL, making this species a relevant animal model for studying the role of PVL in SNP.

Diep et al. developed a convincing rabbit pneumonia model using inocula 10-fold lower than those previously used in mice (34). In this model, nasal instillation of a PVL-positive CA-MRSA USA300 strain is associated with activation of PMN cells and alveolar macrophages, leading to the direct destruction of PMN cells by PVL and intense production of inflammatory mediators, particularly interleukin 8

(IL-8). These mediators attract more and more PMN cells, whose PVL-induced lysis and degranulation liberates proteases and oxidative products. These toxic components provoke vasodilatation, pulmonary edema, and cellular lesions, leading to hemorrhagic necrosis of the pulmonary epithelium (34), mimicking the pathology observed in human necropsies. The central role of PMN cells in the pathogenesis of lesions observed in SNP is emphasized in this model by the absence of Il-8 production, pulmonary edema, and necrotic lesions in neutropenic rabbits exposed to a high concentration of PVL (34). Alveolar macrophages and lung epithelial cells may play a key role in the initiation of inflammation since PVL not only lyses PMN cells directly but also triggers IL-1β release in human alveolar macrophages, which in turn stimulates the massive secretion of the neutrophil-attracting chemokines, IL-8 and monocyte chemotactic protein-1, by lung epithelial cells (35). Interestingly, this inflammatory pathway is amplified by the release of pathogen- and damage-associated molecular patterns from dying neutrophils, possibly explaining the fulminant nature of SNP (36). Of note, the PSMalpha3, beta-, gamma-, and delta-hemolysins and LukDE synergize with PVL to amplify IL-1β release, indicating that these factors cooperate with PVL to trigger inflammation (35). Another issue in deciphering the complex pathophysiology of necrotizing pneumonia is to determine the putative role of influenza virus in the infection. Epidemiological links appear in many studies (11, 17, 22, 37, 38), but interactions between both pathogens remain hypothetical. However, two distinct mechanisms are probably involved: first, influenza infection may favor *S. aureus* attachment, causing epithelial lesions which expose basal layers of mucosae with matrix components to which *S. aureus* adheres (39, 40). Second, animal experiments indicate that influenza may play a role in PMN cell recruitment in the lungs, triggering inflammatory signals (41, 42). Considering the central role of PMN in necrosis induction and the near absence of PMN cells in normal lungs, it is tempting to hypothesize that this PMN recruitment is a key point for necrosis induction mediated by PVL (41).

Altogether, fulminant *S. aureus* pneumonia occurring in patients without known predisposing factors is strongly associated with PVL-producing strains, and most *in vitro* and animal model data reveal that this observation is beyond simple association. However, two major issues remain to be addressed: first, even if the prevalence of the *pvl* genes is low in the general population (8), the carriage rate of *S. aureus* in humans is sufficiently important so that millions of people are exposed to PVL-producing strains; yet the incidence of fulminant *S. aureus* pneumonia remains extremely low, i.e., 30 to 50 cases per year in a 60-million-inhabitant country like France in which there should be roughly 1 million carriers of PVL-positive *S. aureus* (CNR annual report, http://cnr-staphylocoques.univ-lyon1.fr/). It is thus tempting to speculate that factors other than PVL and/or a genetic predisposition to PVL are required for the development of these fulminant presentations of pneumonia. Susceptibility to *S. aureus* infections can be linked to specific human mutations. Rare mutations affecting the Toll-like receptor/IL-1 receptor pathway, the IL-17 pathway, and the NADPH oxidase pathway are strongly associated with recurring or severe *S. aureus* infections (for review see reference 43). Two genome-wide association studies have been performed to identify genes influencing *S. aureus* diseases, highlighting several candidates (44, 45). However, so far, no specific polymorphism has been associated with the development of fulminant *S. aureus* pneumonia. The contributing role of other virulence factors and toxins is another issue to be explored. Indeed, not all fulminant *S. aureus* necrotizing pneumonias are caused by PVL-producing strains, suggesting the involvement of other potentially necrotizing toxins such as alpha-toxin, gamma-hemolysins, or LukGH that are present in most *S. aureus* strains (8, 46). Indeed, alpha toxin is most likely the major pore-forming toxin in *S. aureus* and is known to contribute to the severity of *S. aureus* pneumonia and other infections (47), while the gamma-toxin HlgCB, which targets the C5aR on myeloid cells, as does PVL (48), could be a major contributor. Under what circumstances these toxins could determine fulminant pneumonia remains to be explored, but large variations in the expression of these toxins *in vivo* (49) is an important parameter to be considered.

The existence of staphylococcal lung infection, due to a proven "toxinic" strain, which secretes a large amount of PVL or some other pore-forming toxin, is not necessarily associated with a fulminant course even when clinical evidence of necrosis is observed. Hence, especially in young children, PVL-positive strains are predominant in staphylococcal pneumonia, in both the United States and in Europe and are associated with necrotic abscesses and pleural effusion (50, 51), but in these studies, symptoms of fulminant necrotizing pneumonia were rarely observed and mortality was <5%. In fact, the description of most of the cases matches the classic presentation of staphylococcal pleuro-pneumonia of young children, often caused by the notorious 80-81 epidemic strain, which probably produced PVL, and an unresolved challenge is to understand why in some cases, PVL-positive *S. aureus* lung infection provokes fulminant lung necrosis, whereas in other cases, necrosis is limited to abscess formation, and the outcome is far less devastating. Differential levels of virulence factor expression including that of the core-genome-encoded pore-forming toxins or patient heterogeneity regarding toxin susceptibility may contribute to these differences, but these explanations remain largely hypothetical. Another hypothesis involves the pathophysiology of lung infections. Lung invasion can be secondary to the hematogenous spreading of septic emboli from a distant infection focus; however, *S. aureus* can also directly reach the lung via the inhalation route. In the first case, necrotic effects would involve predominantly conjunctive lung tissue, and the necrotic lesion would be a localized abscess; in cases of inhalation, necrotic lesions should be more diffuse and should involve the respiratory epithelium directly, leading to major and prompt alteration of respiratory function. Currently, this distinction is purely theoretical, although some evidence from the rabbit model favors this pathophysiological model (52).

TOXIC SHOCK SYNDROME

Toxic shock syndrome (TSS) is another rare disease that matches (in some instances) our definition of fulminant staphylococcal infection. TSS was described in 1978 in seven children aged 8 to 17 years (53). Despite the severity of the disease, *S. aureus* was isolated only from mucosal sites, while blood, cerebrospinal fluid, urine, and other potential infectious sites remained sterile, leading a toxin-mediated phenomenon to be suspected. Despite the fact that similar cases had been previously described, J.K. Todd was the first to describe these cases as a specific clinical entity. A few years after the initial description, the United States faced an "outbreak" of TSS in young women who presented typical and often severe forms of TSS during their menses (54–56). This presentation was identified as menstrual TSS (mTSS)

and was initially attributed to the use of hyper-absorbent tampons, but it became rapidly apparent that all kinds of tampons could be involved (57). More recently, mTSS was associated with other devices, such as menstrual cups (58). After the withdrawal from the market of the incriminated tampons and information campaigns about this syndrome in the United States, the incidence of mTSS dramatically fell (although mTSS did not disappear), and TSS not associated with menstruation was therefore "rediscovered" (59). The nonmenstrual forms of TSS (nmTSS) are quite similar in their clinical presentation but lead to higher mortality rates (60). nmTSS are usually associated with a staphylococcal deep-seated focus of infection (61), whereas menstrual cases are associated with vaginal carriage (60).

The clinical features of TSS have been remarkably consistent since the initial description and are associated with several nonspecific features, the combination of which leads to the diagnosis. TSS is thus diagnosed in the face of a rapidly deteriorating condition associate with an infectious syndrome, with muco-cutaneous involvement and hemodynamic failure (61). At the initial stage, the patient presents with a flu-like syndrome associated with fever, headache, chills and muscle pain, sore throat, and often diarrhea (62). Even if they are observed in a young woman during menses, none of these symptoms nor their association are specific, and although not clearly documented, some cases spontaneously resolve by the end of the menstruation and the removal of the tampon (Y. Gillet, unpublished data). A few hours after, in a case progressing toward true TSS, the patient presents with a generalized cutaneous rash, which is sometimes described as "sunburn-like," but even if it may be itchy, the rash is usually not painful (63). The clinical aspect of the cutaneous eruption is not specific but usually involves the extremities with local edema and redness. Such involvement of palms and soles, unusual in other diseases (i.e., in viral rashes), may point toward the diagnosis of TSS. Finally, if the patient survives, rash is followed 10 to 15 days after the onset of the disease by a spectacular desquamation typically involving the whole palms and soles. Concomitant with the rash, mucosal involvement is constant but may be delayed a few days. Lips are dry and bright red, the tongue is red with a "strawberry" aspect, and the eyelids become inflammatory with a bilateral conjunctivitis without purulent exudate (61, 64). Genital mucosae are inflammatory too, usually without purulent discharge. Simultaneously or rapidly after the onset of the rash, the patient's general status worsens and hemodynamic failure appears. As with other infectious shock syndromes, the mechanisms of hypotension are multiple: dehydration due to diarrhea, hypovolemia caused by capillary leakage, and vasodilatation and lowering of cardiac output secondary to direct cardiac dysfunction (63). In the most severe cases, the physiological compensation for circulatory insufficiency may provoke intense vasoconstriction of the skin, leading the erythematous eruption to be unapparent. Diagnosis is particularly difficult in such situations and is often delayed until hemodynamic improvement reveals the rash (Y. Gillet, unpublished data). Moreover, although hypotension is by definition required for the diagnosis of TSS, there are many different clinical presentations in terms of severity, from simple malaise with orthostatic hypotension to refractory shock unresponsive to remediation, even if prompt, and leading to multiorgan failure (61). These potential marked differences in terms of intensity of the hemodynamic failure are unfortunately not always taken into account in studies and may result in misinterpretation, especially in the field of treatment strategies. Obviously, less severe cases are more likely to be self-limited, whereas severely ill patients should benefit more from specific intervention. Therefore, one should keep in mind that the acronym TSS embraces a large diversity of diseases, from self-limited to life-threatening, with only the latter falling into the above definition of fulminant disease. Nevertheless, as mentioned before, none of the symptoms of TSS could be considered specific, and the diagnosis is based on the criteria (65) listed in Table 1.

Interestingly, the presence of *S. aureus* is not required according to the CDC's criteria, since very severe TSS can be observed during *Streptococcus pyogenes* infections with only subtle clinical differences from nmTSS (66).

Despite the above well-defined criteria and the fact that TSS is a reportable disease in many states in the United States (67), the incidence of TSS is not precisely known. Some cases are unrecognized either because they are not severe enough and are self-limited or because of extreme severity leading to prompt death before recognition of the syndrome. However, the incidence in the United States is estimated to be from 1 to 3 per 100,000 inhabitants (56, 59). The incidence is 2-fold higher in women between 13 and 24 years old, and menstrual forms represent 54% of the identified cases (59).

In both mTSS and nmTSS, staphylococcal exotoxins with superantigenic properties are involved. mTSS and, to a lesser extent, nmTSS, are "true toxin-mediated diseases" (toxinoses), meaning that all the observed symptoms can be explained by the action of the exotoxins, without involvement of other staphylococcal virulence factors. *S. aureus* can produce 24 exotoxins with superantigenic properties (68),

TABLE 1 Case definition of staphylococcal TSS

Clinical criteria
Body temperature >38.9°C (102.02°F)
Systolic blood pressure <90 mmHg
Diffuse macular erythroderma
Desquamation (especially of the palms and soles) 1–2 weeks after onset
Involvement of three or more organ systems:
Gastrointestinal (vomiting, diarrhea)
Muscular: severe myalgia or creatine phosphokinase level at least twice the upper limit of normal for laboratory
Mucous membrane hyperemia (vaginal, oral, conjunctival)
Kidney failure (serum creatinine >2 times normal)
Liver inflammation (bilirubin, Aspartate Aminotransferase (AST), or Alanine Aminotransferase (ALT) > 2 times normal)
Low platelet count (platelet count <100,000/mm^3)
Central nervous system involvement (confusion without any focal neurological findings)
Laboratory criteria
Negative results of the following tests, if performed:
Blood, throat, and cerebrospinal fluid cultures for bacteria (besides *S. aureus*)
Serology for *Rickettsia* infection, leptospirosis, and measles
Case classification
Confirmed: All six of the criteria above are met (unless the patient dies before desquamation can occur)
Probable: Five of the six criteria above are met

but most cases of TSS are due to the following four of them, in decreasing order of incidence: toxic shock syndrome toxin-1 (TSST-1), *Staphylococcus* enterotoxin A (SEA), SEC, and SEB (59). The structure of TSST-1 is remarkable by the presence of a peptidic loop dedicated to intraepithelial transportation by a hypothetical carrier and conferring to TSST-1 its unique ability to cross the mucosal barrier (69). Therefore, TSST-1 is associated with >90% of mTSS (59, 60), whereas it is present in only 50% of nmTSS (59). Mucosal lesions induced by staphylococcal cytolysins may also play a role in the transmucosal crossing of TSST-1 (69). Staphylococcal superantigens are active at very low concentrations and induce a polyclonal activation of T lymphocytes by mediating a direct interaction between the major histocompatibility complex class II receptor on antigen presenting cells and the Vβ chain of the T cell receptor on lymphocytes independently of the sequence of the major histocompatibility complex-loaded antigenic peptide. Interestingly, each superantigen is specifically associated with 1 to 8 of the 24 major Vβ chains that can be found in human T cell receptors (70). This "Vβ profile" may be highly specific. For instance, TSST-1 is associated solely with Vβ2 activation, whereas redundancy between different superantigens sharing the same Vβ profile (e.g., SEB and SEC activate lymphocytes sharing the same Vβ chains) is also observed (69). The number of Vβ chains involved has no known consequences in terms of strength of T cell activation. The Vβ specificity of superantigens could therefore be used as a diagnosis tool: by measuring the differential activation of T cells using flow cytometry, it is possible to formally identify the "signature" of the responsible superantigen according to their Vβ chain, allowing the confirmation of atypical cases and the indirect identification of *S. aureus* (71, 72). T cell activation by superantigens bypasses normal major histocompatibility complex-restricted antigen specificity, leading to activation of up to 20% of total T lymphocytes and thus massive cytokine release (73, 74). The resulting inflammatory pathway does not seem to differ markedly from the septic shock pathway and leads to massive production of IL-1, IL-2, IL-6, tumor necrosis factor, gamma interferon, and various chemokines responsible for the enhancement and dysregulation of the inflammatory response (75). The consequences of this cytokine storm are comparable with those observed in septic shock, with even more intense clinical effects due to the initial intensity of the inflammatory response. Moreover, superantigens upregulate the expression of Toll-like receptors 2 and 4 and thus may enhance the inflammatory response by stimulation of these receptors directly by surface-expressed lipoproteins of *S. aureus* and by endotoxins (lipopolysaccharide) translocated from intestinal flora (76, 77). This "two-hit" hypothesis may explain the observed enhanced severity of bacteremic nmTSS compared with mTSS, in which *S. aureus* is absent from the blood circulation.

Nevertheless, conditions that, *in vivo*, lead to such a massive inflammatory response were largely unknown and have been studied essentially for mTSS. *S. aureus* colonization of the vagina is observed in up to 30% of healthy women (78) and may be influenced by various factors including vaginal microbiota (79, 80). Tampons are thought to favor *S. aureus* growth by elevation of the local oxygen concentration and to enhance TSST-1 production (81–83), but considering that 1% to 6.9% of healthy women carry TSST-1$^+$ *S. aureus* during their menses (84, 85), compared to the by far lower incidence of mTSS, it is obvious that TSST-1 production is not sufficient by itself to induce TSS even in favorable conditions. As discussed above for necrotizing pneumonia, various hypotheses are being studied, including genetic predispositions leading to the absence of antibody against the toxins and/or a specific deficiency in immune responses toward superantigens in a context in which 90% of adults are naturally immunized against TSST-1 (84).

STAPHYLOCOCCAL NECROTIZING FASCIITIS

Necrotizing fasciitis (NF) is a rare and severe presentation of skin and soft tissue infection characterized by extensive necrosis of cutaneous and subcutaneous tissue down to the deep fascia and muscles. This infection is often polymicrobial (type 1 NF) with a combination of Gram-positive cocci, Gram-negative rods, and anaerobes. Type 2 NF is usually monomicrobial, classically involving *S. pyogenes*, and is associated with high mortality (86). Recently, *S. aureus*, and especially the CA-MRSA USA300 lineage, has also been recognized as being responsible for type 2 NF (87).

The clinical description of *S. aureus*-associated NF is difficult since most reports are based on retrospective studies. The presentation is thought to be similar to group A *Streptococcus*-associated cases. The classic initial presentation of NF includes severe pain of the skin out of proportion to physical examination findings. Although some patients appear ill, tenderness, erythema, and warm skin are commonly the only signs of early NF. Shock, fever, and altered mental status typically develop during the first 24 to 48 hours of disease progression but are often absent at presentation (88). Local evolution is extremely severe, with uncontrolled extensive necrosis, but due to the deep-seated localization of the necrosis, the overlying skin may be minimally affected, leading sometimes to delayed diagnosis (88). In many streptococcal cases, signs and symptoms of TSS (i.e., erythroderma) are present, whereas TSS symptoms seem less frequent in staphylococcal NF (87). Therefore, staphylococcal NF should be less severe than the group A *Streptococcus*-associated cases, although to the best of our knowledge, no comparative study exists.

Diagnosis and characterization of staphylococcal NF are thus difficult for several reasons: (i) the diagnosis of NF can only be made after surgical debridement reveals pathologic findings indicating necrosis involving fascia and muscles. These findings were confirmed in the 14 cases described in Los Angeles, CA, in 2004 (87) but are not always found in other reports. (ii) In cases of polymicrobial NF, especially when group A *Streptococcus* and *S. aureus* are associated, it is almost impossible to determine which bacterium (or both) is the culprit in terms of severity and, in some cases, it appears possible that one bacterium overwhelms another one, leading to misinterpretation of strain-associated virulence. In relation to this, a recent study revealed that of 35 cases with *S. aureus* and/or Gram-negative bacteria as the only cultured pathogens from swab samples, 21 (60%) had beta-hemolytic *Streptococcus*-confirmed infections and 6 (17%) had probable beta-hemolytic *Streptococcus* disease (89). (iii) *S. aureus* is well known for its ability to induce necrotic skin and soft tissue infection, and PVL-producing strains such as USA300 are associated with primary necrotic cutaneous abscesses (9, 90, 91) involving hair follicles (92). Therefore, severe necrotic skin lesions are common with CA-MRSA (93), and in most cases, the disease description does not fulfill the criteria for necrotizing fasciitis and corresponds to common cutaneous abscesses. Because of these difficulties, staphylococcal NF is less well known than the other fulminant infections described above, but some general differences could be underlined: necrotizing pneumonia and TSS usually occur

in young and previously healthy individuals and appear to be linked to a specific, but to date hypothetical, complex combination of staphylococcal virulence factors and host predisposition. In the case of NF, patients are older (median age, 50 years), and an underlying condition predisposing nonspecifically to bacterial infection is present in most cases. Hence, diabetes is present in 43 to 53% of cases (87, 94), intravenous drug use in 21%, and immunodepression, HIV infection, and cancer each in 7% of cases (87). Thus, the specific virulence profile of the causative staphylococcal strain remains difficult to ascertain in NF.

BACTEREMIA AND INFECTIVE ENDOCARDITIS

S. aureus bacteremia can occasionally present as a fulminant infection, notably when associated with a deep focus of infection. The most frequently reported deep infectious foci are endovascular foci including infective endocarditis (IE) and central nervous system, bone and joint, soft tissue, and intra-abdominal/pelvic infections. *S. aureus* IE occurs in 5 to 25% of *S. aureus* bacteremia (95–98) and is one of the most rapidly progressive etiologies of IE with the worst outcome, often associated with destruction of cardiac valves (99–101). In a study enrolling 2008 consecutive adult bacteremic patients, meningitis (IE, 59%), persistent bacteremia at 48 hours (IE, 25%) and C-reactive protein of >190 mg/liter (IE, 15%) were independently associated with IE (98). The criteria for severe sepsis or septic shock were met in 30% of *S. aureus* bacteremia without IE (overall in-hospital mortality rate, 24%) and in 51% with IE (overall in-hospital mortality rate, 35%).

Numerous *S. aureus* virulence factors (mostly but not exclusively surface-associated proteins) have been shown, from *in vitro* and/or animal studies, to contribute to the pathogenesis of IE. These include clumping factor A-B, fibronectin-binding protein A-B, collagen-binding protein, SdrD/E, the surface factor promoting resistance to oxidative killing, protein A, coagulase, vWFbp (102–107), and superantigenic toxins such as SEA, SEG, and TSST-1 (108, 109). However, with the exception of SEA, which has been specifically associated with septic shock in the course of bacteremia (110), none of these factors have been specifically associated with fulminant progression and worse outcome in either bacteremia or IE in epidemiological studies. The multifactorial nature of *S. aureus* bacteremia severity is reflected in the observation that higher antibody levels against Hla, delta-toxin, PVL, SEC-1, and PSM-α3 may protect against sepsis in patients with invasive *S. aureus* infections (111). Conversely, certain lineages of *S. aureus*, such as clade 3 of CC30, have been associated with attenuated virulence in hematogenous infections (112), likely because of a stop-codon mutation in the major core-genome-encoded pore-forming toxin (alpha-toxin), reduced transcription of the agr effector RNAIII (113), and the expression of a phenol-soluble modulin variant with attenuated toxic and chemotactic activities (114). Interestingly, there is apparently a higher propensity of low-toxicity isolates to cause bacteremia (115).

THERAPEUTIC ASPECTS OF FULMINANT STAPHYLOCOCCAL INFECTIONS

By definition, fulminant staphylococcal infections are potentially lethal and therefore should be managed according to the guidelines of the sepsis survival campaign, i.e., prompt initiation of bactericidal antibiotics, massive fluid remediation, correction of organ failures, and continuous monitoring of objectives (116). However, despite uncertainties about the pathophysiology, fulminant staphylococcal diseases may require specific management due to the involvement of staphylococcal exotoxins. These toxins are produced when bacteria reach a high density, owing to the regulation of their production by quorum-sensing systems. In addition, necrotic lesions prevent adequate antibiotic diffusion at the site of infection. For these two reasons, surgical drainage of any accessible lesions should be systematically undertaken. Moreover, therapeutic approaches targeting toxin production are recommended in TSS (117) despite the lack of strong clinical evidence of efficacy. Furthermore, evidence from animal models and limited clinical series indicates that intravenous immunoglobulins, by their ability to broadly neutralize toxins, have a strong potential to limit casualties in fulminant infections (118, 119). Further, to assess the therapeutic potential of toxin-neutralization strategies in fulminant *S. aureus* infections, studies including larger cohorts and utilizing specific antitoxin monoclonal antibodies (46) remain to be performed. Finally, most fulminant *S. aureus* infections are associated with unleashed inflammation, suggesting that adjunctive immunosuppressive therapies might be beneficial to limit mortality and aftereffects. Yet, based on decades of failed trials involving sepsis, the efficacy of specific immuno-interventions in fulminant *S. aureus* infection remains elusive. Importantly, due to fulminant development of the disease conditions, the choice of an appropriate therapeutic regimen should be made immediately—there is no "second chance." In almost all cases this difficult decision has to be made before any bacteriological documentation, emphasizing the need for advanced knowledge of clinical presentations and severity-associated symptoms to avoid potentially deleterious choices, which are always critical, especially because the diseases are so rare.

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Staphylococcus aureus Colonization of the Human Nose and Interaction with Other Microbiome Members

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45

INTRODUCTION

The human body offers several distinct niches for specific microbiomes, variable consortia of bacterial communities. The skin, for instance, is primarily colonized by members of the genera *Propionibacterium* (now *Cutibacterium*), *Corynebacterium*, and *Staphylococcus* (1). A similar pattern of genera is found in the human nose, which is regarded as a transition zone from the dry skin to the moist, mucoid airways (2). This rather confined area, more specifically, the region from the anterior nasal vestibule to the posterior nasopharyngeal cavity, is the favored colonization site of *Staphylococcus aureus*. While *S. aureus* is a member of the normal nasal microbiome in ca. 30% of the human population, it can also become an aggressive, life-threatening pathogen (3). Nasal carriage is a major risk factor for *S. aureus* infections. Accordingly, *S. aureus* is eradicated from the nose in at-risk patients by treatment with the antibiotic mupirocin. Interestingly, a significant percentage of humans seem never to be colonized by *S. aureus*, for reasons that are currently unknown. Increasing evidence suggests that the composition of the nasal microbiome is an important factor for the exclusion of *S. aureus* from the nose (2).

This article summarizes our knowledge of *S. aureus* nasal colonization and its interaction with other bacterial species that share the same habitat. Understanding naturally occurring *S. aureus* elimination strategies might enable the development of new eradication approaches in the future.

S. AUREUS AS A HUMAN COMMENSAL AND PATHOGEN

The preferred habitat of *S. aureus* is the anterior nares. Approximately 20% of the human population is permanently colonized by *S. aureus*, and another 20% are considered to be noncarriers. The remaining 60% of the population belong to the group of intermittent carriers (4). Although several factors that affect the *S. aureus* carrier status have been described, why only certain people are colonized remains elusive. In general, *S. aureus* occurs particularly frequently in men (5) and in women using hormonal contraception (6). Moreover, diabetic (7), hospitalized, and dialysis patients (4) are more often colonized than healthy humans, and there is a higher carriage rate during childhood compared to adulthood (8). A negative correlation exists between smoking and *S. aureus* carriage (9). Seasonal differences, such as the transition from winter to spring, and temperature and pollen or dust levels, could also affect the nasal microbiome composition (10). Host genetics appear to have only a moderate impact on the shape of the microbiome (11).

S. aureus possesses an extensive arsenal of virulence factors and, therefore, the clinical manifestations of *S. aureus* diseases are diverse and range from rather mild soft skin and tissue infections to severe and life-threatening infections, such as pneumonia, osteomyelitis, endocarditis, and sepsis (12). Although nasal colonization by *S. aureus* is asymptomatic, it increases the risk of invasive infections, especially in immunocompromised and hospitalized patients (4, 13). Analysis of infecting strains revealed that it is usually the nasal strain of a patient that is responsible for subsequent infections (13, 14). Therefore, decolonization of nasal *S. aureus* serves as a very efficacious measure for prevention of invasive infections (13). Since *S. aureus* transmission from the nose to other parts of the body occurs mainly via hand contact, *S. aureus* usually disappears also from other body sites when it is eliminated from the nose (15, 16).

The high risk of *S. aureus* carriers to infection justifies widely applied mupirocin treatment to eradicate *S. aureus* from the nares of patients. Mupirocin interferes with the synthesis of bacterial proteins by reversibly binding to bacterial isoleucyl-tRNA (17). Application of a mupirocin ointment twice a day for 5 consecutive days typically leads to eradication of *S. aureus* from the nose and to a reduced risk of invasive *S. aureus* infections (14, 17). The success rate of mupirocin treatment reaches approximately 90%, yet resistance is increasing, reaching up to 30% in some parts of the United States. Hence, new antimicrobial compounds for *S. aureus* decolonization are urgently needed (17, 18). Recolonization by *S. aureus* frequently occurs a few weeks after the end of mupirocin treatment, probably as a result of *S. aureus* cells using the posterior vestibula as a hidden reservoir, which can hardly be reached by mupirocin treatment (19–21).

Resistance to several common antibiotics has increased in *S. aureus* in the past decades. Resistance to the entire class of β-lactam antibiotics, including methicillin, characterizes the epidemic methicillin-resistant *S. aureus* (MRSA) strains. However, multidrug-resistant MRSA strains, which have acquired resistance to additional antibiotics, such as erythromycin, clindamycin, ciprofloxacin, tetracycline, or mupirocin, have also been observed (22). MRSA infections used to be acquired in hospitals by patients and are called

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hospital-associated MRSA infections. However, in the past decade, increasing numbers of MRSA infections have been acquired outside the health care system by emerging MRSA clones referred to as community-associated MRSA. Many community-associated MRSA clones are much more virulent and transmissible than hospital-associated MRSA clones (23).

DIVERSITY OF THE HUMAN NASAL MICROBIOME

In addition to the genus *Staphylococcus*, which belongs to the phylum *Firmicutes*, the nasal microbiome also contains members of other phyla, in particular, *Actinobacteria* and *Proteobacteria*. Within these phyla, the genera *Corynebacterium*, *Propionibacterium*, *Staphylococcus*, and *Moraxella* are most common in the human nose (24). Other genera are less frequently found (11). Interestingly, the nasal cavities are also colonized by various anaerobic species (19).

To classify different microbiome compositions, seven community state types (CSTs) were defined according to the dominance of specific nasal species, genera, or families. CST1 is dominated by *S. aureus*, and CST2 is defined by the major occurrence of *Enterobacteriaceae*, such as *Escherichia* spp., *Proteus*. spp., and *Klebsiella* spp. *Staphylococcus epidermidis* dominates CST3, whereas *Propionibacterium* spp. are major colonizers in CST4. *Corynebacterium* is the dominating genus of CST5, and *Moraxella* spp. are most prevalent in CST6, whereas CST7 is defined by the predominance of *Dolosigranulum pigrum*. The most common nasal CST is CST4, followed by CST3 and CST1, whereas CST6 is the least prevalent (Table 1). Although the conspicuous presence of various species or genera defines the CSTs, these species can also be identified at lower abundances in other CSTs (11). Comparing the microbiome compositions at various body sites, it is obvious that the nasal bacterial composition overlaps with those of the oral cavity and skin and can therefore be regarded as a bridge between the microbiomes of these two body sites (24, 25).

Human skin areas with mostly dry, sebaceous, or moist properties differ in the abundance of bacterial microbiome members, while the core species are largely the same (1). It remains unclear if the individual differences in the skin microbiomes can also be attributed to specific CSTs. *S. epidermidis*, *Staphylococcus capitis*, *Staphylococcus hominis*, and *Staphylococcus warneri* are the most abundant coagulase-negative staphylococci (CoNS) on the skin (1). *S. aureus* also occurs transiently on the skin of healthy humans, especially in skin areas of the axillae and perineum (26, 27). Notably, the skin of atopic dermatitis patients is often permanently colonized by *S. aureus* on inflamed and non-inflamed skin parts (28).

CoNS not only colonize the human skin; they can also occasionally be detected in the nose (29, 30). Only *S. epidermidis* is a core microbiome member of both the skin and nose (29). In general, the nasal microbiome is less dense and less diverse than, for instance, the gut microbiome (31, 32). *S. epidermidis* is particularly prevalent on moist areas of the body, such as the axillae, inguinal and perineal areas, toe tissue, and conjunctiva, and is also a common resident of the anterior nares (29). In addition, a variety of other CoNS occupy specific regions of the skin. *S. hominis* and *Staphylococcus haemolyticus* are often found in the axillae and pubic areas (29). *S. capitis* can be preferentially isolated from the sebaceous glands on the forehead and scalp (29) and is also found on the skin and in the nares (33). Whereas *Staphylococcus auricularis* is part of the external ear microbiome, *Staphylococcus saprophyticus* often colonizes the rectum and genitourinary tract (29). *Staphylococcus lugdunensis* is also part of the human skin flora and is particularly prevalent in the pelvic and perineal regions, the groin, the axillae, and in the nail bed of the first toe. *S. lugdunensis* has been isolated from the human nose at an incidence rate of 10 to 26% (19, 29, 34, 35). *S. warneri* is typically found at a lower percentage than other CoNS in the nares and on the skin, preferably on the head, arms, and legs (33). Compared to the other CoNS, *S. lugdunensis* and *S. saprophyticus* are considered species with slightly higher pathogenicity (29).

Compared to species such as *S. epidermidis*, which can be present in the nose with multiple strains simultaneously, nasal *S. aureus* isolates typically belong only to one specific clone (36, 37). Notably, the number of *S. aureus* cells, which can be cultivated from carriers by nasal swabbing, varies from only a few to more than 10^7 per swab (38). Attempts to distinguish persistent from intermediate carriers by a single nasal swab indicated that the detection of more than 10^3 *S. aureus* organisms CFU/swab corresponds to a high probability of persistent carriage (39).

The anterior nares are regarded as the primary habitat of *S. aureus* (4), but a recent study showed that this species can be isolated with an even higher incidence from deeper (posterior) areas of the nasal cavity, suggesting that the posterior vestibule or the entire nasal vestibule may be the principle habitat of *S. aureus* (19). Further studies indicated that *S. aureus* can penetrate into the nasal tissue of healthy humans, and individual cells could be visualized even at the stratum basale of the nasal epithelium, which differs in structure between the anterior and posterior areas (40, 41). While the anterior nares are characterized by a keratinized, stratified squamous epithelium, the posterior nares are

TABLE 1 Community state types of the human nose[a]

Community state type	Dominated by	Prevalence (%)
CST1	*S. aureus*	12.4
CST2	*Enterobacteriaceae*, e.g., *Escherichia* spp. (11), *Proteus* spp. (11), *Klebsiella* spp. (11)	9.0
CST3	*S. epidermidis*	22.5
CST4	*Propionibacterium* spp., e.g., *P. acnes* (88)	28.7
CST5	*Corynebacterium* spp., e.g., *C. accolens* (88), *C. pseudodiptheriticum* (88), *C. propinquum* (88)	11.2
CST6	*Moraxella* spp., e.g., *M. lacunata* (88), *M. nonliquefaciens* (88)	5.6
CST7	*Dolosigranulum pigrum*	10.7

[a]Seven major community state types were defined according to the abundance of dominant nasal bacterial species, genera, or families (11).

defined by pseudostratified, columnar ciliated epithelial cells (2, 41). In the anterior vestibule, the moist squamous epithelium on the septum adjacent to the nasal ostium harbors the highest number of *S. aureus* cells. By contrast, CoNS prefer the skin of the nasal septum and the anterior, hair-covered epidermal portion of the lateral wall (5).

METABOLISM IN THE HUMAN NOSE

The nose is an environment with a very low nutrient supply, which might be the reason for the low species diversity of this habitat. Human nasal secretions contain sodium chloride at concentrations found also in other body fluids and small amounts of potassium, magnesium, and phosphate, but they are low in potential nutrients such as sugars, amino acids, and other major building blocks (30). Based on the composition of nasal secretions, a synthetic nasal medium (SNM3) was established that enabled the efficient growth of *S. aureus*, whereas CoNS did not steadily grow in SNM3 (30). This finding indicates that *S. aureus* is better adapted to life in the human nose than many CoNS, which may use the human nose only as a temporary, but not preferred, habitat. *S. aureus* and many other nasal bacteria, such as *S. epidermidis*, *Finegoldia magna*, *Propionibacterium acnes*, and *Streptococcus pyogenes*, secrete proteases that degrade human proteins, such as albumin, lactoferrin, mucin, cytokeratin 10, and hemoglobin. These proteins are present in considerable amounts in human nasal secretions and might serve as nutrient sources for various bacteria (42–44). It is likely that secretory proteases produced by a specific bacterial strain generate peptides and amino acids that can be utilized by many other microbiome members and may have a broad impact on microbiome metabolism.

MECHANISMS OF NASAL EPITHELIAL ATTACHMENT

Efficient attachment mechanisms are a prerequisite for bacterial nasal colonizers to remain in tight contact with epithelial cells and resist clearance by mucociliar movement. *S. aureus* binds to fully keratinized, dead desquamated cells of the anterior nasal cavity and adheres also to live ciliated cells in the posterior nasal cavity. *S. aureus* uses different adhesion mechanisms depending on the specific epithelial characteristics at different parts of the nares (45–48).

The cell wall glycopolymer wall teichoic acid (WTA) mediates the initial attachment of *S. aureus* to epithelial cells, and WTA is crucial for *S. aureus* nasal colonization (41, 47, 49). WTA-deficient *S. aureus* mutants are limited in their ability to bind to nasal epithelial cells. In addition, mutations in the *dltABCD* operon, leading to a loss of D-alanine modification of WTA, also exhibit diminished epithelial binding capacities (47, 49). Similarly, the modification of WTA with *N*-acetylglucosamine (GlcNAc) in α- or β-configuration is essential for efficient binding to nasal epithelial cells, and a lack of WTA glycosylation significantly abolished the ability of *S. aureus* to colonize cotton rat nares *in vivo* (50).

The scavenger receptor class-F member 1 (SREC1) on nasal epithelia in the posterior nasal cavity is a target for the WTA of *S. aureus* and allows *S. aureus* binding to epithelial cells (46). The charged zwitterionic properties of the ribitol-phosphate repeating units of WTA are essential in SREC1 binding (51). The loss of the D-alanine modification of the WTA significantly reduces the SREC1 binding.

The adherence of *S. aureus* to the nasal epithelium is also mediated by cell wall-anchored proteins, which are involved in long-term persistence within the anterior nasal cavity. *S. aureus* expresses a variety of cell wall proteins, which belong to the group of MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) (41). MSCRAMMs such as clumping factor B (ClfB), iron-regulated surface determinant A (IsdA), and serine-aspartate repeat-containing protein D (SdrD) play an important role in nasal colonization by *S. aureus*.

ClfB is a fibrinogen-binding protein produced by *S. aureus* that binds to the matrix proteins cytokeratin 10 and loricrin. These proteins are exposed on human squamous epithelial cells (48, 52, 53). IsdA, which is involved in heme uptake and iron acquisition (54), is expressed during human infections and is responsible for nasal colonization and survival of *S. aureus* on the skin. Similar to ClfB, IsdA interacts with cytokeratin 10 and loricrin, as well as with the extracellular matrix protein involucrin (48, 55). SdrD mediates adhesion to the human squamous epithelium by binding to desmoglein 1, a desmosomal cadherin that is mainly expressed by the epidermis and mucosa (56, 57) (Fig. 1). The connective function of desmoglein 1 is required for the epidermis to maintain its integrity and structure (57). In addition, the surface proteins SdrC and SasG promote adhesion to squamous epithelium cells, but their binding partners are still unknown (45, 58). Interfering with glycopolymer-receptor interaction might become a new strategy for controlling *S. aureus* colonization in the nose (46). Furthermore, ClfB could represent an ideal target molecule for new decolonization strategies (53). In this context, ClfB can be considered a promising component for the development of a vaccine that would also reduce nasal colonization by *S. aureus* (59).

S. epidermidis colonizes the human nose at a higher frequency than *S. aureus*. In contrast to *S. aureus*, *S. epidermidis* has a different WTA structure, and adhesion proteins related to ClfB, IsdA, SdrD, and SdrC, are absent. Therefore, it remains unclear how *S. epidermidis* accomplishes adhesion in the nose (2, 60).

BACTERIAL COMPETITION

Bacteria from habitats with a limited nutrient supply such as the skin and nose have developed mechanisms to increase their fitness in competition with other microbiome members. Competition between bacterial species can be direct or indirect. Direct inhibition can be achieved, for instance, through production of antimicrobials, whereas indirect inhibition may occur via competition for nutrients or modification of living conditions (61).

Many nasal *Staphylococcus* isolates produce antimicrobial substances against bacterial competitors at an unexpectedly high frequency (84%), with *S. epidermidis* as the most frequent producer of antimicrobial activity (62). Importantly, production of many of the antibacterial activities is strongly enhanced or exclusively detectable under specific environmental stress conditions which are present in the human nose, such as hydrogen peroxide release and iron limitation (62). Antimicrobial substances, also called bacteriocins, are categorized into various groups and subgroups (63). Many *Staphylococcus* isolates are producers of lantibiotics, ribosomally synthesized antimicrobial peptides characterized by the presence of the thioether amino acids lanthionine and methyllanthionine. A variety of lantibiotics have been described for *Staphylococcus* strains (64). Epidermin (*S. epidermidis*) (65), Pep5 (*S. epidermidis*) (66), epilancin K7 and 15X (*S. epidermidis*) (67, 68), epicidin 280 (*S. epidermidis*) (69), staphylococcin C55 (*S. aureus*) (70), various nukacins (*S. warneri* [71], *S. epidermidis* [62], and *S. hominis* [72]), and lantibiotic-α and -β (*S. hominis*) (73), were documented. In

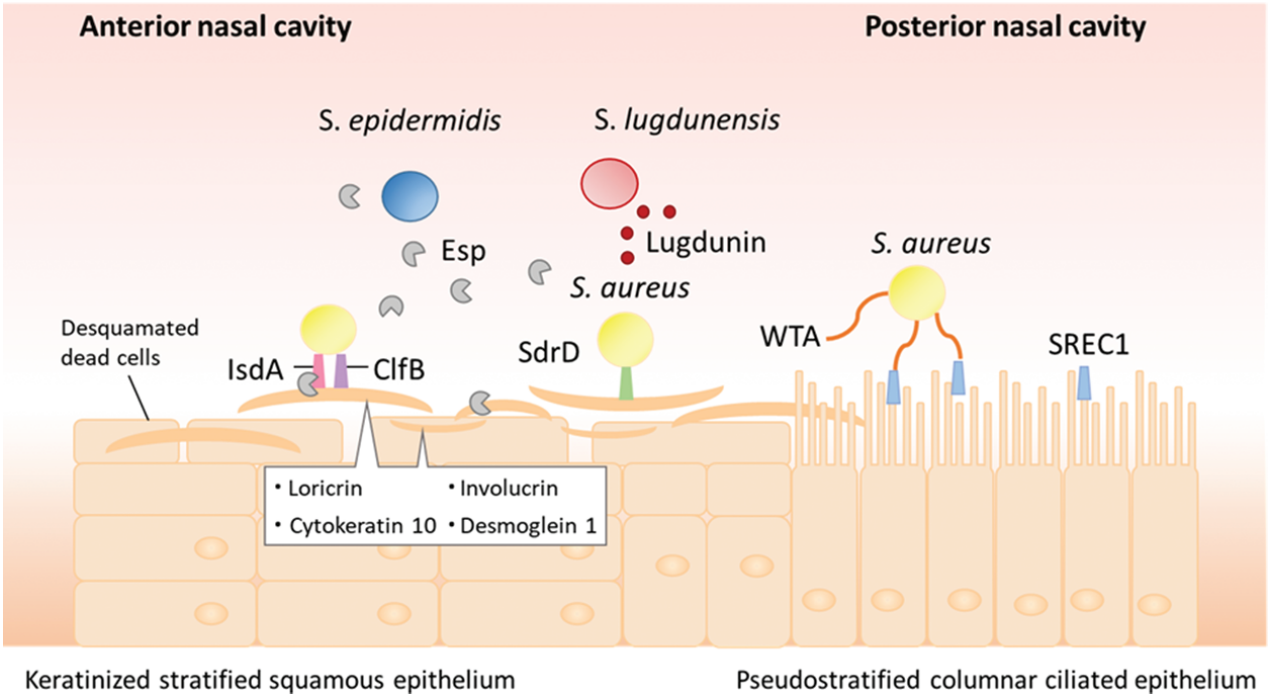


FIGURE 1 Attachment mechanisms of *S. aureus* in the human nasal cavity. The anterior and posterior parts of the human nose are lined by different types of epithelial cell, which require alternative bacterial adhesion mechanisms. For the keratinized stratified squamous epithelium in the anterior nasal cavity, *S. aureus* predominantly uses cell wall-attached surface proteins (MSCRAMMs) (2, 41). In contrast, the primary attachment in the posterior area, composed of a pseudostratified columnar ciliated epithelium, is mediated by specific interaction of the cell-wall linked wall teichoic acid (WTA) with the scavenger receptor class F member 1 (SREC1) (41, 46). The corneocytes (desquamated epithelial cells) in the anterior nasal cavity contain high levels of the proteins loricrin, cytokeratin 10, and involucrin (55). *S. aureus* can express a variety of cell wall proteins, which bind to these matrix proteins. The *S. aureus* adhesin clumping factor B (ClfB) binds to cytokeratin 10 and loricrin (48, 52), whereas the iron-regulated surface determinant A (IsdA) can also interact with involucrin (55). In addition, the *S. aureus* serine-aspartate repeat-containing protein D (SdrD) mediates adhesion to human squamous epithelial cells by binding to desmoglein 1 (56). Some *S. epidermidis* isolates secrete an extracellular serine protease (Esp), which inhibits *S. aureus* colonization by degradation of the surface proteins IsdA and SdrD and host receptors (77, 78). In addition, *S. lugdunensis* can prevent nasal colonization of *S. aureus* by producing the cyclic thiazolidine-containing peptide antibiotic lugdunin (35).

addition, putative lantibiotic-biosynthetic gene clusters were found in the genome of *S. capitis*, which share homology with the biosynthetic systems of epidermin/gallidermin and the nonlantibiotic bacteriocin epidermicin (74). Lantibiotics are usually exclusively active against Gram-positive bacteria but often show no activity against *S. aureus* (62, 71). It is rather uncommon that nasal *S. aureus* is severely affected by lantibiotic-producing CoNS. Interestingly, a systematic analysis of *P. acnes* isolates revealed a frequent capacity to inhibit *S. epidermidis* (75). No compound has been identified that could explain this inhibitory activity; however, the genomes of various *P. acnes* strains encode a putative thiopeptide-biosynthetic gene cluster that is similar to those for antimicrobially active siomycin and berninamycin, which might explain the inhibitory effect (76).

A recent study showed that secretion of the extracellular serine protease Esp by *S. epidermidis* can efficiently inhibit *S. aureus* colonization. Artificial inoculation of Esp-secreting *S. epidermidis* to the nasal cavities of human volunteers was sufficient to eradicate *S. aureus* (77). Nasal colonization of *S. aureus* is probably abolished by Esp via the degradation of both bacterial adhesive surface proteins IsdA and SdrD and host receptor proteins (78). Although most *S. epidermidis* isolates produce Esp and some are producers of lantibiotics, there is no clear correlation of the absence of *S. aureus* with the presence of *S. epidermidis* (20, 64, 77).

Nasal *S. lugdunensis* can prevent colonization by *S. aureus* by producing an unusual antimicrobial compound termed lugdunin, which is a novel, cyclic thiazolidine-containing peptide antibiotic (35). The lugdunin gene cluster is encoded on the *S. lugdunensis* chromosome and is present in almost all *S. lugdunensis* strains. Lugdunin is generated by nonribosomal peptide synthetases. It has bactericidal activity against *S. aureus* and other Gram-positive bacteria, and it was shown to be active *in vivo* in animal models. Analysis of nasal microbiomes of hospitalized patients revealed that *S. lugdunensis* colonization is associated with a 6-fold reduced risk of *S. aureus* carriage in the nose, suggesting that lugdunin or lugdunin-producing commensals could become useful for the prevention of *S. aureus* colonization and infection.

Streptococcus spp. mainly occur in the oropharynx but can also be found at low frequency in the nostrils (79). Importantly, *S. pneumoniae* can modify the nasal habitat by releasing hydrogen peroxide, which induces the SOS response in *S. aureus* and activates DNA repair mechanisms as well as resident prophages. Subsequently, phage-produced lytic enzymes destroy *S. aureus* cells (80, 81). Hence, *S. pneumonia* is negatively correlated with nasal *S. aureus* carriage (82, 83).

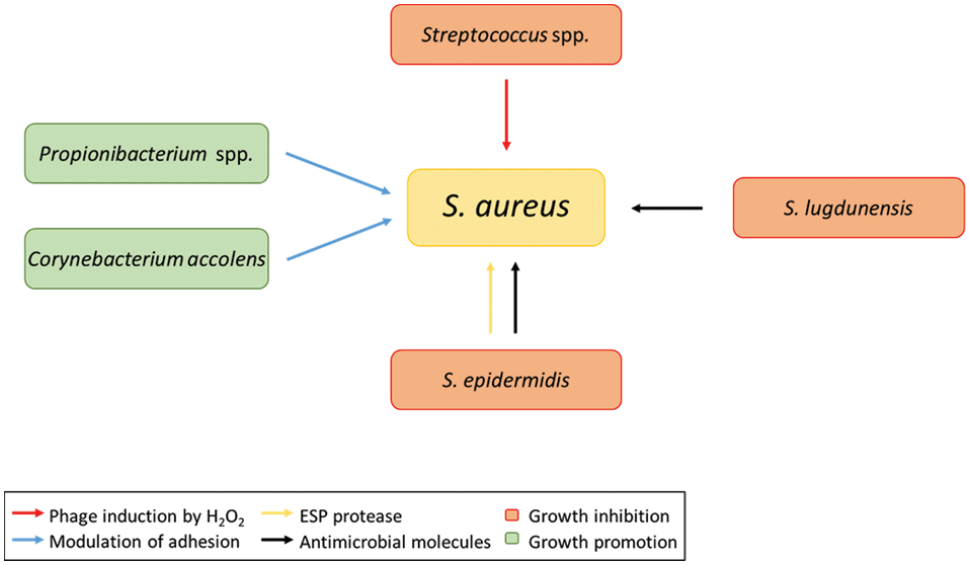


FIGURE 2 Established interactions between nasal bacteria. *C. accolens* and *Propionibacterium* spp. can promote the colonization by *S. aureus* (green boxes) by modulation of its adhesive capacities (20, 85) (blue arrows), while specific clones of *S. epidermidis*, *S. lugdunensis*, and *Streptococcus* spp. can lead to *S. aureus* growth inhibition (orange boxes). Some *S. epidermidis* isolates secrete high levels of extracellular serine protease (Esp), which inhibits *S. aureus* nasal colonization (77) (yellow arrow). In addition, *S. epidermidis* and *S. lugdunensis* can impede *S. aureus* colonization by producing antimicrobial molecules (35, 64) (black arrows). *S. pneumoniae* can release hydrogen peroxide, which leads to prophage activation in *S. aureus* along with phage-mediated lysis of *S. aureus* cells (80, 81) (red arrow).

An ambivalent correlation between *Corynebacterium* spp. and *S. aureus* was reported. While *Corynebacterium accolens* often occurs together with *S. aureus*, *Corynebacterium pseudodiptheriticum* is associated with the absence of *S. aureus*. A mutualistic relationship between *C. accolens* and *S. aureus* might rely on the joint mobilization of nutrients promoting the growth of these strains. In contrast, the competitive interaction between *C. pseudodiptheriticum* and *S. aureus* interferes with *S. aureus* colonization. Thus, the presence of *C. accolens* or *C. pseudodiptheriticum* might become a useful predictor of the propensity for nasal *S. aureus* carriage (20). A bacterial strain replacement study highlighted that nasal inoculation of persistent *S. aureus* carriers with a *Corynebacterium* sp. can lead to complete eradication of the pathogen in more than 70% of the probands (84).

Nostril- and skin-associated *Propionibacterium* spp. release coproporphyrin III, a porphyrin metabolite that promotes the aggregation and nasal colonization of *S. aureus* (85) (Fig. 2). A negative association with *S. aureus* carriage was reported for *Simonsiella* spp., *D. pigrum*, and *F. magna*, which are also regular members of the nasal microbiome (11, 31). The particular sensitivity of *D. pigrum* to a multitude of *Staphylococcus* isolates suggests that *D. pigrum* can only be present when no bacteriocin-producing *Staphylococcus* is in the same habitat (62).

COMPETITION BY INDUCTION OF HOST DEFENSE

In addition to the above-described competition scenarios, the human host can also impact the composition of the nasal microbiome. *S. aureus* expresses surfactant-like phenol-soluble modulin peptides, which mobilize proinflammatory lipoproteins from the staphylococcal cytoplasmic membrane. These lipoproteins activate the Toll-like receptor 2 and, consequently, lead to inflammation (86). The resulting inflammatory response in the nasal epithelium provokes the production of antimicrobial peptides. Although these hardly affect *S. aureus*, with its intrinsic immune evasion factors, they impair growth of other nasal commensal bacteria (5, 87).

CONCLUSION AND OUTLOOK

The mechanisms of microbiome-mediated exclusion of *S. aureus* from the human nose are probably multifactorial. It is likely that in addition to the processes described above, other factors are involved. It will be important to further investigate which other antimicrobial substances are produced by nasal commensals and which other strategies are used in their competition with *S. aureus* for nutrients and adhesion sites. New findings on nasal colonization may clarify why 20% of the human population is permanently colonized by *S. aureus*, while a similar percentage is never colonized. A better understanding could be helpful for developing new *S. aureus* eradication approaches that protect against recurrent *S. aureus* nasal colonization.

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Staphylococcus aureus in Animals

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46

INTRODUCTION

The genus *Staphylococcus* currently comprises 81 species and subspecies (https://www.dsmz.de/bacterial-diversity/prokaryotic-nomenclature-up-to-date/prokaryotic-nomenclature-up-to-date.html), and most members of the genus are mammalian commensals or opportunistic pathogens that colonize niches such as skin, nares, and diverse mucosal membranes. Several species are of significant medical or veterinary importance. *Staphylococcus pseudintermedius* (1) is a leading cause of pyoderma in dogs and is considered to be a significant reservoir of antimicrobial resistance factors for the genus (2, 3). *S. pseudintermedius* is very similar to *Staphylococcus intermedius* and can be distinguished from other coagulase-positive staphylococci by positive arginine dihydrolase and acid production from β-gentiobiose and D-mannitol (4) or by using a multiplex-PCR approach targeting the nuclease gene *nuc* (5). *Staphylococcus saprophyticus* is the second leading cause of uncomplicated urinary tract infections (6). While *Staphylococcus epidermidis* is a normal component of the epidermal microbiota, it is a leading cause of biofilm contamination of medical devices (7). The most promiscuous and most significant human pathogenic staphylococcal species is *Staphylococcus aureus*, which is the causal agent of a variety of disease symptoms that can range from cosmetic to lethal manifestations. *S. aureus* is distinguished from most members of the genus by its abundant production of secreted coagulase, an enzyme which converts serum fibrinogen to fibrin and promotes clotting. However, the *S. intermedius* group and some strains of *Staphylococcus lugdunensis* have coagulase activity (5, 8, 9).

Despite the prevalence of literature characterizing staphylococcal pathogenesis in humans, *S. aureus* is a major cause of infection and disease in a plethora of animal hosts, leading to a significant impact on public health and agriculture (10). Infections in animals are deleterious to animal health, and animals can act as a reservoir for staphylococcal transmission to humans. While about 20 to 30% of the human population carries *S. aureus*, the prevalence of *S. aureus* varies from host species to host species, and up to 90% of chickens, 42% of pigs, 29% of sheep, and between 14 and 35% of cows and heifers are carriers (11, 12). The economic importance of various animal species strongly determines the abundance of available literature on the subject, and as such, it is not surprising that *S. aureus* colonization and infection have only been superficially investigated in wild animals. Nevertheless, *S. aureus* has been isolated from a plethora of wildlife sources such as red squirrels (exudative dermatitis) (13), black bears (endocarditis) (14), zebras (cutaneous granuloma) (15), raccoons (botryomycosis) (16), dolphins (pyogenic meningoencephalitis) (17), harbor seals (systemic infections) (18), black rhinoceroses (skin lesion and sepsis) (19), boars (nasal carriage) (20, 21), rhesus macaques (nasal carriage) (22), great apes (nasal carriage and sepsis) (23), chaffinches (healthy carriage) (24), mallards (sepsis) (25), red deer, griffon vultures, and Iberian ibex (carriage) (21).

Animal isolates of *S. aureus* have been reported to exhibit distinct phenotypic properties that vary depending on the host of origin, and six biotypes have been described: human, β-hemolytic human, bovine, caprine, avian-abattoir, and non-host specific. These biotypes have, by and large, withstood the application of sophisticated characterization methods; isolates from different hosts, characterized by multilocus enzyme electrophoresis, cluster together, suggesting host specificity and a limited ability of strains to be transmitted from one host species to another (10). These observations were further corroborated by genotyping methods such as pulsed-field gel electrophoresis, and strains belonging to specific biotypes grouped in the same or closely related pulsotypes (26).

DNA sequence-based approaches such as multilocus-sequence typing (MLST) (27) have been extensively used to analyze population structures. At present, more than 3,300 sequence types isolated from more than 4,700 *S. aureus* samples have been collated in the MLST reference database (http://saureus.mlst.net/). The database contains isolates from a range of species, with human strains predominating by a large margin. Nevertheless, MLST showed that some clonal complexes (CCs) are predominant in, and associated with, specific hosts. In particular, it was shown that animal-associated strains belonged to specific clonal lineages, whereas human strains did not (28, 29). Today we know that 87% of *S. aureus* isolates from colonization and infections in humans represent 11 widely disseminated clonal complexes: CC1, CC5, CC8, CC12, CC15, CC22, CC25, CC30, CC45, CC51, and CC121. Clonal complexes CC8, CC15, CC22, CC30, CC45, CC30, CC45, and the rarer clonal complexes CC80 and CC152 are primarily associated with isolates from humans (30). MLST-based phylogenetic analysis provided the first long-term picture of the evolution of both human and animal strains (31, 32) and indicated that *S. aureus* has coevolved with its human host over a long time and that it had acquired the ability to infect animals on multiple

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occasions via human-to-animal host jumps. These host jumps eventually led to specific strain lineages spreading and adapting within new animal hosts (32). Animal to human host jumps have also been documented (33) but are less frequent. Additionally, a number of methicillin-resistant *S. aureus* (MRSA) strains with low host specificity attributed to CC130 and CC398 have emerged over the past decades.

The main clonal complexes associated with ruminants are CC97, CC133, CC522, and CC151, while clonal lineage sequence type 385 (ST385) is mainly represented by isolates from poultry (30, 34–38). Comparative analyses of the genomes of methicillin-susceptible *S. aureus* (MSSA) isolates attributed to ST5 from humans and poultry and of MSSA/MRSA of CC398 revealed that the livestock subpopulations of these clonal complexes originated from ancestral populations in humans (39–41). In contrast, human-associated isolates of the ST97 lineage were clearly shown to have originated from ruminants (33). *S. aureus* colonization or infection in companion animals is usually caused by human-related genotypes (42), yet some colonization factors can determine host specificity (38).

S. aureus has colonized diverse animal species following host-switching events and subsequent adaptation through acquisition and/or loss of mobile genetic elements (MGEs) as well as further host-specific mutations, allowing it to expand into new host populations (Fig. 1, Table 1). Close contact between animals and humans can facilitate host-switching events, and there is a significant body of evidence indicating that the beginning of animal domestication in the Neolithic period (10,000 to 2,000 BC), as well as the increased industrialization of livestock farming, provided a platform for animal-to-human transmission of pathogens (43). While host jumps are generally accompanied by the acquisition or loss of larger MGEs, not all host jumps are associated with such large-scale events. Recently, Viana et al. showed that single amino acid substitutions in the *dltB* gene were sufficient to confer infectivity of human ST121 isolates for rabbits (44). Overall, *S. aureus* can readily cross species barriers and infect new hosts. This ability is largely associated with the large proportion of MGEs in the *S. aureus* genome and its capacity to exchange these through contact with its environment. The animal host can provide reservoirs for new virulence traits and antibiotic resistances, and the increased contact between humans and animals through industrialized agriculture coupled with its globalization necessitate tight monitoring of pathogenic animal *S. aureus* strains to understand the development and spread of staphylococcal lineages.

In this article, we will be giving an overview of *S. aureus* in animals, how this bacterial species was, and is, being transferred to new host species, and the key elements thought to be involved in its adaptation to new ecological host niches. We will also highlight animal hosts as a reservoir for the development and transfer of antimicrobial resistance determinants.

S. AUREUS IN RUMINANTS

S. aureus, next to *Escherichia coli* and several streptococcal species such as *Streptococcus uberis*, and *Streptococcus agalactiae*, is a major cause of mastitis in dairy cows and incurs a significant economic loss to the dairy industry. Mastitis in dairy cows results in reduced yields, the need for veterinary intervention, and the loss of milk that has to be discarded due to either pathogen or antibiotic contamination. If treatment of the udder is unsuccessful, the animal is often culled. *S. aureus* is associated with both clinical and, more commonly, subclinical mastitis, both of which frequently result in persistent and recurrent infections with a low cure rate after antibiotic therapy (45). Mastitis leads to the influx of leukocytes into the udder, and various thresholds for leukocyte numbers have been established for categorizing good milk quality. Taking cow milk as an example, milk with more than 200,000 leukocytes per ml is considered to be infected, and in the European Union when more than 400,000 cells per ml are found, the milk is deemed unfit for human consumption. Apart from the considerable economic losses incurred through *S. aureus*-derived mastitis, mammary gland infections pose a considerable public health problem. *S. aureus* can be shed from infected glands, and most staphylococcal isolates from dairy milk possess genes encoding enterotoxins. Thus, contamination of bulk milk can lead to food poisoning from fermented raw milk products (46, 47).

S. aureus can be found in healthy cows (carriers) on the teat skin, nasal cavity, and rectum (11). However, the main reservoirs in a dairy herd are infected udders and teat skin. Infected animals can shed bacteria through their milk, and transmission occurs primarily from udder to udder during milking via contact with contaminated milking machines, farmer's hands, or contaminated bedding (48). Other environmental transmission routes are less frequent; although *S. aureus* can survive in the environment for some time, it requires animal colonization to ensure its survival.

The majority of bovine infections worldwide are caused by a subset of specific, bovine-adapted *S. aureus* strains (28). The substantial genetic variation between different lineages (49, 50) suggests that there might be lineage-specific differences in the molecular mechanisms involved in *S. aureus* pathogenesis.

Animal microbiota provide a reservoir of antibiotic resistance genes that can be acquired from their ecological niches and selected for by the use of antibiotics in agriculture. The ability of some animal-adapted *S. aureus* strains to colonize and infect humans can give rise to the development of new epidemic clones with hitherto uncharacterized virulence capacity (32). This becomes particularly clear in strains of the CC97 lineage, which is one of the major clones associated with bovine mastitis (28). Moreover, an increased number of bovine-to-human transmissions has been reported in recent years (37, 51, 52). A closer analysis revealed that at least two CC97 subclades for human infection had emerged that originated in bovine-to-human host jumps and had thereafter spread through the human population (33). This provided further evidence that animals can provide a reservoir for the development of new *S. aureus* clones that can rapidly spread from animal to human and then through the population. Richardson et al. recently showed, using genomics-based approaches, that cows are a major reservoir for reinfection of humans, and multiple host-switching events, both human-to-cow and cow-to-human, have occurred over the past 3,000 years (43).

Bovine *S. aureus* isolates of the CC8 lineage closely resemble human isolates, and Resch et al. used this observation to further study the genetic basis of host adaptation (53). They compared a total of 14 CC8 isolates from cows with subclinical mastitis, 9 CC8 isolates from colonized or infected human patients, and 9 isolates belonging to typical bovine lineages (CC389, CC71, CC151, CC504, and CC479). They observed that CC8 isolates segregated into a unique group that was separate from typical bovine CCs and that within this group isolates segregated into three subgroups. The main segregating parameter was the content of MGEs within the individual strains, and they showed that strains of the mixed human-bovine isolate clusters contained

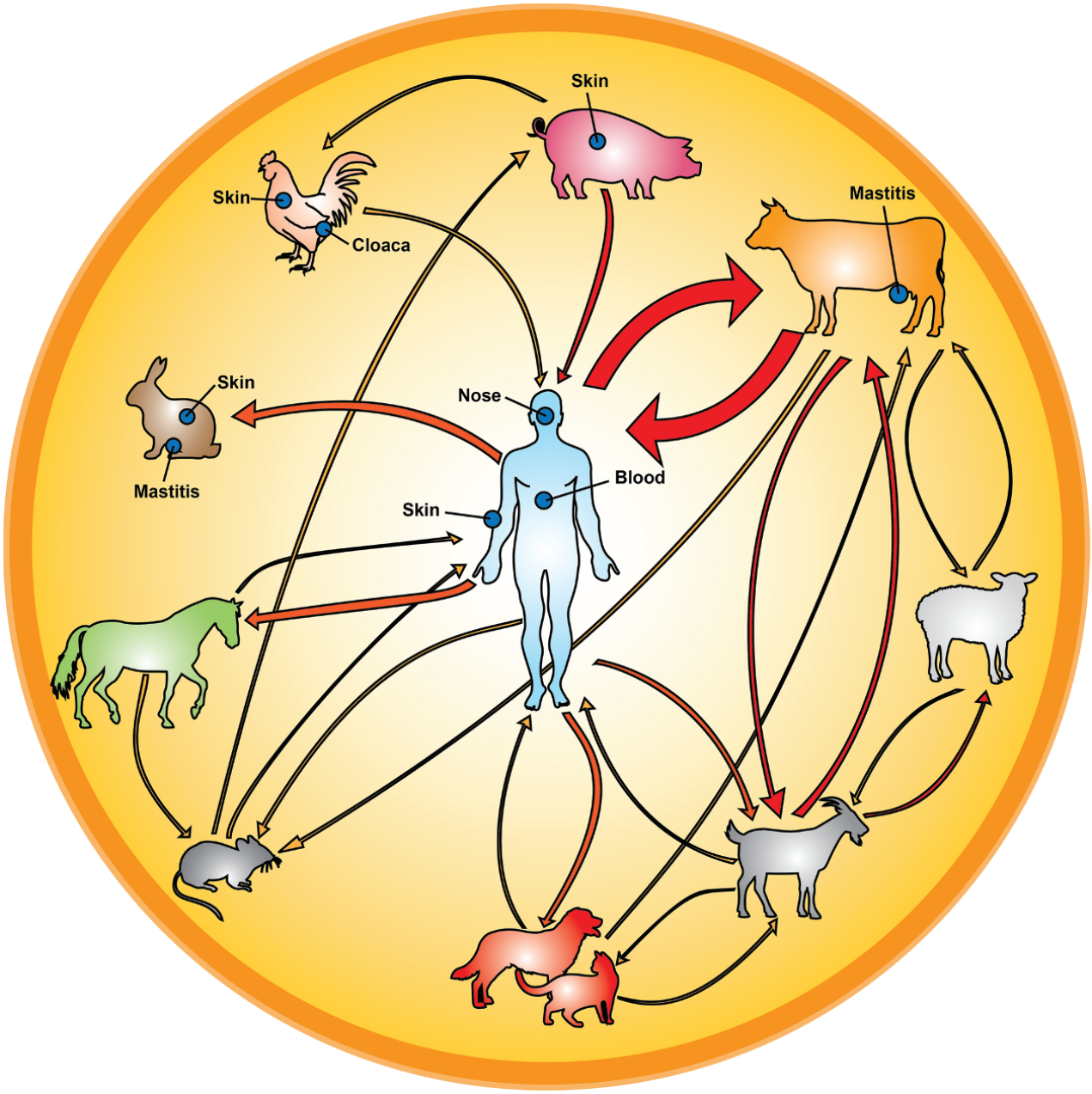


FIGURE 1 Humans act as a major hub for *S. aureus* host jumps. *S. aureus* has been isolated from a plethora of vertebrates and has undergone multiple series of host jumps. A major exchange hub is humans that interact with domesticated livestock and companion animals. Arrow thickness indicates the frequency of host jumps, with colors from yellow to red indicating their likelihood. Figure adapted from reference 43.

β-hemolysin converting prophages. Conversely, the bovine isolates were devoid of this phage and harbored an additional, new non-*mec* staphylococcal cassette chromosome containing an LPXTG-surface protein with similarity to proteins present in environmental bacteria, often found as milk contaminants (53).

Bar-Gal et al. compared pheno- and genotypic characteristics of bovine isolates from Israel, Germany, the United States, and Italy using a Bayesian phylogenetic comparison of several key genes (*nuc*, *coa*, *lukF*, and *clf*A) and *spa* and *agr* typing, followed by CC assignment, and assessed the presence of a broad range of virulence factors and antimicrobial resistance genes (54). This analysis enabled them to cluster different isolates according to their host of origin. Sheep and goat isolates generally showed lower variability and fewer CCs compared to bovine isolates. Within the bovine clade, the authors described two subclades in which isolates matched strains found in Israel or other countries. Their data therefore corroborate other studies suggesting staphylococcal coevolution with its respective host and might indicate the existence of multiple host jumps by bovine *S. aureus* strains that have occurred in diverse geographical locations (54). Overall, the authors found that 27 virulence-associated factors showed a different prevalence in bovine compared to

TABLE 1 Selected staphylococcal elements associated with specific hosts

MGE	MGE-associated determinants putatively involved with virulence/resistance/host specificity[a]	Reference(s)
Human		
ΦSa3 (β-hemolysin converting phage)	*sea*, *sep*, *scn*, *chp*, and *sak* genes encoding staphylococcal enterotoxin A and P, staphylococcal complement inhibitor, chemotaxis inhibitory protein and plasminogen activator staphylokinase, respectively	43
MGE	Type I restriction modification system	43
Ruminant		
SaPIbov	Staphylococcal enterotoxin C (*sec*-bovine) and L (*sel*), toxic shock syndrome toxin *tst* (TSST-1)	171, 172
Enterotoxin gene cluster	Gene cluster encoding 5 enterotoxins (*seg*, *sei*, *sem*, *sen*, *seo*)	171, 173
Not described	Superantigen-like proteins encoded by *ssl07* and *ssl08*	54
SaPIbov4	vWbp with ruminant-specific activity	63
Non-*mec* staphylococcal cassette chromosome	LPXTG-surface protein	53
SCC-*mec*C	*mec*C	79
Equine		
ΦSaeq1	Contains immune modulators with equine-specific activity *scn* gene encoding staphylococcal complement inhibitor	163
	*luk*PQ genes encoding the bipartite leukocidin PQ	162
SaPIeq1	Encodes vWbp able to coagulate equine and ruminant plasma	63
Porcine		
Plasmid	SCC*mec*	43
Plasmid	Resistance to heavy metals	43
SaPI-S0385	Composite of SaPI5 and SaPIbov1 Unique region at 3′ end encoding extracellular proteins with similarity to staphylococcal complement inhibitor and vWbp	146
Avian		
ΦAvβ (β-hemolysin-converting phage)	Putative ornithine cyclodeaminase 38% amino acid identity to ornithine cyclodeaminase made by *Bacillus cereus* HMM match to ornithine cyclodeaminase/mu-crystalin family (PF02423) Putative membrane protease 27% amino acid identity to Plnl (membrane-bound protease of CAAX family) made by *Lactobacillus plantarum*; HMM match to CAAX amino terminal protease family	40
ΦAv1	Ear-like protein *ear* previously identified in pathogenicity islands SaPI1, SaPI3, SaPI5, and SaPImw2 *ear* encodes β-lactamase-like protein	40
SaPIAv	Putative virulence region Novel hypothetical proteins in accessory region A3 where virulence genes such as *tst* and *eta* are located in other SaPIs SAAV_0806: signal peptide, 1 transmembrane helix SAAV_0810: signal peptide, 4 transmembrane helices May suggest role as membrane transporter	40
pAvX	Thiol protease ScpA 99.5% amino acid identity to ScpA (GenBank accession no. AB071596) previously identified among chicken isolates from Japan Suggested role in poultry dermatitis Lysophospholipase 42% amino acid identity to a lysophospholipase encoded by *Bacillus clausii* Bacterial phospholipases are known virulence factors implicated in disease pathogenesis	40

(*Continued on next page*)

TABLE 1 (*Continued*)

MGE	MGE-associated determinants putatively involved with virulence/resistance/host specificity[a]	Reference(s)
pAvY	N/A	
pT181	Tetracycline resistance	102, 108
pT127	Tetracycline resistance	102, 108
pC194	Chloramphenicol resistance	102, 108
pC221	Chloramphenicol resistance	102, 108
pC223	Chloramphenicol resistance	102, 108
pUB112	Chloramphenicol resistance	102, 108

[a]HMM: hidden Markov model; N/A: not applicable.

goat and sheep isolates. The authors noted a higher rate of strains carrying capsule type 8 in sheep and goat isolates compared to cow isolates, where both capsule types 5 and 8 were approximately equally distributed. Superantigen genes *ssl07* and *ssl08* were found in almost all bovine strains (>93%) but were present in less than 44% of sheep and goat strains. Strikingly, all bovine strains carried the *hysA2* gene encoding hyaluronate lyase, while only 48% of goat and sheep strains did. Cow strains showed a higher prevalence of leukocidins D and E, while leukocidins F-P83 and LukM appeared to be more prominent in goat and sheep strains (54). As noted above, infection of the mammary glands triggers the influx of large numbers of leukocytes that are deployed to fight off the infection. Leukocidins play an important role in bovine mastitis and can kill immune cells (leukocytes), thus protecting the pathogen (55–59). In agreement with this, the *lukF*/*lukM* genes are associated with the most prevalent CCs found in mastitic cattle (CC151, CC479, CC133, some CC97, and most CC522) (60). Leukocidins show different specificities for immune cells (particularly phagocytic cells) of various hosts through recognition of different host cell receptor alleles, and this can be related to the formation of hybrids among the different LukF and LukS paralogs. The leukocidins LukMF′ and LukPQ are mainly associated with zoonotic disease and found among animal-derived *S. aureus* strains (61). Several additional virulence factors located primarily on MGEs, such as superantigens (62) and ruminant-specific alleles of the von Willebrand factor-binding protein (vWbp) (63), have also been found to be strongly associated with bovine hosts (43).

S. aureus strains in ruminants appear to be undergoing a significant amount of DNA exchange leading to the emergence of hybrid clones. A recent study by Spoor et al. (64) showed that the CC71 lineage of livestock-associated (LA) *S. aureus* strains evolved from an ancestor belonging to the major bovine lineage CC97. The authors showed that multiple large-scale import and recombination events involving other *S. aureus* lineages occupying the same ruminant niche had occurred and that these affected a 329-kb region surrounding the chromosomal origin of replication. These recombination events resulted in allele replacement and either loss or gain of genes influencing host-pathogen interactions. In particular, the CC71 lineage acquired factors involved in innate immune evasion and bovine extracellular matrix adherence. The ability to take up and integrate large DNA segments from environmental staphylococcal strains highlights the pathogen's capacity for rapid evolution and adaptation.

In small ruminants, *S. aureus* is a major cause of mastitis and septicemia, by infections that may have a thromboembolic origin (65). These infections can also be secondary to parasite infestation, which allows *S. aureus* of the normal skin flora to enter the bloodstream (66) and in lambs can lead to fatal toxemia or to chronic disease with organ dissemination and abscess formation. In goats, staphylococcal infection can be secondary to parapox virus infection, leading to chorioptic mange or contagious pustular dermatitis (67). Morel's disease in sheep and goats is caused by a subspecies of *S. aureus*, *S. aureus* subsp. *anaerobius*, which primarily affects young animals. The disease is manifested through the formation of abscesses in superficial lymph nodes usually located in the mandibular region. This disease is thought to be caused by a single bacterial clone worldwide (ST1465), which has undergone long-term adaptation and is restricted to small ruminants (68).

MRSA in animals was first isolated from the milk of dairy cows with mastitis in Belgium in the early 1970s (69) and has since then been isolated from cows around the globe (70–76). MRSA strains harbor an MGE known as SCC*mec*, containing the *mec* gene, which codes for an additional penicillin binding protein that has low affinity for β-lactam antibiotics and therefore mediates resistance to nearly all compounds of this antibiotic class (besides ceftobiprole and ceftarolin). The mainly pig-related LA-MRSA CC398 has also been isolated from bovine udder infections (77), altogether in line with the elevated host promiscuity of this CC. Several cattle-associated MRSA lineages (ST130, ST425, and ST1943) that had previously been thought to be bovine-restricted have been recently isolated from human disease or carriage in Europe (78). Moreover, a newly identified *mec* determinant, named *mec*C (also known as $mecA_{LGA251}$), which shares 70% homology with *mec*A, was identified among MRSA strains of CC130, CC705, and ST425 recovered from cattle and humans (79). The *mec*C allele is associated with a unique SCC*mec* element designated SCC*mecXI* and is thought to be present in about 1.4% of bovine *S. aureus* isolates in as many as 2.8% of herds.

A recent study investigated the molecular profile of *S. aureus* strains isolated from bovine mastitis in the Shanghai and Zhejiang areas of China (80). The study identified a total of 19 sequence types, with the dominant ones being ST97, ST520, ST188, ST398, ST7, and ST9. The majority of isolates were found to be methicillin-sensitive (198/212), with ST97 being the predominant lineage among MSSA strains and ST9-MRSA-SCC*mecXII* the most common MRSA clone. The study revealed that the molecular virulence profiles of different lineages differed significantly. The predominant lineage causing bovine mastitis in eastern China was the MSSA ST97, but there was some indication that toxigenic MRSA ST9 lineages were also present, and it was suggested that their spread and distribution should be monitored in the future. ST9-MRSA strains containing the SCC*mecXII*

cassette have also been identified in nasal swabs from live pigs in China (80–82), and in isolates from humans in Taiwan (83). These strains were shown to have a specific MGE profile encoding vWbp on an SaPIbov4-like pathogenicity island (83). vWbps are responsible for the activation of host prothrombin and the formation of fibrin strands, thereby promoting the development of infectious lesions. SaPI-borne vWbps are distinct from their genomic homologs and have been shown to be responsible for the coagulation of ruminant plasma (63) and may therefore have an important role in the animal host specificity of *S. aureus*. The vWbp variants encoded by the SaPIbov4-like pathogenicity island shared only between 67 and 93% protein sequence identity with the previously characterized SaPI-vWbp (63). Nevertheless, they were able to coagulate bovine and caprine plasma. However, the ability of these vWbps to coagulate human plasma was not assessed in the study (83).

In a study investigating the prevalence of MRSA strains in contaminated milk and dairy products in southern Italy, 8.3% of all isolates (40/484) were methicillin resistant. Of these MRSA strains, the most prevalent sequence types in this study were ST152 (67.5%) followed by ST398 (25%), ST1 (5%), and ST5 (2.5%) (84). Additionally, 92.5% (37/40) and 5% of isolates harbored SCC*mec* type V and Iva, respectively, while 2.5% of isolates (1/40) harbored a not-further-defined methicillin resistance determinant.

MRSA of CC130, which has recently gained attention, carries *mec*C instead of *mec*A and is primarily associated with ruminants and wildlife that share the same habitats, suggesting that there might be mutual exchange of strains (85). The *mec*C gene is also found in the dairy-associated lineage ST425, which causes mastitis in cows. Both CC130 and ST425 isolates have been isolated from human infections (79, 86, 87).

S. AUREUS IN RABBITS

Staphylococcal infection causes substantial economic losses in commercial cuniculture, and clinical signs of *S. aureus* infection are present in more than 60% of rabbitries (88, 89). Infection of rabbits with *S. aureus* is associated with suppurative dermatitis, abscesses, pododermatitis, and mastitis (90–93), with chronic mastitis being the main reason for culling diseased animals in rabbitries (88, 91). Most chronic staphylococcal infections in rabbits are caused by the ST121 lineage; less common lineages, such as ST96, can also be involved (94, 95). Infection of mammary glands with ST121 strains resulted in elevated levels of granulocytes and reduced numbers of B cells, T cells, $CD4^+$ T cells, and $CD8^+$ T cells compared to mammary glands infected with ST96 strains (96). The authors of the study suggested that this observation might be explained by strain-specific differences in host interactions leading to altered perception by the host's immune system. However, further studies will be required to verify this hypothesis.

Among *S. aureus* strains isolated in rabbitries, two main strain types were initially classified, according to their virulence, into high-virulence strains with the capacity to rapidly spread through entire flocks and low-virulence strains that cause more limited infections (97). In accordance with this, high- and low-virulence strains can induce either severe or mild symptoms, respectively, in a rabbit skin infection model, indicating the presence of either different virulence factors or differences in virulence factor expression levels (98). Interestingly, most low-virulence strains could be grouped into poultry or human biotypes, whereas high-virulence strains were members of a mixed biotype that produced β-hemolysin and showed no staphylokinase activity (99). Classical high-virulence strains belong to the biotype "mixed CV-C" and are sensitive to phages 3A/3C/55/71 of phage group II, suggesting a clonal origin of these high-virulence strains (100). Subsequent molecular typing studies found that the majority of high-virulence strains belonged to ST121 and to a lesser extent to ST425 with *agr* types 4 and 2, respectively (95).

Viana et al. analyzed a total of 178 strains from chronic mastitis in rabbits that presented with a range of disease manifestations including abscesses, suppurative mastitis with a lobular pattern, cellulitis, and mixed lesions. The majority of isolates belonged to the high-virulence ST121 (166/178), with sequence types 398, 96, 45, 1, DVL879, and SLV9 (7, 1, 1, 2, 1, respectively) comprising the rest. However, disease symptoms could not be correlated to any specific genotype or sequence type (94). Rabbit isolates are significantly different from those found in humans and ruminants, suggesting the presence of host-specific factors selective for rabbit-specific sequence types (93). The phylogenetic origin of the ST121 lineage was eventually traced back to a human-to-rabbit host jump approximately 40 years ago (44). Comparative analysis of the accessory genomes of ST121 strains showed that the majority of human strains contained MGEs which encode potent toxins involved in human disease pathogenesis, such as Panton-Valentine leukocidin and exfoliative toxins, and all except one contained a β-hemolysin-converting phage (Φ*Sa*3) encoding the human-specific immune evasion cluster. None of the rabbit strains contained Panton-Valentine leukocidin- or exfoliative toxin-encoding MGEs, indicating that these were dispensable for *S. aureus* infection of rabbits (44). Interestingly, the rabbit strains did not contain any MGEs that were unique to rabbit *S. aureus*, indicating that the acquisition of rabbit-specific MGEs was not required to cause infection. Instead, the authors found that single nonsynonymous mutations at the 5′ end of the *dltB* gene were sufficient to confer rabbit infectivity in human ST121 strains. DltB is an integral membrane protein encoded by the *dltABCD* operon that is likely responsible for the translocation and incorporation of D-alanine into teichoic acids and lipoteichoic acids in *S. aureus* (101). However, Viana et al. (44) showed that neither the D-alanine nor the bacterial cell wall composition was altered in in strains harboring the rabbit-infective *dltB* mutants. This suggests an additional function for DltB during rabbit infections, and the authors propose that DltB, a member of the membrane-bound O-acetyltransferases that transfer organic acids, typically fatty acids, to hydroxyl groups, has a role in signaling that could be responsible (44). Rabbit-associated *S. aureus* strains therefore appear to be representatives of infective strains that require little adaptation to jump between humans and rabbits, and further studies will be required to determine how these relatively recent epidemic strains have evolved.

Rabbitries endeavor to prevent *S. aureus* infection by limiting the introduction of new animals and by reducing contact between rabbit flocks. Unfortunately, antibiotic treatments, disinfection of cages and environments, and vaccinations have so far proved inefficient in eliminating *S. aureus* infections in rabbitries (97). Consequently, culling of entire flocks followed by thorough disinfection of the cages is the only efficient strategy for dealing with *S. aureus* epidemics in cuniculture.

S. AUREUS IN CHICKENS AND OTHER POULTRY

The growth of commercial poultry farming has provided a fertile field for staphylococcal infections and zoonotic

transfer (102). *S. aureus* is among the leading causes of bacterial infections in poultry (10), causing a wide range of diseases including septic arthritis, subdermal abscesses, gangrenous dermatitis, and septicemia (103). As with other hosts, staphylococcal strains associated with poultry cluster into specific clonal complexes that appear to have either evolved together with their avian host or adapted after zoonotic transmission. For example, CC385 has so far been identified only among avian hosts, whereas strains of CC5 and CC398 have been isolated from chickens, humans, and other mammals (39, 104, 105). One of the predominant staphylococcal lineages causing disease in the poultry industry is CC5 (40, 103). Dissemination of CC5 into chickens involved a single transmission from a human approximately 40 years ago (40) followed by a significant number of genetic recombination events leading to host adaptation. At least 44 recombination events in 33 genes have accumulated in poultry isolates, and another 47 genes were found to be more frequent in poultry compared to human isolates. Interestingly, many of these genes were common among chicken isolates from other CCs, indicating that horizontal transfer of these genes between CCs may have a potential role in host adaptation (102). On a phenotypic level, these genetic alterations contribute to *S. aureus* adaptation to poultry hosts, and poultry isolates show enhanced growth at 42°C (the core body temperature of the adult chicken [106]) and greater erythrocyte lysis on chicken blood agar for chicken compared to human isolates (102). Conversely, most human isolates but only around half of the chicken isolates were able to lyse human erythrocytes (102). The improved growth of chicken isolates at 42°C is thought to be related to two poultry-associated genes (SAAV_0062 and SAAV_0064) that share more than 85% nucleotide identity with genes important for growth at elevated temperatures, including *dnaK* and *dnaE* (102).

Furthermore, the host jump from humans to poultry was also accompanied by genetic changes such as the loss of several genes involved in human disease pathogenesis and the acquisition of avian-specific MGEs (40). For example, the poultry strain ED98 had acquired two prophages, two plasmids, and a SaPI, and these MGEs are widely distributed among avian but completely absent from human strains (40). A similar observation has been made with bovine-adapted strains (107).

Plasmids can confer virulence traits and antibiotic resistances (pT181, pT127, pC194, pC221, pC223, and pUB112) (108) and can contribute to the spread of disease. Such plasmids are present in *S. aureus* isolates causing a variety of difficult to treat chicken diseases (102, 103, 109). A recent study focused on identifying bottlenecking and drift-related genetic changes, and on separating them from genetic changes conferring advantages in the poultry niche, and on showing adaptation to the avian host over time (102). By sampling a total of 191 isolates from diseased chickens from the United Kingdom, United States, and the Netherlands, they confirmed that the major staphylococcal lineage in these infections was CC5 and that human and chicken isolates within CC5 clustered in distinct subgroups (102). They further identified an increased recombination frequency within the CC5 poultry isolate relative to human isolate genomes and a tight clustering of chicken isolates once recombination events were compensated for. Changes in chicken-derived genomes localized within 33 genes and consisted of 196 substitution and 44 recombination sites. Forty-seven genes were more frequently present in CC5 chicken isolates compared to human isolates, with 38 of these being shared among CC5 and CC1 and 41 genes shared between CC5 and CC398 poultry isolates. All 47 poultry-associated genes were present in strains of the CC385 lineage. Recombination regions in poultry isolates were associated with both the core genome and plasmids, and many clustered within three distinct genomic regions comprising genes with putative roles in heat shock response, hemolysis, adhesion, mobile elements, and transposons. Furthermore, a total of 58 poultry-associated genes and genetic elements were predicted to be involved in the transfer of MGEs containing genes with predicated function as transposases, in conjugation as well as pathogenicity islands and two hotspot regions containing phage-related elements (102).

Several genes that have so far been found only in poultry isolates are implicated in increased pathogenicity in chickens (41, 110). These include *scpB*, encoding a putative cysteine protease (staphostatin A) (40, 111), which is found on an avian disease-associated plasmid (pAvX) in CC5 and CC385 strains (112). The CC385 lineage has been isolated from various wild and reared birds, suggesting that it has had long-term avian host restriction (40, 43).

MULTIHOST CC398: A MELTING POT AND RESERVOIR FOR VIRULENCE AND RESISTANCE DEVELOPMENT

MRSA strains of the CC398 complex have been studied in detail. This lineage is likely derived from a human MSSA clone that successfully jumped into pigs, where it acquired methicillin resistance and changes to its accessory genome (39). Despite these changes, it has retained the ability to infect humans, and it has been found in other animals, suggesting that CC398 strains are more promiscuous infecting agents than other CCs (43). CC398 is the main lineage of LA-MRSA strains in Europe, whereas other lineages have been isolated frequently in other geographical areas (113–115). CC9 LA-MRSA isolates are predominantly isolated in Asia, whereas CC398 and CC5 are relatively common in North America (116). Methicillin resistance is conferred by the acquisition of SCC*mec* elements that contain various *mec* genes. Presently, at least 13 structural types of SCC*mec* are known (30, 79–83).

The proportion of *S. aureus* infections caused by MRSA increased significantly from the end of the 1980s until 2000 worldwide (30). MRSA infections of humans could be initially grouped into either health care-associated (HA) or community-associated (CA) MRSA based on epidemiological criteria (117). HA-MRSA and CA-MRSA strains can be differentiated by their structural and functional genomic traits (118). However, these epidemiological criteria have become increasingly blurred because HA-MRSA strains have been found within the community and CA-MRSA strains were identified as the causative agents in the hospital setting (119, 120). In addition to these two categories of MRSA, animals can act as a reservoir for the development and transmission of so-called LA-MRSA strains that have been found to cause infections within the human community. All three MRSA types differ from each other in their genotypes and associated genotypic traits, allowing for a clear segregation into specific lineages associated with specific origins of the pathogen.

In pigs, *S. aureus* usually does not cause much disease; skin infections in pigs are typically caused by *Staphylococcus hyicus* and have only been occasionally documented to be caused by *S. aureus* (67, 121, 122). Consequently, *S. aureus* had not been monitored extensively in pigs. However, it has recently been realized that pigs represent a major reservoir for MRSA, after all.

CC398-MRSA and CC398-MSSA staphylococcal strains were first identified among pig farmers in France (122, 123). While CC398 MRSA strains rapidly spread among pigs and other livestock, they are considered to spread only infrequently beyond animals and personnel in direct contact with infected animals (124–126). Most LA-CC398 strains are resistant to β-lactams, macrolides, lincosamides, streptogramins, tetracyclines, and in part to fluoroquinolones as well as to cotrimoxazole. They are susceptible to glycopeptides, daptomycin, tigecycline, rifampicin, fusidic acid, fosfomycin, and with few exceptions also to linezolid (30). Initial studies suggested a possible human origin for LA-CC398 that was transferred to pigs and subsequently acquired methicillin resistance driven by the pressure of antibiotics in animal feed (39). However, a more recent analysis indicated that both human MSSA and LA-CC398 emerged in parallel around 1970 (127).

The CC398 lineage is the most commonly detected MRSA lineage among European livestock and thus was given the name LA-MRSA, with *spa* types t011, t034, and t108 being the most prevalent among the LA-MRSA CC398 strains (128, 129). CC398 MRSA strains are nontypable by *Sma*I pulsed-field gel electrophoresis (130), comprise only a small set of *spa*-types, and harbor a novel *Sau1-hsdS1* type 1 restriction-modification system (131). LA-MRSA isolates typically carry SCC*mec* type IVa or V, which are different from those carried by other MRSA genotypes commonly found in community and health care settings (132). They often exhibit coresistance to many non-β-lactam antimicrobials (e.g., macrolide [70%], trimethoprim [65%], gentamicin [14%], ciprofloxacin [8%], and trimethoprim-sulfamethoxazole [4%]), including those commonly used in animal production (133). The majority of CC398 LA-MRSA isolates do not produce toxins such as Panton-Valentine leukocidin or enterotoxins (134). Following the reduction in cost of next-generation sequencing approaches, further characterization of *S. aureus* CC398 isolates has been possible through the increased availability of whole-genome sequencing data for this CC, allowing more detailed insight into CC398's host adaptation (see sections below).

There is frequent transmission of CC398 LA-MRSA between livestock and farmers (135–139) and, until recently, strains of this lineage were rarely found outside this group (125). However, a rising number of cases of MRSA CC398 have recently been observed in humans within the health care environment (140). These findings show a strong epidemiological link with livestock contact (124). The origin of LA-CC398-MRSA is believed to be a human MSSA strain harboring the Φ*Sa*3 phage. This phage carries a so-called immune evasion cluster that encodes many human-specific immunomodulatory factors, including the *sea*, *sep*, *scn*, *chp*, and *sak* genes (encoding staphylococcal enterotoxin A and P, staphylococcal complement inhibitor, chemotaxis inhibitory protein, and plasminogen activator staphylokinase, respectively) and integrates within the *hlb* gene (141). The *hlb* gene encodes a sphingomyelinase known as β-toxin or β-hemolysin, which can lyse sheep erythrocytes. The factors encoded in the Φ*Sa*3 phage specifically interfere with the human immune response (142, 143), and about 90% of clinical human-derived isolates contain the Φ*Sa*3 phage within their genome (141). Given that the immunomodulatory factors encoded in Φ*Sa*3 specifically target human immune factors, it is not surprising that the Φ*Sa*3 phage is missing from the genomes of CC398 lineages adapted to livestock (30, 39). In general, porcine LA-MRSA CC398 lacks the Φ*Sa*3 phage and is *mec*A positive, while human-specific CC398 is *mec*A negative and Φ*Sa*3 positive (144, 145).

Studies indicate that the adaptation of CC398 to its host is connected to the loss and/or acquisition of MGEs, including Φ*Sa*3, since the major changes that were revealed in these studies occurred within the CC398 accessory genome (134, 146). In particular, a new staphylococcal pathogenicity island (SaPI-S0385) was identified in strain S0385 that appears to be a composite of the 5′ sequence of SaPIbov1 (up to and including the excisionase gene) and SaPI5 (packaging module) and contains a unique region at its 3′ end encoding two putative extracellular proteins with similarity to staphylococcal complement inhibitor and vWbp, respectively. Both proteins also have a conserved homologue in the core genome of S0385, but no studies have been performed to determine whether these conferred advantages to *S. aureus* within the porcine host (146).

In parallel, animal-independent human colonization and infection by CC398 MSSA strains has occurred and spread worldwide with a particularly high incidence rate in China, where this clone accounts for almost 20% of skin and soft tissue infections (147).

While CC398 has spread successfully among pigs in Europe, CC9 is the most commonly isolated lineage in farmed pigs in South-East Asia (82). Strains belonging to this CC are genetically distinct from strains of the CC398 lineage, and their genome is consistent with an independent zoonotic event leading to its emergence. CC9 MSSA strains colonize humans, and transmission between humans and pigs has been reported in the United Kingdom (122). The characterized CC9 isolates were deficient in the type IV restriction modification system, which poses a major restriction barrier for the acquisition of foreign DNA. Loss of the type IV restriction modification system has been observed in *S. aureus* strains prone to acquire the *van*A gene from enterococci conferring vancomycin resistance (148). Furthermore, two novel transposon-like elements containing genes with a high degree of similarity to genes from coagulase-negative staphylococci or enterococci have been identified in CC9 strains but so far have not been found in *S. aureus* strains belonging to other lineages (82). Overall, these observations might suggest that the newly emerged LA-CC9 strains could have an enhanced capacity for the uptake of foreign DNA. However, experimental verification remains to be provided. In line with this observation, the analyzed CC9 strains contained SCC*mec* type XII cassettes with a class C2 *mec* and *ccr*C gene complex (82). Such CC9 strains have also been isolated from cattle in China (81). The genes encoded by the SCC*mec* type XII elements are similar to genes found in coagulase-negative staphylococci, which could represent a potential source of this element. The CC9 MRSA strains thus represent a significant threat to humans as well as livestock, owing to their apparent ability to acquire novel genetic elements and their propensity for interspecies transmission.

MRSA IN COMPANION ANIMALS: CATS, DOGS, AND HORSES

Generally, MRSA strains of companion animals differ from those in livestock and meat production animals. *S. aureus* strains isolated from companion animals are mainly of human origin and are passed between human owners and their animals (38, 149–151). Dogs and cats are not typically colonized by *S. aureus* but, rather, form transient associations that can on occasion lead to severe infections (38, 152). MRSA infections in companion animals are predominantly skin and soft tissue infections; previous antibiotic treatments of human owners, the number of antimicrobial courses, the number of hospitalization days, implant devices, surgical

interventions, and contact with humans who have been previously hospitalized are major risk factors to these animals (153, 154). Overall, these risk factors are similar to those defining HA-MRSA infections in humans (155).

S. aureus MRSA strains isolated from horses and from humans in close contact with horses differ from those spread throughout the human population. A CA-MRSA clone (CC8) was isolated from horses in Canada and was well adapted to the animal host (156). In Europe, CC398 MRSA strains have also been isolated from horses, and horse-to-human transmission has been shown (157, 158). MRSA was first reported in horses in 1999 during a 13-month outbreak in a veterinary teaching hospital in Michigan. These were horses that had undergone surgical procedures and were subsequently infected with MRSA that appeared to have originated from colonized surgical staff (159). MRSA has since then been detected among horses in Europe, the United States, and Asia (160). Consistent with the risk factors, disease presentation in horses mirrors that observed in humans in the clinic. Skin and soft tissue MRSA infections, bacteremia, septic arthritis, osteomyelitis, implant-related infections, metritis, omphalitis, catheter-related infections, and pneumonia have all been reported in horses (160). MRSA infections in horses have been linked to strains carried by clinical personnel (CC1, CC254, and CC398), and nasal colonization of veterinarians, veterinary personnel, and students were also observed, indicating transmission to or from humans (161).

The main CC isolated from horses is CC8, and equine isolates are distinct from human strains of the general population but not from strains isolated from close-contact personnel. A recent study has shown that equine CC8 isolates acquired a phage encoding a novel equine allele of the staphylococcal inhibitor of complement (*scn*) as well as an equine-specific form of the bi-component leukocidins, LukPQ (43, 162, 163). Acquisition of antibiotic resistance determinants influences the clustering of equine and pig isolates, suggesting a role for the acquisition of resistance in host adaptation (43). Adaptation to horses also involves the acquisition of SaPI-encoded paralogues of the vWbp able to coagulate equine plasma (63). Since phages are required for the activation of SaPIs (164), it will be interesting to see whether the newly identified horse-specific phage is also able to activate and transfer this horse-specific SaPI.

MONKEYS IN SUB-SAHARAN AFRICA

Studies of species to species transmission of *S. aureus* have largely focused on LA transmission, yet nonhuman primates are readily colonized by *S. aureus* in captivity and in the wild (165). In a recent study, Senghore and colleagues investigated the transmission of *S. aureus* from humans to green monkeys in The Gambia (166). The study revealed multiple anthroponotic transmissions of *S. aureus* from humans to green monkeys and the emergence of a monkey-associated clade of *S. aureus* approximately 2,700 years ago. Development of this monkey-associated clade was accompanied by the loss of the Φ*Sa3* phage carrying genes known to play important roles in human colonization. More recent anthroponotic transmissions included well-characterized human lineages and are thought to be the result of human encroachment on monkey habitats. However, the authors did not observe any monkey to human transmission (166). Nonhuman primates (and bats) in sub-Saharan Africa are colonized by the related but distinct staphylococcal species *Staphylococcus argentus* and *Staphylococcus schweitzeri*. While *S. schweitzeri* was isolated from monkeys from all study sites, no transmission of these strains to humans was observed. In contrast, human-associated *S. aureus* sequence types (ST1, ST6, ST15) were detected in domestic animals and nonhuman primates, indicating a human-to-monkey transmission in the wild (165).

S. AUREUS HOST SWITCHING AND THE ROLE OF MGES

A recent study by Richardson et al. used a population-genomic approach to better characterize how *S. aureus* adapts to multiple different hosts and causes colonization and disease (43). The study found that humans act as a major hub for the pathogen for both ancient and recent host-switching events leading to the emergence of endemic livestock strains. Cows were shown to be the most frequent recipient of *S. aureus* host jumps but also appeared to be the main animal reservoir for reinfection of humans and the emergence of animal-derived human epidemic clones (33, 43). The study identified 14 host jumps from humans to cows (median number of host jumps per tree as distributions from all subsamples and trees in the study) dating back as early as 2000 BC to as recently as 2012 AD. Cows were also shown to act as a source of *S. aureus* for small ruminants such as goats and sheep. A pan-genome-wide association analysis identified host-specific accessory gene pools specific for birds, pigs, and horses. Accessory genomes from human, cow, sheep, and goat strains also clustered in a host-specific manner but exhibited greater diversity in gene content. The authors suggested that these differences might have been caused either by a range of cryptic host niches occupied by the pathogen or because the time elapsed since the host-switching event had been too short to allow sufficient diversification to result in the clear separation of human and ruminant accessory genome clusters. Alternatively, specific gene sets or combinations of gene sets might confer a more generalist host tropism. However, it was noted that clustering in equine and pig isolates was influenced by the acquisition of host-specific antimicrobial resistance determinants. Host-switching events were shown to be correlated with the acquisition via horizontal gene transfer of host-niche-specific genetic elements that confer selective advantages to the pathogen for survival within the new host. The study identified a total of 36 distinct MGEs (including predicted plasmids, transposons, *S. aureus* pathogenicity islands, and prophages). For instance, the β-hemolysin-converting phage Φ*Sa3*, which encodes modulators of the human innate immune response, was primarily associated with human strains, whereas several pathogenicity islands contain ruminant-specific superantigens or vWbps where associated with ruminants (62, 63). Conversely, equine isolates were shown to contain a prophage, integrated in the lipase precursor gene (*geh*), encoding equine-specific alleles of the staphylococcal inhibitor of complement (*scn*) and a bi-component leukocidin LukPQ (Table 1) (162, 163). The study also identified numerous previously uncharacterized MGEs. A novel plasmid SCC*mec* element encoding resistance to heavy metal ions (a common pig-feed supplement) was linked to human-to-porcine host-switching events. Furthermore, *S. aureus* isolates from animals acquired several gene clusters encoding bacteriocins that would enable them to compete with the resident bacterial flora. Interestingly, the MGEs in *S. aureus* pig isolates showed an increased guanine-cytosine content and reduced codon-adaptation index that indicated a distinct genealogical origin for these MGEs which may be related to pathogenicity islands identified in the pig-associated zoonotic pathogen *Streptococcus suis* (43). Host-switching events are therefore accompanied by the rapid

acquisition of MGEs that confer the capacity for survival within a new host niche, mainly by targeting the host's innate immune response. Acquisition of resistance to antimicrobials and heavy metals allows the pathogen to survive under high selective pressures; subsequent positive selection via point mutations or recombination (102) acts on the core genome to modify metabolic pathways and to further adapt *S. aureus* to its new host.

S. aureus host adaptation was found to coincide, depending on the host, with both gain and loss of gene function. While avian strains contained a higher proportion of functional genes compared to strains from other host species, ruminant strains showed an increase in pseudogenes. Many of these pseudogenes in ruminants were found to be associated with nutrient transport, including carbohydrates, and could indicate metabolic remodeling in response to distinct nutrient availability. *S. aureus* was shown to further adapt to its host niche in response to the availability of distinct nutrients. The authors showed that strains isolated from dairy cattle exhibited an enhanced ability to utilize lactose as a carbon source, supporting the concept that *S. aureus* undergoes genetic diversification in response to the nutrients that differ in availability in different niches (43).

The study also revealed that staphylococcal antibiotic and heavy metal resistance genes are unevenly distributed among isolates from different animal hosts and showed a clear correlation to antibiotic usage practices within medicine and agriculture. For example, human, pig, and ruminant isolates harbored a collection of key resistance determinants that were absent in avian isolates, in line with antimicrobial usage practices (43).

The acquisition of specific MGEs and core genome mutations plays a crucial role in *S. aureus* host adaptation (40, 44, 63). For instance, the presence/acquisition of MGEs not found in human strains could be clearly associated with host jumps from humans to avian and porcine hosts (39, 40, 43). Host-specific functional effectors of *S. aureus* pathogenicity such as leukocidins, superantigens, and vWbps are frequently located on MGEs (61, 162, 163, 167–170).

CONCLUSIONS

In the past decades an increasing number of studies have demonstrated that *S. aureus* is able to colonize and infect a plethora of different hosts. While *S. aureus* can cause severe infections in some animals, others show less severe symptoms and are mainly colonized, acting as a staphylococcal reservoir for human reinfection. This is particularly true for *S. aureus* lineages found in pigs and dairy cows. Due to the use of specific antibiotics and growth-enhancing supplements, these strains have acquired mechanisms of resistance to these agents from various environmental sources. Current farming practices make farm animals ideal breeding grounds for the development and/or acquisition of new resistance mechanisms that can spread into the community and pose a significant risk to the human population. There is an ever-increasing amount of data detailing host-switching events for *S. aureus*, with humans acting as the major exchange hub for strain lineages. These data highlight the ability of *S. aureus* to function as a multihost pathogen and to evolve and adapt to new hosts. The ability of *S. aureus* to readily adapt to new environments and rapidly take up new genetic material via horizontal gene transfer makes the bacterium a most versatile colonizer that is able to spread into new niches. Moreover, it can also rapidly adapt to new stresses and antibiotics with the result of an ever-continuing arms race between *S. aureus* and humankind.

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Antibiotic Resistance and the MRSA Problem

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47

INTRODUCTION

Staphylococcus aureus gives rise to a variety of infections, and treatment of these relies on antibiotics. A number of antibiotics are used to treat staphylococcal infections (Table 1), and they target major bacterial processes including cell wall synthesis, translation, transcription, and DNA synthesis. However, resistance to antibiotics is a growing problem, and treatment failures are associated with enormous human and medical costs. Antibiotic resistance arises by several different mechanisms, such as altered drug targets, enzymatic drug inactivation, increased efflux of antimicrobial compounds, and altered drug accessibility (1), and the spread of resistance is aided by a multitude of mobile genetic elements (reviewed in 2–4). Although resistance has been observed for essentially all compounds, individual strains resistant to all drugs have not appeared. Yet resistance still poses treatment challenges, as exemplified by vancomycin. Fully resistant strains of which are rare, but the more common intermediately resistant (VISA) strains are associated with more severe infections and longer duration of treatment compared to susceptible strains despite the fact that they only display a minor increase in the MIC of vancomycin. This highlights the problem that we only have limited knowledge of how resistance genes and mutations affect the overall biology of resistant strains and of the impact of resistance on pathogenesis. In this article, we focus on the biology of antibiotic resistance in *S. aureus* and on the behavior of resistant strains, and we conclude with a description of some of the new therapeutic approaches that in the future may become treatment options for infections with antibiotic-resistant staphylococci.

CELL ENVELOPE ACTIVE AGENTS

Beta-Lactam Antibiotics

History and mode of action

Alexander Fleming's observation that the growth of *S. aureus* can be inhibited by a contaminating mold led to the groundbreaking discovery of penicillin and initiated the antibiotic era. In the past 75 years beta-lactams have been the most important class of antibiotics for the treatment of *S. aureus* infections. However, even before penicillin was marketed, some *S. aureus* strains were in possession of a powerful resistance mechanism in the form of beta-lactamases. Carried by plasmids (5), the beta-lactamase gene spread rapidly and soon obviated penicillin as the first line of treatment for *S. aureus* infections. As a consequence, in the following years, several hundred antibiotics of four structural subclasses, penicillins, cephalosporins, monobactams, and the more powerful carbapenems, were developed and marketed. The ability of *S. aureus* to become resistant to almost all of these through the acquisition of a single genetic element, the staphylococcal cassette chromosome *mec* (SCC*mec*), giving rise to the notorious methicillin-resistant *S. aureus* (MRSA) strains, is beyond doubt the major resistance concern. The amount of scientific literature covering the epidemiology of MRSA and beta-lactam resistance mechanisms is overwhelming. Nonetheless, important knowledge gaps still exist, as will be highlighted below.

All beta-lactam antibiotics possess the defining four-membered beta-lactam ring that is essential for the biological activity of these compounds. Early work on the mechanism of action of penicillin culminated in the discovery that penicillin inhibits cross-linking of peptidoglycan, the central component of bacterial cell walls (6). In Gram-positive bacteria, the cell wall is composed of glycan chains of *N*-acetylglucosamine and *N*-acetylmuramic acid that are heavily cross-linked by pentapeptides (UDP-MurNAc-penta). Each precursor component is synthesized in the cytoplasm and transported to the division septum of the growing cell, where the structural similarity between penicillin and D-alanyl-D-alanine (D-ala-D-ala) residues of newly synthesized UDP-MurNAc-penta, allows penicillin to inhibit crosslinking of peptidoglycan by acting as a substrate analog (6). The enzymes mediating transpeptidation of peptidoglycan strands, the target of penicillin, were hence designated penicillin binding proteins (PBPs). The binding of beta-lactams to PBPs leads to formation of long-lasting acyl-enzyme complexes, thereby essentially blocking the transpeptidase activity of these enzymes. The realization that penicillin reduces peptidoglycan cross-linking led to the classical model explaining penicillin-mediated cell lysis as a consequence of a mechanically weakened cell wall incapable of withstanding the high internal osmotic pressure (7, 8). The killing effect of beta-lactam antibiotics, however, has turned out to be much more complex, and the downstream effects of blocking transpeptidation that lead to cell death remain an unsolved mystery (8). In staphylococci, the killing mechanism has been shown to be highly cell cycle dependent, and it has been proposed that a mis-coordination in the spatial and temporal deposition of peptidoglycan during cell division leads to cell death and that cell lysis and cell death may be two separate events

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TABLE 1 Antibiotics used against *S. aureus*

Agent class	Agent (examples)	Molecular target	Reference(s)
Cell envelope			
β-lactams (penicillinase sensitive)	Ampicillin, amoxicillin	Penicillin-binding proteins	276
β-lactams + β-lactamase inhibitors	Amoxicillin + clavulanate	Penicillin-binding proteins	276
β-lactams (penicillinase resistant)	Oxacillin, nafcillin	Penicillin-binding proteins	276
Cephalosporins	Ceftaroline	Penicillin-binding proteins	58
Carbapenems	Imipenem	Penicillin-binding proteins	277
Glycopeptides	Vancomycin, teicoplanin	MurNac pentapeptide	278
Lipoglycopeptides	Dalbavancin, telavancin	MurNac pentapeptide and cell membrane	110, 112
Phosphonic acid	Fosfomycin	Enoylpyruvate transferase (MurA)	125
Lipopeptide	Daptomycin	Cell membrane	130
Protein synthesis			
Oxazolidinones	Linezolid, tedizolid	50S ribosomal subunit	279, 280
Macrolides	Erythromycin	50S ribosomal subunit	281
Streptogramins	Quinupristin, dalfopristin	50S ribosomal subunit	282
Lincosamides	Clindamycin	50S ribosomal subunit	283
Chloramphenicols	Chloramphenicol	50S ribosomal subunit	284
Pleuromutilins	Retapamulin	50S ribosomal subunit	194
Aminoglycosides	Gentamicin	30S ribosomal subunit	198
Tetracyclines	Tetracycline, doxycycline	30S ribosomal subunit	208
Glycylcyclines	Tigecycline	30S ribosomal subunit	210
Pseudomonic acid	Mupirocin	Isoleucyl-tRNA synthtase	224
Fusidic acid		Elongation factor G	285
DNA replication			
Fluoroquinolones	Ciprofloxacin, moxifloxacin	DNA gyrase, topoisomerase IV	237
Transcription			
Rifamycins	Rifampicin	RNA polymerase	244
Metabolism			
Trimethoprim		Dihydrofolate reductase	286
Sulfonamides	Sulphamethoxazole	Dihydropteroate synthetase	286

(9, 10). Similar to other bacteria, *S. aureus* possesses several PBPs that in addition to providing transpeptidase activity seem to fulfill distinct functions in coordinating peptidoglycan synthesis (see below). Because different classes of beta-lactams target individual PBPs with variable affinity, this may explain why different beta-lactams introduce various morphological changes (10). Hence, the molecular events underlying the bactericidal effects of beta-lactam antibiotics may depend on the affinity of the specific compounds for the various PBPs.

Resistance

Penicillin-resistant *S. aureus* isolates emerged shortly after penicillin was introduced for clinical treatment of bacterial infections (11), and less than 2 decades later, about 80% had become resistant to penicillin through acquisition of the *blaZ* gene encoding the enzyme beta-lactamase, which inactivates penicillin by hydrolyzing the critical beta-lactam ring (12). The *blaZ* gene is part of a transposable element that can either be integrated into the chromosome or be located on large conjugative plasmids that, in addition to *blaZ*, often harbor resistance to mercury and other heavy metals as well as additional antibiotic resistance genes (13–16). Today as many as 99% of clinical *S. aureus* strains are resistant to penicillin, underscoring the adaptive power of *S. aureus* when exposed to a strong selective pressure such as antibiotics (1).

In response to the worldwide spread of penicillin-resistant *S. aureus*, semisynthetic penicillinase-resistant beta-lactams such as, first, methicillin and, later, oxacillin were developed for clinical use. Methicillin was introduced into clinical practice in 1961, and later the same year, the first clinical MRSA isolates were identified in a UK hospital (1, 17). MRSA strains are resistant to methicillin through horizontal acquisition of the *mec*A gene encoding PBP2a, an alternative transpeptidase with low affinity for most beta-lactam antibiotics, hence conferring resistance not only to methicillin but to virtually all members of the large class of beta-lactams. Despite the broad resistance provided, and even though methicillin is no longer used clinically, MRSA has remained the preferred designation for *mec*A-positive isolates.

The *mec*A gene is present on a family of structurally complex genetic elements designated SCC*mec* that often carry additional genes conferring resistance to other antibiotics and harmful substances such as heavy metals (18, 19). Several lines of evidence suggest that SCC*mec* evolution occurred in the *Staphylococcus sciuri* group, a primitive group

of *Staphylococcus* species that are frequent colonizers of the skin of domestic animals (20–23). PBP2a is very similar to the native PBPs encoded by species belonging to the *S. sciuri* group, but the evolution of the *mecA* ancestor gene into a resistance determinant required numerous genetic changes (23). Consistent with this finding, it seems to be the widespread use of penicillin and other first-generation beta-lactams in the years prior to the introduction of methicillin which selected for *S. aureus* strains carrying the *mecA* resistance determinant (24). A novel genetic determinant, *mecC*, encoding a transpeptidase enzyme with only 63% identity to *mecA*-encoded PBP2a, was recently described (25). Interestingly, *mecC*, similar to *mecA*, seems to have evolved in animal-adapted lineages of *S. aureus*, emphasizing the importance of the animal staphylococci as a reservoir of resistance genes that can potentially contribute to the evolution of antibiotic-resistant human pathogens (26).

The SCC*mec* element

SCC*mec* elements are highly diverse in their structural organization and genetic content, with sizes varying from approximately 20 kb to >60 kb (27). They share a modular structure with two essential gene complexes, the *mec* complex, consisting of *mecA* plus its regulators (*mecR1* and *mecI*), and the *ccr* complex, encoding recombinases of the invertase/resolvase family that mediate site-specific integration of the element (18). The element has a single chromosomal integration site (designated *attB*) located downstream of the *rlmH* gene (previously *orfX*), encoding an rRNA methyltransferase (28). The SCC*mec* elements additionally carry a highly variable number of insertion sequences, transposons, and plasmids inside the so-called joining regions J1 to J3 (also known as junkyard regions). Support for SCC*mec* as a mobile genetic element was provided by the finding that different staphylococcal strains harbor identical SCC*mec* elements (29, 30), but it was only recently that *in vitro* transfer of SCC*mec* by transducing phages or conjugative plasmids was finally demonstrated (31–34). Epidemiological data likewise support the idea that acquisition of SCC*mec* elements by methicillin-susceptible *S. aureus* (MSSA) strains is a relatively rare event (35, 36), and restriction modification barriers between major MRSA clones are thought to limit transfer (37). Thus, the primary spread of MRSA seems to occur via clonal expansion of a relative low number of epidemic clones.

SCC*mec* typing in the epidemiology of MRSA

The structural differences between the *mec* and *ccr* gene complexes form the basis for SCC*mec* typing, a PCR-based typing method that is used worldwide for epidemiological surveillance of MRSA (see 38 for details). To date, 11 SCC*mec* types have been identified (27). Early hospital-acquired MRSA isolates such as the archaic MRSA strain COL typically harbor large SCC*mec* elements belonging to type I, II, or III (>50 kb). In contrast, more recent strains typically possess the much smaller SCC*mec* IV (21 to 24 kb) or V (27 kb). The smaller SCC*mec* cassettes were first identified in community-associated MRSA (CA-MRSA) that emerged outside of hospitals in the mid-1990s (until then MRSA isolates were largely confined to hospitals) (15). Today, the traditional grouping of MRSA isolates into health care-associated MRSA (HA-MRSA) and CA-MRSA is losing relevance, because CA-MRSA has also become established in health care settings, and it has been suggested that these clones, which are more contagious and more virulent than the classic HA-MRSA, will replace the HA-MRSA clones over time (39).

Factors impacting resistance level

A notable feature of most MRSA isolates is that resistance to beta-lactams is expressed in a heterogeneous manner (40, 41). For these strains, populations arising from a single cell display widely different resistance levels, with the majority of cells exhibiting a low level of resistance and a minority of cells being highly resistant. While some HA-MRSA isolates exhibit high-level, homogeneous methicillin resistance, CA-MRSA isolates often exhibit low-level, heterogeneous resistance (42). Insight into the molecular mechanisms underlying this phenomenon has come from the identification of mutations that convert strains expressing low, heterogeneous resistance into homogeneous, highly resistant strains. These mutations map to genes associated with cellular stress responses, such as stringent response signaling via ppGpp and the ClpXP protease controlling the Spx stress response (42–45), indicating a close link between bacterial physiology and resistance levels.

Despite the fact that MRSA strains have become resistant to beta-lactams through acquisition of one specific resistance determinant, the *mecA* gene, clinical MRSA isolates exhibit highly variable levels of resistance: in some MRSA strains resistance is barely above that displayed by susceptible isolates (methicillin MICs <3 μg/ml), while other strains are highly resistant (methicillin MICs up to 1,600 μg/ml) (46). The mechanisms underlying these intriguing differences in resistance level remain poorly understood. In some cases, high resistance levels were attributed to increased expression of PBP2a due to duplication or enhanced transcription of the *mecA* gene (47). Two functionally similar two-component systems, BlaR1/BlaI and MecR1/MecI, control transcription of *mecA* in response to beta-lactams, and because genetic mutations are common in the regulatory elements controlling *mecA* expression, the PBP2a level varies widely between MRSA strains (see 48 for a recent review of *mecA* regulation). In several cases differences in resistance levels did not correlate to PBP2a expression, suggesting that factors other than PBP2a modulate the strain-specific level of beta-lactam resistance (46, 49, 50). Indeed, genetic screens have identified a number of auxiliary factors (also designated "fem-factors") essential for methicillin resistance that are critical for PBP2a-mediated resistance to beta-lactam antibiotics (51, 52). Examples include cell division proteins, native PBPs, and enzymes involved in the synthesis of teichoic acids and peptidoglycan precursors. In some cases, the requirements for these auxiliary factors have been explained. For example, the essential glycosyltransferase domain of the native PBP2 is needed to cooperate with the transpeptidase activity of PBP2a in the building of peptidoglycan (53). The fact that beta-lactam resistance depends on auxiliary factors opens up new possibilities for the treatment of MRSA infections, because drugs that inhibit the functions of auxiliary factors work synergistically with beta-lactams to kill MRSA (52, 54, 55).

Beta-lactams for the treatment of MRSA: future perspectives

The discovery that MRSA strains can be resensitized to beta-lactams by interfering with the activity of auxiliary factors opens up a novel paradigm in the treatment of MRSA, because drug-like leads targeted against these factors could be used in combination with beta-lactams to restore the sensitivity of MRSA to beta-lactams (56). Indeed, a combination agent strategy of pairing beta-lactamase inhibitors such as clavulanic acid with beta-lactams has proven highly successful in restoring beta-lactam efficacy against Gram-negative bacteria that have become resistant to beta-lactams

through acquisition of beta-lactamase enzymes (57). Additionally, a new generation of cephalosporins, fifth-generation cephalosporins such as ceftaroline, with efficacy against MRSA have been marketed (58). The active site of PBP2a is normally protected from beta-lactam antibiotics because it is present in a narrow, inaccessible groove. To accommodate the native peptidoglycan strand, the active site is made accessible via a conformational change regulated by an allosteric switch (59, 60). Ceftaroline binds to the allosteric site of PBP2a and thereby opens the active site for binding of a second molecule of ceftaroline that inhibits transpeptidase activity (60). Despite the short time that ceftaroline has been in clinical use, MRSA strains with reduced susceptibility to ceftaroline (obtained through mutations in PBP2a) have already been isolated (61, 62). Hence, the battle continues.

Vancomycin Resistance and VISA

History and mode of action

Although it was discovered in the late 1950s, it was not until the emergence of MRSA 30 years later that the glycopeptide vancomycin became the choice for treatment of MRSA infections (63). Since then, vancomycin has been one of the preferred drugs for treatment of MRSA infections. The target of vancomycin is the cell wall, where it binds with high affinity to the penultimate D-ala-D-ala residues of newly synthesized UDP-MurNAc-penta, thereby disrupting peptidoglycan assembly (64). Strains of *S. aureus* susceptible to vancomycin (VSSA) have MICs of <2 μg/ml, whereas intermediately resistant (VISA) strains display MICs of 4 to 16 μg/ml, and for the resistant strains the MIC is ≥16 μg/ml. Vancomycin-resistant *S. aureus* (VRSA) carries the *vanA* operon that provides the cell with two important activities, namely, the hydrolysis of the D-ala-D-ala precursors and the synthesis of the D-ala-D-lactate precursor, which cannot bind vancomycin (65). The first case of VRSA was reported in 2002, when during coinfection with *Enterococcus faecalis* a MRSA strain acquired the *vanA* operon from a conjugative *E. faecalis* plasmid and displayed a MIC to vancomycin of >1,000 μg/ml (66, 67). Fortunately, since then, only a handful of VRSA cases have been reported, and usually they involve transfer of the *vanA* operon from enterococci (68). The low prevalence of VRSA may be due to limited fitness of the *vanA*-containing enterococcal plasmids in *S. aureus* (69) or, for the MRSA strains, the incompatibility with methicillin resistance, because the *mecA*-encoded transpeptidase, PBP2a, is not able to cross-link the modified wall precursor in the VRSA wall (70). The incompatibility between glycopeptide and methicillin resistance has been termed the "seesaw" effect and can be exploited clinically (71).

Intermediate resistance

VISA strains are associated with serious clinical complications, such as prolonged hospitalization, persistent infections, prolonged vancomycin treatment, and/or treatment failure (71–75), although they appear compromised when examined in animal model systems (76). Vancomycin treatment failure has even been reported for strains with marginally decreased susceptibility and MIC break- points of 1 to 2 μg/ml, the latter being the concentration of an antibiotic, which defines whether a bacterium is susceptible or resistant to the antibiotic (77). In contrast to VRSA, VISA's reduced susceptibility to vancomycin is not due to an acquired antibiotic resistance gene but results from the accumulation of mutations leading to one or more of a number of characteristic phenotypes. These include increased cell wall thickness, cell wall changes leading to anomalous diffusion of vancomycin through the VISA cell wall, decreased negative cell surface charge, decreased autolysis, increased cell wall synthesis, and decreased peptidoglycan cross-linking resulting in high-affinity binding of vancomycin to nonamidated muropeptides (78–89). The mutations associated with VISA were recently summarized in reference 90 and often involve genes associated with (i) the cell wall stress regulon, e.g., the two-component regulatory systems *graRS*, *vraSR*, and *walRK* (*yycFG*) that stimulate expression of the *dlt* operon and *mprF*, leading to reduced negative cell surface charge and consequently less vancomycin binding; (ii) *agr* encoding the virulence regulatory quorum sensing system; (iii) *rpoB* encoding the RNA polymerase; and (iv) other transcriptional regulators or *clpP* encoding the proteolytic component of the Clp protease. There are multiple evolutionary pathways for a VSSA strain to become VISA (91), and although the number of mutations needed to display the VISA phenotype is usually less than 10 (92), it can be challenging, particularly with respect to clinical isolates, to determine the contribution of individual mutations to the VISA phenotype. In one study of the well-known VISA strain Mu50, Katayama and coworkers introduced mutations of six genes associated with decreased vancomycin susceptibility to VSSA strains and tracked the contribution (93). Importantly, all six mutated genes contributed to the VISA phenotype and were directly or indirectly involved in the regulation of cell physiology (93). In other studies, sequential tracking of strains during infection and chemotherapy has revealed a much greater number of genes involved (80, 94–96), stressing the importance of bacterial processes for the development of VISA strains.

Similar to methicillin resistance, the VISA phenotype is commonly preceded by a hetero-VISA phenotype (hVISA), where multiple mutations in hVISA strains lead to the VISA phenotype (97). Mu3 was the first hVISA to be characterized, and similar to hetero-MRSA, the hVISA phenotype is revealed as an uneven killing of a seemingly homogenous cell population. For hVISA the majority of cells have little or no resistance to vancomycin and are killed by 2 μg/ml, whereas a subpopulation survives vancomycin concentrations of >4 μg/ml and thus behaves like VISA strains (89). The hVISA strains are characterized by a thickened cell wall in the absence of mutations associated with VISA strains, and the phenotype can be triggered by exposure to nonglycopeptide antibiotics such as beta-lactams (98, 99). Interestingly, the hVISA phenotype does not develop in cells carrying a mutation in *trfA* that influences resistance to teicoplanin, another glycopeptide antibiotic (100), and encodes an adaptor of the ClpC ATPase (101) that together with the proteolytic subunit, ClpP, is responsible for the degradation of nonnative proteins (101, 102). Clinically, hVISA strains are associated with persistent infections and attenuated host immune response, and differential gene expression changes seem to underlie their development (103).

Recently, yet another VISA phenotype was discovered, termed slowVISA (sVISA), which is characterized by very slow growth, requiring 72 hours or more for colony formation, relatively high MICs (>8 μg/ml) to vancomycin, an unstable resistance profile, and colony morphology that reverts in the absence of vancomycin (104). Altered expression of the stringent response, as well as mutations in *rpoB* and *rpoC* encoding subunits of the RNA polymerase, are associated with the sVISA phenotype (105, 106). Importantly, low concentrations of mupirocin, a well-known inducer of the stringent response, enabled the isolation of stable sVISA

strains, and this approach was used to demonstrate the presence of sVISA among clinical isolates (107).

A significant challenge with the hVISA and sVISA strains is that traditional susceptibility testing does not reveal their presence. Both forms are induced by vancomycin exposure, but hVISA also arises in response to beta-lactam antibiotics, while stabilization of sVISA occurs upon induction of the stringent response—neither of which are present during susceptibility testing. A related finding was made by Haaber et al., who observed that upon exposure to the antimicrobial peptide antibiotic colistin, *S. aureus* elicited reversible and reduced susceptibility to vancomycin in the absence of genetic change (108). Collectively, these findings indicate that VISA derivatives may develop not only through mutations but also through unrelated phenotypic processes (90) and that such phenotypic tolerance could contribute significantly to the clinical failures of vancomycin chemotherapy.

Lipoglycopeptides

Mode of action

The reduced susceptibility of MRSA strains to vancomycin has called for new treatment options for MRSA infections, leading to the development of the lipoglycopeptides, telavancin, oritavancin, and dalbavancin. They are derivatives of vancomycin that have been modified by lipophilic side chains, where the heptapeptide core inhibits transglycosylation and transpeptidation reactions in cell wall synthesis and the lipophilic side chains prolong half-life and allow interactions with the cell membrane (109). While dalbavancin inhibits the late stages of peptidoglycan synthesis mainly by impairing transglycosylase activity, oritavancin and telavancin anchor in the bacterial membrane by the lipophilic side chain linked to their disaccharide moiety, disrupting membrane integrity and causing lysis (110).

Telavancin is active against both MSSA and MRSA, and it causes rapid, concentration-dependent depolarization of the bacterial plasma membrane, increased permeability, and leakage of cellular ATP and K^+ (111). It is 16- to 32-fold more active than vancomycin and displays activity against hVISA and VISA but reduced activity against VRSA (112). Dalbavancin is also active against VISA and hVISA, but strains with reduced susceptibility to vancomycin also demonstrate reduced susceptibility to dalbavancin (113, 114). Importantly, oritavancin is active against both VISA and VRSA (115). This is likely related to its ability to bind not only to the D-ala-D-ala peptidoglycan termini similar to vancomycin but also to interact with peptides near the terminal D-ala-D-ala (or D-lactate in VRSA), thus maintaining binding affinity for the modified peptidoglycan peptide termini of vancomycin-resistant organisms (116). In comparison to vancomycin; dalbavancin, oritavancin, and telavancin all have longer half-lives, which allow once daily dosing for telavancin, once weekly dosing for dalbavancin, and potentially one dose per treatment course for oritavancin (109, 117, 118).

Resistance

In general, resistance to lipoglycopeptides is remarkably rare. The long half-life of these compounds *in vivo* may contribute to this but may also provide selection for resistance at subinhibitory concentrations. In a study of telavancin, resistance development was assessed by *in vitro* passages, and only 1 out of 10 passaged MRSA clones showed increasing MIC. This was from 0.25 μg/ml to 2 μg/ml after 43 days, and subsequently, no further increase occurred (119). Resistance to dalbavancin could not be obtained by direct selection of MSSA, MRSA, or VISA strains on plates containing dalbavancin (120), whereas passaging in the presence of subinhibitory concentrations increased the dalbavancin MIC of MRSA strains by 2 or 3 dilutions (to 0.25 and 0.5 μg/ml) (120). Recently, a clinical case of decreased susceptibility to dalbavancin was reported, where the use of a dalbavancin and vancomycin therapy led to development of a vancomycin- and dalbavancin-nonsusceptible phenotype. Importantly, these susceptibility changes were accompanied by a 4-fold increase in daptomycin and telavancin MICs, although the patient did not receive treatment with these agents (121). This observation may be explained by the finding that decreased dalvabancin susceptibility was a result of mutations of *yvqF* (*vraT*), which regulates cell wall metabolism and has been implicated in the VISA phenotype (122).

Fosfomycin

Mode of action

Due to the increasing problems with antibiotic resistance, older antibiotics are being re-evaluated for clinical use to expand treatment opportunities against difficult to treat infections (123, 124). Fosfomycin is a phosphoenolpyruvate analogue that inhibits the enzyme MurA, which catalyzes the first step in peptidoglycan biosynthesis, and prevents the formation of *N*-acetylmuramic acid, which is an essential precursor for the peptidoglycan cell wall (125). Fosfomycin is minimally used for other than uncomplicated urinary tract infections. However, it generally displays good activity against MRSA and can be orally administered; hence, this antibiotic may be of clinical importance for difficult to treat *S. aureus* infections (124).

Resistance

Fosfomycin resistance can arise via enzymatic modification of the agent, reduced uptake, or target-site modifications. Enzymatic modification of the antibiotic by a thiol-*S*-transferase encoded by the *fosB* gene is a common resistance mechanism among clinical *S. aureus* isolates (126, 127). Several other enzymes, such as FosA and FosX, have been identified in other bacterial species that can modify fosfomycin, whereas in *S. aureus* only the *fosB* gene has thus far been identified (128). Reduced uptake has been characterized in *E. coli* by chromosomal mutations in two transporter systems, GlpT and UhpT, which enable fosfomycin uptake (129). Mutations in the genes encoding for GlpT and UhpT have also been identified among clinical fosfomycin-resistant *S. aureus* isolates, along with target-site modifications of MurA (127). However, the individual roles of such mutations have not been elucidated in naive strain backgrounds (127).

Daptomycin

History and mode of action

Daptomycin, a cyclic lipopeptide produced by *Streptomyces roseosporus*, was approved by the FDA in 2003 for treatment of complicated skin and soft tissue infections caused by Gram-positive bacteria, for *S. aureus* bacteremia, and in 2006 for right-sided infective endocarditis. (See 130 for an extensive review of the journey of daptomycin from drug discovery to the bedside.) Since then, daptomycin has become an important treatment option for deep-seated infections caused by MRSA (131). Daptomycin targets the cytoplasmic

membrane, but the mechanistic details underlying the bactericidal effect of this binding remain elusive (reviewed in 130). The activity of daptomycin is completely dependent on calcium ions, and it is generally accepted that the positive charge of calcium-complexed daptomycin facilitates binding and insertion of daptomycin into the cytoplasmic membrane, where it oligomerizes to form pore-like structures (130). Demonstrated downstream effects that directly or indirectly may cause cell death involve membrane depolarization, ion leakage, and delocalization of enzymes synthesizing the cell wall (130). Notably, daptomycin, in contrast to most antibiotics, has bactericidal activity even against stationary *S. aureus* cells, supporting the view that it targets a process that is essential in nongrowing cells (132).

Resistance

The development of daptomycin resistance (an official daptomycin resistance breakpoint has not been declared, and therefore the official terminology is "daptomycin nonsusceptibility," but for ease of presentation we will use the term "daptomycin resistance") in staphylococci seems to be a relatively rare phenomenon, and no trend toward increased daptomycin resistance was noted in a worldwide surveillance program (2005 to 2012) (133). Nonetheless, *S. aureus* isolates that have evolved daptomycin resistance during clinical treatment are well described (134–136). Daptomycin resistance is obtained through selection of multiple spontaneous mutations with hotspots in genes affecting the composition or charge of the cell envelope (134, 136). The clinically most significant example of this is mutations increasing the activity of the multipeptide resistance factor (MprF) that catalyzes synthesis of the positively charged lysylphosphatidylglycerol (134–138). The *mprF* mutations correlate with excess lysylphosphatidylglycerol in the cell membrane but not always with augmented positive surface charge, suggesting that electrostatic repulsion alone does not explain the improved ability to withstand daptomycin (138, 139). Other genetic changes frequently implicated in decreased tolerance to daptomycin map to *rpoB* and to genes controlling activity of the YycFG (WalRK) two-component system, which responds to perturbations in cell membrane homeostasis (94, 95, 134, 140, 141). Consistent with these genetic changes, the prevailing phenotypic changes reported for daptomycin-resistant *S. aureus* strains include increased positive charge of the cell membrane and a thickened cell wall (134, 136, 142–144). Additionally, the genetic changes associated with daptomycin resistance often correlate with decreased expression of virulence genes and altered pathogenesis of the isolates (144, 145). An intriguing explanation for this finding was proposed when it was found that mutants lacking the *agr* quorum sensing system survived daptomycin exposure by releasing membrane phospholipids, which bound and inactivated the antibiotic. Although wild-type bacteria also released phospholipid in response to daptomycin, *agr*-triggered secretion of small cytolytic toxins, known as phenol soluble modulins, prevented antibiotic inactivation (146).

PROTEIN SYNTHESIS INHIBITORS

Linezolid and Tedizolid

History and mode of action

Linezolid is an oxazolidinone that since its approval in 2000 has been used to treat nosocomial pneumonia and skin and soft tissue infections caused by both MRSA and MSSA (147). Compared to vancomycin, it has favorable pharmacokinetic properties and is applied to difficult to treat infections, including those caused by strains displaying decreased susceptibility to glycopeptides (148). Linezolid targets the ribosome by interacting with the 23S rRNA and blocks protein synthesis by preventing binding or proper placement of aminoacyl-tRNA in the peptidyltransferase center (149, 150). Linezolid also interacts with tRNA, the ribosomal protein L27, and the ribosomal-associated protein LepA (150). The general effect of linezolid on protein expression leads to reduced expression of toxins (151) and particularly reduced amounts of excreted or membrane-associated proteins (152), which likely contributes to the clinical efficacy of the drug.

Resistance

The primary activity of linezolid is binding to the 23S rRNA, and since there are five to six rRNA (*rrn*) operons in *S. aureus* (153), it was suspected that resistance development would be limited (154). However, resistance has been associated with distinct nucleotide substitutions in domain V of the 23S rRNA gene, particularly G2447T, T2500A, and G2576T, where a gradual increase in the number of mutated 23S rRNA gene copies occasionally combined with copy deletions has been associated with increased resistance levels (155–158). In particular, the first mutation appears to be rate limiting in resistance development as demonstrated in one study, where the first G2576T mutation required by far the longest time to occur (157). Subsequent acquisition of the same nucleotide exchange in the other 23S rRNA gene copies suggested that they are not independent mutational events but arise, for example, by homologous recombination or gene conversion (157, 159). Deletions or mutations in the ribosomal proteins L3 and L4 have also been associated with resistance (158, 160) and sometimes in combination with 23S rRNA mutations (147). The fitness of resistant strains obtained by *in vitro* passage decreases with increasing numbers of mutated 23S rRNA copies and resistance levels (157), but importantly, strains that carry only one mutated copy and are susceptible to linezolid are without apparent fitness defects, and the mutations are stable over extended periods of time (159). In a clinically isolated, linezolid-resistant mutant, the resistance was found to be mediated by a G2576U mutation in all five 23S rRNA copies. The resistance was not accompanied by any growth defect, and passage in antibiotic-free medium did not decrease resistance levels (161). Thus, in successfully resistant clinical strains, compensatory mutations are likely to be present.

Another mechanism by which *S. aureus* may become resistant to linezolid is through the acquisition of the ribosomal methyltransferase *cfr* gene (154, 162, 163). It methylates the A2503 in the peptidyl transferase center of the 23s rRNA (164). The *cfr* gene has been identified in a number of clonal complexes, including the livestock-associated ST398 and ST9 (165), and it is commonly associated with other mobile genetic elements (166). Importantly, the *cfr* gene is present on plasmids also in other Gram-positive pathogens, including *S. epidermidis*, from where it can be transferred to clinical MRSA strains (166, 167), highlighting the importance of the resistance gene pool present in other staphylococcal species. Fortunately, tedizolid, a second-generation oxazolidinone, retains activity against strains carrying *cfr* (168). Tedizolid is 4- to 16-fold more potent toward MRSA compared to linezolid, and this compound causes a dramatic reduction in virulence factor production (169), and resistance is still limited.

Although there are numerous reports of resistance to linezolid (147), generally >98% of *S. aureus* strains remain susceptible. In some patient groups, however, resistance is

significantly more prevalent. For example, in cystic fibrosis patients, 11% of isolates are resistant (170). Surprisingly, linezolid may also stimulate the growth of some staphylococcal strains, as demonstrated for a thymidine-dependent small colony variant of a MRSA strain isolated from the lung of a cystic fibrosis patient (171). In this strain, in the absence of any known linezolid resistance mutations or genes, the presence of linezolid greatly stimulated growth. This finding reflects the complexity and unpredictability of linezolid's effects on ribosomal functions and stresses the need for further investigations.

Macrolides, Lincosamides, and Streptogramins

History and mode of action

Macrolides, lincosamides (including clindamycin [172, 173]), and streptogramins (type A and B) (commonly abbreviated MLS) all inhibit protein synthesis by targeting the peptidyl transferase center of the 50S ribosomal subunit. Macrolides and streptogramin B bind adjacent to the peptidyl transferase center within the exit tunnel and thereby prevent elongation of the peptide chain (174). Binding of lincosamides inhibits peptide-bond formation by preventing correct positioning of the aminoacylated ends of tRNAs in the peptidyl transferase center (174). When macrolides, lincosamides, or streptogramins are used for monotherapy, they are bacteriostatic (175). However, when a type B streptogramin (e.g., quinupristin) is used together with a type A streptogramin (e.g., dalfopristin), the combination, marketed as Synercid, acts synergistically and is bactericidal against staphylococci (176).

Resistance

Resistance to macrolides, lincosamides, and streptogramin can be mediated via target-site mutation, enzymatic target-site modification, enzymatic inactivation of the antibiotic, and active efflux (177). Target-site modification occurs by acquisition of rRNA methylases encoded by *erm* genes, including *erm*(A), *erm*(B), *erm*(C), and related genes, which add methyl groups to an adenine residue in the 23S rRNA (177, 178). Also, the 23S rRNA methyltransferase, encoded by the *cfr* gene, confers resistance to lincosamides and streptogramin A, as well as oxazolidinones, phenicols, and pleuromutilins, but does not confer resistance to macrolides (179).

Ribosomal mutations in the 23S rRNA target site may cause resistance to macrolides, lincosamides, and streptogramins. As is the case for linezolid, the number of *rrn* genes limits resistance development. However, in cystic fibrosis patients treated with a macrolide, strains have been isolated that contain mutations in five of the six *rrn* genes, conferring cross-resistance to lincosamides and streptogramins (180). Mutations in the ribosomal proteins L22 (encoded by *rplV*) and L4 (encoded by *rplD*) may also contribute to the development of resistance to the MLS antibiotics (180, 181). Enzymatic inactivation of macrolides can be mediated by phosphorylases encoded by *mph* genes, and type A streptogramin resistance can be mediated by acetyltransferases (177, 182).

Staphylococci can also acquire genes encoding efflux pumps that can extrude one or more of the MLS antibiotics (183). Acquisition of the *msr*(A) gene encoding for the Msr (A) ABC transporter confers resistance to macrolides and streptogramin B, while lincosamides are not substrates for this pump (184). The plasmid-carried gene *vga*(A) encodes an ABC transporter with specificity for type A streptogramins, along with the pleuromutilin class of antibiotics (184, 185). The LmrS efflux pump can extrude macrolides and lincosamides, as well as several other classes of antibiotics (186). Finally, overexpression of the chromosomally encoded gene *mdeA* reduces susceptibility to the streptogramin virginiamycin (187).

Chloramphenicol

Mode of action

Chloramphenicol is a broad-spectrum, bacteriostatic antibiotic that interferes with protein synthesis by binding to the ribosomal 50S subunit. Chloramphenicol binds at the peptidyl-transferase center and thus inhibits peptide bond formation between the tRNAs at the A- and P-sites (174). The wide use of chloramphenicol has been hindered by its toxicity, because systemic administration of chloramphenicol is associated with irreversible aplastic anemia (188). Due to the severity of the adverse effects of chloramphenicol, it is now used primarily for topical applications, such as a treatment for staphylococcal conjunctivitis (189).

Resistance

Resistance to chloramphenicol is attributed to either enzymatic inactivation of the antibiotic, active efflux, or target-site modification. Inactivation of chloramphenicol occurs through acetylation at the C3 position via various chloramphenicol acetyltransferases, encoded by *cat* genes (190), which often are located on plasmids (191). Active efflux of chloramphenicol is mediated by the FexA (192) or LmrS efflux pump (186). Finally, chloramphenicol resistance can occur through target-site modification of the 23S rRNA at position A2503 by an rRNA methylase encoded by the gene *cfr* (164).

Pleuromutilins

History and mode of action

The pleuromutilin class of antibiotics was first described in 1951, and the first agent in this class, tiamulin, was approved for veterinary use in 1979 (193). In 2007, the pleuromutilin retapamulin was first approved for human use for treatment of skin infections caused by *S. aureus* and *Streptococcus pyogenes* (193, 194). Pleuromutilins inhibit protein synthesis by binding to the ribosomal 50S subunit at the peptidyl transferase center (174).

Resistance

Pleuromutilin resistance mechanisms include reduced binding to the target and active efflux (194). *In vitro* selected pleuromutilin resistance can be conferred by spontaneous mutations in the gene *rplC*, which encodes the ribosomal protein L3. Stepwise mutations in *rplC* confer higher levels of resistance than single mutations in the gene (195, 196). Reduced binding to the target can also occur by acquisition of the *cfr* gene (179). Pleuromutilins are also subjected to active efflux by, for example, Vga(A) (185) and related efflux systems (197).

Aminoglycosides

History and mode of action

Aminoglycosides, such as gentamicin, interfere with protein synthesis by binding to the A-site of the 16S rRNA of the 30S subunit and promote inaccurate codon-anticodon recognition that ultimately decreases fidelity of translation, leading to the generation of mistranslated proteins (198, 199). The bactericidal activity of aminoglycosides is attributed to the

production of mistranslated proteins that may incorporate into the membrane, increasing its permeability and leading to uncontrolled small molecule diffusion and eventually increased uptake of aminoglycosides (198). Aminoglycosides are rarely used as monotherapy; they may be used in combination with other agents for treatment of endocarditis (172).

Resistance

For *S. aureus*, resistance to aminoglycosides is conferred by several mechanisms, including enzymatic modification of the aminoglycoside agents, decreased uptake, and target modifications of the 30S ribosomal subunit. Active efflux of aminoglyocsides has not been reported for *S. aureus*, whereas this mechanism is common among Gram-negative pathogens (200). In contrast, enzymatic modification and inactivation of aminoglycoside agents is common among *S. aureus* clinical isolates. The aminoglycoside-modifying enzymes can be divided into three subclasses based on the chemical type of modification of the aminoglycosides (200–202).

Aminoglycoside uptake is dependent on the membrane potential, and mutants experiencing depolarization of the membrane display low-level resistance to aminoglycosides (203). On agar plates such mutants often display a small colony phenotype and hence are referred to as small colony variants (203). Aminoglycoside-resistant small colony variants are considered to be of clinical importance, because they have been associated with recurrent and persistent staphylococcal infections such as device-associated infections, bone infections, and airway infections in cystic fibrosis (204).

Plasmid-mediated 16S rRNA methyltransferases can confer a high level of resistance to various aminoglycosides by preventing binding of the aminoglycoside to the active site; however, this mechanism has been reported only among clinical Gram-negative bacteria (205). Nonetheless, an *in vitro*-generated *S. aureus* mutant containing the 16S rRNA methyltransferase gene *rmt*C on a plasmid was capable in conferring resistance to gentamicin and kanamycin (206).

Tetracyclines and Tigecycline

History and mode of action

Tetracyclines, including tigecycline, are bacteriostatic antibiotics that inhibit protein synthesis by binding to the ribosomal 30S subunit (207). Tigecycline blocks entry of aminoacyl tRNA molecules into the acceptor site of the ribosome, thus preventing incorporation of amino acids into the elongating peptide chain (174, 208).

The backbone of the tetracycline compound class is characterized by four interlocking six-carbon rings. Due to the emergence of resistance to the marketed tetracyclines, there has been continued development of new analogues that overcome common resistance mechanisms against older versions of tetracyclines (209). In 2005, the FDA approved tigecycline, which belongs to the novel antibiotic class of glycylcyclines. Glycylcyclines are derivatives of the tetracycline class of antibiotics and thus contain the four-ring carbocyclic structure, with a substitution of an *N*-alkylglycylamido group to the D-9 position. This substitution expands the spectrum of activity compared to tetracyclines and overcomes some tetracycline resistance mechanisms (210).

Resistance

Tetracycline resistance of clinical relevance has been attributed to active efflux or target-site protection (211). Active efflux is mediated via the Tet(K) and Tet(L) efflux pumps of the major facilitator superfamily by exchanging a proton for a tetracycline molecule (211). The efflux pump of Tet(K) can be encoded on plasmid pT181, which may be integrated with SCC*mec* elements of MRSA CC398 (212). A chromosomally encoded efflux pump, Tet38, confers tetracycline resistance when the repressor protein MgrA is inactivated (213).

Target-site protection is mediated via TetM or TetO determinants, which are often encoded on chromosomally located conjugative transposons. TetO and TetM bind to the elongation factor G (EF-G) binding site on the ribosome, releasing bound tetracycline from the A site in the presence of GTP (214). Strains harboring both TetK and TetM display higher MICs than strains carrying an individual gene (215) and are selected for during exposure to subinhibitory concentrations (216).

Tigecycline susceptibility is minimally affected by the presence of the tetracycline resistance determinants TetM and/or TetK in *S. aureus* (217). Tigecycline is currently not greatly burdened by resistance in clinical isolates, with only a few reports highlighting this in staphylococci (218). Among 28,278 strains from the United States, more than 99.9% isolated between 2006 and 2012 remained susceptible to tigecycline (219), whereas the susceptibility frequency in Mexico (where prescription control is limited) was down to 91% (220). Mechanisms conferring resistance to tigecycline have been investigated *in vitro* by selection of resistant mutants. Loss-of-function mutations have been identified in the transcriptional repressor *mepR*, leading to derepression of the *mepA*-encoded efflux pump (221, 222). Mutations in *mepA* itself have also been associated with reduced tigecycline susceptibility (222). Furthermore, mutations have been identified in the gene *rpsJ*, which encodes the ribosomal protein S10, that are suspected to reduce the access of tigecycline to the binding site (223).

Mupirocin

Mode of action

Mupirocin is a topical antibiotic widely used to reduce nasal carriage of MRSA and to treat skin infections. Mupirocin arrests protein synthesis by inhibiting the isoleucyl-tRNA synthetase, which is encoded by the *ileS* gene. The isoleucyl-tRNA synthetase (IleRS) catalyzes formation of isoleucyl-tRNA from isoleucine and tRNA and thereby prevents incorporation of isoleucine into protein chains (224).

Resistance

Resistance to mupirocin can arise by chromosomal mutations in the *ileS* gene. Amino acid substitutions in IleRS reduce the affinity for mupirocin and confer low-level resistance to the antibiotic (224). Resistance to mupirocin can also be conferred by acquisition of the gene *mupA*, which encodes a mupirocin-insensitive isoleucyl-tRNA synthetase (224). *mupA* is often located on plasmids that also harbor genes conferring resistance to other antibiotics of greater importance than mupirocin for treatment of *S. aureus* infections, and hence mupirocin may provide selective pressure for maintenance of nonmupirocin resistance genes (225). Recently, it was demonstrated that *mupB* confers high-level resistance, but the mechanism of resistance remains incompletely understood (226).

Fusidic Acid

Mode of action

Fusidic acid is a bacteriostatic antibiotic that inhibits protein synthesis by binding to the EF-G on the ribosome, effectively

stalling the elongation phase of translation. EF-G is encoded by the chromosomally located gene *fusA* (227). For systemic application, fusidic acid often is used in combination with other antistaphylococcal antibiotics to reduce the risk of resistance development, because fusidic acid resistance can be readily selected (227, 228). Fusidic acid has been applied as a monotherapy for topical administration, but consequently has experienced a rapid emergence of resistant variants (227).

Resistance

Fusidic acid resistance can arise due to chromosomal mutations in *fusA*, leading to reduced fusidic acid binding to EF-G due to altered protein conformation (227, 229) or mutations in *rplF* (also termed *fusE*, encoding the ribosome protein L6) (230). *rplF* mutations can be selected for using fusidic acid or aminoglycosides and may be accompanied with secondary mutations in hemin or menaquinone biosynthesis genes conferring the small colony variant phenotype (230, 231). Acquired fusidic acid resistance genes found in *S. aureus* include *fusB*, *fusC*, and *fusD* (232). By binding to EF-G on the ribosome, FusB frees stalled ribosome-EF-G-GDP complexes that form in the presence of fusidic acid, which allow the ribosomes to resume translation (233). *fusC* has been inferred to have a similar mechanism of resistance as *fusB*, based on sequence similarity (231). The *fusD* gene is associated with the intrinsic resistance of *Staphylococcus saprophyticus* to fusidic acid (232). Fusidic acid resistance genes have been identified on SCC*mec*-like elements termed SCC*far*, which lack the *mecA* gene, and these elements may contribute to the horizontal transfer of fusidic acid resistance genes (234). The *fusB* gene has also been identified on mobile elements with similarity to staphylococcal pathogenicity islands. In *S. aureus* the mobile element does not harbor any known virulence determinants, so it is termed the *S. aureus* resistance island carrying *fusB* ($SaRI_{fusB}$) (235), while in *S. epidermidis*, the *fusB* gene is located on the pathogenicity island $SePI_{fusB\text{-}857}$ (236).

INHIBITORS OF DNA REPLICATION

Fluoroquinolones

Mode of action

Fluoroquinolones are bactericidal due to the blocking of bacterial DNA replication. They have two molecular targets, where the primary target in *S. aureus* is topoisomerase IV and the secondary target is DNA gyrase. Topoisomerase IV promotes separation of intertwined daughter chromosomes following replication and is a tetramer complex consisting of two GrlA and two GrlB subunits encoded by *grlA* and *grlB*. DNA gyrase is a tetramer complex consisting of two GyrA and two GyrB subunits encoded by *gyrA* and *gyrB*, and it is essential for viability because it removes positive supercoils that accumulate during replication (237).

Resistance

Resistance to different fluoroquinolones is common, with some reporting a prevalence of up to 60% (238, 239). Due to the high prevalence of fluoroquinolone resistance and the risk of resistance developing during treatment, it is primarily recommended that fluoroquinolones be used in combination with other antibiotics (172). Fluoroquinolone resistance mechanisms include mutational changes in the target site of topoisomerase IV and DNA gyrase and increased expression of endogenous efflux pump systems (240). Target-site resistance mutations have primarily been located in the DNA gyrase gene, *gyrA*, and the topoisomerase IV gene, *grlA* (241). Combinatorial mutations in topoisomerase IV and DNA gyrase produce higher levels of resistance than a single mutation in either of the targets (242). *S. aureus* harbors three chromosomally encoded efflux pump systems, namely, NorA, NorB, and NorC, whose overexpression leads to reduced susceptibility to fluoroquinolones. The affinity of these efflux pump systems varies for the individual fluoroquinolone; e.g., NorA primarily extrudes ciprofloxacin and norfloxacin, and its overexpression can increase the MIC approximately 8-fold (243). Fluoroquinolones generally have great activity against *S. aureus*, so accumulation of multiple mutations altering the target-site and/or conferring overexpression of efflux pump systems is needed to exceed the clinical breakpoint (240).

TRANSCRIPTIONAL INHIBITORS

Rifampicin

Mode of action

The selective antibacterial activity of rifampicin is attributed to the inhibition of RNA synthesis, because it binds to prokaryotic RNA polymerases with much greater affinity than to eukaryotic RNA polymerases. The bacterial RNA polymerase is a multisubunit complex, consisting of the subunits α_2, β, β', and σ. The binding site of rifampicin is located in the β-subunit, which is encoded by the gene *rpoB* (244).

Resistance

Rifampicin-resistant mutants are readily selected (228), and resistance-conferring point mutations have been located within the *rpoB* gene. Alteration of the target decreases affinity for rifampicin and can confer a more than 10,000-fold increase in MIC (245). Rifampicin resistance mutations often confer a fitness cost, but the level of resistance is not correlated with the magnitude of the fitness cost for *in vitro* selected mutants, and rifampicin-resistant mutants from clinical isolates often carry mutations associated with low fitness costs (245). Available online tools can help to determine chromosomal *rpoB* point mutations conferring rifampicin resistance from whole-genome sequences, e.g., the Mykrobe Predictor (246). Due to the extensive risk of resistance development, treatment with rifampicin is recommended only for use in combination with a secondary antibiotic (172).

SMALL MOLECULE BIOSYNTHESIS INHIBITOR

Trimethoprim-Sulfamethoxazole

History and mode of action

Sulphonamides were the first class of antibiotics to be introduced into the clinic (in 1935). Currently, sulfamethoxazole (SMX) is the most widely used sulphonamide. Trimethoprim (TMP) was introduced in 1962 and has been used in combination with SMX since 1968 in a formulation called co-trimoxazole (247). Both of the agents interfere with bacterial metabolism by inhibiting the synthesis of folic acid, a cofactor in amino acid and nucleotide synthesis. SMX inhibits the enzyme dihydropteroate synthase, which catalyzes the formation of dihydrofolate from para-aminobenzoic acid. TMP inhibits dihydrofolate reductase (*dfrB*), which catalyzes the formation of tetrahydrofolate from dihydrofolate (247). By inhibiting two enzymes in the same metabolic pathway, it was anticipated that the risk of resistance

development would be diminished, yet a high prevalence of resistance to both agents has been reported (247).

Resistance

Resistance to SMX in clinical isolates arises from chromosomal mutations in the gene encoding the dihydropteroate synthase enzyme, which is expected to reduce the binding affinity of SMX to the target (248). Similarly, TMP resistance arises from chromosomal mutations in the *dfrB* gene encoding the dihydrofolate reductase enzyme (249). Besides chromosomally encoded TMP resistance, *S. aureus* can acquire *dfr* gene variants, *dfrA*, *dfrG*, and *dfrK*, which are less susceptible to inhibition by TMP (250). The two antibiotics are often used in combination to limit resistance development, but in patients exposed to long-term treatment with TMP-SMX, thymidine-dependent TMP-SMX-resistant small colony variants emerge (251). The thymidine-dependent small colony variants acquire mutations in *thyA* encoding for thymidylate synthase, which is essential for *de novo* thymidylate biosynthesis. Tetrahydrofolic acid acts as a cofactor for thymidylate synthase, and *thyA* mutants are therefore dependent on the uptake of thymidine or its metabolite dTMP (252, 253).

COMPOUNDS AND ALTERNATIVES IN DEVELOPMENT

The World Health Organization has classified MRSA and VRSA as "high priority" for development of new therapeutics (254). The majority of the small molecules in clinical development and those recently approved are modifications of existing chemical scaffolds. Therefore, cross-resistance to earlier versions of the antibiotics may be experienced, as was observed with the recently approved fluoroquinolone delafloxacin (254, 255). However, novel membrane-targeting antibiotics are being developed, such as the nonpeptide defensin mimetic brilacidin, which is in clinical development phase 2 (254, 256), and the antimicrobial peptide LL-37 (257). Although brilacidin is structurally different from daptomycin, the cellular effects of brilacidin are similar to those of daptomycin and LL-37. For thorough reviews on the antibiotic pipeline please see the WHO report (254) and the article by Butler and colleagues (256).

Another avenue being pursued for new clinical options is resensitizing resistant isolates by inhibiting acquired resistance mechanisms or core cellular functions needed for antibiotic resistance (258). The opportunity of expanding the spectrum of activity of polymyxins, an antibiotic class only used for treatment of infections caused by Gram-negative bacteria, has been pursued by searching for inhibitors of the ATP synthase that was identified in a genetic screen for sensitizer targets (259). Compounds affecting expression of PBP2a have also been proposed as ways to increase susceptibility of MRSA to beta-lactams, as demonstrated by the antipsychotic drug thioridazine, which decreases *mecA* and *blaZ* transcription and PBP2a expression (260). Importantly, a combination of thioridazine and dicloxacillin is active against VISA strains (261).

Alternative treatment strategies are also being explored, including but are not limited to antibodies, antivirulence therapy, bacteriophages, and vaccines (262). Two types of antibodies are being explored, namely, those binding to the pathogen to promote opsonization-mediated phagocytosis and those that neutralize virulence factors, such as toxins (263). *S. aureus* possesses a plethora of virulence factors (264), so neutralization of a single factor is generally insufficient against *S. aureus* infections; hence, cocktails of monoclonal antibodies binding to different targets are also under development (254). Antibodies are also being assessed for increasing the intracellular activity of antibiotics that display reduced activity against *S. aureus* residing inside host cells (254, 265). Here, antibody-antibiotic conjugates bound to bacteria are being opsonized by phagosomes, where intracellular proteases release the antibiotic by cleaving the antibody-antibiotic linker and thereby enable the antibiotics to gain intracellular activity and improve intracellular bacterial clearance (265).

In antivirulence therapy, the strategy is to disarm key virulence factors involved in disease progression; bacterial clearance then relies on a functional immune system or coadministered antibiotics (266). For *S. aureus*, compounds have been explored that target the *agr* quorum sensing system and suppress production of major virulence factors such as α-hemolysin by binding to either the AgrC sensor histidine kinase or the AgrA response regulator of the *agr* two-component system (267–270). Such interventions may ultimately prove to be effective against both colonization by and infection of *S. aureus* (271).

Bacteriophages (in short, phages) are viruses that specifically target bacteria; lytic phages were introduced to human medicine a century ago worldwide. With the introduction of antibiotics, the human application of phage therapy was abandoned in Western medicine but has maintained applicability in Eastern Europe, including countries of the former Soviet Union (272, 273). The difficulties imposed by antibiotic resistance have renewed the interest in phage therapy in Western medicine (274). Several phage therapies are currently being tested against *S. aureus*, with a focus on topical decolonization (275). In summary, a line of new therapeutic options are being explored, and future research will reveal their efficacy and clinical potential against infections with *S. aureus*.

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Immunity to *Staphylococcus aureus*: Implications for Vaccine Development

RICHARD A. PROCTOR

48

INTRODUCTION

Most adult humans have high levels of circulating antibodies against many staphylococcal antigens, indicative of prior subclinical infections, but these antibodies are generally not protective, and clinically significant infection with *S. aureus* fails to provide protective immunity. Multiple vaccines have been developed for the prevention of *S. aureus* infections, but none were proven efficacious in the human trials reviewed in references 1–4. All of the vaccine candidates functioned well in animal models, mostly murine models, but also in rabbits and primates. The reliance on murine models can be related to the extensive data available about murine immunity. Based on the large number of failures, a reasonable conclusion is that murine immunity and human preventive immunity against *S. aureus* are significantly different. The divergence of human and murine immunity has been detailed in the recent literature (1–6).

Clearly, one major hurdle in producing an *S. aureus* vaccine is the lack of detailed information about the human protective immune response to *S. aureus* (1–4). Several vaccine candidates that target surface antigens and produce opsonizing antibodies have reached clinical trials, but they have failed to protect or attenuate infections in humans. All of these antigens produced robust humoral immunity that provided protection in animal models. These data indicate that antibodies based on opsonophagoctyic activity are not protective in humans. While these results were very disappointing, they were not entirely surprising, because patients with B cell defects do not show increased frequency or severity of *S. aureus* diseases (2–4). Information from immune defects, clinical trials, and studies of human sepsis are providing important information about the immune response to *S. aureus* in humans, underlining the importance of cellular immunity. This information is examined here.

IMMUNE DEFECTS AND RECURRENT *S. AUREUS* INFECTIONS

The inborn and acquired immune defects that increase the incidence of *S. aureus* help to define the cells and pathways that provide protective immunity, and these are summarized in Table 1. Overall, evidence is mounting that helper T (T_H) cells plus polymorphonuclear neutrophils (PMNs), dendritic cells, and macrophages are important for a protective immune response (7–14).

Vitamin D Deficiency and Dendritic Cell Failure

Patients with vitamin D deficiency have increased numbers of *S. aureus* infections (15, 16). This may relate in part to decreased bactericidal activity of their dendritic cells (17). Indeed, some strains of *S. aureus* were able to reside within bone marrow dendritic cells and macrophages because they expressed high activity of the Agr quorum-sensing system and escaped from phagosomes by producing the lytic proteins phenol-soluble modulins (18). Therefore, having adequate vitamin D levels may help dendritic cells to express maximal bactericidal activity against *S. aureus* as well as to release mediators to activate the immune system.

Mutations in NGFβ and Its Receptor NTRK1 Reduce Phagocyte Activation

Mutations causing loss of nerve growth factor β (NGFβ) or its receptor have been associated with recurrent *S. aureus* infections in humans in skin, joints, bones, and the oral cavity (19). *S. aureus* factors such as peptidoglycan, protein A, α-hemolysin, and phenol-soluble modulins stimulate macrophages through nucleotide-binding oligomerization domain-like receptors to produce NGFβ (19). NGFβ is both a chemoattractant for and an activator of phagocytes. NGFβ activation of neutrophils and macrophages results in enhanced killing of *S. aureus*. Deletion of NGFβ or its receptor NTRK1 results in more severe *S. aureus* infections in animal models (19). Of note, the ability to activate macrophages to produce NGFβ is limited to *S. aureus* because not even *Staphylococcus epidermidis* causes the release of NGFβ. Patients deficient in NGFβ demonstrate the critical importance of phagocytes in the control of *S. aureus* infections.

IRAK-4/MyD88 Deficiency

Defects in interleukin-1 receptor-associated kinase-4 (IRAK-4) or myeloid differentiation factor 88 (MyD88) impair Toll-like receptor 2 (TLR-2)-mediated and interleukin-1 (IL-1) receptor-mediated immunity such that macrophages fail to produce inflammatory cytokines such as IL-1β, IL-6, IL-8, interferons (IFNs), and tumor necrosis factor alpha (TNF-α) (20–23). Without these cytokines, there is a failure to activate a specific subset of T-helper cells known as T_H17 cells, which are critical for the activation of polymorphonuclear

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TABLE 1 Immune defects that increase the incidence of *S. aureus* infections[a]

Immune defect	Biological basis	Clinical presentation	References
Failure of dendritic cells	Vitamin D required for activation	Recurrent and more severe infections	15, 16
Failure to activate macrophages	Mutations in NGFβ and its receptor, NTRK1	Recurrent and more severe *S. aureus* infections	19
Failure to activate PMNs	IRAK-4 or MyD88 deficiency	Failure of PMNs to kill *S. aureus*, *S. pnumoniae*, and *P. aeruginosa* despite Abs; invasive and skin infections; resolving in teenage years	20–23
Failure to differentiate naive T cells into T_H17 cells	IL-6 autoantibodies or IL-6 deficiency	Recurrent *S. aureus* infections	14
Failure of T_H17 cells to recruit PMNs	STAT-3 defect	Recurrent *S. aureus* skin and mucosal infections, starting in neonates	8, 10, 13
T-cell defects, e.g., with HIV or prednisone treatment	Reduced activation of T cells	Recurrent *S. aureus* infections, especially mucocutaneous	34–36
MAIT cell exhaustion (T cell exhaustion)	10–45% of lymphocytes Inactive after SAg exposure	Increased severity and frequency of infections in intensive care units with reduced MIAT cells	39–44, 48

[a]IRAK-4, interleukin-1 receptor-associated kinase-4; MAIT, mucosal-associated invariant T; MyD88, myeloid differentiation factor 88; NGFβ, nerve growth factorβ; STAT-3, signal transducer and activator of transcription 3; T_H17, T helper cell-17.

neutrophils (PMNs). These deficiencies manifest soon after birth with recurrent *S. aureus*, *Streptococcus pneumoniae*, and *Pseudomonas aeruginosa* infections. Prophylactic antibiotics and early aggressive antibiotic therapy are required to treat babies with these defects. While intravenous immunoglobulin is often used, with respect to pneumococcal infection, there is no correlation between the presence or absence of antipneumococcal antibodies and the occurrence of invasive pneumococcal disease in patients with these deficiencies. This demonstrates the critical importance of T_H17 cells and PMNs in the outcome, even though it is well known that anticapsular antibodies are important in host defense against the pneumococcus (20–23). Those children that survive until their teenage years have a resolution of frequent invasive infections.

IL-6 Autoantibodies

A patient described by Puel et al. with recurrent *S. aureus* infections had normal immunoglobulins, suggesting that his infections could not have been the result of immunoglobulin deficiency. Instead, this patient was found to lack the proinflammatory cytokine IL-6 due to anti-IL-6 antibodies (14). The absence of IL-6 causes an immunodeficiency state, which can be assumed to be responsible for his recurrent *S. aureus* infections. IL-6 is normally produced in response to *S. aureus* interactions with macrophages, dendritic cells, and γδ-T cells. IL-6, in conjunction with transforming growth factor β (TGF-β), helps to direct the conversion of naive CD4 T cells to T_H17 cells (24) and the proliferation of T_H1 and T_H2 cells, among many cell types (25). IL-6 is also important for blocking regulatory T cell (Treg) development and for stimulating B cells to produce antibodies (24). Overly active Tregs can produce immunosuppression by reducing T_H1 and T_H17 cellular activation of the bactericidal activity of phagocytes. On the one hand, activation of T_H17 cells is critical for the control of *S. aureus* infections (26); on the other hand, the suppression of Tregs may allow for an overly exuberant inflammatory response to *S. aureus* infections (27, 28). Indeed, loss of Tregs can result in fatal inflammatory disease (29). Thus, IL-6 plays a key role in regulating T_H17/Treg balance so as to control immunity and immunopathology.

Job's Syndrome, a T_H17/STAT3 Defect

Patients with autosomal-dominant hyper-IgE, or Job's syndrome, have dominant-negative mutations in signal transducer and activator of transcription 3 (STAT3) (8, 10, 13), and mutations in STAT3 have a strong association with frequent and severe *S. aureus* mucocutaneous infections, including staphylococcal pneumonia, empyema, and "cold" skin abscesses. STAT3 is an intracellular signal transduction factor in T_H17 cells, which links surface receptors to the activation of genes (30, 31). Activation by IL-6 of its receptor on T_H17 cells plays a key role in the differentiation of naive T cells into T_H17 cells. T_H17 cells are critical for controlling *S. aureus* infections because they release IL-17, which recruits PMNs to the site of infection and helps to activate the PMNs for killing *S. aureus* (31). A key role for PMNs in the protection from *S. aureus* infections has also been established by the observation of recurrent *S. aureus* infections in patients with chronic granulomatous disease (primary defect in the production of reactive oxygen species by their PMNs) (32). However, the role of STAT3 may extend beyond PMNs because STAT3 is also involved in expression of host antimicrobial peptides in keratinocytes (31) and in the production of Reg3γ, an antimicrobial peptide produced by respiratory epithelial cells, whereby these respiratory mucosal cells become bactericidal for *S. aureus* (33). *S. aureus* interaction with respiratory epithelial cells activates them through STAT3 for the production of Reg3γ. Thus, while STAT3 is important for controlling systemic invasion by *S. aureus* by recruiting and activating PMNs, it also plays an important role in activating mucosal and skin cells. Hence, this defect is associated with recurrent *S. aureus* mucocutaneous infections.

Corticosteroids and AIDS

Patients with reduced cell-mediated immunity have an increased incidence of *S. aureus* infections. This can be due

to an underlying disease such as systemic lupus erythematosus, AIDS infection, or the use of corticosteroids (34–37). Even moderate-dose prednisone therapy increases the risk of serious *S. aureus* infection (36). While we frequently think of viral infections as being associated with reduced cell-mediated immunity, the link to *S. aureus* infections comes from the reduced T_H17 cell-PMN axis in prednisone-treated patients (38).

Mucosal-Associated Invariant T (MAIT) Cells, Superantigens, and T Cell Exhaustion

MAIT cells represent up to 10% of T cells in blood and up to 35% in liver and some mucosal sites (39–44), and they may be considered the first T cell responders to invading bacteria because they are activated during the early stages of bacterial infection and act as a bridge between the innate and adaptive immune systems by recognizing bacterial byproducts derived from riboflavin metabolism (42). However, unlike the situation with humans, MAIT cells are infrequent in laboratory mouse strains such as C57BL6, thereby limiting the utility of murine models without humanization (39). MAIT cells have been implicated in protecting the host from multiple species of bacteria (40–42, 44, 45). However, recent studies have shown that they also respond to superantigens (SAgs), such as staphylococcal enterotoxin B (SEB), and have been identified as extremely potent and fast-acting producers of proinflammatory cytokines (39). To quote from Taylor et al. (39), "We have identified a population of innate T-like cells, called MAIT cells, as the most powerful source of proinflammatory cytokines after exposure to SAgs." In addition to being involved in immunopathogenic activity, MAIT cells may also be involved in "a novel mechanism of SAg-associated immunosuppression in humans" (39) wherein widespread activation of MAIT cells by SAgs results in their inability to respond to future challenges. Sandberg et al. (43) report that subsequent to such SAg activation, the MAIT cells remain unresponsive to stimulation with conventional bacterial antigens. This concept of immune exhaustion is supported by the observation that intensive care unit patients with reduced MAIT cells were more vulnerable to severe and subsequent bacterial infections during their stay in the intensive care unit (46). Equally, ill patients' normal MAIT numbers did not show increased bacterial infections (46). Thus, SAg-producing *S. aureus* hijacks MAIT cells by inducing the cytokine storm and leaves them functionally impaired. A mechanism for this is based on the occurrence of anergy via upregulation of inhibitory receptors such as lymphocyte-activation gene 3 (39, 44, 47).

T Cell Exhaustion is Manifested in Humans by Low Levels of IL-2

SAgs induce other modifications to T cells (see Fig. 2). A marker of T cell exhaustion is low levels of IL-2 (39, 43, 48). IL-2 production increases dramatically in SEB-challenged MAIT cells compared to conventional T cells in peripheral blood mononuclear cells (39). This is in contrast to *E. coli* exposure to MAIT cells where IL-2 was not produced (49). These observations are consistent with previous findings where SEB and other SAgs delete or anergize T cells (50, 51). Moreover, exposure of human $CD4^+CD25^-$ T cells to SEA induces proliferation and is followed by a switch to a $CD25^+FoxP3^+$ Treg phenotype that secretes IL-10 but not IL-2 (39). SEA also activates $\alpha\beta$T cells (52). SEB activates $\alpha\delta$T cells and stimulates the conversion of Treg to T_H17-like cells (11, 39, 52–57). In summary, SAgs can induce a cytokine storm, but they are also potent immunomodulators of the T cell immune response. These effects would be anticipated to change during the course of *S. aureus* sepsis.

Summary of Immune Defects and *S. aureus* Infections

In summary, the inborn and acquired immune defects that are associated with an increase in the incidence of *S. aureus* infections show that the macrophage-T cell axis is critical for the activation of phagocytes to control *S. aureus* invasive infections. Conspicuously absent is the need for opsonic antibodies for protection against *S. aureus*, because children with agammaglobulinemia do not show increased numbers of *S. aureus* infections (1–5). The innate activation of macrophages and dendritic cells, as well as the surface factors that activate complement, seem sufficient to control *S. aureus* without opsonization. On the other hand, antibodies against *S. aureus* toxins have been shown to reduce the severity and mortality of *S. aureus* toxin-mediated diseases (6, 58–61). As discussed below, staphylococcal SAgs can play a major role in the modulation of T cell development; therefore, antitoxin antibodies may be crucial in the maintenance of a balanced macrophage-T cell axis.

STAPHYLOCOCCAL SEPSIS AND CYTOKINE PRODUCTION

Some information is emerging about anti-*S. aureus* immunity that is based on studies of patients with sepsis, especially *S. aureus* bacteremia, and this is summarized in Table 2. In septic patients, the profile of cytokines produced during infection is a reflection of the cells that are activated (Table 2). Normally, interaction of invasive *S. aureus* with macrophages and dendritic cells causes the release of proinflammatory cytokines such as IL-1, IL-2, IL-6, IFN-γ, and TNF-α. This is later followed by IL-10, which downregulates the immune response. However, this normal pattern of immune response is not seen in patients that have worse outcomes from *S. aureus* sepsis. Thus, mortality is associated with dysregulation of the immune system in apparently normal individuals. As detailed in Table 2, mortality or more severe disease is associated with low IL-1, IFN-γ, and TNF-α but high IL-6 and IL-10.

A dramatic example of immune dysfunction during *S. aureus* infection came from the V710 staphylococcal vaccine trial, where increased mortality occurred in vaccinated patients (62). V710 was a recombinant *S. aureus* surface protein, IsdB (iron surface determinant protein B), and was given to subjects prior to cardiovascular surgery. Patients that received the vaccine and subsequently developed invasive *S. aureus* infection during the postoperative period showed increased mortality. The cause of the increased mortality was systemic inflammatory response wherein control of the immune response was lost. Posttrial investigation found that low levels of IL-2 were observed not only during sepsis but even before patients received the V710 vaccine (63). There was a 5-fold increased mortality in vaccinees that had low prevaccination levels of IL-2. Thus, low baseline levels of IL-2 could predict a tragic outcome when vaccinees developed a postoperative infection. These patients also showed low levels of IL-17. These unanticipated results show that excess mortality was related to a dysregulated immune response even before the vaccine was given. Because IL-2 is critical for the survival of Tregs (64), one can postulate that the loss of these cells that modulate the immune response may have played a role in the mortality of these vaccinees.

TABLE 2 Cytokines, sepsis, and survival in *S. aureus* infection

Study	Type of infection[a]	Survival or less complicated course	Death or complicated course
Söderquist et al. 1992 (84)	65 patients with sepsis	Rapidly dropping IL-6	Persistently elevated IL-6
van Dissel et al. 1998 (67)	Sepsis	High IL-1 and TNF-α with low IL-10	Low IL-1 and TNF-α with high IL-10
Rose et al. 2012 (68), Rose et al. 2017 (69)	*S. aureus* bacteremia	High IL-1 and low IL-10	Low IL-1β and IFN-ϒ; high IL-10
V710 vaccine trail Fowler 2013 (62); McNeely 2014 (63)	Invasive infections after cardiothoracic surgery	High IL-1, IL-2, and IL-17	Low IL-2 and IL-17 preoperatively and all postoperative testing
McNicholas et al. 2014 (85)	61 patients with SAB[a]	Low levels of IL-6 predict better outcome	High levels of IL-6 predict worse outcome
Gupta et al. 2016 (65)	Posttrauma sepsis (mix of bacterial pathogens)		High IL-4, IL-10, TGF-β, T_H17, and T_H17/Treg Low IL-2 and IFN-γ
Minejima et al. 2016 (66)	196 patients with SAB	Low TNF-α and IL-10; low IL-17A in all patients	High TNF-α and IL-10; low IL-17A in all patients
Chantratita et al. 2017 (86)	327 patients with SAB	Low IL-6 and IL-8 had less respiratory failure	High IL-6 and IL-8 had more respiratory failure

[a]SAB, *S. aureus* bacteremia.

IL-2 is complex in that it produces both pro- and anti-inflammatory effects by downregulating T_H17 but upregulating T_H1 cells. Although increased numbers of T_H17 cells were found in *S. aureus* sepsis in several studies (9, 65), this is usually not accompanied by an increased blood concentration of IL-17 (12, 62, 65, 66) and may help to explain why IL-17 levels were low in clinical studies when one would anticipate higher T_H17 activity. It also raises the possibility that T_H17 cells may act locally rather than systemically (Fig. 1).

The data outlined in Table 2, suggesting that immune dysregulation correlates with more severe disease and mortality, are considered here. IL-1 is released from macrophages and dendritic cells (among other cells) when *S. aureus* is first met while invading tissues (12). However, patients that show low levels of IL-1 have worse outcomes during invasive *S. aureus* infections (67–69). Of the many functions that IL-1 has, perhaps a very important one for invasive *S. aureus* infections comes in directing T_H17 differentiation and activation (70), because T_H17 cells are critical for protection against *S. aureus* infections (10, 13). Therefore, it is striking that patients with *S. aureus* circulating in their bloodstream and interacting with macrophages would fail to produce IL-1, and this is an obvious sign of immune dysregulation.

Another cytokine released early in the course of *S. aureus* infection is IL-2 (71), and low levels have correlated with poor outcomes (62, 65). Broadly speaking, IL-2 ± TGF-β reduces inflammation by directing reduction of T_H1, T_H2, and T_H17 differentiation and increasing Treg expansion (72). The importance of IL-2 in directing cells toward a moderated inflammatory response is underscored by the increased deaths in patients receiving the V710 *S. aureus* vaccine (62), wherein low prevaccination IL-2 levels correlated very strongly with a systemic inflammatory response syndrome (SIRS) response in these patients when an invasive *S. aureus* infection occurred. Moreover, IL-2 levels remained low during the infection. These observations are consistent with data from Gupta et al. (65), who studied sepsis in trauma patients, wherein death was associated with low level of IL-2 in culture supernatant of peripheral blood mononuclear cells from these patients. Of course, severe trauma itself causes changes in the immune response, so the immune dysregulation noted in this study is less clear cut than in the *S. aureus* vaccine study.

Also consistent with the IL-2 data for immune dysregulation are the observations from several studies that strongly correlate high levels of IL-10 with poor outcomes (65, 67–69). IL-10 downregulates the inflammatory response to *S. aureus* invasion. The increased mortality suggests that the balance between pro- and anti-inflammatory responses is incorrect. *S. aureus* itself may actively alter this balance by stimulating production of IL-10 directly. Quantitatively, *S. aureus* interactions with monocytes and macrophages via TLR-2 interactions produce 4- to 20-fold more IL-10 than interactions with dendritic cells (58). Thus, more IL-10 is produced during bacteremia than in cutaneous infections. While the downregulatory effects following interactions of *S. aureus* ligands with the TLR-2 on T cells, monocytes, macrophages, and dendritic cells may be critical for balancing the host response to SAgs (73–76), overly vigorous suppression of the immune response may lead to continued bacteremia, whereas inadequate suppression may result in death due to SIRS. Engagement of TLR-2 on macrophages can also produce the proinflammatory cytokine, IL-1, but in dendritic cells IL-2 is produced. IL-2 production is needed for the development and maintenance of Tregs, which suppress immune responses produced by T_H17 cells (56). In some cases, T_H17 cells, which are not thought of as IL-10 producers, can convert to IL-10 production when given a second *S. aureus* challenge (70), which might relate to the first challenge being vaccination and the second challenge being invasive infection (63). Hence, the inhibitory activity of IL-2 on T_H2 and T_H17 cells and stimulatory activity on Treg cells might be lost when IL-2 levels are low during sepsis and allow for increased IL-10 production. High IL-10 would decrease PMN activation and killing of *S. aureus* (70, 77–81), reduce T_H17 proliferation (78, 82), and reduce Treg activity (78, 82, 83). A balance between too low and too high IL-10 levels in response to *S. aureus* infections has been suggested as needed

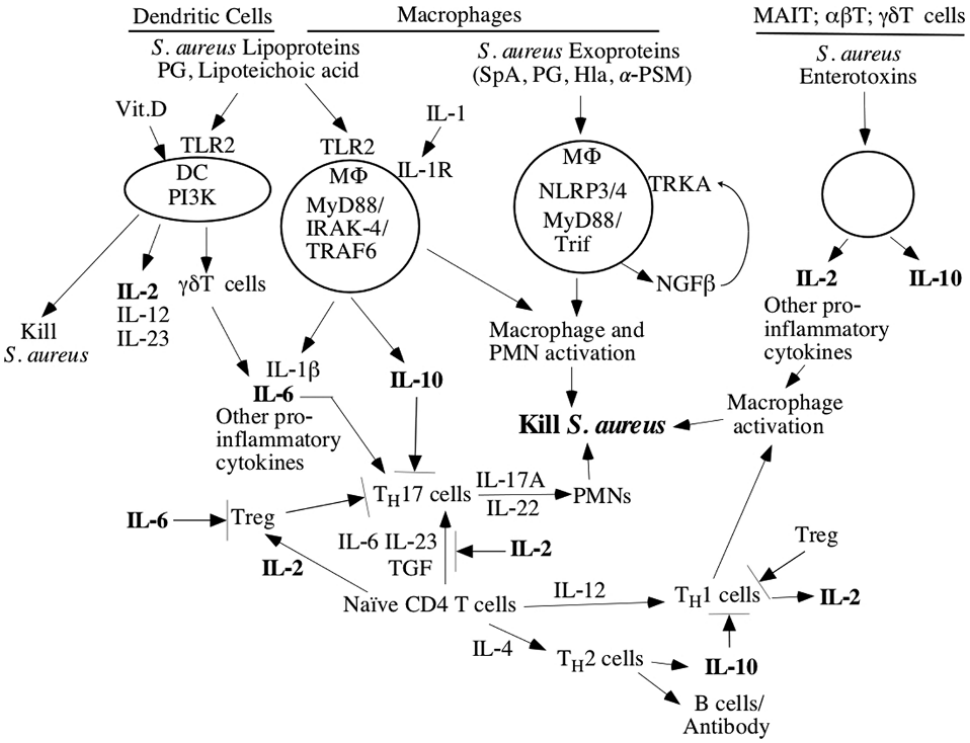


FIGURE 1 Model for human immunity to *S. aureus* based on immune defects and cytokines in sepsis.

to avoid an overly vigorous response (SIRS) or an incomplete activation (persistent bacteremia) (78, 82, 83).

Another consistent finding is that persistently elevated IL-6 correlates with death and/or with another worse outcome with *S. aureus* sepsis (84, 85, 86). IL-6 is produced by macrophages and dendritic cells shortly after *S. aureus* products bind to TLR-2 (14, 24, 27, 30, 81). IL-6 causes differentiation of naive T cells into T_H17 and regular Tregs into proinflammatory Tregs that produce IL-17, IFN-γ, and TNF-α (14, 24, 27, 56). Thus, persistently elevated IL-6 may be linked to an overactive proinflammatory response that damages the host more than it protects it.

Finally, noninterleukins have also been examined during *S. aureus* sepsis in humans. Low levels of IFN-γ correlate with death (65, 68, 69). Early interactions of *S. aureus* with macrophages via TLR-2 lead to IFN-γ release (83). Similarly, when *S. aureus* interacts with dendritic cells via TLR-2, this results in dendritic-cell-mediated activation of T_H1 and γδT cells to produce IFN-γ (18, 87). IFN-γ activates macrophages to kill *S. aureus* (83). MAIT cells can also produce IFN-γ after interactions with SEB (39). Clearly, low levels of IFN-γ might result in poor clearance of *S. aureus* by macrophages. Of interest, immunization of C57Bl/6 mice with killed *S. aureus* resulted in CD4 T-cell-dependent production of high IFN-γ levels with *S. aureus* bacteremia challenge that produced death (88). This provides another example where human and murine immunology diverge.

In contrast to IFN-γ levels, TNF-α levels yielded inconsistent results because high levels of TNF-α were associated with increased (66) or decreased (67) mortality from *S. aureus* bacteremia. TNF-α is important for activation of phagocytes, but it has also been associated with SIRS and death. More studies will be needed to sort this out. Nevertheless, the available studies of cytokine release during *S. aureus* sepsis provide strong evidence for immune dysfunction that leads eventually to death or more severe disease.

IMMUNE DYSREGULATION AND DEATH

Immune dysregulation, either over- or under-activation, of the immune response can be associated with mortality and/or more severe disease. This raises the question of why so many humans have a nonprotective response to *S. aureus* infections that is clearly related to immune dysregulation. We are familiar with *Mycobacterium leprae* and *Trypanosoma cruzi* as causing immunosuppression of the host, and hence one hypothesis is that the balance between the host and *S. aureus* has become lost. This might be due to the production of superantigens (SAgs) that can alter the responses of antigen-presenting cells and T cells (39, 43, 47, 50–53, 57, 74, 89–96). Observations from these papers are summarized in Fig. 2 and are offered as enterotoxins redirecting T cell differentiation. For example, SEB has many interactions with the human immune system. SEB can cause Tregs to develop a T_H17 phenotype and to produce TGF-β (91). The TGF-β enhances naive T cells to differentiate into T_H17 cells, and it inhibits T_H2 cell activation. SEA, SEB, and SEE can activate T_H1 and MAIT cells as superantigens that result in massive macrophage activation and toxic shock (39, 43, 46, 47, 49). After such activation, the T cells and MAIT cells down-regulate and fail to respond to further stimulation, leaving the host immunocompromised for days to weeks (39, 46). In addition, SAgs can even inhibit the primary interactions between staphylococcal products and TLR-2 (89, 90). Thus, SAgs have a very large number of interactions with the human immune system, especially T cells and macrophages (Fig. 2). Notably, cutaneous infections and vaginal toxic shock syndrome with *S. aureus* do not reliably provoke

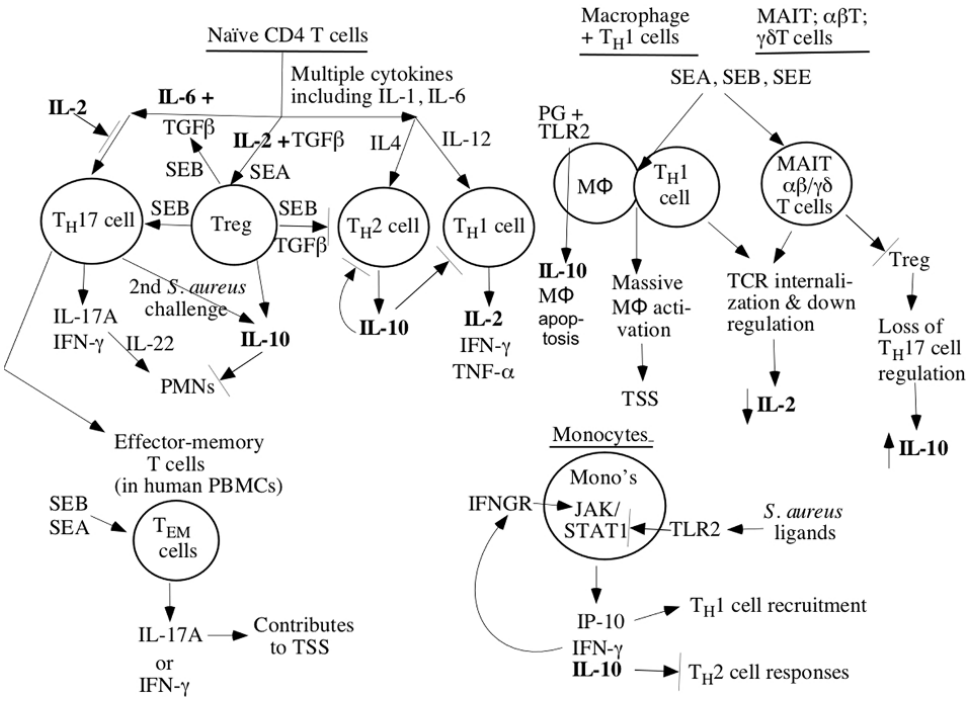


FIGURE 2 *S. aureus* enterotoxin modifications of immune response.

durable, protective immune responses (58, 97), thereby opening the possibility for SAgs and other toxins to influence the immunity response; yet seemingly, they have provoked no immune response. These interactions may underlie failures of humans to respond with protective immunity against *S. aureus* because continued exposure to SAgs is not inhibited without the presence of antitoxin antibodies.

COMMENTS ON THE MODELS

Gaps exist in our knowledge of the construction of the models of human immunity against *S. aureus* presented in Fig. 1 and 2, but these generate testable hypotheses. A major breakthrough for the development of an *S. aureus* vaccine that could prevent disease and death, or at least reduce the severity of infections, would be the discovery of a biomarker. Examples of vaccine biomarkers are anti-hepatitis B viral surface antigen and antimeningococcal capsular antibodies whose presence correlates with protection from infection. Our lack of a biomarker that predicts protection from *S. aureus* infections relates directly to our lack of understanding of protective immunity against *S. aureus*. Many successful bacterial vaccines against *S. pneumoniae*, *H. influenzae*, *Neisseria meningitidis*, and *Clostridium tetani* have been developed when the mechanism of immune protection was known and a biomarker was followed during clinical trials.

While cytokines provide some indication of which cells are activated, the precise cells being activated are not defined because cytokines can be produced by multiple cells and pathways. There is limited information concerning the timing and precise pathways in *S. aureus* human infections, which are not well replicated by murine models (1, 4, 6, 59, 60, 88). Moreover, cytokines may be released just locally, not systemically, so blood levels are not helpful. Finally, the relative balance between T cell subsets may be critical to the outcome. For example, our current state of knowledge does not allow us to know whether T cell exhaustion, reduced action of Tregs, or overactivation of T_H17 cells cause death with multiorgan failure during invasive *S. aureus* infections. In addition, the immune reactions to cutaneous/mucosal invasion versus systemic (bacteremic) infection are probably distinct, wherein γδT and T_H17 cells (IL-17A) are more important for skin and respiratory infections, whereas T_H1 (IFN-γ) responses are more important for survival during bacteremia (12, 98–100). We can see that sustained high levels of IL-10 correlate with death from *S. aureus* infections, yet it is not clear whether this is due to oversuppression of the immune response or a manifestation of the host attempt to reduce an overly exuberant immune response. When human peripheral blood mononuclear cells were differentiated into dendritic cells by culturing with granulocyte-macrophage colony-stimulating factor + IL-4, they produced a primarily T_H1/T_H17 stimulatory response, whereas when they were differentiated with macrophage colony stimulating factor, this triggered primarily an IL-10 response in response to TLR-2 activation by peptidoglycan or lipoteichoic acid (101). Hence, it is conceivable that the high levels of IL-10 during persistent *S. aureus* bacteremia come from continued activation of macrophages; however, it is also conceivable that the high levels of IL-10 downregulate the immune response needed to clear the bacteria.

Similarly, the data from the V710 vaccine trials, wherein low prevaccination IL-2 was strongly associated with SIRS, could be due to be overexpansion of or overly active T_H17 cells, e.g., by reduced Treg activity and loss of suppressive effects on T_H17 cells. Another hypothesis that springs out of Fig. 2 is that some patients may have T cell exhaustion and altered immune response due to colonization with superantigen-producing *S. aureus* strains (especially SEB), which can downregulate T cell receptors (92). For example, if superantigens led to MAIT cell downregulation, then low levels of IL-2 might prime the host for poor Treg and high T_H17 responses when *S. aureus* invasion occurred. It would be anticipated that these patients might be colonized with

SEB-producing strains, lack anti-SEB antibodies, and have low levels of IL-2. Low levels of IL-2 result in reduced Treg and increased T_H17 activity, perhaps adding to an imbalanced immune response to invasive *S. aureus* infections as seen in the V710 clinical vaccine trial. Finally, patients with immunodeficiencies tend to have local infections that rarely develop spillover bacteremia (102), but we have little knowledge about the factors involved in protection from primary *S. aureus* bacteremia. Contrasting the immune responses to bacteremia to cutaneous infections may shed light on the context for T cell activation.

The models for human immune responses to *S. aureus* are really hypotheses (Fig. 1 and 2), and much more detail is needed about the types of T cells being activated, the balance between T cell types, the timing of T cell activation and inactivation, the impact of antibodies, and the correlations to outcomes. Given the multiple possible interpretations of changes in cytokines, a detailed analysis of the types of T cells produced, their state of activation, the time course of activation, and the relative balance between the different T cell subsets prior to and during *S. aureus* invasive infections should shed light on which pathway(s) are critical for immunity to *S. aureus* infections. In addition, prospective data on the types of antibodies being produced (opsonic versus toxin-neutralizing), especially during *S. aureus* bacteremia versus cutaneous infections, that are correlated with outcome will help to inform us about the relative value of these antibodies. Animal models do not substitute for human data. This basic knowledge will be critical as *S. aureus* vaccine trials are designed because patients should be stratified in future clinical trials to evaluate the impact of therapeutic interventions.

SUMMARY OF HUMAN IMMUNITY AGAINST *S. AUREUS*

Cell-mediated immunity seems to be critical for the prevention and resolution of invasive *S. aureus* infections, but an imbalance in this immunity may also produce SIRS and death or an inadequate protective response with prolonged bacteremia and death. This dysregulation is likely at the heart of mortality and severe disease in humans. Antitoxin antibodies may also come into play in reducing the severity of *S. aureus* infections, but these antibodies might also address superantigen-induced immune dysregulation. Thus, while changing intrinsic T cell responses may be therapeutically difficult, monoclonal antibodies against superantigens may have utility in addressing dysfunctional immune responses to *S. aureus*. The models above are hypotheses for examining, and potentially dramatically improving, the immune response to and safety of *S. aureus* vaccines.

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Nonconventional Therapeutics against *Staphylococcus aureus*

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49

INTRODUCTION

Staphylococcus aureus is one of the most important human pathogens, causing a variety of diseases, including skin and soft tissue infections, osteomyelitis, endocarditis, surgical site infections, pneumonia, and sepsis. In recent decades, the treatment of staphylococcal infections has become increasingly difficult as the prevalence of multidrug-resistant strains continues to rise. Penicillin-resistant *S. aureus* emerged in the 1940s, followed by the appearance of methicillin-resistant *S. aureus* (MRSA) in 1961 (1, 2). Subsequent introduction of new antibiotics has been followed by reports of resistance (3). With increasing mortality rates and medical costs associated with MRSA and other drug-resistant strains, there is an urgent need for alternative therapeutic options (4). Therefore, considerable effort has been put forth to identify and develop novel *S. aureus* treatment strategies as alternatives to conventional antibiotics.

PREVENTION AND DISRUPTION OF BIOFILM FORMATION

Biofilms are multicellular, three-dimensional aggregates of bacteria embedded in a matrix composed of polysaccharides, extracellular DNA, proteins, and/or lipids and are formed as an adaptation to environmental stress. *S. aureus* biofilms are notorious for causing chronic infections due to their ability to adhere to living tissues and implanted medical devices (artificial heart valves, catheters, joint prosthetics, etc.), as well as their inherent recalcitrance to antibiotics (5–7). These biofilm-related infections lead to increases in morbidity, mortality, and health care costs, with infected devices often requiring surgical removal. However, antibiotic resistance is adaptive because biofilm-associated resistant bacteria revert to their planktonic susceptible phenotype as they disperse from the established biofilm (8). Thus, considerable effort has been put forth to identify effective antimicrobials that specifically treat *S. aureus* biofilms.

The biofilm extracellular matrix serves as a protective physical barrier that shelters the resident bacteria against antibiotics and host immune defenses. Therefore, approaches to disrupt the matrix by enzymatically degrading the chemical components have been investigated. DNase I-mediated degradation of extracellular DNA appears to be effective in disrupting early *S. aureus* biofilms, and treatment with trypsin or proteinase K disrupts the protein components of the biofilm matrix (9–12). Likewise, dispersin B, a glycoside hydrolase produced by the periodontal pathogen *Actinobacillus actinomycetemcomitans*, is able to break down the polysaccharide components of staphylococcal biofilms and can promote antibiotic penetration, resulting in synergistic killing when combined with the antibiotics cefamandole nafate or triclosan (13–15). However, a number of clinical strains are capable of forming polysaccharide-independent biofilms, and thus *S. aureus* susceptibility to dispersin B can vary widely among strains (16, 17). Additional glycoside hydrolases, α-amylase and cellulase, and lysostaphin, a glycine endopeptidase produced by *Staphylococcus simulans* that cleaves the pentaglycine bridge in the staphylococcal cell wall, have also been shown to significantly reduce the matrix biomass of *S. aureus* biofilms *in vitro* (18, 19). Although these *in vitro* results are promising, the application of exoenzymes as therapeutic drugs may be limited due to the possibility of protein-induced inflammatory responses in the host, toxicity, or immunity. Alternatively, these enzymes could be employed in an approach similar to an "antibiotic lock," where a high concentration is applied to catheter lumens to prevent catheter-associated *S. aureus* infections (12, 20). The efficacy of this strategy was demonstrated when implanted jugular vein catheters in mice preinstilled with lysostaphin provided complete protection against *S. aureus* infection compared to untreated catheters (21).

The release of planktonic cells has been shown to result in increased susceptibility to antimicrobials; thus, combining molecules that induce biofilm dispersal with traditional antibiotics could be another viable strategy to eradicate *S. aureus* infections (22). One such candidate is *cis*-2-decenoic acid, a fatty acid produced by *Pseudomonas aeruginosa* that causes an increase in planktonic bacteria released by *S. aureus* biofilms (23). Although the mechanism by which this occurs is not understood and further studies are needed to confirm these findings, it does suggest that *cis*-2-decenoic acid could be utilized as a dispersal agent. Moreover, *S. aureus* produces a number of endogenous dispersal agents, including the surfactant-like molecules phenol soluble modulins (PSMs). PSMs are intrinsically inflammatory and cytolytic for neutrophils, so repurposing PSMs into therapeutic dispersal agents seems questionable. However, due to the fact that PSMs are key to proper biofilm formation (24, 25), interference with PSM production or secretion could prove to be

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an effective approach to inducing dispersal of *S. aureus* biofilms and enhancing antibiotic killing (26).

Targeting bacterial iron metabolism through the use of chelators and gallium-based therapeutics has been demonstrated to effectively disrupt staphylococcal biofilms (27). Iron is crucial for a variety of cellular processes, including DNA synthesis, energy production, respiration, and biofilm formation, and thus is a potential target for antistaphylococcal therapeutics (28). Due to their structural similarity, gallium is able to serve as an iron analog. In a "Trojan horse" strategy, gallium complexes are imported into the cell through bacterial iron uptake systems, where once inside, gallium competes with iron by binding to iron-dependent enzymes and molecules. This results in disruption of vital iron-dependent activities, including respiration, DNA synthesis, biofilm production, and bacterial proliferation (27). Gallium nitrate [$Ga(NO_3)_3$] has been shown to be effective at reducing bacterial biofilms *in vitro*, and mice treated with gallium maltolate had significantly lower bacterial burdens 48 hours post-treatment in a burn wound model of *S. aureus* infection (29, 30). However, not all gallium-based molecules exhibit antimicrobial effects. Conjugation of gallium to the *S. aureus* siderophore staphyloferrin A failed to effectively inhibit MRSA (31).

Recently, a combination therapy of synthetic gallium-based heme analogs and a metal chelator have shown promise as effective antimicrobials against *S. aureus* biofilms. Heme bound to hemoglobin is the most abundant source of iron within the host and is the preferred iron source for *S. aureus* (32). The metalloporphyrin gallium-protoporphyrin IX (GaPP) is capable of mimicking heme and thus facilitating its uptake (33). Once inside the cell, GaPP can be substituted for heme in heme-containing enzymes, including cytochromes, catalases, and peroxidases, disrupting vital cellular processes (33). *In vitro* studies indicate that treatment with the iron chelator deferiprone and GaPP results in significant reduction of methicillin-susceptible *S. aureus* (MSSA) and MRSA biofilms (34). Similar antimicrobial activity was also observed against biofilms formed by small colony variant *S. aureus* strains, which are linked to increased antibiotic tolerance and resistance (35). Moreover, this combination therapy has the ability to potentiate antibiotic-mediated killing, so combining current antimicrobials with gallium could be a promising strategy for the treatment of biofilm infections (36).

Although current data indicate that GaPP-mediated treatments may prove to be efficacious for abolishing *S. aureus* biofilms, the possibility of cytotoxicity due to interference with host iron metabolism should not be entirely discounted. Loss of cell viability and increased lactate dehydrogenase production, a biomarker for cellular cytotoxicity and cytolysis, have been observed in a number of mammalian cell lines when exposed to high concentrations of GaPP (33, 34, 37). However, concentrations of GaPP that induced cytotoxicity were considerably higher than those needed to significantly inhibit *S. aureus* biofilms (33, 35). Moreover, no health effects or changes in behavior were observed in mice given a single intraperitoneal dose of GaPP (25 to 30 mg/kg) followed by a daily dose (10 to 12 mg/kg) of GaPP given for an additional 4 days (33). This suggests that with optimized dosing of GaPP-based therapeutics, host toxicity could be avoided without compromising GaPP antimicrobial activity.

Antimicrobial peptides (AMPs) have been increasingly recognized for their antibiofilm properties. AMPs are typically small cationic peptides that exhibit a range of antimicrobial and immunological properties. One of the first recognized AMPs with antimicrobial activity against *S. aureus* biofilms was the human cathelicidin peptide, LL-37 (38). This peptide displays bactericidal activities against a wide range of Gram-positive and Gram-negative pathogens by disrupting the bacterial membrane (39). LL-37 synthetic derivative OP-145, when integrated into a medical device coating, was shown to prevent *S. aureus*-induced implant-associated infections in rabbits (40). Moreover, OP-145 has been successfully used in a clinical phase 2 trial for the treatment of chronic otitis media (41). Another LL-37 derivative, SAAP-148, demonstrated significant efficacy against MRSA, where treatment led to complete eradication of established biofilms *in vitro*, as well as *in vivo* in a murine wound model (42). Furthermore, cotreatment of the AMPs IB-367 and BMAP-28 with antibiotics was shown to be highly effective at treating catheter-associated infections, suggesting that AMPs could also be used to potentiate antibiotic killing of *S. aureus* biofilms (43, 44).

Another innovative attempt to effectively eradicate *S. aureus* biofilms includes the use of small-molecule inhibitors. A variety of small molecules with activity against *S. aureus* biofilms *in vitro* have been identified, including aryl rhodanines, d-amino acids, benzimidazole, and metal chelators (12, 45–47). An inhibitor of the essential *S. aureus* protein RnpA (RNP1000) significantly reduced the number of biofilm bacteria in an *in vitro* catheter model and protected against lethal systemic *S. aureus* infection in mice (48). These results are encouraging and suggest that small molecules that exhibit strong antibiofilm activities *in vitro* could be potent antimicrobials. However, very few small-molecule biofilm inhibitors have been tested in animal models, and thus, the ability of these compounds to treat *S. aureus* infections is not yet well defined.

INHIBITION OF VIRULENCE BY TARGETING QUORUM SENSING

Virulence factor production in *S. aureus* is regulated by quorum sensing (QS), a cell-to-cell communication mechanism bacteria use to regulate gene expression in response to cellular density. The *S. aureus* QS system is under the control of the accessory gene regulator (*agr*) system, and activation of the *agr* system by an accumulation of auto-inducing peptide leads to activation of the *agr* regulatory network that controls the expression of virulence factors by RNAIII, the major effector for downstream virulence expression and biofilm dispersal (10, 49–51). Inhibiting QS prevents the production of QS-regulated toxins such as delta-toxin, staphylococcal enterotoxin C, and Panton-Valentine leukocidin, thus restricting *S. aureus*' ability to evade the host immune system, kill host cells, and disseminate (52). Moreover, targeting virulence systems such as QS, rather than systems critical for bacterial survival, may exert less selective pressure for the development of resistance compared to using traditional antibiotics.

A number of synthetic and natural QS quenchers have been evaluated for their efficacy against MSSA and MRSA. Biaryl hydroxyketones were shown to successfully inhibit QS by preventing the interaction between the AgrA transcriptional regulator and the P3 promoter, which drives the transcription of the RNAIII master virulence regulator (53). Follow-up studies with synthesized biaryl hydroxyketones demonstrated that compound F12 was capable of reducing MRSA-induced rabbit erythrocyte hemolysis by 98% *in vitro* (54). In a *Galleria mellonella* insect larvae infection model, F12 treatment led to increased larval survival from 12 hours in untreated controls to 42 hours, and combining biaryl hydroxyketones with the β-lactam antibiotics cephalothin or

nafcillin, both of which MRSA is resistant to, further increased larval survival (55). However, in a murine wound infection model, compounds F12 and F1 promoted only modest increases in wound healing, and there were no significant differences in wound bacterial burdens between treatment groups (55). This suggests that the success of biaryl hydroxyketones to treat MRSA may be highly dependent on the infection model utilized, and therefore it has yet to be proven that biaryl hydroxyketone inhibition of QS will be an effective drug development strategy.

Additional ArgA-targeting molecules include the synthetic small molecule savarin and the natural product ω-hydroxyemodin. Savirin, which is capable of blocking *S. aureus* QS, attenuates *S. aureus* in a murine skin lesion infection model (56). Importantly, extensive passage of *S. aureus* in the presence of savarin does not lead to the development of resistance. ω-Hydroxyemodin, a polyhydroxyanthraquinone isolated from the fungus *Penicillium restrictum*, successfully reduced inflammatory cell recruitment and cytokine production and promoted bacterial cell clearance in a murine model of *S. aureus* skin infection (57, 58). Both molecules show promise for skin and soft tissue infections, but it is unknown if these molecules will be effective in other infection models.

Another promising QS inhibitor is ambuic acid, a fungal small-molecule metabolite that selectively inhibits *S. aureus* auto-inducing peptide production (59). Treatment with ambuic acid resulted in decreased lesion size and reduced weight loss in a murine model of *S. aureus* skin and soft tissue infection (59). Furthermore, plant-derived QS inhibitors such as hamamelitannin and its associated derivatives, ajoene, and cinnamaldehyde exhibit potent killing against *S. aureus* biofilms alone or in combination with antibiotics (60–66).

Pursuing additional components of the staphylococcal QS regulatory network could be an alternative option for development of QS-targeted therapeutics, but further research into the molecular mechanisms of QS regulation is needed. Another biofilm and virulence regulatory locus, staphylococcal accessory regulator (*sar*), is a promising target because it is thought to perform an opposing role to *agr* in *S. aureus* biofilm formation (45, 67–69). Overexpression of *sar* can inhibit biofilm production in some *S. aureus* strains; however, other studies demonstrated that *sarA* facilitates the expression of *agr* (67, 68). Although it is clear that the *agr* and *sar* systems are important regulators of *S. aureus* biofilm activities, a better understanding of the roles and relationships between *agr* and *sar* and how modulators of *agr* or *sar* affect *S. aureus* QS signaling, virulence factor production, and biofilm formation will be necessary to advance the development of drugs targeting QS.

BACTERIOPHAGE-BASED THERAPY

Bacteriophages (phages) have evolved to be the ultimate bacteriocidal agents. Phages are viruses that infect bacteria and multiply via a lytic cycle in which the phage particle attaches to the host, injects its genomic material, and manipulates the host machinery, resulting in intracellular phage multiplication. The cycle is complete when the bacterial cell is lysed, releasing multiple phage progeny. The antimicrobial power of lytic phages against staphylococcal infections was recognized as early as the 1920s, but with the discovery of antibiotics, phage therapy quickly fell out of favor in Western medicine (70–72). However, with the rise of multidrug-resistant bacteria, the use of phage-based therapies as an alternative to antibiotic treatment has garnered renewed interest from the medical and research communities.

A number of factors make phage therapy an attractive therapeutic strategy for *S. aureus* infections. Highly conserved components of the cell wall, such as teichoic acids, serve as phage receptors in *S. aureus*; thus, the likelihood of developing resistance to this type of therapy is reduced, and strains that do develop resistance often exhibit a reduction in virulence or fitness (73). The highly specific nature of phages results in only targeted bacteria being infected and subsequently killed, which prevents the disruption of the resident microbiota and morbidities associated with microbiota dysbiosis (74, 75). Moreover, phage therapy eliminates the potential for toxicity that is associated with many antibiotics, and many phages are capable of targeting multiple strains, including both MRSA and MSSA (76–80).

The efficacy of phage therapy has been explored for a wide range of *S. aureus* diseases, including skin and soft tissue infections, sepsis, pneumonia, and osteomyelitis, utilizing animal models with relatively good success (76, 79–88). An early report examining *S. aureus* skin infections in rabbits demonstrated that simultaneous subcutaneous administration of LS2a phage and *S. aureus* prevented abscess formation in 88% of the rabbits treated (81). Abscess size and bacterial burdens were also shown to significantly decrease compared to untreated infected controls in a dose-dependent response (81). A similar outcome was observed in skin lesions of mice infected with MRSA and injected intraperitoneally with SATA-8505 phage, but in this case lesion size failed to decrease despite a reduction in bacterial burdens (79). Phages have also been shown to protect against lethal doses of *S. aureus* (76, 82). Matsuzaki et al. demonstrated that intraperitoneal treatment with φMR11 phage led to complete protection against an *S. aureus* systemic infection, whereas untreated mice exhibited a mortality rate greater than 90% 24 hours postinfection (76). Phage levels rapidly increased within the blood stream and remained high until 6 hours postinfection, coinciding with a drop in bacterial burdens below detectable limits (76). Moreover, phage therapy has been shown to be effective against chronic *S. aureus* infections. Intravenous injection of M^{sa} phage suspension into mice that were systemically infected with a low dose of *S.* aureus 10 days *a priori* resulted in a significant drop in organ bacterial counts to below the limit of detection compared to infected untreated control mice (82). Combined, these studies provide clear evidence that phages are able to multiply and kill *S. aureus in vivo*, leading to disease reduction. *S. aureus* biofilms on indwelling medical devices and in the sinonasal cavity are notoriously difficult to eradicate, so some phage studies have focused specifically on the treatment of biofilms. Multiple groups have demonstrated that lytic phages are capable of significantly reducing biofilm biomass *in vitro*; however, it is still unclear if this is the case *in vivo* (89–91). Recently, Drilling et al. described a significant reduction in *S. aureus* biofilm mass in the frontal sinuses of sheep that were flushed with a cocktail of *S. aureus*-specific phages compared to those treated with heat-inactivated viruses (92, 93). Moreover, bacteriophage treatment significantly reduced *S. aureus* colonization of an indwelling catheter compared to controls in a rabbit model (94). Although these results are encouraging, questions about the efficacy of phage therapy for biofilm-associated infections, particularly in relation to biofilms in less accessible body sites, such as those coating joint prosthetics or artificial heart valves, have not been extensively addressed. In a single study using a rat orthopedic implant infection model, local injection of phage significantly decreased *S. aureus* colony-forming units and biofilm thickness on the implant compared to the control (95).

Pretreating the surface of such devices with a coating of phages may also prevent *S. aureus* medical device colonization and subsequent disease. Studies examining phage-coated orthopedic implants in mice saw a significant reduction in bacterial adherence to the device (96) and bacterial load in adjoining tissues (97). Combined, these studies suggest that phage therapy could be applicable to a number of *S. aureus* biofilm-associated diseases, and phage prophylaxis could help prevent *S. aureus* infections of indwelling medical devices.

A few attempts have been made to turn phages into drug delivery systems to increase the efficacy of *S. aureus* treatments (98–102). Bacteriophage 75 complex was used to administer a photosensitizer to *S. aureus* cells, which significantly enhanced MRSA and MSSA killing when exposed to red light *in vitro* (102). Additional reports describe the use of phages to transfer the antibiotic chloramphenicol to *S. aureus* cells, but bacterial growth was only partially inhibited due to limitations of drug-loading capacity caused by the drug's hydrophobicity (100, 101). Although the concept of manipulating phages into highly specific drug transfer systems is appealing, additional research is needed to further develop this strategy and determine if it could be applicable to the wide breadth of *S. aureus* disease presentations.

