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Host – Pathogen Interaction

Microbial Metabolism, Pathogenicity and Antiinfectives

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Contents

Preface

Recent developments in microscopy, genomics, molecular biology, and metabolomic analysis allow a detailed analysis of the intracellular lifestyle of endosymbiotic bacteria. The studies showed changes in the cellular organization of the host cells and the bacteria, as well as new structures and cellular functions of the colonizing bacteria. Pathogenic bacteria not only require specific mechanisms for entering the host cell. Rather development of the intracellular and pathogenic lifestyle requires redirecting and adapting of central metabolic routes for successful survival under the changed metabolic conditions and for overcoming defense reactions of the host. Many central metabolic routes have to be redirected and adapted such as to allow their function under conditions of slow growth, limitation in the supply of oxygen, carbon sources, and metal ions, changes of pH and other adverse conditions. Interestingly, various metabolic traits that were known for a long time become obvious in their significance when considered in the context of bacteria/host metabolic interaction. Therefore, studies on the metabolism of bacteria growing in their host gained significant interest. Central metabolism and its adaptation mechanisms turned out to represent important virulence factors for the survival of the bacteria within their host. Understanding the specific metabolic pathways of the bacteria under conditions of host colonization opened new and unexpected views on bacterial physiology. Part A of the book presents some recent examples of this vast area of bacterial physiology. Part B shed lights on fungi–host interactions in human- and plant-pathogenic systems as well as on signaling processes of fungi involved in environmental changes.

The rapidly increasing number and severity of human and plant diseases caused by pathogenic fungi has recently led to many investigations concerning the pathogenic development and physiology of these organisms as well as interactions with their hosts. Most of our knowledge on pathogenic fungi originates from pathogens in terms of pathogenic development, infection, and spread within the host, the treatment of fungal infections, or the reduction of pathogenic effects. In recent years, the elucidation of host–fungus interaction was largely intensified. Fungi need to control their interaction with their hosts in various ways in penetration processes, survival inside hosts, and acquisition of nutrients. In addition, they have to cope with antifungal metabolites, the plant defense or the host immune system. The host may be confronted with toxic fungal metabolites demanding a response to the infection

itself. In addition, this mutual interaction is affected by several parameters such as environmental changes or abiotic stress. In order to adapt to quickly changing environmental conditions, fungal pathogens have to respond to external signals. Understanding the signaling network and the chemical communication within this interaction could lead to new insights and define new targets to control pathogens. New methodologies contribute to understand essential processes during the life cycle of the pathogens and the initiation of host–pathogen interactions. The "omics" approach consisting of genome data, transcriptome analysis, proteomics, and metabolomics leads to many new possibilities to track pathological processes and elucidate their regulation and signaling.

The editors thank the contributing authors for their excellent work and the series editor Paul M. Selzer for his constructive advice and support.

Mainz and Kaiserslautern
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Cover Legend

GFP-picture in the background:

Fluorescent microscopic image of a GFP-expressing mutant of the grapevine trunk disease associated fungus *Phaeomoniella chlamydospora* growing in *Vitis vinifera* root tissue.

Picture: courtesy of the IBWF, Kaiserslautern, Germany.

Metabolic scheme part:

Host-adapted metabolism of *Legionella pneumophila* can be determined by ^{13}C-labeling experiments. On the basis of the unique isotopologue patterns, pathways, and fluxes in the formation of metabolic products and their intermediates are reflected. Thereby, information on the core metabolism of the intracellular pathogen and its adaptation to host organisms is gleaned.

Picture: courtesy of Dr Eisenreich, see chapter 2 for details.

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Part One
Adaptation of Microbial Metabolism in Host/Pathogen Interaction

Host – Pathogen Interaction: Microbial Metabolism, Pathogenicity and Antiinfectives, First Edition.
Edited by Gottfried Unden, Eckhard Thines, and Anja Schüffler.

1
Metabolic Adaptation of Human Pathogenic *Yersiniae*

*Ann Kathrin Heroven and Petra Dersch**

Abstract

Colonization, subsequent penetration of epithelial layers as well as persistence and proliferation in subepithelial tissues of the host by bacterial pathogens demand the expression of special sets of virulence factors. In addition, the bacteria need to adapt their metabolism to survive and replicate within the specific host niches. Activated metabolic functions and physiological adaptation processes during their life cycle and the different stages of the infection reflect the complex and dynamic nutritional resources of their environments, interbacterial competition for energy sources and onslaught of bactericidal host responses. The enteric pathogenic *Yersinia* species *Y. pseudotuberculosis* and *Y. enterocolitica* and the causative agent of plague, *Y. pestis*, have adapted to grow in many different environmental reservoirs (e.g., soil, plants, insects) and in warm-blooded animals (e.g., rodents, pigs, humans) with a preference for lymphatic tissues. In the present book chapter, we discuss metabolic adaptations of human pathogenic *yersiniae* to successfully exploit available nutrients and metabolic functions during infection and illustrate the tight link between carbon metabolism and *Yersinia* virulence. Furthermore, current knowledge about the complex regulatory networks used to coordinate and fine-tune the control of metabolic and virulence functions are presented. Deciphering the mechanisms of the function and control of bacterial metabolism within host tissues will not only increase our understanding of host–pathogen interactions, it will also facilitate the identification of potential novel drug targets for future prevention and therapeutic strategies.

Introduction

Infections of human pathogenic *yersiniae* involves a large number of specific pathogenicity factors that mediate efficient resistance against the host defense systems and enable the bacteria to colonize, invade, and multiply successfully within host tissues. The structure, function, and expression of many of these classical

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virulence factors have been characterized, and their role in pathogenicity has been studied using different animal models. However, to become a successful pathogen, *yersiniae* must also adapt their metabolic functions to the nutrient/ion composition and the physical conditions (e.g., temperature, pH, oxygen tension) of their surrounding and coordinate their metabolism with their life cycle. These unspecific strategies were long neglected, but recent use of global omic-based profiling techniques, phenotypic microarrays, and the *in vivo* analysis of metabolic mutants allowed a deeper insight into nutrient sensing, sequestration, and utilization strategies that optimize the metabolism and biological fitness of *Yersinia* during infection.

Yersinia Life Cycles and Pathogenesis

Of the 17 species of the genus *Yersinia* only *Y. pseudotuberculosis*, *Y. enterocolitica,* and *Y. pestis* are known to cause diseases in mammals [1, 2]. The two enteric pathogens *Y. pseudotuberculosis* and *Y. enterocolitica* are the causative agents of yersiniosis, a gastrointestinal disease with a variety of symptoms such as enteritis, colitis, diarrhea, and mesenteric lymphadenitis, which becomes rarely systemic. Both enteropathogenic species are well adapted to survive long term in external habitats (e.g., ground water, soil, plants, and insects) and are able to persist and replicate in various wild and domestic animals [3, 4]. A recent study analyzing a large number of genomes revealed that they are heterotrophic pathogens that are able to utilize a large variety of C-/N-/energy sources [5]. In contrast, *Y. pestis*, the causal agent of plague, which has evolved as a separate clone from *Y. pseudotuberculosis*, shows a reduced metabolic flexibility based on functional gene loss. This may reflect its unique life cycle: (i) replication within the gastrointestinal tract (proventriculus) of infected fleas and (ii) proliferation in the lymphatic system, blood, or tissues of mammals, in particular rodents [6].

All *yersiniae* are zoonotic pathogens armored with diverse cell envelope–associated virulence structures that either promote host–pathogen interactions or contribute to *Yersinia* pathogenicity by suppression of the host immune response. In case of the enteric *Yersinia* species, initial attachment and invasion of the intestinal layer is mediated by the primary invasion factor invasin (InvA), but other adhesive surface-exposed proteins, for example, homologous Inv-type adhesins (InvB/Ifp, InvC), Ail, the autotransporter adhesin YadA and the PsaA (pH6 antigen)/Myf fimbriae appear to support the dissemination process at later stages of the infection [7, 8]. In *Y. pestis* mainly adhesins Ail and PsaA contribute to host–pathogen interactions, whereas other adhesin/invasin genes, for example, *invA* and *yadA* became unfunctional [9, 10]. Moreover, all pathogenic *yersiniae* evolved mechanisms that mediate resistance against the innate immune response. Several adhesins protect the bacteria against complement killing (e.g., Ail and YadA) or prevent phagocytosis (e.g., PsaA) [7]. Furthermore, they possess a 70-kDa virulence plasmid (pYV/pCD1) that encodes the Ysc (Yersinia secretion)-Yop type III secretion system (T3SS). This needle-like delivery machine (injectisome) enables the bacteria to inject different Yops (*Yersinia* outer proteins) effector toxins from the bacterial cytoplasm

into the cytosol of host cells, in particular professional phagocytes [11]. *Yersinia* pathogenicity relies on the following crucial functions of translocated Yop effector proteins: (i) antiphagocytic activity by manipulation and destruction of the actin cytoskeleton; (ii) suppression of cytokine production by macrophages, dendritic cells, and neutrophils; and (iii) induction of host cell death [11].

Carbon Metabolism and Links to *Yersinia* Pathogenesis

External reservoirs, vector and animal environments colonized by *Yersinia* have likely driven the evolution of metabolic pathways to maximize present nutritional opportunities. Variations in certain metabolic functions might thus be a consequence of the adaptation to a specific host or host niche. A selective advantage can be gained either by acquisition of new metabolic functions, for example, by horizontal gene transfer, or by loss of function mutations that change the metabolic abilities of the pathogen. Furthermore, changes in the control mechanisms implicated in metabolic adaptation and regulatory strategies linking metabolic and virulence traits could manipulate the pathogen's response to varying nutrient availabilities in the environment.

Food Sources, Nutrient Sequestration, and Utilization

Animal tissues contain a large variety of different energy sources (e.g., sugars, amino acids, lipids, proteins) and can be regarded as a rich source of food for bacteria. In particular the digestive tract of mammals is nutrient rich and contains a large diversity of different nutritional substrates, which can be metabolized by enteric *yersiniae*. However, the pathogens have to compete successfully with the perfectly adapted resident microbiota. About 10^{14} bacteria form a complex microbial ecosystem of more than 400 species, in which strictly anaerobic bacteria degrade complex polysaccharides into simple carbohydrates, which are readily absorbed by the mammalian small intestine or used by other (facultative anaerobic) commensals such as *Escherichia coli* [12]. Furthermore, the host can rapidly change the availability of nutrients in host tissues based on the induction of inflammation and hypoxic conditions triggered by the immune response [13], and it can restrict access to essential ions such as magnesium, manganese, zinc, and iron [14, 15]. As a consequence, *Yersinia* needs to sense, retrieve, and metabolize nutrients more efficiently, or alternatively it must grow on available substrates, which are not used by other members of the competing microbiota. An important characteristic of many bacterial pathogens, including *Yersinia*, is their ability to sense and initiate use of readily digestible carbon sources by sophisticated global regulatory systems: (i) **carbon catabolite repression** (CCR) triggered in response of the availability of simple sugars, for example, glucose [16, 17] and (ii) the **carbon storage regulator/regulator of secondary metabolites** system (Csr/Rsm) [18, 19] (see also below: Coordinated control of carbon metabolism and virulence).

Metabolic Pathways of *Yersinia* Crucial for Virulence

All pathogenic *Yersinia* species possess a highly flexible and robust metabolic system with many redundant or alternative catabolic and biosynthetic pathways, which allow them to respond very rapidly and efficiently to changing nutrient concentrations. Simple sugars can be utilized via glycolysis (Embden–Meyerhof pathway), the pentose phosphate pathway and the Entner–Doudoroff pathway. They can further be catabolized by aerobic or anaerobic respiration via a complete tricarboxylic acid (TCA) cycle and a functional glyoxylate bypass, or via fermentation [20–22]. Many enzymes and metabolic pathways are conserved among the different *Yersinia* species, but several characteristic differences were also observed. Due to the loss of multiple metabolic genes, for example, the glucose 6-phosphate dehydrogenase gene *zwf Y. pestis* is unable to use glucose via the pentose phosphate pathway [20]. It further lacks the methionine salvage and the urease pathway, aspartase to mediate catabolism of glutamate to aspartate and is unable to synthesize several amino acids, including glycine, threonine, L-valine and L-isoleucine, L-phenylalanine, and L-methionine [23, 24], which makes the pathogen more dependent on mechanisms accessing host nutrients. An important specific feature of *Y. enterocolitica* is its ability to metabolize 1,2-propanediol and ethanolamine by cobalamin-dependent enzymes under anaerobiosis using tetrathionate as terminal electron acceptor [5]. Tetrathionate production is strongly induced upon inflammation [25], indicating that these metabolic properties are advantageous for *Y. enterocolitica* to outcompete the microbiota of the intestine. In contrast, *Y. pseudotuberculosis* and *Y. pestis* are able to metabolize itaconate by converting it into pyruvate and acetyl-CoA. Itaconate contributes to the antimicrobial activity of macrophages as it inhibits isocitrate lyase, a key enzyme of the glyoxylate cycle. Thus, itaconate degradation could allow *Yersinia* to persist in macrophages [26].

Nutritional Virulence: Nutritional Adaptation Important for Pathogenesis

Various "omic" approaches and transcriptional profiling studies with pathogenic *yersinia*e grown *in vitro* under different virulence-relevant conditions revealed numerous metabolic pathways and adaptive metabolic responses, which could contribute to pathogenesis. Important initial studies addressed temporal changes during a temperature shift from 26 to 37 °C, mimicking transmission of *Y. pestis* from the flea to mammals. They revealed that not only virulence genes but also numerous metabolic functions are under thermal control [27, 28]. Genes encoding for enzymes involved in nitrogen assimilation were strongly downregulated, whereas those required for efficient catabolism of amino acids were induced in *Y. pestis* grown *in vitro* at 37 °C. Some of these enzymes are responsible for the majority of released metabolic ammonia via reactions that directly or indirectly promote deamination during formation of α-keto acids entering the TCA cycle.