At this time, clinical use of therapeutic phages is limited to European countries and the former Soviet Union (71, 103–105). No formal regulations or standards for phage therapy in these countries exist, so well-documented clinical trials including robust controls are lacking (104). Therefore, it is difficult to come to any definitive conclusions, as well as to confidently assess the risks associated with these treatments in humans (106, 107). Nevertheless, multiple reports have described positive clinical outcomes associated with phage therapy for a wide range of *S. aureus* diseases in humans, including respiratory, circulatory, orthopedic, and soft tissue infections (71, 105, 108–110). These clinical reports, taken together with the growing body of literature on *in vitro* and *in vivo* studies, demonstrate that phage therapy could be a feasible strategy for treating *S. aureus* infections.

However, it should be noted that phage therapy is not without potential pitfalls. Due to the high specificity of phages, one significant drawback can be a narrow spectrum of sensitive strains. This issue can be circumvented by selecting polyvalent phages, i.e., those with the capability to infect a large set of strains within a species or combining multiple phages into a therapeutic cocktail (78, 111, 112). Additionally, the health risks associated with *S. aureus* phage therapy are poorly defined. In most cases no adverse effects have been reported, but it is not unforeseeable that a sudden influx of phage or the release of bacterial toxins due to lysis could stimulate a robust inflammatory response (70, 103, 113). Immune induction could also lead to the production of antibodies and subsequent clearance of phages, significantly reducing the efficacy of the treatment (114). Moreover, as viruses are replicating biological agents, it would be extremely difficult to standardize commercial production for clinical use. Nonetheless, the potential of phage therapy may outweigh the drawbacks in the face of increasing staphylococcal antibiotic resistance, and therefore it warrants continued consideration.

STAPHYLOLYTIC ENZYMES AS THERAPEUTICS

The antimicrobial potential for lytic enzymes was first appreciated by Alexander Fleming upon the discovery of the eukaryote-derived cell wall hydrolase, lysozyme; however, more recently, the staphylolytic enzyme lysostaphin, an endopeptidase that cleaves the pentaglycine crosslinking bridges of peptidoglycan, has garnered much attention as a potential antimicrobial agent (115). Since the identification of the enzyme, a growing body of literature indicates that lysostaphin is effective at targeting MSSA, MRSA, and vancomycin-resistant *S. aureus*, as well as *S. aureus* biofilms (19, 116–120). Treatment with lysostaphin systemically or as a material coating has shown promise for eradication of *S. aureus* infections *in vivo* using several animal models (121–123). Moreover, application of lysostaphin reduced *S. aureus* nasal carriage in humans, with no reported toxicity. This suggests that lysostaphin treatment may also be an effective decolonization strategy (124).

Recombinant phage-derived lysins have also been demonstrated to be highly effective antimicrobials *in vitro* and *in vivo* (125). During the lytic phage cycle, viral peptidoglycan hydrolases (endolysins) are produced to facilitate the release of progeny virions by degrading the bacterial cell wall (126). Phage endolysins are particularly attractive as alternative antimicrobial candidates due to a high degree of species and strain specificity (127).

Additionally, endolysins have evolved to bind and cleave highly conserved structures in the cell wall without necessitating intracellular transport of the enzyme, thus decreasing the potential for resistance development and avoiding mechanisms that play a role in conventional antibiotic resistance (e.g., active efflux from the cell) (128). Staphylococcal endolysins can differ significantly at the amino acid sequence level, which is also reflected in their diversity of enzymatic and antibacterial properties (129, 130). Combinations of endolysins have been shown to provide a synergistic treatment effect and would also help decrease the chance of resistance development (131–133). Moreover, recombinant endolysin proteins have the potential to be mass produced for clinical use.

A variety of endolysins and their antistaphylococcal activity have been characterized, with many identified as being highly effective at clearing *S. aureus in vivo* (128). MV-L, originally derived from the staphylococcal phage φMR11, was the first phage endolysin tested in an animal model. These early studies demonstrated that MV-L is capable of killing multiple strains of *S. aureus in vivo*, including those with vancomycin and methicillin resistance (134). Since then, additional endolysins with potent lytic activities against drug-resistant strains of *S. aureus* have been identified, including LysK, an endolysin derived from bacteriophage K, and the LysK derivatives PlyGH15, ClyH, ClyS, CHAPk, and SAL-1 (83, 135–139). Unlike MV-L, where lytic activity is limited to only *S. aureus* strains, LysK has a much broader spectrum of antimicrobial activity that includes the ability to lyse coagulase-negative staphylococci (112). CHAPk, an engineered, truncated version of LysK, has an even broader lytic spectrum that includes members of the *Streptococcus* and *Micrococcus* genera (137).

Numerous reports indicate that endolysins have the potential to be highly effective against skin and soft tissue *S. aureus* infections. Intranasal treatment with MV-L successfully eliminated *S. aureus* in the nares of mice, and similar nasal decolonization outcomes were observed in mice administered CHAPk orally or intranasally (134, 137). Intranasal inoculation with the engineered endolysin fusion protein ClyS resulted in a 2-log reduction in colony-forming units 1 hour postinfection of mice intranasally infected with MRSA (136). In a murine skin infection model, bacterial loads were significantly reduced when ClyS was topically applied compared to mice treated with mupirocin, a com-

monly prescribed antibiotic for the topical treatment of *S. aureus* skin infections, and untreated controls (140). Additionally, endolysin MR-10 combined with the antibiotic minocycline significantly reduced the mortality rate and healing time in a murine burn wound model (141).

Endolysins also hold promise as effective therapeutics against more severe *S. aureus* infections. Mice infected intraperitoneally with MRSA were 100% rescued when MV-L was administered intraperitoneally 30 minutes postinfection. Similar outcomes were observed in mice systemically infected with MRSA and treated intraperitoneally with endolysins phi11, LysK, 80α WMY, and 2638A (129). After 2 days, 100% of the endolysin-treated mice survived, whereas only 25% of vehicle-treated mice survived (129). LysGH15 and SAL-1 have also been shown to be effective against systemic MRSA infections (83, 138). Moreover, intravitreal injection of the engineered endolysin Ply187 significantly reduced bacterial burdens in the eye and preserved retinal function in a murine model of endophthalmitis (50). However, delays in treatment time can significantly reduce the efficacy; thus, endolysin-based therapies may need additional optimization to ensure that they are effective against the most severe *S. aureus* diseases (134).

Recent attempts have been made to enhance the stability and delivery of endolysins utilizing nanoparticles. Nanoparticles containing CHAPk and lysostaphin in the thermoresponsive polymer poly(N-isoporopylacrylamide) allowed for the controlled release of the enzymes upon reaching 37°C (142). Moreover, complexing LysK in polycationic polymers enhanced enzyme stability and lytic activity (143). Nanotechnology could prove to be an effective way to enhance endolysin-based therapies and ensure stability at both storage and physiological temperatures.

Notably, the first generation of staphylococcal phage endolysin-based antimicrobial products is already on the market, and clinical trials are underway for endolysin-based drugs. Staphefekt XDR.300 is an antiseptic solution that is effective against MSSA and MRSA on human skin and is incorporated into a series of creams and gels sold under the Gladskin brand name by the company Micreos Human Health BV (Netherlands). These skin products are for the treatment of skin conditions with an infectious component, such as acne, rosacea, eczema, and skin irritation, and contain the active ingredient Staphefekt. SAL200 is a therapeutic formula containing the endolysin SAL-1, a well-characterized homolog of LysK derived from the *S. aureus* phage SAP-1 (138, 144). It is the first to have undergone a good laboratory practice-compliant safety evaluation including single and repeated dose toxicity and organ function studies in rats and dogs, as well as further pharmokinetics and safety testing in monkeys (145, 146). SAL200 has been shown to be well tolerated with limited side effects observed in these studies. Recently, SAL200 was intravenously administered to healthy male humans as part of a phase 1 clinical trial (147). No serious adverse effects were observed for any of the participants, but there were reports of mild headache, fatigue, and myalgia (147). Additionally, a phase 2 clinical trial is underway for CF-301, an antistaphylococcal endolysin derived from a prophage originally isolated from *Streptococcus suis* (148, 149). Previous work has demonstrated CF-301 to be highly effective at eradicating *S. aureus* biofilms, including biofilms enriched for the more resistant small-colony variants, and it was more effective than antibiotics for the treatment of septicemia in a murine infection model (150, 151). In light of these developments, endolysin-based therapies are likely to be clinically applied in the near future.

RNA GUIDED NUCLEASES

The natural bacterial defense system known as clustered, regularly interspaced, short palindromic repeats (CRISPR) and CRISPR-associated (Cas) genes enables bacteria to recognize and degrade foreign DNA and can serve as an effective, programmable tool for genome editing (152, 153). The Cas9 endonuclease found in the type II CRISPR/Cas system uses a 20-nucleotide small-RNA guide to specify the site of DNA cleavage (154). Recent studies have demonstrated that reprograming Cas9 to target bacterial genomic sequences can result in effective cell killing (155, 156). Therefore, it may be possible to create highly specific, programmable antimicrobials by exploiting the CRISPR system.

Only recently has the antimicrobial power of CRISPR/Cas systems been tested experimentally (157–159). Bikard et al. generated phagemids encoding the packaging site and *rinA*, *terS*, and *terL* genes from the staphylococcal φNM1 bacteriophage with the CRISPR/Cas9 system that were capable of selectively killing *S. aureus* strains depending on the guide RNA sequence provided (158). Application of a phagemid containing RNA-guided Cas9 specific to the methicillin resistance gene, *mecA*, to a mixed culture of MRSA and MSSA strains resulted in a significant reduction in the proportion of MRSA from 50% (pretreatment) to 0.4% (posttreatment), with no differences observed in treated MSSA cells or either strain treated with nonspecific Cas9 targets (158). Similar outcomes were observed when phagemids were topically applied in a murine skin colonization model. Mice colonized with a mixture of kanamycin-resistant and kanamycin-sensitive *S. aureus* saw a significant reduction in kanamycin-resistant but not kanamycin-sensitive cells when treated with RNA-guided Cas9 targeting the kanamycin resistance gene *aph* (158). These results suggest that programming Cas9 nuclease to be a sequence-specific antimicrobial could be an effective treatment strategy, particularly against drug-resistant *S. aureus* infections, or as a decolonization strategy to selectively eliminate *S. aureus* without disturbing the rest of the host's microbiota.

Effective drug delivery remains a significant hurdle to implementation of CRISPR-based antimicrobials (160). As mentioned in the previous section, bacteriophage delivery systems are associated with a number of drawbacks, not limited to reduced host range, poor penetration to areas of infection, and possible adverse health effects. A few attempts have been made to circumvent these issues, including genetically modifying phage tail protein sequences to increase host range and conjugating the CRISPR/Cas9 system to nanoparticles to eliminate the use of the virus altogether (159, 161). Kang et al. described a nonviral delivery system in which CRISPR/Cas9 machinery was covalently modified with the cationic polymer branched polyethylenimine to form a CRISPR nanocomplex (159). These nanocomplexes significantly reduced the growth of MRSA strains *in vitro* compared to the native CRISPR/Cas complex, but their efficacy against *S. aureus* infections *in vivo* remains untested (159). Additional studies examining the use of nanoparticles and other alternative delivery systems are warranted. Future efforts will need to focus on engineering and refining CRISPR/Cas antimicrobial delivery systems, as well as validating these strategies utilizing animal models.

PHOTODYNAMIC THERAPY

Photodynamic therapy (PDT) is a treatment method combining photosensitizers, visible light, and oxygen to induce cell death. Photosensitizers accumulate in the targeted cells,

and upon illumination with light of a specific wavelength, become activated from a ground state to an excited state. The energy produced during excitation is transferred either to a cellular substrate and then to oxygen to form several reactive oxygen species (ROS) (type I mechanism) or directly to molecular oxygen to form a highly reactive singlet oxygen (type II mechanism) (162). Various biomolecules are affected during this process, specifically proteins, nucleic acids, and unsaturated lipids, resulting in irreversible damage and cell death (163, 164). In some cases, the direct mechanism of cytotoxicity has been investigated, which depending on the photosensitizer and its subcellular location, can be attributed to inactivation of enzymes, damage to the cell membrane, or indirect damage to the chromosome (164–168).

Historically, PDT was applied to treat various forms of cancer, but over the past two decades PDT has emerged as an alternative modality for the treatment of localized microbial infections. The nonselective nature of PDT and nonspecific damage triggered by ROS ensures that the development of antimicrobial resistance is unlikely (169–171). Perhaps even more importantly, the effectiveness of PDT against *S. aureus* is independent of a strain's antibiotic resistance profile; thus, PDT treatments can be applied to treat both MSSA and MRSA (172). Moreover, since PDT is exclusively used to treat localized infections, the risk of host cell toxicity and disruption of the microbiota is greatly minimized compared to the use of systemic antibiotics.

A key factor dictating the success of PDT-mediated treatment of *S. aureus* is the selection of a suitable photosensitizer. Consideration should include the propensity of the photosensitizer to preferentially target bacterial cells over host cells, solubility, a long light wavelength absorption band, and high generation of ROS (173). Most antimicrobial photosensitizers tested are organic, aromatic dyes, namely porphyrins, chlorines, phthalocyanine, rose bengal, phenothiazines, and acridines (174). Although a number of photosensitizers have been approved for use in humans, only a select few have been applied clinically to treat microbial infections. These include methylene blue, toluidine blue O, neutral red, PP904 phenothiazium dye, and protoporphyrin IX formed from the porphyrin precursor 5-aminolevulinic acid (ALA) or the ALA-methyl ester, methyl aminolevulinate (175).

Depending on the light source, duration of exposure, and photosensitizer used, PDT can be highly effective at reducing both MSSA and MRSA numbers *in vitro* (166, 172, 176–180). For example, combining 50 μg/ml toluidine blue O and a 15-minute exposure to an 632.8-nm HeNe laser resulted in complete eradication of 8 MRSA isolates, and PDT treatment with aluminum disulfonated phthalocyanine was shown to effectively inactivate 16 epidemic MRSA strains (181, 182). Similar outcomes were observed for the treatment of *S. aureus* biofilms, where PDT treatment with the photosensitizer hypericin significantly reduced biofilm viability (92 to 99%) in all 22 MRSA strains tested (180).

The effectiveness of PDT has also been explored for a number of animal models of localized *S. aureus* infections. In parallel with what is observed *in vitro*, PDT can be highly successful at eradicating *S. aureus* within the host; however, this is greatly dependent on the photosensitizer, light source, and duration of exposure chosen (183). Nonetheless, PDT has been shown to effectively deplete bacterial levels and decrease wound healing time in superficial skin infections using murine skin abrasion and burn wound models, as well as to reduce bacterial burdens in deeper soft tissue abscesses (184–189). Using fiber-optic light delivery systems, PDT treatment has been quite effective against *S. aureus* osteomyelitis in rats (190–193). Administration of the photosensitizer toluidine blue and a red diode laser resulted in an immediate bacterial reduction of 97% within the bone tissues of *S. aureus*-infected rats that was maintained for at least 30 days following treatment (190); moreover, significantly less bone destruction was observed when rats were treated with either toluidine blue or another photosensitizer, Napheophorbide, and the corresponding laser lights (190, 193). Additionally, increased accumulation of neutrophils and bacterial clearance was observed in mice treated with PDT in a murine *S. aureus* septic arthritis model (194, 195).

Despite the nonspecific nature of PDT's ROS-induced killing, PDT sensitivity and resistance levels can vary widely among *S. aureus* strains. A recent study comparing MSSA and MRSA strain susceptibilities to PDT inactivation using protoporphyrin arginate, toluidine blue O, and ALA found that resistance to PDT was independent of antibiotic resistance or virulence profile (196). Moreover, the study also demonstrated that the same bacterial strain could be categorized as PDT sensitive or resistant depending on the photosensitizer used (196). Coupling PDT treatment with ALA and antibiotics has been shown to enhance bacterial killing compared to PDT alone, but this synergistic effect is not necessarily enough to overcome strain differences in PDT resistance (197–199). Although the mechanism that confers strain-dependent resistance to PDT has not yet been fully elucidated, polymorphic differences in the *agr* locus and Agr system functionality have been shown to correlate with *S. aureus* sensitivity to PDT (200, 201). Continued research into the molecular markers that predict strain responses to photo-inactivation will aid in the development of more effective treatment modalities in the future. One significant issue with PDT-based therapies is the potential for ROS to inflict harm on neighboring host cells. Thus, a significant challenge in PDT development is to identify mechanisms in which pathogenic bacteria are efficiently inactivated without damaging the surrounding host tissue. Improving the selectivity of photosensitizers has been an area of intense research within the antimicrobial PDT field. Modification of photosensitizers via antibody conjugation, attachment of peptides, and use of bacteriophage delivery systems have been used to enhance the specificity of antimicrobial PDT (102, 202–204). Moreover, targeting bacteria-specific structures has also been proposed. The addition of two phenothiazinium photosensitizers (EtNBS-COOH) to the side chains of cephalosporin resulted in an enzymatically activated photosensitizer, whereby activation was reliant on the cleavage of the lactam ring by beta-lactamase (205). This novel approach of targeting the antimicrobial resistance mechanism itself resulted in very little nonspecific photosensitizer uptake by host cells *in vitro* (205). Small-molecule activation of the coproporphyrinogen oxidase (CgoX), an enzyme essential for heme biosynthesis and specific to Gram-positive organisms, induced accumulation of the phototoxic heme precursor coproporphyrin III in *S. aureus* and upon light exposure led to a reduction of bacterial burdens *in vitro* and in a murine model of skin and soft tissue infections (206).

Nanotechnology has also been applied to enhance the efficacy of antimicrobial PDT by improving photosensitizer solubility, photochemistry, and photophysics and targeting the pathogen (207, 208). Covalent conjugation of a photosensitizer to a nanostructure or encapsulation in engineered nanoparticles, such as liposomes, micelles, chitosan nanoparticles, and carbon nanotubules, has been proposed to heighten PDT-mediated killing of microbes

(207). Nanostructures with cationic charges have been shown to increase the specificity of PDT photosensitizers due to increased binding to the negatively charged microbial membranes (209). Many photosensitizers are insoluble and tend to aggregate, and thus, nanoparticle-based delivery helps to improve the lethality of PDT via increasing the concentrations of photosensitizer absorbed by the targeted bacterial cells (207, 208, 210). Moreover, some nanoparticles, such as gold, can potentiate PDT by exerting a photothermal effect when exposed to light or, as is the case with silver, have intrinsic antimicrobial properties of their own (208, 211). Additional advantages of using nanoparticles include increased photosensitizer resistance to photobleaching and inactivation, generation of higher concentrations of locally produced ROS resulting in more damage to the targeted bacteria, and low immunogenicity (207, 212).

Another chief obstacle for the advancement of PDT technologies is the limited penetration of light into tissues. Several approaches have been employed to overcome this limitation. Major advancements in fiber optics and microendoscopic technology have allowed PDT to be used with interstitial, endoscopic, intraoperative, or laparoscopic light delivery systems (213). This allows light to be delivered to almost any body site in a minimally invasive manner. Moreover, optical clearing with harmless substances such as glycerol that match the refractive index of tissues can dramatically reduce the effects of tissue scattering of light during PDT (214). Light application with a 2-photon short-pulsed laser has also been shown to result in deeper photopenetration of tissues compared to traditional continuous wave lasers or light sources.

At this time, antimicrobial PDT is predominately applied to treat dental and dermatological infections, but clinical use of antimicrobial PDT has been documented for the treatment of oral infections, acne vulgaris, burn wound infections, and skin ulcers (175). However, clinical trials testing the efficacy of PDT specifically for the treatment of *S. aureus* infections in humans are few. In a randomized, double-blind, placebo-controlled phase 2 trial by Mannucci et al., patients with chronic leg ulcers or chronic diabetic foot ulcers that were treated with a gel containing the photosensiziter RLP086 and 689-nm red light had a significant reduction in total microbial load, with no significant adverse effects reported compared to the placebo-treated group (215). Additionally, a clinical trial in Vancouver, Canada, found that surgical patients that received intranasal PDT during preoperative care had significantly reduced levels of nasal carriage and a 42% reduction in postsurgical site infection rate compared to a 4-year historical average (216). This study led to the development and commercial release of the PDT-based decolonization system MRSAid (Ondine Biomedical, Inc., Vancouver, Canada), which has been approved for clinical use in Canada and is pending approval in the European Union. In the United States, a clinical trial sponsored by the University of Rochester investigating PDT-based treatment of deep tissue bacterial abscesses is expected to be completed by the end of 2018 (217). With the rapid pace at which PDT technologies continue to evolve, it is expected that more clinical trials and PDT-based products will appear in the near future.

ANTIBODIES AND ANTIBODY CONJUGATES

S. aureus expresses many immune evasion and virulence factors that may be potential candidates for antibody therapies and vaccine development. Unfortunately, previous efforts at immunotherapies have failed, due in large part to the functional redundancies of these evasion factors, which is further aggravated when only one antigen is targeted. There have been a number of failed passive immunotherapies against different targets, including lipoteichoic acid (Pagibaximab), clumping factor A (Veronate), capsular polysaccharide (Altastaph), and α-hemolysin (Salvecin, MEDI4893) (218–221). Currently, the development of a promising human monoclonal antibody therapy for the treatment of *S. aureus* bacteremia, 514G3, is ongoing. 514G3 was isolated from the immune repertoire of a healthy human donor and targets the *Staphylococcus* protein A (222). In early 2017, a double-blind, placebo-controlled, phase 1/2 clinical trial of 514G3 involving more than 50 patients in the hospital setting (223) was completed. Another monoclonal antibody treatment against *S. aureus*, ASN100, was developed by Arsanis, Inc., and targets α-hemolysin, Panton-Valentine leukocidin, leukocidin ED, leukocidin GH, and γ-hemolyins AB and CB (224, 225). ASN100 was developed especially to treat patients on respirators who are at risk of developing *S. aureus* pneumonia. The endpoints of a phase 1 trial were met, and a phase 2 clinical trial has been scheduled (226).

SUMMARY

Unfortunately, antibiotic-resistant microorganisms continue to become more and more prevalent, threatening public health and placing a significant economic burden on the health care system. To eliminate drug-resistant infections, novel and effective therapeutic options are desperately needed. Many innovative strategies for alternative drug development are being pursued, including disruption of biofilms, bacteriophage-derived antimicrobials, antistaphylococcal vaccines, and light-based therapies. While many compounds and methods still need further study to determine their feasibility, some are quickly approaching clinical application and may be available in the near future.

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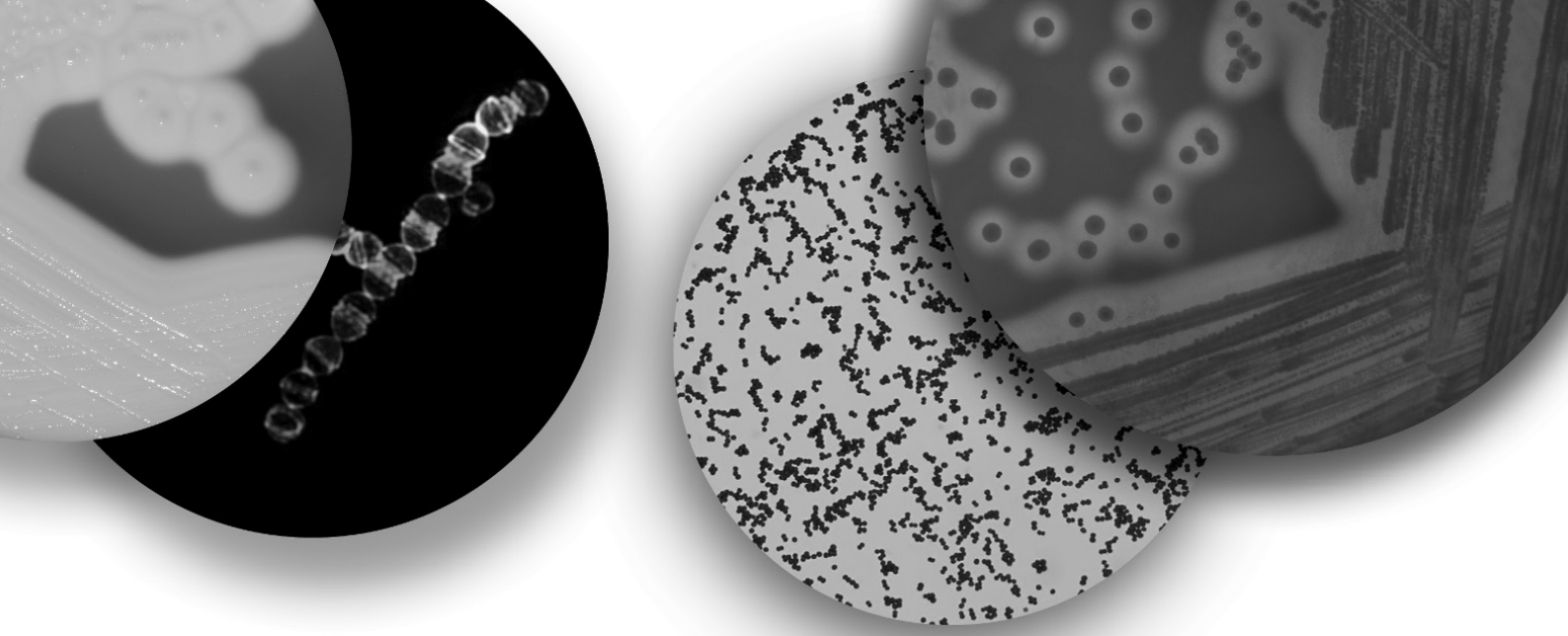
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SECTION 4

The Listeriae

Section Editor: **Daniel A. Portnoy**

Listeriosis and *Listeria monocytogenes* are of interest to a broad range of investigators, including food microbiologists, clinicians, immunologists, medical microbiologists, and cell biologists. While *L. monocytogenes* remains one of the leading causes of mortality from food-borne infections in the United States, it has emerged as an excellent model system to study basic aspects of bacterial pathogenesis and cell-mediated immunity. Indeed, for over five decades, immunologists have exploited the *L. monocytogenes* model to study cell-mediated immunity, and much of our current understanding of cell-mediated immunity has derived from the murine model of listeriosis. It may be true that more is known about the murine response to *L. moncytogenes* than to any other bacterial pathogen; however, it should be noted that the vast majority of these studies have used an intravenous or intraperitoneal route of inoculation, and the relevance of these studies to the natural, oral route of infection is not always clear. Newly developed animal models, including pregnant guinea pigs and transgenic mice, are sure to have a vital impact during the next few years. *L. monocytogenes* has also been utilized as a live, attenuated recombinant vector for the development therapeutic vaccines for cancer immunotherapy. During the past few years, clinical trials have shown promising results.

L. monocytogenes has also emerged as a model system for studying basic aspects of intracellular pathogenesis. Many genetic techniques have been developed, including transposon mutagenesis, allelic exchange, reporter systems, inducible gene expression, integration vectors, and transducing phages. The completion of multiple genome sequences and the subsequent development of transcriptomics have resulted in the identification and characterization of many novel determinants of pathogenesis. The combination of Listeria genomics with mouse genomics, along with an extensive battery of genetic and cell biological tools, should make the next few years truly exciting.

Epidemiology and Clinical Manifestations of *Listeria monocytogenes* Infection

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50

EPIDEMIOLOGY

Listeria monocytogenes is a Gram-positive motile facultative anaerobe that inhabits a broad ecologic niche (1–3). With selective media it can be readily isolated from soil, water, and vegetation, including raw produce designated for human consumption without further processing (4, 5). Newer chromogenic media may offer some advantages in the detection of contaminated foodstuffs (6, 7). Surface contamination of meat and vegetables is relatively common, with up to 15% of these foods harboring the organism. In addition, the organism is a transient inhabitant of both animal and human gastrointestinal tracts (8–10), and intermittent carriage suggests frequent exposure. The gut is the source for the organism in invasive listeriosis when it occurs, and the virulence factor ActA may promote carriage (11). The organism is psychrophilic and enjoys a competitive advantage against other Gram-positive and Gram-negative microorganisms in cold environments, such as refrigerators. It may also be amplified in spoiled food products, particularly when spoilage leads to increased alkalinity. Feeding of spoiled silage with a high pH has resulted in epidemics of listeriosis in sheep and cattle (12).

Several large foodborne outbreaks of listeriosis in humans have parallels to epidemic listeriosis in animals. The first proven foodborne outbreak occurred in Canada in 1980 to 1981 and was caused by ingestion of contaminated coleslaw (13). Subsequently, many other foodstuffs have been implicated in both small and large outbreaks, including unpasteurized and pasteurized cheeses (14–22), pasteurized milk (23, 24), butter (25), various fruits and vegetables (26–30), and several meat products (31–35) (Table 1). A large outbreak of listeriosis with over 900 cases and 200 deaths was reported from South Africa in 2017-2018. The source was a contaminated processed meat called "polony" (36). Recent evidence suggests that hospitalized patients are also at risk of acquiring invasive listeriosis (37). Tracking listeriosis cases and creating linkages to food products is now dependent on typing isolates using pulsed field gel electrophoresis and whole-genome sequencing, which have largely replaced older methods such as serotyping (38, 39). Uncertainty exists as to why outbreaks of listeriosis occur in human populations, although the 50% infective dose in sporadic disease is probably high. Enhancement of organism-specific virulence factors may play a role in epidemic disease, although all isolates of *L. monocytogenes* have the ability to produce all the virulence factors characteristic of the species.

Recent evidence has suggested that sporadic cases of listeriosis are also foodborne. Case-control studies of sporadic listeriosis cases in the absence of epidemic disease have implicated food products, including cold meats, turkey franks, and delicatessen-type foods, as vehicles for the development of sporadic invasive listeriosis in humans (40).

Our current understanding of the epidemiology of human listeriosis suggests that the organism is a common contaminant of food products and that ingestion of small numbers of *L. monocytogenes* occurs frequently in human populations. In one prospective study, a rate of 5 to 9 exposures per person-year was estimated (41). Amplification of the organism in biofilms or on food products undergoing processing but not pasteurization and kept at cold temperatures allows overgrowth of *L. monocytogenes*. Subsequent ingestion of large numbers of the organism may overwhelm innate host-defense systems in the gastrointestinal tract, liver, and spleen with subsequent development of invasive disease. The annual rate of sporadic listeriosis in Europe (42–44) and North America (45) is usually <1/100,000 population per year, and the disease is costly in both human (46) and economic terms (47). Sporadic listeriosis appears to be more common in the spring and summer months. This could be explained by seasonal variations in the types of food products eaten by human populations, with higher-risk products eaten in the warmer months. In addition, data suggest that preexisting damage to the gastrointestinal mucosa by other microorganisms, such as those that are associated with viral gastroenteritis, may allow translocation of *L. monocytogenes* from the gastrointestinal tract with subsequent development of invasive disease (48). These viral pathogens often have seasonal patterns that overlap with those of invasive listeriosis.

Demographic data from surveillance studies indirectly revealed several host-specific risk factors for invasive listeriosis (49, 50). Infection is most commonly seen in the first 30 days of life or in patients older than 60. In the first instance, the fetus is infected during maternal sepsis with *L. monocytogenes* or from perivaginal and perianal colonization of the mother by transition through the birth canal. Host defense against listeriosis is impaired in those infants with underdeveloped macrophage and cell-mediated immune function, and invasive listeriosis is more likely to occur if

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TABLE 1 Some foods implicated in published reports of foodborne listeriosis

Dairy products	Fruits and vegetables	Meat products	Fish products
Pasteurized whole milk	Coleslaw (cabbage)	Delicatessen foods (deli meats)	Tuna salad
Chocolate milk	Lettuce	Pâté	Smoked fish
Mexican-style cheese	Corn	Uncooked hot dogs	Shrimp salad
Soft cheese (different types)	Rice salad	Turkey franks	
Hard cheese	Strawberries	"Rillettes"	
Goat cheese	Cantaloupes	Pork tongue in aspic	
Ice cream	Nectarines	Pork pie	
Fresh cream	Salted mushrooms	Beef	
	Alfalfa tablets	Jellied pork	
	Apples	Cooked ham	
	Blueberries	Foie gras	
	Stone fruit	Ox tongue	
	Sprouts	Undercooked chicken	

colonization of the liver, respiratory tract, or gastrointestinal tract has occurred.

The increased risk of invasive listeriosis in older patients reflects the increasing incidence of immunosuppressive conditions, such as solid tumors and hematologic malignancy, in this age group. Control of early infection in humans and in animal models is highly dependent on an intact gastrointestinal mucosa and effective macrophage function in the liver, spleen, and peritoneum following bacterial translocation from the gastrointestinal tract. Both these protective events can be impaired by the primary disease or by chemotherapy or radiation-induced damage. In addition, treatment of malignancy and the use of immunosuppressive agents with a specific effect on cell-mediated immune function, such as corticosteroids or cyclosporin A (51), predispose to invasive infection by diminishing *L. monocytogenes*-specific host responses that occur after the initial phase of infection. The recent proliferation of biologic treatments with immune modulators such as tumor necrosis factor-alpha inhibitors has also contributed to increases in invasive listeriosis (52–54).

The cell-mediated immune response to *L. monocytogenes* is impaired in pregnant women (55) and, with the decreased gastrointestinal motility (56) seen in pregnancy, may predispose to invasive listeriosis and subsequent transplacental infection of the infant. This results in "early-onset" listeriosis characterized by the delivery of an often premature and severely ill infant. Spontaneous recovery of the mother from *Listeria* sepsis normally occurs following delivery of the infant, although if recognized prepartum, appropriate antibiotic therapy can save the infant.

In "late-onset" listeriosis, the infant is infected through maternal gastrointestinal carriage of *L. monocytogenes* without sepsis, and the infant is infected during transition through a colonized birth canal. In these cases, clinical disease in the infant develops 7 to 14 days later. Direct cutaneous invasion is unlikely, and it is believed that aspiration of the organism into the respiratory tract or swallowing of the organism by the infant may occur during the incubation period. A unique outbreak of neonatal listeriosis in Costa Rica has been described: the vehicle was *L. monocytogenes*-contaminated mineral oil used to clean infants after delivery from healthy mothers, with cross contaminations of shared mineral oil (57). The index case was infected through the placental route of maternal-fetal transmission. Pregnancy-associated listeriosis has been recently reviewed (58, 59).

Several large outbreaks of a febrile gastroenteritis syndrome have further highlighted the importance of *L. monocytogenes* as a foodborne pathogen. In these outbreaks, with an average incubation period of approximately 24 h, attack rates (up to 72%) were much higher than those reported for outbreaks of invasive listeriosis. The reported vehicles for these more typical foodborne infections have included shrimp (60), rice salad (61), chocolate milk (24), corn salad (62), ready-to-eat meats (63, 64), jellied pork (65), and fresh cheese (15). The foods implicated were usually heavily contaminated (>10^9 CFU/ml of *L. monocytogenes*), and the amount of food ingested appeared to correlate with infection, suggesting that the high attack rates are not related to enhanced intrinsic virulence of the particular infecting strain of *L. monocytogenes*. A hospital-acquired outbreak of gastroenteritis has also been described from contaminated meat jelly (66).

While a predisposition to invasive listeriosis is seen in patients with malignancy or organ transplant, human immunodeficiency virus (HIV) infection is also an important risk factor in sporadic listeriosis (67). Earlier studies have reported that attack rates for invasive listeriosis in HIV-positive patients that were 500- to 1,000-fold greater than those in the general population. However, reductions in invasive listeriosis cases in HIV infection has been brought about by widely promulgated dietary recommendations to prevent foodborne illness and by the use of prophylaxis for *Pneumocystis jiroveci* pneumonia, primarily trimethoprim-sulfamethoxazole, to which *L. monocytogenes* is susceptible. Further reductions in HIV-associated cases may be due to better and more widespread antiretroviral therapy (68). Reductions in the overall incidence of listeriosis in non-HIV-positive patients may also be attributed to distribution of dietary recommendations to populations at risk, including pregnant women, patients with malignancies, and organ transplant recipients (69). Perhaps more importantly, the decreased incidence of listeriosis may be due to the promotion of guidelines to promote universal awareness of the problem in the food-processing industry, which has undertaken hazard analysis at critical control point (HACCP) (70–72) and microbial risk assessment (73, 74, 141) programs to reduce contamination of foods with *L. monocytogenes* as well as with other foodborne pathogens such as *Salmonella* spp., *Campylobacter* spp., and *Escherichia coli*. These activities have provided increased protection in the face of increased public demand for fresh, unprocessed food products that may not have been cooked or pasteurized and that by definition present a greater degree of risk for foodborne illness.

In addition to hazard analysis at critical control point programs, regulatory agencies have aggressively pursued the control of *L. monocytogenes* contamination of food. The U.S.

Food and Drug Administration has a zero-tolerance policy for *L. monocytogenes* in its industry sampling programs (75). Other countries have adopted less stringent guidelines, allowing a small amount of contamination ($<10^2$ CFU/g) to strike a balance between protection of public health and needless condemnation of otherwise edible food products. While invasive listeriosis may be more common in some countries in Europe than in the United States, it is not clear whether these differences can be attributed to less stringent standards in Europe that allow more *L. monocytogenes* in the food supply. The debate continues between zero tolerance advocates and those supporting a risk-assessment approach to *Listeria* contamination of food (76). However, these measures have not led to decreases in the incidence of *L. monocytogenes* infections in the developed world (77).

CLINICAL DISEASE DUE TO *L. MONOCYTOGENES*

A wide variety of clinical syndromes have been associated with *L. monocytogenes* infection in both animals and humans (Table 2). The earliest descriptions of *L. monocytogenes* sepsis were described in an epizootic affecting South African rodents (78) and in laboratory colonies of rabbits (79). One distinguishing characteristic of infection in rabbits was the production of monocytosis in blood, which suggested the species name *monocytogenes*. A monocytosis-producing antigen has been described as a virulence factor of *L. monocytogenes* (80), but monocytosis in the peripheral blood is not a characteristic of infection in humans.

Many wild and domesticated animals are subject to invasive listeriosis. Animals acquire the organism from the environment through grazing, amplified by fecal contamination of soil and vegetation. Specific syndromes with parallels in human disease have been recognized in animals. In New Zealand in the 1930s, Gill (81) described "circling disease," a rhombencephalitis of sheep that may effect flocks fed spoiled silage. *L. monocytogenes* has also been implicated as a cause of abortion and prematurity in ruminants. Intravenous and oral models of *L. monocytogenes* infection in rodents can duplicate the illness seen in the natural state in animals, including maternal sepsis and abortion (82).

TABLE 2 Some clinical syndromes associated with *L. monocytogenes* infection

Neonatal meningitis
Meningoencephalitis in adults
Rhombencephalitis
Sepsis (bacteremia) in infants or adults
Native or prosthetic valve endocarditis
Spontaneous bacterial peritonitis
Septic arthritis
Biliary tract disease
Hepatitis
Liver abscess
Cutaneous infections (in animal workers)
Endophthalmitis
Febrile gastroenteritis
Continuous ambulatory peritoneal dialysis peritonitis
Osteomyelitis

The clinical syndromes associated with listeriosis in humans were discovered later. Neonatal listeriosis was initially described in postwar Europe in premature septic newborns in East Germany (83). This description of early-onset listeriosis was followed by reports of neonatal meningitis (late-onset listeriosis) occurring later in the postpartum period. *L. monocytogenes* as a cause of meningitis in neonates is third to group B streptococci and *E. coli* in the developed world (84, 85). The use of antibiotic prophylaxis to prevent group B streptococcal infection may also have reduced cases of neonatal listeriosis (86). In less-developed countries, Gram-negative meningitis with *E. coli* or *Salmonella* spp. is more common, but *Listeria* meningitis still occurs.

PREGNANCY-ASSOCIATED LISTERIOSIS

Pregnant women are at high risk of infection, and occult or overt bacteremia can result in chorioamnionitis producing early-onset neonatal listeriosis (58). These infants have characteristic clinical features, including prematurity, sepsis at birth, fever, a diffuse maculopapular cutaneous eruption, and evidence of significant hepatic involvement with jaundice (87). The mortality rate of early-onset listeriosis, even with treatment, is very high, and stillbirth is also common in this setting. Autopsy findings in cases of early-onset listeriosis show significant chorioamnionitis in placental remnants and granulomas in multiple organs, particularly the liver and spleen, in infected infants. The original descriptions from East Germany characterized the entire syndrome as "granulomatosis infantiseptica." (83).

The mothers of these septic infants may be asymptomatic but commonly have flu-like or pyelonephritis symptoms before the early onset of labor, and their blood cultures are frequently positive for *L. monocytogenes*. Symptoms in the mother include fever, chills, and malaise, which resolve spontaneously following delivery of the infected infant and placenta (87). Anecdotal case reports suggest that early treatment of the mother who has *Listeria* sepsis can prevent transplacental infection or treat the fetus *in utero*, with subsequent delivery of a normal uninfected infant (88). Unfortunately, this usually only happens when a community-based outbreak of *L. monocytogenes* has been identified and physicians are aware of the problem in a particular geographic region through public health alerts.

Late-onset neonatal meningitis due to *L. monocytogenes* has the typical features of the same syndrome caused by other organisms in this setting, including fever, irritability, bulging fontanelle, and meningismus (89). These symptoms usually develop 1 to 2 weeks following delivery. The mother has usually had an uncomplicated pregnancy, delivery, and postpartum course with no signs of sepsis. The clinical syndrome usually dictates a lumbar puncture, and the cerebrospinal fluid (CSF) in 50% of the cases reveals the organism by Gram's stain. CSF cultures are usually positive, although the organism may be isolated simultaneously or only from the blood in some cases. The CSF shows other characteristics of bacterial meningitis, including a high polymorphonuclear leukocyte count, elevated protein, and low glucose with a decrease in the CSF-serum glucose ratio.

ADULT MENINGOENCEPHALITIS

L. monocytogenes is an uncommon cause of bacterial meningitis in adults. There are two major clinical presentations. The first is a typical subacute bacterial meningitis characterized by fever, headache, and neck stiffness (90). Because the organism is not commonly seen on Gram's stain of CSF,

and because the cell counts are lower than in other forms of bacterial meningitis, an initial diagnosis of viral meningitis is commonly made before culture of the organism from CSF or blood. The onset of the syndrome can occur over several days, unlike meningococcal or pneumococcal meningitis, which have more abrupt onsets. During epidemics of foodborne listeriosis, *Listeria* meningitis can occur in apparently healthy individuals of all ages. In sporadic disease, patients more commonly have obvious defects in cell-mediated immune function that predispose them to listeriosis.

The second form of central nervous system listeriosis in adults is a rhombencephalitis that has features characteristic of the same illness in animals, described as circling disease (91). Fever, headache, nausea, and vomiting occur early, with signs of meningeal irritation less commonly present. Subsequently, patients develop multiple cranial nerve abnormalities accompanied by cerebellar dysfunction, including ataxia. Fever may not be present in up to 15% of patients, which makes the diagnosis more difficult and more suggestive of noninfectious disorders. CSF pleocytosis may be minimal, and the organism is rarely seen on Gram's stain. The diagnosis is established by culture of CSF or blood. Magnetic resonance imaging is the best diagnostic study and frequently demonstrates typical multiple microabscesses of the cerebellum and diencephalon (Fig. 1). The mortality rate in this condition approaches 50%, and despite treatment, residual morbidity, including permanent cranial nerve palsies and ataxia, may persist.

L. monocytogenes can also be responsible for cerebritis or typical brain abscess in the supratentorial region (92). In these cases the typical rhombencephalitic symptoms due to microabscesses are absent. Host risk factors reflecting immune deficiency are commonly seen in these cases as they are in *Listeria* sepsis.

LISTERIA SEPSIS

Listeria sepsis, or bacteremia without central nervous system involvement, represents one-third of adult cases of invasive listeriosis. The symptoms are nonspecific but usually include fever and chills. As noted above, in pregnant women *Listeria* sepsis often masquerades as pyelonephritis or "flu" (87). The diagnosis is often established in retrospect following delivery of an infected infant. In nonpregnant adults, *Listeria* sepsis almost always occurs in patients with malignancy, organ transplant, or other immunocompromised states (93–95). In these settings, the presentation is also nonspecific and mimics sepsis with other Gram-positive and Gram-negative pathogens. The mortality rate for *Listeria* sepsis in these series is 25 to 30%.

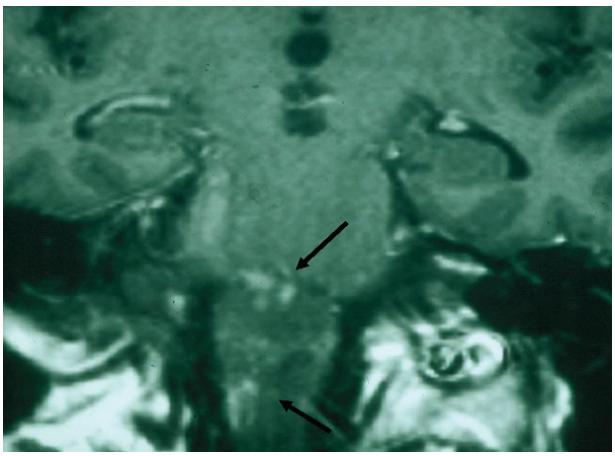


FIGURE 1 Computed tomography scan from a 72-year-old male with *Listeria* rhombencephalitis. The arrows point to multiple microabscesses.

OTHER CLINICAL SYNDROMES

Cutaneous Listeriosis

Cutaneous listeriosis is an occupational hazard of veterinary workers exposed to infected amniotic fluid or placental remnants that are removed from the birth canal of animals (96–98). Occasionally, cutaneous infection, including conjunctivitis, has been seen in laboratory workers (99). Cutaneous listeriosis is characterized by low-grade fever and multiple papulopustular lesions of the skin from which the organism can be isolated. Its appearance is similar to the rash seen in infants with early-onset disseminated listeriosis. In adults, the condition may resolve spontaneously without treatment, but the infection itself should be entirely preventable with appropriate gloving and other protective wear.

Bacterial Endocarditis Caused by *L. monocytogenes*

Bacterial endocarditis presumably follows transient bacteremia from a gastrointestinal source, with subsequent establishment of endovascular infection on an abnormal heart valve. *L. monocytogenes* is an uncommon cause of native valve endocarditis, and over 50% of cases that have been described involve prosthetic valves (100–102). Infection with *L. monocytogenes* is usually found as part of the late prosthetic valve endocarditis syndrome. Diagnostic criteria for *Listeria* endocarditis include the presence of a prosthetic valve with or without vegetation and continuous bacteremia with *L. monocytogenes*. Septic emboli and abscess formations in other organs are relatively frequent. In native valve endocarditis, *L. monocytogenes* can sometimes follow previous episodes of streptococcal bacterial endocarditis or other valvular heart disease. Patients with malignancy, diabetes, steroid therapy, and renal and liver transplantation have been described with *Listeria* endocarditis. Their presentation is nonspecific for *L. monocytogenes* and includes prolonged fever, chills, and ultimately, signs of congestive heart failure. Septic embolization occurs in two-thirds of patients, and aortic and mitral valve involvement are most common. *L. monocytogenes* can also cause arterial infections that involve prosthetic abdominal and aortic grafts or native abdominal aortic aneurysms (103). The mortality of this condition approached 40% before 1985 but has been reduced to 12% with better recognition and surgical management.

Hepatitis and Liver Abscess Due to *L. monocytogenes*

L. monocytogenes has been described as a cause of acute hepatitis in several case reports (104). It occurs as acute onset of fever and jaundice accompanied by positive blood cultures for *L. monocytogenes*. The diagnosis is usually unsuspected. Severe disease with death has been described, and autopsy or liver biopsy generally reveals microabscesses and occasionally granulomas similar to those seen in severe early onset neonatal disease. Predisposing factors include cirrhosis and liver transplantation (105), although *Listeria* hepatitis can occur in a normal host.

Solitary and multiple liver abscesses with fever are also described (106, 107). Bacteremia occurs in half of these patients. Predisposing factors include diabetes mellitus,

transplantation, cirrhosis, and alcoholism. Aspiration of the abscess demonstrates the organism. The mortality rate is 50%, and postmortem examination often reveals abscesses in other organs as well. Patients with multiple abscesses appear to do worse than those with a solitary abscess.

Listeria Peritonitis

L. monocytogenes can also cause isolated episodes of peritonitis (108, 109). It is most commonly seen in patients undergoing continuous ambulatory peritoneal dialysis (110), and the organisms are isolated from the dialysate or from blood culture. The organisms presumably cause infection through translocation from the gastrointestinal tract in patients who have ingested the organism with food. This complication is extremely rare and represents <1% of all cases of continuous ambulatory peritoneal dialysis peritonitis. It can also cause spontaneous bacterial peritonitis in advanced liver disease with ascites (111–113). The mortality rate is low, and laboratory and clinical features are typical of spontaneous bacterial peritonitis due to other organisms. The organism can rarely cause this disease in patients who have undergone liver transplantation.

Biliary Tract Infections

Recent reports have seen *L. monocytogenes* emerge as a biliary tract pathogen (114, 115, 142). Retrograde infection from contaminated food is the likely source. The organism is resistant to bile, and this may play some role in the pathogenesis of biliary disease. Most cases also have medical comorbidities, including immunosuppression from corticosteroid use or newer biologic agents to treat the underlying conditions. A number of deaths have been reported, often due to inappropriate antibiotic therapy.

Musculoskeletal Infection

L. monocytogenes is a very uncommon cause of osteomyelitis (116–118). Reports of *Listeria* osteomyelitis emphasize the role of diabetes mellitus or leukemia as predisposing factors, particularly when long-term corticosteroids are administered. Relapses have been described despite effective antibiotic therapy. Septic arthritis due to *L. monocytogenes* appears to be more common than bone infection, and rheumatoid arthritis appears to be a frequently associated condition. Low-dose methotrexate therapy may predispose to this infection. Infection may follow joint injection with corticosteroids. Infection has been described in prosthetic hips and knees as well as in native joints (119, 120). The organism can also cause vertebral osteomyelitis with epidural abscess (121). With prolonged antibiotic therapy, medical treatment alone, as opposed to removal of the prosthetic joint, may be successful. Deaths are rare and are normally due to the underlying disease.

Gastroenteritis

A febrile gastroenteritis syndrome has been described for listeriosis (15, 24, 25, 60–64, 143). Gastrointestinal prodromal symptoms, such as diarrhea or abdominal pain, have been common in large outbreaks of foodborne adult listeriosis, but sepsis and meningoencephalitis have been the usual presenting syndromes. Population-based attack rates for invasive listeriosis have been low in this setting. In *Listeria* gastroenteritis, a more typical foodborne illness occurs with high attack rates (up to 72%) among the individuals exposed to the vehicle of infection. Large numbers of organisms in the contaminated food may be responsible.

Most patients are well before development of the infection. While bacteremia has occurred in some patients, primary symptoms are diarrhea, fever, fatigue, chills, and myalgias occurring 24 h following exposure. This incubation period is considerably shorter than the 3- to 4-week period for more usual forms of invasive listeriosis.

Pregnant women appear to be more likely to have sepsis in these outbreaks. Isolation of *L. monocytogenes* with selective media from stool has been rare, but serologic tests have been used to help define the extent of the outbreaks. In the outbreaks reported to date, rice salad, shrimp salad, chocolate milk, corn, deli meats, and fresh cheese have been the reported vehicles, and high colony counts of the organism (up to 10^9 CFU/g) appear to be present in the contaminated food. Invasive listeriosis can also be a result of loss of gastrointestinal integrity due to other gastrointestinal tract pathogens such as *Shigella* spp. (122) or to presumed viral gastroenteritis (48). This may account for some sporadic and epidemic cases of *Listeria* sepsis and meningoencephalitis.

DIAGNOSIS OF LISTERIOSIS

Diagnosis of all forms of *L. monocytogenes* infection depends on isolation of the organism from a normally sterile site, usually blood or cerebrospinal fluid. Gram's stain of specimens of sterile spinal fluid, peritoneal fluid, or joint aspirates occasionally, but unpredictably, reveals Gram-positive coccobacilli, characteristic of *L. monocytogenes*. In some forms of central nervous system infection, particularly rhombencephalitis, several samples may have to be obtained to isolate the organism. Focal neurologic findings that are characteristic of rhombencephalitis should prompt computed tomography or magnetic resonance imaging scanning. The finding of multiple microabscesses in the hindbrain is highly suspicious for *Listeria* rhombencephalitis, and empiric treatment for listeriosis should be started.

In situations where antibiotic therapy has already been administered, isolation of the organism from a nonsterile site may support a diagnosis of listeriosis. In pregnant women, stool or vaginal cultures may be positive when selective media for *L. monocytogenes* are used for culture. In febrile gastroenteritis syndromes, where traditional pathogens have not been isolated using standard media, culture of the stool with selective media for *L. monocytogenes* may also demonstrate the organism.

Direct detection of PCR products such as the HlyA from *L. monocytogenes* in CSF and other fluids has been studied. Sensitivity appears to be low but might be useful in patients already on treatment for listeriosis where cultures are negative (123, 124).

TREATMENT OF LISTERIOSIS

L. monocytogenes remains susceptible to most β-lactam antibiotics, with the exception of cephalosporins, to which the organism is usually resistant (125–127). Because newer cephalosporins are commonly used for the treatment of nonspecific sepsis syndromes or for the empiric treatment of bacterial meningitis, specific therapy for listeriosis may be delayed for some patients. Empiric therapy for bacterial meningitis with ampicillin is recommended for older adults but may not be necessary for children beyond the neonatal period (128). When listeriosis is a likely diagnosis, the use of ampicillin or, in penicillin-allergic patients, vancomycin provides empiric coverage for *L. monocytogenes* until the diagnosis is made by culture.

A combination of ampicillin and gentamicin is the current therapy of choice for all forms of listeriosis (129, 130).

However, some data suggest that this combination is not useful and could be harmful (131). There has been a trend toward increasing resistance to penicillins (127). Ampicillin is not bactericidal for *L. monocytogenes*, and *in vitro* and *in vivo* data suggest that an additive or synergistic effect with gentamicin may improve the outcome (132). No randomized, controlled clinical trials of therapy in humans have been carried out, however.

Trimethoprim-sulfamethoxazole, with or without the addition of rifampin, is an alternative treatment regimen that has been recommended. Early "step-down" to oral trimethoprim-sulfamethoxazole has been successful in several cases (133). In one retrospective study, the combination of amoxicillin and cotrimoxazole was found to be more effective than ampicillin and gentamicin (134). High-level resistance to trimethoprim has been recognized, however (127, 135). New quinolones may also be effective, but data are limited to *in vitro* studies (136, 137). Rifampin is active against *Listeria*, but resistance has been found in cases of prosthetic joint infection (138, 139). For central nervous system infection, dexamethasone and phenytoin have been suggested as possible treatment adjuncts (140).

Trimethoprim-sulfamethoxazole has also been used as a prophylactic agent against a number of microorganisms, including *P. jiroveci*, in patients with HIV infection and in patients undergoing chemotherapy for leukemia or lymphoma. This drug would be effective in protecting against *L. monocytogenes* infections. The use of prophylaxis has been temporally associated with a decrease in the incidence of listeriosis in these compromised hosts in combination with dietary guidelines that have been issued for these patients in recent years (68).

The duration of treatment for invasive listeriosis has not been studied. Relapses appear to be uncommon, and 2 to 3 weeks of therapy with ampicillin and gentamicin is sufficient for most forms of listeriosis. Rhombencephalitis with abscess formation in the central nervous system may require more prolonged therapy, but data are not available that support treatment beyond 4 weeks (129, 130).

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Innate and Adaptive Immune Responses during *Listeria monocytogenes* Infection

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51

INNATE IMMUNITY

Mammalian hosts have multiple, redundant ways to rapidly respond to the presence of infection. These innate immune mechanisms are triggered within minutes to hours after infection. Although they are not microbe specific, they do contribute to limiting exponential growth of *L. monocytogenes* and may even reduce bacterial dissemination to secondary infection sites. The role of these rapid immune responses can be clearly demonstrated by examining bacterial loads in various inbred strains of laboratory mice, with CFU burdens differing by 100- to 1,000-fold within the first few days of infection (1, 2). This difference has been attributed largely to multiple innate immune mechanisms, and both the prototypic susceptible strain (BALB/c) and highly resistant C57BL/6 mice can induce robust $CD8^+$ T cell responses and eventually clear sublethal doses of *L. monocytogenes*. Weak or delayed innate immunity allows *L. monocytogenes* to replicate exponentially for several days and thus is a major determinant of disease severity. The innate immune responses generated during the first few days of infection are also critical for proper induction of the adaptive immune mechanisms that lead to immunological memory (3–5).

In humans, *L. monocytogenes* is transmitted by ingestion of contaminated ready-to-eat food products. The attack rate during documented outbreaks can be high, with up to 75% of affected individuals experiencing symptoms of gastrointestinal disease (6–9). However, fewer than 20% of those people had intestinal disease severe enough to require hospitalization, and fewer than 5% had positive blood cultures. These observations suggest that orally acquired *L. monocytogenes* bacteria face several anatomic or innate immune bottlenecks that can severely limit systemic spread and the onset of life-threatening bacteremia or meningoencephalitis. The widely used i.v. infection model, which is meant to mimic systemic listeriosis, has the disadvantage that it completely bypasses the gut phase of infection. Oral infection of mice can be inefficient and much less reproducible than i.v. infection (10); however, technological advances involving humanized mice (11, 12) and mouse-adapted bacterial strains (13, 14) led to the development of a highly reproducible foodborne infection model (10, 15). Because these developments occurred only recently, little has been done at this point to assess host immune responses following oral transmission of *L. monocytogenes* (16). Therefore, the paradigms highlighted in this article come primarily from studies using the i.v. model, unless noted otherwise.

Triggering of Innate Immune Receptors

Innate immune recognition of microbes occurs through a variety of pattern recognition receptors (PRRs) expressed by mammalian cells. Engagement of these receptors initiates signaling cascades that lead to expression of cytokines and chemokines that shape the local microenvironment and dictate the type of immune response that ensues. PRRs have different patterns of cellular localization (e.g., at the cell surface, on phagosomal membranes, in the cytosol) and exhibit differential expression in various cell types. Thus, innate immune recognition of *L. monocytogenes* varies depending on the cell types the bacteria interact with during each phase of infection. Even within a single cell, bi-phasic recognition of *L. monocytogenes* may occur as extracellular bacteria are rapidly sensed by PRRs expressed at the host cell surface, while invasive bacteria may not maximally trigger cytosolic sensors until a few hours later (17). There is significant redundancy among innate immune sensing mechanisms, with multiple receptors able to recognize microbial ligands at each cellular location. Most of these signaling pathways have been studied in isolation, and the net effect of signaling through multiple receptors is not well understood.

The earliest opportunity for innate immune recognition of naturally acquired *L. monocytogenes* occurs at the surface of intestinal epithelial cells that line the gut lumen. InlA-mediated uptake results in rapid translocation across intestinal epithelial cells without escape from membrane-enclosed vacuoles (18), so we would expect to find that only PRRs at the surface or in vacuoles are triggered initially. After reaching the underlying lamina propria, myeloid-derived cells such as monocytes, macrophages, and dendritic cells (DCs) encounter primarily extracellular bacteria (19); as described below, cytosolic invasion is likely to occur only in tissue-resident macrophages. Later in the infection, *L. monocytogenes* may reinvade intestinal epithelial cells from the basolateral side (20), and in this case, escape from vacuoles and cytosolic replication may occur.

Extracellular *L. monocytogenes* at the cell surface

The major families of PRRs expressed at the cell surface are Toll-like receptors (TLRs), which recognize highly

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conserved microbial components found in all bacteria, and C-type lectin receptors, best known for detecting fungal cell wall components. Broad evidence for a significant role for TLR in recognition of *L. monocytogenes* comes from the use of MyD88-deficient mice that lack the signaling adaptor used by most TLR proteins. MyD88$^{-/-}$ mice have at least 1,000-fold increased bacterial burdens compared to wild-type mice (21), and the 50% lethal dose in these animals is decreased by at least three logs (22). Six members of the TLR family (TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11) are expressed at the cell surface, but the two receptors most involved in recognition of *L. monocytogenes* are TLR2 and TLR5.

TLR2 is expressed on a variety of cell types, including intestinal epithelial cells (23) and CD8$^+$ T cells (24, 25), but it is expressed at the highest level on myeloid-derived phagocytes (26, 27). TLR2 binds lipoteichoic acids and lipoproteins found in bacterial cell walls (28). Although it was originally thought that TLR2 could also detect *L. monocytogenes* peptidoglycan, it was later shown that thorough removal of all traces of lipoteichoic acid and other lipoproteins abrogated sensing by TLR2 (29). The receptor forms heterodimers with other TLR proteins, and this results in recognition of specific subsets of lipoproteins. For example, TLR2-TLR1 complexes recognize triacylated lipopeptides, and TLR2-TLR6 detects diacylated lipoproteins (30). TLR2 dimers recruit MyD88 and TIRAP adaptors which activate a signaling cascade that leads to NF-κB activation and the expression of several proinflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor alpha (TNFα), and IL-12 (31). TLR2$^{-/-}$ mice had significantly reduced levels of serum cytokines 24 hours postinfection (22). However, only modest effects of TLR2 deficiency were noted during the later stages of infection (21, 32). TLR2-deficient macrophages internalized fewer *L. monocytogenes* organisms, but there appeared to be little effect on subsequent growth of the bacteria that did reach the cytosol (33, 34). Thus, TLR2 appears to contribute to early recognition of *L. monocytogenes* but is not absolutely required for clearance during the systemic phase of infection.

TLR5 binds flagellin, the structural subunit of bacterial flagella, and is expressed on the basolateral surface of intestinal epithelial cells (35, 36). In the mouse, a subset of intestinal CD11b$^+$ DCs also expresses high levels of TLR5, but most myeloid-derived phagocytes found in other tissues do not (37, 38). TLR5-deficient mice were generated by two groups (39, 40) and have been used to study infection with a variety of Gram-negative flagellated bacterial pathogens. *L. monocytogenes* has peritrichous flagella, but most strains downregulate expression of flagellin at 37°C. However, Way et al. showed that *L. monocytogenes* reference strain 10403s and 20% of the clinical isolates they tested did, in fact, express *flaA* at 37°C, and those bacteria were sensed by TLR5-transfected HeLa cells (41). Furthermore, *flaA* mutant *L. monocytogenes* had a defect in persistence and dissemination in orally infected mice compared with flagellated bacteria (42). These observations suggest that TLR5 is likely to contribute to early recognition of infection following oral transmission and rapid MyD88-mediated production of IL-6 and TNFα in the gut.

The C-type lectin receptor family of proteins is well known for detecting β-glucans expressed by fungi, but more recent work indicates that these receptors are also involved in sensing bacteria and viruses. Chen et al. showed that CLEC5A, a C-type lectin expressed primarily on myeloid-derived cells, was important for innate immunity against *L. monocytogenes* (43). Infection of bone marrow-derived macrophages from CLEC5A$^{-/-}$ mice resulted in decreased IL-1β secretion, and neutrophils from these mice had a defect in killing *L. monocytogenes in vitro*. The authors suggested that *L. monocytogenes* may simultaneously interact with both TLR2 and CLEC5A on the cell surface and that coactivation of the two signaling pathways enhances proinflammatory responses.

L. monocytogenes in phagocytic or endocytic vacuoles

After invading mammalian cells, *L. monocytogenes* resides briefly in phagocytic or endocytic vacuoles before escaping to the cytosol. Cytosolic invasion occurs within 30 minutes in macrophages (44), leaving little time for bacterial products to be sensed by vacuolar PRRs. However, there are circumstances in which *L. monocytogenes* can replicate slowly within vacuoles (45). Leber et al. measured the transcriptional response to vacuolar *L. monocytogenes* by analyzing mRNA expressed in bone marrow-derived macrophages during infection with listeriolysin O (LLO)-deficient bacteria that were unable to escape to the cytosol. They found that the vacuolar response was completely dependent on MyD88 (46), a result that is consistent with sensing only through TLR molecules that are internalized from the cell surface (e.g., TLR1, TLR2, TLR5, or TLR6) or recruited to vacuoles (TLR9). TLR9 detects unmethylated CpG DNA, a motif that is common in bacterial and viral genomes but rare in mammalian cells. Most subsets of DCs, macrophages, and B cells respond to treatment with synthetic CpG oligonucleotides, and plasmacytoid DCs, in particular, express very high levels of TLR9. Stockinger et al. infected TLR9-deficient mice with a lethal dose of *L. monocytogenes* and found no difference in the survival curve compared to that of wild-type mice (47).

Cytosolic *L. monocytogenes*

Intracellular sensing of microbes is a critical function of the innate immune system because commensal bacteria rarely access the host cell cytosol, so detection of microbial components here typically indicates a pathogenic infection. Cytosolic PRRs that sense *L. monocytogenes* can be divided into two large groups: microbial cell wall components and surface proteins are detected by Nod-like receptors (NLRs), and nucleic acids are sensed by either cyclic GMP-AMP synthetase (cGAS)/STING (stimulator of interferon genes) (cyclic dinucleotides), AIM2-like receptors (AT-rich double-stranded DNA [dsDNA]), or retinoic acid-inducible gene 1 (RIG-I)-like receptors (RNA). More than 20 members of the NLR family have been identified to date (48), and cytosolic *L. monocytogenes* can be sensed by several of these proteins. Nucleotide-binding oligomerization domain 1 (NOD1) and NOD2 both detect dipeptide components of bacterial peptidoglycan (49) and are widely expressed by most cell types. Sensing of cytosolic *L. monocytogenes* by either of these receptors activates NF-κB and leads to increased transcription of proinflammatory chemokines (e.g., CXCL2) and cytokines (e.g., IL-6, TNFα). *L. monocytogenes* can avoid detection by NOD1 via the action of PgdA, a deacetylase that modifies *N*-acetylglucosamine residues (50). However, NOD2 appears to be a key PRR for sensing cytosolic *L. monocytogenes*, particularly in the gut. NOD2 deficiency resulted in decreased expression of several antimicrobial peptides in the ileum of infected mice (51) and a reduction in DUOX2-dependent reactive oxygen species production in Caco-2 cells (52). A lack of NOD2 signaling in the gut altered the course of infection, increasing bacterial burdens in orally infected mice, but Nod2$^{-/-}$ mice infected by the intravenous route showed no differences compared to wild-type mice (51).

Cytosolic recognition of bacterial nucleic acids generally leads to the production of type I interferons (IFNαβ). STING is a signaling adaptor that links cytosolic recognition of damaged DNA by cGAS or viral DNA by DDX41 to the (interferon regulatory factor 3) IRF3-dependent transcription of IFNαβ (53, 54). However, STING can also directly sense cyclic di-nucleotides such as c-di-GMP or c-di-AMP (55). These molecules serve as second messengers in bacteria, and for *L. monocytogenes*, c-di-AMP is required for growth in the host cell cytosol (56–58). STING is most highly expressed in myeloid cells and was not found in hepatocytes (59). STING-deficient mice have a blunted type I IFN response but had no differences in bacterial burdens compared to wild-type mice (60, 61). Thus, STING-mediated recognition of cyclic dinucleotides is not absolutely required for innate immune recognition of cytosolic *L. monocytogenes*. However, Archer et al. measured cytokine secretion by macrophages lacking both STING and the TLR adaptor MyD88 and found that the cells made very little IFNβ, IL-12, TNFα, or IL-6 in response to infection (60). RIG-I is generally thought of as a sensor of viral doubled-stranded RNA, and it may also detect small RNAs secreted by actively growing cytosolic *L. monocytogenes* (62). Triphosphorylated RNA released into the cytosol triggered IFNβ expression in a RIG-I-dependent manner in epithelial cells but not in bone marrow-derived myeloid phagocytes (63). In contrast, no role could be demonstrated for the cytosolic RNA sensor MAVS in the induction of type I IFN (64).

Another possible intracellular detection system for *L. monocytogenes* could be TLR10. Much less is known about TLR10 than about other TLRs, and its ligand has not been clearly defined. Regan et al. did an RNA interference screen in *Listeria*-infected human cell lines HT-29 and THP-1 and found that small interfering RNA silencing of TLR10 led to decreased chemokine (CCL20 and IL-8) production and increased intracellular CFU (65). There is no murine homolog for TLR10 (it is a pseudogene in mice), so confirmatory *in vivo* studies using a mouse model of listeriosis are not possible.

Inflammasomes

Many members of the NLR family act as scaffolds for the assembly of unique cytosolic protein complexes called inflammasomes. *L. monocytogenes* can be sensed by multiple inflammasomes. Although each is activated by a different stimulus, all inflammasomes recruit and activate the caspases needed for either secretion of IL-1β and IL-18 or gasdermin-mediated pyroptosis, an inflammatory type of cell death (66, 67). Early work using bone marrow-derived macrophages and human peripheral blood mononuclear cells showed that *L. monocytogenes* was sensed by both NLRP3 (Nalp3) and NLRC4 (Ipaf) (68–71); however, neither of these inflammasomes is essential for protection against infection. NLRP3 is activated by a variety of stimuli but is not constitutively expressed in most resting cells; TLR or NOD signals are required to induce NF-κB-dependent transcription of the gene (67, 72). The secreted proteins p60 and LLO have both been associated with activation of the NLRP3 inflammasome (70, 73), but NLRP3-deficient mice do not have increased bacterial burdens in the spleen or liver 24 hours after i.v. challenge (74). NLRC4 is activated by cytosolic flagellin, but most *L. monocytogenes* strains downregulate the expression of flagella at 37°C. *Listeria* strains engineered to secrete heterotypic flagellin in the cytosol readily activated the NLRC4 inflammasome and were severely attenuated *in vivo* compared to wild-type *L. monocytogenes* (75, 76).

Unlike most inflammasomes that activate caspases, the output of NLRC5 activation is maximal expression of major histocompatibility complex I (MHC-I) genes (77). Two groups showed that *Nlrc5*$^{-/-}$ mice have decreased CD8^{+} T cell responses and significantly higher *L. monocytogenes* burdens than wild-type mice (78, 79). It is not yet clear which cell type is critical for sensing infection or what component of the bacteria is recognized by NLRC5. Neiman-Zenevich et al. recently showed that intracellular infection also indirectly activated the NLRP1B inflammasome by reducing intracellular ATP levels in infected RAW264.7 macrophage-like cells (80). Prior to this study, the only known activator of the NLRP1B inflammasome was anthrax toxin. *L. monocytogenes* can also activate (aim2-like receptors) ALR-dependent inflammasomes (69, 81). It was known that chromosomal DNA was released into the host cell cytosol during *L. monocytogenes* infection (82), and it is now clear that this dsDNA can be sensed by AIM2-like receptors. Like NLRP3, AIM2 expression also requires a signal for induction, but the priming occurs through type I IFN signaling rather than TLR or NOD-mediated NF-κB signaling (83). Studies using macrophages from various knockout mouse strains suggest that caspase-mediated secretion of IL-1β and subsequent cell death by pyroptosis depends mainly on the AIM2 inflammasome rather than NLRP3 or NLRC4 (84, 85).

Portnoy and colleagues have argued that caspase-mediated pyroptosis is actually rare in macrophages infected with wild-type *L. monocytogenes* (4). However, it is clear that inflammasome-mediated cleavage and secretion of IL-18 occur during infection. For example, Schmidt and Lenz showed that the N-terminal domain of p60 could stimulate release of IL-1β and IL-18 from myeloid phagocytes without triggering pyroptosis (73). Seminal work by Shao's lab has made clear that cell death triggered by inflammatory caspases is completely dependent on expression of one or more isoforms of gasdermin, thus providing a mechanism to explain caspase-mediated cytokine secretion in the absence of cell death (86, 87). Further studies will be required to assess the levels of gasdermin expressed in the various cell types encountered by *L. monocytogenes in vivo*. It is also possible that there are negative regulators of innate immune sensing that can dampen inflammatory responses. Anand et al. showed that NLRP6-deficient mice were highly resistant to lethal challenge and had reduced bacterial burdens following intraperitoneal inoculation (88). Caspase-1 activation was unaffected in these animals, but higher numbers of circulating polymorphonuclear neutrophils (PMNs) and monocytes were observed in their blood than in the blood of wild-type mice. This suggests that activation of the NLRP6 inflammasome functions to dampen the proinflammatory signals that release myeloid phagocytes from the bone marrow. NLRP6 is highly expressed in neutrophils but is also found in macrophages, DCs, and epithelial cells (88). Given the large size of the NLR family, it is possible that additional negative regulators will be identified in future studies.

Chemokine and Cytokine Production

The primary output of innate immune signaling pathways is the expression and subsequent secretion of inflammatory mediators that can either act locally or accumulate in the blood and act systemically. Chemokines are small chemoattractant molecules that induce migration of innate immune cells and infiltration of infected tissues, and cytokines are small proteins that alter the behavior of other cells of the immune system. We know that cytosolic invasion of

L. monocytogenes in bone marrow-derived macrophages induces a different profile of cytokine expression compared with bacteria that remain phagosomes (46, 89). This is also true for infection of intestinal epithelial cells such as the human Caco-2 cell line (90). In both cases, the molecular basis for differential cytokine expression is the triggering of distinct innate immune sensing pathways that leads to activation of various transcription factors such as NF-κB, IRF3, or STAT3. In this section, proinflammatory mediators and effector cytokines will be discussed in the approximate order in which they are produced during *L. monocytogenes* infection.

Chemokines attract immune cells to sites of infection

A key function of the innate immune system during bacterial infection is to trigger the release of granulocytes from the bone marrow and to direct migration of circulating neutrophils (PMNs) to infectious foci. Sensing through either TLRs or NODs leads to NF-κB-dependent expression of IL-6, CXCL2 (previously called MIP-2), and CXCL8 (also called IL-8 in humans or KC in mice), and all of these molecules act as chemoattractants for neutrophils. Ebe et al. first showed that a hepatocyte cell line could produce both CXCL2 and CXCL8 within 1 to 2 hours of infection and that treatment of mice with neutralizing antibodies against either of these molecules modestly inhibited PMN infiltration in the liver (91). Later studies showed that cytosolic invasion was required to induce CXCL8 secretion in either human umbilical vein endothelial cells or HEK293 cells (92). Other cell types are slower to induce chemokine secretion. For example, CXCL8 expression peaked at 24 to 48 hours in primary human monocyte-derived macrophages, whether the cells were infected with *L. monocytogenes* or treated with synthetic TLR2 ligands (93).

IL-6 is produced by both myeloid-derived phagocytes and epithelial cells within 0.5 to 3 hours after infection, and the responding cell types with the highest levels of IL-6 receptor on the cell surface are PMNs, monocytes, macrophages, T cells, and hepatocytes (90, 94–97). The need for IL-6 early in infection was demonstrated by the studies of Liu et al., who showed that administration of recombinant IL-6 could protect mice if it was given 4 hours prior to infection, but not if given 24 hours after infection (94). Mice lacking IL-6 are highly susceptible to listeriosis and fail to trigger the release of neutrophils from bone marrow into the blood (98, 99). A recent clinical report described the case of a rheumatoid arthritis patient being treated with anti-IL-6 receptor monoclonal antibody therapy (tocilizumab) who developed meningoencephalitis, one of the more severe forms of systemic listeriosis (100). Inflammatory monocytes and macrophages respond to the presence of IL-6 by increasing expression of CD38, a surface protein that facilitates phagocytosis (101). However, *L. monocytogenes* may have evolved mechanisms to limit the induction of IL-6 *in vivo*. Personnic et al. showed that infection with strains lacking the surface protein InlH induced greater IL-6 production in both spleen and liver without detectable changes in other cytokine levels (102), suggesting that InlH may interact with innate immune cells to dampen the IL-6 response.

Recruitment of monocytes occurs shortly after neutrophil infiltration and is driven primarily by the production of chemokines CCL2, CCL3, and CCL7. CCL2 (also called MCP-1) was produced in the spleen and liver within 1 to 3 hours after i.v. infection (103, 104). CCL7 (also known as MCP-3) was found in blood, liver, and kidney within a similar time frame, with peak levels occurring at 48 hours postinfection (105). Only *L. monocytogenes* organisms that were actively secreting LLO induced chemokine expression in these studies; cytosolic localization of c-di-AMP is the likely stimulus, because STING-deficient mice produced less CCL2 and CCL7 (59). Both of these chemokines bind to the same receptor, CCR2, which is highly expressed on monocytes. Monocytes that lack CCR2 remain sequestered in the bone marrow and do not migrate to sites of infection (106). The overlapping but nonredundant functions of these two chemokines is evidenced by the observation that $CCL2^{-/-}$ and $CCL7^{-/-}$ mice are more susceptible to infection, but neither mouse strain is as susceptible as receptor-deficient ($CCR2^{-/-}$) mice (105). CCL3 (also known as MIP-1α) attracts a variety of leukocyte cell types and is a member of the C-C subfamily of chemokines that all share a common receptor (CCR5). Neither $CCL3^{-/-}$ nor $CCR5^{-/-}$ mice had a defect in bacterial clearance compared to wild-type animals, suggesting that sensing of this subfamily of chemokines is either not necessary or can be easily compensated for by other chemokines during primary infection (107, 108). However, mice treated with anti-CCL3 antibody were much more susceptible to reinfection with *L. monocytogenes* (109). Work by the group of Gregoire Lauvau has shown that CCL3 produced by memory T cells during secondary challenge is indirectly required for TNFα-mediated reactive oxygen species-dependent killing of *L. monocytogenes* by inflammatory myeloid phagocytes (110).

Type I interferons can have either proinflammatory or anti-inflammatory effects

IFNα and IFNβ (commonly referred to as type I IFNs) are produced in response to cytosolic invasion by *L. monocytogenes* (89). Macrophages and DCs are the primary producers of type I IFN, with peak expression in the spleen, liver, or mesenteric lymph nodes occurring approximately 24 hours postinfection (47, 111, 112). Secreted IFNα and IFNβ bind to a common type I interferon receptor (IFNAR). Type I IFN signaling leads to a distinct profile of interferon-stimulated genes 4 to 8 hours after infection of bone marrow-derived macrophages (17), and similar profiles of interferon-stimulated genes have been observed in the blood or spleens of infected mice (113). Most of the transcriptional response to cytosolic bacteria could be reconstituted by transfecting macrophages with *L. monocytogenes* genomic DNA; however, coadministration of muramyl dipeptide to trigger NOD2 signaling did amplify the response (46). The overall proportion of extracellular versus intracellular bacteria in a given tissue is likely to be important since TLR2-mediated signaling can suppress STING/RLR-mediated (RIG-I like receptors) induction of type I IFN (114). Mice that lack the IFNAR have dysregulated infiltration of innate immune cells with an increase in PMNs and a decrease in monocytes 24 hours after intraperitoneal infection (115).

Type I interferons were initially identified as antiviral molecules (116), and secretion of modest amounts of IFNαβ is likely to be important for the initiation of cell-mediated immune responses against all intracellular pathogens. However, excessive production of type I IFN during bacterial infection can promote anti-inflammatory effects such as T cell apoptosis (117), decreased TNFα production (118), downregulation of IFNγR (119), and increased secretion of IL-10 (120, 121). Early studies of $IFNAR1^{-/-}$ mice infected intravenously indicated that sensing of type I IFN was detrimental to the host, since the knockout mice were more resistant to *L. monocytogenes* (118, 122), but more recent work suggests that the magnitude of the type I IFN response depends on the route of bacterial transmission. Kernbauer et al. found that IFNAR-deficient C57BL/6N mice were more susceptible than wild-type mice to *L. monocytogenes*

delivered by intragastric inoculation (123). However, Pitts et al. saw no difference in CFU burdens in wild-type and IFNAR-deficient mice that were infected by the natural feeding route (113). In that study, foodborne transmission of *L. monocytogenes* resulted in significantly reduced IFNβ secretion in the spleen, and no anti-inflammatory effects were observed. Thus, the net effect of IFNαβ production appears to differ depending on the local concentration and the cellular context in which the cytokines are sensed, and the robust type I IFN response previously observed in the spleen may be an artifact of the i.v. model.

Type III interferons (IFNλ1, IFNλ2, IFNλ3, and IFNλ4) are a recently identified family of proteins that respond to different stimuli (TLR ligands) (124) and bind to a different receptor than type I IFN but have a similar functional output of interferon-stimulated genes. Intestinal epithelial cells induced expression of IFNλ within 6 hours of *L. monocytogenes* infection (125). IFNλ binds to a heterodimeric receptor composed of IL-28Rα and IL-10β chains, but unlike IFNAR, the complex is not expressed in hematopoietic cells; it is found primarily on epithelial cells and possibly hepatocytes (126). Given the cell tropism of the IFNλ receptor, it is tempting to speculate that type III IFN is an important component of tissue-specific innate immunity at mucosal surfaces. However, it has been difficult to study the role of type III interferons *in vivo* because two of the IFNλ genes are pseudogenes in mice, and the IFNλ receptor genes are not highly expressed in mouse liver (127). In cell culture, cytosolic *L. monocytogenes* secretes LntA, which translocates to the host cell nucleus and interacts with the chromatin repressor BAHD1 to prevent silencing of interferon-stimulated genes (128). Although it seems counterintuitive for an intracellular bacterium to promote expression of genes involved in innate immunity, one possibility is that IFN signaling can also lead to cell cycle arrest, prolonging the intracellular replication niche (129).

IL-1β is a pyrogen with pleiotropic functions in innate immunity

IL-1 was the first cytokine to be identified, and the original function ascribed to it was induction of fever during infection (130). Fever is part of the early response to *L. monocytogenes* in murine models. Murapa et al. used temperature transponder microchips implanted in the abdominal cavity to show that i.v.-infected mice had a 2°C increase in body temperature that peaked approximately 2 days postinfection (131). However, it is now clear that IL-1 is also involved in modulating neutrophil influx, prostaglandin synthesis, and Th17 T cell responses. IL-1α is constitutively expressed in many cell types and does not require any additional processing to bind to the IL-1 receptor (IL-1R) (132). In contrast, IL-1β is induced by CLEC5A and TLR2 signaling in myeloid phagocytes and is expressed as an inactive proform that must be cleaved by caspase-1 (43, 133, 134). IL-1β does not have a classic secretion signal, and it is not yet clear how it is released from the cell. One possibility is that membrane pores formed when inflammatory caspases activate gasdermin-mediated pyroptosis allow for cytokine release, but other mechanisms have also been proposed (135, 136). *In vivo*, IL-1 did not reach detectable levels in serum during systemic listeriosis, but it was produced locally in the spleen and liver with peak levels occurring 2 to 4 days postinfection (137). Neither IL-1β$^{-/-}$ mice nor IL-1R$^{-/-}$ mice generated on a resistant C57BL/6 background showed a defect in CFU burdens or survival compared to wild-type controls (138, 139). However, antibody-mediated blockade of the receptor in susceptible strains such as BALB/c or SCID mice decreased neutrophil influx (140) and increased bacterial burdens by 50- to 100-fold (137, 141). Thus, IL-1 can enhance innate resistance to listeriosis but is not absolutely required for bacterial clearance.

IL-12 and IL-23 promote the development of proinflammatory T cell responses

The IL-12 gene family consists of five cytokine subunits: p19, p28, p35, p40, and EBI3. Heterodimeric complexes of these proteins form bioactive cytokines: IL-12 (p35 + p40), IL-23 (p19 + p35), IL-27 (p28 + EBI3), and IL-35 (p35 + EBI3) (142). Of these four cytokines, IL-12 is by far the best studied during *L. monocytogenes* infection. IL-12 is produced by phagocytes and is triggered when surface-exposed TLRs encounter either live or heat-killed *L. monocytogenes* (143–145). Secreted IL-12 induces natural killer (NK) cells, NKT cells, and memory phenotype CD8$^+$ T cells to express IFNγ (146–148). If IL-18 is also present, the two cytokines act synergistically to induce IFNγ secretion (22, 146). Using a mouse model with a conditional knockdown of the IFNγ receptor, Lee et al. showed that CD8α$^+$ DCs were an important source of IL-12 that initiated IFNγ responses in the spleen within the first 24 hours of infection (149). However, soon after that, the large number of infiltrating Ly6C^{hi} monocytes become the primary source of IL-12 in the spleen. Although IL-12 levels peak within 12 hours of infection (145), ablating IL-12 does not typically result in changes in bacterial burdens until 3 days postinfection or later (149–151). This highlights the fact that IL-12-dependent IFNγ production is important for shaping the ensuing inflammatory response and generating Th1-type T cells, but IL-12 does not play a direct role in early innate immune clearance of *L. monocytogenes*.

IL-23 is also secreted by phagocytes in response to *L. monocytogenes* infection (152) and is best known for promoting the generation of Th17 cells, a subset of CD4$^+$ T cells that enhance clearance of extracellular bacteria and fungi. In the absence of IL-12 and type I IFN, IL-23 can also drive CD8$^+$ T cells to lose their cytotoxic capacity and begin producing IL-17 (153). A Th17-type inflammatory response could be important during the intestinal phase of infection, when most *L. monocytogenes* organisms are found in the extracellular compartment (19). In fact, Disson et al. recently showed that when *L. monocytogenes* translocated across M cells in the gut mucosa, CX$_3$CR1^{int} "MP" cells rapidly produced IL-23 (154). MP cells are a functionally plastic cell type that shares properties with both macrophages and DCs (155). Increased output of IL-23 in the gut resulted in secretion of IL-22 and IL-17 by innate lymphoid cells (ILCs), which in turn, induced mesenchymal stromal cells to express IL-11 (154). A previous study had found no role for IL-11 in systemic infection (156), but inclusion of IL-11 in the cytokine milieu of the small intestine apparently induced an epithelial response that included increased proliferation, less goblet cell maturation and mucus production, and a decrease in accessible E-cadherin (154). Together, these responses may cause a blockade of InlA-mediated uptake in intestinal villi, which could help to explain why *L. monocytogenes* can persist in the gut lumen for several days after initial clearance from the underlying lamina propria (2). Using the i.v. infection model, p19$^{-/-}$ mice (unable to produce IL-23 but producing normal levels of IL-12) had reduced infiltration of monocytes in the spleen and fewer neutrophils in the liver, leading to increased bacterial burdens in both organs (157, 158). Sieve et al. also reported that splenocytes from p19$^{-/-}$ mice treated exogenously with IL-23 showed a significant reduction in IL-12-dependent IFNγ secretion (159). This

suggests that IL-23 might directly antagonize IL-12 and, thus, serve as a key factor that regulates the switch between Th1 or Th17-type inflammatory responses.

Effector cytokine TNFα is required for direct bacterial killing

TNFα is essential for clearance of *L. monocytogenes*. Mice that lack either the cytokine itself or one of its cognate receptors (TNFR1, also known as p55, and TNFR2, also known as p75) are highly susceptible to either systemic (160–164) or oral (165) *L. monocytogenes* infection. The importance of the TNFα response is also readily observed in humans. TNFα inhibitors are commonly used to reduce chronic inflammation in rheumatoid arthritis and ulcerative colitis patients, and several case reports suggest that this therapy leaves the patients susceptible to developing the more severe forms of systemic listeriosis (166–168). TNFα is produced by a variety of cell types including macrophages, a subset of TNF and nitric oxide synthase-producing myeloid cells referred to as "TipDCs" (169), and effector $CD8^+$ T cells. Secretion can be induced by triggering MyD88-dependent TLR (22) or by the presence of the chemokines IL-6 and CCL3 (109, 170), but a maximal, sustained response requires cytosolic localization of *L. monocytogenes* (104, 171). In the spleens of mice, TNFα was first detected at 24 hours postinfection, and the concentration peaked at 3 days (137). TNFα signaling in conjunction with IFNγ activates macrophages, pushing them toward an inflammatory "M1" phenotype while suppressing the alternatively activated "M2" phenotype (172). Activated macrophages produce increased reactive oxygen species and inducible nitric oxide synthase and display enhanced killing of intracellular *L. monocytogenes* (173, 174). TNFα produced by effector T cells may also have direct killing effects. White and Harty found that perforin-deficient $CD8^+$ T cells killed target cells displaying the appropriate antigen-loaded MHC-I molecules using a TNFα-dependent mechanism (175). Although TNFα is clearly an important cytokine for initiating a proinflammatory, Th1-type immune response, excess production can be detrimental. Murapa et al. showed that heat shock factor 1-deficient mice produced higher levels of TNFα and were significantly more susceptible to infection than wild-type animals (131).

IFNγ stimulates the expression of hundreds of genes and is essential for protective immunity

IFNγ, also known as type II interferon, is a critical cytokine for clearance of intracellular bacterial pathogens such as *L. monocytogenes* and is involved in both the innate and adaptive phases of the immune response. Signaling through the IFNγ receptor activates the JAK-STAT pathway and results in transcriptional regulation of hundreds of genes which are collectively known as interferon-regulated genes (176). Many of these genes are involved in innate immunity and result in increased effector functions such as phagocytosis, production of proinflammatory cytokines, and the production of reactive oxygen and nitrogen intermediates. In addition to enhancing the killing function of phagocytes, IFNγ can increase expression of costimulatory molecules and MHC, resulting in enhanced priming of T cells. Badovinac et al. also showed that the presence of IFNγ can alter the immunodominance hierarchy of specific antigenic epitopes that are processed and presented to T cells (177). For additional information about the specific activities of IFNγ during listeriosis, please see the extensive review by Dussurget et al. (126).

Mice that lack the IFNγ gene are highly susceptible to listeriosis (178), and patients with mutations in the receptor IFNγR1 are prone to develop recurrent infections with intracellular bacteria, including *L. monocytogenes* (179, 180). Furthermore, innate resistance to either orally transmitted or systemic listeriosis, which varies among different laboratory strains of mice, correlates with both the timing and the magnitude of the IFNγ response (181). Serum levels of IFNγ peak at 24 to 48 hours after i.v. infection. Although this important cytokine can be secreted by a variety of cell types (182, 183), the relevant cellular sources are likely to vary throughout the course of infection depending on the localization of infectious foci of bacteria. For example, shortly after i.v. infection, most *L. monocytogenes* organisms in the spleen are found in the red pulp, where they colocalize with NK cells and $CD169^+$ marginal zone macrophages (184). If memory $CD8^+$ T cells are present, they also localize to the red pulp and begin producing IFNγ within 6 hours of infection (185). However, within 24 hours, most of the bacteria have moved to the T cell zone of the spleen, where they encounter primarily DCs and naive T cells (184, 186).

Early IL-10 production may contribute to host susceptibility

IL-10 is an immune-suppressive cytokine with pleiotropic functions that can dampen Th1-type inflammation. Many cell types can produce IL-10 after recognition of bacterial motifs by either MyD88 or (TIR domain containing adaptor-Inducing interFeron beta) TRIF-dependent PRRs (187), but the downstream effects of IL-10 expression vary depending on the time and place that the cytokine is secreted. During the later stages of infection, secretion of IL-10 has an important regulatory function to limit pathology caused by excessive inflammation. For example, γδ T cells produce IL-10 and help to prevent immune-mediated tissue damage in the liver. In mice lacking γδ T cells, TNFα-secreting effector $CD8^+$ T cells persisted in the liver instead of contracting normally once *L. monocytogenes* was cleared, and this resulted in hepatic necrosis (188). NK cells also begin to secrete IL-10 midinfection, as discussed in greater detail below, but robust production of IL-10 early during infection seems to underlie known differences in host innate resistance to *L. monocytogenes*. Pasche et al. observed that gender-specific differences in serum IL-10 concentrations 3 days postinfection correlated with the enhanced susceptibility of females compared to males in certain mouse strains (189). Neonates also have increased serum IL-10 compared to adults early after infection with *L. monocytogenes* (190), and this likely contributes to their enhanced susceptibility compared to adults. In neonatal mice, IL-10 was rapidly produced by both macrophages and dendritic cell precursors (190, 191).

IL-10-deficient mice were more resistant to *L. monocytogenes* infection than wild-type mice and displayed differences in bacterial burdens in the spleen and liver as early as 2 days postinfection (192, 193). Two groups have shown that subsets of regulatory B cells are major producers of IL-10 and that mice lacking these B cells had decreased bacterial loads in the spleen (194, 195). However, it is possible that this early IL-10 response is an artifact of the i.v. model, which results in a large bolus of bacteria all reaching the spleen within just minutes of inoculation (196). Emil Unanue's group has shown that i.v. injection of *L. monocytogenes* induces a robust type I IFN response that leads to massive lymphocyte apoptosis and a concomitant increase in IL-10 production by phagocytes in the spleen (120, 197, 198). Their studies suggest that IL-10 production is integral to creating a proliferative niche in the spleen that allows *L. monocytogenes* to grow exponentially during the first few days of infection. In contrast, Pitts et al. did not see an increase

in T cell death or enhanced IL-10 production in the spleen following oral infection, even when the spleens had bacterial burdens that were similar to those of i.v.-infected mice (113). This suggests that transmission by the oral route, which involves several bottlenecks before reaching the spleen, does not trigger rapid IL-10 production by phagocytes and B cells. Further work will be required to determine if the age- or gender-specific differences in rapid IL-10 production that have been described also occur following natural foodborne transmission in either susceptible mice or humans.

Activation of the Complement System

The complement system is a complex network of soluble proteins present in blood and other body fluids (199). Activation of the complement cascade could aid in the innate immune response to *L. monocytogenes* in three ways: (i) by generating the anaphylatoxins C3a and C5a, which are strong chemoattractants for PMNs, (ii) by generating the opsonin C3b to promote internalization of microbes by phagocytes, and (iii) by direct killing via the C5b-9 membrane attack complex. Gram-positive bacteria such as *L. monocytogenes* are thought to be somewhat resistant to lysis via the membrane attack complex because the thick peptidoglycan layer of the cell wall prevents efficient insertion of C5b-9 proteins (200). However, the cell wall fraction of *L. monocytogenes* does activate complement, most likely via the alternative pathway, and if *Listeria*-specific antibodies bind to the bacteria, the classical pathway will also be activated, resulting in the production of many cleavage products, including C3a, C3b, and C5a (201–204).

The complement mechanism that has been most extensively investigated during listeriosis is promoting uptake by myeloid phagocytes. In the presence of serum from naive mice, C3b-laden bacteria were readily taken up by cells that express the complement receptor CR3 (205). CR3-mediated internalization occurred more frequently in activated macrophages and was associated with bacterial killing (206). Rosen et al. showed that treatment with antibodies specific for CR3 resulted in large increases in CFU burdens and death in mice infected with a dose that is normally sublethal (207). Tissue-resident phagocytes such as Kupffer cells in the liver often express little CR3; however, they do express abundant complement receptor of the immunoglobulin superfamily (CRIg). CRIg binds C3b and iC3b fragments and mediates clearance of opsonized particles from the blood (208). Signaling through CRIg promotes rapid phagocytosis by macrophages and some DCs and induces the subsequent formation of autophagosomes (209). Thus, even *L. monocytogenes* bacteria that are able to escape from phagocytic vacuoles in these cells may be killed by autophagy. Indeed, CRIg-deficient mice had a 100-fold increase in circulating *L. monocytogenes* in the blood compared to wild-type mice (208).

Activation of complement via the alternative pathway also leads to the formation of C3a and C5a fragments, particularly in myeloid-derived cells (monocytes, macrophages, DCs, neutrophils, basophils, and eosinophils). C57BL/6 mice lacking the receptor for either of these anaphylatoxins ($C3aR^{-/-}$ or $C5aR^{-/-}$) were significantly more susceptible to *L. monocytogenes* infection as evidenced by higher CFU burdens and decreased survival (210, 211). In both of these studies, the receptor deficiency led a decrease in innate immune cell infiltration in the spleen at 3 days postinfection. The A/J mouse, an in bred strain that naturally lacks the C5 component of complement, was also moderately more susceptible to *L. monocytogenes* infection (212, 213). These results suggest that complement activation and the production of both C3a and C5a is necessary for optimal chemotaxis of inflammatory phagocytes during *L. monocytogenes* infection.

Recent studies suggest that complement may also have other novel functions during listeriosis. Verschoor et al. found that C3-opsonized *L. monocytogenes* in the bloodstream rapidly associated with platelets and then colocalized with $CD8\alpha^+$ DCs in the spleen (214). In the liver, Kupffer cells also used CRIg to recognize platelet-bound bacteria (215). The authors of these studies proposed that this complement- and platelet-dependent mechanism of clearance is slower than the rapid killing that occurs when phagocytes use scavenger receptors to mediate uptake of bacteria. This would allow for additional time for *L. monocytogenes* antigens to be presented to lymphocytes, thereby promoting adaptive immunity. Complement activation may also directly affect T cells. Nakayama et al. showed that proliferation of both $CD4^+$ and $CD8^+$ T cells was diminished in C3-deficient mice, indicating that complement had a role in promoting optimal expansion of T cells (216). However, Tan et al. found that memory $CD8^+$ T cells were maintained at higher levels in $C3^{-/-}$ mice (217). This suggests that complement may be involved in the contraction phase of the T cell response, although additional studies will be required to understand the exact roles of the various C3 cleavage fragments.

Infiltration of Inflammatory Cells

L. monocytogenes derives its species name from the robust monocytosis first observed in infected rabbits (218), and this feature is replicated in both oral and systemic mouse models of listeriosis (219, 220). Systemic mediators produced during infection generally increase both progenitor development and the release of mature leukocytes from the bone marrow. These circulating cells traffic to sites of infection and, upon recognition of *L. monocytogenes*, begin to secrete additional cytokines that can alter the local microenvironment, shaping the ensuing immune response. This section discusses innate immune cells in the approximate order in which they either infiltrate infectious foci or become activated during the course of infection.

Neutrophils (PMNs) are first responders that directly kill *L. monocytogenes*

During infection, PMNs downregulate expression of CXCR4, a receptor that promotes retention in the bone marrow, and upregulate expression of the receptor CXCR2, which can sense the chemokines CXCL1 and CXCL2 (221, 222). Within just 30 minutes of i.v. inoculation, circulating PMNs begin to infiltrate the *L. monocytogenes*-infected liver, with peak numbers occurring by 4 hours postinfection in C57BL/6 mice (223). This rapid recruitment appears to be at least partially dependent on formyl peptide receptors (FPRs). Murine neutrophils have a low affinity for fMet-Leu-Phe, the prototypic formylated peptide that is commonly used to stimulate human PMNs. However, murine PMNs have strong affinity for other N-formylated peptides derived from *L. monocytogenes* (224). In mice deficient for either FPR1 or FPR2, peak neutrophil recruitment is delayed until 8 hours postinfection, and in doubly deficient mice that cannot sense formylated peptides at all, the total number of PMNs that infiltrate the liver is greatly reduced (223). These results suggest that formylated peptides derived from the N-terminus of *L. monocytogenes* proteins drive much of the early chemotaxis of neutrophils into the liver, although other signals, such as leukotriene B4 and complement component C5a, are also likely to play a role in PMN recruitment. During

sublethal infection, serum granulocyte colony-stimulating factor levels increase, and enhanced granulopoiesis in the bone marrow replenishes lost PMNs. However, in mice challenged with a lethal dose, extensive triggering of PRRs on cells in the bone marrow leads to the depletion of PMN progenitors (225).

Granulocyte colony-stimulating factor-deficient mice have severe neutropenia and when challenged with *L. monocytogenes* have increased bacterial burdens in both the spleen and the liver compared to wild-type animals (226). Likewise, early studies using antibody-mediated depletion of neutrophils suggested that PMNs were critical for limiting *L. monocytogenes* growth during the first few days of infection (227–232). However, the antibodies used in those studies (clone RB6-8C5) were subsequently shown to bind both Ly6G (a surface marker specific for PMNs) and Ly6C (a protein expressed by both PMNs and monocytes). Two more recent studies using newer antibodies with a validated Ly6G specificity (clone 1A8) confirmed that depletion of PMNs resulted in significantly increased bacterial burdens in the liver but not the spleen (233, 234). A third report found no role for neutrophils (235); however, in that study the anti-Ly6G antibodies were administered at the time of infection, rather than as a pretreatment, so it is possible that some PMNs rapidly infiltrated the liver and killed *L. monocytogenes* before the antibodies could deplete the cells. *L. monocytogenes* is not an obligate intracellular pathogen, but the ability to invade host cells and spread from cell to cell to avoid extracellular immune defenses is thought to be the primary virulence strategy during infection of mammalian hosts. However, it is clear that at any given point in time, a significant number of extracellular bacteria are also present in the tissues (19). The ability of PMNs to directly kill those bacteria before they can establish an intracellular niche is likely to be critical for limiting exponential replication of *L. monocytogenes*.

PMNs are abundant in human blood but account for only 15% of circulating leukocytes in mice (236, 237). This, along with the ease of access to large volumes of peripheral blood from human donors, probably explains why most *in vitro* studies of PMN function have used human cells. In mice, bone marrow is a good source of morphologically mature PMNs that release both primary and secondary granules upon stimulation (238, 239). Incubation of human PMNs with either live *L. monocytogenes* or purified LLO caused the cells to degranulate and enhanced production of both leukotrienes and reactive oxygen species (240). This process occurred rapidly, because the granules fused with the plasma membrane before the PMNs had even completed engulfment of the bacteria (241). Arnett showed that matrix metalloproteinase-8 was responsible for the rapid degradation of LLO, leaving *L. monocytogenes* within a phagosome from which it could not escape. Similar results were found in murine PMNs, where a small number of *L. monocytogenes* bacteria were able to persist intracellularly for a short period of time without replicating (242). Pitts et al. demonstrated that murine PMNs use both oxidative and nonoxidative mechanisms to kill serum-opsonized *L. monocytogenes* and that there were no intrinsic differences in the killing ability of PMNs harvested from either susceptible BALB/c mice or resistant C57BL/6 mice (242). However, the level of extracellular superoxide dismutase produced does seem to affect the ability of murine PMNs to kill internalized bacteria (243, 244).

In addition to being laden with granules that allow for rapid degradation of engulfed bacteria, PMNs may also serve other functions in immunity. For example, Yin et al. found that a subset of neutrophils in the spleen was secreting IFNγ 18 hours after *L. monocytogenes* infection (183). This suggests that PMNs can also contribute to the early innate cytokine response to infection. Tvinnereim et al. also reported that depletion of neutrophils reduced $CD8^+$ T cell activation, implicating neutrophils as a source of antigen that could be cross-presented by $CD8\alpha^+$ DCs (245). Unfortunately, those studies were performed with the Ly6G/Ly6C cross-reactive antibodies, so it is not yet clear if PMNs or monocytes were the critical cell type. There is a growing awareness that neutrophils are not homogenous, but it is not yet clear whether there are distinct subsets of dedicated PMNs with different functions or if different states of differentiation exist within one population of cells (246). As this field advances, it will be exciting to see if neutrophils are also involved in regulating other aspects of immunity during listeriosis.

Monocytes patrol tissues and migrate to draining lymph nodes and the spleen

Monocytes were long thought of as simply the circulating blood precursors for tissue macrophages, but we now understand that there are several subtypes of monocytes and that these cells serve both regulatory and antimicrobial functions during infection (247, 248). One regulatory function of monocytes may be to prevent excessive activation of PMNs which could lead to increased tissue damage. Boyartchuk et al. showed that 24 hours after *L. monocytogenes* infection, the number of infiltrating PMNs in the liver was similar in both BALB/c and C57BL/6 mice (249). However, 1 day later, the livers of the more susceptible BALB/c mice still had predominately neutrophilic infiltrates, but the infectious foci in the more resistant C57BL/6 mice were surrounded by monocytes. In mice treated with an anti-CR3b antibody, monocytes did not properly organize into granuloma-like foci in the livers, and increased bacterial burdens were observed (207). Antibodies administered after monocytes had already appeared at the site of infection had much less effect, which suggests that the recruitment of monocytes to neutrophilic infectious foci is critical for limiting exponential growth of *L. monocytogenes*. Monocytes may be uniquely suited to serve a regulatory role because they are more efficient at infiltrating sites of infection where tissue damage already exists. Velásquez et al. used multiphoton intravital microscopy to show that by 3 days postinfection, foci of *L. monocytogenes* in the liver were no longer perfused by blood, yet monocytic cells were still able to enter the foci, while other cell types only accumulated at the periphery (250). These observations are all consistent with the idea that recruitment of monocytes is important for shifting the environment toward a resolving type of inflammation.

Expression of Ly6C distinguishes the two major subsets of monocytes in mice: $Ly6C^{lo}$ cells patrol endothelial surfaces and are functionally equivalent to $CD14^{lo}CD16^+$ cells in humans, while $Ly6C^{hi}$ cells ($CD14^{hi}CD16^-$ cells in humans) are typically thought of as inflammatory monocytes, and these are the cells that increase in number substantially during infection (248, 251, 252). Inflammatory monocytes express high levels of the chemokine receptor CCR2 and are released from the bone marrow during infection in response to the presence of CCL2, CCL7, and CCL12 in the blood (106). Shi et al. reported that recruitment from the bloodstream and subsequent trafficking to infectious foci in the liver was chemokine-independent and instead relied on interactions with CD11b, CD44, and ICAM-1 that were induced near sites of infection (253). However, Jin et al. found that STING-deficient mice had diminished serum

levels of CCL2 and CCL12 during infection and that this led to decreased migration of monocytes to the liver but not the spleen (59). In the spleen, efficient recruitment of Ly6C^{hi} monocytes depends, at least in part, on interactions with CX3CR1 and requires secretion of IL-23 (157, 254). As the infection proceeds, monocyte recruitment declines, perhaps due to negative feedback from IL-10 produced by NK cells (255).

It is difficult to discuss the antimicrobial effector functions of monocytes without acknowledging the confusion that has existed in the field regarding the origin and function of macrophages and DCs (256), since monocytes have been reported to differentiate into both of these cell types. An emerging consensus suggests that activated Ly6C^{hi} monocytes can differentiate into either effector monocytes, monocyte-derived DCs that partially resemble conventional DCs (cDCs), and monocyte-derived phagocytes that resemble tissue-resident macrophages. Although the plasticity of monocytes is widely understood, some debate still exists concerning whether inflammatory cues or the intrinsic properties of precursor subsets determine functional phenotype (257, 258). Regardless, the transcriptional profiles and cell surface phenotypes that are currently being used to distinguish various subsets of monocyte-derived cells are much more extensive and complex than those that were typically used in earlier studies. Therefore, it can be hard to attribute previous results obtained using only a few surface markers to one of these newly defined subsets. Despite this limitation, it is clear that monocytes and monocyte-derived effector cell types have microbiocidal functions that are involved in *L. monocytogenes* clearance. Serbina et al. were the first to show that a subset of Ly6C^{hi} monocytes rapidly secreted TNFα and increased output of nitric oxide (169). Ly6C^{hi} monocytes can also produce high levels of reactive oxygen species, but this seems to be more important during secondary infections.

Another possible function for monocytes during infection is the transport of microbes from one part of the body to another. It is now clear that some subsets of monocytes can migrate through tissues and transport antigen to draining lymph nodes without differentiating into tissue-resident cells (259, 260). However, *L. monocytogenes* does not efficiently invade monocytes. Using a flow cytometric approach, Jones et al. found that the majority of cell-associated *L. monocytogenes* bacteria in either the gut lamina propria or mesenteric lymph nodes (MLNs) were adhered to Ly6C^{hi} monocytes (220). However, they were unable to demonstrate intracellular growth or survival of *L. monocytogenes* in either bone marrow-derived cultured monocytes or Ly6C^{hi} monocytes harvested from the lymph nodes of mice and infected directly *ex vivo*. Likewise, Shi et al. found that very few monocytes in the livers of i.v.-infected mice harbored viable *L. monocytogenes* (253). Drevets et al. published a series of reports demonstrating that association of *L. monocytogenes* with monocytes could promote entry into the brain during systemic listeriosis, but they also failed to identify significant numbers of viable gentamicin-resistant bacteria within these cells (261, 262) Therefore, monocytes appear to be a major cell type that interacts with *L. monocytogenes* during the innate immune response, but they do not serve as an intracellular growth niche.

Macrophages serve as an intracellular growth niche until activated by IFNγ

Tissue macrophages are not technically an infiltrating cell type but are included here because both monocyte-derived and tissue-resident macrophages are involved in the early, innate immune response to *L. monocytogenes*. Resident macrophages are thought to derive from embryonic progenitors that seed tissues during development. They are imprinted with characteristics specific to a given tissue and self-renew throughout the lifespan of the host (263). Resident macrophages differ phenotypically and functionally from monocyte-derived macrophages found in the spleen or lymph nodes, although there is still confusion regarding the extent to which monocyte-derived cells can acquire the properties of tissue-resident cells. Many published *in vitro* studies that examined the interaction of *L. monocytogenes* with macrophages used either transformed cell lines (e.g., J774 or RAW264) or bone marrow-derived macrophages. Culturing bone marrow yields a homogenous population of cells that most closely resembles an early differentiation state of a macrophage, but the cultures generally lack the heterogeneity of macrophage subsets that have been observed *in vivo* (256). Regardless, these cells have been extraordinarily useful for delineating the stages of the *L. monocytogenes* intracellular life cycle since they support extensive bacterial growth in the cytosol (264). Indeed, bone marrow-cultured cells become permissive for intracellular proliferation of *L. monocytogenes* as soon as they start transitioning from a monocyte to a macrophage, as indicated by decreased surface expression of Ly6C and increased CD64 (220).

As the innate immune response to infection proceeds and high levels of proinflammatory cytokines are produced, macrophages become activated and induce multiple killing mechanisms that destroy *L. monocytogenes*. In macrophages pretreated with IFNγ, *L. monocytogenes* was destroyed within phagocytic vacuoles and did not escape into the host cell cytosol (265). TNFα alone did not have the same effect but could synergize with IFNγ to enhance bacterial killing. During primary infection, a major killing mechanism that activated macrophages use is the production of reactive nitrogen intermediates (266). However, during secondary infection, memory T cells are available to rapidly produce CCL3, and this induces phagocytes to produce increased levels of TNFα and reactive oxygen species, and reactive nitrogen intermediates are no longer required (109, 267). Antimicrobial peptides produced by epithelial cells or neutrophils can also contribute to phagosomal killing of *L. monocytogenes* in macrophages. Arnett et al. showed that extracellular α-defensins that were engulfed during phagocytosis blocked phagosomal escape and intracellular proliferation of *L. monocytogenes* in human macrophages (268). Macrophages are professional antigen-presenting cells (APCs) and, thus, are capable of processing and presenting peptide epitopes derived from *L. monocytogenes* proteins in both the MHC-I pathway (for bacteria that reach the host cell cytosol) and the MHC-II pathway (for bacteria killed within phagocytic vacuoles). However, as described below, DCs are thought to be the most efficient APCs involved in priming naive T cell responses.

Following i.v. infection, *L. monocytogenes* is filtered from the blood by specialized subsets of macrophages in the spleen and liver. Two types of macrophages in the marginal zone of the spleen initially engulf the bacteria: highly phagocytic CD209b^{+} marginal zone macrophages and CD169^{+} marginal metallophilic macrophages (186, 269, 270). Both populations of cells appear to restrict intracellular growth of *L. monocytogenes*, although this has not been studied extensively. Selective depletion of marginal zone macrophages and marginal metallophilic macrophages results in the rapid spread of bacteria into the red pulp of the spleen instead of containment within the marginal zone (186, 269). By

24 hours postinfection, *L. monocytogenes* typically moves to the T cell zone in the white pulp of the spleen (184). Perez et al. recently suggested that transfer of *L. monocytogenes* from infected $CD169^+$ macrophages to migratory DCs may facilitate this process (186). Outside of the marginal zone, most macrophages resemble monocyte-derived $CD64^+/F4/80^+$ cells, and it is likely that these cells represent the primary intracellular growth niche in the spleen. In the liver, tissue-resident Kupffer cells also capture *L. monocytogenes* circulating in blood. Kupffer cells are critical for limiting early replication of *L. monocytogenes* in the liver (91, 269), but the mechanism may not involve internalization of the bacteria. Gregory et al. suggested that *L. monocytogenes* did not invade Kupffer cells and that neutrophils recruited to the liver were responsible for killing extracellular adherent bacteria (271). More recently, Blériot demonstrated that Kupffer cells exposed to *L. monocytogenes* rapidly died by necrosis, triggering a Th1-type inflammatory response that recruited monocytes to the infected liver (272). There is also a population of $CX3CR1^{hi}Tim4^-$ macrophages in the liver that is distinct from Kupffer cells (273). These cells patrol the lining of the liver and can engulf any *L. monocytogenes* that may disseminate from the peritoneal cavity. Like other tissues, the gut also has specialized subsets of macrophages that reside either in the lamina propria or the muscularis externa as well as a population of $CX3CR1^{int}$ myeloid phagocytes referred to as MPs that seem to have functional plasticity (274). Interactions between these subsets of cells and *L. monocytogenes* following oral infection have not yet been extensively studied. In one recent report, Disson et al. argued that InlA-dependent invasion of the gut mucosa was immunologically "silent," while uptake via M cells triggered $CX3CR1^{int}$ myeloid phagocytes to produce IL-23 (154). If *L. monocytogenes* organisms disseminate to the brain, they encounter specialized resident macrophages known as microglia. Microglia support intracellular replication of *L. monocytogenes*, and infection induces high-level expression of TNFα, but other inflammatory responses are suppressed in the brain (275).

Antigen-laden DCs migrate to draining lymph nodes to activate T cell responses

cDCs derive from a dedicated precursor in the bone marrow and depend on both the transcription factor Batf3 and the growth factor Flt3 ligand for terminal differentiation (276). Monocytes can also differentiate into $CD11c^+$ cells that resemble cDCs, but there is still debate about the nomenclature of monocyte-derived cells. High expression of CD11c was long used as a defining surface marker for DCs, but we now know that CD11c can be expressed on many myeloid cell types, particularly those that reside outside of lymphoid tissue. Furthermore, the *in vitro*-cultured, bone marrow-derived $CD11c^{hi}$ cells commonly referred to as DCs in the published literature more closely resemble macrophages (277, 278). Both DCs and macrophages express the costimulatory molecules needed to prime naive T cell responses, and both cell types can process and present peptide antigens bound to either MHC-I or MHC-II, but DCs are thought to be the primary cell type that stimulates an initial T cell response. The key functional property that distinguishes DCs from macrophages is their superior ability to traffic to the areas where naive T cells reside in the white pulp of the spleen or the T cell zone of lymph nodes. The migratory nature of DCs allows for acquisition of antigens at sites of infection and transport to lymphoid tissue, but it may also promote dissemination of bacterial pathogens such as *L. monocytogenes*.

Waite et al. used multiphoton intravital imaging to examine the interactions of *L. monocytogenes* with cell types in the subcapsular sinus of the spleen (196). They found that within just minutes, *L. monocytogenes* colocalized with $CD11c^+$ cells that actively extended dendrites towards the bacteria. As early as 9 hours after infection, *L. monocytogenes*-associated DCs begin to move from the marginal zone of the spleen to the periarteriolar sheath (279). Verschoor et al. showed that C3-opsonized bacteria that had associated with platelets in the bloodstream were the microbes preferentially targeted by $CD8\alpha^+$ cDCs in the spleen (214). Association with cDCs appears to be a critical step in colonization of the spleen, since mice depleted of DCs have significantly lower bacterial burdens at 24, 48, and 72 hours postinfection (234, 280). Edelson et al. showed that *L. monocytogenes* failed to move to the white pulp of the spleen in Batf3-deficient mice that lacked $CD8\alpha^+CD103^+$ cDCs (234). This suggests that *L. monocytogenes* that remain in the marginal zone can readily be cleared by macrophages and that exponential growth of *L. monocytogenes* does not happen until cDCs transport the bacteria to the white pulp of the spleen. Once there, DCs form clusters with T cells at the border of the T and B cell zones in the spleen (281). Batf3-deficient mice also have decreased bacterial burdens in the liver following infection with either *L. monocytogenes* or *Listeria ivanovii* (234), but exactly how hepatic $CD103^+$ DCs promote infection in the liver is not yet clear.

Muraille et al. intravenously injected heat-killed *L. monocytogenes* into mice and found that the bacteria were rapidly filtered by the spleen but remained in the marginal zone and red pulp (282). This suggests that active invasion of DCs and cytosolic escape of the bacteria may be required for transport to the white pulp. Multiple studies have used flow cytometric approaches to attempt to identify *L. monocytogenes*-infected subsets of cells in the spleen directly *ex vivo* (278, 280, 282–285). Although the gating strategies used in each study varied somewhat, in the aggregate, this body of work provides evidence that rare DCs do contain live intracellular *L. monocytogenes*. However, when Jones et al. isolated $CD103^+$ and $CD103^-$ cDCs from the MLNs of naive mice and infected the cells directly *ex vivo*, they found no increases in gentamicin-resistant bacteria over time and, instead, observed that the number of live intracellular *L. monocytogenes* bacteria had decreased by 8 hours postinfection (278). Likewise, Westcott et al. found that *L. monocytogenes* did not efficiently escape the phagosome in bone marrow-derived $CD11c^{hi}$ cells (286, 287). Since we know that *L. monocytogenes* organisms do begin growing exponentially once they reach the white pulp of the spleen, there could be a mechanism whereby intracellular *L. monocytogenes* can exit from infected cDCs, or it is possible that only the adherent extracellular bacteria transported by DCs would be able to replicate in the white pulp.

cDCs are more efficient than macrophages at "cross-priming" T cells, a process in which dead or dying cells are phagocytosed and antigens contained within these cells are processed and presented to T cells (288). Thus, cDCs could prime T cells that recognize epitopes from either secreted or nonsecreted proteins expressed by *L. monocytogenes*, even if cDCs themselves are not productively infected with live replicating bacteria (289). All professional APCs, including DCs, can be activated by $CD4^+$ T helper cells through interactions between the costimulatory molecule CD40 expressed on the DC and CD40 ligand (CD40L or CD154) expressed on the T cell. However, in the case of *L. monocytogenes* infection, direct activation of cDCs by exposure to

bacterial microbial associated molecular patterns (MAMP) is sufficient, and $CD4^+$ T cell help is not required to stimulate cDCs to prime $CD8^+$ T cell responses (290). Peak costimulatory molecule expression on splenic dendritic cells occurred 24 to 48 hours after i.v. infection (285). Activated cDCs produce proinflammatory cytokines such as IL-12 and IL-18 (74, 149).

NK cells are a major source of early IFNγ but rapidly switch to production of IL-10

NK cells are protective during many viral infections, so it was a logical assumption that NK cells might also be protective during infection with an intracellular bacterial pathogen such as *L. monocytogenes*. Activated NK cells secrete IFNγ and can directly lyse infected cells if they do not sufficiently engage inhibitory receptors due to low MHC-I expression or if they express high levels of stress-induced host or microbial proteins (291). The p60 autolysin secreted by *L. monocytogenes* indirectly promotes activation of NK cells by a mechanism that involves contact with an infected DC and sensing of IL-18 independent of IL-12 secretion (74, 292, 293). Multiple studies have shown that NK cells are a major source of the early IFNγ produced during listeriosis (146, 182, 255, 294). Early work also envisioned a role for NK cells in the lysis of infected hepatocytes to release intracellular bacteria so they could be cleared by inflammatory phagocytes (295, 296). However, several studies that examined the effect of NK cell deficiency found either little difference in CFU burdens compared to NK-replete animals or significantly increased resistance to *L. monocytogenes* infection (255, 297–299). These results suggested that NK cell activation may actually be detrimental to the host during listeriosis.

Perona-Wright et al. clarified that NK cells have an important regulatory function during infection with *L. monocytogenes* or other pathogens that cause systemic disease (300). Using IL-10 reporter mice, they found that approximately 80% of the NK cells circulating in peripheral blood expressed IL-10 4 days postinfection. They argued that IL-10 production was triggered when a high enough serum concentration of IL-12 was achieved and suggested that this might represent a negative feedback mechanism to prevent extended inflammation and tissue damage during systemic infection. Clark et al. confirmed these findings and further showed that most NK cells stopped producing IFNγ fairly early during infection and began secreting IL-10 instead (255). Depletion of NK cells in IL-10-deficient mice had no effect on bacterial burdens, suggesting that NK-mediated susceptibility was due to secretion of this immune suppressive cytokine rather than a lack of immune stimulatory IFNγ. Mice lacking NK cells did have significantly lower serum levels of IFNγ 24 hours postinfection (255); however, there are a wide variety of other cell types that can be induced to secrete IFNγ during the first few days of infection (182). Early IFNγ levels vary between different strains of mice (181), and the balance of IFNγ produced by these other cell types compared to the level of IL-10 produced by NK cells is likely to have a key role in determining overall susceptibility to infection.

Lymphocytes can also provide protective function during first few days of infection

Lymphocytes mediate the adaptive immune response to infection and are generally thought to require specific antigen stimulation and several days of differentiation before they acquire effector function. One of the hallmarks of adaptive immunity is that lymphocytes can acquire memory and respond rapidly upon secondary exposure, allowing innate and adaptive immune responses to work synergistically within just a few days of infection. However, even in a naive host that has never encountered *L. monocytogenes* before, two different groups of lymphoid cells can respond rapidly in an antigen-independent manner and are therefore part of the innate immune response. The first group of cells are bona fide T lymphocytes that have acquired a memory phenotype without encountering the specific antigen recognized by their T cell receptor (TCR). The second group, termed innate lymphoid cells (ILCs), are tissue-resident cells found in both lymphoid and nonlymphoid organs that resemble lymphocytes but do not express a functional T cell or B cell receptor (301).

Memory phenotype or "virtual memory" $CD8^+$ T cells express high levels of surface markers such as CD44 and CD122 that define memory T cells, but they are found in naive or even germ-free animals that have no known prior exposure to specific antigen (302–304). Early studies showed that IL-12 and IL-18 produced in response to cytosolic invasion of *L. monocytogenes* activated these T cells to rapidly secrete IFNγ within 16 to 24 hours of infection (181, 294, 305–308). Adoptive transfer of virtual memory cells can provide significant protection against *L. monocytogenes* challenge, particularly for the subset of cells that express the chemokine receptor CXCR3 (304, 305, 309). Inbred mouse strains differ with respect to the number of virtual memory cells that can be detected, and the absolute number of virtual memory T cells present in the spleen correlates directly with innate resistance to systemic listeriosis (146, 181). These cells are not just found in mice; Bou Ghanem et al. showed that 85% of human blood donors had circulating memory phenotype T cells that rapidly produced IFNγ after incubation with *L. monocytogenes* (147). High levels of IFNγ would certainly promote clearance of infection by activating phagocytes to kill internalized *L. monocytogenes*. However, the sustained presence of IFNγ produced by innate T cells may also be important for stimulating the programmed death ligand-1 on naive T cells to promote their continued expansion following antigenic stimulation (310).

Another population of lymphocytes involved in innate immunity during *L. monocytogenes* infection is NKT cells, a small subset of T cells that share features of both NK cells and T cells. Most express a TCR that comprises an invariant Vα chain and diverse β chains that recognize several types of lipid antigens instead of peptide. Like memory phenotype T cells, NKT cells are a significant source of IFNγ during the first few days after *L. monocytogenes* infection (146, 294). It is important to note that these cells can express CD8, so they can be misidentified as classical $CD8^+$ T cells if NK1.1 is not used as an additional surface marker (148). NKT cells may be part of a complex feedback mechanism with dendritic cells. Lee et al. showed that TNFα activated NKT cells to secrete IFNγ, which caused $CD8\alpha^+$ cDCs to secrete IL-12 (149). IL-12, IL-15, and IL-18 have all been implicated in the activation of both NK cells and NKT cells (311).

ILCs have been classified into three groups (ILC1, ILC2, and ILC3); however, there is still considerable confusion regarding these cells because they seem to have functional plasticity (312). They are activated by exposure to MAMPs and to "danger" signals released from the cytosol of host cells (DAMPs) and they help to coordinate innate immune responses by secretion of specific cytokines. Disson et al. recently reported a role for ILCs in the intestinal lamina propria of mice orally infected with *L. monocytogenes* (154). They found that bacteria that translocated across specialized

M cells in the gut triggered ILC3 to secrete IL-17 and induced ILC2 to secrete IL-22, and that the dual presence of these two cytokines induced mesenchymal stromal cells to express IL-11. IL-11 had no apparent role in systemic listeriosis (156), but inclusion of this cytokine in the milieu of the small intestine induced an epithelial response that decreased accessible E-cadherin expression (154). These observations suggested that M cells and ILCs work together to provide a sentinel function that ultimately leads to a decrease in further invasion of *L. monocytogenes* in intestinal villi.

Mast cells

Mast cells are granulocytes loaded with secretory granules that are best known for their role in allergic responses. Other physiologic functions have also been attributed to mast cells, but these findings are somewhat controversial because the studies used either a transformed human cell line (HMC-1) or c-kit mutant mice with pleiotropic effects besides mast cell deficiency (313). Mast cells can be activated by a variety of stimuli, leading to degranulation and the local production of chemokines and cytokines that could be important for mobilization of PMNs during early listeriosis. In fact, c-kit specific-antibody-mediated depletion of mast cells resulted in increased bacterial burdens and significantly fewer PMNs infiltrating the spleens and livers of mice (314). Mast cells were also an apparent source of IL-6 *in vivo* (315) and may even be involved in direct antigen presentation to $CD8^+$ T cells (316). *In vitro* studies using HMC-1 cells suggest that *L. monocytogenes* can activate mast cells in an LLO-dependent manner (317). Although the bacteria did not appear to be readily internalized by HMC-1 cells, infection did cause a reactive oxygen species burst and a release of extracellular DNA similar to neutrophil extracellular traps (318). Together, these preliminary reports suggest that mast cells could play a role in innate immunity against *L. monocytogenes*, but further work will be needed to confirm this idea.

ADAPTIVE IMMUNITY

Innate immune responses alone can clear small doses of *L. monocytogenes* and can limit exponential growth in resistant hosts such as C57BL/6 mice. However, an efficient adaptive immune response is required to achieve sterilizing protective immunity. This is readily apparent in animal models such as SCID or RAG-deficient mice, where small differences in CFU burdens observed at early time points grow by orders of magnitude at 10 days postinfection, and the bacteria are never cleared (319, 320). Indeed, in humans, it is deficiencies in adaptive immunity that are associated with high mortality in immunocompromised populations such as neonates and the elderly (321, 322). The primary virulence strategy of *L. monocytogenes* during systemic infection is thought to be using actin-based motility to spread from cell-to-cell, avoiding the extracellular milieu, so it is intuitive that cytotoxic $CD8^+$ T cells are the crucial lymphocyte population needed for clearance (323, 324). As outlined below, $CD4^+$ T cells and B cells play supporting roles but are important for efficient generation of long-lasting memory cells. Because the i.v. model of listeriosis is highly reproducible, immunologists have long used this model system to study the basic biology of $CD8^+$ T cells, and this has been reviewed recently elsewhere (325, 326).

Activation of a naive T cell in the spleen or draining lymph node requires three signals that must be recognized in a specific sequence. A requirement for multiple signals ensures that T cells will not become activated in the absence of danger that indicates the presence of either microbes or tissue damage. Signal 1 occurs when an epitope-specific TCR engages with its cognate antigen (typically a peptide) that has been processed intracellularly and presented on the cell surface by MHC-I or MHC-II proteins. Antigen processing and presentation during *L. monocytogenes* infection are discussed further below. Signal 2, also referred to as "costimulation," involves the binding of stimulatory protein(s) on the surface of the T cell by ligand(s) typically expressed on professional APCs such as macrophages or dendritic cells. Signal 2 costimulatory molecules are typically upregulated only when professional APCs are activated, such as occurs after exposure to MAMPs. The classic signal 2 pairing involves CD28 on the T cell and B7 (also referred to as CD80/86) on the APC. Early studies showed that the immunodominant $CD8^+$ T cell response was markedly reduced in CD28-deficient BALB/c mice after either i.v. or oral *L. monocytogenes* infection (327). However, at least 15 other pairs of costimulatory molecules have now been described, and there are also inhibitory receptors that can counteract the effects of these signaling cascades (328). The third signal, which can alter the outcome of the T cell response, depends on the local concentration of type I IFN and IL-12 (329). The type and duration of both signal 2 and signal 3 can vary depending on the magnitude of the microbial burden and the local tissue microenvironment, and this can influence both T cell survival and differentiation into various subsets of T cells (Th1, Th2, Th17, etc.).

Antigen Processing and Presentation to T Cells

Classical MHC-I (MHC-Ia)

In all nucleated cells of the body, cytosolic proteins are cleaved into peptide-length fragments (8 to 11 amino acids) by the proteasome and transported into the endoplasmic reticulum, where they bind newly synthesized MHC-Ia proteins and are then translocated to the cell surface. MHC-Ia proteins are normally loaded with fragments of self-proteins; however, during intracellular infection, peptides derived from secreted microbial antigens are also displayed on the cell surface, and this serves as a signal to $CD8^+$ T cells that a foreign agent is contained within. *L. monocytogenes* can readily gain access to the cytosol of most cell types, and therefore, any proteins secreted by *L. monocytogenes* can potentially be processed and presented by the MHC-Ia machinery. BALB/c mice ($H\text{-}2^d$ haplotype) express three MHC-Ia proteins (K^d, D^d, and L^d), but the majority of epitopes recognized by $CD8^+$ T cells appear to be presented by the K^d molecule. A peptide derived from LLO ($LLO_{91\text{-}99}$) is by far the immunodominant epitope (330–332). Other subdominant epitopes that induce proliferation of K^d-restricted T cells during primary *L. monocytogenes* infection of BALB/c mice include $p60_{217\text{-}225}$ (333), $p60_{449\text{-}457}$ (334), $p60_{476\text{-}484}$ (335), and $mpl_{84\text{-}92}$ (336). Validation of immunodominant epitopes for C57BL/6 mice has lagged behind these studies, so recombinant *L. monocytogenes* expressing either viral antigens with defined $H\text{-}2^b$-restricted epitopes or the model antigen ovalbumin (SIINFEKL) are often used to study T cell dynamics in C57BL/6 mice. This approach was recently validated by Wolf and Princiotta, who showed that the efficiency of peptide-MHC complex formation in *L. monocytogenes*-infected cells was similar for both endogenously expressed and recombinant secreted antigens (337). The identification of so many different peptide epitopes makes listeriosis a particularly attractive model system to investigate how the abundance and stability of protein antigens produced during infection and the half-life of peptide-MHC complexes on

the surface of APCs contribute to immunodominance (332, 338–342).

Nonclassical MHC-I (MHC-Ib)

MHC-Ia-deficient mice were fully protected against *L. monocytogenes* infection despite the fact that most of the $CD8^+$ T cells in these animals were depleted in the thymus during T cell development (343). This suggested that a residual population of MHC-Ib-restricted $CD8^+$ T cells was sufficient to mediate full clearance of *L. monocytogenes*. Unlike classical MHC-I, MHC-Ib proteins often have lower cell surface expression and may be found only in some tissues (344, 345). Research in this area is limited, mainly because MHC-Ib genes are not well conserved between humans and mice. The best-characterized MHC-Ib molecule during listeriosis is M3, which binds to short N-formylated peptides derived from mitochondrial proteins in the steady state, or during infection, from the N-terminus of bacterial proteins that initiate with a formylated methionine (346). Three distinct peptides derived from *L. monocytogenes* proteins are known to be presented by M3 during infection: fMIVIL, fMIGWII, and fMIVTLF (340, 347–349). However, MHC-Ia-deficient mice infected with *fmt* mutant *L. monocytogenes* that did not stimulate M3-restricted T cells were still capable of generating protective immunity against secondary *Listeria* challenge (350). Likewise, Cho et al. generated MHC-Ia and M3 doubly deficient mice and showed that their $CD8^+$ T cells were also protective (351). These results argue that there are other MHC-Ib proteins that can activate naive $CD8^+$ T cells during listeriosis. One candidate is Qa-1, which has been shown to bind peptides derived from *L. monocytogenes* (352). Neither M3 nor Qa-1 have human counterparts, but humans have more diversity in subtypes of CD1 molecules than mice do (353). Thus, in both systems, there are multiple types of MHC-Ib restricted T cells that can respond to infection.

MHC-II

MHC-II proteins are expressed only by professional APCs such as macrophages, DCs, and B cells. They bind longer peptides (13 to 25 amino acids) derived from extracellular antigens that are processed in degradative vacuoles, and their cell surface levels typically increase during infection. The first MHC-II-binding epitopes derived from *L. monocytogenes* to be identified were $LLO_{215\text{-}226}$ in mice with an $H\text{-}2^k$ haplotype (354) and $P60_{301\text{-}312}$ in BALB/c ($H\text{-}2^d$) mice (355). A follow-up study specifically targeting these two protein antigens found six additional CD4 epitopes in BALB/c mice and five epitopes recognized by C57BL/6 ($H\text{-}2^b$) mice (335). In both mouse strains, peptides derived from LLO appeared to be immunodominant. It is intriguing that the immunodominant antigen produced by *L. monocytogenes* is a pore-forming toxin capable of causing cytotoxicity in APCs; Carrero et al. examined this relationship and found that LLO mutants with reduced toxicity still retained high antigenicity (356). Although most effort has focused on LLO and P60, a recent study revealed that there is considerably more diversity in the repertoire of CD4 epitopes presented by *L. monocytogenes*-infected cells. Graham et al. immunoprecipitated MHC-II from bone marrow-derived, *in vitro*-cultured $CD11c^+$ cells exposed to *L. monocytogenes* and analyzed the bound peptides by liquid chromatography with tandem mass spectrometry (LC-MS/MS) (357). They found 48 distinct peptides (most not previously described) that were derived from 21 proteins. Surprisingly, most of the *L. monocytogenes* antigens were either secreted proteins or cell wall proteins. This suggests that similar to MHC-Ia antigens, the accessibility of the antigen may be a critical factor in determining the likelihood that peptides will be displayed on the cell surface.

Magnitude and Kinetics of the Primary Response

$CD8^+$ T cells

Within 24 hours after i.v. inoculation, most *L. monocytogenes* bacteria have localized to the T cell zone of the spleen (184, 185, 281), and it is likely that most T cell priming occurs within this time frame. Indeed, if mice are treated with antibiotics at 24 hours postinfection to eliminate replicating *L. monocytogenes*, there is no difference in the kinetics of the $CD8^+$ T cell response (358). Although T cells expressing a variety of different TCRs are almost certainly activated at this stage, only those with a high affinity for their cognate peptide::MHC complex expand significantly (359). *Listeria*-specific $CD8^+$ T cells increase in size as they begin to proliferate, downregulate expression of L-selectin (CD62L), and upregulate expression of CD11a, PD-1, and CD69 (281, 360). Based on upregulation of CD11a on the cell surface, Rai et al. estimated that about 30% of the total $CD8^+$ T cells in the BALB/c spleen 7 days after a high-dose i.v. infection were *Listeria* specific (361). In CD11a-deficient mice, the magnitude of the primary $CD8^+$ T cell response was greatly reduced (362). T cell expansion was also impaired in aged C57BL/6 mice (18 months old) compared to younger animals (363), an observation that is clinically relevant given that the elderly are the patient population most at risk for developing life-threatening systemic listeriosis.

Although *Listeria*-specific $CD8^+$ T cells increase to detectable levels within 3 to 4 days, the peak of the MHC-Ia-restricted $CD8^+$ T cell response typically occurs 7 to 8 days after i.v. infection (360). In contrast, M3-restricted T cells peak much sooner, usually within 5 days of infection (364), probably because T cells that undergo positive selection in the thymus by recognizing M3 mature into naive cells that are partially activated (365). The timing of peak activity for M3-restricted T cells suggests that they have a transitional role that bridges innate and adaptive immunity. In fact, Xu et al. showed that M3-restricted T cells have a nonredundant function that could not be compensated for by classically restricted (MHC-Ia) $CD8^+$ T cells (366). Early cytokine secretion by M3-restricted T cells likely contributes to enhanced maturation of cDCs (367). Much less is known about the priming of $CD8^+$ T cell responses following oral transmission of *L. monocytogenes*. The bacteria presumably interact with APCs in either the Peyer's patches of the small intestine or in lymphoid follicles of the large intestine, and subsets of antigen-laden DCs should also migrate to the draining MLNs, but the timing of such events has not yet been determined. However, it is known that the peak number of antigen-specific CD8 T cells in peripheral blood occurs 8 to 9 days postinfection (368, 369). The extra 1 to 2 days required to see peak T cell expansion may be a consequence of the time required for *L. monocytogenes* to reach the MLNs in significant numbers (2). It should be noted that the kinetics of differentiation and the magnitude of expansion for *Listeria*-specific $CD8^+$ T cells is based primarily on work done in either BALB/c or C57BL/6 mice. Rai et al. showed that these parameters varied considerably in outbred mice, raising the possibility that humans might also show the same variation (361).

Extensive proliferation of $CD8^+$ T cells in response to specific antigens results in a large population of effector cells that can traffic throughout the body to promote clearance of *L. monocytogenes*. As the infection subsides, the majority of

those cells die, and if the inflammatory context is appropriate, the remaining cells develop long-lasting memory. This contraction phase occurs with preprogrammed kinetics, regardless of the duration of the infection (330). Porter et al. used an attenuated Δ*act*A strain, which has a much higher infectious dose than wild-type bacteria, to show that $CD8^+$ T cells begin undergoing apoptosis approximately 5 days after the peak antigen display (370). However, the host probably senses the level of inflammation, rather than the bacterial burden, to trigger initiation of the contraction phase. In the absence of significant IFNγ production, no $CD8^+$ T cell death was observed, and mice doubly deficient for TNFα and perforin had greatly reduced levels of T cell contraction (371). In contrast, B cell-deficient mice displayed greater death of $CD8^+$ T cells during the contraction phase (372). B cells are not involved in the activation of naive $CD8^+$ T cells or the expansion of effector cells but can influence the inflammatory milieu.

$CD4^+$ T cells

Helper T cell expansion and contraction during *L. monocytogenes* infection closely mirrors what has been observed for $CD8^+$ T cells. The peak of $CD4^+$ T cell proliferation in the spleen occurs at about the same time in mice at 6 to 8 days postinfection (360). Proliferation seems to proceed with preprogrammed kinetics regardless of the duration of infection or the amount of antigen displayed. However, the total number of $CD4^+$ effector cells with specific functional properties is influenced by the bacterial burden during the first few days of infection (373). Likewise, dysregulated contraction of $CD4^+$ T cells occurs in the absence of IFNγ production and signaling through the IFNγ receptor (373, 374). Paul Allen's laboratory has been using systemic listeriosis as a model system to study how interactions between TCR and peptide MHC-II complexes affect the $CD4^+$ T cell response using cells from two different transgenic mice that both recognize $LLO_{190\text{-}205}$ bound to $I\text{-}A^b$ (375). They showed that clonal T cells can vary in both their proliferative capacity and their half-life even though they recognize the same antigen (376).

Effector Functions of T Cells

Cytotoxicity

$CD8^+$ T cells (sometimes referred to as cytotoxic T lymphocytes) have cytotoxic potential that must be initiated by recognition of a specific MHC::peptide complex on a target cell (364, 377). One of three distinct pathways can then lead to the destruction of that *L. monocytogenes*-infected cell: Fas-FasL-mediated apoptosis (378), TNFα-dependent apoptosis (175), and granule exocytosis (379, 380). The granules of $CD8^+$ T cells contain the pore-forming toxin perforin as well as granzymes and serine proteases that can induce programmed cell death of the host cell. Perforin-deficient mice infected with *L. monocytogenes* generate more antigen-specific $CD8^+$ T cells than wild-type animals, demonstrating a separate role for perforin in regulating T cell expansion (381). At early time points during listeriosis, most of the responding antigen-specific $CD8^+$ cells have the ability to lyse target cells; however, at the peak of T cell expansion, only about 50% of the cells are cytotoxic (363). This may be a feedback mechanism to limit collateral damage to host tissues. $CD4^+$ T cells also have cytotoxic potential. Mice infected with *L. monocytogenes* induced $CD4^+$ T helper cells with high levels of FasL, and it was shown that some of these cells could directly kill B cells displaying the relevant peptide::MHC-II complex on the cell surface (382). However, cytotoxic $CD4^+$ T cells do not contribute significantly to clearance of *L. monocytogenes*, most likely because the bacteria can invade and replicate in many types of cells that do not express MHC-II (383). Lysis of an infected host cell should result in the release of any remaining live intracellular *L. monocytogenes*, which can then be engulfed by neutrophils or activated macrophages and subsequently killed. Direct intracellular killing of *L. monocytogenes* may also be possible in dying cells that have not yet lysed. The lytic granules of human (but not rodent) $CD8^+$ T cells contain granulysin, a pore-forming protein that preferentially targets microbial membranes (384). A recent report by Walch et al. suggests that granulysin can insert into *L. monocytogenes* membranes and cause death of intracellular bacteria within 90 minutes (385).

Secretion of Th1-type cytokines

A primary function for both $CD4^+$ and $CD8^+$ T cells is the secretion of the proinflammatory cytokines IFNγ and TNFα. Indeed, detection of these cytokines by enzyme-linked immunosorbent spot assay or intracellular cytokine staining is often used as a proxy to identify antigen-specific T cells within a bulk population of cells that have been infected or exposed to peptide epitopes. Effector $CD8^+$ T cells can express IFNγ within just a few hours of exposure to antigen, even when the number of peptide::MHC-I complexes on target cells is limited (386). In contrast, the number of $CD8^+$ T cells secreting TNFα is typically lower, unless the target peptide epitope is present at a high concentration. Both IL-12 and type I IFN are thought to be signal 3 molecules that can drive naive T cells toward a Th1 phenotype. Way et al. showed that neither molecule was required for IFNγ production by $CD8^+$ T cells (387). However, defects in either IL-12 or IFNαβ resulted in fewer IFNγ-producing $CD4^+$ T cells, and the loss of both cytokines resulted in an almost complete lack of IFNγ-producing T helper cells. Eicosanoids may also need to be present for full activation of $CD8^+$ T cells. The Sauer lab recently showed that treatment of mice with COX-2 inhibitors such as indomethacin or celecoxib resulted in decreased IFNγ production by CD8 T cells (388). After oral infection, LLO-specific $CD4^+$ T cells were detected in both lymphoid tissues (spleen and MLN) as well as in the lamina propria of the small intestine. In both compartments, the cells were predominantly Th1-type secreting IFNγ, TNFα, and IL-2 (389).

Antigen-independent bystander killing

Although cytolysis, secretion of IFNγ, and secretion of TNFα αre thought of as the major protective functions of $CD8^+$ T cells, none of these mechanisms are absolutely required for resistance to *L. monocytogenes* infection (175, 178, 381). This may be explained by the fact that both primary effector cells and memory $CD8^+$ T cells can also secrete CCL3 (107, 109). CCL3 is a chemokine that can activate monocytes, macrophages, or dendritic cells to secrete TNFα, which then upregulates reactive oxygen species production in all myeloid-derived cells, including neutrophils (109). Thus, $CD8^+$ T cells can also indirectly promote killing of bystander bacteria that are engulfed by activated phagocytes using an antigen-independent mechanism.

Secretion of Th17-type cytokines

Activated $CD4^+$ T cells that produce the proinflammatory cytokine IL-17A (Th17-type cells) could promote bacterial clearance by inducing neutrophil migration to sites of infection. Th17 cells have well-defined roles in protection

against infection with extracellular bacteria and fungi, but their role during infection with a facultative intracellular pathogen like *L. monocytogenes* is not yet clear. Pepper et al. showed that infection via a mucosal route (intranasal), but not i.v. infection, generated IL-17A-producing cells that comprised about 10% of the *Listeria*-specific $CD4^+$ T cells in the spleen (390). Uchiyama et al. also detected a population of $CD4^+$ T cells that produced both IFNγ and IL-17 (Th1/Th17 cells) in the spleens of i.v.-infected mice (391). A strong IL-1β response was required to see an expansion of this subset of cells. The major source of IL-17A during i.v. infection seems to be TCRγδ T cells in the liver (392, 393). These unconventional T cells may play a role in promoting cross-presentation of antigen by cDCs (393). However, multiple studies have shown that mice lacking γδ T cells have a somewhat prolonged infection, but the animals do clear the bacterial burden (394–396); thus, γδ T cells are not absolutely required for protection. The best evidence to date to suggest that IL-17A-producing T cells can contribute to clearance of *L. monocytogenes* resulted from the infection of p35/EBI3 doubly deficient mice, which lack the ability to produce any of the IL-12 family of cytokines (IL-12, IL-23, IL-27 and IL-35). These mice produced significantly higher levels of IL-17 and IL-22 than did wild-type mice, and antibody treatment to deplete the IL-17 resulted in increased bacterial burdens (150). Furthermore, adoptive transfer of LLO-specific Th17 cells greatly reduced *L. monocytogenes* growth in both the spleen and liver. A subset $CD8^+$ T cells can be driven to produce IL-17 if they are primed in the absence of IL-12 and type I IFN (153). This effect appears to be dependent on the presence of IL-23 and resulted in a loss of cytotoxic potential.

Regulatory T cells

A subpopulation of activated $CD4^+$ T cells that express high levels of Foxp3 regulate immunity by inhibiting T cell function. These regulatory T cells (T_{reg}) are critical for avoiding autoimmunity because they actively suppress immune responses directed against self-antigens, and they are often expanded during chronic infections (397). Ertelt et al. showed that as the percentage of antigen-specific $Foxp3^{neg}$ cells expanded following *L. monocytogenes* infection, the T_{reg} population had a corresponding decrease, because the cells did not proliferate (398). Another group suggested that competition for the growth factor IL-2 could explain why regulatory T cells did not expand and could even undergo a partial loss during listeriosis (399). Treating mice with exogenous IL-2 allowed for T_{reg} proliferation but resulted in a corresponding increase in bacterial burdens. Thus, in most situations, T_{reg} activation is detrimental to the host defense against bacterial infections. A notable exception occurs during pregnancy, when regulatory T cells present at the maternal-fetal interface are critical for maintenance of a suppressive environment so that the maternal immune system does not reject the fetus. Sing Sing Way's group has shown that fetal wastage can occur even if *L. monocytogenes* does not directly invade the placenta if the maternal Th1 response to systemic infection is robust enough to disrupt $Foxp3^+$ T cell-mediated tolerance (400).

Memory T Cell Responses During Secondary Infection

Formation of memory T cells

During the primary response to infection, a subset of effector $CD8^+$ T cells differentiates into memory precursor effector cells that express high levels of CD127 and low levels of KLRG1 (401). Further differentiation of memory precursor effector cells results in the generation of long-lasting memory T cells with epigenetic modifications that underlie their enhanced capacities during recall responses (402). The environmental cues that regulate this process have been explored using many model systems, including *L. monocytogenes* infection. The results of several studies suggest that IL-12 is a critical factor needed for efficient memory cell formation and that IL-23 may be able to partially compensate in some contexts (401, 403, 404). IL-2, a cytokine primarily known as a growth factor to promote T cell proliferation, is also required during differentiation to generate memory cells capable of full expansion during secondary challenge (405). Surprisingly, there may also be a role for IL-10 production in memory formation. Foulds et al. showed that IL-10-deficient mice had fewer memory $CD8^+$ T cells and that adoptive transfer of these T cells provided less protection against *L. monocytogenes* than memory T cells from wild-type mice (193). IL-10 involvement was not related to bacterial burden, because similar results were obtained for memory T cells generated in mice treated with antibiotics 24 hours after infection. Upregulation of the integrin chain CD11a typically occurs during the differentiation of primary effector cells, but this seems to hamper the formation of memory cells. Bose et al. showed that mice lacking CD11a had significantly greater numbers of *Listeria*-specific memory T cells and a more robust recall response than wild-type animals (362). $CD4^+$ T cell help is not needed for the generation of memory $CD8^+$ T cells, but the absence of Th1-type helper cells does affect the stable persistence of the memory pool (406). Very little is known about the formation of memory $CD4^+$ T cells, due to the lack of specific surface markers that can be used to define this cell population.

Recall responses involve faster expansion and higher magnitude

Upon secondary challenge with *L. monocytogenes*, memory T cells recognize their cognate peptide::MHC complex and begin to control the infection within just 6 hours (185). T cell expansion generally occurs more rapidly, with the total number of effector cells being substantially higher than during the primary infection. Since memory T cells develop during the contraction phase of the first infection, the magnitude of the primary T cell response directly affects the potential size of the memory T cell pool. For MHC-Ia restricted memory $CD8^+$ T cells, the peak of proliferation occurs 3 to 5 days postinfection (360, 407). M3-restricted $CD8^+$ T cells do not have a characteristic recall response, so peak expansion also occurs at 3 to 5 days postinfection, just as it did during the primary response (408). Cho et al. showed that the residual population of $CD8^+$ T cells present in MHC-Ia and M3 doubly deficient mice also did not display a classic memory response during secondary challenge (351). Thus, if there are other *Listeria*-specific memory $CD8^+$ T cells restricted by novel MHC-Ib molecules, they would contribute to bacterial clearance with the same kinetics as M3-restricted T cells.

Much less is known about $CD4^+$ T cell recall responses during listeriosis. One study that tracked $LLO_{190\text{-}201}$-specific $CD4^+$ T cells during secondary infection found that the peak expansion was similar to the primary response, and then the antigen-specific T cells continuously declined in number over time (409). The magnitude of the $CD4^+$ T cell recall response is thought to be dependent on the duration of the secondary infection and may be influenced by both the inflammatory context and the availability of peptide antigen

(410, 411). It is possible that substantial recognition of peptide antigens by memory $CD8^+$ T cells (which can occur in any nucleated cell that is infected by *L. monocytogenes*) results in rapid bacterial clearance, limiting the availability of antigens that can be processed by professional APCs that express MHC-II.

Effector functions of memory T cells

The key effector mechanisms used by memory T cells are similar to those used by T cells during the primary infection. For memory $CD8^+$ T cells, IFNγ was shown to be critical for activating infiltrating monocyte-derived phagocytes using either i.v. or oral models of infection (412). In contrast, memory $CD4^+$ T cells needed to secrete TNFα, and IFNγ could enhance protection but was not required for clearance of secondary *L. monocytogenes* infection (413). Olson et al. surprisingly showed that a subset of cells displaying markers of effector $CD8^+$ T cells ($CD27^{lo}KLRG1^{hi}$) persisted in the spleens of mice that had cleared a primary *L. monocytogenes* infection. The cytotoxic potential of these cells significantly contributed to optimal control of a secondary *L. monocytogenes* challenge (414). This result underscores the need to define memory $CD8^+$ T cells by expression of differentiation markers and not just function potential. Oral infection (but not i.v. infection) also stimulated multifunctional memory γδ T cells and a tissue-resident population of $CD8^+$ T cells that provided enhanced bacterial clearance in the gut (369, 415).

Even if the ideal conditions are present to allow for efficient development of memory T cells that stably persist, their recall effector function will still be influenced by the inflammatory environment that is present during secondary infection. The Williams lab has been studying the activation of transgenic lymphocytic choriomeningitis virus-specific $CD4^+$ T cells and has shown that boosting by infecting with recombinant *L. monocytogenes* expressing the same lymphocytic choriomeningitis virus epitope results in the induction of increased numbers of memory $CD4^+$ T cells with greater avidity (416). These alterations in memory cell function appeared to be dependent on the local concentration of IL-12 and type I IFN available during the secondary exposure. They further showed that memory $CD4^+$ T cells induced by heterologous *L. monocytogenes* infection provided partial protection even if IFNγ was depleted but showed no protective capacity when TNFα was neutralized (413). Since heterologous boosting is a common immunization strategy, these results could have a significant impact on vaccine design.

Humoral Immunity

Antibodies may have an underappreciated role in the clearance of *L. monocytogenes*. The idea that humoral immunity could be important for protection against facultative intracellular pathogens was championed by Arturo Casadevall 20 years ago (417), but antibody responses are still understudied in listeriosis. The lack of research in this area is probably due to early work showing that passive transfer of sera from immune mice did not transfer resistance to naive animals (418, 419). Indeed, mice that have been intravenously injected with wild-type *L. monocytogenes* do not have a robust antibody response within 2 to 3 weeks of infection (420–422). However, pretreatment of mice with specific antibodies raised against defined *L. monocytogenes* antigens can alter the course of infection. For example, Edelson et al. showed that administration of anti-LLO monoclonal antibodies that neutralized pore formation *in vitro* could reduce bacterial burdens in the spleen and liver by 1,000-fold (420). More recently, Mohamed et al. found that treatment with monoclonal antibodies specific for ferritin-like proteins (Frl) also protected mice from lethal challenge (423). In contrast, mice preimmunized with purified p60 protein mounted a specific antibody response but showed no reduction in bacterial burdens (424). Thus, the paradigm for the mouse model is that strong antibody responses are not naturally induced during infection and antibodies are not required for clearance; however, if certain antibodies are present, they can play a role in limiting bacterial growth.

It is not yet clear why antibody production is diminished during systemic infection of mice. Extracellular *L. monocytogenes* is readily detected in the intestines, spleen, liver, and gall bladder of infected mice (2, 19, 425), so exposure to bacterial protein antigens and subsequent recognition by B cells should not be an issue. However, it is possible that intracellular growth and cell-to-cell-spread of wild-type *L. monocytogenes* limits the ability of mice to mount a productive antibody response. Infection with attenuated Δ*actA* or Δ*hly* mutants induced higher levels of serum antibody than infection with wild-type bacteria (421, 422). Manohar et al. also showed that oral transmission of Δ*actA L. monocytogenes* generated antigen-specific IgA production that could be readily detected in the Peyer's patches and MLNs starting at 3 weeks postinfection (426). However, this phenotype may be unique to the mouse model since Yu et al. readily found both InlA- and InlC2-specific antibodies in the sera of rabbits, guinea pigs, cattle, and sheep that were experimentally infected with a wild-type serotype 4b strain (427).

Humans with confirmed diagnoses of listeriosis also have circulating *Listeria*-specific antibodies. Gentschev et al. showed by immunoblot analysis that the majority of patient sera samples reacted with several secreted *Listeria* proteins (with p60- and LLO-specific antibodies being the most commonly detected), but sera from otherwise healthy donors did not (424). In a larger study using complement fixation as the readout, Leong et al. found that 12 of 74 healthy blood donors (16%) had antibodies that recognized whole bacteria (422). The results of these studies are consistent with the notion that humans frequently ingest small quantities of *L. monocytogenes*, but only a subset of the population is exposed to a high enough dose to induce circulating IgG antibodies.

USE OF ATTENUATED *LISTERIA* AS A VACCINE VECTOR

Generating an effective immunization strategy that can induce cellular immunity, particularly $CD8^+$ T cell responses, remains one of the final frontiers in vaccine research. A critical challenge is to find a safe and effective means to deliver immunogenic proteins or peptides to the cytosol of APCs. Immunization strategies that use nonreplicating vectors typically require an adjuvant and do not efficiently transport antigen to the MHC-I processing pathway. Engineered viruses are great delivery systems but raise safety concerns for use in humans, especially due to the lack of therapeutics that can effectively disable a viral infection. An intracellular bacterium can overcome these issues by naturally gaining access to the class I MHC processing pathway and by being treatable with antibiotics to ensure that there are no adverse outcomes in immunized patients. Immune responses to *Listeria*, *Salmonella*, and *Mycobacteria* have all been extensively studied in mouse models, but of these intracellular pathogens, only *Listeria* consistently induces robust protective immunity. Thus, it is not surprising that investigators have sought to harness the remarkable immune stimulatory properties of *L. monocytogenes* to develop vaccine vectors.

Attenuated *L. monocytogenes* Is Still Highly Immunogenic

Early work focused on identifying mutations that attenuated the pathogenicity of *L. monocytogenes* without losing immunogenicity, with the goal of developing a vaccine strain that could be safely administered to humans. Virulence factors required to gain access to the host cell cytosol (e.g., LLO) could not be altered, because recombinant antigens expressed by a vaccine strain need to be delivered directly to the class I MHC processing pathway. Instead, investigators targeted other virulence factors that promote cell-to-cell spread and persistence of infection. For example, Δ*actA* *L. monocytogenes* bacteria are significantly less virulent than wild-type bacteria, but they do still replicate and survive in mice for up to a week (428). Since the 50% lethal dose of a ΔActA strain is 1,000 times higher than that of wild-type *L. monocytogenes*, immunizing doses can be very large, and this results in substantially greater antigen display and peak T cell responses that occur at least 1 day sooner (370). Thompson et al. demonstrated that further attenuation of *L. monocytogenes* could be achieved while still inducing protective $CD8^+$ T cell responses (429). Their approach was to generate a triple mutant strain (Δ*dal* Δ*dat* Δ*actA*; referred to as "LmddA") that was capable of invading host cells, escaping to the cytosol, and surviving for a short time. The LmddA strain cannot spread from cell to cell due to the lack of ActA, and the bacteria do not replicate in the host because they lack two genes needed for D-alanine biosynthesis, an amino acid that is present in only trace amounts in vertebrates. Although LmddA bacteria were rapidly cleared from the spleens of mice within 48 hours, the infection was sufficient to generate protective immunity against a subsequent lethal challenge (429). Brockstedt et al. developed a Δ*inlB*Δ*actA* vaccine strain (live attenuated double-deleted [LADD]) that selectively infects only phagocytes, thus promoting antigen presentation to T cells while limiting replication in cells such as hepatocytes (430). Phase I safety trials for these attenuated strains administered either intravenously or orally showed no adverse effects in human patients and no prolonged shedding of *L. monocytogenes* in feces (431–434). Given the widespread prevalence of *L. monocytogenes* in the environment and the likelihood that humans may have prior exposure, one concern was that vaccine strains might be cleared too quickly to generate T cell responses against heterologous antigens. However, preexisting immunity to *L. monocytogenes* did not diminish the effectiveness of vaccine strains in either mice or nonhuman primates (422, 435, 436).

Fusing Antigens to Either LLO or ActA Increases Vaccine Efficacy

L. monocytogenes is genetically tractable and has been engineered to express a variety of heterologous proteins, including antigens derived from other pathogens as well as tumor-associated antigens. In many of these studies, known antigens were fused to secreted *L. monocytogenes* proteins to facilitate expression *in vivo*. Although this strategy undoubtedly enhances the ability of a heterologous antigen to be expressed in *L. monocytogenes*, there may be other reasons that fusion proteins increase the protective effect of immunization (337, 437). For example, LLO has an N-terminal sequence that targets the protein for rapid degradation by the proteasome, and vaccine strains that lack this sequence have diminished T cell responses (438). LLO itself can be recognized by PRRs, which may explain its enhanced adjuvant properties as a fusion partner (438, 439). Chen et al. recently showed that immunization with an E7-LLO fusion protein (E7 is an oncoprotein expressed by human papillomavirus) also had the effect of preferentially expanding $CD4^+Foxp3^-$ cells, resulting in a proportional decrease in T_{regs} (440). Since T_{reg} depletion is known to enhance antitumor immunity, this may be another mechanism to explain the superior ability of LLO fusion proteins to elicit a protective effect in cancer models. Antigens fused to ActA also have enhanced protective capacity, although the mechanisms involved are not as well understood (441, 442).

L. monocytogenes Vaccine Strains Could Be Used To Prevent Infectious Disease

In several animal models, infection with recombinant *L. monocytogenes* expressing heterologous microbial antigens resulted in protection against subsequent challenge with the pathogen of interest (443–447). These preliminary studies utilizing antigens derived from *Francisella tularensis*, *Mycobacterium tuberculosis*, and human immunodeficiency virus suggest that attenuated *Listeria* vectors could be used to develop preventative vaccines for either viral or intracellular bacterial pathogens. However, no successful human trials have yet been performed. Johnson et al. orally immunized 22 human volunteers with either Δ*actA*/Δ*plcB* or Δ*actA*/Δ*inlB* attenuated strains expressing influenza A nucleoprotein (432). Although the vaccine generated anti-*Listeria* responses in all of the volunteers, none had an increase in influenza nucleoprotein-specific T cells. The secreted nucleoprotein antigen in that vaccine was not fused to either LLO or ActA.

Infants are exquisitely susceptible to developing life-threatening systemic infections due to the immaturity of their immune systems. However, several vaccines have been developed that can be safely and effectively administered at birth or shortly thereafter, and the Kollmann lab has argued that *L. monocytogenes* vaccines may be ideal for this purpose (448). Five-day-old mice immunized with Δ*actA* *L. monocytogenes* were protected against secondary lethal challenge 3 months later (449). Interestingly, immunization of neonatal mice achieved a maximal $CD8^+$ T cell response after just a single dose, while effective vaccination of adult mice required a booster dose (450). However, optimal stimulation of $CD4^+$ T cell responses required a booster immunization in both neonates and adults. Given the high rate of mortality for systemic listeriosis in neonates, safety remains a significant hurdle to overcome before anti-infective *Listeria* vaccines reach the clinical development stage. One intriguing possibility for vaccine development could be the use of killed but metabolically active bacteria. *L. monocytogenes* deficient in DNA repair can be rendered incompetent to replicate by UV irradiation, and the bacteria remain able to invade the host cell cytosol and to secrete antigens for several hours (451). If the bacteria have a constitutively active PrfA transcriptional regulator to enhance virulence gene expression during these few hours, then $CD8^+$ T cell responses can readily be activated (452). This could be a promising approach for developing vaccines that could be safely administered to neonates.

Multiple Clinical Trials Using *L. monocytogenes* for Cancer Immunotherapy Are Under Way

The most exciting application of *Listeria* vaccine strains is for therapeutic use in the treatment of several types of cancer. Although surgery, chemotherapy, and radiation remain the primary treatments to eliminate tumor burdens, immunotherapies designed to boost a patient's natural immune

response against tumor antigens are now in widespread clinical use. Tumor cells express altered self-antigens that can be processed by the MHC-I pathway and presented to $CD8^+$ T cells, similar to what occurs during infection with intracellular pathogens. Cancer vaccines that elicit memory $CD8^+$ T cells may benefit patients by promoting enhanced recall responses that can quickly eliminate tumor reoccurrences and possibly prevent metastasis. *L. monocytogenes* is a particularly attractive delivery system for tumor antigens because even the attenuated strains elicit such a robust innate immune response that the infection can overcome central tolerance to tumor-associated antigens that are viewed as "self" by the immune system (453). The earliest preclinical studies in mice focused on viral antigens (E6/E7) derived from human papilloma virus (HPV) and demonstrated that immunization with *Listeria* vaccines led to regression of established HPV-16 transformed tumors (437). Importantly, the E7-expressing *L. monocytogenes* strain was able to induce a protective T cell response even in transgenic mice engineered to express the E6 and E7 oncogenes in the thymus, indicating that the vaccine strategy was able to break tolerance to an endogenously expressed protein (454). In the years since then, *L. monocytogenes* has been engineered to stimulate T cells specific for many other tumor-associated antigens, and vaccination with those strains resulted in regression of tumors in small animal models of melanoma (455), hepatocellular carcinoma (456), and prostate (457), pancreatic (458), and breast (459) cancer.

These encouraging results in mouse models have led to the initiation of more than 30 clinical trials testing multiple *L. monocytogenes* cancer vaccines. About half of the trials involve LmddA variants developed by Advaxis, Inc. (Princeton, NJ), and the rest are testing LADD-based strains developed by Aduro BioTech, Inc. (Berkeley, CA). The vaccine that has progressed the farthest to date is ADXS11-001, an LmddA strain expressing the HPV E7 oncogene fused to truncated LLO. The vaccine is currently being tested in conjunction with chemoradiation in a phase III trial with advanced-stage cervical cancer patients (clinicaltrials.gov identifier NCT02853604). The success of this vaccine has led to additional phase I and II trials to test for efficacy in patients with oropharyngeal and anal cancers (460). Another promising LmddA vaccine (ADX S31-164) was engineered to secrete LLO fused to domains of Her2/neu, an epidermal growth factor receptor family member that is overexpressed in many breast cancers with a poor prognosis. Preclinical trials with this strain shifted the ratio of $CD8^+$ T cells and immune suppressive T_{regs} inside tumors (459) and a phase I study in dogs reported an increase in median survival (461). In 2018, the U.S. Department of Agriculture granted approval for use of this vaccine in the treatment of canine osteosarcoma, making it the only *L. monocytogenes* vaccine currently approved for clinical use. The most widely tested LADD-based vaccine (CRS-207) targets mesothelin, a normal host protein that is overexpressed in multiple tumors, including pancreatic, ovarian, and lung cancer. The vaccine has been tested in phase II trials in conjunction with GVAX, a treatment consisting of irradiated pancreatic tumor cells engineered to secrete granulocyte-macrophage colony-stimulating factor (462), and trials are under way to supplement these treatments with immune checkpoint inhibitors. Another promising development from Aduro BioTech is the recent approval (clinicaltrials.gov identifier NCT03189030) to test LADD-based strains that express personalized neoantigens (mutated self-proteins expressed only in tumor cells). If this vaccine proves to be efficacious, it could lead to increased use of personalized medicine in the treatment of colorectal cancer. For more detail about each of the 10 *L. monocytogenes* cancer vaccines that have progressed to either phase I, phase II, or phase III clinical trials over the past decade, please see the comprehensive reviews recently published by Flickinger et al. (463) and Wood and Paterson (453).

CONCLUDING REMARKS

The large body of work highlighted in this article along with the extensive published studies of pathogenic mechanisms encoded by *L. monocytogenes* reviewed elsewhere combine to give us what is perhaps the most complete understanding of a host-pathogen interaction, yet there is still much to learn. One relatively unexplored frontier in the field is the effect of aging on protective immunity (464). The elderly are the patient population most at risk for developing life-threatening systemic listeriosis, yet few studies to date have examined how innate or adaptive immune responses to *L. monocytogenes* differ in aged mice compared to younger animals. Another area of emphasis for the future will be to define the mucosal immune responses that occur in the gut during natural infections acquired by the oral route. Because of the extensive existing knowledge base regarding innate and adaptive immune responses to *L. monocytogenes*, murine infection will likely continue as the favored model system to study how mucosal immunity deals with microbial pathogens that are orally acquired and then disseminate systemically, a category that includes many bacteria and viruses.

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Regulation of *Listeria monocytogenes* Virulence

JÖRGEN JOHANSSON AND NANCY E. FREITAG

52

A VARIETY OF MULTIFUNCTIONAL GENE PRODUCTS CONTRIBUTE TO *LISTERIA MONOCYTOGENES* ENTRY AND SURVIVAL WITHIN MAMMALIAN CELLS

The move from soil to cytosol requires the interplay of *L. monocytogenes* factors that promote survival in the gut, bacterial invasion, phagosomal escape, replication and movement within the cytosol, and spread to adjacent cells (Fig. 1). Bacterial gene products contributing to many key aspects of host infection continue to be identified, and new factors and novel functions often emerge (1, 2). Among the cast of well-known players are surface proteins that promote bacterial attachment to and invasion of nonprofessional phagocytic cells, such as the internalins InlA and InlB as well as Lap and InlP (which appears to be specific for placental invasion) (3–5). Following cell entry, *L. monocytogenes* escapes from host cell vacuoles via the secretion of the pore-forming cytolysin listeriolysin O (LLO) and two phospholipases, a phosphatidylinositol-specific phospholipase C (PI-PLC) encoded by *plcA* and a broad-range phospholipase C (PC-PLC) encoded by *plcB* (6). Entry into the cytosol requires metabolic adaptation as *L. monocytogenes* shifts from glycolysis to the oxidative pentose phosphate pathway and replicates by scavenging phosphorylated sugars, glycerol, lipoic acid, branched-chain amino acids, and peptides from conquered host cells (7). Bacteria spread to neighboring cells by usurping actin polymerization as a motile force, a process dependent upon expression of the bacterial surface protein ActA (8). The breaking and entering of *L. monocytogenes* into adjacent cells is further facilitated by InlC, which relieves cortical tension to allow the extension of membrane protrusions (9). Escape from the double membrane vacuoles formed as a result of *L. monocytogenes* cell-to-cell spread is once again dependent upon the activities of LLO, PC-PLC, and PI-PLC. Protein secretion of a variety of factors thus promotes bacterial survival and life within host cells, and central to the secretion of active protein products is the presence of the secretion chaperone PrsA2, located at the bacterial membrane-cell wall interface, where it contributes to the folding and activity of translocated polypeptide chains (10). *L. monocytogenes* is thereby able to maintain a complex and multifunctional protein arsenal to increase bacterial survival and replication within mammalian host cells.

To better understand bacterial adaptation to the host environment, a number of studies have focused on the identification of bacterial genes expressed within tissue culture cells, within blood, or within infected animals. Microarray analyses of bacterial transcripts induced during *L. monocytogenes* infection of tissue culture cells revealed that approximately 20% of bacterial genes were differentially expressed, including genes with products that have established roles in bacterial virulence (11). Genes with increased expression in cytosolic bacteria included those with roles in general stress responses, cell division, modification of the cell wall, and the use of carbon sources such as glycerol and phosphorylated sugars. Transcriptional profiling of *L. monocytogenes* genes expressed during *in vivo* growth in mouse spleens also indicated that approximately 20% of bacterial genes were differentially expressed (12). Similar to the findings reported for bacteria grown within tissue culture cells, genes induced *in vivo* included those with defined roles in virulence, stress responses, cell wall metabolism, DNA metabolism, RNA/protein synthesis, and cell division. In contrast to tissue culture-based expression studies, transcripts from genes encoding enzymes involved in glycolysis were induced *in vivo*, while those involved in the nonoxidative phase of the pentose phosphate pathway had decreased levels of expression. These contrasting results may reflect differences observed between growth conditions within tissue culture cells versus growth in whole organs and animal tissues.

PrfA, THE MASTER REGULATOR OF *L. MONOCYTOGENES* INTRACELLULAR SURVIVAL

While life is never simple, a transcriptional activator known as PrfA does appear to suffice as the master virulence regulatory protein that enables *L. monocytogenes* to optimize life within a mammalian host while still managing a saprophytic existence in soil. PrfA is a 27-kDa transcriptional activator that is a member of the Crp/Fnr family of transcriptional regulators, and it regulates its target genes via the binding of a 14-bp palindromic DNA binding site, also known as the PrfA box, located in the –40 region of its target promoters (13–17). PrfA regulates the expression of a large number of gene products directly associated with bacterial virulence in mammals, and mutants lacking *prfA* are severely impaired for intracellular growth and are >100,000-fold less virulent in murine infection models (15). In addition to gene products required for host cell invasion, intracellular replication, and cell-to-cell spread, PrfA induces the expression of gene products required for surviving the journey through the

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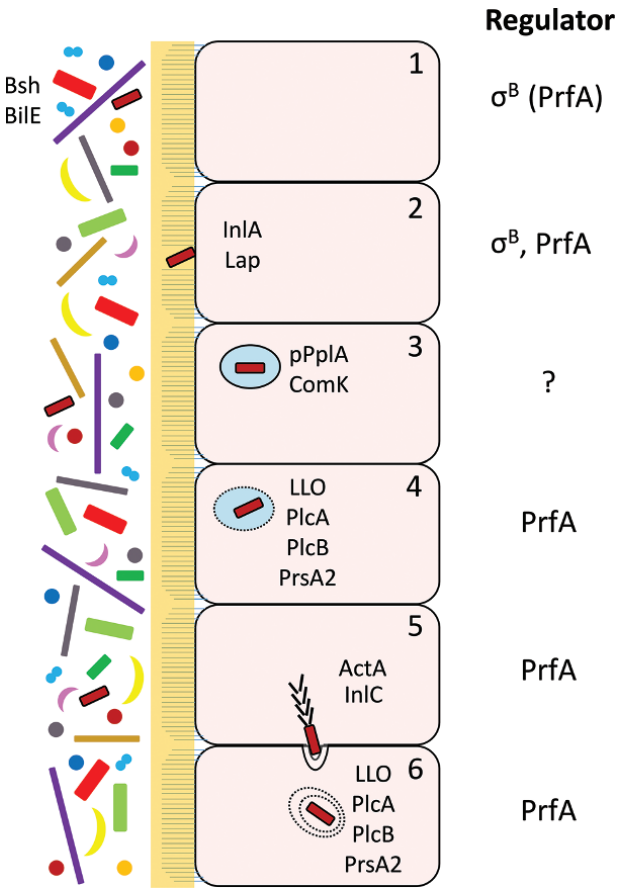


FIGURE 1 Schematic overview of *L. monocytogenes* colonization of the intestine by pathways controlled by σ^B and/or PrfA. The human microbiota is depicted to the left, lying beside the microvilli-containing epithelial cells (1). Survival of *L. monocytogenes* in the gut is mediated by different factors, among them Bsh and BilE (2). The bacterium adheres to cells using different adhesins (e.g., InlA and Lap) (3). Once inside the phagosome, the pPplA and ComK systems become activated, facilitating phagosomal escape (4). Different PrfA-regulated factors mediate lysis of the phagosome (5). Through recruitment of the Arp2/3 complex, ActA allows polymerization of actin at the pole of the bacterium, driving it through the cytoplasm. The entry of *L. monocytogenes* to adjacent cells requires InlC, which relieves cortical tension (6). The escape from the double membrane vacuole requires the action of LLO, PlcA, and PlcB. Regulators (σ^B or PrfA) involved in the different regulatory steps during intestinal passage of *L. monocytogenes* are shown at the right.

gastrointestinal tract, including a bile salt hydrolase (encoded by *bsh*) and a bile exclusion system (encoded by *bilE*) (18, 19). Full PrfA activation occurs following bacterial entry into the cytosol of host cells and results in the expression of gene products that enable intracellular replication and bacterial spread to adjacent cells. A subset of genes directly regulated by PrfA is located on an *L. monocytogenes* pathogenicity island referred to as LIPI-1 (*hly*, *plcA*, *prfA*, *mpl*, *actA*, and *plcB*), while others (*inlA*, *inlB*, *inlC*, *bsh*, *prsA2*, and *hpt*) have distinct chromosomal locations. Comparison of the profiles of wild-type *L. monocytogenes* grown in brain heart infusion broth with those of a strain harboring a constitutively active PrfA protein suggests that the expression of at least 145 genes may be modulated by PrfA (20).

Given that PrfA is critical for enabling *L. monocytogenes* to mediate the balance between life in soil and life inside an infected host, the activity of PrfA is itself carefully regulated by a variety of mechanisms that include transcriptional, posttranscriptional, and posttranslational control. These critical regulatory circuits work together to tightly control *prfA* expression and PrfA activity as outlined below.

Transcriptional Regulation of *prfA* Expression

Transcriptional regulation of *prfA* expression occurs via three promoter elements (Fig. 2). Two promoters, *prfA*P1 and *prfA*P2, are located immediately upstream of the *prfA* translation initiation codon, while the third promoter lies immediately upstream of *plcA* and results in the generation of a *plcA-prfA* bicistronic transcript (14, 21, 22). The *prfA*P1 and *prfA*P2 promoters direct the synthesis of monocistronic transcripts of *prfA* that generate the initial levels of PrfA protein required to activate expression of *hly* and *plcA*, whose gene products are needed for efficient escape of *L. monocytogenes* from host cell phagosomes (22, 23). The *plcA* promoter, which is activated by PrfA, directs the synthesis of the *plcA-prfA* transcripts, resulting in the high levels of PrfA synthesis that are required to induce *actA* expression for efficient bacterial cell-to-cell spread. The *prfA*P1 promoter contains characteristics of a σ^A-dependent promoter, which is the vegetative sigma factor determining RNA polymerase specificity required for transcription in actively growing, unstressed bacterial cells. The *prfA*P2 promoter region contains sequences that resemble a PrfA binding box, a σ^A-dependent promoter, and the general stress response sigma factor σ^B-dependent promoters (23–26). σ^B directs RNA polymerase to the promoter regions of a large number of genes involved in adaptation to general environmental stresses, such as conditions of low pH, high osmolarity, oxidative stress, and carbon starvation (see below). A number of genes coregulated by PrfA and σ^B have been shown to contribute to pathogenesis of *L. monocytogenes* (e.g., *bsh* and *inlAB*), suggesting a cross-talk network between these two regulators and possibly other stress response regulators and alternative sigma factors (18, 27, 28). Interestingly, work by Guldimann et al. (29) suggests that activation of PrfA and σ^B occurs stochastically within individual bacterial cells under defined stress conditions, with evidence that PrfA activation appears population-wide, while σ^B activation is restricted to subpopulations.

In addition to stress-responsive promoter elements, Lobel et al. have recently identified CodY to activate transcription of the *prfA* gene (30). CodY is a regulatory protein that links *prfA* expression with the metabolic status of the bacterium. Intriguingly, CodY binds to a region located 15 bases downstream of the *prfA* start codon (30). CodY responds to the presence of branched chain amino acids and GTP, and *L. monocytogenes* appears to regulate CodY activity by controlling the internal isoleucine pool, a mechanism that has been postulated to have evolved to enable isoleucine to serve as a host signal and virulence effector (31, 32). *L. monocytogenes* thus appears to tie in multiple environmental signals that include both stress and metabolism in its quest to regulate virulence gene expression within host cells.

Posttranscriptional Regulation

It has long been recognized that virulence factors as well as PrfA are mainly expressed at body temperature, 37°C, although the mRNA encoding the PrfA protein is present also at low temperatures (below 30°C) (21, 33). Computer

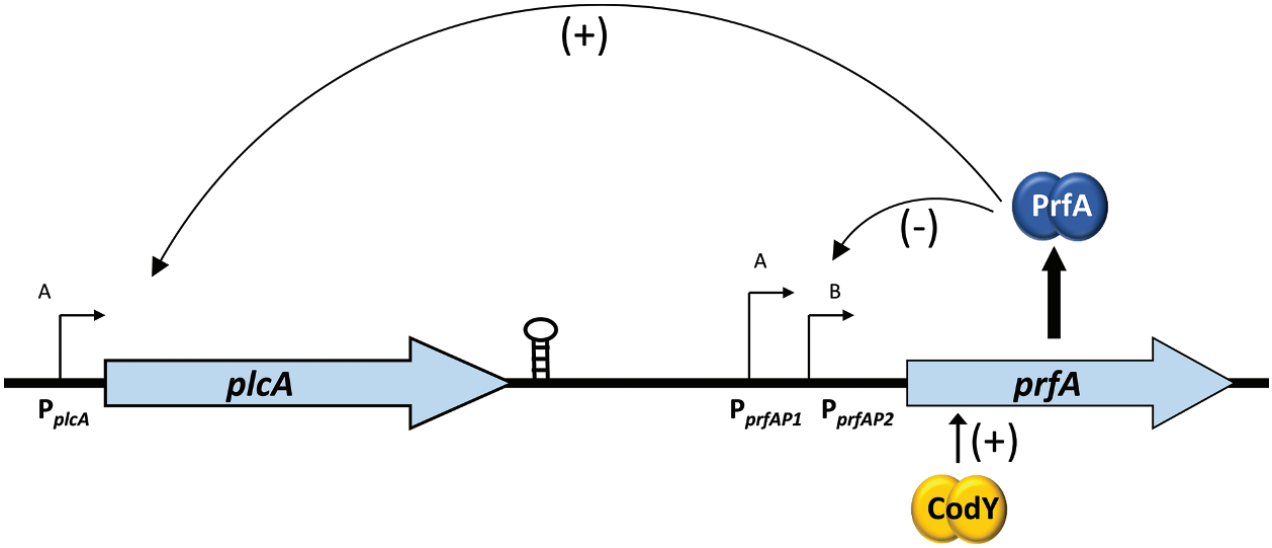


FIGURE 2 Transcriptional regulation of *prfA* expression. The *prfA* transcript is expressed from three different promoters, P1 (σ^A regulated), P2 (σ^A and σ^B regulated), and *plcA* (generating a *plcA-prfA* bicistronic messenger). The *plcA* promoter is positively regulated by active PrfA, creating a positive feedback loop of PrfA expression. CodY positively regulates expression of *prfA* by binding within its coding region. The stem-loop structure shows a putative transcriptional terminator located 3′ of the *plcA* gene.

prediction and chemical probing experiments showed that a 115-nucleotide (nt)-long 5′ untranslated RNA (UTR) present in front of the *prfA* encoding mRNA could form a long hairpin where the Shine-Dalgarno (SD) site was partially masked by an anti-SD site (34) (Fig. 3). *In vitro* translation of the wild-type *prfA* showed a thermoregulated expression, with PrfA mostly expressed at higher temperatures. Mutational analysis suggested that the actual thermosensing occurred in the middle part of the hairpin since mutations that destabilized the *prfA*-UTR structure abolished much of the thermoregulation (34). These data suggested a simple mechanism of thermosensing: at low temperatures, the SD site is closed due to an interaction with an anti-SD site, and binding of the ribosome is prevented. With increasing temperature, the region surrounding the *prfA*-SD site is destabilized, allowing binding of the ribosome and initiation of translation (34–36) (Fig. 3). However, a more complex mechanism of *prfA* translation initiation has been put forward, involving a ribosomal stand-by site inside the *prfA* coding RNA (37).

In addition, *prfA* translation is controlled by at least two prematurely terminated *S*-adenosyl methionine (SAM) responsive riboswitches (38). Riboswitches are RNA elements that are able to control downstream gene expression by directly binding specific metabolites with a high affinity. The metabolite-riboswitch interaction results in RNA restructuring, usually terminating transcription in Gram-positive bacteria (39, 40). When binding SAM, transcription is terminated and short (150 to 250 nt long) transcripts are generated. Two of the prematurely terminated SAM riboswitches in *L. monocytogenes* (SreA and SreB) can base-pair with the 5′ UTR of *prfA* and thus reduce PrfA expression by an unknown mechanism (38) (Fig. 3). Here, the function of the terminated riboswitches resembles the role played by several *trans*-acting small RNAs (41). Interestingly, SreA is unable to inhibit translation at low temperatures, possibly because its interaction site in the *prfA* thermosensor is in a closed conformation, preventing base-pairing with *trans*-acting RNA elements. Expression of PrfA can therefore be affected by SAM riboswitches only at temperatures encountered during infection. The entire SreA is not required for repression; instead, the central core part of the SreA is sufficient for efficient blocking of PrfA translation (S. Krajewski and J. Johansson, unpublished results). Intriguingly, SreA expression is positively controlled by PrfA, creating a negative feedback loop, where high levels of active PrfA turn off its own expression by increasing the level of the repressive element SreA (38). It therefore seems that the 5′ UTR of the *prfA* transcript can act as a sensor, integrating both the environmental temperature and the metabolic state of the bacterium through SAM sensing.

Posttranslational Regulation

The third critical mechanism for regulating PrfA activity occurs via posttranslational modification (Fig. 4). As stated above, PrfA protein is a member of the cyclic AMP receptor protein (Crp)-Fnr family of transcriptional regulators, of which there are approximately 400 members (42). Proteins in this family generally function as dimers and require the binding of small molecule cofactors (for example, cyclic AMP for Crp) or other forms of posttranslational modification (such as the binding of carbon monoxide by the heme moiety of CooA) for full activity. The PrfA cofactor has recently been identified as glutathione, and PrfA-glutathione cocrystals indicate that the cofactor binding site is located at the intraprotein tunnel site, located between the N- and C-terminal domains of the PrfA monomer (43–46). Working models have suggested that once inside the cytoplasm, both uptake of cytosolic glutathione and, most importantly, upregulation of the glutathione synthase, *gshF*, lead to increased glutathione concentrations in *L. monocytogenes* (43, 45). This is required since glutathione only binds PrfA with a modest affinity (0.5 mM), so the increased glutathione concentration thus signals to the bacterium its arrival into the cytosol. Recently, Krypotou et al. (47) proposed that Cys-containing peptides transported into *L. monocytogenes* via the Opp peptide transport systems provide for the generation of glutathione leading to PrfA activation. Interestingly, the authors further suggested that inhibitory peptides transported

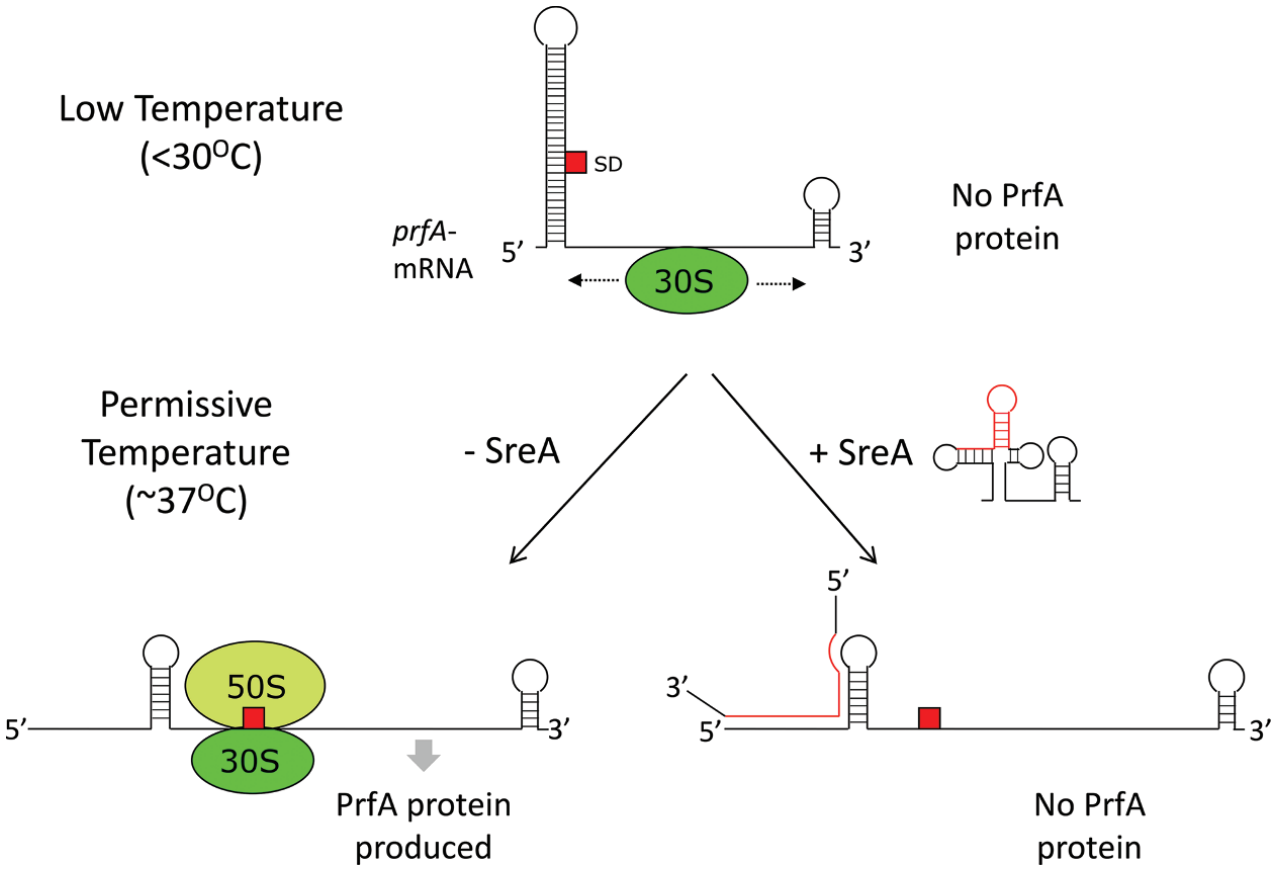


FIGURE 3 Posttranscriptional control of PrfA expression. At low temperatures (<30°C), the Shine-Dalgarno site (SD, red square) is sequestered in an RNA thermosensor, preventing binding of the 30S subunit (green sphere) of the ribosome. Possibly, the binding of the 30S subunit involves a ribosomal standby site, where it primarily binds to the coding region of the *prfA* mRNA, where it scans the transcript. At permissive temperatures (~37°C), the RNA thermosensor dissociates, allowing access of the 30S subunit to the SD, where it can recruit the 50S subunit (light green sphere), and translation can initiate. Under conditions in which a terminated S-adenosylmethionine riboswitch is produced (+SreA), it can bind to the RNA thermosensor and by an unknown mechanism prevent PrfA production.

by this same system bind to PrfA and inhibit PrfA activation by inhibiting the binding of glutathione. The complexity of PrfA activation continues to increase.

Years prior to the identification of glutathione as the PrfA cofactor, Ripio et al. identified an *L. monocytogenes* strain containing a single mutation within *prfA* coding sequences that resulted in the constitutive expression of PrfA-dependent virulence genes in broth culture (48). The substitution of a serine for a glycine at position 145 within PrfA was the first identification of a PrfA* mutation, so named because it appeared analogous to an A144T mutation identified within Crp that resulted in the constitutive expression of Crp-dependent gene products in the absence of cofactor (Crp* mutants) (49). Similar to Crp*, the PrfA G145S mutation was demonstrated by structural analyses to alter PrfA protein confirmation and increase the DNA binding affinity of PrfA for its target promoters via the repositioning of a helix-turn-helix DNA binding motif (50, 51).

There have now been a number of additional mutations identified that confer PrfA activation, albeit at differing levels of activation. Reported *prfA** mutations include G145S, Y63C, S71C, E77K, A94T, L140F, Y154C, L148P, G155S, and P219S substitution mutants (52–61). These mutations in some cases map to very different regions of PrfA in comparison to the original G145S PrfA* mutation, and strains containing these different *prfA** alleles exhibit levels of PrfA-dependent gene expression in broth culture that range from 4-fold to >200-fold greater than the levels of expression observed in wild-type bacteria (62, 63). With the exception of PrfA G145S, for which the structure has been solved using X-ray crystallography (51), the mechanisms by which the other *prfA** mutations confer constitutive activation are not clear.

*prfA** MUTATIONS REVEAL THE DIVERSITY OF GENE PRODUCTS REGULATED BY PrfA ACTIVATION

PrfA* mutants may be considered to phenotypically resemble *L. monocytogenes* under conditions of cytosolic activation (bound to glutathione), and thus the mutant strains have proven useful for the identification of novel virulence factors following transcriptomic and proteomic analyses. Milohanic et al. initially identified substantial overlap between genes whose expression was influenced by PrfA and stress-responsive genes regulated by the stress-responsive alternative sigma factor σ^B (20). However, studies by Ollinger et al. (64) using reverse transcriptase PCR reported that the transcript levels of some of PrfA-associated genes identified by Milohanic et al. (20) were not significantly affected by the presence or absence

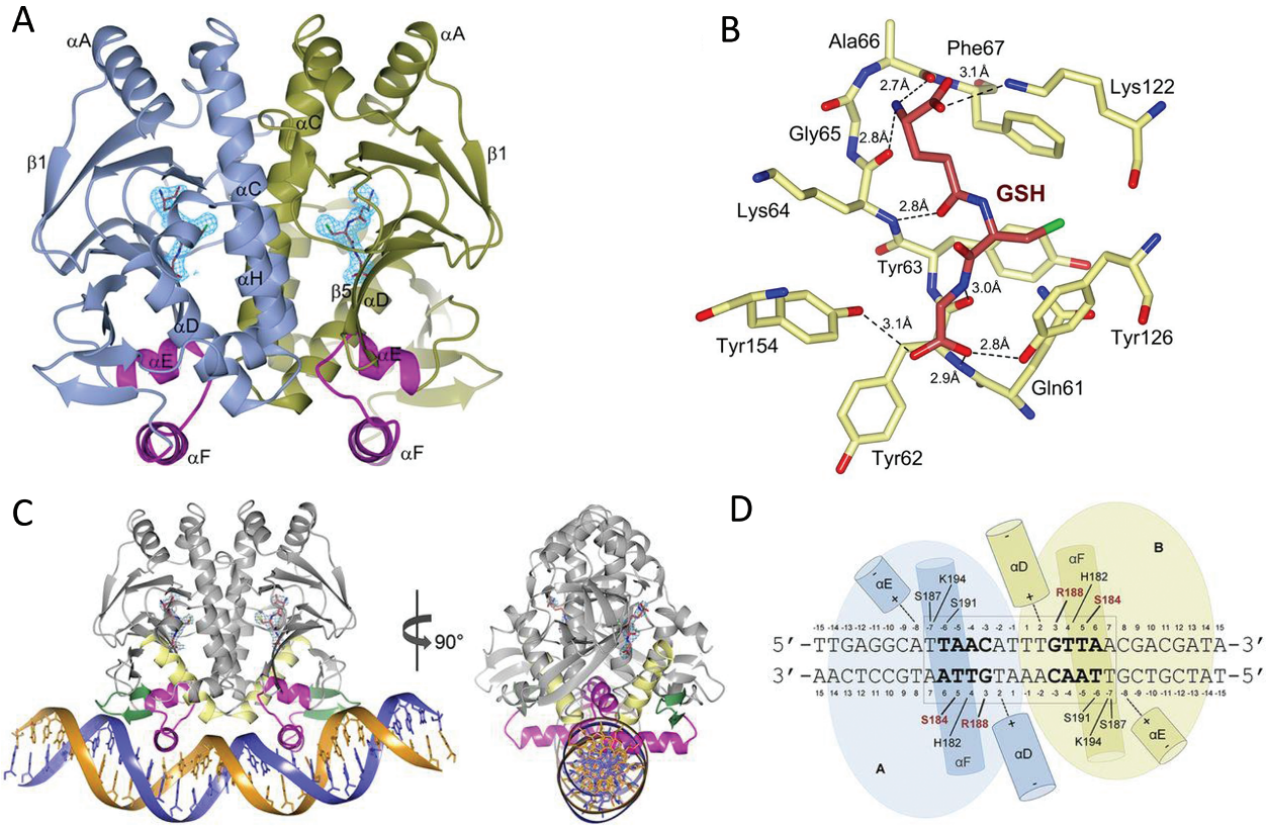


FIGURE 4 Structures of PrfA in complex with glutathione (GSH) and PrfA in complex with GSH and DNA. (A) Ribbon representation of the PrfA homodimer with GSH bound at the tunnel site of each monomer. The monomers A and B are colored blue and green, respectively. The helix-turn-helix motif is highlighted in magenta, and GSH has crimson carbon atoms with light blue electron density. (B) Local amino acid interactions and structural features of GSH-PrfA binding. Direct hydrogen bonds between amino acids and GSH are shown by dashed lines. (C) Ribbon representation of the DNA-bound homodimer in complex with GSH. The monomers A and B are colored gray, the helix-turn-helix motif is magenta, αD is yellow, and the winged β-hairpin is green. GSH is shown as sticks with the carbon atoms in crimson. The 30-bp palindromic operator is shown in dark blue and orange. (D) Schematic drawing of the PrfAWT-DNA interactions. Amino acids S184 and R188 make base-specific contacts and are highlighted in red. Other interactions are nonspecific between protein side chains and the DNA phosphate backbone. Figure adapted with permission from Hall et al. (44).

of PrfA. Discrepancies between these independent studies may reflect disparities between laboratory conditions, variations between strains used for examination (EGDe versus 10403S), or additional undefined complexities associated with PrfA-dependent gene expression.

Secreted proteins are often the first bacterial factors to interact with the host, and a comparison of secreted protein profiles derived from the culture supernatants of wild-type, Δ*prfA*, and *prfA** mutants identified at least 17 proteins that were differentially secreted following PrfA activation (52). The majority of the genes encoding these proteins did not contain recognizable PrfA binding sites in their upstream promoter regions, suggesting that the synthesis and/or secretion of these proteins was indirectly influenced by PrfA activation. Proteins with increased abundance in the supernatants derived from *prfA** cultures included a number of previously identified virulence factors as well as putative ABC transporters, cell wall-modifying enzymes, chitinases, antigenic lipoproteins, and chaperone proteins associated with protein secretion. Many of these PrfA-dependent secreted proteins also depended on the presence of the secretion chaperone PrsA2 for full activity (65). A significant number of secreted gene products that appear indirectly regulated by PrfA have been demonstrated to contribute to *L. monocytogenes* pathogenesis, and these gene products serve as further examples of the expansive influence of PrfA on *L. monocytogenes* life within the host.

THE IMPACT OF CARBON SOURCES ON PrfA ACTIVATION AND THE STIMULATION OF PrfA-DEPENDENT VIRULENCE GENE EXPRESSION

The expression of virulence factors has long been known to respond to the presence of sugars in the medium (66–71). Sugars (e.g., cellobiose and glucose) taken up by the phosphoenolpyruvate):phosphotransferase system (PTS) repress PrfA activity, whereas non-PTS sugars (e.g., glycerol) modestly stimulate PrfA activity. Importantly, the PTS-mediated repression of PrfA activity is not mediated by the catabolite control protein A (CcpA), which controls expression of many genes in different *Firmicutes* in response to PTS sugars (72, 73). Instead, the phosphorylation state of the different PTS permeases appears to dictate the activation level of PrfA (74). In the presence of PTS sugars, the permeases are in an unphosphorylated state as they donate the phosphate group

they received from the phosphoenolpyruvate system to the incoming sugar. In the absence of PTS sugars (and the presence of non-PTS sugars), the permeases are phosphorylated. It has been suggested that unphosphorylated permeases might bind and sequester PrfA directly, thereby blocking PrfA-mediated virulence gene expression (74). Glucose can be transported into *Listeria* by several different permeases. One of the mannose permeases, EIIABMan, was suggested to act as a PrfA-binding partner or, alternatively, to interact with a PrfA-activating molecule (75). In addition, the repressive effect of PTS sugars can be relieved by the addition of charcoal or amberlite to the medium during bacterial growth (76, 77). This suggests that a component released by *L. monocytogenes* could repress PrfA activity, either directly or indirectly. Precisely how the action of PTS-permeases or repressive extracellular molecules such as inhibitory peptides affect the level of glutathione (the PrfA cofactor) or PrfA binding of glutathione remains to be determined.

MAINTAINING THE BALANCE BETWEEN LIFE INSIDE AND OUTSIDE THE HOST: CONSTITUTIVE ACTIVATION OF PrfA IS NOT BENEFICIAL FOR *L. MONOCYTOGENES* SURVIVAL

At first glance, *prfA** strains would appear to exhibit a number of advantages over wild-type bacteria. Strains containing *prfA** are hyperinvasive, mediate more efficient phagosome escape, and initiate bacterial actin-based motility more rapidly. Activation of PrfA also shifts *L. monocytogenes* metabolism toward the preferred use of three carbon sugars and phosphorylated sugars, the principal carbon sources used by *L. monocytogenes* for growth within the cytosol (78). *prfA** mutants are hypervirulent in mouse infection models and exhibit a competitive fitness advantage over wild-type strains during both oral and intravenous mixed infections in mice (78).

The fitness advantage observed for *prfA** strains within the host does not, however, translate into bacterial fitness in the outside environment (78, 79). Constitutively activated *prfA** mutants exhibit impaired flagellum-mediated swimming motility, a defect that would be expected to compromise bacterial fitness in environments where the bacteria must be able to detect and swim toward available nutrient sources and which would additionally impair biofilm formation. *prfA** mutants also exhibit a pronounced fitness defect when grown in the presence of wild-type bacteria in mixed broth culture despite displaying no obvious growth defects in monoculture. Stress conditions such as high osmolarity or low pH exacerbate the competitive defects observed for *prfA** strains in a manner that is independent of the stress-responsive sigma factor σ^B. Lastly, *prfA** mutations appear to negatively impact the ability of *L. monocytogenes* to survive long periods of starvation. Interestingly, a PrfA* strain devoid of most PrfA-regulated genes did not display a fitness cost (80). The multiple mechanisms that thus exist to regulate PrfA activity appear to have evolved to carefully balance gene expression patterns so as to maintain bacterial fitness in the soil as well as in the cytosol.

BEYOND PrfA: OTHER MODES OF *L. MONOCYTOGENES* REGULATION OF GENE EXPRESSION

PrfA is a central factor in the regulation of *L. monocytogenes* virulence gene expression; however, numerous other regulatory factors and mechanisms exist that enable the bacterium to adapt its physiology in response to the diversity of environmental conditions encountered by this ubiquitous organism. A sample of some of these alternative regulatory modalities is presented below.

Posttranscriptional Regulation of Bacterial Gene Expression

Untranslated regions of mRNA (UTRs) have been demonstrated to regulate translation of the associated gene products for multiple genes. The 5′ UTRs located upstream of *inlA*, *actA*, and *hly* have been shown to control expression of their protein products (27, 81, 82). These 5′ UTRs appear, however, to support expression of InlA, ActA, and LLO, respectively, which is in contrast to the previously described *prfA*-UTR, which represses PrfA expression. It has been shown that partial deletions of the 5′ UTRs of *inlA*, *actA*, and *hly* decrease the protein production, but the exact mechanism(s) by which the 5′ UTRs act has not yet been revealed. Interestingly, it was shown that the coding region of *hly* could be important for LLO production (83). Whether this is due to alterations of secondary structures, binding of small regulatory RNAs (sRNAs), or some other factors remains to be investigated.

Regulation by sRNAs

Several hundred sRNAs have been identified in *L. monocytogenes*, ranging in size from less than 100 to more than 500 nt and with different expression patterns (84–88). However, the functions of only a subset of these sRNAs have been revealed, and an even smaller number have been shown to participate in virulence gene expression. Rli27 is an intracellularly induced sRNA able to interact with the 5′ UTR of *lmo0514*, encoding an LPXTG cell wall protein that is also upregulated during intracellular growth (89). Lmo0514 is required for survival of the bacteria in plasma and during infection of mice (90). The 5′ UTR of *lmo0514* contains an inhibitory RNA structure that sequesters the SD region and blocks translation initiation. Binding of Rli27 to the 5′ UTR disrupts the inhibitory structure, thereby allowing intracellular expression of Lmo0514 (89).

Rli38 is a long (514 nt) sRNA that is highly induced when *L. monocytogenes* is exposed to blood (86). A Δ*rli38* mutant strain exhibited reduced bacterial loads in several organs compared to the wild-type strain. The target of Rli38 (RNA or protein) is not yet known, nor is its mechanism of action. Another sRNA induced when *L. monocytogenes* is exposed to blood is RliB (86). In contrast to Rli38, the absence of RliB increased bacterial loads in the liver compared to the wild-type strain. RliB is a substrate for polynucleotide phosphorylase, but it is also a clustered regularly interspaced short palindromic repeat (CRISPR) element, devoid of associated Cas proteins (91). However, presence of *trans*-encoded Cas proteins and polynucleotide phosphorylase allows RliB to exert DNA interference directed against matching protospacers. Why *Listeria* has a degenerated CRISPR system is not clear and deserves further study.

Possibly the most well-known sRNAs in *L. monocytogenes* are the sibling family of LhrC, consisting of seven members, all with varying degrees of conservation (86, 92–95). The LhrCs control expression of at least three virulence factors: *lapB*, encoding an adhesin, *oppA*, encoding an oligopeptide protein, and *tcsA*, encoding a CD4+ T cell-stimulating antigen. The different members of the LhrC family are able to bind to the 5′ UTRs of their targets. For *lapB* and *oppA*, the LhrCs directly interact with their SD region to inhibit translation, whereas the LhrCs destabilizes the *tcsA* transcript

by binding a site distally from the SD region (93, 95, 96). Interestingly, two CU-rich binding sites in LhrC4 were identified to be important for *oppA* binding, giving the possibility of two target mRNAs being sequestered by one sRNA. Another exciting aspect of the LhrC family is that the expression of individual members is controlled by exposure to various environmental cues, such as blood and growth in the intestine and in macrophages through at least one bacterial two-component signaling system (93).

The functions of several sRNAs in bacteria are dependent on RNA chaperones. In Gram-negative bacteria, Hfq plays a vital role, stimulating interaction between short complementary sequences of the sRNA and the target mRNA (97). The role of Hfq appears to be more crucial if the level of complementarity between the RNAs is low. Although the phenotypes of a Δ*hfq* strain are less pronounced in *L. monocytogenes* than a Δ*hfq* strain in Gram-negative bacteria, Hfq has been shown to be associated with sRNAs and to contribute to *L. monocytogenes* virulence (84, 98). However, the interaction between the sRNA LhrA and the target mRNA *lmo0850* is the only example so far described in *L. monocytogenes* when Hfq is needed for a functional regulatory outcome (99). Other RNA chaperones could in some cases replace Hfq.

The SpoVG protein of *L. monocytogenes* is an RNA binding protein that regulates lysozyme resistance, virulence, and swarming motility (100). The sRNA Rli31 was shown to interact with both the 5′ UTR of the *spoVG* transcript and the SpoVG protein (100). Surprisingly, Rli31 did not appear to affect the levels of either the *spoVG* transcript or the SpoVG protein, raising the possibility that Rli31 acts as a "sink" to inactivate SpoVG, in analogy with the CsrA/Rsm systems of posttranscriptional regulators in Gram-negative bacteria. Finally, RNA helicases have been shown to contribute to virulence gene expression in *L. monocytogenes* (101, 102). sRNAs are becoming increasingly recognized for their contributions to diverse aspects of *L. monocytogenes* physiology and virulence.

Regulation Induced in Response to Environmental Stress

During its life as a saprophyte and pathogen, *L. monocytogenes* encounters a diversity of stress-conditions, many of them potentially life-threatening. To survive disparate stresses, the bacterium makes use of a number of strategies. As mentioned previously, the expression of several proteins important for stress survival depends on the stress sigma factor σ^B, which competes with the vegetative sigma factor σ^A for binding to the RNA polymerase (103, 104). Activity of σ^B is controlled by a complex relay of protein interactions and phosphorylation events, ultimately freeing σ^B from the anti-sigma factor RsbW. At the top of this relay hierarchy lies a sensory organelle, the "stressosome," which is a large (~1.8 MDa) multiprotein complex, thus far primarily studied in *Bacillus subtilis* (105). The stressosome is able to detect and integrate environmental cues into a signal transduction pathway that eventually allows the liberation of σ^B and initiation of the expression of σ^B-regulated genes.

In *L. monocytogenes*, it has been suggested that there may be interplay between σ^B and PrfA activity, such that σ^B controls genes important for the initial steps of infection (i.e., during survival in the gut and adhesion to intestinal epithelial cells), whereas PrfA controls the expression of genes involved in subsequent steps (i.e., adhesion and intracellular growth as well as further dissemination in the host) (25, 26, 86, 106, 107). Interestingly, some genes, such as the genes encoding the two important invasins, InlA and InlB, are controlled by both σ^B and PrfA (27, 108). This dual regulation allows the expression of the bicistronic *inlA-inlB* message from two promoters, integrating several external cues and thereby permitting bacterial invasion of several host cell types. Both the σ^B- and PrfA-generated *inlA-inlB* transcripts contain unusually long 5′ UTRs (445 and 396 nt, respectively) possibly acting at the posttranscriptional level to sense environmental factors (27). Whether the stressosome is able to act as a central hub to integrate external signals to control virulence gene expression in concert with other regulators (e.g., PrfA) remains to be determined. Very little is known about the mechanisms underlying stressosome sensory perception and signal integration. It has been shown that a blue-light receptor can sense light and activate σ^B through the stressosome, but the exact mechanism is not known (109–111). A small peptide, Prli42, has been demonstrated to bind the stressosome, and its absence reduces σ^B-dependent gene expression while also affecting expression of virulence factors (112). Prli42 harbors a transmembrane domain and is associated with the membrane, potentially allowing the stressosome to sense stress exerted through membrane disturbance.

Fatty Acids and Regulation of Virulence

Many enterobacteria use fatty acids (FAs) to regulate virulence gene expression. For example, *Vibrio cholerae*, the causative agent of cholera, controls the expression of cholera toxin and toxin coregulated pilus using the transcriptional activator ToxT; unsaturated long-chain FAs were shown to directly bind to and repress activity of ToxT (113). Using the X-ray structure of palmitoleic acid bound to ToxT as a blueprint, highly effective ToxT inhibitors were synthesized that were able to inhibit intestinal colonization in mice (114). FAs have also been shown to affect virulence factor expression in *L. monocytogenes* and have been suggested to interfere with PrfA activity (115, 116). The addition of increasing amounts of non-branched-chain FAs (both saturated and nonsaturated) to bacterial cultures was found to reduce virulence factor expression (116). A similar effect was observed by decreasing the bacterial content of branched-chain FAs (115), possibly because this increases the amount of non-branched-chain FAs. Interestingly, a strain carrying a constitutively active PrfA protein ($PrfA_{G155S}$) also exhibited reduced virulence factor expression in the presence of FA, suggesting that the repressive effect of FAs lies at a level subsequent to PrfA-activation (116). Additional work is required to decipher if FAs interact directly with PrfA or if the effect of the FAs is mediated through another pathway.

Regulation by Two-Component Sensing Systems

To sense and respond to environmental cues, many pathogenic bacteria use two-component systems (TCSs). Typically, the TCS consists of a histidine kinase (HK) that senses an external signal and transfers a phosphor group to activate a response regulator (RR) controlling the expression of genes important for the response of the particular stimulus (117). *L. monocytogenes* harbors 16 TCSs, one of which, DegU, is an orphan RR (118). Several of the *Listeria* TCSs have been implicated in virulence (119–122). One of them is part of the accessory gene regulator (Agr) system of *L. monocytogenes*, consisting of a quorum sensing/TCS system (119). In the Agr system, a small auto-inducing peptide (AIP, derivative of the *agrD* gene product) is processed and transported by AgrB to the outside of the bacterium. The AIP of *L. monocytogenes*

was shown to be a cyclic pentapeptide (CFMFV) (123). Once outside, the AIP is recognized by the HK AgrC, which activates the RR AgrA. Exactly how the Agr system contributes to *L. monocytogenes* virulence is unclear, since the bacterium lacks RNAIII, an sRNA expressed divergently from the *agr* locus and which controls the expression of several virulence factors in *Staphylococcus aureus* (124). Another TCS implicated in *L. monocytogenes* virulence is LisrK (120). Loss of LisK (the HK) reduced bacterial growth in the spleens of mice. Interestingly, LisRK has been shown to be important for expression of the serine-protease HtrA and the sRNA LhrC, both of which contribute to *L. monocytogenes* pathogenesis (93, 125). The flagellin-associated TCSs CheY and CheA are important for listerial adhesion and invasion of cultured Caco-2 cells; however, bacteria lacking this system exhibited normal growth within the spleens of mice (121). The DegU RR appears to be of interest, because it lacks its associated HK, and the absence of DegU impairs *L. monocytogenes* growth in mice (126–128). DegU is also the epistatic regulator of motility by positively activating expression of the antirepressor GmaR, which in turn, inactivates the repressor of motility MogR (129). Having no accompanying kinase, DegU appears to be phosphorylated by intracellular acetyl phosphate (130). The VirRS TCS system was originally identified using a signature tagged mutagenesis screen seeking genes important for mouse infection (122). Surprisingly, VirR can be activated independently of the HK VirS, possibly through variations in bacterial intracellular acetyl phosphate concentrations. VirR controls the expression of several virulence-associated genes upregulated during infection (12). Recently, it was shown that the VirAB ABC transporter controls VirR function in response to the antimicrobial Nisin and/or host factors (131).

Regulation of Virulence via Peptide Pheromones

In addition to the peptide-responsive Agr system discussed above, an additional peptide pheromone system has been recently shown to contribute to *L. monocytogenes* virulence. A PrfA-inducible secreted peptide pheromone-encoding lipoprotein (PplA) was identified that shares significant homology with the *Enterococcus faecalis* Cad lipoprotein encoding the cAD1 peptide pheromone (132, 133). Similar to the cAD1 pheromone of *E. faecalis*, the pPplA peptide is processed from the released PplA lipoprotein N-terminal signal sequence (SS) peptide following secretion of the lipoprotein through the general secretory pathway. In contrast to *E. faecalis*, where the cAD1 pheromone stimulates a mating response between plasmid-containing and plasmid-free cells, the pPplA peptide pheromone has functionally evolved to enhance vacuolar escape of *L. monocytogenes* within nonprofessional phagocytic cells. Studies of mutants lacking both the PplA lipoprotein and its signal sequence-encoded peptide pheromone versus the lipoprotein alone have demonstrated that the pPplA peptide pheromone is a critical virulence factor that contributes to both bacterial aggregation in broth culture and survival in mouse models of infection. The PplA lipoprotein has no apparent role in vacuolar escape; however, its function as an extracellular electron transport protein that contributes to bacterial fitness within the gut has recently been described (134).