A thermal upshift caused a downregulation of glycolysis, whereby terminal oxidation of the available energy sources (carbohydrates, amino acids, and lipids) in the nutrient-rich medium was favored. This first *in vitro* study indicated that, in nature, *Y. pestis* prefers fermentative pathways in the flea vector, while oxidative catabolism is favored during rapid proliferation in the lymphatic systems of the mammalian host [27]. Moreover, differential expression of catabolic enzymes suggests that different sugars (e.g., maltose, gluconate, ribose) are utilized after temperature transition, and this metabolic switch appears to be crucial to trigger virulence. Two equivalent transcriptomic studies were directed to identify metabolic functions of *Y. pestis* required during septicaemic plague in humans and of *Y. pseudotuberculosis* during systemic infections. *In vitro* growth in media containing human plasma showed that in particular genes related to purine/pyrimidine metabolism were upregulated in plague bacilli and supported a previous report demonstrating that purine metabolism is crucial for *Y. pestis* pathogenicity [29, 30]. In *Y. pseudotuberculosis,* genes supporting the consumption of the plasma glucose (e.g., the glucose-specific phosphotransferase system (PTS)) were strongly upregulated [31]. This indicated that high growth rate aerobic cultivations on glucose induce an "overflow metabolism" channeling the carbon flow toward byproduct formation and secretion to balance accumulation of reducing equivalents (NADH) through the TCA cycle. In fact, our recent fluxome approach revealed that *Y. pseudotuberculosis* does not accumulate and excrete acetate like *E. coli* when grown on glucose; it spills large amount of pyruvate (46% of the glucose uptake). Preliminary results indicate that excretion of pyruvate by *Y. pseudotuberculosis* is achieved by a sustained glycolytic flux that is accompanied by a bottleneck in the TCA and a downregulation of acetate formation (Bücker *et al.*, [32]).

Over the past years also *in vivo* gene expression profiling was performed to gain a better insight into host–pathogen interactions and the metabolic activities that support persistence and replication of *Y. pestis* in the flea [33] and the mammalian host [34–36]. Numerous metabolic genes involved in the catabolism of amino acids, in particular the L-glutamate group (e.g., glutamine, histidine, arginine, proline) were found to be upregulated in *Y. pestis* located in the proventriculus of infected fleas [33] (Figure 1.1). This was interpreted as a special adaptation to the flea gut, which contains protein and lipid rich meals with relatively low amount of carbohydrates. Utilization of the L-glutamate group amino acids involves enzymes of the TCA cycle, which are upregulated in the flea vector [33]. In contrast, catabolism of carbohydrates seems less important as most sugar uptake systems are repressed or only slightly expressed. Only chitobiose, a PTS sugar present in the flea's proventriculus spines, is efficiently imported and metabolized (Figure 1.1).

Transcriptional profiling of *Y. pestis* located in the bubo in a rat model as well as in the lung of a murine pneumonic infection model was used to characterize the metabolic adaptation of *Y. pestis* to its mammalian host [34–36]. Notable is the strong induction of genes involved in iron acquisition (e.g., hemin uptake operon) and amino acid biosynthesis (e.g., histidine, glutamate, and aspartate), and downregulation of the TCA cycle and the ATP-proton motive force during pneumonic plague development [35, 36] (Figure 1.1). In parallel, genes encoding the

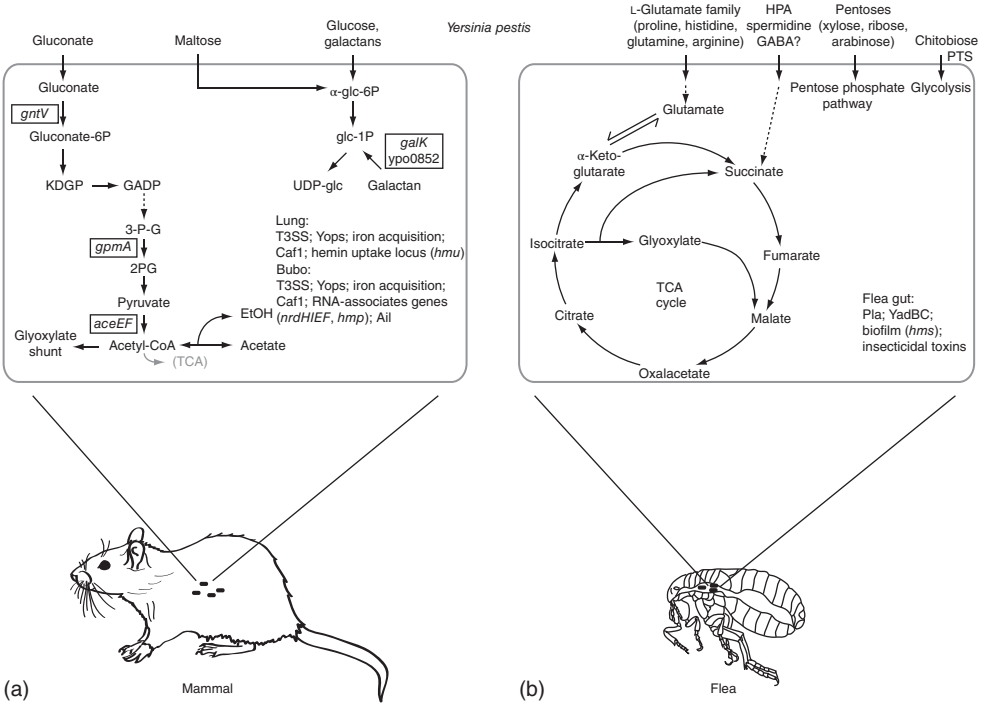

Figure 1.1 Metabolic pathways and virulence factors of *Y. pestis,* which are significantly induced in the mammalian host and the flea gut. Specific metabolic pathways and pathogenicity traits upregulated *in vivo* are presented, which are considered to be crucial for the colonization of the lung or bubo of the mammalian host (a) and the flea gut (b). Abbreviations: BarA/UvrY (nutrient-responsive two-component system); Csr (carbon storage regulator); Crp (cAMP receptor protein); GADP (glyceraldehyde-3P); Hfq: RNA chaperone; KDGP (2-dehydro-3-deoxy-gluconate-6P); M-cell (microfold cell); 3-P-G (3-phosphoglycerate); 2PG (2-phosphoglycerate); PhoP/PhoQ (ion-responsive two-component system), PsaA (pH6 antigen); Yops (*Yersinia* outer proteins); T3SS (type III secretion system); and TCA (tricarboxylic acid cycle).

Y. pestis specific antiphagocytic F1 protein capsule (Caf1), as well as the T3SS/Yop apparatus important for resistance against the innate immune response are highly expressed. A similar strong induction of the Caf1 capsule and the T3SS/Yop machinery was also observed in the rat bubo [34]. Furthermore, *Y. pestis* induces a protective response to reactive nitrogen species (RNS), which are released by polymorphonuclear neutrophils (PMNs) in the buboes [34]. This is reflected by an upregulation of the ribonucleotide reductase genes (*nrdHIEF* operon) and *hmp*, which encodes a flavohemoglobin that detoxifies RNS. To further investigate the importance of genes upregulated during bubonic plague, a mutant library was constructed and tested in a rodent model of bubonic plague [34, 37]. Virulence testing revealed that *Y. pestis* depends mainly on the catabolism of carbohydrates (i.e., glucose, galactans, and gluconate) [37] (Figure 1.1). Since the terminal part

(*gpmA*, *aceEF*), but not the upper part (*pgi*, *pfkA*) of the glycolysis pathway was essential for competition with the wildtype *in vivo*, it was suspected that gluconate is metabolized to glyceraldehyde-3-phosphate, pyruvate, acetyl-CoA, and acetate, whereby the galactans and glucose are most likely channeled toward UDP-glucose synthesis [37] (Figure 1.1). Additional results, demonstrating unimportance of certain TCA cycle genes (e.g., *gltA*, *acnA,* and *fumC*) and constitutive expression of the glyoxylate shunt suggest that *Y. pestis* shifts to anaerobic respiration or fermentation during colonization of rodents [34].

Coordinated Control of Carbon Metabolism and Virulence

Rapid changes in environments encountered by *yersiniae* in their external habitats, during the vector-associated lifestyle and within the intestine/lymphatic tissues in mammals request a fast bacterial response to adjust metabolic and virulence traits. To overcome this challenge, it is no wonder that *Yersinia* and other bacteria use the availability of ions and nutrients as well as certain metabolic cues to coordinately control their metabolism and virulence function. For example, virulence factors can be activated via the stringent response through (p)ppGpp under nutrient-limiting conditions, such as amino acid and fatty acid starvation [38]. Furthermore, the synthesis and activity of certain transcriptional regulators and RNA elements (e.g., Fur, Zur, riboswitches) can be controlled by metal ions or small metabolites to modulate expression of metabolic or virulence functions. Many virulence genes are also under CCR control and are regulated by the global transcription factors cyclic adenosine monophophate (cAMP) receptor protein (Crp) and CsrA. They coordinate the uptake and utilization of alternative carbon sources and enable the bacteria to adjust their pathogenic properties in accordance to the availability of readily utilizable sugars [16, 18].

Importance of Ions

All pathogenic *Yersinia* species are characterized by a strong induction of numerous iron uptake and sequestration systems during the infection of mammals, indicating the importance for *Yersinia* to acquire iron [31, 34–36, 39]. The ferric uptake regulator Fur represses most of the iron uptake systems in the presence of iron and controls genes of various noniron metabolic and physiological functions including biofilm formation in *Y. pestis* [40–42]. Although Fur was also shown to control expression of the T3SS in related pathogens [43, 44], Fur-mediated regulation of T3S in *Yersinia* has not been described. However, most recently, a new regulator, IscR, was found to control expression of LcrF, the major regulator of the T3SS-associated genes in *Y. pseudotuberculosis*. It has been suggested that IscR senses iron, O_2, and/or reactive oxygen species concentrations in order to optimize T3S synthesis [45].

Sensing of magnesium ions is another important feature of *Yersinia* to adapt virulence and metabolic gene expression. The pleiotropic two-component system (TCS) PhoP/PhoQ is composed of the membrane-bound sensor kinase PhoQ that

responds to low magnesium and phosphorylates the cytoplasmic response regulator PhoP. It further recognizes low pH environments and host-secreted cationic antimicrobial peptides (CAMPs) [46]. Transcription of the *Y. pestis phoP* gene is significantly upregulated in the lung in an intranasally challenged plague model in mice [36] and in infected fleas [33, 47] and is essential for the formation of a normal foregut-blocking flea infection [33, 47]. Although the PhoP/PhoQ system was shown to be essential for the survival and proliferation of all pathogenic *Yersinia* species in macrophages and neutrophils *in vitro* [48–50], the role of the PhoP/PhoQ system for *Yersinia* pathogenesis is less clear. *phoP* mutants of *Y. pestis* GB and the *Y. pseudotuberculosis* derivative 32 777 were strongly attenuated in virulence, whereas loss of a functional *phoP* gene did not affect the pathogenicity of *Y. pestis* CO92 and the *Y. pseudotuberculosis* strain YPIII [48, 50–52]. This strongly suggests that the different outcomes are the result of strain-specific differences that remodel regulation and/or composition of the PhoP/PhoQ regulon. This is supported by recent findings from our laboratory, demonstrating the presence of strain-specific variations in the PhoP-mediated control of the Csr system affecting expression of numerous metabolic, stress adaptation, and virulence functions in *Y. pseudotuberculosis* [53].

Importance of the Csr System

The important global posttranscriptional Csr system is composed of the RNA-binding protein CsrA and Csr-type sRNAs (CsrB and CsrC in *Y. pseudotuberculosis*). CsrA recognizes conserved (N)GGA motifs in the loop portions of RNA hairpin structures that are mostly found in close vicinity to the ribosomal binding site in the target mRNA. Binding of CsrA affects translation and/or stability of the mRNA. The Csr-RNAs contain several CsrA-binding sites and can eliminate CsrA function by sequestration of CsrA from its target mRNAs [18, 19]. The Csr system controls many genes involved in metabolism and virulence in *Yersinia* similar to many other pathogens [18, 19]. A recent transcriptomic approach revealed that about 20% of the CsrA-dependent genes of *Y. pseudotuberculosis* are involved in metabolic processes [18] (Figure 1.2). The *Y. pseudotuberculosis* Csr system is further implicated in the first steps of the infection process through regulation of the global virulence gene regulator RovA, which activates the synthesis of the primary entry factor invasin and the PsaA fimbriae (Figure 1.2) [54, 55]. Preliminary data further indicate that the Csr system is also crucial for the expression of the *Yersinia* Ysc-Yop/T3SS machinery (R. Steinmann, unpublished results).

Based on the crucial role of the Csr system, it is not surprising that the expression of the Csr components is tightly regulated in response to environmental parameters. Both Csr-RNAs are controlled by different regulatory mechanisms in response to ions and availability of C-sources. The TCS PhoP/PhoQ activates *csrC* transcription in a Mg^{2+}-dependent manner [53]. Furthermore, CsrC synthesis is repressed in the absence of iron (A.K. Heroven, unpublished results). Expression of the CsrB RNA is induced by the TCS BarA/UvrY [54]. The UvrY/BarA system is activated by metabolic end products such as formate and acetate in *E. coli* or by an imbalance of the TCA cycle in *Pseudomonas* [56, 57]. The signal(s) to which the *Yersinia*

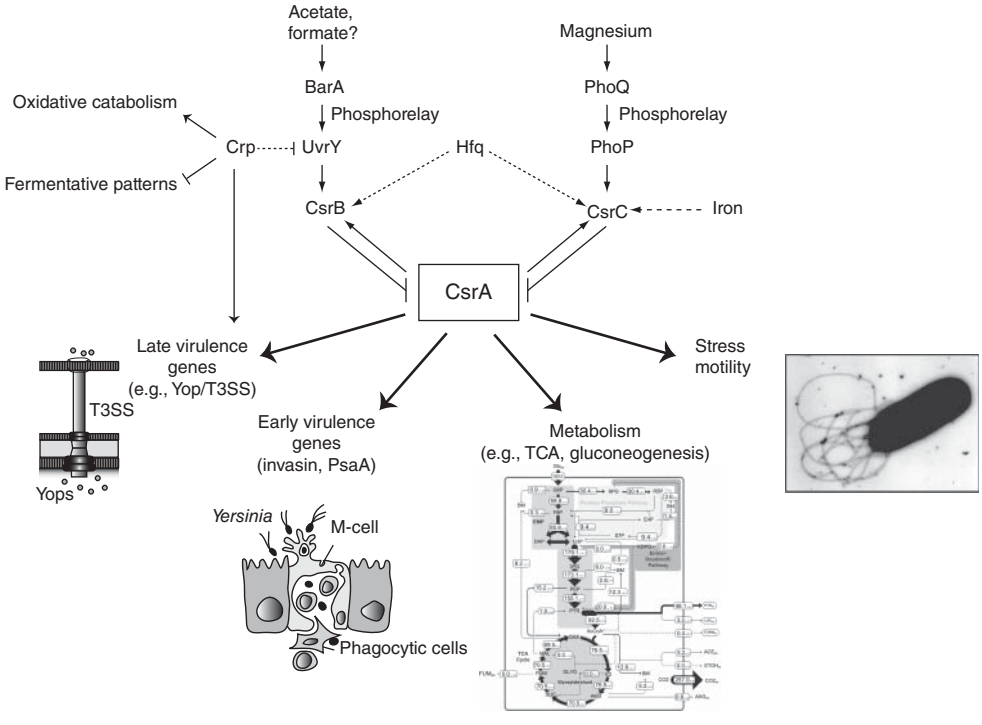

Figure 1.2 Schematic overview of the environmental sensing and signal transduction system and the regulatory cascade with implicated control factors that are known to coordinate expression of metabolic functions and virulence-associated traits of *Y. pseudotuberculosis*. All sensory and regulatory components are also encoded in the other human pathogenic *Yersinia* species, but the function of some of them has still not been experimentally verified.

BarA/UvrY system responds are still unknown, but it has been shown that the *uvrY* gene of *Y. pestis* is highly expressed in the lung, but not in the liver and spleen of infected mice. This indicates that metabolites and/or ions that are present in a certain host niche are able to induce this TCS during infection [36]. Thirdly, Crp regulates expression of *csrC* and *csrB* in an opposite manner, and promotes a tight link between carbon metabolism and regulation of virulence in *Yersinia* [58].

Importance of CCR and the cAMP-Crp Complex

The global transcriptional regulator Crp controls metabolism and pathogenicity in all three human pathogenic *Yersinia* species. The adenylate cyclase catalyzes the synthesis of cAMP in the absence of glucose or other efficiently utilized sugars. Binding of the signal metabolite cAMP activates Crp [59]. At least 6% of the genes in *Y. pestis* and *Y. pseudotuberculosis* are controlled by the cAMP–Crp complex. This includes genes required for growth on different C-sources, survival under carbon, nitrogen, and phosphate limitation as well as virulence [58, 60]. In a recent study, we

could demonstrate that Crp of *Y. pseudotuberculosis* promotes oxidative catabolism of many different C-sources, whereas it represses fermentative patterns [58]. In *Y. pestis*, Crp regulates the T3SS/Yop machinery and the plasminogen activator protease Pla. Consistently, loss of *crp* strongly affects the development of bubonic and pneumonic plague [60–63]. A *Y. enterocolitica crp* mutant strain is severely attenuated in an oral infection model. It has been suggested that the influence of Crp on the expression of the flagellar, Ysc/Yop, and Ysa T3SS might contribute to the loss of virulence [64]. Similarly, mice infected with a *Y. pseudotuberculosis crp* mutant developed no disease symptoms. Crp is required for colonization and/or persistence in the mesenteric lymph nodes (MLNs) and organs later during infection [58].

Importance of Posttranscriptional Regulation Strategies

While numerous transcription factors have been characterized with regard to metabolic and virulence control, only recently attention has been drawn to post-transcriptional control mechanisms involving sensory and regulatory RNAs. A recent study comparing the global transcriptome and proteome response of *Y. pseudotuberculosis* and *Y. pestis* grown under physiologically relevant temperatures revealed that regulation of the metabolism and the translational machinery seems to underlie a conserved posttranscriptional control. This includes proteins of the purine and pyrimidine metabolism, glycolysis/gluconeogenesis, pyruvate metabolism, the TCA cycle, and amino-acyl tRNA biosynthesis [65]. Importance of posttranscriptional control mechanisms became also evident through the analysis of the role of Hfq, an RNA chaperone that controls RNA–RNA and RNA–protein interactions as well as the stability and translation of RNAs [66]. Hfq contributes to virulence of all pathogenic *Yersinia* species, for example, it is implicated in the posttranscriptional regulation of T3SS/Yop machinery in *Y. pestis* and *Y. pseudotuberculosis* [67, 68], and modulates the early stage virulence cascade, including RovA (Figure 1.2), by the control of CsrB and CsrC levels (A.K. Heroven, unpublished results). Hfq influence also seems to occur through Crp as it is required for efficient synthesis of Crp. The underlying posttranscriptional mechanism is still not understood, but it involves the 5′ untranslated region (UTR) of the *crp* mRNA [63].

In addition to the Csr-type sRNAs, other conserved sRNAs could influence *Yersinia* metabolism. Among them are SgrS and Spot42, that are implicated in the regulation of sugar metabolism [69, 70], and GcvB shown to control the amino acid metabolism [71]. Furthermore, *Yersinia* possesses two RyhB homologs. RyhB is a key player for adaptation to iron-limiting conditions in *E. coli* and other *Enterobacteriaceae*, in which it prevents the synthesis of nonessential iron-containing proteins and induces the production of iron-scavenging siderophores [72]. Although the RyhB RNAs are highly expressed in *Y. pestis* within infected lungs (but not in the spleen), their loss had no obvious effect on the dissemination capacity and survival of the bacteria after subcutaneous and intranasal infection. This could be explained by the fact that *Yersinia* possesses several redundant iron uptake systems [73, 74].

The importance of posttranscriptional regulation strategies in the adaptation process of *Yersinia* virulence and metabolism was further supported by observations made in a recent study investigating the regulation of T3SS in *Y. enterocolitica*. Schmid *et al*. could demonstrate that components of the secretion machinery are able to directly interfere with metabolic enzymes [75]. YscM1 (LcrG in *Y. pestis* and *Y. pseudotuberculosis*) and YscM2 are functionally equivalent regulators of the T3SS [76]. Both YscM1 and YscM2 bind to phosphoenolpyruvate carboxylase (PEPC). PEPC is involved in the padding of the oxaloacetate pool in the TCA cycle under virulence conditions. *In vitro*, binding of YscM1 was found to inhibit the function of PEPC [75]. YscM1 and YscM2 participate in the central metabolism of *Y. enterocolitica* as mutants in *yscM1* and *yscM2* displayed increased rates of (i) pyruvate formation via glycolysis or the Entner–Doudoroff pathway, (ii) oxaloacetate formation via the TCA, and (iii) amino acid biosynthesis. It has been suggested that the altered PEPC activity is required for the metabolic adaptation process of *Yersinia* during the infection. In the first phase, *Yersinia* produces massive amounts of Yops to prepare against the phagocytic attack. To do so, PEPC is active in order to refill the TCA for the amino acid synthesis ("loading phase"). After cell contact, the preproduced Yops are rapidly secreted to inhibit the phagocytic cells. In order to maintain the energy charge, anaplerosis is prevented via inhibition of PEPC ("shooting phase"). The cycle starts again when new Yops are needed [75]. *Vice versa*, the availability of amino acids can also influence T3S. Secretion of Yop proteins can be induced by the amino acids glutamate, glutamine, aspartate, and asparagine, feeding into the TCA cycle [77].

Conclusions

Due to the rapid development of antibiotic resistance and emergency of more and more multiresistant bacterial pathogens, new anti-infective strategies are urgently needed. Strategies to adjust the *in vivo* metabolism to nutrient availability in the infected tissues belong to the most fundamental features of bacterial pathogenicity. So far, antivirulence strategies have been developed that inhibit the synthesis or function of crucial virulence factors, such as T3SSs, but important metabolic functions or control systems could also be exploited for antimicrobial therapy since they are a prerequisite for virulence.

Although many aspects of the metabolism of *Yersinia* and related pathogens are already known, this approach is still in its infancy stage. One reason is that our knowledge about the metabolism of these pathogens during the different stages of the infection is still scarce and often inconsistent data have been published that hamper our general understanding. This is based on the fact that identified metabolic genes in *in vivo* high-throughput screens were often not further investigated and the advantage of gene loss was rarely studied. In addition, cultivation conditions, used strain isolates, and animal model systems, as well as the type of infection modes, varied significantly between the studies, which complicate the

identification of important metabolic pathways and regulatory systems. In conclusion, novel approaches and strategies need to be developed in the future, which allow us to follow the metabolism of the pathogen and the host over the course of an infection. Promising techniques are transcriptome profiling approaches, which use deep-sequencing technologies (RNA-Seq), use of radiolabeled C-sources for *in vivo* metabolomics, and the establishment of more "human-like" infection models (e.g., *ex vivo* systems).

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Crosstalk between Metabolism and Virulence of *Legionella pneumophila*

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Abstract
The knowledge of the dynamic interactions between intracellular pathogens and their hosts may identify concepts for future drug discovery against infectious diseases. These interactions involve multiple changes and adaptations in the interlaced metabolic networks of both hosts and pathogens. There is increasing evidence that these metabolic features also determine virulence and differentiation of intracellular bacteria. In this chapter, these emerging items in molecular infection biology are reviewed for the human pathogen *Legionella pneumophila*.

Introduction

Legionella pneumophila is the causative agent of Legionaires' disease, a nontypical pneumonia which can be life-threatening, especially for old and immune-suppressed patients [1]. Fortunately, *Legionella* is still sensitive against established antibiotics [1–7] and the virulent bacteria are not transmitted by human-to-human contacts, but mainly via inhalation of contaminated aerosols. Higher amounts of *Legionella* are predominantly found in human-made aquatic habitats (e.g., hot water systems) at temperatures between 25 and 55 °C [8–10]. After transmission to the human lung, the bacteria are then able to penetrate alveolar macrophages where they actively replicate, finally leading to inflammation and pneumonia. These processes depend on multiple metabolic adaptations of the microbes to the varying environments in their host cells, and intracellular *L. pneumophila* is a useful model for the study of host–pathogen interactions.

The natural habitats of *Legionella* are aquatic habitats in which the bacteria are localized to biofilms or where they replicate within different aquatic protozoa, mainly amoebae such as *Acanthamoeba castellanii* or *Dictyostelium discoideum* [8, 11–15]. In an attempt to perfectly adapt to these niches, *L. pneumophila* has developed a biphasic life style. In the replicative phase, the bacteria are nonmotile, noncytotoxic, and metabolically active [8, 11, 16–18]. After entry into the host cell,

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the effectors of the type 4B secretion system (T4BSS-Dot/Icm) are necessary to establish a vacuole (*Legionella*-containing vacuole, LCV), in which *L. pneumophila* is able to multiply [19–21]. The T4BSS-Dot/Icm system may translocate up to 300 different effector proteins into the host cell cytosol [22, 23]. Accordingly, several genes encoding these factors are highly expressed in *L. pneumophila* replicating in macrophages [24], indicating essential functions of these proteins for efficient replication within the LCV [23, 25]. Notably, the development of the vacuole into a comfortable growth niche for intracellular *L. pneumophila* requires a remodeling of the LCV surface and close interactions with ER-derived vesicles and organelles (mitochondria and ribosomes) [26]. Consequently, an intimate metabolic crosstalk between these vesicles/organelles and the bacteria must be established to provide efficient nutrient supply into the LCV. When these nutrients become limiting, the bacteria switch into the transmissive phase, in which they now become motile and express a variety of virulence factors. Finally, the bacteria differentiate into a spore-like, stress-resistant, mature intracellular form (MIF). This metabolically dormant form is well prepared to survive within the environment or to infect other host cells including human alveolar macrophages [16–18, 27–31].

The nature and origin of the nutrients during the biphasic life-style of *L. pneumophila* are not well known, as holds true for most other intracellular bacteria such as *Salmonella, Mycobacterium, Brucella, Coxiella, Chlamydia, Listeria,* or *Francisella.* For *L. pneumophila,* it is also only partly understood how these nutrients are transported into the LCV, how they are utilized, and, finally, which pathways are modified during the different stages of bacterial development as a response to the changing environmental conditions.

Key Metabolic Features of *L. pneumophila*

Some information can be retrieved from the genome sequence of *L. pneumophila* [32, 33], which displays all genes for the enzymes of the glycolytic pathway, the Entner–Doudoroff (ED) pathway, the pentose phosphate pathway, the TCA cycle, and many genes involved in amino acid biosynthesis and fatty acid metabolism. Surprisingly, homologous genes encoding typical PTS permeases for the import of glucose or other carbohydrates are missing, but glucose and hexose phosphate transporter proteins are apparently present. Moreover, many transporters for amino acids and peptides are present, as well as genes for peptidases, proteases, and enzymes catalyzing the degradation of amino acids.

Early radiotracer experiments showed that amino acids are indeed utilized by *L. pneumophila* as the main carbon and energy source when growing in different media (*in vitro*). On the basis of these observations, serine, threonine, tyrosine, and glutamate were suggested as preferred substrates [34–36]. In line with these data, high activity of glutamate–aspartate transaminase was measured in lysates of *in vitro* grown *L. pneumophila,* which suggested that this reaction is involved in the linkage of the TCA cycle to anabolic and gluconeogenetic pathways [37–39]. Moreover, the activity of serine dehydratase converting serine into pyruvate was

also shown to be high, underlining the role of serine as a potential key substrate and pyruvate as a central intermediate in the metabolic network of *L. pneumophila* [39].

These early studies also revealed that *L. pneumophila* is auxotrophic for arginine, methionine, serine, isoleucine, leucine, valine, phenylalanine, tyrosine, and cysteine [34–36, 40–44]. Notably, cysteine is absolutely required to support the growth of *L. pneumophila* and even rich media must be supplemented with cysteine [45, 46]. Remarkably, amino acid auxotrophy of *L. pneumophila* and its eukaryotic host organisms is quite similar. For example, *L. pneumophila* and *Acanthamoeba* are both auxotrophic for arginine, methionine, isoleucine, leucine, and valine. Synchronization of amino acid auxotrophy has been proposed as an evolutionary trigger for the development of the intracellular life style of pathogens [47]. Strikingly, *in vitro* growth experiments have not yet identified conditions in which the auxotrophy of *L. pneumophila* for these amino acids can be bypassed, although genome analysis revealed the presence of complete or partial routes for the synthesis of some of these amino acids [32, 33].