L. monocytogenes mutants lacking the pPplA peptide exhibit delays in escape from the vacuoles of nonprofessional phagocytic cells but escape with normal kinetics from the phagosomes of macrophage-like cell lines or in bone marrow-derived macrophages (132). Interestingly, loss of the pPplA pheromone did not impair LLO-dependent perforation of the vacuole, only bacterial escape into the cytosol. These data strongly suggest that complete vacuole escape requires an additional mechanism beyond initial pore formation mediated by the activity of the hemolysin LLO. The pPplA peptide was also found to contribute to maintenance of surface-associated and secreted proteins, and it is possible that some of these gene products may contribute to either the stabilization of LLO-induced membrane pores and/or the physical disruption of the vacuole membrane (Fig. 5).

Interestingly, all bacterial virulence defects associated with the loss of the pPplA peptide could be completely compensated for by the introduction of constitutively active PrfA*, suggesting a possible connection between the pPplA signaling pathway and PrfA activation (132). These studies suggest a model in which *L. monocytogenes* senses the confines of the host vacuoles as a result of the accumulation and import of the pPplA peptide, leading to PrfA activation and enhanced bacterial escape into the cytosol. The *L. monocytogenes* pPplA peptide thus appears to have evolved to enable the bacterium to sense the confined environment of the vacuole so as to induce the expression of gene products required for full membrane disruption and entry of the bacterium into the cytosol.

In addition to the pPplA peptide-signaling pathway, another potential peptide-based signaling system related to competence development has been implicated in enhancing escape of *L. monocytogenes* from host cell vacuoles, specifically, the phagosomes of macrophages (135). Competence is a brief physiological state during which the bacterial cells collectively become primed to transport extracellular DNA across the cell wall and bacterial membrane, at times resulting in integration of the newly acquired DNA into the bacterial genome (136). Several gene products, including those encoded by *com* genes, are required for peptide pheromone signaling and DNA uptake. Although it has not been demonstrated to exhibit natural competence, *L. monocytogenes* possesses a number of *com* genes that share homology with those involved in competence development in other bacteria; however, not all the genes required for competence are present (118). Most but not all of the regulatory components and late genes involved in assembly of the Com apparatus required for DNA transport are present in the *Listeria* genome. Because of these missing components, it is unclear if the competence system is functional in *L. monocytogenes*, and natural competence has not been demonstrated under laboratory conditions.

One competence regulator that is encoded by *L. monocytogenes* is ComK, a master transcriptional regulator that induces the expression of late *com* genes involved in assembly of the Com apparatus for DNA uptake, and it is this system which has been reported to be important for *L. monocytogenes* in vacuole escape of professional phagocytic cells (135). Interestingly, the *comK* gene in some strains of *L. monocytogenes* is inactivated by the presence of an A118-like prophage that integrates at a specific attachment site within the *comK* gene. However, Rabinovich et al. have found that the late competence genes regulated by ComK are highly expressed during intracellular growth as a result of prophage excision (135). In addition, mutants missing the specific components of the pseudopilus or the DNA translocation channel were impaired for vacuole escape in macrophages and were attenuated for bacterial virulence in mice, whereas the DNA binding components were dispensable for these processes. A strain cured of the prophage and containing an intact *comK* gene grew similarly to wild-type *L. monocytogenes* in macrophages, and many naturally occurring strains of *L. monocytogenes* lack this prophage and are capable of growth

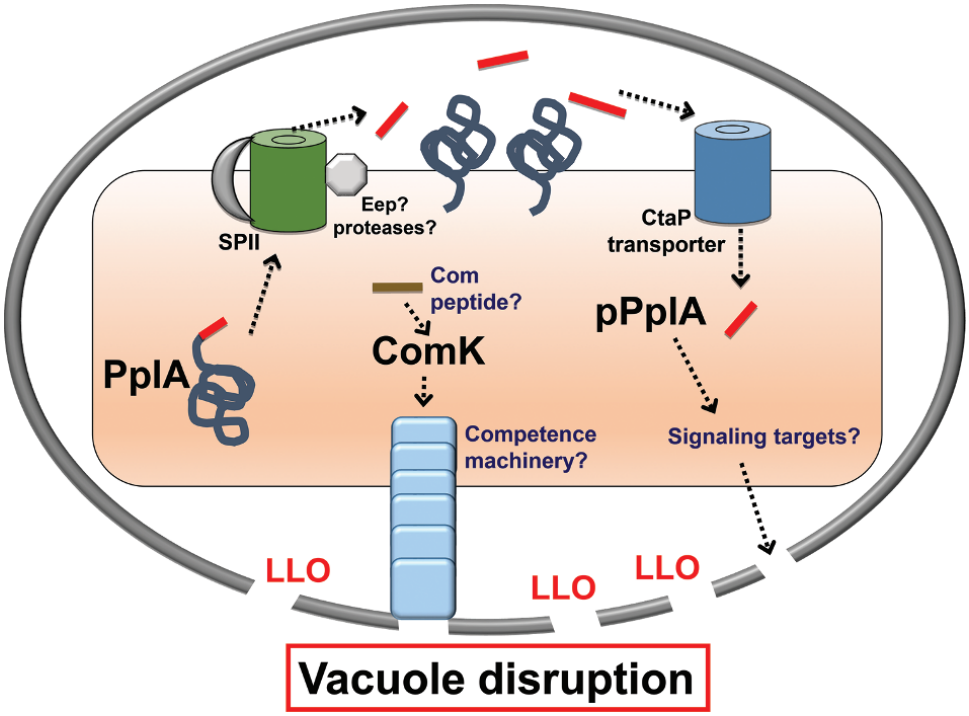


FIGURE 5 *L. monocytogenes* peptide signaling and expression of competence gene products via phage excision within the host vacuole. *pplA* encodes a lipoprotein (PplA) with a peptide pheromone (pPplA) located within the N terminal secretion signal peptide (shown in red). The signal sequence of prePplA is processed by signal peptidase II (SPII), and the released signal peptide is further cleaved by the protease Eep, releasing the pPplA pheromone, while the PplA protein becomes lipid modified and associated with the membrane. The confined space of the vacuole leads to import of the secreted pPplA pheromone, presumably stimulating a signaling cascade that results in the production of an unknown factor(s) that contributes to vacuole lysis. As part of a separate pathway, an unknown signal, potentially also involving a peptide pheromone, leads to the expression of ComK and the expression of a competence-associated pilus that may also aid in vacuole membrane disruption. Select *L. monocytogenes comK* genes harbor a lysogenic phage that must excise to enable ComK expression. The ComK pathway is required for *L. monocytogenes* vacuole escape from professional phagocytic cells; the pPplA system is required for bacterial escape from nonprofessional phagocytic cells.

within macrophages. For strains that do contain prophages, data suggest that prophage integration/excision serves to regulate virulence gene expression and that the ComK system is somehow involved in sensing the presence of *L. monocytogenes* within host cell vacuoles in professional phagocytic cells. Thus, while no peptide pheromone has yet been identified to stimulate the induction of the *L. monocytogenes com* genes within the phagosome, it could be hypothesized that the bacterium has adapted components of the *com* peptide pheromone system for spatial sensing, similar to pPplA (Fig. 5).

The requirement of selected *com* gene products and the pPplA peptide for vacuole escape in different cell types makes it tempting to speculate that a competence-like pseudopilus may help stabilize the pores initially formed by LLO or that maybe the pseudopilus exerts physical pressure on the vacuole membrane to aid in membrane disruption and bacterial entry into the cytosol. It is possible that the pPplA and ComK systems are interconnected in some way and that fundamental differences in the vacuole membranes of professional phagocytic cells versus nonprofessional phagocytic cells necessitate the requirement for differentially regulated components. Additional characterization of the gene products regulated by pPplA and *com* gene products may clarify how these components function to enhance bacterial entry into the cytosol.

MAKING NEW FRIENDS: REGULATION AND THE EXAMINATION OF *L. MONOCYTOGENES* STRAINS BEYOND THE COMMONLY USED ISOLATES

Most studies of *L. monocytogenes* have been performed using EGDe, EGD, or 10403S strains, all belonging to lineage II. However, the most severe outbreaks of listeriosis are caused by *L. monocytogenes* strains belonging to a subset of lineage I (137, 138). These lineage I strains contain an additional pathogenicity island, LIP-III, which appears crucial for the higher virulence potential of lineage I strains. LIP-III encodes eight proteins involved in the synthesis, modification, and transport of listeriolysin S (LLS) (137). LLS was identified as a hemolysin and cytotoxin contributing to *L. monocytogenes* pathogenicity. This finding was challenged by a study suggesting that LLS was not important for infection or killing of eukaryotic cells (139). Instead, LLS was shown to act as a bacteriocin, potentiating survival of *L. monocytogenes* in the microbiota (140, 141). Bacteriocins are peptides capable of killing bacteria closely related to the bacteriocin-producing strain. It should, however, be noted that bacteriocins produced by another bacterium, *Staphylococcus pseudintermedius*, can act both as a bacteriocin and as a cytotoxin (142). An *L. monocytogenes* lineage I strain overexpressing LLS was

shown to kill *Lactococcus lactis* and *S. aureus* as well as *L. monocytogenes* strains of lineage II *in vitro* (140). It was demonstrated that orally administered lineage I strains carrying LLS affected the microbiota in mice, whereas the microbiota remained undisturbed if infected with strains lacking LLS (140). Two of the bacterial genera that were significantly decreased in the gut in the presence of lineage I were *Alloprevotella* and *Allobaculum*. Interestingly, these two genera produced butyric acid, a fatty acid that has been shown to decrease *L. monocytogenes* virulence gene expression, possibly by interfering with PrfA activity (as mentioned earlier in this article). In contrast to LIP-I and LIP-II, expression of genes from LIP-III is not governed by PrfA, thereby allowing expression of LIP-III under conditions that repress PrfA activity (143). It can thus be speculated that LLS-producing *L. monocytogenes* kill bacteria that otherwise would impair PrfA-directed expression of factors needed for passage through the intestinal barrier. The production of LLS in lineage I strains may at least partially explain why this lineage is more often associated with outbreaks than linage II. The induced expression of LIP-III in lineage II strains might answer if other factors in lineage I strains also contribute to infection.

TARGETING REGULATION: THE SEARCH FOR NOVEL ANTIMICROBIALS EFFECTIVE AGAINST *L. MONOCYTOGENES*

Although resistance to traditional antibiotics is fortunately still limited, the increased occurrence of horizontal gene transfer makes the development of new antibacterial compounds targeting *L. monocytogenes* relevant. Stress survival mechanisms are clearly critical for *L. monocytogenes* existence, and a number of attempts have been undertaken to identify novel antibacterial and/or antivirulence chemical compounds targeting facets of *L. monocytogenes* stress resistance and/or virulence. Through a high-throughput screen, a substance (FPSS) was identified that could block the σ^B regulon and thereby also several genes encoding virulence factors (144). The exact mechanism of action of FPSS remains elusive (for example, direct interaction with σ^B or with another upstream component); however, the prospect of being able to inhibit the general stress response and virulence gene expression is exciting.

A different approach using analogs to metabolites that bind and control purine riboswitches led to the identification of 6-aminohydroxyl purine (6-N-HAP) as being able to exhibit both antibacterial and antivirulence properties (145). The target of 6-N-HAP has not yet been revealed, but addition of 6-N-HAP increased the bacterial mutation rate by almost 10,000-fold. Another screening approach identified a natural flavonoid (fisetin) as an efficient inhibitor of LLO activity (146, 147). Molecular modeling and interaction studies suggested that fisetin directly interacts with loops 2 and 3 of LLO, thereby preventing cholesterol binding and LLO oligomerization. Interestingly, fisetin prolonged the survival of mice infected with *L. monocytogenes*. Yet another screening-based approach was used to identify heterocyclic 2-pyridones as efficient inhibitors of *L. monocytogenes* infection and virulence factor expression; the target of the 2-pyridones was shown to be PrfA (148, 149). A 2-pyridone: PrfA cocrystal revealed that the compound bound in an

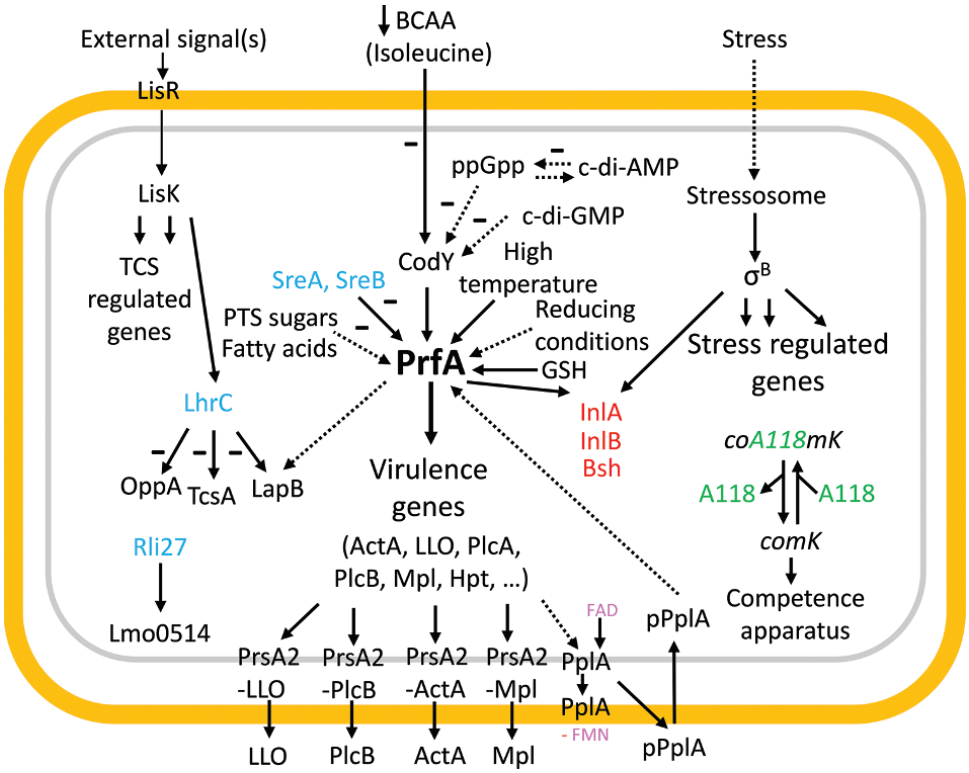


FIGURE 6 A summary of regulatory events underlying stress regulation and virulence factor expression in *L. monocytogenes*, especially highlighting the role of the PrfA system, but also TCS (here represented by LisRK), sRNAs (blue), and σ^B. Gene products regulated by both PrfA and σ^B are shown in red. Consult the text for further details.

intraprotein "tunnel" site, pulling the DNA-binding helix-turn-helix motif in an unfavorable position. The possibility of using tailor-made substances (such as fisetin and 2-pyridones) to inactivate specific targets (LLO and PrfA, respectively) is very appealing since it should only impair *L. monocytogenes* while leaving the remainder of the host microbiota untouched.

CONCLUDING REMARKS AND FUTURE OUTLOOK

L. monocytogenes is notable for its ability to survive and thrive both as an extraordinary pathogen of diverse animal hosts and as a saprophyte in the soil; to accomplish this bacterial gymnastic feat, *L. monocytogenes* utilizes an integrated myriad of regulatory systems (Fig. 6). The most centrally important virulence regulator identified to date is the transcriptional activator PrfA, which controls the expression of the majority of virulence factors in response to environmental cues such as redox status, temperature, energy levels, and potentially in combination with diffusion sensing of the limited space of the vacuole. PrfA is itself regulated by an impressive number of mechanisms that include transcriptional and posttranscriptional regulation as well as translational and posttranslational regulation, each combining to integrate environmental signals into an appropriate regulatory output of bacterial gene expression. It will be intriguing to study single bacterial cells to better understand how the PrfA regulatory network integrates into other sensory systems and how these systems collectively contribute to virulence. Despite being the subject of extensive study for the past 30 years, *L. monocytogenes* still appears to hold regulatory secrets that await discovery.

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Listeria monocytogenes: cell biology of invasion and intracellular growth

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53

Listeria monocytogenes is a facultative intracellular pathogen that has the capacity to actively invade and multiply within mammalian cells. Intracellular replication of *L. monocytogenes* within mononuclear cells was noted in the 1926 publication by Murray and colleagues reporting on this bacterial pathogen for the first time (1). In the 1960s, the seminal work of Mackaness that identified the main actors of cellular immunity against bacterial intracellular pathogens took advantage of the *L. monocytogenes* intracellular lifestyle as a model (2). In the late 1980s and early 1990s, major *L. monocytogenes* virulence factors involved in bacterial adaptation to intracellular life were molecularly characterized (3–7) and the precise stages of the *L. monocytogenes* intracellular life-cycle were morphologically identified (8, 9). Since then, cellular effectors involved in the infection process have been also identified and characterized (10–12). In this article, we review the molecular mechanisms driving *L. monocytogenes* adaptation to the mammalian host cell intracellular environment.

ADAPTATION TO INTRACELLULAR LIFE: GENERALITIES

L. monocytogenes is able to invade and proliferate within macrophages and epithelial nonphagocytic cells. For entry into the latter, bacterial surface molecules (InlA, InlB) interact with cellular ligands, activating signaling cascades that lead to internalization of the pathogen within a membrane-bound compartment (Fig. 1). Residency in the internalization vacuole is prevented by secretion of a pore-forming toxin (listeriolysin O [LLO]) and two phospholipases (PlcA, PlcB) that disrupt the vacuolar membrane, promoting *L. monocytogenes* translocation to the host cell cytoplasm. In this intracellular location, *L. monocytogenes* activates several bacterial metabolic pathways that favor the uptake of cellular resources sustaining bacterial proliferation. The pathogen also displays several strategies to escape cytoplasmic innate immune responses, which include the polymerization of actin by a bacterial surface protein (ActA), allowing *L. monocytogenes* to spread to neighboring cells. In secondary infected cells, *L. monocytogenes* is located in a double-membrane compartment that is disrupted by the same set of secreted enzymes that favor lysis of the primary internalization vacuole. *L. monocytogenes* translocation to the cytoplasm of secondary infected cells leads to a new bacterial replication cycle and further spread to other cells in infected tissues. The intracellular lifestyle is therefore critical for *L. monocytogenes* virulence: it allows escape from extracellular host defense mechanisms, including the complement or antibodies, and hinders detection by patrolling cell populations, e.g., neutrophils; in macrophages, cytoplasmic translocation allows escape from degradative components of the phagocytic cascade, while it provides access to a "Trojan horse" host cell population that can safely transport bacteria to distant locations within the infected organism.

THE CELLULAR INVASION PROCESS

Upon *L. monocytogenes* contact with host target tissues, cellular invasion is morphologically characterized by a localized extension of the plasma membrane around invading bacteria, triggering bacterial internalization within a tight vacuolar compartment. Mechanistically, bacterial surface proteins interact with host cell receptors that are posttranslationally modified (phosphorylation, ubiquitylation), favoring the recruitment of protein adaptors and enzymes that contribute to actin polymerization, the key molecular event required for plasma membrane reorganization. Depending on the invaded cell type, bacterial modulation of the phosphoinositide (PI) metabolism is also critical to trigger cortical actin polymerization.

InlA is the archetypal member of a family of *L. monocytogenes* surface proteins named internalins, which are characterized by the presence of N-terminal leucine-rich repeats (LRR) which mediate interaction with host cell ligands (6, 13). The internalins InlA and InlB, encoded within a single locus in the *L. monocytogenes* genome, are the two major surface molecules driving bacterial entry into host cells. More than 20 other internalins have been identified, but they do not necessarily participate in the cell invasion process, contributing instead to diverse functions including cell-to-cell spread and escape from innate immune responses (i.e., InlC [14, 15]) and escape from autophagy (i.e., InlK [16]). The pore-forming toxin LLO, the actin polymerizing factor ActA, and other bacterial surface proteins have been described as supporting bacterial entry independently of the InlA and InlB invasion pathways (see below).

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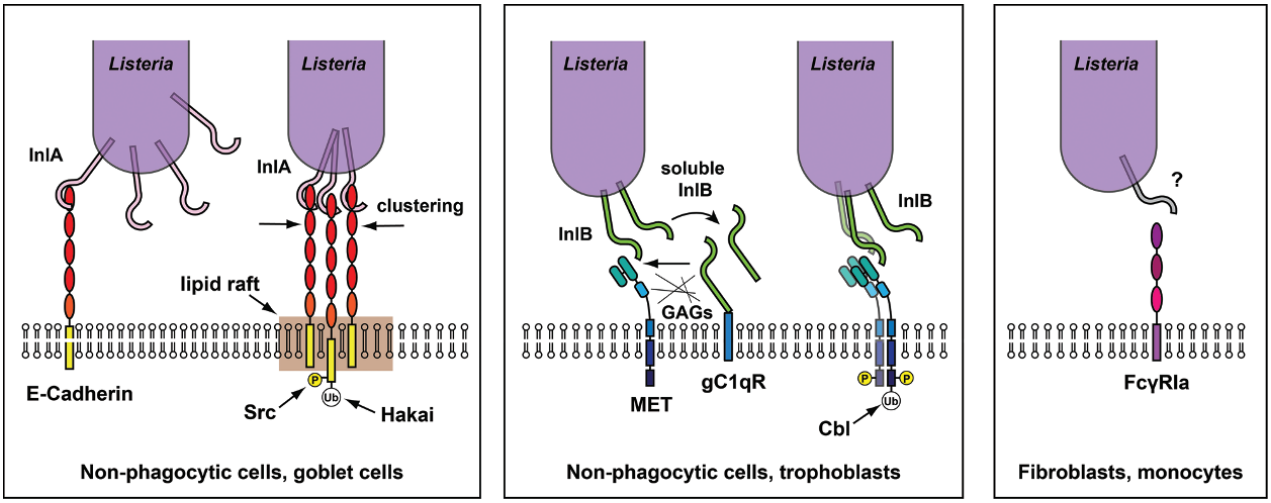


FIGURE 1 Cellular receptors for *L. monocytogenes* in host cells. The receptor for InlA in nonphagocytic polarized cells (including goblet cells) is the transmembrane molecule E-cadherin. Interaction takes place between the InlA leucine-rich repeats (LRRs) and the first extracellular domain of E-cadherin, leading to phosphorylation and ubiquitylation of the cytoplasmic domain of E-cadherin by the kinase Src and the ubiquitin ligase Hakai, respectively. Clustering of E-cadherin requires the presence of lipid rafts (left panel). Via its C-terminal glycine-tryptophan (GW) repeats, InlB interacts with the receptor for the globular part of the C1q complement component (gC1qR) and glycosaminoglycans, which enable interaction of the N-terminal LRRs of InlB with the tyrosine receptor kinase Met in nonphagocytic cells (including trophoblasts). Met dimerization upon interaction with InlB leads to autophosphorylation and recruitment of the ubiquitin ligase Cbl, which ubiquitylates the cytoplasmic tail of Met (center panel). In fibroblasts and monocytes, a function for the FcγRIA receptor has been described for *L. monocytogenes* internalization, via interaction with a still unidentified *L. monocytogenes* surface molecule (right panel). Modified from reference 12.

InlA/E-Cadherin-Mediated Entry

InlA displays a C-terminal LPXTG domain that favors covalent binding to the *L. monocytogenes* cell wall (6). The LRR domain of InlA interacts with the cellular receptor E-cadherin (17, 18), a transmembrane glycoprotein present in the adherens junctions of polarized tissues (e.g., the intestine and the placenta). E-cadherin normally plays a key role in the maintenance of tissue stability, and while the ectodomain participates in most cases in homotypic interactions (E-cadherin/E-cadherin intercellular binding), the cytoplasmic domain interacts with the actin cytoskeleton machinery. By subverting the E-cadherin physiological function, *L. monocytogenes* promotes cortical actin polymerization and plasma membrane rearrangements, favoring cellular invasion and traversal of the intestinal and the feto-maternal barriers (19, 20). InlA access to intestinal E-cadherin mostly occurs at the level of goblet cells, which expose this cellular receptor to bacteria during mucus secretion (21). Exposure of E-cadherin to *L. monocytogenes* during apoptotic cell extrusion at the tip of intestinal villi has also been documented (22). The interaction between InlA and E-cadherin is species-specific (23). A proline at position 16 allows interaction between InlA and human E-cadherin, while a glutamic acid at the same position, as observed in the mouse E-cadherin, does not allow InlA binding. A transgenic mouse model specifically expressing the human E-cadherin in the murine intestine allows a more efficient animal infection through the oral route, demonstrating the pivotal role of InlA in the crossing of the intestinal barrier (19).

In *in vitro* polarized cellular systems, lipid rafts are critical for InlA-dependent E-cadherin clustering (24). InlA binding promotes two successive posttranslational modifications in the cytoplasmic tail of E-cadherin: phosphorylation by the host kinase Src, followed by ubiquitylation by the ubiquitin ligase Hakai (25). These events are critical for the recruitment of a clathrin coat via the adaptor Dab2; the coat is stabilized by tyrosine phosphorylation of the clathrin heavy chain, followed by sequential recruitment of the protein adaptor Hip1R, which in turn coordinates recruitment of actin; myosin VI and unconventional myosin VIIa provide the pulling force that finally leads to bacterial internalization (26, 27). Interestingly, the nonmuscle myosin heavy chain IIA is specifically phosphorylated by Src upon *L. monocytogenes* infection and restricts bacterial entry (28). Several other molecules modulate actin association to the E-cadherin cytoplasmic site during *L. monocytogenes* InlA-dependent invasion: β- and α-catenins provide a physical link between E-cadherin and actin filaments during bacterial entry (29, 30), while cortactin and Src participate in the activation of the Arp2/3 complex, a major actin nucleator (31), highlighting the exploitation of adheren junctions and classical E-cadherin endocytosis components by *L. monocytogenes* during invasion of polarized tissues (32, 33). In the intestinal barrier, the constitutive PI 3-kinase activity is required for promoting actin polymerization during *L. monocytogenes* cell entry; in the placenta, PI 3-kinase activity is not constitutive, and InlB is required for PI 3-kinase activation and InlA-mediated cell invasion (34, 35).

InlB/Met-Mediated Entry

InlB was identified as a second *L. monocytogenes* invasion protein (6, 36). InlB allows *L. monocytogenes* entry into nonpolarized epithelial cells *in vitro* (37), and it cooperates with InlA during placental invasion *in vivo* (34, 35). In nonpregnant animals, InlB expression is associated with an increase of necrotic foci in the liver and spleen (38). The C-terminal region of InlB is characterized by the presence of glycine-tryptophan (GW) repeats that favor loose binding to bacterial membrane-tethered lipoteichoic acids (39, 40) and

pedptidoglycan-bound wall teichoic acids (41). At the surface of host cells, the GW repeats mediate binding to the receptor of the globular part of the complement component C1q (42) and to glycosaminoglycans (43). The N-terminal region displays LRRs that are critical for cell invasion (44, 45) and bind the hepatocyte growth factor receptor Met (46) in a species-specific manner (47). Met expression is modulated by epithelial keratins, which promote InlB-mediated *L. monocytogenes* infection of epithelial cells (48). Met is a tyrosine kinase receptor, and its interaction with InlB leads to Met autophosphorylation and recruitment of the protein adaptors Gab1, Shc, Cbl, and CrkII (49–51), which play key roles in the activation of PI 3-kinase (52–54). Production of $PI(3,4,5)P_3$ and its accumulation in lipid rafts leads to Rac1 activation (24, 55) and recruitment of Ena/VASP, WAVE, and N-WASP, which activate the Arp2/3 complex promoting actin polymerization in a tightly regulated manner (56, 57). The serine/threonine kinases mTOR and protein kinase C-α also control actin polymerization downstream of the InlB/Met interaction (58). The host 5′-phosphatase OCRL restricts *L. monocytogenes* entry by reducing $PI(3,4,5)P_3$ levels and actin polymerization at bacterial entry foci (59). Production of PI 4P by type II PI 4-kinases, downstream of the tetraspanin CD81, is also critical for *L. monocytogenes* entry into host cells (60, 61). The PI 3-kinase adaptor Cbl also displays ubiquitin ligase activity and promotes ubiquitylation of Met upon InlB binding, leading to modulation of actin polymerization via clathrin recruitment (62–64). InlB also modulates exocytosis and favors the delivery of the endocytic GTPase dynamin 2 to bacterial entry sites (65). Finally, the septin cytoskeleton is recruited during cell invasion by *L. monocytogenes* in an InlB-dependent manner (66), and it controls the anchorage of Met to the cortical actin cytoskeleton (67–69).

Additional Adhesion/Entry Effectors

L. monocytogenes displays other surface and secreted molecules that can modulate adhesion and entry into host cells by indirectly affecting the surface exposure of InlA or InlB, by behaving as adhesins, by directly binding putative cellular receptors, and/or by activating cellular pathways that lead to actin rearrangements and bacterial engulfment. For example, the internalins InlE, InlG, and InlH support the InlA-dependent-invasion pathway in Caco-2 cells and might modulate the bacterial cell wall organization, consequently affecting InlA exposure (70). On the other hand, InlJ favors bacterial adhesion but not invasion in a specific subset of polarized epithelial cells (71, 72). A role for InlF in cell adhesion and invasion has been detected only upon inhibition of the RhoA/Rho kinase pathway (73, 74).

Several *L. monocytogenes* autolysins modulate cell adhesion and/or entry processes: Ami is involved in cell adhesion (75, 76), and Auto has been implicated in entry (77), while IspC is required for adhesion and/or invasion in a cell line-dependent manner (78). The lipoteichoic acid modifiers GtcA and DltA (79, 80), the lipoprotein LpeA (81), the prolipoprotein transferase Lgt (82), and the lysylphosphatidylglycerol modifier MprF (83) play roles in host cell adhesion or invasion probably by modulating the bacterial surface charge and/or by altering the organization of bacterial surface proteins. The surface protein ActA, involved in cytoplasmic actin-based motility (see below), has been proposed to favor host cell invasion through interaction with heparan sulfate (84, 85). Additional *L. monocytogenes* surface adhesins or invasins include Vip (86, 87), Lap (88), LapB (89), and FbpA (90).

LLO, a secreted cholesterol-dependent pore-forming toxin that is required for *L. monocytogenes* vacuolar escape (see below), is also secreted by extracellular bacteria and induces a transient influx of extracellular calcium within host cells that correlates with increased cell invasion (91). Mitochondrial fragmentation also correlates with the LLO-dependent calcium influx, and it has been proposed that *L. monocytogenes* modulates the bioenergetic state of resting cells to trigger cell invasion (92). LLO has been recently proposed to mediate *L. monocytogenes* entry into epithelial cells in a $Ca2^+/K^+$-, cholesterol-, dynamin-, tyrosine kinase- and actin-dependent but InlA/InlB- and clathrin-independent manner (93, 94). A broad-range phospholipase C of *L. monocytogenes*, PlcB (see below), has been reported to induce a calcium influx required for efficient bacterial internalization in macrophages (95).

THE VACUOLAR STAGE

The modulation of the actin cytoskeleton and the rearrangements of the plasma membrane upon *L. monocytogenes* interaction with its host cell receptors lead to bacterial engulfment and internalization in a membrane-bound vacuole (Fig. 2). In the intestinal epithelium, and particularly in the goblet cells, *L. monocytogenes* does not escape from this compartment and is directly transcytosed to the lamina propria, where the bacteria disseminate systemically (21). In other cell types, *L. monocytogenes* is able to disrupt its containing compartment and translocates to the host cell cytoplasm. The cholesterol-dependent pore-forming toxin LLO, together with two bacterial phospholipases, are the major bacterial effectors controlling *L. monocytogenes* vacuolar escape. *L. monocytogenes* residency and persistence in vacuolar compartments have also been described (96, 97) (see below).

LLO and Vacuolar Disruption

LLO is able to induce hemolysis *in vitro*, and early on, this activity was correlated with *L. monocytogenes* virulence (98). This toxin is encoded by the *hly* gene, located within a pathogenicity island that also encodes other important virulence factors, including two phospholipases, a metalloprotease, the actin polymerizing factor ActA, and the transcriptional activator PrfA (99). Inactivation of the *hly* gene coupled with electron microscopic observations subsequently demonstrated that LLO is required for bacterial escape from internalization vacuoles (100). LLO belongs to the family of cholesterol-dependent cytolysins which also includes perfringolysin O from *Clostridium perfringens* and streptolysin O from *Streptococcus pyogenes* (101). Perfringolysin O studies indicate that a conserved undecapeptide provides a structural conformation for a threonine-leucine pair at the C-terminal region of cholesterol-dependent cytolysins responsible for cholesterol binding (102, 103). Theoretically predicted to form large pores (20 to 80 monomers), based on initial perfringolysin O studies (104), electron microscopy and atomic force microscopy analyses indicate that LLO oligomers actually form arc-like structures that assemble into lineactants, and these heterogenous structures are responsible for membrane disruption and vacuolar rupture (105, 106). Membrane perforation by LLO not only facilitates *L. monocytogenes* translocation to the cytoplasm, but it also controls the vacuolar pH and calcium concentration, delaying the maturation of the bacteria-containing compartment and inhibiting lysosomal fusion (107, 108).

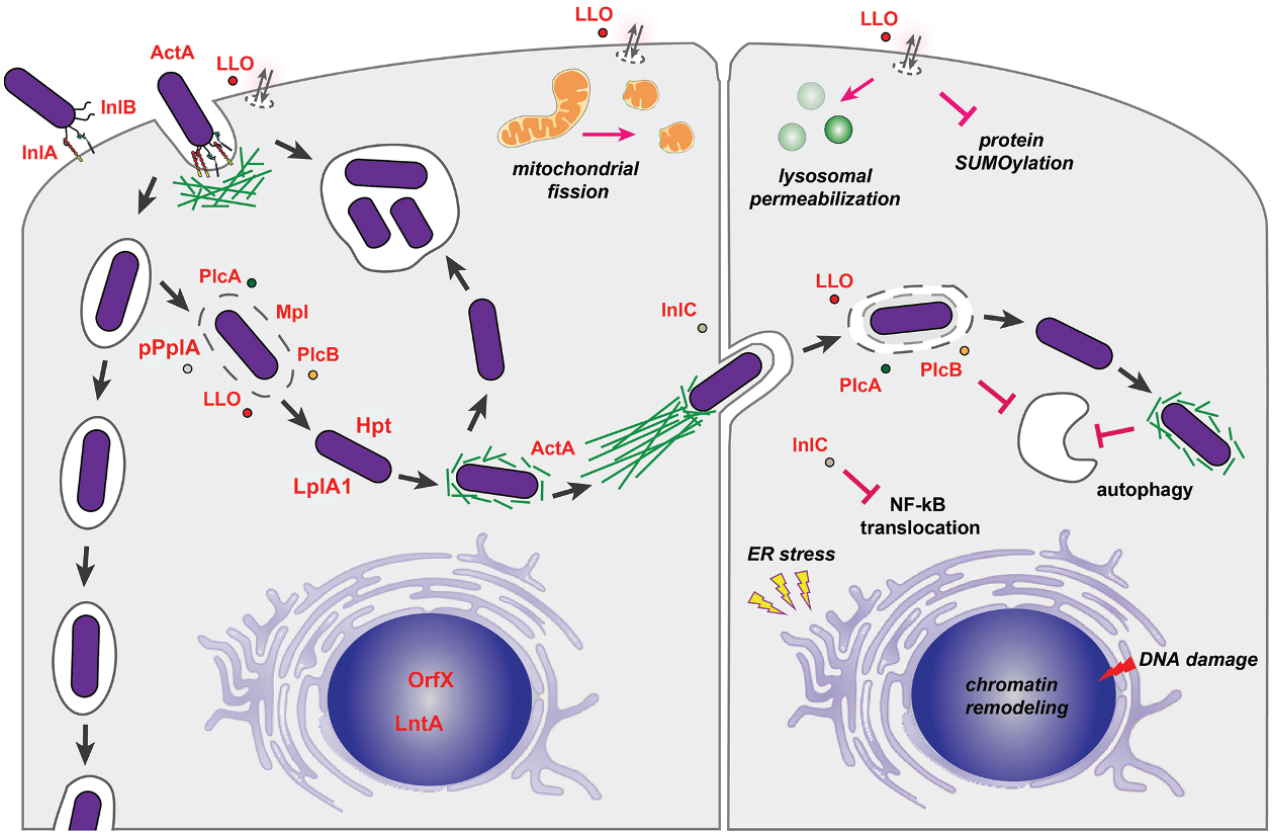


FIGURE 2 *L. monocytogenes* intracellular stages. *L. monocytogenes* is able to induce its entry into nonphagocytic cells mainly via the interaction of InlA and InlB with host cells receptors that promote actin recruitment, remodeling of the plasma membrane, and bacterial engulfment. The surface molecule ActA and the secreted pore-forming toxin LLO have also been implicated in the early *L. monocytogenes* entry steps (left cell, upper left). In goblet cells, upon internalization, *L. monocytogenes* is localized in a vacuole, and through transcytosis the bacterium is translocated to the lamina propria (left cell, left). In other cells, the combined activity of diverse virulence factors, including the pore-forming LLO, the metalloprotease Mpl, the phospholipases PlcA and PlcB, and the pheromone pPplA, favor disruption of the vacuole and *L. monocytogenes* release in the cytosol, where the bacteria takes advantage of host metabolites via the phosphate transporter Hpt and the lipoate protein ligase LplA. The surface protein ActA promotes actin-based motility, and the secreted protein InlC favors reduction of plasma membrane cortical tension, allowing *L. monocytogenes* to form protrusions and to invade neighboring cells. LLO and the phospholipases PlcA and PlcB contribute to the disruption of the double-membrane vacuole (right cell). *L. monocytogenes* has been observed in large spacious compartments that may arise rapidly after internalization of bacteria or upon decrease of ActA expression in already cytoplasmic bacteria (left cell, upper center). Extracellular LLO is able to modulate different cellular functions, including mitochondrial fission, lysosomal permeabilization, protein SUMOylation, ER stress, DNA damage, and chromatin remodeling. The phospholipases PlcA and PlcB, together with actin polymerization by ActA, have been implicated in the resistance to autophagy (195). The secreted molecule InlC prevents NF-κB translocation to the nucleus. Modified from reference 12.

Several physical parameters and host molecules modulate the activity of LLO. Removal of LLO pores from the host cell plasma membrane is mediated by an LLO PEST-like sequence recognized by the clathrin adaptor Ap2a2, favoring pore endocytosis and protection of plasma membrane integrity (109). At 37°C and neutral pH, LLO undergoes denaturation, but it is in a stable conformation at acidic conditions (105). Consequently, within mammalian hosts, the LLO pore-forming activity is compartmentalized to slightly acidified bacteria-containing compartments (110, 111). Cytoplasmic LLO activity, which is cytotoxic to host cells and detrimental to intracellular *L. monocytogenes* (112), is limited by translational regulation of LLO synthesis (113) and by cytoplasmic LLO degradation by the ubiquitin system (114). LLO is activated by reducing agents (115), and within the vacuole of macrophages, the γ-interferon-inducible lysosomal thiol reductase is responsible for reducing and activating LLO (116). The increase in vacuolar chloride concentration mediated by the cystic fibrosis transmembrane conductance regulator has been proposed to enhance LLO oligomerization and *L. monocytogenes* cytoplasmic escape (117). LLO-disrupted vacuoles trigger the recruitment of the protein kinase C (PKC) ε, suggesting that this enzyme is involved in the recognition of damaged membrane organelles (118).

Phospholipases PlcA/PlcB and Vacuolar Disruption

L. monocytogenes secretes two phospholipases, a phosphatidylinositol-specific phospholipase C named PlcA (119) and a broad-range phospholipase C/sphingomyelinase named PlcB (120, 121), which is activated by the metalloprotease Mpl (122–124). Both enzymes have been shown to contribute to *L. monocytogenes* escape from primary vacuoles and

from secondary vacuoles during bacterial cell-to-cell spread (4, 125–128).

PlcA from *L. monocytogenes* is the only bacterialphosphatidylinositol-specific phospholipase C that lacks a Vb β-strand that increases activity toward glycosylphosphatidylinositol-anchored proteins (129); interestingly, expression of this β-strand in PlcA impairs bacterial escape from vacuoles and cell-to-cell spread (130), suggesting that an *L. monocytogenes* adaptation to the intracellular environment requires reduced activity against glycosylphosphatidylinositol-anchored proteins. It has been proposed that PlcA translocating via LLO pores reaches the host cell cytoplasm, and in this compartment PlcA cleaves intracellular phosphatidylinositol into inositol phosphate and diacylglycerol (131); production of diacylglycerol, which might also take place through activation of host phospholipases C and D in an LLO-dependent signaling pathway (132), leads to activation of PKC βI and βII, which are required for vacuolar disruption (133). Because both PKC βI and βII are recruited to the *L. monocytogenes* internalization vacuole (134), it is speculated that the phosphorylation of PKC βI and βII targets at the surface of the bacteria-containing compartment is critical for a still unidentified signaling cascade leading to vacuolar rupture (133).

PlcB maturation and activation by the metalloprotease Mpl requires acidification of the vacuole (135, 136), and as has been observed for LLO, compartmentalization of this phospholipase C activity is critical for intracellular survival of *L. monocytogenes* (137). Both PlcA and PlcB have been found to activate NADPH oxidase during *L. monocytogenes* infection, which might be harmful to internalized bacteria via the production of reactive oxygen species; however, modulation of vacuolar maturation by LLO restricts NADPH oxidase localization to the *L. monocytogenes*-containing compartments (138).

Additional Mechanisms Regulating Vacuolar Disruption

Several other bacteria- and host-related mechanisms have been proposed to modulate the *L. monocytogenes* vacuolar stage. A recent study indicates that *L. monocytogenes* secretes a pheromone, pPplA, that triggers the production of an unknown factor that cooperates with LLO in facilitating vacuolar disruption (139): pPplA is processed from the N-terminal secretion signal sequence of the lipoprotein PplA; pPplA is secreted, accumulates in the space of the *L. monocytogenes* vacuole, and is then exported by the CtaP peptide transporter; cytoplasmic pPplA induces the production of a factor that accelerates vacuolar disruption mediated by LLO in a still unidentified manner (139).

Modulation of bacterial gene expression by "reversible lysogeny" has also been proposed to modulate *L. monocytogenes* vacuolar escape (140). The prophage A118 is inserted within the coding region of the gene *comK*, a master regulator of competence genes that are normally not expressed by *L. monocytogenes*; interestingly, during the bacterial vacuolar stage, A118 is excised, and this event allows reactivation of *comK* and expression of the competence machinery by *L. monocytogenes*; by a still unknown mechanism, the competence system promotes efficient bacterial translocation to the host cell cytoplasm. In this environment, the phage reinserts into *comK* (140).

Additional host factors have been reported to control *L. monocytogenes* vacuolar residency. Rab5a was shown to control the accelerated maturation of *L. monocytogenes*-containing vacuoles (141, 142); the product of the gene *Lmo*2459 was subsequently shown to induce the specific ADP ribosylation of Rab5a, inhibiting its activation and reverting its bactericidal functions (143). The activity of the cytosolic cysteine protease calpain has been shown to be required for efficient *L. monocytogenes* vacuolar escape, but the targets of this protease remain to be identified (144).

THE CYTOPLASMIC STAGE

By translocating from the vacuolar stage toward the cytoplasm, *L. monocytogenes* escapes cellular degradative mechanisms associated with phagosomal pathways. On the other hand, *L. monocytogenes* must adapt its metabolism to nutrients and metabolites found in this novel intracellular compartment and must also escape from additional innate immunity defenses including autophagy. The hexose phosphate transporter Hpt and the actin-polymerizing surface protein ActA play key roles in the survival of *L. monocytogenes* in the host cell cytoplasm.

Utilization of Host Metabolites

Glucose-1-phosphate is the primary degradation product of glycogen and is broadly available within mammalian cells. The observation that *L. monocytogenes* uses glucose-1-phosphate as a carbon source in a PrfA-dependent manner suggested that related hexose phosphates could be important growth substrates for intracellular bacteria (145). *In silico* analysis of the *L. monocytogenes* genome identified the gene *hpt* as encoding a hexose phosphate transporter responsible for the uptake of glucose-6-phosphate in the cytoplasm of host cells, playing a key role in *L. monocytogenes in vivo* virulence (146). A subsequent screen for identification of additional genes required for bacterial intracellular replication recognized lplA1 as a lipoate protein ligase that could potentially use host-derived lipoic acid to modify bacterial substrates (147). LplA1 was later confirmed to be essential for intracellular growth of *L. monocytogenes* under limiting concentrations of available small mammalian lipoylated peptides (148). A genetic screen led to the discovery that the menaquinone synthesis intermediate 1,4-dihydroxy-2-naphtoate is required for *L. monocytogenes* cytosolic survival, but full-length menaquinone is not (149).

Cytoplasmic Innate Immune Responses

Autophagy is a cellular mechanism responsible for protein turnover and removal of abnormal or superfluous subcellular components. The pioneering work of Rich et al. (150) demonstrated that cytoplasmic and metabolically arrested *L. monocytogenes* can be targeted for destruction by the autophagic machinery. Different mechanisms have been proposed to participate in the active escape of cytoplasmic *L. monocytogenes* from autophagy: polymerization of actin by the surface protein ActA favors cytoplasmic motility and avoidance of autophagosomes (151); polymerized actin or Arp2/3 sequestering by ActA may also act as a protective physical barrier preventing the accumulation of signaling molecules (i.e., ubiquitin) that are required for autophagy activation (152, 153). PlcA and PlcB have also been implicated in autophagosomal avoidance (151, 154, 155), and recent studies suggest that these PLCs decrease cytoplasmic levels of PI 3P, causing stalling of preautophagosomal structures and preventing efficient targeting of cytosolic bacteria (156). The surface internalin InlK has also been proposed to recruit the major vault protein and to protect cytoplasmic *L. monocytogenes* from autophagic recognition (16), but these

results have been recently challenged using a different *L. monocytogenes* strain (157).

Cytoplasmic *L. monocytogenes* secretes small molecules leading to activation of an IRF3-dependent cytosolic pathway, resulting in type I interferon activation (158). One of these small molecules, cyclic-di-AMP, is sufficient to activate production of interferon β in macrophages (159). Sensing of tri-phosphorylated RNA via RIG-I and a MAVS-dependent pathway triggers type I interferon production in epithelial cells (160, 161). *L. monocytogenes* cytoplasmic DNA is recognized through STING, TBK1, IRF3, and IRF7, leading to the upregulation of the di-ubiquitin-like protein ISG15 and ISGylation of endoplasmic reticulum (ER) and Golgi proteins, which correlate with increased secretion of cytokines that counteract infection (162). *L. monocytogenes* also activates the type III interferon pathway (163).

Persistence

It is increasingly recognized that bacterial pathogens may persist within host tissues in a dormant state that allows resistance to antibiotics and subsequent reinfection. In macrophages of severe combined immunodeficient (SCID) mice, *L. monocytogenes* can persist in large compartments termed spacious *Listeria*-containing phagosomes (SLAPS), which are formed in an LLO-dependent manner (96). LC3-associated phagocytosis has been proposed to precede the formation of SLAPS (164). A recent study indicates that in epithelial cells, cytoplasmic *L. monocytogenes* bacteria in which ActA production is halted are trapped in acidic vacuoles that are not associated with classical autophagosomal markers and in which bacteria enter a viable but nonculturable state (97). These studies indicate that *L. monocytogenes* may persist in different host cellular populations, favoring the asymptomatic carriage of this pathogen.

CELL-TO-CELL SPREAD

Actin-based motility allows *L. monocytogenes* not only to escape autophagy but also to reach neighboring cells within infected tissues, favoring cell-to-cell spread and bacterial dissemination in organs, avoiding exposure to humoral immunity. Motile *L. monocytogenes* first induces the formation of a membrane protrusion in the primary infected cell that is accompanied by membrane internalization in the neighboring bystander cell, leading to bacterial entrapment in a double-membrane vacuole that is then disrupted (9). Several bacterial virulence factors, including ActA, the internalin InlC, the pore-forming toxin LLO, and the phospholipases PlcA and PlcB, participate at different stages of *L. monocytogenes* cell-to-cell spread.

Cytoplasmic Actin-Based Motility

The surface protein ActA is sufficient to trigger actin polymerization at the surface of *L. monocytogenes* (7). The central region of ActA contains four short proline-rich repeats that bind members of the enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) family; these molecules contribute to the persistence of speed/directionality of bacterial movement by recruiting profilin, which provides polymerization-competent actin monomers (165). The N-terminal region of ActA recruits the Arp2/3 complex which drives actin nucleation (166, 167). The Arp2/3 complex is formed of seven subunits, and it has been traditionally considered a single molecular entity (168). A genome-wide small interfering RNA screen demonstrated that different Arp2/3 complexes are required to control *L. monocytogenes* cell invasion and actin-based motility: the Arp2, Arp3, ARPC2, and ARPC3 subunits are conserved, but the ARPC1B subunit is only involved in cell invasion, while the ARPC1A subunit is required for actin-based motility, and the ARPC4 subunit is dispensable for cell invasion, while the ARPC5 subunit is dispensable both for cell invasion and actin-based motility (169). Multiple actin cross-linking proteins, actin filament-capping or -severing proteins, and protein scaffolds are recruited to the *L. monocytogenes* actin tail (170). Cryo-electron tomography of actin tails has demonstrated that actin bundling is critical for ensuing actin-based motility (171).

Cortical Actin Rearrangements and Protrusion Formation

In mammalian tissues, cortical membrane tension represents a physical barrier for motile *L. monocytogenes*, inhibiting the deformation of the plasma membrane into protrusions. InlC, a secreted member of the internalin family devoid of a cell wall anchoring motif (13), perturbs apical cell junctions by interacting with the protein adaptor Tuba, inhibiting the recruitment of the actin regulatory protein N-WASP and COPII proteins and therefore relieving cortical membrane tension favoring *L. monocytogenes* protrusion formation (14, 172, 173). The downregulation by *L. monocytogenes* of the small GTPase Cdc42, another Tuba interactor, is also required for efficient protrusion formation (174). Within the protrusion, the membrane-cytoskeletal linker ezrin has been proposed to support the formation and stabilization of protrusions (175). Arp2/3 drives actin polymerization at the proximal *L. monocytogenes* rear-end within protrusions, but at distal locations the recruitment of Rho GTPases activate diaphanous-related formins which promote the formation of unbranched actin filaments (176). Inhibition of actin polymerization by components of the AIP1-dependent actin disassembly machinery (177) and ActA processing by the metalloprotease Mpl (178) are proposed mechanisms for the resolution of membrane protrusions into double membrane vacuoles. Efficient cell-to-cell spread can be facilitated by the exofacial exposure of phosphatidylserine at the tip of protrusions promoted by the pore-forming activity of LLO, which leads to phosphatidylserine binding by the TIM-4 receptor in macrophages and protrusion internalization (179).

Lysis of Secondary Vacuoles

Internalization of *L. monocytogenes*-induced protrusions into neighboring bystander cells leads to bacterial localization within a double-membrane compartment (9). Initial studies suggested that phospholipases PlcA and PlcB, together with LLO, contributed to cell-to-cell spread (4, 125, 127), and a more specific contribution of PlcB to double-membrane vacuolar rupture was suggested (128). A current model proposes that PlcA and PlcB contribute to the disruption of the inner membrane of the spreading vacuole, while LLO participates more precisely in the disruption of the outer membrane of this vacuole (180). Bacterial translocation to the cytoplasmic space of neighboring cells allows *L. monocytogenes* to start a new infection process.

MODULATION OF CELLULAR, ORGANELLAR, AND NUCLEAR FUNCTIONS

L. monocytogenes is able to modulate a broad range of cellular functions, even before being internalized within host cells. The pore-forming toxin LLO, which plays a major role in

vacuolar escape (see above) is able to modulate from the extracellular space the function of mitochondria, the ER, lysosomes, protein posttranslational modifications, and DNA stability. Several bacterial nucleomodulins have been identified which directly affect the transcription of host genes involved in the control of immune responses.

LLO Influence on Mitochondria

Mitochondria are critical organelles involved in the generation of chemical energy in eukaryotic cells. As mentioned above, extracellular LLO triggers the influx of calcium, which leads to transient fission of mitochondria, triggering a bioenergetic change of host cells that is beneficial for *L. monocytogenes* host cell invasion (92). Interestingly, atypical mitochondrial fission through a Drp1-independent fragmentation process has been associated with *L. monocytogenes* cellular infection (181).

LLO Influence on the ER

The unfolded protein response is a signaling cascade that maintains the function of the ER under stress conditions. *L. monocytogenes* activates the unfolded protein response in an LLO-dependent manner prior to bacterial entry into host cells (182). The induction of ER stress by drugs such as thapsigargin or tunicamycin leads to a decrease in bacterial intracellular numbers, suggesting that the unfolded protein response represents an innate immune response to bacterial infection (182).

LLO Influence on Lysosomes

The integrity of lysosomes has been shown to be compromised by extracellular LLO, which induces permeabilization and release of lysosome content, including cathepsins, which remain transiently active in the cytoplasm (183). The functional significance of this finding for *L. monocytogenes* infection and survival remains to be identified.

LLO Influence on Protein Posttranslational Modifications

Posttranslational modifications allow the rapid modification of the activity of cellular proteins. Sumoylation is an essential posttranslational modification that is impaired by *L. monocytogenes* through the proteasome-independent degradation of the E2 enzyme Ubc9 following calcium influx mediated by extracellular LLO (184). The downregulation of cellular protein sumoylation, together with the proteasome-dependent degradation of some sumoylated proteins, favors bacterial infection *in vitro* and *in vivo* (184). Histone modifications are also associated with the *L. monocytogenes* infection process (185) (see below).

LLO Influence on DNA Stability

L. monocytogenes modulates general DNA stability in host cells in different manners. *L. monocytogenes* induces DNA strand breaks and simultaneously blocks the DNA damage response through degradation of the sensor Mre11 in an extracellular LLO-dependent manner (186), promoting a cell cycle delay that favors bacterial intracellular replication (187). Interestingly, it has been also reported that LLO-induced calcium influx leads to the proteasomal degradation of the human telomerase reverse transcriptase, an event that is detrimental to bacterial replication (188).

Bacterial Influence on Gene Expression

LntA is the first nucleomodulin discovered in *L. monocytogenes* (189): it targets the chromatin repressor BAHD1 and fine-tunes transcription of interferon-stimulated genes, which is required for efficient *in vivo* infection (190, 191). More recently, the nucleomodulin OrfX has been shown to interact and decrease the levels of the regulatory protein RybP, dampening the oxidative response in macrophages probably through modulation of host transcriptional responses (192). The secreted internalin InlC interferes with innate immune responses by targeting the IκB kinase subunit IKKα, inhibiting NF-κB translocation to the nucleus (15). LLO modulates gene transcription with opposite effects for infection: LLO induces a dramatic dephosphorylation of histone H3 and deacetylation of histone H4 that leads to reduced transcriptional activity of key immunity host genes (185); LLO has also been shown to modulate the functionality of the promyelocytic leukemia protein nuclear bodies, activating a signaling response that decreases *L. monocytogenes* infection (193). Finally, an InlB/PI 3-kinase pathway is required for the SIRT2-dependent deacetylation of histone H3 on lysine 18, which is involved in efficient bacterial infection *in vitro* and *in vivo* (194).

CONCLUSIONS

The study of the interactions of *L. monocytogenes* with eukaryotic host cells during bacterial invasion, intracellular growth, and cell-to-cell spread has proven to be fundamental to better understanding the exquisite adaptation of this bacterial pathogen to mammalian hosts. Indeed, *L. monocytogenes* is able to hijack multiple cellular functions including receptor signaling, membrane trafficking, cytoskeletal rearrangements, organellar dynamics, DNA stability, and gene transcription. The work reviewed in this article also highlights that *L. monocytogenes* is an extraordinary tool to manipulate and to unravel host cell signaling cascades, in particular, innate immune responses that allow us to expand our understanding of the control of bacterial intracellular infections.

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Metabolism of the Gram-Positive Bacterial Pathogen *Listeria monocytogenes*

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54

METABOLIC POTENTIAL

The bacterial pathogen *Listeria monocytogenes* is well adapted to life in the soil and on vegetation as a saprophyte (i.e., extracellular environments) and to life in the cytosol of mammalian cells as a pathogen (i.e., intracellular environment). These environments differ greatly in their metabolite repertoires (e.g., carbon, sulfur, and nitrogen sources) and abundance (e.g., amino acid availability). Therefore, *L. monocytogenes* requires niche-specific adaptations to support growth and uses metabolic cues to trigger virulence mechanisms, such as the master virulence regulator, PrfA (1). The complete metabolic potential of *L. monocytogenes* has been inferred bioinformatically from genomic (2–5) and transcriptomic studies (1, 6–8), as well as experimentally using both defined media as a test for auxotrophy (9–12) and more recently by isotopologue metabolomics (13, 14). While these studies have certainly taught us much about *L. monocytogenes*' metabolic potential, it is important to note that, given the wide array of environments *L. monocytogenes* is capable of inhabiting, our understanding of its metabolic potential and how strain to strain variation might affect this potential, is incomplete.

The first comparative genomic study of *L. monocytogenes* (EGD-e) and *Listeria innocua* (CLIP 11262) demonstrated that complete glycolysis, pentose phosphate, respiratory chain, and multiple fermentation pathways were present in both pathogenic and nonpathogenic *Listeria* isolates (2). In contrast, an incomplete tricarboxylic acid (TCA) cycle was documented due to the lack of α-ketoglutarate dehydrogenase in both organisms, while biosynthetic pathways for vitamins including riboflavin, biotin, thiamine, and lipoate were absent or incomplete, consistent with observed auxotrophies (9). Finally, biosynthetic pathways for all amino acids were present, despite a variety of reported amino acid auxotrophies in various growth conditions, suggesting not surprisingly, that regulation of these metabolic pathways under different growth conditions leads to conditional auxotrophies. Since this original comparative genomics study, more than 100 additional *L. monocytogenes* genomes have been sequenced in addition to many other non-*monocytogenes Listeria* species (4, 15). The core genome of *L. monocytogenes* comprises 1,791 genes, with estimates of up to 6,500 genes in the pan genome of all *Listeria* species, 54% of which are predicted to be dedicated to metabolism in one way or another, highlighting the potential diversity of metabolic adaptations of different *Listeria* species and even potentially different *L. monocytogenes* strains to different environments (3, 4). Defining how all of these accessory metabolic pathways contribute to the colonization of niches, either environmental or host, will be of critical importance over the coming years.

Multiple efforts at developing a minimal medium to define *L. monocytogenes* metabolic requirements have been attempted over the years, dating back to initial efforts by Welshimer in the early 1960s (9). Different strains and different media have yielded some consistent findings and some that are confounding. As noted above, and consistent with genetic analysis, *Listeria* is auxotrophic for a variety of essential vitamins, including thiamine, lipoate, biotin, and riboflavin, which *L. monocytogenes* cannot produce *de novo* (9–11). In addition, multiple growth experiments in minimal defined media have demonstrated that *L. monocytogenes* requires the supplement of methionine and cysteine for growth (i.e., auxotrophic to Met and Cys). Moreover, addition of branched chain amino acids (BCAAs; lle, Leu, and Val), arginine, histidine, phenylalanine, and tryptophan substantially enhance *L. monocytogenes* growth in minimal media, while glutamine can serve as a nitrogen source (which can be replaced with ammonium or ethanolamine) (10–12, 16, 17). The requirement for Met and Cys is explained by the fact that most *L. monocytogenes* genomes are missing the gene(s) for reducing sulfate to sulfide, which is further condensed with *O*-acetylserine to form cysteine (2, 12). Supplementing *L. monocytogenes* bacteria with thiosulfate, which can be alternatively condensed with *O*-acetylserine to form cysteine, does not support *L. monocytogenes* growth, nor does the addition of methionine, indicating the lack of the transsulfuration pathway (converting methionine to cysteine) (12). Notably, strain-specific requirements for Cys and Met are observed. For example, the EGD-e strain requires only cysteine, without the addition of methionine, while the 10403s strain requires both, suggesting a disruption in one or more of the *met* genes, whose gene products convert cysteine to methionine (*metI*, *metC*, and *metD*). The requirement for BCAA, arginine, histidine, phenylalanine, and tryptophan in defined media is more difficult to explain, because *L. monocytogenes* genomes encode for all the biosynthesis genes of these amino acids (more details below). The

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addition of BCAA and arginine appears to be essential for efficient growth, whereas histidine, tryptophan, and phenylalanine are not essential but can improve the growth of distinct *L. monocytogenes* strains in chemically defined media (10–12, 16).

With respect to carbon metabolism, as a facultative aerobe *L. monocytogenes* can both ferment glucose and respire when oxygen is present. *L. monocytogenes* catabolizes glucose through the classical glycolytic (Embden-Meyerhof-Parnas) pathway to pyruvate and generates ATP through substrate-level phosphorylation. Pyruvate can be shunted to acetate, acetoin, and lactate (18) or converted to acetyl-CoA by pyruvate dehydrogenase complex or pyruvate formate lyase. Under anaerobic conditions, fermentation favors the formation of ethanol and formate, and acetyl-CoA can be broken down to produce acetate and ethanol (18). Acetyl-CoA can be funneled into the oxidative branch of the TCA pathway to generate amino acid precursors (19). It is predicted that two putative type II NADH dehydrogenases (*lmo2389* and *lmo2638*), glycerol-3-phosphate (*glpD*), and formate dehydrogenases (*lmo2586*) may shuttle electrons to either cytochrome *bd* oxidase or cytochrome aa_3 oxidase via menaquinone (MK) (20). Electron transfer through the electron transport chain generates a membrane potential and drives oxidative phosphorylation of ATP by F_0F_1-ATPase. In the absence of oxygen or disruption of the electron transport chain, *L. monocytogenes* displays decreased growth, likely due to less ATP generation (20, 21). Interestingly *L. monocytogenes* is missing several key TCA enzymes—α-ketoglutarate dehydrogenase, succinyl-CoA synthase, and malate dehydrogenase (2)—and therefore exhibits a branched variant of the TCA cycle. *L. monocytogenes* is also unable to bypass these steps using the glyoxylate shunt, though it has been proposed that succinate could be generated by the γ-aminobutyric acid shunt (22). The only way to replenish oxaloacetate is through anapleurosis, using pyruvate carboxylase to convert pyruvate and CO_2 to oxaloacetate (23, 24).

LIFE AS A SAPROPHYTE

While *L. monocytogenes* has been extensively studied as a both a human and a livestock pathogen for decades, significantly less is known about its life in the environment as a saprophyte. Seminal early studies by Welshimer demonstrated that *L. monocytogenes* was capable of surviving on soil (25), while a series of studies in the intervening decades demonstrated the ubiquity of *L. monocytogenes*. *L. monocytogenes* has been isolated from environmental samples ranging from uncultivated fields and meadows, where the incidence ranges from 5 to 50% (26, 27), to cultured fields and dairy farms, where the incidence is consistently lower, ranging from 3 to 24% (27–29). Its prevalence varies not only by geography and soil type but also temporally, seasonally, and with meteorological conditions. Importantly, in all of these studies while the frequency of positive soil samples was relatively high, the abundance of *L. monocytogenes* in these samples was very low and required specific enrichment techniques to facilitate identification (30). Not surprisingly, given the diversity of locations from which *L. monocytogenes* can be cultured, a variety of soil types have been demonstrated to support the maintenance of *L. monocytogenes*. In one study, 100 soil samples were tested, and all were capable of maintaining *L. monocytogenes* survival for at least 14 days, while 71% were able to support growth for longer than 80 days (31). A variety of physiochemical characteristics, ranging from water content and pH were identified as contributing to *L. monocytogenes* survival in soil. Finally, the abundance of competing microorganisms is a major determinant in the success of soil colonization by *L. monocytogenes*, as evidenced by the increased growth of *L. monocytogenes* in a variety of autoclaved soils relative to nonautoclaved controls (25, 27, 32). Given the diversity of soil types and geographic locales in which *L. monocytogenes* can be cultured from soil, it is not surprising that there is little information regarding metabolic adaptations required for life in the soil. Much of what we do know about metabolism under saprophytic conditions comes from genomic and transcriptomic studies. For example, the *L. monocytogenes* EGD-e genome encodes over 330 putative transporters, which is 2 and 3 times more transport genes than either *Escherichia coli* or *Bacillus subtilis*, respectively (2). Of these transporters, 84 assemble into 29 complete phosphoenolpyruvate-dependent phosphotransferase systems (PTSs) (33). Despite this abundance of PTSs, they are downregulated during infection and are dispensable for intracellular replication in macrophages, suggesting that they likely have a role facilitating the use of varied carbon sources in the nutrient poor environment (33, 34). Both transport and metabolic genes are overrepresented in both the core genome and accessory genomes (3, 35, 36). In addition to genomic studies suggesting that extreme diversity in transport and metabolic systems likely facilitates survival in diverse environments, a limited number of transcriptomic studies have begun to elucidate gene expression changes that facilitate growth and survival in the soil. In one study, although many genes were differentially regulated at any given time point following introduction of *L. monocytogenes* into the soil, only 94 genes were specifically upregulated at all time points assessed (15 min, 30 min, and 18 hours). Of these 94, 37 genes were involved in various PTS transport systems, while an additional 26 were involved in other metabolic processes, including chitinases and β-glucosidases (37). Similarly, consistent with the limited availability of amino acids, 15 genes of the regulon of CodY, a repressor of BCAA biosynthesis, were upregulated at all time points following instillation of *L. monocytogenes* into soil (37). In a follow-up study with different soil, upregulation of PTS systems was also observed, including those for cellobiose, mannitol, and maltose, among others (38). Finally, both of these studies observed repression of genes regulated by the master virulence regulator, PrfA, consistent with the longstanding observation that in the presence of PTS substrates, PrfA expression is repressed (39).

PrfA also reciprocally regulates the PTS system as Hpr levels are lowered *in vivo* (40), and constitutive PrfA expression results in impaired growth on glucose (14) and impaired fitness in the soil (41). Thus, while *L. monocytogenes* is ubiquitous in the environment, and its core genome accommodates an expanded repertoire of metabolic and transport capabilities to facilitate growth in these diverse environments, our understanding of the metabolic adaptation to any specific nonhost environment remains rather limited.

LIFE INSIDE THE HOST

As a foodborne opportunistic pathogen, *L. monocytogenes* primarily enters host organisms by passage through the gastrointestinal tract (42). For most immunocompetent individuals, commensal microbes may provide a first-line of defense against *L. monocytogenes* colonization and infection, at least in part by competing for available nutrients (43).

L. monocytogenes is competent for growth in anaerobic environments such as the intestinal tract, and a forward genetic screen identified genes encoding components of the F_0F_1-ATPase as essential for anaerobiosis (20). An additional aspect of energy metabolism relevant in the intestinal tract is the recently discovered flavin-based extracellular electron transfer (EET) locus (44). EET supports growth on nonfermentable carbon sources by mediating electron transfer to extracellular acceptors; an EET mutant exhibited a competitive defect within the mouse gastrointestinal tract (44). From within the gut, *L. monocytogenes* is able to traverse the intestinal epithelium to establish an intracellular infection (45), where its intracellular metabolism has been extensively studied.

L. monocytogenes doubles in the host cytosol approximately every 40 min, demonstrating its exquisite metabolic adaptation to the cytosolic niche. Given that cytosolic replication is essential for virulence, metabolism during intracellular growth has been extensively studied. Traditional genetic and biochemical studies of *L. monocytogenes* virulence, coupled with newer transcriptomic and metabolomics approaches, have been used to understand both host and bacterial metabolism during infection. Surprisingly, a comprehensive analysis of the nutritional availability of the cytosol has not been performed and may differ in different cell types. However, based on the observation that only cytosol-adapted pathogens can fully exploit the cytosol as a replication niche, it has been hypothesized that the cytosol is a nutrient-limiting environment (46, 47). The cytosol is a highly reducing (48), neutral pH environment (49) that is low in free amino acids (50) and free metal ions such as magnesium, sodium, calcium, and iron (51). Despite this nutrient-limiting environment, *L. monocytogenes* must acquire nutrients from its host to facilitate replication and must do so in a way that both promotes survival of the host cell and minimizes detection of the pathogen by the innate immune system (52, 53). In addition, *L. monocytogenes* has evolved a metabolic network that not only utilizes nutrients for replication but also integrates signals regarding nutrient availability to regulate virulence gene expression once inside the cell (39, 54–56).

Intracellular Carbon Metabolism

Carbon utilization is one of the most well-described aspects of *L. monocytogenes* cytosolic metabolism where hexose-phosphates and glycerol are the primary carbon sources scavenged from the host. Hexose-phosphates are acquired through the use of a dedicated permease Hpt (gene *uhpT*), which is both required for virulence (55) and directly regulated by the master virulence regulator, PrfA, upon entry into the host cytosol (57). Based on isotopologue metabolomic studies in infected cells, host-derived glucose-6-phosphate is primarily shunted into the pentose phosphate pathway, where it is used to build nucleotides and aromatic compounds but likely contributes little from an energy perspective (24). In contrast, glycerol, the second major intracellular carbon source for *L. monocytogenes*, is the primary energy source during intracellular replication (13). Glycerol does not significantly contribute to gluconeogenesis but, instead, feeds into lower glycolysis and the TCA cycle, and as such it is also used in *de novo* synthesis of some amino acids (13). As expected given its critical role in energy generation and anabolism, glycerol utilization mutants (*glp* and *dha* genes) are also attenuated and defective for intracellular replication (54, 58). Despite the key role of both glycerol and hexose phosphates in *L. monocytogenes* cytosolic metabolism, a combinatorial mutant that can use neither glucose-6-phosphate nor glycerol (Δ*C3*Δ*uhpT*) was still partially able to replicate in macrophages (13), albeit at significantly decreased levels, suggesting that alternative carbon sources are likely available during intracellular replication. Some evidence indicates that lactate, but not pyruvate, could be used as an additional carbon source during cytosolic replication, while amino acids are unlikely to be a major source of carbon and energy generation (13). *L. monocytogenes* lacks genes required for beta-oxidation of fatty acids, likely ruling fatty acids out as another potential carbon source for *L. monocytogenes* in the cytosol (2). However, the breakdown of phosphatidylethanolamine in mammalian membranes could liberate not only glycerol, but also ethanolamine (59, 60), which can be converted into acetaldehyde and ammonia by adenosylcobalamin-dependent ethanolamine lyase (encoded by *eut*) (17). Consistent with phospholipids contributing to carbon metabolism during cytosolic replication, *L. monocytogenes eut* mutants are defective for intracellular replication in epithelial cells (58). Thus, *L. monocytogenes* has diversified the carbon sources that it scavenges from the host cell, likely to promote host cell survival and prevent activation of innate immune defense mechanisms.

Despite the presence of *de novo* synthesis pathways for most amino acids, *L. monocytogenes* relies on both *de novo* synthesis and uptake of amino acids from the host for the promotion of full virulence. Specifically, during intracellular growth in macrophages, *L. monocytogenes* synthesizes *de novo* BCAA, histidine, and arginine, likely reflecting the shortage of these amino acids in the macrophage cytosol. Consistent with this observation, mutants deleted in genes central to these amino acid biosynthetic pathways exhibit intracellular growth defects (56, 58). Additionally, ^{13}C-isotopologue profiling of intracellular *L. monocytogenes* using ^{13}C-glucose demonstrated that *L. monocytogenes de novo* synthesizes many of the other amino acids during intracellular growth (e.g., Ala, Glu, Asp, Ser, Gly, Pro, Thr, and Lys) (13, 14, 24). Nevertheless, despite the importance of *de novo* synthesis, additional ^{13}C-isotopologue profiling with labeled amino acids clearly demonstrated that cytosolic *L. monocytogenes* also imports these amino acids from the host cell, along with most other essential and nonessential amino acids, and that these scavenged amino acids are directly incorporated into newly synthesized proteins with little to no catabolism (13). The one exception is aspartate, which is exclusively synthesized *de novo* (13), consistent with a previous observation that *L. monocytogenes* lacks the ability to take up exogenous aspartate (61). Further demonstrating the importance of amino acid scavenging for intracellular growth, mutants in cysteine, arginine, and glutamine transport systems are each impaired for intracellular replication and attenuated for virulence (61–63), while many other amino acid transporters have yet to be identified and/or characterized. For some scavenged amino acids the amounts required to support the growth of *L. monocytogenes* are consistent with their availability within the host cell cytosol (e.g., glutamine), while for others the requirement exceeds the actual availability (e.g., arginine, cysteine, and methionine) (64). It was therefore speculated that perhaps peptides are acquired from the host cell, where they are then degraded by aminopeptidases for utilization in protein synthesis (65, 66). Indeed, the ability of *L. monocytogenes* to acquire peptides was demonstrated *in vitro* (64, 67, 68), and several peptide transporters contribute to *L. monocytogenes* intracellular growth in macrophage cells or to infection of mice. Among these are the oligopeptide transporter

Lmo0135-0137 (CtaP), which is also associated with cysteine acquisition (3, 61, 69), the oligopeptide transporter OppABCDF (58, 61, 70, 71), and the di-tripeptide transporter, DtpT (72). In addition, the putative peptide transporter Lmo0152 is transcriptionally induced during intracellular growth and therefore may contribute to *L. monocytogenes* pathogenesis (6). Altogether, these studies establish that *L. monocytogenes* relies on both *de novo* synthesis and uptake of amino acids to support intracellular growth (13, 14, 24, 56, 73).

Intracellular Vitamin Metabolism

As previously noted, *L. monocytogenes* is auxotrophic for a variety of essential cofactors, including thiamine, lipoate, biotin, and riboflavin (10, 11), and must acquire them from the host. Biotin and riboflavin scavenging are likely mediated by the putative biotin (BioMNY) and riboflavin transporters (RibU and the ECF complex), respectively (74–76). Interestingly, although *L. monocytogenes* is unable to synthesize thiamine *de novo*, due to the absence of the 4-amino-hydroxymethyl-2-methylpyrimidine phosphate (HMP-P) synthase ThiC, provision of HMP-P/HMP *in vitro* facilitates growth of *L. monocytogenes* in the minimal media lacking thiamine. Furthermore, although the HMP transporter has not been definitively identified, both the thiamine transporter (ThiT) and enzymes for later *de novo* thiamine biosynthesis (ThiD) are required for intracellular replication (77), suggesting that, as is the case with many amino acids, *L. monocytogenes* must concurrently scavenge host-derived thiamine and synthesize additional thiamine from HMP to support robust growth. Finally, *L. monocytogenes* is also auxotrophic for lipoic acid and must acquire it from the host. Two nonredundant lipoate ligase enzymes are required for lipoylation of dehydrogenase complexes, including the pyruvate dehydrogenase complex, branched-chain α-keto acid dehydrogenase (BKD), and glycine cleavage pathway (78). LplA1 is likely the major lipoate host-scavenging enzyme active in the cytosol due to its higher affinity for lipoate (79) and its essentiality for virulence (78, 80). Ultimately, both the pyruvate dehydrogenase complex and BKD are required for virulence, likely explaining the essentiality of LplA1 *in vivo* (21, 81).

Intracellular Fatty Acid Metabolism

Fatty acid synthesis (FAS) is engaged during infection by many Gram-positive bacterial pathogens, due to its role in the biogenesis of phospholipid membranes, lipoproteins, and lipoteichoic acids (82, 83). The FASII pathway used by bacteria, plants, and parasites is structured such that each enzyme of the pathway is encoded by a separate gene that encodes for one protein responsible for the catalysis of a single enzymatic step (84). The initial condensation reaction of the FASII pathway involves short-chain fatty acid thioesters, typically acetyl-CoA and malonyl-ACP (85). This reaction is catalyzed by FabH, with subsequent rounds of condensation and acyl chain elongation catalyzed by FabF. The condensation reactions catalyzed by FabH and FabF result in the formation of β-ketoacyl-ACP products. These products are reduced by FabG to form β-hydroxyacyl-ACP and then dehydrated and reduced to acyl-ACP by FabZ and FabI to complete the cycle (85).

Several key aspects of fatty acid synthesis are relevant to the infectivity and pathogenesis of *L. monocytogenes*. The *L. monocytogenes* FabH enzyme exhibits selectivity toward ante-iso branched chain fatty acid (aiBCFA) precursors (86), resulting in a membrane that is highly enriched in aiBCFA. High aiBCFA content promotes increased membrane fluidity, conferring upon *L. monocytogenes* the unusual property of being able to replicate at refrigeration temperatures (87). Moreover, aiBCFAs contribute to *L. monocytogenes* stress resistance against antimicrobial host mechanisms, including lysozyme and cationic peptides (81). Methicillin-resistant *Staphylococcus aureus*, another Gram-positive pathogen, also displays similar membrane composition, enriched in aiBCFA (88). The high demand for aiBCFA precursors to populate the *L. monocytogenes* membrane results in a reliance on exogenously supplied BCAA, most notably isoleucine, which is the precursor for the predominant membrane BCFA species, despite the capacity for *de novo* synthesis of these amino acids. In defined medium and in the host cytosol, BCAA scavenging is critical for robust replication of *L. monocytogenes* (89). The lipoylated BKD complex is central to conversion of BCAA into the short branched chain thioesters that are the precursors for FASII and is required for virulence (90), likely due at least in part to decreased membrane stress resistance. Thus, *L. monocytogenes* intracellular metabolism is highly dependent on BCAA, particularly isoleucine, not only to provide building blocks for protein synthesis, but also for fatty acid synthesis, which confers resistance to membrane stress.

The Intersection between Metabolism and Virulence

In addition to fulfilling essential metabolic needs for optimal growth, the availability of metabolites such as amino acids and intracellular carbon sources also plays a key role in regulating virulence. As discussed above in the context of environmental PrfA repression, regulation of this essential master virulence regulator is mediated in large part by the presence or absence of carbon sources found in the cytosol of host cells. Both hexose-phosphates and glycerol promote full activity of PrfA, consistent with their function as the primary cytosolic carbon sources (54, 57). In contrast, metabolism of glucose and other PTS substrates not utilized for cytosolic replication actively inhibits PrfA activity, consistent with these PTS systems being dispensable for cytosolic replication (33, 39, 91). Inhibition of PrfA is not mediated by native sugars themselves; instead, one model suggests that uptake and metabolism of PTS sugars induces serine phosphorylation of the phosphocarrier protein Hpr (92) and that Ser-P Hpr directly inhibits PrfA activity (93). Other studies have not observed this effect and instead have proposed that PrfA is regulated by other components of the PTS system (54, 94). PrfA is also responsive to redox conditions in the host cytosol (95, 96), highlighting the importance of this virulence regulator as a key hub for integrating environmental cues with bacterial metabolism.

In addition to carbon source availability, *L. monocytogenes* regulates virulence in response to low BCAA availability and high availability of glutamine as occurs in the host cytosol (56, 62, 97). The response to BCAA is dependent on CodY, a global regulator and a sensor of isoleucine. Whereas under high BCAA conditions, isoleucine-bound CodY acts as a repressor of BCAA biosynthesis pathways, under BCAA-limiting conditions in the cytosol CodY-mediated repression of BCAA biosynthesis is alleviated and isoleucine-unbound CodY instead acts as a transcriptional activator of *prfA*, intimately coupling BCAA sensing to the regulation of virulence gene expression (97). Sensing of BCAA levels is not limited to host BCAA, because a recent study suggests that

BCAA *de novo* synthesis is restricted to sustain virulence gene expression (98). A riboregulator (Rli60), functioning as a ribosome-mediated attenuator, was identified upstream of the BCAA biosynthesis genes that persistently restricts BCAA biosynthesis, even under low BCAA conditions, essentially keeping *L. monocytogenes* starved for BCAA and thus ensuring PrfA expression in the low BCAA environment of the cytosol. In fact, Rli60 was found to be the reason for *L. monocytogenes* partial auxotrophy for BCAA, a phenotype that likely evolved to enable isoleucine to serve as a host signal and effector of virulence (98). In contrast, the mechanism by which L-glutamine regulates virulence gene expression is less clear, because its direct sensor in *L. monocytogenes* is not known, though GlnR is likely a good candidate (62). L-glutamine uptake by *L. monocytogenes* via GlnPQ triggers virulence gene expression only when the internal concentration of L-glutamine in *L. monocytogenes* crosses a certain threshold. Inactivation of GlnPQ arrested L-glutamine uptake and virulence gene expression and led to attenuation of virulence in mice (62). Finally, though not directly tied to PrfA regulation, arginine sensing mediated by the highly conserved ArgR arginine-responsive transcription regulator (99) represses the arginine biosynthesis genes (*arg*C*JBDF* and *arg*G*H*), as well as the arginine deiminase (ADI) pathway (i.e., the ADI genes *arc*A, *arc*B, and *arc*C), the latter of which is required for *L. monocytogenes* acid tolerance and intracellular survival (100, 101). Because both arginine biosynthesis and catabolism genes are regulated during intracellular growth, arginine sensing by ArgR likely plays a key role during *L. monocytogenes* infection (56, 58, 100, 101).

In addition to the classical central metabolic processes required for virulence, many secondary metabolic pathways and their metabolites contribute to virulence both through direct regulation of PrfA and through other, often unknown mechanisms. For example, *S*-adenosylmethionine (SAM) is a methionine precursor that regulates gene expression by binding to SAM-responsive riboswitches (102). The *L. monocytogenes* genome contains seven such riboswitches upstream of Cys-Met biosynthesis genes, which in all cases terminate gene transcription upon SAM binding. Consistent with the theme of tying metabolism to virulence regulation, the prematurely terminated transcripts (i.e., the SAM-bound RNAs) act *in-trans* on *prfA* 5′UTR (via base-pairing), downregulating its transcription specifically under nutrient-rich conditions (102). In contrast, glutathione, an abundant tri-peptide either synthesized by *L. monocytogenes* from cysteine, glutamate, and glycine or acquired directly from the host cytosol, where it is necessary for maintaining redox balance (103), is an allosteric regulator that can increase PrfA activity as much as 150-fold (104, 105). Additionally, glutathione posttranslationally regulates listeriolysin O via *S*-glutathionylation of its conserved cysteine residue, thus rendering it nonactive and minimizing listeriolysin O toxicity in the cytosol and promoting virulence (106). Shikimate biosynthesis, which generates precursors for the biosynthesis of aromatic amino acids (i.e., tyrosine, tryptophan, and phenylalanine), is essential for *L. monocytogenes* intracellular growth (56, 107). However, shikimate biosynthesis is also needed for the generation of both menaquinone (MK) and its biosynthetic precursor 1,4-dihydroxy-2-naphthoate (DHNA), which are also both independently necessary for virulence (21). *L. monocytogenes* genes for the electron transport chain, including genes for MK biosynthesis (*men*) and *L. monocytogenes* cytochrome *bd* and aa_3 oxidases, are induced in cell culture and *in vivo* (6, 70), consistent with a role for the electron transport chain and generation of a membrane potential during infection (21, 66, 107). As discussed previously, utilization of glycerol as a primary energy source requires ATP synthesis through oxidative phosphorylation, at least partially explaining the requirement for MK for virulence. However, the electron transport chain has also been implicated in redox balance (108), oxidative/nitrosative stress responses (109), subcellular localization of cell division machinery (110), nucleotide biosynthesis (111), and solute transport of betaine/carnitine (112) in other bacteria. As such, the requirement for shikimate biosynthesis for virulence is complicated and likely multifactorial, because even the role of MK in infection is not straightforward. To add complexity to ascribing virulence functions to metabolic pathways, it was recently reported that the MK biosynthetic intermediate 1,4-dihydroxynaphthoate (DHNA), is required for virulence and survival in the cytosol independent of full-length MK (21), further emphasizing that the complex portfolio of intermediate products generated through metabolism may be a rich source for signaling and virulence regulation during infection.

Bacterial Metabolism and the Host Immune Response

The metabolic potential of bacterial pathogens such as *L. monocytogenes* is robustly adapted to promote survival and replication within the host, but the highly conserved nature of many products of bacterial metabolism presents an Achilles heel that can be recognized by the host immune system. The capability of the innate immune system to sense microbial ligands has been extensively reviewed elsewhere (113), with the most well-studied paradigm being recognition of bacterial products such as lipopeptides by the Toll-like receptors. However, studies over the past two decades have revealed a surprisingly diverse repertoire of sensing mechanisms within the mammalian host cytosol that are responsive to bacterial virulence factors and metabolites. A striking example is host recognition of the bacterial metabolite, cyclic di-AMP, a cyclic dinucleotide that controls growth, cell wall homeostasis, and virulence (114). *L. monocytogenes* secretion of cyclic di-AMP through multidrug transporters into the host cytosol enables recognition of this bacterial metabolite by a host protein, STING, and then triggering of upregulation of type I interferon and the interferon response (115–117). A recent study suggests that there is a richer diversity of cyclic nucleotides produced by bacteria than previously suspected (118). While only a few *L. monocytogenes* metabolites have been established as ligands for cytosolic pattern recognition, the evolutionary conservation of many metabolic functions implicates these molecules as likely targets for the innate immune system. Evidence points to host recognition of chemically distinct metabolites from other bacterial species, including citrate, heptose-1-7-bisphosphate, and ADP-heptose (53, 119, 120). Thus, bacterial pathogens must evolve to navigate a fine balance between fully exploiting the host niche for nutrients and their visibility to the host immune system through the by-products of their own metabolism.

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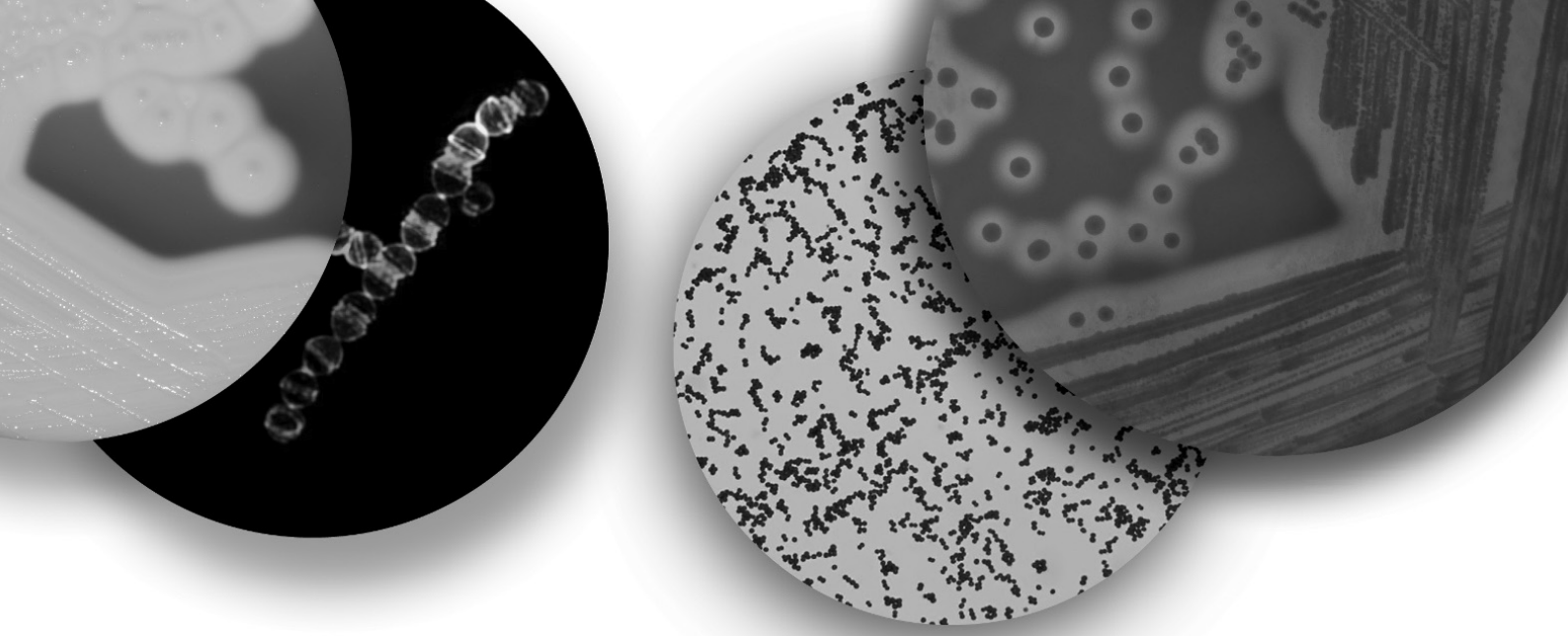
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SECTION 5

Spore-Forming Pathogens

Section Editor: **Julian I. Rood**

Pathogenic bacteria that belong to the *Clostridium* and *Bacillus* genera cause classical diseases, many of which were recognized well before the advent of the science of microbiology and what we now regard as modern medicine. These syndromes were known before the germ theory of disease had been verified and before the concept of spontaneous generation had been disproved. Their analysis has provided some of the paradigm formative studies in microbiology, such as the demonstration by Koch in 1877 that *Bacillus anthracis* was the causative agent of anthrax and the identification of tetanus toxin in 1889 by Kitasato.

In recent years research on these bacterial pathogens has advanced dramatically as a result of multidisciplinary studies involving microbial genetics, bacterial pathogenesis, structural biology and microbial genomics. The crystal structures of key toxins have been elucidated as well as the genetic organization of genes encoding numerous virulence factors and the determination of their functional role in disease pathogenesis. These are exciting times for research on these classical bacterial diseases and other diseases that have been recognized more recently. Wherever possible, the chapters in this edition have been expanded and

reorganized to include recent research that has significantly increased our understanding of fundamental biological processes and disease pathogenesis.

The chapters in this section are organized on the basis of the mechanism of pathogenesis rather than the bacterial species. These diseases all involve the elucidation of powerful protein toxins. Irrespective of which bacterium produces these toxins, they primarily affect either the nervous system or the gastrointestinal tract or cause diseases that involve extensive tissue damage and necrosis. Therefore, it was considered that a more coherent understanding of the pathogenesis of these diseases would result from having separate system-based chapters that discussed the various types of infections. The increasing importance of *Clostridioides* (formerly *Clostridium*) *difficile*-mediated infections, and the depth of research in this field, has been recognized by splitting the previous enterotoxemic infections chapter into two, one dealing exclusively with *C. difficile* infections. The importance of genetic tools for the development of our understanding of clostridial diseases and of plasmids and spores in the dissemination of clostridial toxin genes has warranted separate chapters on these topics, along with a separate chapter on clostridial genomics. By contrast, a different approach has been taken for the discussion of *Bacillus*-mediated infections, with these aspects discussed in a single integrated chapter.

The *Bacillus cereus* Group: *Bacillus* Species with Pathogenic Potential

MONIKA EHLING-SCHULZ, DIDIER LERECLUS AND THERESA M. KOEHLER

55

TAXONOMY: HISTORICAL AND SOCIOECONOMIC ASPECTS

The microorganisms constituting the *Bacillus cereus* group are Gram-positive low-GC-content bacteria belonging to the phylum *Firmicutes*. The group of spore-forming, aerobic, facultative anaerobic, rod-shaped bacteria comprises at least eight closely related species: *B. anthracis*, *B. cereus*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis*, *B. cytotoxicus*, and *B. toyonensis* (1). With the exception of *B. cytotoxicus*, which is the most divergent of the group, with a chromosome of 4.085 Mb (2), the genomes of the *B. cereus* group species are highly conserved, with sizes of 5.2- to 5.9-Mb and very similar 16S rRNA gene sequences.

The definition of these species and the distribution of the strains within them were based on phenotypes and their clinical and economic significance and mainly associated with plasmid content (3, 4). *B. anthracis* harbors two plasmids, pXO1 (182 kb) and pXO2 (95 kb), carrying the structural genes for the toxin proteins and the biosynthetic genes for capsule formation, respectively (5). The emetic *B. cereus* strains harbor plasmid pCER270, encoding enzymatic components required for the nonribosomal biosynthesis of the toxin cereulide (6, 7). *B. thuringiensis* harbors several plasmids encoding a large variety of the insecticidal toxins Cry and Cyt, which form the parasporal crystal characteristic of the species (8–10). The other five species are discriminated by morphological or physiological traits. *B. mycoides* and *B. pseudomycoides* have typical rhizoid growth (11, 12). *B. weihenstephanensis* strains are psychrotolerant. Like the mostly mesophilic members of the *B. cereus* group, the optimal growth temperature for *B. weihenstephanensis* is between 25 and 35°C, but the species is distinguished by its ability to grow at temperatures as low as 4°C (13). On the other hand, *B. cytotoxicus* strains are thermotolerant and can grow in up to 50°C (14). Finally, *B. toyonensis* was characterized for probiotic properties and is used as such in animal feed (15).

Speciation based on plasmid content and on morphologic or certain physiological features is confusing and can result in subjective conclusions. A recent genomic analysis of 224 strains shows that the *B. cereus* group can be divided into 5 major clades which do not match the 8 species described above (see Fig. 1). This lack of congruence between genomic-based phylogenetic analysis and specific phenotypes leads to the conclusion that plasmids carrying toxin genes such as the pXO plasmids of *B. anthracis* and the *cry* plasmids of *B. thuringiensis* cannot serve as signatures for species identification (1). As proposed by Helgason and colleagues (16) and by Rasko and coworkers (4), it is more useful to consider the *B. cereus* group a unique species comprising extremely diverse strains whose properties differ due to plasmid content or gene expression associated with key regulatory genes. For example, the global transcription regulator PlcR is active in *B. cereus* and *B. thuringiensis* but inactive in *B. anthracis*, abolishing the expression of a major regulon in that species (17, 18).

While it is arguably appropriate to refer to members of this highly related group of bacteria as *B. cereus* subsp. *cereus*, *B. cereus* subsps. *anthracis*, *B. cereus* subsp. *thuringiensis*, etc., in keeping with current practice, we will refer to the individual subspecies by their traditional names. We focus here on *B. anthracis*, *B. cereus*, and *B. thuringiensis* because of the abundance of research related to their clinical and socioeconomic importance. We first present some unique and overlapping traits of these bacteria.

B. anthracis

B. anthracis, a major scourge of humanity, was first characterized by Koch and Pasteur as the etiologic agent of anthrax. Their investigations of this bacterium in the late 19th century represented early breakthroughs in microbiology and vaccination. Koch's postulates, criteria designed to establish specific causal relationships between microbes and diseases, were based on studies of *B. anthracis*. In addition, an attenuated strain of *B. anthracis* served as the first live bacterial vaccine (19, 20).

Historically, strains harboring pXO1 and/or pXO2 were designated *B. anthracis*. However, recent reports of closely related species harboring similar plasmids indicate that speciation can be complex. *B. cereus* strains that harbor pXO1- and pXO2-like plasmids, named *B. cereus* bv. anthracis, have been isolated as the causative agents of anthrax-like infections in primates in Cameroon and Côte d'Ivoire (21, 22). Similar *B. cereus* strains that produce the anthrax toxins were identified as the etiological agents of an anthrax-like cutaneous infection and anthrax-like respiratory infections in metalworkers (23–27). The well-studied *B. cereus* strain G9241, isolated from a welder with a severe anthrax-like

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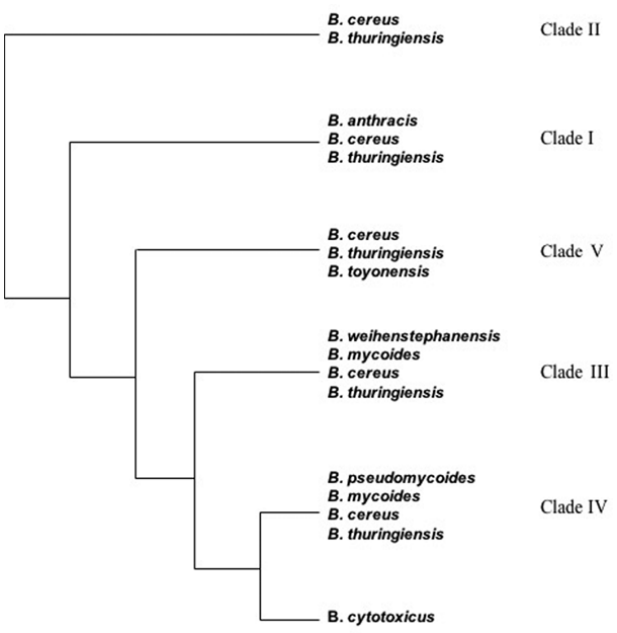


FIGURE 1 The five major phylogenetic clades of the *B. cereus* group. Clade I is known as the *B. anthracis* clade but also includes emetic *B. cereus* and several clinical *B. thuringiensis* isolates. Clade II is known as the *B. cereus*/*B. thuringiensis* clade and includes most of the commercially used *B thurigiensis* as well as the *B. cereus* and *B. thuringiensis* type strains. Clade III is known as the *B. weihenstephanensis* clade and comprises most of the psychrotolerant isolates of the *B. cereus* group. Clade IV includes strains belonging to various *B. cereus* group species. These clades are based on multilocus sequence typing, amplified fragment length polymorphism, and whole-genome sequence data (429–431). The thermotolerant strains belonging to the species *B. cytotoxicus* cluster together in a separate group.

respiratory illness, contains two virulence plasmids, pBCXO1 and pBC210 (also known as pBC218), as well as pBClin29, a linear plasmid that harbors cryptic prophage genes. The plasmid pBCXO1 has high similarity to pXO1 and contains the toxin genes *pag*A, *lef*, and *cya*, encoding toxin proteins with amino acid sequences of 96% or greater identity to their counterparts in *B. anthracis* (24). Plasmid pBC210 encodes a toxin protein paralogue, PA2, and the novel ADP-ribosyltransferase certhrax (24, 28–30). Each plasmid contains genes required for synthesis of a unique polysaccharide capsule; the enzymes needed for hyaluronic acid capsule production are encoded by the *has*ACB operon on pBCXO1, and the proteins required for elaboration of a putative tetrasaccharide capsule are encoded by the *bps* locus on pBC210 (24, 31). Interestingly, although *B. cereus* G9241 and *B. anthracis* Ames both produce capsule(s) and the anthrax toxin proteins, the virulence of G9241 in rabbits is more similar to that of *B. anthracis* Sterne strain 34F2 (pXO2$^-$), which produces the toxins but not capsule (32).

B. cereus

B. cereus was first isolated from air in a cow shed and was characterized as a highly motile bacterium, generally appearing as single cells but occasionally forming longer threads and causing rapid liquefaction of gelatin (33). In the second half of the 20th century, *B. cereus* was recognized as a common food contaminant responsible for two types of poisonings. More recently, *B. cereus* has been increasingly recognized as an etiological agent of localized wound and eye infections as well as systemic infections (34, 35).

In the 1950s, Hauge showed that consumption of *B. cereus*-contaminated vanilla sauce could cause diarrhea (36). Since Hauges' remarkable discovery, two protein toxin complexes (Nhe and Hbl) and a single-protein toxin (CytK) have been described, which are generally thought to contribute to the diarrheal type of food poisoning (37). More recently, several exoproteins with enzymatic activities, such as proteases or phospholipases, have been discussed as putative virulence factors contributing to the diarrheal syndrome (38, 39). Notably, these diarrhea-associated known and putative virulence factors are encoded by chromosome genes. In contrast, genes encoding the biosynthetic machinery for production of the depsipeptide toxin cereulide, which is the causative agent of the emetic type of *B. cereus*-mediated food poisoning, are on the 270-kb megaplasmid pCER270 (35). In contrast to the enterotoxin genes, which are broadly distributed among the members of the *B. cereus* group and show a high degree of molecular diversity (40), the cereulide synthetase gene is restricted to genetically closely related strains and shows rather low molecular diversity (41, 42). The ecological and evolutionary mechanisms driving the diversification processes of *B. cereus* virulence were hitherto poorly understood.

B. thuringiensis

B. thuringiensis was first isolated in 1901 in Japan from infected silkworm larvae (*Bombyx mori*). The bacterium was identified as the causal agent of the sotto disease (sudden-collapse disease), a lethal infection of this economically important insect (43). Subsequently, Berliner isolated the bacterium from the cadavers of flour moth larvae (*Ephestia kuehniella*) collected in a mill in Thuringia (44) and named it *Bacillus thuringiensis*, after the German province.

Based on its entomopathogenic properties, *B. thuringiensis* was rapidly commercialized and used as a biopesticide for pest control. The insecticidal activity spectrum of the bacterium was initially thought to be restricted to a few lepidopteran species. However, in 1977 discovery of the israelensis strain of *B. thuringiensis*, which is active against mosquitoes (45), accelerated a search for additional strains active against several species of insects and other invertebrates.

Today, a large number of strains have been shown to have activity against coleopteran insects or nematodes (46, 47). *B. thuringiensis*-based biopesticides account for approximately 75% of the global bioinsecticide market, representing about 4% of global insecticides (48). Several thousand *B. thuringiensis* strains have been shown to form crystalline Cry protein inclusions during sporulation, which in many cases are key to the insecticidal or nematicidal activity of the bacteria. However, not all *B. thuringiensis* isolates that produce Cry proteins have been found to be toxic. It is possible that some Cry proteins are inactive or have unidentified target hosts.

All the insecticidal toxin genes (*cry*, *cyt*, and *vip*) of *B. thuringiensis* are located on large plasmids (9, 49, 50). Generally, these plasmids are conjugative (51), and the *cry* genes are frequently found in the vicinity of various mobile genetic elements, such as the transposon Tn*4430* and the insertion sequences IS*231* and IS*232* (8, 52, 53). The localization of the insecticidal toxin genes on elements associated with horizontal gene transfer is likely responsible

for the great diversity of these genes and their multiplicity within the *B. thuringiensis* strains.

ECOLOGY: ENVIRONMENTS AND PATHOGENICITY

The *B. cereus* group species are endemic soil bacteria that occupy diverse ecological habitats as depicted in Fig. 2 (41, 54). Due to the formation of heat-, UV-, acid-, and desiccation-resistant endospores, the bacteria can persist in a dormant state, making it difficult to elucidate their primary ecological niches. In addition to soil, the species have been isolated from fresh and stored foods, invertebrates, and plants (55–60). Some reports suggest that the *B. cereus* group species do not germinate and grow in nonsterile soils unless a nutrient source, such as decomposing plants, insects, or animals is present (61–64). Reports of the isolation of *B. cereus* associated with plants may even point toward an endophytic lifestyle as an alternative niche for *B. cereus* (65, 66). Due to their ubiquitous presence, these organisms are hard to control and can enter food production and processing at various points (Fig. 2). Certain *B. cereus* strains have been reported to promote plant growth and to suppress plant diseases (67, 68). Recently, it was reported that the *B. cereus* AR156 strain induces systemic resistance to *Pseudomonas syringae* pv. tomato in *Arabidopsis thaliana* (69), suggesting that plant rhizospheres represent a natural niche for *B. cereus*.

Genome and physiology studies revealed that unlike true soil microorganisms, bacteria of the *B. cereus* group have few genes involved in the degradation of carbohydrate polymers. Conversely, these species contain a wide arsenal of genes encoding extracellular factors such as degradative enzymes (phospholipases, proteases, and chitinases), cytotoxic proteins (hemolysins, enterotoxins, and cytotoxins), and cell surface proteins (70, 71). These factors are potentially involved in the virulence and propagation of the bacteria in an animal host, thus conferring an obvious pathogenic lifestyle to the species (72). Outside of their hosts, the *B. cereus* group species are generally considered to reside as spores. Nevertheless, it is likely that the bacteria undergo some cycling of saprophytic growth, sporulation and germination in the soil. Epidemiological studies of clinical and nonclinical isolates suggest loss and horizontal movement of plasmids (22, 73–75).

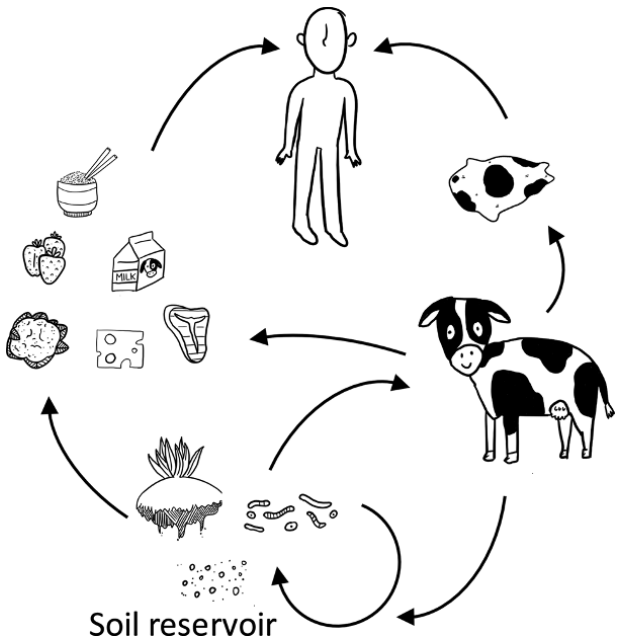


FIGURE 2 Transmission of the *B. cereus* group species from the soil reservoir to humans via food and textile production. Soil and soil-associated organisms including plants, insects, nematodes, and amoebae, serve as the major reservoirs for acquisition of spores. The bacteria are transferred to humans through agricultural products including food and animal-associated textiles, entering humans and other mammals through ingestion, inhalation, and breaks in the skin. Illustration credit: Olive E. Morrison.

The ecology of *B. thuringiensis* remains unclear despite multiple investigations (54, 76), and *B. cereus* ecology is even less explored (66). Studies of *B. anthracis* ecology have been fueled by the severity and worldwide occurrence of anthrax disease. *B. anthracis* can infect all mammals, some birds, and possibly even reptiles. The most severely affected parts of the world are central Asia and western areas of Africa. An "anthrax belt" exists from Turkey to Pakistan. In North America, anthrax is endemic in northern Alberta and the Northwest Territories. In the United States, sporadic outbreaks of anthrax occur in northwest Mississippi/southeast Arkansas and western Texas. The disease is also endemic throughout Mexico, Central America, and many South American countries. "Anthrax seasons" are characterized by hot dry weather which stresses animals and reduces their innate resistance to infection. Data from studies of central Asia support seasonal outbreaks, with the largest number of cases occurring in late summer (77, 78).

The prevalence of *B. anthracis* spores in the soil is affected by mean annual temperature, annual precipitation, elevation, amount of vegetation, soil moisture content, and soil pH (77, 78). Multiplication of *B. anthracis* outside of the host has been controversial. Concentrations of spores following disease outbreaks are thought to result from rains that pool spores in low-lying areas, as opposed to bacterial proliferation. Decomposition of infected animals at anthrax carcass sites leads to increased numbers of vegetative cells, but ultimately the numbers decrease as cells sporulate or die (79). Some studies suggest that *B. anthracis* competes poorly against indigenous bacteria; however, germination and amplification of anthrax spores by soil-dwelling amoebas and in the context of the plant rhizosphere have been reported (63, 80).

Humans and scavenging animals acquire gastrointestinal (GI) anthrax by ingesting spores in meat from infected animals, while wild and domestic herbivores ingest or inhale *B. anthracis* spores from the soil while grazing (81). Acquisition of spores from water appears to be less common (82). The infectious dose and severity of disease vary widely in different hosts. Domestic livestock, including sheep and cattle, and wild herbivores, such as bison, elephants, hippopotami, and kudu, are particularly susceptible to infection (83).

Inhalation anthrax in humans, also known as woolsorter's disease, was once common among textile workers who inhaled spores in dust generated from contaminated wool and animal hides. Today, most naturally acquired human cases of anthrax are cutaneous infections resulting from contact with dead infected animals or their products. Intentional dissemination of *B. anthracis* spores occurred in the United States in 2001. Eleven inhalation and eleven cutaneous cases of anthrax in humans were traced to four envelopes containing *B. anthracis* spores in powder form. The envelopes were sent to different locations via the U.S. Postal

Service, and most of the patients were either mail-handlers or were exposed at worksites where contaminated letters were processed or received. Others are thought to have been exposed to cross-contaminated mail (84). This tragic event demonstrated the apparent ease with which the organism can be dispersed and solidified the placement of *B. anthracis* on the list of biothreat agents.

B. anthracis: the Anthrax Pathogen

Systemic anthrax, generally resulting from inhalation or ingestion of *B. anthracis* spores, has a high fatality rate. When *B. anthracis* spores are inhaled, alveolar macrophages and dendritic cells transport the organism to the proximal lymph nodes. Following spore germination, vegetative cells produce the antiphagocytic capsule and the anthrax toxin proteins, virulence factors which are critical for initiation of systemic disease (85, 86). In humans, bacteremia begins after an asymptomatic incubation period of 1 to 6 days. Following the onset of bacteremia, patients can experience flu-like symptoms for 1 to 5 days prior to an acute disease stage that lasts 1 to 2 days. Ultimately, death results from respiratory failure, sepsis, and shock (84, 87).

B. anthracis virulence is attributed primarily to production of anthrax toxin and capsule. The toxin structural genes and capsule biosynthesis genes are encoded by plasmids pXO1 and pXO2, respectively, and both plasmids are required for full virulence in immunocompetent hosts. The tripartite anthrax toxin, which comprises three secreted proteins—protective antigen (PA), edema factor (EF), and lethal factor (LF)—has been the focus of investigations of anthrax disease. PA facilitates entry of EF and LF into host cells. EF is a calmodulin-dependent adenyl cyclase, and LF is a zinc protease. Together, the toxins disable the host's innate and adaptive immune systems to induce lethal disease (88, 89).

The poly-γ-D-glutamic acid (PGA) capsule of *B. anthracis*, provides protection of the bacterium from phagocytosis, as is true for many bacterial capsules which are more commonly made of polysaccharide. High concentrations of capsule released from *B. anthracis* cells during infection are important also for host-pathogen interactions (90–93). The PGA capsule has been shown to augment lethal toxin-mediated cytotoxicity and induce production of inflammatory cytokines and nitric oxide, which likely contribute to systemic inflammation and sepsis at the terminal stage of infection (94–97).

In addition to toxin and capsule, other secreted and nonsecreted factors have been reported to affect host-pathogen interactions and affect host pathology (98, 99). Among these are extracellular proteases that interfere with the immune response by cleaving antimicrobial peptides (100). The proteases NprB/Npr599 (also designated NprA in *B. thuringiensis* and *B. cereus*) and InhA1 can degrade host tissues, resulting in increasing barrier permeability (101–103). Crossing of the brain barrier is considered to be key to the extensive brain pathology that has been reported for human disease (104).

B. cereus and Gastrointestinal Disease

B. cereus pathogenicity is based on a panoply of virulence factors, which are far from understood, contributing to a range of human disease. *B. cereus* can cause two types of food poisoning, which are primarily characterized by diarrhea and emesis (37, 105, 106). The diarrheal syndrome has been linked to different enterotoxins thought to be produced after outgrowth of spores, taken up with contaminated foods, and active in the small intestine. The emetic syndrome is the result of an intoxication caused by the single depsipeptide toxin cereulide, which is preformed in food. The genes encoding the cereulide synthetase toxin genes are primarily found in a distinct subgroup of *B. cereus*, while the enterotoxin genes are broadly distributed among the members of the *B. cereus* group (41, 107). Usually, these GI-related diseases are self-limiting, but more severe forms require hospitalization, and occasionally fatalities have been reported (58, 108, 109). Intoxication related to cereulide has been linked to severe clinical manifestation, such as acute liver failures and acute encephalopathy (110). Due to the high biological activity of the cereulide toxin, occasionally liver transplantation is necessary as a life saving measure (111, 112). Generally, the pathogenic potential of *B. cereus* strains is highly variable and ranges from strains that show no cytotoxic activity in cell culture assays to strains that are highly cytotoxic (113, 114). In addition to intrinsic strain-associated differences in pathogenic potential, virulence is affected by environmental factors (58, 115, 116).

Apart from its food poisoning potential, *B. cereus* is increasingly recognized as an opportunistic pathogen that can cause local as well as systemic infections. Immunocompromised people and neonates are at special risk for hospital-acquired *B. cereus* infections, such as infections of the central nervous system, endocarditis, respiratory and urinary tract infections, wound infections, and septicemia (34). Outside of hospital-acquired infections, *B. cereus* has been linked to eye infections, such as endophthalmitis and keratitis (117). Furthermore, *B. cereus* can cause severe mammary gland infections in ruminants (118, 119). The virulence factors contributing to infections in ruminants and to most non-GI-related human infections are unknown.

B. thuringiensis Infection of Invertebrates

In addition to virulence genes common to the *B. cereus* group and to specific insecticidal toxin genes, the *B. thuringiensis* genome contains several genes encoding chitinases, suggesting a close relationship between this bacterium and the insects. Many well-studied *B. thuringiensis* strains were isolated from insects (44–46), and large screens of natural environments have shown that insects were more fruitful sources of the bacterium than soil samples (120). About 40% of the invertebrates tested were found to harbor *B. thuringiensis* spores. While insects appear to be the preferred target of *B. thuringiensis*, the bacterium can propagate in other invertebrates (121, 122). Nematode-*B. thuringiensis* association is highly probable in soil and other natural environments, and several *B. thuringiensis* strains harbor *cry* genes that are specifically active against nematodes (123). When nutrients are available, *B. thuringiensis* might be a multihost pathogen (124) with two pathogenic lifestyles: one resulting from its specific toxins (Cry or Vip) encoded by plasmid genes and targeting insects, nematodes, and other invertebrates (49, 125) and one nonspecific, depending on various chromosomal factors providing opportunistic properties. These nonspecific factors may allow the bacteria to propagate and eventually to kill various organisms if they are weakened by any cause, for example, by Cry toxins in the case of insects (126). This second pathogenic lifestyle may be shared with *B. cereus*, which possesses the same chromosome-encoded virulence factors (70). In this point of view, the Cry toxins of *B. thuringiensis* are "public goods" which can act cooperatively and thus profit cheater bacteria such as *B. cereus* (127).

The genome of *B. thuringiensis* contains a 41-gene regulon devoted to its saprophytic or necrotrophic lifestyle (128).

This regulon, which includes genes encoding various degradative enzymes, is activated by the quorum sensing regulator NprR and allows *B. thuringiensis* to survive in the insect cadaver. The NprR regulon is also transcribed in all other *B. cereus* group species, suggesting that the bacteria of this group have similar saprophytic properties (129). Altogether, these pathogenic and saprophytic functions are highly efficient adaptive traits that allow the species to grow and survive in various natural conditions and niches.

CELL STRUCTURE AND DEVELOPMENT

Vegetative cell and spore structures do not vary greatly among the *B. cereus* group species. Generally, vegetative cells appear in chains, with the square ends of the cells fitting closely together. Variations in chain length occur as a normal aspect of growth in different media and environments (34, 130). Interestingly, *B. anthracis* vegetative cell chains in infected tissues are generally shorter than those formed during growth *in vitro*. Chaining of *B. anthracis* cells impedes invasion of host cells and is associated with escape from phagocytic clearance (131). Spores of the three species also have similar morphology. Spore size and morphology can vary between strains of the same species, but most strains produce spores with diameters of approximately 1 μm that are surrounded by a loose-fitting outer layer termed the exosporium (Fig. 3). Fine features of spores can be altered in different growth and sporulation conditions.

Vegetative Cell Envelope

The surface of *B. anthracis*, including secondary cell wall polysaccharides (SCWPs), the surface layer (S-layer), and capsule, has been the most well studied of the *B. cereus* group species. The *B. anthracis* SCWP comprises trisaccharide repeats [→4(-β-ManNAc-(1→4)-β-GlcNAc-(1→6)-α-GlcNAc-)1→] with α-Gal and β-Gal substitutions tethered to the peptidoglycan by acid-labile phosphodiester bonds. SCWP is essential for cell growth, shape, and division.

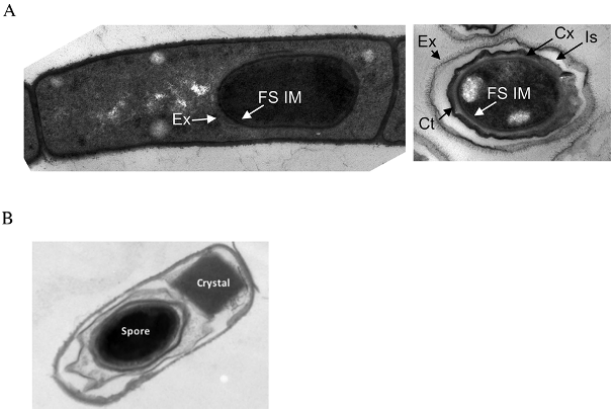


FIGURE 3 (A) Thin-section transmission electron micrograph of *B. anthracis* cell after 5 hours of sporulation (left image) and a mature spore (right image). The forespore inner membrane (FS IM) and nascent exosporium (Ex) (left image), and forespore inner membrane (FS IM), mature exosporium (Ex), coat (Ct), interspace, (Is), and cortex (Cx) (right image) are labeled. (B) *B. thuringiensis* sporulating cell with parasporal crystal. Cells were sporulated, prepared, and imaged as described in reference 432. Image credit: (A) Tyler Boone and Adam Driks. (B) Fuping Song.

Although the enzymes for SCWP synthesis have not been fully characterized, two proteins, WpaA and WpaB, have been proposed as glycosyltransferases that act to assemble the SCWP polymers prior to attachment to the peptidoglycan. The *B. anthracis* SCWP is critical for maintenance of cell shape and for full virulence. Depletion of the glycosyltransferases results in irregularly shaped, nonviable cells (132). In addition, a mutant lacking a UDP-glucose 4-epimerase required for SCWP galactosylation exhibited reduced encapsulation and decreased virulence in a murine model of anthrax (133). Recent investigations of *B. cereus* isolates causing anthrax-like disease indicate SCWP with a structure similar to that of *B. anthracis* but with an additional α-Gal substitution at O3 of *N*-acetylmannosamine (134).

In *B. anthracis* and some pathogenic *B. cereus* strains, S-layer proteins have been shown to attach to the cell wall via noncovalent interactions with SCWPs (135). The S-layers are paracrystalline arrays of protein that serve as protective barriers and as scaffolds for housekeeping enzymes and virulence factors (136). Major S-layer proteins of *B. anthracis* are Sap (for surface associated protein) and EA1 (for extractable antigen 1). Fully assembled and acetylated SCWP is important for assembly of some S-layer proteins. Mutants deleted for putative acetyltransferases are unable to deposit the S-layer-associated murein hydrolase BslO at cell division septa and have altered chain lengths (137).

Finally, a key distinguishing feature of *B. anthracis* is its capsule. The *B. anthracis* capsule comprises PGA. As is true for typical polysaccharide capsules, the *B. anthracis* capsule protects cells from phagocytosis during infection. In addition, the *B. anthracis* capsule renders the bacterium somewhat resistant to mammalian proteases and is a poor immunogen (138, 139). An immunoassay for PGA using monoclonal antibodies has revealed that PGA is shed into the blood during infection of mice and nonhuman primates (91, 140, 141), likely due to the activity of a capsule depolymerizing enzyme, CapD, that is coexpressed with the capsule biosynthesis operon. The capsule degradation products are thought to inhibit the innate immune response through targeting of cytokine pathways (142).

The PGA capsule is also produced by some but not all *B. cereus* isolates that cause anthrax-like disease. These strains harbor plasmids that allow strain-specific differences in synthesis of the PGA capsule, a hyaluronic acid capsule, and a tetrasaccharide capsule. The results of virulence studies to assess the role(s) of the *B. cereus* capsules are somewhat contradictory and incomplete (22, 24, 31, 75). Further investigations are needed to determine the specific functions of these capsules.

Spore Architecture

The architecture of the *B. cereus* group species spore is comparable to the spores of other *Bacillus* species. The innermost compartment of the spore contains the DNA. The DNA is housed with cations and pyridine-2,6-dicarboxylic acid in a 1:1 chelate with divalent calcium ions, which serve to maintain the spore's dormant state and resistance to DNA-damaging agents. Germinant receptors in the inner membrane initiate the signal transduction pathway that leads to outgrowth of the spore and the vegetative cell state (143). Germinants of the *B. cereus* group species can be a subset of sugars, amino acids, and ribonucleosides, as well as other small molecules, depending on the species and strain (144). A shell of peptidoglycan containing two sublayers, the cortex and the germ cell wall, surrounds the inner membrane. The cortex constricts the core's volume to retain the relatively dry

state of the core. At outgrowth of the spore, the germ cell wall becomes the nascent cell wall of the vegetative cell. Surrounding the cortex is the spore coat, which contains approximately 70 proteins arranged in layers to form a somewhat flexible structure (143).

The exosporium, an additional layer surrounding the spore coat of some *Bacillus* species that has been most well studied in *B. cereus* and *B. anthracis* (145), can respond to changes in spore hydration. The exosporium comprises a crystalline basal layer overlaid by a "hairy nap." The composition and assembly process of the exosporium are poorly understood. BclA and related proteins form the hairy nap. Among the approximately 20 proteins of the basal layer is ExsY, which can self-assemble into structures resembling the intact basal layer and thus appears to be a key player in exosporium formation (146). The exosporium excludes large molecules. It also provides resistance to specific chemicals *in vitro* and to nitric oxide in the context of the macrophage (147–149). Removal of the exosporium from *B. anthracis* spores does not impede virulence in several animal models (150–152). However, the exosporium protein BclA binds macrophage receptors to mediate interactions with epithelial cells and extracellular matrix proteins (153, 154), suggesting a role for the exosporium in pathogenesis (145).

Germination Dynamics and Signals

The overall process of germination, in which spores transition to metabolically active vegetative cells, is highly conserved in the *Bacillus* genus, but the specific signals and associated receptors vary among species. *B. anthracis* germinates in response to alanine or purine ribonucleotides, with one or more additional amino acids acting as cogerminants (155–157). Many germinant receptors have been described in the inner membrane of *Bacillus* species. Notably, a unique set of germination genes, the *gerX* operon, is located on pXO1 of *B. anthracis*. Mutants missing the operon are altered for germination *in vivo* (158, 159). Once *Bacillus* spores germinate, spore cortex lytic enzymes degrade the cortex peptidoglycan, allowing core rehydration and cell outgrowth (160). *B. anthracis* differs from other species examined in that it contains an additional spore coat lytic enzyme which appears to work synergistically with the common enzymes (161).

Germination in the Host

Multiple routes of infection with *B. anthracis* spores lead to disease, suggesting that germination can be triggered by signals in different host niches. Studies of *B. anthracis* germination *in vivo* have yielded intriguing results. Inhalation anthrax models have shown that phagocytes and dendritic cells phagocytose spores and transport the bacteria to the lymph nodes (162, 163). Spores and vegetative cells appear in phagocytic cells, but it is unclear whether spores germinate before or after phagocytosis. Moreover, some strains germinate quickly in the lung, while others remain ungerminated for days (164, 165), suggesting that germinants plus additional unknown factors influence development *in vivo*. Finally, germination occurs in cell-free macrophage-conditioned media, indicating that phagocytes are not required for *B. anthracis* spore germination (157).

Studies of *B. thuringiensis* germination include experiments simulating conditions encountered in the insect midgut. The pH of the lepidopteran midgut is 10, and pretreatment of *B. thuringiensis* spores with larval midgut fluid or with an alkaline buffer stimulates germination (166, 167). This alkaline activation of spores requires plasmid-encoded germination genes (168). Cry proteins associated with the spore surface allow *B. thuringiensis* spores to bind brush border membrane vesicles prepared from lepidopteran insects, and the binding has been proposed to be responsible for stimulation of alkali-activated germination (169). It should be noted however, that the spore surface-Cry protein association may simply result from concomitant synthesis rather than from a specific interaction. Finally, germination of *B. thuringiensis* spores in the intestines of nematodes has also been reported (122).

Sporulation

The sporulation signal cascade has been well studied in *Bacillus subtilis*, and the pathways and regulators of sporulation in the *B. cereus* group species appear to be similar to those of *B. subtilis*. Nevertheless, variations among species have evolved with specific niches. *B. anthracis* does not sporulate during infection. In the wild, spores (which are the infectious form of the bacterium) only form when the blood of an infected carcass is exposed to the environment (78). The lack of sporulation in the mammalian host appears to result from a specific configuration of the phosphorylation cascade in this species (170–172). In contrast, it has been shown that part of the *B. thuringiensis* population sporulates during infection in insect larvae, and this process is under the control of the bifunctional regulator NprR (173, 174). The Rap phosphatases negatively control sporulation by stimulating a component of the phosphorelay involved in the phosphorylation of Spo0A, the master regulator of sporulation initiation (175). Rap activity is controlled by the Phr signaling peptides through a quorum sensing system. In *B. anthracis* and *B. thuringiensis*, plasmids harbor *rap-phr* genes involved in sporulation (176). These plasmid-borne Rap-Phr systems may provide advantages for adaptation of these pathogenic bacteria to their respective ecological niches.

TOXINS

Toxins, the first bacterial virulence factors to be described (177), represent the major drivers of pathogenicity within the *B. cereus* group. A variety of chromosome- and plasmid-encoded toxins, some shared among the species and some species specific, have been described. Table 1 shows an overview on the most important *B. cereus* group exotoxins. The species-specific toxins, namely, the *B. anthracis* tripartite anthrax toxin complex and the *B. thuringiensis* insecticidal Cry toxins, as well as the depsipeptide toxin cereulide of the emetic type of *B. cereus*, are encoded on plasmids, while many other *B. cereus* group toxins are encoded by the chromosome. Some *B. anthracis* and *B. cereus* isolates harbor duplicated and/or closely related toxin genes (35, 178), and *B. thuringiensis* strains can harbor several *cry* genes.

B. anthracis Toxins

Anthrax toxin has been a major focus of anthrax research since the initial discovery of the toxigenic potential of *B. anthracis* culture supernates. *B. anthracis* secretes three proteins, PA, LF, and EF, known collectively as anthrax toxin. Purified toxin proteins in binary combinations have distinct effects when injected into experimental animals. Lethal toxin (LeTx), a mixture of LF and PA, causes death, while edema toxin (EdTx), a mixture of EF and PA, produces edema. PA (83 kD) mediates binding and translocation of the enzymatic proteins LF (90 kD) and EF (89 kD) into host cells.

PA is a nonenzymatic but critical component of LeTx and EdTX because it mediates toxin binding to host cells and

TABLE 1 Exotoxins, the major virulence factors of the *B. cereus* group

Toxin	Toxin type	*B. anthracis*	*B. cereus*	*B. thuringiensis*
Anthrax toxin (PA, LF, EF)	AB toxin	+	+[a]	–
Emetic toxin (cereulide)	Heat-stable depsipeptide toxin	–	+	–
Enterotoxins (Nhe, Hbl, CytK)	Pore-forming toxins	–[b]	+	+
Insecticidal toxins (Cry, Cyt, Vip, Sip)	Pore-forming toxins	–	–	+

[a]Some rare strains.
[b]*nhe* genes are present but inactive.

facilitates translocation of LF and EF into cells. PA binds to one of two related proteins, capillary morphogenesis protein 2 (CMG2 or ANTRX2) and tumor endothelial marker 8 (TEM8 or ANTRX1), which are found on cells of many different tissues. CMG-2 is the most important for pathogenesis, while TEM-8 has a minor role (179, 180). In the model of anthrax toxin entry, furin and possibly additional host-associated proteases cleave PA into two fragments, PA63 (63 kDa) and PA20 (20 kDa) (89). The receptor-bound carboxyterminal 63-kDa fragment (PA63) oligomerizes to form a ring-shaped structure comprising seven or eight PA63 molecules. One to three molecules of LF and/or EF bind to the PA ring, and the complex is endocytosed by clathrin-coated vesicles. Endosome acidification induces a conformational change in the ring such that it converts from a "prepore" to a pore (channel) in the membrane, facilitating translocation of EF and LF across the membrane (181).

LF, a zinc-dependent metalloproteinase, is the central effector of shock and death from systemic anthrax. Initial investigations of LF activity revealed that the protein cleaves mitogen-activated protein kinase kinases, resulting in disruption of multiple signaling pathways and causing broad downstream effects on cell physiology (182). More recent reports show that low levels of LeTx inhibit the glucocorticoid receptor, an important component of host defense against inflammation. LF targets the inflammasome sensor NLRP1, causing activation of the caspase-1-dependent cell death signaling pathway (183). Finally, murine susceptibility to lethal toxin has been associated with genetic polymorphisms in the *Ltx* gene on mouse chromosome 11. The *Ltx* gene encodes a kinesin-like motor protein, Kif1C, but the relationship between lethal toxin and Kif1C is not clear (184).

EF is a calmodulin-dependent, Ca^{+2}-dependent adenylyl cyclase (185). As is true for other bacterial toxins that elevate cAMP levels, disruption of normal second-messenger signaling has many effects on host cell physiology. EdTx weakens antibacterial responses of phagocytes, impairing apoptosis, motility, and release of inflammatory cytokines. Human neutrophils treated with the toxin have reduced phagocytic and oxidative burst abilities (186).

A comprehensive model of the molecular basis of anthrax toxin effects on mammalian hosts is still evolving, but a wealth of current information reveals that together, the toxin components severely impinge on the immune functions of susceptible hosts, which are key to the outcome of *B. anthracis* infection (89, 187, 188).

Anthrolysin O is a cholesterol-dependent cytolysin with properties similar to the pore-forming toxins of other Gram-positive pathogens (189). Initially discovered as a hemolysin (190), ALO has lytic activity against phagocytes (191), decreases the barrier function of human polarized epithelial cells (192), and appears to play a role in gut epithelial disruption during *B. anthracis* infection (193). Expression of the *alo* gene is highly responsive to *in vitro* growth conditions (190). During infection, *alo* transcripts are present in newly germinated bacteria after phagocytosis of the spores by mouse macrophages (194, 195). Concurrent deletion of *alo* and phospholipase genes attenuates virulence and reduces growth and survival in phagocytes, suggesting a role for the toxin in destabilization of the phagocytic membrane (196).

B. cereus Toxins

Various *B. cereus* exotoxins have been associated with pathogenicity, but the actual contribution and importance of these toxins for GI and non-GI related diseases is largely unknown. So far, four *B. cereus* exotoxins have been clearly linked to food poisoning. Three pore-forming enterotoxins (Nhe, a three-component nonhemolytic enterototoxin, Hbl, a three-component hemolytic enterotoxin, and the single protein cytotoxin CytK) are thought to provoke diarrhea after toxicoinfections with *B. cereus* spore-contaminated food (37). Hbl enterotoxic activity has been demonstrated *in vivo* on rabbit intestine (197), but direct *in vivo* proof for Nhe and CytK activity is lacking. Nhe was identified from a strain isolated from a foodborne outbreak in Norway (198), and CytK was originally isolated from a *B. cereus* strain that was responsible for a severe foodborne outbreak in which several people developed bloody diarrhea and three elderly people died (199). Enterotoxins may act synergistically with each other and with other virulence factors, making it difficult to predict the overall enterotoxic potential of a given strain (39, 106, 200–204). Nhe, Hbl, and CytK show cytotoxic activity on Vero and Caco2 cells as well as on other human cells. Different cell lines show distinct susceptibilities to the different toxins (205), suggesting different modes of toxin action.

Typically, symptoms occur after 6 to 12 hours and are self-limiting. However, in rare cases the severity of symptoms requires hospitalization, and fatalities among neonates and immunocompromised patients have been reported (108, 109, 199).

The fourth toxin, the so-called cereulide, is linked to the emetic type of *B. cereus* food poisoning (206). Cereulide is a depsipeptide toxin, composed of alternating α-amino and α-hydroxy acids (D-O-Leu–D-Ala–L-O-Val–L-Val)$_3$ (Fig. 4), that is structurally related to the potassium ionophore valinomycin (206). It is produced by the cereulide peptide synthetase Ces (6, 42, 207, 208), which represents a novel type of nonribosomal peptide synthetase (6, 207–209). Cereulide is usually preformed in food, and the onset of symptoms occurs between 15 minutes and 6 hours after uptake of contaminated food (105). In the past, quantification of cereulide has been hampered by the lack of appropriate methods (58). The recently developed ISO method (EN ISO 18465), based on a stable isotope mass spectrometry-based dilution assay (210), will allow accurate cereulide quantitation in the future. Recently, 18 cereulide isoforms were described, with cytotoxic potential ranging from nontoxic to

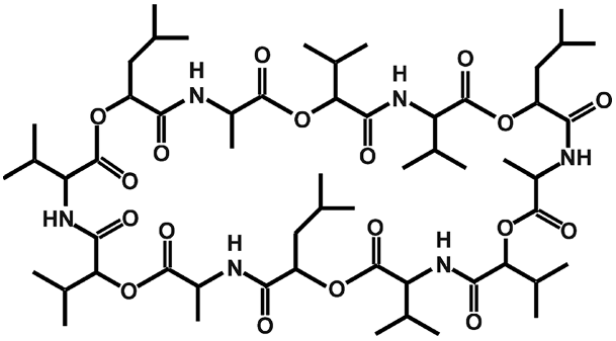


FIGURE 4 Cereulide, the emetic toxin of *B. cereus*.

10-fold more toxic than the classic cereulide (211). Cereulide is not inactivated during stomach passage in the host because it is resistant to cleavage by pepsin and trypsin (105, 212). Due to its hepatotoxic activity, cereulide can cause rhabdomyolysis, liver damage, and severe multiorgan failure (110, 112). Recently, cereulide was linked to the induction of diabetes by causing beta cell dysfunctions (213).

B. thuringiensis Toxins

Except for the cereulide toxin, *B. thuringiensis* strains generally produce the same toxins as *B. cereus* (214). The phenotype distinguishing *B. thuringiensis* from the other species of the *B. cereus* group is the production of a crystal inclusion during sporulation (Fig. 3). This crystal generally appears beside the spore in the mother cell compartment of the bacteria (215), but other situations have been reported in a few strains, including a crystal between the exosporium and the spore coat (216), and production of spores and crystals in two distinct subpopulations of bacterial cells (217). The crystal inclusion can account for up to 25% of the dry weight of *B. thuringiensis* cells, which means that a massive production of proteins should occur during sporulation or stationary phase. This massive protein production results from several mechanisms, including efficient transcription regulation, an exceptionally high stability of *cry* gene mRNA, and the crystallization of the proteins (218). In most of the *B. thuringiensis* strains the expression of the *cry* genes depends on sporulation-specific sigma factors, Sigma E and Sigma K, which are functional in the mother cell compartment during a long period of the sporulation process. Some exceptions have been observed. For example, the *cry3* genes encoding coleopteran-active toxins are expressed during stationary phase independently of the sporulation sigma factors (218).

The crystal inclusions are composed mainly of two types of proteins, the Cry and the Cyt proteins, also called δ-endotoxins (49, 219). Various combinations of toxins can be found on the crystal, depending on the *B. thuringiensis* strain. The commercial strain *kurstaki* HD1 Dipel® contains five *cry* genes (*cry1Aa*, *cry1Ab*, *cry1Ac*, *cry2Aa*, and *cry2Ab*), all encoding proteins active against lepidopteran larvae (220). The strain israelensis contains four *cry* genes (*cry4Aa*, *cry4Ba*, *cry10Aa*, *cry11Aa*) and two *cyt* genes (*cyt1Aa* and *cyt2Ba*), all encoding proteins toxic for the mosquito larvae (9). The Cry proteins are distributed into 75 distinct classes, with less than 45% identity between them (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/). They are pore-forming proteins whose activity requires the binding to specific receptors on the surface of midgut cells (194). The Cyt toxins are cytotoxins specifically active against dipteran larvae (199); they also have a synergistic action by serving as receptors for dipteran-active Cry proteins (221).

In addition to the Cry proteins forming parasporal inclusions, some *B. thuringiensis* strains secrete insecticidal proteins: the Sip (secreted insecticidal protein) and Vip (vegetative insecticidal protein) proteins. Sip1A is active against coleopteran insects and was isolated from a *B. thuringiensis* strain harboring a coleopteran-specific *cry3* gene (207). The toxin Vip3A is specifically toxic against lepidopteran insects and is generally produced by strains that also produce Cry1A lepidopteran-active toxins (209). The insecticidal activity of the exported proteins Vip and Sip and their association with Cry proteins showing related activity spectra suggest that the larvicidal activity of the Cry proteins is reinforced by the secretion of Sip and Vip proteins, thus increasing the entomopathogenicity of the *B. thuringiensis* cells multiplying in the infected insect larvae.

Common Secreted Enzymes of the *B. cereus* Group Species

Phospholipases, sphingomyelinases, hemolysins, proteinases, and peptidases likely represent additional virulence factors of the *B. cereus* group species (39, 106, 202–204). In a murine model of anthrax, protease inhibitors enhance the positive effect of antibiotics, suggesting that one or more proteases contribute to anthrax disease (222).

Immune inhibitor A (InhA1) is a particularly well-studied protease that has homologues in all three species. *B. anthracis*, *B. cereus*, and *B. thuringiensis* each carry multiple apparent *inhA* genes, with transcription of each gene being subject to different regulation (223, 224). Autolytically processed active InhA1 of *B. anthracis* cleaves multiple host proteins and other proteins in the *B. anthracis* secretome (225, 226). A *B. anthracis inhA1*-null mutant is attenuated in a murine model of anthrax (227). The reduced virulence of the mutant likely results from its broad activity on multiple targets, ultimately facilitating bacterial dissemination. InhA1 disturbs the host coagulation machinery by cleaving von Willebrand factor and its regulator ADAMTS13 (102). The enzyme also cleaves zonula occluden-1, resulting in disruption of the blood-brain barrier (103). Degradation of hemoglobin by InhA in the bloodstream has been proposed to allow *B. anthracis* access to essential amino acids required for proliferation in the blood (227). In *B. cereus* and *B. thuringiensis*, deletion of the three *inhA* genes strongly affects virulence (202). InhA1 is required for efficient escape from macrophages (228).

MODELLING HOST INTERACTIONS

Animal Models of Anthrax

Multiple animal models differing in host species, bacterial strain, and delivery route are used to investigate *B. anthracis*-host interactions. The significant variability among laboratory animals in susceptibility to *B. anthracis* infection makes modeling human anthrax particularly challenging. Nonhuman primates are considered superior models for studies of potential therapeutics and vaccines, but the high cost and ethical considerations limit their use. Rabbits serve as good models because the disease pathology is similar to that observed in nonhuman primate and clinical human cases of anthrax. Mice and guinea pig models are used frequently because of experimental ease and moderate cost. For most

animal studies, the spore form of the bacterium is used as the infectious agent, modeling natural infection. Methods for pulmonary delivery of spores include intranasal inoculation, aerosol exposure, and tracheal injection. Spores can also be introduced subcutaneously. To model late-stage disease, vegetative cells are inoculated directly into the bloodstream, bypassing the requirement for *in vivo* germination of spores (229).

Mice are the most common experimental animal for investigations of *B. anthracis*. Inbred mice infected via the parenteral and pulmonary routes develop acute disease, with vegetative cells in multiple tissues. Immunodeficient ($C5^-$) mouse strains such as A/J and DBA/2J are susceptible to relatively low doses (10^3 to 10^4 CFU) of capsule-deficient ($pXO1^+pXO2^-$) Sterne-type strains and are used often for studies of the roles of anthrax toxin *in vivo* and for testing the efficacy of toxin-based therapeutics and vaccines. Exposure of $C5^-$ mice to aerosolized Sterne spores results in pathology similar to that observed in rabbit and nonhuman primate models, with lethal toxin required for dissemination and development of protective immunity (230).

Studies of pathogenesis and host responses related to nontoxin factors have been commonly assessed in infections of immunocompetent mice with nontoxigenic ($pXO1^-$ $pXO2^+$) strains. Interestingly, guinea pigs, rabbits, and nonhuman primates are not susceptible to infection by $pXO1^-$ $pXO2^+$ strains (231). It is also notable that mice are not protected from fully virulent ($pXO1^+$ $pXO2^+$) strains by the PA-based vaccine AVA, whereas AVA is fully protective for rabbits and nonhuman primates but only partially protective for guinea pigs (232, 233).

Mouse models have revealed interesting aspects of germination and dissemination of *B. anthracis* during infection (229). Carr and coworkers (234) demonstrated functional redundancy among the five known germinant receptors, and McKevitt and coworkers (159) showed that the germination inhibitor D-alanine reduced the infectious dose of spores, suggesting that timing of germination is important for disease. Experiments conducted by Glomski and colleagues (235, 236) using luminescent recombinant strains showed that germination and dissemination from pulmonary, skin, and GI entry points does not require transport to lymph nodes, but spore dose and method of delivery can affect the timing and location of germination. Their data also revealed a role for capsule in dissemination.

Inbred mice have also been used extensively to explore the host response to *B. anthracis* infections. Investigations of host systems important for recognition of bacterial factors and activation of immunity have revealed a model in which *B. anthracis* activates Toll-like receptors and the nucleotide-binding oligomerization domain-like receptor (NLR) inflammasome, resulting in tumor necrosis factor alpha production by a mitogen-activated protein kinase signaling pathway. LeTx suppresses this response by cleaving mitogen-activated protein kinase kinases. EdTx-mediated increases in cAMP also disrupt cell signaling pathways. In the battle between host and pathogen, the immunosuppressive effects of LeTx are countered by Nlrp1b, the product of a "lethal toxin sensitivity" locus on chromosome 11. In response to lethal toxin, the lethal toxin-sensitive Nlrp1b allele activates caspase-1, inducing proinflammatory cytokines that recruit neutrophils and mononuclear cells. Thus, in mice, LeTx-mediated Nlrp1b-dependent activation of caspase-1 is a major factor in the control of *B. anthracis* infection (229).

Guinea pigs are keenly susceptible to *B. anthracis* spore infection but not very sensitive to the anthrax toxins. While other animal models show some inconsistencies in terminal loads of bacteria, intramuscular challenge of guinea pigs with a fully virulent ($pXO1^+$ $pXO2^+$) strain leads to specially and temporally consistent infections. Aerosol infection of guinea pigs results in edema and hemorrhage in the spleen, lungs, and lymph nodes, facilitating studies of pathology. Guinea pigs have been used in competitive infection studies employing different strains and mutants. These experiments have revealed roles for biosynthetic genes and surface proteins in disease (229).

Rabbits have been useful to determine virulence differences in *B. anthracis* strains and mutants and to examine pathology, which is similar to that observed in humans and nonhuman primates. Multiple investigations of vaccines and therapeutics have employed rabbit models. Rabbits exhibit a strong immune response to PA and have played a key role in the development of correlates of immunity. A quantitative anti-PA IgG enzyme-linked immunosorbent assay and a toxin neutralization assay developed using sera from vaccinated rabbits are used to evaluate the human immune response to PA-based vaccines (237–240).

Nonhuman primates provide a valuable model for anthrax disease because infection with aerosolized spores results in a disease course that most closely matches human infection. Nonhuman primates, including rhesus macaques, cynomolgus macaques, African green monkeys, and marmosets, represent the only animal models of inhalational anthrax in which anthrax meningitis is consistently observed. Infected nonhuman primates, like humans, also develop bacteremia, lymphadenitis, splenitis, mediastinitis, pneumonia, pleural effusions, hepatitis, adenitis, and vasculitis. Tissues of diseased animals and humans also show hemorrhage, necrosis, and edema. Sera from infected nonhuman primates have been used to develop biomarkers for early diagnosis of infection. Capsule and toxin, detected using enzyme-linked immunosorbent assay, chemiluminescence, and mass spectrometry, serve as reliable markers.

Because nonhuman primate responses to infection most closely resemble human physiological and immune responses, these animals are best suited for immunogenicity, toxicity, and safety testing of vaccines and therapeutics for anthrax. Nonhuman primates have been used extensively for studies of antibiotic efficiency. Investigations of the dose, pharmacokinetics, and time course of antibiotic treatment have shown that antibiotics are only effective against germinating spores and vegetative cells. The long-term persistence of spores and extended time periods to germination mean that complete protection from high doses of aerosolized spores requires prolonged antibiotic treatments over more than 30 days.

Rats are somewhat resistant to challenge with *B. anthracis* spores, but they are exquisitely sensitive to injection of LeTx. Thus, the Fisher 344 strain has been used as a model system for toxin activity. The rat toxin model was key to determining the role of the NLR sensor Nlrp1 in animal susceptibility to LeTx (241, 242). The model also provides a sensitive means to test the efficacy of small-molecule toxin inhibitors, monoclonal antibodies, dominant-negative toxin components, and other potential antitoxin countermeasures (229).

Animal Models of *B. thuringiensis* and *B. cereus* Infection

The opportunistic properties of *B. thuringiensis* and *B. cereus* have been investigated using nasal instillation in mice (243,

244) and intraocular infection in mice and rabbits (245). In all cases, the results highlight the importance of the PlcR regulon in the pathogenic properties of these bacteria. However, established mammalian models of GI infections caused by *B. cereus* are lacking. It has been hypothesized that rodents are not suitable models of GI-related *B. cereus* infection because their gut physiology does not support germination of spores and subsequent enterotoxin production (246). In a more recent study, Rolny et al. (247) used intragastric gavage to test the effect of vegetative *B. cereus* cells on the host immune system. They found some modifications in immune cells in the spleen, Peyer's patches, and mesenteric lymph nodes but did not report any enterotoxin production. Thus, an appropriate mammal model for enterotoxigenic *B. cereus* awaits development.

The emetic activity of the *B. cereus* toxin cereulide was first shown in a rhesus monkey model (248, 249). Results from a study in *Suncus murinus* suggest that cereulide causes emesis through the 5-HT receptor (206). However, it is still unclear if the *S. murinus* model reflects the physiology of the human gut. Thus, further studies will be needed using model organisms, such as pig, more closely resembling the human gut physiology.

Insect Models of *B. cereus* and *B. thuringiensis* Infection

In insects susceptible to *B. thuringiensis* infection, the first stage of the disease results from gut paralysis due to the crystal inclusion (250, 251). Cry toxins are responsible for this initial step. The toxins damage the peritrophic membrane and cause lysis of the intestinal cells by producing osmotic shock (252–254). Depending on the amount of Cry toxins and the susceptibility of the insect larvae to a given Cry toxin, the toxemia resulting from toxin ingestion may result in insect death. This toxemia and the paralysis of the gut allow spore germination. Several studies have shown that the presence of *B. thuringiensis* cells synergizes the toxic effect of the crystals (243, 255–259).

As natural targets of *B. thuringiensis*, insect larvae are a valuable model for studying the virulence of these bacteria. The utility of this infection model is increased by using insects that are poorly susceptible to the ingestion of crystals alone. A commonly used insect is the lepidopteran species *Galleria mellonella* (the greater wax moth) because the larvae are killed by the ingestion of *B. thuringiensis* spore-crystal mixtures but are only weakly affected by the ingestion of crystals alone (243, 258, 259). This model host has also been used to assess the virulence of *B. cereus*. Many other human pathogens are unable to kill *G. mellonella* in this infection model, reflecting the entomopathogenic specificity of these two species (260).

The *G. mellonella* host can be used in two infection methods. In association with Cry toxins to impair the insect defenses, the pathogenicity of *B. thuringiensis* and *B. cereus* can be assayed via the oral route. At the last instar, *G. mellonella* larvae are large enough (2 cm long and 250 mg) to be force-fed, thus allowing the ingestion of defined doses of bacteria (243). Larvae can be raised at various temperatures to mimic different natural conditions, including the mammal environment. This infection model allows determination of a 50% lethal concentration, measurement of bacterial survival, and assessment of the infectious process. The behavior of the bacteria in the intestinal environment, including bacterial factors interacting with the intestinal barrier, can be investigated (261). *G. mellonella* was used to demonstrate the major role of the PlcR regulon in the virulence of *B. thuringiensis* and *B. cereus* (243). The regulon consists of 45 genes encoding a large number of extracellular virulence factors such as degradative enzymes, hemolysins, and cytotoxins (17, 70). The PlcR-regulated metalloproteases InhA2, Bel, and ColB were demonstrated to be involved in *B. thuringiensis* virulence (38, 262, 263). In addition to *G. mellonella*, a nematode oral infection model in association with Cry proteins has also revealed virulence factors of *B. thuringiensis* (264). The ColB protease and another metalloprotease (Bmp1) were found to be involved in the pathogenicity of *B. thuringiensis* against *Caenorhabditis elegans* (262, 265).

A second mode of insect infection is direct injection of the bacteria into the hemocoelic cavity of the larva. This model bypasses the intestinal barrier and therefore does not require the crystal proteins. In addition to *G. mellonella*, the silk worm, *B. mori*, and several other insect species can be used. Bacteria are injected through the intersegmental membrane between the fourth and fifth abdominal legs of fourth-instar larvae (38). Most insect larvae are highly susceptible to the direct injection of vegetative cells or spores of *B. thuringiensis* and *B. cereus* into the hemocoel. This mode of infection led to the identification of multiple virulence factors, including genes associated with (i) iron metabolism and acquisition (266–268), (ii) resistance to the host defenses, including antimicrobial peptides, phagocytic cells, or nitric stress (202, 269–271), (iii) motility (272), and (iv) key transcription factors for *B. cereus* pathogenicity (273). The major role of the sphingomyelinase in the pathogenicity of *B. cereus* was also revealed by this method (39), thus confirming the observation that clinical strains of *B. cereus* overproducing sphingomyelinase were more virulent than environmental strains (203).

Data from these experimental infections clearly demonstrate that *B. thuringiensis* is a true entomopathogenic bacterium (Fig. 5), producing specific toxins that weaken or kill invertebrates and other virulence factors, shared with other *B. cereus* group isolates, that allow bacteria to provoke septicemia and death of the host (72). Moreover, it has been shown that these bacteria harbor a regulon conferring the ability to survive in the insect cadaver through a pathway independent of the sporulation and corresponding to a necrotrophic lifestyle (128, 174). This regulon is activated during the stationary phase of the bacteria by the NprR-NprX quorum sensing system and consists of 41 genes encoding several degradative enzymes, including proteases and chitinases. Although the role of each component is not determined, altogether they should contribute to maximizing the consumption of the nutriments found in the host cadaver. Furthermore, the insect model was successfully employed to demonstrate antibiotic-induced small colony variants, a novel persister phenotype of *B. cereus* (274).

Cell Culture

Immortalized cell lines are employed for testing the cytotoxic potential of *B. cereus* strains. Hep2 cells are used to check strains for the emetic toxin cereulide, while Vero cells are commonly used to check strains for their enterotoxic potential (35). However, more recently, it has been reported that different human cell lines show distinct susceptibility for specific enterotoxins, which must be taken into consideration when functional studies on enterotoxins are carried out (205). These results are in line with a previous study from Doll et al. (39) showing that in polarized colon epithelial cells *B. cereus* sphingomyelinase is a strong inducer of epithelial cell death, by synergistically interacting with Nhe,

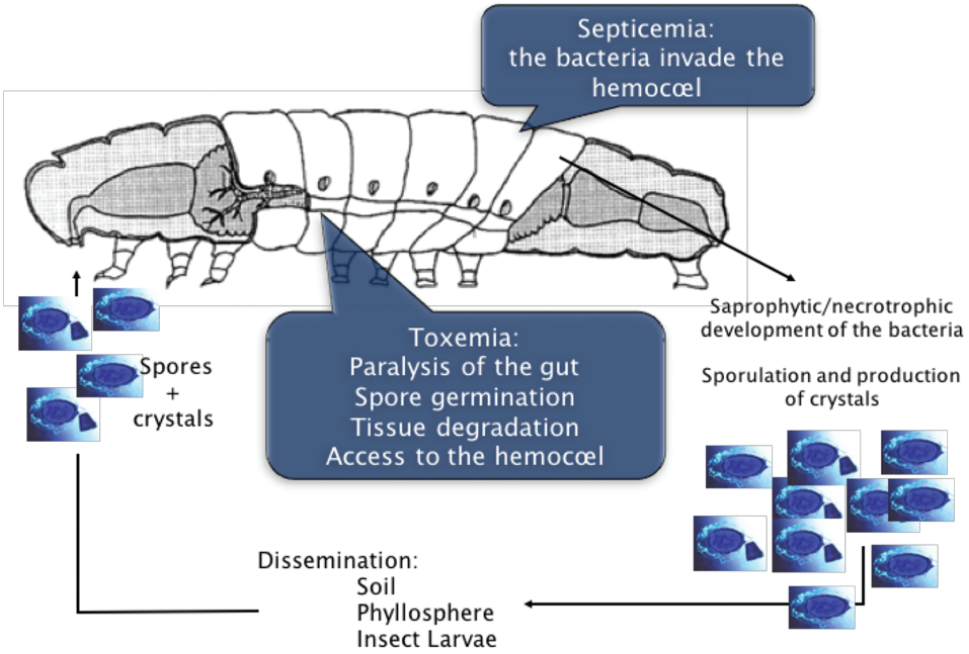


FIGURE 5 The infectious cycle of *B. thuringiensis* in a susceptible insect larva. Ingestion of spores and crystals is followed by the dissolution of the crystal in the alkaline environment of the midgut. Insect proteases activate Cry proteins. Spores germinate in the paralyzed insect gut. Cry toxins degrade the peritrophic membrane. Bacteria multiply and express PlcR-regulated genes. The intestinal barrier is disrupted and bacteria gain access to the hemocoel. The bacteria resist host defenses and cause a fatal septicemia. The NprR regulon is activated, and the bacteria survive in the host cadaver. Finally, spores and vegetative cells are disseminated outside of the host.

while most of the cytotoxic effect on primary endothelial cells can be explained by the cytotoxic activity of Nhe (205). Studies carried out on pancreatic beta cells revealed a detrimental effect of cereulide toxin on these cells, even in low concentrations, rendering it possible that long-term uptake of cereulide could have some implications in diabetes (213). Since beta cell dysfunction and cell death play key roles in the pathophysiology of diabetes, it will be important to study the long-term effects of subemetic doses of cereulide in more detail in appropriate eukaryotic cell models.

Furthermore, boar sperm has been used to show the mitochondrial activity of the *B. cereus* emetic toxin cereulide (275), and the enterotoxic activity of Hbl has been demonstrated in a rabbit ligated ileal loop test (197).

In vitro Simulation of GI Transit

Several systems that mimic gastric passage of *B. cereus* have been described (276–278). *B. cereus* spores, and to a certain extent vegetative cells, survive in these models, but enterotoxin production has not been detected, making these models suboptimal for studies of *B. cereus* enteropathogenicity. Such systems mimicking the physicochemical conditions from mouth to ileum have been successfully used to study probiotic bacteria (279) but lack the influence of host factors, which have been shown to be important for enterotoxin production in *B. cereus* (116).

VIRULENCE GENE EXPRESSION

Virulence gene expression within the *B. cereus* group is controlled by complex regulatory circuits. In addition to global transcription regulators, such as CodY and AbrB, that play key roles in the integration of virulence and bacterial metabolism (273, 280, 281), *B. cereus* group-specific regulators exert control of virulence gene expression on the transcriptional and posttranscriptional levels. The global transcriptional regulator AtxA, encoded by pXO1 of *B. anthracis*, plays a key role in the pathogenicity of *B. anthracis*, while the chromosome-encoded pleiotropic transcriptional regulator PlcR is the key regulator in *B. cereus* virulence (70, 282). Most of the virulence factors in *B. cereus* described so far are at least partially under the control of PlcR, except cereulide toxin synthesis, which belongs to the Spo0A-AbrB regulon (283). In addition, some reports highlight the importance of post-transcriptional regulation of virulence factor expression, which is largely unexplored (114, 284, 285).

Global Transcription Regulators

CodY, a global transcriptional regulator integrating virulence and metabolism, plays an essential role in regulation and timing of virulence gene expression in the *B. cereus* group. CodY, which is highly conserved in low-G+C-content Gram-positive bacteria, senses the nutritional and energetic status of a cell by binding to branched chain amino acids and GTP (286, 287). It often exerts control of virulence factors indirectly by acting on downstream regulators (288, 289). Although the majority of CodY-regulated genes are repressed during rapid growth, CodY is a positive regulator of some virulence genes (286, 290). In *B. cereus*, CodY governs the expression of virulence factors by impacting key regulatory circuits (66). CodY directly represses cereulide toxin synthesis in emetic *B. cereus* during early growth stages but acts indirectly on virulence genes belonging to the PlcR regulon by controlling import of the signaling peptide PapR, which is

required for PlcR activity (273, 281, 433). CodY was also reported to be essential for full virulence of *B. anthracis*. Data suggest that CodY exerts indirect positive control on post-translational regulation of AtxA accumulation (284). By acting, either directly or indirectly, on other virulence regulators, CodY adds an additional level of complexity to the regulatory circuits governing metabolic activities and virulence within the *B. cereus* group.

AbrB is a global transition state regulator that links virulence and cell development. It has been extensively characterized in *B. subtilis* but also highly conserved in other Gram-positive bacteria (291, 292). As is true for *B. subtilis*, AbrB is negatively regulated in the *B. cereus* group by the response regulator Spo0A at the onset of the transition phase. AbrB has been shown to control the expression of the anthrax toxin genes *pag*A, *lef*, and *cya* in *B. anthracis* as well as the cereulide (*ces*) synthetase genes in emetic *B. cereus*. In *B. thuringiensis*, *abrB* represses expression of *inhA1* during vegetative growth (293). During early exponential growth phase, *abrB*-null mutants synthesize elevated levels of anthrax toxin in *B. anthracis* and cereulide levels in emetic *B. cereus* (283, 294). Thus, it can be assumed that AbrB plays a pivotal role in the correct timing of toxin expression in *B. anthracis* as well as in emetic *B. cereus*.

Alternative sigma factors have also been implicated in transcription of virulence factors of the *B. cereus* group. The sporulation gene regulator SigH represents a target of the AbrB repressor in *B. subtilis* (295), and it has been postulated that AbrB acts on anthrax toxin gene expression via control of *sigH* (296). However, the *B. subtilis* consensus sequence for recognition by SigH has not been found upstream of the anthrax toxin genes nor upstream of the *ces* genes in emetic *B. cereus*, and no direct SigH-dependent toxin promotor activity could be shown (283, 297). Therefore, it is tempting to speculate that SigH is not directly linked to toxin gene regulation in *B. anthracis* or emetic *B. cereus*. SigB, another alternative sigma factor, may be a minor regulator of virulence in *B. anthracis*. Although there is no evidence that SigB affects toxin or capsule gene expression, a *sigB* mutant exhibits reduced virulence in a mouse model of anthrax (298).

RNPP Quorum Sensors

A major trait of the *B. cereus* group is the unique presence of two transcriptional regulators, PlcR and NprR, forming two distinct quorum sensing systems in association with their cognate signaling peptides, PapR and NprX, respectively (129, 299). In addition to these specific regulators, the bacteria of the *B. cereus* group produce proteins homologous to the Rap phosphatases of *B. subtilis* (175, 300). These quorum sensors define the RNPP family according to the name of the prototypical members Rap, NprR, PlcR, and PrgX (301). Quorum sensing systems connect gene expression to cell density, thus coordinating the behavior of the whole bacterial community able to respond to the same signaling peptide. In insects, it was shown that the sequential activity of RNPP regulators (PlcR, NprR, and Rap) controls the physiological stages of *B. thuringiensis* throughout the infectious cycle, from virulence to sporulation (302).

PlcR positively regulates 45 genes, most of which encode exported virulence factors such as the enterotoxins Hbl and Nhe, the cytotoxin CytK, the cholesterol-dependent hemolysins, and several degradative enzymes (70). As discussed above, PlcR plays a key role in the virulence of *B. thuringiensis* and *B. cereus* in their invertebrate or mammal hosts (70, 72, 245). The *plcR* gene is present in all *B. cereus* group species but does not always produce a functional protein (303). Notably, in *B. anthracis* a nonsense mutation inactivates the PlcR function (17, 18). PlcR is a 34-kDa helix-turn-helix-type transcription factor activated upon binding of PapR. This binding induces a drastic conformational change of the dimeric form of PlcR, allowing an appropriate positioning of the helix-turn-helix domains and their binding to the PlcR-boxes located upstream from the −10 region of the PlcR-regulated promoters (299, 301, 304). Binding of PapR to PlcR is highly specific, and polymorphisms exist within the *B. cereus* group. Four specificity groups, or pherotypes, can be distinguished (305, 306).

NprR is also found in all *B. cereus* group species and acts as a transcriptional regulator at the onset of sporulation (128). As indicated above, NprR is an RNPP regulator whose transcriptional activity depends on a signaling peptide (NprX) that switches NprR from a dimeric apo form to a tetrameric form (307). Similar to PlcR-PapR, the NprR-NprX system is strain specific, and seven pherotypes were defined within the *B. cereus* group (129). NprR-NprX activates the transcription of a regulon including genes encoding degradative enzymes (proteases, lipases, and chitinases) and a lipopeptide (kurstakin) involved in biofilm formation (128, 308). Using insect larvae as an infection model, it was shown that the NprR regulon allows bacteria to enter in a necrotrophic lifestyle essential for the survival of the bacilli in the cadavers of infected insects. In the absence of its cognate peptide, the apo form of NprR functions as a phosphatase inhibiting the sporulation process (309). It has been shown that sporulation occurs only in bacteria expressing the NprR regulon and following a necrotrophic lifestyle in the insect cadaver (174). These results are in agreement with the dual function of NprR and indicate that its phosphatase activity should be turned off by NprX to allow the sporulation of the bacteria.

AtxA, the Key Regulator in *B. anthracis* Virulence

AtxA, named for its control of toxin gene expression (310) and encoded by a pXO1 gene, is the master virulence regulator of *B. anthracis*. AtxA positively controls transcript levels of the structural genes for anthrax toxin, *pag*A, *lef*, and *cya*, located on pXO1; the capsule biosynthetic operon *cap*BCADE located on pXO2; and multiple other genes on the plasmids and chromosome (5, 282, 311–318). An *atx*A-null *B. anthracis* strain is highly attenuated in a murine model of anthrax disease (318, 319). The *atx*A gene has also been associated with control of *B. anthracis* development. AtxA activates expression of a pXO2-encoded sporulation kinase inhibitor to repress sporulation in conditions in which AtxA is highly expressed and active (320). This activity may be related to the lack of *B. anthracis* sporulation during infection.

Paralogous regulators, AcpA and AcpB encoded by pXO2 genes, were first identified as regulators of *cap*BCADE (321, 322). AcpA and AcpB do not appear to affect toxin gene expression but may affect expression of noncapsule genes (282, 323). Data from experiments employing null mutants reveal that expression of the *atx*A, *acp*A, and *acp*B genes is interdependent and suggest that AcpA and AcpB have similar functions. The primary means for AtxA control of *cap*BCADE is via positive regulation of *acp*A transcription. The *acp*A gene is located upstream of the *cap* locus and in the opposite orientation. Two apparent transcriptional start sites, one expressed at a low constitutive level and one controlled positively by AtxA, drive expression of *acp*A. AcpA can positively affect transcription of *cap*BCADE in the absence of AtxA and AcpB. The *acp*B gene, located downstream of *cap*BCADE and in the same orientation, is expressed as a

monocistronic transcript initiated from a weak constitutive promoter. AcpB, like AcpA, can positively affect *capBCADE* expression in the absence of the other regulators. A weak transcription terminator located between *capE* and *acpB* results in cotranscription of *acpB*, with *capBCADE* in roughly 10% of transcripts, thus forming a positive feedback loop (324).

AtxA expression is also subject to complex regulation. Transcription of *atxA* is affected by temperature, carbohydrate availability, redox potential, metabolic state, and growth phase (325–328). In addition, CodY controls AtxA protein levels posttranscriptionally by an unknown mechanism (284), and AbrB represses *atxA* expression by binding to the *atxA* promoter region upstream of the P1 transcriptional start site (294, 329). Finally, an unknown additional repressor binds to the *atxA* promoter to negatively regulate expression (319).

AtxA, AcpA, and AcpB belong to an emerging class of regulators termed PCVRs (phosphoenolpyruvate-dependent phosphotransferase system regulation domain [PRD]-containing virulence regulators) (330, 331). Generally, PCVRs contain nucleic acid-binding domains and PRDs subject to phosphorylation by the phosphotransferase system (332). AtxA crystalizes as a dimer, and distinct associated folds predicted that DNA-binding domains and PRDs are apparent in each chain. The model for PRD control of AtxA function is that phosphorylation events at two specific histidines, one in each PRD, have opposing effects on AtxA function.

Specific DNA-binding activity of AtxA has not been reported, but phosphorylation of H199 of PRD1 is predicted to affect the interaction of AtxA with DNA, while phosphorylation of H379 in PRD2 disrupts AtxA dimerization and abolishes activity (333). Phosphorylation of AcpA and AcpB has not been demonstrated. Although comparisons of the AcpA and AcpB amino acid sequences and structure predictions are highly suggestive of the presence of PRDs, the placement of histidines within the PRDs differ between the three regulators, suggesting large differences in the potential phosphorylation sites (330). If indeed the AcpA and AcpB PRDs control PCVR function, the relationships between phosphorylation and protein structure may differ between the PCVRs, affecting their target specificity and relative activity.

GENETIC TOOLS

Natural Genetic Exchange: Intra- and Interspecies

Historically, facile identification of the *B. cereus* group species has relied on their plasmid content: plasmids carrying the crystal insecticidal genes of *B. thuringiensis*, the anthrax toxin and capsule genes of *B. anthracis* and more recently *B. cereus* bv. *anthracis*, and the biosynthetic gene cluster for cereulide synthesis of *B. cereus* (6, 16, 49, 334). However, genetic experiments have revealed that plasmids can move among the strains of the *B. cereus* group by conjugation and transduction (335–338), thus complicating the speciation of strains belonging to this bacterial group. Horizontal genetic transfer of plasmid and chromosome DNA has likely contributed to the remarkable diversity of the strains composing the *B. cereus* group.

Transducing phage have been characterized for all three *B. cereus* species. The bacteriophage CP-51, isolated from *B. cereus*, was the first generalized transducing phage discovered for the group and has been the most utilized for genetic manipulation (339). CP-51 and CP-54, a phage with a broader host range than CP-51, also mediate generalized transduction in *B. thuringiensis* (340, 341). They can transduce various plasmids between the *B. cereus* group species and are also useful for intraspecies transduction of chromosomal markers (338, 342). CP-51-mediated transduction of pXO2 from *B. anthracis* to *B. cereus* was instrumental in demonstrating that the plasmid was associated with capsule synthesis (343), while the first genome maps of *B. thuringiensis* were established using this phage (340, 341, 344).

Plasmid conjugation in bacteria of the *B. cereus* group was first demonstrated by showing the transfer of plasmid-borne *cry* genes from *B. thuringiensis* strains to *B. cereus* (51). These observations were then extended to other plasmids and species, including *B. anthracis* (343, 345). Conjugative plasmids native to *B. thuringiensis* have been shown to transfer to *B. anthracis* and *B. cereus* and in some cases to mobilize other non-self-transmissible plasmids. Among these are *B. thuringiensis* plasmids harboring insecticidal toxin genes, such as pXO12 (348) and pHT73 (347). The conjugation experiments performed between *B. anthracis* and *B. thuringiensis* revealed transfer of plasmids by a conduction process involving the formation of cointegrate structures between a conjugative plasmid (i.e., pXO12) and the nonconjugative plasmid pXO1. These experiments showed that the transposon Tn*4430*, located in various conjugative plasmids of *B. thuringiensis* (8, 346), mediates the transfer of nonconjugative plasmids by a conduction process (348). These findings indicate that transposable elements associated with conjugative plasmids may have important adaptive functions by mediating the horizontal transfer of genetic material, including toxin genes, within the *B. cereus* group.

Plasmid transfer among the *B. cereus* group has been reported to occur in various niches, including the insect gut (349–351), gnotobiotic rats (352), the plant rhizosphere (63), soil (61), water (353), and dairy products (354, 355), and a growing database of DNA sequences from clinical and environmental isolates reveals evidence for extensive genetic exchange among the *B. cereus* group. Multiple isolates from soil carry pXO1- and pXO2-like plasmids (24, 355–358). In some cases, clinical isolates from humans and animals have been reported with newly discovered combinations of potential plasmid-borne virulence genes. Interestingly, pAW63, from a *B. thuringiensis kurstaki* strain, shares a common backbone sequence with pXO2 of *B. anthracis* and with pBT9727 from the pathogenic *B. thuringiensis* subspecies *konkukian* (359, 360). *B. cereus* strain G9241, isolated from a patient with life-threatening pneumonia, carries a specific PA gene (*pagA*) on plasmid pBCXO1 and a gene on plasmid pBC218 that encodes a polysaccharide capsule (24).

Some reports have also described genetic exchange between *B. subtilis* and members of the *B. cereus* group. Plasmid pLS20 from *B. subtilis* subspecies *natto* is self-transmissible and facilitates transfer of other plasmids from *B. subtilis* to the *B. cereus* group species (361). Although integrative and conjugative elements (ICEs) have not been reported in the *B. cereus* group, *B. anthracis* has been shown to be an effective recipient of ICE*Bs1* from *B. subtilis* (362). The presence of *cry* genes encoding insecticidal toxins in *Bacillus popilliae* (363) and *Clostridium bifermentans* (364) strongly suggests a horizontal transfer of *B. thuringiensis* plasmids to other spore-forming Gram-positive bacteria.

Generation of Recombinant Strains and Genetic Analysis

B. cereus group members are not naturally transformable, and the commercialization of the BioRad electroporation system in the 1980s was a crucial milestone in the development of

genetic approaches for these bacteria. In 1989, several electroporation procedures were published for the transformation of *B. thuringiensis* and *B. cereus* (365–367, 434) and were followed by procedures functional in *B. anthracis* (311) or in the emetic *B. cereus* strains (42, 370).

Interestingly, it was shown that electrotransformation is significantly improved when plasmids are prepared in DNA methyltransferase-deficient (*dam*) *E. coli* strains or from *B. subtilis* 168, a strain that is not known to possess adenine-methylating activities (371, 372). Plasmids can also be transferred from *Escherichia coli* to the bacteria of the *B. cereus* group by heterogramic conjugation (368, 369, 373). A second important milestone for the development of genetic studies in the *B. cereus* group was the construction of cloning and expression vectors. This was notably achieved thanks to the use of the pHT1030 replicon, which allowed the construction of a series of highly stable vectors (374, 435). Due to the relatively low transformation frequency of the *B. cereus* strains, site-directed mutagenesis and direct allelic exchange is not possible using linear DNA and requires the use of shuttle plasmids such as pMAD or pRN5101, whose replication in Gram-positive bacteria is thermosensitive (375, 436). More recent systems draw upon methods first developed for other bacteria. They include group II intron targeting (376) and systems using tyrosine site-specific recombinases that catalyze cutting and joining reactions between short specific DNA sequences. The systems employing tyrosine site-specific recombinases and their cognate target sequences have been derived from *E. coli* bacteriophage P1 Cre-loxP, *Saccharomyces cerevisiae* Flp-FRT, and a newly discovered IntXO-PSL system from *B. anthracis* plasmid pXO1 (377). A method for conducting I-SceI-mediated allelic exchange in which recombinational loss of a chromosomally integrated allelic exchange vector is stimulated by creation of a double-stranded break within the vector by the homing endonuclease I-SceI has also been described (378, 379). This system has been used to make in-frame deletions in genetic elements of *B. anthracis* (152, 161, 380), *B. cereus* (381, 382), and *B. thuringiensis* (383).

For random mutagenesis, Tn*10*- and *mariner*-based mini-transposons have proved useful for the detection of virulence genes or for the study of genetic systems (384–388). Insertion mutant libraries have also been generated using *Enterococcus faecalis* transposons Tn*916* and Tn*917*. However, insertions of these transposons can be unstable and are sometimes accompanied by local deletions and DNA rearrangements (389, 390).

An *in vivo* expression technology was developed for the identification of *B. cereus* genes specifically expressed during infection in an insect host (266). This genetic tool allows identification of transient promoter activation by using site-specific recombination. The activity of a promoter cloned upstream from a recombinase gene results in the loss of a resistance marker and the activation of a new resistance gene. The *in vivo* screening of a *B. cereus* genomic library led to the characterization of genes expressed during infection in the insect *G. mellonella*. One of these genes was found specifically expressed in the hemocoel and was required for iron acquisition (267), while a five-gene cluster, specifically expressed in the larval gut, was essential for glucose-6-phosphate uptake (391).

Measurements of Gene Expression

Whole-transcriptome sequences of *B. anthracis* and *B. cereus* cultured in different growth conditions have been described (392–394). Not surprisingly, the data predict gene functions and reveal multiple transcripts not associated with previous genome annotations. As is true for other bacteria, the advent of RNA whole transcriptome shotgun sequencing (RNASeq) and quantitative reverse transcriptase PCR (qRT-PCR) has lessened the need for recombinant methods to measure gene expression. Nevertheless, for some studies of the bacteria of the *B. cereus* group, expression of specific genes can be readily monitored using transcriptional fusions employing promoterless genes for β-galactosidase and chloramphenicol acetyltransferase (369, 396, 397). Investigations of transcript and protein function are also aided by the use of recombinant constructs harboring promoters inducible by isopropyl-β-Dthiogalactopyranoside or xylose (223, 273, 299, 398) or by use of recombinant constructs harboring promoters inducible by cold (283).

Bioluminescent reporter systems, based on *luxABCDE* promoter fusions, for real-time monitoring of gene transcription have been described for *B. anthracis* and for *B. cereus*. A *sspBp::lux* plasmid was used for *in vivo* spore germination of *B. anthracis* in mice (164), and a *cesP1::lux* plasmid was successfully employed for monitoring cereulide synthetase (*ces*) promoter activity in complex environments, such as foods (58, 370). Various fluorescent reporters were used to record gene expression at the single-cell level and to follow the physiological stages of *B. thuringiensis* in different growth conditions, including biofilm and infection in insect larvae (174, 399). Furthermore, the *E. coli*/*B. cereus* shuttle vector pAD123 with a promoterless red-shifted green fluorescent protein gene (*gfpmut3a*) was shown to be an effective tool for promoter studies in *B. cereus* (370, 400).

THERAPEUTICS AND VACCINES

Antibiotic therapy is the primary treatment for anthrax and for severe infections caused by *B. cereus and B. thuringiensis*. The *B. cereus* group species have similar antibiotic susceptibility profiles and are susceptible to the majority of front-line antibiotics. Historically, penicillin was the antibiotic of choice for the treatment of known or suspected anthrax. However, intrinsic or inducible resistance to β-lactam antibiotics has been reported in clinical isolates (401–403).

Because *B. cereus* and *B. thuringiensis* infections are relatively rare, are generally treatable with antibiotics, and in the case of *B. cereus* food-poisoning, are self-limiting, vaccines have not been developed for these infections. Anthrax vaccines for humans have been available for decades, but the nature of the vaccine, as described below, provides the impetus for continued research in anthrax vaccine development.

Anthrax Prophylaxis and Treatment

The Centers for Disease Control and Prevention recommends fluoroquinolones, such as ciprofloxacin or doxycycline, as first-line drugs for postexposure prophylaxis and treatment of *B. anthracis* infections. The bactericidal activity and exceptional central nervous system penetration make ciprofloxacin recommended over doxycycline. For treatment of severe systemic and life-threatening disease and for cases with fulminant bacteremia, ciprofloxacin with additional antibiotics, especially clindamycin, is recommended. Clindamycin inhibits protein synthesis, potentially reducing toxin production (404, 405). Treatment with additional antibiotics is also recommended because of reports of ciprofloxacin-resistance generated following serial passage in the presence of increasing concentrations of the antibiotic. Ciprofloxacin resistance is associated with a point mutation in the *gyrA* gene encoding gyrase A (406, 407).

Long courses of antibiotics are recommended for anthrax prophylaxis and treatment because *B. anthracis* spores have been reported to remain viable for up to 60 days in nonhuman primates. Recently, the U.S. Food and Drug Administration approved the anthrax vaccine AVA for postexposure prophylaxis in conjunction with antimicrobials. The efficacy of antibiotic therapy for anthrax decreases as the time between exposure and treatment increases (408–411), likely due to increasing toxin levels, indicating a need for antitoxin therapeutics. Toxin inhibitors in development include a variety of peptides and antibodies (412, 413).

Anthrax Vaccines

Current human vaccines for anthrax are the U.S.-licensed AVA (anthrax vaccine adsorbed, BioThrax) and the United Kingdom-licensed AVP (anthrax vaccine precipitated). The anthrax toxin protein PA is the major immunogenic component of both vaccines. PA elicits toxin-neutralizing antibodies that correlate with protection against anthrax disease in humans and many animal models (414–418). AVA is an aluminum hydroxide-adsorbed, PA-containing supernate from a culture of a toxin-producing ($pXO1^+$) but noncapsulated ($pXO2^-$) strain of *B. anthracis*. The standard AVA vaccination protocol requires five doses administered intramuscularly over 18 months, followed by annual booster injections (419), while a more recently approved regimen requires three doses over 6 months. AVP is precipitated with aluminum potassium sulfate (alum) from culture supernates of a different toxinogenic noncapsulated strain. The AVP vaccine is administered as four doses over 32 weeks with annual boosters. Unlike AVA, AVP contains low levels of LF and EF, and some reports indicate that humoral responses to LF and EF generated by AVP may contribute to toxin neutralization (420, 421).

The prolonged immunization regimen with reports of reactogenicity, cold-storage requirement, and 4-year half-life make the anthrax vaccines nonoptimal for pre-exposure prophylaxis and postexposure therapy. Thus, various expression systems, immunogens, adjuvants, and delivery vehicles are in development to achieve a higher-performing and more user-friendly vaccine.

Efforts include investigations of purified recombinant PA preparations, *Lactobacillus*- and *Salmonella*-producing PA for oral delivery, DNA vaccines, and non-PA-based vaccines with alternative targets such as the *B. anthracis* capsule, cell wall S-layer proteins, spore components, and the toxin proteins EF and LF (422–427). The veterinary anthrax vaccine is a live spore vaccine developed in 1937 by Max Sterne (428). The strain, 34F2 or "Sterne," was first isolated as a stable noncapsulated isolate and was later shown to carry $pXO1^+$ but not $pXO2^-$. Animals injected with the Sterne vaccine develop humoral immunity within 4 weeks, but the immunity is not long-lived, and annual revaccination is recommended. Although the vaccine can be effective in livestock populations, in many parts of the world, underreporting, logistics, and limited resources make implementing vaccination campaigns difficult.

FUTURE DIRECTIONS

Substantial effort has gone into determining biomarkers and distinguishing features of the closely related members of the *B. cereus* group. However, distinctions between the species and between virulent and nonvirulent strains of the species are not always clear. A key issue for *B. cereus* (and for *B. thuringiensis*) is the detection of pathogenic strains responsible for food poisoning resulting in diarrheal syndromes. These syndromes are suspected to be caused by the various cytotoxins and degradative enzymes produced by bacteria under the control of the virulence regulator PlcR. However, most strains produce these factors and are not generally pathogenic, as is likely the case with *B. thuringiensis* strains that are used as biopesticides.

B. thuringiensis is defined by the ability to produce a parasporal inclusion made of Cry proteins. Numerous Cry proteins are insecticidal or nematicidal toxins, but some have no known targets. The search for the presumed targets, whether in arthropods or other organisms, is of scientific and economical interest and will facilitate increased understanding of the expanded ecology of these bacteria. Efforts to identify biomarkers specific for *B. anthracis* are a significant part of biodefense research. While such studies have affirmed some novel features of the species, the recent reports of *B. cereus* isolates causing anthrax-like disease suggest that strains of other species may also present a bioweapon threat. Moreover, increased environmental surveillance and expanded genome sequencing are revealing newly recognized *B. cereus* group isolates with unique physiology, genetics, and virulence capabilities.

The emergence of clinical isolates with novel combinations of plasmids and recombinant forms of well-characterized plasmids suggests an evolving virulence repertoire. It will be interesting to explore the mechanisms by which these changes occur and the frequencies of apparent horizontal gene transfer in nonhost environments. What relationships, if any, exist between plasmid content and specific chromosomes? What happens to the signaling networks controlling gene expression in these atypical isolates? For some *B. cereus* isolates, plasmids can represent up to 20% of the total DNA. With the exception of genes involved in pathogenicity, the adaptive functions of these plasmids are still largely unknown.

Much has been learned about host-pathogen interactions associated with virulent strains of the *B. cereus* group species. What are the core genomic factors of the *B. cereus* group species that are required to cause distinct diseases? Can such factors actually be defined? Do the "virulence" plasmids play roles in the nonhost environments of the *B. cereus* group species? To answer these questions, more advances must be made in the study of the ecology of these organisms, their lifestyles outside of their hosts, and their interactions with each other.

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Sporulation and Germination in Clostridial Pathogens

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56

Notably, different mechanisms exist between these organisms for forming and germinating spores. Some of these differences reflect phylogenetic differences between the *Clostridiaceae* (represented by *C. perfringens* and *C. botulinum*) and the *Peptostreptococcaceae* (represented by *C. difficile*) (1). Other differences reflect genetic diversity within each species (2–4). *C. botulinum* is the most divergent, being divided into four metabolic groups (groups 1 to IV) that effectively represent different species despite their shared production of botulinum toxin (5).

IMPORTANCE OF SPORES TO CLOSTRIDIAL PATHOGENESIS

Disease transmission by clostridial pathogens depends on their ability to form aerotolerant, metabolically dormant spores before exiting their hosts (6). Since spores are highly resistant to extreme temperature and pressure changes, radiation, enzymatic digestion, and oxidizing agents (7), they can persist for long periods of time and serve as environmental reservoirs for these organisms (8). Spores from *C. perfringens*, *C. botulinum*, and *C. difficile* can be isolated from diverse environments, including animal gastrointestinal tracts and carcasses, wastewater, lawns, hospital rooms, and soil (8). Infections by these pathogens typically are initiated upon ingestion of spores, although *C. perfringens* can also enter the body via contaminating wounds. Upon sensing small-molecule germinants, spores from these pathogens germinate and outgrow into toxin-secreting vegetative cells.

C. perfringens

C. perfringens causes two major human diseases: food poisoning and gas gangrene (also known as clostridial myonecrosis [9]). Clostridial myonecrosis occurs when spores from the soil enter muscle tissue, typically through a wound, while food poisoning arises when spores or vegetative cells in contaminated food are ingested. Unlike most clostridial pathogens, *C. perfringens*' vegetative form can initiate infection when present at sufficiently high levels to survive passage through the stomach. *C. perfringens* causes disease by secreting a number of toxins; *C. perfringens* strains are subdivided into toxigenic types A to E based on the combination of the alpha-, beta-, epsilon-, and iota-toxins they produce.

C. perfringens spores can be remarkably heat resistant, with some type A strains surviving boiling for >1 h (10, 11). Spores from food-poisoning isolates exhibit greater resistance to heat (~60-fold), cold, and oxidizing agents (12, 13) than nonfoodborne isolates, suggesting that their resistance properties facilitate their survival in undercooked or improperly held food (10).

C. botulinum

C. botulinum causes a flaccid paralysis known as botulism through the production of the potent neurotoxin, botulinum toxin (BoNT). *C. botulinum* strains are subdivided based on their production of one or more of seven BoNT types (A to G) (14, 15). Botulism typically results from the ingestion of preformed BoNT in contaminated foods, but ingestion of *C. botulinum* spores in contaminated foods such as unpasteurized honey can cause infant paralysis, particularly in those <1 year old (16). Even in cases of botulism intoxication, the spore form is critical for contaminating food, where incomplete sterilization or processing, such as during home canning, can create anaerobic environments that allow *C. botulinum* spores to germinate and form toxin-producing vegetative cells that subsequently intoxicate the food (17).

C. difficile

C. difficile is a leading cause of antibiotic-associated diarrhea and pseudomembranous colitis worldwide (18, 19). *C. difficile*-associated disease occurs when spores, ingested by susceptible hosts, germinate in response to specific bile acids sensed in the mammalian gut; the resulting vegetative cells produced secrete glucosylating toxins that are the primary cause of disease symptoms (20). Notably, antibiotic exposure sensitizes individuals to antibiotic-resistant *C. difficile* by removing the colonization resistance conferred by our gut microflora (21). Spores are critical to this infection process not only because they are essential for transmitting *C. difficile* infections (6) but also because they are inert to antibiotics and resist many commonly used disinfectants in health care settings (22, 23). Accordingly, spores are easily detected in health care-associated environments (24) in addition to numerous other sites (8).

OVERVIEW OF SPORE FORMATION

The first morphological stage of sporulation is the formation of a polar septum, which generates two morphologically distinct,

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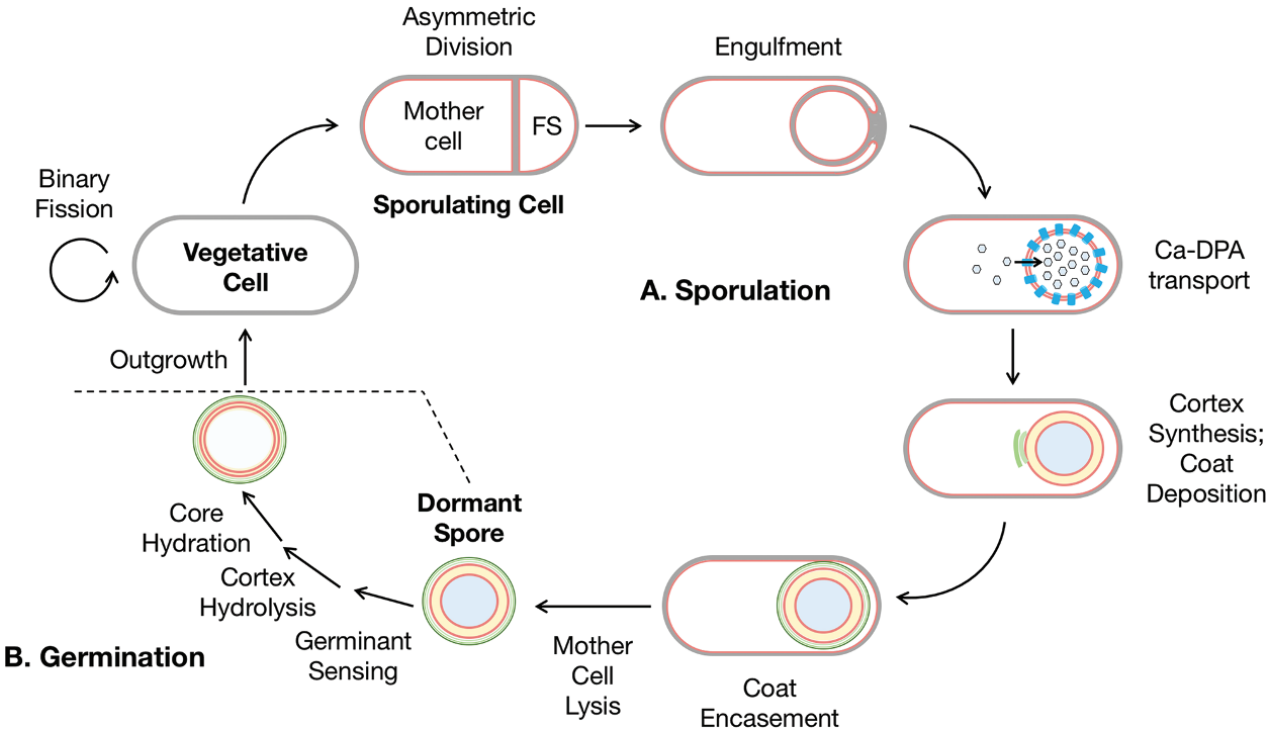


FIGURE 1 Lifecycle of endospore formers. (A) Sporulation. Upon sensing certain environmental conditions, endospore formers activate Spo0A and initiate sporulation. The first morphological event is the formation of a polar septum, which creates a larger mother cell and smaller forespore. The mother cell engulfs the forespore, and the two cells work together to assemble the dormant spore. Calcium dipicolinic acid (Ca-DPA) is synthesized in the mother cell and transported into the forespore in exchange for water. The cortex is formed between the two membranes, and coat proteins polymerize on the surface of the mother cell-derived membrane. Once the spore is mature, the mother cell lyses and releases the dormant spore into the environment. (B) Germination. Upon sensing the appropriate small molecule germinants, the spore initiates a signaling cascade that leads to activation of cortex hydrolases and core hydration, which is necessary for metabolism to resume in the germinating spore.

but genetically identical, cells (Fig. 1) (25). The larger mother cell engulfs the smaller forespore cell, leaving the forespore within the mother cell cytosol surrounded by two membranes. A thick layer of modified peptidoglycan known as the cortex forms between the two membranes, conferring spores with heat and ethanol resistance (26, 27). A series of proteinaceous layers known as the coat assembles around the outer forespore membrane and protects the spore against enzymatic and oxidative insults. In *C. difficile*, an additional layer known as the exosporium assembles on the coat, but this layer is not present in all spore-forming organisms.

The mother cell and forespore also coordinately prepare the forespore for dormancy. The mother cell produces large amounts of dipicolinic acid (pyridine-2,6-dicarboxylic acid) complexed with calcium (Ca-DPA), which it pumps into the developing forespore in exchange for water (28). The resulting partially dehydrated forespore cytosol prevents metabolism. The forespore produces large amounts of small acid-soluble proteins (SASPs), which coat the chromosome, prevent transcription, and protect against DNA damage (26, 28). Once the forespore completes its maturation, the mother cell induces lysis and releases the metabolically dormant spore into the environment.

SPORULATION INITIATION IN CLOSTRIDIAL PATHOGENS

The decision to initiate sporulation requires that vegetative cells recognize specific environmental and nutritional signals and assimilate these cues into a robust response. All sporulating *Firmicutes* use the conserved transcriptional regulator, Spo0A, as a key checkpoint for integrating these signals. The response regulator Spo0A initiates spore formation by activating or repressing the expression of genes encoding early sporulation regulators (6, 29–34), and its DNA-binding activity is directly controlled by phosphorylation. In the model organism *B. subtilis*, Spo0A phosphorylation is orchestrated by a complex regulatory network, known as a phosphorelay, which consists of several orphan sensor histidine kinases (KinA-E; orphan refers to histidine kinases that are not encoded beside a response regulator) and two phosphotransfer proteins (Spo0F and Spo0B) (35). The KinA to KinE kinases directly phosphorylate Spo0F, which subsequently transfers the phosphate to Spo0B and finally to Spo0A (Fig. 2) (36). Antikinases and two classes of phosphatases inhibit Spo0A phosphorylation levels by either blocking kinase activity or stripping Spo0A or Spo0F of its phosphate (37). The complexity of this regulatory pathway functions as a noise generator, creating heterogeneous levels of Spo0A phosphorylation within a population such that only a portion of its population commits to sporulation (38–40).

Notably, the *B. subtilis* phosphorelay is absent in clostridial pathogens, since clostridia lack orthologs of the Spo0F and Spo0B phosphotransfer proteins (41, 42). Thus, Spo0A appears to be directly phosphorylated by histidine kinases in clostridial organisms (32, 33, 43–46), although the kinases, regulatory pathway, and environmental signals used to

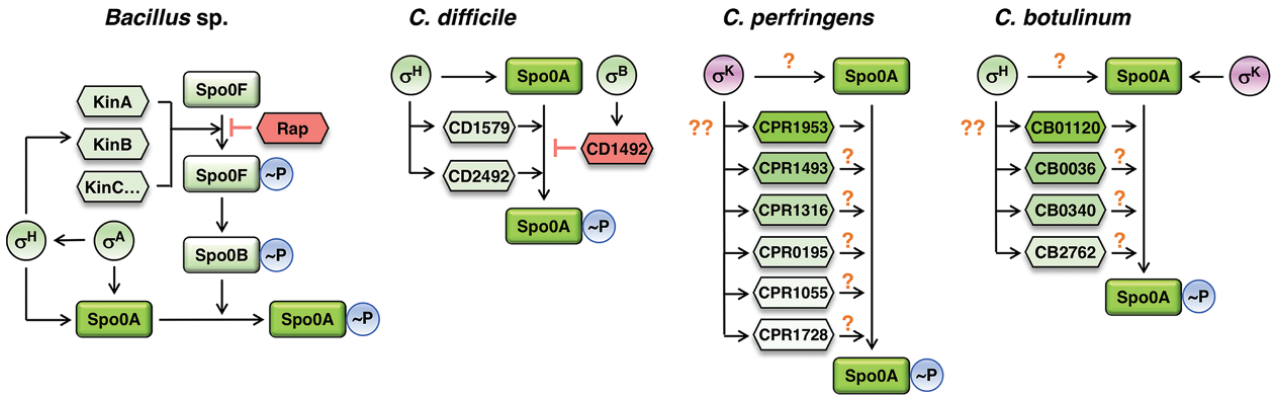


FIGURE 2 Sporulation initiation via Spo0A phosphorylation. Sigma factors are shown as circles, histidine kinases and phosphatases as hexagons (adapted from Al-Hinai et al. [85]). Positive regulators are shown in green (with the exception of σ^K, which is shown in purple), and negative regulators are shown in red. In *B. subtilis*, the KinA-E orphan histidine kinases phosphorylate the phosphotransfer protein Spo0F, the first component in the phosphorelay (25). The Rap phosphatases remove phosphates from phosphorylated Spo0F. The phosphate is transferred from Spo0F to Spo0B to Spo0A. In *C. difficile*, the orphan histidine kinases CD1579 and CD2492 appear to phosphorylate Spo0A (32), while CD1492 likely dephosphorylates Spo0A (47). A more detailed description of *C. difficile* Spo0A regulation is shown in Fig. 3. Although all the putative orphan histidine kinases with the potential to phosphorylate Spo0A in *C. perfringens* and *C. botulinum* are shown, whether they act as positive or negative regulators remains unstudied. The stationary factor σ^H activates expression of *spo0A* in *B. subtilis* and *C. difficile* (101), while σ^K activates *spo0A* transcription in *C. botulinum* (74) and possibly *C. perfringens*, the latter of which induces sporulation during log-phase growth (65).

control clostridial Spo0A phosphorylation remain largely uncharacterized.

C. difficile

Sporulation initiation has been most extensively studied in *C. difficile*, where key Spo0A regulatory proteins have been identified (Fig. 3). An initial study identified three orphan histidine kinases with significant homology to the *B. subtilis* family of sporulation-associated phosphotransfer histidine kinases (32). Loss of one of these putative histidine kinases, CD2492, decreased spore formation ~3-fold in rich liquid media, while another kinase, CD1579, directly phosphorylated Spo0A *in vitro* (32). These results suggest that both CD2492 and CD1579 promote Spo0A activation. Loss of the third putative histidine kinase, CD1492, increases sporulation ~4-fold on solid sporulation media in a manner dependent on its conserved histidine residue (47), suggesting that CD1492 functions as a phosphatase rather than as a kinase.

Interestingly, CD2492 does not necessarily always promote sporulation, since a *CD2492* mutant exhibits increased sporulation on solid sporulation media independent of its conserved histidine residue (A. Edwards and S. McBride, unpublished data). The discrepancy in *CD2492* mutant sporulation phenotypes could be due to differences in growth conditions and/or methods for measuring spore formation. The signals that control kinase versus phosphatase activity of sporulation-associated histidine kinases are largely unknown, even in *B. subtilis*; thus, the activity observed may depend on the presence or absence of unidentified signals. Indeed, one *B. subtilis* kinase mutant exhibits varying sporulation phenotypes depending on the growth conditions used (48, 49). This contradiction highlights the importance of assessing sporulation using multiple conditions and verifying spore frequencies with at least two different methods (50).

Notably, sporulation-associated histidine kinases directly control Spo0A phosphorylation through competing kinase and phosphatase activities in *C. acetobutylicum* and *C. thermocellum*, where at least one sporulation-associated histidine kinase in each species inhibits sporulation *in vivo* (44, 45), and a *C. acetobutylicum* histidine kinase has been shown to dephosphorylate Spo0A *in vitro* (45). Altogether, clostridial sporulation-associated histidine kinases appear to reversibly regulate Spo0A phosphorylation and thus the onset of sporulation.

Spo0A activity in *C. difficile* is further modulated by an RRNPP family ortholog, RstA (regulator of sporulation and toxins) (51), which shares homology with the Rap phosphatases that directly dephosphorylate Spo0F in *B. subtilis*. RRNPP family members have multiple C-terminal tetratricopeptide repeat domains that bind quorum-sensing peptides and regulate the cognate N-terminal helix-turn-helix DNA-binding domain and/or Spo0A/Spo0F-binding domain (52, 53). RstA contains these three conserved regulatory domains, and an *rstA* mutant produces ~20-fold fewer spores than the wild type, indicating that RstA promotes early sporulation events in *C. difficile* (29). Although the helix-turn-helix domain appears to be dispensable for RstA to modulate sporulation (29), the putative Spo0A/Spo0F-binding domain may directly bind and control Spo0A phosphorylation and/or dephosphorylation (A. Edwards and S. McBride, unpublished data). Interestingly, in addition to regulating sporulation, RstA represses motility and toxin production (see below) (51). Homologs of RstA are observed in both pathogenic and nonpathogenic clostridial organisms, including *C. sordellii*, *C. perfringens*, *C. botulinum*, and *C. acetobutylicum* (51).

RRNPP systems in other spore formers use quorum-sensing peptides to control activity of the regulator and, thus, sporulation initiation. For example, *B. subtilis* Rap phosphatase activity is inhibited by quorum-sensing peptides imported by the conserved oligopeptide permeases Opp and App, promoting sporulation (32, 34–36). Further, *C. acetobutylicum* sporulation is affected by the deletion or overexpression of genes encoding RRNPP-type regulators, which are located adjacent to putative quorum-sensing peptide genes (54). However, it is unclear whether *C. difficile* RstA activity is regulated by quorum-sensing peptides. Regardless, peptide transport coordinates the onset of sporulation in *C. difficile*, since the loss of Opp and App increases *C. difficile*

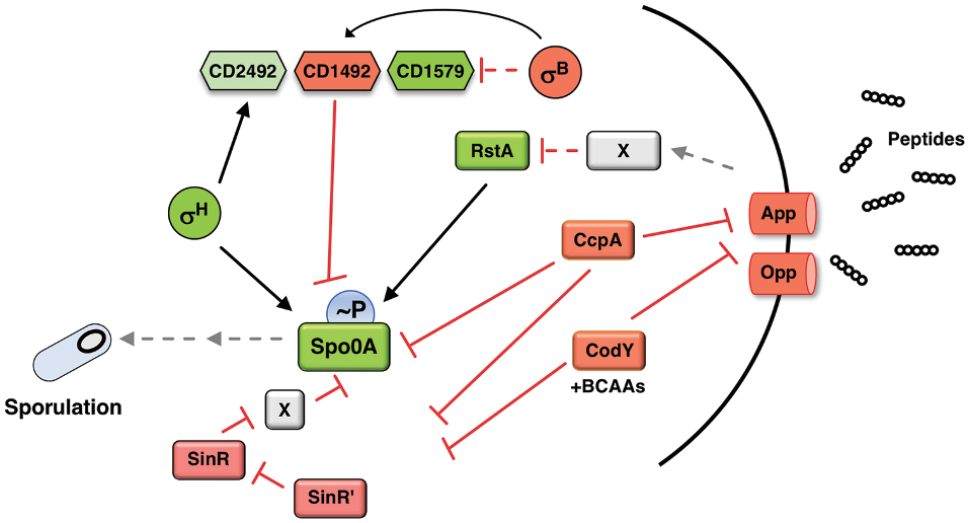


FIGURE 3 Regulatory pathway controlling Spo0A activation in *C. difficile*. Early sporulation factors experimentally determined to function as positive regulators of Spo0A are highlighted in green, and those that inhibit Spo0A are highlighted in red (32, 47, 51, 55, 56, 59, 61, 62, 101). Hexagons indicate histidine kinase/phosphatases, rounded rectangles demarcate transcription factors, and circles highlight sigma factors. Red lines indicate negative regulation, and black lines indicate positive regulation. SinR and SinR′, *C. difficile* orthologs for regulatory proteins characterized in *B. subtilis* (gray), were recently shown to promote sporulation (60). Solid lines indicate defined regulatory interactions, and dashed lines suggest proposed, and potentially indirect, regulatory effects. Branched-chain amino acids are a CodY cofactor (59), and their precursors are likely imported primarily through the Opp and App oligopeptide transporters (55, 61). CcpA-independent carbon-specific regulation is not shown (56). The reciprocal transcriptional regulation of early sporulation factors by Spo0A has also been omitted for simplicity (100).

sporulation ~20-fold (55). These results suggest that the peptides imported by *C. difficile* Opp and App serve as nutrients rather than quorum-sensing molecules such that *C. difficile opp* and *app* mutants may initiate sporulation earlier due to decreased nutrient acquisition.

Metabolic cues regulate the initiation of *C. difficile* sporulation because the global regulators CcpA and CodY directly control the expression of genes encoding early sporulation regulators. CcpA senses carbon availability (56, 57), and CodY senses GTP and branched-chain amino acid levels (58, 59). CcpA directly represses the expression of *spo0A* and the *opp* and *sigF* operons (*sigF* encodes the first sporulation-specific sigma factor to be activated). CcpA also indirectly downregulates transcription of *sinR* (56), which encodes an early sporulation regulator that enhances sporulation (60). Not surprisingly, sporulation is increased ~10-fold in a *ccpA* mutant (56), providing further evidence that *C. difficile* initiates sporulation in response to nutrient deprivation. Similarly, CodY downregulates transcription of *sinR* and the *opp* operon, and a *codY* mutant exhibits increased sporulation (61). However, CodY's effect on sporulation is strain specific, given that an R20291 *codY* mutant produces ~100-fold more spores than the parent strain, but the equivalent mutant in 630Δ*erm* produces only ~2-fold more spores (61). Interestingly, the addition of glucose reduces sporulation frequency in a CcpA-independent manner (56), revealing that additional regulatory pathways impact the timing of sporulation in response to nutritional cues.

C. difficile also uses alternative sigma factors to control sporulation initiation. SigH, which controls the transition to stationary phase, is essential for sporulation and directly drives the expression of *spo0A*, similar to *B. subtilis* (35), and *CD2492*, which modulates Spo0A phosphorylation. In contrast, SigB, the general stress response sigma factor, inhibits predivisional sporulation-specific gene expression and decreases spore formation (62). SigB likely decreases Spo0A phosphorylation by reducing transcription of *CD1579*, which encodes a Spo0A kinase (12), and increasing transcription of *CD1492*, which encodes a putative Spo0A phosphatase (47).

The *C. difficile* genome encodes orthologs of additional *B. subtilis* early sporulation factors, such as SinRI, Spo0J-Soj, Spo0E, and KipI/KipA (see reference 63 for more detail). However, if the trend from recent research holds true, these uncharacterized *C. difficile* orthologs likely function differently from those in *B. subtilis*. Indeed, recent analyses indicate that the *C. difficile sinR* locus encodes two *sinR* homologs, *sinR* and *sinR′*, with SinR′ antagonizing SinR function, analogous to *B. subilis* SinI's negative effect on SinR activity. In *C. difficile*, SinR functions as a DNA-binding transcriptional regulator and enhances sporulation, although the mechanism is unclear. There is some evidence that SinR regulates early sporulation events, because Spo0A-dependent gene expression is significantly decreased in a *sinRR′* mutant and is not rescued by *spo0A* overexpression (60). Furthermore, additional novel *C. difficile* regulators of sporulation initiation are likely to be discovered given that *C. difficile* encounters a diverse array of environmental conditions during growth in the gut, which strongly induces sporulation gene expression relative to broth culture growth to promote survival outside of the host (64).

C. perfringens

In contrast to most spore-forming organisms, *C. perfringens* induces sporulation during the exponential phase (65).

Sporulation induction depends in part on sensing cell density through the Agr-like quorum-sensing system because an *agrB* mutant, which no longer makes a mature quorum-sensing peptide, has reduced Spo0A protein levels and produces ~1,000-fold fewer spores than the wild type (66). Like *C. difficile*, *C. perfringens* CcpA and CodY modulate sporulation (67, 68), although unlike *C. difficile*, *C. perfringens* CcpA activates rather than represses sporulation (68), with a *C. perfringens ccpA* mutant making ~60-fold fewer spores than the wild type. Nevertheless, similar to *C. difficile*, glucose strongly reduces *C. perfringens* sporulation frequency (~2,000-fold) in a CcpA-independent manner.

CodY regulates *C. perfringens* sporulation in a strain-specific manner, again analogous to *C. difficile*. Loss of CodY in the type D strain CN3178 increases spore formation ~10-fold (67, 68), whereas loss of CodY in the food-poisoning strain SM101 reduces sporulation ~1,000-fold relative to the wild type (69). CodY likely regulates sporulation by altering expression of *abrB*, which encodes a negative regulator of sporulation that functions in a strain-specific manner (67). *C. perfringens* also employs a regulatory RNA, *virX*, to repress sporulation by decreasing the expression of genes encoding sporulation-specific factors (70).

While the kinases that phosphorylate *C. perfringens* Spo0A remain unknown, six putative orphan histidine kinases have been identified in BLAST searches (71). Given that the bile acid deoxycholate induces *C. perfringens* Spo0A phosphorylation and thus sporulation (72), these kinases may specifically respond to this bile acid. Similarly, inorganic phosphate induces *C. perfringens* sporulation (73) and could stimulate kinase activity.

C. botulinum

Unlike *C. perfringens*, *C. botulinum* sporulates during the transition from exponential to stationary phase (74). Like *C. perfringens*, the Agr-like quorum-sensing system is important for sporulation initiation, with *agrB* and *agrD* *C. botulinum* mutants producing ~1,000-fold fewer spores (75). While the impact of CcpA and CodY on *C. botulinum* sporulation remains to be studied, five putative orphan kinases have been identified as possible regulators of Spo0A (33). The CB01120 histidine kinase appears to phosphorylate Spo0A based on the observation that production of CB01120 with wild-type Spo0A, but not with a nonphosphorylatable form of Spo0A, causes lethality when heterologously produced in *B. subtilis* (33).

THE LINK BETWEEN SPORULATION AND TOXIN GENE EXPRESSION IN *C. DIFFICILE*

Although most *Clostridium* species initiate sporulation to survive unfavorable conditions, *C. perfringens* and *C. botulinum* directly couple toxin production (10, 30) to sporulation, while *C. difficile* coordinates these processes in a strain-specific manner (76).

C. perfringens Enterotoxin (CPE)

C. perfringens type A causes food-poisoning and nonfoodborne gastrointestinal disease (3). These diseases are caused by the CPE toxin (13), which is encoded either (*cpe*) chromosomally or on a large plasmid: most food-poisoning isolates carry chromosomal *cpe*, while most nonfoodborne isolates carry a plasmid-borne *cpe* (13, 77, 78). Food poisoning typically occurs when chromosomal *cpe* isolates are ingested with food, while nonfoodborne disease is primarily acquired by ingesting spores. Regardless, growth of *C. perfringens* in the small intestine leads to CPE production and thus disease (3).

Numerous studies have demonstrated a strong correlation between spore formation and CPE production (79–82). The first genetic evidence linking *C. perfringens* sporulation with CPE synthesis came from studies showing that mutants blocked at asymmetric division during sporulation failed to produce CPE, while mutants blocked at later stages of sporulation produced CPE (81). For example, a *C. perfringens* strain SM101 *spo0A* mutant cannot produce CPE (29), while inorganic phosphate (Pi) induces both sporulation and CPE production in the wild type (73).

Expression of the *cpe* gene is induced during sporulation, with the *cpe* transcript being detected only in sporulating, but not vegetative, *C. perfringens* cultures (80, 83). *cpe* is transcribed from three promoters, named P1, P2, and P3, that are either σ^E- or σ^K-dependent (83). These promoters induce high levels of CPE production during sporulation, with CPE constituting up to 20% of the total protein present (84). σ^E and σ^K are likely mother cell-specific (85), so CPE production appears to be restricted to the mother cell cytoplasm, where it reaches sufficiently high concentrations to induce paracrystalline inclusion body formation (84). However, rather than being secreted from sporulating cells, CPE is released upon mother cell lysis in the late stage of sporulation (84). Accordingly, *sigK* and *sigE* mutants of the food-poisoning strain SM101 fail to express *cpe* (65), while a *sigF*, but not a *sig*G, mutant exhibit defects in *cpe* expression and CPE production based on reverse transcription PCR (RT-PCR) and Western blot analyses (86). Thus, only σ^F, σ^E, and σ^K are necessary for *cpe* transcription and CPE production (65), even though all four sporulation-specific sigma factors are required for *C. perfringens* sporulation (10, 65).

Since CPE production is strictly sporulation dependent, factors that regulate early events of *C. perfringens* sporulation also regulate CPE production. Accordingly, the Agr-like quorum-sensing system in the nonfoodborne strain F5603 and CodY in the food-poisoning strain SM101 both activate CPE production by activating Spo0A and inducing σ^F production (66, 67). *virX*, a regulatory RNA that represses sporulation, accordingly reduces *cpe* expression and CPE production (70). Notably, while sporulation is crucial for CPE production, CPE is not required for sporulation, because *cpe* mutants sporulate at wild-type levels (13, 87).

C. perfringens TpeL Toxin

Many *C. perfringens* isolates encode a novel toxin named TpeL, which belongs to the family of large clostridial toxins (88) and is encoded both chromosomally and on plasmids (89–91). While the contribution of TpeL to *C. perfringens* pathogenesis is unknown, *tpeL* expression is specifically induced during sporulation based on transcriptional reporter studies (92). The *tpeL* promoter region contains σ^E- and σ^K-dependent sequences, and loss of σ^E strongly reduces *tpeL* expression (~100-fold) (92), indicating that *tpeL* expression also depends on σ^E, similar to *cpe*. More recent analyses indicate that *tpeL* expression is induced by TpeR, a transcriptional regulator encoded in the same pathogenicity locus (PaLoc) as *tpeL*; in these analyses, TpeL production was observed under conditions that promote vegetative cell growth (93).

C. botulinum Type Neurotoxin (BoNT)

Toxin production and sporulation coincide during the transition from exponential to stationary phase, suggesting that

these processes may be coregulated. The Agr-like quorum-sensing system may coordinate these processes as in *C. perfringens*, since toxin and spore formation are reduced in *agrB* and *agrD* mutants (75). In aquatic *C. botulinum* type E strains (94, 95), sporulation and BoNT production are directly linked, because loss of Spo0A prevents spore formation and reduces BoNT production >10-fold relative to the wild type (30). Spo0A likely directly regulates toxin production, because Spo0A directly binds the *botE* promoter *in vitro*, which contains a Spo0A box (30). Interestingly, Spo0A is the first neurotoxin regulator identified in *C. botulinum* type E, which does not encode the alternative sigma factor, BotR, which activates botulinum gene expression in other strains (96).

C. difficile Glucosylating Toxins

C. difficile produces two large exotoxins, toxin A (TcdA) and toxin B (TcdB), which are critical for virulence (97–99). The regulatory links between *C. difficile* toxin production and sporulation are complex and appear to be strain dependent. Spo0A represses toxin expression in epidemic 027 ribotypes (6, 76), does not impact toxin expression in the emerging 078 ribotype (76), and variably impacts toxin expression in the historic 012 ribotype (6, 31, 32, 76, 100). In the 630 background (012 ribotype), the stationary phase sigma factor, SigH, downregulates toxin gene expression (101), and the phosphotransfer protein, CD1492, positively affects toxin production (47), presumably through indirect means. However, as with sporulation, nutrient availability strongly influences toxin gene expression since amino acids and glucose repress toxin gene expression through the global regulators CodY and CcpA, respectively (57, 59, 102).