Radiotracer experiments also suggested that glucose can be shuffled into the central metabolism of the pathogen, although glucose supplements did not support growth of *L. pneumophila* under *in vitro* conditions [34, 36, 48], and it was postulated that glucose did not play a metabolic role during the life cycle of *L. pneumophila* [38, 39, 49]. Mainly stimulated by the progress in metabolic pathway analysis using ^{13}C-labeled precursors, the metabolism of *L. pneumophila* was assessed in more detail during the last decade.

Serine and Glucose Metabolism of *L. pneumophila* under *In Vitro* Conditions

Labeling experiments with *L. pneumophila* growing in defined media containing [U-$^{13}C_3$]serine revealed that the ^{13}C-label of serine was efficiently transferred into some bacterial amino acids (serine > alanine > glutamate > glycine = aspartate) as well as into the carbon and energy storage compound, poly-3-hydroxybutyrate (PHB) [44]. These experiments demonstrated the efficient uptake of serine by *L. pneumophila* and its degradation to pyruvate from where the label was further transferred into alanine by transamination or into acetyl-CoA (Ac-CoA) catalyzed by pyruvate dehydrogenase (Figure 2.1). On the basis of the ^{13}C-enrichments in glutamate and aspartate, ^{13}C-Ac-CoA was shuffled into the complete citrate cycle (yielding labeled α-ketoglutarate/glutamate and oxaloacetate/aspartate, respectively). The detected ^{13}C-pattern in PHB signaled its biosynthesis from labeled Ac-CoA via 3-hydroxybutyryl-CoA (Figure 2.1).

Similar experiments starting from [U-$^{13}C_6$]glucose or [1,2-$^{13}C_2$]glucose revealed that glucose was also used for *de novo* synthesis of amino acids and PHB [44]. The labeling profiles in amino acids (alanine > aspartate > glutamate > serine > glycine) and PHB reflected the incorporation and usage of the glucose supplement via labeled pyruvate and Ac-CoA again entering the TCA cycle or PHB biosynthesis (Figure 2.2). In addition, the positional isotope patterns from [1,2-$^{13}C_2$]glucose indicated that

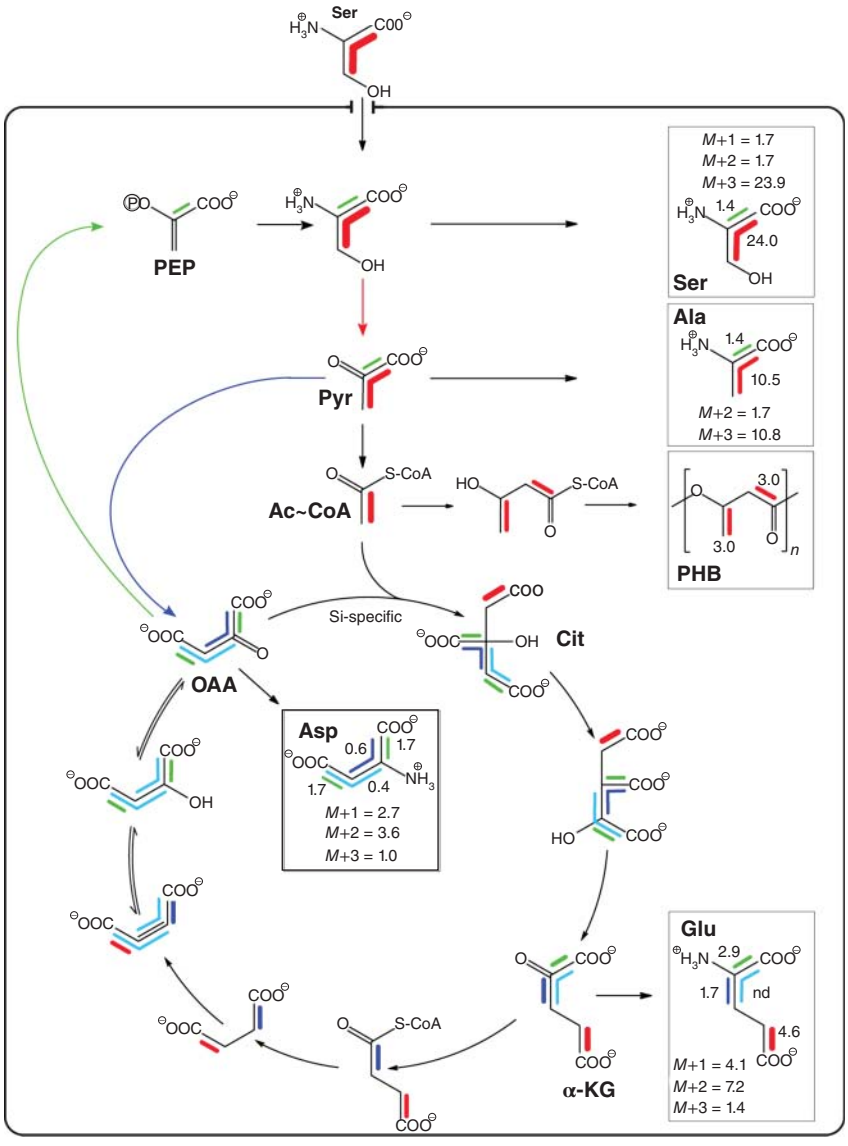

Figure 2.1 Incorporation of [U-$^{13}C_3$]serine into *L. pneumophila* under *in vitro* conditions [44]. The ^{13}C-profiles are indicated by bold lines connecting ^{13}C-labeled atoms in a given molecule. The labeling patterns of the compounds in boxes were determined. The numbers indicate the molar abundances (mol%) of the respective isotopologues as determined by quantitative NMR or MS (M+1, M+2, M+3 indicates isotopomers with 1, 2, or 3 ^{13}C-labels, respectively). The natural molar abundances of M+1, M+2, and M+3 isotopomers are approximately 1.1%, 0.01%, and 0.0001%, respectively. The detected enrichments for the multiply ^{13}C-labeled isotopologues are increased by several orders of magnitude in the labeling experiment and therefore underline the specificity and significance of these data. PEP, phosphoenolpyruvate; OAA, oxaloacetate; and α-KG, α-ketoglutarate.

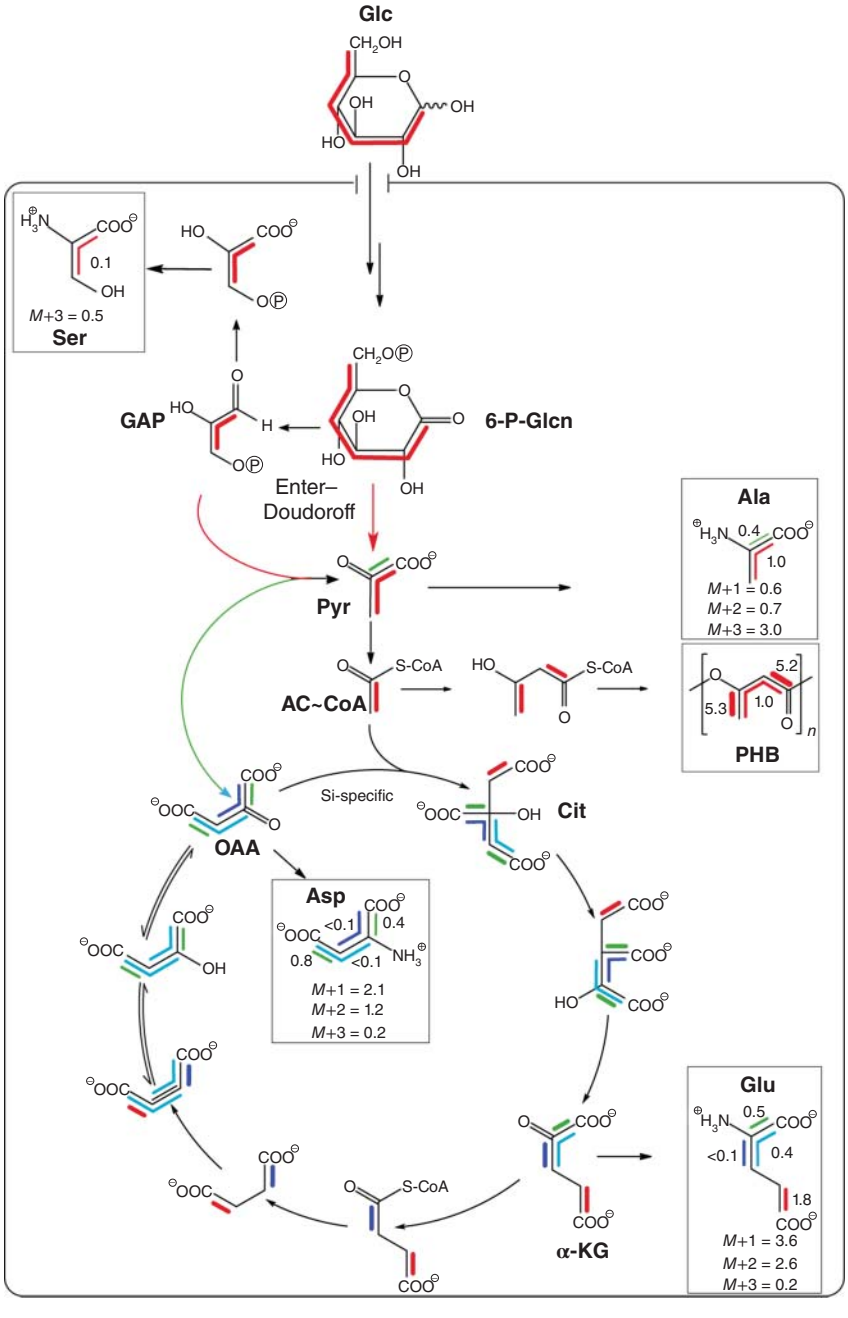

Figure 2.2 Incorporation of [U-$^{13}C_6$]glucose into *L. pneumophila* under *in vitro* conditions [44]. The ^{13}C-profiles are indicated by bold lines connecting ^{13}C-labeled atoms in a given molecule. The labeling patterns of the compounds in boxes were determined. The numbers indicate the molar abundances (mol%) of the respective isotopologues as determined by quantitative NMR or MS. For more details, see legend to Figure 2.1.

glucose was metabolized through the ED pathway and (at minor rates) through the glycolytic pathway (Figure 2.3, blue arrows) [33, 44].

Since the major pathway of glucose degradation in *L. pneumophila* is the ED pathway, a Δ*zwf* mutant strain devoid of glucose 6-phosphate dehydrogenase, the first enzyme of the ED pathway, was also analyzed by labeling experiments using ^{13}C-glucose supplements. Indeed, the carbon flux from glucose into amino acids and PHB was highly reduced in this mutant [44]. Interestingly, however, not only the ^{13}C incorporation of [U-$^{13}C_6$]glucose into PHB from the Δ*zwf* mutant was reduced, but also the total amount of PHB when compared to the wild-type strain [44] and unpublished results. This suggested that the ED pathway (at least the enzyme encoded by the *zwf* gene) influenced the biosynthesis of PHB, probably by the limiting amount of NADPH in the mutant strain, which is normally generated during the ED pathway serving as a cofactor in PHB biosynthesis.

The observation of an active utilization of exogenous glucose by *L. pneumophila* raised the question whether glucose (or glucose phosphate) may be generated by the degradation of glycogen, starch, or cellulose, since polysaccharide-degrading enzymes were also identified in the genome of *L. pneumophila* strains [32, 33, 54]. Further, supporting the potential role of polysaccharides as substrates for *L. pneumophila,* an eukaryotic-like glucoamylase gene (*gamA*) was found to be

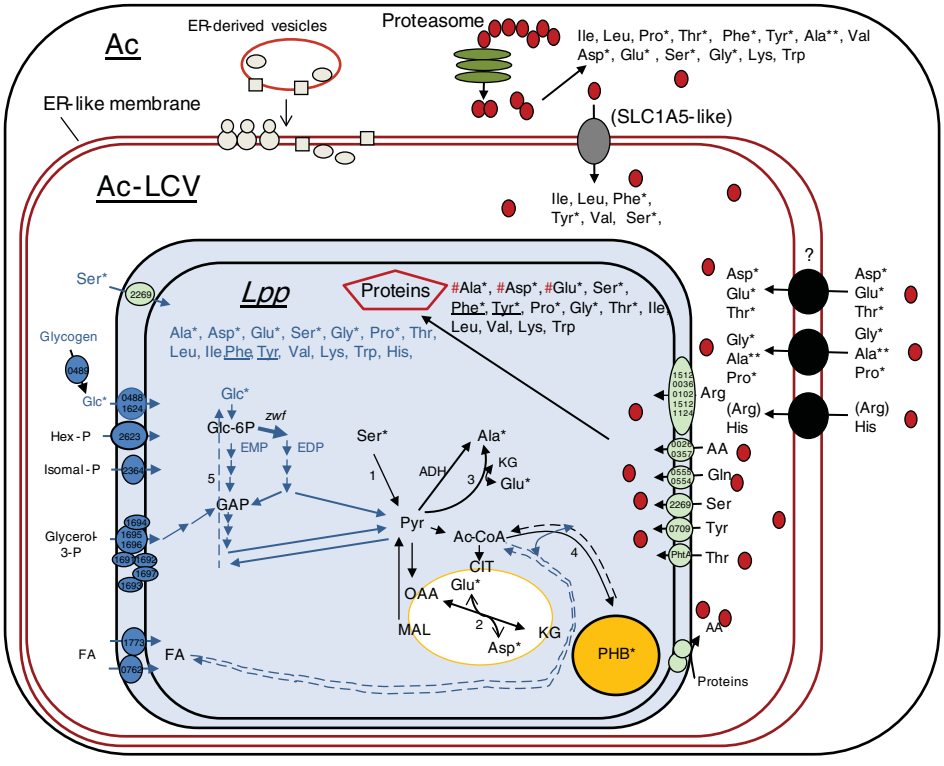

Figure 2.3 Schematic drawing of the metabolism of *L. pneumophila* (*Lpp*) (Schunder et al. [50]; modified). A combination of data of *in silico* analysis (gray arrows), *in vitro*/AYE medium grown *Lpp* (blue arrows), and *in vivo*/*Acanthamoeba castellanii* grown *Lpp* (black arrows) using ^{13}C isotope (*)-labeled substrates, are shown. *In vitro,* glucose is mainly metabolized through the ED pathway and pyruvate enters the citric acid cycle or the PHB synthesis pathway (reaction 4) mainly through acetyl-CoA (Ac-CoA). The Embden–Meyerhof–Parnas (EMP) pathway is present but not used to metabolize glucose *in vitro*, but is thought to be used in gluconeogenesis (reaction 5). The glucoamylase (Lpp0489) is essential for *L. pneumophila* to generate glucose from glycogen [51]. The proteasome is involved in the generation of free amino acids in infected host cells [52]. The amino acids (red circles) reach the *A. castellanii Legionella*-containing vacuole (Ac-LCV) by a SLC1A5-like amino acid transport protein or by other yet not known amino acid transporters (black circles). Many amino acid transport proteins are known for *L. pneumophila* (green circles) and are expressed during intracellular growth. Amino acids of *in vitro* grown bacteria are given in blue and amino acids of *A. castellanii* and of *in vivo* grown *L. pneumophila* are shown in black. The amino acids Gln, Trp, Met, Cys, and Arg could not be analyzed by the isotopologue profiling method. Some amino acids (#) of LCV-grown bacteria showed a significant different isotopologue profile when compared with *L. pneumophila* infected *A. castellanii*-derived amino acids. This seems to be a result of the (co)-metabolism of these amino acids through the Glu-Asp (reaction 2) and the Glu-Pyr (reaction 3) transaminases or by *de novo* synthesis, for example, using Ser dehydratase (reaction 1) to metabolize serine. PHB of *L. pneumophila* (yellow circle) was also found to be labeled in *in vitro* and *in vivo* experiments. That glycerol metabolism is important for intracellular growth of *L. oakridgensis* [53] and a glycerol-3-P transporter complex is present within the genome sequence, as well as carbohydrates and fatty acid (FA) transport proteins [33]. The role of nutrients delivered to the LCV by the cargo of vesicles of the endoplasmic reticulum (ER) is not known yet. Genes (*lpp* gene numbers) are given within the green and blue circles. AAs, amino acids; Ac, *A. castellanii*; Hex-P, hexosephosphate transport protein; KG, ketoglutarate; Pyr, pyruvate; CIT, citrate; MAL, malate; OAA, oxaloacetate; Lpp, *L. pneumophila* strain Paris; reaction 1, serine dehydratase; reaction 2, Glu-Asp transaminase; reaction 3, Glu-Pyr transaminase; and reaction 4, PHB synthesis pathway (β-ketothiolase, acetoacetyl-CoA-reductase, PHB polymerase).

induced during the growth within *A. castellanii* [48]. Indeed, the *gamA* gene product of *L. pneumophila* Paris was shown to be responsible for the starch- and glycogen-degrading activity of the strain [51, 55]. Moreover, using ^{13}C-labeled starch as a supplement to the growth medium of *L. pneumophila*, carbon flux from starch into amino acids and PHB could be demonstrated. As expected, the incorporation of carbon from starch into *L. pneumophila* was strongly reduced in the Δ*gamA* mutant strain.

The capability of *L. pneumophila* to degrade and to use polysaccharides was further supported by the observation that *L. pneumophila* secretes an endoglucanase (CelA), which is able to degrade cellulose [56]. Furthermore, a mutation within a chitinase gene of *L. pneumophila* was shown to reduce the virulence of the mutant strain [57]. Notably, however, the ability to use glucose as a carbon source seems not to be true in the same way for all *Legionella* species. For example, *Legionella oakridgensis*, a strain which is able to cause Legionnaires' disease, but is far less virulent compared to *L. pneumophila* strains, does not contain a homolog of the *gamA* gene and of the glucose transporter gene *lpp0488* [53]. This strain is also negative

for glycogen- and starch-degrading activity. In addition, *L. oakridgensis* was not able to metabolize ^{13}C-glucose in detectable amounts. On the other hand and similar to *L. pneumophila*, ^{13}C from labeled serine was incorporated into amino acids and PHB of *L. oakridgensis* [53]. These results indicate that there are *Legionella* strain specific differences in the ability to use extracellular polysaccharides, maybe also influencing the virulence behavior of the respective strains.

Metabolism of *L. pneumophila* under Intracellular Conditions

Only recently and mainly due to the advances of the bio-analytical tools to study metabolic features in multiple organismic systems, some of the relevant nutrients and their utilization were elucidated for *L. pneumophila* living within host cells. Generally, these results point at complex and dynamic adaptation processes in the bacterial metabolism in response to the changing environmental conditions in the host during the infection process.

The presence of cysteine, glutamine, serine, methionine, valine, glutamate, tyrosine, arginine, and threonine in the cell culture medium for human macrophages stimulated intracellular replication of the pathogen in the LCV [28, 58]. In addition, it was shown that the concentration of free amino acids in *A. castellanii* was increased upon infection with *L. pneumophila* [52, 59–61]. Moreover, many genes encoding transporters for amino acids and oligopeptides, as well as for enzymes involved in the degradation of glutamine, glutamate, threonine, lysine, arginine, and histidine were upregulated in *L. pneumophila* when replicating in macrophage-like THP-1 cells [24]. As prominent examples, the phagosomal transporter A (PhtA) and phagosomal transporter J (PhtJ) encoded by *L. pneumophila* and catalyzing the import of threonine and valine, respectively, were shown to be essential for intracellular replication and differentiation of *L. pneumophila* [28]. In conjunction with the efficient usage of amino acids under *in vitro* conditions (see above), all of these observations strongly indicate that amino acids play important roles as substrates also for *L. pneumophila* replicating in the LCV of host cells. In line with this conclusion is the fact that the eukaryotic amino acid transporter SLC1A5 is upregulated in infected host cells and probably recruited to the membrane of the LCV, benefitting the supply with amino acids for the pathogen [58]. Recently, it was discovered that even the proteasomal degradation of host proteins is stimulated by intracellular *L. pneumophila*, thereby again providing increased amounts of free amino acids as nutrients for the pathogen [52].

The transfer and usage of host amino acids by intracellular *L. pneumophila* was demonstrated directly by advanced ^{13}C-profiling experiments [50, 55]. More specifically, *A. castellanii* cells were ^{13}C-prelabeled with [U-$^{13}C_6$]glucose and then infected for 22 h with *L. pneumophila* Paris. With this setting, the bacteria replicated within the vacuole of the host cells and the majority of the bacteria were shown to be still within the LCV when harvested. Thus, the pathogens were in the replicative and post-exponential phase of growth, and, therefore, still in the nonmotile, metabolically active phase [17, 18, 34, 62]. Isotopologue profiling

of amino acids from bacterial and host proteins displayed highly similar labeling patterns with the exception of aspartate, alanine, and glutamate [50]. As an example, the patterns of phenylalanine and tyrosine from both cell fractions were remarkably similar if not identical, although *L. pneumophila* was not able to *de novo* synthesize these amino acids under *in vitro* conditions [44]. Together, the data clearly indicated that host amino acids (^{13}C-prelabeled in the amoeba from [U-$^{13}C_6$]glucose) were taken up into the LCV and directly used by *L. pneumophila* for bacterial protein biosynthesis. Results are summarized in Figure 2.3 (black arrows).

As already mentioned earlier, the isotope patterns of bacterial alanine, aspartate, and glutamate significantly differed in this experiment when comparing with the respective amino acids from the host fraction [50]. This indicated that only these amino acids were partly *de novo* synthesized and/or cometabolized by *L. pneumophila* replicating within the LCV. This may be partially due to the known activity of the glutamate–aspartate transaminase reaction (Figure 2.3, reaction 2). Recently, this hypothesis was supported by the finding that ^{15}N-label from glutamate was transferred to aspartate and alanine, albeit only shown for *in vitro* conditions (unpublished results).

As mentioned above, genes of the ED pathway and an eukaryotic-like *gamA* were induced during the growth within *A. castellanii* indicating that glucose or other carbohydrates may also play a role for intracellular replicating *L. pneumophila.* Moreover, the *zwf* mutant strain lacking the first enzyme of the ED pathway was outcompeted by the wild type strain in a replication/survival assay [44]. In line with these results, *L. pneumophila* mutants lacking other enzymes of the ED were also defective in growth under intracellular conditions [63]. Together, strong evidence was provided by these experiments that carbohydrates are among the substrates of intracellular *L. pneumophila.*

Surprisingly, however, labeling experiments with the Δ*zwf* mutant strain growing in the ^{13}C-prelabeled *A. castellanii* cells (see above) did not display any significant differences in the isotope patterns of amino acids compared to the wild-type strain [50]. This observation was in sharp contrast to the results obtained during *in vitro* growth [44] suggesting that, under the conditions of the *in vivo* experiment, ^{13}C-labeled glycogen (presumably present in the ^{13}C-prelabeled host cells) did not contribute to the detected labeling profiles of amino acids and, on this basis, was not used as a major carbon substrate.

However, the apparent discrepancy can be explained when labeled glycogen and its downstream products (i.e., glucose or glucose phosphate) were not utilized during the early and logarithmic phases of intracellular growth of the *in vivo* experiments [50, 63] and own unpublished results, but may become relevant only during the subsequent stages (cytosolic phase) of *L. pneumophila* infections.

Indeed, during the late replicative or transmissive phases when the bacteria become motile, leave the LCV, and enter the cytosolic environment of their hosts, sugars might now become accessible at higher amounts. Indeed, about 10% of the dry weight of *A. castellanii* trophozoites is composed of glycogen [64]. In this context, it is also interesting to note that glucose 6-phosphate (deriving from the degradation of polysaccharides; see above) rather than glucose could be the more

effective carbon substrate [36]. Supporting this hypothesis, *L. pneumophila* carries an *uhpC* gene (*lpp2623*) that is highly homologous to the *uhpC* gene of *Chlamydia pneumoniae.* The chlamydial UhpC has already been shown to function as a glucose 6-phosphate transporter [65].

It also appears probable that, next to glucose or glucose phosphate, additional sugars or sugar-like molecules can be incorporated and utilized by intracellular *L. pneumophila.* For example, genes encoding enzymes catalyzing the degradation of glycerol were upregulated during infection of macrophages [24] and the *gpsA* gene (glycerol 3-phosphate dehydrogenase) was detected as a virulence factor of *L. oakridgensis* in a "Scatter screen" using *Acanthamoeba lenticulata* as the host [53]. The concomitant upregulation of genes encoding phosphoenolpyruvate (PEP), carboxylase, and pyruvate carboxylase, respectively, further supports the potential usage of glycerol by intracellular *L. pneumophila*; both enzymes link the conversion of glycerol into PEP/pyruvate with the TCA cycle via oxaloacetate. Notably, oxaloacetate also serves as the precursor in the biosynthesis of aspartate, lysine, and *meso*-diaminopimelate (a key component in peptidoglycane). It is then not surprising that the genes for the biosynthesis of these amino acids were also induced in *L. pneumophila* under intracellular conditions [24]. However, the usage of glycerol and related substrates by intracellular *L. pneumophila* still needs to be proven by more direct experimental observations.

Recently, it was reported that nicotinic acid and thymidine are also needed for full fitness of *L. pneumophila* [66, 67]. In addition, polyamines, such as putrescine and spermidine, seem to support intracellular growth of *L. pneumophila* and spermidine transport proteins are encoded within the genomes of *L. pneumophila* strains [68]. These aspects have been reviewed recently and shall not be repeated here [67].

Metabolic Adaptation during the Life Cycle of Intracellular *L. pneumophila*

The currently available data indicate a tremendous shift in nutrient usage during the life cycle of intracellular *L. pneumophila.* There is increasing evidence that these processes are closely coupled to the changing situation of nutrient supply in the LCV that also triggers the differentiation from the replicative into the transmissive and MIF forms of intracellular *L. pneumophila.* Thereby, these metabolic adaptation processes obviously also determine the virulence potential of *L. pneumophila.* In the following section, some recent results and hypotheses about the linkages of virulence and metabolism are summarized.

Isotopologue profiling of intracellular *L. pneumophila* in the LCV of *A. castellanii* (during the replicative growth phase) have indicated the predominant usage of amino acids from the host organism, but not of carbohydrates [50]. During this developmental stage, the posttranscriptional regulator CsrA suppresses triggers for bacterial transmission and supports efficient replication within the LCV [69]. On the other hand, glucose or glucose phosphate (e.g., derived from polysaccharides of the host cells) might serve as important nutrients for

L. pneumophila when leaving the LCV and entering the cytosolic compartment of host cells, subsequently switching into the metabolically resting MIF forms. Thereby, the pathogen apparently reprograms its metabolism in order to adapt to the changing situation with a limiting supply of amino acids but a higher supply of carbohydrates or related compounds, for example, glycerol. Thus, the differentiation of intracellular *L. pneumophila* from the replicative into the transmissive form could be induced by the decreased supply of amino acids into the LCV. In particular, the amounts of cysteine in the host cell could serve as a nutritional rheostat for the development of *L. pneumophila* [70, 71]. In line with a synchronized metabolic shift from amino acids to sugars and sugar-like substrates is the fact that the biosynthesis of the storage compound PHB, which occurs late in the life cycle of intracellular *L. pneumophila* (i.e., under amino acid-starvation conditions), appears to depend on the usage of carbohydrate nutrients [44].

Furthermore, in *L. pneumophila* the flux of fatty acids is coupled to microbial differentiation and virulence [72]. Probably provoked by amino acid shortage and/or by short-chain fatty acids, guanosine-3′,5′-bis-diphosphate (ppGpp) is produced by the RelA or SpoT enzymes, respectively [27, 73, 74]. This alarmone induces a complex regulatory cascade involving alternative sigma factors such as RpoS and the LetA/LetS two-component system. The stringent response occurs during the switch of replicative *L. pneumophila* into the transmissive form and might therefore again reflect the bacterial adaption to the changing environmental conditions. Notably, fatty acids might become available from the membranes of the decomposing LCV for *L. pneumophila* changing into the transmissive forms. Thus, *L. pneumophila* exhibits various secreted or membrane-associated phospholipases, some of which have been indeed shown to be virulence factors [75–79]. As indicated in Figure 2.3, fatty acid degradation may then lead to Ac-CoA further supporting PHB synthesis when the bacteria switch to the transmissive phase.