The most direct link between toxin gene expression and sporulation is RstA, the RRNPP family member discussed above, which inversely regulates toxin production and sporulation (51). RstA inhibits transcription of *tcdA* and *tcdB* by directly binding to the promoters and inhibiting the transcription of *tcdR* and *sigD* (29; A. Edwards and S. McBride, unpublished data). *tcdR* encodes the sigma factor that directly activates toxin gene expression (103), while *sigD* encodes the flagellar-specific sigma factor that also directs *tcdR* transcription (104, 105). This multitiered regulation suggests that RstA tightly controls toxin production. While RstA-dependent repression of toxin gene expression requires its DNA-binding domain, its regulation of sporulation does not. Thus, RstA regulates sporulation and toxin gene expression through independent mechanisms (51). However, it remains unclear if this bifunctional protein links sporulation and toxin regulation in the same cell: single-cell analyses would reveal whether toxin-producing cells also sporulate or whether these important processes are asynchronous. Interestingly, the regulatory pathways between sporulation and toxin gene expression may be reciprocal in some *C. difficile* strains, because TcdR enhances spore formation in R20291 (027 ribotype) but not 630 (106).

Overall, several *C. difficile* regulators control both sporulation and toxin gene expression, suggesting that the coordinate regulation of both of these processes is important for *C. difficile* survival.

THE SPORULATION TRANSCRIPTIONAL PROGRAM

Once Spo0A is phosphorylated, it induces asymmetric division, which eventually leads to the activation of four sporulation-specific sigma factors: σ^F, σ^E, σ^G, and σ^K. These sigma factors are essential for sporulation (25, 85, 107) because they coordinate the activation of distinct transcriptional programs within the mother cell and forespore, respectively, that culminate in the formation of a metabolically dormant spore. While the regulation of sporulation-specific sigma factors has been extensively analyzed in *B. subtilis*, the conserved sporulation sigma factors exhibit notable differences in their regulation and function in clostridial organisms relative to *B. subtilis* as well as between clostridial organisms. We first describe the activation and functions of sporulation-specific sigma factors in *B. subtilis* and then compare these properties with those in *C. difficile*, *C. perfringens*, and *C. botulinum*.

B. subtilis sporulation-specific sigma factors are posttranslationally activated in a compartment-specific and sequential manner. σ^F is activated in the forespore, followed by σ^E in the mother cell; σ^E then activates σ^G in the forespore, which subsequently activates σ^K in the mother cell (Fig. 4) (25). Intercompartmental signaling regulates sporulation sigma factor activation and couples it to specific morphological changes (108). Spo0A induces the transcription of *sigF* and *sigE* in the predivisional cell such that σ^F and σ^E are present in both the mother cell and forespore, although both sigma factors remain inactive until asymmetric division is complete. σ^F is first activated in the forespore when the preferential activation of the SpoIIE phosphatase in the forespore leads to dephosphorylation of the anti-anti-sigma factor, SpoIIAA (109–111), which antagonizes the anti-sigma factor, SpoIIAB. Inhibition of SpoIIAB frees σ^F to bind its target promoters, which include *sigG* and *spoIIR*. The resulting production of SpoIIR leads to σ^E activation because SpoIIR activates SpoIIGA, the protease that removes σ^E's inhibitory propeptide in the mother cell (112–114).

Activated σ^E directs the transcription of genes (115) required for the mother cell to (i) engulf the forespore in a phagocytic-like process (*spoIID*, *spoIIP*, and *spoIIM* [116–118]), (ii) localize coat proteins to the forespore (119), (iii) activate σ^G in the forespore (*spoIIIA* operon [120]), and (iv) produce and activate σ^K in the mother cell (*sigK*, *spoIIID*, *spoIVFA-B*, *ctpB*, and *spoIVCA* [121–125]). The SpoIIIA proteins form a complex with GerM and the forespore-specific SpoIIQ to form a channel connecting the mother cell and forespore (126–129). This channel, also known as the "feeding tube," is required to maintain transcriptional potential in the forespore (126) and, thus, σ^G activity. SpoIIQ also contributes to σ^G activation by controlling the localization of SpoIIE, which likely antagonizes the σ^G-specific anti-sigma factor, CsfB (130). Thus, discrete anti-sigma factors control the activation of σ^F and σ^G, respectively, in *B. subtilis*.

Engulfment increases σ^G activity, which couples its transcriptional program to morphological changes (131). Activated σ^G directs the transcription of genes in the forespore (132) required to (i) modify the cortex (133), (ii) prepare the forespore for dormancy (7), and (iii) activate σ^K via regulated proteolysis by the mother cell-localized protease SpoIVFB (125). Thus, proteolytic signaling cascades induced by the forespore activate σ^E and σ^K in *B. subtilis*.

σ^K production depends on σ^E because σ^E activates transcription of *spoIIID*, which encodes the transcriptional regulator that induces *sigK* expression (134). σ^E also controls the expression of *spoIVCA*, which encodes the site-specific recombinase necessary to mediate excision of the *skin* element, a 42-kB prophage-like region that disrupts the *sigK* gene (122). Thus, multiple levels of σ^K regulation control the precise timing of its activation in *B. subtilis* to ensure proper spore assembly, since activated σ^K induces cortex and coat assembly genes (135).

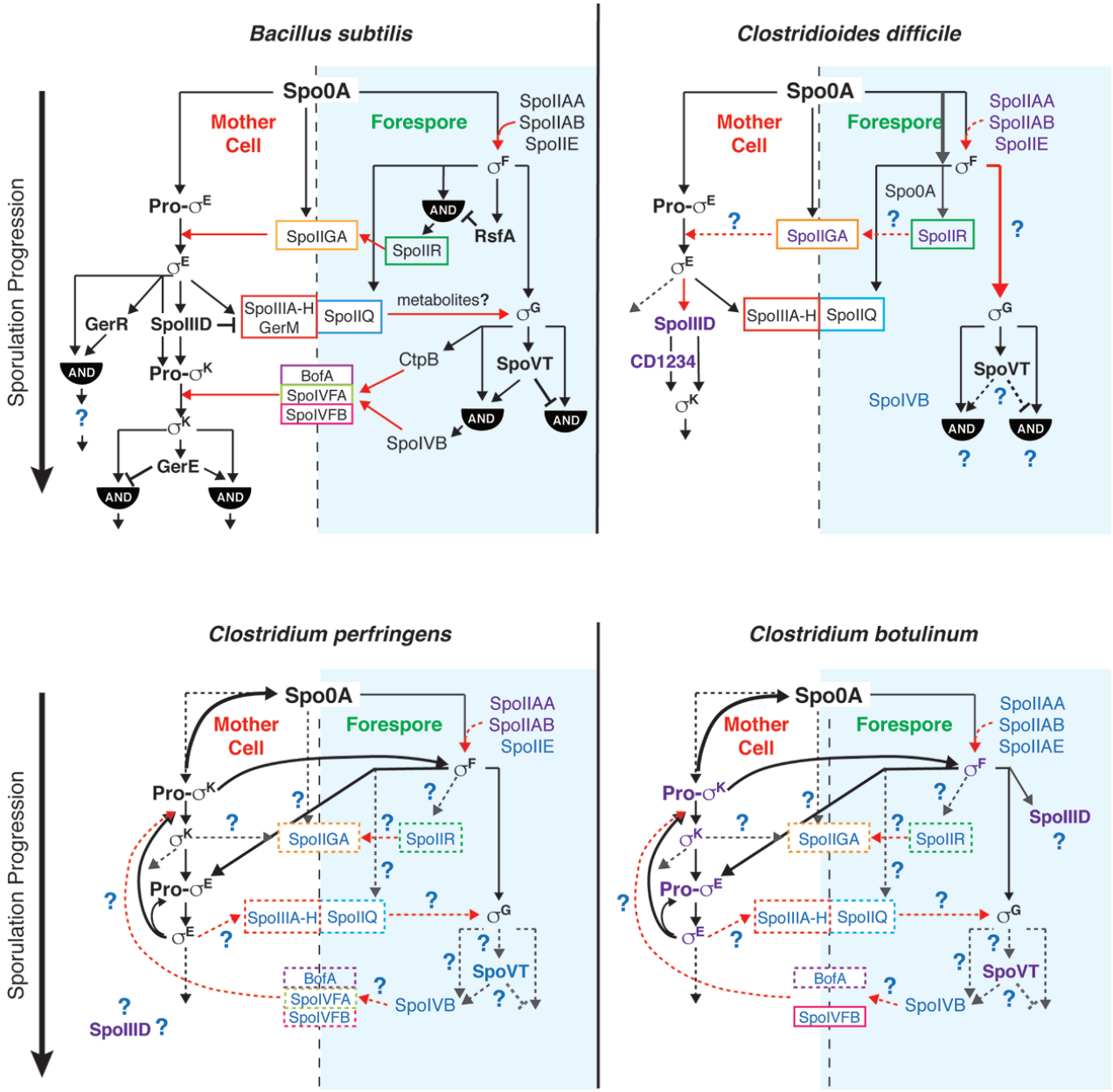


FIGURE 4 Diversity in the regulation of the transcriptional programs controlling sporulation in the *Firmicutes*. The temporal progression of sporulation is shown from top to bottom. Transcription factors and sigma factors are shown in bold, and proteins enclosed in boxes directly participate in signaling between the mother cell and forespore (dashed boxes indicate that trans-septum signaling has not been tested yet). Text color denotes whether the factor has been detected at both the transcript and protein level (black), at either the transcript or protein level (purple), or has not been tested yet at the transcript or protein level (blue). Black arrows delineate transcriptional control of gene expression, red arrows indicate signaling pathways, dashed lines indicate that the regulatory relationship remains unknown, and thick arrows demarcate notable points of divergence from the pathway defined in *B. subtilis*. AND gates are indicated. The figure is adapted from Fimlaid et al. (137) under Creative Commons BY 4.0.

Taken together, *B. subtilis* sporulation gene expression is tightly controlled both spatially and temporally, with events in the forespore being coupled to events in the mother cell. Sporulation sigma factor activation and function are further controlled by additional feedback and feedforward loops to ensure that the timing of sporulation gene expression is tightly coordinated with morphological changes (136).

Notably, while most of the gene products that control sporulation sigma factor activation in *B. subtilis* are conserved in the clostridia, the functions of some of these gene products and the timing of their action are not always conserved. Many of the regulatory loops that fine-tune the timing of sporulation sigma factor activation and function in *B. subtilis* are not conserved in clostridial organisms (42, 71) (Fig. 4), suggesting that (i) sporulation sigma factor activity may not be as tightly regulated in clostridial pathogens relative to *B. subtilis* and/or (ii) additional regulatory pathways exist. While this review focuses on sporulation sigma factor

regulation in pathogenic clostridia, this process has also been examined in detail in *C. acetobutylicum*, which revealed notable differences in the regulation of these sigma factors relative to *B. subtilis* (reviewed by Al-Hinai et al. [85]).

C. difficile

Sporulation sigma factor activation has been best studied in *C. difficile*, where genome-wide transcriptional analyses of sigma factor mutants have defined the regulons of sporulation-specific sigma factors (137, 138). These factors were shown to function in a compartment-specific manner similar to that in *B. subtilis* (Fig. 4) through the development of SNAP tag-based transcriptional reporter constructs by the Henriques group (139). Specifically, *C. difficile* σ^F and σ^G activity is restricted to the forespore, while σ^E and σ^K activity is restricted to the mother cell (139). This regulation resembles *B. subtilis* (25), with the exception that *sig*G expression does not require σ^F and is likely activated by Spo0A (137).

The mechanisms underlying both *C. difficile* σ^F and σ^E activation likely occur through a mechanism similar to that in *B. subtilis* based on gene conservation. Consistent with this notion, the σ^F-activating phosphatase, SpoIIE, and the σ^E-activating protein, SpoIIR, are essential for sporulation based on a transposon screen (140). Similar to *B. subtilis*, *C. difficile* SpoIIR is required for pro-σ^E processing (138). However, unlike *B. subtilis*, *C. difficile* σ^F is not essential for this proteolytic activation event because *spoIIR* is expressed from both Spo0A-dependent and σ^F-dependent promoters (138). Thus, *C. difficile* σ^E is partially active in a *sigF* mutant (137, 138).

C. difficile σ^G and σ^K activation differs markedly from that in *B. subtilis*. Whereas σ^G activation depends on the SpoIIQ-SpoIIIA channel complex in *B. subtilis*, *C. difficile* σ^G is active in the absence of this channel and its associated engulfment defects (141, 142). Notably, σ^G is present but inactive in the absence of σ^F (137), indicating that *C. difficile* σ^G activity is posttranslationally activated in the forespore through an unknown mechanism.

Unlike most spore-forming organisms, *C. difficile* σ^K lacks the N-terminal inhibitory propeptide (143) that tethers *B. subtilis* pro-σ^K to the membrane (123). As a result, *C. difficile* σ^K is active upon translation, since expression of *sigK* from a tet-inducible promoter in vegetative *C. difficile* allows σ^K-dependent genes to be transcribed in contrast to *B. subtilis* (144). However, similar to *B. subtilis*, *C. difficile sigK* transcription depends on the excision of a large *skin* element that disrupts the *sigK* gene (143) and is activated by σ^E and SpoIIID (138, 144). In contrast to *B. subtilis*, the gene encoding the excision recombinase, *CD1231*, is constitutively expressed rather than activated by σ^E (145). Instead, CD1231 activity is posttranslationally activated by the CD1234 recombination directionality factor, whose production depends on both σ^E and SpoIIID. Thus, CD1231 and CD1234 control the timing of SigK production and enhance the fidelity of spore assembly (145), similar to *B. subtilis* (146).

C. difficile sporulation sigma factors generally control morphological processes similar to those in *B. subtilis*, with σ^F and σ^E being required for initiating engulfment, and σ^E and σ^K being required for coat assembly (139). Unlike *B. subtilis*, *C. difficile* σ^G, but not σ^K, is required for cortex production, and *C. difficile* σ^G is required for engulfment completion at least during sporulation on solid media (137).

Taken together, *C. difficile* sporulation sigma factor mutants exhibit less intercompartmental signaling, with the forespore line of gene expression requiring σ^F and σ^G, but not σ^E, and the mother cell line of gene expression requiring σ^E and σ^K, but not σ^G (107, 137–139) (Fig. 4). Furthermore, the timing of sporulation sigma factor activation does not to appear to be as closely coupled to morphological events as in *B. subtilis* (25, 107).

C. perfringens and *C. botulinum*

The regulation of sporulation sigma factors in *C. perfringens* and *C. botulinum* has not been characterized extensively, although gene conservation analyses indicate that the minimal machinery required for activating these sigma factors is conserved (Fig. 4) (136). *C. perfringens* σ^E and σ^K both undergo proteolytic processing during sporulation, similar to *B. subtilis* and *C. difficile* (65). However, in contrast to these organisms, the *sigK* genes in both *C. perfringens* and *C. botulinum* do not contain intervening *skin* elements. Notably, σ^K appears to function at two stages during sporulation in both these organisms, with the first stage regulating sporulation initiation. Indeed, *sigK* mutants in both organisms do not appear to initiate asymmetric engulfment (65, 74), unlike in *B. subtilis* and *C. difficile*. Thus, the function of σ^K as an early and late regulator of sporulation in *C. perfringens* and *C. botulinum* exhibits similarities to *C. acetobutylicum*, which uses σ^K to initiate sporulation through its activation of *spo0A* and during late stage sporulation (147).

In *C. perfringens*, *sigK* is expressed from two promoters at different stages of sporulation: the upstream *CPR_1739* promoter activates transcription during early log phase, in contrast to *B. subtilis* and *C. difficile*, while a σ^E-dependent promoter controls late-stage sporulation gene expression (65), similar to *B. subtilis* and *C. difficile*. *C. botulinum sigK* transcription is similarly biphasic, occurring during late log phase and late-stage sporulation (74). This latter phase of gene expression occurs from σ^F- and σ^E-dependent promoters (148). Consistent with σ^K being required early during sporulation in both these organisms, *C. perfringens* σ^K activates *sigF* and *sigE* expression (65), and *C. botulinum* σ^K activates *spo0A* and *sigF* expression (74). Furthermore, *sigK* mutants in both organisms fail to complete asymmetric engulfment (65, 74), whereas *sigE* mutants are stalled at this stage (65, 148). While transcriptional analyses indicate that σ^K acts upstream of σ^F, Western blot analyses have revealed that σ^K is not detectable in the absence of σ^F (86), suggesting that the production (or stability) of these two factors is interdependent.

Another major difference in sporulation gene regulation relative to *B. subtilis* and *C. difficile* is that σ^E does not activate *spoIIID* expression in *C. perfringens* and *C. botulinum*. Instead, *C. botulinum spoIIID* transcription is σ^F-dependent (149). Whether σ^F regulates *spoIIID* expression in *C. perfringens* is unclear, but immunoblotting indicates that SpoIIID production does not require σ^E or σ^K (65).

These observations raise the possibility that the sporulation sigma factors exhibit differences in their compartment-specific activation in *C. perfringens* and *C. botulinum* relative to *B. subtilis* and *C. difficile*. Indeed, *sigK* is transcribed early during sporulation in *C. perfringens* (65), suggesting that σ^K may also activate sporulation in predivisional *C. botulinum* cells. These observations raise the question of whether σ^K must be proteolytically activated at this early stage. Furthermore, since *sigK* expression is regulated by both σ^F and σ^E in *C. botulinum* (148), is σ^F activity (and by extension SpoIIID activity) restricted to the forespore?

Summary of Sporulation Regulation

Clearly, major differences exist in the functions and regulation of conserved sporulation sigma factors in *C. difficile*,

C. perfringens, and *C. botulinum* relative to the pathways defined in *B. subtilis*. *C. difficile*'s regulatory architecture exhibits greater similarity to *B. subtilis* than to *C. perfringens* and *C. botulinum*, which appear relatively similar to each other. Further study of *C. perfringens* and *C. botulinum* is needed to define the order of sporulation sigma factor activation, their specific regulons, and the location of their activity. Such studies could provide insight into the evolution of diverse sporulation networks.

SPORE ASSEMBLY

The mechanisms by which spores are physically assembled in clostridial organisms also remain poorly defined, although some progress has been made in *C. difficile*. This section focuses on factors required for engulfment, coat, and exosporium assembly in *C. difficile* and the functions of specific coat and exosporium proteins. We also outline the role of the spore-specific small molecule, calcium dipicolonic acid (Ca-DPA), during *C. perfringens* and *C. difficile* spore formation.

Engulfment

The second major morphological event after asymmetric division is engulfment, whereby the smaller forespore is encircled by the mother cell, leaving the forespore free-floating in the mother cell cytosol surrounded by two membranes (Fig. 1). In *B. subtilis*, engulfment is mediated by peptidoglycan synthesis machinery in the inner forespore membrane working in concert with peptidoglycan degradation machinery localized in the mother cell-derived outer forespore membrane (150, 151). The degradation complex consists of the SpoIIP and SpoIID peptidoglycan hydrolases (152, 153) in complex with the transmembrane scaffolding protein, SpoIIM (154). Loss of any of these *B. subtilis* components prevents engulfment completion and thus heat-resistant spore formation (116–118). Interestingly, *C. difficile* SpoIIM is largely dispensable for engulfment and heat-resistant spore formation, in contrast to *B. subtilis*, while *C. difficile* SpoIIP and SpoIID are critical for these processes, similar to *B. subtilis* (155, 156) . While it is unclear why SpoIIM is dispensable for *C. difficile* engulfment, *spoIID* and *spoIIP* are expressed in different cellular compartments in *C. difficile* relative to *B. subtilis* (156), which may obviate the need for SpoIIM to bring SpoIID and SpoIIP together. *C. difficile* SpoIIP and SpoIID nevertheless have conserved enzymatic activities relative to *B. subtilis* (152, 155, 157), although SpoIIP undergoes site-specific cleavage in *C. difficile* (156), in contrast to *B. subtilis* (158).

The conserved SpoIIQ-SpoIIIA channel is also required for *C. difficile* engulfment, unlike in *B. subtilis*. In *B. subtilis*, the channel (also known as the "feeding tube") connects the mother cell and forespore (120, 126, 128) and serves as a back-up mechanism for engulfment (159). However, in both organisms the channel is essential for maintaining forespore health because the forespore becomes deformed on itself in channel mutants (127, 141).

Coat Assembly in *C. difficile*

The coat consists of ~80 proteins in *B. subtilis* (119) that localize to the forespore and form a series of concentric proteinaceous shells around the forespore. However, given that only 25% of these proteins have homologs in *C. difficile* (160), different pathways likely control coat assembly in these two organisms. Furthermore, *C. difficile* encodes only two of the nine coat morphogenetic proteins that function to recruit coat proteins to the *B. subtilis* forespore.

The two coat morphogenetic proteins shared between *B. subtilis* and *C. difficile* are SpoVM and SpoIVA. SpoVM functions as a landmark protein in *B. subtilis* by recognizing the positive curvature of the forespore membrane and embedding itself within this membrane (161, 162). SpoVM recruits SpoIVA to the forespore and is required for SpoIVA to use its ATPase activity (163) to polymerize around the forespore (161). SpoIVA preferentially localizes SpoVM to the outer forespore membrane (164) and recruits the coat morphogenetic protein, SpoVID, to the forespore by binding SpoVID's C-terminal region A (164). These three proteins form the basement layer of the *B. subtilis* coat (165). SpoVID in turn recruits the inner coat morphogenetic protein, SafA (166), and outer coat morphogenetic protein, CotE (167). *B. subtilis spoIVA* and *spoVM* mutants mislocalize the coat and fail to make cortex, so they cannot produce heat- or chloroform-resistant spores (168–170). Furthermore, *spoIVA* and *spoVM* mutants are actively lysed by a bacilli-specific (171) quality control mechanism mediated by CmpA (172).

C. difficile spoIVA mutants resemble a *B. subtilis* IVA mutant in mislocalizing the coat and failing to produce heat-resistant spores (173, 174). However, in contrast to *B. subtilis*, *C. difficile spoIVA* mutants produce cortex (170, 174), consistent with the absence of CmpA in the clostridia (175). Nevertheless, it is unclear why *C. difficile spoIVA* mutants are heat sensitive, given that they produce visible cortex.

Unlike SpoIVA, *C. difficile* SpoVM is largely dispensable for spore formation. *C. difficile spoVM* mutants exhibit an ~3-fold defect in heat- and chloroform-resistant spore formation, make cortex, and properly localize SpoIVA around the forespore in most cells, in contrast to the 6-log defect in heat and chloroform resistance observed in a *B. subtilis spoVM* mutant (169, 173). While it is unclear whether SpoVM encases the *C. difficile* forespore as it does in *B. subtilis* (161), *C. difficile* SpoVM directly binds SpoIVA in recombinant coaffinity purification analyses (173), so *C. difficile* may use a redundant factor to substitute for loss of SpoVM.

While SpoVM is largely dispensable for *C. difficile* spore formation, the clostridia-specific CD3567 was identified as a coat morphogenetic protein because it is essential for proper coat localization and heat-resistant spore formation but not cortex formation (174). CD3567 was identified based on spore proteomic analyses (176) and targeted mutagenesis (174) and directly binds to SpoIVA through CD3567's C-terminal LysM domain, so it was renamed SipL (SpoIVA interacting protein L) (174). Fluorescent protein fusion analyses indicate that SipL is necessary for SpoIVA to encase the forespore, while SpoIVA is necessary for SipL to localize to the forespore (M. Touchette and A. Shen, unpublished data). Thus, even though clostridia-specific SipL (174) lacks sequence homology with bacilli-specific SpoVID (41), aside from their C-terminal LysM domains, the two proteins are functional homologs because they directly bind SpoIVA, encase the forespore, and recruit coat proteins to the forespore (164, 174).

C. difficile SpoIVA and SipL likely comprise the coat basement layer (Fig. 5), but the specific coat proteins they recruit to the forespore are unknown. Analyses of SpoIVA and SipL localization in engulfment mutants indicate that SpoIVA and SipL can localize to the forespore membrane in the absence of engulfment (156), while the outer coat protein CotE (CD1433) localizes to the cytosolic polymerized coat visible by phase-contrast microscopy (141). These

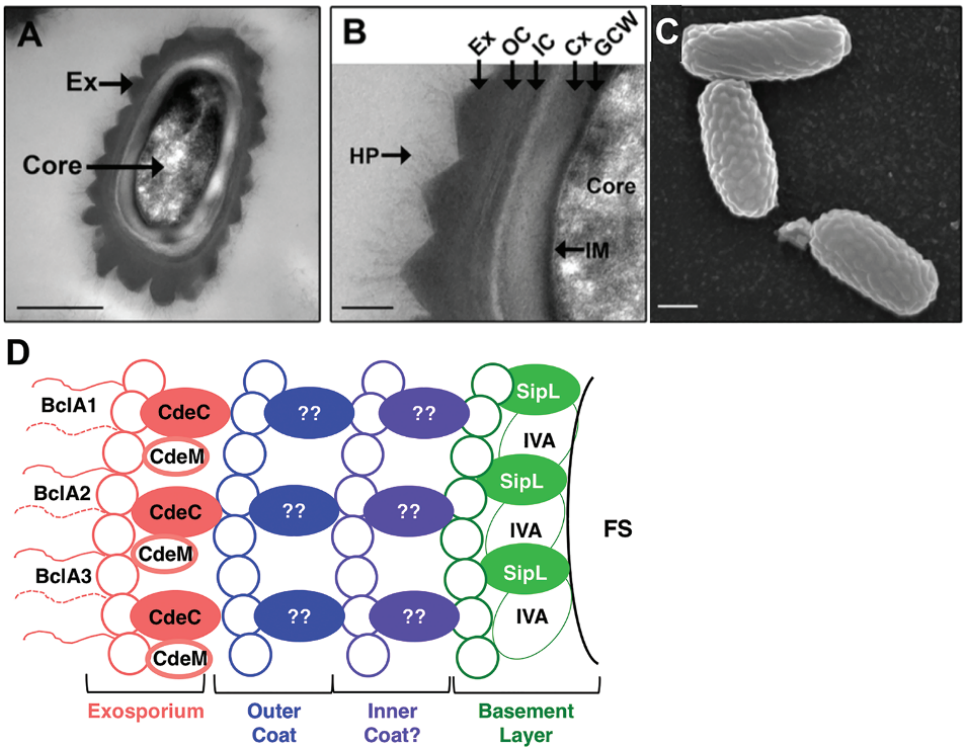


FIGURE 5 Spore coat and exosporium structure in *C. difficile*. (A, B) Transmission electron microscopy sections of *C. difficile* spores highlighting (from outside to inside) the bumpy, outermost exosporium (Ex) layer with its hair-like projections (HPs), outer coat (OC), inner coat (IC), cortex layer (Cx), germ cell wall (GCW), inner forespore membrane (IM), and spore core (cytosol). (C) Scanning electron microscopy of *C. difficile* spores reveals the bumpy surface created by the exosporium. Images used without modification from Rabi et al. (280) under Creative Commons BY 4.0. (D) Schematic of spore coat layers highlighting morphogenetic factors identified as being important for the assembly of specific layers. Assembly of the outermost exosporium depends on the BclA collagen-like proteins, which likely create hair-like projections on the spore surface (186, 187), CdeC (185), and CdeM (D. Paredes-Saja, unpublished data). The proteins that make up the outer and inner coat layers are unknown, but CotA and the mucinase, CotE, have been shown to be surface accessible (180, 182). SpoIVA (IVA) and SipL are interacting coat morphogenetic proteins that are essential for recruiting coat proteins to the forespore and forming heat-resistant spores (173, 174). The specific proteins recruited by SpoIVA, SipL, CdeC, and CdeM remain unknown.

results indicate that SpoIVA and SipL can adhere to the forespore independent of engulfment but that outer layer coat proteins require engulfment completion to stay associated with the forespore (156).

Additional coat proteins have been identified in spore proteomic analyses, some of which have been shown to be surface-localized (176–179). While coat proteins are enriched in these analyses, cytosolic contaminants inevitably become encased as the outer layers are assembled in the mother cell cytosol (176–179). The functions of many of these coat proteins have not been determined. CotA has been implicated in spore assembly and heat and ethanol resistance (180). Nevertheless, enzymatic activities have also been determined for several coat proteins (180, 181). For example, alanine racemase interconverts L- and D-alanine (as well as L- and D-serine) and alters the sensitivity of *C. difficile* spores to the D-alanine cogerminant (181).

C. difficile CotE, which is unrelated to the *B. subtilis* outer coat morphogenetic protein, CotE (119), degrades mucin and promotes spore binding to intestinal epithelial cells (182). Loss of CotE or its C-terminal mucinase domain reduces virulence in a hamster model of infection, indicating that the spore surface actively regulates *C. difficile* colonization and disease (182). Given that *C. difficile cotE* mutant spores do not have obvious ultrastructural or resistance property defects *in vitro* (180), these analyses highlight the importance of analyzing spore function in the context of infection.

Exosporium Assembly in *C. difficile*

The *C. difficile* exosporium layer directly contacts the coat, unlike the exosporium of *Bacillus cereus* group spores, which have an interspace gap between the exosporium and the coat (160, 183, 184). Since the *C. difficile* exosporium is closely associated with the spore coat (Fig. 5), it has been challenging to identify specific components. However, by sonicating spores, the Paredes-Sabja lab enriched for exosporium proteins (178) such as the (i) cysteine-rich proteins, CdeC (CD1067) (185), CdeM (CD1581) (64), and CdeA (CD2375) and (ii) the collagen-like proteins, BclA1, BclA2, and BclA3 (186, 187). Traces of coat proteins such as CotA, CotB, CotD, and CotE were also observed, which may indicate that they are part of the coat/exosporium interface (178).

While it is unclear how the C. *difficile* exosporium is assembled, genetic analyses have identified exosporium morphogenetic proteins. The cysteine-rich proteins, CdeC and CdeM, have been implicated in exosporium morphogenesis (D. Paredes-Sabja, unpublished data). Spores deficient in CdeC have a defective coat that is permeable to lysozyme, have a higher core water content, and are more susceptible to ethanol and heat than wild-type spores (185). Interestingly, cysteine-rich proteins are essential for the morphogenesis of the outer crust of *B. subtilis* and the exosporium of the *B. cereus* group (188–190). These *Bacillus* spp. cysteine-rich proteins self-assemble into two-dimensional crystalline layers (189, 191) that correlate with the two-dimensional crystalline basal layer underneath the hairy nap (extensions) on *B. anthracis* spores. Both CdeC and CdeM form dimers, trimers, and higher molecular weight complexes (178, 185, 192), suggesting that a similar self-assembly mechanism might govern the assembly of the outer layers of C. *difficile* spores.

The C. *difficile* BclA proteins have also been implicated in exosporium assembly: spores lacking either BclA1, BclA2, or BclA3 produce defective exosporiums (186) and exhibit heat-resistance defects (186, 193). The BclA proteins likely compose the hair-like extensions of C. *difficile* spores (187), since these proteins produce hair like-projections on *B. anthracis* spores (160). Furthermore, like *B. anthracis* BclA (194), C. *difficile* BclA3 is glycosylated (193). C. *difficile* BclA orthologues also appear to have a topology similar to that of *B. anthracis* BclA, because both proteins use their N-terminal domains to localize to the spore surface (187).

Exosporium Function in *C. difficile*

As the outermost layer of C. *difficile* spores, the exosporium may contact host component(s) that contribute to the persistence of C. *difficile* spores in the host (195, 196). In transmission electron microscopy analyses of clinically relevant C. *difficile* strains, the exosporium layer appears as electron-dense "bumps" on the spore surface with hair-like extension (185, 196). Notably, two exosporium morphotypes are observed in clonal populations: thin and thick electron-dense layers, although both have hair-like extensions (197, 198). This observation raises the possibility that the different morphotypes may have different roles during C. *difficile* infection, such as spore persistence and immune evasion. Consistent with this hypothesis, loss of the exosporium protein, CdeM (CD1581), decreases C. *difficile* fitness in gnotobiotic mice (64), and loss of individual BclA proteins, particularly BclA1, results in decreased colonization in mice (186).

CLOSTRIDIAL SPORE GERMINATION

Overview of Spore Germination and Outgrowth

During spore germination, metabolically dormant spores lose their resistance properties and transform into metabolically active cells. The low water content of the spore cytosol, known as the core, (~25 to 40%) is critical to this resistance because it prevents metabolism (7). Ca-DPA transport is essential for dehydrating the core, while the modified peptidoglycan cortex layer is essential for maintaining this partially dehydrated state. Thus, spore germination requires the removal of this cortex layer to allow core hydration and metabolism to resume (28).

Spore germination begins when spores sense small molecules termed germinants, which trigger a signaling cascade that leads to cortex degradation, release of Ca-DPA, core hydration, and degradation of SASPs bound to the chromosome. Although many germination-related proteins are conserved in the clostridial pathogens, notable differences in their function and mechanisms of action have been identified in C. *difficile*, C. *perfringens*, and C. *botulinum*. As discussed below, the order in which cortex hydrolysis and core hydration occurs differs between these species and is even strain-specific in the case of C. *botulinum*. Furthermore, C. *difficile* spore germination has several unique features to its signaling pathway that are specific to C. *difficile* and/or *Peptostreptococcaceae* family members.

Germinant Sensing and Signaling

Environmental signals

In most spore-forming bacteria, germinants are nutrient signals such as amino acids, monosacharides, nucleosides, salts, and organic acids (28). In contrast, C. *difficile* responds to cholate-derived bile acids, which are produced exclusively in the mammalian gut (199, 200). Taurocholate is the most potent of the cholate-derived germinants, while chenodeoxycholate is an efficient competitive inhibitor of taurocholate-mediated germination (201). Bile salt-induced spore germination may not be unique to C. *difficile*, since taurocholate can enrich for clostridial species from fecal samples (202), and spores from *Paeniclostridium sordellii*, a *Peptostreptococcaceae* family member, germinate in response to some bile acids (203). Notably, amino acid and calcium ion cogerminants enhance taurocholate-induced C. *difficile* spore germination, with glycine and calcium ions being the most potent of these small molecules (199, 204, 205).

In C. *perfringens* and C. *botulinum*, germinant specificity is species and strain-specific. Universal germinants for C. *perfringens* food-poisoning and nonfoodborne isolate spores include L-cysteine, L-serine, L-threonine, and a mixture of L-asparagine and KCl, while unique germinants for food-poisoning isolate spores are L-asparagine, L-glutamine, KCl, and the cogerminants Na^+ and Pi (206–208). Bicarbonate is a unique cogerminant for nonfoodborne spores (209). L-alanine, L-cysteine, L-methionine, L-serine, L-phenylalanine, and glycine can induce spore germination of group I proteolytic C. *botulinum*, although L-lactate and bicarbonate ions can act as cogerminants (210, 211). Spores of group II non-proteolytic C. *botulinum* germinate in response to L-alanine, L-cysteine, L-serine, L-threonine, and glycine, with L-lactate serving as a cogerminant (211, 212).

Germinant selectivity is likely influenced by adaptation to specific environmental niches. For example, the responsiveness of C. *perfringens* food-poisoning isolate, but not nonfoodborne isolate, spores to KCl or NaPi implies that food-poisoning isolates have adapted to food niches (i.e., processed meat products) where KCl and NaPi are highly abundant (10). Also, the finding that bicarbonate is a unique cogerminant for nonfoodborne spores, which germinate better than food-poisoning isolate spores in the presence of cultured intestinal epithelial cells (196, 210), suggests that nonfoodborne isolate spores are better adapted to germinate in the host's intestinal epithelium environment, where bicarbonate is more prevalent (213). Similarly, the responsiveness of C. *difficile* spores to taurocholate may allow C. *difficile* to sense favorable conditions in the gut, since taurocholate levels are increased in the dysbiotic gut during antibiotic treatment (21).

While classical germinants are directly sensed through germinant receptors, nonnutrient germinants can artificially trigger germination of many bacterial species independent of

germinant receptors. These include the cationic surfactant dodecylamine (28, 214), lysozyme (196), and Ca-DPA (215). While Ca-DPA activates *Clostridium sporogenes* (a proxy for C. *botulinum* group I [5]) spore germination, it does not activate C. *difficile* spore germination (205, 216, 217) and is a relatively weak activator of C. *perfringens* spore germination (218). Interestingly, although high hydrostatic pressure can induce *Bacillus* spp. spore germination independent of germination receptors, high pressure alone is not sufficient to induce spore germination of C. *sporogenes* (219), C. *perfringens* (220), and C. *difficile* (220). For C. *perfringens* and C. *difficile*, the ineffectiveness of high pressure to induce germination is likely due to differences in their germination mechanism relative to *Bacillus* spp. as discussed below.

Transmembrane germinant receptors in *C. perfringens* and *C. botulinum*

Almost all bacterial spores sense environmental signals through germinant receptors localized in the spore's inner membrane; the exception to this rule is C. *difficile* as described below. Germinant receptors usually consist of three protein subunits (A, B, and C) encoded in a tricistronic operon (*gerABC*). GerA and GerB are transmembrane proteins, and GerC is a lipoprotein. Loss of any one of these components generally eliminates germinant receptor function (28, 196).

Many spore-forming bacteria encode multiple tricistronic operons, which are thought to determine the germinant specificity of given strains. While *B. subtilis* encodes three major germinant receptors, there are diverse arrangements for *ger* genes and operons in the *Bacillales* and *Clostridiales* (see 28, 196). For example, C. *perfringens* has a monocistronic *gerAA* that is distant from the *gerK* locus, which encodes a bicistronic *gerKA-KC* operon upstream of the oppositely oriented monocistronic *gerKB* gene (196, 206). While many *Clostridium* species encode monocistronic germinant receptor genes, studies of C. *perfringens* provided the first evidence that functional germinant receptors can consist of a single subunit. Indeed, the lipoprotein GerKC is the sole and essential germinant receptor for all known nutrient- and nonnutrient-induced germination of SM101 and F4969 spores (221, 222), in contrast to *B. subtilis*, in which all three subunits are essential for functional germinant receptor formation. Nevertheless, although GerAA and GerKB can play auxiliary roles in food-poisoning strain SM101 spore germination (206, 222, 223), GerAA is required for nonfoodborne strain F4969 spore germination.

Canonical GerA family germinant receptors localize to the spore's inner membrane in several species, including *B. subtilis* (224) and C. *botulinum* (225). The GerKC subunit is present in the inner membrane of C. *perfringens* spores at ~250 molecules/spore (221); the abundance and relative stoichiometry of the GerKA, GerKB, and GerAA subunits is unclear.

gerA family operons can also contain an additional *gerAD* gene, which encodes a novel protein of 50 to 80 residues with two highly conserved transmembrane sequences in some *Bacillales* and *Clostridiales* species (196). Although GerAD is required for functional germinant receptor formation in *Bacillus* spp. (226, 227), their role in *Clostridiales* spore germination remains unclear.

Genomic analyses of C. *botulinum* strains have identified four *gerABC* subtypes (*gerX* type1 to 4) (211). *gerX* indicates that the germinant recognized by the receptor encoded in the locus is unknown. Notably, although group II C. *botulinum* (gIICb) encodes only one predicted canonical germinant receptor despite being able to respond to many amino acids, deletion of *gerBAC* from gIICb strain NCTC 11219 does not affect germination in response to nutrient or nonnutrient germinants (228). Thus, unidentified germinant receptors appear to mediate spore germination in gIICb in the absence of *gerABC*.

Germinant receptor-mediated spore germination is heat-activatable through a mechanism that is thought to involve conformational changes in the inner membrane germinant receptors and that potentiate their activation (28). Accordingly, both C. *perfringens* and C. *botulinum* spore germination can be enhanced by a transient heat shock (229, 230). In contrast, C. *difficile* spore germination is not heat-activatable, consistent with the absence of germinant receptor genes in its genome (217, 231).

A pseudoprotease, CspC, is the bile salt germinant receptor in *C. difficile*

Since C. *difficile* lacks germinant receptors, it was unclear how it senses bile salt germinants, which are structurally distinct from the nutritional germinants sensed by germinant receptor-encoding spore-formers. Joseph Sorg's group identified the elusive C. *difficile* germinant receptor using a genetic selection for germination-defective mutants (232). This screen identified seven point mutations in *cspC*, which encodes a pseudoprotease from the subtilisin-like serine protease family, and two nonsense mutations in *cspBA*, which encodes a fusion protein consisting of CspB and CspA subtilisin-like serine proteases (Fig. 6). While the CspB protease is catalytically active, both C. *difficile* CspA and CspC are pseudoproteases because they harbor two point mutations in their catalytic triads (233). Subsequent work revealed that CspBA is required for CspC incorporation into spores such that the *cspBA* nonsense mutations identified in the screen effectively lead to loss of CspC (234, 235).

CspC was further implicated as the direct receptor for bile salts using a second genetic screen for altered spore germinant specificity. By selecting for spores that germinate in response to the germination inhibitor, chenodeoxycholate (201), the Sorg group determined that a single point mutation in CspC (G457R) could change the ability of C. *difficile* spores to sense cholate versus deoxycholate derivatives (232). Although direct binding of bile acids to CspC has yet to be demonstrated biochemically, germinant binding to germinant receptors has not been established in any organism.

ACTIVATION OF CORTEX HYDROLYSIS IN CLOSTRIDIAL PATHOGENS

Just as clostridial pathogens use two mechanisms for sensing germinants, these organisms use two mechanisms to degrade their cortex: (i) proteolytic activation of the cortex hydrolase, SleC, and (ii) DPA-mediated activation of the cortex hydrolases CwlJ and/or SleB (Fig. 7) (28). C. *perfringens* and C. *difficile* use the SleC pathway, while C. *botulinum* uses either mechanism in a strain-specific manner, with groups I and III using CwlJ/SleB (211, 230, 236) and groups II and IV presumably using the SleC pathway. While cortex hydrolases are thought to specifically recognize the muramic-δ-lactam modification that characterizes cortex peptidoglycan (237), the CspB-SleC cortex hydrolysis pathway is only found in the clostridia, whereas the CwlJ/SleB system is present in the bacilli and some members of the clostridia (196).

Proteolytic Activation of the SleC Cortex Hydrolase in *C. perfringens* and *C. difficile*

SleC was first identified as a cortex hydrolase in biochemical fractionations of germinating C. *perfringens* exudates (238,

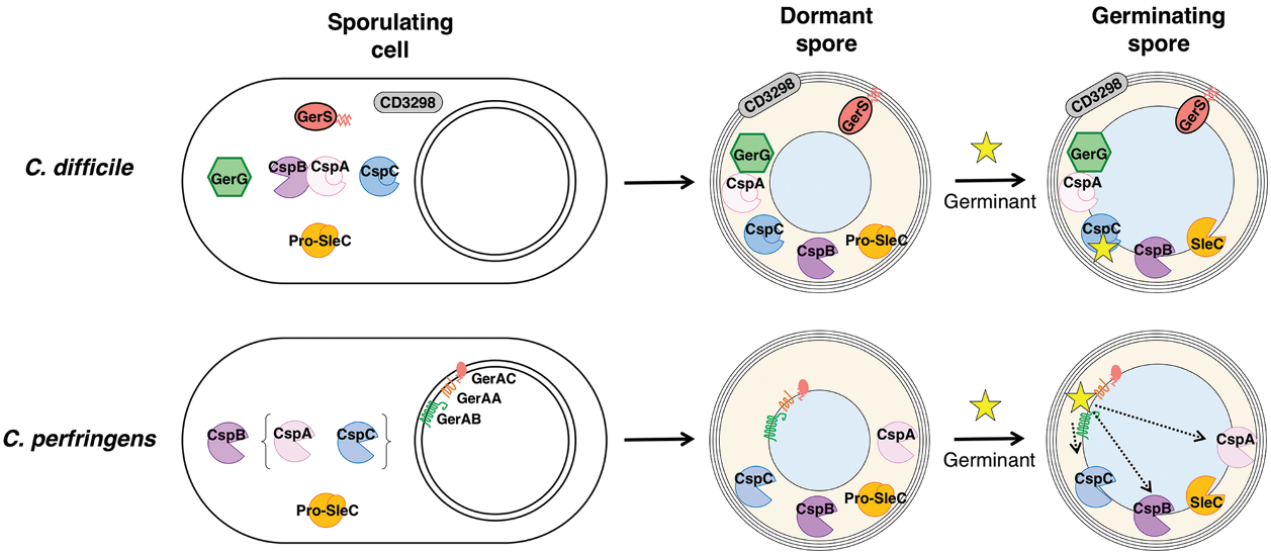


FIGURE 6 Putative locations of germination regulators in C. *difficile* and C. *perfringens*. Germinant signaling proteins, CspC (pseudoprotease and germinant receptor) and its downstream effectors, the CspB protease, and cortex hydrolase, SleC, are all produced in the mother cell under the control of either σ^E or σ^K (137–139). CspB is produced as a fusion to the pseudoprotease, CspA, which is critical for CspC incorporation into mature spores (233–235); all three Csp proteins are incorporated into mature spores. GerG is required for optimal incorporation of CspC, CspB, and CspA into mature spores (253). The GerS lipoprotein (248) is produced in the mother cell and does not directly participate in spore germination (O. Diaz and A. Shen, unpublished data), even though it is required for spore germination to proceed. The ATP/GTP binding protein CD3298 presumably localizes to the cytosolic face of the outer forespore membrane and regulates calcium release and possibly internalization (205). Germinant sensing induces the proteolytic activation of SleC by CspB in both organisms, but CspA and/or CspC can cleave SleC in C. *perfringens* (marked in brackets) (218, 242, 245), since they are active proteases unlike their cognate partners in C. *difficile* (233). C. *perfringens* also produces inner membrane-bound germinant receptors, similar to most spore-forming organisms, in the forespore, in contrast to the soluble CspC protein used by C. *difficile* to sense germinant. The locations of all proteins in mature spores is putative, with the exception of SleC, which has been shown to localize to the C. *perfringens* cortex region by immuno-electron microscopy (251).

239) and was subsequently shown to have lytic transglycosylase and amidase activities (240). SleC's long N-terminal predomain acts as an intramolecular chaperone to ensure proper folding of its hydrolase domain (241). The predomain is removed by proteolysis in a YabG-dependent manner (at least in C. *difficile* [235]) to generate the pro-SleC zymogen. This immature form remains in mature spores until germinant signaling induces the proteolytic removal SleC's inhibitory propeptide to generate active SleC (242).

The proteases responsible for activating SleC were identified as CspA, CspB, and CspC in biochemical fractionations of C. *perfringens* strain S40 germinating exudates (242, 243). These three proteases are members of the subtilisin-like serine protease family (243) and are encoded by a tricistronic operon immediately upstream of the *sle*C gene in C. *perfringens* strain S40 (244). While nonfoodborne C. *perfringens* strains encode this tricistronic operon, C. *perfringens* food-poisoning strain SM101 encodes *cspB* alone upstream of *sle*C (4, 245, 246). Genetic analyses in SM101 revealed that C. *perfringens* CspB is sufficient to proteolytically activate SleC, since *cspB* mutant spores exhibit 10^4-fold germination and cortex hydrolysis defects relative to the wild type (245) and fail to process pro-SleC into active SleC in response to germinants (245).

SleC is the major cortex hydrolase in C. *perfringens*, since *sle*C mutant spores have a 10^3 germination defect relative to the wild type (218). The SleM cortex hydrolase (247) functions as an accessory cortex hydrolase, since a *sle*M mutant exhibits wild-type germination, but a *sle*C *sle*M double mutant has a 100-fold more severe germination defect than the *sle*C single mutant (218).

The CspB protease and SleC cortex hydrolase have similar functions in C. *difficile*, since loss of CspB leads to an $\sim 10^3$ to 10^5 germination defect (233, 234), and complementation with the wild-type *cspB*, but not a catalytic mutant, restores germination and pro-SleC cleavage (233). Loss of *sle*C also results in a similar $\sim 10^3$-fold germination defect relative to the wild type (234) and an inability to hydrolyze cortex (216, 248). However, in contrast with the tricistronic *cspA-cspB-cspC* operon in some C. *perfringens* strains, C. *difficile* strains (and other *Peptostreptococcaceae* family members) universally encode *cspB* fused to *cspA*, with *csp*C encoded downstream of the *csp*BA fusion gene (*cspBA-csp*C) (235).

SleC and Csp proteins are produced in the mother cell of both C. *perfringens* and C. *difficile* (137–139, 249). Western blot analyses of spore fractions in C. *perfringens* and C. *difficile* indicate that CspB and SleC localize to a coat-extractable fraction (248, 250). However, C. *perfringens* SleC can be visualized in the cortex region of mature spores using immune-electron microscopy (251), indicating that fractions previously assumed to comprise coat proteins alone extract proteins from the cortex region. Interestingly, neither C. *perfringens* nor C. *difficile* SleC harbor signal sequences, raising the question of how they are targeted to the intermembrane space (assuming Csp proteins are targeted to this region).

The crystal structure of C. *perfringens* CspB revealed that Csp proteins consist of three main domains: a long N-terminal prodomain, a subtilisin fold, and an internal jelly roll domain (233, 243). Like other subtilisin-like proteases, C. *perfringens* and C. *difficile* CspB have a catalytic triad

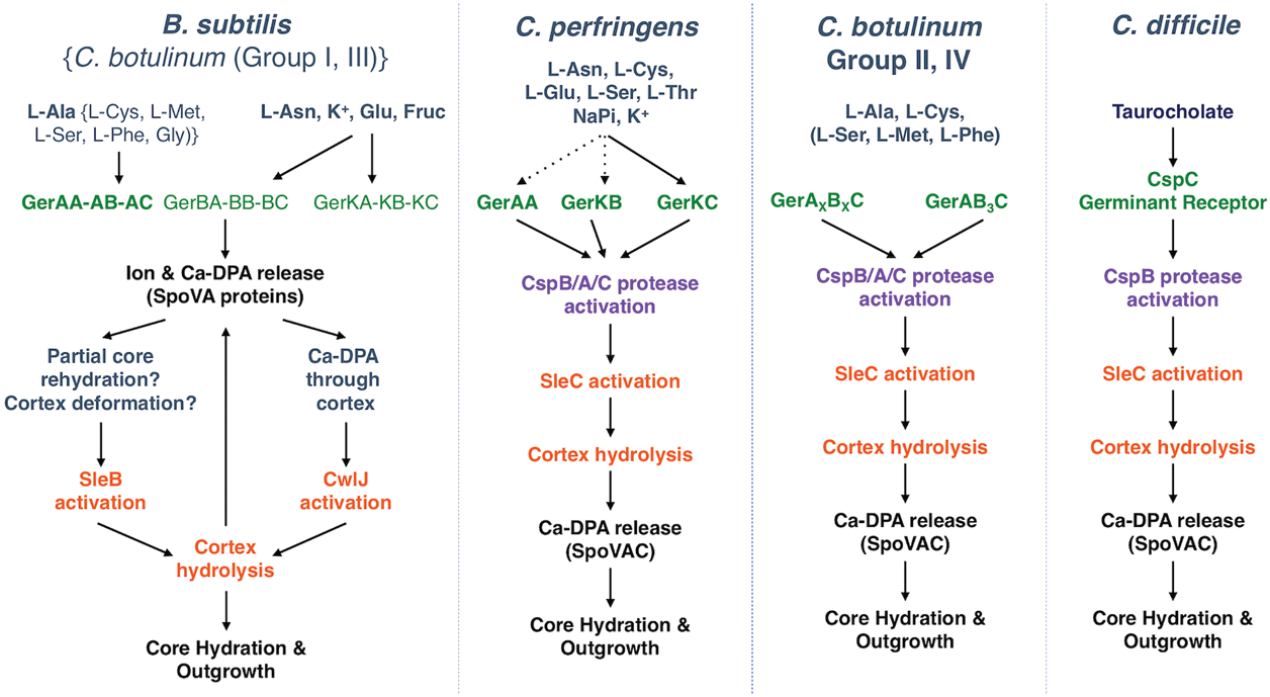


FIGURE 7 Schematic of spore germination signaling pathways. Germinants that are sensed by *B. subtilis*, *C. botulinum*, *C. perfringens*, and *C. difficile* are shown in dark blue; *C. botulinum* group I and III germinants are shown in the brackets (and include L-Ala). Amino acid and calcium ion cogerminants are not pictured for *C. difficile* (199, 204, 205). Germinant receptors are shown in green. The signaling pathway between *B. subtilis* and *C. botulinum* groups 1 and III (far left) differs from the other clostridial organisms mainly with respect to cortex hydrolase (shown in orange) activation mechanisms, with SleC being activated by proteolytic cleavage by Csp proteases, and the CwlJ and SleB cortex hydrolases being activated directly or indirectly by DPA release. Accordingly, the order of cortex hydrolysis and DPA release via SpoVAC differs between these two types of mechanisms. *C. botulinum* groups II and IV encode germinant receptors with variable numbers of A and B components. Adapted from reference 183.

consisting of Asp, His, and Ser, and the prodomain undergoes autoprocessing (233, 243). However, unlike almost all previously studied subtilisin-like serine proteases, both *C. perfringens* and *C. difficile* CspB remain bound to their prodomain following autoprocessing *in vitro* (233, 243). The prodomain sterically inhibits recombinant CspB activity (233), but it is unclear whether it stays bound to CspB in mature spores. Regardless, the prodomain presumably must be removed during spore germination to allow CspB to bind its presumed substrate, SleC. The central jelly roll domain within the subtilase domain is another unique feature of CspB; this domain markedly increases the conformational rigidity of CspB *in vitro* and is necessary for stable CspB production in *C. difficile* (233).

CspB activity has been proposed to require calcium based on the observation that calcium is a critical cogerminant during taurocholate-induced *C. difficile* spore germination (205). Depletion of calcium *in vitro* and in intestinal extracts reduces wild-type *C. difficile* spore germination and abrogates spore germination in a strain lacking CD3298, an ATP/GTP binding protein required for releasing calcium during germination (205). While these data indicate that calcium is a key adjuvant during *C. difficile* spore germination, the precise stage regulated by calcium is unclear, since it could affect CspB activity, CspC activation, germinant permeability, or as-yet-unidentified events. Whether calcium is required for *C. perfringens* spore germination remains to be tested.

Collectively, germinant signaling triggers CspB-dependent processing of inactive pro-SleC into active SleC, which subsequently degrades the cortex in both *C. perfringens* and *C. difficile*. It is unclear how germinant signaling leads to CspB activation, especially since these two organisms use different germinant receptors. The topology of these receptors also differs between these organisms, with Ger receptors being embedded in the *C. perfringens* forespore-derived membrane, and soluble *C. difficile* CspC being made in the mother cell (Fig. 6) (28, 200). *C. difficile* CspC has been hypothesized to be transported across the mother cell-derived membrane into the intermembrane space so that it can activate CspB through protein-protein interactions (200, 234, 252, 253). CspB in *C. perfringens* and *C. difficile* is presumably also transported into the intermembrane space so that it can activate its substrate SleC, which has been localized to this region in *C. perfringens* by immuno-electron microscopy (251). Clearly, many questions regarding the molecular details of this process remain to be addressed.

DPA-Mediated Activation of Cortex Hydrolases in Group I *C. botulinum*

Gene conservation predicts that *C. botulinum* groups II and IV activate cortex hydrolysis via CspB-mediated proteolytic activation of pro-SleC (211), but group I (and likely group III based on homology) use the partially redundant cortex hydrolases, CwlJ and SleB, to remove their cortex layer (211, 230, 236). Both hydrolases are produced in their mature form, but their activation appears to be tied to Ca-DPA release as observed in *B. subtilis* (28, 254). *C. sporogenes* (a proxy for group I *C. botulinum*) *cwlJ* mutant spores do not germinate in response to Ca-DPA, unlike *sleB* mutant spores

(230, 255) and similar to analogous mutants in *B. subtilis* (256). In *B. subtilis*, SleB activity is directly inhibited by YpeB (254), but the mechanism by which SleB is activated during germination is unknown. *C. botulinum* group I *sleB* and *ypeB* mutants exhibit 2- to 3-log defects in spore germination based on colony forming units, whereas *cwlJ* mutant spores produce wild-type levels of colonies on rich media (236). In contrast, *cwlJ* and *sleB* mutant spores in *C. sporogenes* have similar germination defects in single-spore germination analyses (230). Regardless, CwlJ and SleB likely have partially overlapping functions during group I *C. botulinum* spore germination, so a double mutant lacking both enzymes would presumably exhibit a severe germination defect similar to that in *B. subtilis* (257). Interestingly, both group I and group III *C. botulinum* spp. can encode multiple SleB homologs (211), suggesting that there may be additional redundancy in regulating cortex hydrolysis.

Ca-DPA and Spore Formation versus Germination

Ca-DPA is a spore-specific molecule that makes up 5 to 15% of spore dry weight (256). It is synthesized in the mother cell from lysine biosynthesis intermediates (258) and transported across the outer and inner forespore membranes by SpoVV (259) and SpoVAC (260, 261), respectively, in exchange for water. While the partial dehydration of the core by Ca-DPA transport into the forespore confers heat resistance to mature spores (256, 261, 262), Ca-DPA is differentially required for spore formation. In *B. subtilis*, mutants defective in synthesizing or transporting Ca-DPA cannot stably form spores due to premature germination and cortex hydrolase activation (256, 259, 260). Similar to *B. subtilis*, *C. sporogenes spoVA* mutants also produce unstable spores, presumably due to premature SleB activation (211). In contrast, *C. difficile* mutants defective in synthesizing or transporting Ca-DPA can stably form spores, although they are less dense than wild-type spores (261). Interestingly, Ca-DPA synthesis is necessary for *C. perfringens* spore formation (263), but Ca-DPA transport is not (262). Since Ca-DPA does not strongly induce *C. perfringens* germination (262), presumably because it uses the CspB-SleC pathway to activate cortex hydrolysis, it is unclear why DPA is required for spore formation in *C. perfringens* but not in *C. difficile*. This difference may reflect the different mechanisms used by these organisms to synthesize Ca-DPA. While *C. difficile* uses dihydro-dipicolinate synthase (DpaAB or SpoVFAB) to produce Ca-DPA, *C. perfringens* uses an electron transfer flavoprotein, EftA, to make this molecule. Since *C. botulinum* lacks SpoVFAB homologs and likely uses EftA, DPA-less *C. botulinum* spores likely will not be stably produced.

Cortex Hydrolysis Precedes Ca-DPA Release in *C. difficile*

While Ca-DPA release activates cortex hydrolysis in group I *C. botulinum* and other CwlJ/SleB-controlled systems, cortex hydrolysis is necessary for Ca-DPA release in *C. difficile* (261). Cortex hydrolysis activates the mechanosensitive SpoVAC channel (264) to release Ca-DPA from the core, since *C. difficile* spores germinated in high osmolyte solutions degrade their cortex normally but release Ca-DPA more slowly (265). Furthermore, *C. difficile sleC* mutant spores do not release Ca-DPA (216, 217). A similar mechanosensing phenomenon may occur in *C. perfringens*, since a *sleC-sleM* double mutant and a *cspB* mutant exhibit major defects in Ca-DPA release (218, 245). Thus, the CspB-SleC cortex degradation pathway likely induces Ca-DPA release, while the order of these events is reversed in spores that use CwlJ and SleB to degrade their cortex (28).

Events after Cortex Hydrolysis and Ca-DPA Release

While recent studies have provided critical insight into the early stages of germination in clostridial pathogens, namely germinant sensing, cortex hydrolysis, and Ca-DPA release, little is known about subsequent events. Ca-DPA release in *C. difficile* leads to core hydration based on the observation that the optical density of germinating *dpaAB*$^-$ and *spoVAC*$^-$ spores decreases by ~50% relative to the wild type, whereas the optical density of *sleC*$^-$ spores remains unchanged (261). Core hydration allows metabolism to resume, and transcript levels increase within 15 min of germination (231).

In *B. subtilis*, Ca-DPA release activates the Gpr germination protease (266), which degrades SASPs coating the nucleoid. The amino acids liberated by SASP degradation provide substrates for both catabolism and metabolism (267). It is currently unclear whether Ca-DPA activates Gpr homologs in clostridial pathogens.

Loss of Resistance Properties During Germination

The hydration of the core during spore germination allows metabolism to resume while concomitantly leading to loss of resistance. This is because the resistance properties of spores can largely be attributed to (i) the low water content of metabolically dormant spores and (ii) the coating of the genetic material by SASPs, which collectively prevent metabolic activity in the spore core. Indeed, *B. subtilis* mutant spores with higher water content or lacking SASP proteins are more sensitive to heat (256, 268) and in the latter case are more sensitive to UV irradiation (269, 270). Although *B. subtilis* produces three major types of SASP proteins (α, β, and γ), which are encoded by multiple genes (269), SspA and SspB (α and β, respectively) are the major SASPs coating the chromosome and the primary contributors to *B. subtilis* spores' resistance to UV and solar radiation (270).

Similar to *B. subtilis*, *C. difficile* and *C. perfringens* mutant spores with higher spore water content are more susceptible to heat (261, 262, 271) and UV irradiation, possibly because increased hydration reduces SASP binding to DNA (262, 271). Consistent with this idea, *C. perfringens* food-poisoning isolates with extreme resistance properties (e.g., type A chromosomal *cpe* and type C Darmbrand strains) produce an Ssp4 variant (also known as SASP4) that binds DNA with higher affinity than Ssp4 variants from more heat-sensitive *C. perfringens* isolates (272, 273). Mutational analyses indicate that this Ssp4 variant is a major determinant controlling the extreme heat resistance of certain food-poisoning strains. Interestingly, Ssp4 exhibits only ~20% sequence identity relative to the three other SASP proteins (Ssp1 to Ssp3) produced by *C. perfringens* strains, suggesting that this Ssp4 variant may be a specific adaptation of food-poisoning isolates (e.g., SM101). Indeed, it should be noted that Ssp1 to Ssp3 have been shown to confer UV resistance to *C. perfringens* food-poisoning strain SM101 spores using antisense RNA silencing methods (274).

C. botulinum encodes four SASPs, CBO1789, CBO1790, CBO3048, and CBO3145. This latter protein exhibits the lowest sequence conservation and is dispensable for resistance to nitrous acid (275) and thus may function similarly to *B. subtilis* SASP γ in acting as a food source for the outgrowing spore (276). Mutational analyses indicate that CB01789 and CPB01790 are primarily responsible for conferring resistance to nitrous acid, an oxidizing agent (275).

The role of SASPs in conferring resistance to C. *difficile* spores has not been examined, although RNA-Seq analyses suggest that SspA and SspB are the predominant SASPs produced (137). C. *difficile* spores are less resistant to heat than *B. subtilis* and C. *perfringens* spores, since C. *difficile* spores are readily killed at temperatures above 80°C (261).

CONCLUSIONS AND UNIQUE FEATURES OF *C. DIFFICILE* SPORE GERMINATION

Diverse pathways are clearly used by group I C. *botulinum*, C. *perfringens*, and C. *difficile* to mediate spore germination. The pathway used by group I C. *botulinum* spore germination most resembles *Bacillus* spp. germination, with germinant receptors sensing amino acids and stimulating Ca-DPA release followed by cortex hydrolysis by CwlJ and SleB (28). While C. *perfringens* spore germination also uses germinant receptors to sense nutrient germinants such as amino acids, germinant sensing activates the CspB-SleC cortex hydrolysis pathway, after which most Ca-DPA is released. C. *difficile* spore germination is the most divergent, with its unique germinant receptor, the CspC pseudoprotease, sensing bile acids. Like C. *perfringens*, germinant sensing activates the CspB-SleC cortex hydrolysis pathway, and the change in pressure caused by the resulting cortex hydrolysis induces SpoVAC to release Ca-DPA.

Although CspA, CspB, and CspC are conserved in many clostridial organisms, they are produced as single protease domains in the *Clostridiaceae* and *Lachnospiraceae* families and as a CspB-CspA fusion protein in the *Peptostreptococcaceae* family (235). Furthermore, CspB is the only active serine protease, with CspC and the CspA domain being pseudoproteases. Since CspA is critical for incorporating the germinant receptor and pseudoproteases, CspC, into mature spores (234, 235), the catalytic site mutations within CspA and CspC, which are largely conserved across *Peptostreptococcaceae* family members (235), appear to confer unique functions to these proteins.

Recent studies have identified two unique proteins that modulate C. *difficile* spore germination. GerG (CD0311) is a C. *difficile*-specific gel-forming protein that is necessary for incorporating CspA, CspB, and CspC into mature spores, while GerS (CD3464) is a *Peptostreptococcace* family-specific lipoprotein that is necessary for cortex hydrolysis (248) because it is necessary for generating the muramic-δ-lactam required for SleC to recognize its cortex substrate (Diaz and Shen, unpublished data).

While the Csp pseudoproteases, GerG, and GerS are distinguishing features of the C. *difficile* spore germination pathway, it is likely that additional novel regulators specific to certain clostridial families will be identified as this fascinating developmental process is studied using the genetic tools recently developed for studying clostridial pathogens (140, 277–279).

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Clostridial Genetics: Genetic Manipulation of the Pathogenic Clostridia

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57

INTRODUCTION

The *Clostridiaceae* family (and within it the *Clostridium* genus) consists of an extremely diverse group of primarily Gram-positive bacteria that have traditionally been grouped together based on their anaerobic growth requirements and their ability to produce heat-resistant endospores. Historically, the members of the genus were very dissimilar, such that the genus lacked phylogenetic coherence, with over 200 species, at least 35 of which cause disease in humans and animals (1). Two recent studies have proposed a phylogenetic reorganization of the clostridia and subsequently changed the name of *Clostridium difficile* to *Peptoclostridium difficile* (2) and *Clostridioides difficile* (3, 4). However, the earlier name (*P. difficile*) did not comply with the *Internal Journal of Systematic and Evolutionary Microbiology* Bacterial Code and was rejected (3). Also recently, *Clostridium sordellii* has been reclassified as *Paeniclostridium sordellii* (5). For simplicity, in this article pathogenic members of the genera *Clostridium*, *Clostridioides*, and *Paeniclostridium* will all be referred to as pathogenic clostridia.

The most common feature of the pathogenic clostridia is that the cell and tissue damage that they cause primarily results from the production of potent extracellular toxins (6, 7). Although it is somewhat artificial in that it crosses family boundaries, it is useful to divide the pathogenic clostridia into three major groups based on their resultant disease pathology. These groups consist of the neurotoxic clostridia, which produce toxins that affect the nervous system, the enterotoxic clostridia, which produce toxins that affect the gastrointestinal tract, and the histotoxic clostridia, whose necrotic pathology results from the production of one or more toxins that affect the structural and functional integrity of host cells located at or near the site of infection. This article focuses on the genetic manipulation of the pathogenic clostridia and is divided by approaches and techniques rather than individual species.

The genetics and, in particular, the genetic manipulation of the clostridia have been revolutionized in the past decade. In large part, this has been made possible by advances in sequencing technology. The genomes of representatives of all of the pathogenic clostridia have now been completely sequenced, assembled, and annotated. The first *Clostridium perfringens* genome was published in 2002 (8), followed by *Clostridium tetani* in 2003 (9), *C. difficile* in 2006 (10), and *Clostridium botulinum* in 2007 (11). Multiple genome sequences are now available for these pathogens (12–19). The availability of these genome sequences has been important in the development of genetic manipulation technologies because precise sequences are required to design homology arms for homologous recombination, complementation, and use of newer technologies such as clustered regularly interspaced short palindromic repeats (CRISPR).

Genetic manipulation can be broken down into two main categories: forward and reverse genetics. Forward genetics involves random screens to identify genes that are responsible for a particular phenotype and can be employed without knowing the target gene. These methods encompass technologies such as transposon mutagenesis and transposon-directed insertion site sequencing, which are tools of random mutagenesis. Reverse genetics involves the inactivation of a particular gene to study the resulting phenotype and requires the target gene to be identified first. The technologies involved in this approach include insertional mutagenesis, allelic exchange to construct deletions and point mutations, and the insertion of genes or regions of DNA to disrupt genes.

EARLY ADAPTATIONS OF MUTAGENESIS TECHNOLOGIES

Genetic manipulation methods have now been established for most pathogenic clostridia but are most refined for *C. difficile* and *C. perfringens*. The clostridia are not naturally competent, but transformation via electroporation has been described for several of the pathogenic species. Electroporation into *C. botulinum* was demonstrated by Zhou et al. in 1983 (20), and *C. tetani* was transformed with the *Enterococcus faecalis* shuttle vector pAT19 in 1998 (21). So far, however, there have been no further reports of successful transformation (via electroporation) into either of these two species. Whether DNA transfer via electroporation is successful partly depends on the restriction and modification status of the organism and the transformation frequency that can be obtained. Electroporation is not currently feasible for *C. difficile*, despite numerous attempts in many laboratories. It has been successful for *C. perfringens* strain 13 (22, 23), but many other strains of *C. perfringens* are not amenable to DNA transformation.

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The first defined mutants in a pathogenic *Clostridium* species were constructed in C. *perfringens* by allelic exchange utilizing double crossover events (22, 24). These studies made use of the fact that derivatives of the gas gangrene strain 13 have an apparent lack of active restriction and modification systems, thereby making them very amenable to genetic analysis (8, 25). There are several well-characterized shuttle plasmids that can reliably and reproducibly be introduced into C. *perfringens* cells by electroporation-mediated transformation (23) or by conjugation from *Escherichia coli* (26). The most widely used C. *perfringens-E. coli* shuttle vectors for complementation and other genetic studies are pJIR750 and pJIR751 (27), which are derivatives of the shuttle vector pJIR418 (28). These plasmids all contain the origin of replication *lacZ′* gene and multiple cloning sites of the *E. coli* plasmid pUC18 and the origin of replication and *rep* gene of the C. *perfringens* bacteriocin plasmid pIP404. Detection of recombinants can be achieved by X-Gal screening in *E. coli* and transformants selected on chloramphenicol or erythromycin in either *E. coli* or C. *perfringens*, respectively. Subsequently, derivatives of pJIR750 and pJIR751 were constructed that carried the *oriT* site from plasmid RP4, designated pJIR1456 and pJIR1457, respectively (29). The construction of these vectors facilitated RP4-mediated conjugative transfer of these plasmids from *E. coli* to C. *perfringens*, allowing strains that were not amenable to DNA transformation to be genetically manipulated.

The first genetic manipulation of C. *difficile* was successfully performed using the *oriT*-containing C. *perfringens* vectors, allowing regulatory genes to be introduced *in trans* into this bacterium (30). The first mutants generated in C. *difficile* were derived by homologous recombination from single crossover events. In 2006 O'Connor et al. (31) constructed a chromosomal mutant in CD3255 (*virR*) using pJIR1456 (26). This replicon is relatively unstable in C. *difficile*, and hence it can act as a pseudo-suicide plasmid in this bacterium. While this development represented an important step forward in the genetic manipulation of C. *difficile*, the mutants were sometimes unstable when selective pressure was removed, which could lead to the generation of revertants (31, 32).

A prerequisite for the mutagenesis of C. *difficile* was the use of an erythromycin resistance marker, and hence an erythromycin-sensitive recipient strain was required. C. *difficile* 630 has become the primary strain for laboratory manipulation, because it is amenable to DNA transfer (via conjugation, as discussed below), and it was the first C. *difficile* strain to have its genome sequenced (10). However, C. *difficile* 630 is erythromycin resistant. After a number of passages two erythromycin-sensitive strains were eventually obtained, namely 630E or JIR8094 (31) and 630Δ*erm* (33). Recently, it was discovered through whole-genome sequencing that both of these erythromycin-sensitive derivatives obtained additional mutations during their passaging, which differed between the two derivatives (34–36). JIR8094 showed a larger number of seemingly important changes compared to the parental strain. In light of these differences, which are described in more detail by Collery *et al.* (34), 630Δ*erm* is now the strain of choice in studies to elucidate pathways of virulence, regulation, and metabolism in C. *difficile*.

THE ROADMAP TO CLOSTRIDIAL GENETICS

Overcoming Restriction and Modification Systems

As mentioned above, complete genome sequences are now available for all of the pathogenic clostridia, which has opened up the field for the development of more sophisticated genetic tools. Technologies such as Illumina sequencing are now quick and affordable; however, obtaining accurate reads in repetitive regions is difficult with these technologies because of the short reads that they produce. This problem can be overcome by the use of long-read technologies such as MinION and, in particular, PacBio. When these latter methods are used in combination with Illumina sequencing, they can provide an accurately assembled genome, which is crucial to locate genes and identify up- and downstream defined genetic elements and, importantly, potential restriction-modification systems (37).

Restriction-modification systems have held back much of the genetic development of the clostridia, because they can protect the recipient bacterial cell from foreign DNA, making the transfer of foreign DNA for genetic manipulation difficult or impossible (38). A common method used to overcome restriction barriers is *in vitro* or *in vivo* methylation of the DNA to be introduced, so that the recipient recognizes it as its own. To this end, it becomes important again to have knowledge of the genome sequence, which can reveal all the methylases and restriction-modification systems present. For many years pAN2, which contains a bacteriophage methyltransferase gene, has been used to methylate DNA before transformation into *Clostridium acetobutylicum* (39). Recently, similar systems have been developed for *Clostridium pasteurianum* (40), *Clostridium saccharobutylicum*, (41, 42), and *Clostridium cellulovorans* (43).

In all fields of bacteriology, researchers work mostly on a few select laboratory strains, which are for one reason or another easy to manipulate. For example, C. *difficile* 630 has no active restriction-modification systems, so it is easier to conjugate with, and its genome was the first to be sequenced, so most molecular studies have been carried out with this strain and its derivatives. Similarly, C. *perfringens* strain 13 is the most widely used strain of that species, with transformation protocols having been successfully developed at an early stage. Finally, ATCC 9714 is the laboratory strain of choice for *P. sordellii* studies. While these strains are great tools to unravel the molecular basis of pathogenicity and metabolism, it has become evident that they often have phenotypic limitations. For this reason, clinical isolates need to be considered and used for research studies, particularly those related to virulence. Different ribotypes of C. *difficile* and different toxinotypes of C. *perfringens*, for example, display very different phenotypes, causing different diseases or disease severity (44, 45). Therefore, it is important to understand and develop ways to overcome the barrier to DNA transfer into these organisms, and the use of the genetics roadmap suggested in reference 37 can help to facilitate such an analysis. In this workflow, the genome sequence of a strain is determined first, which gives insight into its restriction-modification system. This information in turn helps to overcome the barriers to DNA transfer, as well as providing the genetic blueprint of the strain, making genetic manipulations such as gene deletion, substitutions or additions (46), and further investigation possible.

Plasmid Cloning Vectors

Conjugation as a method of DNA transfer has been very successful in the pathogenic clostridia. C. *perfringens* strains that cannot be transformed by electroporation have successfully been genetically manipulated using conjugation from *E. coli*, using plasmids pJIR1456 or pJIR1457, as discussed earlier (22, 29, 30). Conjugative manipulation was first established for C. *botulinum* using pJIR1457 (47) with

the RP4-carrying *E. coli* strain S17-1 as the donor strain. Conjugation is also used to introduce DNA into *P. sordellii* and has been used successfully to generate numerous mutants in this clostridial species (48–52).

In early studies, *C. perfringens*-based vectors were used for the genetic manipulation of *C. difficile* (30). For example, plasmid pJIR1457 was used to study the functional capacity of the toxin regulator TcdR (then called TxeR) in *C. difficile* employing RP4-mediated conjugative transfer of a *tcdR*-carrying recombinant plasmid from an *E. coli* donor (30). Note that the difficulty of introducing DNA into some strains using the RP4-based system from *E. coli* resulted in the construction of a novel plasmid transfer system that exploited the conjugation apparatus encoded by the broad-host range transposon Tn916 together with the cognate *oriT* site encoded by this element (53). This system, which uses a Tn*916*-carrying *C. perfringens* or *Bacillus subtilis* strain as a donor and $oriT_{Tn916}$-carrying shuttle plasmids, facilitated the genetic manipulation of previously recalcitrant *C. difficile*, *C. perfringens*, *P. sordellii*, and *Clostridium septicum* strains (54). More recently, heat shock has also been used to increase conjugation efficiency in *C. difficile*, and the choice of media was also shown to affect conjugation frequency (55).

The construction of specific *C. difficile* shuttle vectors was realized by Purdy et al. in 2002 (56). The plasmids derived in this work can replicate autonomously in *C. difficile* and contain either replicon CD6, which was amplified from a *C. difficile* plasmid isolated during the same study, the replicon from pCB102, or that from pIP404; the latter two plasmids were derived from *Clostridium beijerinckii* (57) and *C. perfringens* (28), respectively. A much higher frequency of transfer was achieved using the native replicon pCD6, and plasmids carrying this replicon were shown to be maintained more stably in *C. difficile*. The plasmid constructed in this study, containing the pCD6 replicon, a Gram-negative replication site, possibly including *oriT* for transfer and an erythromycin gene for antibiotic selection, was originally called pMTL9301 and was later renamed pMTL960 (56). pMTL960 has been used successfully in numerous studies, including those involving an examination of the function of the *C. difficile* cell wall protein V, the lipoprotein CD0873 (an adhesin), and the cysteine proteases Cwp84 and Cwp13 (58–60).

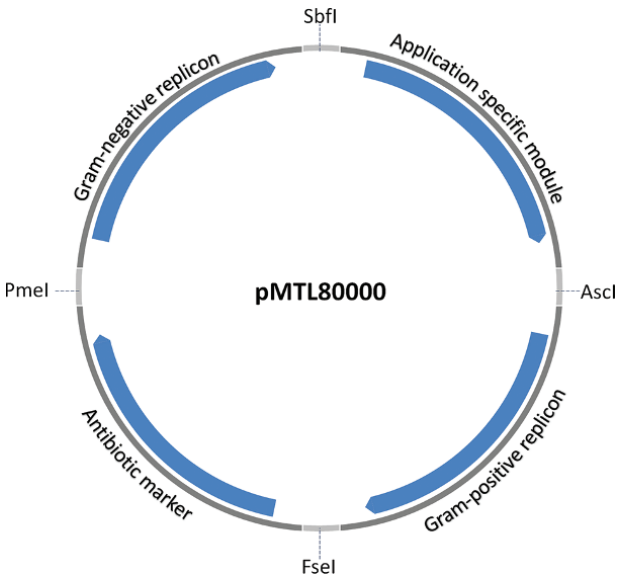


FIGURE 1 Illustration of the pMTL80000 modular vector series. The figure highlights the four modules separated by the unique restriction sites: *Sbf*I, *Asc*I, *Fse*I, and *Pme*I. The modules consist of a Gram-positive replicon module, a selectable marker, a Gram-negative replicon unit with optional transfer (*tra*) genes, and an application-specific module.

TABLE 1 Module choices for pMTL80000 plasmids

Gram+ replicon	Marker	Gram– replicon	Application-specific
0. Spacer*			0. Spacer*
	1. *catP*	1. P15a	1. MCS
2. pBP1	2. *ermB*	2. p15a + *tra*	2. Pthl + MCS
3. pCB102	3. *aad6*		3. Pfdx + MCS
4. pCD6	4. *tetA*	4. ColE1	4. catP reporter
5. pIM13		5. ColE1 + *tra*	

Abbreviations: MCS, multiple cloning site; Pthl, thialase promoter; Pfdx, ferrodoxin promoter

*Spacer: a module may consist of a short spacer in place of a functional component

In 2009, Heap et al. published a modular vector system that has been useful in a number of clostridial species (61). This system includes four main modules: a Gram-negative replicon, a Gram-positive replicon, an antibiotic resistance marker, and an insertion module. All of these modules can easily be swapped and replaced by others, making this system easy to modify depending on the purpose and organism for which it will be used (Fig. 1, Table 1). For example, four different antibiotic resistance markers can be used, and two Gram-negative replicons are included. The use of specific restriction sites that were employed to build the plasmid series allows the straightforward incorporation of new modules incorporating, for example, new Gram-positive replicons. The development of this vector series has helped to standardize the genetic work in many clostridial species. All these vectors can be used as shuttle vectors and can be transferred from *E. coli* via RP4-mediated conjugation, or directly in some species by electroporation, and have successfully been used in many *C. difficile* studies investigating virulence regulation, biofilm formation, colonization, and sporulation (62–64). These plasmids have also been used in other pathogenic clostridial species, including an investigation into the role of two-component systems in neurotoxin regulation of *C. botulinum* (65). If used without the Gram-positive replicon, they act as suicide vectors in the clostridia and have been effectively used in this capacity in studies involving the nonpathogenic species *C. beijerinckii* and *Clostridium autoethanogenum* (66, 67).

Inducible Vector Systems

In many genetic studies it is essential to be able to control or regulate the expression of cloned genes. The usual method is to place the cloned gene behind an inducible or repressible promoter so that expression of the gene of interest is induced by the addition of a low-molecular-weight effector molecule to the culture medium. However, only a limited number of such systems are available for use in the pathogenic clostridia.

In *C. perfringens*, an inducible expression system was made available by the development of the vector pKRAH1 (68). This shuttle plasmid encodes the *C. perfringens* BgaR transcriptional regulator and the inducible promoter, P_{bgaL}, which is activated by BgaR in the presence of lactose.

The addition of lactose to the culture medium therefore leads to the expression of genes cloned downstream of P_{bgaL}. This system initially was validated by cloning the *gusA* reporter gene behind P_{bgaL} and measuring β-glucuronidase (β-GusA) activity in *C. perfringens* SM101 cells with and without lactose induction. Substituting isopropyl β-d-1-thiogalactopyranoside (IPTG) for lactose did not result in increased activity, suggesting that IPTG is either not an inducer or cannot be transported into the cell (68). Subsequently, pKRAH1 was constructed and used to express a *yfp-pilB* construct, which allowed the resultant fusion protein to be localized (68). pKRAH1 has been used in other studies of the biogenesis of type IV fimbriae in *C. perfringens* (69), and the BgaR/P_{bgaL} system was also used to study the role of the endonuclease RNaseY (70) and carbonic anhydrase (71) in *C. perfringens* and for the analysis of acetogenesis in *Clostridium ljungdahlii* (72).

Other workers developed a xylose-inducible gene system for use in *C. perfringens* (73). The vectors, pXCH (confers chloramphenicol resistance) and pXEH (confers erythromycin resistance), encode the XylO transcriptional repressor and the *xylB* promoter from *C. difficile*. Genes are cloned downstream of P_{xylB}, and XylO repression is subsequently relieved by the addition of xylose. The vectors were validated in *C. perfringens* strain 13 by using them to regulate the expression of chloramphenicol acetyltransferase and α-toxin reporter constructs. The xylose-inducible system has been used by other workers in combination with the lactose system (69).

Classic IPTG-inducible systems are not effective in *C. difficile*, but in 2011 Fagan et al. developed an anhydrous tetracycline-inducible system for this bacterium (74). The system is based on pRMC2 from *Staphylococcus aureus* (75), combining the tetracycline-inducible promoter system with a codon-optimized *gusA* gene from pCBR023 on plasmid pRPF185, including the *fdx* terminator from *C. pasteurianum*. The plasmid backbone is taken from the *E. coli-C. difficile* shuttle vector pMTL960. When GusA activity was monitored as a measure for leakiness of the system, no GusA activity was measured in the absence of anhydrous tetracycline (the inducer), providing supporting evidence that the system is tightly regulated (74). This system has been used successfully in other studies involving *C. difficile* to determine the functional role of TcdE (76) and to investigate the spore differentiation pathway, including the creation of an inducible *spo0A* gene most recently (77, 78). Additionally, it has been used to study spore coat proteins of *C. difficile* (79) and has been employed in other clostridial species, such as *P. sordellii*, in which it was used to examine a new conjugation system (51). A second inducible system has also been described and employed in *C. difficile*. Purcell et al. created a nisin-inducible system in which the *cpr* promoter, responsible for the transcription of an ABC transporter, which confers resistance against nisin, is cloned upstream of the target gene. This system was used to study the role of cyclic di-GMP in motility and aggregation of *C. difficile* (80).

Some clostridial genes are toxic when overexpressed in *E. coli* and therefore are difficult to clone, making complementation studies in the original clostridial host problematic. This problem can be overcome by use of the vector pJIR3422 (81, 82). This vector exploits the phenotypic features of the clostridial Tn*4451/3* site-specific recombinase TnpX, specifically, the specificity of binding of this protein to a transposon-derived promoter. TnpX represses expression from its own promoter, P_{attCI}, which is usually located at the joint of the circular form of the Tn*4451/3* elements (83). This promoter is present in pJIR3422, and expression from this promoter is repressed in the presence of a catalytically inactive derivative of TnpX, present in the *E. coli* strain used for cloning, but is not repressed in the clostridial host because of the absence of TnpX. The use of this system facilitated the cloning of the *tcpG* conjugation gene and the *feoB* ferrous uptake gene in *E. coli* and the complementation of mutants of these genes in *C. perfringens*, which could not be achieved without this system (81).

Reporter Assays

Historically, protein reporter systems for the analysis of protein localization have been lacking in most pathogenic clostridia, particularly in *C. difficile*. Widely used reporter proteins such as green fluorescent protein for fluorescence or luciferase for chemiluminescence require oxygen for correct reporter protein folding or full enzymatic activity and hence have been thought to be of limited value in anaerobic bacteria. In the past 5 years, however, a number of assay systems have been established (77, 84, 85), which have made the functional characterization of genes and their encoded products more feasible. Reporters can be split into two main categories: visualizable reporters and enzymatic reporters. The former are utilized to study phenotypic heterogeneity and localizing proteins, whereas the latter serve as read-outs on transcription.

Visualizable reporters

Several of these systems are based on fluorescence. Ransom et al. successfully developed a codon optimized cyan-fluorescent protein and mCherry (mCherryOpt) in *C. difficile* to perform a number of subsequent studies (84, 86). They created two vectors using mCherryOpt, one to study localization, which they used to examine septal location of two cell division proteins, MldA and ZapA, in fixed cells (86). The other vector allows cloning of a promoter upstream of the mCherryOpt gene to study gene expression and has been exemplified by studying the *pdaV* operon, which is required for lysozyme resistance. (84). The variants were successfully expressed in *C. difficile* but required exposure to oxygen for chromophore maturation (84). Recently, work by Ribis et al. used mCherry fusions to establish the importance of SpoIVa and SipL in spore coat assembly and to elucidate the mechanism of engulfment of the forespore by the mother cell in *C. difficile* (87, 88). Other recent examples of the use of mCherry in *C. difficile* include a study of flagellum and toxin phase variation, demonstrating the potential to study single-cell heterogeneity, and work demonstrating that toxin gene expression is bistable in *C. difficile* (89, 90).

Yellow fluorescent protein fusions have recently been used successfully in *C. perfringens* to examine localization and function of PilB, an ATPase responsible for pili assembly (69).

iLOV, a light oxygen voltage domain, has also been optimized for use in *C. difficile*. LOV domains are plant or bacterial blue light receptors and are part of flavin mononucleotide-based fluorescent proteins (FbFPs) (91, 92). These fluorescent reporters can be utilized with and without oxygen, and they are smaller (13 kDa) than green fluorescent protein (25 kDa), which makes them ideal fusion proteins with a reduced probability of disrupting native protein function. iLOV has been used as a fluorescent reporter in *C. difficile* (85), as a translational fusion to study the cell division protein FtsZ. This study also proved that iLOV fusions can be successfully transported out of the cell by constructing a fusion to FliC, the flagella subunit. Functional iLOV fusions

in *P. sordellii* and the nonpathogenic C. *acetobutylicum* were also generated in this study. The brightness of FbFPs is lower than that of green fluorescent protein, and hence a few technical modifications have been suggested to improve their usefulness, such as lowering background noise and improving the quality of the output signal. Flavin-based media, for example, can lead to highly fluorescent backgrounds, which can be reduced by eliminating yeast and beef extract (93). Also, several clostridial species, such as C. *difficile* and C. *acetabutylicum*, have a natural green auto-fluorescence that can increase the background signal (85).

SNAP-tag technology has also been adapted for C. *difficile*. The 20-kDa peptide tags are fused to target genes, and fusions are visualized using a fluorescent microscope by adding the cell-permeable fluorescent substrate TMR-star. This technology has been used to elucidate the role of the sigma factor cascade in the sporulation process in C. *difficile* and, furthermore, a split SNAP-tag has been employed to study protein interactions between the forespore protein SpoIIQ and the mother cell protein SpoIIIAH (77, 94). SNAP-tags have been proven to be versatile and have worked effectively on fixed cells, demonstrating the temporally regulated gene expression of the sigma factors involved in C. *difficile* spore formation and establishing the interactions of C. *difficile* SpoIIQ and SpoIIIA proteins and their control over forespore engulfment and integrity (77, 95).

Enzymatic reporter systems

The widely used β-GusA reporter assay has been used to study the regulation of the large toxins in C. *difficile*. To elucidate the function of the sigma factor TcdR, Mani et al. created *gus*-fusions in C. *perfringens* and then, in a follow-up publication, *gus*-fusions in C. *difficile*, the study of which showed that TcdR positively regulates *tcdA* and *tcdB* expression (30, 96).

An alternative reporter system used in C. *difficile* involves a luciferase gene fusion to the signal sequence of the secreted protein PPEP-1. PPEP-1 is a zinc metalloprotease that has been shown to be one of the most abundant secreted proteins of C. *difficile* (97). Two reporters have been constructed, AmyEopt and sLucopt (98). AmyEopt can be used to measure promoter activity in liquid or plate-based assays due to the production of α-amylase, a codon optimized enzyme from *B. subtilis*, which results in starch degradation. sLucopt is a codon-optimized luciferase based on NanoLuc (99) and has been shown to be detectable in culture supernatants when fused to an inducible promoter (98). Both reporters are assayed under aerobic conditions once the samples have been taken from the anaerobically grown culture (98).

Finally, the *E. faecalis* alkaline phosphatase gene, *phoZ*, has been successfully used in C. *difficile* for the qualitative and quantitative measurement of gene expression using an alkaline phosphatase assay. PhoZ was shown to be active anaerobically and is therefore presumed to fold correctly under these conditions, which makes it a useful reporter gene for anaerobic bacteria (100).

A promoter probe plasmid based on pJIR418 has also been constructed for use in C. *perfringens*. This plasmid, pPSV, was constructed by deletion of the *catP* gene from pJIR418 followed by the addition of a promoterless *catP* gene downstream of the multiple cloning site (101). The *catP* gene has also been used as a reporter system in the promoter probe shuttle vector pTCATT, which contains a promoterless *catP* gene flanked by transcriptional terminators. However, PCR-mediated regeneration of the 5′ terminus of the *catP* gene is required during the construction of promoter fusions in pTCATT, limiting its usefulness (102). Other studies have attempted to develop reporter systems for use in C. *perfringens*. One system involved the construction of a plasmid that contains the *luxAB* genes from *Vibrio fischeri* under the control of the *plc* promoter. Luciferase activity and bioluminescence were obtained from this plasmid in C. *perfringens* (103). In another study, reporter plasmids using the *E. coli gusA* gene cloned into pJIR750 were constructed, and β-GusA production was used successfully to monitor the sporulation-specific regulation of the *cpe* gene in C. *perfringens* (104). More recently, high-level expression of the C. *perfringens* NanI sialidase was achieved by using a ferredoxin promoter-based plasmid, pFF, in C. *perfringens* (105). This system was particularly useful for purification of NanI since the C. *perfringens* expression strain secreted the enzyme, as does the wild type, and seemed to efficiently express the protein, resulting in a 60-fold increased yield in comparison to protein isolation from an *E. coli* host. These results suggest that in C. *perfringens* the pFF plasmid may be very useful for the expression of AT-rich genes from other clostridia and other bacteria (105).

Allelic Exchange and Homologous Recombination

Homologous recombination has been used extensively as a mutagenesis tool for many bacterial species and may involve either single- or double crossover events. Since two crossovers are required for allelic exchange, it is a less efficient process than obtaining mutants by a single crossover event using insertional mutagenesis. However, the disadvantage of single crossover events is that wild-type genes can be regenerated from the mutants by further homologous recombination events. This issue is not a problem with allelic exchange or double crossovers, which result in inherently stable mutants. It is worth noting that the efficiency with which mutants are obtained by allelic exchange depends on the size of the homologous regions that flank the gene of interest, and it is recommended that there be at least 2 kb of flanking DNA present on either side of the gene to be replaced (106). Additionally, a large number of mutants can be generated using homologous recombination, in contrast to a limited number that can be generated with insertional mutagenesis, because of the small number of antibiotic cassettes available, which are required for insertional mutagenesis.

Homologous recombination and allelic exchange methods for the clostridia have also been developed and improved significantly during the past 2 decades. In early studies, allelic exchange was used successfully for genetic analysis in C. *perfringens* (22), and the first defined genetic mutants constructed in the pathogenic clostridia were the *plc* and *pfoA* toxin gene double-crossover mutants that were constructed in C. *perfringens* (22, 24). Since that time many C. *perfringens* mutants have been constructed by allelic exchange, too many to list here, and homologous recombination has become a standard genetic tool for the construction of mutants in this bacterium.

Early homologous recombination studies of C. *difficile* involved the construction of mutants by single crossover events using suicide plasmids that were introduced by conjugation from *E. coli* (31). Subsequently, two improved systems (107, 108) were developed for C. *difficile*, both of which are based on the use of negative selection markers. The first system uses cycloserine deaminase (107) as a counter selection marker and relies on the presence of the *codA* gene on a pseudo-suicide vector (107). Single crossovers are selected on the basis that cells that have the plasmid, which

contains a heterologous *codA* gene from *E. coli*, integrated into their chromosome are able to grow much faster than cells carrying the resistance gene on a plasmid. This is because the plasmid used in this approach is inherently unstable in *C. difficile* and is lost easily from cells. The faster-growing colonies are selected and tested (via PCR) to determine if a single crossover event has occurred. They are then plated on *C. difficile* minimal medium containing 5-fluorocytosine. Only clones in which a second crossover event has occurred, resulting in the loss of the *codA* gene, are able to grow, because the cells still carrying the *codA* gene are killed when 5-fluorocytosine is converted to toxic 5-fluorouracil (97) by CodA. Double crossover events result either in a reversion back to wild type or in the construction of the desired mutant and loss of the plasmid. This system can be used for the construction of DNA deletions or point mutations. Genes can also be complemented by going through another cycle of single and double crossover events, using a different plasmid which contains the intact gene of interest. In this case, genes are reinserted into their original position on the chromosome. It is hence advisable to introduce a watermark or sequence signature into the gene to differentiate it from the wild type (107). This allelic exchange system has successfully been used in a number of studies, including work characterizing two sortase substrates of *C. difficile* and their mechanism, involving cyclic di-GMP, of anchoring proteins to the peptidoglycan layer (109). Another study investigated a dipicolinic acid release mechanism during *C. difficile* spore germination (110).

The second system for allelic exchange in *C. difficile* is based on creating a *pyrE* mutant, carrying a 3′ deletion of the gene, using allele-coupled exchange (111) in the strain of choice and then using a heterologous *pyrE* gene as a negative/counter selection marker. The *pyrE* gene encodes orotate phosphoribosyltransferase, which is involved in pyrimidine biosynthesis. This enzyme is essential for bacterial growth in the absence of exogenous pyrimidines and also acts on 5-fluoro-orotate to generate a toxic derivative of this compound. The *pyrE* mutants are auxotrophs for uracil. A functional *pyrE* gene from *Clostridium sporogenes* is supplied on an allelic exchange plasmid as the counterselection (108). *pyrE*-mutants were generated in *C. difficile* 630Δ*erm* and R20291 (108) and were then used to generate deletion mutants using a process similar to that described for the *codA* methodology. Single crossover clones are selected on the basis of faster-growing colonies once the vector has integrated into the chromosome, since the vector is unstable due to a frameshift mutation in the *oriR*. Double crossover mutants in which the plasmid has been excised from the chromosome are then selected on *C. difficile* minimal medium supplemented with 5-fluoro-orotate and uracil. Once 5-fluoro-orotate-resistant uracil auxotrophs have been isolated and shown to have lost the plasmid, they can easily be restored to prototrophy by conjugative transfer of a plasmid that contains part of the *pyrE* gene that can be utilized to repair the *pyrE* gene in the chromosome using allelic exchange. The resultant transconjugants are streaked on *C. difficile* minimal medium without the addition of uracil, so only bacteria with a repaired *pyrE* gene are able to grow. It is also possible to complement the mutation at the same time as repairing the *pyrE* gene by cloning a copy of the disrupted gene into the plasmid either under the control of its native promoter or through the use of a constitutive promoter for overexpression. Using allele-coupled exchange, the complementation gene is then integrated simultaneously during *pyrE* repair at the *pyrE* locus (108). This technique is very powerful and rapid, allowing for the generation of complementation strains within 5 to 6 days, opposed to obtaining an allelic exchange mutant in 2 to 3 weeks. This approach has been used in several studies; for example, it was used to generate a complete pathogenicity locus deletion in *C. difficile* R20291 to study the role of binary toxin in the absence of the overpowering potency of TcdA and TcdB (112). It was used in a recent study that suggested that the introduction of trehalose into our diets has favored the emergence of so-called hypervirulent *C. difficile* ribotypes, such as RT027 (113). Ribis et al. created the first published quadruple mutant in *C. difficile*, using the *pyrE*-system, in their work studying the roles of putative engulfment regulators, IID (CD0126), IIP (CD2469), and IIM (CD1221), and the known engulfment regulator and putative endopeptidase, IIQ (CD0125) (88).

ClosTron and TargeTron

Two related technologies based on the exploitation of a mobile group II intron were developed for insertional mutagenesis in the clostridia. Mobile group II introns are found in bacterial genomes and are site-specific retroelements. They use a mobility mechanism termed retrohoming to create insertions in DNA, and they do this by inserting an excised intron lariat RNA directly into a DNA target site, which is then reverse transcribed by an intron-encoded enzyme protein (114). The DNA target site is primarily recognized by base pairing of intron RNA, and these bases can be modified to allow intron insertion into any specific DNA target (4). As a result, a mobile group II intron from *Lactococcus lactis, L1.LtrB,* was developed commercially (Sigma-Aldrich) and was used in the development of TargeTron and ClosTron technologies. The use of the TargeTron system in a clostridial species was first described in 2005, when it was used to inactivate the *plc* gene in *C. perfringens* (115). TargeTron was also used as a tool to introduce the simian immunodeficiency virus p27 gene into the *pfoA* gene on the *C. perfringens* chromosome, thereby simultaneously inactivating *pfoA* and introducing the simian immunodeficiency virus p27 gene, which was subsequently shown to be expressed within the mouse gastrointestinal tract (116).

ClosTron technology is an insertional mutagenesis tool similar to TargeTron, but it incorporates the use of a retrotransposition-activated marker (RAM), based on the *ermB* gene, for selective purposes, with successful insertion indicated by the acquisition of resistance to erythromycin (117). The presence of a RAM makes mutant selection much more efficient and less laborious. The retargeted region is designed using an online retargeting algorithm and is then incorporated into the plasmid of choice for subsequent introduction into the clostridial strain (118).

Although the acquisition of a resistance marker in the bacterial genome via ClosTron or similar systems makes mutant selection simpler, it can also be a drawback. In particular, this means that a second mutation using the same marker cannot be obtained. Furthermore, the use of a strong promoter, driving the transcription of the RAM, can lead to unwanted polar effects. While the ClosTron system is very efficient and easy to use, it can, on occasion, be nonspecific, requiring the empirical testing of several insertion sites. To be able to construct double mutants, it is necessary either to use another marker or to remove the initial marker, both of which have been attempted. No other RAM marker other than erythromycin resistance has been described for the clostridia, but markerless ClosTrons or ClosTrons with markers that are not retroposition activated have been used

successfully (115, 119). The alternative and preferred option is marker removal. Flp recombinase has been used successfully in *C. acetobutylicum* to remove the retargeted *ermB* gene from a ClosTron mutant (120). The ability of the Tn*4451/3* site-specific recombinase TnpX to recognize and excise specific DNA fragments has also been exploited for antibiotic resistance marker recycling. Specifically, TnpX was used to remove the *ermB* gene from a marked chromosomal *C. perfringens* mutant, enabling the construction of a double mutant by allowing the removal and subsequent reuse of *ermB* to construct the second mutation (81).

Transposon-Mediated Random Mutagenesis

Transposon mutagenesis has been established in *C. perfringens* for several decades, although the early systems were not optimized and lacked efficiency (22). The most successful early mutagenesis method utilized the conjugative enterococcal tetracycline-resistance transposon Tn*916* (121). In these studies, *C. perfringens* cells were transformed with a suicide plasmid carrying Tn*916*, and transformants were selected on medium containing tetracycline. Hybridization analysis showed that Tn*916* had inserted at different sites on the chromosome but that multiple insertion events were common. However, mutants that had a single Tn*916* insertion and that abrogated the ability to produce perfringolysin O were detected. The insertion site was shown to be located within the *virS* sensor histidine kinase gene, leading to the identification of the VirSR two-component signal transduction system (121). Subsequent studies involved the isolation of Tn*916* insertions in several toxin genes (122), and conjugative transfer or mobilization of Tn*916* from *E. faecalis* and *E. coli*, respectively, has been used to isolate auxotrophic mutants of *C. perfringens*, but again, multiple insertion events were observed (123). Tn*916* mutagenesis also has been used to isolate mutants that have an altered response to oxidative stress (124). In summary, the use of Tn*916* for mutagenesis in *C. perfringens* has been useful, but it is hampered by the presence of multiple insertion and deletion events and subsequently has not been widely adopted.

Other transposon mutagenesis options include transposon mutagenesis of cloned genes in *E. coli* and subsequent transformation and screening in *C. perfringens*. This approach has been used to identify the replication protein of the *C. perfringens* plasmid pCW3 (125). Other workers (126) developed an EZ-Tn*5*-based random mutagenesis system that involved the electroporation of a transposome complex into *C. perfringens* and selection (erythromycin resistance) for the insertion of a modified Tn*5* element. The erythromycin-resistant transformants appeared to contain random single insertions. These experiments led to the mutagenesis and identification of the *agrB* gene, which is involved in the regulation of perfringolysin O production by quorum-sensing (126). Similarly, the bacteriophage Mu-based transposon delivery system has been successfully adapted for *C. perfringens* (127). Electroporation was used to introduce a Mu transpososome complex, and a strain library that contained single transposon inserts was obtained. Finally, the *Himar1* mariner transposon system has been adapted for *C. perfringens* and used to show that the *sagA* gene is required for gliding motility in *C. perfringens* (128). This method utilizes a replicating plasmid that carries an inducible mariner element, but it requires a strain with *galK* and *galT* mutations to ensure that the replicating delivery plasmid can subsequently be selected against in the presence of galactose.

Despite in-depth studies of transposons in *C. difficile* (129), there is only one system for random mutagenesis in this organism. Transposons such as Tn*5397* and Tn*916* (33, 130, 131) have been well characterized, but they either lead to multiple insertion events or show a strong bias to particular target sites. However, the mariner transposon system has been successfully developed for *C. difficile* (132, 133). The transposable element of the mariner system *Himar1* inserts randomly into a TA target site, which lends itself for use in AT-rich organisms such as the clostridia. Furthermore, only the cognate *Himar1* transposase is required for transposition to occur. As mentioned earlier, no transformation system has yet been developed successfully for *C. difficile*, which means that the transposon vectors cannot rely on a plasmid suicide mechanism for their introduction into cells. To overcome this problem, Cartman and Minton (132) developed a pseudo-suicide vector by creating a segregationally unstable plasmid, using the replicon pBP1. This method allowed them to obtain transposition events at a frequency of 4.5×10^{-4} per cell. Drawbacks of this method were the lack of effective control of the time of transposition and lack of plasmid loss, which meant that the size of the library that could be obtained was limited. Library size is important for experiments such as transposon-directed insertion site sequencing, which can be used to determine essential genes under specific conditions. Subsequently, the system was improved by integrating the previously described anhydrous tetracycline system (74) with the transposon vector, generating a plasmid that has tetracycline-dependent conditional replication. The new transposon vector not only has a conditional replication phenotype, but it also has tight control over transposition of the *ermB* transposon. This tetracycline-inducible system consists of two promoters (133); one promoter drives expression of *tetR* (74), and the other promoter drives expression of the codon-optimized Hinmar1 transposase. Upon induction, Dembek et al. showed near complete plasmid loss within 13 generations, whereas 40% of bacteria in the uninduced culture retained the plasmid. (133). The transposon system has been demonstrated in *C. difficile* 630Δ*erm* and in the epidemic strain R20291. Transposon libraries have been created during *in vitro* growth and during sporulation, followed by transposon-directed insertion site sequencing, which resulted in the identification of 404 and 798 essential genes, respectively, for these conditions (133).

Newer Technologies for Genetic Manipulation

New and improved technologies for genetic analysis and manipulation of the pathogenic clostridia are being reported all the time. Most *C. difficile* genomes contain prophages and lysogenic bacteriophages, which have been characterized in some detail (134–136), but to date, no lytic phages have been described. There has been one report of transduction, showing that a novel transposon, Tn*6215*, carrying an erythromycin resistance gene, could be transferred from a donor strain to a recipient strain and integrated into the recipient genome if the bacteriophage ΦC2 was integrated into the recipient genome. The study was unable to show transduction into another three recipient strains, which were all susceptible to the phage, suggesting that conditions for transduction need to be optimized further before it can be used as a reliable genetic tool (137).

Antisense RNA technology has successfully been used to repress gene expression in *C. difficile* and to show that the S-layer proteins and cell wall protein V are translocated across the cell membrane through an accessory Sec-system

(SecA2). Most recently, CRISPR-gene editing technologies were successfully used in the clostridia. Initially developed in nonpathogenic clostridia, such as *C. pasteurianum* (138), *C. beijerinckii* (139), and *C. cellulolyticum* (140), this emerging technology was used in 2017 to generate deletion mutants in *C. difficile* (141). The authors developed a CRISPR-Cas9 system with a reported mutation frequency of 20 to 50%, exemplifying it by deleting *selD*, a selenophosphate synthetase that is essential for the specific incorporation of selenium into selenoproteins. They showed that deletion of *selD* leads to growth reduction and a lack of selenium incorporation, suggesting that the Stickland reaction could be a target for future antimicrobial therapy development. Since then, two more papers were published (142, 143), further developing and refining the technology for use in *C. difficile*. Wang et al. used a plasmid-based CRISPR-Cas9 system for mutagenesis, reporting 100% mutation efficiency and knocking out the sporulation master regulator Spo0A (142). The latest study by Hong et al. developed a CRISPR-Cpf1 system capable not only of targeted deletion from the *C. difficile* genome, including the largest deletion to date of 49.2 kb, but also of simultaneous deletion of at least two targets located on different parts of the genome. This new toolkit could greatly improve the genetic analysis of *C. difficile* and advance the development of new therapeutics and diagnostics by identifying new targets for these strategies (143).

CONCLUSIONS

The pathogenic clostridia are a diverse group of anaerobic, Gram-positive spore formers, all of which can cause human disease. Pathogenesis is toxin mediated, but many auxiliary virulence factors have been described and are still being discovered. Historically, it has been extremely difficult to study the clostridia at a molecular level owing to their anaerobic nature, but also because of a number of other difficulties such as the presence of multiple restriction modification systems, which preclude the uptake of foreign DNA into the bacterial cell. With the advent of next-generation sequencing and, consequently, the availability and analysis of whole-genome sequences, the development of clostridial molecular tools has taken an unprecedented leap. The technologies presented and reviewed in this article open up unique opportunities to study the pathogenic clostridia and their disease mechanisms in detail, with the objectives of improved diagnostics and the development of novel disease therapies.

There is, however, more work to be done. Recent years have seen a surge in interest in the nonpathogenic clostridia, particularly because of their utility in producing a range of biochemicals. Technologies have been developed that could be adapted for use in the pathogenic clostridia, such as a range of allelic exchange systems. Examples include the I-SceI system used in *C. acetobutylicum* and *C. beijerinckii* (144) and the MazF toxin-antitoxin system, which has been used in *C. acetobutylicum* and *C. cellulolyticum* (145, 146).

Additionally, there is an ever-growing interest in the healthy microbiota and in particular clostridial species within that microbiota (147, 148). To date, no mechanistic studies have been conducted, despite studies showing compelling data that suggest benefits to the host or microflora from certain of these species (147). The main reason for this information shortfall is the lack of available genetic tools. With the newest approaches, described in this article, we are likely to see this area of research develop rapidly in the very near future (38, 142).

In conclusion, the past 10 years have seen a rapid increase in mechanistic studies of the pathogenic clostridia owing to the development of novel, sophisticated tools, the outcomes of which are being translated into the clinic through improved diagnostics, prevention strategies, and novel treatments. The next decade will undoubtedly see even more significant advances in clostridial genetics and their subsequent application to provide a more detailed understanding of this important genus of bacteria.

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Genomics of the Pathogenic Clostridia

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58

INTRODUCTION

The earliest DNA-based studies of the clostridia investigated the guanine + cytosine (G+C) content of DNA and measured hybridization as an indication of the relatedness of strains (1–3). Since those early "genome" studies, a range of other DNA-based typing methods, such as pulsed field gel electrophoresis, amplified fragment-length polymorphism analysis, 16S rRNA gene sequence comparisons, multilocus variable-number tandem-repeat analysis, and multilocus sequence typing (MLST), have been used to classify, group, and differentiate clostridial isolates (4). However, these methods have now been largely superseded. With the rapid advances in DNA sequencing technologies and the subsequent drop in the price and effort required to acquire whole-genome sequence (WGS) data, the comparative analysis of strains is now commonly based on core genome single-nucleotide polymorphisms (SNPs), gene content analysis, MLST on a large group of genes, or average nucleotide identity (ANI) across whole genomes. Whole-genome analysis is the tool of choice for comparing isolates and informs our understanding of the pathogenic mechanisms that are deployed by the clostridia, the epidemiology of disease outbreaks, and the evolutionary histories of the organisms. Analysis of WGS allows the identification of metabolic pathways or the lack thereof (e.g., amino acid metabolism and substrate degradation pathways) and thus indicates how clostridia are adapted to their lifestyles within the ecological niches that they occupy. The quality of WGS information available in sequence databases is variable, so some caution is needed in how it is interpreted when investigating genome structure and absolute gene content. Complete closed genomes are particularly valuable and can be used as scaffolds around which the more usual draft assemblies can be made. Like WGS data from all species, the clostridial WGS annotations need to undergo continual refinement as gene functions are experimentally confirmed and extended. Commonly, a quarter to a third of the open reading frames identified in genomes encode putative proteins of unknown function. Whole-genome analysis provides a much better tool for taxonomic classification than the traditional biochemical, phenotypic, and limited molecular characterization methods (e.g., 16S rRNA gene sequencing) that were previously applied.

The pathogenic clostridial species with the greatest impacts on human and animal health are *C. difficile*, *C. perfringens*, *C. tetani*, *C. botulinum*, and other species that produce botulinum toxins. Among the clostridial species, *C. difficile* has attracted the most active and broad research effort because of its clinical impact and the need for improved treatment options for infections. Therefore, the most intensive genomic research effort has been applied to *C. difficile*, and this is reflected in the number of WGSs that have been submitted to public databases (Table 1) and the depth of information that has been generated regarding strain diversity, transmission, and evolution. The clostridial species that produce botulinum toxins are also well studied, but there is only limited genomic information available for other clostridia, such as the clostridial species for which clinical cases are only rarely seen and the predominantly animal pathogens that, in most cases, are well controlled by vaccines.

Many of the clostridia are ubiquitously found in soil and in the gastrointestinal tract of healthy animals, including humans. Disease generally occurs when there is some tissue damage to the skin or gastrointestinal tract or environmental disruption within the gastrointestinal tract resulting in clostridial proliferation. This bimodal lifestyle, divided between a free-living, often saprophytic existence in the environment and host colonization and pathogenesis, is reflected in the genomes of the clostridia. The spore-forming machinery encoded by all the clostridia may increase resilience and survival in the environment, and the wide array of toxins and degradative enzymes that are encoded assist in nutrient scavenging. Many of the toxins produced by clostridia are encoded on large plasmids, at least some of which have been shown to be conjugative. The main chromosomes of clostridia range in size from 2.4 to 7.2 Mb (Table 1), and hence their genetic potential is quite variable. In general, across all bacteria, smaller genomes tend to be found in bacteria that occupy limited specialist environmental niches, whereas those with larger genomes, such as many of the clostridia, are generalists that can survive in a range of ecological niches and use a wider variety of substrates for growth (5).

CLOSTRIDIAL TAXONOMY IS INFORMED BY GENOMICS

The bacteria that have historically been given the genus name *Clostridium* represent a genetically disparate collection of organisms. The original classifications were based on the phenotypic characterization of isolates as Gram-positive, endospore-forming, obligate anaerobes. However, genomic information, first from 16S rRNA gene sequences and more

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TABLE 1 Pathogenic clostridial genomes available in GenBank

Species	Example reference(s)	G+C%	No. of sequences in GenBank	Size range (Mb)
C. argentinense	104	28.4–28.7	3	3.9–4.8
C. baratii	104, 105	28.0–28.3	10	3.1–3.3
C. bolteae	87	48.6–49.4	9	6.2–6.6
C. botulinum	20, 104, 106, 107	27.1–29.0	225	2.4–4.5
C. butyricum	105, 108	28.4–28.9	18	3.8–4.9
C. chauvoei	92	28.3	3	2.9
C. clostridioforme	87	38.1–49.2	16	4.9–7.2
C. difficile	45, 63, 77, 78, 109	28.2–29.2	1263	3.5–4.35
C. haemolyticum	110	27.4–27.8	3	2.5–2.9
Hathewaya histolyticum (*C. histolyticum*)	GenBank BioProject no. PRJEB18332	34	1	2.8
C. novyi	111	27.6–28.9	9	2.5–2.8
C. perfringens	36, 39, 40, 43, 44, 112	27.8–28.6	59	2.9–4.1
C. septicum	96	27.5	1	3.3
Paeniclostridium sordellii (*C. sordellii*)	82, 83	27.3–27.9	47	3.4–3.7
C. spiroforme	GenBank BioProjects no. PRJNA18925 and PRJNA377666	28.6–28.9	4	2.5–2.8
C. tetani	29, 98, 113	28.4–28.7	12	2.8–2.9
Clostridia that are rarely isolated pathogens or species generally regarded as nonpathogenic but occasionally found in clinical cases				
Paraclostridium bifermentans (*C. bifermentans*)	99	28.0–28.4	4	3.5–3.6
C. cadaveris	98	31.1	3	3.5
C. celerecrescens	114	43.5–43.9	2	5.0–5.3
C. fallax	GenBank BioProject no. PRJEB18188 and PRJEB6403	26.5–27.1	2	2.8
C. innocuum	115	43.3–44.0	6	4.2–4.8
C. paraputrificum	98	29.7	7	3.5–3.8
Erysipelatoclostridium ramosum (*C. ramosum*)	Genbank BioProject PRJNA19663	31.4	1	3.2
C. sphenoides	GenBank BioProject no. PRJEB16486, PRJDB859, and PRJEB6403	43.7–43.8	3	5.2–5.3
C. sporogenes	116	28	30	2.7–4.2
C. taeniosporum	117	26.6	1	3.3
C. tertium	98	27.8	2	3.9–4.0

recently from WGS, has clearly shown that many of the species categorized within the genus *Clostridium* are only distantly related and by all currently accepted taxonomic principles should not be considered as belonging to the same genus (6, 7). Among the major pathogenic clostridial species, *C. difficile* and the taxonomically related species *Clostridium sordellii*, fall outside the main cluster of true clostridial species (Fig. 1). These two species have been grouped within clostridial cluster XI, whereas most of the other important clostridial pathogens (e.g., *C. botulinum*, *C. perfringens*, and *C. tetani*) fall within the "true" clostridia classified within cluster I (Fig. 2) with the type species of the clostridia, *C. butyricum* (8). In recognizing this taxonomic issue, Ezaki reclassified the cluster XI species, including *C. difficile*, *C. sordellii*, *C. sticklandii*, *C. bartlettii*, *C. bifermentans*, and *C. glycolicum*, to the family *Peptostreptococcaceae* (9). The cluster XI clostridia have been assigned to a number of genera within this family, including *Intestinibacter* (e.g., *I. bartlettii*), *Paeniclostridium* (e.g., *P. sordellii*), and *Paraclostridium* (e.g., *P. bifermentans*) (7, 10, 11). Lawson et al. suggested changing the name of *Clostridium difficile* to *Clostridioides difficile* (12), and this new name is now officially recognized (13) and will be used in this review.

The dichotomy between these different groups of clostridia can also be clearly seen in the relative degree of genomic plasticity observed within cluster I species compared with that noted in *C. difficile*. Cluster I species exhibit low genome plasticity compared to many other bacterial genera, whereas *C. difficile* has an extremely plastic genome (see discussion below). Within cluster I the most significant intraspecies genomic variation tends to be in plasmid carriage, prophage sequences, and divergent flagellar genes, whereas the cluster XI intraspecies differences are, in addition, driven by a wider array of mobile elements and high

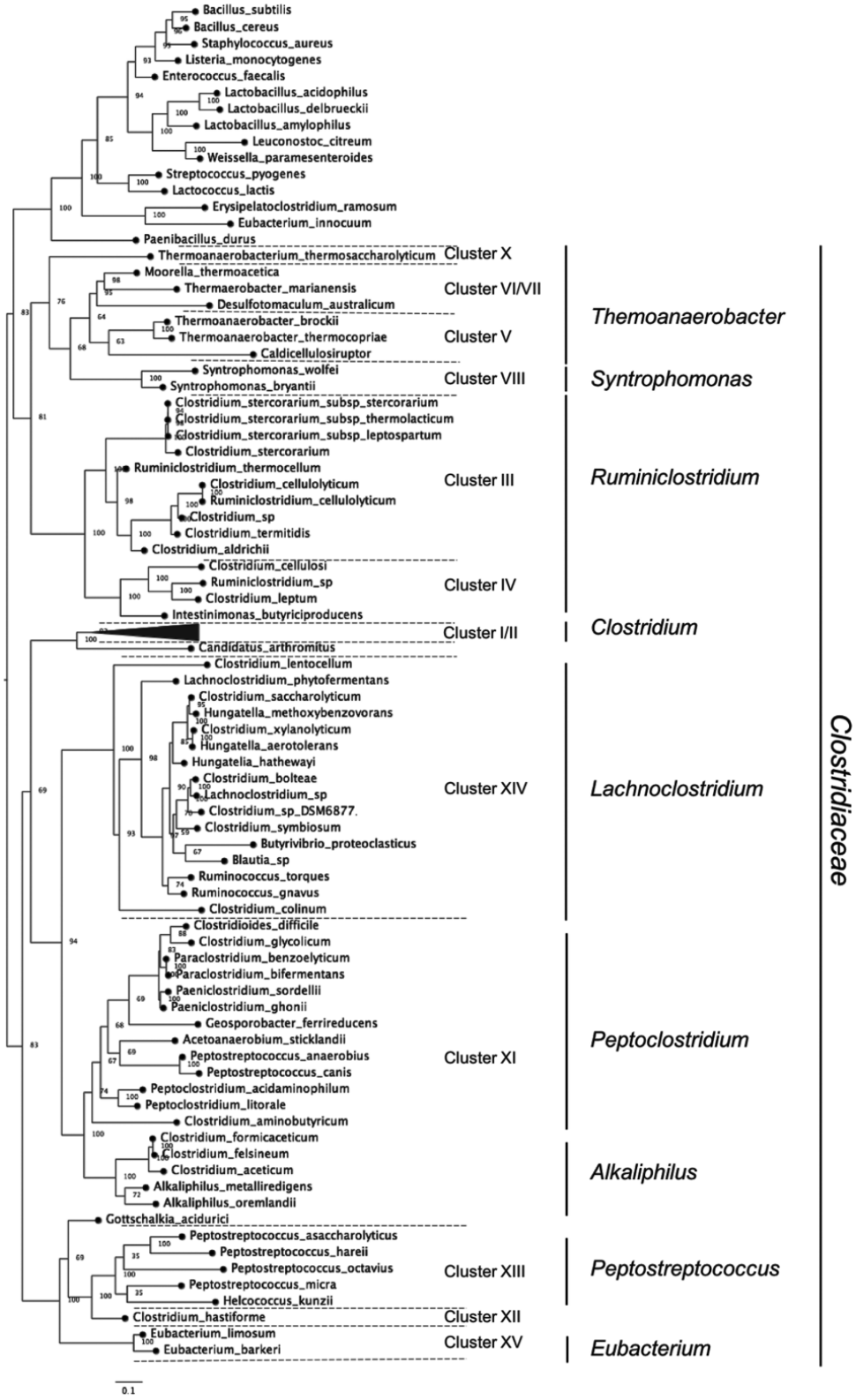


FIGURE 1 Relationships within the *Clostridiaceae*. Maximum likelihood tree of 16S rRNA genes of members of the *Clostridiaceae* family. 16S small subunit rRNA gene sequences were retrieved from RDP (release 11) and NCBI:GenBank (http://www.ncbi.nlm.nih.gov/). Sequences were aligned using Clustal Omega. The multiple sequence alignment was used to build a maximum likelihood tree using IQ-TREE with the general time reversible model (GTR+F+G6) and rapid bootstrapping (-bb 2000) nonparametric bootstraps (-v) (101–103). Clusters are labeled corresponding to the groups proposed in Collins et al. and genus groups as proposed by Parks et al. (7, 8). Clusters from Collins et al. that are not included are due to reclassification outside of the *Clostridiaceae* family.

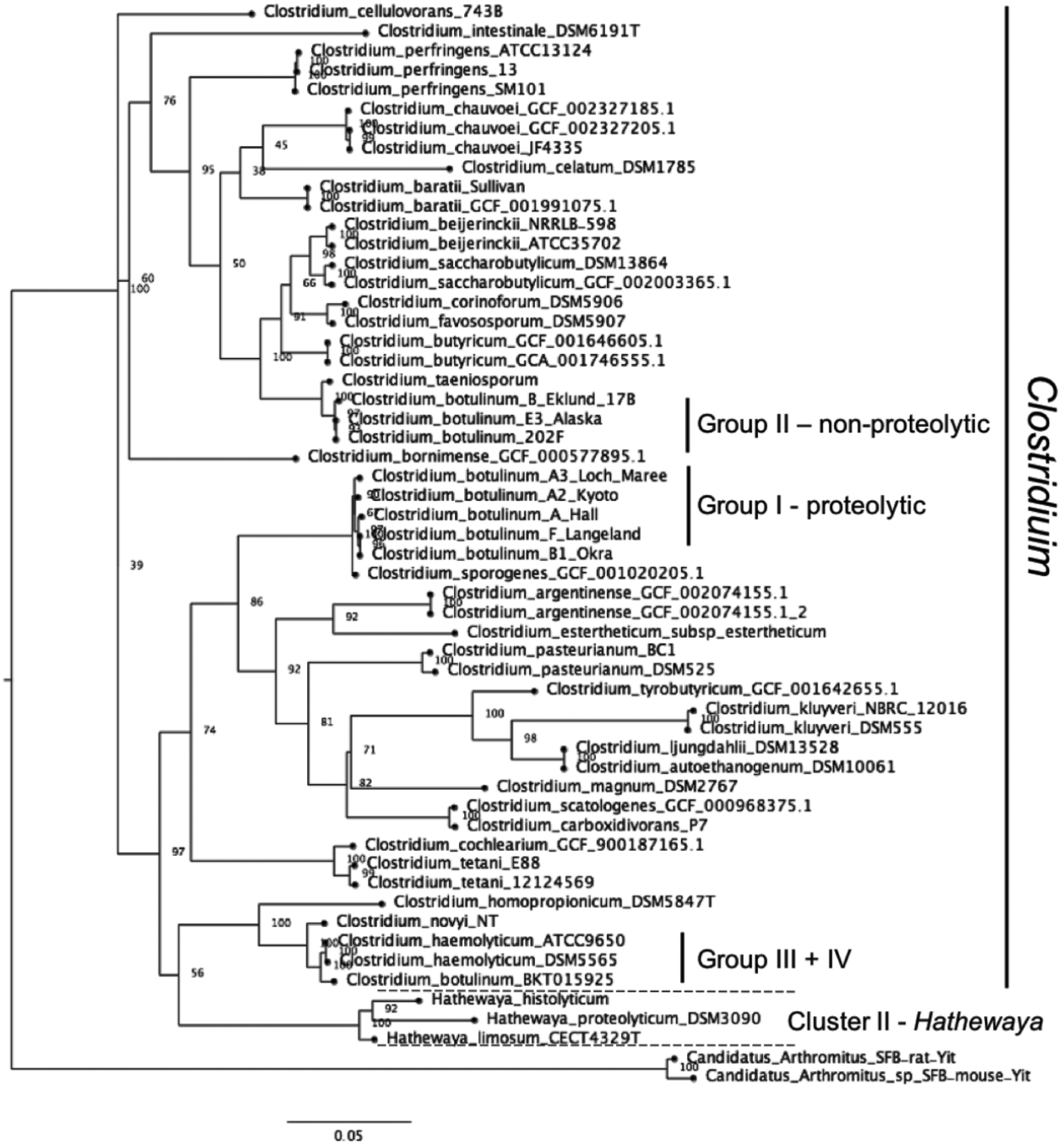


FIGURE 2 Relationships within *Clostridium*. Maximum likelihood tree of 16s rRNA genes of members of the genus *Clostridium*. 16S small subunit rRNA gene sequences were retrieved from RDP (release 11) and NCBI:GenBank (http://www.ncbi.nlm.nih.gov/). Sequences were aligned using Clustal Omega. The multiple sequence alignment was used to build a maximum likelihood tree using IQ-TREE with the general time reversible model (GTR+F+G6) and rapid bootstrapping (-bb 2000) nonparametric bootstraps (-v) (101–103). Clusters are labeled corresponding to the groups proposed in Collins et al. (8). *C. botulinum* groups I to IV are labeled.

levels of recombination, chromosomal rearrangement, and gene acquisition and loss.

Genomic analysis of other non-cluster I clostridial species, such as *C. bolteae*, *C. clostridioforme*, *C. histolyticum*, *C. celerecrescens*, *C. innocuum*, and *C. sphenoides*, clearly shows, just on the basis of G+C content, that these species should not be classified within the genus *Clostridium* (Table 1). *C. histolyticum* has been formally renamed *Hathewaya histolyticum*, but the others remain to be formally renamed.

CLOSTRIDIAL STRAINS THAT PRODUCE BOTULINUM NEUROTOXIN

A phylogenetically diverse collection of bacteria, representing at least four species (*C. botulinum*, *C. butyricum*,

C. argentinense, and *C. baratii*), express botulinum neurotoxins (BoNTs). BoNT-expressing isolates are further classified into six groups (I to VI) depending on which toxin types they produce. Groups I to IV encompass the *C. botulinum* isolates (Table 2), group V is the *C. baratii* isolates that encode type F BoNTs, and group VI covers the *C. butyricum* isolates that encode type E BoNTs (14). There are multiple clades within groups I, II, and III, and by normal phylogenetic considerations the genomically disparate groups of *C. botulinum* should not all have the same species names (Fig. 2). Each group encodes between one and three BoNTs from among the seven BoNT serotypes known (BoNT/A to BoNT/G), and there are also many subtypes (15, 16). Recently, other novel and hybrid BoNT variants have been characterized (17, 18). The BoNT serotype-encoding genes differ by ~25 to 45% at the nucleotide level and have diverse locations in the genome, on either chromosomes, plasmids, or bacteriophage loci, depending on the *C. botulinum* type (16, 19).

The genomes of BoNT-expressing isolates vary widely in size, with some as small as 2.4 Mb and others as large as 4.5 Mb (Table 1), indicating large differences in gene-coding potential. Williamson et al. carried out a comparative analysis of 170 genomes of BoNT-producing bacteria and other clostridial species (20). Proteolytic group I isolates are the most broadly represented bacteria among the whole-genome-sequenced *C. botulinum*. Using a variety of analytical approaches, including 16S rRNA gene, core genome, MLST, and concatenated marker gene comparisons, it has been demonstrated that there is a large amount of phylogenetic diversity not only across isolates carrying genes encoding the various BoNT serotypes but also between isolates of the same toxin type. For example, group III isolates were found to be evolutionarily distant from groups I and II (Fig. 2). Whole-genome comparisons using ANI found that some strains within groups I, II, and III have identities below the 95 to 96% level that is generally regarded as defining relatedness at the species level. Some strains within groups I and III have ANI values of less than 93% compared to other strains classified in the same group, indicating greater species-level diversity than had previously been recognized using conventional phenotypic classification methods. Other toxigenic clostridial species are much more closely related within the designated species; for example, the lowest ANI value for isolates classified as *C. perfringens* is 97.02%, and for *C. tetani* isolates, the lowest is 99.13%, indicating much tighter and coherent classification of these species than is the case for *C. botulinum* isolates (20).

Analyses comparing phylogenetic of *C. botulinum* genome sequences with those of other clostridial species have demonstrated a close genetic relationship between some strains that do not produce BoNTs and the toxigenic strains. For example, there is a high degree of similarity between *C. botulinum* group I strains and *C. sporogenes* and *C. tetani* strains; *C. taeniosporum* is genomically related to group II strains, and *C. novyi* is related to group III strains. The group II, V, and VI strains are more closely related to *C. perfringens* strains than to group III strains (20). There are also nontoxigenic representatives of BoNT-expressing species; i.e., there are toxigenic and nontoxigenic representatives of *C. argentinense*, *C. baratii* and *C. butyricum* (21).

Mechanisms such as conjugative plasmid transfer and phage infection and replication instability may account for some elements of the diverse BoNT carriage noted across this phylogenetically diverse collection of bacteria. Conjugative transfer of a large group I BoNT plasmid (pCLJ) has recently been demonstrated (22). Group III *C. botulinum* isolates are generally associated with cases of animal botulism, and they encode *bont* genes for types C or D BoNTs or chimeric C/D or D/C BoNTs. The *bont* genes are encoded by bacteriophages that replicate as unstable plasmids that are frequently lost during primary isolation and subsequent *in vitro* culture (23, 24). Recombination and mutation also shape the genomes, and SNP analysis of the core genomes indicates that recombination is more prevalent in group I isolates compared to group II isolates (20). It is not clear whether this dichotomy is due to actual differences in recombination rates or is a result of the varied evolutionary genesis and history of the groups.

Spore formation is important for *C. botulinum* survival in the environment, and efficient spore germination, leading to vegetative growth and BoNT production, occurs under favorable conditions. WGS analysis has demonstrated that each *C. botulinum* group encodes distinctive spore germination pathways. Groups I and III encode similar germination pathways that differ from the pathways of groups II and IV (25). Germination can be induced by a variety of amino acids and L-lactate. The genome analysis indicates a more diverse set of germinant receptor encoding genes in group I strains compared to the other groups, and this is in agreement with a greater range of amino acids that are known to be effective germinants (25).

C. tetani

C. tetani produces the tetanus neurotoxin (TeNT), the gene for which is encoded on a plasmid (26). The toxin has a similar mechanism of action and some structural similarity to the BoNTs, but the two types of toxin differ in the types of paralysis that they induce; TeNT causes rigidity of skeletal muscles, resulting in death in both humans and other

TABLE 2 Phenotypic groups of *C. botulinum*[a]

Property	Group I	Group II	Group III	Group IV
Toxin types	A,B, F	B,E,F	C,D	G
Proteolysis	+	–	Weak	–
Saccharolysis	–	+	–	–
Lipase	+	+	+	–
Disease host	Human	Human	Animal	–
Toxin genes	Chromosome/plasmid	Chromosome/plasmid	bacteriophage	plasmid
Close relatives	*C. sporogenes*	*C. beijerinckii*	*C. haemolyticum*, *C. novyi*	*C. haemolyticum*

[a]+, present; –, absent;.

animals, whereas the BoNTs cause flaccid paralysis (27). Although effective vaccines against tetanus have been available for many years, there are still estimated to be several hundred thousand human deaths from tetanus each year (28). Phylogenomic analyses have shown that *C. tetani* is closely related to group I proteolytic *C. botulinum* strains (20). From the small number of WGSs available for *C. tetani*, it has been deduced that they are more highly conserved than *C. botulinum* genomes (29). WGS studies have investigated the genetic relatedness of vaccine strains from different laboratories and wild-type strains (29, 30). The original vaccine strain was isolated in the United States around 1917 to 1919 and was distributed to various vaccine manufacturers and laboratories around the world, so all vaccine strains are believed to have a common origin (29). WGS studies have demonstrated how these strains have changed in the intervening period, with the accumulation of varying numbers of SNPs, ranging from 25 to 281, among the 5 vaccine strains sequenced. More recent wild-type clinical isolates, unsurprisingly, are more divergent from the vaccine strains, with several thousand SNPs and diverse phage insertion sites and other genomic rearrangements. The chromosome of one strain, ATCC 19406, was very similar to the vaccine strains, but the strain was nontoxigenic due to the absence of the tetanus toxin-encoding plasmid (29). There appears to be more variation in the sequence of the large toxin-encoding plasmid than in the chromosome, with significant deletions and/or insertions in many areas.

C. novyi and *C. haemolyticum*

Comparison of 16S rRNA sequences indicated that both *C. novyi* and *C. haemolyticum* are closely related to group III *C. botulinum*, and this has been confirmed by WGS analysis, and hence these species have been referred to by the collective genospecies name *C. novyi sensu lato* (31, 32). *C. novyi* can cause gas gangrene/blackleg (myonecrosis) and toxic shock-like presentation. *C. haemolyticum* causes bacillary hemoglobinuria. There are pathogenic and nonpathogenic isolates of *C. novyi* that are genomically differentiated by the chromosomal carriage of beta-toxin (phospholipase C) and gamma-toxin (nontoxic phospholipase C) encoding genes respectively. The major virulence factor of *C. haemolyticum* isolates is a phospholipase similar to that found in beta-toxin of *C. novyi*. The similarity of the genomes within the *C. novyi sensu lato* group means that loss or acquisition of some mobile elements, such as the phages that encode BoNTs, can effectively result in conversion between the different species (33).

C. perfringens

C. perfringens is widely distributed in the environment. It can be isolated from soil and water samples and is often found within the gut microbiota of healthy animals. *C. perfringens* isolates can cause a variety of histotoxic and intestinal diseases in a wide variety of animals, including humans (34). *C. perfringens* isolates have been classified according to the combination of the four typing toxins (alpha, beta, epsilon, and iota) they carry. For many years five toxinotypes (A to E) were recognized, but the toxinotyping scheme has recently been expanded to include two more toxins, enterotoxin (CPE) and NetB, and two new toxinotypes, F and G, that these toxins are associated with (Table 3) (35). The typing toxins are just six of the many extracellular toxins and hydrolytic enzymes that are encoded within the *C. perfringens* pangenome. Because clostridial diseases are largely driven by the array of toxins that various isolates can produce, the toxinotyping scheme has some utility in differentiating isolates associated with particular diseases. All *C. perfringens* isolates carry the chromosomally encoded alpha-toxin gene, and those isolates that do not carry any of the other typing toxins are classified as toxinotype A isolates.

WGSs are available for strains of each toxinotype, although it is only poultry isolates of toxinotype A and the newly defined toxinotype G that have been studied in any depth. *C. perfringens* genomes range in size from 2.9 to 4.1 Mb (Table 1). A comparison of 51 *C. perfringens* genomes identified a core genome of 1,192 genes and a pangenome of 8,102 genes (36). The core genome is likely to be fairly well defined, although the sequencing statistics suggest that the number of genes within the pangenome has not plateaued and hence would be expected to expand as more isolates are sequenced. Comparative genomic studies have shown that there are no chromosomal differences which clearly delineate the different groups identified by toxinotyping, so a phylogenetic tree is not particularly informative regarding the pathogenic potential of isolates. The key differentiating feature of the toxinotypes is their carriage of large plasmids that encode the beta, epsilon, iota, CPE, and NetB toxins. These plasmids have large conserved gene clusters encoding conjugation machinery, and a number of the large clostridial plasmids, including those encoding typing toxins, nontyping toxins, and antibiotic resistance genes, have been shown to be conjugative (37, 38). Some nonpathogenic strains carry no large plasmids, whereas some pathogenic isolates have up to five large plasmids (32). A WGS study that compared pathogenic poultry isolates to nonpoultry isolates identified three DNA regions that were more often found in pathogenic chicken isolates and may therefore carry genes involved in

TABLE 3 Presence and absence of toxins within *C. perfringens* toxinotypes[a]

Toxinotype	Typing toxins						Nontyping toxins				
	plc	*cpb*	*etx*	*Iap/ibp*	*cpe*	*netB*	*cpb2*	*pfo*	*netF*	*cpd*	*tpeL*
A	+	–	–	–	–	–	+/–	+	–	–	–
B	+	+	+	–	–	–	+/–	+	–	+/–	–
C	+	+	–	–	+/–	–	+/–	+	–	+/–	+/–
D	+	–	+	–	+/–	–	+/–	+	–	–	–
E	+	–	–	+	+/–	–	+/–	+	–	–	–
F	+	–	–	–	+	–	+/–	+	+	–	–
G	+	–	–	–	–	+	+/–	+	–	–	+/–

[a]+, present; –, absent; +/–, present in some isolates, absent in others.

necrotic enteritis pathogenesis. Two of the regions were on large plasmids, and a third was chromosomal (39). A survey of WGSs of *C. perfringens* isolates from necrotic enteritis-diseased turkeys found that only one of the chicken-associated regions was commonly found in the turkey isolates, but a putative collagen adhesin was identified as having potential relevance to pathogenesis (40). It appears from the comparative genomic surveys that necrotic enteritis in chickens and turkeys is likely driven by different genetic factors.

The picture that is emerging from WGSs, whole-genome microarrays and MLST, multilocus variable-number tandem-repeat analysis, and pulsed field gel electrophoresis genomic typing studies is that of a species that can be characterized by a number of evolutionary lineages, each of which has variable carriage of a range of large plasmids (36, 41–43). Plasmid carriage, and hence the toxins encoded, is the most important determinant of disease-causing potential, but at least in the case of necrotic enteritis-causing isolates, genomic analysis and mutation studies have shown that chromosomally encoded genes are also critical determinants of strain virulence (41). Strains isolated from poultry include at least four evolutionarily distinct clades, and all carry a chromosomally encoded alpha-toxin gene. The few toxinotype B-E isolates that have been sequenced group within two of the toxinotype A clades and are only clearly differentiated by the toxin-encoding plasmids that they carry. Isolates can potentially interconvert between the different toxinotypes by acquisition and loss of toxin-encoding plasmids (37). Within toxinotype G isolates the plasmid-encoded genes are more highly conserved than chromosomal genes, indicating that there is either stronger selective pressure on their maintenance or they have a more recent origin (41, 42). The relative levels of sequence conservation between chromosomal and plasmid genes in *C. perfringens* are opposite to that seen in *C. tetani*, where plasmid sequences are less conserved (29).

There is a large pangenome, so each of the toxinotype strains that have been sequenced does carry unique genes, but a much larger collection of diverse *C. perfringens* isolates would need to be sequenced to determine which genes reliably characterize particular lineages and which may influence animal and tissue tropisms. A number of variable genomic regions have putative or known roles in capsular polysaccharide biogenesis, motility, antimicrobial activity, adhesion, and alternative metabolic pathways, and many of these regions are associated with mobile genetic elements such as transposase and recombinase genes and insertion elements (41, 42, 44). A significant proportion of the interisolate differences result from a large and diverse collection of phage insertion events, with some apparently intact prophages and many phage remnants throughout the genomes (41). Some nonpathogenic chicken isolates form a separate clade which is very distinct from the clades which include pathogenic chicken isolates and the toxinotypes isolated from different animals and diseases (36). Interestingly a nonpathogenic isolate from this divergent clade can be transformed into a pathogenic strain by conjugative transfer of the pNetB plasmid (37). It would be interesting to sequence a range of isolates from other animals and the environment to determine if this chicken nonpathogenic clade is an unusual outlier or whether each environment contains specialized isolates that have diverged from the other evolutionary lines.

Clostridioides difficile (*Clostridium difficile*)

C. difficile is the leading cause of antibiotic-associated diarrheal disease, and because of its clinical importance, the comparative genomics of *C. difficile* isolates have been extensively studied, as evidenced by the more than 1,000 genome sequences deposited in GenBank (Table 1) and some individual studies that have sequenced over 1,000 isolates (45). *C. difficile* has a diverse population structure with hundreds of strain types spread across at least six phylogenetic clades (46, 47). The clade structure was first defined by MLST studies (47–49) and was subsequently confirmed by WGS studies (46, 47, 51, 52). Recently, highly divergent isolates from animals and the environment have been assigned to three additional "cryptic" clades (53). Disease-causing isolates are found in a number of distinct lineages, indicating that virulence has evolved independently in the highly epidemic lineages, and it has been concluded that environmental and anthropogenic activities have influenced the rapid emergence and worldwide dissemination of virulent clonal lineages (47, 51). The *C. difficile* genomes range in size from 3.5 to 4.35 Mb. WGS studies have defined a large pangenome of almost 10,000 genes. Estimates of the size of the core genome have varied greatly, depending on the methods that have been used for its definition. Some studies have estimated the core genome to be comprised of approximately 1,000 genes, whereas others have estimated as many as 3,000 genes within the core genome (46, 54, 55). Although a large and diverse pangenome might indicate a specialized ability of isolates to respond to different environments in specific and different ways, a transcriptomics study found that the vast majority of genes that were differentially expressed following two different *in vitro* stress treatments were part of the core genome, indicating that the response to stress is a largely conserved process despite the great sequence plasticity of *C. difficile* (56).

WGS analysis of *C. difficile* isolates has resulted in an increasingly sophisticated understanding of the emergence and evolution of pathogenic strains. The first *C. difficile* comparative genomics study compared three strains: non-epidemic, epidemic, and hyper-virulent strains (57). The epidemic strain was found to have five unique regions, including a novel phage island, a two-component regulatory system, and transcription regulators. It was estimated that some of these genes were acquired over the past 20 years. Broader studies of animal and human isolates, including hyper-virulent isolates, have defined a large pangenome that has been formed by extensive horizontal gene transfer and recombination and has resulted in divergent *C. difficile* genomes in which there has been a high incidence of genome rearrangement and gene acquisition and loss (47, 51). These studies confirmed the important role of both homologous recombination and acquisition of mobile genetic elements in the evolution of the *C. difficile* species. Phylogenetic analysis has been used to estimate that the highly dynamic genome of *C. difficile* evolved within the last 1.1 to 85 million years and continues to rapidly adapt to environmental pressures and anthropogenic activities (51). Interestingly, a WGS study of *C. difficile* isolates from Costa Rica, which investigated microdiversification of isolates, suggested that for NAP1/ST01 strains, typically associated with high morbidity and mortality rates, mutation was an important driver, whereas in NAP_{CR1}/ST54 strains, also isolated from the outbreaks, lateral gene transfer played a more prominent role (58).

Disease-associated isolates have emerged from multiple lineages. Numerous point mutations have been found throughout the genomes of epidemic strains, including mutations in important genes such as that encoding the DNA gyrase, resulting in resistance to fluoroquinolone. WGS analysis of 151 isolates of *C. difficile* RT027, a clinically important ribotype, used 536 SNPs within the core genome to build a phylogenetic tree of strain relatedness (59). It was

found that the accumulation of SNPs was the main driving force in shaping the phylogeny, with no major role for genome rearrangements or gene loss or acquisition, indicating recent emergence of this *C. difficile* population, although acquisition of mobile elements associated with antibiotic resistance was also noted. The analysis indicated that fluoroquinolone resistance had arisen at least twice in North America to establish different epidemic lineages, one of which spread widely to the United Kingdom, Europe, and Australia (59). Other specific mutations have been suggested as driving forces in the emergence of more virulent epidemic strains. For example, Collins et al. showed that low concentrations of trehalose increase the virulence of some RT027 strains, due to mutation of a trehalose repressor gene (60). They noted that the emergence of RT027 and RT078 strains as major epidemic lineages occurred shortly after trehalose was introduced as a food additive, leading them to postulate that this helped to select for the emergence of these hypervirulent epidemic strains.

Isolates of ribotype RT017 have also been subjected to detailed WGS analysis. This ribotype is unusual in that isolates express only toxin A, rather than toxins A and B as commonly found in other lineages. Cairns et al. sequenced 277 RT017 isolates sourced from around the globe and from human and animal sources (61). They identified genetically diverse lineages, SL1 and SL2. RT017 had been thought to have arisen in Asia (62), but this detailed sequence study indicated that it probably had its origins in North America and then spread globally (61).

Mobile genetic elements account for a significant proportion of the genetic diversity of *C. difficile*, with as much as 11% of the genome of some isolates attributed to such elements (63). Mobile elements commonly found in the *C. difficile* genomes include a large and diverse group of intact and fragmented bacteriophage genomes, group I introns, insertion sequences, and transposons, including conjugative and mobilizable transposons, plasmids, clustered regularly interspersed short palindromic repeat (CRISPR)-cas elements, sigK intervening elements, and genomic islands (63–66). All these mobile elements occur in very diverse forms, and isolate surveys have indicated that insertion and rearrangement events have occurred many times in a wide range of lineages. For example, many inducible prophages have been identified, and at least some of them can have significant impacts on host biology. *C. difficile* phages have been shown to repress and induce toxin production, encode elements of quorum sensing systems, and contribute to drug resistance (67, 68). The pathogenicity locus (PaLoc) genomic island, encoding the major virulence factors toxins A and B, is flanked by phage-like integrase genes, and hence it has been suggested that it may have a phage origin (69).

The PaLoc is a key feature of the genomes of toxigenic isolates. In its normal configuration it is a 19.6-kb region that carries the *tcdA* and *tcdB* genes, encoding toxins A and B, as well as four other genes: *tcdR* encodes a sigma factor, *tcdE* encodes a holin-like protein, *tcdC* encodes a negative regulator of toxin expression, and there is a gene encoding a putative amidase (70, 71). The PaLoc is absent in nontoxigenic isolates, and instead there is a 115-bp sequence known as the integration site (72). The PaLoc region of RT017 isolates is unusual in that it contains mutations in the *tcdA* gene, rendering it inactive; therefore, RT017 isolates express only TcdB (61, 73).

Genome studies have also been conducted to investigate the transmission of *C. difficile* in both animal-human contacts and hospital settings. Didelot et al. (74) undertook WGS analysis of 486 *C. difficile* isolates taken from serial samples from patients over a 4-year period to determine the mutation rate and estimated the rate of microevolution to be 1.4 mutations per genome per year. This information was then used to construct time-scaled genealogies that could be used to identify potential transmission of infection between patients in the hospital setting. A similar but larger study of 1,223 sequenced isolates from hospital patients estimated the evolutionary rate to be 0.74 mutations per genome per year (45). WGS analysis of isolates from another hospital was used, in combination with patient movement records, to identify transmission events likely to have occurred within hospital wards and by direct donor-recipient contact, as well as recurrent infections in some patients (75). WGS analysis of isolates from recurrent infections indicated that almost 80% of cases could be attributed to the original infecting isolate (18). This tracking of very closely related strains is not possible with the other, previously used, typing methods (e.g., ribotyping, MLST, pulsed field gel electrophoresis, etc.); it is only with the use of WGS that such high-resolution identification of differences between isolates is possible. The epidemiological story emerging from these studies is that there appears to be a lower rate of direct transmission (35%) of *C. difficile* in the hospital environment than previously thought, and many infections (45%) involve genetically distinct isolates that are not obviously sourced from the immediate hospital environment. WGSs of isolates from asymptomatic carriers of *C. difficile* have also been investigated to estimate transmission rates. It was concluded that although transmission from asymptomatic carriers is probably rare, it is still probably of significant clinical importance because there are so many carriers within the population and many of the isolates found were toxigenic (76). A WGS comparison of *C. difficile* isolates from humans and animals on Dutch pig farms (77) estimated a mutation rate (1.1 mutations per genome per year) similar to that estimated in the human hospital isolates mentioned above. In some instances the human and pig isolates from the same farms were identical or nearly so, supporting the view that *C. difficile* infection is zoonotic, although not identifying the direction of transmission. An Australian WGS study has also found that some human isolates showed a clonal relationship with pig isolates, further supporting the transfer of isolates between humans and animals (78).

Groß et al. sequenced the genomes of three *C. difficile* strains isolated from a single patient with a mixed infection (79). They found that there was evidence of horizontal gene transfer, including transfer of virulence-related genes and antibiotic resistance genes between the strains within the mixed infection. The analysis of *C. difficile* isolates from a patient with recurrent infections over 13 months identified 10 *C. difficile* isolates of 6 ribotypes. Relapses and reinfections were indicated, and at one sampling point three isolates were recovered, indicating a mixed infection, but with little indication of any microevolution within the patient (80). Another interesting use of WGSs of isolates obtained from repeat investigation of particular patients was directed toward understanding the comparative value of two antibiotic treatments in preventing reinfection and relapse. Investigation of paired isolates from 93 patients before and after antibiotic treatment demonstrated that fidaxomicin was more effective than vancomycin in reducing both relapse and reinfection (81).

Paeniclostridium sordellii (*C. sordellii*)

P. sordellii infections can result in toxic shock-like presentation. The genome size ranges from 3.4 to 3.7 Mb, and

phylogenetic analysis of WGSs has placed isolates into four clades, with a number of isolates that do not belong to any of the clades (82). Toxigenic isolates of *P. sordellii* produce two large clostridial cytotoxins (LCCs); the lethal toxin is encoded by *tcsL*, and a hemorrhagic toxin is encoded by *tcsH*. In addition to the LCCs, *P. sordellii* also encodes a number of other probable exotoxins and virulence factors, including a collagenase, a neuraminidase, a phospholipase C, a cholesterol-dependent cytolysin, a secreted metalloprotease, and an antimicrobial peptide, LL-37. *P. sordellii* is unusual among the clostridia in that most isolates carry a urease operon, although to date, it has not been shown to be a virulence factor such as is the case in *Helicobacter pylori* (82, 83).

Although *P. sordellii* is phylogenetically related to *C. difficile*, a comparison of the coding potential of the genomes of the two species, supported by phenotypic analysis, revealed differences in the metabolism of the related species, with *C. difficile* being more capable of using carbohydrates as carbon sources and *P. sordellii* able to utilize amino acids and fatty acids as carbon sources (83). Genomic differences in pathogenicity loci, substrate utilization genes, and pH tolerance mechanisms indicate the genetic basis of the broader tissue tropisms of *P. sordellii* compared to *C. difficile*. WGS analysis of 44 animal- and human-derived isolates from 3 continents classified the *P. sordellii* isolates into 4 clades, with some outlier isolates that could not be assigned to a clade (82). There were no apparent correlations between clade and geographic or animal origin.

The major virulence factors, the LCCs (lethal toxin and hemorrhagic toxin), are localized on plasmids, in contrast to the chromosomal location found in *P. difficile* for the two LCCs which it produces (toxins A and B) (84). However, the LCC genes were only found to be present in a few *P. sordellii* isolates, and it was speculated that the large plasmids that carry the LCCs may be susceptible to loss during primary isolation or continued culturing in the laboratory (82). Such loss of toxin plasmids during primary isolation of bacteria has also been speculated to occur in chicken necrotic enteritis-causing strains of *C. perfringens* (85). The LLC-carrying plasmids of *P. sordellii* were recently shown to be conjugative (86).

GENOMICS OF OTHER PATHOGENIC CLOSTRIDIA

A number of other pathogenic clostridia are either well controlled by vaccination or are only occasionally isolated from clinical cases. Hence, there is a less pressing need to understand the virulence and survival mechanisms of the organisms, and therefore there are generally few genomic studies available beyond the sequencing of type strains and small isolate collections.

Clostridium bolteae and *Clostridium clostridioforme*

These two species can be found in the normal intestinal microbiota of humans and can cause disease when normal gut barrier function is compromised and they escape the confines of the gut to cause intra-abdominal infections. A comparative genomic analysis of *C. bolteae* and *C. clostridioforme* isolates was recently reported (87). Six isolates of each species were sequenced; the genomes were approximately 6 Mb, with the *C. bolteaea* genomes being larger than the *C. clostridioforme* genomes (Table 1). The two species are similar as determined by normal phenotypic methods used to classify bacteria but differ in their ability to ferment lactose. This difference in metabolic potential is evident in the coding potential of the genomes, with *C. bolteae* containing a lactose utilization operon which is absent from the *C. clostridioforme* genome. *C. clostridioforme* encodes an acetyl-CoA pathway and hence is likely to produce more butyrate than *C. bolteae*. Both species are notable for carrying antimicrobial resistance genes to a broad array of antibiotics (87).

Clostridium chauvoei

C. chauvoei can be highly pathogenic and causes blackleg, a histotoxic myonecrotic disease of ruminants (88). Simple bacterin or toxoid vaccines have been used to try to control the disease, but their effectiveness has been questioned (89). Human infections have been reported but are rare (90). The first complete genome sequence of *C. chauvoei* identified an absence of genes for the citric acid cycle and some amino acid metabolism paths, but there was a rich collection of genes for tissue-damaging toxins and pathways involved in protein degradation and amino acid assimilation (91). Presumably, some amino acid metabolism pathways have been lost because the bacteria can scavenge the necessary amino acids from damaged host tissue. The *C. chauvoei* genome is most closely related to the *C. septicum* genome, with gene homologies for key sporulation genes ranging between 77 and 99% (92). A recent analysis of the genomes of a geographically and temporally diverse collection of 20 *C. chauvoei* isolates showed that the genomes are highly homologous (93). The isolate collection spanned 64 years and 5 continents, yet there were very few differences in the genomes, with most genes encoding metabolic pathways and major virulence factors highly conserved. There was an array of single nucleotide variants throughout the genomes, but mainly located in intergenic areas, a few allelic variants in genes encoding flagellin proteins, and differences in some CRISPR sequences, with a large number of variable spacer elements. The WGS analysis indicated that *C. chauvoei* is highly specialized for survival in its host animals (sheep and cattle) and in the pastures in which they graze. The *C. chauvoei* genome (~2.9 Mb) is one of the smaller clostridial genomes. Smaller genomes are typical of bacteria that have evolved to occupy specific narrow environmental niches (5). Interestingly, from WGS analysis Rychener et al. (93) concluded that "*C. chauvoei* is a successful pathogen whose evolution has reached a dead end."

C. septicum

C. septicum is a rarer cause of gas gangrene (myonecrosis) following traumatic injury than *C. perfringens* but is becoming the leading cause of spontaneous gas gangrene in humans (94). *C. septicum* can also cause blackleg in animals. It produces four main toxins: a hemolytic alpha-toxin, a DNase beta-toxin, a hyaluronidase gamma-toxin, and the septicolysin delta-toxin (95). The single WGS that has been published reported a genome size of 3.27 Mb with a G+C content of 27.5% (96). Notably, the genome was found to have 22 rRNA operons, making it the clostridial genome with the highest rRNA operon count. The pathogenic clostridial species generally have approximately 10 rRNA operons, and there are no other bacterial species reported with such a high number of rRNA operons (97).

Other Clostridial Species

Some clostridial species that are only occasionally seen as pathogens in humans or animals, such as *C. carnis*, *C. colinum*, and *C. limosum* (*Hathewaya limosum*), have still not had any whole genomes sequenced. There are a number of other clostridial species that may be pathogenic in humans

and/or animals but have been only rarely documented or are generally regarded as nonpathogenic species but have, rarely, caused human infections. These bacteria have attracted little research, but WGSs are available for some of them, some published and some just as GenBank entries. These include *C. ramosum* (*Erysipelatoclostridium ramosum*), *C. bifermentans* (*Paraclostridium bifermentans*), *C. cadaveris*, *C. celerecrescens*, *C. fallax*, *C. innocuum*, *C. paraputrificum*, *C. sphenoides*, *C. spiroforme*, *C. taeniosporum*, and *C. tertium* (97–99). These genomes are useful resources for the comparative analysis of clostridial gene content and for investigating features such as genes encoding antibiotic resistance, toxins, degradative enzymes, sporulation, and phages. A comparison of 40 clostridial genomes, including both pathogenic and nonpathogenic species, concluded that there were only 169 orthologous gene groups shared by all strains, and a pangenome of 22,668 genes (100). It was concluded that this was consistent with a group of bacteria that live in very diverse environments and hence have evolved a large and diverse gene pool allowing specific adaptation to different environments.

CONCLUSIONS

Advances in DNA sequencing technology have precipitated an avalanche of bacterial WGS information. The study of pathogenic clostridia has benefited enormously from this information explosion. Thousands of clostridial genomes have been sequenced, with most of the effort directed toward those species that present the most serious or intractable clinical challenges. Comparative genomic analysis within species has informed our understanding of strain evolution. By estimating mutation rates and then applying this to the level of variation found within species, evolutionary timelines can be hypothesized, and the likely origins of clades can be deduced. A particularly valuable application of within-species comparative genomics has been its use in epidemiological studies to trace the movement of isolates within the hospital environment, between patients, and in zoonotic transfers between humans and animals. With *C. difficile*, this has provided valuable information about bacterial spread and evolution in the clinical environment and the relative importance of new infections and recurrence of existing infections in recurrently infected patients.

Genomic analysis clearly shows the polyphyletic nature of the pathogenic clostridia. This issue is being addressed by renaming many of the clostridia, but there is still some way to go with this rationalization. Different levels of genomic plasticity have been noted across the phylogenetic lineages, with the cluster XI species, most notably *C. difficile*, showing high levels of sequence variation driven by mobile elements, chromosomal rearrangement, deletions and insertions, and SNPs. The genomes of true clostridia (cluster I) appear to be more stable, with most variation driven by mobile elements and the accumulation of SNPs. Plasmids play a major role in strain variation, with the large conjugative toxin gene-carrying plasmids often determining the virulence of isolates.

The gene contents of isolates provide clues to the survival strategies that are used. For example, species adapted to survival in the gastrointestinal tract differ from those that have a more saprophytic lifestyle in the carriage of adhesion factors, extracellular degradative enzymes (e.g., proteases, chitinases), sporulation and germination pathways, and toxins involved in rapid killing (e.g., neurotoxins), and this determines the biotic and abiotic environments in which they can survive and proliferate. The WGSs of clostridia provide a wealth of information about the metabolic capabilities and the regulatory pathways (e.g., alternative sigma factors, two-component regulators) that these organisms utilize to adapt to varying environments and growth substrates. The genomes can be bioinformatically mined to identify the secretome, surface-exposed proteins, and other gene products involved in pathogenesis. These represent targets that may be used for therapeutic and vaccine development. There is a wealth of information within the already available genomes that is yet to be fully exploited, and of course, the volume of new WGSs available for analysis and exploitation will greatly expand in the coming years. This, complemented with laboratory studies, will drive our knowledge of the pathogenesis of the clostridia and help in the control and treatment of infections.

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Virulence Plasmids of the Pathogenic Clostridia

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59

Many clostridial species are ubiquitous in the environment and in the intestinal tracts of birds, fish, and mammals. Commensal species are often carried asymptomatically within a host. However, if the immune status of the host is compromised, due to either age, illness or a change in diet, disease can result from toxigenic strains. Alternatively, some clostridial species or strains don't require predisposing factors. They can cause disease simply if they gain entry into the host either through damage to the skin or through the gastrointestinal tract, often *via* poorly prepared or incorrectly stored food. These clostridia then overgrow and cause cell and tissue damage. Diseases mediated by the clostridial species discussed in this review are predominantly mediated by potent protein toxins, many of which are located extrachromosomally. These toxins have diverse mechanisms of action and include pore-forming cytotoxins, phospholipases, metalloproteases, ADP-ribosyltransferases and large glycosyltransferases. This review focuses on these toxins and the elements that carry the toxin structural genes. For ease of discussion it has been structured on a bacterial species-specific basis.

PAENICLOSTRIDIUM (CLOSTRIDIUM) SORDELLII

Virulence Properties of *P. sordellii*

Paeniclostridium (formerly *Clostridium*) *sordellii* causes several severe diseases in both humans and animals. Most documented animal infections caused by *P. sordellii* are edemic or enterotoxic diseases of livestock and poultry, but they also include rare myonecrotic infections of other animals (1). Human infections are infrequent, but severe in nature, with mortality rates approaching 70% overall (2). The majority of *P. sordellii* human infections are soft tissue infections that occur after trauma or surgery (2). Sporadic, yet severe cases of post-partum and post-medical abortion uterine *P. sordellii* infections have occurred over the last 20 years, with almost every case resulting in the death of the patient (1, 2).

Due to the rare nature of *P. sordellii* infections, the pathogenesis of disease is still poorly understood. The major virulence factors involved in *P. sordellii* infection, however, are the production of two large clostridial toxins (LCTs): TcsH (hemorrhagic toxin) and TcsL (lethal toxin) (1). The LCTs enter mammalian host cells and glycosylate small GTPases such as Ras, leading to an altered cytoskeletal arrangement, cellular rounding and eventual death of the cell (3). Interestingly, while being highly potent, both *P. sordellii* LCTs are produced by only ~5- to 13% of isolates tested (4–6), with the majority of toxigenic isolates producing only TcsL and containing a truncated *tcsH* gene (6). Despite these findings, TcsL has been shown to be the major virulence factor responsible for the production of uterine-infection related toxic shock using an animal model of infection (7). In *P. sordellii*, the LCTs are encoded within a region called the pathogenicity locus (PaLoc), alongside genes that are likely to be required for their regulation and release (8). A recent phylogenetic analysis found that in all toxigenic isolates the PaLoc is carried on a group of related plasmids called the pCS1 family (6), which is the major focus of this section. Other virulence factors of *P. sordellii* include a pore-forming phospholipase SDL, the sialidase NanS, and the production of unique endospores (1, 9–11).

Bioinformatic Characterization of Plasmids in *P. sordellii*

The pCS1 family is a group of large plasmids carried by both toxigenic and non-toxigenic *P. sordellii* isolates. Seven such plasmids have been identified to date (12). The pCS1 plasmids encode a putative replication initiation (Rep) protein (Fig. 1), which, based on conserved amino acid motifs, falls within the RepA family that was first identified from the theta-replicating *Escherichia coli* plasmid P1 (6, 13). However, neither the function of this protein nor the location of an origin of replication (*ori*V) region has been experimentally determined. All members of the pCS1 family also encode a number of predicted surface exposed proteins that may behave as adhesion molecules (6). A number of these predicted surface proteins contain an LPxTG-like sortase motif (6, 13). Sortases mediate the attachment of specific proteins via the LPxTG motif to the peptidoglycan of the cell wall (14). A type B sortase enzyme is encoded (*srtB*, Fig. 1) in the vicinity of the majority of these surface protein genes and may therefore facilitate their attachment to the *P. sordellii* cell wall (6, 12), however, the role of these surface proteins is yet to be explored.

While there is a large degree of nucleotide sequence identity between the characterized plasmids (Fig. 1), significant differences also have been observed, both in plasmid size and in the presence of unique open reading frames (ORFs) (6, 12). The paradigm element is pCS1-1, which is encoded by the *P. sordellii* type strain ATCC 9714. This plasmid is 103 kb

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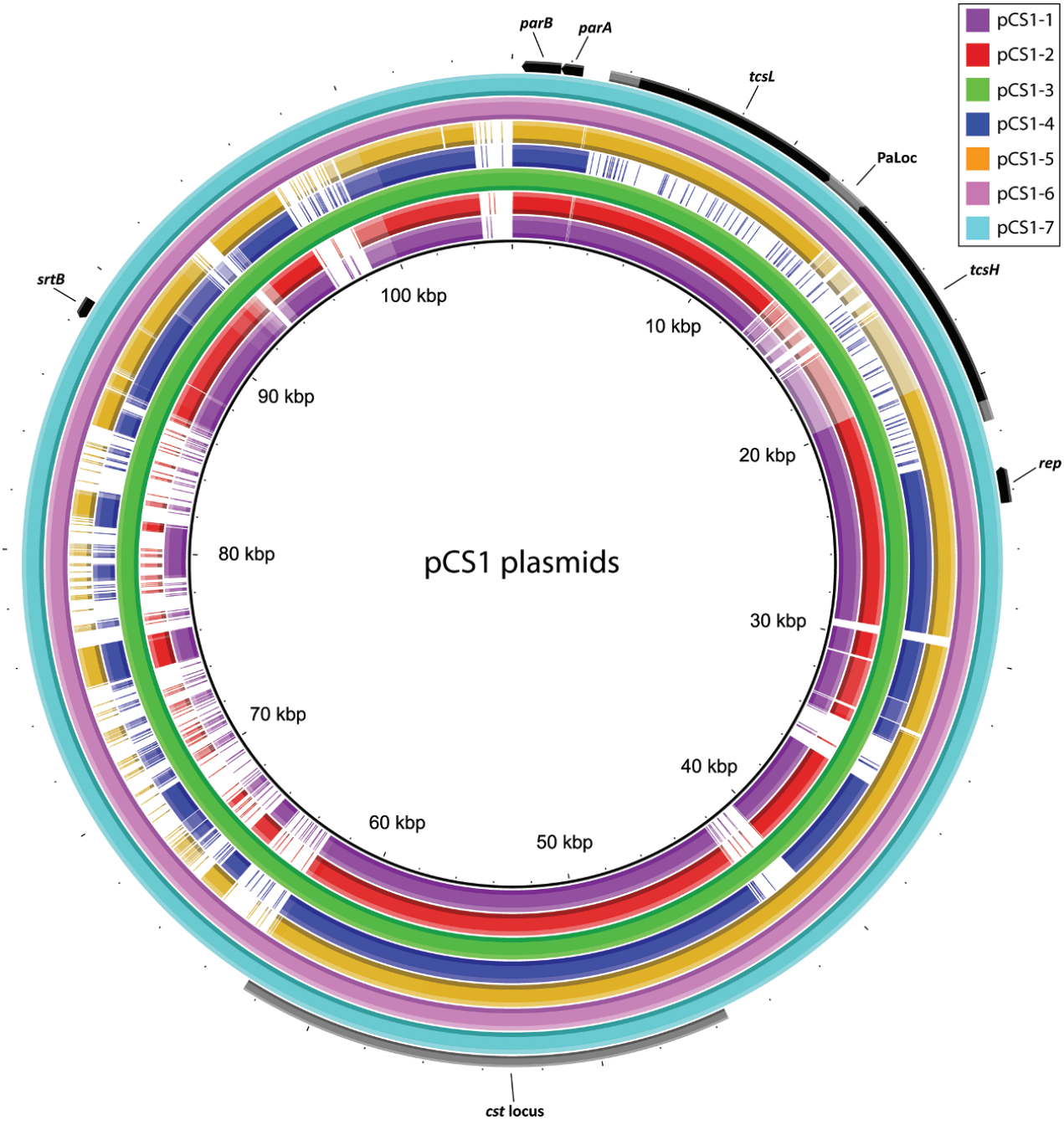


FIGURE 1 The pCS1 family plasmids of *P. sordellii*. Shown is a visual representation of a blastn analysis comparing each of the seven sequenced pCS1 plasmids to the reference sequence of pCS1-3 from strain JGS6382. The third ring from the center (green) and the coordinates (in kb) correspond to pCS1-3. Plasmids displaying 70 to 100% identity to pCS1-3 at a particular locus are shown with a solid block of colour on their respective ring. Identity to pCS1-3 between 50 and 70% is represented as a pale block of colour and if the identity is lower than 50% it is represented as a gap in the corresponding ring. Conserved loci on pCS1-3 are indicated as a gray arc on the outermost ring and labeled. Genes of interest are annotated as black arrows on the outermost ring and also labeled. Sequences analyzed and accession numbers: pCS1-1 (LN679999), pCS1-2 (LN681232), pCS1-3 (LN681235), pCS1-4 (LN681233), pCS1-5 (MG205643), pCS1-6 (MG205642), pCS1-7 (MG205641). Produced using BRIG (224).

in size and carries the PaLoc with a truncated version of the *tcsH* gene, just upstream of the hypothetical *rep* gene (6). The pCS1-2 and pCS1-5 plasmids carry a PaLoc almost identical to that of pCS1-1 (6, 12). pCS1-2 is the largest member of the family at 112 kb and while their PaLocs are identical, pCS1-2 is missing a number of genes, including genes encoding a transcriptional regulator and a cold shock protein (6). A large insertion that carries a putative lantibiotic synthesis cluster is present on pCS1-2 (6). On the other end of the spectrum is the smallest plasmid member, pCS1-5, at 92 kb (12). pCS1-5 displays regions of high identity to the other pCS1 members, but also contains two small unique

regions (12). The plasmids that carry a full-length *tcsH* gene, pCS1-3, -6 and -7, are very closely related (Fig. 1), displaying 99% identity across their ~106-kb sequences (12). Major differences between these plasmids and the other pCS1 members include an absence of the genes encoding subunits of an anaerobic sulfite reductase and the presence of a number of putative transposases (6). Non-toxigenic isolates of *P. sordellii* also carry pCS1 family plasmids, such as pCS1-4 from strain UMC2 (6). This plasmid is 100 kb in size and in place of the PaLoc is a string of ORFS with unknown functions, with the exception of a single putative transposase-encoding gene (6).

A second type of extrachromosomal element, named pCS2, has been identified in *P. sordellii* (6). This ~37-kb circular element has not been as thoroughly characterized as the pCS1 plasmids and appears to encode a number of bacteriophage-related genes (6). Putative plasmid-related genes are present, however, in the form of partitioning and replication initiation genes (6). Related elements appear to be present in another four strains of *P. sordellii*, two of which also carry a pCS1-like plasmid (6). Whether pCS2-like elements represent plasmids, cryptic plasmids, or phage is currently unclear, but no virulence genes have been identified within this plasmid family to date.

Stability and Conjugative Transfer of the pCS1 Family of Plasmids

A recent study has functionally characterized conserved genes of the pCS1 family plasmids, using pCS1-1 as a model (12). All pCS1 plasmids carry *parA* and *parB* genes, which are putative members of a ParABS plasmid partitioning system (6). Such systems are utilized by low-copy-number plasmids to ensure stable inheritance of the plasmid in each daughter cell upon cellular division (15). Stability assays conducted on a marked version of pCS1-1 in different strain backgrounds showed that the plasmid is stable across ~120 generations (12). Independent insertional inactivation mutants of the pCS1-1 *parB* gene displayed gradual loss of the plasmid during continual subculture, providing evidence that ParA and ParB represent a true plasmid partitioning system (12).

Bioinformatic analysis of pCS1-1 also leads to the identification of an ~17.3-kb region, subsequently named the *cst* (for *C. sordellii* transfer) locus (Fig. 1), that encoded proteins with similarity to components of a plasmid conjugation system (12). The conjugative transfer of a marked derivative of pCS1-1, from type strain ATCC 9714 to a *P. sordellii* isolate from a distant clade, was subsequently demonstrated (12). The *cst* locus is conserved between all members of the pCS1 family so far characterized (Fig. 1) (12). Encoded within the *cst* region is a putative relaxase protein belonging to the MobMG family of relaxases, along with a cognate origin of transfer (*oriT*) located upstream of the relaxase gene (12). A number of genes encoding components of a type IV secretion system are also located within the *cst* locus. Two of these, the putative coupling protein, CstD4, and the putative ATPase, CstB4, were demonstrated experimentally to be required for conjugative transfer, confirming the *cst* region to be a true conjugation locus (12). Regions with significant similarity to the *cst* locus also have been identified in plasmids of *Clostridium perfringens* and *Clostridium botulinum*, and within plasmid or conjugative transposon-like regions of the genomes of *Clostridioides* (previously *Clostridium*) *difficile* isolates, indicating that *cst* represents a variant of a common clostridial conjugation locus (12).

CLOSTRIDIUM PERFRINGENS

C. perfringens is ubiquitously distributed among animals, humans and the environment (16, 17), but is also the causative agent of numerous opportunistic infections (16). The diseases caused by *C. perfringens* range from typically mild, self-limiting human food poisoning, to the more severe pathologies of human gas gangrene, and enterotoxemia and necrotic enteritis in domestic livestock (18). It is this broad spectrum of disease that makes this bacterium of particular interest to both veterinary and human medicine. Enteric disease caused by *C. perfringens* is estimated to cost the poultry industry in excess of $6 billion globally as well as $466 million in human health service costs in the United States alone (19, 20). What makes *C. perfringens* such a successful pathogen is its ability to encode and produce up to 20 toxins, many of which are associated with conjugative plasmids (21, 22).

The *C. perfringens* Typing Toxins

Strains differ in their carriage and expression of toxins, and this forms the basis of the *C. perfringens* toxinotype classification scheme (23). This scheme previously categorized *C. perfringens* strains into one of five toxinotypes based on the expression of four lethal typing toxins: alpha, beta, epsilon and iota (23). However, this scheme recently was expanded to include two new typing toxins, *C. perfringens* enterotoxin (CPE) and NetB toxin (Table 1) (24). This toxinotype classification is unique because particular "types" are often associated with specific diseases in humans and animals, for

TABLE 1 The *C. perfringens* toxinotype classification scheme

type	Toxins Produced[a]						Associated Diseases
	Alpha	Beta	Epsilon	Iota	CPE	NetB	
A	+	–	–	–	–	–	Gas gangrene in humans and animals Canine and equine enteric disease
B	+	+	+	–	–	–	Lamb dysentery
C	+	+	–	–	+/–	–	Necrotic enteritis in humans, sheep, horse, cattle and pigs
D	+	–	+	–	+/–	–	Enterotoxemia of sheep and goats
E	+	–	–	+	+/–	–	Enterotoxemias of rabbits, canines and cattle
F	+	–	–	–	+	–	Food poisoning and nonfoodborne gastrointestinal disease in humans
G	+	–	–	–	–	+	Chicken necrotic enteritis

[a]+, Produced; –, not produced; +/–, as produced by some strains.

example; type A strains typically cause gas-gangrene, whereas type D strains are typically associated with enterotoxemia in sheep and goats (25).

Alpha toxin is encoded by all strains of C. *perfringens* and is the major toxin involved in the pathogenesis of gas gangrene infections (26). In all C. *perfringens* strains, the alpha toxin structural gene, *plc*, is chromosomally encoded with basal levels of *plc* mRNA always transcribed (27). The relative amount of toxin produced varies based on the strain background, with type A strains producing the highest levels of alpha toxin *in vitro* (28, 29). This toxinotype-specific control of toxin expression is said to be dependent on strain-specific global control elements, such as the two-component regulatory system VirR/VirS, as well as the structure of the promoter sequences preceding the *plc* gene (27, 28, 30, 31). Alpha toxin is a zinc-metalloenzyme that possesses both phospholipase C and sphingomyelinase activity, and functions by cleaving the phosphatidylcholine and ceramide head groups from the components of membrane phospholipid bilayers (32, 33). It is this targeted membrane disruption that leads to the severe disease often seen in gas gangrene infections (32, 34, 35).

Beta toxin, like many of the C. *perfringens* toxins, is a pore-forming toxin and is produced by both type B and C strains of C. *perfringens* (36). Beta toxin is responsible for disease development in C. *perfringens* type C infections; with beta toxin null mutant strains displaying avirulent phenotypes in animal models of enterotoxemia and necrotizing enteritis (37–39). The precise mechanism of action of beta toxin has not been fully elucidated, but, the hypothesis is that the toxin forms pores in the membranes of susceptible cells leading to cell lysis (40–42).

Beta toxin is encoded by the *cpb* gene, which is located on large conjugative plasmids (43–45). In type B stains, *cpb* is encoded on one of two plasmids: either ~65 kb or ~90 kb in size (44). More commonly the *cpb* gene is found on the larger ~90-kb plasmid (44). Preceding the *cpb* gene is the insertion sequence (IS) element, IS*1151* (44, 45). The presence of IS elements co-located in the toxin gene region may facilitate mobilization of these gene regions and may explain the presence of this toxin gene on multiple different plasmids (44, 45). The potential for mobilization is further supported by the detection of toxin-encoding circular intermediates in these strains (44, 45). In type B strains of C. *perfringens* there appears to be a strong association between carriage of *cpb* and the gene encoding the LCT, TpeL, which has no demonstrated role in disease (44). This study found that in all type B strains examined, the *tpeL* gene was always encoded ~3 kb after the *cpb* gene. The *cpb* plasmids of type B strains co-exist with at least one other virulence plasmid, an epsilon toxin (*etx*)-encoding plasmid, and sometimes another accessory toxin plasmid, encoding lambda toxin (*lam*) and a putative urease operon (44). In these strains the size of the *etx*-encoding plasmid and other accessory plasmids is consistent, with the only variable plasmid being the *cpb*-encoding plasmid (44). The limited variation of these plasmid combinations may be due to incompatibility issues with resident plasmids present in type B isolates. No type B plasmids have been identified that encode both the *cpb* and *etx* genes (44).

In contrast, the *cpb*-encoding plasmids of type C strains demonstrate much greater size variation (45). In these strains, the beta toxin gene is encoded on plasmids ranging in size from 65 kb to 110 kb (45). Restriction analysis suggests that some type C isolates may carry the same *cpb* plasmids found in type B strains (90- and 65-kb plasmids) (45). However, in addition to size differences, the *cpb*-encoding plasmids of type C strains also differ in their carriage of accessory toxin genes (45). As in type B strains, *cpb* is often associated with *tpeL* (45), although *tpeL* and *cpb* are not always encoded on the same plasmid (45). This result suggests potential movement of either of these IS-associated toxin genes (45). In addition to *tpeL*, *cpb*-plasmids can also encode other toxin genes, such as the CPE structural gene, *cpe* (45).

Epsilon toxin is the most potent of all toxins produced by C. *perfringens* (40, 46). It is the third most potent bacterial toxin, after tetanus and botulinum toxins (47). During infection, epsilon toxin is secreted as a pro-toxin from type B and D C. *perfringens* strains and is proteolytically cleaved to produce the active toxin (48, 49). Activation of epsilon toxin can be facilitated by proteolytic cleavage catalyzed by the C. *perfringens* protease lambda toxin (*lam*), but has been shown to be more commonly activated by chymotrypsin, trypsin or other carboxypeptidases encountered within the gut of the host organism (49–52). Once active, epsilon toxin primarily targets endothelial cells near the intestinal border, forming a prepore complex before inserting itself into cell membrane regions following binding to specific cellular receptors (53, 54). The toxin oligomerizes, forming a pore in the cell wall and inducing cellular damage (55). These damaged endothelial cells then allow epsilon toxin to be absorbed, where it systemically targets organs such as the lungs, and kidneys and in the brain, where it induces the release of the excitatory neurotransmitter, glutamate (47, 56, 57). Excessive glutamate release and subsequent overstimulation of neurons results in altered neurological capabilities, which are commonly observed in animals suffering from epsilon toxin-induced enterotoxemia (47, 57, 58).

Epsilon toxin is encoded by the *etx* gene (57). The *etx* gene is harbored on conjugative plasmids found in both type B and D strains of C. *perfringens* (44, 59, 60). The *etx* plasmids of type B strains again appear to have a limited degree of plasmid diversity, with the *etx* gene only encoded on an ~65-kb plasmid (44). In contrast, the *etx* plasmids of type D strains are more variable in size, ranging from ~45 to ~110 kb (60). In addition to the size variability observed for type D *etx* plasmids, they are also more diverse in their toxin gene carriage (60). Some of the larger *etx* plasmids have been found to encode numerous other toxin genes such as *cpe*, *lam*, and *cpb2*, which encodes beta2 toxin (60). Whether the toxin genotype of a type D strain is simple (carrying only *plc* and *etx*) or more complex (possessing other toxin genes such as *cpe* or *cpb2*) appears to delineate the size of the plasmid on which the *etx* gene is located (44). 'Simple' strains tend to encode *etx* on plasmids ranging in size from ~45 to ~75 kb. Conversely, more 'complex' strains tend to encode *etx* on larger plasmids of roughly 75 to 110kb (44).

Iota toxin is a binary toxin encoded by type E strains of C. *perfringens*, which infrequently cause enterotoxemia in rabbits, lambs and calves (61). It comprises two protein components: Ia -(the enzymatic component) and Ib -(the binding component) (62, 63). Each component alone is non-toxic, but when combined the intact toxin produces rapid cytotoxic effects that often result in death (64, 65).

Iota toxin is encoded by two genes, *iap* and *ibp*, which form an operon (62). These genes are located on large virulence plasmid that range in size from 65 kb to ~135 kb (66, 67). Like many other C. *perfringens* toxins, the genes encoding iota toxin can be associated with the insertion sequence element, IS*1151* (66), with a complete element upstream of *iap* and *ibp* and a partial element downstream (66). The association of the toxin genes with IS*1151* may explain the existence of a highly conserved silent *cpe* gene upstream of the iota toxin operon in some strains (68). Current theories suggest that an insertion event on a *cpe*-encoding plasmid

with the IS*1151*-associated iota toxin operon resulted in the formation of the type E iota toxin plasmids (66, 68). However, surveys of environmental strains and isolates from healthy humans has indicated that some type E strains derived from these sources encode an intact, but variant *cpe* gene that is not associated with IS elements (67).

Many type E strains carry additional genes, such as those encoding for urease and lambda toxin, on their iota plasmid (66). The components of iota toxin, like epsilon toxin, are activated by proteolytic processing (64), and the lambda toxin protease is known to process iota toxin into an active form (64, 69). The carriage of both toxin genes on a single plasmid would be considered advantageous because it would be beneficial for cells expressing iota toxin to also encode its activator, lambda toxin (66). In all strains characterized both *lam* and *iap/ibp* were encoded on the same large conjugative plasmid (66).

CPE is responsible for C. *perfringens*-mediated food poisoning in humans (70, 71) and is produced by the newly designated C. *perfringens* type F strains during sporulation in the small intestine, following the ingestion of a large number of C. *perfringens* cells from contaminated food (24, 72). Once released, CPE binds to enterocytes and forms a range of complexes on the cell surface (70). These complexes then form pores in the cell membrane, activating cell death pathways that result in cellular destruction (71). Like epsilon and iota toxins, CPE requires proteolytic cleavage for activation (73). It is encoded by the *cpe* gene, which can be located both chromosomally and on conjugative plasmids (74, 75), which appears to play a role in the epidemiology of disease (75, 76). C. *perfringens* type F food poisoning isolates generally possess chromosomally-encoded *cpe* genes, whereas isolates from nonfood borne gastrointestinal infections, such as antibiotic-associated diarrhea, have plasmid-encoded *cpe* genes (75). In fact, the *cpe* encoding plasmid from strain CPF4969, pCPF4969, was the first toxin plasmid of C. *perfringens* demonstrated to be conjugative (74). It is thought that the carriage of *cpe* on conjugative plasmids is a key factor in the disease phenotype observed during nonfood born gastrointestinal infections (40). In this syndrome, unlike C. *perfringens*-food poisoning, only a small number of *cpe*-positive C. *perfringens* cells are required to be ingested for disease establishment, which is postulated to be facilitated by the conjugative transfer of the plasmid to commensal C. *perfringens* strains in the gastrointestinal tract (40, 74).

The *cpe* gene is encoded on numerous plasmids that range in size from ~70 kb up to ~110 kb (45, 60, 66, 77). Plasmids encoding *cpe* have been found in type C, D, E and F strains, but not in any type B isolates (45, 60, 66, 77). In the newly designated type F strains (24), whether chromosomal or plasmid-borne, there is an IS*1469* element directly upstream of the *cpe* gene (78). However, the sequence downstream of *cpe* differs greatly, depending on the different genetic locations (78). Chromosomal *cpe* loci possess flanking IS*1470* sequences, which are proposed to comprise a larger mobile element (74, 77, 78). Plasmid-encoded *cpe* genes can have either a downstream IS*1151* or IS*1470*-like element, which forms the basis of the two *cpe* plasmid families: pCPF4969-like (IS*1470*-like) and pCPF5603-like (IS*1151* and *cpb2*) (77). The *cpe* plasmids show significant diversity and can differ in their carriage of other toxin genes (45, 60). In some type C strains *cpe* can either be carried on the same plasmid as *cpb* or on a separate plasmid (45). The larger *etx*-encoding plasmids characterized in type D strains tend also to carry *cpe* genes (60).

NetB is a β-pore forming toxin which is the causative agent of necrotic enteritis in chickens and forms the basis for the new toxinotype G. NetB was discovered after C. *perfringens* strains isolated from necrotic lesions were found to still induce disease after inactivation of the *plc* gene, which previously was thought to be the primary toxin involved in this syndrome (79, 80). Sequencing of these strains identified a putative toxin which was denoted NetB, Necrotic Enteritis Toxin B-like (80). Deletion of *netB* resulted in the formation of a strain that was unable to produce necrotic lesions in chickens (80). Complementation with the *netB* gene *in trans* restored the strain to wild-type virulence (80). NetB is produced by type G strains of C. *perfringens* and is encoded on large plasmids ranging in size from 82 to 95 kb (81), which like pCPF4969, have been shown to be conjugative (81, 82). Strains harboring *netB* plasmids can also contain two other closely related conjugative plasmids, encoding *cpb2* and tetracycline resistance (80–82).

Other Plasmid-Encoded *C. perfringens* Toxins

C. *perfringens* strains can harbor and express numerous other toxins and extracellular enzymes. The toxins of C. *perfringens* are functionally classified into four broad categories: pore-forming toxins, intracellular toxins, membrane damaging enzymes and hydrolytic enzymes (36). With some exceptions, most of the toxins and hydrolytic enzymes produced by C. *perfringens* are encoded on large conjugative plasmids (36, 83).

The beta2 toxin gene, *cpb2*, is possibly the most promiscuous of all toxin genes, because it can be found on plasmids from all C. *perfringens* strain types (44, 45, 60, 66). Despite its name, beta2 toxin has little amino acid sequence identity (less than 15%) to beta toxin (84). The involvement of beta2 toxin in disease is often debated because there is no direct evidence of beta2 toxin-induced virulence (85). However, there is a correlation between the prevalence of C. *perfringens* strains expressing beta2 toxin among animals with enteritis, particularly piglets, suggesting that it may play a role in the pathogenesis of this disease, but no genetic studies have been carried out (85).

The *cpb2* gene is carried by numerous plasmids ranging in size from 45 to 90 kb (44, 45, 60, 81, 82). These plasmids can carry numerous other toxin genes such as *cpe* and *etx*, with one plasmid characterized in a type D strain carrying all three toxin genes (60). In many strains *cpb2* is carried on a conjugative plasmid distinct from other toxin plasmids, as is seen in many type B to G strains (44, 45, 66, 81, 82).

Delta toxin is another β-pore forming toxin produced by C. *perfringens* (86, 87). Although it has not been shown to be involved in disease, it is thought that there may be a synergistic effect between delta and beta toxins, because they are often produced by the same strains (88, 89). Delta toxin is produced primarily by type C strains and possibly some type B strains (89, 90). Delta toxin is encoded by the *cpd* gene, which has so far only been characterized from two type C strains, CP24-03 and NCTC8131 (88). Not much information regarding the genetics of delta toxin is currently available, however, Southern blot analysis has shown that a *cpd* probe hybridizes to both total DNA and plasmid DNA preparations, suggesting that the *cpd* gene is likely carried on a plasmid (88). Further sequencing determined that *cpd* was encoded within a region flanked by ORFs that could make up a mobile genetic element, as is seen for other C. *perfringens* toxins (88).

NetE, NetF and NetG are putative β-pore forming toxins recently discovered in C. *perfringens* type A strains from dogs and foals presenting with enteric disease (91). Sequencing of the strains identified three genes—*netE, netF*, and *net*G—that were predicted to encode pore forming toxins with

sequence similarity to NetB (91). In the first *netE/F/G*-positive type A strain characterized, both *netE* and *netF* were found to be encoded on the same large conjugative plasmid (92, 93), whereas *net*G was located on a second large conjugative plasmid that also carried the *cpe* gene (91). Sequencing of other *netF*-positive strains showed that co-carriage of *netE* and *netF* on the same plasmid remained consistent; however, carriage of the *net*G plasmid was variable among these isolates (92, 93).

NetF is cytolytic for equine ovary cells (91). There is also a strong association of *netF*-positive strains isolated from diseased dogs and foals which, along with the cytotoxicity assays, suggests that NetF is involved in these diseases (91). Further genetic characterization of *netF*-positive strains has demonstrated that *netE/netF* and *net*G are encoded on two distinct and relatively large pathogenicity loci on their respective plasmids (92, 93).

TpeL is a large glycosylating toxin, closely related to the LCTs, toxin A and toxin B from *C. difficile*, as well as TcsH and TcsL from *P. sordellii*. A role for TpeL in *C. perfringens*-associated disease has not yet been established (94). However, due to the presence of TpeL in some hypervirulent type G avian necrotic enteritis strains it has been suggested that TpeL may play a synergistic role with other toxins such as NetB (95). TpeL is encoded by the *tpeL* gene, which is located on a series of large conjugative plasmids from type B, C and G strains (44, 45, 96, 97). To date, *tpeL* has not been found in any type D or type E strains (96). In type B strains, *tpeL* is consistently encoded on plasmids in a location downstream of the *cpb* gene (44). In contrast, in type C strains that also carry the *cpb* and *tpeL* genes plasmid carriage is more diverse, and TpeL can be encoded on plasmids with *cpb*, or *cpb2* or on entirely separate plasmids (45).

Lambda toxin is a thermolysin-like metalloprotease produced by type B, D and E strains of *C. perfringens* (69). Lambda toxin possesses casein-hydrolyzing activity and has been demonstrated to degrade biological substances such as fibrinogen, collagen and complement component C3 (69). Purified lambda toxin increases vascular permeability, resulting in edema in an *in vivo* model, suggesting that the toxin is potentially involved in pathogenesis of *C. perfringens* infections (69). Lambda toxin is encoded by the *lam* gene, which is located on large conjugative plasmids of varying size (44, 69). In many type B strains it is encoded with the urease genes on a plasmid of approximately 80 kb (44). The carriage of lambda toxin is not as prevalent in type D strains, even though it has been shown experimentally that lambda toxin can activate epsilon toxin, however, host proteases also can activate epsilon toxin (60). The majority of type E strains carry lambda toxin on the same plasmid as the iota toxin and urease genes (66).

Numerous type B, D and E strains possess the ability to produce urease, which is encoded by a plasmid-encoded *ure*ABC operon (98). These plasmids also may carry toxin genes, such as *cpe*, *lam*, *iap*, *ibp*, and *etx* (44, 60, 66, 98).

Toxin Association with Insertion Sequences

A common theme among the toxin genes of *C. perfringens* is the strong association with insertion sequence elements even though none of these genes have been shown to be located on a genetically confirmed transposon (60, 66). Frequently, toxin genes are located in close proximity to elements such as IS*1151*, IS*1470*, IS*1469*, and IS*406* (44, 45, 60, 68). The presence of these elements near toxin genes raises the possibility that toxin genes of *C. perfringens* can be easily mobilized and indeed circular DNA molecules that encompass toxin genes have been detected (44, 60, 66, 74). This theory could explain why several toxin genes, such as *cpb*, are found on numerous diverse plasmids and why toxin genes such as *cpe* are encoded both chromosomally and on plasmids (45, 74, 76).

Other *C. perfringens* Plasmids

Plasmids were first described in *C. perfringens* in 1973 (99) with the identification of a small bacteriocin-encoding plasmid, pIP404, in a type A strain (100). This plasmid forms the basis for most of the current *C. perfringens* cloning vectors (101). Other studies then focused on the identification and characterization of plasmids encoding antibiotic resistance determinants and bacteriocins (102–105). The first conjugative plasmid to be identified in *C. perfringens* was the tetracycline and chloramphenicol resistance plasmid pIP401 (102), in which the chloramphenicol resistance gene was later shown to be encoded on the integrative mobilizable element Tn*4451* (106, 107). Early studies also led to the identification and characterization of the conjugative tetracycline resistance plasmid pCW3 (108) and a series of plasmids that are closely related to pCW3 and pIP401 (109–112). Toxins were first associated with plasmids after a beta toxin-producing strain lost the ability to produce toxin after the loss of an uncharacterized plasmid (113).

Many of the antibiotic resistance and toxin plasmids of *C. perfringens* were initially characterised by methods such as pulsed-field gel electrophoresis, restriction analysis and Southern hybridization (44, 45, 60, 66, 105, 109–111, 114). It was through this analysis that the great diversity of toxin plasmids harbored by *C. perfringens* strains was demonstrated. The first known conjugative *C. perfringens* antibiotic resistance plasmid to be sequenced was the 47.3 kb plasmid pCW3 (115). Comparative analysis of this sequence revealed that there was a region that was common to many large *C. perfringens* plasmids (115). The first toxin plasmids to be sequenced were the *cpe*-encoding plasmids, pCPF4969 and pCPF5603, which were derived from two type F food poisoning isolates (77). Sequence analysis showed that the *cpe* plasmids had ~35 kb of conserved sequence, indicating that these two plasmids may have been derived from a single common ancestor (77). Subsequently, several other toxin and antibiotic resistance plasmids of *C. perfringens* have been sequenced and characterized (74, 81, 82, 91, 97, 115, 116). As was seen for the *cpe*-encoding plasmids, approximately 35 to 40 kb of plasmid sequence remained highly conserved, despite these plasmids arising from different strain types. This analysis demonstrated that there was a significant similarity between the toxin plasmids and the tetracycline resistance plasmid, pCW3 (83, 115) and this plasmid has become the paradigm for the genetic analysis of this family of toxin and antibiotic resistance plasmids (Fig. 2).

Sequencing of pCW3 identified novel replication and conjugation regions within the 35- to 40-kb conserved *C. perfringens* plasmid backbone (115). Within this conserved backbone there were regions identified with sequence similarity to another *C. perfringens* plasmid, pCP13 (115, 117). pCP13 is a resident plasmid of *C. perfringens* strain 13 and carries a *cpb2* gene (117). Sequence analysis showed that these two plasmids are unrelated, apart from nine similar genes (115). Until recently pCP13 was not considered important to toxin gene carriage, because the *cpb2* gene located on this plasmid was defective, due to the presence of a premature stop codon (117). However, it is now apparent that pCP13-like plasmids can encode other toxin genes, including a new *C. perfringens* binary toxin, BEC (also called

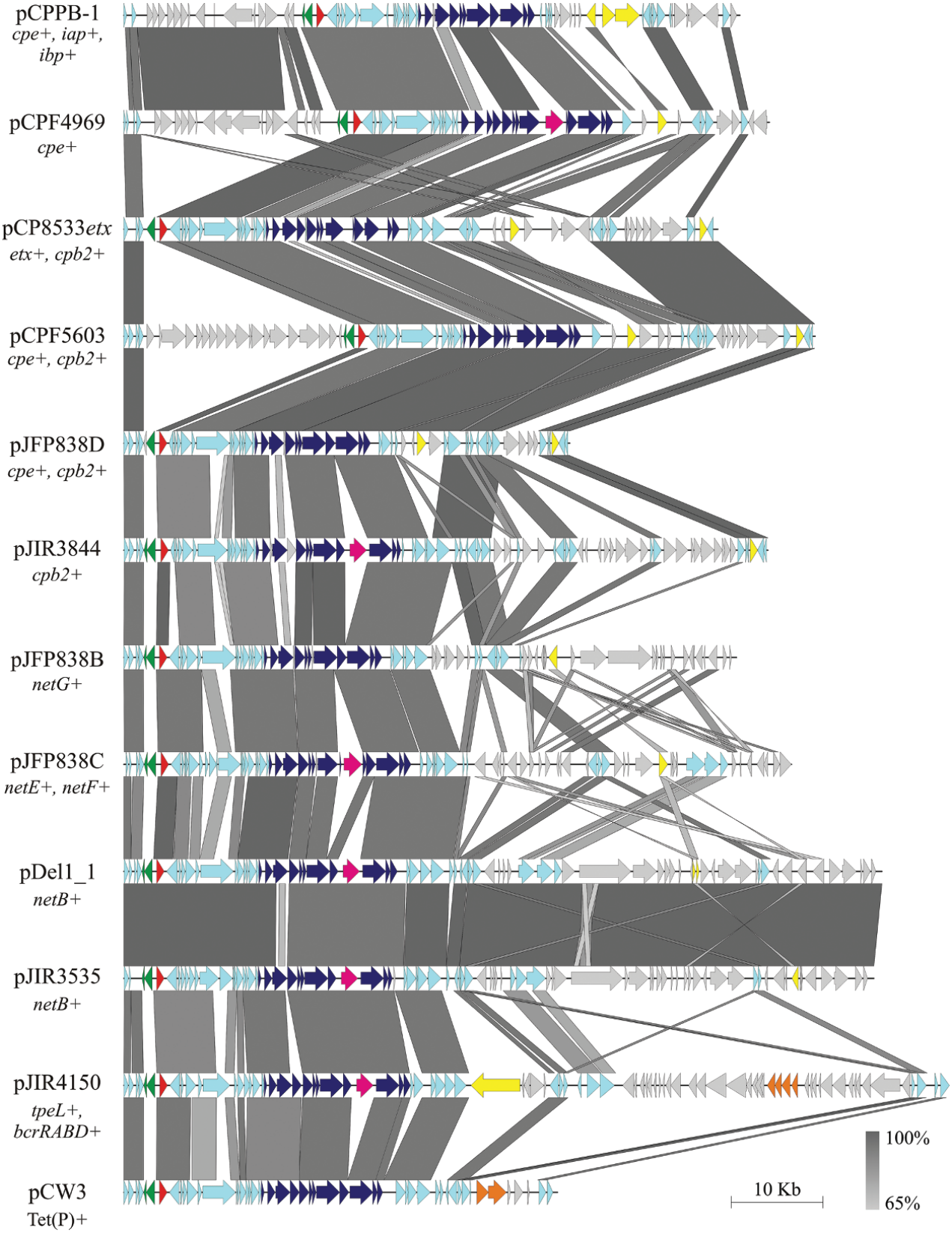


FIGURE 2 Nucleotide alignment of pCW3-like plasmids from *C. perfringens*. Full plasmid sequences were aligned using the blastn algorithm (225) and Easyfig for visualization (226). The plasmid names are noted on the left along with the toxin or antibiotic resistance determinants encoded within each plasmid sequence. Predicted (ORFs) are indicated by arrows with the following color code: conserved ORFs (light blue), *tcp* conjugation genes, dark blue; toxin genes, yellow; *parMR* partitioning genes, green; *rep* red; group II introns, pink; antibiotic resistance genes (orange) less-conserved ORFs, gray. The scale bar and key for nucleotide identity are shown.

CPILE), which appears to be involved in non-CPE mediated human food poisoning in Japan (118, 119). Now, *C. perfringens* toxin plasmids can be broadly categorized into two families: the well characterized pCW3-like plasmids and the less well-characterized pCP13-like plasmids. All the toxin plasmids mentioned so far, other than the BEC plasmids, belong to the pCW3-like plasmid family.

Comparative Analysis of pCW3-Like Antibiotic Resistance and Toxin Plasmids

Two key genetic loci were identified on the conserved backbone of pCW3 following sequence analysis: the plasmid replication and maintenance region (81, 82, 115, 120) and the putative conjugation locus, denoted as the transfer clostridial plasmid, or *tcp* locus (115). The *tcp* locus encodes a novel transfer mechanism that is common to pCW3-like plasmids of *C. perfringens* (Fig. 2) (115, 121–127). To date, six large toxin plasmids (*cpe, netB, cpb2, etx, net* G, and the plasmid carrying the *netE, and netF* genes) and many antibiotic resistance plasmids have been shown experimentally to be conjugative (59, 74, 82, 91, 97, 106, 108, 109, 111). Furthermore, sequencing, pulsed-field gel electrophoresis, and subsequent Southern hybridization have demonstrated that virtually all large toxin plasmids carry a *tcp* locus that is closely related to that of pCW3, albeit with some minor variations in genetic organization and sequence, meaning that these plasmids are highly likely to be conjugative (44, 59, 60, 66, 74, 77, 82, 116).

The biological importance of the toxins being encoded on conjugative plasmids is the potential of *C. perfringens* strains, particularly non-toxigenic commensals, to be converted to virulent isolates following the acquisition of a toxin plasmid(s) (59, 74, 128). Recently, this concept was validated *in vivo* in the gastrointestinal tracts of chickens, whereby non-pathogenic strains of *C. perfringens* were converted to disease causing isolates, following the horizontal transfer of a NetB toxin-encoding plasmid (129).

Current Model for Tcp-Mediated Conjugation

pCW3 is the smallest sequenced conjugative plasmid in *C. perfringens*, it contains a complete *tcp* locus and its transfer can easily be tracked by monitoring the acquisition of tetracycline resistance. Therefore, it has become the archetypal plasmid for conjugation studies in this bacterium (115, 128). The *tcp* locus of pCW3 encodes 11 genes, *tcp*M and *tcp*A to *tcpJ* (115). Many of the genes within the *tcp* locus were predicted to encode functional or structural components of the mating pair formation or transferosome complex. By contrast, no relaxase or other relaxosome components were identified from these initial investigations. Functional mutagenesis and complementation studies on each of the *tcp* encoded genes showed that most of these genes were required for efficient pCW3 conjugation (115, 121–127).

The pCW3 relaxosome

Prior to transport into the recipient cell pCW3 DNA must first be processed by the relaxosome. Typically, the relaxosome complex comprises a relaxase enzyme, which nicks DNA at the origin of transfer (*oriT*) of the plasmid to be transferred; in most cases, accessory proteins are also required (130). Initial analysis of the pCW3 *tcp* locus did not identify a typical relaxase gene, nor were any sequences identified with similarity to known *oriT* sites (115). However, the first gene encoded in the putative *tcp* operon, *tcp*M, encodes a protein showing limited similarity to tyrosine recombinase enzymes and was hypothesized to function as an atypical relaxase (83, 115, 127). Mutagenesis and complementation studies have shown that TcpM is necessary for efficient transfer (127). Subsequent complementation with site-directed mutants revealed that a single C-terminal tyrosine (Y259) residue was essential for TcpM function, in contrast to typical tyrosine recombinases, which require seven conserved arginine, histidine and tyrosine residues for catalytic function (127, 131). Therefore, it was concluded that TcpM was a unique relaxase enzyme with Y259 postulated to be responsible for the essential nucleophilic attack on the sugar-phosphate backbone at the *oriT* site of pCW3 (127).

In many conjugation systems, the *oriT* site is located adjacent to genes involved in the relaxosome complex (132). It was postulated that the pCW3 *oriT* was located within a 391-bp intergenic region located upstream of *tcp*M (127). Mobilization assays using the 391-bp region cloned into a non-conjugative shuttle-vector confirmed that the *oriT* sequence was located within this region and defined the minimal *oriT* site (127).

The pCW3 transferosome

Transferosomes are large multi-protein membrane-associated complexes, or type 4 secretion systems, that physically transfer the relaxase-plasmid DNA complex into a recipient cell using ATPase activity to power the process (133). Initial bioinformatic analysis of the pCW3 *tcp* locus identified several putative proteins predicted to assemble as part of the transferosome complex (115). TcpF is a putative hexameric ATPase (115); ATPases are common components of conjugation systems, and are required to power DNA translocation and to assist in the formation of the transferosome complex (134, 135). TcpH is a putative structural protein with eight putative transmembrane domains; it is predicted to comprise a large proportion of the transmembrane channel that spans from the donor cell into the recipient (115). TcpF and TcpH were the first proteins tested for their involvement in pCW3 conjugation. Mutation by allelic exchange of the genes encoding these proteins and subsequent complementation and conjugation analysis demonstrated that the TcpF and TcpH proteins are essential for the conjugative transfer of pCW3 (115). In a later study, functional domains of TcpH were determined by deletion and site-directed mutagenesis. It was concluded that a region located between amino acids 514 and 581 was essential for TcpH function *in vivo* (125). Despite being essential for pCW3 transfer, TcpF remains to be functionally characterized.

*tcp*G and *tcpI* are both predicted to encode peptidoglycan hydrolase enzymes, which are predicted to function by hydrolyzing local regions of the thick peptidoglycan layers of the donor and recipient cells to allow for the formation of the transferosome. Mutation and complementation analysis determined that TcpG, but not TcpI, was required for efficient pCW3 transfer (121). Further analysis confirmed that TcpG had peptidoglycan hydrolase activity (121).

*tcp*C, *tcpD*, *tcpE*, and *tcpJ* all encode hypothetical proteins of unknown function (115, 123, 126). With the exception of *tcpJ*, they all are required for maximal pCW3 transfer. Analysis of the crystal structure of the C-terminal portion of TcpC (123) showed that although it had no conserved domains or similarity to other protein sequences, TcpC had a structure similar to VirB8, a conserved structural protein of the *Agrobacterium tumefaciens* type 4 secretion system (123). The structural similarities between VirB8 and TcpC suggest that TcpC plays a structural role in the pCW3 transferosome (123). The small transmembrane proteins TcpD and TcpE are essential for pCW3 transfer, because plasmids with mutations

in either of these genes are non-conjugative (126). Their function remains to be elucidated, but immunofluorescence imaging showed that these proteins localize to the cellular poles, such as TcpF and TcpH (125, 126). It is highly likely that TcpD and TcpE also compose part of the transferosome.

The second gene in the *tcp* operon, *tcp*A, encodes a putative integral membrane protein, with a conserved FtsK/SpoIIIE domain (122). Proteins within the FtsK/SpoIIIE family bind DNA in cellular processes such as chromosomal segregation and the transfer of genetic material into the forespore during sporulation (136). Therefore, TcpA was postulated to function as a coupling protein in pCW3 conjugative transfer (122). Subsequent mutation and complementation analysis confirmed that TcpA was essential for pCW3 transfer (122). As the coupling protein, it was assumed that TcpA mediated a range of protein-protein interactions with other Tcp proteins in both the relaxosome and transferosome complexes (124). Through chemical cross-linking and bacterial two-hybrid analysis, TcpA was found to self-associate, as well as to interact with the transferosome components TcpC, TcpG and TcpH (124). At the time of this study no relaxosome components had been identified, and thus, interactions with pCW3 relaxosome components have not yet been assessed (124). Based on these studies and other protein-protein interaction studies, including structural studies (121–125) a model for the conjugative apparatus utilized by pCW3 has been constructed (Fig. 3) (128).

Although a large body of work has been conducted on the *tcp* locus of pCW3, there are still large gaps in our knowledge and understanding of the structure and function of the conjugation apparatus. To date, it is still not certain whether pCW3 DNA transport occurs in a single-stranded or double-stranded manner. Additionally, the precise processing reactions that are catalyzed by the relaxosome remain uncharacterized and the protein composition of this structure remains to be elucidated. Finally, the mechanism of direct cell-to-cell contact between *C. perfringens* cells remains elusive. These genetic and functional studies of pCW3 are important because they serve as a model system for the analysis of the pCW3-like toxin plasmids.

Multiple pCW3-Like Toxin Plasmids Are Often Present in the Same Strain

Plasmid incompatibility is defined as the inability of two plasmids to coexist in the same cell without the application of

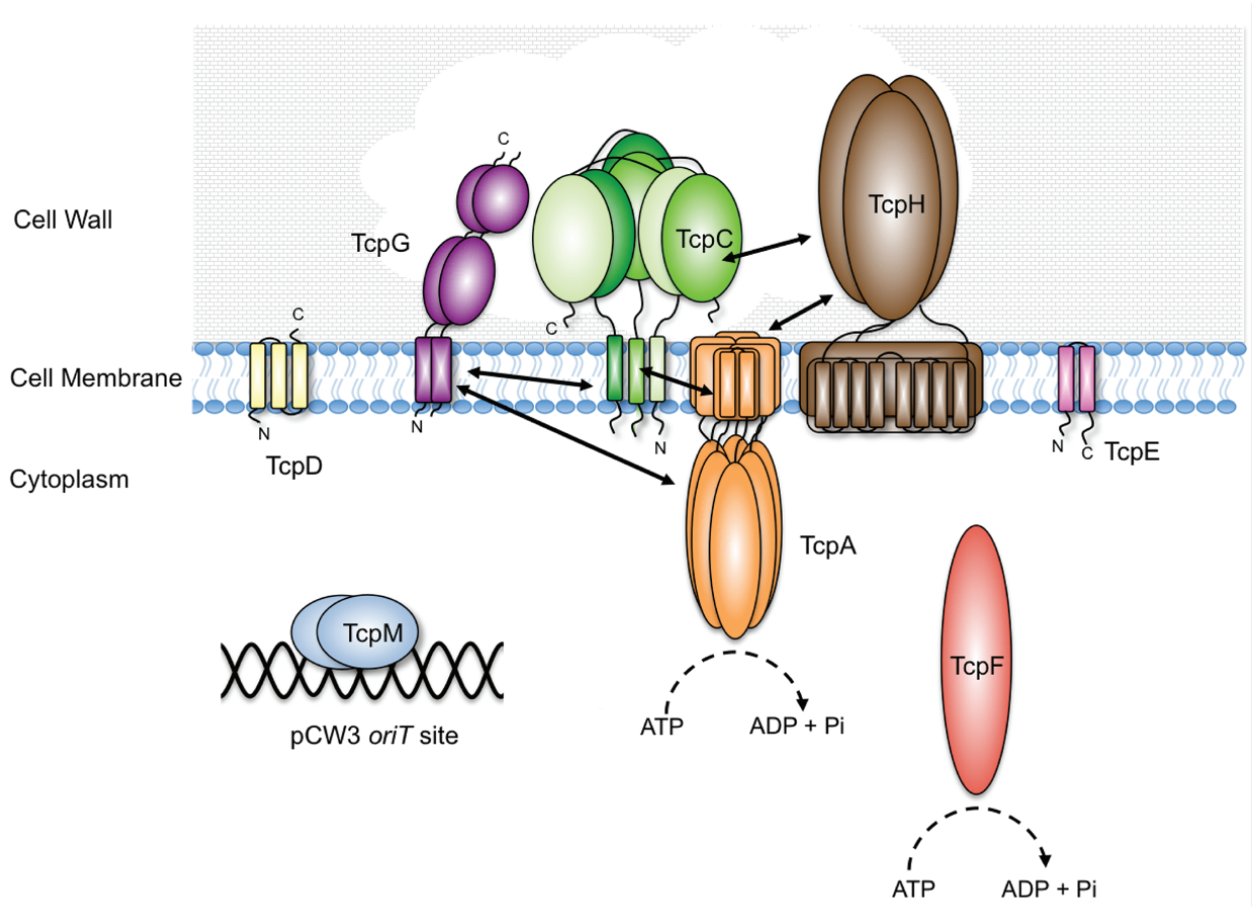


FIGURE 3 Model of the pCW3 conjugation apparatus. The arrangement of the proteins within the model is based on protein localization studies and bioinformatics analysis. Black arrows indicate confirmed protein interactions between Tcp proteins and only Tcp proteins required for wild-type transfer of pCW3 are shown. Indicated within the membrane are the integral membrane proteins TcpH (brown), the peptidoglycan hydrolase TcpG (purple), the assembly factor TcpC (green; monomers as different shades), the proteins of unknown function, TcpD (yellow) and TcpE (pink) and the putative coupling protein TcpA (orange). Within the cytoplasm are a putative ATPase TcpF (red) and the novel relaxase TcpM (blue) in complex with the double stranded pCW3 *oriT* site. Dotted arrows indicate putative ATPase activity. Reproduced with permission from Wisniewski and Rood (2017).

external selection (137). Plasmid incompatibility usually arises when two plasmids encode similar essential plasmid encoded factors such as replication initiation factors, replication control mechanisms or partitioning machineries.