Metabolic Host Cell Responses Triggered by Intracellular *L. pneumophila*

In the meantime, a solid body of data shows that *L. pneumophila* specifically manipulates the metabolism of host cells in order to benefit its replication and development. This capability of *L. pneumophila* and other intracellular pathogens has been described recently as an emerging paradigm by the term *nutritional virulence* [70].

As already mentioned above, the amino acids transporter SLC1A5 (specific for neutral amino acids) is highly induced in the human monocyte cell line MM6 upon infection by *L. pneumophila* [58]. Indeed, the presence of this transporter is essential for bacterial proliferation in these host cells. Indirect evidence suggested that SLC1A5 may be recruited to the LCV membrane thereby again supporting the uptake of neutral amino acids into the LCV (cf. Figure 2.3).

In addition, the supply of amino acids is manipulated by several effector proteins translocated by the T4SS machinery of *L. pneumophila* [80]. As an example, the

effector protein AnkB stimulates the degradation of host proteins by activation of the ubiquitin/proteasome pathway [52, 81].

As another example, activation of the NF-κB signaling pathway by the effector protein LnaB was noticed in *L. pneumophila*-infected human macrophage-like cells [82, 83]. NF-κB is a well-known key regulator of eukaryotic cell development, but also directly linked to metabolic processes such as the metabolism of glutamine [84]. Among the Dot/Icm-dependent effector proteins, putative serine/threonine protein kinases and phosphatases can also be found, which may be involved in the regulation of enzyme activities thereby modulating metabolic host processes [85]. However, the downstream reactions controlled by these mechanisms are not yet known.

Conclusion

The switch of carbon utilization during the life cycle of *L. pneumophila* can be considered as a prime example for the metabolic adaptation of an intracellular pathogen to the changing environmental conditions in the host cell. During the replicative phase, when *L. pneumophila* grows within the LCV, amino acids serve as the major nutrients. During this developmental stage, efficient supply of amino acids from the host cell is triggered, for example, by activating the ubiquitin/proteasome pathway of host protein degradation. Upon amino acid starvation, however, the intracellular bacteria switch to the transmissive and MIF forms where *L. pneumophila* leaves the LCV. This developmental change appears to be connected with the usage of glucose or glucose phosphate and related compounds (e.g., glycerol) as substrates. It can be expected that inhibitors targeting the signaling proteins, transporters, and catabolic enzymes essential for these metabolic processes are promising targets for the treatment of Legionaires' disease and related disorders. It can also be expected that the crosstalk between metabolism and virulence of *L. pneumophila* can serve as a model for other intracellular pathogens interacting with their hosts.

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3
Metabolism of Intracellular *Salmonella enterica*

*Peter Holtkötter and Michael Hensel**

Abstract
Salmonella enterica is an important human food-borne pathogen that initially enters the lumen of the intestinal tract. The bacteria are invasive, facultative intracellular pathogens that survive and replicate in a unique membrane compartment, termed *Salmonella*-containing vacuole or SCV. *S. enterica* is a metabolic generalist that is equipped with a broad repertoire of metabolic pathways for utilization of various carbon and energy sources. In the last years, there was a remarkable increase in understanding of the cellular microbiology of *Salmonella* infections and the molecular functions of virulence factors required for intracellular life, but the nutritional basis of life of *Salmonella* within the SCV is still not completely understood. How *Salmonella* gets access to nutrients could open new avenues to therapeutic interference with *Salmonella* infections. The redirection of host cell endosomal transport may be one means of accessing host-derived nutrient for growth in the SCV. This chapter provides an overview of recent research on metabolism of intracellular *Salmonella*. We also compare the metabolic adaption of *S. enterica* to other important gastrointestinal or intracellular pathogens.

Introduction

Salmonella enterica is an important human food-borne pathogen that initially enters the lumen of the intestinal tract. *S. enterica* can infect the intestinal epithelium through an active invasion process of polarized epithelial cells, which is dependent on the *Salmonella* pathogenicity island (SPI1)-encoded type 3 secretion system (T3SS) and the SPI4-encoded giant adhesion SiiE [1]. A further possibility is the penetration of the epithelial cell layer using a T3SS-independent mechanism, whereby bacteria are taken up by dendritic cells that open the tight junctions and migrate into the intestinal lumen [2]. After crossing the intestinal epithelium, bacteria are internalized by phagocytic cells, such as dendritic cells and macrophages [3]. *S. enterica* has an invasive and facultative intracellular lifestyle [4]. Among the

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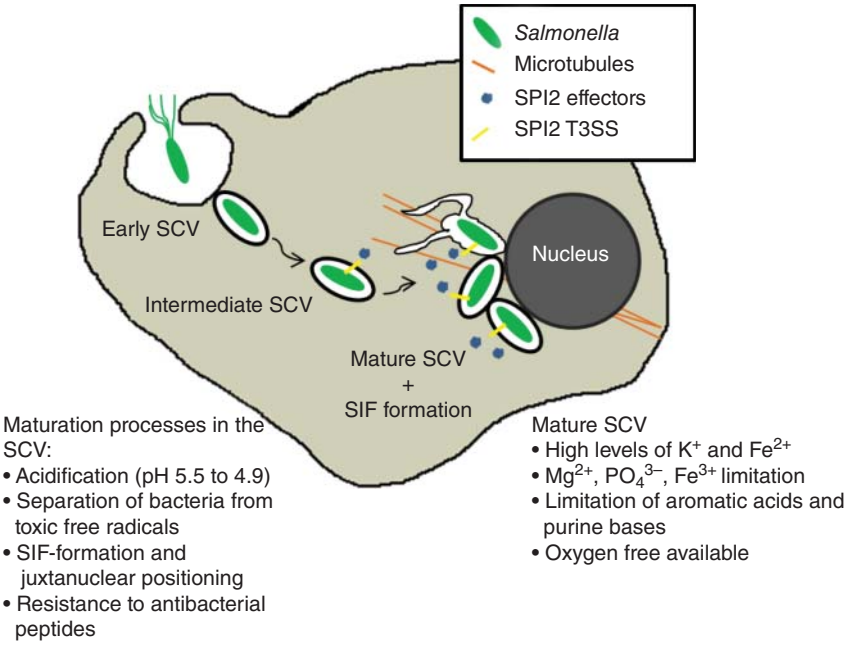

Figure 3.1 Intracellular lifestyle of *Salmonella enterica*. *Salmonella* is taken up by host cells either by *Salmonella*-induced invasion (SPI1-T3SS-induced macropinocytosis) or by phagocytosis. By translocating effector proteins via the SPI2-T3SS into the host cell, the SCV undergoes an altered maturation process. The formation of *Salmonella*-induced filaments (SIFs) takes place with the start of *Salmonella* replication between 4 and 6 h after infection. SIF develop on a microtubule scaffold.

various habitats that can be colonized by *Salmonella*, the adaptation to life inside the host cell is of specific interest, since this ability is considered as crucial for systemic infections with fatal outcome [5]. Infection models using mammalian cell lines or *Salmonella*-susceptible mice with *S. enterica* serovar Typhimurium are used as a model for human systemic infections by *S. enterica* serovar Typhi.

During intracellular life, *Salmonella* mainly resides in a membrane-bound compartment, termed *Salmonella*-containing vacuole (SCV). This unique compartment is formed by the combined action of a number of bacterial virulence factors (Figure 3.1). The SCV can be modified by virulent *Salmonella*, resulting in the protection of *Salmonella* from killing by antimicrobial host cell activities and allowing proliferation within host cells [10]. The ability to survive and to replicate within host cells is closely related to the systematic pathogenesis of *Salmonella* in a murine model of typhoid fever. *Salmonella* mutant strains with defects in intracellular replication due to auxotrophies are attenuated also in virulence in the murine model of typhoid fever [11]. Furthermore, comparison of results from *in vitro* and *in vivo* infection models indicates that proliferation appears to be far less rapid within cells in the tissue of infected hosts than in various eukaryotic cell lines. This leads to the assumption that conditions *in vivo* are more restrictive for *Salmonella*

proliferation [12]. The mammalian hosts possess a variety of differentiated cells with distinct overall metabolic activities and, accordingly, distinctly regulated metabolic pathways. In contrast to most cancer cells, which are often used as model host cells for the study of intracellular bacteria, the metabolic activity of tissue cells is often low. In cancer cells, some catabolic and anabolic pathways are highly upregulated, while aerobic respiration is strongly reduced even in the presence of oxygen [13]. Results obtained by phenotypes of auxotrophic strains, bacterial reporter strains, and microarray analyses of intracellular *Salmonella* indicate that the SCV is a nutritional deprived environment. This fact explains the need of *Salmonella* for successful adaptation to the intracellular environment for replication.

In the past years, there was a remarkable increase in understanding of the cellular microbiology of *Salmonella* infections and the molecular functions of virulence factors required for intracellular life, but the nutritional basis of life of *Salmonella* within the SCV is still not completely understood. How *Salmonella* gets access to nutrients could open new avenues to therapeutic interference with *Salmonella* infections. This chapter provides an overview of recent research on metabolism of intracellular *Salmonella*.

S. enterica – A Metabolic Generalist

S. enterica is a metabolic generalist that is equipped with a broad repertoire of metabolic pathways for utilization of various carbon (C) and energy sources. This flexibility may be exemplified by various uptake systems for sugars and amino acids, the prototrophic use of simple C-sources for biosynthesis of all cellular macromolecules, and the ability to switch from aerobic respiration to anaerobic life involving fermentative metabolism or various alternative electron acceptors. This metabolic flexibility allows *Salmonella* to adapt to various environmental niches outside the host and to diverse situations in colonized host organisms (Table 3.1). Niches for life of *Salmonella* in the host can be as diverse as the intestinal lumen with its competing microbiome, the inflamed intestine, biofilm formation on gallstones, or the presence inside mammalian host cells. Depending on the environment, *S. enterica* can rapidly adjust its metabolism. Work on the metabolism of intracellular *S. enterica* in infection models using mammalian cell lines or animal models of systemic infection indicated a key role of the utilization of glucose. The following section describes the central metabolism of *S. enterica* based on glucose as C-source.

Metabolism of Glucose by *S. enterica*

Salmonella can use three routes for the catabolism of glucose: (i) glycolysis, (ii) the pentose phosphate pathway (PPP), and (iii) the Entner–Doudoroff pathway also known as the 2-keto-3-desoxy-6-phosphogluconate pathway (KDPGP) (Figure 3.2). The last two pathways for glucose utilization seem to be of lesser importance for *Salmonella.* Mutant strains with defects in PPP and KDPGP are not attenuated in proliferation in the murine macrophage cell line RAW 264.7 [5]. A *Salmonella*

Table 3.1 Nutrients that can be metabolized by *Salmonella* in infected tissues.

Different nutrients	Experimental evidence	References
Carbohydrates		
Fucose	Proteomics of *Salmonella* in infected mouse tissues	[6]
Gluconate	DGEP-studies, mutants with utilization defects	[6–8]
Glucose	^{13}C-IPA data, auxotroph mutants	[9]
Glycerol	DGEP-studies, mutants with utilization defects	[6–8]
Lactate	Proteomics, mutants with utilization defects	[6]
Mannitol	Proteomics of *Salmonella* in infected mouse tissues	[6]
Mannose	Proteomics of *Salmonella* in infected mouse tissues	[6]
N-Acetyl-D-glucosamine	Proteomics, mutants with utilization defects	[6]
Ribose	Proteomics of *Salmonella* in infected mouse tissues	[6]
Trehalose	Proteomics of *Salmonella* in infected mouse tissues	[6]
β-D-Galactose	Proteomics of *Salmonella* in infected mouse tissues	[6]
Amino acids/proteins		
4-Aminobutyrate	Proteomics of *Salmonella* in infected mouse tissues	[6]
Arginine	Proteomics, mutants with utilization defects	[6]
Aspartate	Proteomics of *Salmonella* in infected mouse tissues	[6]
Ethanolamine	Proteomics of *Salmonella* in infected mouse tissues	[6]
Glutamate	Proteomics of *Salmonella* in infected mouse tissues	[6]
Lysine	Proteomics, auxotroph mutants	[6]
Phenylalanine	Proteomics, auxotroph mutants	[6]
Serine	Proteomics of *Salmonella* in infected mouse tissues	[6]
Threonine	Proteomics, auxotroph mutants	[6]
Valine	Proteomics, auxotroph mutants	[6]
Nucleosides		
Adenosine	Proteomics of *Salmonella* in infected mouse tissues	[6]
Cytidine	Proteomics of *Salmonella* in infected mouse tissues	[6]
Guanosine	Proteomics of *Salmonella* in infected mouse tissues	[6]
Inosine	Proteomics of *Salmonella* in infected mouse tissues	[6]
Thymidine	Proteomics of *Salmonella* in infected mouse tissues	[6]
Uridine	Proteomics of *Salmonella* in infected mouse tissues	[6]
Fatty acids	Proteomics of *Salmonella* in infected mouse tissues	[6]

mutant strain deficient in *zwf* (encoding glucose-6-phosphate dehydrogenase), catalyzing the first step of both PPP and KDPGP, shows reduced virulence in a mouse model of systemic infection [14]. The authors refer to the importance of NADPH production by the PPP, which is used as electron donor for reductases required for oxidative stress response. In RAW 264.7 cells, the superoxide levels should be lower than in infected tissues, and this may explain the decreased need for such reductases and for NADPH [5]. Isotopologue profiling experiments in Caco-2 cells showed that the internalized glucose is mainly converted by glycolysis and/or KDPGP and excluded PPP as a major route for glucose catabolism [15]. Furthermore, *Salmonella* studied in epithelial cell lines are significantly less challenged with reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates responses; hence, the generation of NADPH would be less important than in animal models. The activity

of the PPP may become important if *Salmonella* is challenged with higher degrees of oxidative stress [11]. Bowden *et al.* analyzed a set of glycolysis mutant strains and showed that *eno*, *fba*, *pgk*, *gapA*, or *tpiA* deficient strains are strongly attenuated in intracellular replication and survival in RAW 264.7 cells. These results show the significance of glucose as one of the major C-sources and glycolysis as the main route for utilization [15].

Connection to the TCA Cycle by Oxidative Decarboxylation of Pyruvate

Pyruvate is the final product of glycolysis and the KDPGP. The enzyme pyruvate dehydrogenase metabolize pyruvate to acetyl-CoA; through this the connection to

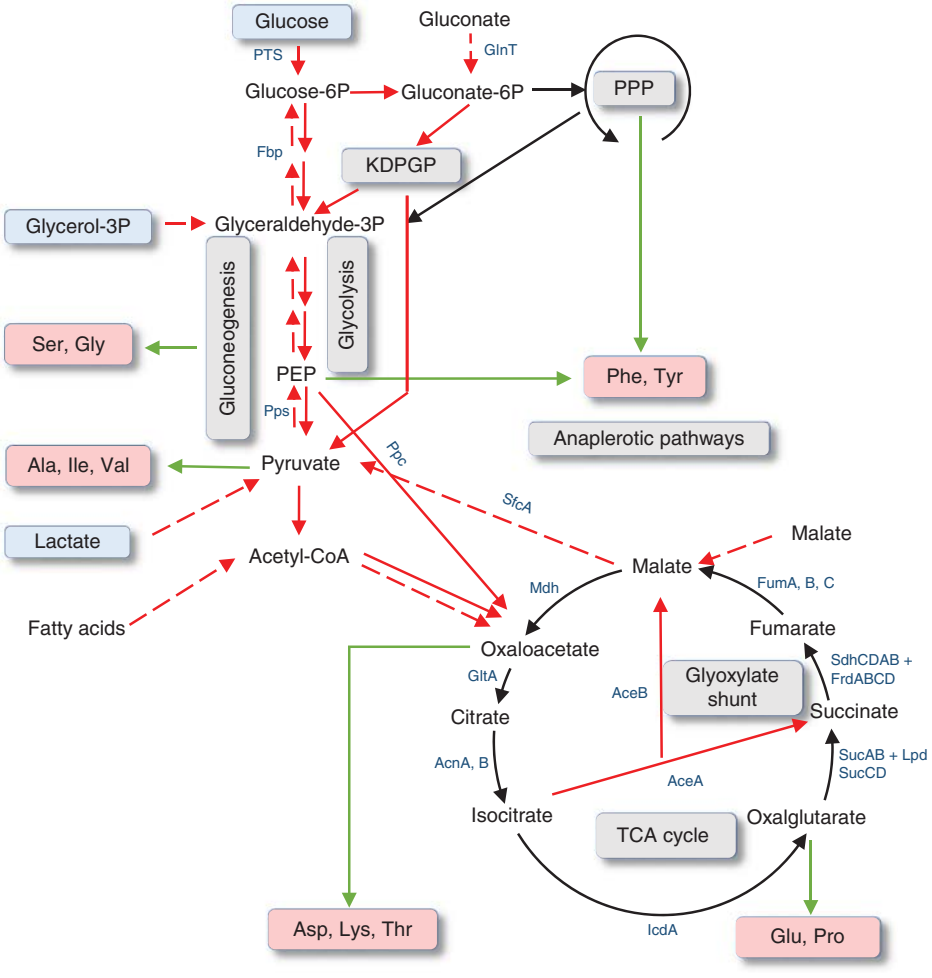

Figure 3.2 Model of intracellular metabolism of *Salmonella enterica*. Carbon substrates, catabolic reactions, and *de novo* synthesized amino acids deduced from differential genes expression profiling (DGEP) data and ^{13}C-isotopologue-profiling analysis (^{13}C-IPA) data as well as *in vivo* studies are shown for intracellular growing *S. enterica*. Solid black arrows indicate pathways and reactions that may be required under all conditions, but the extent of their contribution to the metabolism of intracellular bacteria cannot be deduced from the data available. The blue boxes indicate carbon sources used for *S. enterica* and the red arrows show the active catabolic reactions for the major carbon source, which is glucose for *S. enterica*. Red boxes indicate amino acids that are synthesized *de novo*, according to the ^{13}C-IPA data. In *S. enterica* Ser, Gly, Ala, Val, Asp, and Glu are *de novo* synthesized more efficiently than the other amino acids. Dashed red arrows indicate metabolic reactions and pathways suggested from DGEP and ^{13}C-IPA data from mutants defective for the major carbon source and by mouse infection studies. Mutants defective for utilization of major carbon sources had to use nonglycogenic carbon substrates such as glycerol-3P or pyruvate for carbon metabolism. Besides the reversible reactions from glycolysis, this requires anaplerotic reactions and gluconeogenesis. Gluconeogenesis involves the reactions generating PEP from pyruvate (by PEP synthase, Pps) and fructose-6-phosphate from fructose-1,6-bisphosphate (by fructose-1,6-biphosphatase, Fbp). Green arrows show the anabolic step and the major catabolic intermediates from which they derive. Central metabolic pathways (blue boxes) comprise glycolysis and gluconeogenesis, the KDPGP, the pentose phosphate pathway (PPP), the TCA cycle, and various anaplerotic reactions that replenish metabolic gaps, such as the generation of oxaloacetate by phosphoenolpyruvate carboxylase (Ppc), the glyoxylate shunt (which uses isocitrate lyase, AceA, and malate synthase, AceB), and the generation of pyruvate by decarboxylating malate dehydrogenase (SfcA). PTS, PEP-dependent phosphotransferase system; GlnT, gluconate transferase; Mdh, malate dehydrogenase; GltA, citrate synthase; AcnAB, aconitase AB; IcdA, isocitrate dehydrogenase A; SucAB, 2-Keto-glutarate dehydogenase (E1, E2 subunits); SucCD, succinyl-CoA synthetase (alpha, beta subunits); Lpd, 2-Ketoglutarate dehydrogenase (E3 subunit); SdhCDAB, succinate dehydrogenase; FrdABCD, fumarate reductase; and FumA, B, C, fumarases A, B, C [4, 5].

←

the TCA cycle is achieved. The production of acetyl-CoA is of great importance as indicated by the reduced growth of a pyruvate dehydrogenase subunit I-deficient strain (Δ*aceE*) in rich media such a Luria Broth (LB) and strong attenuation of intracellular replication [5]. The *aceE* mutant of *S. enterica* serovar Enteritidis analyzed in a chicken infection model had a retarded growth rate compared to the wild type (WT) strain, due to a lower ability to survive within a chicken macrophage cell line and less resistance to ROI [16]. This result indicating that loss of *aceE* may also influence the expression of virulence genes important for defense against ROI.

The Important Role of the TCA Cycle and Anaplerotic Reactions

The TCA cycle plays an important role as a source for precursors for anabolic pathways, like amino acids and reducing agents used as electron donors in the respiratory chain or for biosynthesis. It was shown that mutants with defects in the TCA cycle lost virulence in a murine model [17, 18], concluding that the ability to run the full TCA cycle is critical for virulence. Surprisingly, some TCA cycle mutant strains showed a reduced virulence in the murine model (*mdh*, *sdhCDAB*, and *sucCD*), but

an increased replication in resting and activated macrophages (RAW 264.7). This observation may be explained by difference in available nutrients in the *in vitro* and *in vivo* environment [18].

Anaplerotic reactions are required for survival in macrophages. A strong attenuation was observed for a TCA cycle mutant strain with an *icdA* deletion [5]. This strong attenuation could be due to the accumulation of an inhibitory product like citrate or isocitrate as was shown for an *Escherichia coli icd* mutant [19]. The glyoxylate shunt, which is used during growth on acetate and fatty acids, seems to be important for chronic infections, but not for acute infections [17, 20], otherwise citrate or isocitrate was metabolized. This observation supports that acetate and fatty acids are not important as C-sources in acute infections, either due to their absence or the presence of more favorable C-sources such as glucose [5]. Transcriptome analyses showed that no upregulation occurs in expression of isocitrate lyase gene *aceA* [21]. *Salmonella* strains with a defect in *β*-oxidation of fatty acids (*fadD*) showed the same virulence as the WT strain in a murine model [17].

A further anaplerotic reaction is the direct conversion of phosphoenolpyruvate (PEP) to oxaloacetate, an important precursor for amino acids aspartate and asparagine. This reaction prevents the TCA cycle from idling and is catalyzed by PEP carboxylase [22]. It was shown that loss of this enzyme did not attenuate virulence in a murine model [17], indicating the presence of C-sources other than glucose, for example, amino acids, in amounts required for intracellular replication of *Salmonella.* The replenishment of the TCA cycle is not necessary by this route. Taken together *S. enterica* has multiple possibilities to use a broad range of carbon substrates for the metabolism. How *Salmonella* get access to this broad range of carbon substrates will be extended in the next section.

Metabolism of Intracellular *S. enterica*

Glucose represents a major C-source for *Salmonella* during infection. By combining various pathways, *Salmonella* can easily metabolize glucose to generate both energy and amino acids. The same applies for most nutrient sources. Further C-sources are gluconate and glucose-6P based on differential gene expression profiling (DGEP) studies [7, 8], which compared transcripts from *Salmonella* grown intracellular with those derived from LB-grown bacteria. This assumption is based mainly on the fact that genes encoding enzymes for glycolysis and the KDPGP are upregulated in these bacteria. Based on ^{13}C-isotopologue profiling analyses (IPAs) data [23], glucose is the preferential carbon substrate. The ^{13}C from glucose is incorporated into amino acids very efficiently, suggesting that there is extensive *de novo* synthesis of their precursors from glucose under intracellular conditions. In *Salmonella* Ser, Gly, Ala, Val, Asp, and Glu are *de novo* synthesized more efficiently than the other amino acids [4]. Analysis of mutants defective in glycolysis and glucose uptake was severely attenuated for replication and survival in macrophages, so that glucose is needed for efficient intracellular growth of *S. enterica* in the SCV [9]. Another analysis showed that a mutant strain impaired in glucose uptake is still able to replicate in Caco-2 cells, although at a reduced growth rate, so that *S. enterica* has the possibility to

switch to other carbon sources in the absence of glucose [23]. When *S. enterica* replicates in systemically infected mice, bacterial growth depends on a complete TCA cycle [17]. It was shown that a mutant unable to convert succinate to oxaloacetate is avirulent [24], another mutant defective in the conversion of malate to pyruvate is also strongly attenuated, whereas an *aceA* mutant (lacking isocitrate lyase) is only weakly attenuated [24]. The last result excludes the need for the glyoxylate shunt in systemic *S. enterica* infections, but this anaplerotic reaction might have a role in persistent infections of animals [20]. Another analysis tested mutants blocked in gluconeogenesis in systemic mouse infection, these mutants were fully virulent [17]. These data indicate that *S. enterica* grows in mouse phagocytes on a limited supply of glycolytic carbon sources, presumably glucose or other C_6 carbohydrates [9, 24]. Further, it could be noted that mutants blocked in the biosynthesis of purines, especially adenine, and aromatic amino acids, including histidine, were strongly attenuated in proliferation; while pyrimidine and methionine mutants were moderately attenuated. Taken together *S. enterica* has a strong dependency on these anabolic monomers in the SCV, a conclusion in line with DGEP and ^{13}C-IPA data [11, 25, 26]. An overview of the intracellular metabolism is given in Figure 3.2. The combination of different pathways guarantees a fine-tuned balance of internal metabolites. These findings explain why inhibition of growth of *Salmonella* by antibiotics targeted against key metabolic enzymes of primary metabolism is problematic [27].

The Metabolism of *S. enterica* Compared to Other Important Intracellular and Gastrointestinal Pathogens

S. enterica, *Listeria monocytogenes, Shigella flexneri,* and *Campylobacter jejuni* are important food-borne pathogens. They get access to the host cells over crossing the intestinal epithelium and following internalization by phagocytic cells such as dendritic cells, macrophages, and granulocytes. In contrast to *S. enterica* and *L. monocytogenes, S. flexneri* quickly triggers apoptosis of the infected phagocytes, leading to release of the bacteria and their subsequent basolateral invasion of mucosal epithelial cells [28]. *S. enterica* and *L. monocytogenes* reside and replicate mainly in macrophages and dendritic cells, but both are able to invade nonphagocytic cells during infection [10, 29]. *L. monocytogenes* and *S. flexneri* lyse their pathogen-containing compartment and enter the host cell cytosol. Here they get access to nutrients, mostly catabolic intermediates and anabolic compounds in the cytosol of the host cell. In principle, cytosolic bacteria have direct access to all of these host cell metabolites. In contrast, *S. enterica* resides in the SCV and has to deploy other mechanism to obtain substrates for growth. Yet the composition of host cell metabolites is varying in different cell types and host cells and bacteria may use similar nutrients, so these bacteria may need well-balanced nutrient uptake systems to compete for these often limited host-cell-derived nutrients [4]. *Mycobacterium tuberculosis* and *Legionella pneumophila,* both causing lung diseases, enter the respiratory tract through aerosols.

M. tuberculosis and *L. pneumophila* get access to the intracellular environment through internalization by alveolar macrophages of the lung. For *M. tuberculosis* it is known that infected macrophages are unable to kill intracellular bacteria. Other immune cells encapsulate infected macrophages thus forming a granuloma in which *M. tuberculosis* is able to persist in a latent, metabolically quiescent state for a long time [30, 31].

An intracellular pathogen with a complex developmental cycle is *Chlamydia trachomatis*, growing and differentiating within an intracellular vacuole [32]. The developmental cycle begins with a metabolically inactive infectious form called the elementary body (EB) that, after entry into the target cell, differentiates into a metabolically active form called reticulate body (RB). After multiple rounds of division, RB then differentiates into the EB developmental form. After lyses of the host cell, the infectious EBs are released to initiate new rounds of infection [33]. Analysis of the genome of *Chlamydia* suggested that host-derived glucose-6-phosphate is the primary carbon and energy source used to support growth [34]. Furthermore, the genome contains key gluconeogenic enzymes, suggesting that host-derived glutamate or dicarboxylic acids may also support growth [34]. Microarray analysis showed that *C. trachomatis* is transcriptionally unresponsive or inflexible to nutrient-based environmental changes, such as carbon source availability. The situation is different in *S. enterica*, where a change from growth in media rich in glucose to growth in macrophages leads to a massive switch in global gene expression [21]. In addition to virulence factors such as the SPI2-encoded T3SS, several metabolic pathways are affected (see below).