All plasmids must replicate and maintain a stable copy number relative to the host chromosome to be faithfully inherited by the daughter cells at cell division (138). The copy number of a plasmid is related to the properties of the replication machinery and other factors. Initial attempts to identify the pCW3 replication gene could not identify an ORF with significant similarity to any known plasmid replication proteins (115). Therefore, a functional genetics approach was employed to determine which plasmid encoded regions were involved in plasmid replication. A combination of deletion and transposon mutagenesis studies led to the identification of the *rep* gene, which was essential for the replication of pCW3 (115). The intergenic region upstream of the *rep* gene had five pairs of inverted repeats and a series of conserved 17-bp direct repeats that were postulated to act as iterons or as a centromeric binding site for partitioning proteins (115). Comparative bioinformatic analysis of the available *C. perfringens* conjugative plasmid sequences revealed that the pCW3-like replication protein is highly conserved (120).

As already mentioned, many strains of *C. perfringens* carry more than one pCW3-like toxin or antibiotic resistance plasmid, each of which encodes a similar replication protein (82, 115, 120). These plasmids are stably maintained in the same cell, which is unexpected given the conventional relationship between plasmid incompatibility and plasmid replication functions (137). The best studied example of this phenomenon is the avian necrotic enteritis type G isolate, EHE-NE18. This strain stably harbours three highly similar pCW3-like conjugative plasmids: a NetB plasmid, a beta2-toxin plasmid and a tetracycline resistance plasmid. These plasmids each encode an almost identical replication protein (≥ 98% amino acid sequence identity) (82, 120). The apparent stability of this plasmid combination, and other toxin plasmid combinations in strains from other toxinotypes, suggested that factors other than the replication machinery were involved in the determination of plasmid incompatibility in *C. perfringens*.

pCW3 is a low-copy number plasmid (~5 plasmid copies/chromosome) (T. Stent, X. Han, R. Moore, V. Adams and J. Rood, personal communication) and therefore most likely requires an active maintenance system to ensure that it is inherited correctly (139). Closer inspection of the pCW3 sequence revealed two genes that were adjacent to, but divergently transcribed from, the *rep* gene. These genes encoded putative partitioning system homologues, ParM and ParR, and a putative *par*C centromere site (115).

Partitioning systems act as positioning systems to ensure that sister plasmids are positioned at the cell poles thus ensuring that the plasmid is maintained in daughter cells when cell division occurs (139). Type II or ParMRC-like plasmid partitioning systems encode three components: ParM, an actin-like ATPase that polymerizes to form filaments, ParR, a DNA-binding adaptor protein, and *par*C, a centromeric ParR binding region that usually comprises a series of direct repeats located upstream of the *par*M gene (140). ParMRC systems segregate plasmids via a pushing mechanism in which two sister plasmids are linked through a bundle of ParM filaments that recognize and interact with ParR adaptor proteins bound to the *par*C centromere sites (140). *C. perfringens* ParMRC-like plasmid partitioning systems have not been thoroughly investigated, although a *parMRC*$_B$ family partitioning system from the *C. perfringens* isolate JGS1987 has been demonstrated to stabilize a mini-replicon in *E. coli* (141).

An extensive survey of sequenced pCW3-like plasmids showed that there were at least 10 distinct families of *parMRC*-like partitioning systems (*parMRC*$_{A\text{-}J}$) present in *C. perfringens* (81, 82, 115, 120, 142). Comparative analysis showed that ParM homologues had upwards of 90% amino acid sequence identity within a family, but only 15- to 54% identity between the various groups (120). ParR homologues clustered into the same phylogenetic groups as the ParM homologues, but showed more amino acid sequence variation within a family (120). The *par*C centromere sites also clustered with their cognate ParM and ParR family groups, although the *par*C sites are very AT-rich and have a lot more sequence variability between the 10 groups than the ParM and ParR components (120). It was observed that *C. perfringens* strains that house multiple pCW3-like plasmids generally do not have plasmids that encode the same *parMRC* allele (120). This observation suggested that *parMRC* partitioning families were responsible for maintenance within the same strain of multiple pCW3-like plasmids that encode a highly similar replication region.

There now is experimental evidence to support this hypothesis (143). In this study different combinations of genetically marked, *parMRC*-encoding, pCW3-like plasmids were introduced into the same *C. perfringens* strain. These strains were constructed so that they housed either two plasmids with the same *parMRC* locus, or two plasmids with *parMRC* loci from different families. Analysis of these isolates showed that plasmids with the same *parMRC* families were unable to stably coexist in the same cell, whereas plasmids with different *parMRC* families could stably coexist even though they encoded similar Rep proteins (143).

pCP13-Like Plasmids

Most studies of *C. perfringens* plasmids have focused on the pCW3-like family of toxin and antimicrobial resistance plasmids, but another class of toxin plasmids is beginning to garner more interest in the field (144). Genome mapping and whole genome sequencing of the *C. perfringens* type A isolate, strain 13, revealed the presence of a 54.3 kb plasmid called pCP13 (117, 145). pCP13 encodes 63 putative ORFs, many of which encode proteins of unknown function. Several ORFs could be assigned a predicted function including ORFs encoding a defective, consensus beta2-toxin, a predicted collagen adhesin called CnaB (146), and plasmid partitioning homologues, ParA (Soj) and ParB (117). Both the consensus and atypical beta2-toxins have been implicated as accessory virulence factors becuase they are found in various *C. perfringens* disease isolates (81, 82, 84). However, beta2-toxin has no confirmed association with disease. Cna proteins are known to act as adhesins and virulence factors in other Gram positive pathogens such as *Staphylococcus aureus* (147–149). A Cna homologue, CnaC, encoded on pCW3 and recently CnaA, which is produced by several avian necrotic enteritis isolates of *C. perfringens*, was shown to be important for gastrointestinal colonization and subsequent infection in chickens (146, 150).

pCP13-like BEC (CPILE)-encoding plasmids

Recently, three *cpe*-negative *C. perfringens* strains, OS1, TS1 and W5052, were isolated from three separate food poisoning outbreaks in Japan (118, 119). The supernatants of two of these isolates showed enterotoxic activity in a rabbit ileal loop model, even though no CPE was produced (119). Sequence analysis led to the discovery of two 54.5 kb plas-

mids, pCP-OS1 and pCP-TS1, each of which had 38 kb sequence identity with pCP13 (Fig. 4). Sequencing of these pCP13-like plasmids led to the identification of a novel clostridial binary toxin designated BEC (or CPILE, see reference 119 for a discussion of the terminology). BEC comprises two components, BECa and BECb, which show 43% and 44% amino acid identity to iota-toxin components Ia and Ib, respectively (118, 119). The BECb component is responsible for some of the enterotoxic effects, however its activity is enhanced by the BECa component, which has been shown to have ADP-ribosyltransferase activity on purified actin (119). In addition, both BECa and BECb components are required to cause Vero cell rounding *in vitro* (119). X-ray crystal structures of BECa have been solved, revealing a fold and substrate recognition mechanism similar to other ADP-ribosyltransferase toxins (151, 152).

Comparative analysis of pCP13 plasmids

The BEC plasmids, pCP-TS1 and pCP-OS1, were isolated from different strains, but are almost identical at the nucleotide sequence level (99% identity) (Fig. 4), which suggests that a recent horizontal gene transfer event occurred (119). The original sequencing of pCP13 did not initially reveal any ORFs with homology to known conjugation proteins (117). However, more recent comparative analysis of clostridial plasmid sequences has shown that pCP13 has several genes that encode proteins with amino acid sequence identity to conjugation proteins from the conjugative toxin plasmids pCS1-1 (12) and pCLL (153) from *P. sordellii* and C. *botulinum*, respectively. These proteins include a putative coupling protein with VirB4 domains, a putative essential ATPase with predicted VirD4 domains and a potential membrane scaffold protein with predicted VirB6-like domains (12). The presence of these conjugation machinery homologues on pCP13 together with the identification of almost identical BEC plasmids pCP-TS1 and pCP-OS1 in two distinct strains suggested that pCP13-like plasmids may be conjugative. We now have experimental evidence that pCP13 is indeed conjugative (T. Watts, C. Vidor, M. Awad, D. Lyras, J Rood and V. Adams, unpublished results). These studies highlighted the importance of the pCP13 plasmid family to the toxin armory of C. *perfringens* and to the dissemination of the toxin genes.

The most recent addition to the pCP13-like family is the bacteriocin-encoding plasmid pBCNF5603, a plasmid found to coexist with a pCW3-like, CPE-plasmid in the type F isolate F5603 (144). Bacteriocins are antibacterial factors produced by bacteria to inhibit the growth of similar bacterial strains (154). C. *perfringens* strains often produce bacteriocins (155), some of which have been implicated in strain competition in the gastrointestinal tracts of broiler chickens (129, 144, 156, 157). pBCNF5603 was recently sequenced and found to have genes with a high level of similarity to homologues on pCP13 and on the small bacteriocin plasmid pIP404, suggesting that pBCNF5603 arose from a recombination event between these two C. *perfringens* plasmids (144) (Fig. 4). Sequencing of this plasmid has provided insight into the replication functions of pCP13-like plasmids by identifying two potential replication regions (144). The first replication region showed homology to the *rep* and *cop* genes of pIP404, whereas the second region displayed similarity to the *parA* and *parB* gene regions of pCP13, pCP-TS1 and pCP-OS1. The 5.5 kb gene cluster with similarity to pCP13 supported replication of a recombinant plasmid in C. *perfringens*, whereas the replication region with similarity to pIP404 did not (144). Analysis of subsequent deletion derivatives showed that the homologue to PCP63 may be the pBCNF5603 Rep protein (144). The region upstream of the putative *rep* gene was AT-rich, with a series of inverted repeats that are likely to be important for replication initiation.

THE TOXIN PLASMID OF *C. TETANI*

The neurotoxic clostridia comprise the C. *botulinum* complex, the causative agents of botulism, and C. *tetani*, which causes tetanus in both humans and animals. Both the

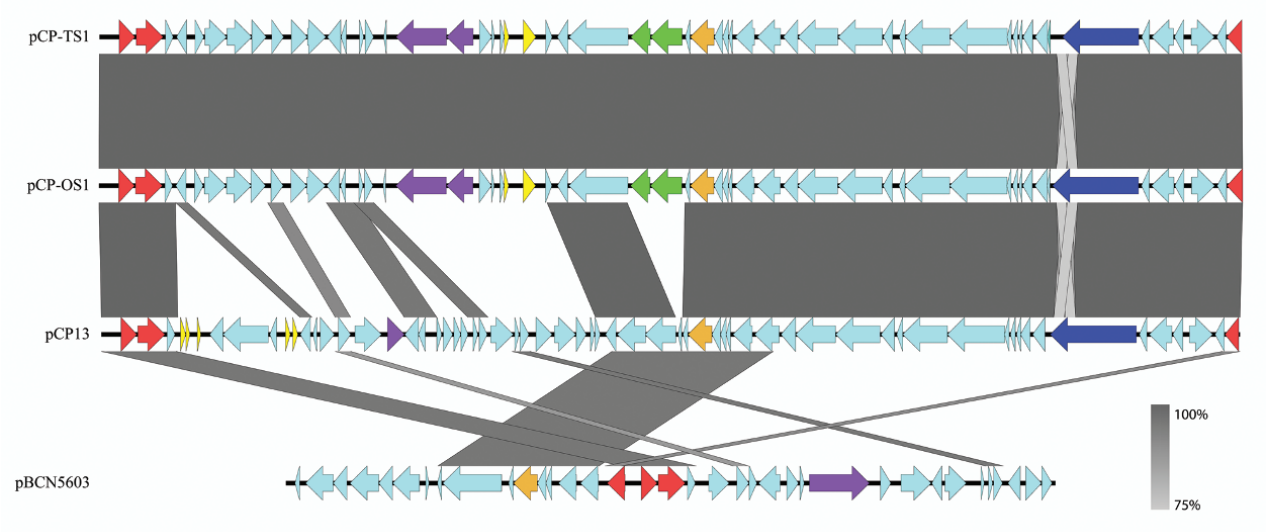


FIGURE 4 Sequence alignment of pCP13-like plasmids from C. *perfringens*. The plasmid sequences of pCP-TS1, pCP-OS1, pCP13 and pBCNF5603 were aligned using the Blastn algorithm using Easyfig (226). The percentage identity is indicated by the scale bar at the bottom right and each sequence is compared separately to the sequences above and below. ORFs are indicated by arrows and ORFs of particular interest are colored as follows: green, restriction modification systems; red, replication and maintenance; purple, toxin genes; yellow, transposase genes; dark blue, putative collagen adhesins; orange, putative relaxase enzymes.

clostridial species that comprise the C. *botulinum* complex and C. *tetani* are renowned for their ability to produce potent neurotoxins, botulinum toxin (BoNT) and tetanus toxin (TeNT), respectively (158). The various BoNT structural genes can be encoded on the chromosome, on a temperate bacteriophage or on a plasmid, whereas the TeNT structural gene is plasmid encoded (159).

TeNT is a zinc metalloprotease that acts on the SNARE protein VAMP/synaptobrevin at the synaptic junction of the nerve relaxation pathway in the spinal cord. Cleavage of VAMP/synaptobrevin means that it is unable to form a complex with other SNARE proteins, preventing fusion of the synaptic vesicle with the cellular membrane at the synaptic nerve junction and preventing release of the neurotransmitter from the inhibitory interneurons to the motor neurons in the spinal cord. The net effect is inhibition of the muscle relaxation pathway and a rigid muscular paralysis that may be fatal (158, 160).

The first C. *tetani* strain that was sequenced, the Harvard vaccine strain E88, carries a 74,082 bp plasmid, pE88, that encodes 61 putative genes (159). These genes include the TeNT structural gene, *tetX*, and the *tetR* gene, which encodes an alternative sigma factor that is required for *tetX* expression and consequently for TeNT production (161). TetR belongs to a family of alternative sigma factors together with BotR from C. *botulinum*, TcdR from C. *difficile*, TcsR from *P. sordellii*, and UviA and TpeR from C. *perfringens* and (162, 163). Little is known about the function of other genes encoded on the TeNT plasmid, although there is another potential virulence factor gene, the *colT* collagenase gene, and genes encoding five distinct ABC transporter complexes (159). The regulatory proteins encoded by pE88 include TetR, two other putative alternative sigma factors, a putative two-component signal transduction system (CTP21/22) and a homologue of the bacteriocin plasmid pIP404-encoded UviB protein from C. *perfringens*. pE88 encodes two IS*200*-family transposases and an IS*605*-like transposase. It has *parMR*-like plasmid partitioning genes and a duplicated replication gene, the product of which is related to the replication protein encoded by pIP404. The structure of the actin-like ParM protein, Alp12, has been determined and shown to be compose of a four-stranded filament that is in a different conformation than other actin-like proteins (164, 165). Note that there is no genetic or bioinformatic evidence that pE88 or its homologs are conjugative or mobilizable.

A transcriptomics study that included plasmid-encoded genes has been carried out on strain E88 grown in fermentation bioreactors (166). The results showed that most of the pE88 genes have a transcriptional profile similar to the *tetX* and *tetR* genes, with gene expression increasing over time. No significant expression of the *colT* collagenase gene is observed under these conditions.

Since the publication of the genome sequence of strain E88 (159), the sequences of 10 other C. *tetani* strains have been reported (167–169). The chromosomes of these strains are highly conserved, with variations occurring at putative bacteriophage insertion sites, and CRISPR loci and within a flagellar glycosylation genomic island. Therefore, the C. *tetani* pan-genome can be considered closed, supporting the concept that C. *tetani* has only limited genetic exchange with other bacteria (170).

All of the sequenced strains, with the exception of the non-toxigenic isolate ATCC 19406, carry a pE88-like plasmid (168, 169) (Fig. 5). Another non-toxigenic strain, ATCC 454, carries a plasmid that has >95% identity to pE88, but has a 20 kb deletion that encompasses both the *tetX* and *tetR* genes (Fig. 5). The remaining plasmids have considerable conservation as well as specific pE88 regions that are not present (Fig. 5). The *tetX* genes are highly conserved (99.3 to 99.4% identity) and the *tetR* genes are all identical (169). The lack of genetic tools for the analysis of C. *tetani*, combined with the inherent difficulties in working with this anaerobic, very motile and highly toxigenic pathogen, have clearly limited the genetic and functional studies that can be carried out on these important plasmids.

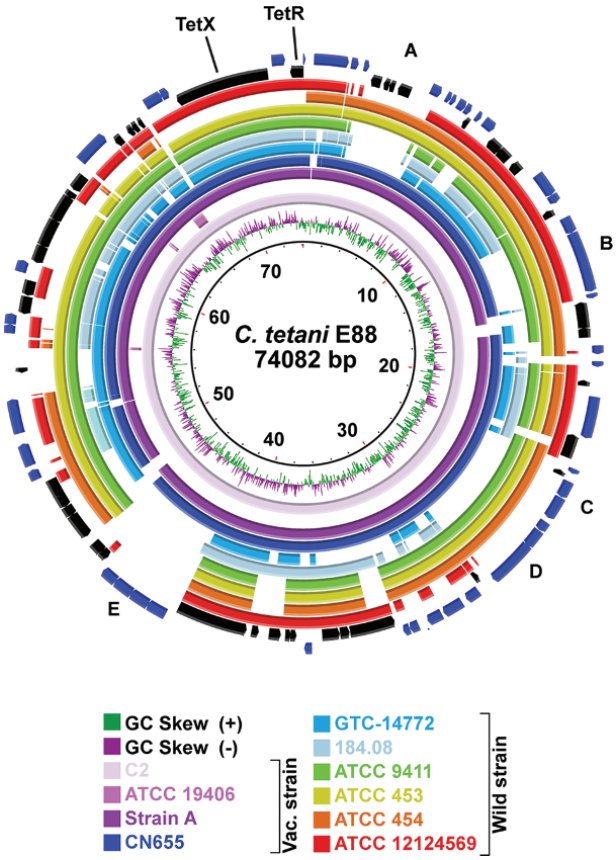


FIGURE 5 Blast analysis of the tetanus toxin plasmid family. Plasmid sequences derived from C. *tetani* were compared to the 74 kb pE88 plasmid (GenBank accession number NC 004565). The two outermost rings show predicted (ORFs) for both the (+) and (-) DNA strands. The position of the tetanus toxin gene (*tetX*) and the regulator (*tetR*) are shown. DNA regions with less than 80% sequence identity are indicated by gaps. Strain ATCC 454 does not encode either the *tetX* or *tetR* genes. The regions denoted A through E indicate deletions within plasmids found in wildtype strains and include *uviB* and DNA directed RNA polymerase sigma-70 factor (A), *ftsX* and an ABC anti-microbial transporter system (B), an *ompR*/*baeS* two-component system (C), *ftsX* and an ABC-type lipoprotein export system/permease complex (D), and another ABC multidrug-resistance transporter/permease complex (E). Reproduced with permission from Cohen *et al* (2017).

BOTULINUM TOXIN-ENCODING MOBILE GENETIC ELEMENTS

The production of BoNT neurotoxin is one of the defining features of the clostridial species C. *botulinum*. However, unlike TeNT-producing C. *tetani*, C. *botulinum* constitutes a highly heterologous group of organisms (171–175). This collection of strains consists of six groups (I- to VI), with

groups I- to III generally designated C. *botulinum* (171, 172, 175). Groups IV, V, and VI consist of BoNT-producing strains of C. *argentinense*, C. *baratii*, and C. *butyricum*, respectively (175). This highly divergent spread of related toxin genes suggested early on that mobile genetic elements may be involved in toxin gene dissemination and subsequent studies have demonstrated that multiple types of elements are involved (171, 173).

All of these different species are able to produce BoNT, which is related to TeNT in both sequence, structure and function (176, 177). Both BoNT and TeNT are highly specific zinc metalloproteases. However, unlike TeNT, BoNT associates with non-toxigenic proteins to form large complexes (178), which is related to the different routes of intoxication between TeNT and BoNT (wound versus intestinal absorption). The non-toxic components are involved in the protection and transport of the active proteolytic component of BoNT from the gut to its site of action within the synaptic vesicles of excitatory neurones, where it blocks the release of neurotransmitters at the neuromuscular junction (179). The resultant flaccid paralysis can be fatal without appropriate supportive treatment (180). Many species, including humans, livestock, aquatic birds and fish, have been demonstrated to be susceptible to botulism (171).

The BoNT protein sequences have conserved functional domains, but are still highly variable (between 29 and 64% amino acid sequence identity), and historically have been separated into seven serologically distinct types (A- to G) (161, 179, 181). Many of the BoNT types have been further segregated into subtypes, designated by a number (179, 182), for example, BoNT/F6. An eighth serotype designated H, was reported in 2014, but there is controversy about whether this serotype constitutes a chimeric protein between serotypes BoNT/F5 and BoNT/A or a distinct group (172, 183, 184). Subsequently, another BoNT type has been identified and named BoNT/X. This neurotoxin appears not to be a mosaic enzyme and constitutes a new class of BoNTs (185). A ninth class of BoNT, BoNT/En, has recently been identified after genome sequencing of an *Enterococcus faecium* isolate derived from a manure sample in South Carolina and appears most closely related to BoNT/X (38.7%) (181). The heterogeneity exhibited by BoNTs is intriguing because it has resulted in differences in the complexes that are formed prior to cellular uptake and in the mechanisms of transportation. Most importantly, they have different host targets, catalyzing the proteolytic cleavage of different SNARE proteins, which facilitate neurotransmitter vesicle fusion and therefore neurotransmitter release and propagation of the electrical signal from the nerve cell to the muscle cell (186). In spite of this diversity, the result is the same: inhibition of neurotransmitter vesicle fusion, leading to flaccid paralysis (186).

The BoNT structural (*bont*) genes are encoded within two distinct genetic regions, the *orfX+* (function unknown) and *ha+* (hemoglutinin) loci (Fig. 6) (173). Both loci contain conserved ORF's (*orfs*), including *bont*, found downstream, and in the same orientation, as the conserved *ntnh* (non-toxigenic, non-hemagglutinin) gene as well as the *botR* gene mentioned above (161, 178, 187). All BotR-related proteins are responsible for positively regulating the expression of toxin genes in their native hosts. Likewise, BotR has been shown to regulate transcription of the *bont* gene (161, 162, 188).

The variable regions of the *bont* loci encode either the *orfX1-3* genes (*orfX+* loci) or the *ha17*, *-33*, and *-70* genes (*ha+* loci; Fig. 6, Table 2). The organization of these two loci is largely conserved between different strains, the surrounding nucleotide sequences having co-evolved with the variable *bont* gene (173). In addition to the nine serological BoNT types, hybrid *bont* genes have also been identified, such as serotype C/D and D/C derivatives (189). Certain BoNT positive strains encode multiple *bont* loci (173, 190), suggesting an easy mechanism for genetic exchange and explaining how recombination between *bont* loci could readily

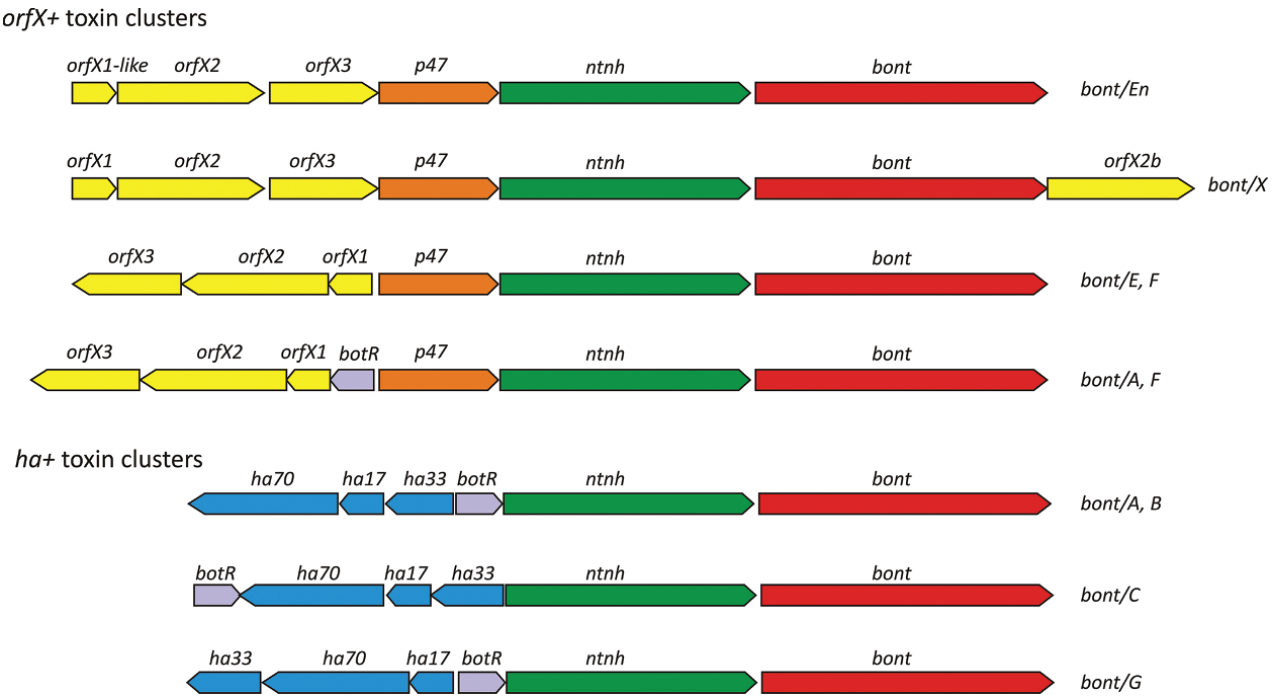


FIGURE 6 Gene arrangement for *bont* toxin loci. The two basic loci types, *orfX+* and *ha+*, are indicated. Similar genes are designated with the same color and the toxin gene serotype designation is given on the right.

TABLE 2 Summary of BoNT-producing species and toxin gene locations

Bacterial clade or species[a]	I					II			III		IV	V	VI	*Enterococcus faecium*
BoNT type[b]	A(8)	B(8)	F(7)	H	X	B(8)	E(12)	F(7)	C	D	G	F(7)	E(12)	En
bont locus[c]	*orfX* *ha*	*ha*	*orfX*	*orfX*	*orfX*	*ha*	*orfX*	*orfX*	*ha*	*ha*	*ha*	*ha*	*orfX*	*orfX*
Chromosome[d]	*ars*C (*orfX*) *oppA*/*brnQ* (*ha*)	*oppA*/ *brnQ*	*ars*C*pul*C (*F4*)	IS*110* flanking *bont*/*H*	Cys. biosyn. Pathway		*rarA* (MGE)	*topB* (MGE) (*F6*)			Strain CDC2741	IS*1182* flanks *bont*/*F7* locus Compound Transposon?	*rarA* (MGE) (*E4*, *E5*)	
Plasmid	150-270 kb A/F site E (*orfX*)	150-270 kb B site	150-270 kb A/F site			47-63 kb (*B4*)	133-144 kb Helicase (*E1*, *E3*, *E10*)				140 kb Strain 89G			207 kb Putative Conjugative Plasmid
Bacteriophage encoded									Yes	Yes				
Species[e]	Humans Chickens	Humans Horses Cattle	Humans	Human (Bivalent B2h)	Human (Bivalent B2x)	Human Horses Cattle	Human Fish Birds	Human	Birds Cattle Horses	Birds Cattle Human	Soil Humans?	Humans	Human	Cow Manure

[a]Bacterial clade designation: I-III *C. botulinum*, IV *C. argentinense*, V *C. baratii*, VI *C. butyricum*.
[b]Designation of serologically distinct BoNT proteins, the number of toxin subtypes are in parentheses.
[c]Genes encoding BoNT toxins are found associated with one of two distinct genetic loci; *orfX*+ or the *ha*+ loci (see Fig. 6).
[d]Chromosomal location of *bont* locus, often associated with a mobile genetic element (MGE). F6 is the *bont* subtype.
[e]The vertebrate species in which each toxin type is most frequently demonstrated to cause Botulism. Clade IV strains have not been definitively associated with botulism but have been associated with multiple sudden deaths (199).

occur. It has been shown that when multiple *bont* loci are present within the same strain, one particular BoNT toxin is expressed to a higher degree than the other(s) and is thereby designated with uppercase (indicating the more abundant toxin type) and lower case toxin types, for example BoNT/Ba (191).

Group I: — Proteolytic *C. botulinum*

Bacterial species belonging to this clade or group include C. *botulinum* as well as the non-neurotoxigenic, C*lostridium sporogenes* (173, 175). The toxin serotypes that have been identified within this clade include BoNT/A, /B, /F, /H (F5/A) and /X (172, 173, 184, 185) (Table 2). The serotype B *bont* loci (*ha*+ operon) has a chromosomal location within the *oppA*/*brnQ* site (172). Alternatively, some B subtypes such as *bont*/*B1*, */B2*, and */B5* are found within the "B" site of large plasmids (172, 191). Very similar plasmids have been shown to harbor *orfX*+ operons encoding either *bont*/*A2-4* or */F2* toxin genes integrated at another site known as the "A/F" site (172). Bivalent strains often carry one or even two plasmid encoded *bont* loci, where both the "B" site and the "A/F" sites are occupied (such as pCLJ from the bivalent strain 657 Ba) (191), for example, the *bont*/*F5* locus from the trivalent strain, Af84, also was found to be plasmid located (190, 192).

All clade I plasmids identified appear to be related and range in size from 149- to270 kb (173, 191, 193–195). A significant number of genes are conserved, including multiple genes involved in DNA replication (173, 194). In addition, there appear to be many genes show similarity to bacterial type II and IV secretion systems and two clade I plasmids, pBotCDC-A3 and pCLJ, are conjugative, with transfer demonstrated between plasmid-carrying donors and two different clade I recipients (153). The plasmids derived from clade I strains exhibit different degrees of stability, impacting the stability of toxin production as a result (194, 196). It is clear that the *bont* toxin genes of serotypes A, B, and F are commonly carried by highly related clade I conjugative plasmids capable of transfer between C. *botulinum* strains, but also to non-toxigenic C. *sporogenes* (197). Given the ability of the toxin loci to both recombine and move in the context of mobile genetic elements, there is significant capacity for the further spread either to other strains or to other locations within the same strain.

Group II: – Non-proteolytic *C. botulinum*

Group II strains produce BoNT serotypes B4, F6 and E (multiple subtypes; Table 2) (198, 199). The *bont*/*F6* loci are chromosomally located and found within what is thought to constitute a mobile genetic element that carries a recombinase gene, *topB* (172, 200). The *bont*/*B4* loci found within this clade are believed to be exclusively plasmid-borne (193, 198, 199). The first group II plasmid sequenced is derived from strain Eklund 17B and carries the *bont*/*B4* locus on the 48-kb plasmid, pCLL (173, 174). Subsequent studies demonstrated that *bont*/*B4* loci are carried by plasmids 47 to 63 kb in size that are unrelated to group I plasmids (198). These plasmids form two distinct plasmid groups, perhaps sharing a common ancestor (198). One group (class 1) is related to pCLL and its members are of similar size, whereas class 2 plasmids only have about 33% similarity to class 1 plasmids; this similarity is limited to the large neurotoxin gene cluster (198). Class 2 plasmids range in size from 58 to 63 kb (198). A third plasmid appears to be a hybrid of the two other plasmid classes (198). All plasmid types encoding *bont*/*B4* loci were found only in group II strains and related plasmids that were non-toxigenic have subsequently been found by analysis of genome sequence data from other group II strains (199).

Serotype E strains belonging to group II had previously been reported to encode only chromosomal *bont*/*E* loci, but recent pulsed-field gel electrophoresis studies showed that a proportion of type E strains (6-10%) carry *bont*/*E* loci on large plasmids in group II strains (199, 201). These plasmids range in size from 134 to 144 kb and are closely related (199). Functional CRISPR systems were identified on plasmids carrying *bont*/*E1* and *bont*/*E10* loci, but not on plasmids carrying the *bont*/*E3* loci (199). The type E toxigenic plasmids appear to be very low copy number as judged from sequencing read abundance, approximately one copy per chromosome (199). The *bont*/*E* plasmid loci are very similar to the chromosomal *bont*/*E* toxin clusters that have integrated into a chromosomal copy of the resolvase gene, *rarA*. The toxin loci in both cases consists of a 24-kb cassette that include genes outside of the normal toxin cluster. This region includes a second, related *rarA* gene that remains intact (199). The plasmid-borne type E toxin cassette is completely conserved, but the integration site is different. The plasmid gene that has been interrupted is a helicase gene and the insertion site appears to be a 6-bp sequence that is conserved between the chromosomal *rarA* gene and the plasmid-encoded helicase gene (199).

All of the toxin plasmids so far identified from group II organisms (class1 and 2 *bont*/*B4* and *bont*/*E* plasmids) have a significant number of ORFs whose predicted function involves conjugative transfer (198, 199). The smallest plasmid characterized so far, pCLL, which contains the least putative conjugation genes (198), is the only group II plasmid to have a demonstrated ability to transfer, remarkably, into a group I recipient (153). No group II- like plasmids have been detected in any other related strain and they show little conservation between themselves or plasmids from other groups, suggesting that these plasmids have a narrow host range (199). pCLL has a low level of similarity to pCS1-family plasmids from *P. sordellii* and pCP13-like plasmids from C. *perfringens* (12). The plasmid location of *bont* genes in this bacterial group explains how the *bont* genes have been so widely disseminated.

Group III: – *Clostridium novyi sensu lato*?

The clade designated type III includes a number of clostridial species and recently it was proposed that it be re-named from C. *botulinum* to C. *novyi sensu lato* (189). This designation includes three species depending on the toxins that each strain produces (189): C *novyi, Clostridium haemolyticum* and C. *botulinum*. C. *novyi* produces the lethal alpha toxin, which is related to the alpha toxin produced by *Clostridium septicum*. In C. *novyi*, the alpha toxin gene is located on a bacteriophage (202) and any particular C. *novyi* strain may also produce one of two phospholipases (beta and gamma) (189). C. *haemolyticum* produces a phospholipase serologically indistinguishable from the beta phospholipase of C. *novyi* (189). Group III C. *botulinum* strains are associated with the expression of BoNT/C and BoNT/D and the chimeric derivatives, C/D and D/C (203) (Table 2). The location of the *bont* locus within group III strains of C. *botulinum* has been known for many decades to be within temperate bacteriophage, called pseudolysogens, that could be readily cured from toxigenic strains (203). The curing mechanism was shown to be due to the unusual nature of the lysogenic state — that of a replicating plasmid (203). For example, the *bont*-encoding

bacteriophage from strain C-ST has a large (186-kb) linear genome with 404-bp terminal direct repeats when packaged within the phage particle (203), but the genome is found as a circular, self-replicating molecule within the bacterial cell (189, 203). Many other plasmid and phage-like molecules can be found not just in C. *botulinum* group III, but in all C. *novyi sensu lato* strains, indicating that this group is highly dependent on the accessory genome to determine the virulence of any particular strain (189, 202–205).

Group IV: – *Clostridium argentinense*

This group was renamed C. *argentinense* because it had very little similarity to other C. *botulinum* strains other than the production of BoNT (171). Only BoNT/G has been detected in C. *argentinense* and the *bont*/G toxin gene was demonstrated to be located on large plasmids in at least two strains (206) (Table 2). The first type G strain sequenced, CDC 2741, remains a draft assembly, but analysis of the sequence data indicated that the *bont*/G cluster was located on a large 1.1-Mb contig and was thought to be chromosomally located (172, 207). Recently, another completed genome sequence for a BoNT/G-expressing strain was released, strain 89G (208), and a circular plasmid sequence was obtained and shown to carry the *bont*/G locus. This plasmid, pRSJ17_1, is 140 kb in size and, although no further analysis of this sequence has been published (208), it appears to share little or no similarity to plasmids of group I or II C. *botulinum* strains. However, the entire pRSJ17-1 sequence appears to be located within the 1.1 Mb contig assembled from the genome sequence of strain CDC 2741 so it is possible that either the *bont*/G plasmid has become integrated into the chromosome in this strain or that the CDC 2741 draft assembly is erroneous and CDC 2741 contains a 140-kb plasmid almost identical to pRSJ17-1. Further analysis is required to confirm the toxin cluster location in strain CDC 2741.

Group V: – Neurotoxigenic *Clostridium baratii*

Type V BoNT-expressing strains are not designated as C. *botulinum* but are members of the non-neurotoxigenic species, C. *baratii* (172, 173). Again only one serotype of BoNT has been detected from this species: BoNT/F7, and the location of this gene is chromosomal, where it is flanked by directly repeated IS*1182* elements (207) (Table 2). The context of the *bont*/F7 locus in this strain suggests that it has been acquired by horizontal gene transfer (207).

Group VI: – Neurotoxigenic *Clostridium butyricum*

Neurotoxigenic C. *butyricum* strains have only been demonstrated to carry *bont*/*E* genes within *orfX*+ loci (173) (Table 2). The neurotoxin gene cluster was originally thought to be plasmid determined rather than chromosomal (209). However, more recent data have clearly indicated that the *bont*/*E* genes in C. *butyricum* are chromosomally located (173, 210, 211). The *rarA* gene is the position of insertion for the *bont*/*E* locus in both group II C. *botulinum* strains that carry *bont*/*E* as well as neurotoxigenic C. *butyricum* strains (172, 173, 199), which suggests that the *bont*/*E* locus has most likely been inserted *via* the same mechanism in both organisms, possibly via the recombinase RarA (172, 173).

CLOSTRIDIOIDES (*CLOSTRIDIUM*) *DIFFICILE* VIRULENCE PLASMIDS

C. *difficile* is a genomically diverse species, that consists of eight phylogenomic clades (212), and it contains many integrated and extrachromosomal genetic elements (213). The presence of numerous potentially mobile genetic elements suggests that horizontal gene transfer has played an important role in the evolution of C. *difficile*. Although C. *difficile* pathogenesis involves multiple factors (214), disease resulting from infection with this microbe is primarily toxin-mediated, and strains can encode up to three toxins. These toxins include toxin A (TcdA) and toxin B (TcdB), which are both encoded within the Pathogenicity Locus (PaLoc), and the C. *difficile* transferase or binary toxin (CDT), encoded within the CDT locus (CdtLoc) (215). Both the PaLoc (216) and CdtLoc (217, 218) were found in chromosomal locations when first identified. However, the chromosomal PaLoc is mobilizable, and can be transferred to a non-toxigenic recipient thereby converting it to a toxigenic strain, but neither a defined mobilizable or conjugative PaLoc element nor the mechanism of transfer have been determined (219).

Few studies have investigated plasmids in C. *difficile* and only a small number of native plasmids have been described or characterized to date. Unlike many other clostridial species, in which toxin genes are often encoded on extrachromosomal elements, as discussed earlier in this review, C. *difficile* toxins appeared to be encoded exclusively on the host chromosome (219, 220). However, a recent study using single-molecule real-time sequencing technology showed that the genome of a bacteriophage, designated phiSemix9P1, carries a complete functional binary toxin locus within its genome (221). The location of the CDT genes on this 56.6 kb bacteriophage suggests that a transduction-mediated mechanism may be involved in transferring these toxin genes across different C. *difficile* lineages. Of relevance to this review, the genome of phiSemix9P1 was found to encode a putative ParA-like protein and a gene for a protein that might play a role in the initiation of plasmid replication in addition to typical bacteriophage-encoded genes involved in DNA packaging, head and tail morphogenesis or host cell lysis, suggesting that this element may represent a bacteriophage-plasmid hybrid (221). Such hybrid elements have recently been identified in C. *difficile* (221), and the presence of these elements can sometimes modulate the virulence of strains by regulating the expression of the chromosomally located toxin genes (222). Genome sequencing studies are continuing to identify new and novel hybrids (223) which may impact phenotypes that influence strain virulence or lateral gene transfer processes, and therefore the evolution and dissemination of C. *difficile* variant strains. The ubiquity of mobile elements in C. *difficile* suggests that horizontal gene transfer plays an important role in the movement of DNA between strains, and the identification of plasmid-bacteriophage fusions may suggest that intra- and inter-species DNA exchange has occurred, probably within the diverse bacterial populations found in the gastrointestinal environment (213).

CONCLUSIONS

The importance of mobile genetic elements to toxin dissemination within the clostridia is clearly evident. These genera produce a large variety of protein toxins, including the three most potent toxins known to man: botulinum, tetanus and epsilon toxins. Many of these toxin genes are not only located on extrachromosomal elements, but also may be associated with mobile genetic elements that may facilitate their spread from one plasmid or bacteriophage to another. The carriage of these toxins on extrachromosomal elements greatly facilitates the spread of toxin genes either within a species or between species. No doubt the extent of the spread

detected to date is limited only by our survey capabilities and the advent of next generation sequencing will facilitate the identification of an even greater range of elements that can facilitate the movement of the genes encoding these important, and sometimes devastating, toxins.

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Enterotoxic Clostridia: *Clostridium perfringens* Enteric Diseases

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60

INTRODUCTION

Clostridium perfringens is a Gram-positive, spore-forming rod with ubiquitous environmental distribution, including a presence in soil and sewage (1–3). Given its anaerobic nature, it is unsurprising that C. *perfringens* is also a component of the normal gastrointestinal (GI) tract microbiota of humans and other animals (1, 2, 4). Additionally, this bacterium is an important cause of intestinal and histotoxic infections in humans and other animals (2, 5, 6).

The pathogenicity of C. *perfringens* largely reflects the toxin-producing proficiency of this bacterium, which can make at least 20 different toxins, including many with GI tract activity (6–10). However, toxin production patterns vary greatly among different strains. This variability permits classification of C. *perfringens* isolates based on their production of certain toxins referred to as typing toxins. Historically, five C. *perfringens* types were recognized, but this system was recently expanded (11) to include seven types (A to G), as shown in Table 1.

Due to their medical importance, this article will primarily focus on type F strains of C. *perfringens*. It will also briefly mention other C. *perfringens* strains that cause human or livestock GI diseases.

C. PERFRINGENS TYPE F FOOD POISONING

Epidemiology

Type F strains are the causative agents of C. *perfringens* type F food poisoning (formerly referred to as C. *perfringens* type A food poisoning prior to the recent typing system revision), which is the second most common bacterial foodborne illness (12, 13). A million cases of this disease occur annually in the United States, involving ~$400 million in economic losses (12–14). The high prevalence of C. *perfringens* type F food poisoning is attributable to several factors (1). First, type F strains are common in foods (15, 16), providing ample opportunity for infection if that food is incompletely cooked or held prior to ingestion. Second, this bacterium has a very short doubling time of ~10 minutes, which allows it to grow quickly in contaminated food to reach the bacterial load (>10^6 vegetative cells/gram of food) needed to initiate GI disease. Last, most C. *perfringens* type F food poisoning strains have an outstanding ability to survive in incompletely cooked or improperly held foods due to (i) the heat tolerance of their vegetative cells, which grow in up to at least 50°C (17) and (ii) the even greater heat, cold, and food preservative resistance of their spores, as discussed below.

C. *perfringens* type F food poisoning outbreaks are typically large, averaging ~100 cases (1). This large outbreak size is attributable to at least two factors. First, C. *perfringens* type F food poisoning is strongly associated with institutional settings because such facilities need to prepare and store foods in advance of serving, which provides sufficient time for C. *perfringens* to grow and reach pathogenic loads in improperly cooked or held foods. Second, public health officials often overlook small food poisoning outbreaks not involving fatalities; therefore, small outbreaks of this type of food poisoning typically go unidentified.

The most important contributing factor to C. *perfringens* type F food poisoning outbreaks is holding foods at improper temperatures (1, 13). Because of the relative heat tolerance of C. *perfringens* vegetative cells, food should either be stored under refrigeration or held at temperatures >65°C to prevent growth of this bacterium. Incomplete cooking is the second leading contributor to development of this type of food poisoning since it allows C. *perfringens* spores to survive and then germinate in undercooked foods. Beef roasts and turkey are among the most common vehicles for this type of food poisoning because those large food items are difficult to cook thoroughly (1, 13).

The laboratory plays an important role in identifying C. *perfringens* type F food poisoning outbreaks (1). The most reliable approach involves serologic demonstration of C. *perfringens* enterotoxin (CPE) in feces from food poisoning victims. Several commercial assays for fecal CPE detection are available. Alternatively, it can also be informative to demonstrate, by PCR, the presence of *cpe*-positive C. *perfringens* in foods or feces.

Pathogenesis

C. *perfringens* type F food poisoning begins with the ingestion of food contaminated with vegetative cells of a type F strain (1). Most of those bacteria die upon exposure to gastric acid but, if the ingested food was heavily contaminated (i.e., >10^6 to 10^7 vegetative cells/gram of food), some survive to pass into the intestines. After initial growth, those C. *perfringens* cells then undergo *in vivo* sporulation. The trigger for this response is unclear but may involve exposure of ingested

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TABLE 1 Revised C. *perfringens* toxin-based typing scheme[a]

Toxinotype	Toxin production					
	α-Toxin (*plc* or *cpa*)	β-Toxin (*cpb*)	ε-Toxin (*etx*)	ι-Toxin (*iap* and *ipb*)	CPE (*cpe*)	NetB (*netB*)
A	+	–	–	–	–	–
B	+	+	+	–	–	–
C	+	+	–	–	+/–	–
D	+	–	+	–	+/–	–
E	+	–	–	+	+/–	–
F	+	–	–	–	+	–
G	+	–	–	–	–	+

[a]NetB, necrotic enteritis B-like toxin; +, toxin is produced; –, toxin is not produced.

C. *perfringens* cells to low pH in the stomach or to bile salts or phosphate in the intestines (18–20).

Considerable evidence implicates CPE as the toxin responsible for the GI symptoms of C. *perfringens* type F food poisoning (reviewed in reference 1). First, this toxin is detectable in the feces of nearly all C. *perfringens* food poisoning victims but is absent from the feces of healthy people. Second, the CPE concentrations present in the feces of food poisoning victims are similar to the CPE concentrations that cause GI effects in animal models. Third, type F strains are much more virulent in animal intestinal disease models than are most CPE-negative type A strains, which (like type F strains) produce alpha-toxin but (unlike type F strains) do not produce CPE. Importantly, the GI effects of type F strains can be neutralized with a CPE antibody. Fourth, human volunteers fed purified CPE developed the GI symptoms of C. *perfringens* type F food poisoning. Last, and perhaps most persuasive, are studies fulfilling molecular Koch's postulates, which showed that an isogenic *cpe* null mutant of a type F food poisoning strain is fully attenuated in a rabbit small intestinal loop model of GI disease, and complementation of that mutant to regain CPE production also fully restored virulence (21).

Only sporulating C. *perfringens* cells produce CPE (1, 22, 23). Once made, this toxin is not secreted but is instead freed into the intestinal lumen only when the mother cell lyses to release its mature endospore upon the completion of sporulation (24). CPE then binds to the intestinal epithelium and exerts its cytotoxic action, described below, causing villus shortening and intestinal epithelial desquamation. Animal model studies suggest that this intestinal damage is responsible for the onset of the GI symptoms associated with C. *perfringens* type F food poisoning (25–27), although contributions by other processes (such as CPE effects on paracellular permeability) cannot be excluded.

All regions of the small intestine respond to CPE, but the ileum is particularly sensitive (28, 29). Interestingly, while CPE binds to, and initially damages, villus tips in the small intestine, the toxin eventually damages the entire villus (25). Recent cell culture studies, described below, identified a bystander killing effect that may help to explain this *in vivo* observation (30).

Based upon an initial study in which CPE did not affect the rabbit colon (31), it was originally believed that C. *perfringens* type F food poisoning only involves the small intestine. However, later studies demonstrated that purified CPE does affect the rabbit colon (32) and it can also damage human colonic tissue *ex vivo* (33). Furthermore, colonic damage has been observed in some human C. *perfringens* type F food poisoning victims (34). Recent studies suggest that CPE acts focally in the human colon, mainly damaging cells with exposed receptors (35).

Clinically, C. *perfringens* type F food poisoning usually involves diarrhea and abdominal cramps that develop within ~8 to 16 h of ingesting contaminated food (1). This incubation period largely reflects the ~8 to 12 h needed for C. *perfringens* to complete sporulation and then release CPE into the intestinal lumen. In most cases, the GI symptoms of this type of food poisoning persist for 12 to 24 h before self-resolving. However, it has long been recognized that this disease can be fatal in the elderly or debilitated; it is estimated that ~26 people die from C. *perfringens* type F food poisoning every year in the United States (12).

In addition, two unusually severe C. *perfringens* type F food poisoning outbreaks occurred during the past 15 years, resulting in the deaths of several relatively healthy and younger people (34, 36). Both outbreaks involved psychiatric institutions, which is relevant because the severity of these outbreaks appears attributable to the victims having predisposing medical conditions prior to contracting C. *perfringens* type F food poisoning. Specifically, the severely affected individuals in both outbreaks had been receiving psychoactive medications that can induce severe constipation or fecal impaction side effects. Those side effects likely prevented the GI tract flushing effects of the diarrhea that typically occurs during C. *perfringens* type F food poisoning. The absence of diarrhea in these cases is thought to have prolonged intestinal contact with CPE, thus enhancing disease severity. Studies with a mouse model (37) supported this hypothesis by showing that prolonged intestinal contact with purified CPE increases uptake of this toxin into the circulation. This allowed CPE to damage internal organs, such as the liver and kidneys, resulting in a hyperpotassemia that induces cardiac arrest. Those mouse model studies suggest that pre-existing severe constipation or fecal impaction can facilitate development of a lethal enterotoxemia following C. *perfringens* type F food poisoning (37).

The CPE Protein

CPE is a single 319-amino acid polypeptide (M_r 35,317; pI, 4.3) (38). Its primary amino acid sequence lacks significant sequence homology with other proteins, except for some limited sequence homology (of unknown significance) with a nonneurotoxic protein of C. *botulinum* (39). The secondary structure of CPE is ~80% beta-pleated sheet and ~20% random coil (28).

The structure of CPE was solved in a series of studies that began with crystallization and X-ray diffraction analysis of the C-terminal domain of CPE, which mediates binding of the toxin to receptors, as discussed below. That initial study (40) showed that this CPE domain consists of a nine-stranded beta-sandwich, with most strands in an antiparallel arrangement. Tyrosine and leucine residues implicated (41) (described below) in receptor binding line a pocket located on the surface of this C-terminal CPE domain, where they can readily interact with receptors (40).

The complete structure of native CPE was solved independently by two groups in 2011 (42, 43). Those structural analyses (Fig. 1A) revealed that, in addition to the C-terminal domain mediating receptor binding activity, this pore-forming enterotoxin also has an N-terminal domain required for CPE oligomerization and pore formation. The 34 N-terminal amino acids of CPE appear to be disordered, explaining why their presence reduces CPE-induced cytotoxicity (see below). Although lacking primary sequence homology with other proteins, CPE structurally belongs to the aerolysin pore-forming toxin family, which also includes *C. perfringens* epsilon toxin. A region in the N-terminal domain named TM1 (for transmembrane 1), which is important for pore formation (as discussed below), is largely present in an α helix when the native CPE monomer is in solution. This is distinctive since comparable sequences in other members of the aerolysin pore-forming toxin family are present in a beta-strand (42, 43).

Structure versus function relationships have been extensively mapped for the CPE protein. Early deletion mutagenesis research (44), coupled with chemical cleavage analyses (45), revealed that the C-terminal domain of the toxin is sufficient for receptor binding. That research also showed that the N-terminal domain must also be present to obtain cytotoxicity and cause GI tract pathology. Later studies using recombinant CPE fragments and synthetic peptides showed that even the 30 C-terminal amino acids of CPE alone possess some receptor binding activity (46). Subsequent site-directed mutagenesis results identified three tyrosine residues (Y306/Y310/Y312) and a leucine triplet (L223/L254/L315) in the C-terminal half of CPE as being important for receptor binding (41).

While full-length CPE (amino acids 1 to 319) is cytotoxic, removing up to the first 45 amino acids from CPE by deletion mutagenesis increases cytotoxic activity by 2- to 3-fold (47). This activation effect, which does not correspond to removal of a leader sequence since CPE is not a secreted protein, may have pathologic relevance; i.e., purified trypsin and chymotrypsin also induce a 2- to 3-fold increase in CPE-induced cytotoxic activity by removing the first 25 or 37, respectively, N-terminal amino acids from the native toxin (48, 49). However, deleting amino acids beyond residue 45 renders CPE noncytotoxic (50). Subsequent site-directed mutagenesis studies identified several amino acids located between residues D45 and G53 of the native toxin as mediating CPE oligomerization (51). In particular, residue D48A is required for both CPE oligomerization and cytotoxicity (51).

Besides oligomerization, the N-terminal domain of CPE mediates a second function during CPE-induced cytotoxicity. This CPE domain contains TM1, a region consisting of alternating hydrophobic and hydrophilic residues spanning from amino acids 80 to 106 of native CPE (52, 53). As mentioned earlier, the TM1 region is mainly localized within an alpha-helix in the N-terminal domain of native CPE. However, once CPE oligomerizes, the TM1 α-helix is thought to unwind to form a beta-hairpin loop that participates in pore formation (as described in the next section).

CPE-Induced Cytotoxicity

CPE binding

The cytotoxic action of CPE begins (Fig. 2) with binding of this toxin to its receptors (54). Conclusive insights into CPE

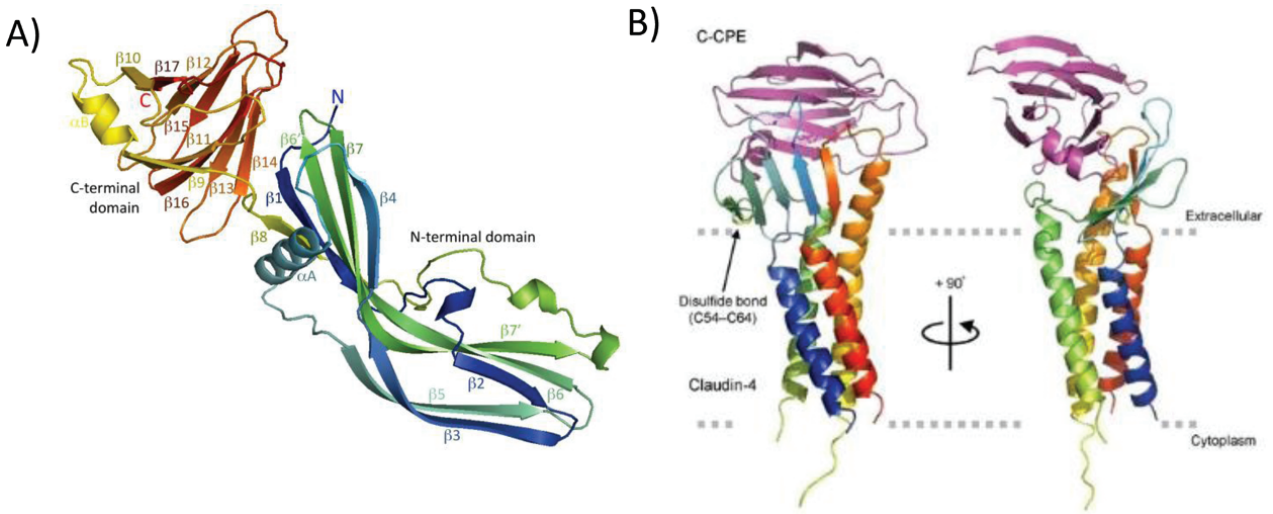


FIGURE 1 Structure of CPE and the C-terminal CPE structure bound to a claudin-4 receptor. (A) Structure of the CPE monomer, colored from blue at the N-terminus to red at the C-terminus. Note the presence of two distinct domains, including the C-terminal domain (red/yellow) mediating receptor binding and the blue-green N-terminal domain mediating oligomerization, membrane insertion, and pore formation. The alpha helix labeled αA is located at the TM1 region that becomes a beta-hairpin when CPE is assembled in the prepore. Beta-hairpins from the seven CPE molecules in the prepore are thought to then form a beta-barrel that inserts into membranes to form the active pore. (B) Structure of the C-terminal CPE region (C-CPE) bound to the human claudin-4 receptor. The claudin receptor is rainbow-colored and the C-CPE is purple. The membrane orientation of the claudin receptor, including the transmembrane helices, is also shown. Panels A and B are reproduced with permission from references 42 and 66, respectively.

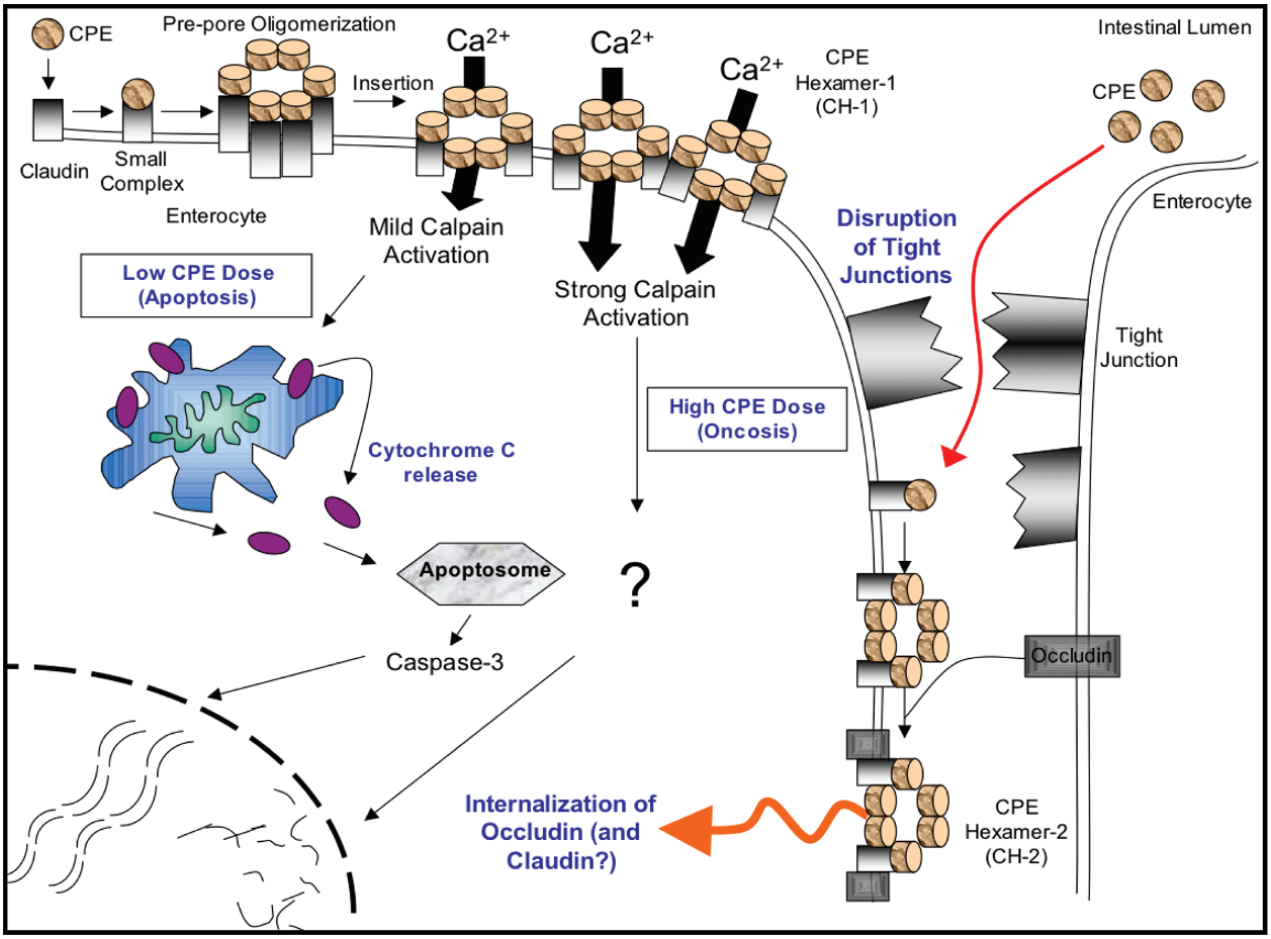


FIGURE 2 Model of the CPE mechanism of action. At the top left, CPE binds to receptors forming a small complex that contains both receptor claudins and nonreceptor claudins, as well as CPE. At 37°C, several small complexes interact to form a prepore on the membrane surface. Portions of CPE in the prepore insert into the membrane to form a pore that allows Ca^{2+} entry into the cytoplasm. With high CPE doses, there is a massive Ca^{+2} entry that causes strong calpain activation to trigger oncosis (a form of necrotic cell death). At low CPE doses, there is a more limited Ca^{2+} entry that causes a mild calpain activation; this results in mitochondrial membrane depolarization, cytochrome C release, and caspase-3 activation to cause death by apoptosis. Dying CPE-treated cells undergo morphologic damage that exposes their basolateral surface to CPE, resulting in formation of a second large complex containing occludin (as well as CPE and claudins), which induces internalization of these molecules. This effect may contribute to paracellular permeability changes, at least in cultured cells. Reproduced with permission from reference 54.

receptors were provided by expression cloning studies that identified two CPE-binding host proteins, later determined to be claudins-3 and -4 (55, 56). Claudins are a family of ~21- to 27-kDa single polypeptides that play a critical role in maintaining the normal barrier and fence properties of the tight junction (54). Humans are thought to produce 27 different claudins (54). Structural analyses (57) revealed that claudins consist of a four-transmembrane domain bundle and two extracellular loops, referred to as ECL-1 and ECL-2. Claudins also possess a short (~7-amino acid) N-terminal sequence and a C-terminal tail (~20 to 60 amino acids), both of which are located in the host cell cytoplasm. The sequence of the C-terminal tail is variable, allowing discrimination between different claudins using antibodies raised against synthetic peptides with a claudin tail sequence.

Experiments established that, in addition to claudin-3 and -4, CPE can use claudin-6, -7, -8-, -9, and -14 as receptors (54, 58, 59). The CPE binding affinity among those claudin receptors varies greatly, with some (e.g., claudin-3 and -4) binding CPE strongly but others (e.g., claudin-8 and -14) binding CPE with only moderate affinity (59, 60). Furthermore, not all claudins are CPE receptors, because claudin-1, -2, -5, -10, -13, and -20 to -24 cannot bind significant amounts of CPE at physiologically relevant toxin concentrations (54, 58, 59, 61, 62).

Substantial research has focused on the basis for the different CPE binding abilities among claudins. Early studies used chimeric claudins (54, 58) to demonstrate the importance of ECL-2 for CPE binding. Later work (54, 59, 62) identified specific amino acids in ECL-2 that contribute to CPE binding. In particular, an N residue is conserved in the ECL-2 of receptor binding claudins, while the corresponding residue is never an N (usually it is a D or S) in claudins that are unable to bind the toxin (62). Site-directed mutagenesis studies (62) directly confirmed the importance of this conserved N residue for CPE binding. ECL-2 residues adjacent to the essential N residue vary among the receptor claudins, and those variations modulate the CPE binding affinity of different receptor claudins (62–64). Structural biology studies demonstrated that the N residue and adjacent P and L/S

residues dock tightly in the tyrosine pocket of the C-terminal CPE domain (57, 65).

Recently, the structure of the CPE C-terminal domain bound to a claudin receptor was solved (57, 66) (Fig. 1B), which provided a major surprise by showing that CPE interacts with ECL-1, as well as with ECL-2. ECL-1 sequences are conserved in all claudins, explaining why previous chimeric claudin studies did not pick up CPE interactions with ECL-1. The strong sequence conservation among the ECL-1 of different claudins is also consistent with a model where ECL-2 is the primary determinant of CPE binding ability among the claudins, although CPE also interacts with the conserved ECL-1 in receptor claudins. Supporting the importance of CPE interactions with ECL-1 for receptor binding were site-directed mutagenesis studies showing that mutation of ECL-1 residues A39 to I41 affects CPE binding to claudin receptors (57).

Postbinding steps in CPE action

Binding of CPE to a claudin receptor results in the formation of a small complex of ~90 kDa (67). Coimmunoprecipitation and electroelution results (68) suggest that this small complex can contain a nonreceptor claudin (e.g., claudin-1), as well as CPE bound to the claudin receptor. Heteromer gel shift studies (68) indicated that 6 small CPE complexes then oligomerize to form the CH-1 large complex, with a molecular mass of ~425 to 500 kDa. It is believed that CH-1 is initially present as a prepore on the membrane surface of host cells, but each CPE in the prepore then unwinds its TM1 α-helix to form a beta-hairpin loop (52, 53). Similar loops likely extend from each of the 6 CPE molecules present in the prepore oligomer, forming a beta-barrel that inserts into the membrane to create an active pore. In support of this model, site-directed mutagenesis identified several TM1 variants that have normal binding and oligomerization properties but are impaired for membrane insertion or for formation of a functional pore (52).

CPE pore formation leads to a rapid efflux of some cytoplasmic ions (e.g., potassium) but an influx of other extracellular ions, including calcium (28, 69–72). CPE-induced calcium influx is particularly important since it activates, in a toxin dose-dependent manner, calpain and calmodulin to cause cell death (70, 73). At low CPE doses (~1 μg/ml), modest pore formation occurs to cause a limited calcium influx and a mild activation of calpain that triggers caspase-3 dependent apoptosis (70, 73). However, high CPE doses (~10 μg/ml) form large numbers of pores, allowing a massive calcium influx that results in strong calpain activation to cause necrotic death involving oncosis (70, 73).

Cells affected by CPE develop morphologic damage (71, 74). This allows even more CPE binding to the abundant claudin receptors present on the basolateral cell surface (75). In Caco-2 cells, CPE-induced cell damage also permits some receptor-bound CPE to interact with another tight junction protein named occludin to form an ~600-kDa complex named CH-2 (68, 75, 76). The importance of this complex for CPE activity, particularly *in vivo*, is not clear since only the CH-1, (i.e., not the CH-2) complex is detectable in CPE-treated rabbit small intestine (37).

CPE Induces a Bystander Killing Effect

The human body is a complex mixture of host cells, including cells that produce claudin CPE receptors and respond to this toxin, as well as other cells that do not make CPE receptors and are not directly sensitive to this toxin. Therefore, a recent study (30) asked whether CPE effects on sensitive host cells might impact nearby CPE-insensitive host cells. When a coculture of CPE-sensitive and CPE-insensitive cells was treated *in vitro* with the enterotoxin, many of the CPE-insensitive cells died. This bystander killing effect was shown to involve a factor, possibly a 10- to 30-kDa serine protease, that is released from CPE-sensitive cells when they undergo CPE-induced, caspase 3-mediated apoptosis. The cytotoxic host factor released from those dying cells then induces a caspase 3-mediated apoptosis in the naturally CPE-insensitive cells. Collectively, these results suggest that a similar bystander killing effect may amplify CPE action *in vivo* and thus contribute to disease.

CPE Genetics

Less than 5% of global *C. perfringens* isolates carry the gene (*cpe*) encoding the enterotoxin (77). Those *cpe*-positive strains belong to types C, D, and E, as well as type F (77–81). In type F strains, the *cpe* gene can be present on either the chromosome or large plasmids (82–84); no type F strain has been found that carries both a chromosomal and plasmid-borne *cpe* gene. In other types, the *cpe* gene is always present on large (>70-kb) plasmids (78, 79, 82, 85).

Whether located on plasmids or the chromosome, the *cpe* open reading frame sequence is highly conserved among type C, D, and F strains (84, 86). However, type E strains can possess either silent *cpe* sequences (due to loss of the *cpe* promoter and the presence of mutations, including a nonsense mutation, in the *cpe* ORF) or a functional *cpe* gene that encodes a variant CPE with ~10 amino acid sequence differences from the classical *cpe* ORF found in type C, D, and F strains (85, 87). Whether those amino acid sequence differences affect CPE cytotoxic activity is unknown.

Most (~70%) type F food poisoning strains carry a chromosomal *cpe* gene (1, 88–91). This association is not due to the chromosomal *cpe* strains producing either more CPE or a CPE variant with greater cytotoxicity (84). Instead, it is attributable to at least two factors: (i) the exceptional resistance of the spores made by most chromosomal *cpe* strains (17), which likely facilitates their survival in incompletely cooked foods, and (ii) the vast majority of *cpe*-positive strains present in retail raw meat/fish having a chromosomal as opposed to a plasmid-borne *cpe* gene, providing the chromosomal *cpe* strains with much greater opportunity to cause food poisoning (1, 16). The reason for the greater prevalence of chromosomal *cpe* strains in meat/fish is unknown but may be related, in part,to their better survival in foods (as explained above).

Genome sequencing of strain SM101, a transformable derivative of the type F food poisoning strain NCTC8798, showed that its *cpe* gene is located in a highly variable region of the chromosome (92). Other chromosomal *cpe* strains appear to carry a similarly organized *cpe* locus (86, 93, 94). The presence of the *cpe* gene in a highly variable chromosomal region is likely due to the integration of Tn*5565*, a putative 6.4 kb-transposon where the *cpe* gene and an upstream IS*1469* sequence are both flanked by IS*1470* sequences (95, 96). The putative Tn*5565* insertion event apparently occurred in the chromosomal locus between *nad*C, encoding a putative quinolate phosphoryltransferase gene, and *uap*C, encoding a putative purine permease (Fig. 3). The ability of adjacent insertion sequences to mobilize the chromosomal *cpe* gene is suggested by PCR studies, which detected circular DNA species carrying the *cpe* gene in DNA extracted from chromosomal *cpe* strains; those circular DNAs may be transposition intermediates carrying the *cpe* gene (95).

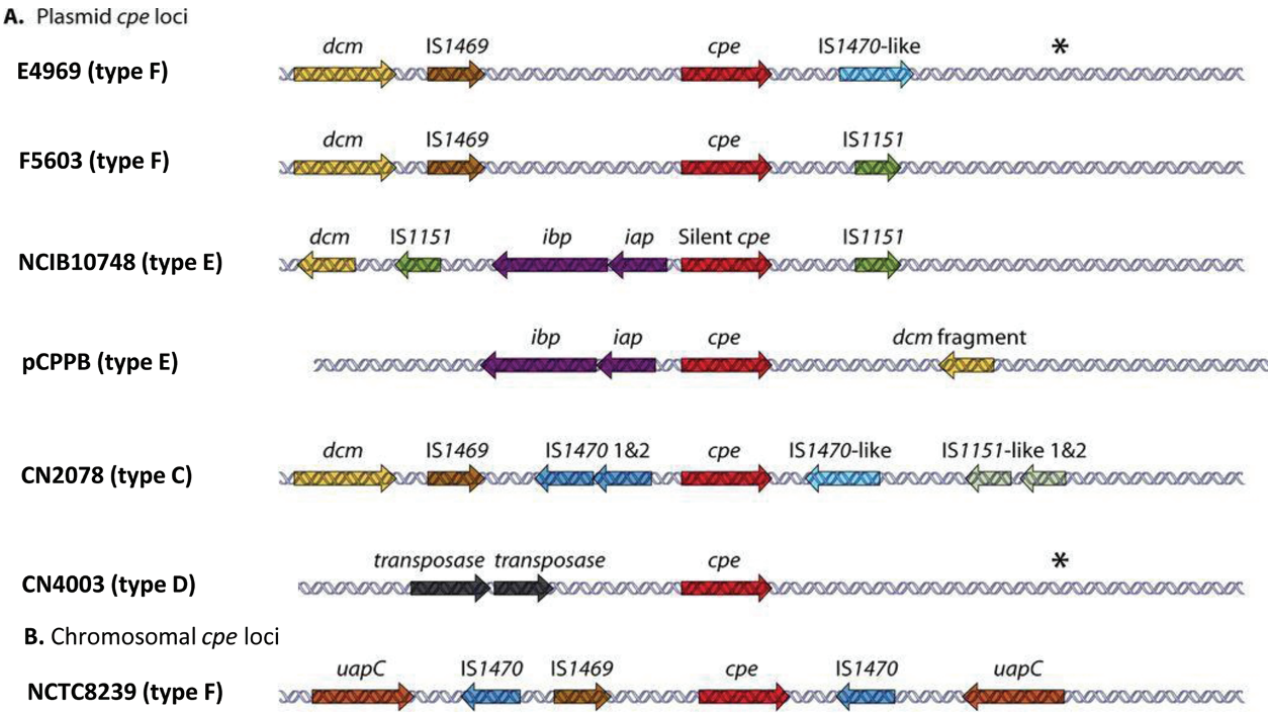


FIGURE 3 Organization of *cpe* loci in type C, D, E, and F strains of C. *perfringens*. (A) Organization of plasmid-borne *cpe* loci in type F, E, C, and D strains. (B) Organization of the type F chromosome *cpe* locus. Asterisks indicate a region with similarity to sequences present downstream of the *cpe* gene in F4969, except for the absence of an IS*1470*-like gene. Modified with permission from reference 82.

Multilocus sequence type studies (80, 97) showed that, beyond their *cpe* gene location, chromosomal *cpe* type F strains differ phylogenetically from type F plasmid *cpe* strains and most other C. *perfringens* strains, with the exception of the type C strains causing a form of enteritis necroticans (EN) known as Darmbrand in post-World War II Germany (discussed further below). Type F chromosomal *cpe* strains and type C Darmbrand strains are both also unusual (80) among C. *perfringens* strains in that they (i) lack the *pfoA* gene that encodes perfringolysin O and (ii) carry a *sasp4* gene that encodes a variant small acid-soluble protein-4 to impart exceptional spore heat resistance (described further below). However, an important distinction between the type F chromosomal *cpe* strains and the type C Darmbrand strains is that when Darmbrand strains carry a *cpe* gene, it is located on a large plasmid rather than on the chromosome (80).

The *cpe* plasmids of type F strains belong predominantly to the pCPF5603 and pCPF4969 plasmid families (82, 98). Both of those plasmid families likely evolved from a common precursor plasmid. The pCPF5603 family later acquired a cluster of metabolic genes, as well as the gene encoding beta2-toxin, while the pCPF4969 family picked up genes encoding a VirS/VirR-like two-component regulatory system and a putative bacteriocin. The *cpe* gene in both of these *cpe* plasmid families is associated with insertion sequences (Fig. 3), including the presence of a 5′ IS*1469* sequence near their *cpe* genes. However, the *cpe* loci in these two *cpe* plasmids lack the upstream IS*1470* sequence upstream found in the chromosomal *cpe* locus. In addition, while an IS*1470* sequence is located downstream of the *cpe* gene in pCPF4969 plasmids, that insertion sequence is absent from the *cpe* locus of the pCPF5603 plasmids. Instead, pCPF5603 plasmids carry an IS*1151* sequence downstream of their *cpe* gene. The presence of these insertion sequences near these *cpe* genes may help to mobilize this toxin gene into circular forms that could be transposition intermediates, as shown for type C and D strains carrying *cpe* plasmids (86).

Insertion sequences are also located adjacent to the plasmid *cpe* gene (Fig. 2) in type C, D, and E isolates (85, 86), as well as the silent *cpe* sequences present on plasmids in some type E strains (86). However, the *cpe* locus is organized differently in those strains compared to type F strains (Fig. 2). In addition, the plasmids carrying *cpe* genes or silent *cpe* sequences in non-type F strains are often larger, ranging up to ~135 Kb, than the pCPF5603 or pCPF4949 plasmids (82, 99). Some *cpe* plasmids in type C, D, and E strains share partial similarity with pCPF5603 or pCPF4969, and they can also carry other toxin genes (78, 79, 99), e.g., the *cpb* gene encoding beta-toxin, the *etx* gene encoding epsilon-toxin, or the *iap* and *ibp* genes encoding iota-toxin (78, 79, 82, 99).

Nearly all *cpe* plasmids, including the pCPF5603 and pCPF4969 plasmids, carry a region named *tcp*, for transfer of clostridial plasmids (82). This carriage is important because the *tcp* locus has been shown to mediate conjugative transfer of another C. *perfringens* plasmid, named pCP13 (82, 100). Those pCP13 results predicted that the pCPF5603 and pCPF4969 *cpe* plasmids should also be conjugative. This postulate was confirmed for pCPF4969 when mixed mating studies demonstrated conjugative plasmid transfer of this *cpe* plasmid (83, 101).

In summary, both the chromosomal and plasmid *cpe* genes are associated with insertion sequences, which may facilitate mobilization of the *cpe* gene within a C. *perfringens* cell. Additionally, the *cpe* gene is often present on conjugative plasmids, which allows transfer of the *cpe* gene from one C. *perfringens* strain to another. If this conjugative transfer

happens during infection, it could contribute to pathogenesis, as explained below in the nonfoodborne human GI disease section.

Regulation of CPE Production

When CPE is produced during sporulation, it can account for >15% of the total protein inside the sporulating C. *perfringens* cell (1). This abundant CPE production is not attributable to a gene dosage effect since most food poisoning strains carry only a single copy of the *cpe* gene. Instead, Northern blotting and reporter studies indicated that *cpe* expression is primarily regulated at the transcriptional level, with the *cpe* gene transcribed as a monocistronic message that becomes detectable soon after the onset of sporulation (1, 28, 102–104).

The presence of three promoters upstream of the *cpe* start codon likely explains the strong production of CPE during sporulation (1, 103). The close association between CPE production and sporulation is attributable to those *cpe* promoters sharing significant homology with SigK-or SigE-dependent promoters in *Bacillus subtilis* (103). Those promoter sequence homologies are important because SigK and SigF are sporulation-associated sigma factors in many *Bacillus* and *Clostridium* spp. (105). A direct role for SigK and SigE in regulating both CPE production and C. *perfringens* sporulation was demonstrated experimentally using *sigK* and *sigE* null mutants (106).

A later study (107) established a role for SigF, another sporulation-associated sigma factor, in regulating both CPE production and C. *perfringens* sporulation. In contrast to early PCR studies (106) suggesting that SigK is the first sporulation-associated sigma factor made during C. *perfringens* sporulation, the later study (107) using Western blotting detected no SigK or SigE production by a *sigF* null mutant. Those Western blot results indicate that during early sporulation, either (i) SigF controls production of both SigK and SigE or (ii) only very low (below Western blot levels of detection) SigK levels are produced before SigF is made. In addition, those Western blot studies (107) demonstrated that SigF controls production of a fourth sporulation-associated sigma factor, named SigG. It was also shown that SigG is necessary for C. *perfringens* sporulation but not for CPE production (107). An emerging model from these and other studies is shown in Fig. 4. Briefly, C. *perfringens* sporulation initiates with phosphorylation of Spo0A by an unknown kinase. Phosphorylation of Spo0A (108), a master regulator

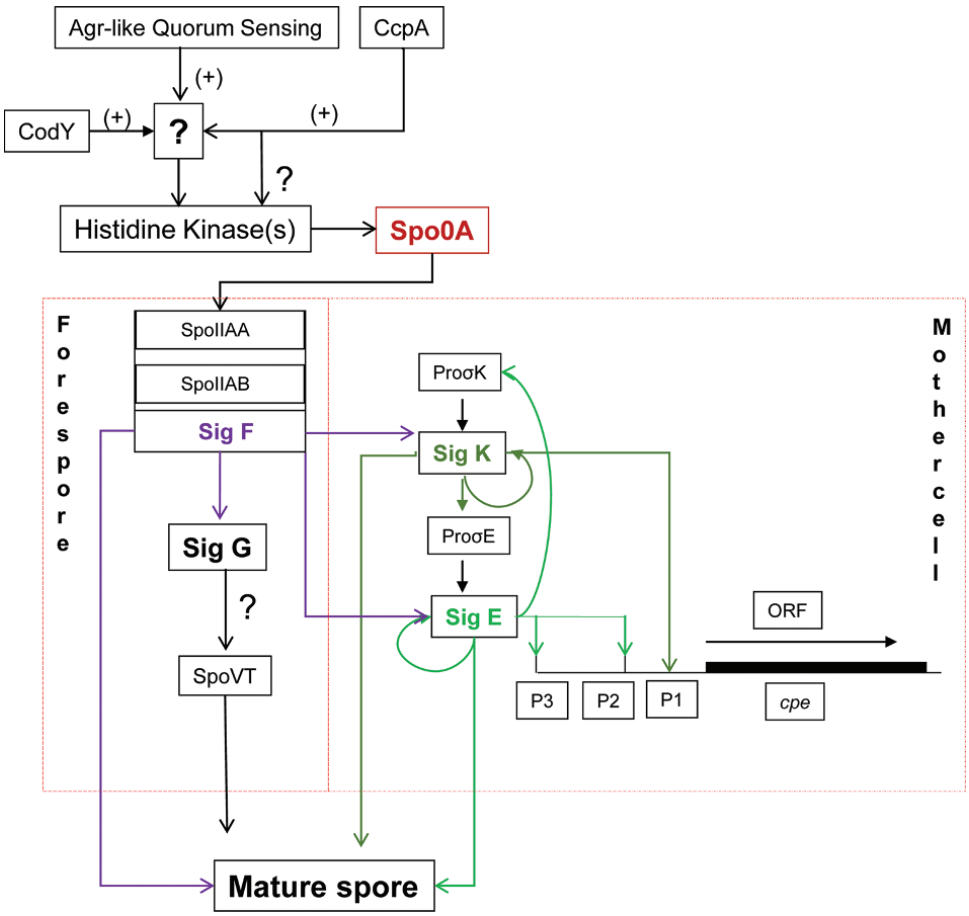


FIGURE 4 Model of C. *perfringens* sporulation and enterotoxin production. Via the Agr-like quorum sensing system, CcpA and CodY, and unidentified intermediates, a histidine kinase(s) affects spoOA expression and/or possibly Spo0A phosphorylation to initiate sporulation. This triggers a sigma factor cascade, where SigF controls production of three other sporulation-associated sigma factors (SigE, SigG, and SigK). SigE and SigK then regulate CPE production during sporulation by enhancing *cpe* expression. All four sigma factors are needed for sporulation. Reproduced with permission from reference 142.

of stationary phase gene expression, leads to production of SigF (107). Once produced, SigF signals the sporulating cell to make SigE, SigG, and SigK. SigE and SigK then direct expression of the *cpe* gene in the sporulating C. *perfringens* cell. All four sporulation-associated sigma factors contribute to the gene expression needed for sporulation (106, 107).

Several other regulators, i.e., CcpA, CodY, and the Agr-like quorum sensing system, have been shown to regulate sporulation and CPE production, but the precise nature of their involvement is unclear at present (109–111).

Other Virulence Factors: Small Acid-Soluble Protein 4

The vegetative cells and, especially, spores of type F strains carrying a chromosomal *cpe* gene are usually much more resistant to heating (cooking), cold (storage in refrigerators/freezers), and food preservatives than cells and spores of most other C. *perfringens* strains (17, 112, 113). The exceptions are type C Darmbrand strains, which also form highly resistant spores and cause human food-borne disease (80). For example, the spores of both type F chromosomal *cpe* strains and type C Darmbrand strains have a D_{100} value (the time needed at 100°C to reduce viability by 1 log) of ~1 h versus a D_{100} value of only ~1 to 2 min for spores made by other C. *perfringens* strains (17, 80).

Small acid-soluble proteins (SASPs) provide important protection to spore DNA. C. *perfringens* produces four SASPs (114, 115). Early studies (114) showed that differences in SASPs 1 to 3 do not explain the exceptional heat resistance properties of spores made by type A chromosomal *cpe* strains or type C Darmbrand strains. Instead, later studies demonstrated that those chromosomal *cpe* type F strains and type C Darmbrand strains forming highly heat-resistant spores produce a variant SASP-4 with an Asp at residue 36, unlike the SASP-4 of other C. *perfringens* strains, which have a Gly at this residue (80, 115). Studies with isogenic mutants and complementing strains confirmed the importance of this variant SASP-4 for the extreme spore resistance properties of type F chromosomal *cpe* stains and, presumably, type C Darmbrand strains (114). The greater protection offered by the SASP-4 Asp_{36} variant appears to involve tighter DNA binding, particularly to AT-rich DNA sequences, by the Asp_{36} compared to the Gly_{36} SASP-4 variant (116). While their SASP-4 variant plays a major role in the strong spore resistance properties of type A chromosomal *cpe* strains, other factors may also contribute (117, 118).

Immunology

There is no evidence that immune responses contribute substantially to the rapid resolution of most C. *perfringens* type F food poisoning cases. Instead, symptoms of this illness are thought to quickly abate due to CPE-induced diarrhea flushing unbound toxin and C. *perfringens* from the GI tract. Most people have CPE antibodies in their serum, but there is no evidence that prior exposure to C. *perfringens* type F food poisoning provides long-term protection against future acquisition of this illness (119, 120).

Treatment and Prevention

Treatment of C. *perfringens* type F food poisoning is symptomatic. However, recent studies showed that mepacrine could be a potential therapeutic since it can inhibit CPE action *in vitro* (121).

As mentioned earlier, the best approach to prevent this type of food poisoning is to thoroughly cook foods and then hold them at temperatures of <4°C or >65°C. No vaccine is licensed for prevention of C. *perfringens* type F food poisoning. However, the C-terminal domain of CPE, which is noncytotoxic and does not cause GI tract effects, contains a neutralizing epitope (1, 48). Immunization of mice with a conjugate consisting of a peptide containing this CPE epitope coupled to a thyroglobulin carrier induced high titers of CPE-neutralizing antibodies in mice, suggesting that this epitope could be a vaccine candidate (122).

CPE-ASSOCIATED NONFOODBORNE HUMAN GI DISEASES

C. *perfringens* type F isolates are also responsible for 5 to 15% of all nonfoodborne human GI disease cases (2, 123, 124), which include antibiotic-associated diarrhea and sporadic diarrhea. In CPE-associated antibiotic-associated diarrhea, patients receiving antibiotics become colonized, often from the nosocomial environment, by type F strains of C. *perfringens* (124–126). CPE-associated nonfoodborne GI disease cases often last longer (up to several weeks) and are typically more severe than most cases of C. *perfringens* type F food poisoning, particularly in the elderly (124–126).

The more chronic duration of CPE-associated nonfoodborne GI diseases may be attributable to the organisms' superior intestinal colonizing ability compared to type F food poisoning strains. *In vitro* studies (127) showed that nonfoodborne human GI disease strains adhere much better than type A chromosomal *cpe* strains to human enterocyte-like Caco-2 cells. This enhanced adherence correlates with the ability of the CPE-associated nonfoodborne GI disease strains, but not chromosomal *cpe* type F food poisoning strains, to produce NanI sialidase (127). Studies (127) of *nanI* null mutants of a nonfoodborne human GI disease strain confirmed a role for this sialidase in Caco-2 cell adherence, where it likely trims the host cell surface to promote bacterial adherence via a combination of surface charge reduction and exposure of adhesin receptors. In addition, sialic acid generated by NanI from host sources such as enterocyte/colonocyte surfaces or GI tract mucus may facilitate the intestinal growth of C. *perfringens* (128). Interestingly, NanI can also increase the activity of CPE and several other intestinally active C. *perfringens* toxins by enhancing their binding to host cells (129, 130).

As mentioned earlier, virtually all C. *perfringens* type F isolates causing CPE-associated nonfoodborne human GI diseases carry a plasmid *cpe* gene, in contrast to the chromosomal *cpe* gene present in most C. *perfringens* type F food poisoning isolates (82, 83, 89, 93, 94). The presence of the *cpe* gene on plasmids, which are conjugative (101), may contribute to the development of CPE-associated nonfoodborne diseases. Unlike C. *perfringens* type F food poisoning, which develops following the ingestion of massive numbers of bacteria, CPE associated nonfoodborne GI diseases likely involve ingestion of low numbers of C. *perfringens* (2). Therefore, inside the GI tract, the presence of the *cpe* gene on plasmids may result in conjugative transfer of this toxin gene to C. *perfringens* in the normal intestinal microbiota, thereby helping to establish and amplify the infection during CPE-associated nonfoodborne GI disease.

OTHER HUMAN GI DISEASES CAUSED BY *C. PERFRINGENS*

Enteritis Necroticans

C. *perfringens* type C strains cause a disease named enteritis necroticans (EN), which was first identified in malnourished

TABLE 2 C. *perfringens* enteric diseases of livestock and poultry

Type	Main enteric disease	Toxin
A	Possible necrotizing and hemorrhagic enteritis in dogs and foals	NetF? (necrotic enteritis F-like toxin)
B	Lamb dysentery; possible necrotizing enteritis in horses	CPB (beta-toxin), ETX (epsilon-toxin)
C	Necrotizing enteritis in neonatal calves, foals, lambs, piglets, and possibly other species	CPB (CPE may be synergistic in some cases)
D	Enterotoxemia in sheep, goats, and cattle	ETX
E	Possible hemorrhagic enteritis in cattle and rabbits	ITX (iota-toxin)
F	Possible enteric disease in horses, dogs, and other species	CPE (enterotoxin?)
G	Necrotic enteritis in chickens, turkeys, and possibly other avian species	NetB (necrotic enteritis B-like toxin)

people in post-World War II Germany, where it was known as Darmbrand (80). EN, known locally as pigbel, was the leading cause of childhood death in Papua New Guinea during the 1960s to 1970s (131, 132). For special occasions, villagers in the Papua New Guinea highlands would barbecue a pig in a pit dug in the ground, thus allowing contamination of the meat with type C strains present in the environment (soil or pigs). When that food was ingested, beta-toxin (CPB) was produced in the intestines and then caused both a necrohemorrhagic enteritis and enterotoxemia. While the presence of high trypsin levels in the intestines of well-fed people with a normal diet rapidly inactivates CPB, the diet in the highlands population was poor in protein (which reduces trypsin production) but rich in sweet potatoes (which have a trypsin inhibitor), rendering the local people (especially children) highly susceptible to CPB. Type C-induced EN outbreaks occur sporadically in malnourished populations in other developing countries (131). Individual cases are observed occasionally in developed countries, particularly in people with underlying pancreatic diseases such as diabetes (133).

CPB is necessary for type C strains to cause EN (134). However, some strains (including many Darmbrand strains) produce only low amounts of CPB, often along with low amounts of CPE (135). For those strains, it has been shown (135) that CPB and CPE can act synergistically in the intestines to cause pathology. CPB, also a pore-forming toxin (6), has been poorly studied. For example, the receptor for this toxin remains unknown.

The *cpb* gene is always plasmid borne in type C strains; occasionally this gene is located on the same plasmid carrying a *cpe* gene (79, 82). Plasmids carrying the *cpb* gene are predicted to be conjugative since they typically carry the *tcp* locus; however, this has not yet been evaluated (82). Adjacent to the *cpb* gene are insertion sequences that may mobilize the *cpb* gene (79, 82).

The only treatment for EN is prompt intestinal surgery to remove affected portions of the small intestine (131). Immunization with a CPB toxoid during the 1980s to 1990s greatly reduced the prevalence of EN in Papua New Guinea (131, 132).

Another *C. perfringens* Foodborne Human Disease?

Recently, there were reports (7, 8) of several Japanese food poisoning outbreaks that apparently involved *cpe*-negative C. *perfringens* type A strains. Analysis of those strains determined that they produce a binary toxin whose A and B components share 44% and 37% identity, respectively, with C. *perfringens* iota-toxin. This new binary toxin (named CPILE or BEC by different groups) structurally resembles iota-toxin and, like iota-toxin, can ADP-ribosylate actin (136, 137). It causes fluid accumulation in rabbit ileal loops, and null mutants that do not produce the binary toxin lose their intestinal virulence, but complementation of those mutants has not yet been performed (7). Additional research is needed to address the overall importance of strains producing CPILE/BEC for human foodborne disease; e.g., how common are outbreaks caused by these bacteria both inside and outside of Japan?

C. PERFRINGENS ANIMAL DISEASES OF INTESTINAL ORIGIN

C. *perfringens* type B, C, and D strains, and perhaps certain type A and E strains, can cause enteritis, colitis, enterocolitis, and/or enterotoxemia in livestock, while type G strains are responsible for necrotic enteritis of poultry (Table 2). Like C. *perfringens* human enteric diseases, these animal diseases are not intoxications but true infections resulting from C. *perfringens* producing toxins in the intestines. Molecular Koch's postulate analyses have confirmed a role for epsilon-toxin in type D infections, CPB in type C infections, and necrotic enteritis B-like toxin in avian necrotic enteritis (9, 134, 138). Each of those pore-forming toxins is plasmid encoded (82). Detailed discussion of these animal diseases is beyond the scope of this chapter, so readers are directed to several recent reviews on this subject (6, 139–141).

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Because of page limitations, this chapter has cited published reviews for many older studies.

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Enterotoxic Clostridia: *Clostridioides difficile* Infections

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61

INTRODUCTION

Clostridioides difficile is a Gram-positive anaerobic, spore-forming bacterium and the most common identifiable infectious agent of nosocomial antibiotic-associated diarrhea (AAD) (1, 2). This bacterium is also linked to several life-threatening syndromes in humans, including pseudomembranous colitis and toxic megacolon (1, 2). Disease symptoms associated with *C. difficile* infection (CDI), including diarrhea, fluid loss, and inflammation, result from the production and activity of two exotoxins, toxin A (TcdA) and toxin B (TcdB) (2). These toxins disrupt the Rho family of GTPases within the host cell, which eventually results in cell rounding and death (3, 4). *C. difficile* was originally discovered by Hall and O'Toole as *Bacillus difficilis* during their 1935 study of neonatal fecal microbiota and was named "*difficilis*" due to the difficulty of its cultivation and isolation (5). At the time of its initial isolation, *C. difficile* was not considered pathogenic, with recognition of *C. difficile* as a human pathogen occurring roughly 40 years after its initial discovery (5). With time, identification of the obligately anaerobic nature of this organism ultimately resulted in reclassification from *B. difficilis* to *Clostridium difficile*. More recently, with an update to the classification of the clostridia, it was first suggested that *C. difficile* be reclassified as *Peptoclostridium difficile* (6) and, more recently, to *Clostridioides difficile* (7). However, as recently highlighted in Smits et al. (8), there is reluctance for reclassification, because there currently exists a large knowledge base surrounding *Clostridium difficile*, in addition to a strong public awareness which could be lost upon reclassification. Despite these reservations, *Clostridioides difficile* has now been officially approved by the *International Journal of Systematic and Evolutionary Microbiology* and International Committee on Systematics of Prokaryotes and has begun to be used more commonly (9–11).

An increase in clinical CDI rates has been occurring for over a decade, coinciding with the emergence of new epidemic strains, including the BI/NAP1/027 group (referred to as ribotype 027 strains from here on), which appear to have enhanced virulence properties in comparison to historical isolates (8, 12, 13). The emergence of these epidemic *C. difficile* strains has resulted in increased disease severity in infected patients which has been recorded globally and has placed a great financial and administrative burden on hospitals worldwide (14–18).

BACTERIOLOGY, DIAGNOSIS, AND TYPING

Bacteriology and Diagnosis

As a strict anaerobe, the ability of *C. difficile* to form spores is important for disease transmission and recurrence (2). Spores facilitate the survival of *C. difficile* in harsh conditions, including in aerobic environments or in the acidic stomach during transit to the large intestine. Sporulation and spore persistence are major challenges, particularly in nosocomial environments, resulting from the resistance of spores to many commonly used disinfection regimens (19). Spores of *C. difficile* can be induced to germinate at a higher frequency when cultured in media supplemented with bile salts that are usually found in the intestine, which also greatly improves organism recovery from clinical samples (20).

Although *C. difficile* has been established as the leading identifiable cause of infectious AAD in the hospital setting, disease diagnosis remains a contentious issue (21, 22). CDI is defined as acute diarrhea accompanied by the presence of either toxigenic *C. difficile* or its toxins in stool samples (23). However, the lack of a clear distinction between specific clinical CDI and more general non-CDI symptoms in all patients prevents the accurate diagnosis of *C. difficile* disease. Current diagnostic methods for *C. difficile* disease include bacterial culture, enzyme immunoassays (EIAs), cell cytotoxicity neutralization assays (CCNAs), toxin detection tests, and nucleic acid amplification tests (NAATs).

Isolation of *C. difficile* from stool samples requires bacterial culture in an anaerobic environment on selective media such as cycloserine-cefoxitin fructose agar (CCFA) (24). The addition of sodium taurocholate and horse blood to CCFA results in enhanced spore germination and enables colony fluorescence, respectively, allowing for accurate identification (25, 26). However, nontoxigenic *C. difficile* strains also grow in these conditions, and culturing on CCFA can take up to 48 hours, delaying the reporting of results.

Toxigenic and nontoxigenic strains of *C. difficile* constitutively produce the cell wall-associated enzyme glutamate dehydrogenase (GDH), which is highly conserved across different ribotypes (27). A recent large-scale meta-analysis indicated that GDH EIAs are highly sensitive, accurate, and relatively cheap to conduct as screening tests, returning positive results for all strains of *C. difficile*, regardless of the presence or absence of toxin in stool samples (28). These

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assays are often utilized as initial screening tools for the presence of C. *difficile* because they are quicker and more sensitive than toxin EIAs (29). However, GDH EIAs are not recommended as the sole form of diagnosis (30, 31).

CCNAs have historically been viewed as the "gold standard" of CDI detection because they can detect the presence of TcdA and TcdB at picogram levels (31, 32). Cell lines such as Vero (African green monkey kidney epithelial cells) and HT29 (human colorectal adenocarcinoma cells) are cultured in the presence of fecal filtrates, with and without toxin-neutralizing antibodies, and observed for characteristic toxin-induced cell rounding over 24 to 48 hours (33, 34). Although the sensitivity of these assays is very high, CCNAs are more expensive than EIAs and bacterial culture, can take between 72 and 96 hours to complete, and require a high level of technical expertise to perform, making them impractical for routine diagnostic use or during CDI outbreaks (35).

NAATs detect the presence of C. *difficile*-specific nucleic acid sequences in fecal samples, primarily through PCR amplification of conserved regions within the genes that produce TcdA and TcdB (35). In recent years, NAATs have been commonly accepted as the most sensitive form of diagnosis relative to the other methods discussed above and are increasingly used in hospitals despite being more expensive than bacterial culture and GDH EIAs (36). However, NAATs are less specific for CDI compared to CCNAs, because they detect toxin genes instead of active toxin within fecal samples and thus cannot differentiate between asymptomatic carriage of C. *difficile* and CDI, potentially leading to overdiagnosis and overtreatment (36, 37).

Due to the differing degrees of sensitivity and specificity between these individual diagnostic tests, a multistep algorithm is likely to be the most effective strategy for accurate CDI diagnosis and case surveillance. Guidelines released in 2016 for Europe and the United Kingdom recommend the use of the more specific TcdA or TcdB EIAs to confirm CDI in patients that initially yield a positive result for the more sensitive NAATs or GDH EIAs and suggest the consideration of other factors such as the onset of disease symptoms and progression prior to diagnosis and subsequent treatment of CDI (38). Correlating disease symptoms with positive molecular tests could prove vital for accurate CDI diagnosis, because asymptomatic C. *difficile* colonization in otherwise healthy individuals has been reported to be between 4 and 15% via PCR ribotyping and stool culture in geographically distinct regions (39–41). The use of such algorithms is proposed to provide a quicker and more accurate diagnosis of positive CDI cases and to decrease false-positive results. Importantly, reducing false-positive cases decreases the risk of susceptible patients being physically grouped together with true CDI patients in hospitals, thereby exposing them to C. *difficile* (36). This also prevents unnecessary antibiotic treatment for CDI, which may lead to the rise of multidrug-resistant organisms such as vancomycin-resistant enterococci (42). Overall, algorithm-based diagnoses provide a more accurate representation of C. *difficile*-induced diarrhea, although the composition of each algorithm may vary between hospitals (22).

Strain Typing

Typing of C. *difficile* isolates is essential for identifying the strains responsible for CDI outbreaks and for monitoring the spread and emergence of new strain types. Several typing schemes have been developed for C. *difficile*, utilizing the molecular and serological differences that exist between strains (43, 44). Although several schemes have been developed, in recent years molecular techniques, specifically PCR-based methodologies, have been favored due to their reproducibility and relative low cost (44).

With respect to the recent epidemic ribotype 027 strains, three molecular techniques have primarily been used in their typing (44). The first technique, restriction endonuclease analysis (REA), identifies ribotype 027 strains as the REA type BI (44). In this typing scheme, whole genomic DNA extracts are fragmented by the restriction enzyme *Hin*dIII, followed by agarose gel electrophoresis to separate the resulting DNA fragments (45). The banding pattern obtained is then compared to that of a reference strain, and the strain under question is typed accordingly, utilizing the results obtained through other typing methods to further confirm the strain type (45). The second technique uses pulsed-field gel electrophoresis and results in the ribotype 027 strain belonging to the NAP1 (North American pulsed-field type 1) pulsotype (44). This technique works in a similar way to REA, but it uses the *Sma*I or *Sac*II restriction enzymes to fragment whole genomic DNA, after which pulsed-field gel electrophoresis is performed (44). As with REA, analysis of the resulting banding pattern occurs through comparison to known reference strains, which is then used to classify each isolate (46). The third technique is ribotyping, which has become the typing method of choice in recent times because it is quick, sensitive, and reproducible, effectively distinguishing between different strain types (44, 47). Ribotyping relies on differences in the number of copies and overall length of the rRNA genes within the genome of C. *difficile* strains (47, 48). Due to these differences, amplification by PCR between the 16S and 23S rRNA intergenic spaces results in the production of distinct and characteristic banding or fingerprint patterns which are compared to those produced by reference strains to identify the ribotype to which each isolate belongs (48, 49). Over 400 ribotypes have been identified and, as with the other two typing schemes just described, comparative analysis between the typing schemes can be used to confirm each strain type (47–49).

In addition to the three major schemes used to type C. *difficile* strains, toxinotyping may be used to differentiate between strains (47). Toxinotyping assesses the genetic differences within a discrete 19.6-kb genomic region defined as the pathogenicity locus (PaLoc) (Fig. 1a), with a focus on the *tcdA* and *tcdB* genes, and a comparison is generally made to the PaLoc region of the reference strain VPI10463, which is classed as toxinotype 0 (50, 51). Toxinotypes range from I to XXXI, and new toxinotypes are frequently identified; some of these toxinotypes contain subsets which serve to subdivide toxinotypes with a small number of differences between them (52).

Another technique, multilocus sequence typing, analyzes several C. *difficile* housekeeping and toxin genes to define strains based on their sequence type, which characterizes C. *difficile* strains into 6 distinct phylogenetic clades: clades 1 to 5 and clade C-I (53–58). Each clade represents a variety of both toxigenic and nontoxigenic C. *difficile* isolates, from many different ribotypes (53–58). Clade 1 is the most diverse of the 6 clades, with over 100 sequence types, including several ribotypes of clinical significance, such as ribotypes 001, 012, and 014, which are frequently associated with CDI throughout Europe (59) and throughout Asia and the United States (60, 61). Clade 2 includes the epidemic ribotype 027 strains associated with recent outbreaks and the related ribotype 244, which has a variant TcdB that shows cytopathic effects more similar to *Paeniclostridium sordellii* (previously *Clostridium sordellii*) TcsL, and is associated with more

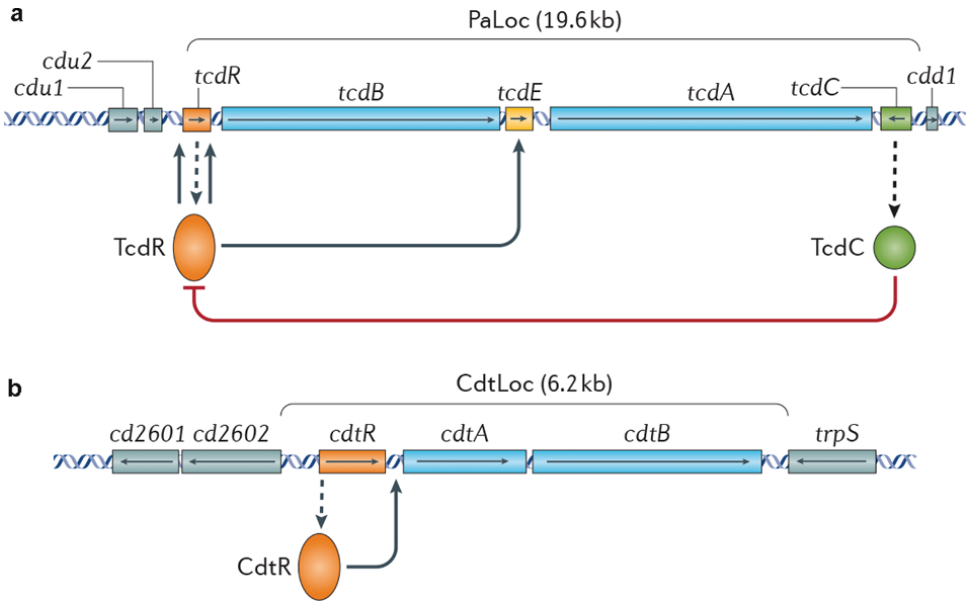


FIGURE 1 Schematic representation of the C. *difficile* pathogenicity locus (PaLoc) and C. *difficile* transferase locus (CDTLoc) and flanking genes. (a) The PaLoc chromosomal region is responsible for both the production and regulation of the major toxins TcdA and TcdB (encoded by *tcdA* and *tcdB*), which are critical for pathogenesis, virulence, and the disease symptoms of CDI. PaLoc also encodes genes involved in the positive (*tcdR*) and negative (*tcdC*) regulation of toxin production, as well as a gene (*tcdE*) encoding a holin-like protein, TcdE, which may be involved in the release of toxins from the cell. Nontoxigenic strains lack this region and are consequently avirulent. (b) The CDTLoc chromosomal region is responsible for both the production and regulation of CDT/binary toxin (encoded by *cdtA* and *cdtB*). CDTLoc also encodes a gene involved in the positive regulation of CDT (*cdtR*). Modified from *Nature Reviews Disease Primers* (8).

severe disease (62, 63). Analysis of clade 3 is less comprehensive, and it appears to be less diverse (62, 63). Clade 4 includes strains from ribotype 017, a $TcdA^{-}TcdB^{+}CDT^{-}$ variant strain that has caused significant disease throughout Asia, as well as being a predominant strain throughout Europe (64–68). Like the ribotype 244 TcdB variant, TcdB from ribotype 017 strains also has similarity to TcsL from *P. sordellii*, specifically in the enzymatic domain of the variant TcdB, which alters the cytopathic effects of the toxin (69). Clade 5 contains C. *difficile* strains from ribotype 078, a ribotype commonly associated with disease in animals, particularly livestock, including pigs and cows; however, in recent times ribotype 078 strains have also been associated with community-acquired cases of human CDI (59, 70, 71).

Clade C-I is a more significantly divergent clade than the other 5 clades and was originally thought to encompass several nontoxigenic strains; however, some recent variant toxigenic strains have been found to cluster with other clade C-I strains (56, 72). Recent work analyzing environmental C. *difficile* isolates has suggested a need for further classification of the clades, with the considerable divergence of clade C-I resulting in 3 distinct clades, C-I, C-II, and C-III (73). Strains belonging to clade C-I/II/III also show considerable divergence from the remaining 5 clades, which has brought into question if these strains are indeed C. *difficile* or if they represent a new species that is distinct from the other 5 clades (74).

Other typing schemes for C. *difficile* include multilocus variable-number tandem repeat analysis, whole-genome sequencing, and single nucleotide polymorphism analysis, all of which provide different genetic perspectives and degrees of sensitivity for strain typing (48). Use of the different typing schemes available for C. *difficile* has shown that a broad diversity of strains exists, and this is true for human clinical and animal isolates. In particular, whole-genome sequencing has provided new insights into the epidemiology and spread of C. *difficile* strains and has changed our understanding of the dissemination and transmission of this bacterium, allowing new and more effective infection control strategies to be developed (75).

EPIDEMIOLOGY

Over the last 2 decades the incidence of CDI has increased dramatically, correlating with the emergence of epidemic isolates of C. *difficile* in hospitals worldwide, in particular, ribotype 027 strains which have gained fluoroquinolone resistance (76). Nosocomial outbreaks of ribotype 027 C. *difficile* strains have undoubtedly contributed to the global increase in CDI rates (13, 77). In late 2002, an outbreak of C. *difficile* occurred in Quebec, Canada, which resulted in a dramatic increase in CDI rates (78). In Quebec, the overall incidence of CDI had increased nearly 4-fold from 1991 to 2003 (78). Furthermore, rates of infection in those above the age of 65 increased roughly 4-fold from 2002 to 2003 during the Quebec outbreak, which resulted in CDI rates increasing 8-fold from 1991 to 2003 in this age group (78). Complications from infection and mortality also increased during this time (78). The ribotype 027 strain responsible for the outbreak spread quickly, leading to several outbreaks across the United States, the United Kingdom, and Europe and highlighting the difficulty in containing CDIs and preventing the spread of disease (17, 77, 79). Interestingly, the increasing use of dietary trehalose has contributed to the dissemination of ribotype 027 strains because their ability to metabolize this

sugar may be an additional factor contributing to the changing epidemiology of this strain type (80).

REA and pulsed-field gel electrophoresis analysis of isolates collected from clinical outbreaks across the United States from 2000 to 2003 highlighted the prevalence of BI/NAP1/027 strains, with an average of 51% of isolates classified as belonging to this class (13). Although the current incidence of ribotype 027 strains appears to be declining in the United States, indicated by surveillance of 10 of the Centers for Disease Control and Prevention (CDC) Emerging Infections Program sites in 2011, the prevalence of this strain type remains high (81). In this study, ribotype 027 strains accounted for 20.6% of community-associated CDI and 33.9% of hospital-acquired CDI in the United States, with ribotype 002, 020, and 106 strains also contributing considerably to CDI, albeit at lower rates of 10 to 10.7%, 10.3 to 11.4%, and 4.3 to 7.6%, respectively (81). Nevertheless, infection rates and mortality resulting from CDI still remain high in the United States. In 2013, the CDC estimated that *C. difficile* caused 250,000 infections per year and 14,000 deaths in the United States, but these numbers are likely underestimates (18). Estimates from CDC data in 2015 by Lessa et al. suggest that over 450,000 CDIs occurred in the United States, accounting for over 29,000 deaths annually (81). The disparity between the two estimates highlights an ever-growing need for more stringent detection of infections and subsequent analysis of CDI rates.

Similar to the United States, in the United Kingdom *C. difficile* disease rates were reported to be four times greater than those seen in the late 1990s in both England and Wales, reaching a peak between 2007 and 2010 and coinciding with outbreaks of ribotype 027 strains (82–84). From late 2003 to 2005, two severe and devastating outbreaks of *C. difficile* were documented in the Stoke Mandeville Hospital in England, in which a total of 334 cases of CDI were recorded, resulting in 38 fatalities (85). Strain R20291 was isolated during this outbreak and was identified as a ribotype 027 strain (86). These outbreaks were associated with several contributing factors, including insufficient isolation of infected individuals and poor implementation of infection control measures and practices by staff, which would have minimized and prevented *C. difficile* spread and contamination (85). In the years following this outbreak, rates of CDI appeared to be declining in the United Kingdom, from 164.8 deaths per million people in 2008 to 34.3 deaths per million people in 2015, as a result of increased awareness and infection control management; however, infections and mortalities resulting from CDI still occur at high rates in the United Kingdom (82).

Several other European countries, including France, Germany, and the Netherlands, have experienced similar recent outbreaks of *C. difficile*, with ribotype 027 strains found to be prevalent during these outbreaks (87, 88). Although ribotype 027 isolates are still highly prevalent throughout Europe, accounting for 19% of the reported CDIs, a wide diversity of *C. difficile* ribotypes also contribute to these infections, highlighting the diverse global epidemiology of *C. difficile* (89). The diversity of *C. difficile* throughout Asia is dramatically different and is dominated by a high number of variant *C. difficile* strains, in particular, ribotype 017 *C. difficile*, a $TcdA^{-}TcdB^{+}CDT^{-}$ variant strain which, for example, comprises up to 40% of all strains isolated in Korea and China (65, 67, 68, 90).

Although *C. difficile* is an important nosocomial pathogen, it can also cause community-acquired infections, which occur without recent exposure to *C. difficile* during hospitalization or during a hospital stay. Interestingly, community-acquired CDIs often occur without the presence of risk factors commonly associated with nosocomial CDI, such as advanced age or prior antibiotic use (8, 70). Furthermore, community-acquired CDI rates appear to be on the rise, accounting for 20 to 27% of all CDI cases, with an increasing number of cases detected in the United States and Europe (70). Although ribotype 027 strains do contribute to community-acquired CDI, other isolates, including ribotype 078 strains which are often isolated from pigs and cows, also contribute to community-acquired CDI in high numbers (59, 70, 71). This may be linked to the shedding of spores within feces of livestock and domesticated animals, which contributes to the transmission and spread of disease from animal to animal (91) and may also aid in potential zoonotic transmission from animals to humans (92). This potential zoonotic transmission probably occurs via the ingestion of spore-contaminated meat products derived from *C. difficile*-infected livestock (92). In the United States, *C. difficile* spores have been detected in up to 42.4% and 41.3% of tested beef and pork products, respectively, with ribotype 078 and 027 strains being isolated in high frequencies from these samples. Similar *C. difficile* spore contamination of meat products occurs in Canada, albeit at lower levels, with levels of detection ranging from 6.1 to 20% for beef and 1.8 to 12.2% for pork products (93). Detection of *C. difficile* spores in meat products throughout Europe is considerably lower, with less than 5% of tested samples yielding a positive *C. difficile* culture (93). Nevertheless, detection of *C. difficile* spores within retail meat products has the potential to aid in transmission from animal to human host and may account for the emergence of typically animal-associated *C. difficile* strains in humans (70, 71).

PATHOGENESIS

Susceptibility to CDI

Following the ingestion of *C. difficile* spores, infection may be established within the host upon spore germination and vegetative cell colonization of the colon (94). However, susceptibility to colonization and disease is generally not seen in healthy individuals who have an established and diverse gastrointestinal microbiota, which provides a physical barrier to colonization and contributes to the induction and maintenance of a protective immune response to invading pathogens (95–97). The commensal microbiota provides colonization resistance by occupying and interacting with the colonic niche but becomes disrupted in individuals undergoing antibiotic treatment (98).

A number of factors may contribute to reduced colonization resistance in antibiotic-treated hosts. Disruption to the microbiota may change the nutrient profile within the gastrointestinal tract, which may create a niche that is more suitable for *C. difficile* (99). Furthermore, with reduced microbiota-mediated priming of the innate immune system following antibiotic disruption, recognition of invading pathogens such as *C. difficile* may be compromised, thereby increasing host susceptibility to opportunistic infection (98). Under normal conditions when the microbiota is intact, immune responses occur within the host which promote the production of several gastric and immune molecules, including mucus/mucin and antimicrobial peptides such as defensins and immunoglobulin A (IgA), which help to prevent gut colonization by pathogens (98). Although significant disruption of the host microbiota may occur following multiple doses of antibiotics, a single dose is sufficient to "shift" the microbiota and promote CDI (98, 100). For example, depletion of the microbiota can change the cleavage of sugar substrates within the gut, resulting in higher

levels of sialic acid and succinate, promoting the growth of *C. difficile* (99). Antibiotic resistance of *C. difficile* strains exacerbates this problem, as has been seen with fluoroquinolone resistance in the epidemic ribotype 027 strains following the overuse of this group of antibiotics (101).

Colonization and infection with *C. difficile* occurs in people from a range of age groups, but increased risk of infection, infection rates, and disease severity occurs in elderly and in immune-compromised patients (23, 76, 102). *C. difficile* often presents as a nosocomial infection and is the leading cause of infectious AAD, accounting for about 25% of all cases (21). More recently, substantial increases in CDI rates have been recorded, with the persistence of *C. difficile* in hospitals and spore-mediated transmission likely to play a fundamental role in this phenomenon (13, 76). In addition to this, rates of community-acquired CDI are also increasing and often occur in patients that are not typically at risk of infection such as children, pregnant women, and individuals with no recent antibiotic use (8, 70).

Disease Associated with CDI

CDI is associated with a variety of disease symptoms, ranging from asymptomatic carriage of *C. difficile* to severe and life-threatening disease complications (2). The spectrum of disease that is observed in CDI patients begins as mild diarrhea and may increase in severity with the progression of infection. Mild diarrhea associated with CDI often resolves following the cessation of antibiotic therapy; however, it may progress to severe diarrhea, leading to dehydration and mild inflammation of the colon with the possible development of fever (103). The progression of disease in severe cases of CDI may lead to the development of pseudomembranous colitis, with toxin-mediated damage resulting in the formation of plaques throughout the colon, consisting of cellular debris, fibrin, and neutrophils (104). Patients may also present with fever, abdominal pain, and elevated white blood cell counts (104).

A rare yet significant complication of severe disease is toxic megacolon, which results in detrimental inflammation and swelling of the colon, colonic perforation, sepsis, and in many individuals, death (105). Disease complications may be compounded by the onset of multiple organ dysfunction syndrome, promoting patient fatality (106). This syndrome can be defined as "the development of potentially reversible physiologic derangement involving two or more organ systems not involved in the disorder that resulted in ICU admission," which includes damage to the lungs, kidneys, heart, liver, or brain (107). Extraintestinal infections may occur following CDI, presenting as abdominal abscess and wounds, ascites, bacteremia, brain abscess, catheter infection, perianal abscess, peritonitis, and toxemia (106, 108–110). Recent work by Carter et al. (111) has shown that damage and architectural disorganization occurs in the kidneys, lymph nodes, spleen, and thymus during murine CDI. Toxemia, or escape of toxins into the blood, in *C. difficile*-infected piglets has also been shown to result in systemic pathologies of the lungs, which seemingly occurs via TcdB-mediated damage of the colonic epithelium, because neutralizing antibodies to TcdB, but not TcdA, were able to block colonic damage and systemic pathology progression (112). Although this level of disease severity is considered rare in humans, the increasing incidence of CDI and the more severe disease symptoms associated with ribotype 027 strain infections has resulted in more cases involving life-threatening complications (105).

Disease in *C. difficile*-infected animals shares many of the characteristics seen in human disease. One of the most well-characterized CDI-associated diseases in animals is *C. difficile*-associated enteritis in piglets (113). As in humans, CDI often leads to diarrhea in piglets, which may be yellow and pus-like, and may progress into severe life-threatening disease (113). As a result of these disease symptoms, weight-loss, difficulties digesting food, dehydration, and weakness often occur, which over time may result in piglet death (114). On examination of the colon tissue, swelling and edema of the epithelium may also be observed, with colonic dilation and hemorrhage also common (114). As seen in humans, neutrophil infiltration and pseudomembranous formations throughout the colon are common, particularly in severe enteritis (114). Disease in animals is not restricted to pigs, with infection and disease occurring in other animals including horses, cows, and domesticated dogs, as well as exotic and wild animals including penguins and elephants (115–117).

Animal Models of CDI

Studies of *C. difficile* and CDI have used specific animal models to provide key insights into the role played by virulence factors in disease pathogenesis (33, 118, 119). The hamster model of *C. difficile*-associated disease was used extensively in early research because of the sensitivity of hamsters to the *C. difficile* toxins, with infections almost always resulting in hamster death (120). Most other animals (including humans) are not as sensitive to the effects of the toxins (120); thus, the extreme sensitivity of hamsters to the *C. difficile* toxins has prevented the more subtle aspects of infection and disease from being teased out. The use of hamsters in infection studies is also limiting because of a lack of reagents such as specific antibodies, or hamster genetic variant strains, making more detailed research using this animal model difficult (120).

The more recent development of a murine model of CDI by Chen et al. in 2008 (120), which mimics *C. difficile* susceptibility and disease in humans, has overcome many of the limitations associated with the hamster model of CDI (121). The murine CDI model provides the opportunity to analyze *C. difficile* disease from a new and more detailed perspective (120). In the model by Chen et al. mice are pretreated with an antibiotic cocktail prior to infection challenge and, following infection, animals develop many of the characteristic disease pathologies associated with clinical CDI, including diarrhea and weight loss (120). Infection also induces severe colonic pathology, with epithelial damage, submucosal inflammation, and neutrophil influx seen, similar to human disease, further supporting the utility of this model in developing a better understanding of *C. difficile* disease (120). *C. difficile*-infected mice also succumb to disease quickly and exhibit observable signs of disease 2 to 3 days postinfection. This model of CDI and the availability of reagents and genetically manipulated mice has facilitated recent studies focused on defining the contribution of key immune pathways to *C. difficile* disease, which are described in more detail below, providing important new insights into the progression of CDI and the host response (120, 122–125).

Following the development of the Chen et al. model, additional murine models of CDI were developed which utilize different pretreatment regimes and result in different disease pathology and outcomes (126). In 2009, Lawley et al. developed a less severe CDI model through disruption of the intestinal microbiota via oral administration of clindamycin for four consecutive days prior to infection (122). Infection in these mice was established following exposure to "carrier state" mice, which were induced to carry *C. difficile* in their

microbiota through the administration of neomycin 24 hours prior to challenge. Mice infected with *C. difficile* in this model show some weight loss, diarrhea, and damage to the intestinal epithelium, albeit at lower levels than in the Chen et al. model (122). In 2012, Buffie et al. showed that a single dosage of clindamycin by intraperitoneal injection was sufficient to induce CDI with disease pathology and outcome similar to the other models (127). The third murine CDI model, developed by Theriot et al. in 2011, induces *C. difficile* susceptibility through the oral administration of cefoperazone for 10 days, 12 days prior to infection (128). Dependent on the *C. difficile* strain and dosage used for infection, mice developed severe disease within 2 days of infection, characterized by weight loss, diarrhea, and damage/inflammation of the intestinal epithelium (128). More recently, the oral administration of either metronidazole or streptomycin to mice has been shown to induce susceptibility to CDI, allowing for high levels of *C. difficile* colonization (129). Gnotobiotic mice, which are void of colonic microbiota, have also been used to model CDI, presenting similar disease profiles to the aforementioned models; however, since a complete lack of colonic microbiota is unlikely in human CDI, this model does not accurately represent human CDI (130). More recently, a mouse model of CDI has been established in mice with humanized microbiota, allowing for the analysis of disease under conditions more similar to human CDI (131). Together, these different murine models of CDI have provided valuable insights into CDI and disease progression under different circumstances and conditions.

Other *C. difficile* disease models include the porcine model, which emulates human disease symptoms and severity and which has provided new insights into the understanding of *C. difficile* disease (114). Through the infection of gnotobiotic piglets with *C. difficile* spores, Steele et al. (114) were able to replicate natural *C. difficile* disease, with infected pigs developing diarrhea 48 hours after infection. Severe colonic pathologies and lesions were also observed, leading to the formation of pseudomembranes in some animals, much like those seen during severe CDI in humans. With these similarities to human disease, this model provides a valuable resource for the development of our understanding of CDI and provides an appropriate model for the testing of potential therapeutics and treatments for CDI (114).

C. difficile Toxins

TcdA and TcdB

Several *C. difficile* virulence factors have been identified, the most important of which are TcdA and TcdB (33, 111, 118, 132). Typically, the 19.6-kb PaLoc encodes both the *tcdA* and *tcdB* genes, producing TcdA (308 kD) and TcdB (270 kD), respectively, and also encodes genes involved in the positive (*tcdR*) and negative (*tcdC*) regulation of toxin production (Fig. 1a) (133, 134). TcdR is an alternative RNA polymerase sigma factor, which shares similarity to the alternative sigma factors BotR in *Clostridium botulinum* (135), TetR in *Clostridium tetani* (136), TpeR and UviA in *Clostridium perfringens* (137–139), and TcsR in *P. sordellii* (140). TcdR positively regulates the transcription of *tcdA*, *tcdB*, and *tcdR*; however, this process is influenced by various environmental stimuli, including changes in the metabolism of amino acids via CodY (141) and carbon sources such as glucose via CcpA (142). Other regulatory proteins involved in aspects of the *C. difficile* life cycle, including Spo0A, a master regulator of sporulation, and SigD, a regulator of flagella, also regulate toxin transcription (143, 144). TcdC is an anti-sigma factor, which is thought to negatively regulate toxin transcription by disrupting the function of TcdR, but this occurs with considerable variability across different strain backgrounds (145–147). PaLoc also carries a gene (*tcdE*) encoding a holin-like protein, TcdE, which may be involved in the release of toxins from the cell, but this hypothesis is contentious (148–150). Variants of the *C. difficile* PaLoc exist, which contain mutations or deletions within the locus, altering the toxin profile of these strains (72). More recently, the PaLoc has been found in alternate genomic locations in specific strains, although such strains do not appear to be common (72).

TcdA and TcdB are members of the large clostridial glucosylating toxin family, members of which are also found in several other pathogenic clostridia (132). These include the hemorrhagic (TcsH) and lethal (TcsL) toxins produced by *P. sordellii* (151), α-toxin (TcnA) produced by *Clostridium novyi* (152), and TpeL produced by *C. perfringens* (153), all of which function in a similar way to the *C. difficile* toxins and inactivate Rho, Rac, or Ras small GTPases. These small GTPases play a vital role in maintaining cellular architecture and are involved in the maintenance of the actin cytoskeleton, cellular migration, and division (154). Inactivation of these small GTPases via the covalent transfer of a glucose moiety by one of the large clostridial toxins, or an acetylglucosamine moiety only by TcnA and TpeL, leads to the disruption of their normal function and consequently results in cell rounding and cell death (152, 153, 155, 156).

TcdA and TcdB, which have 63% amino acid similarity, share an overall conserved structure (Fig. 2), with four functional domains that are involved in cellular binding, entry, and enzymatic activity of the toxins (157–159). The C-terminal ends of TcdA and TcdB, consisting of the combined repetitive oligo-peptides (CROPS) domain, are thought to facilitate receptor binding for each toxin (157); however, toxins which lack this domain can still induce cell rounding in tissue culture, suggesting that there are CROPS-dependent and -independent receptor binding regions within each toxin (160–165). CROPS-mediated receptor binding has been shown to increase TcdA-mediated cytotoxicity compared to a CROPS deletion mutant (165), while recombinant C-terminal-TcdA (REP321) blocks cytotoxicity in F9 cells when used to saturate the cell surface prior to intoxication (166). Immunization of mice with REP231 also protected mice against subcutaneous lethal dosing with TcdA (166), highlighting the importance of the CROPS in receptor binding and cell entry. Several receptors have been proposed for TcdA, including the α-Gal-(1,3)-β-Gal-(1,4)-β-GlcNAcO$(CH_2)_8CO_2CH_3$ carbohydrate moiety (167) and glycoprotein 96 (gp96) (160) on human cells, but the exact mechanism of binding still remains unclear. Three potential host receptors have been identified for TcdB: poliovirus receptor-like protein 3 (162), chondroitin sulfate proteoglycan 4 (161), and members of the frizzled family receptors (163). Binding to these receptors occurs in a CROPS-independent manner at regions adjacent to the CROPS domain; however, further *in vivo* characterization of the role for these receptors in CDI is required (161–164). Thus, it appears that receptor-mediated binding of TcdA and TcdB is a complex process involving multiple binding regions within the C-terminal portion of each toxin, which interact with an array of receptors that are not yet fully characterized.

Following receptor binding, both TcdA and TcdB enter the cell via clathrin-mediated endocytosis, with acidification of the endosome resulting in a conformational change in the pore-forming domain of the toxin, allowing insertion of the toxin into the membrane and facilitating toxin entry into

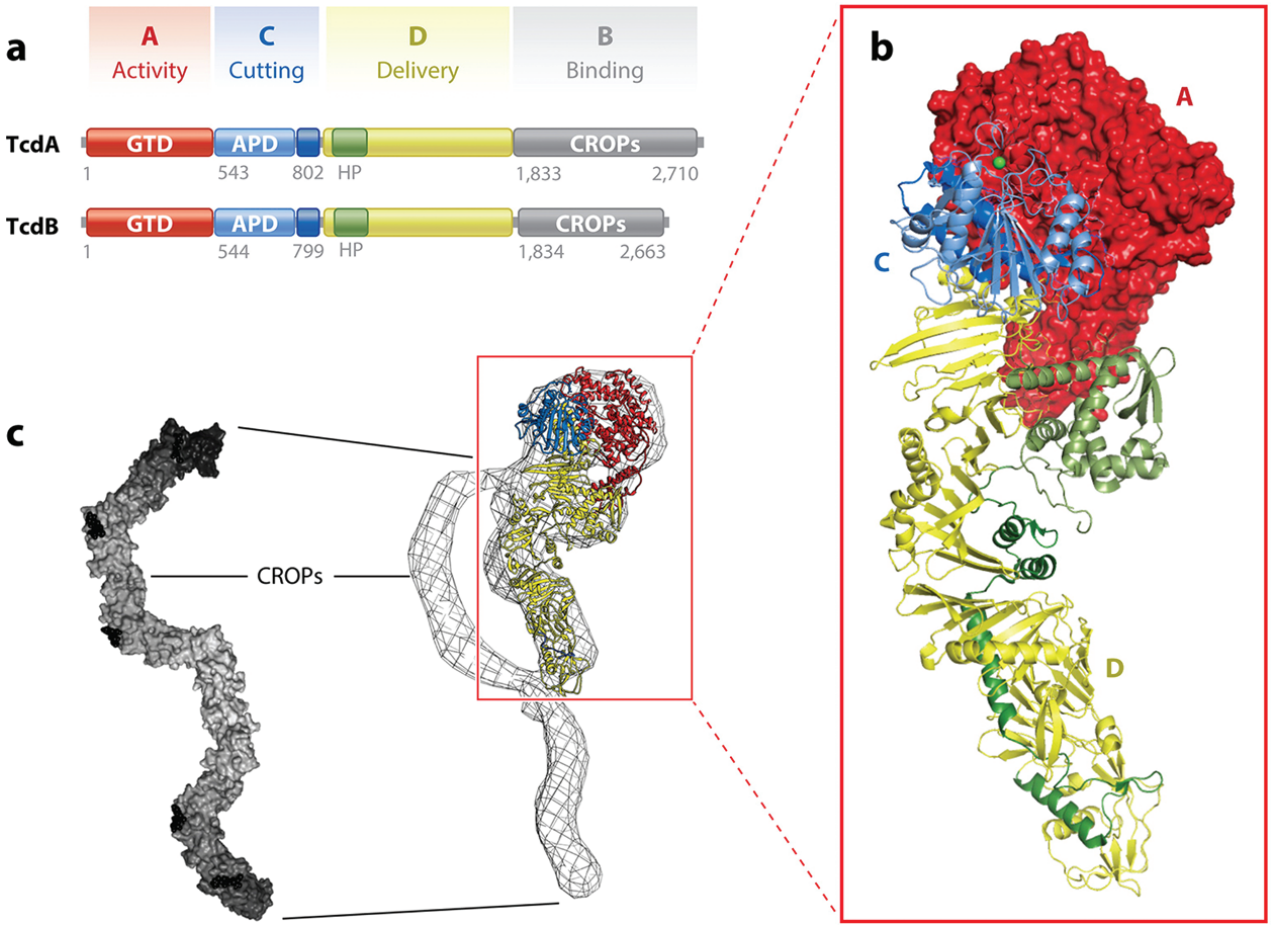


FIGURE 2 Schematic representation of C. *difficile* TcdA and TcdB. (a) Comparison of TcdA and TcdB functional domains. Comparison of the four functional domains of the toxins, including the glucosyltransferase domain (GTD) (red), auto-protease domain (APD) (blue), delivery domain (yellow), and combined repetitive oligo-peptides (CROPS) domain (gray). (b) The crystal structure of the TcdA GTD (red), APD (blue), and delivery domains (yellow) in the absence of the binding domain. (c) Three-dimensional structure of TcdA, superimposed with the crystal structure of TcdA. Reprinted from *Annual Review of Microbiology* (254).

the cytosol (Fig. 3) (157, 168). Once translocated across the endosomal membrane, the glucosyltransferase domain (GTD) of each toxin is cleaved off by the auto-protease domain, following activation of this domain by host inositol hexakisphosphate (InsP6), which releases the enzymatic GTD into the cytosol (169, 170). Once in the cytosol, the GTD then inactivates host cell Rho family GTPases. A conserved tryptophan residue at amino acid position 102 and a DXD motif at position 286 to 288 are located in the GTD of TcdA and TcdB and are essential for binding to UDP-glucose and for the catalytic function of the monoglucosyltransferase domain (171). Inactivation of Rho family GTPases induces cell rounding and death (172). These outcomes occur because of a lack of regulation in actin polymerization within the intoxicated cell, resulting in the disruption of a number of essential host cell features, including the actin cytoskeleton, stress fibers, and tight junctions (173). TcdB has also been shown to promote the formation of NADPH oxidase complexes, releasing reactive oxygen and leading to necrotic cell death (174). TcdB from ribotype 027 strains appears dissimilar to historical TcdB in some aspects, with variations in the CROPS domain (175). The differences between the binding domains of ribotype 027 TcdB and historical TcdB are thought to provide an increase in tissue tropism, because ribotype 027 TcdB induced more severe and extensive pathologies when used to treat embryonic zebra fish (175). Specifically, ribotype 027 TcdB displayed increased cardiotoxicity, resulting in severe cardiac damage and necrosis after 24 hours compared to historical TcdB in this model, which induced swelling and bleeding of the cardiac tissue at a slower rate (175).

Recent work by Chumbler et al. (176) has also shown that TcdA and TcdB induce cell damage in distinct ways, with TcdA inducing apoptosis via glucosyltransferase activity and TcdB via two modes of action that are dependent on toxin concentration. At low concentrations, TcdB induced apoptosis in young adult mouse colonic cells, in a similar way to TcdA (176). Unlike TcdA, which only induced apoptosis, high concentrations of TcdB (≥100 pM) induced necrosis, which, from analysis using an enzymatically inactive TcdB, occurs independently of glucosyltransferase activity (176). Despite these insights, the exact mechanism for the vastly different roles of TcdA and TcdB in disease still remains unclear. Notably, TcdB has a substantially smaller CROPS domain than TcdA, which may confer a largely different three-dimensional structure, as depicted in modeling of the CROPS region from smaller crystalized fragments (Fig. 4) (157, 177). The difference in physical size between these

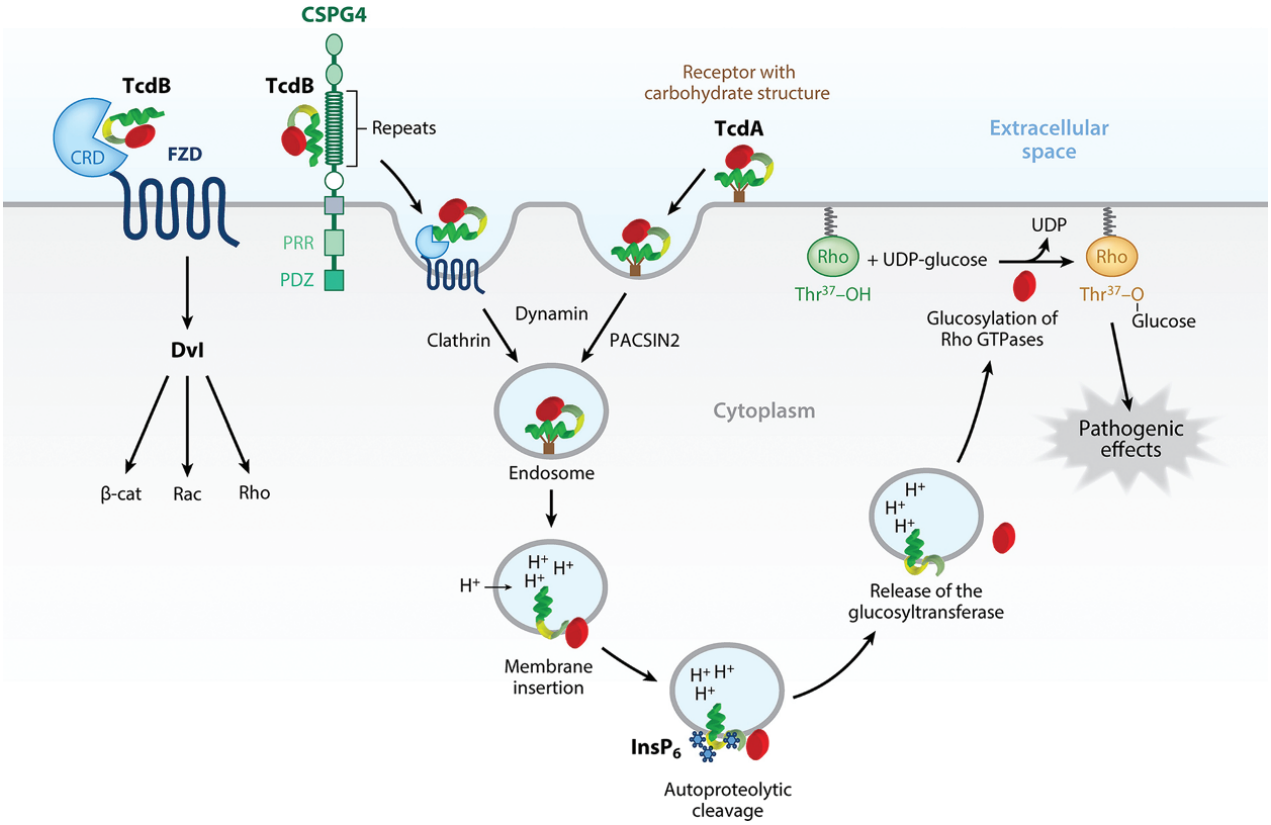


FIGURE 3 Binding and entry of *C. difficile* toxins into colonic epithelial cells. TcdA and TcdB bind to the cell surface via interaction of the binding region and one or more cell surface receptors, including the α-Gal-(1,3)-β-Gal-(1,4)-β-GlcNAcO$(CH_2)_8CO_2CH_3$ carbohydrate moiety and glycoprotein 96 (gp96) or surface receptors including frizzled (FZD), chondroitin sulfate proteoglycan 4 (CSPG4), and poliovirus-like receptor 3 (PVLR3) respectively, resulting in receptor-mediated endocytosis. Binding can occur via CROPS (green)-dependent and -independent mechanisms. Once internalized, the endosome acidifies, resulting in a conformational change and membrane insertion of the delivery domain and translocation of the auto-protease domain (APD; gray) and glucosyltransferase (GTD; red) into the cytosol. The APD then interacts with host inositol hexakisphosphate (InsP6; blue), cleaving the GTD, which can interact with the Rho family of GTPases. Reprinted from *Annual Review of Microbiology* (254).

domains may account for some of the disparity in disease associated with the two toxins, either altering the binding of each toxin to its receptors or resulting in steric hindrance differences which may influence functional capacity within the complex gastrointestinal compartment.

The role of TcdA and TcdB in the development and progression of *C. difficile* disease has been contentious. Early research in this area, involving the intragastric administration of TcdA and TcdB to hamsters, suggested that TcdA was the major virulence factor in the development of severe *C. difficile* disease, with TcdB thought to be unable to induce disease in the absence of TcdA despite being a potent cytotoxin in cell-based *in vitro* assays (119). In hamsters, toxin coadministration, using sublethal doses of TcdA together with TcdB, was able to induce severe disease symptoms, indicating possible synergy between TcdA and TcdB (119). Similar results were seen in a rabbit ileal loop model, where exposure to TcdA but not TcdB was able to induce intestinal hemorrhage, fluid secretion, and inflammation (4). However, the isolation of clinical *C. difficile* variant strains that encoded only TcdB suggested that, at least in humans, TcdB alone is sufficient to induce disease, raising questions about the clinical relevance of previous studies using purified toxins (178, 179). Early studies did not assess the role of the toxins in the context of infection but instead used purified toxins and the intragastric administration of the toxins to animals to a site that is not where colonization and infection occur. A true representation of CDI and disease was therefore not achieved in these studies.

More recently, multiple laboratories have used various CDI animal models together with genetically engineered isogenic *C. difficile* toxin mutants to elucidate the role of TcdA and TcdB in disease. Each of these studies, which utilized hamsters and mice together with historical or epidemic *C. difficile* isolates and their isogenic toxin gene mutants, showed that TcdB induced severe disease in both the absence and presence of TcdA, highlighting the important role that TcdB plays in disease (33, 111, 118, 180). Although these studies have redefined TcdB as a key mediator of *C. difficile* disease, the role of TcdA in disease remains somewhat unclear. In two of the studies, TcdA was shown to have a minor role in disease, with hamsters or mice infected with TcdA-producing ($TcdA^+B^-$) strains not progressing to severe diarrhea or mortality and with mild pathology and damage seen in mouse colonic tissue (111, 118). In the other two studies, TcdA appeared to have a more intermediate role in disease, with $TcdA^+B^-$*C. difficile* strains inducing some disease or mortality but at a slower rate in comparison to the wild-type (WT) and $TcdA^-B^+$ strains (33, 180). Nevertheless, a key finding from these studies was that TcdB plays a

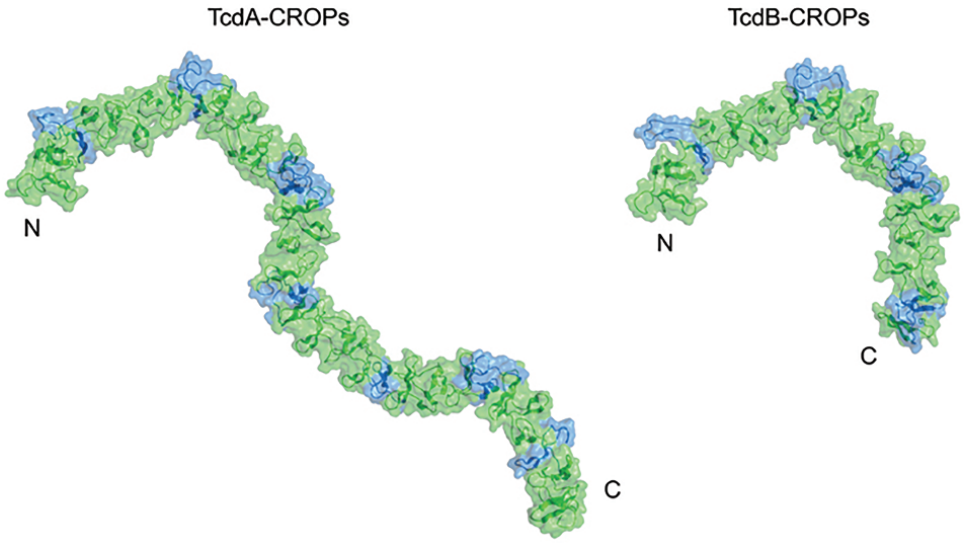


FIGURE 4 Comparison of the TcdA and TcdB combined repetitive oligopeptides (CROPS) domains. Three-dimensional models of the TcdA and TcdB CROPS domains based on the crystal structure of a 127-amino acid fragment from the C-terminal region of TcdA characterized in reference 177. Long repeats are in green; short repeats are in blue. N, N terminus; C, C terminus. Modified from *Frontiers in Cellular and Infection Microbiology* (157).

key role in disease, which is supported by the high number of clinical $TcdA^{-}B^{+}$ *C. difficile* strains that are isolated from patients with CDI, which currently comprise between 0.2 to 40% of all strains isolated, dependent on geographical location (64–68, 90). The recent emergence of a single $TcdA^{+}B^{-}$ *C. difficile* isolate is interesting since historically no such strains have been isolated; the clinical importance of this strain type remains to be determined, but future epidemiological tracking will be important to determine if such a strain type disseminates further (72).

C. difficile transferase

Some *C. difficile* strains, including ribotype 027 strains, produce a third toxin known as binary toxin or *C. difficile* transferase (CDT) (77, 181). CDT is an ADP-ribosyltransferase encoded by a 6.2-kb chromosomal locus, defined as the CdtLoc, which is independent of the PaLoc region (Fig. 1b) (77, 182, 183). CDT shares similarity with other clostridial binary toxins including *C. botulinum* C2 toxin, *C. perfringens* iota toxin and the *Clostridium spiroforme* CST toxin (184, 185). The locus encodes the *cdtA* and *cdtB* genes, which produce the enzymatic (CDTa) and binding (CDTb) components of the toxin, respectively. Similar to other clostridial binary toxins, CDTb is proteolytically activated prior to oligomerization into a predicted heptamer, which may occur prior to attachment to the cell surface via lipolysis-stimulated lipoprotein receptor or upon monomer binding (185). Once oligomerized, CDTa may bind, allowing for entry into the cell via endocytosis (185). Upon endosome acidification, CDTa is translocated into the cytosol, where it ADP-ribosylates actin, disrupting the cell cytoskeleton and inducing microtubule protrusions in intoxicated cells (181).

CDTLoc also encodes *cdtR*, producing CdtR, an orphan LytTR family response regulator responsible for regulation of CDT production (Fig. 1b) (183, 186). Interestingly, CdtR has recently been shown to also regulate the production of TcdA and TcdB in some strains of *C. difficile*, because insertional inactivation of *cdtR* resulted in reduced levels of TcdA/TcdB production and significantly reduced virulence in a mouse model of CDI (187). Although CdtR appears to regulate toxin production and virulence in some CDT-producing strains, the exact role of CDT in disease remains unclear.

Overall, CDT was found to be present in approximately 5% of historical isolates, but the prevalence of this toxin in *C. difficile* has steadily increased over the last 2 decades, with CDT detected in clinical strains at levels ranging from 17 to 45% (185). CDT was found to be highly prevalent in animal strains and is encoded by 23 to 100% of such isolates (92, 183). Importantly, CDT is also now found in approximately one-third of human clinical isolates (187, 188). CDT alone was thought to be unable to induce disease since clinical isolates producing only CDT, but not TcdA or TcdB, were unable to induce diarrhea or death in hamsters (189). However, the isolation of clinical strains that only produce CDT has raised questions about the role of this toxin in disease.

Since infection with strains producing only CDT and not TcdA or TcdB appears able to induce diarrhea in some patients, the role of this toxin in disease is still unclear (190). It may be that CDT acts synergistically with TcdA and TcdB to promote colonization and induce severe disease (181, 189, 191). Work by Schwan et al. (181) suggests that CDT may act as a colonization factor by inducing the formation of microtubule structures at the host cell surface, aiding in the adherence of the bacterium to the host cell. CDT also appears to increase the virulence of a TcdB mutant of strain R20291 in a hamster model of disease, but the mechanism by which this occurs is unknown (180). Recent work by Cowardin et al. (191) has also shown that CDT can enhance virulence to some degree, with CDTa mutants of two *C. difficile* isolates and a single CDTb mutant inducing less severe disease than the WT strains in a mouse model of CDI. This difference in disease profile associated with the CDT mutants was linked with increased levels of eosinophils in the colons of mice infected with the CDTb mutant of strain R20291 and suggests that eosinophils may play a protective role in CDI (191). Thus, it appears that CDT may play a synergistic role

with TcdA and TcdB to enhance virulence by decreasing eosinophil recruitment. Analysis of eosinophil involvement in disease progression in naturally occurring CDT-negative strains has yet to be investigated, and may provide further insights into this phenomenon. With the prevalence of CDT-positive animal isolates and the increasing emergence of these CDT-positive strains in humans (185), the enhanced virulence associated with CDT could have severe consequences on disease profiles and severity.

Immune Response to *C. difficile*

Our current understanding of the role of the host immune system during CDI is limited, particularly with respect to the cytokine response and the changes that occur to immune cell populations in gut tissues during infection in response to each toxin. Clinical observations show that humoral immunity is induced during CDI, specifically, increased levels of IgG and IgM are detected in response to infection with strains producing TcdA and TcdB and in response to nontoxin antigens (192, 193). An additional hallmark of CDI seen in humans, mice, and pigs is an abundant influx of polymorphonuclear leukocytes into infected gut tissues (114, 120).

Cytokine and Chemokine Responses to Toxins

The immune response to *C. difficile* and its toxins has been previously investigated, but most of this work was performed through the application of purified toxins or *C. difficile* antigens to cell lines or rabbit and mouse ileal loop models (194–200). In several studies, purified TcdA was shown to induce the release of the key neutrophil and monocyte chemoattractants interleukin 8 (IL-8) and monocyte-chemoattractant protein 1 from HT-29 cells, a human colonic adenocarcinoma cell line (195, 201, 202), and T84 cells, a human intestinal epithelial cell line (197, 201). TcdA also induced tumor necrosis factor α (TNF-α) release in murine and human monocytes (198, 203), and TcdA stimulation of human monocytes induced IL-1 and IL-6 production (203). Purified TcdA has also been shown to induce the synthesis and release of IL-8 in a human xenograft ileal loop intoxication model (199). Exposure of tissues in a mouse ileal loop intoxication model to purified TcdA also promotes the transcription of proinflammatory cytokines and chemokines, including interferon-γ, macrophage inflammatory protein 1α (MIP-1α), MIP-2, and TNF-α (200), and in a rat ileal loop intoxication model, MIP-2, within 2 to 6 hours of toxin exposure (194). When injected directly into the cecum, TcdA also alters the expression of several chemokines involved in neutrophil chemotaxis, including chemokine (C-X-C motif) ligand 1 (CXCL1), IL-1β, and IL-6 (204, 205). Taken together, these data suggest that the production of TcdA during infection will alter the cytokine milieu to induce a highly inflammatory environment, most likely contributing to the inflammatory pathology observed during CDI. However, infection studies of the immune response that occurs with strains that only produce TcdA have not been performed.

Less is known about the immunomodulatory role of TcdB compared to knowledge of this role for TcdA. TcdB can induce IL-8 release in a human xenograft ileal loop model (199), IL-1β release in a mouse ileal loop model (196), TNF-α in rat macrophages (206), and TNF-α, IL-1, and IL-6 production in human macrophages (203). TcdB has also been shown to induce IL-1β via the pyrin inflammasome in mouse bone marrow-derived macrophages (207). As for TcdA, infection studies on the immune response that occurs with TcdB-producing strains in the absence of the other toxins have not been performed.

Cytokine and Chemokine Responses to Nontoxin Antigens

Nontoxin antigens of *C. difficile* have also been investigated for their role in the induction of the host immune response. A recent investigation into the interaction between nontoxin *C. difficile* antigens, including surface layer proteins (SLPs), flagella, and heat shock proteins 42 and 60, isolated from several *C. difficile* isolates, and the human macrophage cell line THP-1 showed that a number of proinflammatory cytokines were induced (208). These nontoxin antigens were isolated from a diverse group of strains belonging to ribotypes 012, 087, 027, 011, and 106, and antigens from all isolates promoted IL-1β, IL-6, IL-8, IL-10, IL-12p70, and TNF-α synthesis in THP-1 cells, to similar levels (208). However, a comparison to an untreated control was not included in this study (208); these results therefore require validation through additional research. SLPs isolated from *C. difficile* strains have also been shown to induce the release of several cytokines, including IL-1β, IL-6, IL-10, IL-12, IL-23, monocyte-chemoattractant protein, MIP-2, and TNF-α (125, 209–211). *C. difficile*-mediated cytokine induction during infection is not the result of toxin action alone and results from stimulation of the host immune response to numerous *C. difficile* factors. Further investigation of these effects is clearly needed.

Immune Response During CDI

The role of myeloid differentiation factor 88 (MyD88), an adaptor molecule critical for Toll-like receptor (TLR) signaling, in CDI was assessed using $MyD88^{-/-}$ mice (122). When compared to WT ($MyD88^{+/+}$) mice, $MyD88^{-/-}$ mice infected with *C. difficile* strain M68, a TcdA- and TcdB-producing virulent human isolate, developed more severe disease with pronounced weight loss and a moribund appearance, together with edema and epithelial hyperplasia detected upon microscopic examination of cecal tissues. Furthermore, the survival rate of $MyD88^{-/-}$ infected mice was significantly less than WT infected mice, with 48% of the $MyD88^{-/-}$ infected mice not surviving the infection, compared to 100% survival in the WT mice (122). Considering the critical role that MyD88 plays in the downstream signaling of several TLRs and nuclear factor-κB activation, it is not surprising that infected $MyD88^{-/-}$ mice developed more severe disease pathology (212). This important study confirmed the critical role that the innate immune system plays in CDI, providing an appropriate platform on which to base other studies of the immune response to CDI.

Building on the MyD88 studies, recent investigations have explored the mechanism by which MyD88 deficiency increased disease severity and mortality during CDI (213). A detailed analysis of cecal and colonic histopathology of WT ($MyD88^{+/+}$) and $MyD88^{-/-}$ mice following *C. difficile* challenge was performed, which showed a significant reduction in neutrophil and monocyte infiltration into the colonic lamina propria of $MyD88^{-/-}$ mice (213). Furthermore, $MyD88^{-/-}$ mice had impaired CXCL1 transcription, which is an important observation since CXCL1 is a mouse homologue of the human chemokine IL-8, which is responsible for neutrophil trafficking (213). To study the role of neutrophils in CDI, neutrophils were depleted from mice by injection of a neutrophil-specific, Ly6G monoclonal antibody 1A8 (213). Mice depleted of neutrophils showed a 70% mortality rate

following C. *difficile* challenge compared to 25% mortality in mice with intact neutrophils (213). By contrast, little mortality was observed in C-C chemokine receptor type 2-deficient mice ($CCR2^{-/-}$, which are deficient in monocyte chemotaxis) infected with C. *difficile*, suggesting that CCR2 and, in turn, monocyte recruitment, are not important for protection from fulminant C. *difficile* disease progression (213). Collectively, these results suggest that neutrophils, and neutrophil chemotaxis mediated by CXCL1, play an important role in preventing severe CDI (213, 214). The increased disease severity and mortality associated with neutrophil depletion during CDI was speculated to be linked with the translocation of microbiota to extraintestinal sites. Depletion of neutrophils and monocytes via monoclonal antibody blockage of their receptor, Gr-1, resulted in substantial increases in microbiota translocation to the mesenteric lymph nodes of C. *difficile*-infected mice compared to untreated infected mice (213). Together, these data suggested that neutrophil, and to a lesser extent, monocyte, recruitment to the colon plays a crucial role in preventing excessive microbial dissemination, thereby reducing the broader host effects of CDI and highlighting the complex nature of this infectious disease (213).

An earlier study also showed a link between reduced neutrophil infiltration and the dissemination of the commensal microbiota during severe CDI, under conditions in which the host was immunocompromised (124). In this study, the involvement of the intracellular pattern recognition receptor nucleotide-binding oligomerization domain 1 (NOD1) in C. *difficile* disease was assessed (124). NOD1 detects *meso*-diaminopimelic acid peptidoglycans common to Gram-negative bacteria, while a second receptor, NOD2, detects muramyl dipeptide, a component of Gram-positive and Gram-negative peptidoglycans (215). Strikingly, NOD1 is critical in protection from life-threatening CDI, with $NOD1^{-/-}$ mice succumbing to infection at a more rapid rate than WT mice (124). By contrast, $NOD2^{-/-}$ mice were not more susceptible to C. *difficile* disease despite the association between Gram-positive bacteria and NOD2 (124). Increased mortality in C. *difficile*-infected $NOD1^{-/-}$ mice was associated with reduced neutrophil trafficking to the intestine as well as reduced CXCL1 expression in comparison to infected WT ($NOD1^{+/+}$) mice (124). The reduction in CXCL1 expression coincided with an increase in commensal microbiota contamination in the lungs, kidneys, mesenteric lymph nodes, and spleen of $NOD1^{-/-}$ mice, suggesting that neutrophilic infiltration of gut tissues plays an important role in preventing the dissemination of the commensal microbiota when C. *difficile*-mediated tissue damage occurs (124). The association between NOD1 and C. *difficile* defined in this study was unexpected since, at the time, Gram-positive bacteria were not believed to produce *meso*-diaminopimelic acid peptidoglycans. However, it now appears that *Bacillus* and many other clostridia, including C. *difficile*, do produce *meso*-diaminopimelic acid peptidoglycans, so this phenotype now appears not to be exclusive to Gram-negative bacteria and may provide an explanation for the link between NOD1 and CDI (124, 215, 216).

The association between severe C. *difficile* disease, reduced neutrophil infiltration, and commensal microbiota dissemination has also been shown in infection studies involving apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (CARD) $(ASC)^{-/-}$ mice, which lack a key component of the inflammasome, an immune factor which is involved in CDI (123, 196). The inflammasome is an intracellular caspase-activating complex often consisting of the pattern recognition receptor NOD-like receptor family, pyrin domain-containing 1 (NLRP1) or NLRP3, the adaptor protein ASC, and caspase-1, which is responsible for the cleavage of pro-IL-1β and pro-IL-18 into their active forms (196, 217). $ASC^{-/-}$ mice infected with C. *difficile* display impaired neutrophil recruitment to the cecum and colon in addition to higher levels of commensal microbiota dissemination to the spleen, liver, and lungs upon development of fulminant disease compared to WT ($ASC^{+/+}$) infected mice (123). These differences in pathology resulted in an increased mortality in $ASC^{-/-}$ mice, with 54% of infected mice succumbing to disease, compared to mortality in 3% of $ASC^{+/+}$ infected mice (123). The reduction in neutrophil recruitment in this study was linked to a deficiency in CXCL1 production in $ASC^{-/-}$ mice following C. *difficile* challenge (123). Work performed prior to these studies had shown that the NLRP3 inflammasome was involved in TcdA- and TcdB-mediated IL-1β release in a mouse ileal loop intoxication model (196). It was therefore not surprising that $ASC^{-/-}$ mice infected with C. *difficile* had reduced IL-1β levels compared to WT ($ASC^{+/+}$) infected mice, and it has been suggested that the reduction in CXCL1 expression in $ASC^{-/-}$ infected mice results from the disruption of an IL-1β-mediated positive feedback loop for CXCL1 release (123).

In addition to NOD1 and NLRP3, TLRs also appear to be important in the host response to CDI. TLR4 interacts with C. *difficile* SLPs and induces the release of several cytokines, including IL-23, IL-1β, and TNF-α (125). $TLR4^{-/-}$ mice develop severe C. *difficile* disease, with dramatic weight loss and increased mortality occurring following CDI, compared to WT ($TLR4^{+/+}$) infected mice, which showed moderate weight loss and pathology (125). TLR5 also plays an important role in the prevention of severe CDI, with stimulation of TLR5 via exogenous flagellin, a TLR5 ligand, promoting increased survival following C. *difficile* challenge (218). More recently, infection of mice with an R20291 *fliC* mutant, which lacks flagella, resulted in less severe disease than WT ($fliC^{+/+}$) CDI, characterized by less goblet cell loss and epithelial damage, and a 50% increase in survival (219). Furthermore, infection of $TLR5^{-/-}$ mice with WT R20291 resulted in little epithelial damage, further supporting the role of TLR5 in CDI and in the induction of the inflammatory response (219). This was confirmed on cytokine gene analysis, which showed that WT infection induced significant upregulation of CXCL1, IL-6, IL-1β, IL-22, CXCL10, and TNF-α expression compared to infection with the *fliC* mutant, which only induced a significant change in CXCL1 expression (219).

Several immune cells, including eosinophils (220), neutrophils (221), and innate lymphoid cells (222), have also been shown to play a key role in modulating disease severity during CDI. Reduced levels of eosinophil infiltration to the colon in mice (linked to reduced IL-25 signaling) during CDI were shown to promote severe disease, resulting in greater than 50% mortality, compared to mice pretreated with recombinant IL-25 (to induce eosinophil infiltration), which showed ~20% mortality following infection (220). Neutrophil recruitment to the site of infection during CDI has been shown to have various effects on disease severity during CDI in different studies. Chronic neutrophil inflammation exacerbates disease by inducing nonspecific damage to the colon (221), but neutropenia patients have a higher incidence of severe and potentially fatal CDI (223). Furthermore, blocking neutrophil recruitment to the colon promotes systemic dissemination of the gut microbiota and increased mortality during CDI (213). The role of neutrophils in disease is therefore complex. With respect to other immune cell types,

C. difficile-infection of Rag$\gamma c^{-/-}$ mice, which lack innate lymphoid cells, results in ~90% mortality, compared to an ~30% survival rate in WT mice, suggesting that innate lymphoid cells play a critical role in protecting mice from severe *C. difficile* disease (222).

Recent clinical data have also provided insights into the human host response during CDI. Diarrheal specimens from CDI patients were compared to those collected from non-CDI AAD patients and healthy people and were found to have higher levels of complement component 5a, granulocyte-colony stimulating factor, IL-8, IL-13, IL-16, IL-27, monocyte-chemoattractant protein 1, and TNF-α (224), suggesting that the human host immune response to CDI involves many different pathways, most of which have not been examined in detail. In a separate study, a comparative analysis of serum cytokine levels in CDI and uninfected patients showed that CDI induced a significant increase in cytokine release into the blood system (225). Infection with *C. difficile* resulted in a greater than 2-fold increase in IL-1β, IL-2, IL-5, IL-6, IL-8, IL-10, IL-13, IL-15, IL-16, IL-17A, and TNF-α release into the blood when compared to uninfected donors, with elevated levels of IL-2 and IL-15 associated with increased disease severity (225). Collectively, these studies highlight the importance of the innate immune response in CDI and disease.

TREATMENT, PREVENTION, AND DISEASE RECCURENCE

Antibiotic Treatment of CDI

Depending on disease severity, CDI may require antibiotic treatment to ensure infection clearance. Antibiotic interventions for CDI include oral metronidazole, vancomycin, and more recently, fidaxomicin, in most cases for 10 days (226). For cases of mild and moderate CDI, the European Society of Clinical Microbiology and Infectious Diseases recommends oral metronidazole (either 250 mg four times a day or 500 mg three times a day), because it is less expensive than vancomycin and fidaxomicin while reducing the risk of developing antibiotic resistance in other pathogens associated with the use of the clinically important vancomycin (226). When treating severe CDI, vancomycin is preferred over metronidazole (226). Vancomycin is usually administered at >125 mg at least two to four times a day and has a reduced oral availability and colonic absorption compared to metronidazole, enabling retention in the gut lumen, which facilitates more efficient *C. difficile* clearance (226). Treatment of CDI with fidaxomicin is as effective as vancomycin treatment, with dosage between 50 mg to 200 mg twice a day being sufficient to clear infection (226). Importantly, treatment with fidaxomicin is associated with less recurrent disease compared to metronidazole and vancomycin treatment of CDI. This is because it has a high level of activity against *C. difficile* but a narrow spectrum of activity against other bacteria, particularly Gram-negative bacteria, which results in a reduced impact on the protective colonic microbiota (227, 228).

Prevention

Because there is currently no effective vaccine against *C. difficile* disease, prevention strategies are mainly focused on controlling spread in high-risk environments, including hospitals and nursing homes, and antibiotic stewardship. Although *C. difficile* spores are nearly ubiquitous in health care facilities, infection control guidelines suggested by Vonberg et al. (229) indicate that early diagnosis, extensive education of hospital staff, isolation of infected patients, proper hand hygiene, and hospital surface and equipment disinfection are measures that limit the spread of *C. difficile*. Improving true-positive diagnosis also reduces the unnecessary use of antibiotics, which has been successful in reducing CDI incidence in hospitalized patients (229).

The normal microbiota of the colon prevents overgrowth of *C. difficile* in colonized individuals, although this protective effect can be disrupted following treatment with antibiotics (230). *C. difficile* cell proliferation and toxin production follows, resulting in the symptoms associated with CDI (230). Probiotics have been investigated as a potential preventative therapy for cases of noninfectious AAD with some success, yet studies in the context of primary CDI have proven inconclusive (231). Patients age 65 and older that received a multistrain preparation of lactobacilli and bifidobacteria in a multicenter trial exhibited no reduction in AAD and *C. difficile*-associated diarrhea compared to a placebo group (232). Similarly, provision of the yeast *Saccharomyces boulardii* to hospitalized patients age 50 or over along with prophylactic antibiotic treatment resulted in *C. difficile*-associated diarrhea in 2.1% of patients, compared to 1.5% receiving the placebo, in a single-center trial (233).

Recurrent CDI and Alternative Therapeutics

Although antibiotic treatment of *C. difficile* can effectively clear a primary infection, recurrence of disease occurs in roughly 25% of patients (234). Antibiotic treatment, which depletes colonic microbiota, is a key risk factor for CDI (234). CDI recurrence may occur through infection relapse, either with the same infectious strain causing disease or through infection with a different strain not related to the first infection (235–237). In both cases recurrence is related to disruption of the host microbiota and probably also due to damage that has occurred to the colonic epithelium, the latter of which may contribute to patient susceptibility (238, 239).

The broad-spectrum microbial activity of both metronidazole and vancomycin, although efficient at treating CDI, also allows these antibiotics to continue to disrupt the protective microbiota (240), consequently resulting in patient relapse rates of 19 to 23% and 18 to 32%, respectively (234). Following an initial recurrence of CDI, the risk of a second recurrence increases to approximately 45%, which is also likely to be linked to treatment-mediated depletion of the colonic microbiota (234). The risk of a third CDI recurrence increases to roughly 65% when vancomycin or metronidazole are used to treat the earlier CDI episodes (234). With recurrence rates of roughly 13% and a narrower spectrum of activity on the microbiota compared to vancomycin and metronidazole, fidaxomicin has emerged as a more promising treatment for CDI. The high cost of this antibiotic has unfortunately prevented broad clinical implementation, with a cost more than two times that of vancomycin and over 100 times that of generic metronidazole (241).

The relatively high levels of relapse associated with antibiotic treatment of CDI have resulted in the investigation of alternative therapeutics which aim to treat CDI without disrupting the microbiota. One such therapy, fecal microbiota transplant (FMT), aims to replace or replenish the microbiota of the gastrointestinal tract in times of dysbiosis and is used almost exclusively to treat CDI, particularly after several relapse events (8). By reintroducing "healthy" microbiota from a donor to the colon of CDI patients, in most cases following a failure to treat CDI with antibiotics, the resolution of symptoms and eradication of

C. difficile can be achieved in approximately 80 to 100% of patients (242). However, with a majority of previous FMT work being conducted without effective controls, its efficacy as a treatment for CDI has been a contentious subject. In a recent randomized controlled trial, FMT was highly superior to standard antibiotic treatment of CDI (243). However, because FMT is inherently difficult to standardize and control, due to variation in the microbiota of each individual, Food and Drug Administration (FDA) approval can be difficult to achieve (244). Despite the effectiveness of FMT at reducing recurrent disease, this treatment may not be suitable for all patients, including some immunocompromised individuals for whom organisms from another individual's microbiota may present as pathogenic under these conditions. However, several recent cases have used FMT to treat recurrent CDI in immunocompromised individuals, including solid organ transplant patients, without substantial consequences (245), suggesting that the use of FMT may be a viable treatment for recurrent CDI in select cases. There is undoubtedly a need for alternative treatments for CDI with fewer secondary consequences on the microbiota, and FMT is one such treatment, which has provided a starting point for the development and optimization of microbiota therapy-based approaches (246).

Interestingly, patients with low serum antibody levels are more prone to severe CDI and disease recurrence, while asymptomatic carriers of *C. difficile* have elevated serum IgG levels (247, 248). This observation led to studies of the provision of passive immunization via intravenous immunoglobulin (IVIG) supplementation in the treatment of both severe primary CDI and recurrent disease. This potential therapeutic is prepared from pooled human serum and contains *C. difficile* toxin antibodies (249). Leung et al. (250) demonstrated that IVIG treatment of five children experiencing recurrent CDI who had low serum IgG levels against TcdA resulted in resolution of all clinical signs of colitis with treatment every 3 weeks for up to 6 months, with a rise in IgG TcdA antibodies following therapy. Furthermore, clinical symptoms of CDI were resolved in two adult patients with severe pseudomembranous colitis that did not respond to standard-of-care metronidazole and vancomycin treatment when treated with pooled normal human immunoglobulin (251). However, the mechanism of IVIG-mediated symptom relief is still unknown because only a handful of small-scale case studies have been conducted. IVIG could be beneficial as a therapeutic option for patients that do not respond to treatment with traditional antibiotics or those that cannot be given FMT, although there is an urgent need for randomized trials to understand dosage and timing of treatment, as well as possible side effects.

More recent alternative therapies include the TcdB-specific monoclonal antibody therapy bezlotoxumab, which has recently obtained FDA approval and has been suggested for use in patients with relapsing disease since it does not disrupt the protective microbiota like antibiotics do (238). More recently, work by Hutton et al. (252) has shown that hyper-immune bovine colostrum directed toward TcdB, as well as a combination of TcdB, SLP, and spore exosporium-directed colostrum, is effective at treating and preventing severe disease progression in primary CDI in approximately 70 to 75% of *C. difficile*-infected mice, compared to mice treated with nonimmune colostrum, of which >90% succumbed to disease. Furthermore, the combination therapy also prevented relapse of CDI in roughly 80% of mice, compared to the roughly 90% of mice that relapsed without secondary treatment (252). Both bezlotoxumab and colostrum antibody approaches represent viable alternative therapies for CDI that may help to minimize disease and the recurrence of CDI, which has become increasingly urgent since the emergence of the epidemic 027 strains. The clinical link between increased CDI recurrence and the emergence of ribotype 027 strains is supported by studies identifying higher recurrence rates in patients infected with ribotype 027 strains compared to infections with other strains (253). Disease recurrence has therefore become a focus of CDI treatment and prevention strategies in recent years.

CONCLUSION

Although there has been a substantial increase in our knowledge of *C. difficile* pathogenesis and treatment of infection which has resulted from the development of suitable animal disease models and advances in technology, several key aspects of disease and recurrent infection have yet to be investigated in detail. With the ever-changing epidemiology of CDI and the complexity of *C. difficile*-mediated disease, coupled with the recent listing of *C. difficile* as an urgent public health threat by the CDC, there is a need for an improvement of our understanding of disease pathogenesis and for the development of effective alternative therapeutics for the treatment of infection to provide a better standard-of-care for patients suffering from CDI.

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Histotoxic Clostridial Infections

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62

GENERAL CHARACTERISTICS OF HISTOTOXIC CLOSTRIDIAL INFECTIONS

Many pathogenic clostridial species cause potentially fatal soft tissue infections in humans and animals because of their ability to produce extracellular protein toxins (1, 2). The ability of these pathogens to produce spores that are resistant to environmental stress is an important factor in the epidemiology of these diseases. Disease pathogenesis involves the growth of the clostridial pathogen in the tissues and extensive tissue destruction, which is the result of the action of extracellular toxins. Typical histotoxic clostridial diseases include human gas gangrene or myonecrosis and blackleg in cattle. Although there are several clostridial species that are responsible for these syndromes (Table 1), this review will focus on histotoxic infections caused by *Clostridium perfringens* and *Clostridium septicum*, primarily because these are the species that have been the subject of the most extensive molecular and functional studies.

Although the histotoxic clostridia cause severe, rapidly developing infections, these potent toxigenic bacteria still must be regarded as opportunistic pathogens. All of these infections require predisposing conditions to cause disease, often a traumatic wound that enables the entry of spores from the soil or gastrointestinal tract to gain access to ischemic internal organs or soft tissues of the body. Alternatively, in nontraumatic myonecrosis caused by *C. septicum*, gastrointestinal malignancy is commonly associated with the onset of disease, with tumor development leading to ulceration and necrosis of the gastrointestinal mucosa and entry of the gastrointestinal microbiota into the circulation and subsequent infection at distal sites (3, 4). Clostridial necrotizing soft tissue infections, especially those caused by *C. perfringens*, *Clostridium sordellii*, and *Clostridium novyi*, also have resulted from the subcutaneous injection of contaminated black tar heroin (5–7).

PATHOGENESIS OF CLOSTRIDIAL MYONECROSIS CAUSED BY *C. PERFRINGENS* AND *C. SEPTICUM*

As previously described (8), the pathogenesis of these infections involves three distinct stages, irrespective of whether the source of infection involves a traumatic wound. Stage 1 involves an interruption to the blood supply such that the redox potential in the tissues drops to a level that facilitates spore germination and/or the growth of the infecting clostridial cells. Stage 2 involves bacterial growth and establishment of the conditions for toxin production. In *C. perfringens*, regulation of toxin production primarily utilizes the VirSR two-component signal transduction system (9) and an accessory growth regulator-like quorum sensing system (10, 11), as well as other regulatory networks. Stage 3 encompasses the toxin-mediated cell and tissue damage that leads to necrosis, systemic toxicity, and clinical disease (8).

C. perfringens type A is the major cause of traumatic gas gangrene, although other histotoxic clostridia may also be responsible for this sporadic, but fulminant and often fatal, disease (Table 1) (1). The major toxin produced by gas gangrene strains of *C. perfringens* is α-toxin, a zinc metallophospholipase that has both phospholipase C and sphingomyelinase activity (12). α-Toxin was the first bacterial toxin to be shown to have enzymatic activity (13). Two independent studies using different experimental approaches have shown that α-toxin is essential for *C. perfringens*-mediated myonecrosis. First, immunization studies in mice using recombinant α-toxin variants purified from *Escherichia coli*, and therefore devoid of any other *C. perfringens* toxins, showed that the C-terminal domain of α-toxin was immunoprotective (14). Second, mutation of the α-toxin structural gene (*plc* or *cpa*) abrogated the ability of the bacterium to cause clostridial myonecrosis in a murine model. Virulence was restored by complementation *in trans* with a recombinant plasmid containing the wild-type *plc* gene (15), thereby fulfilling molecular Koch's postulates and providing proof of the essential role of this toxin in disease.

The other major toxin produced by *C. perfringens* type A is perfringolysin O, or θ-toxin, a pore-forming toxin that is a member of the cholesterol-dependent cytolysin family (16, 17). Although mutation of the perfringolysin O structural gene, *pfoA*, did not eliminate the ability to cause disease (18, 19) subsequent studies of *plcpfoA* double mutants (20) provided evidence that perfringolysin O has a synergistic effect with α-toxin on disease pathogenesis (20, 21).

Recent studies have involved the concurrent analysis of the transcriptomes of both the host and *C. perfringens* in a murine myonecrosis infection (22). The results showed that many host genes involved in the innate immune response to infection were upregulated in *C. perfringens*-infected muscle tissues. In the *C. perfringens* cells, upregulated genes included those encoding potential adhesins and proteins associated with the cell envelope.

C. septicum is the major causative agent of nontraumatic gas gangrene, often associated with a gastrointestinal

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TABLE 1 Histotoxic clostridial infections

Syndrome[a]	Causative agent	Host	Major toxins[b]	References
Traumatic gas gangrene	C. *perfringens* type A	Humans	α-toxin	12, 17
			Perfringolysin O (θ-toxin)	
Traumatic gas gangrene	C. *novyi*	Humans	α-toxin	151
	C. *septicum*		α-toxin	129
	C. *histolyticum*		Lethal factor	152
	C. *sordellii*		Lethal toxin (TcsL)	153
			Hemorrhagic toxin (TcsH)	
Nontraumatic gas gangrene	C. *septicum*	Humans	α-toxin	3, 4
	C. *fallax*	Humans and animals	Not known	154
Necrotizing pancreatitis	C. *perfringens* type A	Humans	α-toxin	155
Clostridial myonecrosis	C. *perfringens* types A, B, C, and D	Sheep, cattle, and other animals	α-toxin	29
			β-toxin	
			ε-toxin	
Blackleg	C. *chauvoei*	Cattle, sheep, and other ruminants	Cytolysin A (CctA)	156

[a]The terms "gas gangrene" and "clostridial myonecrosis" are used interchangeably in this review.
[b]Note that the different α-toxins referred to in this column are not necessarily structurally or functionally related.

malignancy (4). The major toxin produced by C. *septicum* is also called α-toxin, but it is not related to C. *perfringens* α-toxin. C. *septicum* α-toxin is an aerolysin-like pore-forming toxin that is secreted as an inactive prototoxin that is cleaved near the C terminus by membrane-bound furin or furin-like proteases to form the active toxin (23). The toxin then oligomerizes on the membrane and subsequently inserts to form a membrane pore, which ultimately leads to cell lysis (24). Genetic studies have shown that α-toxin is essential for C. *septicum*-mediated myonecrosis in mice (25). Mutation of the α-toxin structural gene, *csa*, leads to a loss of virulence, which is restored by complementation with the wild-type *csa* gene.

STRUCTURE AND FUNCTION OF *C. PERFRINGENS* α-TOXIN

α-Toxin is a 43-kDa single polypeptide that is a hemolytic, cytotoxic, dermonecrotic, and lethal extracellular toxin (26–29). Moreover, it induces contractions of blood vessels and smooth muscle, aggregation of platelets, superoxide production, and cytokine release. Importantly, together with perfringolysin O, it is responsible for the hallmark histopathological feature of C. *perfringens*-mediated myonecrosis, the paucity of the polymorphonuclear leukocyte (PMNL) influx into the infected lesion (15, 19–21, 30).

Determination of the crystal structure of α-toxin revealed that it has an α-helical N-terminal domain (amino acids 1 to 246) that is responsible for its enzymatic activity, a flexible linker (amino acids 247 to 255), and a C-terminal (amino acids 256 to 370) β-sandwich membrane-binding domain (31) (Fig. 1). The two domains also are linked by hydrophobic surface interactions between their neighboring faces. The N-terminal domain has significant sequence similarity to phospholipase C enzymes from *Bacillus cereus* (32), *Clostridium bifermentans* (33), and *Listeria monocytogenes* (34). It comprises nine densely packed α-helices and resembles the structure of *B. cereus* phospholipase C (31, 35), which has enzymatic activity, but is neither toxic nor hemolytic. There are three loops in the N-terminal domain: loop 1 (residues 70 to 90), loop 2 (residues 135 to 150), and loop 3 (residues 205 to 215). These loops serve a vital role in the direct interaction between the toxin and the membrane-binding component. The C-terminal domain comprises eight-stranded antiparallel β-sheets (31). This domain plays a role in membrane binding and is required for hemolytic and toxic activity (28, 36). It is absent from nontoxic phospholipase C enzymes (31). It has structural similarity to the C-terminal domain of pancreatic lipase, the N-terminal domain of soybean lipoxygenase, and the C2 phospholipid-binding domains of

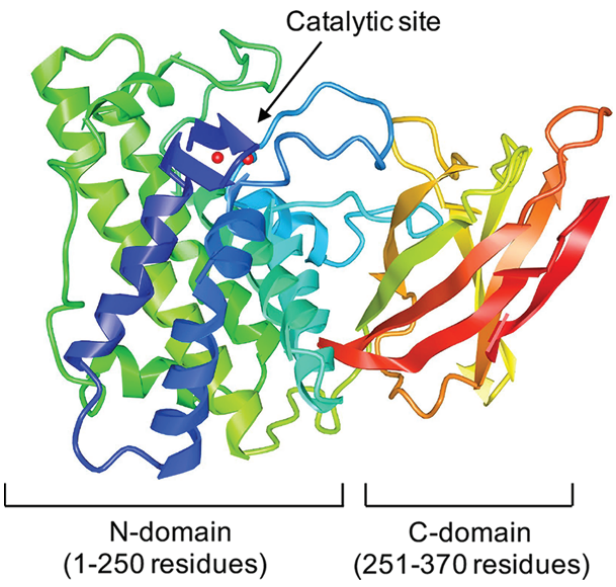


FIGURE 1 Structure of α-toxin. The structure of α-toxin was obtained from the Protein Data Bank (code 1QM6) and was created using MacPyMOL. A ribbon representation is shown, colored from blue (N-terminal) to red (C-terminal). Two zinc metal ions in the active site are shown as red spheres.

eukaryotic intracellular proteins, such as synaptotagmin I, which are involved in signal transduction and vesicular transport (37, 38).

The active site in the N-terminal domain is situated within a solvent-accessible cleft that contains two zinc ions and an exchangeable divalent cation. His-148 and Glu-152 dock with one zinc ion, which is critical for the catalytic site of the toxin. His-11 and Asp-130 tightly dock with the other zinc ion, which is needed for maintenance of the structure. His-68, His-126, and His-136 dock with an exchangeable divalent cation, which is required for binding to target cell membranes (39). The external loop regions (residues 70 to 90, 135 to 150, 205 to 215, 265 to 275, 290 to 300, and 330 to 340) bind to phospholipids in the host cell membranes (31). Thr-74 is present in one of these loops, and although the substitution derivative T74I is still active and can cleave a water-soluble substrate, its membrane-damaging activity toward red blood cells and phosphatidylcholine-cholesterol liposomes is decreased compared to wild-type toxin (40, 41). However, the structure of the T74I variant is identical to that of the wild-type toxin (41). The T74I substitution results in altered recognition of the glycerol moiety in the phospholipid, without affecting the function of the active site, supporting the hypothesis that the amino acid 70 to 90 loop is important for phospholipid recognition (42, 43).

Computational modeling of α-toxin and phosphatidylcholine revealed that Tyr-57 and Tyr-65, which exist at an external site near the catalytic cleft and are in proximity with the 70 to 90 loop, are responsible for substrate interaction (44). The replacement of these Tyr residues with Ala did not have any effect on phospholipase C and sphingomyelinase activity but had a marked effect on the membrane-damaging activity (hemolysis of sheep red cells and disruption of liposomes). These residues exist in or near the entrance of the catalytic cleft and are essential for the membrane-damaging effect of α-toxin, because they are required for entry of the catalytic cleft into the hydrophobic region of plasma membranes (44).

Several studies have shown that the calcium-binding C-terminal domain is responsible for membrane binding (28, 31, 36, 37). Two acrylodan-labeled C-terminal domain derivatives, S263C and S365C, bound to lipid bilayers and displayed a chromogenic shift, demonstrating entry of the C-domain of the toxin into the hydrophobic regions of the liposomes (36). The data provide evidence that the C-terminal domain of the toxin binds to the membrane and that the amino acid 70 to 90 loop and Tyr-57 and Tyr-65 serve an important role in the recognition of target membranes (Fig. 2). Other studies have shown that substitution of the C-terminal domain residues Asp-269, Asp-336, Tyr-275, and Tyr-331, which were predicted to be involved in membrane binding (31), were required for hemolytic and cytotoxic activity (45).

INTERACTION OF *C. PERFRINGENS* α-TOXIN WITH HOST CELLS

Membrane-Damaging Activity on Phospholipid Bilayers

The effect of α-toxin on host tissues is caused by the phospholipase C- and sphingomyelinase-mediated cleavage of

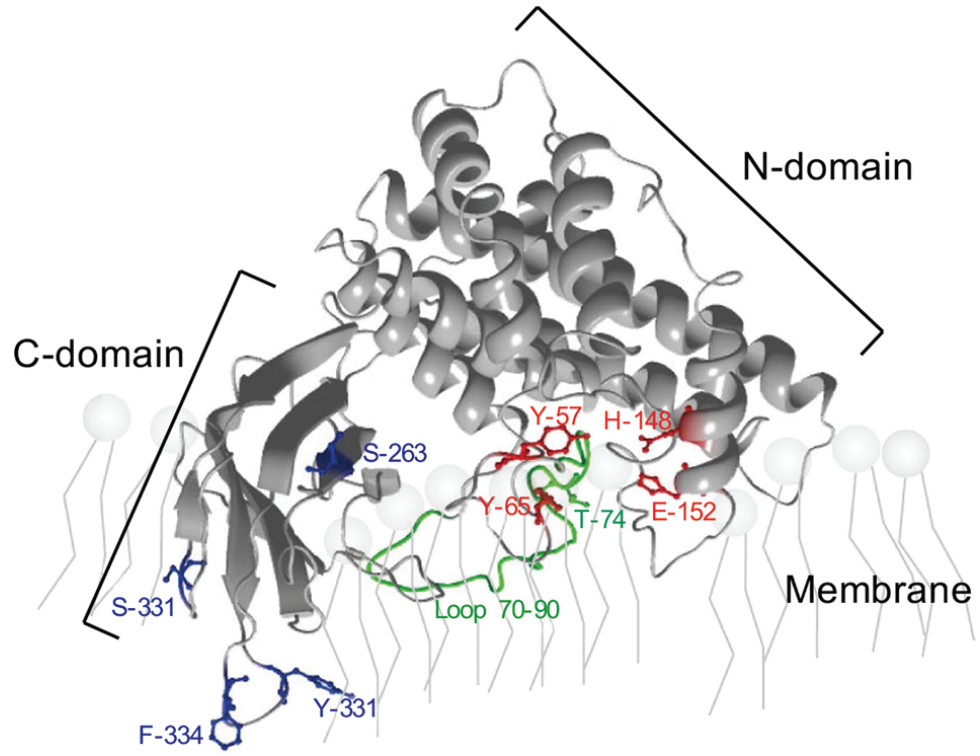


FIGURE 2 Binding model of α-toxin and membrane phospholipids. The structure of α-toxin was obtained from the Protein Data Bank (code 1QM6) and was created using MacPyMOL. Phospholipids are shown as light gray spheres for head groups and light gray lines for tail groups. The head group of a phospholipid, from the outer membrane into the active site of α-toxin, is displayed. Amino acids that may play a role in membrane interaction are shown.

phospholipids in the host cell membranes. The resultant disruption of the phospholipid bilayer together with the end products of these cleavage reactions, diacylglycerol and ceramide, respectively, activates lipid metabolism in host cell membranes and plays a role in host cell damage (27, 28).

The composition of the phospholipid bilayer modulates the extent of the α-toxin-mediated membrane disruption (46). In another study that measured the release of carboxyfluorescein from various liposomes, α-toxin-mediated release, or liposomal leakage, decreased with increasing chain length of the hydrocarbon residues in the phospholipid bilayer (40). It was concluded that membrane disruption was related to the fluidity of the membrane and double bonds in the acyl chain. Subsequently, several phosphatidylcholines (C18:0/C18:1) with a double bond in the *sn*-2 fatty acyl chain were prepared (47). The phase transition temperature value was lowest when the *cis*-double bond was positioned at the center of the C18:1 *sn*-2 acyl chain and increased continuously as the double bond was moved toward either end of the acyl chain. α-toxin-mediated carboxyfluorescein release from various liposomes was highest when the *cis*-double bond was positioned at the center of the *sn*-2 fatty acyl chain, resulting in a bell-shaped curve (47). Accordingly, the membrane-disrupting activity of the toxin is strongly associated with the membrane fluidity of target membranes.

There is evidence that α-toxin also promotes perfringolysin O-induced membrane damage in phospholipid bilayers. Hydrolysis of phosphatidylcholine residues of liposomes that contained cholesterol increased the amount of free cholesterol in the lipid bilayer and enhanced perfringolysin O-dependent cytolytic activity (48). These data may at least partly explain the synergistic effects of α-toxin and perfringolysin O that were previously observed in C. *perfringens* infections of mice (20).

Activation of Phospholipid Metabolism and Hemolysis

α-toxin provokes contraction of isolated rat ileum and aorta tissues in a dose-dependent manner through stimulation of the phospholipid metabolic pathway (49, 50). In isolated rat organs, α-toxin activates the phosphatidylinositol metabolic pathway and then the arachidonic acid cascade, thereby promoting thromboxane production. Contraction caused by the toxin was evoked by thromboxane A2 generation induced by arachidonic acid (49, 50). The metabolism of arachidonic acid results in the production of bioactive lipid mediators, including leukotrienes, thromboxanes, and prostaglandins (27). These bioactive lipid mediators play key roles in enhancing the local inflammatory reaction and lead to vasoconstriction, which is responsible for inducing anoxia in the early stages of bacterial infections. These effects presumably contribute to the capacity of C. *perfringens* to proliferate in the host and cause disease.

Treatment of rabbit neutrophils with α-toxin promotes the formation of diacylglycerol, which is followed by adhesion of the cells to extracellular matrix proteins and the generation of superoxide ions (Fig. 3B) (51). α-Toxin

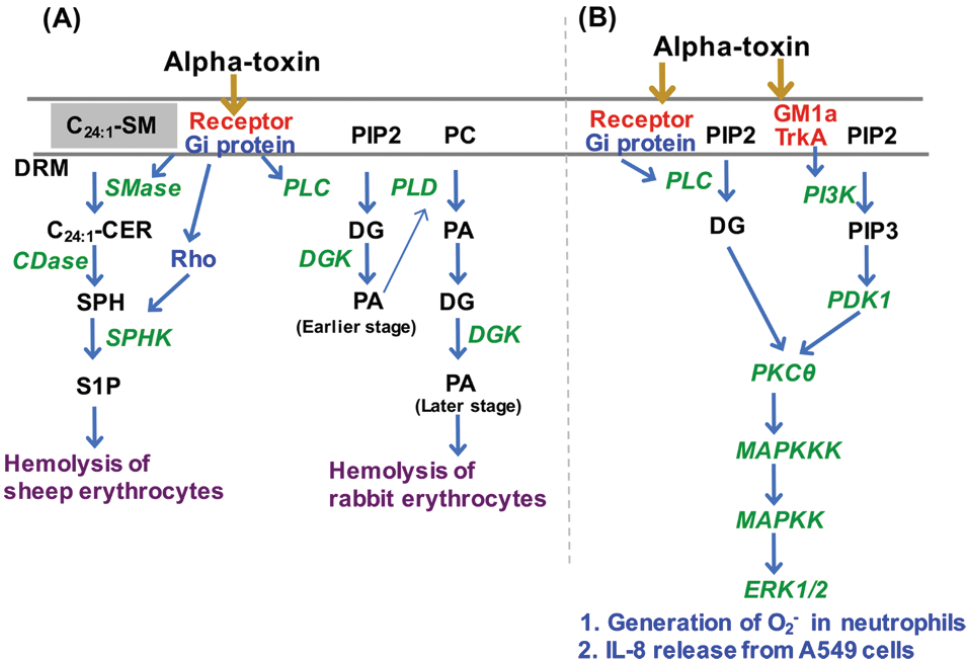


FIGURE 3 Relationship between phospholipid metabolism and the biological activities of α-toxin. (A) Signaling events involved in α-toxin-induced hemolysis of sheep or rabbit erythrocytes. (B) Signaling events involved in α-toxin-activated generation of O_2^- in neutrophils and in α-toxin-mediated release of IL-8 from A549 cells. Abbreviations: SM, sphingomyelin; PIP2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; DG, diacylglycerol; PC, phosphatidylcholine; SMase, sphingomyelinase; PLD, phospholipase D; PA, phosphatidic acid; PKCθ, protein kinase Cθ; PI3K, phosphatidylinositol 3-kinase; PIP3, phosphatidylinositol 3,4,5-trisphosphate; CDase, ceramidase; CER, ceramide; SPH, sphingosine; S1P, sphingosine 1-phosphate; DGK, DG kinase; PDK1, phosphatidylinositide dependent kinase 1; DRM, detergent-resistant membrane; TrkA, neurotrophic tyrosine kinase receptor type1; MAPKK, mitogen-activated protein kinase kinase; Erk1/2, extracellular signal-regulated kinase 1/2.

induces diacylglycerol generation through activation of intrinsic phospholipase C enzymes by a pertussis toxin-sensitive GTP-binding protein. Moreover, it increases the phosphorylation of neurotrophic tyrosine kinase receptor type 1 (TrkA), leading to 3-phosphoinositide-dependent protein kinase 1 activation (52). Therefore, α-toxin independently causes stimulation of intrinsic phospholipase C and protein kinase 1 activity through TrkA receptor activation. Both phenomena synergistically augment the activity of protein kinase C θ, leading to the formation of superoxide ions via activation of the mitogen-activated protein kinase (MAPK) cascade (Fig. 3B) (52). In human lung carcinoma (A549) cells, α-toxin mediates the activation of the p38 MAPK, NF-κB, and extracellular signal-regulated kinase 1/2 (ERK1/2) through TrkA activation, which leads to interleukin-8 (IL-8) release (53) (Fig. 3B).

Treatment of rabbit erythrocytes with α-toxin induces the biphasic formation of phosphatidic acid (54) (Fig. 3A). α-Toxin-induced formation of phosphatidic acid is activated in the earlier stage (30 s) by intrinsic phospholipase C and in the later stage (ca. 25 min) by intrinsic phospholipase D. Both processes involve stimulation of the G-protein-signaling pathway by toxin-mediated membrane phospholipid cleavage (55) (Fig. 3A).

Sheep erythrocytes have higher amounts of sphingomyelin but do not contain phosphatidylcholine. Cleavage of sphingomyelin by α-toxin leads to increased levels of ceramide and sphingosine 1-phosphate (56), which is essential for toxin-induced hemolysis (57) and activates the sphingomyelin metabolism system (Fig. 3A) (56). The resultant cleavage of N-nervonoic sphingomyelin ($C_{24:1}$-SM) occurs in a detergent-resistant membrane component (57) (Fig. 3A). Pertussis toxin blocks the production of $C_{24:1}$-ceramide caused by α-toxin and blocks red cell hemolysis, which indicates that intrinsic sphingomyelinase, which can cleave $C_{24:1}$-SM to $C_{24:1}$-ceramide, is regulated by a pertussis toxin-sensitive G-protein in plasma membranes. In addition, the activation of Rho protein by α-toxin increases sphingosine kinase activity. Subsequently, the ceramides are quickly metabolized to sphingosine, indicating that the toxin also activates ceramidases (57). These results indicate that the cleavage of $C_{24:1}$-SM to sphingosine 1-phosphate by α-toxin in lipid rafts is essential for toxin-induced hemolysis of sheep red cells (Fig. 3A).

Interaction of α-Toxin with Gangliosides

Amino acid sequence analysis of botulinum toxin and tetanus toxin showed that the ganglioside-GT1b- and lactose-binding site is determined by the existence of the conserved peptide motif, H.....SXWY.....G (ca. 30 amino acids [aa]), with the Tyr and Trp residues being particularly critical (58). A similar motif (H.....SWY.....G, amino acids 68 to 93) is present in α-toxin (53), in the region (amino acids 55 to 93) that is located at the interface between the *N-terminal* and C-terminal domains (42). It has been demonstrated that the 70 to 90 loop in α-toxin plays a role in binding to the cell surface (43) (Fig. 2). Computational docking studies indicated that Trp-84 binds to the sialic acid residue on ganglioside GM1a via an aromatic stacking interaction and a hydrogen-bonding interaction and that Tyr-85 associates with the galactosamine residue on GM1a via an α-glycosidic bonding interaction (59). Replacement of Trp-84 and Tyr-85 in α-toxin, which correspond to the ganglioside recognition site of botulinum and tetanus toxins, significantly reduced binding to ganglioside GM1a-containing liposomes. W84A and Y85A derivatives had markedly decreased capabilities to stimulate TrkA (59). Ganglioside GM1a assembles with a TrkA molecule on the cell surface and stimulates TrkA and ERK1/2 (60). It has been reported that ganglioside GM1 clustering leads to the condensation of TrkA in lipid rafts and the stimulation of downstream signaling targets (61). α-Toxin-mediated diacylglycerol production leads to diacylglycerol flip-flop movement that affects the dynamics of cell membranes (Fig. 4). Therefore, it serves in GM1a clustering and intrinsic phospholipase C-γ1 activation through TrkA, triggering various signal transduction activities (62–64).

α-toxin causes higher cytotoxicity in a ganglioside-deficient cell line (Don Q) (65) and can be internalized by dynamin-mediated endocytosis (66, 67). The toxin binds to caveolin-1 both on the cell membranes and in endosomal vesicles, and it also induces ceramide production in the cell membrane (28), which generates negative membrane curvature and facilitates endocytosis (68). Internalized α-toxin is transported to the early and late endosomes and lysosomes. Lysosomes are damaged during progressive α-toxin internalization into Don Q cells. In addition, the internalization of α-toxin is also mediated through the stimulation of MEK/ERK signaling (66, 67). It has been reported that the toxin causes reactive oxygen species generation via PKC, MEK/ERK, and NF-κB signaling activities in Don Q cells (66, 67). Therefore, α-toxin perturbs the host cell signaling cascade and acts not only on the cell membrane, but also in the cytoplasm.

Effect of α-Toxin on Hematopoietic Cells

The first line of defense of the innate immune system involves neutrophils, which play an important role in the phagocytosis and subsequent elimination of pathogenic bacteria (69, 70). Normally, the number of neutrophils is sustained in a steady state through granulopoiesis, which is accelerated to replenish neutrophils during bacterial infection (71–73). Pattern recognition receptors, such as Toll-like receptors (TLRs), are responsible for the recognition of structural components of microorganisms (74, 75). TLR2 and TLR4 have been identified as being pivotal for the recognition of Gram-positive or Gram-negative bacteria by recognizing cell wall components such as peptidoglycan and bacterial endotoxin (lipopolysaccharide), respectively (76–79). During bacterial infection, these bacterial components stimulate the production of granulocyte colony-stimulating factor (G-CSF), which is a glycoprotein that promotes the proliferation, survival, and differentiation of neutrophils and their progenitor cells (80) from endothelial cells and monocytes, resulting in the acceleration of granulopoiesis (81). G-CSF-deficient and G-CSF receptor-deficient mice exhibit chronic neutropenia and reduced infection-driven granulopoiesis, leading to impaired ability to eliminate infecting bacteria (82, 83). Thus, granulopoiesis is precisely regulated to replenish neutrophils during bacterial infection. However, *C. perfringens* can still cause life-threatening infections, and the mechanisms by which *C. perfringens* avoids these host defenses are still not completely understood.

C. perfringens-mediated myonecrosis progresses so rapidly that death, which is associated with muscle destruction, shock, and multiple organ failure, sometimes precedes an accurate diagnosis (84). *C. perfringens* infection is characterized by a greatly reduced PMNL influx to the site of the infection (19, 85–87), suggesting that the bacteria disrupt neutrophil-based innate immunity to evade host defenses. α-Toxin mediates the formation of platelet-leukocyte aggregates, leading to vascular occlusion, and induces a marked

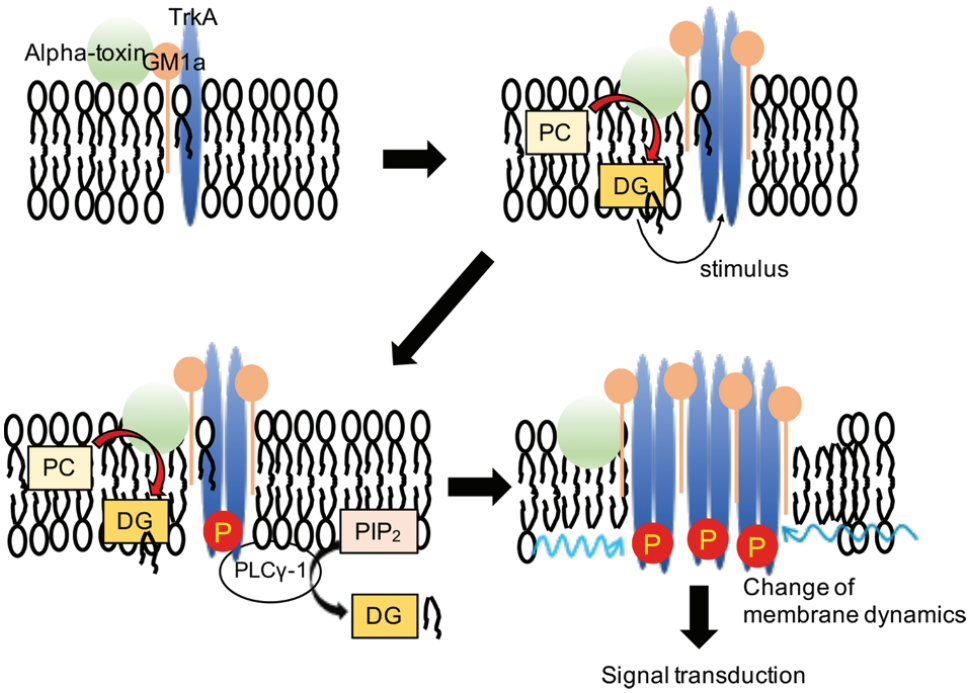


FIGURE 4 Model of α-toxin-induced membrane dynamics and clustering of the GM1a and TrkA complex. α-Toxin binds to GM1a and, through the phospholipase C activity of the toxin itself, causes diacylglycerol (DG) production at the outer membrane. The flip-flop movement of DG affects membrane dynamics, facilitating clustering of GM1a and TrkA activation by phosphorylation. Activation of TrkA results in the activation of PLCγ-1, leading to the increased production of DG. Finally, DG formation results in the enhanced activation of TrkA, triggering activation of signal transduction pathways, which induce neutrophil activation. Abbreviations: PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; DG, diacylglycerol; PC, phosphatidylcholine; TrkA, neurotrophic tyrosine kinase receptor type 1.

reduction in microvascular perfusion (45, 86, 88), which is thought to impede neutrophil extravasation (89). Recently, it was shown using a mouse model of infection that *C. perfringens* infection reduced mature neutrophils in bone marrow, in peripheral blood, and in *C. perfringens*-infected muscle, in an α-toxin-dependent manner (90). α-Toxin inhibits neutrophil differentiation and impairs the replenishment of differentiated mature neutrophils in the peripheral circulation, resulting in reduced recruitment of neutrophils to the *C. perfringens* infection site. The half-life of peripheral neutrophils is less than 12 hours *in vivo* (91), meaning that the number of mature neutrophils required to counter a bacterial infection is sustained through continuous granulopoiesis. Therefore, blockage of both granulopoiesis and PMNL diapedesis into the tissues, as a result of platelet aggregation, leads to a reduction in cell numbers in a short period of time. Thus, *C. perfringens* infection impairs the host immune system, which could explain the both scarcity of PMNL at the site of infection and a propensity for polymicrobial infections in many patients (Fig. 5).

α-Toxin is essential for disease pathogenesis (18–21, 30), and blockage of neutrophil differentiation is dependent on its phospholipase C and sphingomyelinase activities (90). In human lymphocytes, *S. aureus* sphingomyelinase disrupts lipid rafts, which are cholesterol-rich plasma membrane microdomains (92). Lipid rafts function as platforms of signaling molecules involved in the regulation of cell differentiation in many cell types (93, 94). Ceramide, the hydrolysis product of sphingomyelinase, also is known to be a lipid messenger implicated in various cellular responses, including apoptosis, differentiation, and cell growth (95). Recently, *C. perfringens* α-toxin was reported to perturb the integrity of lipid rafts, leading to the blockage of neutrophil differentiation (90). The detailed molecular mechanism behind this blockage has not been elucidated, but it may be important to focus on the relationship between the role of sphingomyelinase and cell differentiation to shed further light on host-pathogen interactions in clostridial myonecrosis.

Effect of α-Toxin on Erythropoiesis

α-Toxin has been reported to lyse erythrocytes from various species. It activates T-type Ca^{2+} channels and increases intracellular Ca^{2+}, which plays an important role in the hemolysis of horse erythrocytes (96). The toxin activates the sphingomyelin metabolic system, leading to the hemolysis of sheep erythrocytes (56, 57), and the lysis of rabbit erythrocytes involves α-toxin-mediated activation of endogenous phospholipase C activity (54, 55). Recently, *Bacillus anthracis* lethal toxin was reported not only to induce hemolysis, but also to inhibit erythroid differentiation of cord blood-derived $CD34^+$ hematopoietic stem cells, leading to the suppression of erythropoiesis (97). The toxin-induced suppression of erythropoiesis is potentially part of a lethal toxin-mediated pathophysiology in anemia and hypoxia. Similarly, it was reported that mature erythroblasts were preferentially decreased when isolated mouse bone marrow cells were treated with α-toxin, and subsequent experiments revealed

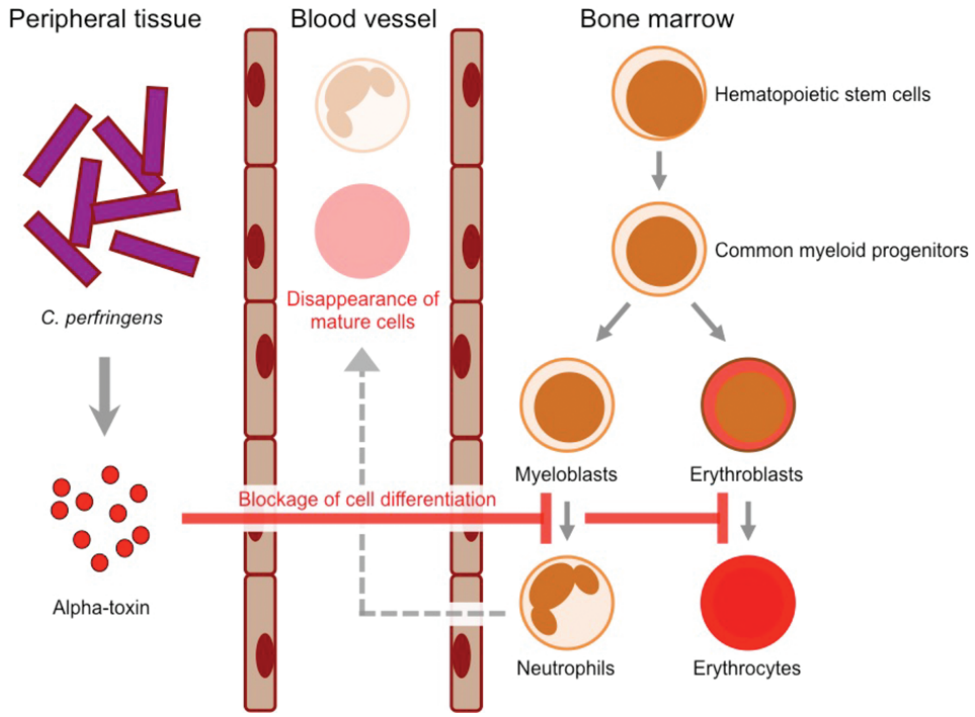


FIGURE 5 Inhibition of granulopoiesis and erythropoiesis in a C. *perfringens*-infected host. Infection with C. *perfringens* diminishes mature hematopoietic cells in the bone marrow, leading to impairment of replenishment of differentiated neutrophils and erythrocytes in the peripheral circulation and resulting in host innate immune deficiency. The major virulence factor of C. *perfringens*, α-toxin, plays an important role in this phenomenon by interfering with cell differentiation of myeloblasts and erythroblasts.

that the blockage of erythroid differentiation was involved in the reduction in mature erythroblasts by α-toxin (98). Massive intravascular hemolysis and severe anemia have been reported in C. *perfringens*-infected patients (99), although it is a relatively uncommon event. The detailed mechanisms by which C. *perfringens* infection causes severe anemia have not been elucidated, but blockage of erythropoiesis by α-toxin may be important (Fig. 5).

Experiments carried out using an inactive α-toxin H148G variant revealed that its enzymatic activity was required for the α-toxin-mediated reduction of erythroblast levels (98). Furthermore, the cell surface expression of a lipid raft marker, GM1, decreased in association with erythroid differentiation, and α-toxin affected lipid raft integrity in erythroid progenitors, leading to the impairment of erythroid differentiation. During erythroblast enucleation, the clustering of lipid rafts at the furrow between incipient reticulocytes and pyrenocytes is observed, and an inhibitor of lipid rafts impedes enucleation (100). Therefore, lipid rafts play an important role in the regulation of erythropoiesis, but the detailed mechanism by which the disturbance in lipid raft integrity by α-toxin impairs erythroid differentiation remains unclear.

STRUCTURE AND HOST CELL INTERACTIONS OF *C. PERFRINGENS* PERFRINGOLYSIN O

Perfringolysin O (θ-toxin) is a pore-forming hemolytic toxin that belongs to the cholesterol-dependent cytolysin (CDC) family (16, 101), which includes streptolysin O from *Streptococcus pyogenes*, listeriolysin O from *L. monocytogenes*, pneumolysin from *Streptococcus pneumoniae* (17), and related cytolysins produced by several other clostridia (102). The perfringolysin O structural gene, *pfo*A, is located on the chromosome (103, 104). Perfringolysin O is elaborated by most, but not all, strains of C. *perfringens* (104–107), and mature perfringolysin O (53 kDa, 473 aa) (108) is secreted as a water-soluble monomer and binds to host cell membranes in a cholesterol-dependent manner (102). Binding to the cell membrane initiates oligomerization and the formation of a pore complex that can contain up to 50 monomeric subunits and is responsible for cell lysis.

Perfringolysin O monomers have a hydrophilic, β-sheet-rich structure that can be divided into four distinct domains (109, 110) (Fig. 6). Domain 1 contains a seven-stranded antiparallel β-sheet flanked by helices and loops. Domain 2 forms an elongated β-sheet structure. Domain 3 comprises α-helices and β-sheets, and domain 4 (the C-terminal region) is divided into two distinct β-sandwich regions. Domain 4 also contains a highly conserved 11-aa Trp-rich motif (ECTGLAWEWWR) or undecapeptide loop that is near the C-terminus. This structure subsequently was shown to be similar to that of members of the eukaryotic membrane attack complex/perforin family (111).

Perfringolysin O and most of the other cholesterol-dependent cytolysins bind directly to cholesterol in the host cell membrane. Contrary to what was thought for many years, the undecapeptide is not the receptor-binding motif (112). Instead, there is a Thr-Ile pair located in a nearby loop (L1) of domain 4 that is responsible for binding to cholesterol (113). The role of the undecapeptide is to assist in anchoring the toxin monomer to the membrane and to couple membrane insertion with oligomerization (112). The next step in the

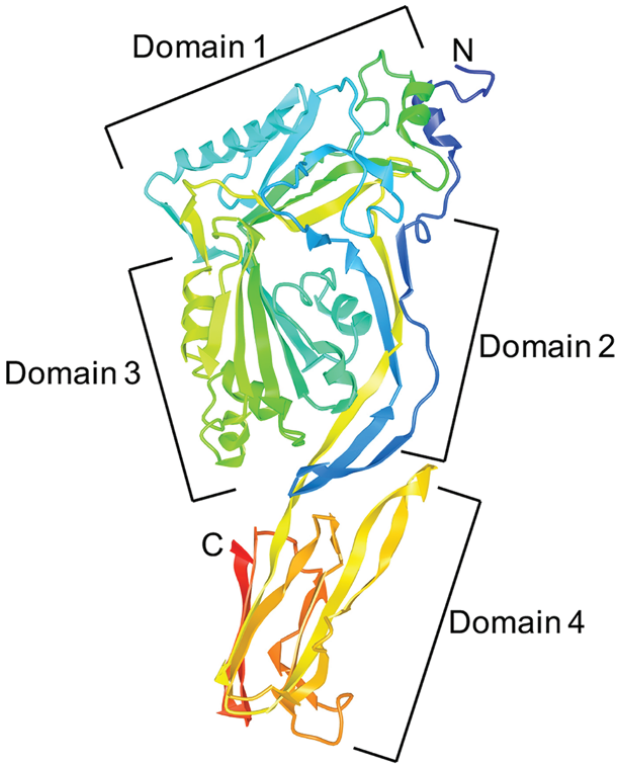


FIGURE 6 Structure of perfringolysin O. The structure of perfringolysin O was obtained from the Protein Data Bank (code 1PFO) and was created using MacPyMOL. A ribbon representation is shown, colored from blue (N-terminal) to red (C-terminal).

oligomerization process is a conformational change of two α-helical bundles in domain 3 to form two longer amphipathic β-hairpins; this extended β-hairpin structure inserts into the host cell membrane, initially to form toxin dimers and then to form a large oligomeric β-barrel pore that contains 35 to 45 monomers. The biological effect of large pore formation is to induce cell lysis by osmotic stress (114). In addition, perfringolysin O can interfere with cell signaling events by activating TLR4 and inducing inducible nitric oxide synthase expression and tumor necrosis factor-α (TNFα) secretion in bone marrow-derived macrophages (115) and by stimulating the degradation of Ubc9, thereby reducing the posttranslational SUMOylation of host proteins (116). Therefore, perfringolysin O has effects on both membrane integrity and internal cell signaling.

Perfringolysin O works synergistically with α-toxin to cause gas gangrene by promoting leukostasis and intravascular coagulation (19–21, 117). The result is decreased blood flow (87, 88) and the accumulation of PMNL and macrophages in the blood vessels around the site of infection, which leads to vascular leukostasis and contributes to the paucity of leukocyte infiltration to the site of infection (20, 21). Similarly, intramuscular injection of a crude *C. perfringens* toxin preparation causes and promotes the formation of intravascular platelet aggregates (87). Leukocytes and fibrin accumulate rapidly in the aggregate, leading to obstruction of the blood vessels and the production of thrombi that reduce local blood flow (87, 88).

Perfringolysin O upregulates the expression of adherence molecules, including platelet-activating factor, CD11b, and endothelial adherence molecules (118–120). In addition, it stimulates the expression of intercellular adherence molecule 1 and platelet-activating factor in endothelial cells (19, 118, 119, 121). The toxin triggers platelet/leukocyte and platelet/platelet aggregation by activating the platelet fibrinogen receptor gpIIb/IIIa synergistically with α-toxin (86, 122). Moreover, it has been reported to impair the polymerization of actin filaments in leukocytes and the migration of neutrophils in response to chemoattractants (118, 123). These events lead to the formation of intravascular platelet/leucocyte/fibrin aggregates, and the adherence of the aggregates to the vascular endothelial cells results in vascular injury, vessel obstruction, hypoxia, and tissue destruction (1, 30, 124). In addition, a direct cytotoxic effect of perfringolysin O on endothelial cells has been demonstrated, which also impairs leukocyte transmigration (30, 88). Disruption of the endothelium contributes to progressive edema (30). α-Toxin impairs granulopoiesis, leading to the reduction of neutrophils, and a direct cytolytic effect of perfringolysin O on leukocytes has been reported (123). Therefore, perfringolysin O works synergistically with α-toxin to reduce the level of leukocytes. Finally, *C. perfringens* cells can escape from the phagosome of macrophages and survive, and perfringolysin O is an important factor in this escape process (125, 126). These data are in agreement with genetic studies that showed that α-toxin and perfringolysin O act synergistically in the disease process (19, 20).

In the latter stage of clostridial gas gangrene, cardiovascular collapse, tachycardia, and multiorgan failure are observed in patients (1). Toxins such as perfringolysin O and α-toxin are released into the blood and can act at a distance from the site of infection. Perfringolysin O reduces systemic vascular resistance, increases cardiac output, and decreases the heart rate without a drop in mean arterial pressure (117, 127). In addition, it promotes the release of inflammatory cytokines, such as TNFα, IL-1, IL-6, platelet-activating factor, and prostaglandin I2, by acting synergistically with α-toxin, which contributes to the symptoms of toxic shock, including hypotension, hypoxia, and reduced cardiac output (30, 128).

FUNCTION AND HOST CELL INTERACTIONS OF *C. SEPTICUM* α-TOXIN

C. septicum is the primary causative agent of nontraumatic clostridial myonecrosis and enumerates an α-toxin that is essential for disease and is responsible for reduced PMNL influx into the infected lesions (25). *C. septicum* α-toxin is a member of the aerolysin family of pore-forming toxins (129, 130). These toxins include aerolysin from *Aeromonas hydrophila*, ε-toxin and *C. perfringens* enterotoxin (CPE) from *C. perfringens*, and parasporin toxins produced by *Bacillus thuringiensis*. These toxins all form small, often heptameric, β-barrel pores in host cell membranes (130). Although the crystal structures of several members of the aerolysin family have been determined, there is no such structure available for α-toxin. However, molecular modelling of α-toxin shows that it does not have the small-lobe lectin-binding domain (D1) of aerolysin, known as the aerolysin pertussis toxin domain (131), but has homologous domains to the other three aerolysin domains (Fig. 7) (132).

α-Toxin is synthesized as a preprototoxin, with a 31-aa leader sequence that is cleaved upon secretion from the bacterial cell and a 45-aa C-terminal extension that is cleaved by host proteases such as furin prior to pore formation (23, 102, 133). The resultant cleaved propeptide prevents premature oligomerization (134). The 46-kDa prototoxin

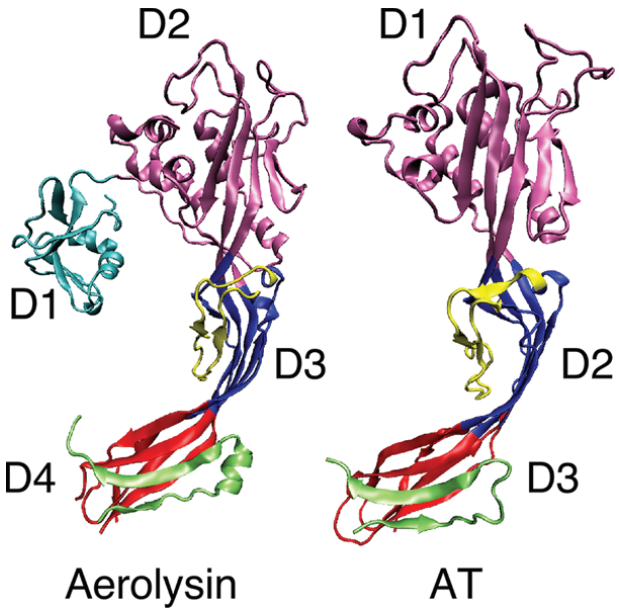


FIGURE 7 Crystal structure of aerolysin and molecular model of *C. septicum* α-toxin. Domains are indicated. Note that domain 1 (D1) of aerolysin is not present in α-toxin. AT, *C. septicum* α-toxin. Reprinted with permission from reference 137. Copyright (2006) American Chemical Society.

binds to glycophosphatidylinositol-anchored protein receptors located in lipid rafts in the host cell membrane (135). After protease cleavage on the cell membrane, the 43-kDa mature toxin undergoes a conformational change and oligomerizes to form a heptameric prepore complex that is required for pore formation (24). Cleavage of the C-terminal extension enables the formation of a transmembrane β-barrel structure, with each monomer contributing one extended β-hairpin loop to the β-barrel that forms the transmembrane pore (130, 132).

The analysis of substitution derivatives of α-toxin has yielded insights into the functional domains of the toxin (132, 136–138). Y155F and S189C α-toxin derivatives had reduced toxicity for CHO cells but were still cleaved by host proteases and could bind to glycophosphatidylinositol-anchored receptors and form oligomers. A S189C/S238C derivative retained its ability to bind, act as a substrate for furin cleavage, and form oligomers but was not toxic (136). Like aerolysin, α-toxin has a Trp-rich motif (amino acids 302 to 312) in its C-terminal domain (138). Several of the residues in this motif, including three Trp residues, are essential for binding and/or cytotoxic activity (137, 138). Fluorimetric studies using Cys-substituted derivatives led to the identification of the amphipathic transmembrane β-hairpin (Lys-203 to Gln-232) that spans the host cell membrane. Deletion of this region eliminated the toxin's ability to form pores and kill cells, but the resultant protein could still bind, undergo cleavage, and form oligomers (132). Subsequently, saturation Ala or Cys substitutions were made across the remaining residues in the α-toxin molecule, and the resultant proteins were purified and analyzed for their biological and biophysical properties (137). The results showed that the only α-toxin Cys residue, Cys-86 in domain 1, was required for receptor binding. In summary, derivatives altered in receptor binding all had substitutions in domain 1, but proteins with altered oligomerization properties had changes in domains 1 and 3. Several of these mutants have been examined in the mouse myonecrosis model (139). The results showed that both the amphipathic β-hairpin and Cys-86 were essential for disease, providing evidence that receptor binding and pore formation are important in pathogenesis.

Pore formation is essential for *C. septicum*-mediated myonecrosis (129, 140), but pore-mediated osmotic cell lysis is not the only consequence of intoxication by α-toxin. Treatment of C2C12 mouse myoblast cells with α-toxin leads to a rapid Ca^{2+} influx into the cell but does not induce apoptosis. Instead, α-toxin induces programmed cellular necrosis (140), which is consistent with the fact that it is more active against nucleated cells (141, 142). α-Toxin-mediated Ca^{2+} influx resulted in the activation of proteolytic calpain activity, a reduction in lysosomal integrity, leading to cathepsin release, mitochondrial dysfunction and ATP depletion, and release from the nucleus of the histone-binding protein HMGB1 (140). Induction of this programmed necrotic pathway was dependent on the ability to form pores; treatment with a substituted α-toxin lacking the β-hairpin loop did not induce a Ca^{2+} influx or programmed necrosis. All of these changes are consistent with α-toxin-mediated pore formation causing the induction of the programmed cellular necrosis pathway (140). Other studies have shown that α-toxin treatment of Madin-Darby Canine Kidney (MCDK) cells causes a rapid K^+ efflux, ATP depletion, and caspase-3-independent necrosis and nonapoptotic cell death (129). Subsequently, it was shown that pore formation by α-toxin led to ERK, c-Jun N-terminal protein kinase, and p38 phosphorylation and activation of their resultant MAPK pathways and the resultant dose-dependent release of TNFα (143). *C. septicum* α-toxin also leads to a significant reduction in vascular perfusion of muscle tissues, as shown by comparative intravital microscopy studies using culture supernatants from an isogenic series of *C. septicum* strains (88).

CONTROL AND TREATMENT OF HISTOTOXIC CLOSTRIDIAL INFECTIONS

The treatment of myonecrotic infections caused by *C. perfringens* and *C. septicum* is complicated by the rapid onset and progression of disease. Death can result within 24 hours of initial symptoms. Standard treatment regimens involve surgical debridement of the infected tissues, which needs to occur as early as possible (2), and treatment with penicillin and clindamycin (144). The effectiveness of the penicillin on its own has been questioned (145), and there is experimental evidence that clindamycin and metronidazole, alone or in combination with penicillin are more effective in mice (145, 146). Often, surgical removal of an infected limb is the only option available in life-threatening infections.

In other studies, mice lacking TNF-α were protected from *C. perfringens* α-toxin-induced lethality (147), demonstrating that TNF-α release is essential for the lethal effect of the toxin. Treatment with erythromycin blocked α-toxin-induced release of TNF-α from peripheral neutrophils and the activation of TrkA and ERK1/2, demonstrating that toxin-induced TNF-α release is related to stimulation of the ERK/MAPK signaling pathway through TrkA. Anti-TNF-α antibodies, but not anti-IL-1β or anti-IL-6 antibodies, blocked the death of mice and intravascular hemolysis by α-toxin. Compounds that block the release of TNF-α therefore may be valuable in treating patients with *C. perfringens* infection. Recently, experiments with *C. perfringens* infections in mice provided evidence that treatment with opioids such as buprenorphine stop the development of

disease (148), but this treatment option has not been tested in clinical settings.

Several studies have investigated the use of recombinant α-toxin variants to vaccinate against C. *perfringens*-mediated gas gangrene, primarily from a military or biosecurity perspective or for elderly patients that are at risk. Initial studies showed that the isolated nontoxic C-terminal C2 lipid-binding domain of α-toxin (amino acids 247 to 370) was highly immunoprotective in mice (14). Mice immunized with this formaldehyde-treated recombinant antigen were protected from the effects of α-toxin and from challenge with a 10× LD_{50} dose of C. *perfringens*. By contrast, mice immunized with the equivalent N-terminal enzymatic domain (amino acids 1 to 249) produced immunoreactive antibodies, but these antibodies did not protect against the toxin or disease. These results were confirmed in a subsequent, more detailed, vaccination study that also showed that mice vaccinated with the C-terminal domain and then challenged in a murine infection model had reduced thrombosis and an increased PMNL influx into the site of infection (149). To further define the immunoprotective region of the C-terminal domain, the potency of several recombinant fragments (amino acids 251 to 370, 281 to 370, and 311 to 370) fused to glutathione S-transferase (GST) were examined in an active vaccination model (150). Vaccination with the 251 to 370 and 281 to 370 GST-fusions protected against the toxicity of α-toxin and against C. *perfringens* challenge, whereas the amino acid 311 to 370 derivative yielded only partial protection. Despite these three promising studies, no commercial C. *perfringens* vaccine is currently available.

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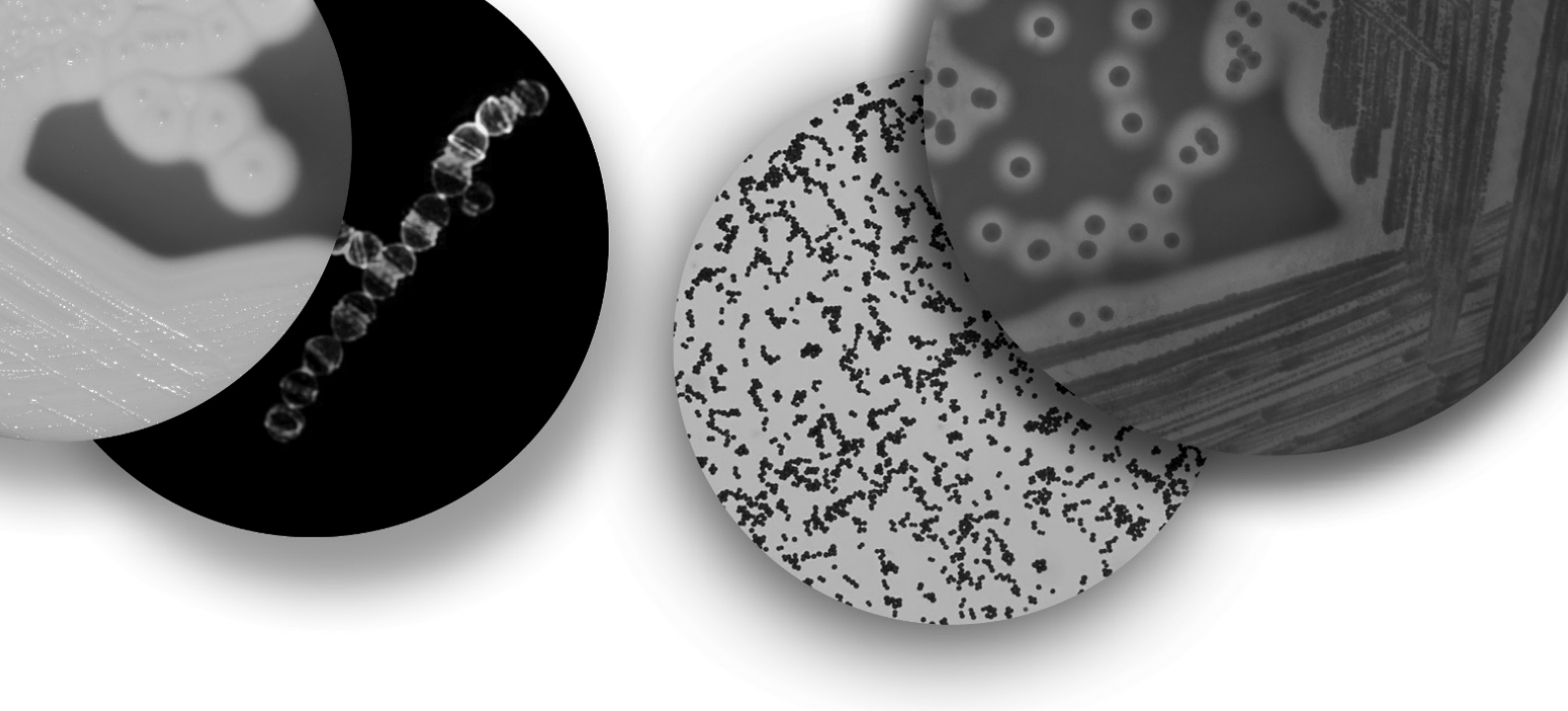
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SECTION 6

Mycobacteria and Corynebacteria

Section Editor: **Miriam Braunstein**

Mycobacteria and corynebacteria are high-GC Gram-positive bacteria of the *Actinobacteria* phylum and *Corynebacteriales* order. Mycobacteria and corynebacteria share a novel cell envelope containing a highly impermeable crosslinked network of peptidoglycan, arabinogalactan and long-chain mycolic acids. In conjunction with non-covalently linked lipids and glycolipids, mycolic acids form an outer membrane bilayer, called the mycomembrane. The double-membrane, diderm, cell envelope of mycobacteria and corynebacteria is dramatically different from the monoderm cell envelope of most Gram-positive bacteria. Moreover, the mycomembrane is physically similar to that of Gram-negative bacteria, although its molecular composition differs completely from that of the Gram-negative outer membrane. Along with the distinctive cell envelope structure, mycobacteria and corynebacteria, are increasingly recognized as having novel mechanisms for critical processes such as cell division, protein export, and metabolism.

Among the diverse array of mycobacterial and corynebacterial species are two notorious bacterial pathogens: *Mycobacterium tuberculosis* (etiologic agent of tuberculosis) and *Corynebacterium diptheriae* (etiologic agent of diptheria). The chapters that follow cover a mix of topics on the physiology and pathogenesis of mycobacteria and corynebacteria. As a result of increased research attention and new molecular technologies, our understanding of the distinctive features of these bacteria has expanded greatly in recent years.

Mycobacteriophages

GRAHAM F. HATFULL

63

INTRODUCTION

Because of the importance of mycobacterial diseases such as tuberculosis and leprosy, mycobacteriophages have long been studied, with the first ones being isolated in the 1940s (1–3). At that time, it was recognized that bacteriophages display particular profiles of specificity for their bacterial hosts, nearly always distinguishing between bacteria in different genera and sometimes distinguishing between strains of the same bacterial species (4–7). Mycobacteriophages thus presented a plausible means of identifying (i.e., typing) clinical isolates by scoring for phage sensitivity profiles, with the prospects of obtaining data considerably faster than standard protocols that required extensive growth of the host. *Mycobacterium tuberculosis*—the causative agent of human tuberculosis—has a remarkably slow growth rate (24-hour doubling time), so phage typing can speed up diagnosis by several weeks. This general concept of exploiting the relatively rapid propagation of mycobacteriophages has been a common theme in their subsequent development (8–10).

Although the important role of *M. tuberculosis* in human health was known from the early studies by Koch, our knowledge of the organism was limited for many years because of the inability to genetically transform it with the uptake of DNA (11). An important breakthrough in the late 1980s occurred when it was demonstrated that phage DNA could be taken up by mycobacterial spheroplasts, followed by conversion into infectious units and plaque formation (12). The approach illustrated three additional aspects of working with the mycobacteria and their phages. First, Jacobs and colleagues used *Mycobacterium smegmatis* as a surrogate host, because of its much faster growth rate (~3-hour doubling time), coupled with mycobacteriophages which had been shown to infect both *M. smegmatis* and *M. tuberculosis*. Second, the construction of shuttle phasmids, which replicate as a large plasmid in *Escherichia coli* and as a phage in mycobacteria, facilitated the introduction of foreign DNA, because the shuttle phasmids could be manipulated readily in *E. coli* and then used as vectors in *M. smegmatis* and *M. tuberculosis*. Shuttle phasmids have found extensive uses for introduction of reporter genes, transposons, and allelic exchange substrates into a variety of mycobacterial hosts (11). Lastly, shuttle phasmids built with temperate phages enable stable introduction of gene cassettes that can be used to evaluate expression and activities of foreign genes such as those for antibiotic resistance (13).

Mycobacteriophages entered the genomics era in the early 1990s (14), opening up a variety of new tools that can be applied to studying the mycobacteria (15). As sequenced mycobacteriophage genomes emerged over the next 25 years, the extent of their considerable genetic diversity became apparent, culminating in a current collection of over 9,600 individual phages, of which 1,500 have been completely sequenced (16). Because all of these (with the exception of DS6A) infect a single bacterial strain (*M. smegmatis* mc^2155), they represent a unique perspective on viral diversity and bacteriophage evolution. These also encompass more than 150,000 genes and together provide a tremendous resource for advancing our understanding and control of their mycobacterial hosts.

In this article I will review our current understanding of mycobacteriophages, their roles in mycobacterial genetics, and the insights gained from their diversity. A number of other reviews of mycobacteriophages are available (8, 9, 15, 17–26) that provide a variety of perspectives, including a historical view of this field. Here, I will focus primarily on recent developments that have not been extensively covered in the earlier reviews, the most recent of which was 4 years ago (27).

GENERAL MYCOBACTERIOPHAGE FEATURES

Current Genomic Scope

As noted above, the total number of mycobacteriophages isolated is nearing 10,000, and over 1,500 have been completely sequenced. Further details about how these were isolated and characterized are provided below, but for the most part we will constrain the discussion here to the subset of genomes that are available in the public databases, totaling 899 genomes. As should be apparent from the discrepancies in these numbers, at the time of writing there are almost 700 genomes that have been sequenced but are not yet fully annotated and submitted to GenBank. The complete and up-to-date list of sequenced phages and their current status is available at the PhagesDB (http://phagesdb.org) and Phamerator (http://phamerator.org) databases (16). It should be noted that the annotation, quality control review, revision, and GenBank submission represents a current bottleneck in the pipeline and is relatively time-consuming compared to sequence determination. Nonetheless, the dataset of 899 genomes offers a representative view of the overall diversity, which does not change substantially if the larger dataset is considered.

It is also worth noting that there is a growing collection of genomically sequenced phages that infect other bacterial

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hosts within the phylum *Actinobacteria*. This includes substantial numbers of phages of *Arthrobacter*, *Corynebacterium*, *Gordonia*, *Microbacterium*, *Propionibacterium*, *Rhodococcus*, and *Streptomyces* (16). These will not be discussed in detail here but are especially relevant in considering the processes by which bacteriophages evolve.

Phage Discovery and Genomics as a Platform for Advancing Science Education

Isolation of bacteriophages using a simple plaque assay has been performed successfully for the past 100 years (28). However, genomic analyses of these phages have only been available since about the early 1990s (14), and DNA sequencing technology has advanced rapidly since then. The coupling of phage isolation and genomic analysis facilitates defining viral diversity and provides insights into understanding their evolution (29). The finding that there are so many different phage genomes underpins the use of phage isolation and genomics as a powerful platform for integrating our missions in science education with those in phage exploration (30).

The Phage Hunters Integrating Research and Education (PHIRE) program was developed at the University of Pittsburgh in 2002 with the intent of using phage discovery and genomics to introduce high school and undergraduate students to authentic scientific research (31). The PHIRE program provides strong evidence not only that students can readily successfully engage in research, but that it is inclusive and does not require either prerequisite understanding of bacteriophages or technical skills in microbiology (30, 32). Since its inception, students in the PHIRE program have isolated over 300 individual mycobacteriophages, about one-half of which have been sequenced. However, PHIRE is essentially a local program mostly confined to the Pittsburgh region where our research laboratory is located.

The demonstration that this discovery-rich platform is well suited to scientific novices provided the rationale for broader dissemination and expansion to a nationwide program organized as a course-based research experience for first-year undergraduate students. In 2008, in collaboration with the Science Education Alliance (SEA) program at the Howard Hughes Medical Institute (HHMI), we developed the Science Education Alliance Phage Hunters Advancing Genomics and Evolutionary Science (SEA-PHAGES) program (33). SEA-PHAGES is an example of a general model for transforming science education—an inclusive research education community—in which a program infrastructure supports inclusion of different types of institutions and students from all types of backgrounds (Fig. 1). In its first year (2008) there were 12 participating institutions, but additional schools have joined SEA-PHAGES each year, and currently there are over 120 member schools, with more than 4,500 student researchers each year (33, 34). The SEA-PHAGES experience strongly promotes student engagement in studying science, with project ownership and networking being prominent contributing elements (34). It has also had a major impact on our understanding of bacteriophages, and SEA-PHAGES students and faculty have isolated a total of 9,600 actinobacteriophages, of which 1,880 have been sequenced. Of these, 8,800 are mycobacteriophages, of which over 1,200 have been sequenced (16).

In general, we have adopted a nonsystematic scheme for naming these newly isolated phages. The reasons are threefold. First, the genomes are clearly constructed as architectural mosaics, with individual genomes composed of specific assemblages of individual genes (35) (see below). It is thus unclear how to use a systematic naming system that reflects the genomic constitution of the phages. Second, naming a newly discovered phage is interesting and stimulating for PHIRE and SEA-PHAGES students and provides an opportunity for them to define their discoveries and their contributions, expanding the sense of project ownership that is important to the impact of these programs (34). Third, from a purely practical perspective, it is much easier to remember names such as Corndog, Rosebush, Patience, Daenerys, RedRock, Phayonce, or Snazzy than some tedious series of meaningless letters and numbers. Science doesn't have to be dull and boring all the time.

Databases

The large sizes of both the SEA-PHAGES program and the growing phage collections require data management that is accomplished with three interconnected databases with front-end web interfaces. One is available at http://seaphages.org and contains information about the SEA-PHAGES program, including institutional participation, contact information, protocols, forums, news events, etc. The second is at http://phagesdb.org (16) and contains all of the information associated with individual phages, including genome sequences, GPS location coordinates, BLAST functions, and many other resources. It is a place where information on newly isolated phages is deposited and where data on all of the phages is available.

The third is the Phamerator database at http://phamerator.org. The database was designed as a means of organizing mycobacteriophage-encoded genes into "phamilies," or sequence-related groups, together with the nucleotide sequence data (36). This information can then be used for comparative genomics, such as displaying sets of genome maps in which the genes are colored according to their phamily membership and pairwise nucleotide sequence similarity can be displayed. The Phamerator database includes some genome-associated information that is not present in the other databases, and a web browser interface provides facile database usage. It liaises with both phagesdb.org and GenBank to help maintain data consistency and is also used by other programs such as Starterator and the Phage Evidence Collection and Annotation Network (PECAAN). As such, Phamerator plays a central role in the annotation pipeline, from genome sequence to GenBank submission (Fig. 1). Phamerator databases containing different numbers of genomes can be downloaded and "frozen" so that they can be archived and used for publication preparation.

Mycobacteriophage Morphologies

All of the mycobacteriophages isolated to date are tailed phages with double-stranded DNA (dsDNA) genomes; no single-stranded DNA (ssDNA) or RNA phages have been found. Three families of such *Caudovirales* have been described for other bacterial hosts (*Siphoviridae*, *Myoviridae*, and *Podoviridae*) but only siphoviral and myoviral morphotypes are present among the mycobacteriophages (Fig. 2). Because such a large number have been morphologically examined, it seems unlikely that mycobacteriophages with podoviral morphologies exist. Interestingly, phages with podoviral morphologies have been described for other actinobacterial hosts, including *Arthrobacter* and *Streptomyces* (37, 38), suggesting that the absence of podoviral mycobacteriophages is due to a physical blockage presented by mycobacterial cell walls, rather than being evolutionarily inaccessible.

The mycobacteriophage myoviruses, which have contractile tails, are all confined to a single genomic type, with

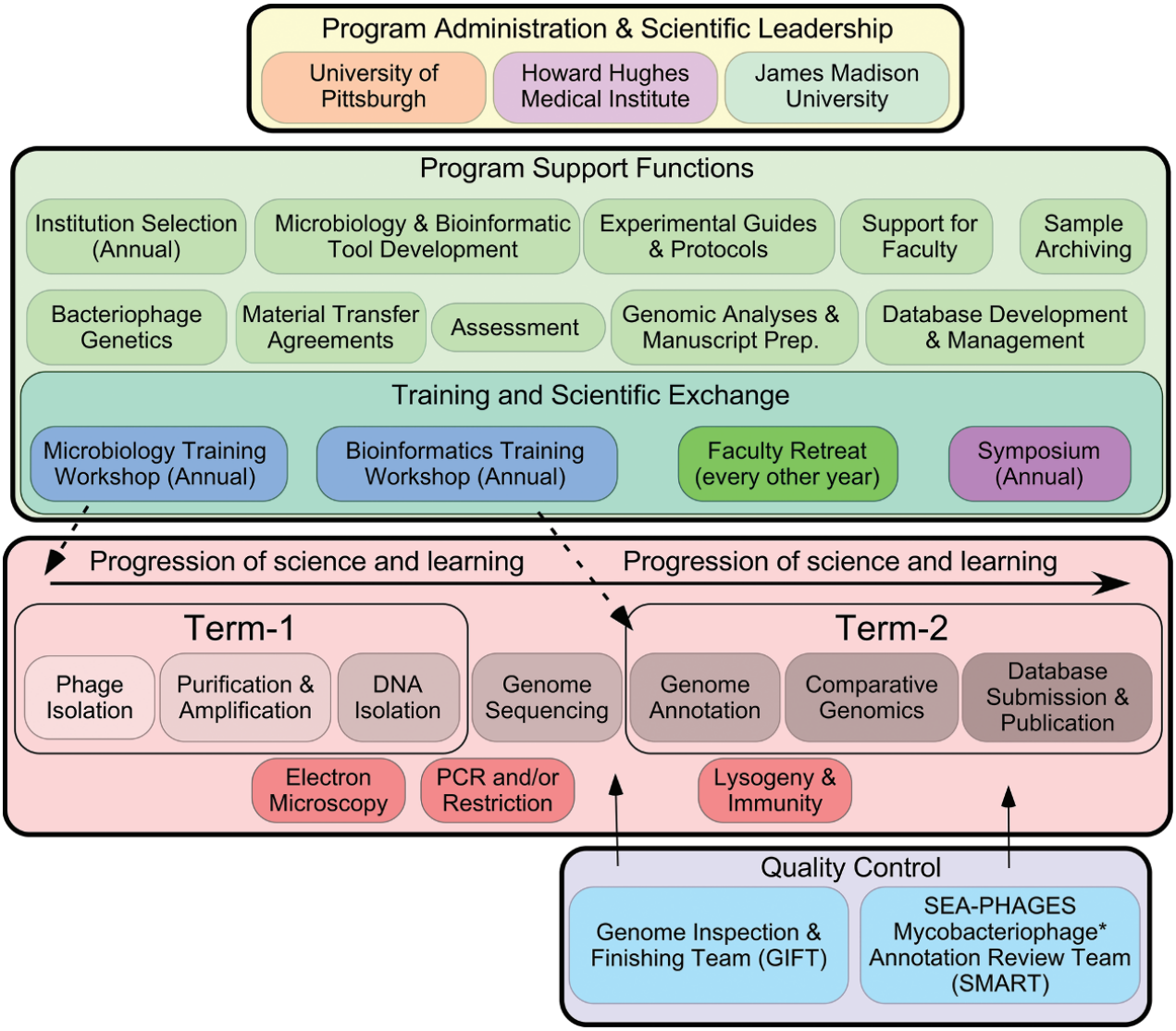


FIGURE 1 Organization and structure of the SEA-PHAGES program. SEA-PHAGES program administrators (yellow box, top) oversee support components critical to program implementation (green box, upper middle). The typical two-term course structure (red box, lower middle) includes phage isolation through comparative genomics; additional characterization includes electron microscopy, PCR/restriction analysis, and lysogeny assays (red ovals). Sequence and annotation quality control is shared with SEA-PHAGES faculty teams (purple box, bottom right). Reproduced with permission from reference 34.

phages grouped within cluster C (Fig. 2; see below). All of the other mycobacteriophages are siphoviruses, with long flexible noncontractile tails, and these span considerable genetic diversity (Fig. 2). Most mycobacteriophages have isometric capsids (i.e., like a soccer ball) with sizes varying from a diameter of about 40 nm to about 80 nm, correlating with their genome sizes. However, some have prolate heads with the length:width ratios varying from about 2.5:1 to about 4:1 (Fig. 2). Tail length also varies considerably, ranging from about 135 nm to more than 350 nm.

MYCOBACTERIOPHAGE GENOMIC DIVERSITY

Clusters, Subclusters, and Singletons

When the first few mycobacteriophage genome sequences were determined in the 1990s, it became apparent that they do not simply represent variants of a common sequence type (14, 35, 39–41). As the number of sequenced genomes grew, it became clear that these could be grouped according to their overall nucleotide sequence similarity. These groups are referred to as "clusters," and in general, phages within a cluster share evident DNA sequence similarity to each other that spans more than 50% of their genome lengths; phages in different clusters share little if any DNA sequence similarity (42). When 30 genome sequences were available (42), these could be assembled into six clusters (clusters A to F); nine phages were not closely related to the others and are referred to as singletons. Within 4 years, the number of genomes had doubled, and these 60 phages were grouped into 9 clusters (clusters A to I), and five singletons (43). It also became clear that within some clusters there are boundaries justifying a separation into subclusters, and average nucleotide identity values reflect these divisions. Thus, for the 9 clusters formed with 60 sequenced mycobacteriophages, 5 could be divided

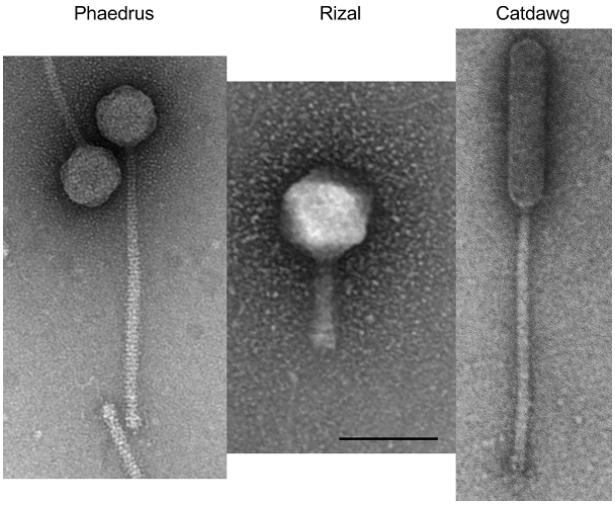


FIGURE 2 Mycobacteriophage viral morphotypes. Mycobacteriophages generally fall into two morphological families, the *Siphoviridae* with long flexible tails (e.g., Phaedrus and Catdawg) and the *Myoviridae* with contractile tails (e.g., Rizal). Most of the siphoviral phages have isometric heads (e.g., Phaedrus), but some (e.g., Catdawg) have prolate heads. Scale marker is 100 nm.

into subclusters. For example, cluster A contained a total of 13 phages at that time, 8 of which are in subcluster A1, and 5 are in subcluster A2 (43). As noted at the time (43), the nature of the relationships within clusters is not uniform, such that the apparent divisions revolve around different ANI values for different clusters. A similar pattern has been reported for phages of the *Enterobacteriaceae* (44).

The 899 mycobacteriophages considered here assemble into a total of 28 clusters together with 5 singletons (Table 1). These clusters are designated clusters A to Z and clusters AB and AC (note that cluster AA is constituted with genomes that are yet to be submitted to GenBank and are thus excluded from the current list). Twelve of these clusters (A, B, C, D, F, G, H, I, K, L, M, and P) are currently divided into subclusters (Table 1). The largest of the clusters is cluster A with 322 phages, and this is split into 18 subclusters (A1 to A14 and A16 to A19; note that A15 is made up solely of *Gordonia* phages). The largest of the clusters that is not subdivided is cluster E, with 52 members (Table 1). These all share substantial nucleotide sequence similarity but are far from identical and differ with numerous small insertions/deletions/substitutions relative to each other, often constrained to a single gene or a small group of genes. Although it typically requires two or more members to constitute a cluster, three of the clusters in the dataset discussed here (X, Y, AC) contain only a single member, and the other members of these are sequenced but are not yet publicly available in GenBank (Table 1).

Cluster assignments simplify the analyses and descriptions of phage genomes, but clusters represent groupings of convenience rather than hard biological boundaries, consistent with them having mosaic architectures (45). As more phage genomes are sequenced, the boundaries between different clusters and subclusters have become more obscure. Thus, these divisions likely reflect unequal sampling of phages from an underlying continual spectrum of diversity, of which not all parts are equally prevalent in the biosphere (45). Thus, while the vast majority of mycobacteriophages within a cluster do indeed share nucleotide sequence similarity spanning greater than 50% of the genome lengths, some phages have genomes that fall only just below that, complicating their cluster assignment. This has become especially apparent in the comparative genomic analyses of *Gordonia* phages (46, 47). As a consequence, for those genomes that have partially shared sequences where nucleotide-based assignments are difficult, it is recommended that the encoded genes be first grouped into phamilies using Phamerator and the proportions of shared genes be calculated (http://phagesdb.org). Phages sharing more than 35% of their genes should be grouped into the same cluster, and those with fewer than 35% should be in different clusters (46).

Allocation of Cluster Designations

When only a relatively modest number of mycobacteriophages were sequenced and the first few clusters were designated, their naming was simple, using ordered alphabetical names (cluster A, cluster B, etc.). Three events prompted further consideration of this naming system. First, as the numbers of clusters grew, the possibility arose that new clusters would be designated even after cluster Z was named. How should these be designated? Second, as our attention shifted to actinobacterial hosts in other genera, how should these be termed? Third, phages isolated on different host genera might group into the same cluster either frequently or very rarely. How should this be accommodated?

We decided to implement a scheme in which blocks of letters are set aside for clusters of phages isolated on particular hosts (Table 2). In brief, the alphabetical series is extended beyond clusters A to Z into two-letter (e.g., AA, AB, AC, etc.) and then three-letter (AAA, AAB, AAC, etc.) names as needed, and a block is assigned to each host. For example, phages isolated on mycobacteria have a reserved block of clusters A to Z and AA to AJ. Currently, only clusters up to AC are actually used, so there are seven unoccupied clusters. The size of the initial block (40 for mycobacteriophages; Table 2) is mostly a guess based on an estimate of how many phages may be isolated for that host and their predicted diversity. For example, a relatively small block is set aside for *Cutibacterium* (including the formerly named *Propionibacterium acnes*) phages, because the phages of *Cutibacterium acnes* are very similar to each other and are all in a single cluster (48). If a block of cluster allocations becomes fully used, then additional blocks can be added, even though they are likely to be nonsequential. For example, phages of *Arthrobacter* were initially assigned clusters AK to AZ, but these were quickly used, and an additional block of FA to FZ was added (Table 2). Similarly, *Gordonia* phages were found to be quite diverse, and the initial allocation of clusters CQ to CZ was quickly populated. However, this could be easily extended to clusters DA to DZ, because these had not been previously assigned (Table 2), preserving a series of consecutive letters.

This scheme works in large part because most of the phages isolated on a host of a different genus form new clusters from those previously described. However, we do not expect a strict correlation between the genus of the host and the phage cluster designation. An excellent example is provided by cluster A. Cluster A is highly populated (Table 1) with individual phages, by far the majority of which were isolated on *M. smegmatis*. However, some cluster A phages were isolated on *Gordonia* strains, all of which constitute subcluster A15; it is noteworthy, however, that in spite of the genomic relationships, subcluster A15 phages do not infect *M. smegmatis*, and other cluster A phages that have been

TABLE 1 Mycobacteriophage clusters and subclusters

Cluster	No. of Phages	No. of Subclusters[a]	Avg genome length	Avg G+C%
A	318	18	51,656	63.3
B	147	6	68,705	67.2
C	49	2	155,858	64.7
D	10	2	64,965	59.4
E	52		75,446	63.0
F	84	3	57,427	61.5
G	28	4	42,429	66.9
H	6	2	69,292	57.3
I	5	2	51,129	66.3
J	17		109,962	61.0
K	71	6	60,000	66.8
L	27	3	74,978	59.0
M	7	2	81,539	61.2
N	14		43,200	66.2
O	9		70,805	65.4
P	17	2	47,901	67.0
Q	6		53,753	67.5
R	5		71,424	56.0
S	3		64,989	63.4
T	5		42,847	66.2
U	2		69,942	50.4
V	2		78,262	56.8
W	3		60,820	67.5
X	1		90,460	56.8
Y	1		77,832	67.3
Z	2		50,806	66.0
AB	2		47,976	58.7
AC	1		68,869	49.1
Dori[b]		N/A	64,613	66.0
Kumao[b]		N/A	70,373	62.1
MooMoo[b]		N/A	55,178	62.0
DS6A[b]		N/A	60,588	68.4
Sparky[b]		N/A	63,334	65.2

[a]The numbers of subclusters are shown but are not applicable (N/A) for singleton phages.
[b]Singleton phages with no close relatives. Phage names are provided.

tested do not infect *Gordonia terrae*. Likewise, we note that several *Gordonia* phages described previously also infect *Nocardia* strains (49). In general, we predict that the more closely related the bacterial hosts are phylogenetically, the more likely it is that their phages may be grouped into the same clusters.

We have constrained these cluster allocations to phages of hosts in the phylum *Actinobacteria*. However, this could plausibly be extended to phages of hosts in other phyla using a similar scheme and designations, using a prefix to denote the phylum in circumstances where confusion might arise.

Mycobacteriophage Gene Phamilies

The central questions in deciphering these mycobacteriophage genomes are how are they architecturally structured and how have they evolved? To simplify the comparative genomic analyses, we can take the total gene set, compare each gene to all other genes pairwise at the amino acid sequence level, and place similar genes into groups that we call phamilies (43), often abbreviated to phams. This is a different strategy to using a hidden Markov model, in part in an attempt to accommodate the complexity arising from the substantial number of modular genes. When the dataset was small, phamilies were constructed using BlastP and ClustalW, using cutoff values of 10^{-50} (E value) and 32.5% (amino acid identity), respectively (36). These values were set to capture distantly related homologues into the same phamily but to minimize false-positive inclusion of non-homologues in phamilies (36). As the dataset expanded, the use of BlastP and ClustalW became computationally prohibitive, and they were substituted with a kmer-based

TABLE 2 Allocation of cluster designations

Host phylum	Host genus	Designations	Number
Actinobacteria	*Mycobacterium*	A–Z, AA–AJ	40
	Arthrobacter	AK–AZ, FA–FZ	42
	Streptomyces	BA–BT	20
	Propionibacterium	BU–BZ	6
	Rhodococcus	CA–CP	14
	Gordonia	CQ–DZ	36
	Microbacterium	EA–EM	13
	Corynebacterium	EN–EZ	13

method for phamily assembly (45). Different phams are identified by numbers (pham 1, pham 2, pham 3, etc.), and we attempt to maintain pham designations as additional phages are added to the databases. The program Phamerator uses k-clust to sort genes into phamilies and displays genome maps that include pham designations and other genome and gene information (36). The web-based version of Phamerator is available at http://phamerator.org, which uses a default database(Actino_Draft) that contains information on all sequenced phages of actinobacterial hosts. The Actino_Draft database is constantly updated as new phages are isolated and their genomes sequenced, and as such contains genomes of several different status classes including "draft" (auto-annotated) annotations, "final" annotations that have been manually reviewed and revised, and "Gbk," whose annotation history is unknown.

A Phamerator database (Mycobacteriophage_899) was constructed for the 899 phages discussed here, which contains only genomes that are available in GenBank. This database contains 96,979 genes, which are sorted into 6,046 phamilies. The average number of genes per phamily is 16.03, and approximately 25% (1,506) contain only a single gene and are designated as "orphams." The largest phamily has 337 gene members.

MYCOBACTERIOPHAGE GENOME ORGANIZATION AND EXPRESSION

Genome Annotation and Gene Naming

When phage genomes are sequenced within the PHIRE or SEA-PHAGES program, they can be autoannotated simply with Glimmer (50) and GeneMark (51) using the program DNA Master (http://cobamide2.bio.pitt.edu/). Although these programs are quite good at identifying coding regions and predicting translational start sites, they do this incorrectly for about 10% of the genes. To obtain as well-supported an annotation as possible, each of the predicted genes is manually inspected, and putative functional assignments are made. The genomes are also inspected for missing genes and for tRNA genes and other features. All annotated genomes are reviewed through a quality control process prior to submission to GenBank (Fig. 1).

To simplify the gene naming process, each open reading frame or RNA gene is assigned a gene name as a number, sequentially starting with 1 at the left end of the genome and proceeding rightward. It is important to note that gene names (e.g., 1, 2, 3, etc.) and their gene products (e.g., gp1, gp2, gp3, etc.) have no functional meaning. Thus, for example, both phage Corndog and phage Rosebush have a gene *20*, each coding for a protein, gp20, but Corndog gp20 and Rosebush gp20 are unrelated at the sequence level and are not functionally related.

Genome Organization

Although there is great sequence diversity among the mycobacteriophages, there are several common themes arising in their overall organization and genomic architecture. An example is shown in Fig. 3. First, there is a common organization of virion structural genes in all of the phages with siphoviral morphologies, which includes all of the clusters and singletons, with the exception of cluster C. The genomes are typically presented such that these structural genes are at the left end of the genome and transcribed rightward (Fig. 3). The gene order is typically terminase (small and large subunit genes), portal, capsid maturation protease, scaffold, capsid, tail capping and head-tail connectors, major tail subunit, tail assembly chaperones (typically expressed via a programmed translational frameshift), tape measure protein, and minor tail genes (of which there may be 4 to 10) (Fig. 3).

In some genomes, usually those that are relatively short (40 to 45 kbp), the 20 to 25 closely packed virion structure and assembly genes span about 22 to 23 kbp, occupying ~50% of the whole genome (Fig. 3). In such phages, the terminase small subunit gene is usually very close to the physical left end of the genome (in phage Taheera, the translation start site of the small terminase subunit genes is predicted to be at coordinate 47). In other genomes, the gene order may be conserved, but the virion structure and assembly genes are interspersed with nonstructural genes. In phage Omega and its relatives (52), the virion structure and assembly genes span over 35 kbp, and the inserted genes include a glycosyl transferase, an *O*-methyl transferase, and an IS*110*-like transposon. In phage Marvin and its relatives two of the minor tail protein genes are atypically located near the right end of the genome, separated by about 20 kbp from the other structural genes (53).

All of the phages code for a lysis system that typically includes three components: a holin, an endolysin (lysin A), and a serine-esterase, referred to as lysin B or lysterase (see Fig. 3). The lysis cassette is located in one of two genomic positions. In most phages it is immediately downstream (to the right) of the virion structural genes (Fig. 3). However, in the cluster A phages it is positioned to the left of the structural genes, between the leftmost *cos* end and the terminase genes (14, 54). Within the lysis cassettes, the holin gene is sometimes difficult to assign, because of there being several genes with predicted transmembrane domains near the lysin A gene; it is plausible that several of them play some role in the regulation of lysis. The endolysin genes are constructed modularly, with individual modules shared between otherwise distantly related phages (55). The lysin B genes code for esterases that cleave the mycolic acids from the cell wall to promote efficient lysis, and at least in phage Giles the lysin B is not essential for plaque formation, although it forms smaller plaques with fewer particles (56). Some of the mycobacteriophages appear to lack a lysin B gene altogether (e.g., Rosebush), and it is unclear if other genes substitute for its function (56).

Over one-half of the clusters of mycobacteriophages appear to be temperate and contain an integration cassette (e.g., clusters A, E, F, G, I, J, K, L, M, N, O, P, Q, T, X, Y, Z; singletons DS6A, Dori, Kumao, MooMoo, and Sparky). Typically, the integration cassette is located near the center of the genome and contains an integrase gene and an *attP* site

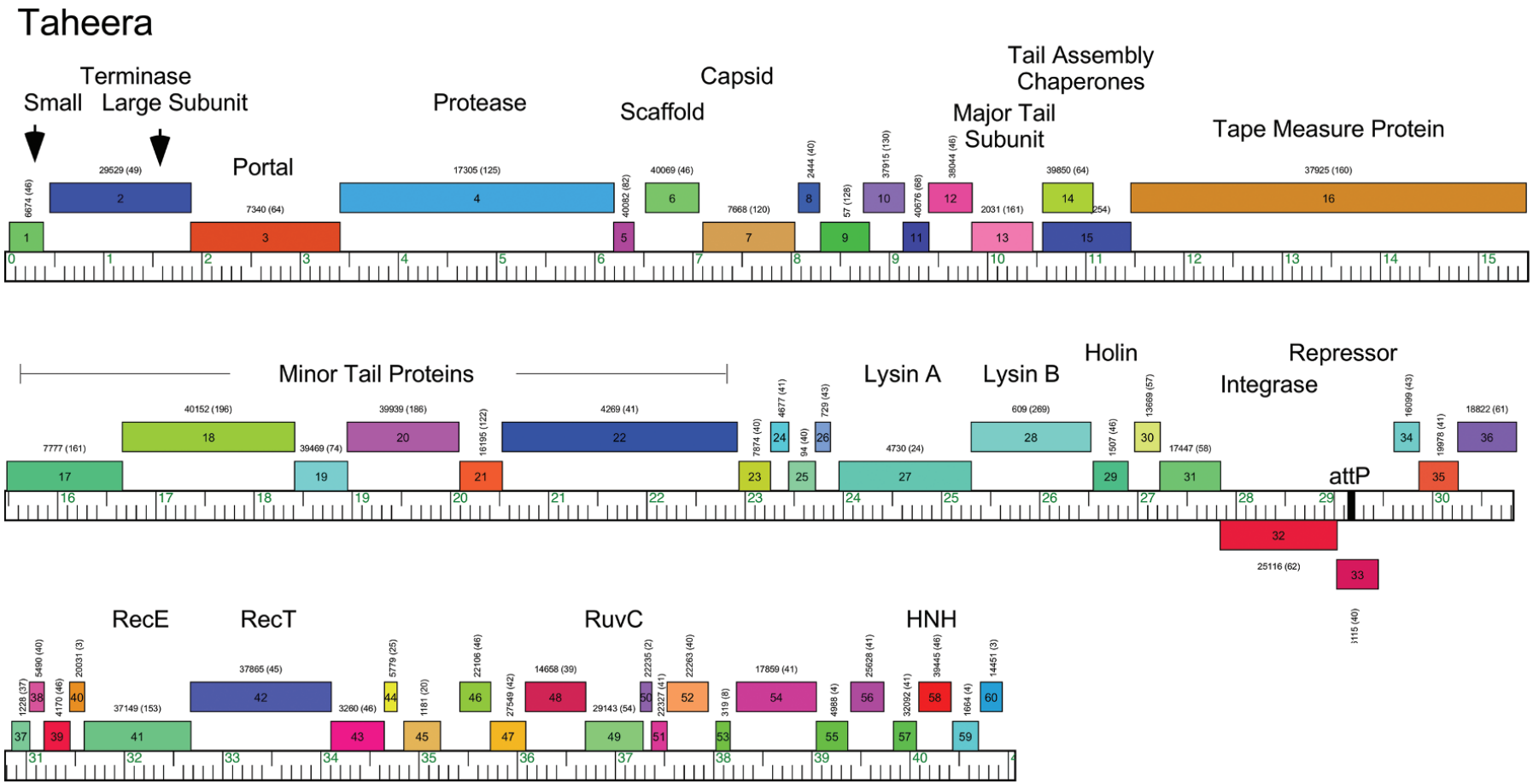


FIGURE 3 Genome organization of mycobacteriophage Taheera. The genome of phage Taheera (cluster G) is represented as a scale bar (major intervals: 1 kbp) with predicted genes shown as boxes either above (rightward transcribed) or below (leftward transcribed). Gene numbers are shown within each box, and the phamily designation is shown either above or below with the number of phamily members shown in parentheses. Putative gene functions are indicated.

(see Fig. 3). Both major families of integrases—tyrosine integrases and serine-integrases—are represented, although the serine-integrases are typically found in cluster A and K genomes. Over a dozen *attB* sites are predicted to be used for prophage integration (22). Interestingly, some phages—predominantly members of cluster A—do not have an integration cassette and instead code for a *parABS* partitioning system (57, 58). In lysogeny, these phages replicate extrachromosomally at low copy number, although the replication functions have yet to be determined (58).

The mycobacteriophages have a variable number of genes that are not involved in virion structure and assembly, lysis, or integration, depending on the overall genome size. Thus, the smallest of the mycobacteriophages (e.g., clusters G, N, T) may have only 25 to 30 additional genes (Fig. 3), whereas larger genomes (e.g., cluster J) may have over 150 such genes. A few of these have predicted functions in DNA metabolism, including replication and recombination, but the vast majority are relatively small and are of unknown function. In phage Giles, it has been shown that about half of the nonstructural genes are dispensable for lytic growth (59). Little is known about the essentiality of any of the predicted DNA replication functions, including the Pol I type genes and Pol III alpha genes present in some of the phages. Many mycobacteriophages do not encode any DNA polymerase, so those that do may need them to synthesize genomes with altered chemistry or because the host polymerases are degraded or inactivated. However, this has yet to be investigated.

Mycobacteriophage Gene Expression

A general picture of mycobacteriophage gene expression is emerging, although only a rather small subset of phages has been examined. Lytic gene expression has been described for several cluster A phages including L5, D29, StarStuff, Kampy, and SWU1 (54, 60, 61); Giles (cluster Q) (59); Fruitloop (cluster F) (62); and several cluster N phages (63). In general, there appear to be two major types of transcription that occur, in early and late periods. Early gene expression occurs in the first 30 minutes, with late transcription initiating about 30 minutes after infection and continuing until lysis, typically about 180 minutes after infection. These general profiles are in agreement with patterns of phage protein synthesis detected by radiolabeling (14, 40).

Early gene transcription usually encompasses nonstructural genes in the right arm, and the virion structure and assembly genes and the lysis cassette are expressed late. In phages such as Fruitloop (Fig. 4), late transcription starts ~5 kbp from the right genome end (in the 113-bp intergenic region between genes *91* and *92*) and proceeds rightward through *cos* into the structural genes (62) (Fig. 4). Of the 11 right arm genes (*92* to *102*) at the beginning of the late operon, 4 of the gene products have predicted functions. One (gp92) is a predicted transcriptional regulator, two (gp99 and gp102) are putative glycosyltransferases, and gp100 is a putative kinase. It is plausible that several of these may be involved in postreplication DNA modification as protection against host defenses. Currently, very little is understood about the factors needed to activate late transcription in any of the mycobacteriophages. Presumably, they are encoded as early genes, but they have not been identified.

Curiously, in the cluster A phages that have been transcriptionally characterized (L5, D29, StarStuff, RedRock, Kampe, SWU1), a noncoding region near the right genome end is transcribed extremely high very early in lytic growth (54, 58, 60, 61). Several small transcripts may be made, but their functions are not known. However, they appear to be quite toxic to *M. smegmatis*, and even *E. coli*, somewhat complicating their study (54). It was previously shown that host gene expression is shut down during L5 early lytic growth (14), and a role for these early RNAs in host shutdown would be consistent with their severe toxicity.

Lysogenic gene expression has been explored in a few mycobacteriophages, primarily those in clusters A, F, Q, and N (58, 59, 62, 63). Not unexpectedly, the repressor is typically expressed, and the lytic genes are switched off. However, it is not uncommon for additional genes—typically located near the repressor—to also be expressed (see below). The cluster A phages are unusual in that they contain a large number of putative repressor binding sites (54, 64–66), spanning the entire genome. These sites are 13 to 14 bp long, asymmetric, and predominantly present in short intergenic regions. Furthermore, the sites are mostly oriented in one direction relative to the direction of transcription (64). It is proposed that at least one of these sites acts as a true operator site for regulation of the early lytic promoter P_{left} (67), whereas the others, termed "stoperators," act to promote transcription termination, primarily to prevent expression of toxic genes during lysogeny (64).

Unusual Life Cycle Regulation

Some of the mycobacteriophages employ an interesting and noncanonical system for the establishment of lysogeny that differs from the well-studied paradigms such as in coliphage lambda. Examples of integration-dependent immunity are found in mycobacteriophages in clusters G, N, and P, and the best studied is that of phage BPs (cluster G) (68). However, these systems have specific bioinformatic features that are readily recognizable in a variety of other temperate phages (46).

Perhaps the most striking feature of the integration-dependent immunity systems is that the phage attachment site (*attP*) is located within the coding region of the immunity repressor (Fig. 5). At first, this is seemingly a bizarre organization, because integrase-mediated site-specific recombination between the phage *attP* and host *attB* sites results in disruption of the repressor gene, even though repressor synthesis is absolutely required for maintenance of lysogeny (68). A rationalization of this emerges from a second common feature, a C-terminal tag in the virally encoded repressor gene product that promotes degradation, typically via *ssr*A-like proteolysis. Because of this *ssr*A-like tag, the virally encoded form of the repressor is rapidly turned over, and insufficient repressor is made to maintain lysogeny or confer superinfection immunity (68). Integration results in removal of the C-terminal 30 to 35 amino acids, including the *ssr*A-like tag, such that the prophage-encoded form of the repressor is active for maintaining lysogeny and immunity to superinfection (Fig. 5). These phages thus require integration as a prerequisite for lysogeny, and the efficiency of lysogeny is determined by the level of integration, which in turn is modulated by a tag for proteolysis at the C-terminus of integrase (68).

These features impose constraints on other aspects of immunity regulation and are accompanied by some notable consequences. First, we should note that in all of the systems studied to date, the integrases are of the tyrosine-recombinase family and use an *attB* site that overlaps a host tRNA gene (Fig. 5). The common core, a region of sequence identity between *attP* and *attB*, is typically 25 to 45 bp and contains not only the region at which strand-exchange occurs, but also

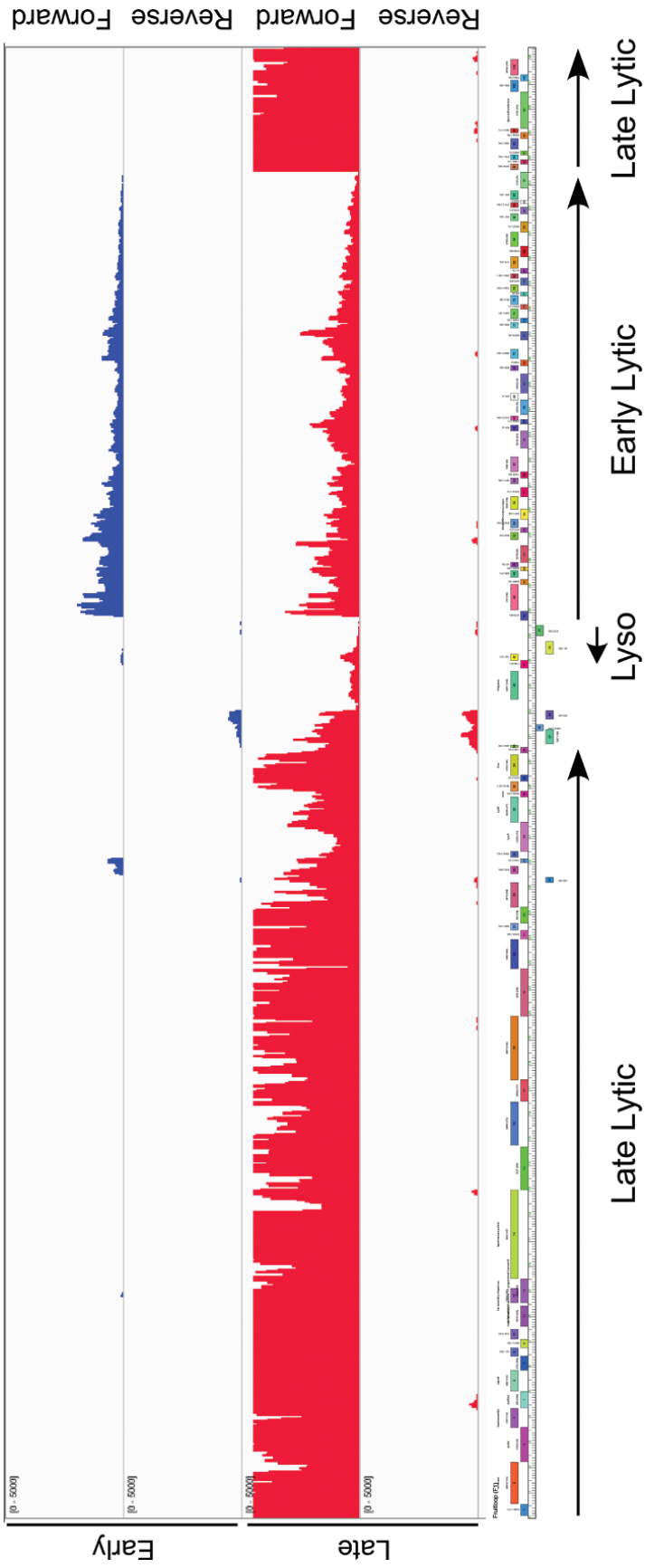


FIGURE 4 Gene expression in mycobacteriophage Fruitloop. RNA was isolated from *M. smegmatis* infected with phage Fruitloop at either early (30 min) or late (150 min) time points after infection. Following strand-specific RNAseq analysis, sequence reads were mapped to the Fruitloop genome (shown below). Reads mapping to the forward and reverse strands are indicated on the right. Scales for the numbers of sequence reads are from 0 to 5,000. Reproduced with permission from reference 62.

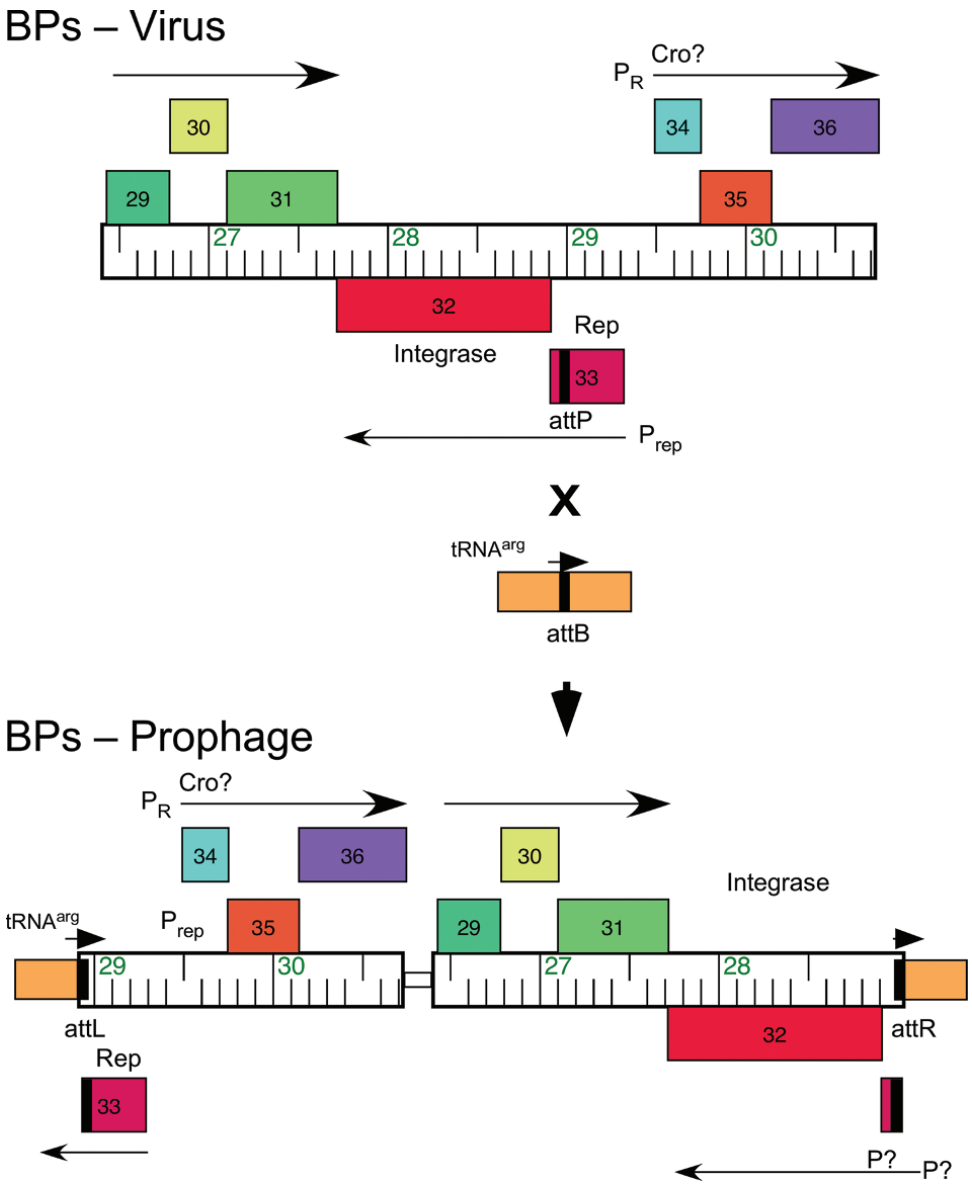


FIGURE 5 Integration-dependent immunity regulation. BPs is a cluster G phage, and a central ~4 kbp segment of the genome is shown at the top. Genes are shown as colored boxes, transcribed rightward or leftward as depicted above or below the genome, respectively. The repressor and integrase genes (*33* and *32*, respectively) are transcribed leftward from the P_{rep} promoter. The *attP* site (black bar) is located within the repressor open reading frame and can recombine with an *attB* site overlapping an *M. smegmatis* $tRNA^{arg}$ gene. Establishment of lysogeny involves formation of an integrated prophage in which an active form of the repressor is expressed from near *attL*, and the 3′ end of the gene is near *attR*. The virally encoded form of the repressor contains a C-terminal *ssrA*-like tag that targets it for degradation, and it fails to confer immunity. Integration results in removal of the C-terminal tag such that the prophage-expressed form of the repressor is stable and confers immunity.

the sequences corresponding to the 3′ end of the tRNA gene; tRNA gene integrity is thus maintained at the attachment junction (*attL* or *attR*) following integration (Fig. 5). In phage BPs, the common core is 35 bp long and positioned 33 codons from the 3′ end of the viral repressor gene. Following integration, the first base of the bacterial sequence joined at *attL* changes a cysteine codon (5′-TGT) to a translation terminal codon (5′-TGA), so the prophage-encoded form of the repressor lacks the C-terminal 33 amino acid residues of the virally encoded form (68).

Canonical tyrosine integration systems such as in phage lambda and mycobacteriophage L5 (69, 70) use complex *attP* sites containing secondary integrase-binding sites flanking the common core (i.e., arm-type Int-binding sites) and thus span up to 250 bp. Not only is there no evidence of similar arm-type binding sites in BPs or other integration-dependent systems, but the integrase proteins lack an N-terminal arm-type binding domain (68). As such, these are simple site-specific recombination systems reminiscent of FLP or Cre. This in turn raises the question of how directionality (i.e., integration

versus excision) is regulated, because without the ability to use arm-type binding to form higher-order protein-DNA architectures, there is no obvious role for a recombination directionality factor such as Xis (71). It is likely that directionality is regulated at least in part by the availability of active integrase, because the integrases in these systems also contain an *ssr*A-like C-terminal proteolysis tag. Removing the tag leads to higher rates of excisive recombination even in the absence of additional phage-encoded proteins (68).

The relationship between the *attP* site and the repressor gene also results in an unusual expression pattern (Fig. 6). This is illustrated by transcriptome sequencing (RNAseq) analysis of an *M. smegmatis* lysogen carrying a prophage, the cluster N phage Panchino, which integrates at the *attB*-4 site overlapping a tRNA-lys gene (22). The truncated prophage form of the repressor (gene *33*) is expressed as expected, but the host tRNA-lys gene is also expressed, generating RNA on both strands within the repressor gene (Fig. 6). These rightward transcripts terminate before they proceed through to gene *34*, which is a candidate for a *cro* gene, whose expression could result in interference with lysogeny and induction of lytic growth. It is somewhat surprising that the transcripts originating from the tRNA do not act as antisense inhibitors of repressor expression. This unusual expression pattern is observed in all the integration-dependent systems that have been examined by RNAseq (63).

Finally, these immunity systems can be used to construct integration vectors for inserting genes into mycobacterial chromosomes at *attB* loci, as has been described for other integration vectors (72–74). However, they transform at very low frequency because of the *ssr*A-like tags on the integrase. Removal of these tags gives higher transformation frequencies, but this may be accompanied by instability of the integrated vector if the integrase gene is still present in the recombinant strains (68).

INSIGHTS INTO PHAGE EVOLUTION

Mosaicism: The Hallmark of Phage Genomes

Comparative genomic analysis of phages clearly shows them to have modular structures, with segments assembled to form distinct genomes (35, 75). This is evident at the nucleotide sequence level when comparing phages grouped within particular clusters or subclusters, where otherwise closely related segments are interspersed with regions that are unrelated or more distantly related. An example illustrated by some genomes grouped in subcluster A2 is shown in Fig. 7. It is strikingly common for the boundaries between shared and nonshared sequences to coincide with gene boundaries. A vast number of discontinuities are identifiable, and rarely are there signs of sequence homology that could have promoted recombination through a homology-dependent mechanism, although such events have been described (54). This has led to the suggestion that the recombination events are likely to predominantly occur without extensive sequence dependence but with selection for function (76). It is plausible that very small regions of homology (3 to 5 bp) are favored, and these could be mediated by phage-encoded recombinases, which often have relaxed sequence requirements (77–79).

Genomic mosaicism is also evident when comparing genes at the amino acid sequence level, where distant homologues can be identified (Fig. 8). There are numerous examples in which gene members of a phamily are present in two otherwise unrelated genomes, such that the flanking genes to its left and its right are quite different (Fig. 8).

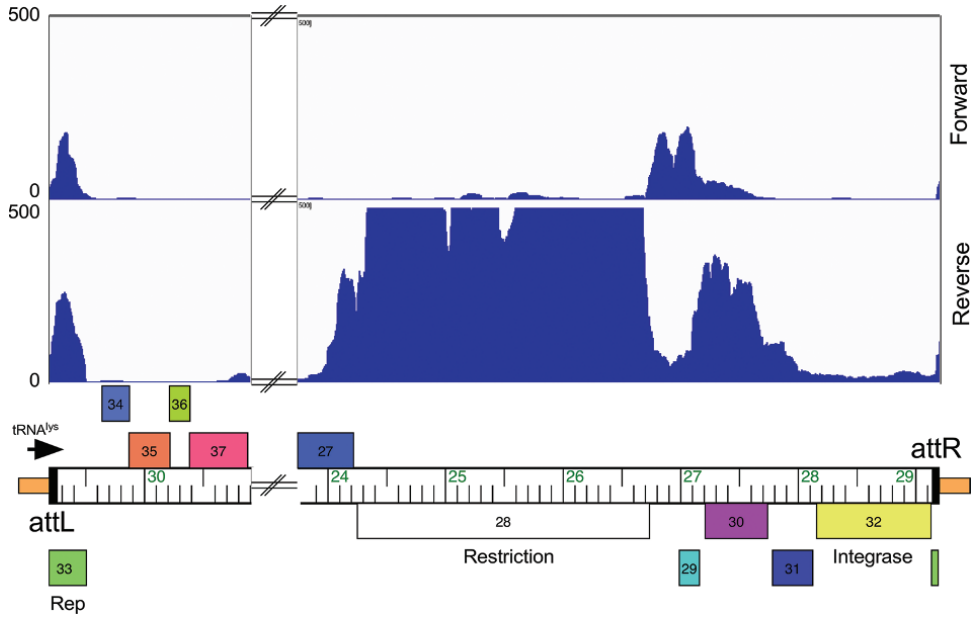


FIGURE 6 Gene expression in a Panchino lysogen of *M. smegmatis*. RNA was isolated from a lysogen of mycobacteriophage Panchino and strand-specific RNAseq reads were mapped to the Panchino prophage. Only the genome segments at the ends of the prophage near *attL* and *attR* are shown. Sequence reads for forward and reverse DNA direction are shown, and the scales are 0 to 500 reads. Genes below the genome are transcribed leftward, and those above are transcribed rightward. Panchino uses an integration-dependent immunity system similar to that in phage BPs (see Fig. 5). Genes *31* to *28* are also expressed; the lytic genes are not expressed (not shown). Reproduced with permission from reference 63.

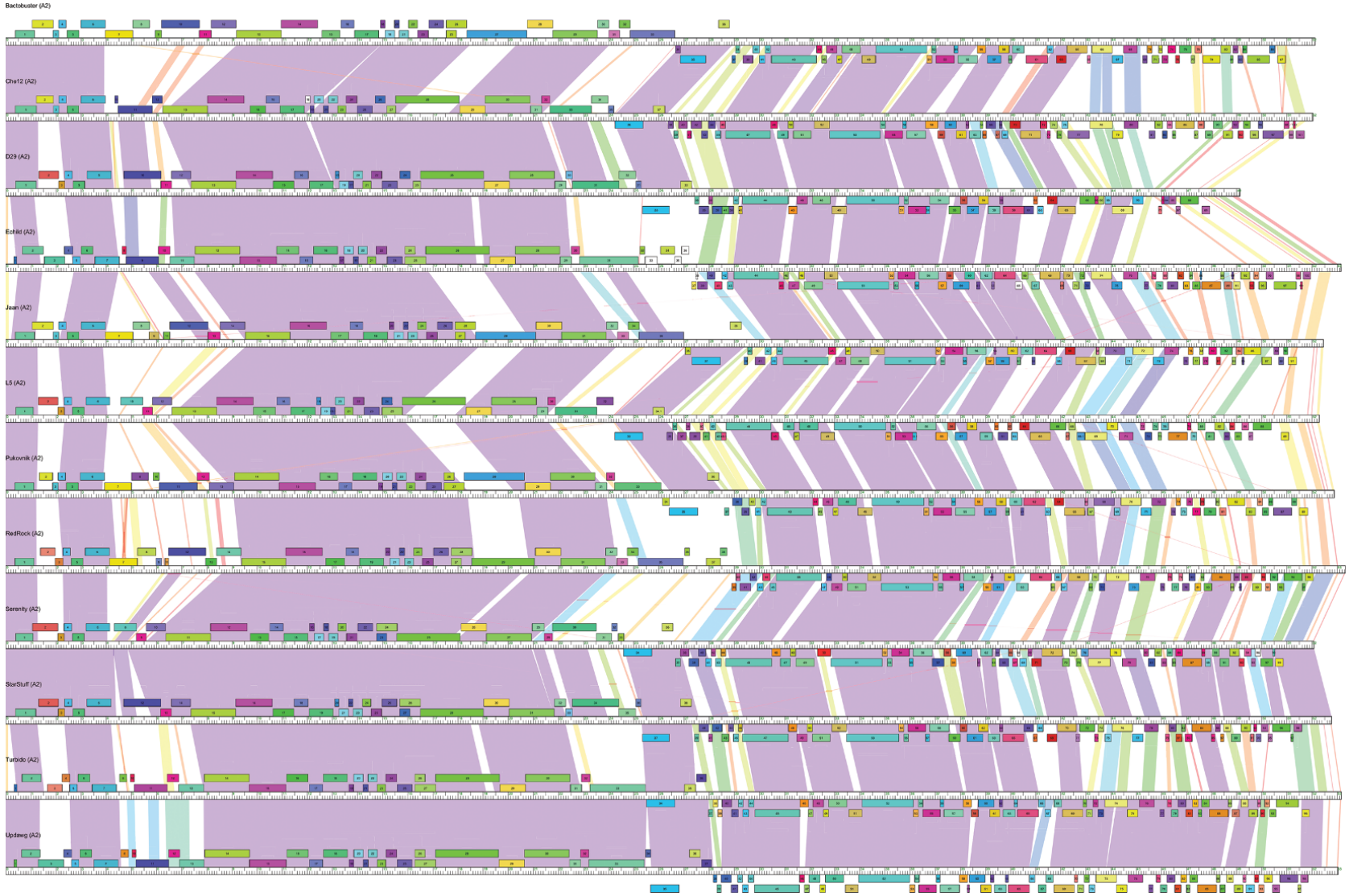
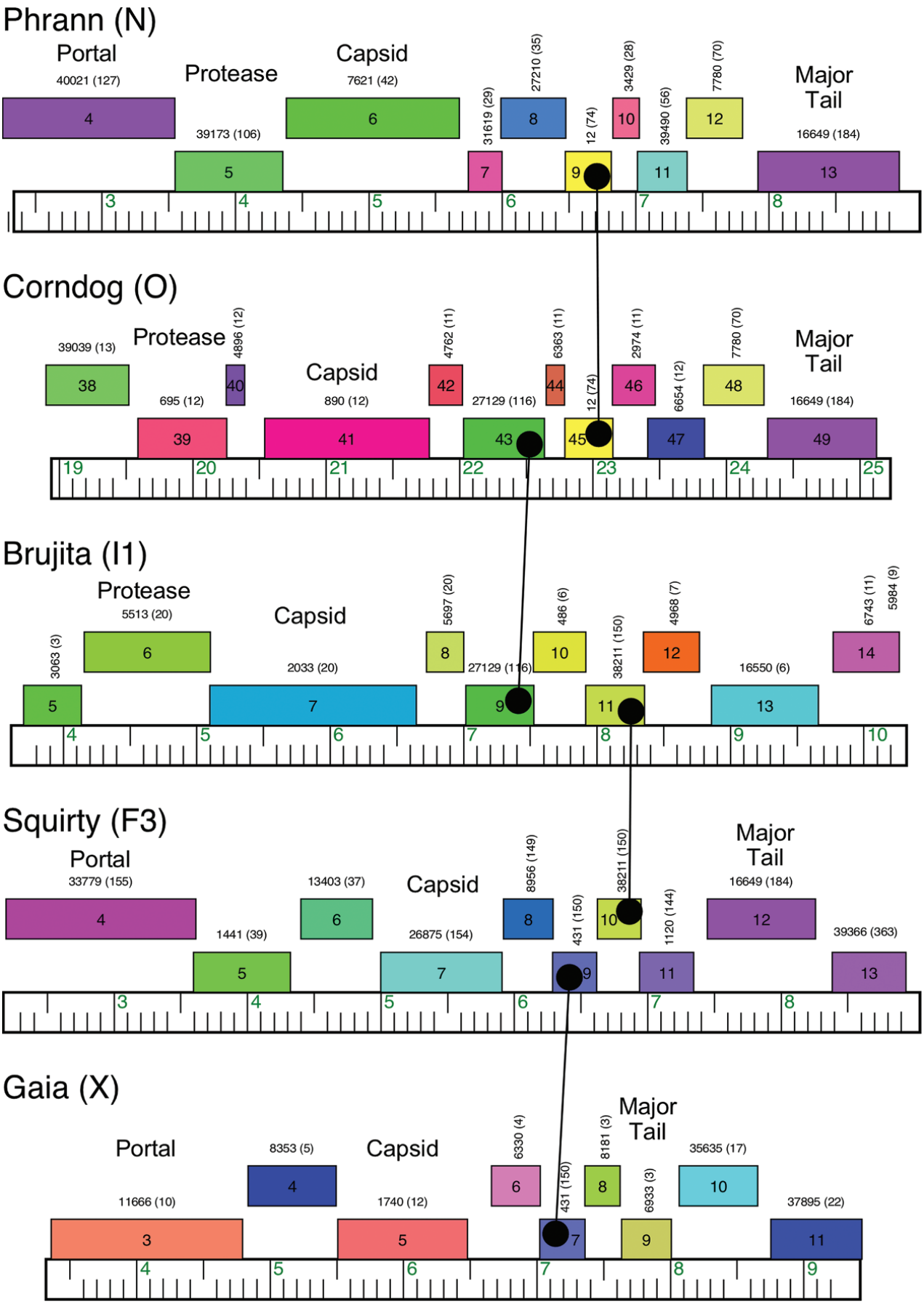


FIGURE 7 Genomic mosaicism in subcluster A2 phages. Mycobacteriophages are characteristically mosaic, with shared genome segments interspersed with nonhomologous regions. The genomes of 12 subcluster A2 phages are shown (Bactobuster, Che12, D29, Echild, Jaan, L5, Pukovnik, RedRock, Serenity, StarStuff, Turbido, and Updawg), with pairwise nucleotide sequence similarities shown as shadings between the genomes; shading is spectrum colored, with violet being the most closely related and red being just above the threshold E value of 10^{-5}. Genes are shown as colored boxes, and genes within the same phamily are similarly colored. Note the segments with close sequence similarity (violet-shaded regions) interspersed with dissimilar regions (white-shaded segments).


Phrann (N)
Portal
40021 (127)
4
Protease
39173 (106)
5
Capsid
7621 (42)
6
31619 (29)
7
27210 (35)
8
12 (74)
9
3429 (28)
10
39490 (56)
11
7780 (70)
12
Major Tail
16649 (184)
13
3
4
5
6
7
8
Corndog (O)
39039 (13)
38
Protease
4896 (12)
40
695 (12)
39
Capsid
890 (12)
41
4762 (11)
42
27129 (116)
43
6363 (11)
44
12 (74)
45
2974 (11)
46
6654 (12)
47
7780 (70)
48
Major Tail
16649 (184)
49
19
20
21
22
23
24
25
Brujita (I1)
Protease
5513 (20)
6
3063 (3)
5
Capsid
2033 (20)
7
5697 (20)
8
27129 (116)
9
486 (6)
10
38211 (150)
11
4968 (7)
12
16550 (6)
13
6743 (11)
5984 (9)
14
4
5
6
7
8
9
10
Squirty (F3)
Portal
33779 (155)
4
13403 (37)
6
1441 (39)
5
Capsid
26875 (154)
7
8956 (149)
8
431 (150)
9
38211 (150)
10
1120 (144)
11
Major Tail
16649 (184)
12
39366 (363)
13
3
4
5
6
7
8
Gaia (X)
Portal
11666 (10)
3
8353 (5)
4
Capsid
1740 (12)
5
6330 (4)
6
431 (150)
7
8181 (3)
8
Major Tail
6933 (3)
9
35635 (17)
10
37895 (22)
11
4
5
6
7
8
9

Although sometimes two or more genes may be coevolving and are shared between genomes, it is very common to observe single genes as units within the mosaic architecture. This mosaicism is particularly pervasive among the large number of small nonstructural genes, most of which are of unknown function. However, it is also evident in some of the virion structure and assembly genes, especially those located between the capsid and major tail subunit genes (Fig. 8).

Mobile Elements

Although it is anticipated that mobile genetic elements contribute to mosaicism, transposons, introns, and inteins are relatively rare in mycobacteriophage genomes. There is an IS*110*-like element in phage Omega and some other cluster J phages (52), IS*605*-like elements in phage Llij and several other subcluster F1 phages (although inserted at several distinct locations), an IS*1096*-like element in the cluster G phage LouisV14, and an IS*1380*-like element in Guillsminger (subcluster K5). There are instances of introns (e.g., in Omega and other cluster J phages) (52) and inteins, especially in terminase large subunit genes (65). However, there are more genes appearing to code for stand-alone homing endonucleases or HNH proteins (65). These are expected to be mobile, and it is not unusual to find HNH genes sharing high levels of amino acid sequence and sometimes nucleotide sequence similarity located in different genomic contexts, indicative of recent mobility.

The most common mobile elements are small mycobacteriophage mobile elements (MPMEs) that form two main classes, MPME1 and MPME2, which share about 85% nucleotide identity with each other (80). These were first identified by comparison of genomes in cluster G, where some pairs of genomes are very closely related and differ primarily by insertion of the MPME (80). These comparisons reveal the sequence of the preintegration site and thus the consequences of insertion. The MPMEs are small (~440 bp) and have short (11 bp) imperfectly conserved inverted repeats (IRs) at each end (IR right [IR-R] and IR left [IR-L]), and IR-R is joined to the target DNA. However, the junction at IR-L contains six additional base pairs that are not evidently part of the transposon and are not derived from the target sequence. Neither the origin of these nucleotides nor the mechanism giving rise to their inclusion is known. The MPMEs contain a small opening reading frame coding for a putative 125-residue protein, although it has no motifs commonly found in other transposases or recombinases (80). Many instances of these MPMEs have been identified (over 120 MPME1 and over 35 MPME2) in a variety of genomes (including those in clusters F, G, I, O, T, and W); within a group they are either completely identical or differ by one nucleotide difference. Thus, even though there has been no experimental demonstration of mobility, there is strong evidence that they are indeed mobile, and their small size and unusual features suggest that they warrant further investigation.

Multiple Modes of Evolution

The pervasive mosaicism of the genomes likely arises because of a relatively high level of horizontal genetic exchange relative to the mutational clock. However, the rates have not been simple to quantify, in part because of the challenges in determining the number of horizontal genetic exchange events and estimating mutation rates that likely differ among different phages, especially those that may deploy their own DNA replication machinery. Pairwise comparisons of NCBI phage reference sequences (whose annotations should be reasonably reliable), determining both nucleotide distances and shared gene content, shed some light on these parameters. Phages of all hosts were included (not just mycobacteriophages) (81). Traditional comparison methods are too computationally intensive to measure nucleotide distances, so a kmer-based Mash method was used (82). Although there is not universal agreement that Mash distances reflect nucleotide divergence, a variety of validation strategies supported its application in this analysis (81). Because phage genome diversity is high, most genomes are not related, and pairwise comparisons have Mash scores above 0.5 (Fig. 9) and are insufficiently similar to be informative (81). The second parameter is an estimate of gene content similarity (Fig. 9), which was determined by grouping the genes into phams and then determining what proportion of phams are shared pairwise between genome pairs (81).

This analysis provides an interesting insight, in that there appear to be two major modes of phage genome evolution, one in which gene content varies broadly in proportion to nucleotide distances (low gene content flux [LGCF]) and a second in which gene content appears to vary substantially more over shorter nucleotide distance changes (high gene content flux [HGCF]) (81) (Fig. 9A). In general, lytic phages follow the LGCF evolutionary mode, whereas temperate phages distribute between the LGCF and HGCF modes. Moreover, phage genomes fall into one of the two modes based on their genomic type, such that clusters of phages tend to follow one or the other but not both modes (81). This is well illustrated by three clusters of mycobacteriophages (Fig. 9B,C,D).Cluster B phages are lytic, do not form stable lysogens, and do not contain integrase or repressor genes. These generally distribute throughout the LGCF profile (Fig. 9B). In contrast, clusters F and K are both temperate (Fig. 9C,D), stable lysogens can be recovered, and repressors and integrases have been characterized (73, 83). Cluster F genomes clearly distribute in the HGCF mode (Fig. 9C), whereas cluster K phages lie in the LGCF mode (Fig. 9D). The drivers of these evolutionary modes are unclear, but it is plausible that the HGCF temperate phages strongly participate in the acquisition of genes that benefit their host when expressed in lysogeny and may contribute directly to pathogenesis (81).

Evolution and Host Range

The great genetic diversity of the mycobacteriophages, with many types sharing little or no DNA sequence information, is unexpected, given that they all infect the same host bacterial strain and therefore should be in genetic communication with each other. A simple model to explain this is that phage host preferences are quite mutable, and there is a low barrier to switching from one host to another (84). Moreover, there must be strong pressure to do so, because phages must mutate

FIGURE 8 Single-gene mosaics in mycobacteriophage genomes. Segments of five phage genomes (Phrann, Corndog, Brujita, Squirty, and Gaia) are shown, which are unrelated at the nucleotide sequence level and are grouped in different clusters, as shown (in clusters, N, O, I1, F3, and X, respectively). Genes are shown as colored boxes, with genes in the same phamilies colored similarly and pham numbers shown above with the number of phamily members in parentheses. Pairwise-shared homologues are indicated with dumbbells. Note that the homologues are situated in different genomic contexts and the flanking genes are not homologous.

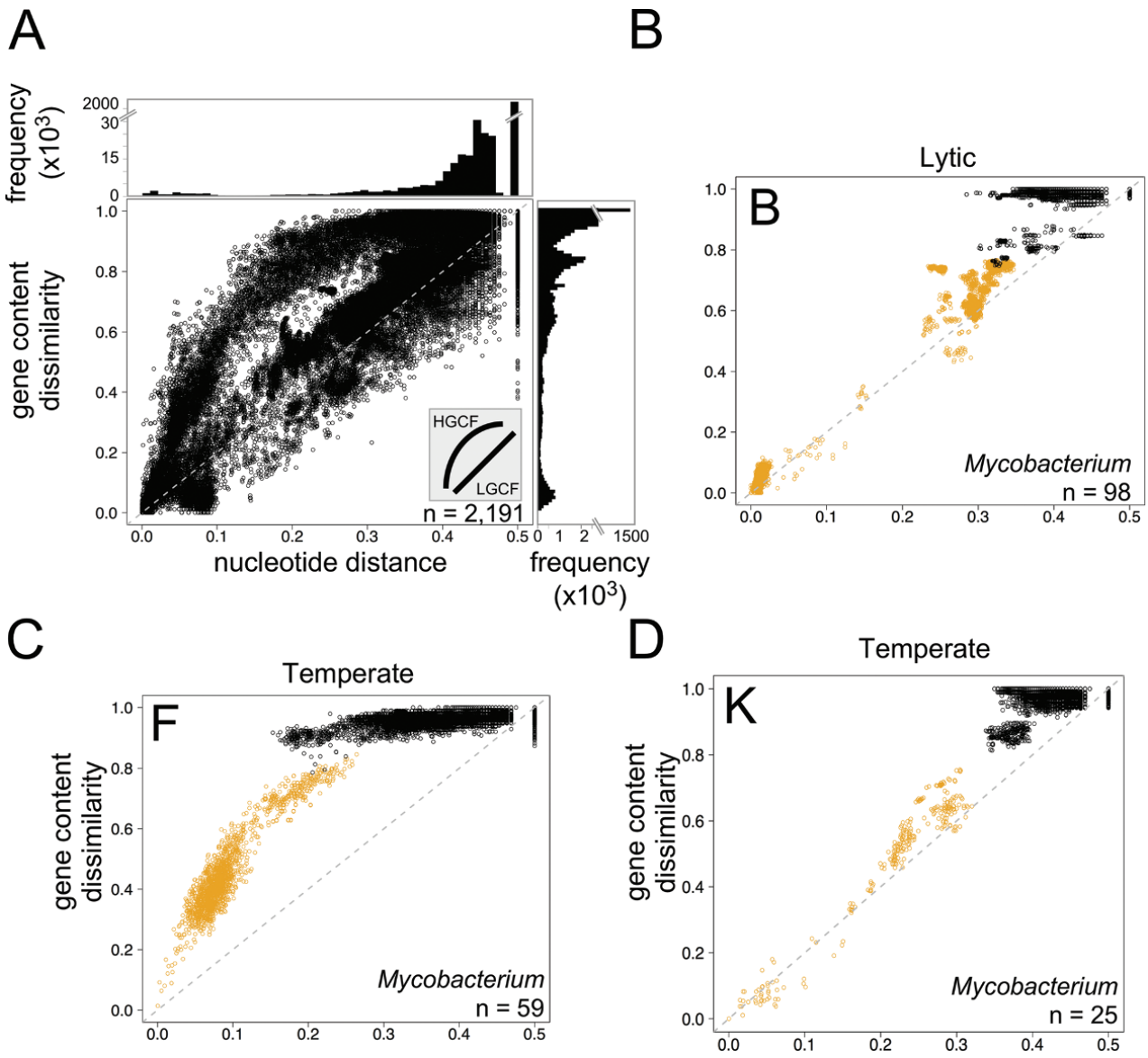


FIGURE 9 Modes of phage evolution. (A) Nucleotide distance (using Mash) and gene content dissimilarity (using phams from Phamerator) are plotted for ~2.4 × 10^6 dsDNA phage comparisons to reveal two evolutionary modes, HGCF and LGCF (inset). The line at y = 2x is plotted for reference. Marginal frequency histograms emphasize densely plotted regions, with truncated y axes for viewability. (B–D) Cluster-specific intracluster (orange) and intercluster (black) comparisons are plotted as in Fig. 1A for actinobacteriophage clusters B (panel B), F (panel C), and K (panel D). Phages in clusters F and K are temperate, and phages in cluster B are lytic, as indicated. n, number of phages present in the specific cluster. Reproduced with permission from reference 81.

to survive viral attack, and phages must coevolve to access hosts for replication. We thus envisage pathways by which phages migrate across bacterial host landscapes, such that phages pursuing different paths have different access to the large common gene pool (Fig. 10).

Several lines of evidence support this model. First, several examples demonstrate that mycobacteriophages can expand their host ranges at reasonably high frequencies (e.g., 10^{-5}), including acquiring the ability to infect M. *tuberculosis* or a different strain of M. *smegmatis* (84). In each case, this requires just a single base pair change resulting in single amino acid substitutions in phage tail proteins. Second, characterization of the cluster U phage Patience identified features consistent with the hypothesis that it acquired the ability to infect mycobacteriophage hosts relatively recently (85). Patience has an unusually low G+C content (50.3%), substantially different than most other mycobacteriophages (Table 1) and its M. *smegmatis* host (67.3%). Perhaps not surprisingly, it also has a radically different codon usage profile than M. *smegmatis*, including preferred use of some codons that are only rarely used in the host. A seemingly obvious route to adapting to this situation would be for Patience to acquire tRNA genes that would recognize the rare codons, but this has not happened, and Patience has just a single tRNA gene (tRNA-gln). Instead, the genome appears to be in the process of adapting to its new host, such that the most highly expressed genes are more quickly adapting to match the coding profiles of host genes (85).

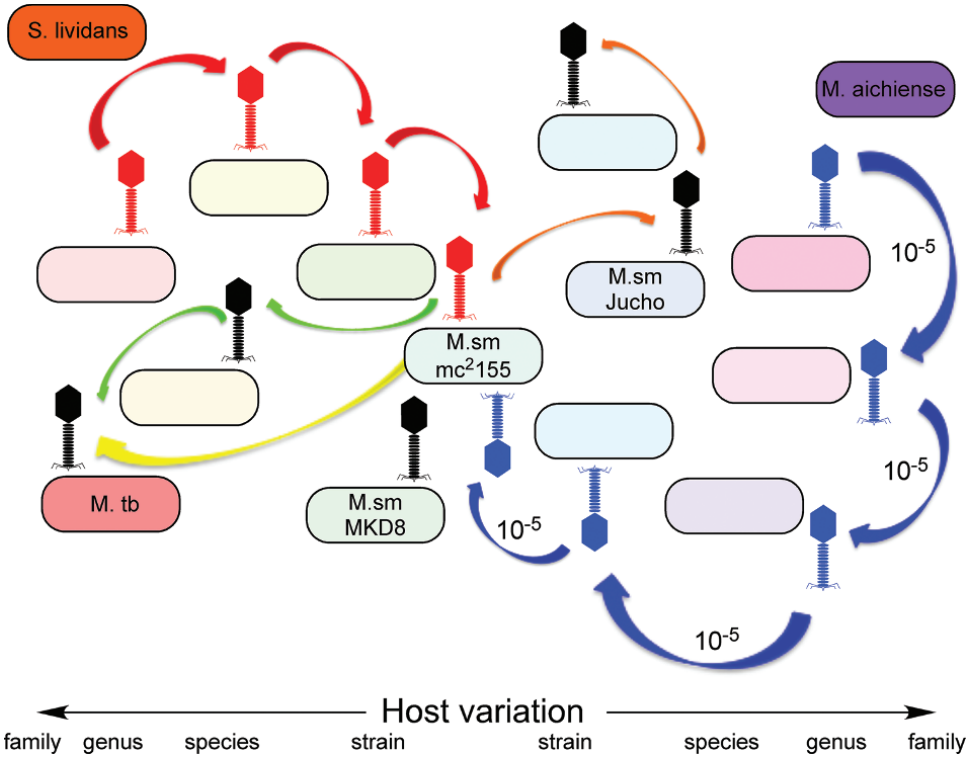


FIGURE 10 A model for mycobacteriophage diversity. The diversity of mycobacteriophages isolated on *M. smegmatis* mc^{2}155 can be explained by a model in which (i) phages can readily infect a new bacterial host, by either a switch or an expansion of host range, and (ii) there is a highly diverse bacterial population, including many closely related strains, in the environments from which the phages are isolated. As such, phages with distinctly different genome sequences and GC contents infecting distantly related bacterial hosts, such as those to the left (red) or right (blue) extremes of a spectrum of hosts, can migrate across a microbial landscape through multiple steps. Each host switch occurs at a relatively high frequency (~1 in 10^5 particles, or an average of about one every 10^3 bursts of lytic growth) and much faster than either amelioration of phage GC content to its new host or genetic recombination. Two phages (such as those shown in red and blue) can thus "arrive" at a common host (*M. smegmatis* mc^{2}155) but be of distinctly different types (clusters, subclusters, and singletons). The variety of hosts is shown two-dimensionally for simplicity; the actual relationships among bacteria in environments such as soil and compost is likely to be considerably more complicated. Because host range variation is a common feature of bacteriophages, the model predicts that a high degree of phage diversity will be seen for any particular host if the microbial population from which the phages are isolated is also highly diverse and rich in closely related strains. Reproduced with permission from reference 84.

DRIVERS OF MYCOBACTERIAL AND MYCOBACTERIOPHAGE DYNAMICS

Prophage-Mediated Defense against Viral Attack

Because bacteria are under constant attack from viral infection, there is a strong selection for survival and acquisition of systems for viral defense. These include the well-studied restriction-modification and CRISPR-Cas systems, but there are numerous examples of defense by abortive infection, and a plethora of new defense systems have been identified (86, 87). Some mycobacterial strains—including *M. tuberculosis*—have CRISPR-Cas-like arrays, although their functionality in defense and spacer acquisition is unclear (88); mycobacterial restriction-modifications have also been described (89–92). However, *M. smegmatis* mc^{2}155, which has been used extensively for phage isolation, appears to be free of both restriction and CRISPR-Cas systems (93).

Mycobacteriophages have been useful for demonstrating that phage defense systems can also be expressed from the prophages of temperate phages (63). This phenomenon has also been described for *Pseudomonas* phages and is likely to be widespread (94). Although it has been known for some time that phages and plasmids can carry restriction and abortive infection systems (95, 96), the number and variety of prophage-encoded systems is quite impressive. These have been best characterized in the mycobacteriophages within cluster N but are predicted to be present in many other temperate phages and other phages of actinobacterial hosts (63). It is simple to envisage how such defense systems would play key roles in microbial dynamics, because lysogeny is common, and prophage acquisition and recruitment of critical defense systems can occur at relatively high frequency in bacterial populations (97).

The presence of prophage-mediated defense systems is suggested from the expression patterns of lysogenic strains, as indicated by RNAseq analysis. The expression pattern of part of the Panchino genome is shown in Fig. 6, which clearly illustrates that the repressor is not the only phage gene being

expressed during lysogeny. Up to four additional genes are expressed (*28* to *31*), and the expression of gene *28* is several times greater than the repressor (Fig. 6). Bioinformatic analysis strongly predicts the gp28 is a multisubunit restriction system, containing hsdR-, hsdM-, and hsdS-like domains (63). Genes *30* and *31* are expressed at more modest levels and are of unknown function, although gp31 is a predicted membrane protein. Although prophage-expressed genes could mediate a multitude of physiological responses in the general category of lysogenic conversion, the restriction system is strongly implicated in viral defense.

The availability of the large collection of diverse phages that infect *M. smegmatis* mc^2155 facilitates the characterization of such defense systems. For example, determination of the plating efficiencies of about 100 phages shows that about one-third of them have a reduction of 10^{-5}-fold or greater plating efficiency on the Panchino lysogen relative to the parent strain. When Panchino *28* alone is expressed from a plasmid, the plating efficiencies of the Panchino lysogen are largely reproduced (63). The cluster N phages are homoimmune and show strong superinfection immunity to each other (Fig. 11A), and we note that Panchino gp28 does not alter infection of Panchino itself or other cluster N phages; thus, this defense system is heterotypic with regard to its specificity for the attacking phages. Presumably, genes *29*, *30*, and *31* could be involved in viral defense too, but if a phage(s) targeted by these is not present in the set that was tested, it could be readily overlooked.

The Panchino *28* to *31* genes are centrally located in the genome between the immunity cassette and the lysis genes (63). Even though the genomes in cluster N are closely related to each other, this central region is highly variable, and many of these genes are expressed from the prophages (63). Approximately 3 to 8 genes are expressed from each of 11 cluster N genomes, corresponding to over 25 phamilies, and the determination of plating efficiencies shows that all of the 11 prophages defend against at least one other phage (63). In the case of phage Charlie, the specificity is quite impressive, and the Charlie prophage defends against only one of the tested phages, Che9c. A Charlie derivative in which gene *32* has been deleted using Bacteriophage Recombineering of Electroporated DNA (BRED) engineering (98) loses its defense against Che9c, and a recombinant plasmid expressing gp32 recapitulates the defense (63). Charlie gp32 is a small (121-residue) protein that is predicted to be membrane localized and could plausibly act by exclusion, preventing Che9c DNA injection. Consistent with this is the observation that Charlie gp32 not only inhibits Che9c plaque formation, but also interferes with Che9c lysogenization. It is plausible that Charlie gp32 would defend against other phages that use similar host proteins for DNA entry, but these are rare in our collection.

Several of the cluster N prophages defend against phage Tweety (cluster F), including Phrann, and three closely related phages: Xerxes, Carcharodon, and MichelleMyBell (Fig. 11) (63). For this second group, a responsible gene was suggested from comparative genomic analysis, because Pipsqueaks is a closely related phage, but has a small deletion in the region corresponding to MichelleMyBell gene *29* (and its relatives), and Pipsqueaks does not defend against Tweety. Deletion of genes *29* and *30* from MichelleMyBell abrogates Tweety defense, and a plasmid carrying genes *29* and *30* reproduces the defense. When using extrachromosomal vectors, it was not possible to clone MichelleMyBell gene *29* without gene *30*, suggesting that high levels of gp29 are toxic and that gp30 alleviates the toxicity, akin to the behaviors of toxin-antitoxin systems. MichelleMyBell gp30 is predicted to be membrane localized, but the biochemical function of gp30 is unclear.

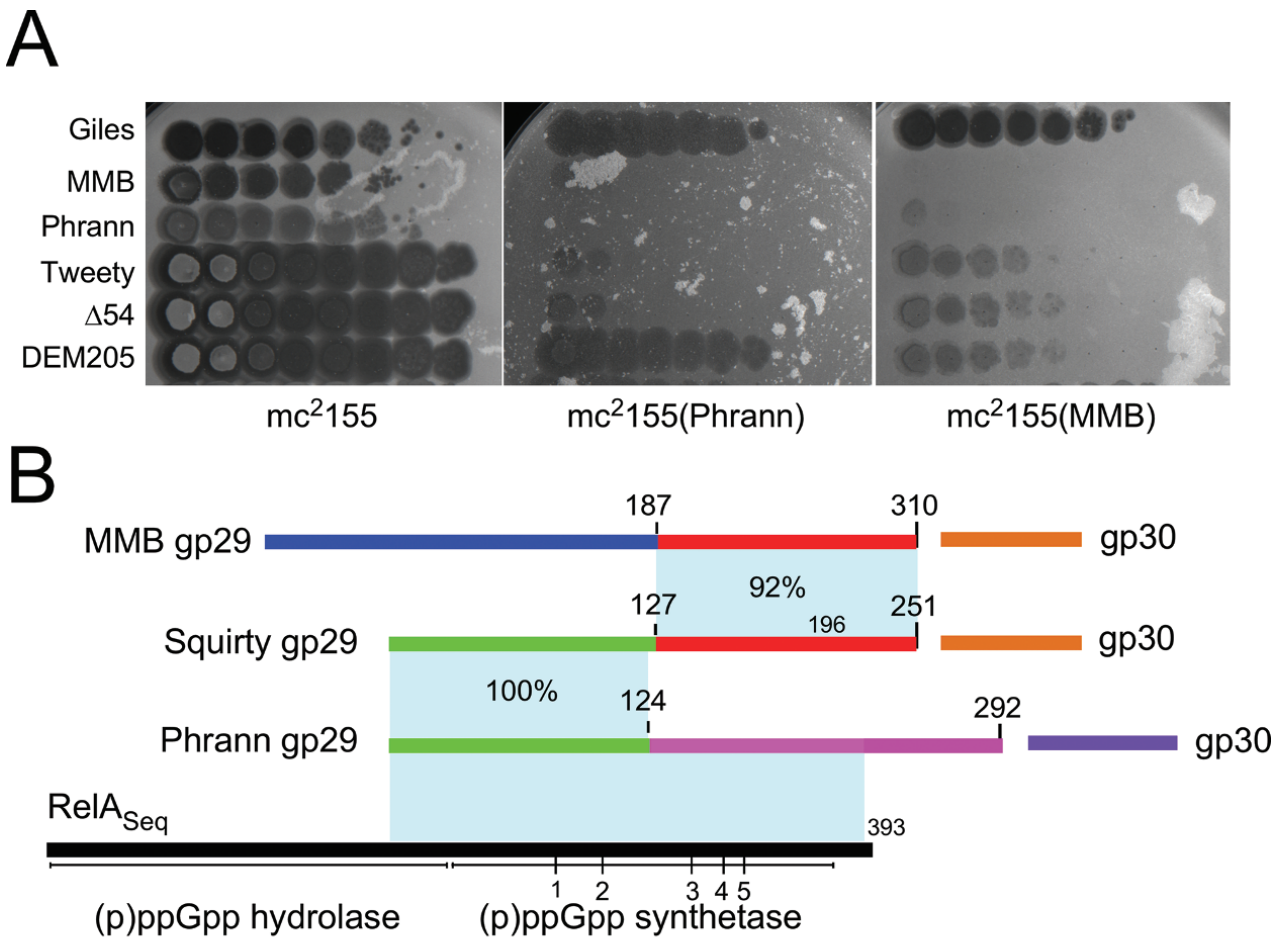
Genetic analysis of phage Phrann showed that Phrann *29* and *30* are required for defense against Tweety (note that the similarity of gene names is coincidental and does not indicate homology) (Fig. 11). These share some of the behaviors of the MichelleMyBell defense genes in that Phrann gp29 is toxic in the absence of Phrann gp30, but they are not related at the sequence level. Phrann gp29 is strongly predicted to be a (p)ppGpp synthetase with similarity to RelA family proteins (63). This poses a conundrum in that (p)ppGpp is used by bacteria as an alarmone to promote arrest of cell growth, and yet it is expressed well in a Phrann lysogen (63). However, the lysogen grows normally, and it is probable that gp30 interacts with gp29 and maintains it in an inactive state, consistent with its anti-toxic behavior. This model is attractive because the Phrann gp29/30 proteins would then be poised to be activated upon infection by an attacking phage (Fig. 11). Because the defense is specific to only a subset of the phages tested (including TM4 and Gaia, in addition to Tweety), these phages presumably code for a function that dissociates Phrann gp29 and gp30, resulting in activation of gp29, synthesis of (p)ppGpp, growth arrest, and inhibition of phage replication (63). Interestingly, Phrann gp29/30 defends against Tweety lytic growth but does not interfere with Tweety lysogenization, consistent with a post-DNA injection sensing mechanism. It is unclear what genes or sequences identify Tweety, TM4, and Gaia for targeting by this defense system, but early lytic genes are implicated (see below) (63).

MichelleMyBell gp29/30 likely acts similarly to Phrann, in spite of the lack of sequence similarity between the products (Fig. 11B). In an unusual set of relationships, the C-terminal half of MichelleMyBell gp29 is closely related (92% amino acid identity) to gp29 encoded by phage Squirty (cluster F). However, the N-terminal half of Squirty gp29 is 100% identical to Phrann gp29 (Fig. 11B). Thus, although Phrann gp29 and MichelleMyBell gp29 are not related to each other, it is plausible that MichelleMyBell is also involved in alarmone synthesis (63).

Mycobacteriophage Counterdefense

Phages often carry counterdefense systems such as anti-restriction and anti-CRISPR genes (99, 100), so it should not be surprising that mycobacteriophages have systems to counter prophage-mediated defenses. A particularly intriguing system is encoded by Tweety, which was revealed by isolating defense escape mutants (DEMs) that overcome either Phrann-mediated or MichelleMyBell-mediated defense (63). Many of these DEMs have genomic changes in gene *54*, a peculiar gene coding for a protein with multiple copies of a tetrapeptide repeat (Fig. 12). The DEMs typically have changes in the numbers of repeats, usually fewer but sometimes more (63). DEMs isolated for their escape of Phrann defense remain subject to MichelleMyBell defense, and vice versa (C. Gauthier and G.F. Hatfull, unpublished observations), suggesting that gp54 is being "tuned" to specifically counter the different defenses. The hypothesis that these mutants are gain-of-function derivatives rather than loss-of-activation mutants is illustrated by the observation that gene *54* can be deleted and Tweety is still subject to both Phrann and MichelleMyBell defenses (63).

Tweety gp54 plays no known role other than in its counterdefense capacity, but homologues are present in about 40 other cluster F phages, all with different numbers of tetrapeptide repeats. The repeats are composed of the


A
Giles
MMB
Phrann
Tweety
Δ54
DEM205
mc²155
mc²155(Phrann)
mc²155(MMB)
B
MMB gp29
187
310
gp30
92%
Squirty gp29
127
196
251
gp30
100%
Phrann gp29
124
292
gp30
RelASeq
393
1 2 3 4 5
(p)ppGpp hydrolase
(p)ppGpp synthetase
C
Exclusion (heterotypic)
Counter-defense
Restriction
Lytic growth
Activation
Recovery
Exclusion (homotypic)
ppGpp
Immunity
Lysogeny (integration)
Prophage
CRISPR
Bacterial chromosome
RNA
DNA

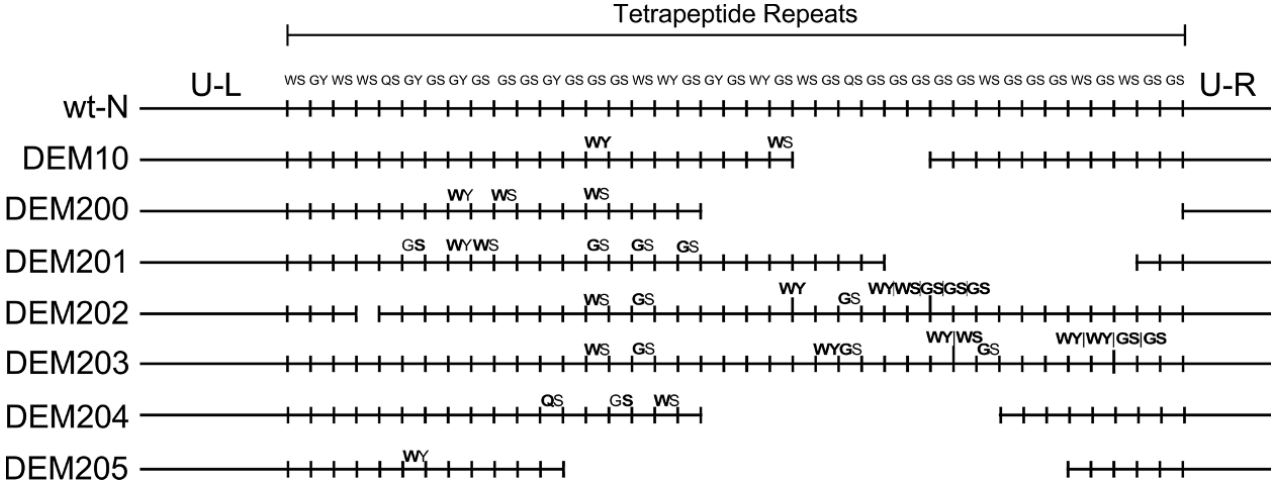


FIGURE 12 Variations in tetrapeptide repeats in Tweety gp54. Gene *54* of phage Tweety codes for a 374-residue protein in which a unique N-terminal (U-L) and C-terminal (U-R) flank a long series of tetrapeptide repeats. The number of repeats in the original sequenced genome is 48, although the derivative used for defense studies (wt-N) shown here only has 40. Each of the repeats has a sequence AAXX, where XX is either WS, GY, QS, GS, or WY, and the sequence at each repeat is shown. The organization of nine defense escape mutants (DEMs) are shown below, each of which efficiently overcomes defense mediated by the Phrann prophage. They all remain subject to defense mediated by the MichelleMyBell prophage. Six of the DEM mutants (DEM10, DEM200, DEM201, DEM202, DEM204, DEM205) have lost some of the repeats but also have altered repeat sequences, as indicated. DEM202 also has an insertion of five additional repeat copies. DEM203 has two insertions, one with two repeat units and the other with four repeat units.

sequence alanine-alanine-XX, where XX is either WS, GY, QS, GS, or WY (Fig. 12). The three-dimensional structure of this type of repeat is not known, but because the DEMs have variations in the numbers of repeats, it is plausible that it forms an extended structure, positioning the unique N- and C-terminal domains in configurations that can bind to gp29 and gp30 and prevent their dissociation. The mechanism by which expansion and contraction of the repeat number happens is also not known, but it is noteworthy that in addition to changes in repeat number, many of the mutants also have changes in the sequence of the repeat itself (Fig. 12). However, the changes in the repeat are always in the XX motif and always substituted by one of the tetrapeptide repeats found elsewhere. For example, DEM201, a mutant that overcomes Phrann defense, has lost 11 copies of the repeat, but 6 of the individual repeats are altered (Fig. 12). Not only are the 6 repeats not contiguous, but only 1 pair of the substituted sequences is present elsewhere, suggesting that 5 distinct mutational events created that derivative, in addition to the deletion of 11 copies. Because the repeated nature is also reflected as a 12-bp nucleotide sequence repeat, these variations could arise from replication errors and slippage, although it would appear to occur at a relatively high frequency. Whether the repeat substitutions play any functional role in counterdefense is unclear.

Finally, we note that although Tweety *54* homologues are predominantly found in cluster F phages, there is a related gene in the subcluster A1 phage, Nerujay (Nerujay gene *74*)

FIGURE 11 Prophage-mediated defense against phage attack. (A) Phage defense plaque assays. Ten-fold serial dilutions of phages Giles (control), MichelleMyBell (MMB), Phrann, and Tweety were plated onto lawns of a nonlysogen (mc^2155) and *M. smegmatis* lysogens of Phrann [mc^2155(Phrann)] and MichelleMyBell [mc^2155(MMB)]. The Phrann and MMB lysogens confer superinfection immunity to Phrann and MMB, because these are part of cluster N, and all of the cluster N phages are homoimmune, coding for closely related repressors. Tweety (cluster F) is unrelated to Phrann and MMB, and the Phrann and MMB lysogens defend against Tweety, and the efficiency of plating is greatly reduced relative to the wild-type strain. A defense escape mutant (DEM205) overcomes the Phrann defense and plates efficiently on the Phrann lysogen but remains subject to MMB-mediated defense. DEM205 contains a mutational change in gene *54*, but it is a gain offunction mutant, because a Tweety mutant in which gene 54 has been deleted (Δ54) is still targeted by the Phrann defense. (B) Relationships among phage-encoded (p)ppGpp synthetase-like proteins. Phrann gp29 is a homologue of RelA/SpoT proteins with similarity to the (p)ppGpp synthetase domain of *Streptococcus equisimilis* RelA, including five conserved motifs (1 to 5). The cluster F phage Squirty encodes a related protein (gp29) sharing the N-terminal 124 residues with Phrann gp29 but with divergent C-termini. MMB gp29 is not predicted to be related to Rel_{Seq} but shares its C-terminus with Squirty gp29. MMB gp30 and Squirty gp30 are closely related and are both predicted to be membrane localized. (C) Models for prophage-mediated viral defense. An integrated prophage (red line) confers defense against viral attack through numerous mechanisms, either homotypically (i.e., against the same or closely related viruses) or heterotypically (i.e., against unrelated phages). Homotypic defense includes repressor-mediated immunity (repressor, red circle) and superinfection exclusion (blue circle) against itself (red phage). Heterotypic defense includes an exclusion-like system illustrated by Charlie gp32 defense against Che9c (blue phage) and restriction against many viruses (illustrated by the green phage) by Panchino gp28. Defense is also mediated by a predicted (p)ppGpp synthetase (e.g., Phrann gp29; gold circle), which we propose is kept in an inactive form (gold circle with crossed lines) by an inhibitor (purple circle), which for Squirty gp30 is membrane localized. Lytic growth by specific phages activates the defense through early lytic protein, which is proposed to dissociate the (p)ppGpp synthetase from its inhibitor, enabling rapid accumulation of (p)ppGpp and growth arrest. Tweety encodes a counterdefense system (gp54) that may prevent activation of (p)ppGpp synthesis. Reproduced with permission from reference 63.

(Fig. 13). The unique N- and C-terminal regions are closely related to Tweety gp54 (90% and 80% amino acid identity, respectively), but Nerujay contains only 10 copies of the repeat, and only 2 tetrapeptide sequences are present, AAWS and AAGS. Nerujay is the only cluster A phage to carry such a gene, and it is notably absent from phage DD5, which is closely related in this region at the DNA sequence level (Fig. 13). A simple evolutionary explanation is that Nerujay acquired gene *74* relatively recently as insertion of a 796-bp fragment, perhaps derived from a cluster F phage similar to Ibhubesi, whose homologue (*54*) is identical in length to Nerujay *74* and shares 90% nucleotide sequence identity. Nerujay gp74 may also be active in counterdefense, but identifying which defense system it counters is a substantial challenge.

Exclusion during Lytic Growth

Many viruses have been reported to exclude superinfection of other viruses during either lytic or lysogenic growth. Some

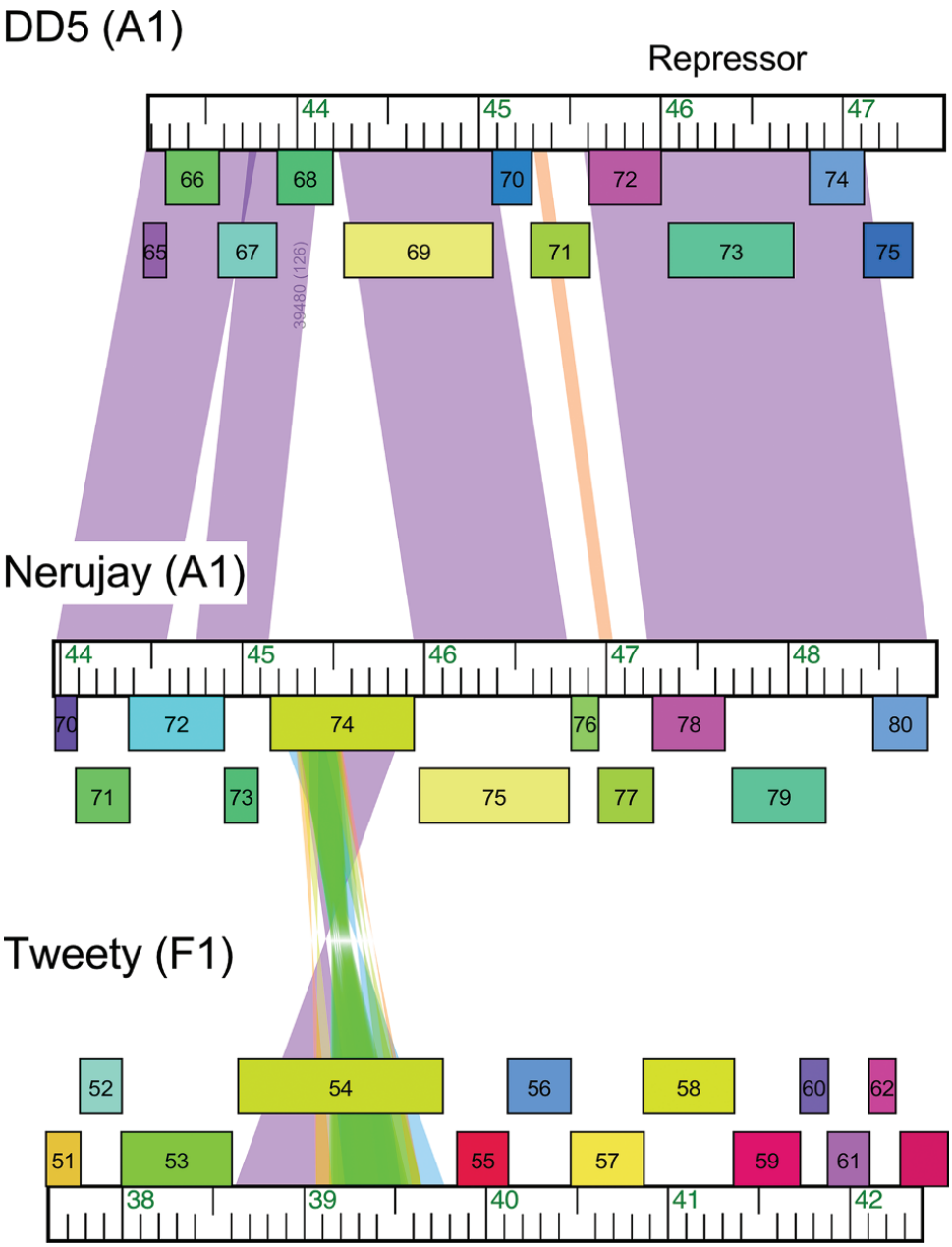


FIGURE 13 Genomes with potential counterdefense systems. Tweety gene *54* is active in counterdefense against Phrann (see Figs. 11, 12), and there are homologues in many other cluster F phages. The only phage outside of cluster F with a closely related homologue is the subcluster A1 phage, Nerujay. All other cluster A phages, including DD5, lack a Tweety *54* homologue. Segments of the genome maps are aligned with pairwise nucleotide sequence similarity shown as colored shading, as described for Fig. 7. In this region, all of the DD5 and Nerujay genes are transcribed leftward, and the Tweety genes are transcribed rightward.

well-studied examples include the T4 *imm* gene and P22 *sieA*, both of which are small membrane proteins that confer homotypic exclusion (101, 102). Coliphage HK97 also codes for a small membrane protein that is prophage expressed and prevents HK97 superinfection (103). It is not unusual for some of the small genes of unknown function in mycobacteriophages to be predicted membrane proteins that could perform such functions (e.g., there are eight predicted membrane proteins in the right arm of phage Nerujay), but a specific role for these in exclusion has not been shown. However, a WhiB-like regulator encoded in phage TM4 is implicated in homotypic exclusion, plausibly through regulation of host genes (104). In a recent example, superinfection exclusion was shown to be mediated by interference of a host protein used for efficient infection by some other phages (Fig. 14) (62).

This superinfection exclusion system was discovered through use of a screen for mycobacteriophage genes that are toxic to *M. smegmatis* when overexpressed (62). Approximately 5 to 10% of genes are expected to have this toxic property (105–108), and we identified gene *52* of phage Fruitloop (cluster F) as being highly toxic when overexpressed. Fruitloop gp52 interacts directly with the essential host protein Wag31 (DivIVA) and inactivates it, resulting in cell death (Fig. 14). Fruitloop *52* is expressed early in lytic growth and is not essential for plaque formation, and its toxicity is unlikely to be relevant for phage growth. Rather, it was reasoned that some mycobacteriophages depend on Wag31 for efficient infection, because it is polar localized and present both cytoplasmically and extracytoplasmically (109, 110). A search for phages inhibited by Fruitloop gp52 expression identified two subcluster B2 phages, Hedgerow and Rosebush, that are affected and presumably use Wag31 for efficient infection (62). Hedgerow and Rosebush are not related to Fruitloop at the nucleotide sequence level, and it is not evident that they have followed similar evolutionary paths as Fruitloop. It is plausible that mycobacteriophages code for many similar systems, targeting different aspects of mycobacteria that other phages exploit to gain entry.

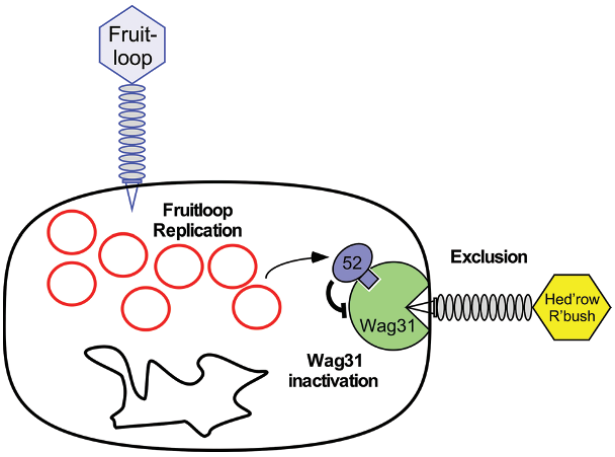


FIGURE 14 A model for Fruitloop gp52-mediated superinfection exclusion of Wag31-dependent phages. This model proposes a role for gp52 expressed by lytically growing phage Fruitloop in binding to and inactivating the host Wag31 (DivIVA) protein, which is essential and localizes to the growing pole of the cell. Some phages, such as subcluster B2 Hedgerow and Rosebush depend on Wag31 for efficient infection, such that gp52-mediated inactivation of Wag31 excludes them from superinfecting. Replicating Fruitloop genomes are shown as red circles alongside the bacterial chromosome (black circle), and Fruitloop gp52 is shown in blue, binding to Wag31, shown in green. Not drawn to scale. Reproduced with permission from reference 62.

Compatibility and Incompatibility

Most of the temperate mycobacteriophages can be grouped into homoimmune classes and have closely related repressors. The large majority of these carry integration systems, targeting a variety of *attB* sites. In general, if two phages are heteroimmune and use different *attB* sites for integration, they are fully compatible and there is no obvious impediment to the formation of double lysogens. This is not unexpected, and the sequences of large numbers of bacterial genomes show that they commonly carry two or more prophages. If phages are heteroimmune but use the same *attB* site, then they may compete with each other for stable lysogeny.

A subset of temperate mycobacteriophages do not have integration systems but instead encode *parABS* systems similar to those used by plasmids to promote segregation into daughter cells at division (57, 58). These phages, all of which group in cluster A but span 10 subclusters, replicate extrachromosomally at low copy number and, as such, are subject to incompatibility mediated potentially by both the prophage replication system and the partitioning systems themselves. None of the replication systems have yet to be described, but there is good evidence of incompatibility among phages with similar *parABS* systems (58). For example, a double lysogen of phages RedRock and Alma, both of which have *parABS* systems, can be constructed but not stably maintained without loss of one of the prophages (58). In contrast, a double lysogen of RedRock and Bxb1, which has a canonical integration system, stably maintains both prophages.

Repressor Theft

The immunity repressors have been well studied in the cluster A phages (107), although there is substantial sequence variation reflecting a variety of heteroimmune groups. Unexpectedly, a variety of other phages in other clusters carry closely related copies of some of these repressors (65). These related gene copies are found in lytic phages that do not form lysogens, including some cluster C phages, and also in temperate phages (e.g., cluster F and cluster J), where they are present in addition to their canonical repressors. For example, Fruitloop (cluster F1) has a canonical repressor (gene *44*), which confers immunity to itself and some other cluster F1 phages, and the stolen repressor (*37*), which shares 97% amino acid identity with the Bxb1 repressor. Fruitloop gene *37* is expressed in a lysogen, although at a somewhat lower level than the canonical repressor (62), but confers immunity to at least some cluster A1 phages such as SkiPole and Pinto (Deborah Jacobs-Sera and GFH, unpublished observations). Because the cluster C phages do not form lysogens, the repressor may block superinfection of cluster A1-like phages during lytic growth.

EXPLOITATION OF MYCOBACTERIOPHAGES

Using Recombinant Phages for Efficient Delivery

Bacteriophages have the inherent ability to efficiently inject their DNA into host bacterial cells. Mycobacteria do not undergo natural transformation at high frequency, and electroporation is effective, but only a subset of cells take up

DNA. In contrast, infection with mycobacteriophages at a multiplicity of infection greater than three generally results in DNA injection into every cell in the culture. This property was exploited early on in the development of mycobacterial genetic systems (12) and has been further developed for the delivery of transposons, reporter genes, and allelic exchange substrates (111–115). Phages are useful tools for the delivery of reporter genes such as luciferase or florescent proteins in rapid diagnostic assays for tuberculosis drug susceptibility in the clinic (116–119) but are also useful for understanding mycobacterial physiology. For example, dual reporter phages have been exploited to identify and analyze persister cells in *M. tuberculosis* (120).

Mycobacteriophage Tools for Mycobacterial Genetics

Mycobacteriophages also offer a rich reservoir of genes and cassettes for use in mycobacterial genetics. One application that has found widespread use is the development of integration-proficient vectors that transform mycobacteria by stable chromosomal integration using phage integrases. Integration vectors have been constructed from a variety of phages, including Adephagia, Brujita, L5, Bxb1, Tweety, Giles, Omega, and Ms6 (52, 68, 72–74, 83, 121, 122). These typically transform efficiently, and the transformants are quite stable, except if newly introduced genes are deleterious to growth and the integrase gene is retained in the recombinants. Most of these vectors work in both fast- and slow-growing strains, because the *attB* sites—especially those used by the tyrosine integrases—tend to be well conserved.

A second tool with broad applicability is a mycobacteria-specific recombineering system utilizing mycobacteriophage-encoded recombination systems (123). The most widely used system is that derived from mycobacteriophage Che9c, which has been used for both dsDNA and ssDNA recombineering (78, 79, 124). For dsDNA recombineering, both the exonuclease and DNA pairing genes (Che9c *60* and *61*, respectively) are required, but for ssDNA recombineering, only *61* is needed. The recombineering systems work in both *M. smegmatis* and *M. tuberculosis*, and recombination is sufficiently efficient that it can be used to introduce point mutations into the genome of *M. tuberculosis* (125). Many newly identified recombination systems are present in the multitude of mycobacteriophages sequenced since the Che9c system was developed, and some of these may be worth investigating for improved ease of use or efficiency.

The recombineering system has been especially useful for engineering the phages themselves (98, 123, 126). When phage DNA and a mutagenic DNA substrate are coelectroporated into recombineering-proficient cells, recombination is efficient, and following recovery, individual plaques can be screened by PCR for the presence of the mutant allele. Typically ~10% of progeny are mixed and contain both wild-type and mutant alleles, and a homogenous mutant derivative can be identified by PCR of plaques after replating. Point mutations can be introduced relatively easily, provided a PCR scheme is used that can distinguish between the mutant and wild-type alleles (68). The main limitation of the BRED approach is the question of whether plaques can be recovered following transfection in *M. smegmatis* (98). Many phages do this quite efficiently, and sufficient plaques can be readily recovered for screening. However, some phages transfect at very low efficiency or not at all, and these tend to be phages with larger genomes. Whether genome size *per se* is the principle factor or whether some of these phages use encapsidated phage proteins for infection is unclear.

Mycobacteriophages have also provided non-antibiotic-based selectable markers for mycobacteria, taking advantage of immunity repressor genes that confer immunity to superinfection, and lytic derivatives of the temperate phages that efficiently kill mycobacteria. The most well-developed system is based on the cluster K phage Adephagia, which infects both *M. smegmatis* and *M. tuberculosis* (127). Plasmids carrying the Adephagia repressor (gene *43*) can be introduced by electroporation and selected in the presence of an Adephagia derivative from which gene *43* has been deleted. The transformants carry an integrated copy of the Adephagia genome, with the additional advantage that the plasmid cannot be lost without lytic induction and death, thus ensuring plasmid stability. If the presence of the prophage is undesirable, a second phage derivative can be used in which both the repressor (*43*) and the integrase gene (*41*) have been deleted (127).

Mycobacterial plasmids based of the *oriM* origin of replication derived from plasmid pAL5000 (128–131) are somewhat unstable in the absence of selection (72), especially if recombinant genes they are carrying are not well tolerated. Addition of the phage-encoded *parABS* systems to these plasmids adds considerable stability to their maintenance (58).

Therapeutic Potential

The possibility of using mycobacteriophages for the treatment of mycobacterial infections has been postulated for some time, although little progress has been made (10). Although tuberculosis remains a major public health concern in many parts of the world, effective treatment could be achieved with current antibiotics, a robust health care infrastructure, and monitoring. However, the emergence of multiply and extensively drug-resistant tuberculosis infections demands new approaches, and phage applications may warrant attention. It would seem unlikely that mycobacteriophages could achieve sterilization from an *M. tuberculosis* infection and a long-term cure. But some gains might be achieved from combinations with antibiotics that could extend their effectiveness, lower required dosages, reduce the prevalence of resistance, provide relief in late stages of infection, or help prevent transmission. An alternative approach would be to use phages prophylactically, to protect coworkers or family members from infection when in close contact with an infected patient (10).

Therapeutic utility is likely to require a cocktail of phages to minimize the incidence of phage resistance, just as antibiotics must be used in combination for tuberculosis treatment. However, the key property of phages in such a cocktail is that resistance to one phage does not confer resistance to the other phages. Unfortunately, very little is known about either the specific receptors used for mycobacteriophage infection or the mechanisms of resistance (132), and this is an area that needs further investigation. Furthermore, relatively few of the phages isolated on *M. smegmatis* infect *M. tuberculosis*, and they generally group in a small number of clusters or subclusters (84). As such, they may use a rather limited set of receptors, and cross-resistance may limit the numbers that could be combined together into a suitable cocktail. Moreover, early phage typing studies suggested differences in phage sensitivities among clinical isolates of *M. tuberculosis*, and this needs to be revisited now that we have a deeper understanding of the genetic diversity of *M. tuberculosis* strains (133).

Infections of nontuberculosis mycobacteria may also represent possible targets for phage therapy. Attractive

targets are *Mycobacterium abscessus* infections associated with cystic fibrosis, which are often highly refractory to antibiotic therapies. Relatively little is known about the phage susceptibility profiles of *M. abscessus* clinical isolates, but a substantial proportion of sequenced *M. abscessus* strains carry one or more prophages (134, 135), which could code for viral defense systems. Suitable mycobacteriophage cocktails could conceivably be used to reduce the bacterial load in cystic fibrosis patients or to prevent infection following lung transplantation.

PERSPECTIVES

Mycobacteriophages have provided a wealth of insights into viral diversity and evolution and played indispensable roles in the development of mycobacterial genetics. We anticipate that the advances in mycobacteriophage genomics will slow somewhat, in large part because we are nearing saturation in the isolation of phages that infect *M. smegmatis*. However, as the emphasis shifts to phages that infect other mycobacterial strains and other *Actinobacteria*, new and different types of genomes are likely to be discovered. We anticipate that the coming years will see some progress toward defining the functions of more mycobacteriophage genes. The number of uncharacterized genes is vast, but the methods are available for their genetic, biochemical, and structural characterization, and the limiting factor may be the development of suitable assays. Surrogate approaches such as screening for toxicity (62, 106) and finding interacting partners (136, 137) should be useful strategies. Because many of the phage genes may play roles in microbial dynamics, the availability of such a large collection offers a very substantial resource.

Finally, the mycobacteriophages occupy a unique niche in how they have been isolated and characterized, mostly by early-career undergraduate students and faculty at institutions spread across the United States and the world (34). These students have not only made fantastic contributions to this field, but the fun, curious, and sometimes entertaining names of the phages they discovered will continue to amuse. The number of phages that infect actinobacterial hosts likely far exceeds the numbers of students who will isolate and characterize them over the coming years, and I can't wait to see the novelties, surprises, and potential utilities that emerge.

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Immunology of *Mycobacterium tuberculosis* Infections

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64

INTRODUCTION

Mycobacterium tuberculosis, the etiologic agent of tuberculosis (TB), remains a significant global public health burden (1). In 2016, there were 10.4 million new TB cases reported globally and nearly 1.7 million TB-related deaths (1). Understanding the host response to *M. tuberculosis* infection is a key aspect of efforts to eradicate TB through the development of effective vaccines and immune therapeutics. *M. tuberculosis* is an intracellular pathogen transmitted via inhalation of aerosolized, bacteria-containing droplets. Innate immune cells in the lungs, primarily macrophages, dendritic cells, monocytes, and neutrophils, readily phagocytose *M. tuberculosis* and are the earliest defenders against the pathogen. The transformation of bacteria-containing phagosomes into acidified, antimicrobial compartments is a central tenet of defense against *M. tuberculosis*. In this regard, the production of interferon-γ (IFN-γ), which can activate infected myeloid cells and inhibit bacterial replication, is a well-known antimycobacterial contribution by adaptive immune cells such as CD4 and CD8 T cells. Despite pressures from host immunity, *M. tuberculosis* is able to persist in the host. *M. tuberculosis* infection results in hallmark lesions called granulomas, which are initially aggregates of infected and uninfected myeloid cells circumscribed by a lymphocytic cuff. The granuloma is thought to prevent bacterial dissemination to extrapulmonary sites but can also become a niche for long-term bacterial persistence. *M. tuberculosis* has evolved myriad strategies to evade and subvert immune responses to persist within a host, and it is becoming increasingly clear that the immune response to *M. tuberculosis* infection involves contributions from a wide variety of innate and adaptive immune cells. A clearer understanding of the complex cross talk between *M. tuberculosis* and host immunity is essential for the development of efficacious TB vaccines. Despite being developed nearly a century ago, *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), an attenuated strain of *M. bovis*, remains the only licensed vaccine against TB. Vaccination with BCG provides protection against severe forms of disseminated TB in children but has variable efficacy in preventing pulmonary disease in children and adults (2–4). However, the immunological basis for the poor efficacy of BCG remains unclear. Moreover, long-held concepts regarding the nature of desired immune responses in an ideal TB vaccine, namely, the induction of antigen-specific CD4 T cells producing IFN-γ, are being updated to reflect the expanding knowledge of host immunity to *M. tuberculosis* infection gathered from animal models and human cohort studies. Advances in imaging and single-cell technologies combined with high-throughput approaches and systems-based analyses are providing more information on the immune response to *M. tuberculosis* infection at increasingly higher resolutions. As our understanding of the host response to *M. tuberculosis* infection grows, opportunities to leverage knowledge of the immunology of *M. tuberculosis* infection toward improving therapeutics and vaccines for TB are increasing.

This article will cover integral features of the innate and adaptive immune response to *M. tuberculosis* infection. Additionally, it will highlight recent findings on the hallmark granuloma and novel cellular players contributing to the host response to *M. tuberculosis* infection. Finally, it will provide an overview of the state of TB vaccine research, including a summary of BCG-based vaccines and the TB vaccine pipeline.

IMMUNOPATHOGENESIS OF TB IN HUMANS AND ANIMAL MODELS

Overview of Human TB Disease and Comorbidities

Transmission of *M. tuberculosis* occurs after inhalation of aerosolized droplets containing live bacteria into the lungs. Successful transmission is influenced by a variety of conditions, including proximity and duration of contact with an individual with active TB (ATB) disease and the immunecompetency of the individual infected with *M. tuberculosis* (5–7). We now appreciate that in a clinical setting, *M. tuberculosis* infection presents as a continuum of diseased/infected states ranging from asymptomatic latent TB infection (LTBI) to ATB disease. This complexity, combined with remarkable heterogeneity in lesions within a single patient, has presented unique challenges to the eradication of TB (8). While the majority of individuals exposed to *M. tuberculosis* are able to control infection in the form of LTBI, an estimated 5 to 10% of people exposed to *M. tuberculosis* develop ATB, which is characterized by a persistent cough accompanied by sputum production, weight loss, weakness, and night sweats (9). Clinical diagnosis and treatment of *M. tuberculosis* infection is complicated by a variety of coinfections and comorbidities.

Comorbidities that modulate immune function can exacerbate TB disease or contribute to progression of individuals

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with LTBI to ATB. HIV coinfection in latently infected individuals increases the risk of developing TB from a 5 to 10% lifetime risk to a 10% annual risk, and HIV infection is the single greatest risk factor for the development of TB (10–14). The relevance of HIV coinfection to global TB mortality is highlighted by the fact that more than a fifth of all TB-related deaths in 2016 were in HIV-positive individuals (1). Progressive depletion and dysfunction of CD4 T cells following HIV infection leads to immune suppression and negatively impacts immunity to *M. tuberculosis*. Specific depletion of *M. tuberculosis*-specific CD4 T cells has been reported in the peripheral blood (15, 16) and bronchoalveolar lavage (BAL) samples (17, 18) of HIV-infected individuals with LTBI. Several studies indicate that specific depletion may be a consequence of enhanced HIV coreceptor expression in CD4 T cells, particularly CCR5, in TB patients (15, 19–24). Alternative hypotheses to explain specific depletion of *M. tuberculosis*-specific CD4 T cells include differential functionality of specific T cells. In HIV coinfected LTBI, *M. tuberculosis*-specific CD4 T cells are reported to secrete interleukin 2 (IL-2) in contrast to MIP-1β (macrophage inflammatory protein 1 beta) secreted by cytomegalovirus-specific CD4 T cells (16). Analysis of viral loads in HIV coinfected LTBI showed an inverse correlation between viral load and the frequency of *M. tuberculosis*-specific CD4 T cells secreting IL-2 (25), suggesting that IL-2 producing *M. tuberculosis*-specific CD4 T cells may be specifically depleted in the context of HIV coinfection. Relatedly, HIV-coinfected individuals have lower frequencies of cytokine-producing *M. tuberculosis*-specific CD4 T cells with impaired proliferative capacity compared to HIV-uninfected individuals with LTBI (26–28), suggesting *M. tuberculosis*-specific CD4 T-cell dysfunction during HIV-infection. The relative contributions of depletion versus dysfunction of *M. tuberculosis*-specific CD4 T cells to enhanced TB risk following HIV infection remains unclear. Further, HIV infection may perturb protective immunity to *M. tuberculosis* in other immune compartments, such as CD8 T cells. For instance, *M. tuberculosis*-specific CD8 T cells from individuals with LTBI are reported to have impaired proliferation and degranulation in HIV-infected compared to HIV-uninfected individuals (29). Studies have also described associations between TB and many other conditions or activities, including smoking, malnutrition, diabetes, helminth infections, chronic lung diseases, and cancer (30, 31). Further investigations will be required to fully understand the basis of identified associations with other infections and morbidities.

Animal Models of Infection

Knowledge of the host response to *M. tuberculosis* infection has benefited greatly from the development of animal models of infection. The variable outcomes of *M. tuberculosis* infection in humans are challenging to model in a single animal model. Many experimental animals are susceptible to *M. tuberculosis* infection and can inform us about aspects of human disease. The mouse model for TB benefits from many advantages: ease of manipulation and housing, availability of well-characterized inbred strains, sophisticated techniques for the generation of mutant strains, availability of immunological and other reagents, and relatively low cost. Mice have been utilized to model host responses to *M. tuberculosis* infection, to evaluate drug and vaccine candidates, and to study the immune response to mutant strains of mycobacteria. Experimental infection can be delivered through multiple routes: intravenously, intraperitoneally, intratracheally, or via aerosolized particles. The latter method, especially low-dose aerosol infection, is the most physiologically relevant and has become the preferred method. Different mouse strains have well-characterized lung pathologies and levels of susceptibility (32–36). Typically, following bacterial deposition into the lungs, it takes approximately 2 weeks to begin priming adaptive immune responses in the lung-draining lymph nodes and a further 1 to 2 weeks for robust participation in the lungs by adaptive immune cells, but bacterial burdens continue to be maintained at a high level in the lungs of *M. tuberculosis*-infected mice. There are limitations to what can be gleaned from mouse models of *M. tuberculosis* infection due to the differences in lung pathology between mice and humans. Further, true latent infection and significant immune control of infection are difficult to establish in the mouse model, though chemotherapeutically induced models of paucibacillary disease in mice exist (37, 38). The development of humanized mice that can recapitulate the heterogeneity of human lung pathology may extend the advantages of the mouse model, but humanized mice are also reported to display aberrant T-cell responses and be unable to control bacterial burden (39, 40).

Other animal models of *M. tuberculosis* infection include guinea pigs, rabbits, fish, and non-human primates (NHP). Each has distinct advantages and disadvantages that make their use particularly suitable for different types of research questions. Following infection, guinea pigs exhibit pathological features, such as the organization and development of caseous necrotic granulomas, that more accurately recapitulate the human granulomatous response compared to mice (41). Further, guinea pigs are very susceptible to *M. tuberculosis* infection and, thus, are a good choice for testing candidate drugs and vaccines and studying dissemination dynamics. Similarly, rabbits develop a well-organized granuloma that can become necrotic following mycobacterial infection. However, rabbits are resistant to *M. tuberculosis*, and high numbers of bacteria during inoculation or use of more virulent strains are needed (42–45). Nevertheless, the rabbit model has been leveraged to study relatively rarer forms of TB, such as cutaneous and meningeal TB (46, 47). The usefulness of both the guinea pig and rabbit models is hampered by the scarcity of immunologic reagents relative to mice. The zebrafish model has provided novel insights into the establishment of the mycobacterial granuloma. Infection of transparent zebrafish larvae with the natural fish pathogen *Mycobacterium marinum* leads to the establishment of well-organized granulomas that become necrotic and can be visually monitored (48). The primary advantage of the zebrafish model is the transparency of the zebrafish larvae, which, alongside facile manipulation of host and bacterial genetics, has been leveraged for insight into early innate immune events leading to the formation of the granuloma as well as insights into human disease. Adaptive immunity is present in adult zebrafish, and different populations of CD4 T cells have recently been described (49, 50), but these animals are no longer transparent, and relevance of the adult zebrafish immune response to human TB have yet to be established.

The NHP model of *M. tuberculosis* infection reflects much of the heterogeneity observed in human TB. Infection of NHPs is typically performed by aerosol or direct bronchoscopic deposition into the lungs of rhesus or cynomolgus macaques and, depending on the dose of the inoculation and the strain of bacteria utilized, leads to symptomatic ATB disease or asymptomatic infection in which bacteria persist at low levels akin to LTBI. The NHP model accurately recapitulates many of the hallmark granulomas seen in humans, including the heterogeneity of granulomas that can be present in the same animal (51), and presents clinical

symptoms similar to those seen in humans (52–57). The NHP model is regarded as an important preclinical model for TB research and is an excellent model for studying immunity to *M. tuberculosis* and assessing candidate drug and vaccine efficacies (58–63). Further, the NHP model can be used to study reactivation in the setting of simian immunodeficiency virus coinfection or other types of immune modulation, such as anti-tumor necrosis-α (anti-TNF-α) treatment, CD4 depletion, or inhibition of indoleamine 2,3-dioxygenase (IDO) (64–71).

INNATE IMMUNITY TO *M. TUBERCULOSIS* INFECTION

The earliest encounter between host and pathogen in TB occurs at the interface between innate immune cells and *M. tuberculosis*. While innate immunity is critical for early antimycobacterial responses, it is also important for the progression of infection and long-term control of *M. tuberculosis* by continually priming and educating adaptive immune responses and by regulating inflammation. However, innate immune cells are often niches for bacterial replication, and *M. tuberculosis* utilizes a variety of strategies that subvert innate immune responses to establish a chronic infection. Here, we will detail key features of the innate immune response to *M. tuberculosis* infection, starting from recognition of the bacterium and phagosomal defenses within infected macrophages to priming of adaptive immune responses by professional antigen-presenting cells. In between, we will highlight how neutrophils and monocytes are mobilized after *M. tuberculosis* infection, the role that natural killer (NK) cells play during infection, how the balance of inflammation is regulated by the innate immune system, and how cell death affects the immune response. In each section, we will also highlight some of the myriad strategies that *M. tuberculosis* utilizes to subvert or evade the host innate immune response.

Recognition of *M. tuberculosis* by Pattern Recognition Receptors

Pathogen-associated molecular patterns on *M. tuberculosis* are recognized via a variety of receptors to mediate opsonic and nonopsonic bacterial uptake: C-type lectins (e.g., mannose receptors, DC-SIGN, Dectin-1, Dectin-2, Mincle), complement receptors (e.g., complement receptor 3), collectins (e.g., surfactant proteins A and D, mannose-binding lectin), scavenger receptors (e.g., MARCO, SR-A1, CD36, SR-B1), Fc receptors (e.g., FcgR), glycophosphatidylinositol-anchored membrane receptors (e.g., CD14), and Toll-like receptors (TLRs) (e.g., TLR-2, TLR-4, TLR-9) (72–74). Mannosylated lipoarabinomannan, phosphatidyl-inositol mannosides, phthiocerol dimycocerosates, phenolic glycolipids (PGLs), trehalose dimycolate, peptidoglycan, and other mycobacterial components are recognized by an array of cell surface and intracellular receptors that mediate phagocytosis and/or antimicrobial defenses. *M. tuberculosis* DNA (75, 76) or bacterial second messengers (77) can be recognized by cytosolic pattern recognition receptors (PRRs), such as cGAS and STING (78, 79), to induce downstream cytokine production and autophagy. Further, nucleotide oligomerization domain-like receptors (NLRs) are cytosolic PRRs that recognize *M. tuberculosis* pathogen-associated molecular patterns, such as muramyl dipeptide, to activate a multiprotein complex termed the inflammasome. Functional redundancies for many of the receptors are likely to exist due to promiscuous ligand binding by different receptors and the wide array of available ligands on *M. tuberculosis*. Indeed, single or double knockouts for canonical scavenger receptors and C-type lectin receptors did not modulate susceptibility or attenuate immune responses following *M. tuberculosis* infection (80). However, increased susceptibility to *M. tuberculosis* infection in a variety of knockout mice demonstrate that a number of PRRs and their associated signaling pathways also play important, nonredundant roles in host defense against *M. tuberculosis* infection.

M. tuberculosis expresses a variety of known or putative TLR ligands, and TLR-2, TLR-4, and TLR-9 have been implicated in host recognition of *M. tuberculosis* (reviewed in 73, 74). Polymorphisms in specific TLRs or TLR signaling proteins have also been strongly associated with pulmonary TB in humans and have been shown to influence immunity against *M. tuberculosis* (81–84). The contribution of individual TLRs to immunity against *M. tuberculosis* infection is variable, but the importance of the TLR signaling pathway to antimycobacterial immunity is evident in studies showing that mice lacking the common TLR adaptor protein, myeloid differentiation factor 88 (MyD88), quickly succumb to *M. tuberculosis* infection (85, 86). Susceptibility of *MyD88*$^{-/-}$ mice to *M. tuberculosis* infection has been attributed to deficient expression of NOS2 (86), impaired ability to activate the IL-1β or IL-1 receptor (IL1R) pathway (87, 88), impaired receptivity of macrophages to IFN-γ signaling (89), and impaired IL-12 and TNF-α responses in macrophages and dendritic cells (DCs) (85). Gene-deletion studies in single TLRs have revealed that innate immune responses to *M. tuberculosis* are likely the result of the complex activation of multiple signaling pathways. For instance, mice lacking both TLR-2 and TLR-9 are more susceptible to *M. tuberculosis* infection than mice lacking the ability to signal through either TLR by itself (90). The susceptibility of *MyD88*$^{-/-}$ mice to *M. tuberculosis* infection is an example of the importance of common adaptor molecules that integrate signals from multiple PRRs and other innate immune pathways for the induction of antimycobacterial immunity. Further evidence for this concept is demonstrated by the increased susceptibility of *M. tuberculosis*-infected mice lacking CARD9, an adapter molecule integrating signals from C-type lectin receptors, or PYCARD/ASC, an adapter molecule integrating signals from nucleotide oligomerization domain-like receptors for the induction of the inflammasome (91, 92).

MyD88 signaling in innate immunity integrates signaling from TLR and IL-1 receptor families by bridging ligand-receptor binding to IL-1-receptor-associated kinases and the activation of multiple downstream pathways, including NF-κB, mitogen-activated protein kinases, and activator protein 1. The IL-1 signaling pathway is clearly required for resistance to *M. tuberculosis* infection in mouse models and is supported by human immunogenetics studies (93–96). In mice, the absence of IL-1 signaling led to severe susceptibility to *M. tuberculosis* infection. Both IL-1α and IL-1β, as well as their common receptor, IL-1R1, have been implicated in immunity to *M. tuberculosis* (87, 88, 97–101). Secretion of the mature form of IL-1β requires cleavage by the terminal inflammasome effector, caspase-1, but *M. tuberculosis*-infected mice lacking MyD88, ASC, or caspase-1 signaling do not display impaired IL-1β levels (87). Further, mice deficient in IL-1β are considerably more susceptible to *M. tuberculosis* infection than mice lacking ASC or caspase-1 (87). These findings suggest that IL-1β is a key mediator of resistance to *M. tuberculosis* infection but also indicate that the basis for resistance conferred by MyD88, CARD9, and PYCARD/ASC likely depend on additional factors beyond IL-1β.

While host recognition of *M. tuberculosis* leads to the activation of innate immunity, *M. tuberculosis* has also evolved strategies that evade innate immune responses mediated by PRRs. Strain-specific expression of cell envelope components may be associated with differential immune responses. For example, the W-Beijing lineage strain, HN878, has been found to express polyketide synthase-derived phenolic glycolipids that are missing in lab-adapted H37Rv or other clinical isolates (i.e., CDC1551) (102). Expression of PGL by HN878 has been found to diminish production of multiple innate immune cytokines and chemokines (102, 103), though its role in the increased virulence of HN878 remains controversial. Modulation of innate immune responses by *M. tuberculosis* is also accomplished through the presence of immune-inhibitory lipid components that compete with immune-activating mycobacterial components for the same receptors. For example, expression of tetra-acylated sulfoglycolipids by the W-Beijing strain GC1237 can competitively bind TLR-2 to attenuate responses to canonical TLR-2 agonists, including mycobacterial lipomannans (104). Lastly, *M. tuberculosis* can also impair innate immune responses to cell-envelope components through enzymatic means. For instance, an *M. tuberculosis* serine-hydrolase, Hip1, was found to cleave multimeric, cell wall-associated GroEL2 to a secreted monomeric form to mediate attenuated macrophage and DC responses (105–109). Additionally, *M. tuberculosis* mutants lacking *hip1* or a putative mycobacterial metalloprotease, *zmp1*, display enhanced inflammasome activation (106, 110), suggesting that *M. tuberculosis* has multiple strategies for dampening activation of the inflammasome.

Thus, in addition to the array of host receptors that mediate recognition of *M. tuberculosis*, innate immune responses to infection likely depend on the strain of *M. tuberculosis*, the presence of cell wall components that can competitively inhibit the activation of PRRs, and the presence of *M. tuberculosis* enzymes that modify the immunogenicity of cell envelope components.

Phagosomal Defense in Macrophages

Macrophages are the first immune cells to encounter *M. tuberculosis* during infection and also represent the primary replicative niche for *M. tuberculosis*. Recognition of *M. tuberculosis* by macrophages leads to phagocytosis and sequestration of the bacterium in phagosomes, which typically eradicate pathogens via fusion with lysosomes and consequent acidification of the pathogen-containing phagolysosome. However, *M. tuberculosis* is able to survive and replicate in the phagosome by inhibiting phagosomal maturation and phagolysosomal generation through a variety of mechanisms (reviewed in 72, 111). Further, transcriptional profiling of intraphagosomal bacteria indicated that *M. tuberculosis* readily counters the nitrosative, oxidative, hypoxic, and nutrient-poor phagosomal environment through the expression of stress-adaptive genes (112), though a genome-wide transposon site hybridization screen for *M. tuberculosis* survival in macrophages suggested that *M. tuberculosis* constitutively expresses genes required for its survival (113). Nevertheless, it is clear that *M. tuberculosis* has adapted for a lifestyle inside the macrophage and employs many strategies to survive within these cells.

M. tuberculosis glycolipids can prevent accumulation of phosphatidylinositol 3-phosphate on phagosomal membranes and prevent phagolysosome biosynthesis (114). *M. tuberculosis* also secretes phosphatases (SapM and PtpA) and serine/threonine kinases (PknG) that are proposed to interfere with phagosomal maturation (115–121). There is also evidence that *M. tuberculosis* lipids, in particular, phthiocerol dimycocerosates, can mediate escape from the phagosome and host cell death (122). An *M. tuberculosis* secretion system, ESX-1, is also known for mediating disruptions in phagosomal integrity and preventing phagosome maturation. Promotion of aberrant phagosomal integrity and bacterial replication by *M. tuberculosis* ESX-1 is countered by IFN-γ-induced, Rab20-mediated phagosomal maturation (123). ESX-1-mediated phagosomal escape of bacteria is hypothesized to work through disruption of the phagosome by the 6-kDa early secretory antigenic target (ESAT-6) (124–127), though recent evidence proposes a contact-dependent, ESAT-6-independent mechanism for ESX-1-mediated phagosomal permeabilization (128). Nevertheless, ESX-1-mediated permeabilization of the phagosome exposes *M. tuberculosis* pathogen-associated molecular patterns, such as *N*-glycolyl-muramyl dipeptide, to cytoplasmic nucleotide oligomerization domain 2 receptors to induce type I IFNs (129, 130). ESX-1-mediated permeabilization of the phagosome also exposes extracellular bacterial DNA to the cytosolic DNA-sensing pathway, which leads to targeting of *M. tuberculosis* to autophagosomes for subsequent killing (75). *M. tuberculosis* ESX-3 has also been implicated in modulating intracellular trafficking of bacteria to avoid phagosomal maturation through inhibition of the host endosomal sorting complex required for transport (131–133). Thus, studies of the *M. tuberculosis* ESX secretion system have provided evidence for its role in both bacterial evasion of phagosomal pressures and host sensing of bacterial components. In addition to the ESX system, *M. tuberculosis* also expresses two SecA ATPase protein homologues (SecA1 and SecA2) involved in protein export (134). SecA2, in particular, has been implicated in virulence and intracellular growth (135, 136). Interestingly, both *M. tuberculosis* and BCG Δ*secA2* mutants are enriched in acidified phagosomes, indicating that mycobacterial SecA2 is required for arrest of phagosome maturation (137).

M. tuberculosis entry into macrophages through different receptors can lead to distinct activation of pathways that can inhibit or promote bacterial replication. The overall effect of multiple receptors engaging distinct or overlapping *M. tuberculosis* ligands is a complex and dynamic issue. For example, *M. tuberculosis* uptake by complement receptor 3 depended on host cholesterol, which mediated phagosomal association with coronin-1 and consequent inhibition of phagolysosome formation through activation of host calcineurin (138, 139). Alternatively, TLR-2 recognition of mycobacterial mannosylated lipoarabinomannan activates NF-κB and *NOS2* gene transcription that leads to antimycobacterial nitric oxide (NO) production (140). NO production is strongly associated with resistance to *M. tuberculosis*, though evidence for the antimycobacterial effects of NO is stronger in the mouse model. In mice, reactive nitrogen intermediates are toxic to mycobacteria *in vitro* (141–143), and infection can be exacerbated by the inhibition of NOS *in vitro* (144, 145) or *in vivo* (146–148). NO production following IFN-γ signaling has also been reported to limit overt inflammation by inhibiting processing of IL-1β by the inflammasome (149). Relatedly, mice with disrupted NOS2 alleles display exacerbated disease following *M. tuberculosis* infection (146, 150). Although *in vitro* studies using human alveolar macrophages and primary monocytes did not find an antimycobacterial role for NO (151–153), specific staining for NOS2 in the BAL of TB patients reveals upregulation in infected individuals compared to healthy controls (154). Nevertheless, *M. tuberculosis* has several strategies to cope

with otherwise damaging reactive nitrogen and oxygen intermediates: M. *tuberculosis* KatG, a catalase-peroxidase, can inactivate phagosomal reactive oxygen (155), and the M. *tuberculosis* proteasome can mediate resistance to nitrosative stresses (156). Promiscuous recognition of mycobacterial antigens by the same receptor may also have convergent outcomes as in the case for TLR-2-mediated recognition of M. *tuberculosis* cell wall fractions leading to TNF-α production in murine macrophages (157). TLR-mediated recognition of M. *tuberculosis* is also reported to synergize with the vitamin D pathway to induce the antimicrobial peptide (AMP), cathelicidin, in human macrophages (158, 159). The biologically active vitamin D metabolite, calcitriol, induces hCAP-18, a gene encoding the pro-form of cathelicidin, following TLR ligation of macrophages (158–160). In addition to direct antimicrobial activity, cathelicidin has been shown to exert antimicrobial functions by activating transcription of host autophagy genes *Beclin-1* and *Atg5* (161). The vitamin D pathway also synergizes with IFN-γ secreted by T cells to induce IL-15 autocrine signaling to promote autophagy and phagosomal maturation in M. *tuberculosis*-infected human macrophages (162).

Autophagy is the process whereby cytoplasmic constituents are degraded or recycled. A role for autophagy in antimycobacterial immunity in macrophages has been extensively characterized. Initial studies utilizing M. *bovis* suggested that autophagy plays a role in promoting phagosomal maturation to enhance bacterial killing (163). Moreover, LRG-47, an IFN-γ inducible p47 GTPase reported to be critical for phagosomal maturation and control of M. *tuberculosis* (164), is also involved in the induction of autophagy in M. *bovis*-infected macrophages (165). Autophagy-related genes were revealed to be involved in regulating the intracellular bacterial load of lab-adapted and clinical isolates of M. *tuberculosis* in a genome-wide small interfering RNA screen in infected human macrophage-like THP-1 cells (166). Accumulating evidence indicates that autophagy is integrated into the host response to M. *tuberculosis* infection by synergizing with pathogen sensing, phagosomal maturation, and IFN-γ inducible pathways to mediate antimycobacterial immunity: STING-dependent cytosolic sensing of M. *tuberculosis* DNA is required to deliver bacteria to autophagosomes and restrict bacterial replication (75); knockdown of cGAS in infected macrophages attenuated the induction of autophagy and survival during chronic M. *tuberculosis* infection (78); detection of cyclic-di-AMP secreted by M. *tuberculosis* in macrophages induced type I IFN production and autophagy to limit bacterial virulence (77); PARKIN, a conserved ubiquitin ligase, was shown to ubiquitinate M. *tuberculosis*-containing phagosomes to facilitate ubiquitin-mediated autophagy and restrict bacterial replication (167); IFN-γ-induced host ubiquilin-1 colocalizes with M. *tuberculosis* and mediates trafficking of bacteria to autophagosomes (168); IFN-γ receptor signaling mediated by the MyD88 adaptor-like (Mal) molecule induced autophagy and killing of intracellular M. *tuberculosis* in macrophages (169). Several studies have also delineated strategies employed by M. *tuberculosis* to evade autophagy. M. *tuberculosis* is reported to induce the expression of microRNA-33 to inhibit autophagy and regulate intracellular lipid metabolism to benefit bacterial replication (170). Further, a screen of M. *tuberculosis* cosmid clones in search of genes that inhibited bone marrow-derived DC antigen presentation revealed M. *tuberculosis* PE_PGRS47 (*Rv2741*) as an inhibitor of autophagy-mediated antigen presentation (171), suggesting that M. *tuberculosis*-mediated impairment of innate immunity can also negatively impact the generation of adaptive immunity. It is also becoming clear that autophagy-related proteins are likely to perform multiple functions, and care must be taken when interpreting specific knockouts or knockdowns of individual genes. For instance, myeloid cell-specific ablation of *Atg5*, but not other autophagy genes, compromised control of M. *tuberculosis* (172, 173). Deletion of the autophagy-related genes *Ulk1*, *Ulk2*, *Atg4B*, or *p62* compromised the ability to induce autophagy, but they were dispensable for the control of M. *tuberculosis* (173). Analysis of lung sections from M. *tuberculosis*-infected *Atg5* knockout mice indicated that Atg5 may be involved in regulation of neutrophil responses during infection, suggesting autophagy-independent roles for Atg5. Further, a recently described role for Atg5 in LC3-associated phagocytosis during M. *tuberculosis* infection supports the notion that specific components of autophagy can also overlap with other phagosomal pathways in immunity against mycobacteria (174).

Taken together, it is clear that macrophage recognition and phagocytosis of M. *tuberculosis* lead to a dynamic tug of war between antimycobacterial defenses and M. *tuberculosis* immune evasion. Macrophage defenses include AMPs, nitrosative stresses, phagolysosomal fusion, and autophagy and may operate independently of or subsequent to IFN-γ signaling. On the other hand, M. *tuberculosis* can subvert macrophage defenses at the level of the bacterial cell wall components that limit phagosomal maturation and the bacterial genes that combat or allow adaptation to intracellular immune pressure.

Recruitment and Function of Neutrophils and Monocytes Following *M. tuberculosis* Infection

Secretion of cytokines and chemokines early during infection recruits additional phagocytes to the site of infection. Early secretion of chemoattractants may be attributed to infected alveolar macrophages as well as lung epithelial cells (175–177). Moreover, a recent study suggests that cross talk between primary bronchial epithelial cells and infected macrophages may also promote secretion of chemokines (178). Trafficking of additional monocytes and granulocytes to the lung exerts immune pressure on M. *tuberculosis* and is crucial for the initiation of adaptive immune responses, but it may also promote M. *tuberculosis* cell-to-cell transmission and dissemination.

Recruitment of neutrophils serves as an early line of defense against M. *tuberculosis* infection via secretion of antimicrobial molecules and inflammatory mediators, but neutrophils also serve as niches for bacterial replication and can impede immunity against M. *tuberculosis*. In humans with active pulmonary TB, neutrophils have been found to be a significant population of M. *tuberculosis*-infected phagocytes in the BAL and sputum (179). Whole blood transcriptional profiling also identified a neutrophil signature in ATB patients that is associated with type I and type II IFN-inducible genes (180) and expression of the inhibitory molecule PD-L1 (181), suggesting that neutrophils may play an immunomodulatory role in human TB. In mice, the kinetics and magnitude of neutrophil recruitment following M. *tuberculosis* infection depends on the strain of mouse infected. Evidence for a pathogenic role for neutrophils is shown in studies comparing neutrophil recruitment in resistant versus susceptible mouse strains (182, 183). When comparing resistant C57BL/6 mice to susceptible DBA/2 mice after M. *tuberculosis* infection, a study found that neutrophils were rapidly recruited into the broncheoalveolar space at higher magnitudes in susceptible mice. Depletion of neutrophils at the onset of M. *tuberculosis* infection specifically extended the life spans of DBA/2 mice, suggesting that

early neutrophil involvement was pathogenic in genetically susceptible mice (183). Similarly, neutrophil depletion in susceptible I/St mice shortly after M. *tuberculosis* infection reduced lung pathology and bacterial growth and improved survival compared to C57BL/6 mice (184). In a separate study, depletion of neutrophils 5 weeks after aerosol M. *tuberculosis* infection of resistant BALB/c mice enhanced the levels of lung IL-6 and IL-17 without impacting IFN-γ and modestly enhanced control of bacterial burden (185). Neutrophil depletion in the first 4 days following intravenous M. *tuberculosis* infection of BALB/c mice, however, led to enhanced bacterial growth at extrapulmonary sites, suggesting that antimycobacterial immunity conferred by neutrophils may be dependent on the route of infection and the kinetics of neutrophil involvement (186). Utilizing fluorescently labeled bacteria, a recent study demonstrated that bacterial distribution in myeloid cells shifts from CD11b+ Ly6G– monocytes and macrophages to CD11b+Ly6G+ neutrophils in *Nos2*$^{-/-}$ animals infected with M. *tuberculosis*, suggesting that neutrophil influx can create a growth-permissive environment for M. *tuberculosis* under NO-deficient conditions (187). Evidence for beneficial roles that neutrophils play in antimycobacterial defense focus on neutrophil secretion of AMPs such as cathelicidin and lipocalin-2 to restrict bacterial replication (188) or via uptake of AMP-containing apoptotic neutrophils by M. *tuberculosis*-infected macrophages (189). Neutrophils can also release chromatin scaffolds that trap extracellular bacteria in an AMP-containing mesh. M. *tuberculosis* has been shown to induce the formation of neutrophil extracellular traps *in vitro* (190), and levels of neutrophil extracellular traps detected in the plasma of ATB patients were associated with disease severity and decreased with antibiotic therapy (191). Further, as discussed later in the chapter, dysregulation of neutrophil recruitment by unrestrained IL-17 responses during M. *tuberculosis* infection can incur pathological consequences by driving lung-damaging inflammation. Thus, the overall effect of neutrophil recruitment to the site of M. *tuberculosis* infection may be determined by host genetics, the context of infection (pulmonary versus extrapulmonary), or timing and duration of neutrophil activity.

In addition to neutrophils, monocytes are recruited to the site of M. *tuberculosis* infection. Similar to neutrophils, monocyte recruitment is important for innate immunity during M. *tuberculosis* infection but may also inadvertently promote M. *tuberculosis* dissemination. C-C chemokine receptor type 2 (CCR2) is a chemokine receptor expressed on monocytes and is responsible for CCL2-mediated recruitment of monocytes to sites of bacterial infection (192). CCR2 was found to mediate immunity against M. *tuberculosis* depending on the dose of infection. CCR2 knockout mice were more susceptible to high-dose intravenous M. *tuberculosis* infection (193), but not after low-dose infection (194). Monocytes have been shown to differentiate into macrophages and DCs following M. *tuberculosis* infection, and monocytes transferred into M. *tuberculosis*-infected mice were shown to be the predominant population of innate immune cells producing iNOS (195). Additionally, monocyte delivery of M. *tuberculosis* to pulmonary lymph nodes can coordinate with DCs to prime CD4 T cells after infection (196). Monocytes may therefore represent a recruited population of innate cells that combat M. *tuberculosis* infection through the production of reactive nitrogen intermediates and priming of adaptive immunity. However, monocyte recruitment following M. *tuberculosis* infection may also be detrimental to the host by providing an environment full of permissive cells. Treatment of M. *tuberculosis*-infected mice with polyinosinic-polycytidylic acid (polyIC) led to CCR2-dependent recruitment of a population of M. *tuberculosis*-permissive monocytes, severe susceptibility, and early mortality (197). Interestingly, susceptibility of polyIC-treated mice to M. *tuberculosis* infection was dependent on type I IFN signaling and was not due to any particular alteration to the T-cell response. The recruitment of neutrophils and monocytes to the site of M. *tuberculosis* infection represents a host strategy to contain bacterial replication that is co-opted by the bacterium to facilitate its growth and dissemination.

NK Cells in *M. tuberculosis* Infection

NK cells are innate lymphocytes with the capacity to secrete IFN-γ and perform cytolytic functions to mediate control of a variety of pathogens, including M. *tuberculosis*. Various components of the M. *tuberculosis* cell wall can bind directly to NKp44 found on NK cells (198), and NK cells can also recognize stress molecules upregulated on the surface of M. *tuberculosis*-infected cells (199). NK cells can mediate direct killing of M. *tuberculosis*-infected macrophages (199) but can also restrict intracellular bacterial replication via secretion of IL-22 (200) and IFN-γ (201) to increase phagolysosomal fusion of M. *tuberculosis*-containing phagosomes. Additionally, NK cells can enhance immunity against M. *tuberculosis* indirectly by enhancing CD8 T-cell production of IFN-γ (202) by promoting the expansion of γδ T cells (203) and by lysing M. *tuberculosis*-expanded regulatory T cells (204). The cytolytic capacity of NK cells is diminished in ATB patients relative to healthy controls and can be reconstituted following antibiotic therapy (205). Further, NK cell function in TB patients can be attenuated by monocyte-derived IL-10 (201). Interestingly, a population of IL-21-dependent NK cells that appears following BCG vaccination has been shown to expand following M. *tuberculosis* challenge (206), suggesting that NK cells may also display some hallmark characteristics of memory cells.

Inflammation and Cell Death During *M. tuberculosis* Infection

The regulation of inflammation is a critical factor that determines the outcome of M. *tuberculosis* infection. Overexuberant inflammation impairs cellular immunity, damages lung tissue, and can lead to lung cavitation and enhanced transmission. Inversely, too little inflammation can impair control of bacterial burden by delaying the induction of innate and adaptive immunity. While neutrophil recruitment and activity during M. *tuberculosis* infection can help contain bacterial replication, sustained neutrophilic inflammation can mediate damaging inflammation and promote disease. Importantly, whole blood transcriptomics identified a neutrophil-driven type I IFN-inducible signature in human TB that decreased upon treatment (180). Excessive type I IFN signaling has been shown to promote disease in mouse models and human samples. Mice lacking type I IFN signaling are more resistant to M. *tuberculosis* infection (207–210), though signaling through type I IFNs may play a protective role in the absence of IFN-γ (211, 212). Mechanisms underlying the pathogenic role of type I IFNs during M. *tuberculosis* infection include inhibition of IL-1β production (213, 214), induction of IL-10 to impair innate cytokine production (215), and loss of IFN-γ responsiveness in infected macrophages (215). In addition to induction of type I IFNs, neutrophils have also been reported to drive lung destruction through the secretion of matrix metalloproteinase 8 (216). The matrix metalloproteinase

family of enzymes has been implicated in lung tissue destruction during M. *tuberculosis* infection (217–220) but has also been shown to promote macrophage recruitment and bacterial dissemination during infection of zebrafish (221).

Eicosanoids are lipid mediators of inflammation derived from the oxidation of arachidonic acid. The balance between proinflammatory prostaglandin E2 (PGE2) and anti-inflammatory lipoxin A4 (LXA4), two members of the eicosanoid family of signaling molecules, can determine the outcome of M. *tuberculosis* infection (222–224). During M. *tuberculosis* infection, mice incapable of synthesizing PGE2 display increased susceptibility (223), and absence of the enzyme 5-lipoxygenase, which metabolizes arachidonic acid to LXA4, confers resistance (222). Importantly, therapeutic correction of low PGE2 levels can confer enhanced survival in highly susceptible mice infected with M. *tuberculosis* (100). Leukotriene A4 hydrolase is an enzyme that catalyzes the production of proinflammatory leukotriene B4 from leukotriene A4, which can also be converted to anti-inflammatory LXA4 as a counterbalance. In zebrafish, *LTA4H* mutants were found to be hypersusceptible to M. *marinum* infection due to dysregulation of the balance between leukotrienes and lipoxins; increased levels of LXA4 in *LTA4H* mutants impaired TNF-α responses and promoted susceptibility (225). The relevance of this finding to humans is highlighted in a TB meningitis cohort in Vietnam where heterozygosity for six *LTA4H* polymorphisms conferred a survival advantage over homozygosity (225). Indeed, anti-inflammatory glucocorticoid treatment efficacy in TB meningitis patients can be differentiated by a single nucleotide polymorphism in the *LTA4H* promoter controlling transcriptional activity, which suggests that the balance of inflammation is critical to disease progression and treatment outcomes in TB meningitis (226).

TNF-α is a critical proinflammatory cytokine in immunity against M. *tuberculosis* infection and can be secreted by a number of innate and adaptive immune cells. The importance of TNF-α in antimycobacterial immunity is clearly demonstrated by heightened susceptibility of TNF-α antibody-depleted animals or in animals lacking TNF receptor signaling following M. *tuberculosis* infection (227). TNF-α is also a critical mediator of immunity against TB in humans. This is demonstrated by increased rates of progression to ATB in LTBI patients receiving anti-TNF treatment for inflammatory disorders (228), which can be recapitulated in the NHP model of infection (70). The effects of anti-TNF treatment in humans and NHPs, as well as in mice (229–231), suggests that TNF-α is critical for maintaining sequestration of M. *tuberculosis* in the granuloma. Histopathological evidence from gene-disrupted or antibody-depleted mice infected with M. *tuberculosis* also suggests that TNF-α signaling may be playing a role in modulating apoptotic or necrotic cell death following infection (229, 231).

Cell death can be a means of restricting bacterial replication by the host or a way to disseminate to secondary loci of infection for M. *tuberculosis*. Apoptosis of M. *tuberculosis*-infected cells leads to fewer viable bacteria and effective cross-presentation of bacterial antigens (224, 232, 233), whereas necrosis of M. *tuberculosis*-infected cells allows viable bacteria to exit and disseminate (223, 234, 235). Proapoptotic M. *tuberculosis* mutants lacking *secA2* (236) and *nuo*G (237) were attenuated *in vivo*, and mice infected with these strains displayed enhanced priming of adaptive immunity compared to infection with wild-type M. *tuberculosis*, suggesting that prevention of host cell apoptosis is an M. *tuberculosis* virulence strategy. Relatedly, M. *tuberculosis*-infected murine neutrophils can aid in DC trafficking to the draining lymph nodes to initiate antigen-specific CD4 T-cell responses (238), but M. *tuberculosis* delays CD4 T-cell priming by inhibiting neutrophil apoptosis (239). Infection with the proapoptotic *nuo*G mutant M. *tuberculosis* resulted in earlier DC trafficking to lung-draining lymph nodes and earlier priming of antigen-specific CD4 T cells, but enhanced priming was abrogated upon neutrophil depletion (239). Additionally, uninfected macrophages performing a constitutive housekeeping function called efferocytosis can uptake M. *tuberculosis*-containing apoptotic bodies, which leads to delivery and killing of bacteria in lysosomes (240). This suggests that apoptosis may be a host strategy to limit bacterial replication by sequestering bacteria in vesicles that can be safely degraded by nearby innate immune cells. Inhibition of apoptosis by M. *tuberculosis* is driven by host intrinsic factors following infection with virulent strains. The proinflammatory eicosanoid PGE2 has been demonstrated to regulate synaptotagmin-7, a calcium sensor that maintains plasma membrane integrity (241). Human macrophages infected with virulent M. *tuberculosis* H37Rv, but not avirulent H37Ra, promote LXA4 production and inhibition of PGE2 biosynthesis, which impairs resealing of plasma membrane disruptions to preferentially induce host cell necrosis instead of apoptosis (241). Mice lacking PGE2 also suffered from increased lung bacterial burden following low-dose aerosol infection with virulent M. *tuberculosis* (223). Host cell necrosis following M. *tuberculosis* infection can be induced through activation of the cytosolic receptor interacting protein kinase 3 pathway, which inhibits apoptosis of infected macrophages through Bcl-xL and promotes necrosis through upregulation of ROS (242). Additionally, macrophages infected with virulent H37Rv, but not avirulent H37Ra, undergo proteolysis at the N-terminal of annexin-1, which prevents the completion of the apoptotic envelope and drives macrophage necrosis (235). Taken together, apoptosis represents a strategy by the host to limit infection through the combination of bacterial sequestration in apoptotic vesicles and the induction of adaptive immune responses, but M. *tuberculosis* may delay apoptosis or promote necrosis to facilitate replication and dissemination.

Initiation of Adaptive Immunity to *M. tuberculosis* by DCs

An important function of innate immunity during M. *tuberculosis* infection is the priming of adaptive immune responses. DCs are professional antigen-presenting cells that initiate adaptive immunity by presenting M. *tuberculosis* antigens in the context of major histocompatibility complex (MHC), costimulatory molecules, and cytokines. Depletion of cells expressing the pan-DC marker, CD11c, following M. *tuberculosis* infection impaired control of bacterial burden and delayed the initiation of adaptive immunity, illustrating the importance of DCs in mobilizing adaptive immune responses that can control bacterial replication (243). There is abundant evidence that M. *tuberculosis* is able to infect murine (244–246) and human DCs (247–249). In mice infected with green fluorescent protein GFP-expressing M. *tuberculosis*, DCs were found to be the major population of phagocytes infected by bacteria after 4 weeks (246). Upon M. *tuberculosis* infection, DCs mature and migrate to the lung-draining lymph nodes to initiate antigen-specific T-cell responses, which depended on the chemokine receptor CCR7 and its corresponding chemokines CCL19 and CCL21 (250–252). Further, IL-12, a cytokine secreted by myeloid cells and important for the induction of IFN-γ responses, is required for DC migration during M. *tuberculosis* infection (253). Priming of adaptive immune responses requires the transport of live

bacteria to the lung-draining lymph nodes (246, 250), but antigen-specific T cells can be primed by both the infected migratory DC and uninfected lymph node resident DC. A study demonstrated that infected DCs migrate to the lung-draining lymph nodes, where they secrete soluble, unprocessed *M. tuberculosis* antigens that are summarily phagocytosed by uninfected lymph node resident DCs (254). The exportation of *M. tuberculosis* antigens was initially proposed to benefit the host by circumventing inefficient antigen presentation by infected DCs. However, secretion of *M. tuberculosis* antigens by infected DCs may also benefit the pathogen by diverting antigen away from MHC class II antigen presentation (255).

Effective interaction between DCs and T cells is dependent on appropriate function of antigen presentation machinery, including expression of MHC, costimulatory molecules, and cytokines following *M. tuberculosis* infection. However, there is abundant evidence that *M. tuberculosis* infection impairs antigen presentation to evade antigen-specific T-cell responses. It is well recognized that *M. tuberculosis* infection leads to impaired MHC class II antigen presentation by macrophages (reviewed in 256). *M. tuberculosis*-mediated inhibition of phagosomal maturation has been implicated in attenuating processing of *M. tuberculosis* antigen 85 (Ag85) and the MHC class II-associated invariant chain (257). Multiple studies have also reported that *M. tuberculosis* infection impairs MHC class II expression in macrophages through inhibition of class II transactivator, a master transcriptional regulator controlling expression of MHC class II molecules (258–261), although there is little evidence of similar inhibition of MHC class II in DCs. Nevertheless, *M. tuberculosis* infection of DCs leads to functional impairment of antigen presentation. *M. tuberculosis* infection has been shown to impair DC maturation of human (reviewed in 262) and murine DC functions (reviewed in 263, 264). Studies examining proliferation of T-cell receptor transgenic CD4 T cells specific for *M. tuberculosis* Ag85 as a proxy for functional antigen presentation have demonstrated that *M. tuberculosis* EsxH can impair antigen processing through inhibition of the host endosomal sorting complex required for transport (ESCRT) (265). Additionally, *M. tuberculosis* promotes suboptimal antigen presentation *in vitro* and *in vivo* without detectable differences in the expression levels of costimulatory molecules when compared to BCG-infected DCs (266). Interestingly, studies using a mutant *M. tuberculosis* strain lacking *hip1* (discussed above) indicate that *M. tuberculosis* readily impairs DC costimulation and cytokine production to evade antigen-specific CD4 T-cell responses (107, 109), and a recent study demonstrated that BCG *hip1* retains similar immune evasion functions (267). Taken together, the initiation of the adaptive immune response requires the participation of DCs, which themselves are readily infected and subverted by *M. tuberculosis* infection. *M. tuberculosis* subversion of DC functions can interfere with antigen presentation and delay or impair the initiation of the adaptive immune response. Improving DC functions during *M. tuberculosis* infection may improve innate and adaptive immunity and enhance immune control of bacterial burden. A study that exogenously engaged the CD40 costimulation pathway in *M. tuberculosis*-infected DCs improved DC functions and promoted antigen-specific CD4 T-cell responses that augmented control of lung bacterial burden (268). Further, mucosal transfer of Ag85B-loaded DCs following challenge with *M. tuberculosis* augmented the efficacy of BCG vaccination (269), suggesting that early antigen presentation by DCs is an important component that determines the efficacy of vaccine-induced immunity. DCs are critical players that initiate adaptive immune responses to *M. tuberculosis* and determine the outcome of infection. Interventions or therapies that improve DC functions may provide benefits by augmenting cross talk between DCs and antigen-specific T cells.

ADAPTIVE IMMUNITY AGAINST *M. TUBERCULOSIS*

Protective immunity to *M. tuberculosis* and control of bacterial replication requires adaptive immune responses. This is best exemplified by the extreme susceptibility to mycobacterial infections of lymphopenic HIV patients and gene-deleted mice lacking MHC class II or T cells in general. Cytokine secretion and direct antimicrobial actions of antigen-specific T cells are key features of the adaptive immune response against *M. tuberculosis* infection. Further, the long-lived nature of antigen-specific memory T cells provides the basis for developing vaccines that induce antimycobacterial immunity. There are also expanding roles for B cells, γδ T cells, and CD1-restricted T cells that provide specific responses to a diverse set of *M. tuberculosis* antigens that complement antigens classically presented through MHC class I and II. However, adaptive immune responses can also become malignant by promoting excessive inflammation or be rendered ineffective from chronic antigen exposure. Here, we cover the importance of timing, location, and quality of CD4 T-cell responses during *M. tuberculosis* infection, how CD8 T cells contribute to immunity against *M. tuberculosis*, the roles that inhibitory receptors play during infection, the phenotypes and functions of memory T cells, and the roles that B cells, γδ T cells, CD1-restricted lymphocytes, and mucosal associated invariant T (MAIT) cells play in immunity against *M. tuberculosis*.

Kinetics and Homing of CD4 T Cells after *M. tuberculosis* Infection

In the mouse model of infection, CD4 T-cell responses are absolutely required to control bacterial replication, and animals lacking such responses succumb rapidly (270, 271). MHC class II knockout mice or CD4 depletion led to abrupt mortality following *M. tuberculosis* infection (270, 271). CD8 T cells play a key role in immunity against *M. tuberculosis* but cannot compensate for CD4 deficiency (270). Similarly, antibody depletion of CD4 in cynomolgus macaques severely compromised control of *M. tuberculosis* and led to reactivation in latently infected animals (69). Thus, the initiation of the CD4 T-cell response is a key feature defining the outcome of *M. tuberculosis* infection. There is a widely recognized delay in the initiation of antigen-specific CD4 T-cell responses following low-dose aerosol infection of mice (250, 272–275) and NHPs (276). *M. tuberculosis*-infected cynomolgus macaques had detectable antigen-specific responses 4 weeks postinfection (276). In mouse models of infection, antigen-specific CD4 T-cell responses are first detected in the lung-draining lymph nodes 2 weeks after infection. Significant antigen-specific lung CD4 T-cell responses are subsequently detected in the lungs 3 weeks after infection. This is in stark contrast to antigen-specific responses to other bacterial (277) or viral (278) pathogens, which are detected swiftly after infection. Adoptive transfer of ESAT-6-specific CD4 T cells prior to aerosol *M. tuberculosis* infection have demonstrated an apparent kinetic bottleneck whereby lung antigen-specific activation occurs only 7 days after infection despite the presence of antigen-specific T cells (279), suggesting that antigen-specific responses are delayed by

mechanisms other than trafficking of CD4 T cells from the mediastinal lymph nodes to the lungs. Delay in the initiation of adaptive immune responses to M. *tuberculosis* infection may be due to a variety of factors, including slow growth of the bacterium, inhibited apoptosis of infected macrophages and neutrophils, and delayed activation and migration of DCs, which cumulatively allow M. *tuberculosis* to establish a persistent infection in the lung.

CD4 T cells interact with infected macrophages to restrict intracellular M. *tuberculosis* replication. Thus, the effectiveness of the CD4 T-cell response depends on proper homing of antigen-specific CD4 T cells from lymphoid tissues to M. *tuberculosis*-infected cells in the lung. In M. *tuberculosis*-infected mice, antigen-specific CD4 T cells expressing CXCR3 localized to the lung parenchyma and were more efficient at controlling bacteria following M. *tuberculosis* infection when compared to vasculature-restricted CD4 T cells that expressed CX3CR1 (280). Interestingly, cells retained in the lung vasculature secreted the highest amount of IFN-γ during infection (280). Adoptive transfer studies demonstrated that IFN-γ accounted for greater control of bacterial burden in the spleen over the lung and drove immunopathology when overexpressed (281), suggesting that the function of IFN-γ may be to mediate control of bacterial dissemination to extrapulmonary sites and that IFN-γ may be detrimental when unrestrained. The distinction between vasculature-restricted and parenchyma-localizing CD4 T cells seems less important in rhesus macaques (282), where the majority of antigen-specific CD4 T cells can be found in the lung parenchyma but are restricted to the outer lymphocytic cuff of granulomas. Notably, studies have demonstrated that expression of IDO by cells in the granulomas of M. *tuberculosis*-infected rhesus macaques can mediate inhibition of T cell entrance into granuloma, and biochemical inhibition of IDO led to reorganization of the granuloma to include T cells localizing into the macrophage core (66, 283). Taken together, there is strong evidence that localization of antigen-specific CD4 T cells into the lung tissues where M. *tuberculosis*-infected myeloid cells reside is an important feature of protective immunity to M. *tuberculosis*.

Quality and Specificity of the CD4 T-Cell Response to *M. tuberculosis*

The quality of the T-cell response is an important feature determining the outcome of M. *tuberculosis* infection. Canonically, the production of IFN-γ by Th1 cells, CD8 T cells, and other lymphocytes is considered essential for protection against mycobacterial infections. In human immunogenetics studies, Mendelian susceptibility to mycobacterial disease (MSMD) is a spectrum of genetic mutations in five autosomal genes (*IFNGR1*, *IFNGR2*, *STAT1*, *IL12B*, *IL12RB*) and an X-linked gene that confer susceptibility to avirulent environmental mycobacteria and BCG (284). Deficiencies related to IFN-γ signaling in young patients with mutations in *IFNGR1* and *IFNGR2* confer fatal susceptibility to mycobacterial infections (285–288). STAT1 is an intracellular molecule important for IFN-γ signaling, and individuals with heterozygous germline *STAT1* mutations lose gamma-interferon activating factor (GAF) expression (289). GAF is an important transcription factor that facilitates IFN-γ-induced gene expression. Individuals with heterozygous *STAT1* mutations have impaired nuclear accumulation of GAF and suffer from recurrent mycobacterial infections (289). Additionally, mutations affecting IL-12 expression levels and signaling also confer susceptibility to mycobacterial infections. Two mutations in the leucine zipper domain of NEMO, an intracellular protein involved in NF-κB activation, impairs CD40-mediated IL-12 production in monocytes and DCs (290) and leads to recurrent mycobacterial infections. Similarly, defects that impair IL-12p40 lead to decreased IFN-γ levels and confer susceptibility to mycobacterial infections (291–294). Mutations in *IL12RB* are the most frequent genetic factors associated with Mendelian susceptibility to mycobacterial disease, but recurrent mycobacterial susceptibility in individuals with *IL12RB* mutations can be mitigated with BCG vaccination or primary BCG disease (291, 292, 295–298), suggesting that IL-12/IL-23 signaling may not be completely required for secondary immunity. IFN-γ is readily detected in human BAL in patients with TB disease and decreases following therapy (299), which is likely a consequence of decreasing bacterial loads. In contrast, studies of human peripheral blood mononuclear cells show a decrease in IFN-γ responses in ATB patients compared to controls (300–305). Lower frequencies of M. *tuberculosis*-specific IFN-γ responses in ATB patients may reflect trafficking of these cells to the lungs, resulting in specific depletion from the periphery. IFN-γ secretion is also an important tool leveraged for the detection of M. *tuberculosis*-specific CD4 T-cell responses in humans and in animal models. Genome-wide analysis of M. *tuberculosis*-specific CD4 T-cell epitopes in LTBI individuals revealed three broadly immunodominant antigenic islands related to bacterial secretion systems recognized by IFN-γ secreting CD4 T cells (306). Animal models of TB also demonstrate a key role for IFN-γ in immunity against M. *tuberculosis* infection. Mice deficient in IFN-γ succumb to low-dose M. *tuberculosis* infection (307, 308). Correspondingly, mice lacking IL-12 are also unable to control M. *tuberculosis* infection (253, 309, 310). The antimycobacterial effects of IFN-γ in mouse models are broadly related to the induction of AMPs, iNOS, and cytokines that activate infected macrophages to restrict intracellular bacterial replication, though other mechanisms underlying IFN-γ-mediated immunity to M. *tuberculosis* infection are still being elucidated. IL-10-deficient mice are less susceptible to M. *tuberculosis* infection due to an enhanced Th1 response (311), suggesting that IL-10 limits Th1 immunity during M. *tuberculosis* infection. However, Th1 cells secreting IL-10 can also impair host control of M. *tuberculosis* infection (312), and CD4 T cells producing both IFN-γ and IL-10 are detected in the BAL of ATB patients (313). Given that IL-10 secretion by Th1 cells has been shown to be a result of high antigen dose (314), it is possible that adaptive immunity at stages of infection when bacterial burden is high may be compromised by T cell-derived IL-10. T-bet is a member of the T-box family of transcription factors that is encoded by *Tbx21* and is the master transcriptional regulator for lineage commitment to the Th1 subset (315). Interestingly, adoptive transfer of T-bet knockout ESAT-6-specific T-cell receptor-transgenic CD4 T cells skewed toward Th1 *in vitro* retains the capacity for early protection against M. *tuberculosis* infection (316), suggesting that protection conferred by Th1 cells may be independent of T-bet or IFN-γ production. Taken together, these studies demonstrate a clear requirement for the IL-12/IFN-γ axis in immunity against M. *tuberculosis* infection in humans and animal models. Further studies delineating the mechanisms underlying IFN-γ- and Th1-mediated immunity against M. *tuberculosis* are warranted.

Although Th1 responses are important for immunity against TB, studies have also demonstrated that CD4 T-cell subsets secreting IL-17 (Th17) and FoxP3+ regulatory CD4 T cells contribute to the response against M. *tuberculosis* infection. There are context-dependent beneficial or

detrimental roles for Th17s during infection with *M. tuberculosis*. Infection with a W-Beijing lineage strain of *M. tuberculosis*, HN878, induce Th17 responses, and mice deficient in IL-17 display increased bacterial burden following infection (317). IL-17 receptor A subunit knockout mice (318) and IL-17A knockout mice (319) also displayed impaired long-term control of high-dose infection with H37Rv. Transfer of BCG-specific, IFN-γ knockout Th17 cells into *M. tuberculosis* infected, T cell-deficient mice conferred enhanced protection and prolonged survival compared to transfer of naive IFN-γ knockout CD4 T cells (320), suggesting that Th17 cells can mediate protection independently of IFN-γ. In humans, significant frequencies of IL-17-producing CD4 T cells were found in the peripheral blood mononuclear cells and BAL of BCG-vaccinated healthy individuals and declined in patients with active disease (321). Further, individuals with bi-allelic *RORC* loss-of-function mutations displayed impaired IL-17 and IFN-γ responses and were susceptible to mycobacterial disease and candidiasis (322). The generation of Th17 responses to *M. tuberculosis in vitro* requires costimulation through the CD40-CD40L pathway since the absence of CD40 on DCs or CD40L on CD4 T cells attenuates antigen-specific IL-17 responses (268). Activation of *M. tuberculosis*-infected DCs through CD40 promoted enhanced antigen-specific Th1 and Th17 responses that contributed to better control of bacterial burden *in vivo* (268), suggesting that a balanced Th1 and Th17 response is desirable for immunity against *M. tuberculosis*. The precise role of Th17 cells in protective immunity to *M. tuberculosis* remains unclear but may be related to their role in the development of less hypoxic granulomas (323), in the recruitment of Th1 cells (324), or in the induction of CXC-chemokines and B-cell follicles (325). However, unrestrained IL-17 responses have also been shown to promote detrimental immunopathology, typically through pathological neutrophilia. IFN-γR1 knockout animals (326) or IFN-γR1 bone marrow chimeric mice selectively lacking the receptor in nonhematopoietic cells (327) display amplified Th17 responses following *M. tuberculosis* infection that lead to a pathogenic accumulation of neutrophils detrimental to the host, suggesting that IFN-γ signaling serves a regulatory role by limiting excessive IL-17-mediated neutrophilia.

FoxP3+ CD4 T cells, or T-regulatory cells (T-regs), can impair antimycobacterial T-cell responses and contribute to disease but can also limit overt inflammation. FoxP3+ T-regs can be found in the peripheral blood and airways of *M. tuberculosis*-infected macaques (328) and humans (329–334). In mice, T-regs accumulate in the lung-draining lymph nodes and the lungs following low-dose aerosol *M. tuberculosis* infection (335). Importantly, FoxP3+ T-regs localized to pulmonary areas adjacent to effector CD4 T cells and depletion of T-regs before and early after infection-enhanced control of bacterial burden (335). Further, *M. tuberculosis*-specific T-regs delay the expansion of antimycobacterial CD4 and CD8 T cells and, consequently, transfer of *M. tuberculosis*-specific T-regs confers increased susceptibility to infection (336). Regulation of T-regs during *M. tuberculosis* infection may be mediated by Th1 responses since *M. tuberculosis*-specific T-regs are selectively eliminated following IL-12 driven T-bet expression (337). The functional properties of T-regs responsible for limiting antimycobacterial CD4 and CD8 responses remains unclear. IL-10 was not found to be secreted by T-regs in mice infected with H37Rv (335). In contrast, T-regs from mice infected with the W-Beijing strain, HN878, were found to secrete IL-10, express inhibitory receptors, and expand to greater degrees compared to infection with H37Rv (209), suggesting that IL-10 secretion by T-regs may be dependent on bacterial strain. Notably, the expansion of T-regs in the lungs of mice and outbred guinea pigs infected with W-Beijing strains occurred concurrently with a loss of Th1 responses and is associated with severe pulmonary pathology (209, 338). However, progressive loss of T-regs in chronically infected TLR-2 knockout mice was associated with increased pulmonary inflammation (339), highlighting a role for TLR-2-mediated recruitment of T-regs in limiting tissue pathology at chronic stages of disease. Taken together, these results suggest that the functional contribution of T-regs to immunity against *M. tuberculosis* infection and outcome of disease may be dependent on multiple factors, including strain of bacteria and stage of infection.

In humans and animal models, *M. tuberculosis* establishes a persistent infection despite the induction of adaptive immune responses. Persistent inflammation and chronic antigen exposure precedes functional exhaustion due to chronic antigenic stimulation. In contrast to the expression of Ag85B, which decreases early following infection, ESAT-6 is expressed by *M. tuberculosis* throughout infection (340, 341). Multiple studies examining CD4 T-cell responses to ESAT-6 and Ag85B have suggested that antigen-specific responses are dictated by bacterial expression of those antigens throughout infection. CD4 T cells specific for ESAT-6 display a terminally differentiated phenotype with evidence for functional exhaustion, which runs in contrast to Ag85B-specific CD4 T cells that appear functional but are quickly diminished (272, 342–347). Indeed, a vaccine that contains ESAT-6, Ag85B, and Rv2660c, which is expressed at late stages of infection, demonstrated enhanced efficacy compared to BCG or to a vaccine containing ESAT-6 and Ag85B (348), suggesting that rational incorporation of antigens present at different stages of infection may improve vaccine efficacy. A clearer understanding of protective CD4 T-cell immunity will require further studies of the spectrum of antigens recognized by CD4 T cells following infection with *M. tuberculosis* in animal models and in humans.

Role of CD8 T Cells in *M. tuberculosis* Infection

Mice with gene deletion of β2 microglobulin, which abrogates MHC class I antigen presentation, or mice depleted of CD8 T cells live longer than corresponding disruptions to the MHC class II pathway or CD4 T-cell responses following *M. tuberculosis* infection (270). Regardless, CD8 T cells contribute significantly to immunity against *M. tuberculosis* infection. Mice lacking TAP-1 (transporter associated with antigen processing 1) antigen presentation molecules have deficient CD8 T-cell responses and succumb more rapidly following *M. tuberculosis* infection compared to wild-type controls (349, 350). Depletion of CD8 T cells in rhesus macaques compromises protective immunity from BCG vaccination or chemotherapeutic interventions (57), suggesting that CD8 T cells are important components of recall responses to *M. tuberculosis* infection. Similarly, in a mouse model of latency induced by antibiotic treatment, CD8 T-cell responses were found to be important in preventing reactivation (351). The importance of CD8 T cells during *M. tuberculosis* infection is related to their secretion of cytokines and cytolytic effector molecules that can limit bacterial replication. In addition to IFN-γ and TNF-α, CD8 T cells secrete perforin to lyse *M. tuberculosis*-infected macrophages (352). CD8 T cells can also release granulysin in cytotoxic granules to directly kill intracellular *M. tuberculosis* (353, 354). The use of anti-TNF-α therapy in patients with rheumatoid arthritis depletes a subset of effector memory

CD8 T cells that secrete granulysin and express cell surface TNF (355), which may partially explain the increased progression from LTBI to ATB in patients undergoing anti-TNF-α therapy. Human CD8 T cells respond to epitopes in CFP10 (356), ESAT-6 (357, 358), and the Ag85 complex (359, 360). A variety of human CD8 T-cell clones tested against a panel of synthetic peptides derived from immunodominant *M. tuberculosis* antigens revealed that CD8 T-cell responses are concentrated toward a limited set of epitopes and are generally restricted by the HLA-B allele (361, 362). *M. tuberculosis* escape from the phagosome and induction of apoptosis by *M. tuberculosis*-infected macrophages can promote cross-presentation of *M. tuberculosis* antigens to CD8 T cells. However, as previously discussed, virulent *M. tuberculosis* has been shown to inhibit host apoptosis and favor necrosis to circumvent efficient induction of CD8 T-cell responses. All considered, CD8 T cells are a critical component of adaptive immunity to *M. tuberculosis* infection and play an important role in different disease contexts by limiting reactivation during latency and by directly participating in antimicrobial functions during active infections.

Inhibitory Receptors During *M. tuberculosis* Infection

Chronic viral infections, such as HIV, induce the expression of coinhibitory receptors on the surface of T cells that can dampen T-cell functionality. Abrogation of coinhibitory receptor ligation has been shown to be a viable strategy to revitalize functionally exhausted virus-specific T-cell responses. The evidence for the importance of coinhibitory receptors during *M. tuberculosis* infection in animal models and in human samples varies between human and small animal models and between the specific inhibitory receptor studied. PD-1, CD160, and 2B4 are inhibitory receptors associated with CD8 T-cell dysfunction in chronic viral infections (363, 364) but are expressed at low levels on *M. tuberculosis*-specific CD8 T cells (365). Expression of inhibitory molecules, including PD-1 and CTLA-4, among *M. tuberculosis*-specific CD4 T cells has been shown to decrease following treatment (366, 367). Importantly, expression of PD-1 on antigen-specific CD4 T cells from LTBI was not associated with decreased effector functions, and these cells proved to be polyfunctional with respect to cytokine production upon antigen restimulation (368), suggesting that PD-1 may be an indicator of bacterial burden and CD4 T-cell activation rather than functional exhaustion. However, there is some *in vitro* evidence from human samples suggesting that blockade of PD-1/PD-L1 interaction can prevent *M. tuberculosis*-specific CD4 T-cell apoptosis (369) and enhance CD8 T-cell degranulation and antigen-specific IFN-γ responses from the peripheral blood mononuclear cells of a subset of high-responding ATB patients (370). There is evidence that T-cell responses during ATB disease are less polyfunctional and have limited proliferative capacity compared to LTBI individuals (371, 372), but whether this functional impairment is mediated by inhibitory receptors such as PD-1 remains unclear. PD-1-deficient mice infected with *M. tuberculosis* have increased bacterial burden, neutrophilic infiltration, overt inflammation, tissue necrosis, and diminished lifespan compared to wild-type mice (373), suggesting that PD-1 is required to prevent aberrant inflammation during *M. tuberculosis* infection. Further, adoptive transfer studies demonstrated that PD-1-expressing CD4 T cells are highly proliferative (342, 346), and CD4 T cells lacking PD-1 can drive pathology and mortality following *M. tuberculosis* infection (374), together suggesting that PD-1 may mark functional CD4 T cells with intrinsic capacity for immunoregulation. T-cell immunoglobulin and mucin domain-containing 3 (Tim-3) is another inhibitory receptor shown to play a role in mediating antimicrobial responses by binding to one of its ligands, galectin-9 (375), and inducing the production of IL-1β by human and murine macrophages infected with *M. tuberculosis* (375, 376). In contrast to PD-1, Tim-3-deficient mice were less susceptible to *M. tuberculosis* infection, and Tim-3 blockade was shown to improve antigen-specific CD4 and CD8 T-cell cytokine expression (377), suggesting that Tim-3 may play a role in limiting T-cell responses by promoting functional exhaustion. However, Tim-3-expressing, *M. tuberculosis*-specific T cells from ATB patients were functionally superior to T cells expressing low levels of Tim-3. Further, small interfering RNA- or antibody-mediated disruption of Tim-3 signaling on the T cells from ATB patients led to attenuated IFN-γ and TNF-α production by Tim-3-expressing T cells, while Tim-3 ligation augmented IFN-γ production (378). The mechanisms underlying the roles of receptors such as PD-1 and Tim-3 require further study and may deviate from their role in viral immunity. The evidence accumulated thus far suggests that these molecules mark functional T cells that play important roles in antimicrobial activity and prevention of uncontrolled inflammation following *M. tuberculosis* infection.

Memory T-Cell Responses

In humans, antigen-specific memory T-cell responses have been detected in individuals with LTBI and in TB patients following successful treatment and cure. Memory T-cell subsets can be identified according to their cell surface phenotype and functional properties, and distinct populations of antigen-specific memory T cells can be categorized based on their expression of a panel of cell surface activation markers and chemokine receptors (379). Characterization of *M. tuberculosis*-specific memory CD4 T cells in LTBI indicated that these cells did not express activation markers and were largely of a CD45RA-CCR7 phenotype descriptive of T effector memory cells (368, 380). In contrast, analysis of LTBI individuals using MHC class II tetramers revealed a population of tetramer+CD45RA-CCR7+ central memory CD4 T cells that further expressed CXCR3+CCR6+ (306), highlighting the heterogeneity of memory CD4 T-cell phenotypes that can vary based on antigen specificity, disease status, and manner in which specific responses are identified. Human memory CD8 T cells are predominantly terminally differentiated effector memory T cells in individuals with LTBI (365, 381). Memory T-cell responses have also been studied in the context of "memory-immune" mice, which are *M. tuberculosis*-infected mice that subsequently receive antibiotic treatment. In this context, both memory CD4 (382, 383) and CD8 (384, 385) T cells play a role in immunity against *M. tuberculosis* infection. T cells from memory-immune mice expanded rapidly, secreted IFN-γ, and conferred a significant level of protection at early timepoints after infection (383, 386–388) but are ultimately unable to confer long-term protection (389), suggesting that memory T cells generated after primary *M. tuberculosis* infection have limited capacity to protect from reinfection.

B-Cell and Antibody Responses During *M. tuberculosis* Infection

There is a body of evidence suggesting that humoral immunity plays a role in defense against *M. tuberculosis* infection (reviewed in 390). B cells can be found alongside T cells in

the lymphocytic cuff in human granulomas (391–393), and whole blood gene expression analysis revealed significant changes in B-cell-associated genes in TB patients after initiation of TB treatment (394). Notably, antibodies to *M. tuberculosis* proteins have been reported in the sera of TB patients (395), and antibodies identified in a subset of health care workers exposed to *M. tuberculosis* provide modest protection *in vitro* and in a mouse model of infection (396). Utilization of a high-throughput approach to identifying antibody targets in the *M. tuberculosis* proteome revealed a set of extracellular antigens recognized by antibodies in the plasma of patients with ATB (397), suggesting that B cells are active participants in immunity to *M. tuberculosis* infection. B-cell-deficient mice have elevated neutrophilic recruitment and exacerbated lung immunopathology following *M. tuberculosis* infection (398), which is mediated through enhanced IL-17 responses in *M. tuberculosis*-infected B-cell-deficient or B-cell-depleted animals (399). These studies suggest that B cells can influence the outcome of *M. tuberculosis* infection by moderating inflammatory responses. Antibody production by B cells can promote divergent outcomes (400). Binding of antibody to the inhibitory Fc gamma receptor II B attenuates macrophage IL-12 production and negatively impacts Th1 responses (401), while passive transfer of monoclonal antibodies specific for *M. tuberculosis* cell wall components can improve the outcome of infection in mice (390). B-cell secretion of cytokines can also influence *M. tuberculosis*-infected macrophages. Type I IFN expression by murine B cells and B cells from pleural effusion of TB patients altered macrophage polarization toward an anti-inflammatory phenotype (402). Taken together, these studies highlight a role for B cells, which constitute a significant population of lymphocytes around lung granulomas in the adaptive immune response to *M. tuberculosis* infection by modulating inflammation through the secretion of antibodies and cytokines.

γδ, CD1-Restricted T Cells, and MAIT Cells in Immunity against *M. tuberculosis*

γδ T cells are a population of T cells that express a restricted repertoire of T-cell receptor genes, recognize nonpeptide antigens such as microbial metabolites and phosphoantigens (403), and can be found at mucosal surfaces including the lung (404). γδ T cells proliferate when exposed to *M. tuberculosis*-infected monocytes (405). Multiple *M. tuberculosis* metabolites, including pyrophosphate, prenyl pyrophosphate derivatives (406, 407), and triphosphorylated thymidine-containing compounds (408), are recognized by human γδ T cells. Human γδ T cells can also respond to mycobacterial heat shock proteins (409), though this response may be dependent on BCG immunization (410). Vγ9Vδ2-expressing γδ T cells represent a significant proportion of *M. tuberculosis*-reactive T cells in peripheral blood (411–413) and can restrict intracellular *M. tuberculosis* replication in macrophages (414). Interestingly, Vγ9Vδ2 T cells can function as antigen presentation cells via provision of CD40 costimulation to promote the expansion of αβ T cells with enhanced capacity to restrict intracellular BCG replication (415). Additionally, human Vγ2Vδ2 T cells recognize *M. tuberculosis* (416), and in NHPs, Vγ2Vδ2 T cells are expanded by phosphoantigen and IL-2 administration (417). Adoptively transferred Vγ2Vδ2 T cells into naive animals confer protection against *M. tuberculosis* infection (418). γδ T cells have been shown to mediate direct killing of *M. tuberculosis* via secretion of granulysin and perforin (419) or through the induction of TNF-α by monocytes (420). There is also evidence that γδ T cells can influence DC cross talk with T cells by promoting DC maturation and expression of costimulatory molecules (421). In mice, γδ T cells accumulate in the lung-draining lymph nodes, are responsive to *M. tuberculosis* antigen independent of MHC class II (422), and are significant sources of early IL-17 production following *M. tuberculosis* infection (423).

Due to the large repertoire of glycolipids present on the mycobacterial cell wall, a significant T-cell response is directed at glycolipid antigens presented by the CD1 family of molecules. CD1 molecules are a family of MHC class I-like antigen presentation molecules that present glycolipid antigens to T cells. There are five CD1 family members in humans, split into two groups based on sequence homology. Group 1 molecules include CD1a, CD1b, CD1c, and CD1e. CD1d is the sole inclusion in group 2 (424). Mycobacterial lipids are readily presented by CD1 molecules in human cells, but mechanistic studies of this family of molecules is limited because mice only express two orthologs of CD1d and do not express group 1 molecules. Nevertheless, studies in human cells revealed that mycobacterial lipids presented by group 1 CD1 molecules promote T-cell proliferation and cytokine production (425–432). Mycobacterial glycerol monomycolate, glucose monomycolate, sulphoglycolipids, and mycolic acid can be presented through CD1b (426–428, 433). CD1b-restricted T cells expand and secrete IFN-γ and IL-2 upon interaction with cognate antigen and contract following anti-TB therapy (430). Interestingly, use of CD1b tetramers loaded with glucose monomycolate revealed that CD1b-restricted T cells are antigen-specific and also express CD4 (429, 434). *M. tuberculosis* lipids presented through CD1a and CD1c have also been identified. A family of *M. tuberculosis* lipopeptides called didehydroxymycobactins are presented by CD1a (435), and a variety of phospholipid antigens are presented by CD1c (425). The precise role of CD1-restricted T cells in immunity during *M. tuberculosis* infection remains unclear, and further studies of their function in the periphery and especially in BAL would inform their potential as targets for TB vaccines.

MAIT cells are a subset of T cells with innate-like qualities enriched in mucosal tissues, including the intestinal mucosa, lung, and liver (436–438). These cells recognize antigen through a nonpolymorphic MHC class I-related molecule 1 (439) presenting pterin-containing byproducts of riboflavin synthesis in bacteria and fungi (440). In humans, MAIT cells express a semi-invariant Vα7.2 and CD161 and can either be double negative for CD4 and CD8 or CD4-CD8+ (436, 441). MAIT cells have been described in the peripheral blood of healthy individuals and are depleted in ATB patients (442), possibly reflecting migration into the lung. These cells produce IFN-γ and TNF-α upon activation (442, 443), but their contribution to the immune response to *M. tuberculosis* infection requires further study.

INITIATION AND HETEROGENEITY OF THE GRANULOMA

The granuloma is a hallmark histopathological structure in TB. It represents host sequestration of bacteria to limit dissemination as well as a niche for long-term persistence of *M. tuberculosis*. Further, the selectively drug-permeable nature of the TB granuloma can diminish the efficacy of drugs meant to treat persistent bacteria (444). The granuloma is composed of an aggregate of *M. tuberculosis*-infected and -uninfected macrophages in varying stages of maturation and differentiation (445–447). Macrophages in the granuloma can undergo an epithelioid transformation, become

lipid-filled foamy macrophages, or merge into multinucleated giant cells. This central core of macrophages is accompanied by neutrophils, DCs, and fibroblasts circumscribed by T and B lymphocytes and progressively becomes a hypoxic environment where many cells undergo necrotic death to form an acellular core termed the caseum (448). The granuloma is a hallmark structure in human TB that is modeled variably among available animal models. C57BL/6 and BALB/C mice do not naturally recapitulate the human granuloma in that lung lesions are rarely necrotic and caseating. The animal models that most closely recapitulate the heterogeneity of human granulomas include certain susceptible inbred mouse strains that present with necrotizing granulomas (C3HeB/FeJ, DBA/2, CBA/J, I/St), guinea pigs, rabbits, and the NHP model. Additionally, the zebrafish model has also yielded fundamental insights into the initiation and dynamics of the tuberculous granuloma.

The transparency of zebrafish larvae has made direct visualization of the initiation of the granuloma possible following infection with *M. marinum* (48). Studies based on this model have revealed that the innate immune response is sufficient to initiate the granuloma following infection. Recruitment of additional macrophages mediated, in part, by mycobacterial ESX-1 proteins initiates a cascade of events that leads to the establishment of the mycobacterial granuloma (221, 449). Importantly, recruited macrophages can traffic through the initial granuloma to phagocytose apoptotic infected macrophages and egress to form distal secondary granulomas (450). Mycobacterial lipids play a key role in establishing the granuloma by limiting macrophage effector functions and promoting the recruitment of additional macrophages to facilitate dissemination. In particular, mycobacterial phthiocerol dimycocerosate can mask TLR-signaling and prevent induction of nitrosative stresses (451), and mycobacterial PGL can induce macrophage production of CCL2 to recruit CCR2+ monocytes that permit bacterial dissemination (452). These studies collectively indicate that the initiation of the mycobacterial granuloma is dependent on recruitment of bacteria-permissive macrophages and monocytes following initial infection and can be mediated by mycobacterial secreted factors and membrane lipids.

TB granulomas can vary in their cellular composition, oxygenation levels, inflammatory milieu, and bacterial burden. This heterogeneity can exist between and within infected hosts. Infection of cynomolgus macaques with a panel of *M. tuberculosis* isolates that differed by a single nucleotide polymorphism revealed that individual granulomas can be founded by a single bacterium and can vary in their bacterial burden compared to other granulomas within the same host (51). Analysis of T-cell functionality between sterile and nonsterile granulomas revealed a modest association between IL-10 and IL-17 responses and clearance of *M. tuberculosis* in sterile granulomas (453). However, in the context of TNF-α neutralization in latently infected macaques, IL-10 and IL-17 responses were associated with animals at higher risk of reactivation (71). Proteome analysis of laser-capture microdissected human and rabbit lung lesions suggests that inflammatory responses typical of the center of the TB granuloma are physically segregated from anti-inflammatory responses in adjacent lung tissue (454). T-cell functionality in the granuloma may therefore be a function of disease status and proximity to the bacteria-containing, hypoxic, and necrotic core of the TB granuloma. Additionally, T cells near the granuloma can be negatively impacted by the depletion of key amino acids required for proper function. As mentioned previously, IDO, an enzyme that functions in the catabolism of tryptophan, is expressed by cells in the core of the granulomas of rhesus macaques infected with *M. tuberculosis* (283), and inhibition of IDO promoted granuloma reorganization and attenuated disease (66). The functionality of T cells within granulomas may also be regulated by direct cross talk with infected myeloid cells, including macrophages and DCs. Intravital imaging of mycobacteria-induced liver granulomas revealed limited antigen-specific T-cell migration arrest in response to infected myeloid cells (345), suggesting that T cells do not interact meaningfully with infected cells in granulomas. Taken together, these studies highlight the vast complexity and heterogeneity of the TB granuloma.

IMMUNOLOGY OF TB DIAGNOSTICS

TB diagnosis relies on evaluation of clinical symptoms and patient history combined with radiographic examination and detection of bacteria in sputum (9). The presence of acid-fast bacilli in sputum smears by microscopy does not specifically indicate infection with *M. tuberculosis*; microbiological culture and nucleic acid amplification-based tests are required to confirm the presence of *M. tuberculosis* infection. Xpert MTB/RIF, a cartridge-based near-patient diagnostic assay utilizing real-time nucleic acid amplification of *M. tuberculosis* DNA, which also detects drug resistance to the first-line drug rifampicin, is recommended by the World Health Organization for TB diagnosis (455, 456). IFN-γ release assays (IGRAs), which leverage the specificity of the immune response to *M. tuberculosis*, are the basis of the QuantiFERON-TB Gold In-Tube and T-SPOT.TB diagnostic assays. IGRAs measure IFN-γ produced by antigen-specific T cells in blood that recognize *M. tuberculosis* antigens (ESAT-6, CFP-10, TB7.7) (457). IGRAs provide increased specificity over traditional Mantoux skin tests that depend on delayed-type hypersensitivity reactions to purified protein derivative, which is not specific to *M. tuberculosis* infection, and positive results may be due to BCG vaccination or exposure to environmental mycobacteria. However, IGRAs do not differentiate between active and latent TB and cannot be used to diagnose TB disease. While sputum-based smear and culture techniques are established worldwide for clinical indication of *M. tuberculosis* infection, collection of sputum, especially from children, can be challenging and is not completely reliable. Therefore, there is interest in developing non-sputum-based diagnostic approaches for TB. Detection of urinary lipoarabinomannan in suspected TB cases is being investigated in HIV-infected (458) and -uninfected (459) individuals. Blood-based biomarkers discriminating between LTBI and ATB are being investigated for potential application to TB diagnostics and treatment response (460–462). HLA-DR, CD38, and Ki67 expression on *M. tuberculosis*-specific CD4 T cells from peripheral blood is reported to be a highly specific and sensitive method to discriminate LTBI and ATB and evaluate treatment response (460). A recent study suggests that HLA-DR could function as a robust marker distinguishing LTBI and ATB in HIV-infected populations (461). Further understanding of the spectrum of antigen-specific responses to *M. tuberculosis* infection can be leveraged to develop diagnostics that can monitor infection and treatment response.

TB VACCINES

The only currently licensed vaccine against TB is bacillus Calmette-Guérin (BCG), an attenuated strain of *M. bovis* (463, 464). BCG confers protection against severe forms of TB, including miliary TB and TB meningitis (465), but does

not reliably protect against pulmonary TB in children or adults (3, 4, 466). The lack of validated correlates of protection against TB is a severe limitation to TB vaccine development. Despite the importance of IFN-γ responses in resistance against *M. tuberculosis* infection in humans and animal models, accumulating evidence suggests that induction of enhanced IFN-γ responses is not sufficient to obtain a more efficacious TB vaccine. Indeed, the frequency and functional profile of BCG-specific CD4, CD8, and γδ T cells from whole blood, including IFN-γ-producing T cells, did not correlate with protection against TB in newborns (467). As of 2017, there are 14 TB vaccine candidates in varying phases of clinical development representing three broad strategies: subunit vaccines pairing *M. tuberculosis* antigens with adjuvants, viral-vectored vaccines utilizing an attenuated virus for antigen delivery, and whole-cell vaccines utilizing attenuated *M. tuberculosis* or related mycobacterial species. Protein subunit vaccines currently under clinical development include M72/AS01E (468), H4:IC31 (469), H56:IC31 (470), and ID93/GLA-SE (471). Among viral-vectored vaccines, results from the MVA85A phase IIb clinical trial have prompted reevaluation of immune correlates to aim for in a TB vaccine. MVA85A is a modified vaccinia Ankara virus expressing Ag85A from *M. tuberculosis* that was utilized as a booster vaccine in infants previously vaccinated with BCG (472). Notably, vaccination with MVA85A enhanced frequencies of antigen-specific, polyfunctional CD4 T cells co-expressing IFN-γ, IL-2, and TNF-α (472). Although MVA85A vaccination enhanced antigen-specific CD4 T-cell responses, it did not provide added protection against TB disease in infants (472). Other viral-vectored vaccines in various stages of development include Ad5Ag85A (473), ChAdOx1.85A + MVA85A (474), MVA85A-IMX313 (475), and TB/FLU-04L. Additionally, a recent study utilizing a recombinant cytomegalovirus demonstrated protection in rhesus macaques (476). While viral vectors do not require the use of adjuvants, previous exposure to the vector may attenuate vaccine-induced responses and represents a potential complication to the use of viral vectors. Whole-cell vaccines currently under development include killed *Mycobacterium vaccae*, DAR-901 (477), VPM1002, MTBVAC, and RUTI. VPM1002 is an approach to improve BCG immunogenicity and vaccine potential by engineering BCG to express lysteriolysin from *Listeria monocytogenes* to escape the phagosome and carry a urease deletion mutation that facilitates phagosomal acidification, thereby enhancing MHC class I antigen presentation to CD8 T cells (478). MTBVAC is a genetically attenuated *M. tuberculosis* strain lacking *phoP* and *fadD26* that abrogates synthesis of various surface lipids (479). Lastly, the therapeutic vaccine candidate RUTI was developed by growing *M. tuberculosis* under stress prior to fragmentation, detoxification, and delivery in liposomes to individuals with LTBI to prevent progression to ATB (480–482).

There have been substantial advances in our understanding of immunity against *M. tuberculosis* from the days of Drs. Calmette and Guérin. Nevertheless, the absence of suitable alternatives to BCG highlights the challenges before us. *M. tuberculosis* is adept at subverting the cross talk between innate and adaptive immunity, and it will be important to understand that cross talk for the rational development of better vaccines. Even in the absence of protective correlates and in the face of disappointing preliminary results for MVA85A, the state of TB vaccine development is resurgent now more than ever and provides cause for optimism for the development of more efficacious vaccines and therapeutics against TB.

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Unraveling the Structure of the Mycobacterial Envelope

MAMADOU DAFFÉ AND HEDIA MARRAKCHI

65

INTRODUCTION

The bacterial cell envelope, defined as the structure that surrounds the cytosol, is critical for bacterial physiology, because many crucial processes take place in this compartment. The cell wall contributes to the bacterial shape, mechanical resistance of the cells, and their protection against hostile environments. In addition, the envelope constituents are involved in cell division, transport of molecules (solutes and ions) and macromolecules (proteins), cell motility, adhesion, and protection, being in direct contact with the environment etc. The genus *Mycobacterium* belongs to the *Corynebacteriales* order, which also includes corynebacteria, nocardia, rhodococci, and other related microorganisms. Mycobacteria are probably the most successful microorganisms to parasitize animals and humans. Among the more than 200 valid species described to date in the genus *Mycobacterium*, only three are strict pathogens for humans: *Mycobacterium tuberculosis* (the Koch's bacillus), *M. leprae*, and *M. lepromatosis*. Tuberculosis still represents a major public health problem worldwide, remaining one of the leading causes of death from an infectious agent; about one-quarter of the world's population is infected by the Koch's bacillus and thus susceptible to develop the disease. In addition, two-thirds of mycobacteria species are opportunistic pathogens for humans, and all mycobacteria produce granulomatous lesions in experimental animals with large enough inoculum (1). Most mycobacterial pathogenic species grow very slowly (e.g., generation times of 24 hours and 13 days for the tubercle and leprosy bacilli, respectively), whereas saprophytes are relatively rapid growers (e.g., doubling time of 6 hours for the widely used genetically tractable *M. smegmatis*).

Mycobacteria and related genera are classified among the *Actinobacteria*, a division of Gram-positive bacteria, although they do not reliably retain the stain, a clear indication of the uniqueness of their cell envelope composition (2). Dissection of their fascinating coat reveals an atypical architecture of their envelope, different from those of both Gram-positive and Gram-negative bacteria (Fig. 1B). Unlike those of the former group of bacteria, with a reputed lipid-poor cell envelope (less than 10%), mycobacterial cell envelopes are extremely rich in lipids, representing up to 40% of their cell dry mass, more than the recorded 20% in Gram-negative bacteria (3). This high lipid content is in agreement with the huge number of genes devoted to the synthesis and regulation of this class of compounds (4). This hallmark would explain the tendency of mycobacteria to grow in clumps and their distinctive property of acid-fastness, but also their impermeability to nutrients and small molecules and their resistance to alkali, dilute acid, and infected mammalian cells (3).

Reviews have been recently published on the biogenesis (5), structures (6), and transport (2, 7, 8) of mycobacterial cell envelope constituents. This chapter highlights major advances in deciphering key biological and structural features of this unique and fascinating mycobacterial coat.

THE MICROSCOPIC APPEARANCE OF THE MYCOBACTERIAL CELL ENVELOPE

When studied by transmission electron microscopy (TEM), the mycobacterial cell envelope recurrently displays a multilayered structure (Fig. 1A). In TEM, the specimen is fixed prior to being embedded in a hydrophobic resin, followed by ultrathin sectioning and staining with heavy-metal salts. Consequently, fixation protocols and the use of different solvents can lead to extraction and shrinkage of its different constituents. Lipids noncovalently attached to cell wall polymers may be extracted by solvents during the dehydration step, and water-rich structures may collapse (9, 10). In ultrathin conventional sections (11), the cell envelope of mycobacteria looks more like that of Gram-positive bacteria (Fig. 1). It consists of a plasma membrane (PM), a thick electron-dense layer, an electron-transparent layer, and an outer layer of variable density and thickness (Fig. 1). The PM appears asymmetrical in images of some conventionally prepared cells, with the accumulation of material in its outer leaflet (12–14). This asymmetry, however, is dependent upon conditions of fixation (15). To circumvent these disadvantages, freeze-substitution techniques have been used (16, 17), but with little success, because the resulting images were diffuse and not resolved as expected (Fig. 1A).

More recently, cryo-electron microscopy of vitreous sections (CEMOVIS) and cryo-electron microscopy (cryoEM) of whole-mount cells have been applied to mycobacteria (Fig. 1B). In these techniques, the specimens are observed at liquid nitrogen temperature, which allows them to be imaged in their fully hydrated and unstained state. As expected, images observed for the mycobacterial cell envelope look significantly different from those seen by TEM (11, 18, 19). They revealed a symmetrical PM, a periplasm containing a granular layer presumably consisting of proteins, and for the first time, the presence of a long-predicted outer membrane (Fig. 1B). The existence of an outer membrane was supported

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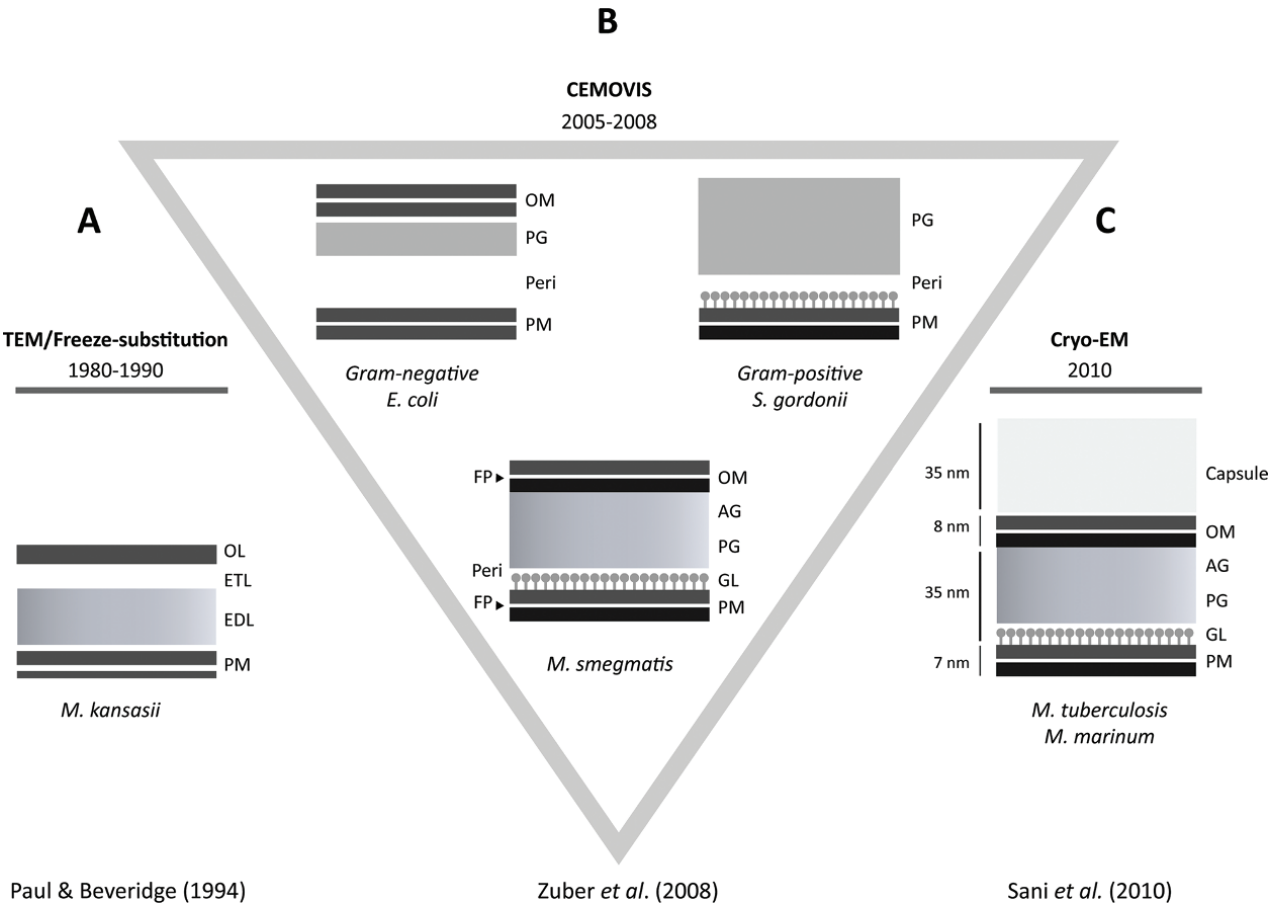


FIGURE 1 Diagrams (adapted from references 11 and 17–19) of the cell envelopes extracted from images obtained by (A) TEM, (B) CEMOVIS of various bacterial cells, and (C) cryoEM of whole-mount mycobacteria. AG, arabinogalactan; CEMOVIS, cryo-electron microscopy of vitreous sections; cryo-EM, cryo-electron microscopy; EDL, electron-dense layer; ETL, electron-transparent layer; FP, fracture plane; GL, granular layer; OL, outer layer; OM, outer membrane; PG, peptidoglycan; PM, plasma membrane; peri, periplasm; TEM, transmission electron microscopy.

by data from freeze-fracture electron microscopy, which clearly showed the occurrence of a major fracture plane in the outer part of the envelope, in addition to the expected plane that typifies the PM (Fig. 1B). However, no direct observations of the hypothetical outer membrane were available using TEM. The demonstration of the existence of an outer membrane fits nicely with the very low permeability of mycobacteria to nutrients and antibacterial drugs, 10- to 100-fold lower than that of the notably impermeable bacillus *Pseudomonas aeruginosa* (20). Consistently, mycobacteria and other members of the *Corynebacteriales* order contain pore-forming proteins (porins) in their cell walls (21).

During eukaryotic cell infection, a space between the wall of *M. lepraemurium* and the phagosomal membrane was observed in electron microscopic pictures, called the "capsular space" (22). Almost concomitantly, when studying the permeability of mycobacteria to dyes using light microscopic techniques, a tentative correlation was made between the capsule space and an unstainable halo observed only around some pathogenic mycobacteria (23–25). According to Hanks (26), this halo would also correspond to the "electron-transparent zone" that is seen in several published electron micrographs surrounding intracellular mycobacterial pathogens but not around nonpathogenic mycobacteria or pathogenic mycobacteria grown *in vitro* under standard conditions (27, 28). Consistently, a capsule-like structure was observed around *in vitro*-grown pathogenic mycobacteria that were first either coated with specific antibodies or preembedded in gelatin prior to the different drastic treatments involved in conventional electron microscopy studies (29, 30). Under these conditions, the capsule-like structure was not seen around the nonpathogenic *M. smegmatis* and *M. aurum* (30). Definitive proof of the existence of a capsule in pathogenic mycobacteria came from whole-mount plunge-frozen mycobacteria imaged by cryoEM (Fig. 1C), which does not require the use of a cryo-protectant and under conditions where they were grown without detergent or shaking (19). The layer was not observed in CEMOVIS pictures (Fig. 1B) (11, 18), probably because the density of the capsule matches that of the cryo-protectant-containing freezing solution. Likewise, the capsule was not observed in pictures of mycobacteria grown in detergent-containing culture media, which is routinely used to prevent mycobacterial cell clumping (19). When the same bacteria are instead processed by conventional methods commonly used to observe mycobacteria by TEM, the outer layer probably collapses by dehydration to give a dark layer (outer layer, Fig. 1A).

From the combination of chemical and microscopic data, it is now well established that the mycobacterial envelope consists of a PM surrounded by a complex cell wall composed

of peptidoglycan (PG) covalently linked to arabinogalactan (AG) esterified by mycolic acids (MAs), which form the inner leaflet of an outer membrane bilayer. MAs are very-long-chain, up to 100 carbons, 2-alkyl 3-hydroxylated fatty acids. An outermost layer, called "capsule" in the case of pathogenic species (9), in turn surrounds the cell wall (Fig. 1C). A periplasmic space, defined as the region located between the PM and the cell wall PG exists in mycobacteria, as revealed by CEMOVIS (Fig. 1B).

THE PLASMA MEMBRANE

The bilayer structure of the mycobacterial PM is similar to that of other Gram-positive and -negative microorganisms (Fig. 1), consistent with the published chemical composition, comprising phospholipids and proteins, with specific metabolic functions (9). Its asymmetrical appearance in images of some conventionally prepared cells (12, 15) has been attributed to the presence of excess glycoconjugates, e.g., phosphatidylinositol mannosides (PIMs), lipoarabinomannan (LAM), and lipomannan, in the thicker outer leaflet. Although glycoconjugates were identified in the PM (see below), their distribution between the two leaflets is not known. Alternatively, the observed apparent thicker outer leaflet in some conventional preparations may be due to the collapse of the granular layer, presumably composed of proteins, by a few nanometers beyond the outer leaflet of the PM (11), as shown by CEMOVIS (see Fig. 1).

The native PM is typically and easily obtained by breaking the cells by mechanical stress, e.g., sonication or shearing in the French pressure cell, followed by fractionation using differential centrifugation or density gradients (31, 32). The determination of specific enzyme activities, e.g., NADH oxidase (31), generally follows the purification process. Further chemical and biochemical analyses of the fractions are necessary to check their purity (31, 32). As far as lipid composition of the purified PM is concerned, no significant difference between those of fast- and slow-growing *Mycobacterium* species examined have been observed. Polar lipids, mainly phospholipids, assemble in association with proteins into a lipid bilayer (see 33). The polar lipids of the mycobacterial PM are composed of hydrophilic head groups and fatty acid chains that usually consist of mixtures of straight chain, unsaturated and mono-methyl branched fatty acid residues fewer than 20 carbons. Palmitic ($C_{16:0}$), oleic (cis $\Delta 9$ $C_{18:1}$), and 10-methyloctadecanoic (called tuberculostearic) acids are the major fatty acid constituents of the isolated PMs. The main phospholipids of the PM are PIM, phosphatidyl glycerol, cardiolipid, and phosphatidyl ethanolamine, while phosphatidyl inositol occurs in small amounts. Incidentally, PIM and phosphatidyl ethanolamine have also been identified among the lipids extracted from the mycobacterial cell surface by means of gentle mechanical treatment of cells with glass beads (34), yet the function of phospholipids in those layers is still unknown. Besides phospholipids, other lipids, such as menaquinones, may be present in the PM (see 33). The mycobacterial lipopolysaccharides LAM and lipomannan are partly located in the PM, and the relative contents of both lipopolysaccharides are growth-phase dependent (31, 35). The lipid composition of the PM of *M. smegmatis* also has been determined using detergent extraction, namely, with dioctyl sulfosuccinate sodium (36). The composition of the PM of *M. smegmatis* isolated by the latter method differs significantly from that obtained by breaking the cells by mechanical stress followed by fractionation using differential centrifugation or density gradients (31, 32). Notably, trehalose monomycolate (TMM), a lipid never reported in isolated mycobacterial PMs (9), was found in the PM using the detergent extraction method (36). Rather, TMM has been isolated from the cell surface of several mycobacterial species by mechanical treatment (34).

Bottom-up quantitative proteomics analysis allowed the identification of more than 2,000 membrane proteins in the purified PM of *M. smegmatis* (31). These include the canonical membrane ATP synthase, cytochrome P450, and ABC transporter YjfF, as well as enzymes involved in the biosynthesis of PIM, e.g., the mannosyltransferase PimB (37) and galactofuranosyltransferase GlfT2 (38). To assemble their cell wall components, which include PG, AG, lipids, and lipoproteins, as well as surface and outer membrane proteins (OMPs) (see below), mycobacteria must transport them or their precursors. Although little is known about the transport of these constituents from the cytosolic compartment to the exterior of the cell, mechanisms similar to those functioning in Gram-negative bacteria have been postulated (2, 7). Consistently, several transmembrane proteins such as MmpL (mycobacterial membrane protein large), involved in the transport of various lipids (2, 7), have been located, at least partly, in the PM (Fig. 2). Among the MmpLs of *M. tuberculosis*, MmpL3 is involved in the transport of TMM, and MmpL7 mediates the transport of phthiocerol dimycocerosate, whereas MmpL10 was reported to participate in the translocation of acylated trehaloses. All these lipids contribute to the integrity of the cell envelope, regulate membrane permeability, and have important physiological functions. The PM also contains the sophisticated secretion systems, named 6-kDa early secretory antigenic target (ESAT6) protein family secretion systems (also known as type VII secretion systems), used to export a set of effector proteins that help the pathogen resist or evade the host immune response (8).

THE CELL WALL

The cell wall of mycobacteria and related genera differs in composition from those of both Gram-positive and Gram-negative bacteria. It is a giant complex constituted of the outer membrane bilayer, AG, and PG. The mycobacterial outer membrane, also known as mycomembrane (MM), exhibits a 7- to 8-nm thickness (Fig. 1B), similar to that of the PM, and contains MAs. MAs form the inner leaflet of the MM, covalently linked to AG, which in turn is covalently attached to PG, a complex polymer classified as A1γ, as in *Escherichia coli* and *Bacillus* spp. This represents the so-called mAGP (mycoloyl-arabinogalactan-peptidoglycan), formerly known as the cell wall skeleton, defined as the material remaining after removal of all non-covalently bound wall-associated substances (39). The outer leaflet of the MM is presumably composed of various lipids (31).

Mycobacterial walls are readily isolated and purified from fragments of PM and cytosolic material by breaking the cells with mechanical stress, followed by purification using differential centrifugation or density gradients to remove unbroken cells (see 31, 32). The mycobacterial cell wall appears in conventional TEM (40) as an electron-dense layer surrounded by an electron-transparent layer (Fig. 1A). The occurrence of the latter in sections of *Corynebacteriales* devoid of MAs, e.g., *Corynebacterium amycolatum* (41), calls into question the long-assumed interpretation that the low density of the electron-transparent layer is attributed to lipids, notably MA.

The cell wall can be dissected into its constituent parts by relatively gentle methods so that each part may be studied separately (see 42–44). The PG is composed of repeating

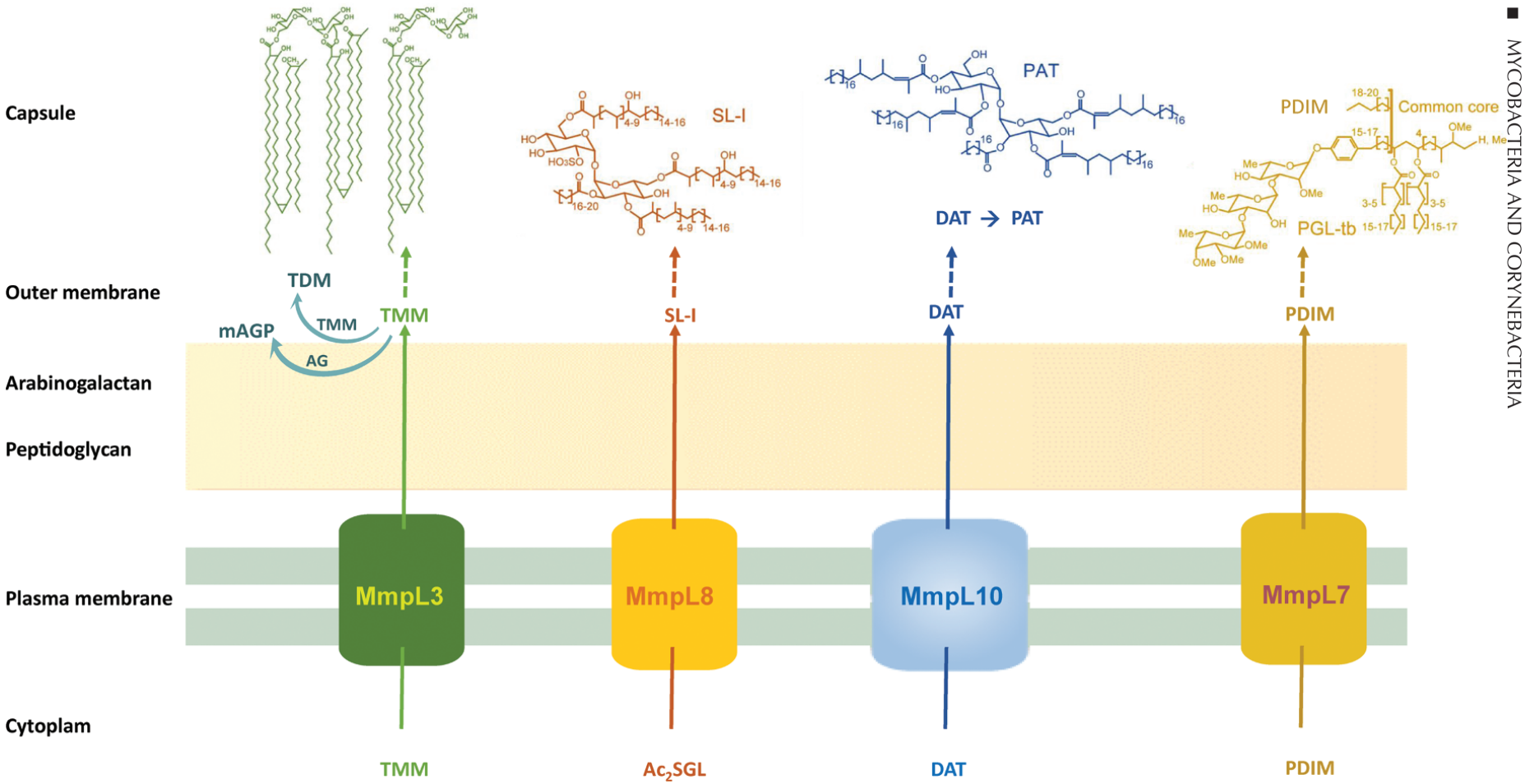


FIGURE 2 Chemical structures of representative mycobacterial lipids and their export systems through the plasma membrane to reach their final locations in the outer membrane (mycomembrane) or/and capsular compartment of the cell envelope. Some of the lipids are ubiquitous (e.g., TMM and trehalose dimycolate), whereas other are species- or type species-specific (e.g., PAT, phthiocerol dimycocerosate, PGL-tb), found in selective mycobacterial species or strains. TMM is used to transfer its mycoloyl residue onto both the arabinan termini of AG and TMM to yield the cell wall skeleton mAGP and trehalose dimycolate, respectively. Ac_2SGL, diacylated sulfoglycolipid; AG, arabinogalactan; DAT, diacyl trehalose; mAGP, mycoloyl-AG-peptidoglycan; mAGP, mycoloyl-AG-peptidoglycan; MmpL, mycobacterial membrane protein large; PAT, polyacyltrehalose; PDIM, phthiocerol dimycocerosate; PGL-tb, phenol glycolipid of *M. tuberculosis*; SL-I, sulfolipid I, the major sulfolipid of *M. tuberculosis*; TDM, trehalose dimycolate; TMM, trehalose monomycolate.

units of *N*-acetylglucosamine and *N*-acetyl/glycolylmuramic acid cross-linked with a peptide side chain that may be cross-linked to that of other glycan strands. The tetrapeptide side chains of PG consist of L-alanyl-D-isoglutaminyl-*meso*-diaminopimelyl-D-alanine. These peptide chains are heavily cross-linked, with up to 80% cross-linking, compared with 50% in *E. coli* (3). Interestingly, the PG of *M. leprae* has a glycine residue substituted for the L-alanine residue found in the PG of *Mycobacterium* spp. examined to date.

The AG is a branched heteropolysaccharide that contains a galactan chain composed of alternating 5- and 6-linked D-galactofuranosyl residues; three D-arabinan chains substituted the D-galactan chain in *M. tuberculosis*, of which MAs (45) esterify two-thirds of the nonreducing termini of the penta-arabinosyl motifs. In fact, MAs (Fig. 2) esterify the four-hydroxyl groups at position 5 of both terminal and 2-linked D-arabinofuranosyl of the penta-arabinosyl motifs of AG. The cell wall of the *in vivo*-grown leprosy bacillus (a yet *in vitro* noncultivable bacterium) was found to contain significantly more AG-MA attached to PG than that of *in vitro*-grown *M. tuberculosis*, and only half of these are occupied by mycoloyl residues (46).

In 1982, Minnikin proposed an original model of an outer membrane analogous to that found in Gram-negative bacteria. In this model, the very-long-chain MAs that esterified the AG arranged themselves to form the inner leaflet of an asymmetric outer membrane, the outer leaflet being constituted of free polar mycolate trehaloses (33). This suggested the existence of a second hydrophobic layer in addition to the PM and could explain the very low permeability to polar molecules. It could also provide a good explanation for the additional and main fracture plane observed in freeze fractures of mycobacteria (47–51): the fracture would occur between the two leaflets of the outer membrane (Fig. 1B). This model was later supported by experimental data (see 52), notably the work of Nikaido and his colleagues, who showed that an aqueous suspension of purified walls of *M. chelonae* produced an X-ray diffraction pattern with reflections characteristic of close-packed hydrocarbon chains in a semicrystalline monolayer (53). However, the complete lack of observation of this outer membrane bilayer in transmission electron micrographs of sectioned mycobacterial cells seriously called into question the validity of this model.

Using CEMOVIS, three research groups have independently clearly visualized a symmetrical 7- to 8-nm-thick bilayer whose thickness was comparable to that of PM in the mycobacterial species analyzed (11, 18, 19). The thickness of the outer membrane MM was even less in corynebacteria, around 4 to 5 nm (11, 18). The fact that the outer membrane contains MA was proved by the absence of this structure in a mycolate-less *Corynebacterium* mutant. LAM and lipomannan located in the MM would presumably be associated with its outer leaflet (Fig. 3), comparable to the location of LPS in Gram-negative organisms (31, 35). Among the lipids proposed to compose the outer leaflet of the MM in various models, TMM, trehalose dimycolate (Fig. 2), and phospholipids (cardiolipid/phosphatidyl glycerol, phosphatidyl ethanolamine, and phosphatidyl inositol, plus a few PIMs) have been clearly identified in the MM-containing fractions of *M. smegmatis* (31). The 7- to 8-nm thickness of the outer membrane layer contradicts the arrangement of MA, whose long alkyl chains would be organized parallel to one another to yield a >40-nm-thick asymmetrical bilayer (33). In view of the 7- to 8-nm thickness of the MM in the observed micrographs (Fig. 1B), the alkyl chains of MA have to be arranged in a folded manner (as shown for TMM and trehalose dimycolate in Fig. 2). Consistent with the published chemical structures and fold of mycobacterial MAs (54, 55), a model was proposed in which mycoloyl chains are intercalated in a zipper-like manner and the long mycoloyl chains are folded in an ω-shape (11).

Like Gram-negative bacteria, mycobacteria need to transport and assemble their outer membrane and to import small polar molecules across their outer membrane, notably those needed for nutrition. In Gram-negative bacteria, the transport of lipids, lipoproteins, and outer membrane proteins (OMPs) involves a combination of ATP-dependent transporters in the PM, chaperones in the periplasm, and β-barrel proteins that facilitate the insertion and translocation/folding into the outer membrane (2). The nutrition issue is solved by producing specialized proteins called porins, which form hydrophilic pores through the structure (Fig. 3). These pore-forming proteins have been identified in the walls of both fast-growing mycobacterial species, such as *M. chelonae* and *M. smegmatis* (21, 56, 57), and *M. tuberculosis* (58, 59). However, as Rv0899 (OmpA-Tb) adopts a mixed alpha/beta-structure and does not form a transmembrane beta-barrel (60), its status as a porin is a matter of debate (21). As expected, the crystal structure of the major OMP of *M. smegmatis*, MspA, reveals a β-barrel transmembrane channel (61). MspA forms an octameric composite β-barrel where each monomer comprises two β-strands. In Gram-negative bacteria, such as *E. coli*, there are about 60 OMPs. Using *in silico* analysis, two independent studies have proposed a list of putative OMPs in *M. tuberculosis* based essentially on β-barrel computational predictions (21). Out of 100 putative mycobacterial OMPs, only a few have been identified and characterized. These include mycoloyl transferases (originally called Fbp for fibronectin-binding proteins or antigen85) (63), some glycosylated proteins (Rpf proteins) (62), the outer membrane channel protein CpnT (63), and sphingomyelinase Rv0888 SpmT protein (64). Proteomic analysis confirmed the presence of Fbps and porins (MspA in the case of *M. smegmatis*) in the cell wall fraction, but also putative transporters, e.g., the mycobacterial cell entry protein family (31).

THE CAPSULE

The first mycobacterial capsules defined, namely, those of *M. leprae* and *M. lepraemurium*, were composed of lipids, but this turns out to be the exception. The fibrillar substance that surrounds *M. lepraemurium* inside the phagocytic vacuoles of the host cell (50) was composed of glycopeptidolipids (GPLs) (9), and massive amounts of phenolic glycolipids (PGLs) (Fig. 2) were isolated from cells infected with *M. leprae* (65, 66). However, both GPL-positive and GPL-negative strains of the *M. avium-M. intracellulare* complex elaborate capsules (67), indicating that the capsule is not always composed of lipids. Moreover, most strains of *M. tuberculosis* are devoid of PGL (68).

Knowing that *in vitro*-grown pathogenic mycobacterial species secrete abundant amounts of polysaccharides and proteins (69, 70), we postulated that the material shed into the culture medium of *in vitro*-grown mycobacteria is retained by the phagosomal membrane to form the electron-transparent zone seen around pathogenic species in phagocytic cells (9). Consistently, more extracellular material was recovered from the culture filtrates of the pathogenic species, e.g. *M. tuberculosis* (71) and *M. kansasii* (69), than from those of saprophytic and nonpathogenic strains such as *M. smegmatis* and *M. aurum* (69). Accordingly, the nature of the capsular constituents of various mycobacterial species were determined by analyzing the composition of their extracellular materials and those extracted by gentle shaking

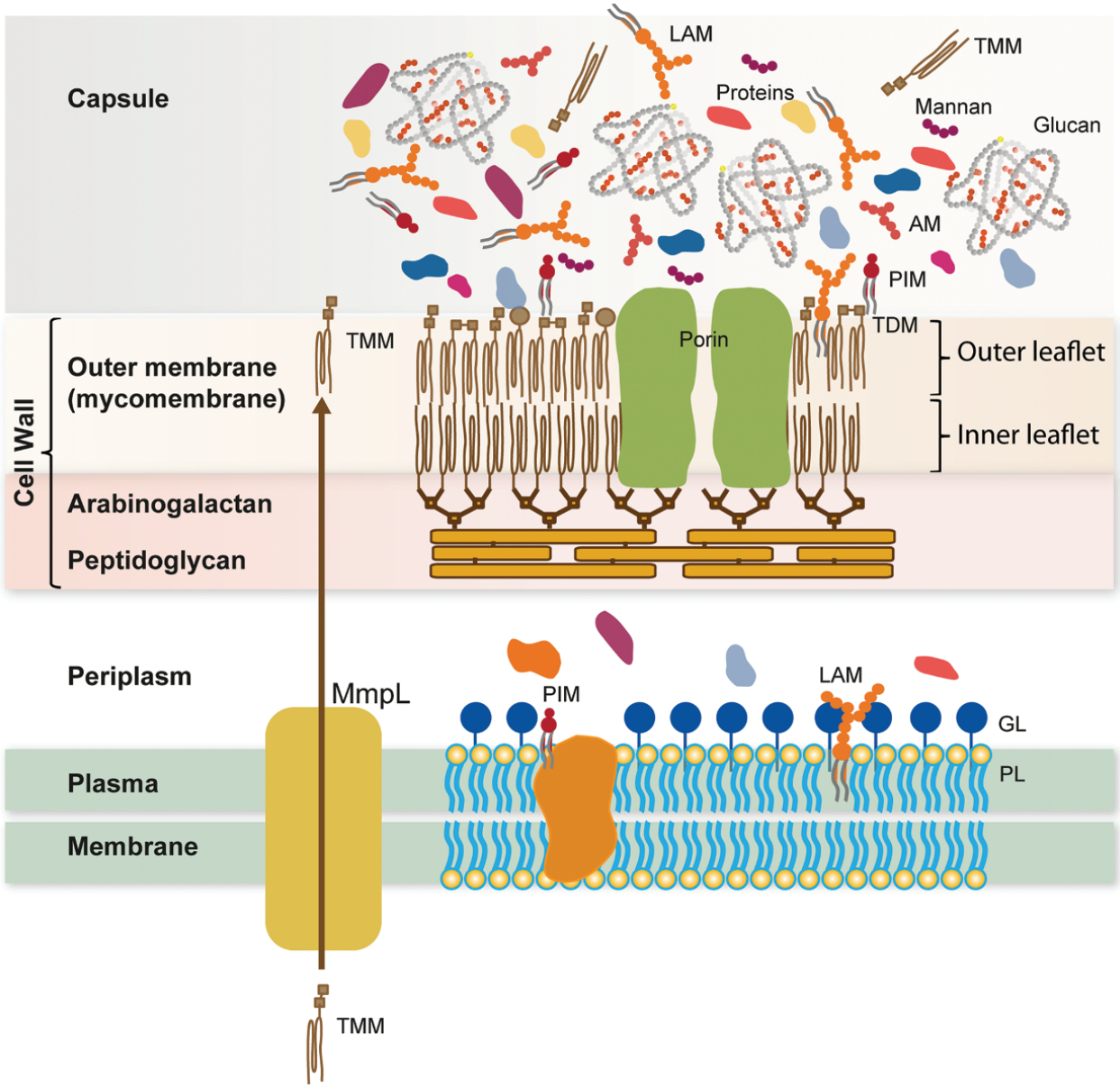


FIGURE 3 Model of the mycobacterial cell envelope. The cell envelope consists of a plasma membrane, a periplasm, the cell wall skeleton, and the outermost layer, called capsule in the case of pathogenic species. The plasma membrane is composed of phospholipids (e.g., PIM), lipopolysaccharides (e.g., LAM), and proteins, which include MmpL involved in the transport of lipids. The periplasmic space contains the GL and, presumably other proteins. The cell wall skeleton is made of peptidoglycan, arabinogalactan and mycolic acid residues that form the inner leaflet of the outer membrane (mycomembrane). The outer leaflet of the latter membrane is composed of various lipids, notably, trehalose mycolates. The capsular layer is a matrix of glucan that contains proteins, (lipo)polysaccharides, and small amounts of lipids. AM, arabinomannan; LAM, lipoAM; GL, granular layer; MmpL, mycobacterial membrane protein large; PIM, phosphatidyl inositol mannosides; PL, phospholipids; TDM, trehalose dimycolate; TMM, trehalose monomycolate.

of bacterial pellicles with glass beads and/or by detergent extraction for short periods of time (34, 69, 72). Importantly, these treatments, aiming at de-clumping the cells, do not affect their viability, as judged by scanning electron microscopy (34, 70).

The main components of the outermost capsular layer of slow-growing mycobacterial species, most of which are pathogens (e.g., *M. tuberculosis* and *M. kansasii*), are polysaccharides (69, 70), whereas the major components of the outer layer of rapid growers (e.g., *M. phlei* and *M. smegmatis*) are proteins (69). The major extracellular and capsular component of slow-growing mycobacterial species is a glucan (Fig. 3) that is composed of repeating units of five or six α-(1→4) linked D-glucosyl residues substituted at position 6 with mono- or oligoglucosyl residues (69, 70, 71, 73). The surface-exposed and extracellular materials also contain a D-arabino-D-mannan, a heteropolysaccharide that exhibits an apparent molecular mass of 13 kDa, and a mannan chain composed of an α–(1→6)-D-mannosyl core, with some units substituted with α-D-mannose at position 2. Importantly, glucan was identified in the capsule of *M. tuberculosis* inside the phagocytic vacuoles of the host cell (74).

The outermost part of the mycobacterial cell envelope contains only a tiny amount of lipid (2 to 3% of the surface-exposed material). Some of the species- and type-specific lipids and glycolipids, e.g., phthiocerol dimycocerosate, PGL, and GPL, can be found on the surface of the capsule, in agreement with serological and ultrastructural findings (see 9). Progressive extraction of the capsular material with glass beads and short-time treatment with Tween 80 detergent shows that most of the lipids are in the inner rather than in the outer part of the capsule (34).

The outermost capsular proteins of *in vitro*-grown *M. tuberculosis* are a complex mixture of polypeptides (70). Some proteins seem to correspond to secreted polypeptides found in short-term culture filtrates, whereas others appear to be cell wall associated (29). In fact, most of the hundreds of proteins identified in the short-term culture filtrate of *M. tuberculosis* by two-dimensional SDS-PAGE (75) are also found in significant amounts in the surface-exposed material extracted by mild mechanical treatment of cells. This observation supports the concept that the compounds found in culture filtrates of *in vitro*-grown cells are probably shed from the surface of the bacilli and, in an *in vivo* context, would be confined around the cells in the capsular layer (9). Components of the early secretory antigen transport system were recently localized to the capsule by immuno-electron microscopy of cryosectioned mycobacteria (19).

CONCLUDING REMARKS

The chemical structure of most of the core components of the mycobacterial cell envelope was determined a long time ago, establishing its extreme richness in lipid. However, it is only recently that a combination of CEMOVIS and whole-cell cryoEM has revealed the true structure of the mycobacterial cell envelope, i.e., the existence of a Gram-negative-like (diderm) outer membrane in these Gram-positive bacteria and an outermost capsular layer of protein and carbohydrate (Fig. 3). Determining the composition, localization, and supramolecular organization of the capsular and cell wall constituents and understanding how these factors make the envelope essential to *M. tuberculosis* survival, virulence, and pathogenesis remain major challenges. This will require synergistic expertise in analytical and structural biochemistry and biophysics to fill the gap in understanding the MM compartment by isolating and defining the full lipid and protein compositions of the capsule and cell wall. Deciphering the supramolecular organization of the capsule and MM-containing cell wall represent an additional exciting goal. The discovery of how mycobacteria select and organize their envelope constituents to resist killing should help decode the mechanisms involved in the mycobacterial pathogenicity and provide new potential targets for antituberculosis chemotherapy. In this respect, the biogenesis of MM constitutes a highly relevant and validated pharmaceutical target for antituberculosis drug discovery.

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The Dream of a Mycobacterium

CATHERINE BARANOWSKI, E. HESPER REGO AND ERIC J. RUBIN

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> A toutes ces activités, il faut une cohésion rigoureuse pour que puisse se réaliser le rêve de la bactérie: produire deux bactéries. [To all these activities, it takes a rigorous cohesion to realize the dream of the bacterium: produce two bacteria.]
>
> FRANÇOIS JACOB, 1965 (1)

Bacterial psychology is a challenging field. Like many psychologists, Jacob impressed the bacterium with our own value system. And part of that is defining what it is to be alive. As microbiologists, we generally define bacterial life as the ability to form progeny, to become two bacteria through cell division. Like all reproductive processes, bacterial division uses a combination of mechanics and mystery. Here, we will focus on the mechanics (and mystery) of cell division in acid-fast mycobacteria, a genus that includes the major human pathogens *Mycobacterium tuberculosis* and *Mycobacterium leprae*.

From the 1968 study in which Hirota, Ryter, and Jacob classified temperature-sensitive mutants blocked for various steps of cell division in *Escherichia coli* (2) to the discovery of the FtsZ ring (Z-ring) in 1991 by Bi and Lutkenhaus, microbiologists have launched into an era of genetic, microscopic, biochemical, and structural work to understand bacterial cell growth and division (3). (For a review on the history of cell division research see reference 4). This topic has been studied most in the model rod-shaped bacteria, the Gram-negative *E. coli* and the Gram-positive *Bacillus subtilis*. Work on cell growth and division in mycobacteria is not as extensive as in *E. coli* or *B. subtilis*. However, mycobacteria face the same challenge as these other organisms: organizing, both spatially and temporally, the factors that are responsible for making two cells from one. This process requires the concerted effort of many molecules to elongate the cell, replicate and separate the chromosome, build the septal cell wall that separates the two daughter cells, and separate the now distinct daughter cells. As you will see in this article, one common theme in the nascent field of mycobacterial cell division is that mycobacteria have often solved these challenges differently, sometimes vastly so, than either *E.coli* or *B. subtilis*.

We will take a comparative approach. Throughout this article, we will describe distinct stages of cell division in *B. subtilis* and follow with the current knowledge of this process in mycobacteria. Mycobacteria are quite different than *B. subtilis*, and for many described proteins in *B. subtilis*, mycobacteria either lack homologs or use unique factors and protein interactions to fill these voids.

Cell elongation and division are intimately associated processes that require both synthesis and remodeling of the complex mycobacterial cell wall. The mycobacterial cell wall consists of covalently linked layers of fatty acids (mycolic acid), a carbohydrate polymer known as arabinogalactan, and an innermost layer of peptidoglycan (PG) (5). The envelope of most other bacteria, like *B. subtilis*, is comprised of PG, but these other polymers are unique to mycobacteria and related bacteria. We favor a model whereby the synthesis and remodeling of the distinct layers is coordinated, but there is currently little direct evidence to support this model.

MYCOBACTERIAL GROWTH: A DIFFERENT BALLGAME

The first obvious difference between *B. subtilis* and mycobacteria is in the geometry of cell division. To increase in size, mycobacteria insert new material at or near their poles, rather than along their side walls (Fig. 1) like *B. subtilis* and *E. coli*. However, where exactly cell wall synthesis occurs is still unclear. Many cell wall synthetic enzymes localize to the peripolar region (6), but pulse chase experiments with dyes that covalently bind the cell wall suggest that new cell wall material is inserted at the very distal end of the cell (Fig. 2, bottom panel) (7). Supporting this last observation are labeling experiments with dyes that incorporate into different layers of the cell wall via enzymatic processes. These dyes brightly label the cell poles (8–10) (Fig. 2, top panel), as does a fluorescent analog of vancomycin (11, 12) which labels unprocessed, un-crosslinked PG. Thus, it seems most likely that mycobacteria insert a new cell wall at the distal ends of the poles. But how that is coordinated by enzymes that are most abundant at adjacent cellular regions will need to be reconciled in future research.

In general, polar growth leads to a different set of challenges not encountered by side-wall growers. First, the site of division in pole growers becomes the site of elongation. This means that division and elongation might need to be more tightly controlled temporally in mycobacteria than in *B. subtilis*, so that growth from the new poles does not occur before the cells completely divide. This raises a question: Are the components of the mycobacterial division complex (divisome) distinct from those of the elongation machinery (elongasome)? Throughout this review, you will see that mycobacterial cells blocked in certain points of division are elongated and form ectopic poles, suggesting that division and elongation can be uncoupled. This does not necessarily mean that the divisome and elongasome contain mutually

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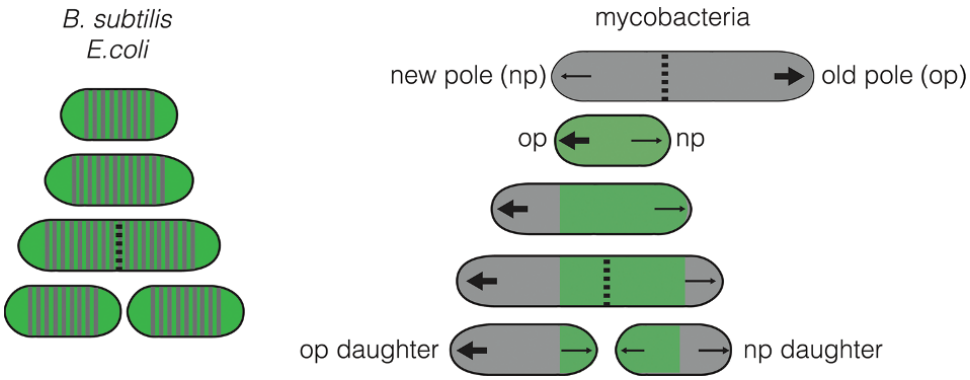


FIGURE 1 Characteristics of growth and division in *B. subtilis* and mycobacteria. *B. subtilis* and *E. coli* grow by adding new cell wall (gray) along the lateral cell body. Mycobacteria grow only at the polar regions, and do so at unequal amounts depending on the identity of the pole. This is observed by using a cell wall dye (green) to stain the existing cell wall and observe outgrowth of the newly synthesized, unstained cell wall (7). Arrows, polar location of new cell wall synthesis (a large arrow indicates more growth); dotted line, septum; green portion, old cell wall; gray portion, new cell wall.

exclusive proteins; however, it does support a model where division and elongation are temporally, and potentially spatially, separate. For example, there are localization, phenotypic, and protein-protein interaction data that place PonA1, a cell wall synthase, in both the elongasome and divisome. However, we lack a precise understanding of when the divisome becomes the elongasome at the new growth pole. Therefore, assigning proteins to either the divisome or elongasome based on localization at a single time point could be misleading, because factors involved in elongation also localize to the site of division/new pole and may belong to both machineries.

Second, polar growth leads to the spatial segregation of the cell wall with different ages, while lateral cell growth leads to the mixing of new and old material (7, 10, 13) (Fig. 2). In *Mycobacterium smegmatis*, it has been shown that the daughter cell inheriting the pole created at the most recent division, the so-called new pole, inherits the oldest cell wall (7) (Fig. 2, bottom panel). The functional consequence of this remains unknown, but it may contribute to the distinct characteristics of the daughter cells.

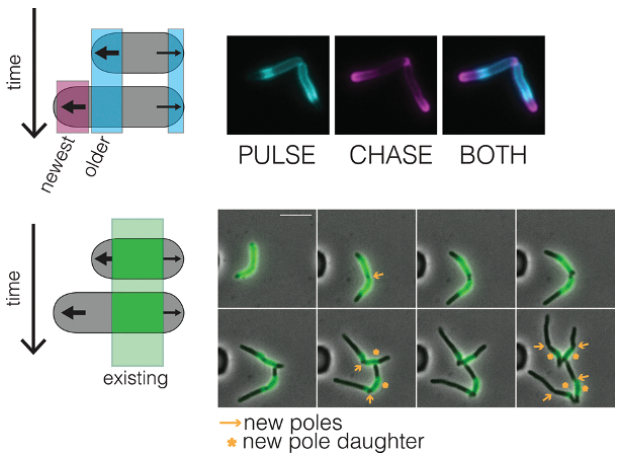


FIGURE 2 Polar growth segregates the cell wall based on age. (Top) Fluorescent D-amino acids are thought to incorporate into nascent PG. Pulse chase with these shows how the new and old cell walls are spatially segregated in *M. smegmatis* (Baranowski C, Rego EH, and Rubin EJ, unpublished images). (Bottom) Alexa-488 NHS ester stains the existing cell wall (green). New cell wall is unstained and can be monitored using time-lapse microscopy. After two divisions, the oldest cell wall is inherited by the new pole daughter cells (*) in *M. smegmatis*. (Baranowski, Rego, and Rubin. unpublished images; 7).

Lastly, the manner by which mycobacteria maintain their rod shape in the face of turgor pressure must be completely different than in the model rod-shaped organisms *B. subtilis* and *E. coli*. In *B. subtilis*, MreB, a homolog of eukaryotic actin, dictates the location of new cell wall synthesis along the side wall, incorporating new material at sites of negative curvature to aid in rod shape maintenance as the cell grows (14). This rod shape propagates in an MreB-dependent manner along the greatest membrane curvature (circumferentially around the cell) (15). Not only do mycobacteria lack an obvious homolog of MreB, but they grow from a much smaller portion of the cell (11). It remains a mystery how cell shape can be maintained across the entire cell. However, we do have a few clues. Mycobacteria encode a homolog of *B. subtilis* DivIVA called Wag31. In *B. subtilis*, depletion of DivIVA results in elongated cells that cannot divide, indicating a role in division (16). In contrast, depletion of Wag31 in mycobacteria leads to cells that lose their rod shape first at the pole, and eventually throughout the entire cell, resulting in a spherical cell. Further, dysregulation of Wag31 results in polar growth from incorrect sites (6). Thus, it appears that Wag31 in mycobacteria works to establish the site of polar growth, unlike its counterpart in *B. subtilis*, which has a role in dictating septum placement. Additionally, depletion of enzymes responsible for synthesizing or remodeling PG results in loss of rod shape either at the cell poles (17, 18) or along the mid-cell (6). Thus, it may be that Wag31 is responsible for directing formation of the poles and that the structure and rigidity of the cell wall itself, established at the time of cell wall synthesis at the pole, aids in shape maintenance as the cell grows.

THE MYSTERY AND MECHANICS OF DIVISION

Once a cell grows, it must divide to make two cells. The factors involved in cell division are referred to collectively as

the divisome, and they work in concert at the mid-cell. The purpose of the divisome is to (i) identify the mid-cell for Z-ring placement; (ii) recruit Z-ring anchors, stabilizers, and regulators; (iii) build the septal cell wall; and (iv) split the two halves of the partitioned cell. The bacterial chromosome must be replicated and segregated in concert with cell division; however, this process will not be covered in this review.

Identify the Mid-Cell for Z-Ring Placement

FtsZ, a tubulin homolog, polymerizes into a ring (Z-ring) at the mid-cell to begin cell division (3; reviewed in 19). The mid-cell placement and stabilization of the Z-ring are the critical first stages of bacterial cell division. Recent work using metabolic labeling of PG, the crucial innermost cell wall layer, as well as fluorescent fusions to FtsZ and other well-characterized septal proteins in *B. subtilis* shows that FtsZ treadmilling directs septal synthesis in an inward-spiral fashion (you can also imagine a bullseye) (20). Thus, the Z-ring is the figurative and literal ringleader of bacterial cell division. Once placed, the Z-ring recruits a cascade of factors, both structural and enzymatic, required for synthesizing and splitting the septum (which becomes the new cell poles) to create two daughter cells (for review of FtsZ see references 19 and 21).

An (a)side on (a)symmetry

B. subtilis grows along the lateral cell body and divides at the mid-cell with striking precision (22), resulting in two nearly identical daughter cells. However, many observers (though not all) have observed that, on average, mycobacteria grow asymmetrically, with growth occurring in unequal amounts from the two poles (5). Multiple reports show that the old pole continues to grow during and after septation but that the new pole—the pole established at septation—takes some time before it grows. Thus, the overall amount of elongation from the poles in one cell cycle is different. This can easily be visualized by staining the existing cell wall with a dye and following the outgrowth of a new, unstained cell wall (6, 7, 23, 24). Likewise, the amount of incorporated PG-specific fluorescent D-amino acids supports this finding (10) (Fig. 2). While the total amount of growth appears different between the poles, the rate of cell wall addition may be the same between the poles at the end of division (10, 25, 26).

Besides potential differences in growth from the distinct poles, divisome placement may also be asymmetric. Fluorescent fusions to FtsZ show that septal placement in mycobacteria occurs over a range of the mid-cell, skewed toward the new pole (26–28). As a result of asymmetry in growth and Z-ring placement, mycobacterial daughter cells are phenotypically distinct—cells inheriting the new pole are smaller, while old-pole daughters are larger (5). The consequences and molecular details of this heterogeneity are poorly understood. Recently, however, a mycobacteria-specific protein, LamA (loss of asymmetry mutant A), was found to actively promote asymmetry by inhibiting growth at the new pole (23). Loss of LamA leads to a population of cells that can be more uniformly killed by certain antibiotics, suggesting that heterogeneity generated through cell growth and division confers a survival advantage in some conditions.

Identify the Mid-Cell for Z-Ring Placement (continued)

FtsZ is highly conserved in nearly all bacteria, including mycobacteria. One unique feature of mycobacterial FtsZ is that its polymerization can be inhibited, at least *in vitro*, via phosphorylation by the serine/threonine protein kinase, PknA (29, 30). In general, phosphorylation by PknA and PknB, both of which are essential for growth, plays a key role in regulating many mycobacterial divisome components. Depletion of either of these kinases results in elongated cells, implying a defect in division (31). Throughout this review, we will highlight specific ways in which division is regulated via phosphorylation by these kinases.

Where not to put the Z-ring: over the chromosome and at the poles (see also 32). Division occurring over the chromosome is lethal, and creation of a daughter cell without DNA is not useful. Therefore, mechanisms exist to prevent Z-ring formation over the chromosome and at the poles. *B. subtilis* has two systems that prevent Z-ring formation from occurring at these nonoptimal locations—Noc and Min. The Noc protein (nucleoid occlusion) binds along the chromosome, avoiding the replication terminus (which ends up orienting at mid-cell) (32). It then spreads from primary sites and associates with the membrane to inhibit Z-ring formation over the nucleoid (32). The Min system prevents the Z-ring from forming at the poles. The *B. subtilis* Min system comprises MinC,D,J and DivIVA. MinC is recruited and activated by membrane-bound MinD to interact with and inhibit FtsZ. This MinCD complex is recruited to the poles by the DivIVA interacting partner, MinJ (33). DivIVA is believed to localize to the septum/new pole late during division, at sites of negative membrane curvature, where it begins the recruitment of the Min system (34). To date, the mycobacterial DivIVA homolog, Wag31, has no described function in septal placement and, instead, functions in late septation/early pole establishment through interactions with non-Min-related septal and polar machinery.

Mycobacteria do not have a known Noc homolog, and there has been only one report of a single Min protein distantly related to MinD (Ssd). Cells overexpressing ssd are longer, and transcriptional profiling shows numerous genes involved in growth and division to be repressed (35). However, we do not yet know whether this protein acts in a system akin to *B. subtilis* Min.

While the exact molecular mechanism of septal placement in mycobacteria remains unknown, it appears that the mycobacterial cell wall again plays a role. A recent study utilizing atomic force microscopy identified troughs on the mycobacterial cell surface that formed at the poles and, eventually, became the sites of division one or two generations later. This suggests that potential Z-ring placement sites are created and inherited well before division has begun, the earliest described marker of Z-ring placement in bacteria to date. The nature of the troughs and the mechanism of their formation are not yet known, but this might represent a novel Z-ring placement strategy in bacteria (28).

Recruitment of Z-Ring Anchors, Stabilizers, and Regulators

FtsZ resides in the cytoplasm yet must trigger events occurring outside of the plasma membrane. Accordingly, is must be anchored to the membrane, an action achieved through various "early" interacting proteins. Once the Z-ring is anchored, the septal wall must be synthesized and daughter cells must then be split. Thus, the "late" divisome is comprised of structural proteins that recruit PG synthesis enzymes and these enzymes themselves. While in *B. subtilis*, there is clear distinction between the early and late divisome (~20% of the cell cycle passes between recruitment of these portions of the complex), this distinction is less well defined in mycobacteria but only perhaps due to a lack of data (36).

Early divisome

The goal in the early steps of divisome assembly is to help the Z-ring polymerize and to anchor it to the membrane. The FtsZ ring is dynamic and requires both stabilizing and destabilizing proteins to function properly (21). Division in *B. subtilis* begins with Z-ring formation at the mid-cell, which recruits FtsA (37), SepF, ZapA, and EzrA (21). FtsA, an actin homolog, and SepF help anchor the Z-ring to the membrane (21). ZapA interacts with FtsZ and promotes its polymerization and stability (21, 32). A mutant of the transmembrane protein EzrA produces extra Z-rings (hence the name). The resultant mutant cells are longer, suggesting that negative regulation of the Z-ring by EzrA is necessary for successful division. This protein has been shown to inhibit Z-ring polymerization *in vitro* (21, 34). EzrA also works to recruit PBP1, a PG synthase, to the divisome (38).

Thus far, only SepF from *B. subtilis*'s early, FtsZ-interacting divisome has been identified in mycobacteria. *B. subtilis* SepF localizes to the septum in an FtsZ-dependent fashion and anchors and stabilizes the Z-ring (21, 39). In addition, in *B. subtilis*, *sepF* is synthetically lethal with *ftsA* and *ezrA*, perhaps due to overlapping functions (21). Likewise, mycobacterial SepF localizes to the septum in an FtsZ-dependent manner. Depletion and overexpression of *sepF* leads to filaments and branching—canonical phenotypes of cell division inhibition in mycobacteria (39).

In *B. subtilis*, ClpX, the ATPase chaperone/substrate recognition portion of the ClpXP protease, inhibits FtsZ assembly. Intriguingly, this appears to be independent of its ATPase function (40, 41). Similarly, mycobacterial ClpX interacts directly (*in vivo*) and inhibits FtsZ polymerization (*in vitro*). Overexpression of ClpX leads to multinucleate, elongated cells, suggesting a defect in septal assembly and division (42).

Connecting the cytoplasmic steps to the periplasmic steps

After the early *B. subtilis* divisome is assembled (the Z-ring anchored and stabilized), "late" divisome proteins are recruited (43). These proteins connect the cytoplasmic steps of division to the periplasmic steps required to build the septal cell wall (reviewed in 21). To summarize decades of work briefly, a complex of structural proteins—FtsQ (DivIB), FtsL, and FtsB (DivIC), transmembrane proteins that are critical in connecting Z-ring formation to septal synthesis—and FtsW, a PG transglycosylase, localize to the septum to recruit other PG synthases. The timing and dependency of septal protein localization in *B. subtilis* reveals interdependency between members of the FtsQLB complex, FtsW and Pbp2B (a monofunctional PG transpeptidase) (36). This is in contrast to the more linear dependency of localization in *E. coli*: FtsK→FtsQLB→FtsW→PBP3 (34).

Mycobacterial homologs of the essential *ftsL* and *ftsB* have been identified and experimentally validated through localization, depletion, and *in silico* structural predictions (44). Intriguingly, these homologs have substantial N- and C-terminal expansions compared to their *B. subtilis* counterparts, presumably for mycobacteria-specific interaction partners. In mycobacteria, depletion of either *ftsL* or *ftsB* causes cells to filament and branch, a phenocopy of known septal protein depletions (*ftsZ*, *pbpB*). FtsL and B, along with FtsQ and FtsK (a DNA translocase), localize to the septum. Depletion studies allow inferred localization dependencies of these structural divisome components in mycobacteria. Localization of FtsK, FtsQ, FtsL, or FtsB depend upon FtsZ. FtsK localization does not require FtsQ, L, or B. FtsQ can partially localize in the depletion of FtsL or FtsB, supporting a preliminary dependency hierarchy: FtsZ→FtsK→FtsQ→FtsLB (44). Thus, finding counterparts to known *B. subtilis* divisome members provides a handle to identify and study previously uncharacterized mycobacteria-specific divisome proteins (Fig. 3, brown dashed lines).

One potential mycobacterial "work-around" in the absence of canonical Z-ring anchors like FtsA is the unique interaction between mycobacterial FtsZ and FtsW. FtsW is the divisome-specific homolog of the SEDS (shape, elongation, division, sporulation) family protein RodA, whose function is to polymerize glycan strands of PG through its transglycosylase function (45). Mycobacterial FtsW and FtsZ have non-canonical C-terminal extensions that are proposed to facilitate their interaction (46). They localize at the septum in a manner suggesting that FtsZ localizes before FtsW (47). FtsW also interacts with and appears to recruit PbpB, a monofunctional PG transpeptidase, to the septum (48). In cells depleted of FtsW, FtsZ localizes between nucleoids, but these cells are not proficient at division (48). By interacting with early divisome-founding FtsZ and late divisome septal PG synthase PbpB, FtsW appears to link beginning cytoplasmic steps with late periplasmic wall synthesis steps in mycobacteria.

In actinomycetes, the cascade of interactions is facilitated by the small transmembrane protein, CrgA. CrgA is involved in nearly all steps of cell division: from the start of division, to septal wall synthesis, to establishment of the new growth poles. CrgA interacts with FtsZ and two PG transpeptidases, PbpA and PbpB. It localizes to the septum after the Z-ring and may be required for proper recruitment of PbpB, similarly to FtsW (49). CrgA also interacts with the mycobacteria-specific CwsA, a transmembrane protein that interacts with Wag31 (50). Expression of *cwsA* is directly linked to Wag31 abundance at the poles, suggesting that it is an important factor in either recruiting or maintaining Wag31 at the poles (50). Since Wag31 is associated with new polar growth, the information flow from CrgA through CwsA to Wag31 may coordinate establishing the new pole at the site of division.

Another transmembrane protein, FhaB (also called FipA), interacts with FtsZ. This relationship depends on phosphorylation of FhaB by PknA. FhaB is required for interactions between FtsZ and FtsQ during oxidative stress, a condition that pathogenic mycobacteria experience in the

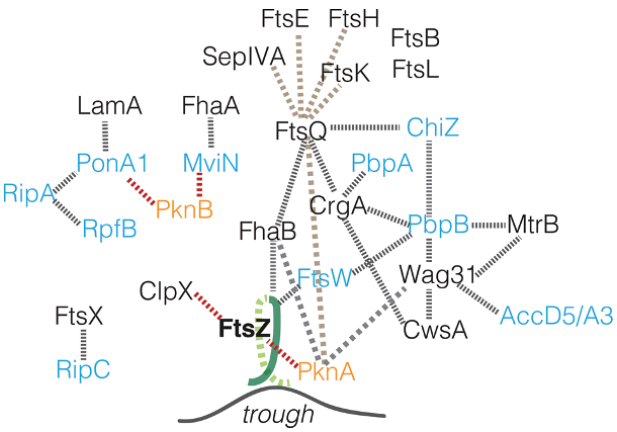


FIGURE 3 Mycobacterial divisome interactions. A schematic of mycobacterial divisome protein interactions. Note that interactions are not necessarily direct given the available data. Gray dotted lines, physical interactions; red dotted lines, negative regulation; brown lines, FtsQ pulldown proteins (44); blue text, cell wall enzymes; orange text, kinases.

host, suggesting that it may be a signal transducing interaction between the Z-ring and the structural divisome protein FtsQ during intracellular division (5, 30).

While these examples are understood in some detail, several other proteins produce morphologic changes when disrupted. Loss of proteins such as the *whiB2* homolog *whmD* and the MinD like protein Ssd result in branching and chaining morphologies, phenotypes associated with disrupted cell division (35, 51). The functions of these proteins remain unknown.

Call to Arms: Septal Cell Wall Synthetic Machinery To Build a Wall

Constructing the septal wall

Division requires synthesis of a wall between two halves of the cell and physical separation of the two halves into independent daughter cells. The cell wall of mycobacteria is multifaceted, consisting of linked layers of PG, arabinogalactan, and mycolic acids (5). The coordination between layers seems likely, but evidence of this is currently slim. This discussion of septal cell wall synthesis will focus on the PG layer.

PG, a netlike structure comprising disaccharide chains cross-linked by peptide bridges, is located outside of the plasma membrane. PG synthesis begins in the cytoplasm, where the disaccharide backbone and pentapeptide side chain are linked to a lipid and flipped across the membrane by the lipidII flippase, MurJ (MviN in mycobacteria) (52) (Fig. 4). This unit is added to the existing glycan strand by the transglycosylase function of bifunctional penicillin-binding proteins (class A PBPs or aPBPs) and by SEDS proteins such as RodA (45, 53). To covalently close the PG network into a cage-like molecule, the peptide bridges are cross-linked between either the fourth and third (4-3) or the third and

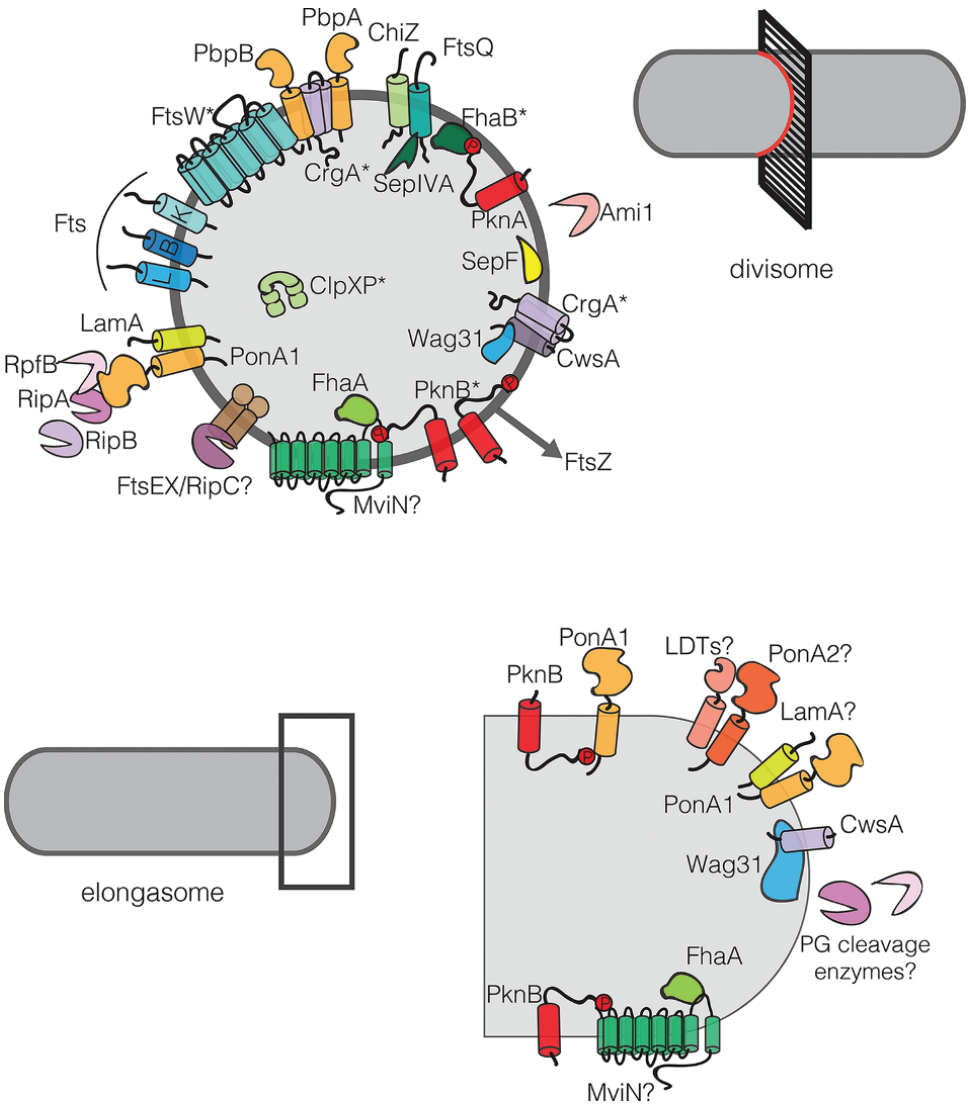


FIGURE 4 Mycobacterial divisome and elongasome members. (Top) Schematic of proteins involved in mycobacterial cell division. Proteins marked with an asterisk (*) have been shown to interact with FtsZ. The FtsZ ring is illustrated as a dark gray circle upon which the divisome members are arranged. (Bottom) Schematic of proteins involved in mycobacterial elongation. Interacting proteins are depicted touching, and proteins with a question mark (?) may belong in these complexes, but data are limited.

third (3-3) residues of opposite PG strands. The 4-3 cross-links, those commonly found in most well-studied PG, are catalyzed by the transpeptidation function of aPBPs and/or by mono-functional class B PBPs (bPBPs), which possess only transpeptidase activity (53). The 3-3 cross-links, a variety of connection that is rare in the PG of other model rods but highly enriched in mycobacteria, are made by L,D-transpeptidases (5, 54, 55) (Fig. 4).

Mycobacteria encode homologs of the *B. subtilis* proteins that perform the essential cytoplasmic steps of PG synthesis, but there are important differences in their regulation. For example, the first committed step of PG synthesis, performed in the cytoplasm by MurA, is regulated by a CwlM, a degenerate PG amidase homolog (56). Phosphorylation of CwlM by PknB results in binding and activation of MurA to initiate PG synthesis. This regulatory cascade functions in times of starvation when CwlM is dephosphorylated, resulting in a halt of PG synthesis and reductive cell division.

Another difference is MviN, which is also regulated via phosphorylation in mycobacteria. MviN's phosphorylation by PknB recruits FhaA to a pseudokinase domain of MviN. Depletion of MviN causes cells to arrest PG synthesis as measured by precursor abundance in the cytoplasm. FhaA depletion leads to an accumulation of PG illustrated with a fluorescent derivative of vancomycin. FhaA localizes to the poles and septum. Together, these data suggest that FhaA may work to inhibit PG synthesis at the MviN step. Alternatively, FhaA may be required for a PG processing event required to remove the substrate for fluorescent vancomycin binding (57).

After lipid II is flipped across the plasma membrane, it must be incorporated into the existing PG network by both transglyosylases and transpeptidases. In *B. subtilis*, it is possible to delete all of the aPBPs, suggesting the presence of another glycosyltransferase (58). In *B. subtilis*, this was recently identified to be RodA in the elongation complex (45) and is likely FtsW, a homolog of RodA, in the division complex. In mycobacteria, the story again appears slightly different. PonA1, an aPBP, is an essential member of the *M. smegmatis* elongation complex and is synthetically lethal with PonA2, another aPBP, in *M. tuberculosis* (59). PonA1 is essential because of its transglycosylase activity (24). RodA is not annotated essential in either organism. On the other hand, FtsW is essential and localizes to the site of division. Thus, these data suggest that, in contrast to *B. subtilis*, the bifunctional PBPs are the major transglycosylases involved in elongation in mycobacteria. During division, the story may be more similar to *B. subtilis*, with FtsW fulfilling the major transglycosylase role.

Working in concert with the PG transglycosylases are transpeptidases. Again, this role can be filled by either aPBPs or bPBPs. There are two main divisome-associated bPBPs in *B. subtilis*, Pbp1 and Pbp2B. Pbp2B (also known as FtsI or PbpB in mycobacteria) localizes to the septum in an FtsQLB-dependent manner (60) and works in partnership with FtsW (21, 36). Pbp1 is thought to shuttle in a cell cycle dependent between old septa (poles), to the sidewall, and then to the division complex via interactions with GspB and EzrA. (38, 61).

In mycobacteria, there is evidence for both an aPBP (PonA1) and bPBPs (PbpB, PbpA) being involved in division. PbpB interacts with divisome members FtsW, MtrB, and CrgA (see above; Fig. 3). Depletion of *pbpB* leads to branching, filamenting cells, clearly blocked in cell division (44, 62). Cells lacking *pbpA* are longer than the wild type, and PbpA may localize to the septum (63). While PonA1 plays a critical role in elongation, it may also have a role during division. The best evidence for this is that PonA1 localizes to both the septum and poles and interacts with RipA, a PG hydrolase important for daughter cell separation (64). However, given the difficulty of assigning proteins to the division or elongation complex based on protein localization at a single time point (see "Mycobacterial Growth: a Different Ballgame"), it could be that PonA1's appearance at the septum reflects recruitment to the site of the nascent poles. Indeed, genetic modulation of PonA1's function results in elongation defects rather than canonical cell division defects (24). Further, its overexpression results in the appearance of atopic poles, suggesting an early role in the establishment of polar elongation.

Breaking the cell wall

Once the wall is made between the two halves of the cell,it must be split to create two independent daughter cells. This is achieved in *B. subtilis* through hydrolysis of PG by LytC, D, E, and F (65–67). Localization studies found LytE and LytF of *B. subtilis* at the septum and poles (68).

Currently, three PG hydrolases have been implicated in mycobacterial cell division: RipA/B, ChiZ, and Ami1. RipA and B are operonic D,L-endopeptidases that cleave within PG peptide side chains (69). These proteins have been shown to be synthetically lethal with one another (70). RipA interacts with resuscitation promoting factor B (RpfB), a lytic transglycosylase, as well as with PonA1. It is cleaved and activated by the MarP protease, specifically in acidic conditions like those found in host phagosomes (10). RipA localizes to the poles and the septum (64, 71). Cells depleted of the *ripAB* operon chain and branch (septa are made but not cleaved), suggesting that this operon is required for daughter cell separation. RipA PG cleavage is synergistic with RpfB (72). Intriguingly, RipA and PonA1 interact in the same domain as RipA/RpfB. Adding PonA1 to a mixture of RipA and RpfB reduces the rate PG hydrolysis by the RipA/RpfB complex but does not inhibit RipA activity alone, suggesting a that the interaction of RipA with PonA1 and RpfB helps coordinate the synthesis and breakdown of the septal wall (5, 17).

ChiZ, like many PG hydrolases, contains a LysM domain known to bind PG (73). ChiZ shares some similarities with *B. subtilis* YneA, a protein whose expression is induced by DNA damage, which curbs division by inhibiting the Z-ring and cell separation (74, 75). Overexpression of *yneA* leads to filaments, but intriguingly, these filaments can be bypassed by a *divIB* (*ftsQ*) mutation further linking YneA with cell division (75). Similarly, overexpression of *chiZ* leads to filamentation, and Z-ring assembly appears partially defective; some normal Z-rings appear to form, but much of FtsZ appears distributed throughout the cell. ChiZ does not appear to inhibit FtsZ polymerization *in vitro* (74). It interacts with PbpB and FtsQ but does not appear to affect their localization (76). The mechanism by which ChiZ and YneA act in division remains unclear.

Ami1, a putative PG amidase, was recently found to be involved in mycobacterial division. In *Δami1* cells, division is blocked, leading to filamentation and lateral/polar branching. Stability of Z-rings appears compromised in these cells, and lysis at the site of attempted septation is observed (77).

Notably, PG hydrolysis is a dangerous game for a bacterial cell, so hydrolase enzymes must be carefully regulated. FtsEX, an ABC transporter complex, is required for proper cell division in *E. coli* through recruitment of EnvC, a regulator of PG amidases at the septum and also through interaction with early divisome anchor FtsA (78, 79). However, FtsEX appears to regulate the CwlO hydrolase during cell

elongation and not division in *B. subtilis* (80). In mycobacteria it has been shown that FtsX interacts with a RipA homolog, RipC, but the role of these proteins in division is yet to be explored (81).

Physical separation of daughter cells in *B. subtilis* occurs slowly. In contrast, mycobacterial cell separation has been described as "V-snapping," where daughter cells literally snap away from each other at the septum but often retain a point of contact (like the quick opening of a door) (82). High-speed imaging has recently revealed that actinobacteria, like mycobacteria, split rapidly, in approximately 10 milliseconds (83). While some mycobacterial cells V-snap, some cells quickly push away from each other at the septum but fail to V-snap; rather, they "straight" snap. This is likely due to the complexity of the mycobacterial cell wall, which must be quickly sheared for full V-snapping (83). In fact, all layers of the cell wall, including PG, arabinogalactan, and mycolic acids, are present at the site of the septum by metabolic labeling (8, 10).

THE CIRCLE OF LIFE: CREATING A NEW GROWTH POLE

In *B. subtilis*, the late septum, post-separation, becomes a synthesis-inert pole. In stark contrast, the pole created at division in mycobacteria becomes the location of cell wall synthesis, the hub of growth for these bacteria.

As previously mentioned, *B. subtilis* DivIVA is a cooperative partner of the Min system to prevent septation over the nucleoid. This can be classified as an early division inhibitor at the stage of Z-ring placement. Conversely, the mycobacterial DivIVA homologue, known as Wag31 or Antigen84, plays a critical role in late stage septation/new pole establishment. It is essential for survival, and its misregulation results in distinct division and elongation impairment phenotypes—branching cells in *wag31* overexpression (84) and polar bulges when depleted (6, 12). Like *B. subtilis* DivIVA, the N-terminus appears to direct Wag31 to polar curvature that may help it localize to the poles (6). Wag31 is phosphorylated by PknA/B (31, 85), but it is unclear how phosphorylation regulates its function (62, 85).

Wag31 localizes primarily to the old pole (i.e., the pole that existed before the most recent division [Fig. 1]) and is observed at the very late septum after the membrane has come down and separated daughter cells (12, 26). This postmembrane septal localization may indicate a role for Wag31 in the transition from a septum to a pole (26). In line with this, Wag31 interacts with members of both division (septal PG synthase PbpB (62, 86), MtrB-discussed below) and elongation (CwsA) machinery. The interaction of Wag31 and PbpB prevents protease cleavage of PbpB in oxidative stress conditions such as growth in macrophages. This highlights a critical link between *in vivo* stresses and cell division (86).

In addition, Wag31 has been shown to interact with the mycolic acid synthesis enzymes AccA3 and AccD5 (6), supporting the notion of coordination between the mycobacterial layers. Interestingly, these enzymes localize in a manner like Wag31, primarily at the old pole but also at the transitioning septum (6), again suggesting that Wag31 may be involved in the creation of new poles at the site of division.

Wag31 is not an enzyme, and yet, it must somehow coordinate cell wall synthases to the pole. How this is accomplished is unknown. We do know that one major PG synthase involved in elongating the cells, PonA1, is also regulated by phosphorylation in mycobacteria. PonA1 is phosphorylated by PknB, an essential Ser/Thr eukaryotic-like kinase (24). A phosphoablative mutation in PonA1 increases the rate of cell elongation. Thus, PonA1's activity is negatively regulated by phosphorylation (24). PonA1 further interacts, either directly or indirectly, with LamA, a mycobacteria-specific protein that actively inhibits growth from the new pole to create asymmetry in polar growth, at the late divisome (23) (see "An (a) side on (a)symmetry" above). Like FtsZ ring assembly, there may be both stimulatory and repressive forces at play to maintain balance in cell wall synthesis.

MYCOBACTERIAL GROWTH RATE: DOES SLOW AND STEADY WIN THE RACE?

Many laboratory bacteria are famous for their ability to rapidly multiply. Mycobacteria, however, grow and divide relatively slowly or, in some cases, not at all under laboratory conditions. There is diversity even among the mycobacterial genus. For example, soil-dwelling *M. smegmatis* are "fast growers," dividing once every ~2.5 hours. On the other side of the spectrum are the human pathogens *M. tuberculosis* and *M. leprae*, which grow extremely slowly during infection (between 20 hours to a few days for *M. tuberculosis* and ~2 weeks for *M. leprae* (87)). Cultured *M. tuberculosis* grows at a similar rate (~20 hours) (88), suggesting that slow growth is not simply a matter of host restriction. Why do mycobacteria grow slowly? The short answer is that we do not know, but that will not stop us from speculating.

One possibility is that polar growth leads to a different set of rules governing bacterial growth. At what point does a bacterium divide? Is it based on time, on size, or on amount of growth? These three hypotheses are known as the sizer, the timer, and the adder models, respectively. Recent work supports the idea that bacteria such as *E. coli*, *Caulobacter crescentus*, and *B. subtilis* may adhere to the adder model, whereby cells add a constant amount of volume during a cell cycle, independent of cell size (89, 90).

The adder model relies on exponential growth. In lateral growers such as *B. subtilis* this is an easy feat to accomplish: as new cell wall is inserted along the entirety of the cell body, the area of new addition also grows. Polar growth, however, does not so simply allow for exponential addition because expansion is restrained to a specific, and proportionally small, geographic area of the cell. Indeed, at first it seemed that mycobacteria might be governed by a different principle. Early work suggested that mycobacteria employ a timer mechanism, dividing after a certain amount of time independent of cell size (7). However, two recent studies strongly suggest that mycobacteria are governed by the same adder principle as other bacteria, albeit with modifications (91, 92). One surprising conclusion from these studies is that single mycobacterial cells appear to grow exponentially (26, 92).

Another possible factor in slow growth is the formidable nutrient barrier provided by the mycobacterial cell wall. And, of course, there is a strong causal relationship between nutrient availability and growth rate: in nutrient-rich environments, bacteria grow faster and, to an extent, become larger. Is the availability of nutrients rate-limiting in mycobacteria? *M. smegmatis*, the most well-studied fast-growing mycobacteria, encodes a porin, MspA, which aids in the uptake of nutrients across the myco-membrane. Notably, MspA is absent in *M. tuberculosis* (93). Expressing *mspA* in BCG, a slow-growing mycobacteria naturally lacking *mspA*, caused the cells to slightly but reproducibly increase their doubling rate, supporting a link between nutrient uptake and growth rate in mycobacteria (93–95). Likewise, porin knockdowns in the fast-growing mycobacteria *Mycobacterium fortuitum*, lead to a decrease in colony size, suggesting

that nutrient uptake plays some role in growth rate (96). However, this must only be part of the story, because expression of MspA only marginally increased the growth rate of BCG.

An additional putative factor in the slow growth of mycobacteria is the rate of DNA replication. In many other species, the demands of fast growth necessitate mutlifork DNA replication because DNA replication is slower than the process of growth and division in nutrient-rich conditions (97). Until recently, it was thought that mycobacteria were not capable of multifork DNA replication, thus decreasing the potential to increase the growth rate beyond this rate-limiting step. However, a recent study challenges this idea, showing multifork replication using time-lapse microscopy and fluorescent reporters of replication-related proteins (98). Intriguingly, the authors found that multifork replication occurred in nutrient-rich and nutrient-poor conditions. Thus, it remains unclear what role multifork replication plays in mycobacteria. Perhaps mycobacterial DNA polymerization is inherently slow. *M. smegmatis* has an estimated DNA synthesis rate of 400 bases/second. This is quite rapid compared to *M. tuberculosis* with a proposed rate of 50 bases/second (98, 99). However, these rates are sluggish in relation to the 600 to 1,000 bases/second that *E. coli* and *B. subtilis* can accomplish (100). DNA replication, the so-called "C" period of the cell cycle, takes up ~75% of the *M. smegmatis* cell cycle (26, 98). Oddly, the *in vitro* rate of nucleotide incorporation by mycobacterial DNA polymerase DnaE1 is faster than that of *E. coli* PolIIIα (101). This suggests that conditions within the cell, for example, limited nucleotide pools, restrict the rate of new chromosomal synthesis (100).

Yet another possibility is that the construction of the intricate cell wall itself limits the growth rate of mycobacteria. Supporting this idea is the recent *E. coli* study by Vadia et al. (102). They proposed an "outside-in" model, in which *E. coli* cell size is limited by fatty-acid synthesis so that cell volume does not increase at a rate faster than can be accommodated by the cell envelope. Certainly, cell wall fatty acid synthesis could be a contributing factor to mycobacterial slow growth since mycobacteria must synthesize their complex and long mycolic acids, and there are substantial cell wall differences between fast- and slow-growing mycobacteria (93).

MORE DEEP THOUGHTS ON MYCOBACTERIAL DIVISION

While bacteria dream of dividing, pathogens dream of dividing within the host. And mycobacteria seem to be exquisitely tuned for doing so: *M. tuberculosis* is an obligate pathogen with essentially no environmental niche. And it is highly successful. In 2016 there were over 10 million new cases of *M. tuberculosis* infection estimated and nearly 2 million deaths (103). Thus, an important part of thinking about the growth of this organism is to consider its adaptations to the host. The slow growth rate is likely optimized for surviving in humans, though why this should be remains unclear. And the ability to survive for decades as an asymptomatic infection, perhaps growing slowly or not at all, permits a reservoir of infection that ensures the long-term survival of the species. The cell wall is appropriately adapted to interface with the host (5). And while the pattern of growth has been inherited from the soil-dwelling organisms from which it has likely evolved, *M. tuberculosis* takes full advantage of it to avoid clearance during infection.

The outcome of these adaptations is crucial when we consider therapy for tuberculosis. The thick cell wall and its multiple embedded efflux pumps present a formidable barrier for many drugs that are effective against other bacteria (104). At the same time, it creates new opportunities to target structures and processes that are absent in model organisms. For example, antibiotics such as ethambutol and isoniazid that inhibit the formation of arabinogalactan and mycolic acid synthesis, respectively, are absolute mainstays of tuberculosis therapy. With a better understanding of the underlying molecular mechanisms, other unique aspects of mycobacterial cell division could provide effective and specific targets for tuberculosis therapy.

Those of us who study mycobacteria are indebted to the groundbreaking research conducted in *B. subtilis* and *E. coli*—work that has created a template on which to base our thinking about our own organisms. But mycobacteria create their own, unique paradigms. Model bacteria might dream alike, but each unusual bacterium dreams in its own way. C'est la vie!

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Mycobacterium tuberculosis Metabolism

GABRIEL T. MASHABELA*, TIMOTHY J. DE WET AND DIGBY F. WARNER

67

INTRODUCTION

The notion that metabolic capacity might correlate with lifestyle has largely been confirmed by comparative genomics analyses of different bacterial species: free-living bacteria tend to have larger, more complex genomes than pathogens and symbionts (1), with obligate intracellular organisms often representing the extreme limit of small genome size (2). This correlation exists also in *Mycobacterium*, a genus which includes environmental organisms as well as a select group of animal and human pathogens—primarily of the *Mycobacterium tuberculosis* complex (MTBC) (3)—whose genome sizes and compositions relate broadly to habitat and host range (4, 5). Most notable among these from a public health perspective is *M. tuberculosis*, the causative agent of tuberculosis (TB), an airborne infectious disease which continues to result in over 10 million new cases and more than 1.5 million deaths globally each year (6). This is despite the widespread use of a neonatal anti-TB vaccine (7) and the existence of an effective combination chemotherapy (8), the insufficiency of which points to systemic failures in TB diagnosis and treatment (9, 10), as well as the complexities encountered in eliminating an organism that has coevolved with its human host (11) and, throughout modern history, has proven adept at exploiting urbanization (12), economic disparity (13, 14), societal upheaval (15), and incarceration (16–18) in maintaining a global prevalence characterized by high-burden endemic regions (6).

M. tuberculosis is an obligate human pathogen; the bacillus can infect other animals, but evidence for zoonotic transmission (back) to humans from these "spillover" hosts (11) is scarce (19). Consistent with its near exclusive association with a single "maintenance host" (11), the *M. tuberculosis* genome has undergone significant downsizing during its evolution from a generalist ancestor. Nevertheless, *M. tuberculosis* retains a large metabolic repertoire, much of which is shared with other mycobacteria with very different lifestyles (20–22); moreover, horizontal gene transfer during the early evolution of the MTBC has enabled the acquisition of additional functions that are critical for pathogenicity (23–25). In other words, the organism has harnessed, adapted, and even augmented the metabolic pathways of its environmental ancestor in evolving the capacity to persist and propagate within its obligate human host (21, 22, 26).

The development of specialist *M. tuberculosis* sublineages from more generalist precursors (11, 27)—in some cases through the inactivation of certain genes (28)—appears, therefore, to signal natural evolution driven by key structural changes in human population demographics and geographies. These include the shift to urbanization, the introduction and widespread use of anti-TB drugs and, possibly, BCG vaccination (29). Greater host population density and the emergence of comorbidities such as diabetes and HIV might additionally have favored the selection of more virulent strains (30, 31), though this inference is not always clear-cut (32). A common theme, however, is that gain or loss of different metabolic functions appears to be a major factor in the modern evolution of the *M. tuberculosis* bacillus (21, 22, 28, 33–36).

We noted previously (37) that the topic "*M. tuberculosis* metabolism" is very broad, encompassing all the dynamic processes and chemical transformations which enable bacillary cellular function and physiology, including those involved in maintaining structural integrity, energy production, generation of biomass, and elimination of waste. It is not possible, therefore, to cover these in any detail in a single review. Instead, this article will attempt to situate *M. tuberculosis* metabolism within the framework of an intimate host-pathogen coevolution while providing some insights into the special challenges faced in elucidating metabolic function (as distinct from metabolic capacity) in a pathogen with no known reservoir outside its obligate human host. Where relevant, we will highlight recent advances in our understanding of different aspects of the metabolism of *M. tuberculosis*, focusing primarily on those which represent unusual adaptations or departures from the model intracellular pathogens; that is, the key observations implicating specific metabolic functions in mycobacterial pathogenicity and the host-pathogen interaction. In addition, given the strong interest in the development of new (shorter) combination regimens for both drug-susceptible and drug-resistant TB, we will consider the impact of metabolism on the susceptibility of *M. tuberculosis* to existing and experimental anti-TB drugs. As with other summaries of this subject, the reader is advised not to consider this a standalone piece. Rather, any of a number of specialist reviews should be consulted for further

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TABLE 1 Selected readings

Metabolic pathway	Recommended reviews
Cell wall biosynthesis and maintenance	253, 254, 329
Energy metabolism and respiration	230, 231
Central carbon metabolism	26, 66
Nitrogen metabolism	330–333
Sulfur metabolism	334, 335
Metallobiology	275, 336
Cofactors (molybdenum, coenzyme A, riboflavin, coenzyme F420, cytochrome P450, vitamin B_{12}, etc.)	145, 337–340
Nucleic acid metabolism	341–343
PE/PPE proteins	77

details on those aspects covered only superficially here, as well as for different perspectives on the key gaps in current knowledge of fundamental mycobacterial metabolism and the implications thereof for TB prevention and new TB drug development (for a list of suggested readings, please see Table 1).

MYCOBACTERIAL METABOLISM AND HUMAN TB DISEASE

Although the precise duration of the *M. tuberculosis*-human coevolution remains subject to contention (11)—estimates range from fewer than 6,000 years (38) to around 70,000 years (39)—there is general acceptance that the interaction extends across millennia. It was striking, therefore, when the first whole-genome sequence of *M. tuberculosis* revealed the genetic potential for synthesis of all essential amino acids, vitamins, and cofactors, with a disproportionate number of pathways dedicated to lipid and fatty acid metabolism (40). This large metabolic capacity (often conflated with "metabolic flexibility" [41–43]) represents a key departure from many other obligate pathogens which tend to rely on the host to complement their limited (and often diminishing) metabolic repertoires (44) and is critical to understanding the defining characteristics of this organism and the disease it causes (26, 45–48). Such characteristics include (i) long-term coevolution of *M. tuberculosis* with humans (38, 39), leading to sympatric associations of specific lineages with geographically distinct human populations (11, 49), (ii) the ability to persist for months to decades in a subclinical state (in some cases characterized by undetected disease [50]) which might develop later to cause active TB (26, 47, 51–53), (iii) the inertness to clearance by the host immune response or eradication by antibiotics (54, 55), (iv) the requirement for extended-duration combination therapy to effect symptom-free cure (8, 56) and, linked to that, (v) the difficulties inherent in developing new drugs and combination regimens to accelerate therapy (57, 58), and (vi) the capacity to acquire—and adapt to—drug resistance mutations that might otherwise be expected to incur a crippling fitness cost, particularly where sequential mutations confer multidrug resistance (59–61). Each of these characteristics reinforces the durability of *M. tuberculosis* in preserving its special niche within humans and, by implication, the challenges inherent in disrupting this association, whether by vaccines, chemotherapy, or other interventions (Fig. 1).

Pathogenicity and Metabolism

That physiology and pathogenicity cannot be separated in an obligate pathogen such as *M. tuberculosis* seems axiomatic (26, 62). However, the relevance of this precept to efforts to counter TB might be less evident and so bears restating: an understanding of mycobacterial metabolic capacity supports the inference that *M. tuberculosis* is a "survival machine," able to exploit its human host as both a source of nutrients for increased biomass and a reservoir for long-term persistence (26, 37, 63). Reinforcing this premise is the observation that the infecting population does not necessarily drive a metabolic program aimed at maximizing growth rate (64, 65) or biomass (26, 37, 63, 66). Instead, as observed by others (66), the frequent requirement for bacilli to exit the standard bacterial cell cycle owing to the pressures imposed by the host immune response and, in the modern history of *M. tuberculosis*, antimycobacterial chemotherapy, demands the capacity to maintain viability in a state of rapidly reactive and reversible metabolic quiescence for extended periods.

While evidence exists for decades-long survival of bacilli in an asymptomatic host (67, 68), most cases of clinical disease are thought to arise within 1 to 2 years of infection (53, 69). During this period, those bacilli which survive the attempts at clearance by the host's innate immunity generally maintain a state of paucibacillary, asymptomatic infection in which host lipids and mycobacterial antigens are accumulated within alveoli in the lung apices. There is a spread of bacilli which, depending on the preferred model, is thought to occur via bronchi (69) or via the lymphatics (70). And, although most new lesions regress spontaneously, necrosis can result in caseous pneumonia, a heterogenous outcome which can be paucibacillary or multibacillary, with or without significant neutrophil involvement. Importantly, this manifestation—known as post-primary TB disease—depends on the acquisition of systemic immunity by the infected host. Its sequelae are considered the key determinant of disease outcome: softening and fragmentation of the caseous pneumonia triggers its expulsion by coughing, in turn leaving a hole which can become the site of active bacillary growth and the primary source of organisms for transmission; in contrast, those sites which are not removed by coughing are sources of ongoing inflammation, ultimately leading to fibrocaseous disease (69). Though central to disease pathogenesis, these events in post-primary TB, in particular, the development during latent infection of an asymptomatic obstructive lobular pneumonia, have not been adequately studied, owing to the unavailability of suitable clinical specimens and the failure of animal models to recapitulate many of the key features. Inevitably, mycobacterial metabolism under these conditions, too, remains very poorly understood.

The quiescent state adopted by *M. tuberculosis* is distinct from "true dormancy," a very specific physiological and morphological condition often involving sporulation, for which the bacillus appears to lack the necessary genetic machinery (71). Physiologically, though, it is interesting to note that a deficiency in α-crystallin—a major TB antigen (72), bacterial protectant, and chaperone protein which has been implicated in long-term bacillary survival (55)—is associated with hypervirulence *in vivo* (73). This seems to imply a disturbance of the natural host-pathogen interaction where a genetic lesion has deprived the bacillus of its natural capacity for persistence. The observation, noted previously (37), that mutations in regulatory genes are often associated with hypervirulence (74–76) appears to buttress this conclusion. So, too, does recent evidence (28) implicating disrupted secretion of the large class of *Mycobacterium*-specific

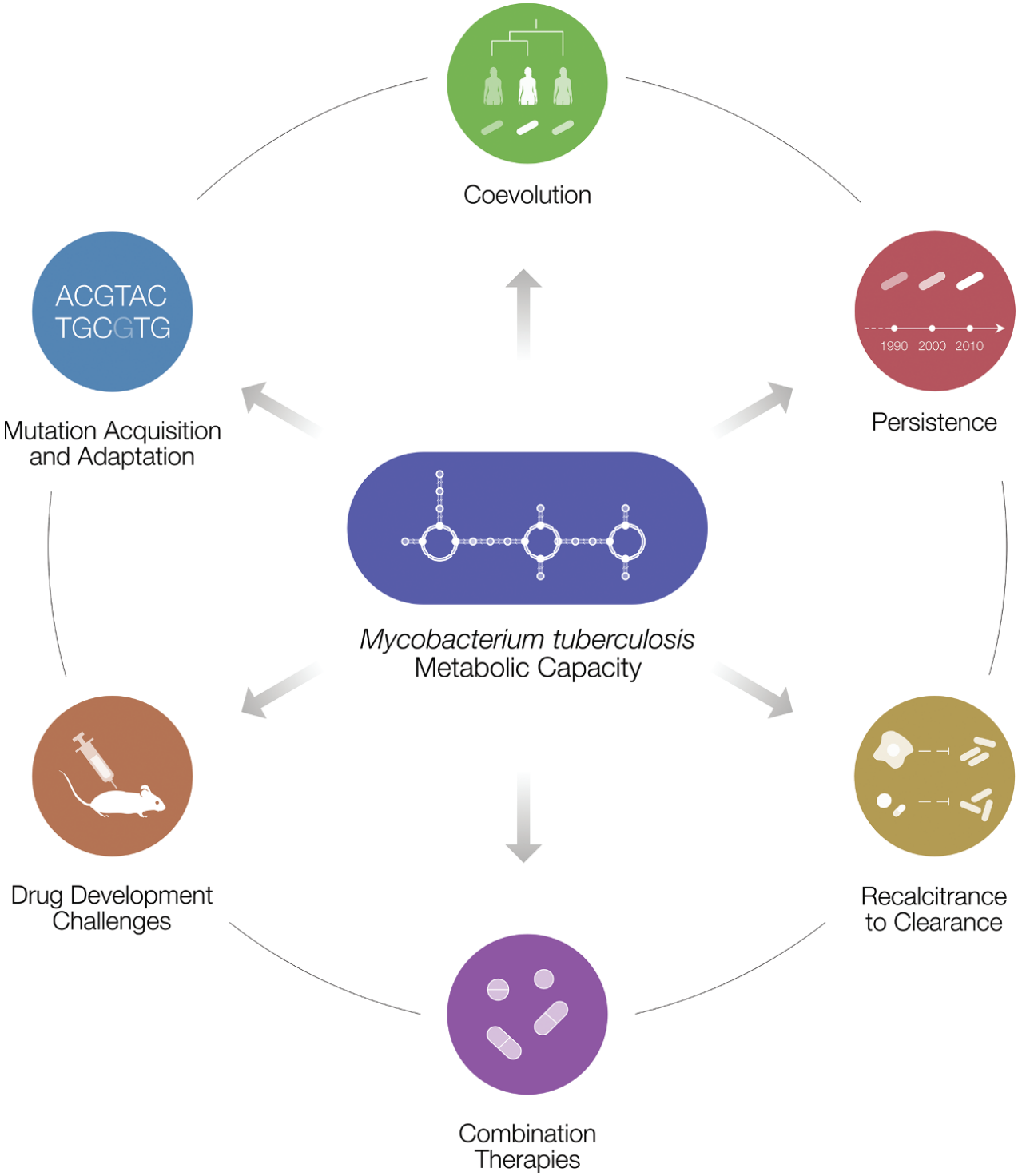


FIGURE 1 The metabolic capacity of *M. tuberculosis* is critical to understanding key features of mycobacterial pathogenicity, which include (i) long-term coevolution with humans, (ii) the ability to persist for months to decades in a subclinical state, (iii) inertness to clearance by the host immune response or eradication by antibiotics, (iv) the requirement for extended duration combination therapy to effect symptom-free cure and, linked to that, (v) the difficulties inherent in developing new drugs and combination regimens to accelerate therapy, as well as (vi) the capacity to acquire—and adapt to—multiple drug resistance mutations without incurring a crippling fitness cost.

PPE-MPTR and PE_PGRS proteins (whose functions remain largely unknown [77]) in enhanced growth and virulence of modern Beijing lineage strains *in vivo*. The inferred centrality of a conserved host-pathogen interaction in TB pathogenesis is reinforced in the "hyper conservation" of T cell epitopes (39, 78), a feature which might be integral to the development of post-primary TB (69, 79).

At an epidemiological level, the inference that *M. tuberculosis* is hard-wired for persistence is supported by the fact that the number of active TB cases globally (~10 million) (6) is dwarfed by the estimated annual incidence (thought to exceed tens of millions [53]) and global prevalence of infections (estimated at ~25% of the world's population [80] but subject to contention [53]). As Rhee and colleagues have cogently argued, this entails that the bacillus must occupy a range of physiological states book-ended by "unrestrained replication" and "perpetual quiescence" (26), extremes which are incompatible with the natural history of the species (11, 49). In turn, the notion that *M. tuberculosis* cellular function defaults to persistence immediately suggests the corollary: host status, whether genetic (81), immunological (82), nutritional (83, 84), environmental (85, 86), or a combination of some or all of these, is critical in determining who develops, and succumbs to, TB disease (a seemingly obvious conclusion, perhaps, but one which reinforces the potential pitfalls in attempting to define—and quantify—mycobacterial "virulence" as a solely bacterial property).

The intimate, dynamic, and durable interaction between *M. tuberculosis* and humans defines the framework within which any new anti-TB interventions must be considered. That is, tackling any single aspect—for example, drug regimens to treat active cases, novel preventative chemotherapies or vaccines to forestall new disease, or strategies to block transmission—is unlikely to prove sufficient on its own (87). Importantly, it also underscores an acknowledged limitation of many studies which aim to elucidate fundamental aspects of *M. tuberculosis* metabolism: namely, that the potential disconnect separating bacillary metabolic functions active during host infection (whether in the presence or absence of manifest TB disease) versus those actuated—and therefore described—*in vitro* and/or *in vivo* in the various disease models risks a growing phenomenology of metabolic capacity distinct from direct evidence of metabolic function in the context of the complete biological system which defines TB (37, 88).

Investigating Metabolism *in Vitro*

While the majority of genome-wide mutagenesis studies recognize the importance of, and even exploit, the applied *in vitro* conditions (or infection model) in inferring conditional essentiality (89–93), the impact of growth conditions on experimental observations has often been ignored in fundamental mycobacteriology. This is despite an increasing number of reports of the profound impact of the growth medium and various additives on the described phenotypes (94–97, 344). Aside from auxotrophy (92, 98, 99), perhaps the most illustrative example of how even apparently absolute concepts such as essentiality (100) can be condition-dependent is provided by the paradoxical tolerance of cell wall-targeting antibiotics in cell wall-deficient L-form bacteria (101), a phenotype which demonstrates the potential for wholesale loss of a fundamental macromolecular structure under the appropriate conditions—in that case, *in vivo* residence in a suitably osmoprotective intracellular environment.

Similarly, practical considerations tend to favor the use of culture conditions that enable optimal (that is, the most rapid) growth. For *M. tuberculosis*, this generally means aerobic incubation in standard medium (usually Middlebrook 7H9 base or equivalent) comprising glycerol and glucose as the primary carbon sources (102). The disconnect between *in vitro* and *in vivo* models used in experimental investigations of mycobacterial metabolism and those environments which are likely to prevail in the natural human host is not unique to *M. tuberculosis* (103). Therefore, in addition to appreciating that logarithmic aerobic growth represents just another condition and that mycobacterial physiology in this state will necessarily influence assessments of gene essentiality or metabolic pathway vulnerability, there is a pressing need to develop experimental systems that target slow-growing and nongrowing states, perhaps in the context of more advanced cellular scaffolds (104). Though often performed in the context of TB drug discovery, the application of advanced analytical technologies to determine the precise molecular content (proteins, lipids, metabolites) of a number of environments, including macrophage organelles (105), lipid droplets (106), and tissue samples from animal models and patients (107, 108), represents an exciting area of development and might be complemented by recent advances in generating more complex tissue culture models (109).

Many researchers have made pioneering efforts to develop other complex models of *M. tuberculosis* growth and survival, and the strategies adopted to model environments that are far less favorable for unchecked generation of biomass have yielded important insights into metabolic function under the applied conditions, as well as revealing condition-selective antimycobacterial compounds. These include manipulating the pH of growth media, the inclusion of peroxide and/or nitric oxide stresses, oxygen and nutrient deprivation, and combinations thereof (110–115), many of which have been applied in screening programs aimed at identifying compounds with condition-specific activities or those able to inhibit survival of nonreplicating bacilli (116, 117). Again, while each is intuitively attractive, the difficulties inherent in translating inferences derived from these *in vitro* systems to human TB disease remain daunting; the field urgently requires a proof-of-concept demonstration of enhanced therapeutic efficacy achieved using a novel small molecule whose development has been prioritized owing to its condition-selective activity in one of more *in vitro* models.

Moving to More Complex Models

Like for many other pathogens, investigations of cellular functions in the context of the host-pathogen interface of *M. tuberculosis* have tended to focus primarily on the effectors of immunity (host) or virulence (bacillus), with the metabolic consequences of infection often ignored (44). Systems biology and its tools, including the analysis of labeled metabolites (118–122) and other mass spectrometry-based technologies (123–127), have eroded this bias, enabling key insights into the biochemical compositions and metabolic fluxes which characterize *M. tuberculosis* bacilli under different conditions and in different infection models (128). These and related approaches have also been used to elucidate the regulators of metabolic adaptation and function (129–131), the mechanisms of action of existing and experimental anti-TB drugs (43, 131–134), in assigning functions to proteins of unknown or predicted function (133) and in functional genomics of clinical *M. tuberculosis* isolates (36). Except for sputum bacilli, though, the number of studies directly investigating *M. tuberculosis* metabolic function and physiological state(s) *in situ* during host infection remains very small (135–137).

In some cases, results from experimental systems *in vitro* and *in vivo* have been combined with analyses of human TB pathology to construct sophisticated models of mycobacterial metabolism (138, 139). Though compelling, the *in vivo* relevance of these is largely untested; for this reason, determining the metabolic and physiological states adopted by the tubercle bacillus in the infected human host represents the ultimate, though daunting, scientific goal. Knowledge that the functions of approximately 40% of the *M. tuberculosis* proteome remains predicted or obscure (140, 141)—so, too, the speculative reasons the bacillus possesses numerous examples of apparently redundant or duplicate pathways (142–149)—exacerbates the size of the task. Moreover, growing evidence indicating key functional differences between (and even among different laboratory stocks of) the laboratory strain, *M. tuberculosis* H37Rv, clinical *M. tuberculosis* isolates, and other species of the MTBC (33–35, 150, 151), implies that diverse phenotypes will inevitably be recorded when different species, and even strains (152), are tested in the same assay. That is, while prevailing approaches might enable some generalizable insights into core *M. tuberculosis* metabolism, the next phase demands innovative approaches to study bacilli of different genotypes in the context of natural host infection (153). Here, understanding the features which differentiate infection outcomes in sympatric versus allopatric hosts (154) might provide some important insights (151).

Of course, the fact that the diversity which characterizes *M. tuberculosis* lineages and strains is matched by the genetic and phenotypic variety of host immune cells further complicates these analyses. For example, different macrophages and macrophage-like cell lines are commonly explored to understand mycobacterial function in the intracellular environment (155); for TB, these are usually immortalized human and mouse cell lines (e.g., THP-1s, J774s), mouse bone marrow-derived macrophages, and human peripheral blood mononuclear cells (156), with some recent reports proposing alternative, more complex, systems (109, 157). Macrophages themselves are functionally diverse, however, and follow different developmental trajectories and respond to a variety of activation signals (158). Consequently, there are significant risks inherent in a rigid dependence on standard models, a pitfall underscored by recent observations that alveolar macrophages might offer a more favorable intracellular environment for *M. tuberculosis* survival and dissemination than interstitial macrophages (159, 160). Moreover, evidence that a shift to aerobic glycolysis in infected cells can enable proliferation of intracellular bacilli (161, 162) requires that caution should be exercised in drawing conclusions about mycobacterial metabolic function from experiments utilizing the different macrophage lines. To enable rapid anabolism, proliferating cells (as is the case for the various cell lines) naturally drive a strong Warburg metabolism which is independent of intracellular infection but must inevitably impact the metabolic response of the infecting bacillus (161). A further complication is that this effect can be species-specific, with recent evidence highlighting differential metabolic responses in human versus mouse macrophages (163). In this light, the proposal that intracellular pathogens might have evolved to take advantage of specific immune cell subsets locked into promicrobial metabolic programs (44) seems apposite. Therefore, special emphasis must be placed on the need for much greater functional and ontogenic information about the host cells in which bacilli preferentially reside during infection. And this should be coupled with a better understanding of the extent to which immunometabolic responses of host cells to *M. tuberculosis* are cell-type specific, host species specific, and/or strain specific (45, 161).

M. tuberculosis Metabolism and the TB Disease Life Cycle

Other than during transient periods of transmission, when bacilli are released into the environment in droplet aerosols, the entire *M. tuberculosis* life cycle occurs within the infected host (26, 164). This concept—encapsulated in the notion of humans as the "maintenance host" (11)—is critical to understanding *M. tuberculosis* pathogenicity: the ability to infect, persist, cause disease, and trigger production of infectious aerosols demands the capacity for survival and propagation in different environments and under a variety of stresses. Current knowledge of *M. tuberculosis* infection reinforces this notion. Most experimental models, ranging from intramacrophage systems *in vitro* (165, 166) through small animals (159, 167, 168) to nonhuman primates (NHPs) (169, 170), indicate that bacilli are able to replicate rapidly immediately after infection, with evidence of early dissemination in NHPs (170) until host-mediated killing results in a decline in replication rates and total bacillary load. Even then, it appears from the mouse model (167) that replication might continue in discrete microenvironments throughout infection (68), a possibility recapitulated in genomic data from bacilli isolated from NHPs which revealed the acquisition of chromosomal mutations during subclinical disease (171). Moreover, although its relevance to *in vivo* infection remains to be established, the observation that *M. tuberculosis* replicates rapidly inside dead macrophages (172) further argues that the transient growth rates (and fates) of infecting bacilli in different lesions in the same host are likely to be dynamic and unlinked.

The heterogeneity which characterizes host infection supports a model in which infecting bacilli must establish an early foothold within the new host before seeding a number of discrete lesions, the fate of which appears to be determined for each lesion at a local level, independent of events in other microenvironments (173). For *M. tuberculosis* as a species, each new infection represents a potential dead-end if transmission is prevented. Therefore, long-term survival within a host—and by any mechanism—appears to represent the best hedge against this risk. Loss of immune control at any stage during the host's lifetime might then allow for stimulation of the destructive pathology that could enable (or enhance) infectious aerosol release, renewing the infection cycle.

This is the lens through which mycobacterial metabolism must be viewed. Transient opportunity for generation of biomass means that preservation of viability under varied and multiple stresses represents the driving metabolic imperative. While contrary to popular views which conflate bacterial virulence with rapid growth, the enforced (and sustained) quiescence which likely characterizes human *M. tuberculosis* infections is a feature of most bacteria in their preferred niches, whether pathogens or not (103). The apparently "surprising" retention of a multifunctional metabolism in a successful obligate pathogen appears then to be just the opposite: metabolic flexibility is, instead, precisely the attribute which enables *M. tuberculosis* to occupy its preferred environment—the human host—without recourse to a separate (environmental) reservoir. For *M. tuberculosis*, the absence of any competing microorganisms within its "ultranarrow ecologic niche" (66) additionally ensures a privileged, albeit transiently hostile, space in which replication can occur subject to prevailing conditions, largely determined by the host immune response. Again, this highlights the need for

greater understanding of the metabolic functions which ensure prolonged viability under nonreplicating persistence. Here, it is interesting to consider recent work with other bacteria which has demonstrated the conditional essentiality of many genes, including a large number of proteins of unknown function, in long-term fitness under growth arrest (174, 175), as well as the potential for selective targeting of pathogens under these conditions (176).

The example of *pat* mutants, engineered strains of *M. tuberculosis* which achieve population numbers similar to wild-type bacilli *in vivo* but do not cause the same degree of pathology (177), has been cited as evidence of the fact that disease outcome is not necessarily linked to the bacillary population size (37). Rather, it is the interplay between the host response and the infecting *M. tuberculosis* population—and the antigens it produces (69)—which determines infection outcomes (178). This tenet is perhaps best captured in the aphorism that *M. tuberculosis* is necessary, but not sufficient, for TB disease (82, 179).

Again, evidence from pioneering studies in NHPs reinforces this conclusion. For both diseased and clinically latent monkeys, the population of culturable organisms commonly numbers around 2×10^5 bacilli per lesion prior to the onset of the adaptive immune response and stabilizes at a median of $\sim 10^2$ bacilli per lesion during active disease (169). That is, the total numbers of bacilli present in active versus latent disease can overlap. Moreover, while there can be microenvironments in which the mycobacterial population achieves large numbers during active disease, the total bacillary burden generally seems much lower than might be expected given the extent of pathology (180). This observation is reiterated in human studies (69, 82) and contrasts with recent conclusions from an unrelated infectious disease model which demonstrated that infection outcome was directly attributable to bacterial load (181).

It is important, though, to differentiate TB disease in HIV-infected versus HIV-uninfected individuals. The enhanced propensity for extrapulmonary disease and TB bacteremia in HIV-TB cases argues that infection outcomes in the context of HIV-mediated immunodeficiency might correlate much more closely with the mycobacterial burden (182). Moreover, while the precise cause of death in disseminated TB disease in HIV-infected individuals remains speculative (183, 345), for pulmonary TB in individuals who are not immune-suppressed, it is clear that most patients ultimately succumb to advanced pathology (signaling a systemic failure in TB diagnosis and treatment [184]), a distinction which reinforces the intimate interplay between host and pathogen in the context of a functional immune response (185). Therefore, to consider TB disease as a single entity and, by extension, to categorize *M. tuberculosis* metabolic states (and, by implication, metabolic vulnerabilities) according to a single framework that assumes a handful of possible infection outcomes (51, 52) imposes a conceptual constraint that, albeit practically appealing, is potentially crippling in its failure to accommodate the complexity inherent in an infectious organism adapted to persistence, not virulence.

TB as Polymicrobial Infection of Humans

The continual acquisition of new sequence data from different mycobacterial species and strains has enabled comparative genomics analyses to determine the evolutionary histories of the different members of the MTBC with greater resolution (3). Importantly, when key genetic differences are considered against the backdrop of the very specific host tropisms of the different MTBC members, it becomes increasingly evident that laboratory-determined virulence is not only insufficient to understand pathogenicity, but likely inappropriate. As noted elsewhere (186), each species has diverged from a common ancestor through coevolution with its own cognate host, resulting in genetically related but phenotypically distinct pathogens specifically suited to hosts with disparate social and biological features.

M. tuberculosis causes TB, a disease which manifests primarily as damage of the lungs, yet extrapulmonary forms are also known which affect other organs and sites, including the pleura, abdomen, genitourinary tract, meninges, skin, joints, and bones (187). Lymph nodes, too, are often cited among lists of "extrapulmonary" sites, but this modifier is likely inaccurate given significant evidence that infection of lymph nodes constitutes a critical event in TB pathogenesis (70, 188, 189). Also distinct from extrapulmonary TB is the observation that *M. tuberculosis* can occupy diverse anatomical and cellular niches during latent host infection (190). Even in "simple" cases of pulmonary TB, infecting *M. tuberculosis* bacilli are not restricted to specific environments but can be found in multiple different host compartments, including cellular and necrotic granulomas and the inner surfaces of open cavities (58, 191–193). In each of these, bacilli can be sequestered within host cells or located in extracellular niches (194), reinforcing the suggestion that TB disease might usefully be considered a polymicrobial infection (195).

The occupation of diverse host microenvironments—in many cases, by paucibacillary populations—with independent disease trajectories constitutes a major obstacle to elucidating the metabolic adaptations demanded of bacilli located within (or transitioning between) the different niches (26). From a pharmacological perspective, this uncertainty presents a clear challenge to developing approaches that will ensure optimal drug delivery to bacilli in dynamic and disparate physical loci and metabolic states (57, 58, 196) and to understanding which (sub)populations must be controlled (or eliminated) for favorable therapeutic outcomes (55, 189, 197–199). For drug discovery, such uncertainty undermines efforts to identify unequivocally the pathways and functions which are critical to mycobacterial survival and so might represent viable antibiotic targets, that is, the organism's "metabolic vulnerabilities." In addition, it renders ambiguous the potential utility of compounds known to be selectively active against either proliferating or quiescent organisms, growing or subsisting on different nutrient sources (conversely, such molecules are of unquestionable utility as probes of the distinct metabolic states adopted by *M. tuberculosis* in those environments). Therefore, for those wanting (simply) to understand the link between bacillary metabolism and host disease, the lack of granularity informing available descriptions of *in situ* metabolism poses unresolved challenges regarding the assays and approaches—available and required—that might best elucidate mycobacterial metabolic function *in vitro*, in infection models, and in clinical disease.

Immunometabolism and Pathometabolism at the Host-Pathogen Interface

Like all intracellular pathogens, the bacillus must adapt its metabolism to the host cell, whose own metabolic status might correspondingly be altered as a consequence of infection (200). Though poorly understood, this interplay between immunometabolism (201) and pathometabolism (202) is critical to infection outcomes (45). There is increasing evidence that successful intracellular pathogens such as *M. tuberculosis* have evolved to exploit the reciprocal

determinism linking host cellular function and metabolic status (161). Ergo, this interplay might offer a solution to the apparent paradox that the very immune cells designed to eliminate the invading pathogen can function as a preferred niche (44). Consistent with their role as early responders, innate immune cells appear much more vulnerable to infection than adaptive immune cells, reinforcing the inference that organisms such as *M. tuberculosis* have adapted to the tightly regulated responses of innate immune cells to enable long-term intracellular survival. The observation that a core transcriptional profile unites species and lineages across the MTBC in response to intracellular infection (151) strengthens this conclusion and is supported by the presence of metabolic enzymes among the hyperconserved T-cell epitopes in *M. tuberculosis* (203). That is, through millennia of coevolution, the bacillus is likely to have adapted to the complex network of metabolic and immune alterations and signals triggered in its host.

The corollary also appears to hold true: in response to *M. tuberculosis* infection, some host macrophages shift to a "Warburg-like" (161) program of aerobic glycolysis (162, 204–207) in which oxidative phosphorylation is reduced and cytosolic glycolysis and the mitochondrial tricarboxylic acid cycle are redirected primarily to the biosynthesis of fatty acids. In an example of "bipartite metabolism" (44), these are utilized as energy sources by the intracellular bacillus (120) without imposing excessive metabolic stress on the host macrophage, which is able to drive an anabolic program utilizing the available glucose. Notably, a metabolic shift in the infected macrophage has been implicated in the accumulation of intracellular lipid bodies characteristic of foamy macrophages. In turn, this phenotype has been closely linked with mycobacterial persistence and disease progression (208–211), though recent observations suggest that it might be more nuanced (212). There is also evidence that altered metabolism at the site of *M. tuberculosis* infection influences cytokine production (213), reinforcing the connection between cellular metabolic state and immune function. Here, it is useful to consider the key observation that, at a metabolic level, the host-pathogen interaction is likely to possess emergent properties: specifically, the metabolic responses of host cells to living bacteria are likely to be more subtle and complex than the sum of metabolic responses to individual bacterial components (161).

Given the major recent impact of positron emission tomography and computed tomography imaging in providing unprecedented insights into TB disease pathogenesis and heterogeneity, both within and between individuals in different disease models and human TB patients (50, 169, 180, 189), it is worth noting here that the technique utilizes a [^{18}F]fluoro-2-deoxy-2-D-glucose ([^{18}F]-FDG) probe which exploits the enhanced glucose utilization of inflammatory cells. Recent work investigating the potential for alternative probes to provide cell-specific information ([^{18}F]-FDG is taken up by all glycolytically active cells and does not distinguish subsets) has revealed the dynamic nature of granuloma composition and metabolism over the course of infection (214), again reinforcing the critical concept that lesional progression with an individual does not follow a fixed temporal program (173).

M. TUBERCULOSIS METABOLISM

As noted at the outset, it is not possible to cover all aspects of mycobacterial metabolism within a single review. Instead, this and subsequent sections will provide a very brief update on research supporting four key concepts which are critical to understanding *M. tuberculosis* metabolic function and regulation, namely, (i) metabolic and respiratory flexibility, (ii) the capacity for cocatabolism of different nutrient sources, (iii) the potential for metabolic anticipation, and (iv) the heavy investment in cell wall metabolism.

Central Metabolism

"Not a picky eater, at least not for carbon and nitrogen sources" (Luiz Pedro De Carvalho, Twitter, 1 March 2019). Despite its levity, this declaration, made recently in promotion of research demonstrating the utilization by *M. tuberculosis* of amino acids as nitrogen sources (215), perhaps best summarizes a sizeable literature on (mostly *in vitro*) mycobacterial metabolic function (see Table 1 for selected references). Unsurprisingly, the predominant focus over the past 2 decades has been on carbon utilization, and consistent with this bias, this aspect of mycobacterial metabolism has been extensively reviewed (26, 45). Among the central tenets emerging from this work are that *M. tuberculosis* can cocatabolize different carbon sources (216), an observation which signals a key departure from classic bacterial metabolism in which diauxic growth regulated via carbon catabolite repression constitutes the dominant model.

A number of subsequent studies have reinforced carbon cocatabolism as critical to *M. tuberculosis* cellular function and pathogenicity, identifying specific roles for fructose-1,6-bisphosphate aldolase (217) and pyruvate kinase (218) in enabling this capacity. Moreover, the persistence defect observed in an *M. tuberculosis* mutant lacking both glucokinase homologues—in other words, functionally depleted of glycolytic potential—supports the conclusion that both carbohydrates and fatty acids are required for long-term survival *in vivo* (219). More recent research has extended the capacity for cocatabolism to nitrogen sources *in vitro* (215) and in an intracellular model (220), strongly suggesting the generalizability of this principle to mycobacterial metabolism. In this context, recent evidence of the power of techniques such as cryo-electron microscopy in revealing cytoplasmic organization and the presence of microcompartments in different prokaryotes (221, 222) suggests the intriguing possibility of investigating the potential that cocatabolism is spatially regulated.

At least one codicil to the inferred absence of catabolite repression in *M. tuberculosis* might exist in the observation that cholesterol-dependent growth inhibition cannot be alleviated by the provision of glucose as an alternative carbon source (223). This implies that preferential utilization of cholesterol—predicted to be a feature of *M. tuberculosis* pathogenicity (224)—might represent a critical constraint to mycobacterial metabolic flexibility (223). In some ways, this phenomenon is reminiscent of the very well-described differential (often poor) growth of clinical *M. tuberculosis* isolates in specialist laboratory medium following isolation from patient samples, including sputum (54, 225). In turn, it highlights the fact that, while *M. tuberculosis* H37Rv is the workhorse of experimental mycobacteriology, this strain has become adapted to growth in laboratory media through decades of serial passage *in vitro* (152).

The concept that growth arrest represents a key stress response in *M. tuberculosis* and, importantly, might be triggered even when the organism retains the metabolic capacity to grow (65) is also central to understanding mycobacterial pathogenicity. This preservation strategy appears to be enabled, at least partially, by the capacity for "anticipation" of future events, an example of which was provided by the observation that *M. tuberculosis* exposed to hypoxia remodels

its own cell envelope to generate building blocks for later reentry into the cell cycle (226). Notably, this capacity is enabled by the catabolism of trehalose mycolates, from which the bacillus generates pentose phosphate intermediates that are incorporated into peptidoglycan biosynthesis much later, when environmental conditions improve, that is, contingent on re-aeration at an indeterminate future time. This illustrates an inherent capacity for preservation that might be hard-wired through long-term adaptation to predictable host environments and stimuli (227). Reinforcing this idea, very recent work has demonstrated that multiple stresses induce expression of the small regulatory RNA, MrsI, which functions to preserve intrabacillary iron reserves, perhaps in anticipation of iron deprivation in the infected macrophage (228). Although not involving metabolic anticipation *per se*, the observation that utilization of nitrate as the sole nitrogen source requires a functional proteasome (229) is analogous in demonstrating the very tight connection between metabolic pathway utilization and regulation of cellular macromolecular synthesis and recycling, perhaps offering insights into the organism's intrinsic responses to *in vivo* nutrient availabilities.

Respiration

Like all mycobacteria, *M. tuberculosis* is an obligate aerobe. Therefore, survival in growth-inhibitory hypoxic environments demands sufficient energy generation for maintenance functions, while aerobic conditions enable full metabolic activity for bacillary replication (230). From comparative genomics of different mycobacteria, it is apparent that, whereas major biosynthetic pathways are reasonably conserved, occurring within core orthologous clusters, the respective complements of genes involved in energy metabolism are more diverse. This observation is consistent with the adaptation of individual species to specific environmental and host niches and, perhaps, replication rates (21, 22).

M. tuberculosis possesses multiple branched pathways for electron transfer from a variety of low-potential reductants to oxygen during aerobic growth (230) and to alternative electron acceptors under hypoxic conditions (231). The potential flexibility suggested by the expanded complement of primary dehydrogenases and oxidoreductases (232)—the function and regulation of which remain poorly understood (230)—is, however, tempered by the observation that aerobic respiration in *M. tuberculosis* appears to depend on a single lipoquinone, menaquinone, and two terminal respiratory oxidases, the aa_3-type cytochrome *c* oxidase and the cytochrome *bd* menaquinol oxidase (231). Moreover, evidence suggests the obligatory coupling of ATP generation to the electron transport chain and the membrane-bound F_1F_0-ATP synthase, irrespective of the oxygen concentration or the proton motive force (230). And, whereas some bacteria are able to utilize ATP to energize the cytomembrane via coupling of ATP hydrolysis to proton pumping, the *M. tuberculosis* F_1F_0 ATP synthase lacks ATP hydrolysis activity (233). These limitations are unusual for mycobacteria and have been exploited in the development of the diarylquinoline bedaquiline (Sirturo), the first new FDA-approved anti-TB drug in over 40 years, whose initial use has been restricted to multidrug-resistant TB pending the results of ongoing phase 2 and phase 3 trials (234, 235).

As noted elsewhere (230), the *M. tuberculosis* genome encodes expanded complements of both electron donors and electron acceptors whose roles in pathogenicity are largely unexplored. It is perhaps no exaggeration to suggest that, of all the core metabolic functions, respiration and energy metabolism are those which have been most prone to very selective investigation given the practical difficulties associated with manipulating the bacillus under variable conditions of hypoxia and anoxia, the default reliance on standard, glucose-based growth media *in vitro*, and the general use of mouse models which recapitulate only certain aspects of TB pathology (236). A salient example is provided by the likely contribution of the assimilatory/respiratory nitrate reductase to bacillary survival under various stresses, a notion supported by strong functional and comparative genomics evidence that the acquisition of molybdopterin biosynthetic and salvage genes (molybdenum cofactor is essential for multiple enzymes in carbon, sulfur, and nitrogen metabolism, including nitrate reductase [145, 237]) differentiates the pathogenic *M. tuberculosis* from precursor (nonpathogenic) mycobacteria (22).

The predicted capacity of infecting bacilli to subsist under different conditions and in various microenvironments offering very different substrates for energy generation argues for further research into the consequences of disrupted mycobacterial bioenergetics for host infection outcomes (238, 239). Certainly, early clinical reports of the benefits of bedaquiline for multidrug-resistant and extensively drug-resistant TB are very encouraging (234, 235). Moreover, in addition to highlighting the possible benefits of targeting components of the oxidative phosphorylation machinery for new TB drug development, knowledge about the precise mechanism of action of this compound has proved very instructive. By binding the *atpE*-encoded F_1F_0 oligomeric c-ring subunits, bedaquiline effectively functions as a nonclassic uncoupler: exposure of bacilli to the drug stimulates respiration owing to the electroneutral uncoupling of proton transport and ATP synthesis (240, 241). This is important because it suggests the potential to target the proton motive force, a property that appears to be shared by known antimycobacterial compounds (242), as well as verapamil (243). However, caution is required given recent observations that inhibition of energy metabolism might have unintended consequences in some combinations (244).

Cell Wall Homeostasis

Consistent with its dominant role in the biology of *M. tuberculosis*, the cell wall has been the subject of intensive study for decades (Table 1): as a primary site of the host-pathogen interface (245–247), as a target of—and impermeability barrier to—many current and experimental antimycobacterial drugs (248–251), and as a major determinant of the robustness and durability of the bacillus throughout its life cycle (252). The molecular composition and arrangement of the mycobacterial cell envelope, classified as Gram positive but comprising an outer membrane and a periplasmic space, is quite well understood and broadly comprises three distinct layers (251, 253, 254). Exterior to the plasma membrane, a peptidoglycan layer links covalently to a mycolyl-arabinogalactan layer which is decorated with noncovalently linked lipids and lipoglycans such as the phosphatidylinositol mannosides, phthiocerol dimycocerosates, phenolic glycolipids, and lipoarabinomannan, which have been investigated for their roles in mycobacterial pathogenicity (255) and as diagnostic markers (256, 257). This outer membrane or "mycomembrane" is, in turn, surrounded by a loosely attached capsular layer predominantly comprising polysaccharides and proteins (95, 253).

Less well understood is how the construction, maintenance, and remodeling of the cell envelope are regulated and accomplished in concert with the mycobacterial cell cycle,

particularly *in vivo* in different host microenvironments (258, 259). Here, an emerging theme is the application of advanced, live-cell and time-lapse imaging in combination with elegant fluorescent bioreporters, in most cases fluorophore-linked analogs of natural cell wall precursors which are incorporated into the cell wall without significant impairment of mycobacterial growth (260–265). Moreover, advances in mass spectrometry have enabled the cataloguing of cell wall lipids, fatty acids, and proteins with ever greater resolution (266), including under different growth conditions (267), in response to drug treatment (268), and in drug resistance and drug tolerance (94, 269).

Accessing Nutrients from the Host

The number of M. *tuberculosis* organisms required to initiate successful host infection remains unknown. Though deep sequencing suggests the possibility for transmission of multiple genotypes (270), most historical estimates are that a single bacillus might be adequate (271). This prediction has been reinforced most convincingly in NHP models in which the elegant use of genetic "barcodes" has shown that individual lesions can arise from a single seeding M. *tuberculosis* genotype (169, 170). Whatever the number, proliferation depends on the ability of the invading pathogen to assimilate host nutrients for energy consumption and macromolecular synthesis. Precisely which host-derived nutrients M. *tuberculosis* accesses, where this occurs, and how are subjects of ongoing investigations (41, 272–277). A core paradox in M. *tuberculosis* research is that, whereas the promiscuity in the nutrients and cofactors potentially assimilated from the host seems to complement M. *tuberculosis*'s autarkic metabolism (41), the same promiscuity is difficult to reconcile with the inferred impermeability of the mycobacterial cell wall. This apparent incongruity has driven the search for potential transport and porin proteins in the mycobacterial outer membrane (278, 279).

An intriguing mechanism for nutrient acquisition was proposed in a series of studies characterizing the tuberculosis necrotizing toxin (TNT), an enzyme which intracellular M. *tuberculosis* utilizes to kill the host macrophage and gain access to cytoplasmic nutrients (280–283). Synthesizing multiple lines of experimental evidence, the prevailing model holds that bacilli contained within the macrophage phagolysosome deploy CpnT, a bifunctional protein comprising an N-terminal pore-forming domain and C-terminal NAD^+ glycohydrolase (TNT), via the mycobacterial ESX-1 type VII secretion system (284) to effect macrophage death. Notably, release of TNT into the macrophage cytoplasm (the glycohydrolase is pH sensitive, and so, inactive in the acidified phagolysosome) is dependent on the ability of the other ESX-1 substrate, EsxB (also known as ESAT-6/CFP-10), to puncture the phagolysosomal membrane, therefore requiring the neat coordination of mycobacterial effectors to ensure that intraphagosomal bacilli (which, from the inserted CpnT N-terminal domain, now possess the membrane pores necessary for maximal nutrient uptake) are poised to take advantage of the inactivated host cell. In an intriguing twist, parallel work has suggested that, contrary to the connotations of its explosive moniker, the mycobacterial toxin might deliver a "blast without power" (285), resulting in necrotic cell death that might deliberately limit immunogenicity. Given the implied importance of this mechanism to the ability of M. *tuberculosis* to exploit the host macrophage, perhaps while minimizing host immune detection, it would be interesting to know whether the enhanced growth in dead macrophages described recently (172) also depends on functional TNT and, moreover, whether loss of TNT impacts pathogenicity *in vivo*.

MYCOBACTERIAL METABOLISM AND NEW TB DRUG DISCOVERY

We noted previously (286) that investigations of core metabolic processes in M. *tuberculosis* are generally framed within the context of drug discovery—whether looking specifically for new active molecules or vulnerable targets or attempting to elucidate mechanisms of action or to reveal reasons for compound failure or bypass. Examining the current TB drug pipeline, it is evident that significant progress has been made in expanding not only the number of different mechanistic classes represented in the most promising drug-target pairs, but also in validating new high-value targets for future discovery programs (134, 287). Nevertheless, the challenges for new TB drug discovery remain significant. Compound permeation (288), xenobiotic metabolism (289), and efflux (290–292) are three major innate mechanisms of drug resistance, all of which are poorly understood and correspondingly difficult to demonstrate or quantify. Impermeability of the complex mycobacterial cell envelope is often cited as a major confounder to new TB drug development. Though intuitively attractive, the evidence to support this conclusion remains scarce. In contrast, the formidable capacity of M. *tuberculosis* for biotransformation of xenobiotics is much better established (289) and, with the mostly theoretical contribution of efflux (293), might represent the major innate barrier to antibiotic activity (294).

The increasing appreciation of the organism's capacity for biotransformation has exposed the lack of rapid, medium-throughput strategies to measure metabolism of xenobiotics (289). Similarly, despite the frequency with which the inactivity of many compounds against M. *tuberculosis* is assumed to be a function of poor or failed permeation, proving so remains enormously difficult. The inability to determine quickly and reliably whether a compound—and its analogs in a structure-activity relationship analysis—has lost activity owing to differential (lost) target engagement, inactivation via metabolic transformation, extrusion by efflux, or failure to penetrate severely undermines medicinal chemistry efforts. Without complete information about the fate of the applied molecule, efforts to optimize physicochemical properties toward a drug-like lead are based almost entirely on experience, intuition, and empiricism (i.e., that which works is clearly evident in the inhibition of bacillary growth in a specific assay, usually an MIC determination *in vitro* [295]). The scarcity of techniques to enable rapid determination of the intrabacillary accumulation and metabolism of compounds, perhaps even independent of inhibitory activity, therefore presents a significant obstacle to rational drug development and suggests that strategies employed in other model systems (296, 297) might be usefully adapted to this end.

Recent work elucidating the impact of cell wall antibiotics on membrane fluidity and biogenesis (298, 299), as well as the observation that many compounds with antimycobacterial activity function as protonophores (242, 243)—in some cases, in addition to interacting directly with their cellular target activity (300)—and so operate at the cytoplasmic membrane (the site of many so-called promiscuous targets, including DprE1, QcrB, and MmpL3 [250, 301]), reinforces the sense that outer membrane impermeability might not present the major obstacle to drug efficacy. This impression will, however, require confirmation via the use of advanced metabolomics approaches to elucidate the

(differential) penetration of different compounds into the mycobacterial cytoplasm (302).

Efflux, too, presents a potentially sizeable but largely undefined obstacle. The M. *tuberculosis* genome encodes more than 40 predicted efflux pumps, only a handful of which have been implicated in innate drug resistance (232, 292, 303, 304). Recent evidence that the antihypertensive verapamil does not function as an efflux pump inhibitor but instead disrupts membrane energetics in M. *tuberculosis* (243) reinforces the perils inherent in making inferences from phenotypic observations in the absence of compelling mechanistic insight. Even ostensibly clear-cut drug-target interactions can have surprising locations (305), while the mechanism of resistance does not always correlate with the mechanism of action (306, 307).

From a metabolic perspective, incomplete understanding of M. *tuberculosis* metabolism in its human host can have very practical implications for TB drug discovery. For example, two separate studies elucidating the *guaB2*-encoded mycobacterial inosine monophosphate dehydrogenase (IMPDH) as the target of related compound series took very different views of the potential of their respective molecules for advancement as candidate drugs and, by implication, the attractiveness of *de novo* purine biosynthesis as an anti-TB target. The first (149) propounded IMPDH as "vulnerable" and, consequently, worth pursuing. In contrast, challenging pharmacokinetics, coupled with the observation that concentrations of guanine in resected lung tissue from TB patients achieve levels in excess of those required to allow bypass of IMPDH through salvage, prompted the authors of the second study to conclude the opposite. They argued that IMPDH might be "essential" but is "not vulnerable" (107), thus consigning GuaB2 and its chemical inhibitors to red flag status for new TB drug development, at least in their estimation (270). These conflicting views reinforce the fact that, even where a molecular catalogue of individual tissues, lesions, or microcompartments is available, it might not necessarily enable an unequivocal prediction of what the bacillus is able to access and assimilate from its surroundings. That is, the *availability* of any nutrient must be distinguished from its *accessibility* to the infecting M. *tuberculosis* population. Of course, when prioritizing resources for new drug candidates, it is prudent to assume that all nutrients, if present, are (potentially) accessible. The risk that salvage might overcome antibiotic-mediated nutrient starvation therefore ensures that the bar is set very high for new TB drug development (308).

Harnessing Metabolism to Potentiate Drug Activity

Earlier sections highlighted some of the ways in which different intrinsic metabolic functions contribute to the innate resistance of M. *tuberculosis* to antibiotics, reinforcing a theme which has been discussed extensively elsewhere (65, 309, 310). Increasingly, however, knowledge of metabolic function is being exploited not only to circumvent innate resistance mechanisms, but also to understand the metabolic functions which might subvert antibiotic efficacy so as to identify novel countermeasures (64, 311, 312). Recent work highlighting the contribution of altered propionate metabolism to the emergence of drug resistance in M. *tuberculosis* (313) provides a very important example of the potential for metabolic deficiency to confer a tolerant phenotype that enables the acquisition and fixation of genetic drug resistance. Moreover, the growing number of studies demonstrating altered metabolic function in drug-resistant organisms, including M. *tuberculosis* (269, 314), suggests that this phenomenon might even be more widespread.

A corollary to the above is that metabolism might also be harnessed to improve drug action. For example, the inferred mechanism of action of the repurposed agent, clofazimine, which triggers a redox cycling mechanism that generates toxic reactive oxygen species (315), reinforces the notion that bacilli might be trapped in a futile and ultimately destructive metabolism. Notably, by combining clofazimine with other compounds targeting components of the mycobacterial electron transport chain, Steyn and colleagues demonstrated the potential to enhance killing of M. *tuberculosis* in an intracellular model (43). In an analogous example of this approach, inactivation of the essential maltosyltransferase, GlgE, was shown to trigger an amplification loop which resulted in rapid mycobacterial death owing to self-poisoning by maltose 1-phosphate accumulation (316). Though the precise mechanism differs in each case, these observations suggest the potential for combination regimens based on drug targets and/or mechanisms of action which incorporate inhibition of growth (target/drug A) and prevention of ordered metabolic shutdown (target/drug B); that is, combinations designed to prevent metabolic escape. This strategy is distinct from both metabolic bypass (in which an alternative, perhaps less efficient or autonomous, mechanism exists to enable generation of the same essential metabolite, e.g., via scavenging from the host) and metabolic buffering (in which partially redundant mechanisms enable the organism to maintain homeostasis). Instead, it entails eliminating the organism's inherent capacity for surviving through replicative and metabolic quiescence and so might add a further element to the three models for antimycobacterial drug synergy proposed previously (65). A recent report describing the identification of chemical inhibitors of the stringent response enzyme, Rel_{Mtb}, a (p)ppGpp synthetase, provides a cogent demonstration of the potential of this therapeutic approach (317).

To some extent, this strategy overlaps with the idea, summarized in a recent review, of developing "metabolism-targeted adjuvant therapies" to prevent antibiotic tolerance (311). In support of that proposal, the authors cite the targeted disruption of regulatory mechanisms, for example, through the identification of artemisinin analogs as inhibitors of the mycobacterial DosRST system (318), an ~50-gene regulon that has been implicated in antibiotic tolerance in infection models *in vitro* and *in vivo* (319). In addition, they provide several illustrations of the potential to achieve or enhance antimycobacterial activity by interfering with bacillary metabolism. These include the direct inhibition of specific "tolerance/persistence" effectors such as isocitrate lyase (320) or nicotinamide adenine dinucleotide synthetase, NadE (321). Of particular interest, though, is the proposition that stimulating bacillary respiration and energy metabolism, for example, by providing a glycolytic substrate together with an alternative terminal electron acceptor such as fumarate (322), can increase efficacy of known antibacterial agents against apparently recalcitrant subpopulations. This concept not only reinforces the impact of metabolic state on antibiotic efficacy (an obvious conclusion), but it also suggests the potential to manipulate bacillary metabolism artificially through the deliberate provision of nutrients which might drive a defined metabolic program that ensures susceptibility to the applied agent. That is, it opens the door for a combination comprising an (inhibitory) antibiotic and a (stimulatory) metabolite.

FUTURE PERSPECTIVES

This review has highlighted the complications inherent in working with an infectious pathogen whose preferred

anatomical target (i.e., the human lung) is difficult to access. The result is that most studies, rather than revealing mycobacterial metabolic function in the context of TB disease, have tended to elucidate the metabolic capacity of the bacillus, either *in vitro* in different growth media and under various stresses thought to mimic the intrahost environment or *in vivo* in experimental models of infection (37). Those attempts, much fewer in number, to investigate M. *tuberculosis* pathogenicity (including metabolism) *in situ* or closer to the site of disease have predominantly relied on bacilli captured in sputum or bronchoalveolar lavage fluid, lymph node or other biopsies, or lung resections of treated patients. One consequence is that the potential distinction between primary and post-primary TB as separate disease entities (and therefore likely invoking different mycobacterial metabolic functions) has been obscured (69, 79).

As noted above, determining the metabolic and physiological states adopted by the tubercle bacillus in the infected human host represents the ultimate goal and, though still a significant challenge, seems increasingly attainable with the development of new technologies. Recent advances such as the ability to obtain transcriptional data from very-low-abundance RNA (258, 323) and at the single-cell level (324) promise to provide much greater insight into the exchange of stimuli and responses that occurs between M. *tuberculosis* and its host cell during intracellular infection and which shapes the metabolic adaptation of the invading bacilli to the available nutrients in this and other microenvironments. Exciting advances in other bacterial models, including the combined application of RNA profiling with stable isotope labelling (325, 326), reinforce the sense that elucidating the growth rates—and metabolic states—of even slow-growing pathogens such as M. *tuberculosis* will become feasible (327).

A recent systems pharmacology study (198) suggests another approach to this problem, namely, the potential to combine empirical data derived from *in vitro* and *in vivo* experiments—ideally including clinical samples (197, 328)—to generate a complex mathematical model that simulates disease progression within a single locus. In that case, a shift was made from the M. *tuberculosis*-infected macrophage as the "unit of infection" (155) to the granuloma in modeling the efficacies of different fluoroquinolone antibiotics as anti-TB agents. Their approach enabled a compelling demonstration of the capacity to incorporate both host and bacillary factors in building the model and, at the same time, raised the possibility that this strategy could be adapted to (or informed by) metabolomic, proteomic, and lipidomic data. Notably, the study also highlighted the emergent properties of a complex system comprising infecting bacilli and different types of host immune cells (the variety was necessarily limited to a handful of T cell subsets and macrophage phenotypes owing to the demands of computational modeling). Again, this suggests the potential to enable key insights into the dominant metabolic pathways active in different lesions, how these might influence infection trajectories and pathological outcomes, and how they might be exploited for improved chemotherapy.

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Protein Export into and across the Atypical Diderm Cell Envelope of Mycobacteria

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68

INTRODUCTION

Mycobacteria are *Actinobacteria*, which is a phylum of high-GC Gram-positive bacteria. Among the wide range of *Mycobacterium* spp. are several important pathogens. Most notable of these is *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB). Although the number of TB cases and deaths show a declining trend in the past decade, the World Health Organization recently warned that "current actions and investments in research are falling far short" (http://www.who.int/mediacentre/news/releases/2016/tuberculosis-investments-short/en/). Over 1.3 million deaths and up to 10.0 million new infections were attributed to *M. tuberculosis* in 2017, which makes *M. tuberculosis* the most deadly infectious agent in the world, surpassing HIV and the malaria parasite (1). One issue facing efforts to control TB is that the live attenuated bacillus Calmette-Guérin (BCG) vaccine strain is not able to provide lifelong protection against *M. tuberculosis* (2). Another problem is the increased incidence of infections caused by multidrug-resistant *M. tuberculosis* strains. Consequently, major research efforts focus on understanding *M. tuberculosis* pathogenesis and physiology to advance the development of new anti-TB vaccines and antibiotics.

M. tuberculosis is a member of the order *Corynebacteriales*, which is an order that is characterized by its resistance to many classic antibiotics and its ability to withstand highly stressful environments, such as oxidative stress, desiccants, and common disinfectants (3, 4). These features can be explained by the presence of a unique cell envelope, which differs from that of both typical Gram-positive (low-GC), bacteria also called *Firmicutes*, and Gram-negative bacteria. While members of the *Corynebacteriales* have a traditional inner membrane, their peptidoglycan layer is connected to an additional polymer of arabinose and galactose (i.e., an arabinogalactan layer), which in turn is linked to mycolic acids, unusually long fatty acids that can contain up to 90 carbon atoms (Fig. 1) (5–8). It is now widely accepted that these mycolic acids are the main constituents of a second (outer) membrane, which also contains noncovalently linked (free) lipids such as phthiocerol dimycocerosates and phenolic glycolipids. Finally, mycobacteria have a more loosely attached capsular layer, which mainly consists of polysaccharides and proteins (9). Although the presence of an outer membrane in mycobacteria was initially proposed in 1982 (10), its existence was not confirmed until 2008 by electron microscopy (EM) imaging (7, 11). Thus, although the order *Corynebacteriales* belongs to the group of high-GC Gram-positive bacteria, their double-membrane, or diderm, cell envelope is physically more similar to the inner and outer membrane-containing cell envelope of Gram-negative bacteria, because EM images of the two reveal a resemblance. However, despite the resemblance, the mycolic acid outer membrane of *Corynebacteriales* differs dramatically from the Gram-negative outer membrane, the latter being composed of phospholipids and lipopolysaccharides.

The firm bond of the long mycolic acids to the underlying arabinogalactan and peptidoglycan structures results in very low fluidity of the outer membrane, which contributes to the high impermeability of the *Corynebacteriales* cell envelope (5). Although this characteristic provides excellent protection from extreme environmental conditions, it also requires *Corynebacteriales* species to have pathways to import and export molecules. In line with the notion that the mycolic acid-containing membrane is functionally comparable to the outer membrane of Gram-negative bacteria, both mycobacteria and corynebacteria possess outer membrane porins for importing hydrophilic nutrients through the outer membrane (12–14). At the same time, the unique cell envelope of *Corynebacteriales* necessitates the existence of special pathways for secreting proteins into the environment.

In this article, we will review the different pathways mycobacteria use to export proteins from the cytoplasm into the cell envelope or beyond into the extracellular environment (i.e., to secrete them across the outer membrane) (Fig. 1). Four types of protein export pathways are currently known in mycobacteria: two highly conserved systems that exist across Gram-positive and Gram-negative bacteria (the Sec and Tat pathways) and two specialized systems that exist in mycobacteria, corynebacteria, and a subset of low-GC Gram-positive bacteria (the SecA2 and type VII secretion [T7S] pathways). The Sec, Tat, and SecA2 pathways serve to transport proteins across the inner membrane, and whether an exported protein remains in the putative periplasmic space between the two membranes or reaches the capsule or extracellular environment is determined by currently unknown secondary mechanisms. T7S pathways differ in that, so far, periplasmic localization of substrates has not been observed, suggesting that this pathway may secrete proteins beyond the outer membrane. However, here too,

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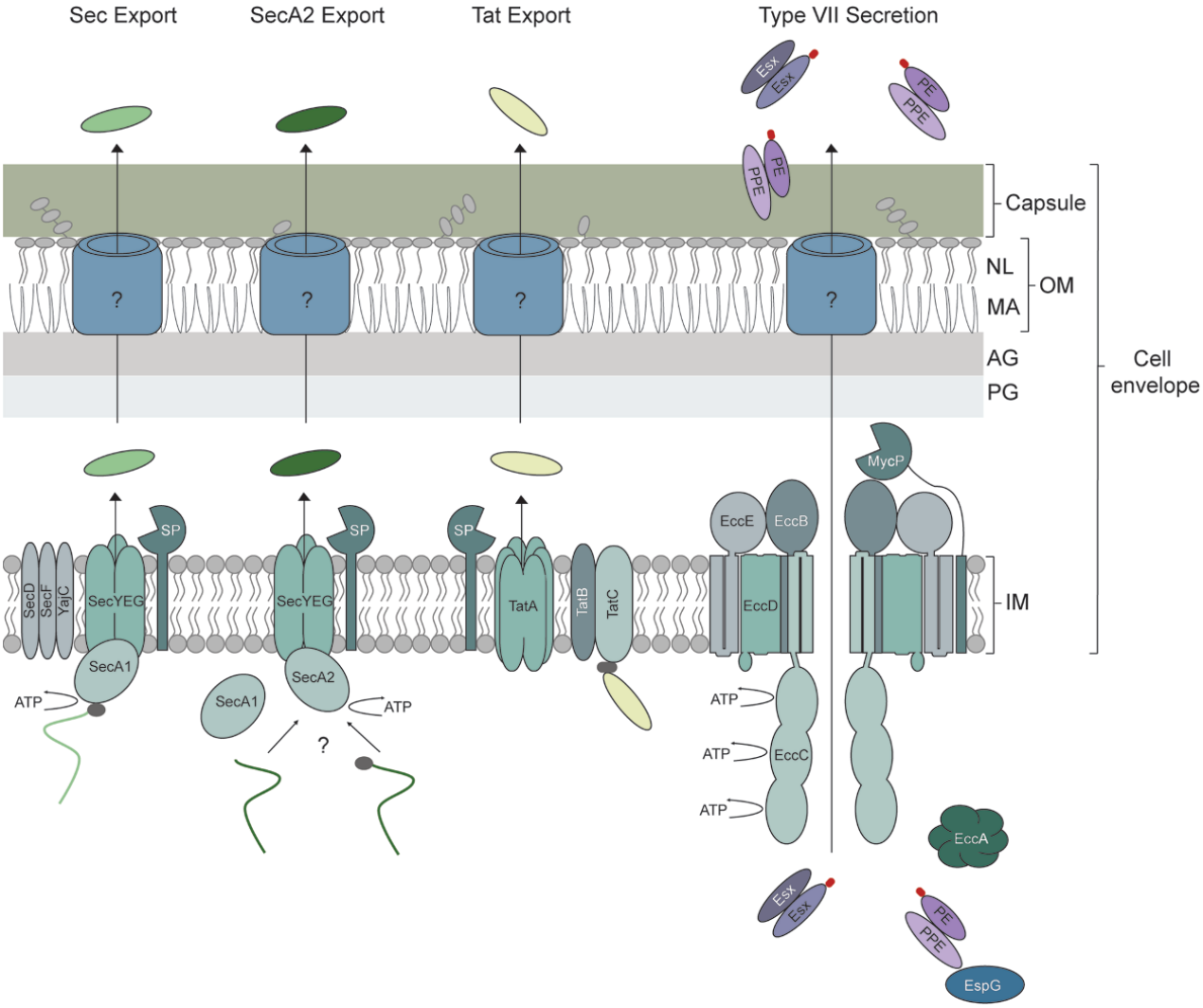


FIGURE 1 Model of the mycobacterial cell envelope and export systems. Although the mycobacterial cell envelope contains a traditional cytosolic (inner) membrane, the peptidoglycan layer is covalently linked to an arabinogalactan layer consisting of arabinose and galactose, which in turn is covalently linked to mycolic acids. These unusually long fatty acids (up to 90 carbon atoms) are one of the main components of the outer membrane, which also contains noncovalently bound (free) lipids. The final layer of the cell envelope is the capsule, mainly consisting of polysaccharides and proteins. The cell envelope is highly impermeable and unique to other Gram-positive bacteria. To export proteins into and across the cell envelope, mycobacteria have four systems, Sec, SecA2, Tat, and type VII secretion (T7S). The Sec pathway exports unfolded proteins across the inner membrane, and the substrates bind to an ATPase, SecA1, which targets the substrates to the translocation channel consisting of SecYEG and provides energy for translocation. The additional membrane components SecD, SecF, and YajC increase the efficiency of export. Upon translocation across the inner membrane, the N-terminal signal peptide (depicted in gray) is removed by a signal peptidase (SP). Less is known about the SecA2 pathway. Substrates are dependent on the ATPase SecA2; however, studies show that they also utilize the SecYEG channel and possibly SecA1, as well. The list of SecA2-dependent exported proteins includes examples with and without a signal peptide. The third export system, the Tat pathway, exports folded proteins. The Tat substrates, containing an N-terminal signal peptide, with a pair of arginine residues, is targeted to the membrane components TatBC, which then recruit homo-oligomers of TatA for subsequent transportation across the membrane. Similar to Sec, the signal peptide is removed by a signal peptidase. The T7S system consists of five conserved membrane components, of which EccBCDE form the secretion complex. EccC is the ATPase, providing the required energy for the secretion process. Although the mycosin protease (MycP) is not an integral component of the secretion complex, it associates with the complex and is essential for successful secretion. The substrates (Esx and PE-PPE) are secreted as heterodimers and are targeted to the secretion complex by a conserved secretion signal that includes a YxxxD/E motif (depicted in red), whereas the cytosolic chaperone EspG is required for directing the PE-PPE pairs to their specific T7S. The role of the second conserved cytosolic component, EccA, remains uncertain. It is currently unknown how Sec, SecA2, Tat, and T7S substrates are secreted across the outer membrane into the capsular layer or culture supernatant. IM, inner membrane; PG, peptidoglycan layer; AG, arabinogalactan layer; MA, mycolic acids; NL, noncovalently bound lipids; OM, outer membrane.

the mechanism for crossing the outer membrane remains a mystery.

THE MYCOBACTERIAL SEC PATHWAY

Bacteria export a large proportion of their proteome to the cell envelope or extracellular environment (~20 to 35%) (15, 16). In all bacteria, the majority of this protein export is carried out by the general Sec pathway (16). Mycobacteria have ~1,000 predicted Sec-exported proteins (this number includes proteins exported across as well as into the inner membrane), many of which have been experimentally demonstrated to be exported (17, 18). Among the many proteins exported by the Sec pathway are proteins with essential functions in the cell envelope (i.e., nutrient acquisition, cell wall biogenesis); consequently, in all bacteria the Sec pathway is essential for viability (19). Further, there are Sec-exported proteins with critical roles in host-pathogen interactions, making the Sec pathway critical for bacterial pathogenesis as well (17, 18, 20). The Sec pathway is highly conserved in bacteria, and most of our understanding of this pathway comes from studies of the *Escherichia coli* system (for recent reviews of the Sec system see references 21 and 22). Mycobacteria possess conserved orthologues of all the critical Sec pathway components, and as expected, these Sec orthologues are either proven or predicted by saturating mutagenesis transposon-site hybridization or transposon insertion sequencing studies to be essential (23). While only a few mycobacterial Sec proteins have been directly studied, the results so far indicate that the Sec pathway of mycobacteria functions similarly to the Sec pathways of other bacteria.

The Sec pathway transports proteins across the inner membrane. Sec-exported proteins can remain in the cell envelope or through subsequent mechanisms transit across the additional cell envelope layers and be fully secreted. Sec-exported proteins travel across the inner membrane through a channel composed of the integral membrane proteins SecY, SecE, and SecG (Fig. 1), in which SecY forms the central core (24). Sec-exported proteins are in an unfolded state during their translocation through the channel (25). SecY and SecE are sufficient to achieve translocation, while SecG improves the efficiency of export (26–28). There are also additional membrane components that improve Sec export efficiency: SecD, SecF, and YajC (29). The SecA ATPase provides energy for protein translocation (30, 31). SecA binds to Sec-exported proteins in the cytoplasm, targets them to SecYEG in the membrane, and harnesses energy from repeated rounds of ATP binding and hydrolysis to drive stepwise export of unfolded proteins through the SecYEG channel in the inner membrane (32). For mycobacteria, the second step a Sec-exported protein may take to cross the outer membrane remains completely unknown.

While the majority of bacteria have a single essential SecA, mycobacteria and a subset of other Gram-positive bacteria have two SecA proteins (named SecA1 and SecA2) (33–35). As demonstrated in mycobacteria, the two SecAs have unique functions. SecA1 functions in the general Sec pathway, as described above. SecA2 is a specialized SecA and is discussed later in the article. Consistent with SecA1 being the SecA of the essential general Sec pathway, SecA1 of *Mycobacterium smegmatis* and *M. tuberculosis* is essential for viability (33, 36; L. Rank and M. Braunstein, unpublished). SecA1 exports proteins with Sec signal peptides (introduced below) (36) and has ATPase activity, as expected for it being the canonical SecA (37).

Sec export is a posttranslational process. Following protein synthesis, Sec-exported proteins exist in the cytoplasm as unfolded preproteins with N-terminal signal peptides that are ~25 to 30 amino acids long (38). The Sec signal peptide is composed of a positively charged N-terminus, a hydrophobic central domain, and an uncharged polar C-terminus containing a signal peptidase cleavage site (39). For lipoproteins exported by the Sec pathway, there is also a lipobox motif at the C-terminus of the signal peptide with an invariant cysteine that serves as the site of lipid attachment and anchoring of the protein to the cell envelope postexport (40). In association with export, the signal peptide is removed from the preprotein by one of two signal peptidases (LepB for the majority of Sec preproteins and LspA for lipoprotein preproteins specifically), which serves to produce the cleaved, mature protein species on the extracytoplasmic side of the membrane (39). As is the case in all other bacteria studied to date, LepB of *M. tuberculosis* is essential (41). On the other hand, LspA of *M. tuberculosis* is not essential, although it is important for pathogenesis (42, 43). The dispensability of LspA is also the case for Gram-positive *Firmicutes*, while in Gram-negative bacteria, LspA is essential (44).

The general principles of signal peptides established for other bacteria also apply to mycobacterial proteins exported by the Sec pathway, as shown by *in silico* prediction programs for Sec signal peptides being good predictors of *M. tuberculosis*-exported proteins (18) and by comparing *in silico* predictions to experimentally determined N-terminal signal peptides of *M. tuberculosis* (45).

In addition to possessing a signal peptide, another required feature of Sec-exported proteins is that they must be unfolded to be exported (25, 46, 47). Cytosolic chaperones can help preproteins maintain an unfolded and translocation-competent state, and the SecA-SecYEG translocase may also play a role (48, 49). Gram-negative bacteria have a SecB chaperone that maintains a subset of preproteins in an unfolded state compatible with transport through SecYEG (49, 50). SecB also has a role in delivering preproteins to SecA (51). However, SecB is not present in Gram-positive bacteria. In *M. tuberculosis*, there is a SecB-like protein (Rv1957); however, Rv1957 is not conserved across mycobacteria, and the available data indicate that Rv1957 is a chaperone for the HigBA toxin-antitoxin system and not a protein export chaperone (52).

In addition to the above described posttranslational export of proteins across the inner membrane, the Sec pathway also participates in cotranslational export of transmembrane domain (TMD)-containing integral membrane proteins into the membrane (53). With membrane proteins, once the TMD emerges from the ribosome, the nascent polypeptide is recognized by the signal recognition particle (SRP), (comprising the Ffh protein and the 4.5S RNA), which then delivers the ribosome-mRNA-nascent protein complex to the SRP receptor FtsY (54). The SRP receptor in turn delivers the nascent protein to the SecYEG channel for translocation. For integral membrane proteins, there is a lateral gate in SecY that allows TMDs to transfer out of the channel and integrate into the inner membrane through a process that is facilitated by the integral membrane YidC protein (55). Specifically, for membrane proteins with large periplasmic domains, SecA may assist in exporting these domains across the membrane (56). Both Ffh and FtsY are GTPases, and GTP hydrolysis is required for membrane protein delivery (57). Using purified proteins, *M. tuberculosis* Ffh and FtsY are shown to possess functions of their *E. coli* counterparts; they possess GTPase activity and interact with one another and with the 4.5S RNA of *M. tuberculosis* (58, 59). Working with a conditional *yidC* depletion strain of *M. tuberculosis*, YidC was demonstrated to be required for

growth and membrane protein localization, as expected for a YidC ortholog (60). However, in the fast-growing *M. smegmatis*, *yidC* depletion has no effect on growth (60).

THE MYCOBACTERIAL Tat PATHWAY

The twin-arginine translocase (Tat) pathway is another conserved export system of bacteria. Again, our understanding of the Tat pathway comes largely from studies of the *E. coli* system (for a recent review see reference 61). Similar to the Sec pathway, after transport from the cytoplasm, Tat-exported proteins can either remain in the cell envelope or be fully secreted via a subsequent mechanism. Here too, the pathway for crossing the outer membrane remains a mystery. Proteins exported by the Tat pathway are synthesized as preproteins with cleavable N-terminal signal peptides. However, as the name of the pathway reflects, Tat signal peptides are distinguished from Sec signal peptides by a Tat motif containing a pair of twin arginine residues (see below) (62). Compared to the Sec pathway, fewer total proteins are exported by the Tat pathway. Some *Streptomyces* strains are estimated to have ~150 proteins exported by the Tat pathway, and *M. tuberculosis* may have as many as 95 proteins (63), whereas other bacteria have far fewer or lack a Tat pathway completely (64, 65). Most notably, the Tat pathway is fundamentally different from the Sec pathway in that folded, rather than unfolded, proteins are exported (66). The studies conducted so far of the mechanism of Tat export in mycobacteria indicate that the Tat system of mycobacteria functions similarly to the Tat pathways of other bacteria.

Like in *E. coli*, in high-GC Gram-positive actinobacteria, including mycobacteria, the Tat pathway is made up of three integral membrane proteins: TatA, TatB, and TatC (Fig. 1) (67–69). TatA and TatB are small homologous membrane proteins, each containing a single TMD. TatC is a larger integral membrane protein (70). In Firmicutes, however, the Tat pathway is a streamlined system with only TatA and TatC (65).

Tat preproteins are recognized by a complex of TatB and TatC, with TatC recognizing the twin arginine signal peptide (71, 72). Once a preprotein is bound to TatBC, homo-oligomers of TatA are recruited to the complex (71) and the Tat preprotein is transported across the membrane. Tat export is a posttranslational process, and the energy required to drive Tat transport comes from the proton motive force (73, 74). The mechanism of transit across the membrane remains a significant unknown. Some models propose that TatA oligomers form a substrate-fitted protein-conducting channel (75) that accommodates folded proteins of different sizes and shapes. An alternative model proposes that TatA multimers lead to destabilization of the membrane in the vicinity of the TatBC-substrate complex and thereby allow TatC-driven translocation of the substrate without the need of a channel (76). Following export, Tat preproteins undergo signal peptide cleavage by the same signal peptidases that work with the Sec pathway: LepB or LspA (77, 78).

Tat signal peptides resemble Sec signal peptides in having a positively charged N-terminus, a hydrophobic central domain, and an uncharged polar C-terminus containing a cleavage site. However, Tat signal peptides are distinguished from Sec signal peptides by the presence of a Tat motif with twin arginines (R-R-X-Φ-Φ [Φ = hydrophobic]) (62). Substitution of the twin arginines with other residues prevents export of most Tat-dependent proteins, but there are a few examples of Tat-exported proteins with only one arginine (79–81). In mycobacteria, Tat signal peptides appear to follow the same rules established in other bacteria. So far, 18 *M. tuberculosis* proteins are confirmed as having functional Tat signal peptides (for a list see reference 23). Working with the endogenous β-lactamase of *M. tuberculosis* (BlaC), which is a Tat-exported protein, the requirement of the twin RR pair for mycobacterial Tat export was confirmed (63, 67). Since β-lactamases must be exported to protect bacteria against β-lactam antibiotics, the requirement of the Tat pathway for BlaC export contributes to the natural resistance of *M. tuberculosis* to β-lactams.

In addition to a Tat signal peptide, the second required feature of Tat-dependent proteins is that they are folded prior to export. Unfolded or misfolded proteins are generally unacceptable for export, suggesting that the Tat export system has a "proofreading" ability that restricts export to properly folded proteins (66, 82).

In addition to fully exporting proteins across the membrane, the Tat pathway plays a role in the delivery of a small subset of integral membrane proteins into the inner membrane. These cases either involve a protein with a Tat signal peptide and a C-terminal TMD, with the latter becoming integrated into the membrane during translocation, or a protein with a noncleavable N-terminal Tat signal peptide that remains anchored in the membrane (65, 83, 84). Most recently, examples of integral membrane proteins that require both the Sec and Tat pathways to deliver and transport across the membrane multiple periplasmic domains were identified. The role of the Tat pathway in this interesting subset of proteins is to translocate periplasmic domains that, similar to many soluble Tat substrates, must bind a cofactor in the cytoplasm and, therefore, fold prior to export. An internal Tat motif is present in such Tat-dependent periplasmic domains. The first example of a dual Sec- and Tat-dependent membrane protein was the *Streptomyces* Rieske iron-sulfur binding protein, which is a subunit of the cytochrome bc_1 complex (85).

In general, the Tat pathway is not essential for bacterial viability, and *tat* mutants are viable, at least in standard laboratory media (65). This is not the case, however, for *M. tuberculosis*, which provides one of the few examples of a bacterium with an essential Tat pathway (69). In fact, even in the fast-growing and nonpathogenic mycobacterium *M. smegmatis*, the Tat system is not essential, although *tatA*, *tatB*, and *tatC* deletion mutants have *in vitro* growth defects (67, 68). A possible explanation for the essentiality of the *M. tuberculosis* Tat pathway is that, like the case in *Streptomyces*, the mycobacterial Tat pathway may be required for export of the iron-sulfur-bound periplasmic domain of the Rieske membrane protein (i.e., QcrA) subunit of cytochrome bc_1 since in *M. tuberculosis* the cytochrome bc_1 complex is essential (86). In support of this possibility, the iron-sulfur binding domain of *M. tuberculosis* QcrA has an internal Tat motif (RRKLI).

THE MYCOBACTERIAL SECA2 PATHWAY

Mycobacteria are unusual in having two SecA homologues, but they are not the only bacteria with two SecAs. Along with other *Actinobacteria* (e.g., corynebacteria) (87), there is a subset of *Firmicutes*, including *Listeria* (88), *Clostridioides* (89), *Staphylococcus* (90), some *Streptococci* (91–93), and some *Bacilli* (94) with two SecAs. In all these cases, SecA1 is the name given to the canonical and essential SecA ATPase of the general Sec pathway, and SecA2 refers to a specialized SecA that promotes export of a relatively small set of proteins. It is important to note that not all SecA2 systems are similar or evolutionarily conserved (35, 95). SecA2 systems can be divided into at least two groups: mycobacteria,

Listeria monocytogenes, and *Clostridioides difficile* provide examples of SecA2 systems in which SecA2 appears to work with the canonical SecYEG channel of the general Sec pathway to export a small but diverse set of proteins (89, 96, 97). These systems are called multisubstrate SecA2 systems or SecA2-only systems, the latter name referring to their lack of a second SecY (SecY2) in the system. *Staphylococcus* and *Streptococci* are examples of bacteria with SecA2 systems that include an accessory SecY (SecY2) and additional accessory Sec proteins (98). These latter systems export a single large glycosylated substrate, and they are called accessory Sec (aSec) or SecA2-SecY2 systems and will not be discussed further here.

Composition of the Mycobacterial SecA2 Pathway

The SecA2 pathway exists in all mycobacteria. In contrast to SecA1 of the general Sec pathway, the role of SecA2 in mycobacterial protein export is limited to a smaller set of proteins, and SecA2 is not essential for mycobacterial growth *in vitro* (99–102). In pathogenic mycobacteria, SecA2 is required for pathogenesis (99–101). Early studies of the SecAs of mycobacteria demonstrated that SecA2 and SecA1 are functionally distinct proteins. Even if SecA2 is overexpressed, it cannot replace the requirement for SecA1, and if SecA1 is overexpressed, it cannot carry out the function of SecA2 (33). The functional differences between SecA1 and SecA2 remain a big unknown. Both SecAs have ATPase activity (37), and the crystal structures of *M. tuberculosis* SecA1 and SecA2 reveal a surprisingly high level of structural similarity, with both SecAs possessing the functional domains of canonical SecAs (103).

As shown with SecA2 variants harboring amino acid substitutions in the ATP binding site of *M. smegmatis* SecA2 (K129R) or *M. tuberculosis* SecA2 (K115R), ATP binding is required for SecA2 to carry out its function in protein export and pathogenesis (37, 104). Interestingly, *secA2 K129R* in *M. smegmatis* is dominant negative and associated with more severe phenotypes than a *secA2* null mutant. Moreover, SecA2 K129R is associated with reduced levels of the sole SecY of mycobacteria, and suppressors of the severe *secA2 K129R* phenotypes map to *secY* (96). These results support a model whereby the severe phenotypes of SecA2 K129R result from a detrimental interaction with the canonical SecYEG channel of the general Sec pathway. By extension, these effects of SecA2 K129R argue for SecA2 exporting proteins through the same SecYEG channel that is used by SecA1 (Fig. 1). Data from *L. monocytogenes* and *C. difficile* are consistent with SecA2 working with the canonical SecYEG channel in these other multisubstrate SecA2-only systems as well (89, 97).

Along with SecY, SecA1 may also be important for mycobacterial SecA2-dependent export. SecA proteins are known to dimerize, and a recent study demonstrated heterodimer (as well as homodimer) formation *in vitro* with *M. tuberculosis* SecA1 and SecA2 proteins purified from *E. coli* (105). While it remains to be demonstrated if SecA1-SecA2 heterodimers are biologically relevant, another finding in support of a role of SecA1 in the SecA2 pathway is that export of SecA2-dependent substrates in *M. smegmatis* is compromised when SecA1 is depleted. However, it is also possible that this latter result reflects a role for SecA1 in delivering components of the SecYEG channel to their membrane location (104).

The convergence of SecA1 and SecA2 on the same translocation channel raises the question of what distinguishes the SecA2 pathway from the general Sec pathway. We expect there are distinctive features of SecA2 substrates and additional components of the SecA2 pathway that distinguish it from the general Sec pathway.

The Multisubstrates of the Mycobacterial SecA2 Pathway

Through a combination of discovery proteomics and direct testing of candidates, proteins that are exported by the SecA2 pathway have been identified in *M. tuberculosis*, *Mycobacterium marinum*, and *M. smegmatis* (99, 101, 102, 106). Across these species, there are currently 15 validated examples of proteins that are exported in a SecA2-dependent manner (Table 1). Some of these proteins localize to the cell envelope, and others are fully secreted. From proteomics experiments, there are additional candidates for SecA2-dependent proteins that remain to be validated. The current list of SecA2-dependent proteins includes examples of proteins with or without predicted N-terminal signal peptides or TMDs. In the SecA2-only system of *L. monocytogenes*, the list of proteins identified as SecA2 dependent also includes examples with or without signal peptides (107). The list of proteins exported by the mycobacterial SecA2 pathway reveals functional themes.

Solute binding proteins (SBPs), a category of transporter proteins, are exported by the SecA2 pathways of *M. tuberculosis*, *M. marinum*, and *M. smegmatis* (99, 101, 102, 106). SBPs are exported into the cell envelope to carry out functions in delivering solutes to membrane-localized ABC (ATP-binding cassette) transporters for import into the cell. The first SBPs identified as exported by the SecA2 pathway are the *M. smegmatis* Msmeg1704 and Msmeg1712 proteins (106), which are predicted to function in sugar import. SBPs are also identified as being exported by the SecA2 pathways of *M. tuberculosis* and *M. marinum* (101, 102). Of 15 SBPs identified by quantitative proteomics of *M. tuberculosis*, 13 are detected as being exported in a SecA2-dependent manner (102). SBPs are also on the list of SecA2-exported proteins for the *L. monocytogenes* SecA2-only system (107). It is worth noting that SBPs exist across bacteria (i.e., they are not always exported by SecA2 systems). The SecA2-dependent SBPs of mycobacteria possess recognizable N-terminal signal peptides containing a lipobox motif, with the majority having predicted Sec signal peptides. However, 4 of the 13 SecA2-dependent SBPs of *M. tuberculosis* are predicted to have Tat signal peptides (102).

Mce proteins, another category of transporter proteins, are also exported in a SecA2-dependent manner in mycobacteria (101, 102). Comparative proteomics of cell envelope fractions of wild-type versus *secA2* mutant *M. tuberculosis* and *M. marinum* strains identified numerous components of cell envelope-localized Mce transporters as SecA2-dependent; Mce components are also SecA2-dependent in *M. smegmatis* (L. Rank and M. Braunstein, unpublished). These exported Mce components possess either a signal peptide or transmembrane domain. Mce transporters are thought to function similarly to ABC transporters in that they recognize an extracytoplasmic solute (in this case, a lipid) and deliver it to a membrane complex that imports it across the membrane using ATP hydrolysis (108). In an interesting similarity to SecA2-dependent SBPs, each Mce transporter (*M. tuberculosis* has four Mce transporters) includes six exported Mce proteins that are thought to function like SBPs. It remains to be resolved if SecA2 is required for export of numerous individual components of Mce transporter complexes or if the role of SecA2 is to export one or a small number of Mce proteins that are required to stabilize the entire Mce complex

TABLE 1 Validated examples of SecA2-dependent exported proteins of mycobacteria

Protein	Signal peptide/ transmembrane[a]	Species	Function	Reference
Ms1704	SP	*M. smegmatis*	SBP	106
Ms1712	SP	*M. smegmatis*	SBP	106
PhoS1 (Rv0928)	SP	*M. tuberculosis*	SBP	102
DppA (Mmar_5154)	SP	*M. marinum*	SBP	101
Mce1A (Rv0169)	TM	*M. tuberculosis*	Mce transporter component	102
Mce1C (Rv0171)	SP	*M. tuberculosis*	Mce transporter component	102
Mce1E (Rv0173)	SP	*M. tuberculosis*	Mce transporter component	102
Mce1F (Rv0174)	TM	*M. tuberculosis*	Mce transporter component	102
SapM (Rv3310)	SP	*M. tuberculosis*	Phosphatase effector	111
PknG (Rv0410c)	None	*M. tuberculosis* *M. marinum*	Kinase effector	101, 102
LipO (Rv1426c)	SP	*M. tuberculosis*	Predicted esterase (possible effector)	102
SodA (Rv3846)	None	*M. tuberculosis*	Superoxide dismutase	99, 293, 294
KatG (Rv1908c)	None	*M. tuberculosis*	Catalase-peroxidase	99
LipA (Mmar_2284)	SP	*M. marinum*	Cell wall hydrolase	99
MMAR_3060	SP	*M. marinum*	Conserved hypothetical	101

[a]SP, Signal peptide; TM, transmembrane.

in the cell envelope. In the latter scenario, a defect in the export of a single Mce protein may account for the observed reduction in abundance of numerous Mce components in a *secA2* mutant.

Effectors of phagosome maturation arrest are another category of protein exported by the SecA2 pathway. A hallmark of pathogenic mycobacteria that survive in macrophages, such as *M. tuberculosis* and *M. marinum*, is their ability to arrest the phagosomes in which they reside from maturing and fusing with degradative lysosomes (109). As a result, pathogenic mycobacteria are in a phagosomal environment that is permissive for replication and sequestered from antigen-presenting compartments that drive immune responses. Phagosome maturation arrest is a complex process involving numerous *M. tuberculosis* proteins (i.e., effectors) that must be exported to reach their site of action at the host-pathogen interface. In *M. tuberculosis* and *M. marinum*, the SecA2 pathway is required for phagosome maturation arrest (101, 110), and SapM and PknG are two SecA2-dependent effectors of this process (101, 111). SapM is a secreted phosphatase that dephosphorylates phosphatidyl inositol-3-phosphate and thereby limits phagosomal recruitment of host factors that drive downstream phagosome fusion and maturation events (112). PknG is a eukaryotic-like serine threonine kinase that is found in both the cytoplasm and cell envelope of pathogenic mycobacteria (113). From its exported location, PknG has a poorly defined role in phagosome maturation arrest (114), and from its cytoplasmic location it has better-characterized roles in metabolism and redox homeostasis (113, 115, 116). Through experiments in which wild-type levels of exported SapM and/or PknG were restored to the *secA2* mutant of *M. tuberculosis* or *M. marinum*, the role of the SecA2 pathway in exporting these proteins was proven to be required for phagosome maturation arrest (101, 111). However, since the combined effect of restored SapM and PknG export is not sufficient to fully restore phagosome maturation arrest to the *secA2* mutant, additional SecA2-dependent effectors must exist. A candidate for an additional factor is the *M. tuberculosis* lipoprotein LipO, which was identified in a screen for phagosome maturation arrest effectors of *M. tuberculosis* (117). LipO, which was initially detected as being SecA2 dependent by comparative proteomics (102), was recently validated as being exported in a SecA2-dependent manner (K. Zulauf and M. Braunstein, unpublished). Although there is a clear role for the SecA2 pathway in exporting effectors of phagosome maturation arrest, it is important to note that not all effectors of this process are exported by the SecA2 pathway.

Proteins lacking signal peptides are another category of SecA2-dependent protein. PknG, SodA (superoxide dismutase), and KatG (catalase-peroxidase) make up this subset of SecA2-dependent proteins (99, 101, 102). Although there are multiple reports of all three of these proteins being exported by mycobacteria (i.e., localized to the cell envelope or secreted fractions), they lack any obvious signal peptide or TMD, which makes them unconventional exported proteins (113, 114, 118–120). At the same time, these proteins are also known to reside in the mycobacterial cytoplasm, where they have physiological functions (113, 115, 116, 118, 121). Interestingly, the SecA2-only pathway of *L. monocytogenes* is also reported to export a SodA protein lacking a signal peptide (122). However, nothing is known about the recognition of proteins lacking signal peptides by the SecA2 pathway, and it remains possible that the effect of SecA2 on their export is indirect. For example, there may be signal peptide-containing proteins exported by SecA2 that are themselves responsible for export of these unconventional proteins.

The Defining Features of SecA2 Substrates

While there are exceptions, the majority of SecA2-exported proteins possess N-terminal signal peptides. The role of the signal peptide in export of SecA2 substrates was evaluated with Msmeg1704, Msmeg1712 (123), and SapM (K. Zulauf and M. Braunstein, unpublished). As is the case for proteins exported by the general Sec pathway, the signal peptide on

these SecA2 substrates is necessary for protein export to occur, and the signal peptide is cleaved in association with export to release the mature protein (106). Experiments in which the signal peptide of a SecA2-dependent and SecA1-dependent substrate are swapped demonstrate that the signal peptide does not determine whether a protein is exported by the SecA2 pathway (123). Rather, the mature domains of SecA2-exported proteins impart the requirement for SecA2 in their export.

The defining feature of the mature domain that dictates the need for SecA2 in export may be a propensity to fold in the cytoplasm prior to export. In support of this idea, when the Sec signal peptide of Msmeg1704 is exchanged for a signal peptide that directs preproteins for export through the Tat pathway, Msmeg1704 is exported by the Tat pathway (123). Since the Tat pathway requires preproteins to be folded prior to export, this suggests that the mature domain of a SecA2 substrate can fold in the cytoplasm prior to export.

Since there is no SecB protein export chaperone in mycobacteria, one possibility is that the SecA2 pathway is adapted to facilitate export through the SecYEG channel of problematic substrates that can fold in the cytoplasm. Along these lines, a mycobacterial protein export chaperone named SatS was recently identified as playing a role in the SecA2 pathway (124). SatS stabilizes and prevents aggregation of a subset of SecA2 substrates. Thus, SatS may serve to keep SecA2 substrates in an unfolded state compatible with transport through SecYEG and thereby help the SecA2 pathway export proteins that have the potential for folding in the cytoplasm.

MYCOBACTERIAL T7S PATHWAYS

Substrates of the T7S pathway are among the most abundantly secreted proteins, indicating their ability to efficiently cross the mycobacterial outer membrane (125). Although conserved throughout the phylum *Actinobacteria* (see below), this secretion pathway was first described in mycobacteria, and in earlier literature T7S was referred to by multiple names, such as ESAT-6 secretion system (Ess), WXG100 secretion system (Wss), and Snm (secretion in mycobacteria) system (126, 127). However, with the visualization of the diderm cell envelope and the observation that T7S substrates are efficiently secreted into the extracellular environment and therefore need to pass two membranes, the term T7S, in line with the specialized secretion systems found in Gram-negative bacteria, became the accepted standard name (128, 129). Mycobacteria can contain up to five T7S systems, named ESX-1 through ESX-5 (125, 130). Of these five systems, the ESX-1 system was the first one to be discovered, being responsible for the secretion of the EsxA and EsxB proteins, also known as ESAT-6 and CFP-10, respectively, in *M. tuberculosis* (131, 132). The importance of the ESX-1 system for the virulence of *M. tuberculosis* is clearly demonstrated by the observation that deletion of a large portion of the *esx-1* gene cluster, resulting in a nonfunctional system, is the decisive determinant for attenuation of the vaccine strain *Mycobacterium bovis* BCG (133–135). Further studies, in both *M. tuberculosis* and the closely related fish pathogen *M. marinum*, showed that the ESX-1 system is crucial for intracellular survival in macrophages by mediating lysis of the phagosomal membrane (136). This membrane permeabilization has been linked to phagosomal escape (137, 138). Additionally, phagosome permeabilization by the ESX-1 system is likely required for the delivery of secreted effectors from the phagosomal lumen to the host cell cytosol, including effectors secreted from mycobacteria by other ESX systems, SecA, SecA2, or Tat. Although this permeabilization has been mainly attributed to the ESX-1-secreted protein EsxA, different studies provide conflicting results (138–142). Further research is therefore required to pinpoint the ESX-1 substrate that is responsible for the phagosomal membrane rupture (138–142). Besides its pivotal role in the virulence of pathogenic mycobacteria, ESX-1 is also present and functional in nonpathogenic mycobacteria such as the fast-growing *M. smegmatis*. Intriguingly, the ESX-1 system of *M. smegmatis* seems to have a very different role, of promoting DNA conjugation, which will be discussed later (143). The diverse roles of ESX-1 are probably caused by a difference in the ESX-1 substrates that are secreted by different species. Indeed, *M. smegmatis* lacks several ESX-1 substrates that are present in *M. tuberculosis* and *M. marinum* and have been linked to virulence (136).

The role of the ESX-1 system in virulence and pathogenicity has been a major focus of TB-related research for more than a decade. The function and importance of several of the other ESX systems have only been described in the past few years. Strikingly, while the ESX-1 system is dispensable for growth in culture, both the ESX-3 and ESX-5 systems are necessary for bacterial viability in *M. tuberculosis*. The essentiality of the ESX-3 system is due to its role in iron and zinc uptake (144, 145). Possibly linked to its role in iron acquisition, ESX-3 is one of the most conserved ESX systems, being present in all mycobacterial species, of which genome sequences are available. In addition to this, the ESX-3 substrates EsxH, PE15, and PPE20 also play a role in immune modulation, e.g., EsxH is required for inhibition of phagosome maturation (146, 147). The ESX-5 system, on the other hand, is only present in the subgroup of so-called slow-growing mycobacteria, which contains most pathogenic species, such as *M. tuberculosis*, *Mycobacterium leprae*, and *M. marinum* (148). Although it is still unknown what the exact role of the ESX-5 system is, its essentiality for growth has been linked to outer membrane permeability and nutrient uptake in *M. marinum* (149). Furthermore, the ESX-5 system has been shown to be involved in immune modulation of the host as well (150–154). The characterization of the exact roles of the ESX-5 system is complicated by the large number (>100) of substrates that it secretes.

The roles of the ESX-2 and ESX-4 systems and the substrates they secrete remain unknown. Several studies have shown that these systems are not essential for virulence or *in vitro* growth of *M. tuberculosis* (155, 156), although a transposon insertion sequencing study found that mutations in the ESX-2 and ESX-4 systems resulted in a reduced fitness in dendritic cells (157). In contrast to this, a recent study by Laencina et al. showed that the ESX-4 system of the opportunistic pathogen *Mycobacterium abscessus* is not only functional but even plays a critical role in virulence (158), because ESX-4 transposon and deletion mutants showed reduced survival both in amoeba and murine cells. This phenotype was suggested to be the result of impaired phagosomal rupture, a feature generally attributed to the ESX-1 system in *M. tuberculosis* and *M. marinum*. Because *M. abscessus* lacks the ESX-1 system, ESX-4 may carry out its role in this species. Finally, in *M. smegmatis*, ESX-4 seems to be, together with ESX-1, involved in DNA conjugation as well (see below) (159, 160).

In addition to these five ESX systems that are genomically encoded, many other *esx* loci have been identified that are located on plasmids (161–163). In addition, homologous *esx* gene clusters are present throughout the phylum *Actinobacteria*, not only in species with a diderm cell envelope similar to that in mycobacteria, such as in corynebacteria and

nocardia, but also in species that lack a mycolic acid-containing outer membrane (130, 164, 165). Lastly, more distantly related systems are present and functional in a subset of *Firmicutes*, such as *Staphylococcus aureus*, *Streptococcus intermedius*, and *Bacillus subtilis*. Because most T7S components present in *Actinobacteria* are not conserved in the *Firmicutes* systems, *Firmicutes* systems are referred to as type VIIb secretion systems, opposed to the type VIIa systems of *Actinobacteria* (130, 166, 167).

Composition and Mechanism of Mycobacterial ESX Systems

The ESX systems of mycobacteria contain a set of conserved components. During the early years of T7S-related research, genome annotation numbers were often used to refer to these components. To avoid confusion, researchers in the T7S field proposed a systematic nomenclature based on the genetic composition of the systems, which is now the accepted standard (128). Based on this nomenclature, genes that are conserved in at least four of the mycobacterial ESX systems are defined as *ecc*, for *esx* conserved component. The system-specific genes, in most cases encoding secreted substrates, are designated *esp*, for *esx*-specific protein. There are two exceptions to this rule, *mycP* and *esp*G. Although the mycosin proteases (MycP) are conserved in all ESX-systems, the term "mycosin" was already an established name and was therefore not altered. For the second exception, EspG was originally not identified as a conserved component due to low homology between the different *esp*G genes. However, the EspG proteins, present in four of the ESX systems, have now been shown to be functional homologues (168, 169). For the major substrate groups, the previously defined terms were kept. The first of these groups are called the Esx proteins, because "EsxA" (ESAT-6) and "EsxB" (CFP-10) were already frequently used terms for two members of this group. The second, larger group contains the so-called PE and PPE proteins, referring to the presence of a proline-glutamic acid (PE) or proline-proline-glutamic acid (PPE) N-terminal amino acid motif (128, 130, 154, 170).

ESX substrates

All mycobacterial ESX systems are encoded by genes that are clustered in distinctive genomic loci. In the middle of these gene clusters is always a pair of *esx* genes. These *esx* genes, which are cotranscribed, encode small proteins of the WXG100 family, named after the conservation of a tryptophan and glycine residue separated by a random amino acid and their length of roughly 100 amino acids (166, 171). The *esx* genes are not limited to the ESX loci; additional *esx* gene pairs can be found throughout the genome sequence (130, 148). The two Esx proteins that are encoded from the bicistronic transcript form heterodimers in the cytosol and are codependent on each other for secretion (Fig. 1) (172). For example, the ESX-1 system contains the well-described protein pair EsxA-EsxB, which are believed to be secreted as a dimer (173, 174). However, whereas EsxA has been shown to play a major role in virulence, this has not been shown for EsxB (138, 175). From this observation, it is postulated that EsxB is the chaperone for EsxA and that their interaction is required for successful secretion. Because their interaction is required for successful secretion, an *esxB* mutant shows a similar phenotype as an *esx*A mutant (139, 176). The heterodimer EsxA-EsxB is the first Esx pair for which a high-resolution structure has been solved (174). The structure shows the presence of a helix-turn-helix motif in both proteins, where the turn contains the WXG motif and the double helices interact in an antiparallel orientation with the double helices of the partner. Furthermore, it shows that the N- and C-terminal tails are flexible. Predicted structural similarities suggest that the EsxA-EsxB structure can serve as a model for the other Esx proteins, which was confirmed by the crystal structures of the ESX-3-associated heterodimeric substrates EsxG-EsxH and EsxR-EsxS (177, 178).

Genes encoding the second substrate group, the PE and PPE proteins, are also present in four of the *esx* loci; only the ESX-4 gene cluster does not contain any *pe* or *ppe* genes. However, most *pe* and *ppe* genes are not associated with these loci (130, 148, 154, 179). In fact, pathogenic mycobacteria contain up to 99 *pe* and 69 *ppe* genes and, e.g., in *M. tuberculosis* approximately 10% of the encoding genome belongs to *pe* and *ppe* genes. While the ESX-1 and ESX-3 systems are responsible for the secretion of some PE and PPE proteins (147, 154, 180), the majority of the PE and PPE proteins are secreted through the ESX-5 system (150). Similar to the Esx proteins, several *pe* and *ppe* genes are clustered in a bicistronic operon, consisting of one *pe* and one *ppe* gene, and at least some of the corresponding proteins form heterodimers (169, 181). These heterodimers are often dependent on each other for stability and solubility (Fig. 1) (169, 181, 182).

Substrates belonging to the PE/PPE protein group are often bound to the cell surface, which is in contrast to the Esx proteins that are mainly secreted into the culture medium (183, 184). These substrates consist of a distinctive N- and C-terminal domain: whereas the N-terminal PE and PPE domains, with a length of ~110 and 180 amino acids, respectively, are highly conserved and are involved in the secretion process, the C-terminal domains are less conserved and are thought to be involved in protein-specific functions. The resolved crystal structures of PE25-PPE41 and PE18-PPE15 reveal structural similarities between Esx and PE-PPE heterodimers (182, 185). While the PE domain only consists of two alpha helices, the PPE domain contains five alpha-helices, of which helices $\alpha 2$ and $\alpha 3$ mediate dimerization with the PE partner, forming a four-helix bundle similar to Esx pairs. Unlike Esx dimers, only the PPE protein contains a WXG motif in the turn between these two helices. The fourth and fifth alpha helices of the PPE domain form an extending hydrophobic tip that is recognized by the EspG component, which will be discussed below (169, 180, 181, 185). The size of the more variable C-terminal domains of PE and PPE proteins can be large; for example, the subgroup of PE_PGRS (polymorphic GC-rich repetitive sequence) proteins can have C-terminal domains of over 1,400 amino acids (148). Only a minority of these C-termini contain predicted functional domains, e.g., executing predicted lipase activity. Consequently, a function has been described for only a few PE-PPE proteins (186, 187).

The final group of substrates, the Esp proteins, are specific for the ESX-1 system (188–191). Although less is currently known about the secretion of these substrates, EspA and EspC are cotranscribed and have been shown to be codependent on each other for secretion and interact to form a heterodimer (188, 192), similar to what is likely the case for EspE and EspF (184, 188, 193). In contrast to this, another ESX-1 substrate, EspB, has a PE-PPE dimer-like fold, forming a double helix-turn-helix by itself, suggesting that it is secreted as a monomer (194, 195). Whether the final Esp substrates, EspK and EspJ, are secreted as monomers or heterodimers remains unknown (190, 191).

An intriguing feature of ESX-1 substrates is that there are some cases of unpaired substrates being codependent on each other for secretion; i.e., the secretion of EspA-EspC is affected by mutations in *esxA-esxB*, and vice versa (176, 188, 196).

This codependence of ESX-1 substrates complicates the understanding of the mechanism of secretion by the ESX-1 system, because a mutation in, e.g., specific chaperones, will also affect the secretion of ESX-1 substrates that do not interact with this chaperone. Furthermore, it makes it difficult to pinpoint specific functions for individual ESX-1 substrates (132, 176, 197). Some level of codependence of substrates for secretion has also been reported for ESX-3 and ESX-5 substrates. For ESX-3, deletion of *esxH* has been shown to abolish secretion of ESX-3-dependent PE-PPE proteins, although this does not happen the other way around (147). The ESX-5 substrate, PPE38, is required for secretion of the two largest ESX-5 substrate groups, PPE_MPTR and PE_PGRS (198). Although the mechanism for this dependency on PPE38 is currently unknown, PPE38 appears to be of great importance because the emerging *M. tuberculosis* Beijing isolates share a polymorphism in the *ppe38* locus (198). In addition, the ESX-5 substrate PPE10 affects the integrity of the capsular layer of mycobacteria (see below) (199), indirectly affecting the proper localization of other substrates, not only of ESX-5 but also of the ESX-1 system (199).

ESX secretion signal and the cytosolic chaperone EspG

Unlike Sec or Tat substrates, the ESX substrates do not contain an N-terminal signal peptide; instead, the Esx, PE, and Esp substrates have a C-terminal secretion signal directly downstream of a helix-turn-helix. In the case of the Esx and PE-PPE heterodimers, only one of the proteins has this secretion signal: EsxB of the EsxA-EsxB pair (172) and the PE protein in the case of the PE-PPE pairs (195). Two elements of the secretion signal have been identified. One is a conserved motif, consisting of a tyrosine residue and a residue with a negative charge separated by three random amino acids (YxxxD/E). This motif is required for secretion of T7S substrates (195). The other element is downstream of the YxxxD/E motif at the extreme C-terminus of the protein. These approximately seven amino acids, which include additional hydrophobic residues, are also required for secretion (172). In addition, these C-terminal seven amino acids interact with the C-terminal domain of the ESX-1 component EccCb1 of *M. tuberculosis*, as demonstrated by yeast two-hybrid analysis and immunoprecipitation (172). Structural studies demonstrate a similar interaction between a peptide containing the last 23 residues of EsxB and EccC of the T7S system of *Thermomonospora curvata* (200). It is noteworthy that only the last seven amino acids of this peptide were visible in the structure. The YxxxD/E motif was disordered in the structure, suggesting that the motif is not involved in the binding of this peptide to EccC (200). However, it remains unclear how the entire signal interacts with EccC in the context of the full substrate and the complete secretion system. Sequence alignments across the wider array of WXG100 subfamilies also identify a C-terminal consensus sequence, aligned to the side of alpha helix 2, consisting of a conserved HxxxD/E motif (like that of YxxxD/E) and hydrophobic residues downstream of this motif (201). Swapping the C-terminal 15 amino acids, including the YxxxD/E secretion motif of two PE substrates, secreted by ESX-1 or ESX-5 did not affect their secretion or by which system they were transported (195). This shows that, although the C-terminal secretion signal is essential for secretion, it is not involved in system specificity. Instead, a recent study by Phan et al. showed that the fourth and fifth helix of the PPE domain, recognized by the EspG chaperone, contains the signal for system-specific recognition (180).

Although EspG was at first considered an ESX-1-specific protein, it has now been established to be a conserved component; in fact, only the ESX-4 system does not contain an *esp*G gene. Although the EspG proteins share a low amino acid conservation, the solved tertiary structures of $EspG_1$, $EspG_3$, and $EspG_5$ are very similar (169, 181, 202). Previous studies illustrated the importance of $EspG_5$ in the general secretion process, because it is required for the secretion of several ESX-5 substrates in *M. marinum* (151). More information about its role in the secretion process was obtained by the observation that the deletion of $espG_1$ decreases the stability of PPE68 in *M. tuberculosis* (203), which indicated that the EspG proteins might function as a chaperone. Importantly, the chaperone of the ESX-5 system, $EspG_5$, only interacts with PE-PPE pairs secreted by the ESX-5 system in *M. marinum* (169). Similar to this, $EspG_1$ was shown to only bind to an ESX-1 dependent PE-PPE heterodimer and not to PE-PPE pairs secreted by the ESX-5 system (168). This is in line with the observation that $EspG_1$ and $EspG_5$ increase the solubility of their cognate PE-PPE substrates *in vitro* (181). Because EspG is strictly cytoplasmic, it was hypothesized that EspG is required for the recruitment of the PE-PPE pairs to the membrane-embedded secretion complex, after which it dissociates from these substrates (Fig. 1) (168).

The regions involved in the interaction of EspG and PE-PPE dimers were shown by the crystal structure of $EspG_5$ in complex with the PE25-PPE41 dimer (169, 181). The structure showed that $EspG_5$ only binds to the hydrophobic tip of helices $\alpha4$ and $\alpha5$ of the PPE domain of PPE41 and does not interact with the PE protein, including the general secretion signal. This corresponds with an earlier study showing that the YxxxD/E motif of the PE protein is not required for binding of the PE-PPE pair to EspG (195). The observation that binding of $EspG_5$ does not introduce conformational changes to the PE-PPE dimer, but increases the solubility of the protein pair (181), suggests that EspG prevents self-aggregation via the hydrophobic tip of the PPE protein (169, 181).

Because EspG proteins specifically interact with PE-PPE pairs of their respective system, it was already postulated that EspG might direct system specificity of these substrates (169). To confirm this, Phan et al. investigated the role of the region involved in $EspG_1$ binding, hereafter called the $EspG_1$ binding domain, of the ESX-1 substrate PE35-PPE68_1 in *M. marinum* (180). Indeed, PE35-PPE68_1 could be rerouted to the ESX-5 system by replacing the $EspG_1$ binding domain of PPE68_1 with the equivalent $EspG_5$ binding domain of the ESX-5 substrate PPE18. This domain replacement makes the PE35-PPE68_1 protein pair independent of both $EspG_1$ and the ESX-1 membrane complex but, instead, dependent on $EspG_5$ and the ESX-5 complex for secretion. These findings show not only that EspG is required for the solubility of PE-PPE complexes, but also that it specifically directs these protein pairs to their respective ESX system. The additional observation in several studies that $EspG_1$ is also required for the secretion of Esx and Esp proteins in *M. marinum* is probably an indirect effect of the substrate codependence, because Esx pairs lack an EspG binding domain (180, 204). Finally, recent findings show that the ESX-1 substrate EspE interacts with EspH, a small ESX-1-associated protein that is not secreted, in the cytosol of *M. marinum*, which is required for the stable expression and secretion of EspE-EspF (204). Interestingly, several ESX-1-associated proteins show homology and conserved predicted structural features to EspH (i.e., EspD and EspL), indicating that these Esp proteins might serve as specific chaperones for specific Esp substrates.

EccA

The second ESX conserved cytosolic component is EccA, belonging to the family of AAA+ (ATPases associated with diverse cellular activities) proteins. AAA+ ATPases have been linked to a wide range of mechanisms, such as protein homeostasis, signaling pathways, and the assembly and disassembly of protein complexes (205, 206). A distinctive feature of AAA+ proteins is that they usually form hexameric rings with an opening in the center. Although not a common feature of prokaryotic secretion systems, the type VI secretion system also contains an AAA+ ATPase, which is involved in the disassembly of the secretion apparatus (205). EccA is absent in the ESX-4 system, similar to the cytosolic chaperone EspG. All EccA homologs have an identical composition, consisting of distinctive N- and C-terminal domains separated by a linker region. The C-terminal domain contains the typical AAA+ ATPase characteristics, such as the walker and oligomerization motifs, whereas the N-terminal domain contains 12 antiparallel α helices arranged into 6 tandem tetratricopeptide repeat motifs, known for their involvement in protein-protein interactions (207–209). Full-length $EccA_1$ indeed has been shown to form hexamers, although it has (relatively low) ATPase activity *in vitro* (207). Furthermore, it was confirmed that oligomerization and ATPase activity are mediated by the C-terminal domain. Because expression of only the C-terminal domain results in higher ATPase activity than full-length $EccA_1$, it is thought that the N-terminal domain plays a regulatory role in this activity (207).

The role of EccA in secretion remains unclear. Early studies, showing that $EccA_1$ is essential for secretion of EsxA and EsxB in *M. tuberculosis* and *M. marinum*, suggested that EccA proteins might play a role as a chaperone in the ESX secretion process (140, 210). Similarly, $EccA_5$ was also found to be required for the secretion of ESX-5 substrates in *M. marinum* (153, 211). However, other studies of *M. tuberculosis*, *M. marinum*, and *M. smegmatis* showed that $EccA_1$ and $EccA_5$ are dispensable for ESX-1- and ESX-5-mediated secretion, respectively, suggesting that the involvement of this component varies between different strains and/or growth conditions (127, 212). The latter notion is supported by the recent finding that the importance of $EccA_1$ for secretion of EsxA in *M. marinum* is dependent on the growth condition (204). Although yeast two- hybrid experiments indicated that a peptide corresponding to the C-terminus of EsxB interacts with $EccA_1$ (213), this interaction could not be observed with full-length EsxB, nor does $EccA_1$ affect the conformation or solubility of EsxB (207). On the other hand, both yeast two-hybrid assays and *in vivo* protein pull-downs in *M. marinum* showed that $EccA_1$ is able to interact with EspC (188). This interaction can be blocked by the removal of the C-terminus of EspC, which also blocks the secretion of EspC. However, $EccA_1$ could not be shown to interact with EsxA or EsxB in this same study. Although this could mean that $EccA_1$ exclusively interacts with the Esp proteins and targets them to the membrane-embedded secretion machinery, the role of the EccA component in the other ESX systems that do not secrete Esp substrates remains unknown.

ESX membrane components, complex formation, and structure

The ESX systems of mycobacteria contain five conserved membrane components, EccB, EccC, EccD, EccE, and MycP, which are expected to be involved in the transport of ESX substrates across the cell envelope. All these components have been shown to be essential for secretion for ESX-1 (132, 214, 215), ESX-3 (216), and ESX-5 (149, 153, 212, 217). EccD is the most hydrophobic of these five components, predicted to have up to 11 TMDs, whereas the other components have only one or two TMDs and contain relatively large N- or C-terminal hydrophilic domains. EccD is postulated to be the core membrane pore of the secretion machinery due to its hydrophobicity. The structure of the ~110-amino acid N-terminal soluble domain of $EccD_1$ has been solved and shows a ubiquitin-like fold with a negatively charged groove, suggesting a role in the recruitment of other components of the secretion complex or possibly as a gating element of the secretion channel (218). The structure of the C-terminal soluble domain of $EccB_1$ shows an elongated shape with a hydrophobic core, suggesting a more structural role of this protein that likely forms a hexamer (218, 219). In addition to this, the C-terminal domain of $EccB_1$ was shown to have ATPase activity *in vitro*, although it is uncertain whether this has functional relevance because this domain is predicted to be located in the periplasm, where ATP is absent (219). There is no crystal structure available for EccE; however, it is predicted to have two TMDs and a C-terminal soluble domain of unclear localization.

EccC is the most conserved of all T7S components, because it is the only component that is also found in the type VIIb secretion systems in *Firmicutes* (see below) (220). EccC contains three predicted functional domains, which all show homology to the family of FtsK-SpoIIIE ATPases (221), named after the two best-studied members, FtsK and SpoIIIE (222), both mediating DNA transport. Another notable protein of this family is the type IV coupling protein, involved in the recognition of substrates and protein transport in type IV secretion systems (223). Just like the AAA+ ATPases, a major part of the FtsK-SpoIIIE ATPases also forms ring-like hexamers. However, because EccC contains three nucleotide binding domains (NBDs), opposed to one, it might have different structural features.

Although EccC has been proposed to be the motor of the secretion complex, energizing the translocation of substrates across the cell envelope, its function remains unclear. Of the different EccC proteins, $EccC_1$ has been most extensively investigated. In contrast to other EccC proteins, which are encoded by a single gene, $EccC_1$ has the unusual feature that it is encoded by two genes, *eccCa*$_1$ and *eccCb*$_1$. However, both yeast two-hybrid experiments and protein pull-downs showed that it most likely functions as a single unit (132, 212, 224). $EccCb_1$ has been shown by the Cox lab to interact with the C-terminus of EsxB that contains the general secretion motif (188). This suggests that it could play a similar role as the type IV coupling protein in mediating substrate recognition and subsequent transport. The same group subsequently solved the crystal structure of the cytoplasmic domain of EccC from the actinobacterium *T. curvata* (200). The structure revealed that NBD2 and NBD3 were both bound to ATP, while NBD1 was in a nucleotide-free state, indicating a low ATPase activity of NBD2 and NBD3. They additionally produced a cocrystal of a truncated version of EccC with a peptide representing the last 23 amino acids of the C-terminus of EsxB bound to NBD3. The ATPase activity of EccC, shown to be held in an auto-inhibited state of NBD1 by interactions with NBD2, could not be induced by binding of the C-terminus of EsxB, suggesting that other substrates or components are required for this (200). Interestingly, they did find that EsxB binding resulted in the multimerization of EccC in both dimers and higher-order oligomers. From these findings the authors proposed a working model in which binding of multiple substrates induces EccC multimerization and subsequent activation of NBD1. The suggestion that NBD1 is the main domain

responsible for energizing protein translocation is supported by another study that found that ATP binding to NBD1 of $EccC_5$ of *M. marinum* is essential for secretion, while mutations in the ATP pocket of NBD2 and NBD3 only reduced secretion efficiency (149).

Evidence that the conserved membrane components form a large membrane-embedded secretion complex was first provided for the ESX-5 system of both *M. marinum* and *M. bovis* BCG (212). First, the five components with predicted TMDs were indeed shown to be localized to the cell envelope, of which four, $EccB_5$, $EccC_5$, $EccD_5$, and $EccE_5$, were shown to be part of a 1.5-MDa membrane-embedded complex, postulated to form the ESX-5 membrane channel (Fig. 1). Subsequent antibody pull-downs on $EccB_5$ confirmed that these four proteins are the main constituents of this complex, with a notable absence of the fifth membrane component, $MycP_5$. Finally, limited proteolytic digests indicated that the N-terminal part of $EccE_5$ and the middle and C-terminal part of $EccC_5$ are the most exposed domains of the complex. Additional studies using protein pull-downs of Strep-tagged $EccCb_1$ and $EccC_5$ of *M. marinum* showed that the composition of the ESX-1 complex is comparable to that of the ESX-5 complex, also forming a complex of approximately 1.5 MDa (224). Mass spectrometry analysis confirmed the presence of the four Ecc components in both ESX complexes. Although $MycP_1$ and $MycP_5$ were present in the ESX-1 and ESX-5 complexes, respectively, they were both below the set threshold levels, indicating that the MycP proteins are not integral components of these assemblies.

More recently, the first image of a mycobacterial T7S system was published, determined by negative-stain single-particle analysis (225). For this, the ESX-5 system of the slightly thermophilic and slow-growing *Mycobacterium xenopi* was overproduced in and purified from *M. smegmatis*, which does not naturally contain an ESX-5 system. The overproduction in *M. smegmatis* resulted in a relatively stable and functional system with a similar composition and size as the complexes that were previously isolated for *M. bovis* BCG and *M. marinum* (212, 224). Furthermore, the reconstituted system in *M. smegmatis* allowed the investigation of the minimal requirements for complex formation. This showed that the ESX-5 complex assembles independently of the presence of ESX-5 substrates, opposed to the previously proposed mechanism of EsxB-dependent multimerization of EccC (200).

The EM images of negatively stained purified complexes and subsequent three-dimensional reconstruction resulted in a 13-Å structure, revealing a 6-fold symmetry and a size of 28 (width) × 16 (height) nm, with a 5-nm-wide central pore. Interestingly, based on previously solved crystal structures of Esx and PE-PPE heterodimers, the central pore is wide enough to allow the secretion of folded, dimeric ESX-substrates (201). The combination of the observed symmetry with mass spectrometry-based stoichiometry determination suggests that the four individual components are each present in 6 copies, resulting in a 24-subunit complex with a mass of 1.8 MDa, opposed to the previously estimated 1.5 MDa (212, 224).

The raw EM images of single particles showed "beads-on-a-string-like" densities extending from the main body of the complex. These extrusions have a similar shape as that previously shown for the cytosolic portion of EccC of *T. curvata* (200), and specific gold labeling confirmed them to be the EccC subunits. The seemingly random orientation of these EccC extrusions suggests that they are flexible, which was also observed by small-angle X-ray analysis, resulting in their absence in averaged complexes. Segmentation analysis in combination with the available crystal structure of the soluble domain of $EccB_1$ allowed for the fitting of $EccB_5$ at the periplasmic side of the complex, while gold-labeling showed that the C-terminal domain of $EccE_5$ is also periplasmic and located at the periphery of the complex (218, 225).

A distinct and interesting feature is that the dimensions of the ESX-5 complex dictate that it can only span the inner membrane of mycobacteria, because it is not tall enough to span both the inner and mycobacterial outer membrane (184). This is in contrast to the complexes of type I, II, III, IV, and VI secretion systems in Gram-negative bacteria, which span both the inner and outer membrane. It therefore remains unknown how T7S substrates can cross the outer membrane of mycobacteria. However, several possibilities have been proposed, which will be discussed below.

The fifth membrane component, the mycosin protease, or MycP, is not an integral part of the core ESX secretion complex, as shown by protein pull-down analysis (212, 224, 225). However, it is highly conserved in T7S systems throughout the phylum *Actinobacteria* (4), indicating that it does play an important role within T7S. Mycosins are subtilisin-like serine proteases, which had been identified and indicated to be cell envelope associated for *M. tuberculosis* before T7S was discovered (226, 227). Because mycosins have a predicted N-terminal signal sequence and a C-terminal TMD, the protease domain is most likely located in the periplasm.

Crystal structures of $MycP_1$ from *Mycobacterium thermoresistible* and *M. smegmatis* and a structure of $MycP_3$ from *M. smegmatis* all show a similar subtilisin-like alpha/beta structure, consisting of a seven-stranded beta sheet surrounded by eight alpha helices (228, 229). The active site, consisting of an aspartic acid, histidine, and serine residue, is in the active conformation in all mycosin structures (228). One interesting feature is the presence of an atypical N-terminal extension. Subtilisins usually contain an N-terminal propeptide that blocks the active site of the protease and is cleaved and degraded upon protein maturation (230). In contrast, the "propeptide" of mycosins does not block the active site of the protease, but it is instead tightly wrapped around the protease domain. *In vivo* data furthermore showed that the N-terminal domain remains attached to the protease without blocking protease activity (228), while *in vitro* analysis showed that the N-terminal extension is most likely involved in protein stability (231, 232). Additional unique features of mycosins are the presence of three extended loops in the protease domain, surrounding the active site and extending the active site pocket (228, 229). Finally, the substrate binding grooves of $MycP_1$ and $MycP_3$ show significant differences to each other, in both the surface hydrophobicity and the depth of interacting sites, which is also influenced by differences in the three extended loops of the protease domain (228). These differences are probably the basis for substrate specificity of the mycosins.

Although the catalytic site is conserved in mycosins, as is their predicted active conformation, only one mycosin substrate has so far been identified: $MycP_1$ has been shown to process the ESX-1 substrate EspB near its C-terminus, both *in vitro* and *in vivo*, in *M. marinum* and *M. tuberculosis* (214, 224, 228). Interestingly, the proteolytic inactive mutant showed an increased secretion of ESX-1 substrates, indicating that although $MycP_1$ is required for secretion, its proteolytic activity is not (214, 224). In line with this observation, while $MycP_1$ is crucial for intracellular growth and virulence, a proteolytic inactive mutant of $MycP_1$ resulted in only a slight attenuated phenotype in a mouse infection model (214). This disparity suggests that mycosins have an additional role in ESX systems, besides being involved in the processing of

substrates. This second role has been identified to be in the stabilization of the core membrane-embedded ESX-secretion complex, because in the absence of MycP, its respective ESX complex is highly unstable (224). Furthermore, it was shown that although mycosins are not a stable component of the ESX complex, they do loosely associate with it (Fig. 1). It is currently unknown whether this decreased stability is the only way that mycosins directly affect secretion; hopefully, additional interaction studies between the ESX complex and MycP can shed more light on this. Although proteolytic activity is not required for secretion, the decreased virulence of an active site mutant of $MycP_1$ could potentially be of interest for the development of novel drugs that block protease activity (233).

Outer membrane translocation of substrates

Because the EM structure of the isolated ESX-5 membrane complex indicates that this core secretion channel only spans the inner membrane (225), the outer membrane channel in T7S remains unknown. While these findings suggest that T7S is a two-step process in mycobacteria, i.e., mediated by two separate membrane channels, one in the cytoplasmic membrane and one in the mycobacterial outer membrane, a one-step mechanism for T7S, i.e., a single membrane channel that mediates protein export across the complete cell envelope, is still possible, because outer membrane components could be lost during the complex purifications. However, the five conserved membrane components EccB, EccC, EccD, EccE, and MycP are most likely not involved in outer membrane translocation, because all these components can also be widely found in T7S systems of *Actinobacteria* that lack mycolic acids, the major constituent of the mycobacterial outer membrane. Therefore, in both scenarios, a one-step and a two-step mechanism, the proteins mediating outer membrane translocation remain unknown. Notably, there is no conserved component within the different *esx* clusters that could be involved in this second translocation step, with the exception of the PE and PPE proteins, which are present in most ESX systems and have only been found in *Corynebacteriales*.

The identification of outer membrane proteins involved in T7S secretion is complicated by the fact that very little is known about mycobacterial outer membrane proteins in general (234). In fact, for mycobacteria, only the outer membrane porin MspA of *M. smegmatis*, which is not present in slow-growing species, has been properly characterized and its crystal structure solved (12, 235). The 96-Å-tall MspA porin is an octamer, showing an 8-fold rotational symmetry with a central pore (12). The individual subunits contain a 134-amino acid domain forming the bulk of the porin. This globular domain consists of two 4-stranded antiparallel beta sheets connected by a 50-residue loop at the base of the structure, forming two 16-stranded beta-barrels. While four highly homologous Msp porins, MspA, B, C, and D, are critical for the sufficient uptake of nutrients such as glucose and phosphate in *M. smegmatis*, no such porins are present in *M. tuberculosis*. In *M. tuberculosis*, CpnT and SpmT are the best candidates for outer membrane proteins, because they have been shown to be involved in nutrient and metabolite uptake (236–240) and possibly form a channel or porin (241, 242). However, thus far, the structures of these channels remain unknown. Most attention has been given to the identification of mycobacterial outer membrane proteins that form a beta-barrel, analogous to the Gram-negative bacteria. However, research on PorB, one of the porins present in *Corynebacterium glutamicum* (243–245), shows a very different structure for an outer membrane porin (14). The core of this protein consists of 70 residues forming 4 alpha helices connected by disulfide bridges, showing that mycolic acid-embedded proteins do not necessarily need to form beta-barrel-like structures.

None of the mycobacterial outer membrane proteins identified so far have been linked to protein export; however, there are indications that specific ESX substrates may be involved in the outer membrane translocation step. For ESX-1, there are currently two substrates suggested to be involved in this second secretion step: EspB and EspC. Both EspB and EspC have been shown to be required for secretion of EsxA/EsxB in pathogenic mycobacteria (246, 247), whereas EspB is still secreted in the absence of EspA-EspC or when EsxA-EsxB is not secreted (188, 246).

As already mentioned, EspB has the specific feature that it forms a PE-PPE-like fold by itself and is therefore probably secreted as a monomer (194, 248). Interestingly, this substrate has been shown to multimerize upon secretion and to form heptameric tubular-shaped particles after overexpression in *E. coli*, as visualized by negative-stain EM (248). Modeling the solved crystal structure of monomeric EspB without the C-terminal portion that is cleaved off by $MycP_1$ results in an arrangement where the hydrophobic helical tips of EspB are tightly packed, possibly allowing membrane-embedding activity. This has led to the hypothesis that EspB might be within the (outer) membrane and involved in the secretion of ESX substrates. The cleaved off C-terminal fragment of EspB was shown to be dispensable for the formation of these ring-shaped oligomers. By direct comparison of structures with and without this region, the C-terminus was visualized as an appendage protruding from the top of the ring (248). A model was subsequently proposed in which the C-terminal cleavage of EspB by $MycP_1$ has a regulatory function for passage through this potential channel, which could help to explain the increased secretion of ESX-1 substrates in an active site mutant of *mycP*$_1$ (214, 224).

EspC is the second ESX-1 substrate proposed to be involved in the outer membrane translocation. EspC, which is secreted as a heterodimer together with EspA, has been shown to localize to the cell surface of *M. tuberculosis* and forms filamentous structures when overproduced in *E. coli* (192). Based on these findings, EspC was postulated to form filaments that have a role analogous to the type III injectisome needle proteins, mediating contact-dependent phagosomal lysis (142, 249). However, while EspC is required for the secretion of EsxA and EsxB in *M. tuberculosis* (188), EspC is absent in *M. smegmatis*, while this species does secrete EsxA-EsxB. Possibly another unknown protein could replace the function of EspC in *M. smegmatis*.

It still needs to be seen whether EspB and/or EspC are involved in the outer membrane translocation step within T7S. In any case, this would only provide a model for the secretion of ESX-1 proteins, because EspB is not required for secretion of ESX-5 substrates (250). This would suggest that each ESX system has its own specific outer membrane components. The only current hypothesis for the outer membrane channels of ESX-3 and ESX-5 is that this role is performed by members of the PE/PPE families. This hypothesis is supported by their localization to the cell envelope and the notion that they are not widely found outside mycolic acid-containing species.

Role of Mycobacterial ESX Systems in Conjugation and Cell Physiology

While the mycobacterial ESX systems are known to play critical roles in virulence, e.g., in immune modulation

(ESX-3 and ESX-5) and phagosomal escape (ESX-1), more recently, several crucial roles in cell physiology and nutrient uptake have been additionally found and described. Here, we will discuss several of those roles and the involved ESX systems (Fig. 2).

One of the first nonvirulent roles attributed to an ESX-system was the role of the ESX-1 system in horizontal gene transfer in *M. smegmatis* (143). The DNA transfer is unidirectional, chromosomally encoded, and allows for the transfer of large genomic fragments. This form of conjugation is called distributive conjugal transfer and has so far not been described for other mycobacteria, including *M. tuberculosis* (159). An interesting observation is that, while recipient strains are dependent on the ESX-1 system for DNA uptake (143), *esx-1* mutants in the donor strains are hyper-conjugative (251). In addition to the ESX-1 system, the ESX-4 system is also required for receiving DNA by the recipient, because a deletion of $eccC_4$ or $eccD_4$ blocked conjugation (159). The presence of ESX-4 was only required for the recipient strain, because the same genes could be deleted in the donor strain without affecting DNA transfer. A more recent study showed that SigM, the regulator of ESX-4, is highly upregulated in the recipient when in contact with a donor cell, suggesting that ESX-4 is specifically induced upon cell-cell contact (160). The exact role of ESX-1 and ESX-4 in conjugation by *M. smegmatis* remains unclear. The fact that conjugation takes place in a contact-dependent manner (159) might indicate that putative surface-localized ESX-1 and ESX-4 substrates might be required for initial donor-acceptor engagement and/or communication.

More recently, a conjugative plasmid has been described for *M. marinum*, the pRAW plasmid (161). This plasmid

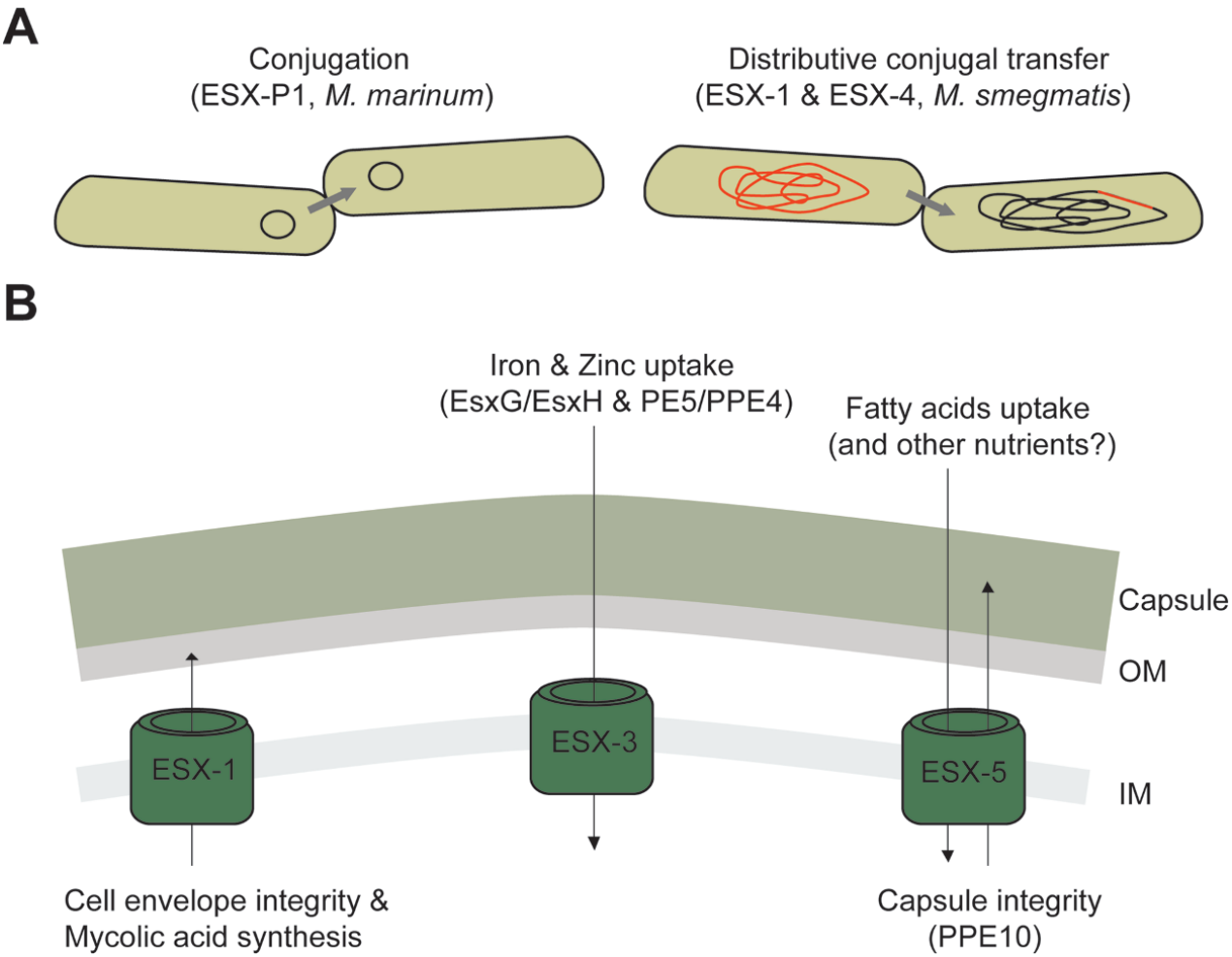


FIGURE 2 Roles of ESX systems in DNA transfer, and cell physiology and nutrient uptake. (A) The pRAW plasmid-encoded ESX-P1 system, present in certain strains of *M. marinum*, is involved in DNA conjugation, allowing the transfer of the plasmid to other slow-growing mycobacteria (161). Similar plasmids are found in other mycobacteria, and they have been linked to the evolution and distribution of *esx* gene clusters within mycobacteria (162, 256). In addition, the ESX-1 and ESX-4 systems of *M. smegmatis* are involved in distributive conjugal transfer, allowing the horizontal gene transfer of random large genomic fragments (143, 159). (B) A second major role of the ESX-systems is in cell physiology. While the ESX-1 system has been linked to the integrity of the cell envelope and the biosynthesis of mycolic acids (196, 210, 264), the ESX-3 and ESX-5 systems are involved in the uptake of metabolites and nutrients, respectively. The ESX-3 system and two of its substrate pairs (EsxG-EsxH and PE5-PPE4) have been specifically linked to the uptake of iron and zinc (144, 145, 147), while the ESX-5 system has been linked to the utilization of fatty acids and possibly other nutrients (149) Finally, the ESX-5 system secretes a substrate (PPE10) that is required for the integrity of the capsular layer (199). IM, inner membrane; OM, outer membrane.

contains not only an *esx* gene cluster, designated *esx-P1*, but also genes predicted to encode type IV secretion-like proteins, known to be involved in DNA conjugation in Gram-negative and Gram-positive bacteria (161, 252, 253). The pRAW plasmid could be transferred very efficiently, but only between different slow-growing species, whereas no transfer was observed to fast-growing mycobacteria, such as *M. smegmatis* (161). Conjugation of the pRAW plasmid was shown to depend on the presence of an intact *esx-P1* cluster and on the type IV-like genes (161). Interestingly, since then, similar *esx* gene cluster-containing plasmids have been found in various nontuberculous mycobacteria, both pathogenic and nonpathogenic (161, 254, 255). The wide distribution of these *esx*-containing plasmids has been linked to a possible role of plasmids in the evolution and distribution of *esx* gene clusters within mycobacteria (162, 256). Because ESX-P1 is most closely related to the ESX-5 system, pRAW-like plasmids might have been involved in acquiring the ESX-5 system in slow-growing mycobacteria.

DNA exchange and/or cell-to-cell contact is not the only function assigned to ESX systems. Another critical role of these systems is in the uptake of nutrients. In fact, both the ESX-3 and ESX-5 systems are involved in this, explaining why these systems are required for bacterial viability in slow-growing species (149, 155, 217, 257). The ESX-3 system is transcriptionally controlled by the iron-dependent transcriptional repressor (IdeR), and in *M. tuberculosis*, additionally by the zinc uptake repressor (Zur), indicating that the ESX-3 system is linked to the uptake of metal ions (258, 259). Indeed, the ESX-3 system is upregulated at low iron levels (145, 257, 260), and more recent work confirmed that the ESX-3 system is actively involved in the uptake of iron and, in the case of *M. tuberculosis*, also of zinc (144). The ESX-3 system was additionally shown to be essential for growth of *M. tuberculosis*, not only in low-iron or -zinc conditions, but also for optimal growth in high-iron and -zinc concentrations (144). For *M. tuberculosis*, six substrates of the ESX-3 system have been identified: EsxG-EsxH, PE5-PPE4, and PE15-PPE20. Interestingly, only EsxG-EsxH and PE5-PPE4 appear to be involved in iron uptake, while PE15-PPE20 is involved in immune modulation (147). In contrast to *M. tuberculosis*, the deletion of the ESX-3 system of *M. smegmatis* only affected bacterial growth when *fxbA* was also deleted. FxbA is an enzyme required for the biosynthesis of the high-affinity iron chelator or siderophore exochelin, which is not produced by *M. tuberculosis* (144, 145, 216). Importantly, the production of a second siderophore, mycobactin, only affected growth in this *fxbA* mutant, linking the function of ESX-3 to iron uptake through mycobactin (147). Later, it was shown that the addition of hemin rescues the growth of an *esx-3* mutant in *M. tuberculosis* (144), supporting the hypothesis that ESX-3 is involved in iron uptake (145).

Both directed knockout experiments and transposon insertion sequencing experiments have shown that the central components of the ESX-5 system are required for growth of *M. marinum* and *M. tuberculosis* (149, 217). Using a modified screen, transposon mutants could be identified that allowed the deletion of ESX-5 (149). These mutants contained a transposon in either the *mas* or *ppsD* gene, both involved in the synthesis of the phthiocerol dimycocerosates and phenolic glycolipids. The absence of these outer membrane lipids results in a more permeable cell envelope (149). To test whether a more permeable outer membrane was responsible for the observed difference in *esx-5* essentiality, an independent mechanism was devised. Indeed, heterologous expression of the outer membrane porin MspA from *M. smegmatis* in *M. marinum* also allowed the generation of *esx-5* mutants (149). Because the ESX-5 system is only present in slow-growing mycobacteria, while MspA-like outer membrane porins are only present in fast-growing species, it was proposed that the ESX-5 system is able to substitute the function of these porins in nutrient uptake (149). Growth assays utilizing different carbon sources showed a difference in the growth rate of wild-type *M. marinum* compared to an *esx-5* mutant for Tween-80, suggesting that the ESX-5 system is involved in the utilization of fatty acids (149). ESX-5 mutants were subsequently shown to be unable to internalize fluorescently labeled fatty acids (149). In addition to its role in uptake, the ESX-5 system could additionally be involved in the processing of Tween-80, releasing the oleic acid, as one of its substrates, LipY, degrades long-chain triacylglycerols (261). The role of the ESX-5 system in nutrient uptake is further substantiated by its upregulation under phosphate-limiting conditions by the Pst-SenX3-RegX3 system (262). This would also mean that specific ESX-3 and ESX-5 substrates might form the outer membrane channels that mediate the uptake of specific nutrients.

The ESX-5 system has a second physiological role, because it is required for the integrity of the capsule of *M. marinum* via its substrate PPE10 (199). Mutations in the *esx-5* system components or in *ppe10* resulted in a reduced capsular layer and, subsequently, reduced amounts of glycolipids and surface-localized proteins, such as EspE and PE_PGRS proteins (199). As a result of these morphological differences, a *ppe10* mutant showed a reduced hemolytic activity and reduced ubiquitin binding during infection of host cells, indicative of phagosomal rupture and cytosolic translocation, respectively, as read-outs for ESX-1 functionality (199). A study also found a link between the ESX-1 system and cell envelope integrity, because *esx-1* mutants in *M. tuberculosis* have been shown to have an increased susceptibility to detergents (196), although another study showed that ESX-1 mutations did not impact cell envelope integrity (263). Finally, studies of *M. marinum* have suggested a link between the ESX-1 system and mycolic acid biosynthesis (210, 264). In addition to these physiological roles, substrates of ESX-5, mainly the PE_PGRS proteins, have been implicated in host-pathogen interactions by various groups, although some of these results are still ambiguous (265).

T7S in Other Species

As already mentioned, T7S is also found outside of the mycobacterial family, not only throughout the phylum of *Actinobacteria*, including corynebacteria, nocardia, and *Streptomyces* species, but also in more distantly related systems that can be found in the *Firmicutes*. The T7S systems found in *Actinobacteria* are highly similar to the ESX systems of mycobacteria, all containing an Esx protein pair and four of the five conserved membrane components, EccB, EccC, EccD, and MycP; the membrane component EccE is only present in some T7S systems. In many cases *pe/ppe* gene pairs can also be identified (4). Interestingly, although EccE has been shown to be an integral component of both the ESX-1 and ESX-5 membrane complexes, the structure of the ESX-5 complex of *M. xenopi* showed that $EccE_5$ is present at the periphery of this complex, and in the absence of $EccE_5$, complexes could still successfully be purified (225). This makes it reasonable that secretion complexes can be functional without the EccE component in some T7S systems of *Actinobacteria*. The strong conservation of MycP is also rather unexpected, because MycP is not an integral component of the core secretion complex in mycobacterial ESX systems of

(212, 224, 225). However, because MycP is required for a stable ESX complex and successful secretion in mycobacteria (214, 224), it is possible that this is a conserved feature in all *Actinobacteria*. In addition to this, the association of MycP to the core complex might be stronger in the T7S systems of other *Actinobacteria*. This hypothesis is supported by the T7S gene cluster found in *Bifidobacterium dentium*, where there are two *mycP* copies, one of which is part of a chimeric gene together with a complete homologue of *eccB*. This gene potentially produces a chimeric protein, in which the periplasmic domain of EccB is fused to the protease domain of MycP (4).

Within the *Actinobacteria*, T7S is not limited to species containing mycolic acids, i.e., to species with a diderm cell envelope, because it is also present, for instance, in *Streptomyces* species such as *S. coelicolor*. These mycolic acid-lacking bacteria can also contain the five conserved membrane components (164), supporting the model of the ESX membrane complex mediating secretion across the inner membrane only (225). Consequently, components, or substrates, that mediate secretion across the mycobacterial outer membrane should not be present in these monoderm species.

The T7S systems found in the *Firmicutes* show a lower similarity to the ESX systems of mycobacteria. In fact, only the FtsK-SpoIIIE ATPase and (potential) substrates belonging to the WXG100 protein family are conserved (128, 129, 166). In addition to these conserved features, the *Firmicutes* systems contain a set of unique genes, encoding either substrates or system-specific (functional) components. The T7S systems within different *Firmicutes* species also show relatively high variation among each other, both in substrates and in (predicted) structural components.

While functional systems have been confirmed for *L. monocytogenes*, *Streptococcus agalactiae*, *S. intermedius*, *Bacillus anthracis*, and *B. subtilis* (166, 220, 266–269), most information on T7S in *Firmicutes* has been obtained for the T7S system of *S. aureus*, also called Ess (ESAT-6 secretion system) (220). The Ess system contains five structural components, conserved throughout different *S. aureus* strains (270), i.e., EssA, EssB, EssC, EsaA (Esa, ESAT-6 secretion accessory), and EsaB. Whereas EsaB is cytoplasmic, the other four components are membrane proteins (270), with EssC being the conserved FtsK-SpoIIIE ATPase. Interestingly, the C-terminal ATPase domain of EssC shows four variants within different *S. aureus* strains that cluster with genes encoding different potential substrates. It was recently shown that this domain is indeed involved in specific substrate recognition (270, 271). The role of EsaB is uncertain, because it is not required for Ess secretion in all *S. aureus* strains. An interesting feature of EsaB is the ubiquitin-like fold, resembling the soluble domain of EccD of mycobacteria (218, 272, 273). However, it is unknown how and if these proteins share a comparable function.

Recently, several papers were published describing the first structural data on the Ess secretion complex. Whereas one study only found homo-oligomers of EsaA, EssB, and EssC (274), other studies showed a complex consisting of EssC, EsaA, EssB, and EssA (275, 276). It is possible that both the heteromeric and homo-oligomeric complexes are present in *S. aureus*, because Ess secretion in *S. aureus* is highly variable both in different strains and at different growth phases (272). In addition, a flotillin-like protein has been found to catalyze the heteromeric oligomerization of the Ess complex (275). This opens the possibility that scaffold proteins are involved in complex assembly upon their specific upregulation during, e.g., different growth phases or during infection (272, 277). Based on these three studies, the Ess complex seems less stable than the ESX-complexes found in mycobacteria, as for instance, shown by the strong effect of different mild detergents on the oligomerization state (274). This considerably complicates the isolation and characterization of the Ess secretion complex.

The Ess system of *S. aureus* contains two proteins of the WXG100 family, EsxA and EsxB, which are, unlike the homologous proteins in mycobacteria, not produced from bicistronic transcripts and not cosecreted as a heterodimer (278). Interestingly, EsxA is reported to either self-interact or interact with the substrate EsxC, and EsxB interacts with EsxD (279, 280). All four of these secreted proteins have been linked to the pathogenicity of *S. aureus* (272, 273, 279). Inactivation of the Ess system results in a less persistent infection and reduced bacterial survival (220, 272, 273); more specifically, Ess-mediated secretion of EsxA and EsxB is required for the formation of staphylococcal abscesses and the blocking of apoptosis, indicating a role in intracellular survival (220, 273, 281, 282). Unlike the ESX-3 and ESX-5 systems of mycobacteria, which are required for bacterial viability, the Ess system of *S. aureus* is dispensable for *in vitro* growth (220, 273).

The *B. subtilis* T7S system (also known in the literature as the YukE-Yue system) resembles the Ess system of *S. aureus*. The notable difference is that it only seems to secrete a single substrate, the WXG100 protein YukE, which forms a homodimer (167, 283, 284). Similar to the Ess system, the YukE-Yue system also contains five functional components, the membrane proteins YukC, YukBA, YueB, and YueC and the cytoplasmic YukD, the latter resembling EsaB of *S. aureus* (167, 283, 285). YukBA is the FtsK/SpoIIIE ATPase of the system. A *yueB* mutant shows reduced conjugative DNA transfer, similar to the ESX-1 system *M. smegmatis* (286). WXG100 genes have also been found in the pathogenic strains *Bacillus cereus*, *Bacillus thuringiensis*, and *B. anthracis*, and at least several of their protein products are actively secreted (167, 267, 283). For example, *B. anthracis* contains six WXG100 genes, of which the gene products EsxB and EsxW are secreted during *in vitro* growth, while the other genes might only be expressed during infection (267).

One distinguishing feature of the T7S systems of *Firmicutes* compared to the ESX systems of mycobacteria is their involvement in bacterial competition by mediating the secretion of toxins. This intriguingly shows that *Firmicutes* use T7S systems in bacterial warfare similarly to how Gram-negative bacteria use type VI secretion systems. For example, *S. aureus* secretes a fifth substrate, EsaD, which functions as an antibacterial DNase (287). The toxic effects of EsaD are blocked by EsaG, an antitoxin produced also by *S. aureus* strains lacking EsaD (287). A T7S system in *S. intermedius* secretes so-called LXG proteins, with N-termini homologous to WXG100 proteins, that function as antibacterial toxins in a contact-dependent manner (266). LXG toxins with RNase activity have also been found in *B. subtilis* (288) and shown to have antibacterial properties when produced in *E. coli*. Although they contain a typical N-terminal WXG100 motif, it is not known whether these proteins are dependent on the YukE-Yue system for secretion (288). A genomic study found that predicted T7S substrate genes with putative toxin activities are present in a range of different species, indicating that T7S systems might be widely used by *Firmicutes* in bacterial competition (289).

OUTLOOK

In the past 15 years, considerable effort has been made to understand the different export pathways of mycobacteria. A major focus of research in this area has been the identification

of substrates of the different systems and their roles in mycobacterial physiology and virulence. In parallel, we are steadily obtaining insight into the components of these systems and their mechanisms of substrate recognition and transport. However, a major topic that needs to be addressed is the issue of how proteins transit the specific mycolic acid-containing second membrane for release into the extracellular environment. While T7S substrates are among the most abundant proteins found in culture supernatants, there are also signal peptide-containing proteins that are efficiently secreted into the extracellular environment (17, 18, 63, 120, 290–292), emphasizing the importance of an outer membrane translocation step. Protein transport across this highly impermeable outer membrane should be tightly regulated to maintain the permeability barrier, because this is crucial for pathogenic mycobacteria to withstand the harsh conditions of their intracellular niche. As discussed in this article, for T7S, some specific substrates might be involved in the second membrane transport event; however, the mechanism of outer membrane transport for the Sec, Tat, and SecA2 substrates is a complete mystery. Research focused on identifying and characterizing the proteins that are localized to this special mycobacterial outer membrane is urgently needed to gain insight into these processes.

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Corynebacterium diphtheriae: Diphtheria Toxin, the *tox* Operon, and Its Regulation by $Fe2^+$ Activation of apo-DtxR

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69

INTRODUCTION

Over the past 4 decades, perhaps no other infectious disease has been as successfully studied as diphtheria (1, 2). Indeed, the study of diphtheria toxin established the structure-function paradigm for the study of other toxins in the bacterial protein toxin field. Moreover, when coupled with the molecular genetic study of the iron-activated regulatory element, DtxR, that controls the expression of diphtheria toxin, we now have a detailed understanding of the entire *tox* genetic system, from the regulation of expression to the molecular mechanism of diphtheria toxin action. In this article, we review the development of our current understanding of diphtheria toxin, from its structure-function relationships to its mechanism of entry into the eukaryotic cell cytosol, the molecular mechanism of transition metal ion activation of DtxR and its regulation of *tox* expression, and finally, the protein engineering of diphtheria toxin for the development of highly potent and selective cell-surface receptor-targeted fusion protein toxins for the treatment of human diseases.

It is well known that to cause clinical disease, the etiologic agent of diphtheria, *Corynebacterium diphtheriae*, must first be infected with one of a closely related family of bacteriophages that carry the structural gene for the toxin. Humans are the only natural host for diphtheria, and disease is readily spread through close contact and aerosolized droplets. While clinical diphtheria remains prevalent in tropical developing countries where immunization with diphtheria toxoid remains sporadic, diphtheria is almost nonexistent in industrialized countries that have continued to maintain a comprehensive immunization program with diphtheria toxoid. Since diphtheria toxin is secreted into the culture medium, the high-yield-producing Park-Williams 8 strain of *C. diphtheriae* has been used almost worldwide to produce diphtheria toxin in sufficient yields to produce diphtheria toxoid (3). Once purified from the spent culture medium, native diphtheria toxin is then treated with formalin at 30 to 38°C for several weeks to produce the nontoxic toxoid. To produce the vaccine, diphtheria toxoid is absorbed to alumina, which acts as an adjuvant, and it is then combined with tetanus toxoid and either heat-killed *Bordetella pertussis* to produce the DPT vaccine or purified pertussis antigens to produce the diphtheria-tetanus-acellular pertussis vaccine.

Along with botulinum, tetanus, and Shiga, diphtheria toxin is one of the most potent bacterial protein toxins known. In humans the 50% lethal dose for diphtheria toxin was found to be approximately 100 ng/kg. This value was determined after a catastrophic accident in Kyoto, Japan, in 1948. Shortly after the end of World War II, a mass immunization program against diphtheria was initiated in Japan. After the production of diphtheria toxin and its distribution into vials for formalin detoxification in the preparation of toxoid, one vial of toxin was either inadvertently not treated or reverted to full toxicity after formalin treatment and was sent into the community as part of the program to immunize children. Of 606 children that were "immunized" from this vial, 68 children, mostly between the ages of 1 and 2, died of diphtheria intoxication (4, 5). Since the concentration of toxin in the vial, the dose that was administered, and the body weight of the children were all known, the human 50% lethal dose for diphtheria toxin could be calculated.

While diphtheria toxoid as a vaccine was available as early as the 1920s, investigations into the development of a simplified medium for toxin production led Alwin M. Pappenheimer, Jr., to make one of the earliest observations that the concentration of iron in the growth medium was critical to obtain maximal yields of the toxin (6). In 1935, Pappenheimer, then a Bradford Fellow at the Harvard Medical School, started his laboratory at the Massachusetts Antitoxin and Vaccine Laboratory to work on diphtheria toxin production. At that time Pyrex glassware was newly available, and he used Pyrex flasks in his first attempts to produce toxin. While he followed established protocols for medium preparation and incubation temperature and time for maximal yields, Pappenheimer was able to produce only half as much toxin as that reported by his predecessors who used the older soft glassware flasks in the laboratory. The question was obvious: Why did the glassware make a difference in toxin production? Pappenheimer's experiment to address the question was equally obvious: He broke a flask made of soft glass and ground it into a powder. He then added varying amounts of powdered glass to the Pyrex flasks he was using and determined its effect on toxin production. Remarkably, Pappenheimer found that the addition of as little as 300 μg powdered soft glass resulted in a stimulation of both *C. diphtheriae* growth and toxin yield. Equally importantly, he found that the addition of 5 or 10 mg of powdered glass resulted in the almost complete inhibition of toxin production without a change in the growth of the diphtheria

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bacillus. The irony (pun intended) of this story is that Pappenheimer wrote his Ph.D. dissertation at Harvard College on the effect of organic bases on the oxidation potential of heme. It is no surprise then that his first experiments were focused on the effect of adding increasing amounts of inorganic iron to the growth medium as a function of diphtheria toxin yield. As shown in his classic 1936 paper (6), the stimulation and subsequent inhibition of toxin production following the addition of either powdered glass or iron salts to the media were superimposable. The ability to produce a maximal yield of diphtheria toxin in the soft glass resulted from the leaching of small levels of iron from the glass into the culture medium. We now know that maximal yields of diphtheria toxin are produced only when iron becomes the growth-rate-limiting substrate. So, as early as 1936, it was realized that with respect to iron, an essential nutrient for the growth of the bacillus, the physiologic state of toxigenic *C. diphtheriae* was a determining factor in the production of diphtheria toxin.

TEMPERATE CORYNEBACTERIOPHAGES CARRY THE STRUCTURAL GENE FOR DIPHTHERIA TOXIN, *tox*, IN LYSOGENIC *C. DIPHTHERIAE*

While it had been known since the earliest observations of *C. diphtheriae* that there were both virulent and nonvirulent strains that could be isolated from healthy individuals, it was not until the remarkable discovery by Freeman (7) that one could isolate diphtheria toxin-producing strains following the exposure of nontoxigenic strains to corynebacteriophages. Thus, it became clear that not only the physiologic state of *C. diphtheriae* with respect to the concentration of iron in the growth medium but also its lysogenic state determined the ability of a given isolate to produce diphtheria toxin. As important as Freeman's observation was, the question of whether it was the host bacterium or the lysogenic phage that carried the structural gene for the toxin still remained.

Twenty years after Freeman's initial report, Uchida et al. (8) described the isolation of corynebacteriophage β mutant lysogens of *C. diphtheriae* that produced nontoxic proteins that were serologically related to diphtheria toxin. The discovery of these mutant serologically related cross-reacting materials (CRMs) was extremely important because they not only demonstrated that the structural gene for diphtheria toxin, *tox*, was carried by β-phage, but the isolation of CRMs that were the result of both nonsense (e.g., CRM45) and missense (e.g., CRM197) mutations established both the genetic orientation of the toxin, N- to C-terminal, and began to give both biochemical and genetic definition to its structural-functional domains.

While corynebacteriophage β has not been nearly as well studied as coliphage λ, it is a temperate phage and, as such, may either enter a vegetative lytic cycle or lysogenize a sensitive host (e.g., the nontoxigenic, nonlysogenic $C7_s(-)^{tox-}$ strain of *C. diphtheriae*) and convert it from a *tox*– to a *tox*+ phenotype. Both genetic and molecular genetic evidence suggest that the linear β-genome circularizes by ligation of its cohesive ends (*cos*) and integrates into the host chromosome as a prophage in a manner that corresponds to that for the integration of λ-phage into the *Escherichia coli* genome (9–11). It has been shown that the prophage map is a circular permutation of the vegetative map and that the *imm* marker is positioned at one end of the β-genome and *tox* is at the other end (Fig. 1). The $C_s7(-)^{tox-}$ genome carries two

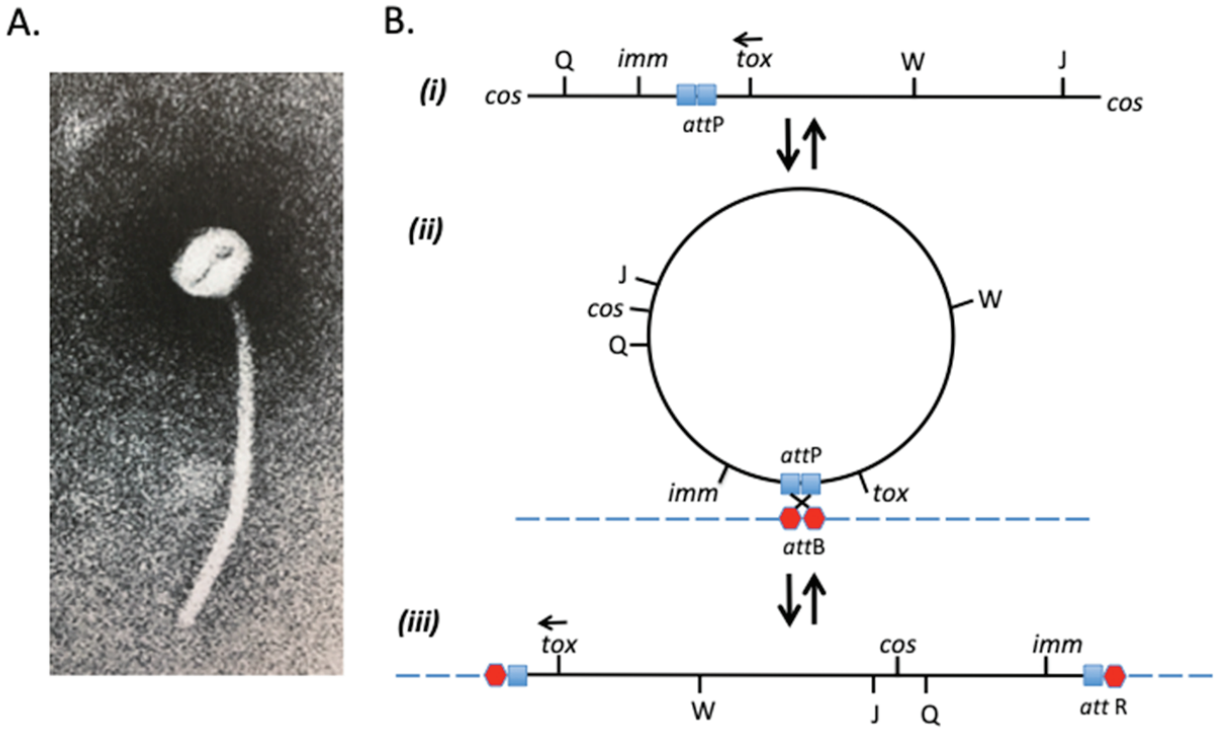


FIGURE 1 (A) Electron micrograph of corynebacteriophage β, which has a polyhedral head ~52 nm in diameter and a 270 nm long tail. (B) Genetic map of β-phage in its (i) vegetative phase, (ii) circularized form, and (iii) prophage state. (Modified from reference 9.)

separate *attB* sites, *attB1* and *attB2*, and as a result, β-phage can integrate and form single lysogens, double lysogens, and in rare instances, triple lysogens (12). In its lysogenic state, most β-phage genes appear to be repressed, and the lysogen is immune to superinfection by homoimmune corynebacteriophages. While triple lysogens are unstable and revert to stable double lysogens, under iron-limiting conditions, the final yield of diphtheria toxin that may be produced is directly related to the number of integrated prophage genomes (12).

CORYNEBACTERIAL DETERMINED DtxR REGULATES EXPRESSION OF THE CORYNEBACTERIOPHAGE *tox* GENE

As noted above, the discovery by Uchida et al. (8) that the structural gene encoding diphtheria toxin was carried by corynebacteriophage β raised the question of whether the iron-sensitive regulation of *tox* expression was mediated by either a corynebacteriophage or corynebacterial determinant. To address that question, Murphy et al. (13) used S-30 extracts of *E. coli* in a coupled transcription-translation system programmed with β-phage DNA to synthesize diphtheria toxin *in vitro*. In this system, diphtheria toxin was expressed in good yield, and remarkably, the *in vitro* synthesis of the toxin was not inhibited by the addition of iron. In contrast, the addition of cell-free extracts of the nontoxigenic, nonlysogenic $C7_s(-)^{tox-}$ strain of *C. diphtheriae* to the *E. coli* system resulted in the inhibition of *tox* expression but not that of other phage gene products. These results clearly suggested that the inhibitory effect of iron was mediated through a bacterial host-determined factor. Furthermore, the subsequent isolation of *C. diphtheriae* mutants which when lysogenized by β-phage constitutively expressed diphtheria toxin in the presence of excess iron (14), as well as the isolation of phage mutants which constitutively expressed diphtheria toxin when lysogenized in wild-type *C. diphtheriae* (15, 16), ultimately led to a hypothetical model for the regulation of *tox* expression (Fig. 2).

DNA sequence analysis of the structural gene for diphtheria toxin revealed a 27-bp interrupted palindromic sequence that overlapped the putative –10 region of the *tox* promoter (17–20). Based on these observations, this upstream region of the gene encoding diphtheria toxin was then designated the *tox* promoter-operator, *toxPO*. In 1989, Fourel et al. (21) used DNase protection assays to show that an element(s) found in crude cell-free extracts of *C. diphtheriae* specifically bound to *toxO*. Mutant strains of *C. diphtheriae* that are *cis*-dominant for toxin production were later found to carry mutations only in the *tox* operator (22–24).

To clone the *trans*-acting putative *tox* repressor, Boyd et al. (25) screened genomic libraries of the $C7_s(-)^{tox-}$ strain of *C. diphtheriae* in recombinant *E. coli* that carried a transcriptional fusion between *toxPO* and *lacZ*. This work led to the isolation of the structural gene for the diphtheria *tox* repressor, *dtxR*, which encoded a 226-amino acid protein. DtxR was shown to repress the expression of β-galactosidase from the *toxPO-lacZ* fusion in *E. coli* in an iron-dependent fashion. Schmitt and Holmes (23) subsequently demonstrated the functional activity of DtxR in *C. diphtheriae* by showing that expression of wild-type *dtxR* in the iron-insensitive host-mutant C7hm723(β^{tox+}) strain resulted in conversion to the wild-type iron-sensitive phenotype.

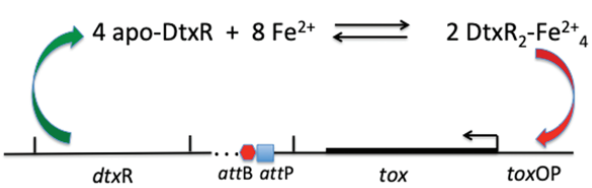


FIGURE 2 Schematic representation of Fe^{2+} activation of apo-DtxR and the binding of two dimers to the *tox* operator, thereby repressing expression of the diphtheria toxin structural gene, *tox*.

TRANSITION METAL IONS ACTIVATE DtxR BINDING TO THE *tox* OPERATOR

By using gel mobility shift assays, Tao et al. (26) demonstrated that the activation of apo-DtxR and its subsequent binding to a [^{32}P]-labeled *toxO* probe required the addition of divalent transition metal ions to both the binding reaction mixture and the gel matrix. Since binding of DtxR to the labeled *toxO* probe was blocked by the addition of excess unlabeled probe, anti-DtxR antibodies, or the chelator 2,2′-dipyridyl, DtxR binding was specific and dependent on metal ion activation. Both Tao and Murphy (27) and Schmitt and Holmes (28) demonstrated that the addition of Co^{2+}, Fe^{2+}, Ni2+, Cd^{2+}, or Mn^{2+} activated apo-DtxR and resulted in the protection of the *toxPO* probe in DNase protection assays. Zinc was found to be only a weak activator of apo-DtxR, and Cu^{2+} failed to activate repressor activity.

While apo-DtxR could be activated by a number of divalent transition metal ions, Tao and Murphy (29) demonstrated that the activated DtxR could also bind to a number of closely related DNA target sequences. To determine the consensus operator sequence for DtxR binding, Tao and Murphy used a gel electrophoresis mobility-shift assay and PCR amplification for *in vitro* affinity selection of randomized DNA sequences from a universe of $>6 \times 10^{10}$ variants. After 10 rounds of *in vitro* selection, each with 30 cycles of PCR amplification, a family of DNA sequences that functioned as DtxR-responsive elements both *in vitro* and *in vivo* was isolated and characterized. The consensus sequence for DtxR binding was found to be as follows: T.AGGTTAGC/GCTAACCT.A. Moreover, since this family of related target sequences was found to bind DtxR with the same apparent affinity as the 27-bp *tox* operator, it was clear that the iron-activated repressor was most likely to function as a global regulatory element in the regulation of iron-sensitive genes in *C. diphtheriae*. Indeed, a number of genes which have upstream DtxR-binding sites, including the operon essential for the expression of siderophores for iron acquisition, have been isolated and characterized (23, 28).

Since the cloning and characterization of DtxR from *C. diphtheriae*, homologous metal ion-activated repressors have been identified in a number of other Gram-positive prokaryotes. IdeR (iron-dependent regulator) which has been found in several species of *Mycobacterium* and is 78% identical and 90% homologous has been isolated and characterized (30). Remarkably, expression of the *C. diphtheriae* hyperactive repressor, DtxR(E175K), in merodiploid strains of both *Mycobacterium tuberculosis* and *Staphylococcus aureus* has been shown to attenuate virulence *in vivo*, presumably by repression of iron-sensitive genes (31–33). These results clearly suggest that there is a high degree of both function and DNA sequence target specificity between DtxR and IdeR. In addition, DtxR homologs have been identified in a number of genera, including the species *Enterococcus faecalis* (34), *Streptococcus mutans* (35), *S. aureus* (36), *Streptomyces pilosus* (37), and *Rhodococcus equi* (38). It is of interest to note that

both *M. tuberculosis* and *C. diphtheriae* also express additional DtxR-like proteins which are likely to be activated by different transition metal ions and have different DNA target sequences (39), including the MntR protein in *C. diphtheriae*, which is responsive to Mn^{2+}.

STRUCTURAL BASIS OF TRANSITION METAL ION ACTIVATION OF DtxR REPRESSOR ACTIVITY

As noted above, DtxR is a 226-amino acid protein. DNA sequence analysis revealed a single cysteine residue, Cys102, and disulfide-linked dimers are inactive. The substitution of Cys102 with all 20 amino acids, except for Asp, by site-directed mutagenesis results in the complete loss of repressor activity. Further characterization of the wild type and individual mutants demonstrated that Cys102 plays an essential role in the coordination of Fe^{2+} in the activation of apo-DtxR (40). In addition, Wang et al. (24) used bisulfite mutagenesis to inactivate DtxR and found that a high percentage of mutations were found in regions of DtxR that exhibited homology with other repressors. In addition, a number of mutations were also isolated in a predicted α-helical region with the sequence of His98-Cys102-His106 that resembled metal ion-binding motifs in other proteins.

A more complete understanding of the structural basis of metal ion-activated DtxR repressor activity was revealed by the solution of the crystal structures of apo-DtxR and holo-DtxR in complex with *toxPO* (41–44). As shown in Fig. 3, the structure of the C-terminal end of DtxR was found to fold into a SRC homology 3 (SH3)-like domain (45). The SH3 domain was first described as a conserved sequence in the viral adaptor protein v-Crk but has also been found to be present in phospholipases and the cytoplasmic tyrosine kinases, Abl and Src. In general, SH3 domains are found in proteins that either interact with other proteins or mediate the formation of specific protein complexes by binding to proline-rich peptide sequences.

DtxR contains a total of eight α-helices, six of which are contained in the N-terminal two-thirds of the protein. Helices B and C and the loop connecting them form the helix-turn-helix DNA-binding motif. Interestingly, Ni^{2+}-activated DtxR was shown to bind to the *toxPO* oligonucleotide as two pairs of dimers (44). In this instance, each DtxR dimer was found to bind to almost opposite faces of the palindromic *tox*O sequence (Fig. 4). As one would anticipate, the solution of the X-ray structures of DtxR and the ternary complex that forms with its binding to *tox*O confirms and extends the earlier observation that its footprint compasses a region of 30 bp immediately upstream of the transcription initiation signal (46).

While the overall mechanism of DtxR binding to *tox*O is similar to that of other prokaryotic repressors, there are some unique interactions that should be noted. The C-helix of DtxR is responsible for most interactions with DNA and inserts into the major groove of the DNA double helix. Each helix-turn-helix in the dimer makes a total of nine interactions with backbone phosphate groups. In addition, the guanidinium group of Arg60 binds in the minor groove of DNA and thereby makes a bridge to additional phosphate groups. Since there is a structural rearrangement of activated DtxR upon binding to the *tox* operator, Thr7 in helix A also interacts with a backbone phosphate group, and Ser37 and Pro39 contact methyl groups on thymine bases in the *tox* operator through van der Waals interactions (47).

While saturation and equilibrium dialysis experiments suggested that DtxR contained a single metal ion-binding site with an apparent dissociation constant of 2×10^{-6} to 9×10^{-7} M (46), X-ray crystallographic analysis of transition metal ion-DtxR complexes clearly revealed two metal ions bound to each monomer (41, 43). Using site-directed mutagenesis, Ding et al. (48) demonstrated that the primary metal ion-binding site of DtxR is composed of Met10, Cys102, Glu105, His106, and a water molecule form an octahedral coordination center. The second metal ion-binding site, or ancillary site, is composed of five residues: His79, His98, Glu83, Glu170, and Gln173 (41, 43, 49). The role played by the ancillary metal ion-binding site was elucidated through the analysis of DtxR(E175K), a hyperactive mutant that remained active *in vivo* even in the presence of the chelator 2,2′dipyridyl (50). *In vitro* studies with purified apo-DtxR(E175K) demonstrated that this mutant required very low levels of metal ion to transit from an inactive apo- to

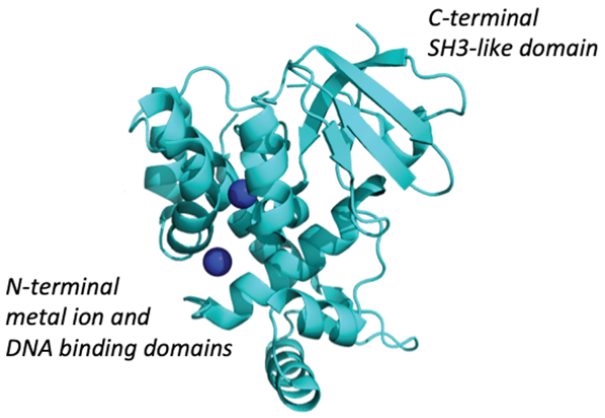


FIGURE 3 X-ray crystal structure of Co^{2+}-activated DtxR showing its N-terminal metal ion and DNA-binding domain and the C-terminal SH3-like domain. (Modified from reference 45 [Protein Data Bank ID 1C0W].)

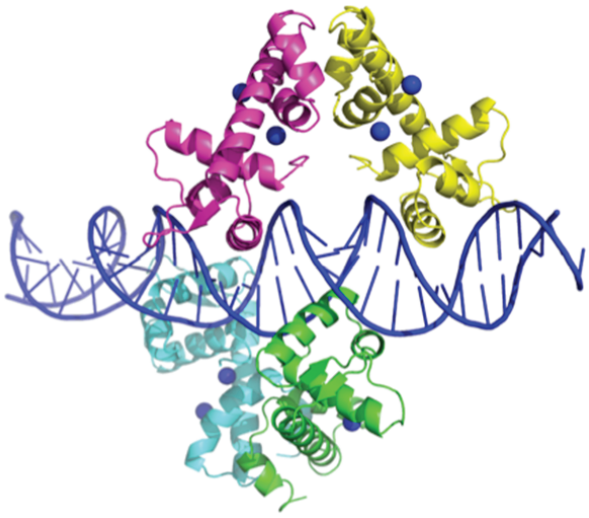


FIGURE 4 X-ray crystal structure of Ni^{2+}-activated DtxR (C102D) bound to the *tox* operator. Due to high thermal values, the C-terminal SH3-like domain is not shown in this Ni^{2+} activated structure. (Modified from reference 44 [Protein Data Bank ID 1DDN].)

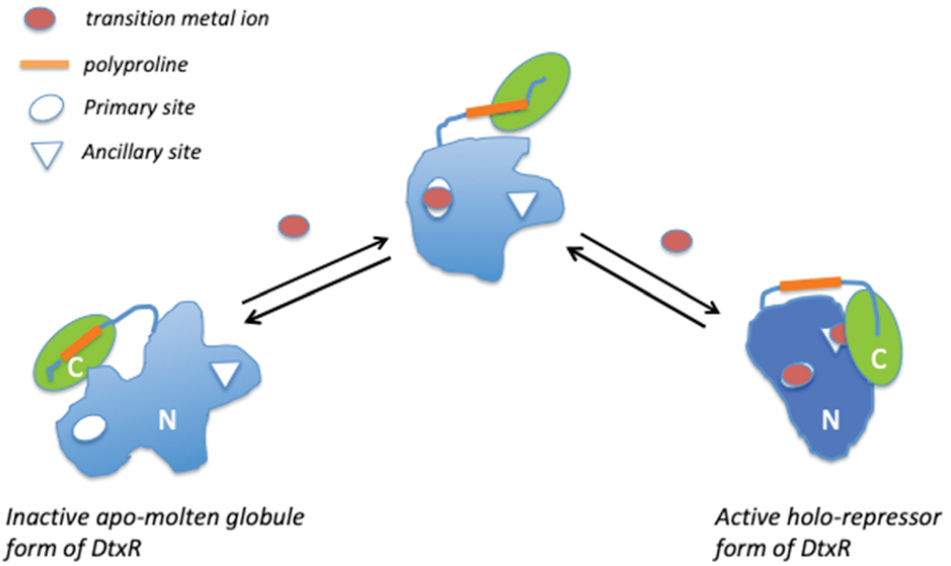


FIGURE 5 DtxR is a two-domain protein that is composed of the N-terminal [blue] and C-terminal SH3-like [green] regions and functionally distinct transition metal ion-binding sites (primary and ancillary). An activating transition metal ion first binds to the primary site. This binding event orients the DNA-binding helices and begins to induce the folding of the disordered N-terminal domain. Subsequent binding of a second metal ion to the ancillary site disassociates and reorients the SH3-like domain away from the poly-proline region of the repressor and completes the folding of the N-terminal domain, resulting in the formation of the dimer interface of the active holo-repressor. (Modified from reference 52.)

the active form of the repressor. Love et al. (51) demonstrated that the hyperactive phenotype of DtxR(E175K) was dependent on an intact ancillary metal ion-binding site and that this site facilitated the conversion of the inactive apo-repressor to its active *tox*O binding conformation.

Nuclear magnetic resonance solution structures combined with other biophysical studies have suggested that apo-DtxR exists in a partially unstructured molten globule, which upon coordination with divalent transition metal ions undergoes a structural conversion to a discrete ordered tertiary structure that both dimerizes and is able to bind to the *tox* operator (52). As shown schematically in Fig. 5, in its inactive molten globule apo-form, the SH3-like domain forms an intramolecular complex with a proline-rich peptide segment of the repressor and, in doing so, destabilizes the ancillary metal ion-binding site of the repressor, and this leads to the complete inactivation of the repressor. Upon metal ion-binding to the primary site, the SH3-like domain disassociates from this proline-rich region, thereby allowing a structural conversion and stabilization of the ancillary metal ion-binding site. The resulting conformational changes then allow the binding of the second metal ion to the ancillary site and subsequent dimerization of DtxR and the formation of an active repressor (53).

STRUCTURE-FUNCTION RELATIONSHIPS OF DIPHTHERIA TOXIN AND THE MOLECULAR MECHANISM OF ITS ACTION

Diphtheria toxin is expressed and secreted into the culture medium as a single polypeptide chain of 62 kDa by *C. diphtheriae*. The toxin is readily purified from the spent culture supernatant by ammonium sulfate precipitation followed by ion exchange chromatography on a diethylaminoethyl matrix. While the intact toxin is enzymatically inactive, exposure of purified toxin to trypsin or other serine proteases results in "nicking" of the protein and the subsequent activation of adenosine diphosphate ribosyl (ADPR) transferase. Under reducing and denaturing conditions, nicked diphtheria toxin may be separated into an enzymatically active N-terminal 24-kDa fragment A and its 38-kDa C-terminal fragment B (54, 55). Honjo et al. (56) and Gill et al. (57) demonstrated that nicked diphtheria toxin and fragment A were enzymatically active in catalyzing the following reaction:

$$\text{NAD}^+ + \text{elongation factor 2} \leftrightarrow \text{ADPR-elongation factor 2} + \text{nicotinamide} + \text{H}^+$$

Yamaizumi et al. (58), in a series of elegant experiments, loaded erythrocyte ghosts with various concentrations of purified fragment A and a known concentration of fluorescein isothiocyanate-labeled bovine serum albumin. These preloaded red cell ghosts were then fused to diphtheria toxin-resistant mouse L-cells by Sendai virus. Using a fluorescence-activated cell sorter, L-cells that fused with a single red cell ghost were then isolated and grown for 7 days. Careful analysis of the colony-forming ability of the recipient cells compared to the control cells clearly demonstrated that the delivery of a single molecule of fragment A to the cytosol was sufficient to kill that cell.

Comparison of the nontoxic mutants CRM197 and CRM45 revealed that the C-terminal 38 kDa fragment B of diphtheria toxin carried two functional domains: a hydrophobic domain (59), which under denaturing conditions behaved like an integral membrane protein, and the native receptor-binding domain. As such, it was realized through biochemical and genetic analyses that native diphtheria toxin was a protein with at least three structural-functional domains: (i) catalytic, (ii) transmembrane or translocation, and (iii) receptor-binding domains. As shown in Fig. 2, this prediction

proved to be correct with the determination of the crystal structure of diphtheria toxin (60, 61). While DNA sequence analysis of the *tox* structural gene revealed the presence of a 21-amino acid signal sequence (18–20), the mature form of diphtheria toxin is a 535-amino acid polypeptide. The N-terminal catalytic domain consists of amino acids Gly1 to Cys186 and is composed of eight β-strands that form two β-sheets forming a core structure that is surrounded by seven short α-helices. The β-sheets that form the central core also form the active site cleft. The catalytic domain is connected to the centrally positioned translocation domain by a 14-amino acid loop that is subtended by a disulfide bond between Cys186 and Cys201. This exposed loop carries an ArgArgValArg protease recognition site for either furin or other trypsin-like proteases. The proteolytic cleavage of this site is essential for the release of the catalytic domain into the eukaryotic cell cytosol (62). The translocation domain encompasses amino acids Cys186 to K385 and is composed of nine α-helices and their connecting loops. The translocation domain plays an essential role in the intoxication process by forming an 18- to 22-Å channel, or pore, through which the catalytic domain is threaded from the lumen of acidified endocytic vesicles into the cytosol. This channel is formed by translocation helices 5 to 7 and 8 to 9, which are highly hydrophobic and form two membrane-soluble "daggers" (61). The loops connecting translocation helices 8 to 9 and 5 to 6 are highly acidic and carry a total of six Asp and Glu residues. Upon internalization of the toxin into an endosomal compartment and the acidification of the vesicle lumen by the vacuolar ATPase, the carboxylic acid groups on these Asp and Glu residues become protonated and lose their respective charges, allowing for membrane insertion and channel formation.

Ratts et al. (3) developed an *in vitro* assay system composed of partially purified endosomes that were preloaded with either diphtheria toxin or the fusion protein toxin DAB389IL-2 in the presence of the vacuolar ATPase inhibitor bafilomycin A1 to probe the requirements necessary for catalytic domain translocation from the endosomal lumen to the external medium. Upon removal of bafilomycin A1 and addition of ATP, the endosomal lumen becomes rapidly acidified; however, catalytic domain translocation to the external medium was shown to require the addition of eukaryotic cytosolic fractions to the assay mixture. Using this assay system, Ratts et al. (3) demonstrated that both coatomer complex 1 (COPI) and thioredoxin reductase were essential for *in vitro* translocation of the catalytic domain. Ratts et al. (63) also described a highly conserved motif, T1, in translocation helix 1 of diphtheria toxin that played an essential role in the delivery of the catalytic domain to the cytosol. In 2010, Trujillo et al. (64) used both site-directed mutagenesis and a COPI complex precipitation assay to demonstrate that the interaction(s) between at least three lysine residues in the T1 motif are essential for both COPI binding and delivery of the catalytic domain to the eukaryotic cell cytosol. Further, Trujillo et al. (64) demonstrated that substitution of the lysine-rich region in translocation helix 1 with the COPI binding portion of the p23 adaptor cytoplasmic tail results in a mutant form of toxin that displays full wild-type activity against sensitive eukaryotic cells.

The above-described studies demonstrate that diphtheria toxin has evolved to become an extremely efficient nanomachine in which all of its structural domains play an essential role in the intoxication of sensitive eukaryotic cells. The molecular mechanisms leading toward intoxication require at least the following steps: (i) binding of the toxin to its cell surface receptor, the heparin-binding epidermal growth factor precursor, (ii) furin-mediated nicking of the protease-sensitive RVRR site in the 14-amino acid exposed loop between the catalytic and translocation domains, (iii) internalization of toxin into an endosomal compartment that becomes acidified through the action of its vacuolar ATPase, (iv) insertion of translocation helical loops formed by TH5-7 and TH8-9 into a vesicle membrane forming a channel or pore, (v) the insertion of the disulfide-linked C-terminal end of the catalytic domain and N-terminal end of the translocation domain into the channel, (vi) protein::protein interactions between the dibasic KXKXX signatures in translocation helix 1 with COPI, which facilitates the threading and translocation of the catalytic domain through the channel, (vii) reduction of the disulfide bond between the catalytic and translocation domains by thioredoxin reductase, (viii) the release and refolding of the catalytic domain into an enzymatically active ADP-ribosyltransferase, and (ix) the NAD^+-dependent ADP-ribosylation of elongation factor 2, which results in the cessation of protein synthesis and death of the cell (65).

MOLECULAR EPIDEMIOLOGY OF DIPHTHERIA AND DtxR

Outbreaks of clinical diphtheria almost always occur in individuals who have not been immunized with either the DPT or diphtheria-tetanus-acellular pertussis vaccine and who have been exposed to a "carrier" (a person who carries a toxigenic strain of *C. diphtheriae* as part of their normal flora) who has recently traveled to a country where mass immunization against diphtheria is not performed and disease is still prevalent. *C. diphtheriae* is usually transmitted via the aerosol route; close contact with an individual presenting with cutaneous diphtheria may also result in transmission. While colonization of a susceptible individual with a toxigenic strain certainly plays a role in pathogenesis, Pappenheimer and Murphy (66) demonstrated that transmission of toxigenicity may also occur by *in situ* lysogenic conversion of an autochthonous nontoxigenic strain of *C. diphtheriae* to toxigenicity. It is remarkable that immunization with diphtheria toxoid results in the production of neutralizing antibodies that not only block diphtheria toxin from binding to its cell surface receptor, but also protects against clinical disease (67, 68). It is well known that mass immunization of populations with diphtheria toxoid has led to a dramatic decrease in the incidence of clinical disease (69). For example, in 1958, before mass immunization with diphtheria toxoid in Romania, only 60% of the population was immune to diphtheria toxin, whereas by 1979, the percentage dramatically increased to 97%. At the same time, diphtheria morbidity dropped from ca. 600 per 100,000 in 1958 to only 1 per 100,000 in 1972 (67, 70).

The consequence of either stopping or dramatically reducing a mass immunization program against diphtheria is also known. During the 1990s, the Newly Independent States (NIS) of the former Soviet Union experienced a sweeping epidemic of clinical diphtheria. With the breakup of the Soviet Union, public health immunization programs were dramatically affected and by 1990 only 68% of Russian children had received the appropriate vaccination regimen (71). As a result, by 1995 the incidence of clinical diphtheria in the NIS peaked at over 50,000 cases. From the onset of the diphtheria outbreak in 1990 through 1999, more than 157,000 cases and 5,000 deaths were reported.

Given the increased incidence of clinical diphtheria in the NIS, Kolodkina et al. (72) characterized 129-bp single-strand conformational polymorphism DNA fragments that contained the *toxPO* region from 81 strains of *C. diphtheriae*.

From this group, only two strains had mutations. Nucleotide sequence analysis revealed T to C mutations in positions –54 and –184 of the *tox* operon. The –54 mutation was found in the 9-bp interrupted palindromic sequence of *toxPO*, and these two strains had the highest level of toxin production out of the 81 strains characterized. In addition, these investigators also analyzed the nucleotide sequence of the *dtxR* gene. Fifteen strains in this collection were found to carry two missense mutations in DtxR, A147V and L214I in the C-terminal region of the repressor. Interestingly, the *dtxR* alleles in the two strains that expressed the highest levels of diphtheria toxin were not found to carry a nucleotide substitution. This initial study was extended by Kombarova et al. (73), who found seven point mutations in the structural gene for diphtheria toxin. While most of these mutations did not result in an amino acid substitution, two strains from the Otchakov ribotype carried a G393R mutation in the native receptor-binding domain of the toxin. In contrast, 16 point mutations in *dtxR* were found. Of these, the A147V mutation is characteristic of the epidemic clonal group Sankt-Peterburg/Rossija, which was the dominant strain isolated in the Russian diphtheria epidemic in the 1990s.

It is remarkable that the amino acid sequence of diphtheria toxin encoded by the omega-phage in the Park-Williams strain of *C. diphtheriae*, PW8, which was isolated in 1896 (74), is identical to the amino acid sequence of the toxin produced by all but two of the clinical isolates carrying the G393R mutation that have been characterized from the NIS diphtheria epidemic. While several missense mutations within the *tox* gene have been described, they encode the same amino acid as found in the PW8 allele. It is interesting to note that in the single exception, the G393R substitution falls early on in the toxin's receptor-binding domain and is far removed from the region of that domain which interacts with the toxin's eukaryotic cell surface receptor-binding region. This extraordinary conservation of amino acid sequence strongly suggests the highly evolved nature of the toxin's structure-function relationships. This conservation of amino acid sequence for at least 100 years suggests that the evolution of new *tox* alleles that might encode a toxin sufficiently different to avoid neutralization by antitoxoid antibodies is highly unlikely.

In contrast, this high degree of conservation does not hold for DtxR. In the case of this metal ion-activated repressor, the N-terminal region of *dtxR* was found to carry several missense mutations that did not give rise to an amino acid substitution. In contrast, missense mutations in the C-terminal region of the repressor were often found to encode amino acid substitutions that appeared to subtly affect DtxR activity (51, 75–77).

TURNING THE SWORD OF DIPHTHERIA INTO THE PLOWSHARE OF TARGETED THERAPEUTICS

Shortly after Köhler and Milstein (78) described the methodology to produce monoclonal antibodies, many in the bacterial protein toxin field seized on this technology in an attempt to fulfill Paul Ehrlich's idea of developing "magic bullets," therapeutic compounds that were selectively targeted toward a disease-causing organism or cell (79). In an early series of experiments, Gilliland et al. (50) independently coupled ricin A chain and diphtheria toxin fragment A to monoclonal antibodies directed against a cell surface antigen on colorectal carcinoma cells. While the ricin A chain-coupled immunotoxin was highly potent (i.e., the half maximal inhibitory concentration [IC_{50}] < 10^{-12} M), the diphtheria toxin fragment A immunotoxin, surprisingly, was found to be at least 1,000-fold less potent (i.e., $IC_{50} \sim 5 \times 10^{-9}$ M). These results prompted Bacha et al. (80) to ask whether fragment A alone contained sufficient structural information to deliver the catalytic domain across the endocytic vesicle membrane. Since Boquet et al. (59) had previously demonstrated that under denaturing conditions, the hydrophobic domain in the B fragment of CRM45 displayed properties of an integral membrane protein, Bacha et al. (80) independently coupled fragment A (CRM26) and CRM45 to thyrotropin-releasing hormone (TRH) and assessed their relative cytotoxic potency against GH3 rat pituitary cells. While the fragment A-TRH construct was nontoxic even at concentrations of 10^{-7} M, the CRM45-TRH construct was highly active ($IC_{50} \sim 3 \times 10^{-9}$ M). These results clearly demonstrated that fragment B translocation domain sequences were required to facilitate the delivery of fragment A, the catalytic domain, into the eukaryotic cell cytosol.

Since additional preparations of CRM45-TRH varied by greater than 10-fold in cytotoxic potency, Murphy et al. (81) turned to recombinant DNA and protein engineering methods to redesign native diphtheria toxin by receptor-binding domain substitution. The hypothesis was that by choosing a surrogate receptor-binding domain that bound to a cell surface receptor that was both limited in distribution and was internalized into an endosomal compartment that became acidified, it might be possible to develop a family of fusion protein toxins that could potentially serve as a new class of targeted toxins for the treatment of human diseases. Moreover, we reasoned that, if successful, these genetically engineered proteins would constitute a unique family of reagents to deplete discrete subsets of cells and to study the molecular process by which the diphtheria toxin catalytic domain was delivered to the cytosol of targeted eukaryotic cells.

To begin to test this hypothesis, Leong et al. (82, 83) demonstrated that cloned fragment A of diphtheria toxin was expressed and secreted by the SecA apparatus in recombinant *E. coli*. Despite the controversial nature of deliberately designing and constructing a recombinant gene that encoded a toxic protein that the world had not seen previously, in 1985, we were finally granted approval to genetically construct, express, and evaluate the biosafety of the first fusion protein toxin under biosafety level 4 (BSL-4) containment. Murphy et al. (81) described the assembly of a fusion gene that was composed of a portion of diphtheria *tox* that encoded first 486 amino acids of diphtheria toxin and a synthetic gene encoding the 13-amino acid polypeptide α-melanocyte-stimulating hormone (α-MSH). While this fusion protein was expressed and secreted into the periplasmic space of recombinant *E. coli*, the yield of full-length fusion protein toxin was extremely low due to proteolytic degradation. Since this degradation appeared to have occurred close to the fusion junction between diphtheria and α-MSH sequences, we reasoned that increasing the molecular size of the substitute receptor-binding domain with a larger polypeptide might at least partially solve the degradation problem by stearic hindrance. In 1987, Williams et al. (84) described the genetic construction and properties of a fusion protein toxin in which the 133-amino acid sequence of human interleukin-2 (IL-2) was genetically fused to amino acid 486 of diphtheria toxin. Surprisingly, DAB486IL-2 was expressed, and full-length fusion protein toxin was secreted into the periplasmic space in good yield.

Since the DAB486IL-2 construct used a naturally occurring *SphI* restriction endonuclease site in the *tox* gene as the fusion junction between diphtheria toxin and IL-2 sequences, we then asked how much fragment B sequence was required to deliver the catalytic domain of the fusion

protein into the target cell cytosol. Williams et al. (85) constructed a series of in-frame deletion mutants and found that the removal of 97 amino acids from Thr387 to His485 increased both the cytotoxic potency and the apparent dissociation constant of the resulting fusion toxin, DAB389IL-2, for cells that expressed the high-affinity IL-2 receptors (IL-2R). Upon the subsequent solution of the diphtheria toxin X-ray structure (Fig. 6), we realized that quite by chance our internal in-frame deletion of 97 amino acids resulted in an almost perfect receptor-binding domain substitution (Fig. 7). Since that time, more than a dozen diphtheria-based fusion protein toxins have been constructed and characterized using amino acid 388 or 389 as the junction between diphtheria toxin and surrogate receptor-binding domain polypeptides.

In 1999, the U.S. Food and Drug Administration (FDA) approved denileukin diftitox (Ontak; DAB389IL-2) for the treatment of refractory cutaneous T cell lymphoma (CTCL), making it the first-in-class recombinant targeted biologic to achieve approval. Phase III trials conducted with CTCL patients who had failed other therapeutic interventions demonstrated that 30% of the total number of patients had a 50% or greater reduction in their tumor burden for at least 6 weeks following treatment (86, 87). In the case of peripheral T cell lymphomas, an aggressive form of non-Hodgkin's lymphoma with a median overall survival of 5.5 months, Ontak has been used both as a monotherapy and in combination with traditional chemotherapy (88). Both Fuentes et al. (89) and Wong et al. (90) reported individual cases where Ontak therapy, when used as a long-term maintenance therapy over a span of 1 to 2 years, resulted in sustained remissions of 9 and 4 years that are ongoing. In addition to its effective use in T cell malignancies, Ontak has also been successfully used in the treatment of steroid-resistant graft-versus-host disease. Ho et al. (91) conducted a phase I trial of Ontak in 30 patients presenting with steroid-resistant graft-versus-host disease and reported that 50% of the patients responded with complete resolution, and an additional 21% of the total number of patients responded with partial resolution of their disease.

While Ontak selectively targets the high and intermediate IL-2R on malignant T cells in both CTCL and peripheral T cell lymphomas, as well as on activated T effector cells in graft-versus-host disease, in recent years it has also shown promise as an immunotherapeutic in the transient depletion of T regulatory cells in solid tumors. Both phase I (92) and phase II (93) studies have shown that Ontak is also effective as an immunotherapeutic as a monotherapy in patients presenting with unresectable stage IV malignant melanoma. In these instances, Ontak was shown to transiently deplete T regulatory cells and, as such, apparently break tolerance and allow for a more robust host-mounted T-effector cell antitumor response.

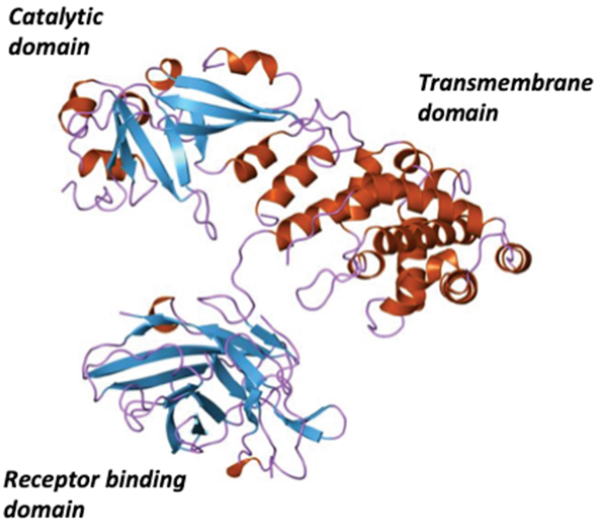


FIGURE 6 X-ray structure of native diphtheria toxin showing its catalytic, translocation, and receptor-binding domains.

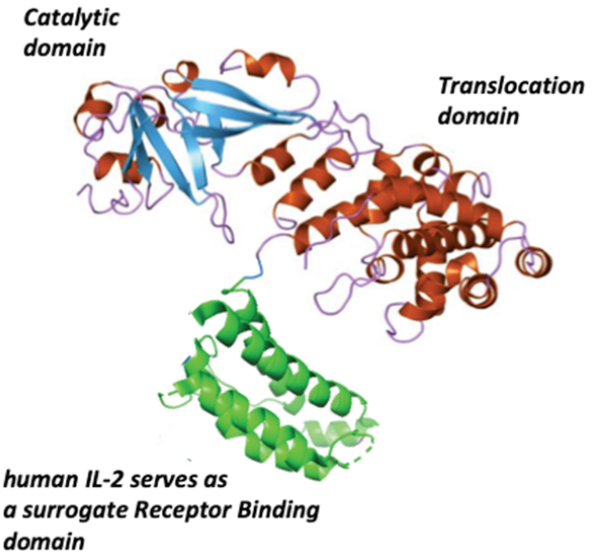


FIGURE 7 Molecular model of denileukin diftitox (Ontak, DAB389IL-2). The catalytic and translocation domains are composed of amino acids Gly1 through Thr387 of native diphtheria toxin, to which the 133-amino acid polypeptide of human IL-2 is genetically fused. The additional two amino acids in the construct are the result of the introduction of a unique *SphI* restriction endonuclease site at the fusion junction between diphtheria toxin and human (h)IL-2 sequences.

Despite its clinical effectiveness, Ontak was placed on clinical hold in 2011 because of the presence of drug aggregates, contaminating DNA, varying concentrations of Tween20, and batch-to-batch variations in its final formulation. Since Ontak was expressed as inclusion bodies in recombinant *E. coli*, the agent had to be completely denatured and refolded into a biologically active conformation. During the refolding process, it was necessary to add Tween20 to partially prevent the formation of insoluble drug aggregates and allow for refolding of the drug into a biologically active conformation.

We (94) recently solved the production issues associated with refolding of the drug by recloning the structural gene for Ontak, DAB389IL-2, in an *E. coli*-*C. diphtheriae* shuttle vector. Furthermore, we modified the gene by adding back the native *tox* signal sequence so that the fusion protein toxin would be secreted into the culture medium. Finally, we introduced mutations into the downstream half of the palindromic *tox* operator so that expression was constitutive even in medium with high iron concentrations. Thus, this second-generation Ontak, s-DAB389IL-2, is expressed and secreted from recombinant *C. diphtheriae* in good yield as a fully folded monomeric protein that may be readily isolated and purified from the culture medium. Cheung et al. (94) also demonstrated that s-DAB389IL2 has great potential when used in a sequential combination regimen with anti-PD-1 in

the treatment of B16F10 melanoma in the mouse. In these studies, either s-DAB389IL-2 or s-DAB389IL-2(V6A), a vascular leak motif modified mutant, was first used to transiently deplete T regulatory cells, thereby removing one of the immune suppression signals that dampen a T effector cell antitumor response. Anti-PD-1 was then administered to block the PDL-1–PD-1 interaction between the tumor and the immune system, thereby blocking a second suppressive signal to the T effector arm of the immune system. The combined effect of this sequential therapeutic regimen was found to be at least additive and perhaps synergistic in the treatment of this aggressive murine melanoma. These results are encouraging and prestage the use of s-DAB389IL2(V6A) in clinical medicine in a sequential checkpoint inhibitor blockade for the treatment of solid tumors.

Ontak (denileukin diftitox, DAB389IL-2) and the second-generation form of the biologic, s-DAB389IL-2 (V6A), while not magic and certainly not bullets, have shown remarkable efficacy in the treatment of human malignancies and in murine models of melanoma and colon and renal cell carcinomas, both as a targeted monotherapy against the tumor itself and as an immunotherapeutic to reset immunologic balance by transient depletion of T regulatory cells. In the latter instance, the transient depletion of T regulatory cells appears to break tolerance and thereby allows a robust patient-mounted antitumor response. Thus, through the biochemical and biochemical genetic study of diphtheria toxin, the diphtheria *tox* operon, and the iron-activated repressor DtxR, which regulates expression, and through modern molecular biologic methods, one of nature's most potent bacterial protein toxins has been tamed. Diphtheria toxin, once the primary virulence determinant of a dreaded infectious disease of children, has now been retargeted to selectively eliminate specific disease-causing cells as a protein therapeutic, thereby at least partially fulfilling Paul Erlich's magic bullet concept of a "chemical that binds to and specifically kills microbes or tumor cells" (79).

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INDEX

Accessory genes
  pathogenicity islands, 544–545
  role for *Staphylococcus aureus*, 526–527
Acinetobacter baumannii, field emission scanning electron microscopy (FESEM), 11
Actin-based motility, cell-to-cell spread, 856
Actinobacillus actinomycetemcomitans, 706, 776
Actinobacteria spp., 724, 1027, 1030, 1087, 1129
  ESX system, 1135
  oral microbiome, 428
  T7S system, 1136, 1142, 1143
Actinomyces naeslundii, 28
Active Bacterial Core program, 186
Acute glomerulonephritis, *Streptococcus dysgalactiae* subsp. *equisimilis* (SDSE), 276–277
Acute poststreptococcal glomerulonephritis, M proteins and, 95
Acute rheumatic fever (ARF), 86
  adhesion and extravasation of T lymphocytes in ARF valve in valvulitis, 90
  epidemiology, 188
  group A streptococci and, 86, 90
  M proteins and, 89–92
  potential mechanisms of pathogenic antibodies in, 88
Adaptive immunity against *Mycobacterium tuberculosis*
  B-cell and antibody response during, 1066–1067
  CD1-restricted T cells, 1067
  γδ T cells, 1067
  inhibitory receptors during, 1066
  kinetics and homing of CD4 T cells after, 1063–1064
  MAIT cells, 1067
  memory T-cell responses, 1066
  quality and specificity of CD4 T-cell response, 1064–1065
  role of CD8 T cells in, 1065–1066
Adaptive immunity of *Listeria monocytogenes*, 814–818
  antigen processing and presentation of T cells, 814–815
  effector functions of T cells, 816–817
  humoral immunity, 818
  magnitude and kinetics of primary response, 815–816
  memory T cell responses during secondary infection, 817–818
Adhesins, 108
  binding streptococcal, to host cell receptors, 126–128
Adults
  meningoencephalitis, 795–796
  Streptococcus agalactiae in, 229–230
Aduro BioTech, Inc., 820
Advaxis, Inc., 820
Aeromonas hydrophia, 1019
Aggregatibacter actinomycetemcomitans, 452
Agmatine deiminase system (ADS), 439
Agr quorum-sensing system
  agr regulon, 670–672
  AIP types (autoinducing peptides), 670, 672
  architecture of, 669–670
  environmental cues, 672
  host-derived factors, 672
  impact of environment and host stimuli on, 672–673
  importance of *agr* during infection, 673
  regulators impacting, 673
  S. aureus, 669–673
  schematic of, 671
AIDS patients
  corticosteroids and *S. aureus* infection, 767–768
  infection risk, 700
Alkaliphilus, relationships within *Clostridiaceae*, 942
Allobaculum, 845
Alloprevotella, 845
American Type Culture Collection (ATCC), 158
Aminoglycosides, 748
  history and mode of action, 753–754
  resistance, 754
  resistance in enterococci, 379
Amniotes, associated with enterococcal colonization, 379
Ampicillin
  invasive GBS disease, 232
  treatment of listeriosis, 797–798
Animal diseases, *Clostridium perfringens*, 985
Animal models
  anthrax, 882–883
  Bacillus thuringiensis and *B. cereus*, 883–884
  Clostridioides difficile infections (CDI), 995–996
  enterococci pathogenicity, 388
  hyaluronic acid enhancing virulence of group A streptococci, 49
  Mycobacterium tuberculosis infection, 1057–1058
Anthrax, 873
  animal models of, 882–883
  Bacillus anthracis, 878
  prophylaxis and treatment, 888–889
  vaccines, 889
Antibiotic resistance
  enterococci, 378–380
  staphylococcal biofilm formation for, 704–706
Antibiotic resistance of *S. aureus*
  aminoglycosides, 753–754
  beta-lactam antibiotics, 747–750
  cell envelope active agents, 747–752
  chloramphenicol, 753
  compounds and alternatives in development, 756
  daptomycin, 751–752
  fluoroquinolones, 755
  fosfomycin, 751
  fusidic acid, 754–755
  linezolid and tedizolid, 752–753
  lipoglycopeptides, 751

macrolides, lincosamides and streptogramins, 753
mupirocin, 754
pleuromutilins, 753
protein synthesis inhibitors, 752–755
rifampicin, 755
small molecule biosynthesis inhibitor, 755–756
tetracyclines, 754
tigecycline, 754
transcription inhibitors, 755
trimethoprim-sulfamethoxazole, 755–756
vancomycin resistance and VISA, 750–751
Antibiotic resistance reservoir, *Streptococcus sanguinis*, 453–454
Antibiotics
enterococcal resistance, 404
small colony variants (SCV), 556
treatment of *Clostridioides difficile* infection, 1002
vancomycin resistance, 379, 402–404
Antibodies and antibody conjugates, *S. aureus* infection, 782
Anticancer strategies, peptides from evasion molecules, 631
Antigenicity, lactococci, 474
Antimicrobial peptides (AMPs)
biofilms, 777
microbiocidal activities of, 125
neutrophils interaction with *S. pyogenes*, 129, 131
staphylococcal biofilm-associated infection, 706
Streptococcus mutans and, 443
Antimicrobial resistance, methycillin-resistant *Staphylococcus aureus* (MRSA), 490–491
Antimicrobials, targeting *L. monocytogenes* regulation, 845–846
Apoptosis
group A streptococcal-mediated, 135–138
term, 137
Arabidopsis thaliana, 877
Arginine Catabolic Mobile Element (ACME), 713
Arginine deiminase system (ADS), 430–431, 439
Artemis Comparison Tool (ACT), 487
Arthrobacter, 1030, 1032
Arthropods, associated with enterococcal colonization, 379
Artificial transformation, *S. pneumoniae*, 351
Atomic force microscopy (AFM), 4
Atopobium spp., oral microbiome, 428
AureoWiki, 487
Autophagy, 855
Autophagy (xenophagy), *Streptococcus pyogenes*-mediated, 133–135

Bacillaceae, 565
Bacillithiol, biosynthesis in *S. aureus*, 690
Bacillus spp., 873
germination, 918
rolling circle replicating (RCR) plasmids, 500
T7S system, 1143
Bacillus anthracis
anthrax pathogen, 878
capsule of, 7
future directions, 889
germination dynamics and signals, 880
germination in host, 880
inhalation anthrax in humans, 877–878
prevalence in soil, 877
spore architecture, 880
sporulation, 880
T7S system, 1143
taxonomy, 875–876
toxins, 880–881
vegetative cell envelope, 879
virulence, 878
virulence regulation, 886–887
Bacillus Calmette-Guérin (BCG), vaccine against TB, 1056, 1068–1069, 1129
Bacillus cereus
animal models of infection, 883–884
cell culture, 884–885
cereulide as emetic toxin in, 881, 882
future directions, 889
insect models, 884
taxonomy, 876
toxins, 881–882
Bacillus cereus group
animal models of infection, 883–884
cell culture, 884–885
cell structure and development, 879–880
clades of, 876
common secreted enzymes, 882
ecology, 877–879
future directions, 889
generation of recombinant strains, 887–888
genetic analysis, 887–888
genetic expression measurement, 888
genetic tools, 887–888
germination dynamics and signals, 880
germination in host, 880
insect models of *B. cereus* and *B. thuringiensis* infection, 884
modeling host interactions, 882–885
natural genetic exchange, 887
spore architecture, 879–880
sporulation, 880
taxonomy, 875–877
therapeutics and vaccines, 888–889
toxins, 880–882
transmission from soil to humans, 877
vaccines, 888–889
virulence gene expression, 885–887
Bacillus difficilis, 991
Bacillus megaterium, bacterial cell wall turnover, 5
Bacillus pumilus, chloramphenicol acetyltransferase, 80
Bacillus subtilis, 28
bacteriology studies, 443
bacteriophages, 524
cell growth, 1096, 1097
cell wall constituents, 6
cryo-studies, 15
DNA replication, 398, 398–399
environmental stress, 842
exponential growth, 1102
expression strains, 474
Fst toxin sequence, 409
genetic organization of ICEBs*1*, 509
global transcription regulators, 886
growth pole, 1102
peptidoglycan global structural parameters, 576
phage, 521
respiration, 468
schematic of spore germination, 916
sporulation, 880
sporulation initiation, 905
T7S system, 1136, 1143
transcriptional programs controlling sporulation, 909
transcription factors, 565
transport genes, 865
Bacillus thuringiensis, 1019
animal models of infection, 883–884
ecology of, 877
future directions, 889
germination in host, 880
infection of invertebrates, 878–879
infectious cycle of, in insect larva, 885
insect models, 884
sporulation, 880
taxonomy, 875, 876–877
toxin-antitoxin (TA) system, 412
toxins, 882
Bacteremia, 45, 55
Bacillus anthracis, 878
enterococci, 382–383
Staphylococcus aureus, 717
Streptococcus dysgalactiae subsp. *equisimilis* (SDSE), 278
Bacterial cell envelope, 1087. *See also* Mycobacterial cell envelope

Bacterial cell wall, 4–6
backbone of peptidoglycan, 5
biochemical synthesis of peptidoglycan layer, 4–5
capsule, 7
chemistry of, backbone, 4
lipoteichoic acid and wall teichoic acid as major constituents of, 6–7
specialized, in mycobacteria, 9
turnover of peptidoglycan, 5–6
Bacterial competition
interactions between nasal bacteria, 727
nasal environment, 725–727
Bacterial endocarditis, by *Listeria monocytogenes*, 796
Bacterial psychology, 1096
Bacteriocin production, *Streptococcus sanguinis* and, 453
Bacteriophage-based therapy, *S. aureus* infection, 778–779
Bacteriophage Recombineering of Electroporated DNA (BRED), 1045
Bacteriophages
CRISPR-Cas system, 473
enterococcal, 413–414
S. pneumoniae, 352–353
staphylococcal biofilm-associated infection, 706
Staphylococcus aureus, 488
see also Staphylococcus aureus phages
Bacteriophages of *Streptococcus pyogenes*
assembled and annotated genomes of S. pyogenes, 161
bacteriophage A25, 158–159
biology and distribution of lytic phages, 158
diversity of lysogenic phages, 163, 165–166, 168
DNA replication and modification, 163
genome of bacteriophage A25, 159
genome prophages, distribution and attachment sites, 160–161, 164–165
horizontal transfer of genes, 165–166
identity matrix of genome prophages by M type, 171
lysogenic phages, 160–166
lysogeny module, 162–163
lytic phages, 158–160
morphology and genome organization, 161–163, 167
morphology of streptococcal lysogenic phages, 166
phage-like elements carrying antimicrobial resistance genes, 169
phylogenetic relationships of, 168
phylogenetic tree of SpyCI DNA sequences, 172
prophage attachment sites in *S. pyogenes* genome, 162
prophage hyaluronidase, 163, 165
prophages and host phenotype, 166–172
prophages as agents of genome plasticity, 170, 172
prophages as vectors for virulence genes, 166, 169
prophages regulating host gene expression, 169–170
shared genetic modules of T12-related prophage family, 170
SpyCIM1 regulation of MMR operon, 172
transduction of *S. pyogenes*, 159–160
virulence genes, 163
Bacteroides fragilis, 331, 339
B cell superantigens, 647
Beachey, Edwin, 150
Beta-lactam antibiotics, 748
history and mode of action, 747–748
resistance, 748–749
SCC*mec* elements, 749
SCC*mec* typing in epidemiology of MRSA, 749
treatment of listeriosis, 797
treatment of MRSA, 749–750
Bicomponent pore-forming toxins (PFTs), 640–645
model for pore formation, 642
phylogenic tree of *S. aureus* PFTs, 645
receptor, species, and cell type specificities, 644
structure for, 643
Biliary tract infections, *Listeria monocytogenes*, 797
Biofilm formation
prevention and disruption of, 776–777
Streptococcus mutans, 437–439
Streptococcus sanguinis, 449, 451–452
see also Staphylococcal biofilms
Biological warfare, 632
Biotin, metabolism, 867
Birds, staphylococcal elements, 734, 735
Blood-brain barrier, pneumococci at, 321
Bloodstream
enterococci from gut into, 382
pneumococci in, 320–321
survival of bacteria in, 321
Borrelia hermsii, 137
Botulinum neurotoxin, clostridial strains producing, 943–948
Branched chain amino acids (BCAAs), 864, 865, 866
intracellular metabolism, 867
metabolism and virulence of *L. monocytogenes*, 867–868
Bullous pneumonia, 712
Burkholderia cepacia, 137

Caenorhabditis elegans
S. aureus and, 492
testing SCVs, 553
CAMP factor, group C and G streptococci, 251
Campylobacter spp., 794
Campylobacter jejuni, phase variation, 331
Campylobacter upsaliensis, 339
Cancer immunotherapy, clinical trials using *L. monocytogenes* for, 819–820
Candida albicans
bacterial killing, 627
biofilm formation and, 451
biofilms, 430
fibrinogen and, 117
Streptococcus mutans and, 443
surface glycolytic enzymes, 27
Candida tropicalis, bacterial killing, 627
Capsular polysaccharides (CPSs)
cell wall complex of pneumonococci, 285
chair models of, 207–210
CPS-based vaccines, 305–306
CPS-protein conjugate vaccines, 218–220
CPS-GBS (group B streptococci) protein conjugate vaccines, 221
GBS, 205–206, 210
genetics of, 211, 212
phase variation and quorum sensing, 311
regulation of CPS production, 309–311
repeat unit structures of *Streptococcus pneumoniae* serotypes, 305
role in pathogenesis, 304–305
role of *cpsABCD* in production, 309–310
S. pneumoniae, 304
transcriptional regulation of *cps* locus, 310–311
vaccines, 217–218, 362–363
variation in, 333
Capsule
bacterial cell wall, 7
CD44-mediated tissue invasion, 51
experimental infection models, 49–50
host immune defenses and, 48–49
immunogenicity of hyaluronic acid, 48–49
mucosal colonization and, 50
resistance to phagocytosis, 49
Streptococcus pyogenes attachment to epithelial cells, 50–51
Streptococcus pyogenes entry into epithelial cells, 51
as virulence factor, 49–51
Capsule production
hyaluronic acid biosynthesis, 45–47
preventing phagocytosis, 623
regulation of hyaluronic acid biosynthesis, 47–48
schematic of biosynthetic pathway, 46
Carbapenems, invasive GBS disease, 232
Carbohydrate antigens
capsular polysaccharides (CPSs), 205–206, 210
chair models of repeating units GBS CPSs, 207–210

electron micrographs of immunogold-labeled GBS, 206
GBS (group B streptococci), 204–211
group B carbohydrate, 204–205
proposed structure of GBS carbohydrate, 205
Carbohydrate fermentation, *Lactococcus lactis*, 464–465
Carbohydrate metabolism, *Streptococcus mutans*, 436–437
Carbon/energy sources, *Staphylococcus aureus*, 687–688
Carbon metabolism, intracellular *L. monocytogenes*, 866–867
Catabolic ornithine transcarbamylase (cOTC), death health, 430
Catch and release, *Streptococcus mutans*, 436–437
Cats, methicillin-resistant *S. aureus* (MRSA), 738–739
Caudovirales, 161, 1030
Cell cycle, cell growth regulation during, 293–298
Cell death
Cell-signaling events regulating, 136
group A streptococcal-mediated apoptosis, 135–138
inflammasome-associated, 137
Cell lysis system, *Lactococcus lactis*, 474
Cell physiology, role of mycobacterial ESX systems, 1140–1142
Cell structure and development, *Bacillus cereus* group, 879–880
Cell-to-cell spread
cortical actin rearrangements, 856
cytoplasmic actin-based motility, 856
Listeria monocytogenes, 856
lysis of secondary vacuoles, 856
protrusion formation, 856
Cellular invasion process
adhesion/entry effectors, 853
InlA/E-cadharin-mediated entry, 852
InlB/Met-mediated entry, 852–853
Listeria monocytogenes, 851–853
Cell wall(s)
anatomy of *S. aureus*, 575
antibiotic resistance, 582
atomic force microscopy (AFM) of cell division, 584
cell division, 583, 593
cell growth and division models, 584
changing image of, 574
envelope assembly, 592
femX, femA and femB genes, 581
functions of, 582–585
historical overview, 574
methicillin-conditional mutants, 580
microbacterial envelope, 1089, 1091, 1092
murein hydrolases in cell division, 582, 583
penicillin binding proteins (PBPs), 579–580
protein anchoring to, 593–594
protein secretion across, 593
protein secretion into cross-wall compartment, 594–595
regulation and transcriptomic studies, 581–582
release of surface proteins from envelope, 595
role of stringent response in β-lactam resistance mechanism, 580–581
scanning electron micrograph (SEM) of division site, 584
secondary modifications, 581
staphylococcal, 574, 585–586
staphylococcal protein A, 595
surface proteins and heme-iron transport, 595
synthesis, 592
synthesis of, 579–582
variation in polysaccharides, 333–334
see also Peptidoglycan
Cell wall-anchored (CWA) proteins, 599
adaptive immune responses, 611
contributing to virulence of *Staphylococcus aureus*, 608–609, 610
G5-E repeat proteins, 600, 603, 606–607, 608
immune evasion by, 611
innate immune responses, 611
MSCRAMM family, 599, 600, 601, 604–606
NEAT motif family, 600, 602, 607
potential antigens for vaccination against *S. aureus*, 611–612
promoting colonization of host, 610–611
structural motifs for classification, 600
structure and function, 599–608
three-helical bundle, 600, 603, 607
see also Surface proteins of *Staphylococcus aureus*
Cell wall of *S. pneumoniae*, 284–298
cartoon of growth and division complex, 295
cell growth regulation during cell cycle, 293–298
diagram, 285
division site selection (MapZ/LocZ), 298
functional anatomy of, 284–285
functions of choline residues, 293
growth and cell division complexes, 294–296
lipases MurM and MurN in branching of, 287
murMN operon, 288–290
origin of replication, 298
penicillin resistance, 288
peptidoglycan biosynthesis and remodeling, 286–288
peptidoglycan composition, 288
regulators of growth and division, 296–298
role of phosphorylation, 296–298
segregation, 293–294
structure of peptidoglycan, 285–286
structure of WTA and LTA, 290–292
structures of stem peptides in peptidoglycan, 290
synthesis of teichoic acids and modifications, 292–293
zone growth and cell wall segregation, 293–294
Centers for Disease Control and Prevention (CDC), 362, 363, 888, 994
Central venous catheters (CVC), prone to infection, 699–700, 705
Cephalosporins, invasive GBS disease, 232
Cereulide, *Bacillus cereus* toxin, 881, 882
Chemokine and cytokine production, *Listeria monocytogenes*, 805–809
Chickens, *Staphylococcus aureus* in, 736–737
Chlamydia, 248
Chloramphenicol, 748
mode of action, 753
resistance, 753
Choline-binding proteins
cell wall complex of pneumonococci, 285
functions of, 293
immunohistochemical and schematic of pneumococcal surface, 317
nutritional requirement for, 293
pneumococcal vaccines, 365–366
Chromosomal integration, *S. pneumoniae*, 351
Clarke, J., 435
Clindamycin, invasive GBS disease, 232
Clostridiaceae, 918, 927
Clostridial genetics, 927
allelic exchange and homologous recombination, 931–932
Clos Tron technology, 932–933
enzymatic reporter systems, 931
illustration of pMTL80000 modular vector series, 929
inducible vector systems, 929–930
module choices for pMTL80000 plasmids, 929
newer technologies for genetic manipulation, 933–934
overcoming restriction and modification systems, 928
plasmid cloning vectors, 928–929
reporter assays, 930–931
roadmap to, 928–934
Targe Tron system, 932
transposon-mediated random mutagenesis, 933
visualizable reporters, 930–931
Clostridial infections. *See* Histotoxic clostridial infections
Clostridial pathogens
activation of cortex hydrolysis in, 914–918
importance of spores, 903
linking sporulation and toxin gene expression in *C. difficile*, 907–908
spore assembly, 911–913
spore formation, 903–904
spore germination, 913–914
spore germination features of *C. difficile*, 918
sporulation initiation in, 904–907

sporulation transcriptional program, 908–911
strains that produce botulinum neurotoxin, 943–948
taxonomy of, 940–941, 943
see also Genomics of pathogenic clostridia
Clostridioides, 927
Clostridioides (formerly Clostridium) difficile, 874
activation of cortex hydrolysis in, 914–916
allelic exchange and homologous recombination, 931–932
bacteriology and diagnosis, 991–992
coat assembly in, 911–912
cortex hydrolysis preceding Ca–DPA release in, 917
description, 991
engulfment, 911
epidemiology, 993–994
exosporium assembly in, 912–913
exosporium function in, 913
exosporium structure in, 912
germination regulators, 915
glucosylating toxins, 908
importance of spores, 903
inducible vector systems, 929–930
pathogenesis, 994–1002
proteolytic activation of cortex hydrolase in, 914–916
pseudoprotease (CspC) as germinant receptor in, 914
regulatory pathway controlling Spo0A activation in, 906
schematic of spore germination, 916
spore coat, 912
spore formation, 903–904
spore germination features, 918
sporulation initiation in, 905–906
strain typing, 992–993
transcriptional programs controlling sporulation, 909, 910
transposon-mediated random mutagenesis, 933
virulence plasmids, 969
whole-genome sequence (WGS) analysis of isolates, 946–947
see also Clostridial genetics; *Clostridioides difficile* infections (CDI)
Clostridioides difficile infections (CDI)
alternative therapeutics, 1002–1003
animal models of, 995–996
antibiotic treatment of, 1002
C. difficile transferase (CDT), 999–1000
cytokine and chemokine responses to nontoxin antigens, 1000
cytokine and chemokine responses to toxins, 1000
disease associated with, 995
immune response during CDI, 1000–1002
immune response to *C. difficile*, 1000
prevention, 1002
recurrent therapeutics, 1002–1003
schematic of *C. difficile* toxins, 997
susceptibility to, 994–995
TcdA and TcdB toxins, 996–999
toxins, 996–1000
Clostridium spp., 873, 927, 928
relationships within, 943
relationships within *Clostridiaceae*, 942
Clostridium acetobutylicum, 928, 934
Clostridium argentinense, 969
Clostridium baratii, 969
Clostridium bolteae, 948
Clostridium botulinum
activation of cortex hydrolases in, 916–917
germination, 918
importance of spores, 903
neurotoxin (BoNT), 907–908, 965–966, 967, 968–969
non-proteolytic, 968
proteolytic, 968
schematic of spore germination, 916
sporulation initiation in, 905, 907
toxin-encoding mobile genetic elements (MGE), 965–966, 968–969
transcriptional programs controlling sporulation, 909, 910
transmembrane germinant receptors in, 914
Clostridium butyricum, 969
Clostridium cellulolyticum, 934
Clostridium cellulovorans, 928
Clostridium chauvoei, 948
Clostridium haemolyticum, 945
Clostridium novyi, 945
Clostridium novyi sensu lato, 968–969
Clostridium perfringens
activation of cortex hydrolysis in, 914–916
allelic exchange and homologous recombination, 931
clostridial myonecrosis by, 1012–1013
comparative analysis of pCW3-like antibiotic resistance and toxin plasmids, 961
current model for Tcp-mediated conjugation, 961–962
E. coli and, 928
enterotoxin (CPE), 907
germination, 918
germination regulators, 915
importance of spores, 903
inducible vector systems, 929–930
model of pCW3 conjugation apparatus, 962
multiple pCW3-like toxin plasmids, 962–963
nucleotide alignment of pCW3-like plasmids from, 960
pCP13-like plasmids, 963–964
perfringolysin O, 853
plasmid cloning vectors, 928–929
plasmid-encoded toxins, 958–959
plasmids, 959, 961
proteolytic activation of cortex hydrolase, 914–916
schematic of spore germination, 916
sequence alignment of pCP13-like plasmids from, 964
sporulation initiation in, 906–907
toxin association with insertion sequences, 959
toxins withih toxinotypes, 945
TpeL toxin, 907
transcriptional programs controlling sporulation, 909, 910
transmembrane germinant receptors in, 914
transposon-mediated random mutagenesis, 933
type F food poisoning, 977–984
typing toxins, 945–946, 956–958
see also Clostridial genetics; *Clostridium perfringens* enterotoxin (CPE)
Clostridium perfringens α-toxin
activation of phospholipid metabolism and hemolysis, 1015–1016
binding model of, and membrane phospholipids, 1014
biological activities of, 1015
effect on erythropoiesis, 1017–1018
effect on hematopoietic cells, 1016–1017
function of, 1013–1014
inhibition of granulopoiesis and erythropoiesis, 1018
interaction with gangliosides, 1016
interaction with host cells, 1014–1018
membrane-damaging activity on phospholipid bilayers, 1014–1015
model of α-toxin-induced membrane dynamics, 1017
structure of, 1013
Clostridium perfringens enterotoxin (CPE), 1019
chromosomal *cpe* loci, 982
CPE binding, 979–981
CPE-induced cytotoxicity, 979–981
enteritis necroticans, 984–985
epidemiology, 977
genetics, 981–983
immunology, 984
inducing bystander killing effect, 981
model of mechanism of action, 980
model of sporulation and production, 983
nonfoodborne human GI diseases, 984
pathogenesis, 977–978
plasmid *cpe* loci, 982
postbinding steps in CPE action, 981
protein, 978–979
regulation of CPE production, 983–984
small acid-soluble protein 4, 984
structure of, 979
treatment and prevention, 984

Clostridium perfringens perfringolysin O, 1018
host cell interactions, 1018–1019
structure of, 1019
Clostridium saccharobutylicum, 928
Clostridium septicum, 948, 1012
clostridial myonecrosis by, 1012–1013
crystal structure of aerolysin, 1020
function and host cell interactions of α-toxin of, 1019–1020
molecular model of α-toxin, 1020
Clostridium tetani, 771
blast analysis of tetanus toxin plasmid family, 965
tetanus neurotoxin (TeNT), 944–945
toxin plasmid of, 964–965
Clos Tron technology, clostridial genetics, 932–933
Collagen-building proteins (CBPs), *Streptococcus mutans*, 436
Collagens
Gram-positive pathogens and, 113–114
structure and function of, 109–110
Colonization mechanisms
oral streptococci, 429–430
Streptococcus sanguinis as commensal colonizer, 449–451
Combination protein vaccines, combination, 368–369
Commensal oral flora, 430–431
Community-associated methicillin-resistant *S. aureus* (CA-MRSA), 713, 737, 749
Community development, *Streptococcus sanguinis* in, 452–453
Companion animals, methicillin-resistant *S. aureus* (MRSA), 738–739
Competence development, *S. pneumoniae*, 348–350
Competence-stimulating peptide (CSP), death health, 431
Complement receptor of immunoglobulin superfamily (CRIg), 809
Complement system
main pathways and components of, 624
opsonization by, 622–623
proteins targeting, 624–625
Conjugation, *S. pneumoniae*, 351–352
Conjugative transposons
enterococci, 410–412
genetic organization of Tn916, 411
integrated conjugative elements (ICEs), 404, 410–412
Conventional embedding, electron microscopy, 12–13, 14
Cortical actin, rearrangements, 856
Corticosteroids and AIDS, *S. aureus* infections, 767–768
Corynebacteriales, 1027, 1087, 1088, 1129
Corynebacterium, 723, 724, 1027–1028, 1030
Corynebacterium accolens, interactions with nasal bacteria, 727
Corynebacterium diphtheriae, 3, 1028
agent of diphtheria, 1154
corynebacteriophages carrying gene for diphtheria toxin (*tox*), 1155–1156
corynebacteriophage *tox* gene, 1156
denileukin diftitox (Ontak), 1161–1162
DtxR two-domain protein, 1158
electron micrograph of corynebacteriophage β, 1155
genetic map of β-phage, 1155
molecular epidemiology of diphtheria and DtxR, 1159–1160
molecular mechanism of action of *tox*, 1158–1159
molecular model of denileukin diftitox, 1161
schematic of Fe^{2+} activation of apo-DtxR, 1156
structural basis of transition metal ion activation of DtxR repressor activity, 1157–1158
structure-function relationships of *tox*, 1158–1159
targeted therapeutics for diphtheria, 1160–1162
transition metal ions activating DtxR binding to *tox* operator, 1156–1157
X-ray structure of native diphtheria toxin, 1161
Corynebacterium striatum, nasal spray of, 569
Cows, *Staphylococcus aureus* in, 732–733, 735–736, 739
CPE. *See Clostridium perfringens* enterotoxin (CPE)
CPILE/BEC toxin, *Clostridium perfringens*, 985
CRISPR (clustered regularly interspaced short palindromic repeat)-associated system (Cas) function
adaptive immunity systems in *Staphylococcus aureus*, 566–568
antibacterial drones (ABDs), 546
enterococcal defense system, 413–414
future for infection models, 138
genetic manipulation, 934
genome sequences, 927
immune system against phages, 473
mycobacteriophages, 1044
S. aureus infection, 780
Cross-feeding, *Lactococcus lactis* and interbacterial, 472
Cross-inhibition, *Lactococcus lactis* and interbacterial, 472–473
Cross-reactive antigens
advent of monoclonal antibodies (mAbs), 86
in Group A streptococci, 90, 95
human and murine antistreptococcal/antimyosin mAbs, 88
human cardiac myosin peptide epitopes in rheumatic heart disease, 94–95
mAbs group A streptococci and human heart tissues, 87–88
mAbs identifying streptococcal, 88–95
model of valvular heart disease by Group A or Group G streptococcal M proteins in Lewis rat, 94
molecular basis of mimicry in streptococcal M protein, 92
mouse antistreptococcal mAb with human tissue, 87
M proteins and cross-reactive T cells, 92–94
M proteins and poststreptococcal acute glomerulonephritis, 95
M proteins and rheumatic fever, 89–92, 101
potential mechanism of antibody in pathogenesis of rheumatic heart disease, 88
Cryo-electron microscopy (cryoEM), 1087, 1088
Cryo-electron microscopy of vitreous sections (CEMOVIS), 1087–1089
Cryo-electron tomography (CET), 3, 9–10, 14–15
Cryo-focused ion beam (cryo-FIB), 3
Cryo-focused ion beam with scanning electron microscope (cryo-FIB-SEM), 3, 10, 13
Cryo-methods, 13–15
CEMOVIS (cryo-electron microscopy of vitreus sections), 13–14, 15
cryo-electron tomography (CET), 14–15
freeze-fracturing and freeze–etching, 13
high-pressure freezing and freeze–substitution, 13
outlook of new, 15
C-terminal anchored (LPxTG motif), 20, 23, 24
C-terminal-anchored surface proteins
characteristics of, 25
conformational analysis, 25, 26
seven–residue periodicity, 25–26
Cutaneous listeriosis, 796
Cutaneous T cell lymphoma (CTCL), denileukin diftitox for, 1161
Cutaneous wound infections, ecology of, 178
Cutibacterium, 723, 1032
Cytochrome C, 691
Cytokine production, staphylococcal sepsis and, 768–770
Cytolytic toxins, of *Streptococcus pyogenes*, 55–56
Cytoplasmic stage
cytoplasmic innate immune responses, 855–856
Listeria monocytogenes, 855–856
persistence, 856
utilization of host metabolites, 855
Cytotoxicity, pneumococcus, 322–324
Cytotoxins, β-barrel pore-forming toxins, 640–645

Dale, James, 150
Daptomycin
enterococci resistance, 379
history and mode of action, 751–752
resistance, 752
Dendritic cell failure, *S. aureus* infections, 766
Dendritic cells (DCs), 803
antigen-laden, activating T cell responses, 812–813
Denileukin diftitox (Ontak), 1161–1162
Dental caries, oral streptococci, 428, 430
Dental health, good streptococci for, 430–431
Dental plaque
oral streptococci, 428
Streptococcus mutans in, 439–440

Dichelobacter nodosus, bacterial attachment sites for prophages, 161
Diphtheria
epidemic in Newly Independent States (NIS), 1159
molecular epidemiology of, 1159–1160
targeted therapeutics for, 1160–1162
see also Corynebacterium diphtheriae
Directed mutagenesis
allelic replacement using linear DNA, 70, 72
considerations for, 76
counterselection markers, 74
directed insertional inactivation, 72–74
in-frame deletion, 74, 75
in-frame deletion using CRISPR/Cas9, 74–76
Streptococcus pyogenes, 70, 72–76
DNA (deoxyribonucleic acid)
influence of listeriolysin O (LLO) on stability of, 857
pathogenic clostridia, 940
randomly amplified polymorphic (RAPD) fingerprinting, 240
repair of *S. pneumoniae*, 353–354
replication and modification in *S. pyogenes* genome, 163
role of mycobacterial ESX systems in DNA transfer, 1141
DNA Master, 1034
Dogs, methicillin-resistant *S. aureus* (MRSA), 738–739
Dolosigranulum pigrum, 724
Drosophila melanogaster, alginate, 246
Dynamin, 132

Eagle effect, 279
Ecology of group A streptococcus (GAS)
GAS (agent) factors, 179–180
GAS-microenvironment factors, 179
host tissues and diseases, 177–179
human (host) factors, 179
macroenvironment risk factors, 179
transmission, 179–180
transmission routes for, 178
Ectopic expression, techniques for, 81
Ehrlich, Paul, 1160
Elastin
Gram-positive pathogens and, 114
structure and function of, 110
Electrococcus faecium, field emission scanning electron microscopy (FESEM), 11
Electron microscopic techniques, 9–15
conventional embedding, 12–13
cryo-electron tomography (CET), 9–10
cryo-methods, 13–15
field emission scanning electron microscopy (FESEM), 10–11
heavy metal coating or shadowing, 11
negative staining, 11–12
surface proteins of Gram-positive bacteria, 20–21
Electron transparent zone, 1088
Electron transport, metabolic pathways and mutations, 551
Embden-Meyerhof-Parnas glycolytic pathway, 688, 865
Endocarditis
enterococci, 383–384
Streptococcus dysgalactiae subsp. equisimilis (SDSE), 279
Endoplasmic reticulum (ER), influence of listeriolysin O (LLO), 857
Endosomal sorting complex required for transport (ESCRT), 1063
Enteric diseases. *See Clostridium perfringens* enterotoxin (CPE)
Enteritis necroticans (EN), *Clostridium perfringens*, 984–985
Enterobacter cloacae, conjugative plasmid, 505
Enterobacteriaceae, 724
Enterococcal genetics
aggregation substance (AS), 404, 406–407
bacteriophage, 413–414
chromosomal replication, partition and cell division, 398–399
conjugation apparatus, 406–407
conjugative transposons (ICES), 410–412
genome defense systems, 413–414
landmark discoveries, 398
mobilome features, 412–414
model for EutV- and EutX-mediated regulation of *eut* regulon, 401
model of RNAI-RNAII interaction, 409
non-pheromone-responding plasmids, 412
organization of pheromone-responsive plasmid replicon, 408
organization of transposon Tn916, 411
organization of VanA and VanB vancomycin resistance genes, 403
PAIs (pathogenicity islands) and intercellular transfer by Hfr formation, 412, 413
pheromone-responsive plasmids, 404–410
regulating pheromone signal transduction circuit, 404–406
regulation of gene expression, 399–402
regulatory circuitry of pheromone response, 405
replication and stable inheritance, 407–410
resistance to antibiotics, 404
TA (toxin-antitoxin) systems, 408–410, 412–413
transposon Tn916, 410–412
two-component signal transduction system (TCSTS), 399–400, 402–403
vancomycin resistance, 402–404
Enterococci
animal models for study of pathogenic mechanisms, 388
animals associated with colonization of, 379
antibiotic resistance, 378–380
bacteremia, 382–383
biology and characteristics, 378
comparing genomics of *E. faecalis* and *E. faecium*, 387–388
endocarditis, 383–384
environmental persistence, 380
human colonization, 380–382
immune evasion, 384–385
invertebrate models, 388
pathogenic mechanisms, 380
population structure of *E. faecium* and *E. faecalis*, 386
root canal failure, 384
surgical site infections, 384
tissue damage, 385, 387
toxic metabolites, 387
translocation from gut into bloodstream, 382
urinary tract infection, 383
vertebrate models, 388
virulence factors, 381
see also Enterococcal genetics
Enterococcus, term, 378
Enterococcus spp. interbacterial cross-feeding, 472
Enterococcus faecalis, 28, 69, 137, 378
alkaline phosphatase of, 80
biofilm formation and, 451
conjugative plasmids, 503
DtxR repressor in, 1156
Fst toxin sequence, 409
gene expression, 399–402
genetics, 398, 414
genomics of, 387–388
human colonization, 380–382
hyaluronic acid synthesis in, 46
population structure, 386
S. aureus and, 492
transposon mutagenesis, 933
transposon Tn916, 410–412
Enterococcus faecium, 378
gene expression, 399–402
genetics, 398, 414
genomics of, 387–388
human colonization, 380–382
population structure, 386
Enterococcus hirae, 400
Entner-Doudoroff pathway, 429
Envelope assembly
cell wall synthesis and, 592
protein secretion across cell wall, 593
teichoic acid synthesis and, 592
Environment
anthrax and *Bacillus anthracis*, 878
Bacillus cereus group, 877–879

B. cereus and gastrointestinal disease, 878
B. thuringiensis infection of invertebrates, 878–879
peptidoglycan recycling, 585
Environmental persistence, enterococci, 380
Environmental stress
lactococci and, 470–471
regulation of *L. monocytogenes* in response to, 842
Enzymes
Clostridioides difficile transferase, 999–1000
dental health, 430–431
staphylococcal biofilm-associated infection, 706
see also Exoenzymes
Epidemiology
acute rheumatic fever (ARF), 188
building evolutionary models, 190–191
Clostridioides difficile, 993–994
core housekeeping genes, 186
diversity within host communities, 188
evolution of markers (EMM), 188–190
global trends, 186–188
infection at epithelium of throat and skin, 186–187
invasive disease, 187–188
Listeria monocytogenes, 793–795
molecular, of diphtheria and DtxR, 1159–1160
molecular markers, 180–186
M protein and GAS infection, 180–183
multilocus sequence typing, 186
serum opacity factor, 183
strategies for M protein-based vaccines, 190
Streptococcus anginosus group (SAG), 275
Streptococcus dysgalactiae subsp. *equisimilis* (SDSE), 275–276
T-antigens, 183–186
typing methods for group A streptococci (GAS), 181
whole-genome sequences, 186
Erysipelothrix rhusiopathiae, sequenced surface proteins, 21
Erythromycin
invasive GBS disease, 232
plasmids encoding resistance to, 71
resistance, 35
Escherichia coli, 45, 204, 246, 794
bacterial attachment sites for prophages, 161
bacteriology studies, 443
capsid modification, 543
cell envelope diagram, 1088
cell growth, 1096, 1097
cell wall, 574
conjugation from, 928
DNA replication, 398
expression strains, 474
field emission scanning electron microscopy (FESEM), 11
Fst toxin sequence, 409
gene expression, 1036
genetic analysis, 888
growth rate, 1103
hyaluronic acid synthesis in, 46
peptidoglycan global structural parameters, 576
phage, 521
phase variation, 331, 332
plasmid propagation in, 68–69
plasmids, 1029
protein secretion, 593
respiration, 468
sequence comparison with *Streptococcus pyogenes*, 249
TEM image of peptidoglycan sacculus of, 5
TEM of negatively stained bacteria, 12
Toxin-antitoxin (TA) system, 412
transport genes, 865
transposon mutagenesis, 933
vectors encoding erythromycin resistance, 70, 71
ESX systems
protein export, 1136–1142
see also Type VII secretion (T7S) pathway
Etiological agent, *Streptococcus sanguinis* as, 455–456
Eubacterium, relationships within *Clostridiaceae*, 942
Eukaryotes, associated with enterococcal colonization, 379
European Nucleotide Archive, 486
Evolution
host range and, 1042–1043
mosaicism of mycobacteriophages, 1039, 1040–1041, 1042
multiple modes of, 1042, 1043
Evolution of group A streptococci
core housekeeping genes, 193
full complement of epidemiological markers, 193–194
lateral movement of *emm*, 193
long term, 193–194
molecular mechanisms of genetic change, 191–193
population dynamics and, 195
resurgence of scarlet fever, 195
short term, 194–195
trends in distribution of epidemiological markers, 194
Evolution of markers (EMM), epidemiology, 188–190
Exfoliative toxins (ETs), 648–649
Exoenzymes
cofactors for host enzyme activation, 649–651
cysteine proteases: staphopains, 652–653
degrading host tissue components, 651–656
fatty acid-modifying enzyme (FAME), 655–656
glycerol ester hydrolases (lipases), 655
hyaluronidase, 654
lipases, 655
metalloprotease: aureolysin, 651–652
nucleases, 651
phospholipases, 655
proteases, 651–654
S. aureus, 649–656
schematic of *S. aureus* secretions, 656
serine proteases (serine protease-like proteins), 653–654
serine proteases (SspA), 652
staphylococcal coagulases, 649–650
staphylokinase, 650
Exotoxins
α-toxin (prototypic pore forming toxin, PFT), 640
β-toxin (β-hemolysin), 647–648
B cell superantigens, 647
bicomponent PFTs, 640–641
current models of PFT pore formation, 642
cytotoxic enzymes, 647–649
cytotoxins, 640–645
ε-toxin, 645
exfoliative toxins, 648–649
γ-hemolysin (HlgAB, HlgCB), 641–642
LukAB (LukHG), 642–644
LukED, 642
LukMF, 644
LukPQ, 644–645
LukSF-PV (PVL), 641
major, by *S. aureus*, 641
phenol soluble modulins (PSMs), 645
schematic of *S. aureus* secretions, 656
staphylococcal enterotoxins, 646
staphylococcal superantigens, 645–647
structures of α-toxin and bicomponent PFT, 643
T cell superantigens, 645–647
toxic shock syndrome toxin-1 (TSST), 646–647
Extracellular DNA (eDNA), 438, 439
Extracellular matrix (ECM), 108
collagens, 109–110
collagens and Gram-positive pathogens, 113–114
degradation by Gram-positive pathogens, 117–119
elastin, 110
elastin and Gram-positive bacteria, 114
fibrinogen, 111
fibrinogen and Gram-positive bacteria, 116–117
fibronectin, 110–111
fibronectin and Gram-positive pathogens, 114–116
interactions of Gram-positive pathogens with, 113–119
laminin and Gram-positive bacteria, 114
laminins, 110

matricellular glycoproteins, 110–111
Staphylococcus aureus surface proteins recognizing, 109
Streptococcus pneumoniae surface proteins recognizing, 109
structure and function of ECM molecules, 109–110
thrombospondin, 111, 113
thrombospondin and Gram-positive bacteria, 117
vitronectin, 111
vitronectin and Gram-positive bacteria, 116
Extracellular polysaccharide matrix (EPS), 438
Extracellular vesicles, of Gram-positive bacteria, 7–9
Extraoral diseases, *Streptococcus sanguinis* as etiological agent, 455–456

Fatty acid metabolism, intracellular *L. monocytogenes*, 867
Fatty acid-modifying enzyme (FAME), 655–656
Fatty acids, regulation of *L. monocytogenes* virulence, 842
Fatty acid synthesis (FAS), 867
Fermentation, *Staphylococcus aureus*, 690–692
Fibrinogen
Gram-positive pathogens and, 116–117
structure and function of, 111
Fibrinogen binding protein of G streptococci (FOG), 267
Fibronectin
Gram-positive pathogens and, 114–116
structure and function of, 110–111
Fibronectin-binding proteins
group C and G streptococci, 244–245, 266–267
Streptococcus pyogenes, 35, 37–38
Field emission scanning electron microscopy (FESEM), 8, 10–11
Firmicutes, 404, 461, 724, 875, 1143
protein export, 474
respiration, 468
transcriptional programs controlling sporulation in, 909
Flavobacterium, 248
Fleming, Alexander, 747, 779
Fluorescence microscopy, surface proteins of Gram-positive bacteria, 20–21
Fluoroquinolones, 748
invasive GBS disease, 232
mode of action, 755
resistance, 755
Focal adhesion kinase (FAK), streptococcal invasion, 38–40
Food and Drug Administration, 153
Foodborne listeriosis, 794
Food-grade strain, *Lactococcus lactis*, 474
Food poisoning
Clostridium perfringens type F, 977–984
see also Clostridium perfringens enterotoxin (CPE)
Formyl peptide receptors (FPRs), 621
Fosfomycin
mode of action, 751
resistance, 751
Freeze-etching, 13
Freeze-fracturing, 13
Freeze-substitution, 13
French National Reference Center for Staphylococci, 712
Fulminant, 712
Fulminant infections, 712
bacteremia, 717
infective endocarditis, 717
staphylococcal necrotizing fasciitis, 716–717
staphylococcal necrotizing pneumonia, 712–714
therapeutic aspects of, 717
toxic shock syndrome, 714–716
Fusidic acid, 748
mode of action, 754–755
resistance, 755
Fusobacterium nucleatum, community development, 452

Galectins, *S. pyogenes* and, 134, 135
Galleria mellonella, 777
Gallium-based therapeutics, *S. aureus*, 777
Gastroenteritis, *Listeria*, 797
Gastrointestinal disease, *Bacillus cereus* and, 878
Gastrointestinal transit, *in vitro* simulation of *Bacillus cereus*, 885
Gemella, 248
GenBank, 158, 344, 483, 486
clostridial genomes, 941, 949
mycobacteriophages, 1029, 1030, 1032, 1034
pathogenic clostridial genomes, 941
Gene expression
analysis of, 79–81
extracytoplasmic family (ECF) factors, 399–400
listeriolysin O (LLO) and bacterial influence on, 857
measurements for *Bacillus anthracis* and *B. cereus*, 888
model for EutV- and EutX-mediated regulation of *eut* regulon, 401
mycobacteriophages, 1036, 1037
posttranscriptional regulation, 401–402
regulation for enterococci, 399–402
two-component signal transduction system (TCSTS), 399–400, 402–403
virulence of *Bacillus cereus* group, 885–887
GeneMark, 1034
Genetic exchange, 67–70
mechanisms of, 499
plasmid technology, 68–70
transduction, 67–68
transformation, 68
see also Genetics of Group A streptococci
Genetic manipulation
newer technologies for, 933–934
S. pneumoniae, 354–355
see also Clostridial genetics
Genetics
Clostridium perfringens enterotoxin (CPE), 981–983
group B streptococci (GBS) capsular polysaccharides (CPSs), 211, 212
see also Clostridial genetics; Enterococcal genetics
Genetics of Group A streptococci, 67, 81–82
allelic replacement using linear DNA, 70, 72
analysis of gene expression, 79–81
analysis of transposon mutants, 79
common plasmids for S. pyogenes genome engineering, 69
counterselection markers, 74
directed insertional inactivation, 72–74
directed mutagenesis, 70, 72, 76
genetic exchange, 67–70
*Himar*1-based mariner transposon systems, 79
in-frame deleting using CRISPR/Cas9, 74–76
in-frame deletion, 74, 75
plasmids encoding resistance to erythromycin, 71
plasmid technology, 68–70
strategy for allelic replacement mutagenesis, 72
techniques for ectopic expression, 81
transduction, 67–68
transformation, 68
transposome mutagenesis, 79
transposon mutagenesis, 76–79
transposon mutagenesis of *S. pyogenes*, 78
Genetics of lactococci
genome plasticity, 463–464
phylogenetic tree comparing, and streptococcal pathogens, 462
Genetics of *Streptococcus pneumoniae*
DNA repair, 353–354
genetic manipulation, 354–355
horizontal gene transfer, 348–353
insertion-duplication mutagenesis and restoration, 355
restriction-modification systems, 354
transcription promoters, 354
Genetic tools, *Bacillus cereus* groups, 887–888
Genome engineering, plasmids utilized for *Streptococcus pyogenes*, 69
Genomic islands (GIs), virulence gene clusters, 545
Genomics
comparing *Enterococcus faecalis* and *E. faecium*, 387–388
see also Pneumococcal genomes

Genomics of mycobacteriophage
allocation of cluster designations, 1032–1033, 1034
annotation and gene naming, 1034
cluster, subclusters and singletons, 1031–1032, 1033
diversity, 1031–1034
gene expression, 1036, 1037
gene phamilies, 1033–1034
mosaicism of genomes, 1039, 1040–1041, 1042
organization, 1034–1036
see also Mycobacteriophages
Genomics of pathogenic clostridia, 940
Clostridioides difficile, 946–947
Clostridium boltaea, 948
Clostridium chauvoei, 948
Clostridium clostridioforme, 948
Clostridium haemolyticum, 945
Clostridium novyi, 945
Clostridium perfringens 945–946
Clostridium septicum, 948
Clostridium tetani, 944–945
GenBank, 941, 949
Paeniclostridium sordellii (*C. sordellii*), 947–948
relationships within *Clostridiaceae*, 942
strains that produce botulinum neurotoxin, 943–948
taxonomy, 940–941, 943
Glimmer, 1034
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 246
Glycopeptide resistance-associated (GraSR) system, cell wall synthesis, 581–582
Glycosyltransferases, cell wall branching, 287
Glycosyltransferases (GTFs), 438
Gordonia, 1030, 1032
Gordonia terrae, 1033
G protein-coupled receptors (GPCRs), activation of, 620–621
Gram, Hans Christian, 3
Gram-negative bacteria, 3
Gram-negative cell wall, schematic drawing of, 6
Gram-positive bacteria, 3
appearance of surface of, 28
characteristics of surface molecules from, 26
extracellular vesicles of, 7–9
mycobacteria as, 9
Gram-positive cell wall, schematic drawing of, 6
Gram-positive pathogens
collagens and, 113–114
degradation of extracellular matrix (ECM) by, 117–119
elastin and, 114
fibrinogen and, 116–117
fibronectin and, 114–116
human diseases causing, 119
laminin and, 114
thrombospondin and, 117
vitronectin and, 116
Gram staining, 3
Granulomatosis infantiseptica, 795
Green fluorescent protein (GFP), reporter of gene expression, 80
Group A polysaccharides
pediatric autoimmune neuropsychiatric disorder associated with streptococcal infection (PANDAS), 97, 100
rheumatic heart disease, 96–97
structure of, 95–96
Sydenham chorea, 97, 99
Group A streptococcal-mediated signaling, 125–126, 138
apoptosis and apoptotic signaling, 135–138
autophagy in *Streptococcus pyogenes* infection, 133–135
binding of streptoccoal adhesins to host cell receptors, 126–128
events responsible for inflammation, 129, 130
interaction of *S. pyogenes* with neutrophils, 129, 131
noncanonical inflammasome, 132–133
pyroptosis, 130, 133
role of integrins in, 128–129
S. pyogenes infection, 131–132
streptococcal surface dehydrogenase (SDH), 126–128
Streptococcus with mucin on pharyngeal cell mucosa, 126
Group A streptococci (GAS), 45, 239, 264
animal models with hyaluronic acid enhancing virulence, 49
cross-reactive antigens in, 86–87, 95
DNases of *S. pyogenes*, 59–60
future perspectives, 60–61
future research on, 60–61
IgG-targeting enzymes of *S. pyogenes*, 60
lysogenic phages of, 160–166
lytic phages of, 158–160
model of valvular heart disease by M proteins, 94, 96
S. pyogenes prophages, 166–172
streptococcal inhibitor of complement, 60
streptococcal pyrogenic exotoxin B, 59
streptococcal superantigens, 56–59
streptokinase, 60
see also Bacteriophages of *Streptococcus pyogenes*; Capsule; Genetics of Group A streptococci; *Streptococcus pyogenes*
Group B streptococci (GBS), 49, 204
capsular polysaccharides (CPSs), 204, 205–206, 210
carbohydrate antigens of, 204–211
chair models of CPSs, 207–210
clinical trials of vaccines, 220
disease in humans, 204
genetics of CPS, 211
mouse models of GBS disease, 218
sequenced surface proteins, 21
sialic acid in, 210–211
vaccines, 217–221
Group B streptococci (GBS) disease
neonatal, 230–232
nonpregnant adults and, 229–230
older adults and, 230
rates in pregnant women, 229
Group C streptococci (GCS)
antiphagocytic factors, 267–268
cell surface-associated proteins, 242–247
complement fragment C5a peptidase, 247
cytoplasmic membrane–associated enzymes, 247–248
enzymes and toxins, 268–269
epidemiology, 275–276
extracellular proteins, 248–253
fibronectin (FN)-binding proteins, 244–245
history and taxonomy, 275
human disease and diagnosis, 264, 266
hyaluronan synthase, 247–248
M and M-like proteins, 242–243
mechanisms for adherence, 266–267
molecular taxonomic approaches to classification, 239–241
pathogenicity factors of, 265
phylogenetic relationships, 241
phylogenomic findings implicating, 241–242
plasmin(ogen)-binding proteins, 245–246
properties of species, 240
prospects of, 254–255
protein G, 268
protein G and related Ig-binding proteins, 243–244
species of, 266
targeting alginate, 246–247
taxonomy and identification, 264
Group G streptococci (GGS)
antiphagocytic factors, 267–268
cell surface-associated proteins, 242–247
complement fragment C5a peptidase, 247
cytoplasmic membrane-associated enzymes, 247–248
enzymes and toxins, 268–269
epidemiology, 275–276
extracellular proteins, 248–253
fibronectin (FN)-binding proteins, 244–245
history and taxonomy, 275
human disease and diagnosis, 264, 266
hyaluronan synthase, 247–248
M and M-like proteins, 242–243
mechanisms for adherence, 266–267
model of valvular heart disease by M proteins, 94, 96

molecular taxonomic approaches to classification, 239–241
pathogenicity factors of, 265
phylogenetic relationships, 241
phylogenomic findings implicating, 241–242
plasmin(ogen)-binding proteins, 245–246
properties of species, 240
prospects of, 254–255
protein G, 268
protein G and related Ig-binding proteins, 243–244
responses of *S. dysgalactiae* subsp. *equisimilis*, 253–254
sequenced surface proteins, 21
species of, 266
taxonomy and identification, 264
Growth. *See* Microbacterium (growth)
Guinea pigs
models for anthrax, 882–883
models for *M. tuberculosis* infection, 1057

Haemophilus spp., 248, 320
Haemophilus influenzae, 454, 771
conjugate vaccines against, 218
otitis media (OM) and, 318
phase variation, 331, 332
Haemophilus influenzae type b, 363
Hazard analysis at critical control point (HACCP), 794
Health care-associated methicillin-resistant *S. aureus* (HA-MRSA), 737, 749
Heart, pneumococci in, 321
Helicobacter, 248
Helicobacter pylori, variation in proteins, 334
α-Hemolysin, 640
β-Hemolysin, 647–648
γ-Hemolysin, 641–642
Hepatitis, *Listeria monocytogenes*, 796–797
Herd immunity, 306
High-performance liquid chromatography (HPLC), 285
High-pressure freezing, 13, 14
Histotoxic clostridial infections
agents, hosts and toxins of, 1013
clostridial myonecrosis, 1012–1013
control and treatment of, 1020–1021
function and host cell interactions of *C. septicum* α-toxin, 1019–1020
general characteristics of, 1012
interactions of *C. perfringens* α-toxin with host cells, 1014–1018
structure and function of *C. perfringens* α-toxin, 1013–1014
structure and host cell interactions of *C. perfringens* perfringolysin O, 1018–1019
HIV (human immunodeficiency virus)
listeriosis and, 794
tuberculosis in patients with, 1068, 1112
Horizontal gene transfer
conjugation, 351–352
insertion sequences in *S. pneumoniae*, 349
lysogenic phages of *S. pyogenes*, 165–166
plasmids and bacteriophages, 352–353
pseudotransduction, 352
staphylococci, 489–490
Staphylococcus aureus phages mediating, 529–530
streptococci, 34
Streptococcus pneumoniae 348–353
Streptococcus sanguinis, 453–454
transformation, 348–351
Horses
methicillin-resistant *S. aureus* (MRSA), 738–739
staphylococcal elements, 734
Host cell
intracellular carbon metabolism, 866–867
intracellular fatty acid metabolism, 867
intracellular vitamin metabolism, 867
Listeria monocytogenes in, 865–868
metabolism and virulence intersecting in, 867–868
see also Group A streptococcal-mediated signaling
Human cardiac myosin
peptide epitopes in rheumatic heart disease, 94–95
reactivity of antistreptococcal M5 peptide sera, 92
rheumatic fever and M proteins, 89–92
sequence alignment of streptococcal M6 protein and tropomyosin, 91
Human colonization, enterococci, 380–382
Human gastrointestinal diseases, *Clostridium perfringens*, 984–985
Human immune response, models for, 771–772
Human immunity, *S. aureus* infection, 772
Human nose
bacterial competition in, 725–727
community state types of, 724
diversity of nasal microbiome, 724–725
mechanisms of nasal epithelial attachment, 725, 726
metabolism in, 725
outlook, 727
Human T lymphocytes, M proteins and cross-reactive, 92–94
Humoral immunity, *Listeria monocytogenes*, 818
Hyaluronan synthase, group C and G streptococci, 247–248
Hyaluronic acid
animal models with, enhancing virulence, 49
biosynthesis, 45–47
capsule and resistance to phagocytosis, 49
chromosomal locus encoding regulatory system for, 48
immunogenicity of, 48–49
map of *S. pyogenes* chromosomal region encoding enzymes for synthesis, 47
regulation of biosynthesis of, 47–48
schematic diagram of biosynthetic pathway, 46
see also Capsule; Capsule production
Hyaluronidase, 654
Hyaluronidase gene, genome prophages, 163, 165
Hydrogen peroxide (H_2O_2)
cytotoxicity mediator, 323, 324
dental health, 430–431
Streptococcus sanguinis and, 449, 453

Immune defects, *S. aureus* infections, 766–768
Immune dysregulation and death, *S. aureus* infection, 770–771
Immune evasion
abbreviations of staphylococcal proteins for, 619
bacterial killing by neutrophils, 626–627
biological warfare, 632
cell wall-anchored (CWA) proteins, 611
cluster 1/φSa3, 631–632
enterococci, 384–385
escape by toxins, 629
evading killing, 627–629
evading net formation, 626
evading neutrophil extravasation to infection site, 620
evading opsonization and phagocytosis, 623–625
evading oxygen-dependent killing by neutrophils, 627–628
evading oxygen-independent killing by neutrophils, 628–629
evading priming, chemotaxis, and activation of neutrophils, 622
evasion proteins and therapeutic strategies, 630–631
neutrophil extracellular trap formation, 625–626
opsonization, 622–623
overview of evasion proteins, 627
oxygen-dependent killing by neutrophils, 626–627
oxygen-independent killing by neutrophils, 626
phagocytosis, 623
priming, chemotaxis, and activation of neutrophils, 620–621
schematic of *S. aureus* evading opsonization and phagocytosis, 624
schematic overview of *S. aureus*, 621
staphylococcal biofilm formation for, 704–706
staphylococcal innate, 629–630
by staphylococcal toxins, 628
Staphylococcus aureus, 618–619
Immune inhibitor A (InhA1), *Bacillus cereus* group, 882
Immunofluorescence microscopy, 50

Immunoglobulin binding, streptococcal protein G, 268
Immunoglobulins
  opsonization by, 623
  proteins targeting, 623–624
Impetigo
  ecology of, 177–178
  emm pattern assignments from population–based surveys, 187
  emm type diversity from population–based surveys, 189
  epidemiology, 186–187
  nonbullous, 188
Inducible vector systems, Clostridial genetics, 929–930
Infectious diseases, *Listeria monocytogenes* vaccine strains for, 819
Infective endocarditis (IE), 712
  Staphylococcus aureus 717
  Streptococcus sanguinis, 455
Inflammasomes
  Listeria monocytogenes, 805
  noncanonical, 132–133
  S. pyogenes infection, 131–132
Inflammation
  Group A *Streptococcus*-mediated signaling events for, 129, 130
  pneumococcal cell wall, 322, 323
Inflammatory cells, infiltration of, 809–814
Influenza A virus (IAV), bacterial adherence, 41, 42
Innate immune system, 618
Innate immunity of *Listeria monocytogenes*, 803–814
  activation of complement system, 809
  antigen-laden DCs activating T cell responses, 812–813
  chemokine and cytokine production, 805–809
  chemokines attracting immune cells to infection sites, 806
  cytosolic *L. monocytogenes*, 804–805
  effector cytokine TNFα, 808
  extracellular *L. monocytogenes* at cell surface, 803–804
  host susceptibility and IL-10 production, 808–809
  IFNγ stimulating gene expression, 808
  infiltration of inflammatory cells, 809–814
  inflammasomes, 805
  lymphocytes protecting during early infection, 813–814
  macrophages as intracellular growth niche, 811–812
  mast cells, 814
  monocytes patrolling tissues, 810–811
  neutrophils killing L. monocytogenes, 809–810
  NK (natural killer) cells and IL-10 production, 813
  in phagocytic or endocytic vacuoles, 804
  pleiotropic functions of IL-1β pyrogen, 807
  proinflammatory or anti-inflammatory effects of type 1 interferons, 806–807
  proinflammatory T cell responses of IL-12 and IL-23, 807–808
  triggering of innate immune responses, 803–805
Innate immunity to *Mycobacterium tuberculosis* infection
  dendritic cells (DCs) for initiation of adaptive immunity, 1062–1063
  inflammation and cell death during, 1061–1062
  NK cells in, 1061
  pattern recognition receptors (PRRs), 1058–1059
  phagosomal defense in macrophages, 1059–1060
  recruitment and function of neutrophils and monocytes, 1060–1061
Insect models, *Bacillus cereus* and *B. thuringiensis* infection, 884, 885
Institute for Genomic Research (TIGR), 344
Institute of Medicine, 204
Integrin-linked kinase (ILK), streptococcal invasion, 38–40
Integrins, role in streptococcal-mediated signaling events, 128–129
Interbacterial cross-feeding, *Lactococcus lactis* and, 472
Interbacterial cross-inhibition, *Lactococcus lactis* and, 472–473
Interleukin-1-receptor-associated kinase-4 (IRAK-4) deficiency, *S. aureus* infections, 766–767
Interleukin-6 (IL-6) autoantibodies, *S. aureus* infections, 767
International Journal of Systematic and Evolutionary Microbiology, 927, 991
Intracellular pathogenesis, Listeria monocytogenes as model, 792
Invasive disease, epidemiology, 187–188
Invasive group A streptococci (iGAS), ecology of, 178
Invasive pneumococcal disease (IPD), 316
Invertebrate models, enterococci, 388
Invertebrates, *Bacillus thuringiensis* infection, 878–879
Iron, *Staphylococcus aureus*, 689
Iron transporter lipoproteins, pneumococcal vaccine, 367

Jacob, Francois, 1096
Job's syndrome (TH17/STAT3 defect), *S. aureus* infections, 767
Joint and bone infections, *Streptococcus dysgalactiae* subsp. *equisimilis* (SDSE), 277

Kitten, Todd, 455
Klebsiella, 724
Koch's bacillus, 1087
Krebs cycle, 549–550
Kupffer cells, 809, 812

Lachnoclostridium, relationships within *Clostridiaceae*, 942
Lachnospiraceae, 918
β-Lactam resistance, cell wall synthesis, 580–581
Lactic acid bacteria (LAB), varied lifestyle of, 461, 463
Lactobacillus spp.
  oral microbiome, 428
  rolling circle replicating (RCR) plasmids, 500
Lactobacillus salivarius, oral microbiome, 428
Lactocobacillus paracasei, variation in proteins, 334
Lactococci, 461, 463, 474
  applications and tools of, 473–474
  basic features of, 463–464
  cell lysis systems, 474
  containment and food-grade strains, 474
  CRISPR-Cas system, 473
  expression strains, 474
  higher antigenicity, 474
  immune system against phages, 473
  metabolic options of, 464–469
  nitrogen metabolism, 468
  nucleotide metabolism, 468–469
  phylogenetic tree comparing, and streptococcal pathogens, 462
  protein transport and display systems, 473–474
  respiration metabolism, 471
  site-specific single–copy integration, 473
  stress responses of, 469–471
  see also Genetics of lactococci
Lactococcus, interbacterial cross-feeding, 472
Lactococcus lactis, 81, 166, 474, 845
  adaptation to environmental stress, 470–471
  basics of fermentation, 465
  basics of respiration, 467
  characteristics of, 463
  conjugative plasmids, 503
  cyclic-di-AMP in bacterial adaptation, 469–470
  description of, 463
  fermentation in, 464–465
  genome of, 461
  genome plasticity, 463–464
  heme homeostasis and respiration, 466, 468
  heme sensitivity, 469–470
  interbacterial dialogue, 471–473
  nonrespiration processes, 468
  plasmid, 407
  regulation by nucleotide pools, 469
  respiration in, 465–468
  as respiration prototype, 468
  role of regulator CcpA in respiration, 466
  Spx family of regulators, 469
  survival strategies in stress conditions, 470
Laminins
  Gram-positive pathogens and, 114
  structure and function of, 110
Lancefield, Rebecca, 45, 204, 221, 228

Latent TB infection (LTBI), 1056–1057, 1064
Leloir pathway, capsular polysaccharides, 311
Leuconostoc, interbacterial cross-feeding, 472
Lewis rats, valvular heart disease by streptococcal M proteins, 94, 96
Lincosamides, 748
  history and mode of action, 753
  resistance, 753
Linezolid
  history and mode of action, 752
  resistance, 752–753
  resistance in enterococci, 379
Lipases
  glycerol ester hydrolases, 655
  phospholipases, 655
Lipoate, metabolism, 867
Lipoglycopeptides, 748
  mode of action, 751
  resistance, 751
Lipoproteins, pneumococcal vaccines, 366–367
Lipoteichoic acids (LTA), 3
  constituent of bacterial cell wall, 6–7
  S. aureus, 524–526
  structure of, 290–292
Listeria, 36
  blocking killing mechanisms of macrophages, 40
  metabolic potential, 864–865
  use of attenuated, as vaccine vector, 818–820
Listeria monocytogenes, 137
  adaptation to intracellular life, 851
  adaptive immunity, 814–818
  adhesion/entry effectors, 853
  adult meningoencephalitis, 795–796
  bacterial endocarditis by, 796
  biliary tract infections, 797
  cell-to-cell spread, 856
  cellular invasion process, 851–853
  cellular receptors for, in host cells, 852
  clinical disease due to, 795
  cytoplasmic stage, 855–856
  diagnosis of listeriosis, 797
  epidemiology, 793–795
  gastroenteritis, 797
  hepatitis and liver abscess by, 796–797
  innate immunity, 803–814
  intracellular stages of, 854
  life as a saprophyte, 865
  life inside the host, 865–868
  listeria peritonitis, 797
  listeriosis and, 791–792
  membrane vesicles (MVs) of, 8
  metabolic potential, 864–865
  modulation of cellular, organellar and nuclear functions, 856–857
  musculoskeletal infection, 797
  pregnancy-associated listeriosis, 795
  sequenced surface proteins, 21
  T7S system, 1143
  treatment of listeriosis, 797–798
  vacuolar stage, 853–855
  see also Metabolism of *Listeria monocytogenes*; Regulation of virulence (*Listeria monocytogenes*)
Listeria peritonitis, *Listeria monocytogenes*, 797
Listeria sepsis, 796
Listeriolysin O (LLO), 836
  bacterial influence on gene expression, 857
  DNA stability and, 857
  influence on endoplasmic reticulum (ER), 857
  influence on lysosomes, 857
  influence on mitochondria, 857
  intracellular life, 851
  lysis of secondary vacuoles, 856
  modulating cellular functions, 856–857
  protein posttranslational modifications, 857
  vacuolar disruption, 853–854
Listeriolysin S (LLS), 844–845
Listeriosis
  cutaneous, 796
  diagnosis of, 797
  foods implicated in foodborne, 794
  Listeria monocytogenes and, 791
  treatment of, 797–798
Liver abscess, Listeria monocytogenes, 796–797
Livestock
  Clostridium perfringens enteric diseases, 985
  livestock-associated methicillin-resistant *S. aureus* (LA-MRSA), 735, 737–738
Lymphocytes, *L. monocytogenes* infection, 813–814
Lysins, staphylococcal biofilm-associated infection, 706
Lysosomes, influence of listeriolysin O (LLO), 857

Macaques, models for anthrax, 883
Macrolides, 748
  history and mode of action, 753
  resistance, 753
Manganese, *Staphylococcus aureus*, 689
Massachusetts Antitoxin and Vaccine Laboratory, 1154
Mast cells, *L. monocytogenes* infection, 814
Maternal infections, *Streptococcus dysgalactiae* subsp. *equisimilis* (SDSE), 278
Matricellular glycoproteins, 110–111
Matrix metalloproteinases (MMPs)
  bacterial killing, 626
  controlling inflammation, 621
Medical devices
  prone to infection, 699–700
  staphylococcal biofilm-associated infection, 705
Membrane vesicles (MVs), of Gram-positive bacteria, 7–9
Mendelian susceptibility to mycobacterial disease (MSMD), 1064
Meningitis, *Streptococcus agalactiae*, 228, 232
Meningoencephalitis, 795–796
Metabolic gene regulation, *Staphylococcus aureus*, 692–694
Metabolism, *Staphylococcus aureus*, 688–694
Metabolism of *Listeria monocytogenes*
  bacterial, and host immune response, 868
  intersection with virulence, 867–868
  intracellular carbon metabolism, 866–867
  intracellular fatty acid metabolism, 867
  intracellular vitamin metabolism, 867
  life inside host, 865–868
  potential, 864–865
Methaquinones, interbacterial cross-feeding, 472
Methicillin-conditional mutants, cell walls, 580
Methicillin-resistant *Staphylococcus aureus* (MRSA), 485, 488, 732, 776
  in companion animals, 738–739
  epidemiology, 618
  livestock-associated (LA-MRSA), 527
  peptidoglycan of, 575
  S. aureus phage in, 527
  staphylolytic enzymes as therapy, 779–780
Methicillin-susceptible *S. aureus* (MSSA), 577, 732
  biofilms, 777
  staphylolytic enzymes as therapy, 779–780
MHC-I (major histocompatibility complex) pathway, 820
  classical, 814–815
  nonclassical, 815
MHC-II (major histocompatibility complex), *Mycobacterium tuberculosis* infection, 1062–1063
MHC-II pathway, antigen processing, 815
Microbacterium, 1030
Microbial Nucleotide BLAST, 344
Micrococcus zymogenes, 378
Micronutrients, *Staphylococcus aureus*, 688–694
Microreact project, 487
Mitochondria, influence of listeriolysin O (LLO), 857
Models
  animals for anthrax, 882–883
  animals for *Bacillus cereus* infection, 883–884

insect, for *B. cereus* and *B. thuringiensis*, 884
S. aureus infection, 771–772
Moesin, streptococcal surface dehydrogenase (SDH) and, 127
Molecular mimicry, 86
cross-reactive antibodies, 100–101
identification in streptococcal M protein, 92
types, 87
Monkeys
models for anthrax, 883
Staphylococcus aureus in, 739
Moraxella, 724
Moraxella catarrhalis, otitis media (OM) and, 318
Mosaicism
mycobacteriophages, 1040–1041
phage genomes, 1039, 1042
Mouse models
anthrax, 882–883
group B streptococci (GBS) disease, 218
M proteins
candidate for streptococcal vaccine, 148
candidate for type-specific protection, 149–150
cross-reactive T cells, 92–94
epidemiology of group A streptococci (GAS) infection, 180–183
fibrinogen and, 117
group C and G streptococci, 242–243
poststreptococcal acute glomerulonephritis, 95
proposed model of (M6 strain), 149
rheumatic fever and, 89–92
role in excessive plasma leakage, 127
size variation, 26
strategies for vaccines based on, 190
Streptococcus pyogenes, 35, 37–38
structure and function, 148–149
type-specific protection, 149–150
see also Vaccine(s)
M protein sequence, computer-generated model of C-terminal end of, 25
MSCRAMM (microbial surface components recognizing adhesive matrix molecules), 108, 700, 701
Mucins, interactions with *Streptococcus*, 126
Mucosal-associated invariant T (MAIT) cells, *S. aureus* infections, 768
Mucosal immunity, pneumococcal vaccines, 369
Multilocus enzyme electrophoresis (MLEE), 240
Multilocus sequence typing (MLST), 731
epidemiology of group A streptococci (GAS) infection, 186
evolutionary models, 190–191
Mupirocin
mode of action, 754
resistance, 754
Musculoskeletal infection, *Listeria monocytogenes*, 797
Mutagenesis technologies, early adaptations of, 927–928
Mycobacteria, 1027–1028
schematic drawing of cell wall, 10
specialized cell wall in, 9
Mycobacterial cell envelope, 1087–1093
capsule, 1091–1093
cell wall, 1089, 1091
diagrams of, 1088
export system, 1130
microscopic appearance of, 1087–1089
model of, 1092, 1130
outlook, 1143–1144
plasma membrane, 1089, 1090
SecA2 pathway, 1132–1135
Sec pathway, 1131–1132
T7S pathway, 1135–1143
Tat (twin-arginine translocase) pathway, 1132
see also Protein export
Mycobacterial metabolism
complex models, 1110–1111
harnessing, to potentiate drug activity, 1116
human TB disease and, 1108, 1110–1113
investigating metabolism *in vitro*, 1110
new TB drug discovery, 1115–1116
pathogenicity and, 1108, 1110
TB life cycle, 1111–1112
see also Mycobacterium tuberculosis metabolism
Mycobacteria smegmatis
gene expression, 1036, 1039
regulation, 1039
Mycobacteriophages, 1029
allocation of cluster designations, 1032–1033, 1034
clusters, subclusters and singletons, 1031–1032
compatibility and incompatibility, 1049
counterdefense, 1045, 1047–1048
current genomic scope, 1029–1030
databases, 1030
discovery and genomics as platform for advancing science, 1030
exclusion during lytic growth, 1048–1049
exploitation of, 1049–1051
features, 1029–1031
gene expression, 1036, 1037
gene phamilies, 1033–1034
genome annotation and gene naming, 1034
genome organization, 1034–1036
genomic diversity, 1031–1034
mobile elements, 1042
model for diversity of, 1044
modes of phage evolution, 1043
morphologies, 1030–1031
mosaicism, 1039, 1040–1041, 1942
organization and structure of SEA-PHAGES program, 1030, 1031
perspectives, 1051
phage evolution, 1039–1043
prophage-mediated defense against viral attack, 1044–1045, 1046
recombinant phages for efficient delivery, 1049–1050
repressor theft, 1049
therapeutic potential, 1050–1051
tools for mycobacterial genetics, 1050
unusual life cycle regulation, 1036, 1038–1039
see also Genomics of mycobacteriophage
Mycobacterium, 1087
Mycobacterium (growth)
breaking cell wall, 1101–1102
connecting cytoplasmic steps to periplasmic steps, 1099–1100
constructing septal wall, 1100–1101
creating new growth pole, 1102
division, 1103
divisome interactions, 1099
early divisome, 1099, 1100
elongasome, 1100
geometry of cell division, 1096
growth rate of, 1102–1103
identifying mid-cell for Z-ring placement, 1098
mechanics of division, 1097–1102
polar growth of, 1096–1097
recruitment of Z-ring anchors, stabilizers and regulators, 1098–1100
Mycobacterium abscessus
ESX-4 system, 1135
therapeutic potential, 1051
Mycobacterium avium, surface glycolytic enzymes, 27
Mycobacterium avium spp. *paratuberculosis*, structure of cell wall, 11
Mycobacterium bovis, 1056, 1135
Mycobacterium fortuitum, 1102
Mycobacterium kansasii
capsule, 1091, 1092
cell envelope diagram, 1088
Mycobacterium leprae, 770, 1087, 1091
Mycobacterium lepraemurium, 1088, 1091
Mycobacterium smegmatis, 1029, 1032
cell envelope diagram, 1088
defense against viral attack, 1044–1045
evolution, 1043
growth rate, 1102–1103
recombineering systems in, 1050

Mycobacterium tuberculosis, 1028, 1029, 1044, 1087
blocking killing mechanisms of macrophages, 40
cell wall, 28
DtxR repressor in, 1156
life cycle of TB, 1111–1112
metabolic capacity, 1109
recombineering systems in, 1050
therapeutic potential, 1050
tuberculosis (TB), 1087, 1129
Mycobacterium tuberculosis complex (MTBC), 1107, 1111, 1112
Mycobacterium tuberculosis infections
adaptive immunity against, 1063–1067
animal models of, 1057–1058
human TB disease and comorbidities, 1056–1057
immunology of TB diagnostics, 1068
initiation and heterogeneity of granuloma, 1067–1068
innate immunity to, 1058–1063
TB vaccines, 1068–1069
Mycobacterium tuberculosis metabolism, 1107–1108
accessing nutrients from host, 1115
cell wall homeostasis, 1114–1115
central metabolism, 1113–1114
future perspectives, 1116–1117
implications for TB drug discovery, 1116
respiration, 1114
see also Mycobacterial metabolism
Mycoplasma bovis, 339
Mycoplasma pulmonis, 339
Myeloid differentiation factor 88 (MyD88) deficiency, *S. aureus* infection, 766–767
Mykrobe predictor, 487
Myocarditis, M5 protein sequences producing, in mice, 93
Myosin, cross-reactive T cell epitopes of streptococcal M5 protein, 94
Myoviridae, 1030
S. aureus phages, 521, 528

N-acetyl-glucosamine, streptococcal infection and, 154
Nasopharyngeal infections, *S. pyogenes* and, 148
NCBI Sequence Read Archive, 486
Nebraska Transposon Library, 487
Necrotizing fasciitis (NF), 45, 50, 55, 239
ecology of, 178
staphylococcal, 716–717
Necrotizing pneumonia, staphylococcal, 712–714
Neisseria spp., 320
oral microbiome, 428
phase variation, 331
Neisseria gonorrhoeae, phase variation, 331
Neisseria meningitides, phase variation, 332
Neisseria meningitidis, 206, 771
Neisseria meningitis, 135
Neonatal disease
group B streptococci (GBS), 230–231
prevention of, 231–232
treatment, 232
Neonatal infections, *Streptococcus dysgalactiae* subsp. *equisimilis* (SDSE), 278
Neutrophil(s)
bacterial killing by, 626–627
evading bacterial killing by, 627–629
evading extravasation, 620
evading oxygen-dependent killing by, 627–628
evading oxygen-independent killing by, 628–629
evading priming, chemotaxis and activation of, 621, 622
extravasation through the endothelium, 619–620
impaired function, 618
interaction of *S. pyogenes* with, 129, 131
killing *L. monocytogenes*, 809–810
migration to infection, 806
Mycobacterium tuberculosis infection, 1060–1061
overview of evasion proteins, 627
oxygen-dependent killing by, 626–627
oxygen-independent killing by, 626
priming, chemotaxis and activation of, 620–621
see also Immune evasion
Neutrophil extracellular traps (NETS)
evading NET formation, 626
formation, 625–626
interaction with *S. pyogenes*, 129, 131
Newly Independent States (NIS) of former Soviet Union, diphtheria epidemic, 1159
Nitric oxide (NO), role in cell signaling, 135
Nitrogen metabolism, lactococci, 468
NK (natural killer) cells
Mycobacterium tuberculosis infection, 1058, 1061
protection against infection, 813
Nocardia, 1033
Noncoding RNA, 562
antisense RNA (asRNAs) in transcription or acting as antitoxins, 564–565
crRNA, tracrRNA and CRISPR-Cas adaptive immunity systems in *S. aureus*, 566–568
mechanisms of RNA regulation in *S. aureus*, 563
mRNA 3' untranslated regions (UTRs), 564
mRNA 5' untranslated regions (UTRs), 562, 564
role of sRNAs in physiopathology, 568–569
Staphylococcus aureus, 562
transcriptional factors and sRNAs building regulatory networks, 565–566
Nonconventional therapeutics against *S. aureus*
antibodies and antibody conjugates, 782
bacteriophage-based therapy, 778–779
photodynamic therapy, 780–782
prevention and disruption of biofilm formation, 776–777
quorum sensing for inhibition of virulence, 777–778
RNA guided nucleases, 780
staphylolytic enzymes as, 779–780
Nonfoodborne human GI diseases, *C. perfringens* enterotoxin (CPE), 984
Nonhuman primates
models for anthrax, 883
models for *M. tuberculosis* infection, 1057–1058, 1063, 1068, 1111, 1112
Non-pheromone-responding plasmids, enterococcal mobilome, 412
Nose. *See* Human nose
N-terminal-anchored lipoproteins, 27–28
Nuclear magnetic resonance (NMR), 206
Nuclear magnetic resonance (NMR) spectroscopy, cell wall, 577, 578
Nucleases, 651
Nucleotide metabolism
lactococci, 468–469
purine biosynthesis, 469
pyrimidine biosynthesis, 469

Olsenella spp., oral microbiome, 428
Ontak, denileukin diftitox, 1161–1162
Opsonization
by complement system, 622–623
evading, 623–625
by immunoglobulins, 623
proteins targeting the complement system, 624–625
schematic of *S. aureus* evading, 624
Oral environment, 426
Oral microbiome, microbiome and metagenomics, 428
Oral streptococci
commensal oral flora, 430–431
dental caries or simple cavities in, 430
dental plaque, 428
mechanisms of colonization, 429–430
microbiome and metagenomics studies, 428
oral health, 428, 430, 435
phylogeny of streptococcal species, 427
schematic of homolactic and heterolactic fermentation, 428

schematic of phosphoenolpyruvate sugar:phosphotransferase system, 429
Streptococcus sanguinis and oral health, 449
sugar metabolism of, 428–429
taxonomy of, 426
Osteomyelitis, *Streptococcus agalactiae*, 228, 232
Otitis media (OM), 316, 318
Outer membrane vesicles (OMVs), 7–8

Paeniclostridium, 927
Paeniclostridium sordellii (*C. sordellii*), 947–948, 992–993
bioinformatic characterization of plasmids in, 954–956
pCS1 family plasmids of, 955
stability and conjugative transfer of pCS1 family of plasmids, 956
virulence properties of, 954
Panton-Valentine leukocidin (PVL), 712, 777, 782
Pappenheimer, Alwin M., Jr., 1154–1155
Parascardovia denticolens, oral microbiome, 428
Passive immunization, *Staphylococcus aureus* infection, 631
Pathogen (danger)-associated molecular patterns (PAMPs/DAMPs), *S. pyogenes* infection, 131
Pathogenesis, role of capsular polysaccharides in *S. pneumoniae*, 304–305
Pathogenicity islands (PIs)
accessory genes, 544–545
antibacterial drones (ABDs), 546–547
capsid morphogenesis and packaging, 543
de-repressor proteins and their targets, 542
diagrammatic of ABD backbone, 546
enterococcal mobilome, 412, 413
existing and proposed ABDs, 546
features of two SaPI types, 540–541
genomic islands (GIs) of *Staphylococcus aureus* (SaPIs), 536–538
history of SaPIs, 537
host cell protection by operon 1, 544
integration-excision, 542–543
interference mechanisms, 543–544
regulation of SaPI, 538–539
replication, 543
role in host cell biology, 544
SaPI1 subset, 537
SaPIbov5 subset, 538
SaPI encapsulated, 536–537
SaPI evolution, 545–546
SaPI genome, 537, 538
SaPI genome pattern, 539
SaPI life cycle, 538–539, 542–544
SaPI-mediated gene transfer, 544
SaPI-phage war, 539, 542
term, 536
virulence genes, 544–545
Paxillin, streptococcal invasion, 38–40
Pediatric autoimmune neuropsychiatric disorder associated with streptococcal infection (PANDAS), 97, 100
Penicillin, pneumococcal cell wall and inflammation, 323
Penicillin binding proteins (PBPs), 4
cell wall branching, 287, 579–580
cell wall of Streptococcus pneumoniae, 284
peptidoglycan biosynthesis, 286
trimming of peptides by carboxypeptidases, 286
Penicillin G, invasive GBS disease, 232
Penicillium restrictum, 778
Peptide pheromones, regulation of *L. monocytogenes* virulence, 843–844
Peptidoglycan
analysis of, of methicillin-susceptible *S. aureus*, 577
antibiotics and, 578
bacterial cell wall backbone, 5
biochemical synthesis of layer, 4–5
biosynthesis and remodeling, 286–288
cell wall of pneumococci, 285
composition, 288
fluorescence microscopy, 578
high-resolution analysis of *S. aureus*, 575–578
HPLC elution profiles of stem peptides of, 289
hydrolases involved in septum remodeling and cleavage, 287–288
interaction with environment, 585
interaction with host, 585
model for cross-linking of *S. aureus*, 577
modifcations of precursor of, 286
N-acetylglucosamine deacetylase PgdA, 288
O-acetyltransferase, 288
penicillin-binding proteins (PBPs), 286
putative lytic transglycosylase mltG, 287
recycling, 585
sortase A-dependent covalent attachment of proteins to cell wall, 288
stability of muropeptide composition, 578
structure of, 285–286
structures of cell wall stem peptides in, 290
three-dimensional structure of staphylococcal, 576
turnover of, 5–6
ultrastructural analysis of *S. aureus*, 578
vancomycin and teicoplanin resistance, 578
vancomycin-intermediate *S. aureus* (VISA) isolates, 575, 578–579
vancomycin-resistant *S. aureus* (VRSA), 579
variation in composition, 578–579
variation in composition of, 578–579
see also Cell walls
Peptoanaerobacter, 248
Peptoclostridium, relationships within *Clostridiaceae*, 942
Peptoclostridium difficile, 927, 991
Peptostreptococcaceae, 918
Peptostreptococcus, relationships within *Clostridiaceae*, 942
Peptostreptococcus magnu, sequenced surface proteins, 21
Peritonitis, *Listeria monocytogenes*, 797
Persisters, small colony variants (SCVs), 553, 555
Phage(s)
therapy for *S. aureus* infection, 778–779
see also Staphylococcus aureus phages
Phage Evidence Collection and Annotation Network (PECAAN), 1030
Phage Hunters Integrating Research and Education (PHIRE), 1030, 1034
PhagesDB, 1029
Phagocytosis, 623
capsule and resistance to, 49
capsule production, 623
evading, 623–625
schematic of *S. aureus* evading, 624
Phamerator, 1029, 1030
Pharyngitis, 45
emm pattern assignments from population-based surveys, 187
emm type diversity from population-based surveys, 189
epidemiology, 186–187
Streptococcus dysgalactiae subsp. *equisimilis* (SDSE), 276–277
Phase variation, 331
by differential DNA methylation, 332
by DNA inversion, 331, 332
by programmed homologous recombination, 331, 332
by slipped-strand mispairing, 331–332
see also Pneumococcal phase variation
Phenol soluble modulins (PSMs), 645
Pheromone-responsive plasmids
conjugation apparatus, 406–407
enterococcal genetics, 404–410
genetic organization of plasmid replicon, 408
regulation of pheromone signal transduction circuit, 404–406
regulatory circuitry, 405
replication and stable inheritance, 407–410
TA (toxin-antitoxin) module, 407, 408–410, 412–413
Phosphoenolpyruvate transport system (PTS), 687
Phospholipases (PlcA/P)lcB)
intracellular life, 851
lysis of secondary vacuoles, 856
vacuolar disruption, 854–855
Phosphotransferase systems (PTSs), 399, 436, 865
Photodynamic therapy (PDT), *S. aureus* infection, 780–782

Placenta and fetus, pneumococci interactions with, 321–322
Plasma membrane, microbacterial envelope, 1089, 1090, 1092
Plasmid cloning vectors, clostridial genetics, 928–929
Plasmids
  S. pneumoniae, 352–353
  staphylococcal, 489
  see also Staphylococcal plasmids
Plasmid technology, *Streptococcus pyogenes*, 68–70
Plasmid transformation, *S. pneumoniae*, 351
Plasmin(ogen)-binding proteins, group C and G streptococci, 245–246
Plasminogen-mediated invasion, 40
Platelet activating factor receptor (PAFr), 320, 323
Pleuromutilins, 748
  history and mode of action, 753
  resistance, 753
Pneumococcal genomes, 344–348
  complete, 346–347
  population structure, 345, 348
  properties of *S. pneumoniae* laboratory strains, 345
Pneumococcal histidine triad (Pht) proteins, 367
Pneumococcal meningitis, 137
Pneumococcal phase variation, 332–333
  in capsular polysaccharides, 333
  in cell wall polysaccharides, 333–334
  cod-like systems in other bacteria, 339
  diversification of pneumococcal methylomes by DNA inversions, 336–337
  epigenetic switch, 337–338
  genetic organization of *cod* locus of *S. pneumoniae*, 335
  mechanisms of, 336–339
  phase-locked pneumococci, 338–339
Pneumococcal surface antigen A (PsaA), 366–367
Pneumococcal vaccine(s), 362, 369–370
  capsular polysaccharides, 362–363
  choline binding proteins, 365–366
  combination protein, 368–369
  lipoproteins, 366–367
  mucosalo immunity, 369
  noncapsular polysaccharide, 364–369
  pneumococcal histidine triad proteins (Pht), 367
  pneumolysin, 365
  polysaccharide-protein conjugate, 363–364
  protein candidates, 368
  PspA (pneumococcal surface protein A), 364–365
  PspC (pneumococcal surface protein C), 364–365
  sortase-dependent surface proteins, 367–368
  whole-cell vaccines (WCV), 369
  see also Vaccine(s)
Pneumococcus, 128
  vaccines, 362
  vitronectin and, 116
Pneumolysin
  cytotoxicity mediator, 322–324
  domain structure of, 324
  pneumococcal vaccines, 365
Podoviridae, 1030
Podoviridae C1 phage, 161
Podoviridae class, *S. aureus* phages, 521, 528
Polysaccharide intercellular adhesin (PIA), biosynthesis in biofilm formation, 700–701, 702
Polysaccharide-protein conjugate vaccines, pneumococcus, 363–364
Porphyromonas gingivalis, 26
  community development, 452–453
  oral microbiome, 428
Posttranscriptional regulation
  gene expression, 401–402, 841
  Listeria monocytogenes virulence, 837–838
Posttranslational regulation, *Listeria monocytogenes* virulence, 838–839
Poultry
  Clostridium perfringens enteric diseases, 985
  Staphylococcus aureus in, 736–737
Pregnancy
  Listeria monocytogenes, 794, 795
  pneumococci with placenta and fetus, 321–322
  Streptococcus agalactiae in pregnant women, 229
Prevotella spp., oral microbiome, 428
Propionibacterium, 723, 724, 727, 1030
Propionibacterium acnes, 1032
Proteases, 651
  cystein proteases (staphopains), 652–653
  metalloprotease (aureolysin), 651–652
  serine (protease-like proteins), 653–654
  serine proteases (SspA), 652
  staphylococcal cascade, 652
Protective immunity, group A streptococcal infection, 148–149
Protein antigens
  alpha C protein, 213–215, 221
  beta C protein, 215, 221
  BibA and FbsA, 216
  BPS (B protective surface) protein, 215
  C5a peptidase (ScpB), 215–216, 221
  evaluation as protective antigens, 213
  GBS67, GBS80 and GBS104, 216–217
  group B streptocci (GBS), 211–217
  LrrG protein, 216
  ornithine carbamoyltransferase, 216
  phosphoglycerate kinase, 216
  pilus proteins, 216, 217
  Rib protein, 214
  R proteins, 214–215
  SAN-1485, 216
  Sip (surface immunogenic protein), 216
Protein export
  ESX systems of mycobacteria, 1136–1142
  model of, 1130
  SecA2 pathway, 1130, 1132–1135
  Sec pathway, 1130, 1131–1132
  T7S (type VII secretion) pathways, 1130, 1135–1142, 1142–1143
  Tat (twin-arginine translocase) pathway, 1130, 1132
  types in mycobacteria, 1129
  see also Mycobacterial cell envelope
Protein G, group C and G streptococci, 243–244
Protein posttranslational modifications, influence of listeriolysin O (LLO), 857
Proteins, variation in, 334
Protein secretion
  into cross-wall compartment, 594–595
  Staphylococcus aureus, 593
Protein vaccines
  candidates, 368
  combination, 368–369
Proteobacteria, 724
Pseudomonas spp., 320
Pseudomonas aeruginosa, 126, 1088
  alginate, 246
  bacterial killing, 627
  biofilms, 699
  cystic fibrosis, 456
  S. aureus and, 492
  S. aureus biofilms, 776
Pseudomonas syringae, 877
Pseudotransduction, *S. pneumoniae*, 352
Puerperal sepsis, 55
Purine biosynthesis, *Lactococcus lactis*, 469
Pyrimidine biosynthesis, *Lactococcus lactis*, 469
Pyrogenic exotoxins, group C and G streptococci, 251, 253
Pyroptosis, group A streptococcal-mediated signaling, 130, 133

Quorum sensing (QS)
  Bacillus cereus group, 886
  biofilm formation, 452
  inhibition of virulence by targeting, 777–778
  Streptococcus mutans, 441, 442

Quorum-sensing blockers, staphylococcal biofilm-associated infection, 706–707

Rabbits
anthrax model, 883
models for *M. tuberculosis* infection, 1057
Staphylococcus aureus in, 736
Rapidly amplified polymorphic DNA (RAPD) fingerprinting, 240
Reactive oxygen species (ROS)
formation of, 618
microbiocidal activities of, 125–126
neutrophils interaction with *S. pyogenes*, 129
Recombination systems, streptococci, 34
Regulation of virulence (*Bacillus cereus* group), 885–887
AtxA as regulator in *B. anthracis*, 886–887
global transcription regulators, 885–886
RNPP quorum sensors, 886
Regulation of virulence (*Listeria monocytogenes*)
balance between life inside and outside the host, 841
examining new *L. monocytogenes* strains, 844–845
fatty acids and, 842
future outlook, 846
impact of carbon sources on PrfA activation, 840–841
intersection with metabolism, 867–868
peptide pheromones for, 843–844
PfrA as master regulator of *L. monocytogenes* survival, 836–839
posttranscriptional regulation, 837–838
posttranslational regulation, 838–839
prfA mutations and diversity of gene products, 839–840
responding to environmental stress, 842
by small regulatory RNAs (sRNAs), 841–842
targeting novel antimicrobials against, 845–846
transcriptional regulation of *prfA* expression, 837
two-component sensing system for, 842–843
Regulation of virulence (*Staphylococcus aureus*)
agr quorum-sensing system, 669–673
alternative sigma factors, 678–679
factors for *Staphylococcus aureus*, 669, 670
global regulators for *S. aureus*, 679
metabolic genes of *S. aureus*, 692–694
SarA protein family, 675–677
SigB, 678
SigH, 678–679
staphylococcal biofilm formation, 703–704
two-component systems (TCS), 673–675
Regulatory nucleotides, *Streptococcus mutans*, 441–442
Reporter assays, clostridial genetics, 930–931
Resistance, vancomycin in enterococci, 379, 402–404
Resistance mechanisms, phage interference, 543–544
Respiration
basics of *Lactococcus. lactis*, 467
branched scheme in *S. aureus*, 691
chain components in nonrespiration processes, 468
components of electron transport chain, 465–466
heme homeostasis in *L. lactis*, 466, 468
interbacterial cross-feeding, 472
L. lactis, 465–468
L. lactis as prototype, 468
metabolism increasing survival capacity of lactococci, 471
role of regulator CcpA, 466
staphylococcal metabolism and, 549
Staphylococcus aureus, 690–692
TCA (tricarboxylic acid) cycle (Krebs cycle), 549–550
Respiratory tract
alveoli and pneumococcus, 319–320
epithelial cells of nasopharynx, 316–318
middle ear and otitis media (OM), 316, 318
pneumococci through, 316–320
pneumococcus in lower, 318–319
pneumococcus in upper, 318
Restriction-modification systems
clostridial genetics, 928
S. pneumoniae, 354
Reversible lysogeny, vacuolar disruption, 855
Rheumatic fever, 45
M proteins and, 89–92
United States outbreaks, 148
see also Acute rheumatic fever (ARF)
Rheumatic heart disease (RHD)
cross-reactive antibodies, 101
epidemiology, 188
monitoring progression of, 94–95
N-acetyl-B-D-glucosamine of group A polysaccharide, 96–97
potential mechanism of antibody in pathogenesis of, 88
streptoccal pharyngitis and, 148
Rhizobium meliloti, 45
Rhodococcus, 1030
Rhodococcus equi, DtxR repressor in, 1156
Riboflavin, metabolism, 867
Rifampicin, 748
mode of action, 755
resistance, 755
RNA (ribonucleic acid). *See* Noncoding RNA
RNA guided nucleases, *S. aureus* infection, 780
Root canal failure, enterococci, 384
Ruminants
staphylococcal elements, 734
Staphylococcus aureus in, 732–733, 735–736, 740
Ruminiclostridium, relationships within *Clostridiaceae*, 942

Saliva, oral environment, 426
Salmonella spp., 135, 320, 794
invasion of epithelial cells, 39
Salmonella ryphimurium, phase variation, 331
Saprophyte, Listeria monocytogenes as, 865
SarA protein family, 675–677
MgrA, 677, 678
regulatory network, 676
Rot (repressor of toxins), 677
SarA, 675–676
SarH1, 677
SarR, 676
SarT, 676–677
SarU, 676–677
Scanning electron microscopy (SEM), streptococci–HeLa cell interactions, 36, 37
Scardovia wiggsiae, oral microbiome, 428
Scarlet fever, 60, 67, 195
Science Education Alliance (SEA), 1030
Science Education Alliance Phage Hunters Advancing Genomics and Evolutionary Science (SEA-PHAGES), 1030, 1031, 1034
Serum opacity factor, 40, 183
Shigella ssp., 320
Sialic acid, group B streptocci (GBS), 210–211
Signaling events. *See* Group A streptococcal-mediated signaling
Signal recognition particle (SRP) pathway, *S. mutans*, 443
Simpsons diversity index, 188
Single nucleotide variations (SNVs), staphylococci, 485, 487
Siphoviridae, 161, 166, 1030
S. aureus phages, 521, 522–523, 528
Skin infection
epidemiology, 186–187
Streptococcus dysgalactiae subsp. *equisimilis* (SDSE), 277
Small acid-soluble proteins (SASPs), *C. perfringens* virulence, 984
Small colony variants (SCVs)
antibiotics and, 556
changes in redox and ATP-producing reactions in, 554, 555
clinical laboratory identification, of *S. aureus*, 555–556
compensatory metabolic pathways in *S. aureus*, 552
cooperation of *P. aeruginosa-S. aureus*, 555
persistence of respiratory-defective, 553, 555
respiration and virulence factor production in, 552–553
Staphylococcus aureus, 549
Small-molecule inhibitors, biofilms, 777

Small regulatory RNAs (sRNAs), regulation of *Listeria monocytogenes* virulence, 841–842
Sneathia, 248
Sortase, cleaving LPxTG motif, 24
Sortase-dependent surface proteins, pneumococcal vaccine, 367–368
Spore formation
  assembly, 911–913
  coat assembly in C. *difficile*, 911–912
  engulfment, 911
  exosporium assembly in C. *difficile*, 912–913
  exosporium function in C. *difficile*, 913
  lifecycle, 904
  overview, 903–904
Spore-forming pathogens, 873–874
  Bacillus cereus group, 875–889
  see also Bacillus cereus group
Sporulation
  architecture of spores, 887–890
  Bacillus cereus group, 880
  diversity in regulation of transcriptional programs, 909
  link with toxin gene expression in C. *difficile*, 907–908
  model of *Clostridium perfringens*, 983
  transcriptional program, 908–911
  transcriptional programs controlling, 908–911
SP-STP virulence factor, 137
Staphylococcaceae, 565
Staphylococcal biofilms, 699–707
  antibiotic resistance mechanism, 704–706
  antimicrobial peptides, 706
  attachment phase, 700
  bacteriophages and lysins, 706
  biofilm-degrading enzymes, 706
  during infection, 699–700
  formation as immune evasion, 704–706
  infection on medical devices, 705
  in vivo roles of factors, 704
  material alterations, 706
  molecular basis of formation, 700–703
  in natural habitat, 699–700
  predominant matrix components, 702
  proliferation and matrix formation, 700–703
  quorum-sensing blockers, 706–707
  regulation of formation, 703–704
  schematic of development, 701
  structuring and detachment, 703
  therapeutic strategies for infections, 706–707
Staphylococcal cassette chromosome (SCC) elements, 488–489, 510–511, 512
Staphylococcal coagulases, 649–650
Staphylococcal enterotoxins, 646
Staphylococcal necrotizing pneumonia (SNP), 712–714
Staphylococcal plasmids, 499–505
  conjugative, 503–505
  diffusible pigment (DiP), 505
  maps of conjugative theta-replicating, 504
  maps of nonconjugative theta-replicating, 502
  maps of rolling circle replicating (RCR), 501
  small rolling circle replicating (RCR), 500, 501
  theta replicating, 500–501, 502, 503
Staphylococcal scalded skin syndrome (SSSS), 648, 649
Staphylococcal sepsis, cytokine production and, 768–770
Staphylococcal superantigens, 645–647
  crystal structures of *S. aureus*, 647
Staphylococci, 485–486, 492
  antimicrobial resistance, 490–491
  bacteriophages, 488
  biological warfare remarks, 632
  cassette chromosome, 512
  composite transposons, 505, 506
  composite transposon Tn*4001*, 502, 504, 505, 507
  genomes, 486–487
  genomic islands (GIs) in, 536, 537
  horizontal gene transfer, 485, 489–490
  host, 491–492
  immune system and, 483–484
  insertion sequence IS*257*, 507–508
  insertion sequences, 489, 505, 506
  insertion sequences and composite transposons, 505, 506
  integrative and conjugative elements, 511–512
  microbiome and competing bacteria, 492
  mobile genetic elements (MGEs), 488–489
  nitrate use, 549
  pathogenicity islands, 488
  perspective, 512
  phage movement between species, 528–529
  plasmids, 489, 499–505
  S. aureus lineages, 487–488
  SCC*mec* elements, 488–489
  selection and adaptation, 490–492
  staphylococcal cassette chromosome *mec* (SCC*mec*) elements, 488–489, 508, 510–511
  transposable elements, 505–511
  transposons, 489
  unit transposons, 505, 507
  variation of core genome, 486, 487
  virulence and pathogenicity, 490
Staphylococcud sciuri, 512
Staphylococcus, 248, 483–484, 723
Staphylococcus aureus, 126
  agr quorum-sensing system, 669–673
  antibiotics and small colony variants (SCVs), 556
  antibiotics used against, 748
  bacterial adhesins of, 108
  bacteriophages, 488
  biofilms, 699–704
  carbohydrate carbon/energy sources, 687–688
  cell wall, 574
  cell wall anatomy, 575
  cell wall constituents, 6
  cell wall envelope of, 592–595
  collagens and, 113
  colonization in human nose, 723–727
  colonizer in humans, 485, 618, 669
  competing bacteria, 492
  competition by induction of host defense, 727
  CRISPR-Cas adaptive immunity systems in, 566–568
  cryo-studies, 15
  cytotoxic enzymes, 647–649
  cytotoxins, 640–645
  daptomycin resistance, 379
  dehydrogenases of, 693
  DtxR repressor in, 1156
  elastin and, 114
  electron transport and small colony variant phenotype in, 556
  electron transport in, 550, 552
  embedded in low-viscosity resin, 12
  embedded in LR White resin, 12
  enterotoxin modifications of immune response, 771
  evading neutrophil extravasation to infection site, 620
  evasion proteins and therapeutic strategies, 630–631
  exoenzymes, 649–656
  exotoxins, 640–649
  fatty acid metabolism, 867
  fibronectin and, 114–116
  Fst toxin sequence, 409
  genetic exchange, 499
  genetic organization of Tn*916*, Tn*5801*, and ICE*6013*, 509
  genome, 483
  genomes illustrating core variation, 486
  genomic organization for type III-A CRISPR system of, 568
  host adaptations, 492
  as human commensal and pathogen, 723–724
  immunity to, 766–772
  infection in companion and livestock animals, 485
  infective endocarditis (IE), 455
  interactions with nasal bacteria, 727

invasion mechanisms of methicillin-resistant, 118
lineages, 487–488
LPxTGase from, 24
metabolic gene regulation, 692–694
metabolic pathways and mutations in SCV's, 551
metabolism and respiration, 549
micronutrients, 688–694
network between sRNAs and transcriptional factors in, 566
noncarbohydrate carbon/energy sources, 688
pathogenicity islands, 488, 536–538
peptidoglycan global structural parameters, 576
peptidoglycan layer, 4
plasmids, 499–504
pneumococcal conjugate vaccine, 363
protein A, 19
protein export, 473
recurrent infections, 766–768
regulation of virulence, 669
regulatory RNAs, 562
respiration versus fermentation, 690–692
role of sRNAs in physiopathology, 568–569
schematic of toxins and enzymes secreted, 656
sequenced surface proteins, 21
small colony variants (SCVs), 549, 550, 552–553
staphylococcal superantigens, 645–647
surface glycolytic enzymes, 27
surface proteins recognizing ECM molecules, 109
T7S pathway, 1143
T7S system, 1136
thrombospondin and, 117
virulence and pathogenicity, 485, 490
virulence gene clusters, 545
vitronectin and, 116
see also Cell walls; Immune evasion; Noncoding RNA; Nonconventional therapeutics against *S. aureus*; Pathogenicity islands (PIs); RNA (ribonucleic acid); Staphylococcal biofilms; Surface proteins of *Staphylococcus aureus*

Staphylococcus aureus in animals, 731–732, 740
chickens, 736–737
host switching and role of MGEs, 739–740
humans as hub for host jumps, 733
monkeys in sub-Saharan Africa, 739
MRSA in companion animals, 738–739
multihost CC398, 737–738
poultry, 736–737
rabbits, 736
ruminants, 732–733, 735–736

Staphylococcus aureus phages
bacterial recognition, 524–526
classification of, 521, 522–523, 524
dual control of phage-encoded virulence genes, 528
integration and excision, 524
mediating horizontal gene transfer, 529–530
movement within and between staphylococcal species, 528–529
Myoviridae, 521, 528
occurrence and role of accessory genes, 526–527
Podoviridae, 521, 528
receptor specificity of, 525
role in human adaptation of animal isolates, 527–528
Siphoviridae class, 521, 522–523, 528

Staphylococcus capitis, 724

Staphylococcus epidermidis, 126, 731
biofilms, 699–704
energy sources, 688
in human nasal cavity, 726
interactions with nasal bacteria, 727
theta-replicating plasmids, 503
thrombospondin and, 117
vitronectin and, 116
see also Staphylococcal biofilms

Staphylococcus fleurettii, 512

Staphylococcus hominis, 724

Staphylococcus lugdunensis, 485, 492, 724
in human nasal cavity, 726
interactions with nasal bacteria, 727

Staphylococcus pseudintermedius, 485, 731, 844

Staphylococcus saprophyticus, 485, 724, 731

Staphylococcus staphylolyticus, 706

Staphylococcus vitulinus, 512

Staphylococcus warneri, 724

Staphylokinase, 650–651

Starterator, 1030

Strep throat, 148

Streptococcaceae
interbacterial cross-inhibition, 472
respiration, 468

Streptococcal inhibitor of complement (Sic), 127

Streptococcal-mediated signaling events
role of integrins in, 128–129
see also Group A streptococcal-mediated signaling

Streptococcal pharyngitis, 148
active immunization with conserved region peptides, 151–152
ecology of, 177–178
Gram-positive commensals as vaccine vectors, 153
new generation streptococcal vaccine, 153–154
non-M-protein approaches to protection, 154
occurrence of, 150–151
passive protection, 151
pilus-based vaccine, 152
vaccinia virus as vector, 152–153
see also Vaccine(s)

Streptococcal pyogenic toxins (spes), group C and G streptococci, 269

Streptococcal pyrogenic exotoxin B (SpeB), streptococcal infection and, 154

Streptococcal superantigens, 56–59
function of, 56
infection models for studying, 58–59
interaction with MHC class II, 57
models of T cell activation complexes for, 57, 58
phylogenetic relationships of, 56, 57
streptococcal pyrogenic exotoxin B (SpeB), 59
structural conservation of, 57
as virulence factors for streptococcal disease, 58

Streptococcal surface enolase (SEN), 127

Streptococcal toxic shock syndrome, 45, 55, 67
ecology of, 178
see also Toxic shock syndrome (TSS)

Streptococci, 33–34
phylogeny of species in oral cavity, 427
taxonomy of oral, 426
see also Oral streptococci

Streptococcus genus
group C streptococci (GCS), 239
group G streptococci (GGS), 239
interactions with nasal bacteria, 727
rolling circle replicating (RCR) plasmids, 500

Streptococcus agalactiae, 204
clinical manifestations, 228–229
conjugative plasmids, 503
meningitis, 228
neonatal disease and, 230–231
nonpregnant adults and, 229–230
older adults, 230
phage, 165
pregnant women and, 229
prevention of neonatal disease, 231–232
serotypes, 228
T7S system, 1143
treatment, 232
see also Group B streptococci (GBS)

Streptococcus anginosus, 170
collagens and, 113
human oral environment, 426, 427
laminin and, 114

Streptococcus anginosus group (SAG)
epidemiology, 275
history and taxonomy, 275
human infections, 276
see also Streptococcus dysgalactiae subsp. *equisimilis* (SDSE)
Streptococcus bovis, human oral environment, 426, 427
Streptococcus canis, 170
disease and diagnosis, 264
pathogenicity factors, 265
Streptococcus constellatus, oral microbiome, 428
Streptococcus cristatus, colonization mechanisms, 429–430
Streptococcus downei, human oral environment, 426, 427
Streptococcus dysgalactiae
fibronectin and, 115
pathogenicity factors, 265
sequenced surface proteins, 21
Streptococcus dysgalactiae subsp. *Equisimilis* (SDSE), 179, 239
bacteremia, 278
endocarditis, 279
epidemiology, 275–276
history and taxonomy, 275
human infections, 276–279
joint and bone infections, 277
maternal and neonatal infections, 278
organization of streptokinase-M protein gee region, 247
pharyngitis and acute glomerulonephritis, 276–277
skin and soft tissue infections, 277
stringent and relaxed responses of, 253–254
treatment, 279
Streptococcus equi, 163
pathogenicity factors, 265
sequenced surface proteins, 21
Streptococcus equisimilis
disease and diagnosis, 264, 266
pathogenicity factors, 265
Streptococcus gordonii
biofilm formation and, 452
cell envelope, 1088
colonization mechanisms, 429–430
fibronectin and, 112, 113, 115
laminin and, 114
new-generation vaccine, 153–154
Streptococcus mutans and, 442–443
TEM of negatively stained bacteria, 12
Streptococcus infantis, oral microbiome, 428
Streptococcus intermedius, 170
oral microbiome, 428
T7S system, 1136, 1143
Streptococcus mitis
human oral environment, 426, 427
oral microbiome, 428
phage, 165
Streptococcus mutans
biofilm formation, 437–439
carbohydrate metabolism, 436–437
carbon catabolite repression, 436
catch and release, 436–437
collagens and, 113
colonization mechanisms, 430
DtxR repressor in, 1156
future perspectives for, 444
genetic and phenotypic heterogeneity, 435–436
human oral environment, 426, 427
laminin and, 114
as model organism, 443
N-terminal-anchored lipoproteins, 28
oral microbiome, 428
quorum sensing (QS) system, 441, 442
regulatory nucleotides, 441–442
sequenced surface proteins, 21
signaling pathways, 441–442
stress tolerance, 439–440
as therapeutic target in caries prevention, 443–444
two-component signal transduction systems (TCSTSs), 441
Streptococcus parasanguinis
alginate, 246
colonization mechanisms, 430
oral microbiome, 428
Streptococcus parauberis, 170
Streptococcus pneumoniae
adhesion and invasion of, 118
bacterial adhesins of, 108
bacterial cell wall backbone, 4
at blood-brain barrier, 321
in bloodstream, 320–321
capsular polysaccharides (CPSs) of, 304
capsule of, 7, 8
cell growth regulation during cell cycle, 293–298
comparing *cps* loci, 308–309
complete genomes of, 346–347
CPS-based vaccines, 305–306
CPS biosynthesis genes, 306–308
cryo-studies, 15
cytotoxicity and, 322–324
dissolution of fibrin clot by, 118
fibronectin and, 115
functional anatomy of cell wall, 284–285
functions of cell wall choline residues, 293
GBS genome and, 212
in heart, 321
horizontal gene transfer, 348–353
image of conventional embedded, 12
inflammation and, 233, 323
membrane vesicles (MVs) of, 8
murMN operon, 288–290
penicillin resistance, 288
peptidoglycan biosynthesis and remodeling, 286–288
peptidoglycan composition, 288
phage, 165
phase variation of, 332–333
in placenta and fetus, 321–322
polysaccharide structures, 206
preservation under in vivo conditions, 8
PspA surface protein, 27
regulation of CPS production, 309–311
in respiratory tract, 316–320
role of CPS in pathogenesis, 304–305
schematic of spread and progression of infection, 319
sequenced surface proteins, 21
sialic acid and, 210–211
size variation of M protein, 26
structure of peptidoglycan, 285–286
structure of WTA and LTA, 290–292
surface glycolytic enzymes, 27
surface proteins recognizing ECM molecules, 109
synthesis of teichoic acids and modifications, 292–293
see also Cell wall of *S. pneumoniae*; Pneumococcal genomes; Pneumococcal phase variation
Streptococcus pseudopneumoniae, 170
Streptococcus pyogenes, 26, 33, 35
analysis of gene expression, 79–81
autophagy and infection, 133–135
bacterial adhesins of, 108
capsule and, attachment to epithelial cells, 50–51
capsule and, entry into epithelial cells, 51
capsule and host immune defenses, 48–49
capsule as virulence factor, 49–51
collagens and, 113–114
cytolytic toxins of, 55–56
diseases by pathogen, 148
diseases in humans, 67
DNases of, 59–60
experimental infection models, 49–50
fate of intracellular, 40–41
fibronectin (Fn)-binding proteins as invasins, 37–38, 39
field emission scanning electron microscopy (FESEM), 11
focal adhesion and signal transduction, 38–40
GBS genome and, 212

growth factor TGF-β1 activation, 41–42
human oral environment, 426, 427
hyaluronic acid synthesis in, 45–47
IgG-targeting enzymes of, 60
infection and IL-1β/inflammasome signaling pathways, 131–132
initial interaction with neutrophils and neutrophil extracellular traps (NETS), 129, 131
intracellular invasion of, 35–36
invasins and host receptors for, 36
laminin and, 114
LPxTGase from, 24
map of region of chromosome for hyaluronic acid synthesis, 47
mechanisms for ingestion, 36–37, 41–42
membrane vesicles on surface of, 9
M protein, 19, 20, 37–38
noncanonical inflammasome, 132–133
protein export, 474
regulation of hyaluronic acid biosynthesis, 47–48
sequence comparison with *Escherichia coli*, 249
sequenced surface proteins, 21
streptolysin O from, 853
superantigens of, 56–59
tonsils and, 36
zipper-like mechanism, 36
see also Bacteriophages of *Streptococcus pyogenes*; Genetics of Group A streptococci
Streptococcus salivarius
colonization mechanisms, 429–430
human oral environment, 426, 427
Streptococcus sanguinis, 428
antibiotic resistance reservoir, 453–454
bacteriocin production and, 453
in biofilm formation, 449, 451–452
competitive behavior, 453
components for commensalism, 450
as etiological agent of extraoral diseases, 455–456
horizontal gene transfer, 453–454
human oral environment, 426, 427
hydrogen peroxide (H_2O_2) production, 449, 453
as modulator of host, 454–455
molecular determinants of, as commensal pioneer colonizer, 449–451
oral biofilm, 449
oral microbiome, 428
outlook for, 456
Streptococcus sanguis, 28
Streptococcus sobrinus, dental caries, 430
Streptococcus suis, 21, 170
Streptococcus thermophilus, 163, 170, 468
Streptococcus zooepidemicus
disease and diagnosis, 264, 266
pathogenicity factors, 265
sequenced surface proteins, 21
Streptodornase, group C and G streptococci, 251
Streptogramins, 748
history and mode of action, 753
resistance, 753
Streptokinase
activating host protein plasminogen, 60
adaptive evolution of SK alleles, 250–251
expression control of SK gene, 248–250
group C and G streptococci, 248–251, 268–269
structure-function relationships of SK, 250
Streptolysin O (SLO), 127, 132
exotoxin of group A streptococci, 55–56
group C and G streptococci, 251, 269
survival of *S. pyogenes*, 134, 135
Streptolysin S (SLS), 132
exotoxin of group A streptococci, 55, 56
group C and G streptococci, 269
S. pyogenes and, 135
Streptomyces spp., 1030, 1142
Streptomyces pilosus, DtxR repressor in, 1156
Stress responses, lactococcal, 469–471
Sugar metabolism, of oral streptococci, 428–429
Sulfur, *Staphylococcus aureus*, 689–690
Summers, David, 536
Superantigens
S. aureus infections, 768
staphylococcal and streptococcal exotoxins as, 126–128
streptococcal, 56–59
see also Streptococcal superantigens
Surface dehydrogenase (SDH), 126, 128
mediating binding activity, 127–128
streptococcal, 126
Surface proteins of Gram-positive bacteria
cell wall, 19
complete M6 protein sequence, 22
conformational characteristics of surface molecules from, 26
C-terminal-anchored proteins, 19–27
C-terminal anchor region, 21–23
electron and fluorescence microscopy, 20–21
enzymes cleaving LPxTG motif, 24
fluorescein-labeled anti-M6 antibody analysis, 23
major surface structures of cell wall, 20
model of anchoring of M protein and SfbI, 24
M protein structure, 19–20
multifunctional proteins, 26–27
N-terminal-anchored lipoproteins, 27–28
proteins anchored by charge and/or hydrophobic interactions, 27
schematic of organization of nine well-described M proteins, 23
sequenced surface proteins from, 21
seven-residue periodicity, 25–26
surface-exposed region, 25–26
surface glycolytic enzymes, 27
wall-associated region, 24–25
Surface proteins of *Staphylococcus aureus*, 599–612
anchoring to cell wall, 593–594
biofilm formation, 607
cell wall-anchored (CWA) proteins, 599–608
collagen binding, 601, 606
contributing to virulence, 608–609
CWA classified based on structural motifs, 600
CWA proteins promoting colonization of host, 610–611
fibronectin binding by FnBPs, 609
flexible stalks, 604
G5-E repeat proteins, 600, 606–607
Heme-iron transport and, 595
homophilic interactions, 607
immune evasion by CWA proteins, 611
legume lectin domain of SraP, 607–608
ligand binding, 601
MSCRAMM family (microbial surface components recognizing adhesive matrix molecules), 599, 600, 601, 602, 604–606
NEAT motif family, 600, 602, 607
nucleotidase motif, 608
posttranslational modifications, 604–606
properties of CSA surface proteins, 602–603
release from staphylococcal envelope, 595
schematic diagram of MSCRAMMS before/after ligand binding, 605
tandem β-zipper binding to fibronectin, 608
three-helical bundle, 600, 603, 607
Surgical site infections, enterococci, 384
SWAN (streptococcal wall-anchored nuclease), 456
Sydenham chorea, 97, 99, 100
Syntrophomonas, relationships within *Clostridiaceae*, 942

T-antigens, epidemiology of group A streptococci (GAS) infection, 183–186
Targe Tron system, clostridial genetics, 932
T cell exhaustion in humans, *S. aureus* infections, 768
T cells
$CD4^+$, 816
$CD8^+$, 815–816
cytotoxicity of $CD8^+$, 816
effector functions of, 816–817

effector functions of memory, 818
formation of memory, 817
memory responses during secondary infection, 817–818
recall responses, 817–818
regulatory, 817
secretion of Th17-type cytokines, 816–817
secretion of Th1-type cytokines, 816
T cell superantigens, 645–647
Tedizolid
history and mode of action, 752
resistance, 752–753
Teichoic acids (TAs), 3
structure of, 291–292
synthesis of, 292–293
Tetanus toxin, 873
Tetracyclines, 748
history and mode of action, 754
resistance, 754
Themoanaerobacter, relationships within *Clostridiaceae*, 942
Therapeutics. *See* Nonconventional therapeutics against *S. aureus*
Thiamine, metabolism, 867
Throat infection, epidemiology, 186–187
Thrombospondin
Gram-positive pathogens and, 117
structure and function of, 111, 113
Tigecycline
enterococci resistance, 379–380
history and mode of action, 754
resistance, 754
Tissue damage, enterococci, 385, 387
Todd, J. K., 714
Toll-like receptors (TLRs), innate immune, 621
Tonsils, Streptococcus pyogenes and, 36
Toxemia, *Clostridioides difficile* infections, 995
Toxic shock, 35–36, 45
Toxic shock syndrome (TSS), 239, 712
case definition of, 715
fulminant infection, 714–716
menstrual TSS (mTSS), 714–715
nonmenstrual TSS (nmTSS), 715
Toxic shock syndrome toxin-1 (TSST-1), 537, 646–647, 716
Toxin(s)
Bacillus cereus group, 880–882
Clostridioides difficile, 996–999
escape by, 629
see also Exotoxins
α-Toxin, 640
model for pore formation, 642
structure for, 643
ε-Toxin, 645
Toxin-antitoxin (TA) system, enterococcal genetics, 407, 408–410, 412–413
Transcription promoters, S. pneumoniae, 354
Transcriptomic analyses, gene expression, 401–402
Transduction
group A streptococci, 67–68
in Streptococcus pyogenes, 159–160
Transformation
artificial, 351
chromosomal integration, 351
competence development, 348–350
DNA release, binding and update, 350
model, 350
plasmid, 351
Streptococcus pneumoniae, 348–351
Streptococcus pyogenes, 68
Transmission, group A streptococci (GAS), 179–180
Transmission electron microscopy (TEM), 3, 1087, 1088
Transpeptidases, cell wall branching, 287
Transposon mutagenesis
analysis of, 79
*Himar*1-based mariner transposon systems, 79
pOSKAR/pKRMIT, 78, 79
Streptococcus pyogenes, 76–79
Tn916, 77, 78
Tn917, 77, 78
TnFuZ, 77, 78, 79
Treponema denticola, 266, 339
Treponema medium, 339
Tricarboxylic acid (TCA) cycle, 549–550, 864
host nitric oxide redirecting metabolism, 550
incomplete with *Listeria* isolates, 864
NADH levels and control of, 550
Trimethoprim-sulfamethoxazole, 748
history and mode of action, 755–756
resistance, 756
treatment of listeriosis, 798
Trypanosoma cruzi, 770
Tuberculosis (TB), 1087, 1129
future perspectives, 1116–1117
immunology of TB diagnostics, 1068
immunometabolism and pathometabolism at host-pathogen interface, 1112–1113
M. tuberculosis metabolism and TB life cycle, 1111–1112
new TB drug discovery, 1115–1116
polymicrobial infection of humans, 1112
unit of infection, 1117
vaccines, 1068–1069
see also Adaptive immunity against *Mycobacterium tuberculosis*; Innate immunity to *Mycobacterium tuberculosis* infection; *Mycobacterium tuberculosis* infections
Two-component sensing systems, regulation of *L. monocytogenes* virulence, 842–843
Two-component signaling systems (TCS), biofilm formation, 452
Two-component signal transduction system (TCSTS)
gene expression, 399–400
Streptococcus mutans, 441
vancomycin resistance, 402–403
Two-component systems
ArlRS TCS, 675, 678
SaeRS TCS, 673–674
S. aureus virulence, 673–675
schematic of SaeRS, 674
SrrAB TCS, 674–675
Twort, Frederick, 158
Type 4 secretion systems (T4SSs), 407
Type VII secretion (T7S) pathway, 1135–1136, 1142–1143
composition and mechanism of ESX systems, 1136–1140
cytosolic chaperone EspG, 1137
ESX conserved cytosolic component (EccA), 1138
ESX membrane components, complex formation and structure, 1138–1140
ESX secretion signal, 1137
ESX substrates, 1136–1137
ESX system in DNA transfer, 1141
ESX systems, 1136–1142
ESX systems in conjugation and cell physiology, 1140–1142
mycobacterial cell, 1135–1142
other species, 1142–1143
outer membrane translocation of substrates, 1140
see also Protein export

University of Minnesota, 185
Urease system, death health, 431
Urinary tract infection
enterococci, 383
Staphylococcus saprophyticus, 485
Streptococcus agalactiae, 229
U.S. Food and Drug Administration, 794–795, 889, 1003, 1161
U.S. Postal Service, anthrax, 877–878

Vaccination, *Staphylococcus aureus* infection , 630–631
Vaccine(s)
active immunization with conserved region peptides, 151–152
anthrax, 889
candidates, 150

capsular polysaccharides (CPS), 217–218, 305–306
clinical trials of GBS vaccines, 220
CPS-protein conjugate, 218–220
GBS CPS-GBS protein conjugate, 221
GBS protein, 221
Gram-positive commensals as vaccine vectors, 153
group B streptococci (GBS), 217–221
mucosal, for non-type-specific protection, 150–151
mucosal immunity, 369
multivalent type-specific, 150
new-generation streptococcal, 153–154
non-M-protein approach to streptococcal infection protection, 154
passive protection, 151
pilus-based, 152
staphylococcal biofilm-associated infection, 706
strategies for M protein-based, 190
tuberculosis (TB), 1056, 1068–1069, 1129
vaccinia virus as vector, 152–153
see also Pneumococcal vaccine(s)
Vaccine vector
fusing antigens for vaccine efficacy, 819
immunogenicity of, 819
strains for preventing infectious disease, 819
trials of *L. monocytogenes* for cancer immunotherapy, 819–820
use of attenuated *Listeria* as, 818–820
Vaccinia virus, as vector for streptococcal vaccine, 152–153
Vacuolar stage
Listeria monocytogenes, 853–855
LLO and disruption, 853–854
phospholipases PlcA/PlcBV and disruption, 854–855
Vancomycin
history and mode of action, 750
intermediate resistance, 750–751
invasive GBS disease, 232
organization of VanA and VanB resistance genes, 403
resistance in enterococci, 379, 402–404
Vancomycin-intermediately resistant *S. aureus* (VISA), 747, 750–751
Vancomycin resistance-associated (VraSR) two-component system, cell wall synthesis, 581
Vancomycin-resistant *S. aureus* (VRSA), 750
Vancomycin-susceptible *S. aureus* (VSSA), 750
Vegetative cell envelope, Bacillus anthracis, 879
Veillonella spp.
community development, 452
Streptococcus mutans and, 442–443
Vertebrate models, enterococci, 388
Vertebrates, associated with enterococcal colonization, 379
Vibrio cholerae, bacterial attachment sites for prophages, 161
Vibrio fischeri, luciferase reporter, 80
Virginia Commonwealth University, 455
Virulence, *Staphylococcus aureus*, 687–694
Virulence factors, enterococci, 381
Virulence gene expression, *Bacillus cereus* group, 885–887
Virulence genes
pathogenicity islands, 544–545
prophages as vectors for, 166, 169
S. pyogenes genome, 163
Vitamin C deficiency, *S. aureus* infections, 766
Vitamin metabolism, intracellular *L. monocytogenes*, 867
Vitronectin
Gram-positive pathogens and, 116
group C and G streptococci, 267
structure and function of, 111

WalKR two-component system, cell wall synthesis, 582
Wall teichoic acid (WTA)
cell wall complex of pneumococci, 285
constituent of bacterial cell wall, 6–7
nasal epithelial attachment, 725, 726
S. aureus, 524, 524–526
structure of, 290–292
surface attachment of biofilms, 700
Warfare, remarks on biological, 632
Weiser, Jeffery, 333
Whole-cell vaccine (WCV), pneumococcal, 369
Whole-genome sequence (WGS)
Clostridioides difficile, 946–947
epidemiology of group A streptococci (GAS) infection, 186
pathogenic clostridia, 940
Wilson, Armine, 45
Woolsorter's disease, 877
World Health Organization, 316, 362, 756, 1068, 1129

Xenophagy (autophagy), *Streptococcus pyogenes*-mediated, 133–135

Yersinia pseudotuberculosis, integrins binding to, 36

Zebrafish, models for *M. tuberculosis* infection, 1057, 1068
Zinc, *Staphylococcus aureus*, 689
Z-ring
discovery of, 1096
identifying mid-cell for placement, 1098
recruitment of anchors, stabilizers and regulators, 1098–1100