Intracellular *S. enterica* induce a complex network of tubular vesicular structures, [35] and these so-called *Salmonella*-induced filaments (SIFs) may contribute to provide the intracellular bacteria with nutrients. In contrast, *C. trachomatis* only consume the energy sources until they are depleted. A contemplated hypothesis is that depletion of such energy stores could serve as the molecular switch behind the morphological changes that occur during the developmental cycle; however, carbon source depletion does not appear to serve as a signal for developmental regulation of *Chlamydia* [36].

The genome sequence [37] and *in vitro* growth studies of *L. monocytogenes* [38, 39] show that this species has an interrupted citrate cycle, lacks most of the enzymes involved in anaplerotic reactions and fatty acid degradation, and does not have the biosynthetic pathways for riboflavin, thiamine, lipoate, and biotin. A mutant impaired in glucose uptake replicates as efficiently as the wild-type strain [40], suggesting that glucose does not serve as a major substrate for the carbon metabolism of intracellular *L. monocytogenes*, as in *S. enterica*. Transcription profiles of *L. monocytogenes* grown in different cell lines show that there is a substantial upregulation of all genes in the facilitated uptake and catabolism of glycerol; furthermore, the phosphotransferase system (PTS)-system for glucose uptake is not upregulated [40]. Together with the observed downregulation of glycolysis genes and the upregulation of genes essential for gluconeogenesis, the data suggest that glycerol may be a major carbon source for carbon metabolism in intracellular bacteria. ^{13}C-IPA studies confirm that host-cell derived

C_3 substrates are major carbon sources for carbon metabolism in intracellular *L. monocytogenes* [41].

The genome of *S. flexneri* contains complete gene sets for the major catabolic and anabolic pathways, so *S. flexneri* represents a typical heterotrophic and prototrophic microorganism able to grow on a large range of carbon substrates, as well as *S. enterica*. DGEP studies of *S. flexneri* show transcriptional repression of the genes encoding glucose uptake proteins and induction of most genes for glycerol uptake. The glycolysis genes are downregulated, whereas those for gluconeogenesis are upregulated, again favoring C_3 substrates such as glycerol and glycerol-3P as the major carbon sources for cytosolic growth [8], as in *L. monocytogenes* [42]. ^{13}C-IPA of a cytosolic replicating EIEC (enteroinvasive *Escherichia coli*, which is closely related to *S. flexneri*) imply that glucose, but not glucose-6P, serves as the primary carbon source during intracellular replication of this strain. This assumption is based on the observation that most of the ^{13}C in amino acids that are synthesized *de novo* are derived directly from [^{13}C] glucose and not from a $^{13}C_3$ source [23]. Another EIEC strain analyzed in this study confirms a carbon metabolism using C_3 substrates in intracellular bacteria, as suggested by the DGEP data [23]. A mutant defective in the major glucose-specific PTS permeases shows a strong reduction in ^{13}C incorporation into newly synthesized amino acids; however, this mutant is still able to replicate (although at a lower growth rate) in the host cell cytosol and the ^{13}C pattern of those amino acids that do become labeled points to the use of a host cell-derived C_3 substrate in this mutant [23]. Taken together, these data suggest that EIEC and possibly also *S. flexneri* may exhibit strain-dependent preferences for carbon substrates and, hence, carbon metabolism when growing intracellular [4].

Unlike *Salmonella*, *C. jejuni* is limited in its ability to conserve energy for growth and maintenance via metabolism of carbohydrates. These bacteria lack 6-phosphofructokinase, which is a key enzyme in glycolysis [43]. Moreover, *C. jejuni* is further limited in its ability to conserve energy for growth via catabolism of carbohydrates due to the absence of an active PTS system, which would function to transport and phosphorylate sugars simultaneously [43]. Energy is conserved via respiration, oxidizing hydrogen, and formate for the reduction of electron acceptors (fumarate, nitrate, sulfites) and, if at low concentrations, oxygen, to generate proton motive force for electron transport phosphorylation [43–45]. *C. jejuni* relies on the use of amino acids and the TCA cycle intermediates as carbon sources [46]. The current knowledge of *C. jejuni in vivo* metabolism is based on amino acid utilization to support growth and establishment of colonization in the host intestines. In comparison to *Salmonella,* the metabolism of *C. jejuni* is very restrictive.

As *S. enterica*, also *M. tuberculosis* is a prototrophic organism, but despite their basic metabolic similarities, these pathogens exhibit characteristic physiological differences. *M. tuberculosis* has a special waxy cell envelope, which might explain the unusual transporters that are involved in its uptake of nutrients [47]. Other than *S. enterica*, which preferred glucose as the major carbon source, *M. tuberculosis* possesses numerous *fad* genes encoding the enzymes for fatty acid uptake and degradation by β-oxidation, as well as genes for the uptake and catabolism of cholesterol

and all genes for the glyoxylate shunt. *M. tuberculosis* can therefore use fatty acids and cholesterol as carbon sources [48]. DGEP data from *M. tuberculosis* grown in resting and activated BMMs (bone-marrow-derived macrophages) compared with *M. tuberculosis* grown in standard medium showed substantial upregulation of all of the genes characteristic for a C_2-based metabolism [49]. Together with the upregulation of many *fad* genes and the genes for glycerol-3P uptake and metabolism, there is good evidence that fatty acids and possibly glycerol or glycerol-3P are the preferred carbon sources of *M. tuberculosis* in proliferating BMMs [4]. A further discovery was the need for an ABC (ATP-binding cassette) disaccharide transporter for *M. tuberculosis* growth in a mouse infection model [50], suggesting that either carbohydrates serve as additional carbon sources early in infection or that a switch from carbohydrates to lipids occurs later during infection. Furthermore, *M. tuberculosis* has a dependency on fatty acids in acute and persistent *M. tuberculosis* infections in mice [51].

A comparison between *S. enterica* and *L. pneumophila* shows that both pathogens use glucose for metabolism. Previously *L. pneumophila* was supposed to solely feed on amino acids while residing in host alveolar macrophages [52]. Indeed, amino acids are efficiently used as carbon and energy sources *in vivo* [53]. However, recent studies show that also glucose is metabolized by *Legionella* during infection of eukaryotic cells. Glucose is predominantly degraded by the KDPGP and only in small quantities by glycolysis. The nonoxidative branch of PPP also accounts for small amounts of glucose catabolism [54]. Furthermore, ^{13}C-IPA results indicate that a complete and active TCA cycle occurs in *Legionella* and that the ability to synthesize amino acids *de novo* applied for Ile, Leu, Val, Phe, Met Arg, and Tyr. ^{13}C-IPA further dismisses a functional glyoxylate shunt [55].

Taken together, *S. enterica* prefers C_6 substrates such as glucose, glucose-6P, and gluconate for intracellular metabolism and live in the SCV in the host cell. *C. trachomatis* has a very restrictive metabolism, unable to react on nutrient-based environmental changes, and only consume energy sources until they are depleted, but has a complex developmental cycle. *L. monocytogenes* prefers C_3 substrates such as glycerol and glycerol-3P and lives cytosolic. For *S. flexneri* there is an interesting difference, it could be that the preferred carbon substrates and the carbon metabolism are strain specific, but also lives cytosolic and has direct access to host nutrients such as *L. monocytogenes*. *C. jejuni* has very restrictive metabolism in contrast to *S. enterica* and uses amino acids and TCA cycle intermediates as carbon sources. *M. tuberculosis* prefers C_2 substrates such as fatty acids and C_3 substrates such as glycerol and glycerol-3P for intracellular metabolism, but it could be that *M. tuberculosis* changes the carbon metabolism during infection from C_6 carbon substrates (early state of infection) to C_3, C_2 carbon substrates (later state of infection). Similarly to *S. enterica*, *M. tuberculosis* also lives in an own compartment (modified phagosome) in the host cell. *L. pneumophila* uses glucose similarly to *S. enterica* for intracellular metabolism, but glucose is predominantly degraded by the KDPGP (*S. enterica* uses glycolysis) and only small quantities by glycolysis. Besides glucose, *L. pneumophila* efficiently degrades amino acids for intracellular metabolism.

Salmonella Induce Networks of Tubular Structures – Access to Host-Derived Nutrients?

Regulated by different transcription factors [4, 15], *Salmonella* metabolism adapts to the intracellular lifestyle in host cells. Gene expression analyses suggest upregulation of genes for uptake of magnesium, phosphate, and iron. Furthermore, it could be shown that glycolysis, KDPGP, and TCA cycle are highly expressed by *Salmonella* in the SCV of macrophages and epithelial cells [7]. In the early phases of infection, the SCV is a nutrient poor, underfed place [7, 21], so that intracellular *Salmonella* has to deploy specific mechanisms to redirect vacuolar transport to make use of host-derived nutrients [56–58]. During formation of the SCV, acidification of the intravacuolar environment takes place [59] and sensing acidification is considered as stimulus for activation of virulence functions. It is known that *Salmonella* in the SCV interfere with the microtubule (MT) cytoskeleton and cellular transport. Following various trafficking tracer in the infected host cell, it was observed that virulent intracellular *Salmonella* alter exocytic transport and recruit exocytic transport vesicles to the SCV. The recruitment of post-Golgi vesicles suggests that the SCV can interact with exocytic pathway of the host cell, mediated by interference of *Salmonella* with MT-dependent trafficking events. The interchange with the secretory pathway may be important for the supply of the proliferating bacterial population with nutrients and to provide membrane material to the growing SCV [56]. Microtubules also guide the formation of SIFs, that is, tubular extensions of the SCV. SIF are tubular membrane structures that contain various late endosomal/lysosomal markers (e.g., LAMP1) that are also characteristic for the SCV [58]. These structures display, in the early phase of infection (4–5 h), highly dynamic properties in various types of host cells. Initially SIF extend, branch, and contract rapidly, and later (≥8 h after infection) form a stabilized network. Mutant strains defective in the SPI2-T3SS and unable to translocate effector proteins do not induce SIF formation. It is known that a subset of SPI2 effector proteins is involved in SIF formation consisting SifA, SseF, SseG, SopD2, and PipB2 [35, 60–63]. Further, it was demonstrated that the function of MT motor proteins is necessary for SIF formation. A model was proposed for dynamic extension and contraction of SIF and how SIF recruit membrane vesicles to and fuse with the SCV (Figure 3.3) [58]. As mentioned, during the early phase of SIF formation, there is a rapid extension and contraction or collapse of SIF. SIF grow out from the SCV both by continual fusion of vesicles with the tip of SIF and by pulling force generated by MT motors associated with the tips of SIF. SIF growth can be directed toward minus ends or plus ends, depending on the proportion of dynein and kinesin motors, respectively, that are recruited [58]. In the model every fusion step introduces membrane material into the SIF and partially and temporarily relaxes the internal stress. However, when the tip of SIF is pulled by MT motors too far without fusion, the elastic stress in the membrane increases and reaches a critical threshold. If MT motors detach from MT or from SIF membranes, the SIF collapses rapidly. An explanation is the higher velocity of SIF contraction compared with extension. The availability of such a stabilized SIF network in later phase indicates consumption

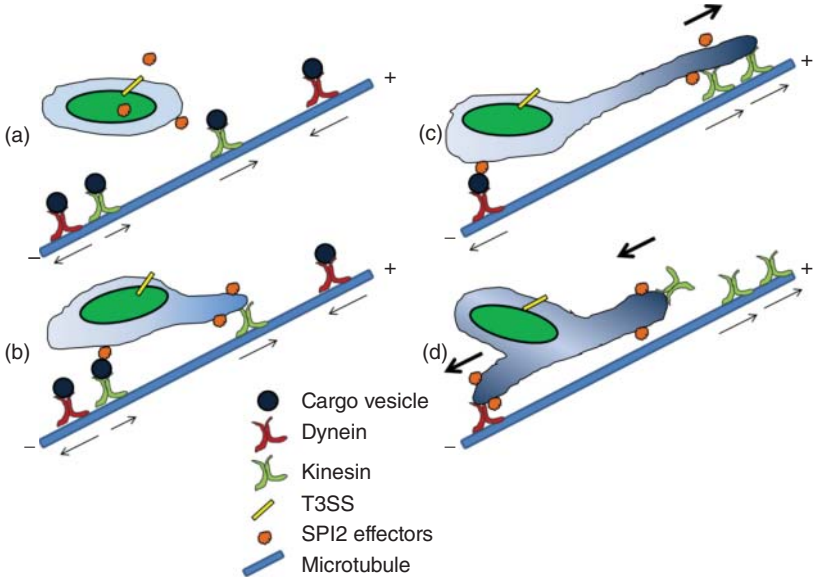

Figure 3.3 Model of the dynamic extension and contraction of SIF and possible accession to membrane vesicles. (Reproduced from Rajashekar *et al.* [58], with the permission of John Wiley and Sons.) (a) *Salmonella* within the SCV translocate effector proteins of the SPI2-T3SS (orange circles). (b) Membrane vesicles transported on microtubules (MTs) are recruited and fuse with the SCV by the activity of effector proteins. These events not only allow the enlargement of the SCV and delivery of luminal content to the SCV (indicating blue shading) but also lead to accumulation of motor proteins on the SCV. (c) Increased accumulation of motor proteins results in a pulling force on the SCV membrane and formation of tubular extension. (d) If pulling forces are too high, motor proteins could lose contact to SIF membranes or MT, resulting in contraction of SIF. Depending on the nature of the motor protein recruited, SIF extend toward the minus (–) or plus (+) end of MT. The extension toward the plus end appears to be dominant [58].

of vesicles available for fusion [58]. It was observed that the SCV interacts continuously with endocytosed material [57]. Recent studies showed a massive fusion of endocytic material with the SCV over the SIF. In addition to the vacuolar lifestyle, a cytosolic form of intracellular life was discovered, which is shown in the following.

S. enterica Has a Bimodal Lifestyle in Epithelial Cells

Salmonella can replicate in the SCV as well as in the cytosol of host cells [10, 64]. The replication in the SCV is dependent on SPI2-T3SS function [10]. Bacteria lacking a functional SPI2-T3SS and therefore unable to translocate any effectors remain within an immature SCV, which does not form membrane tubules and is defective in juxtanuclear positioning [65]. These mutants also have an intracellular replication defect, in both macrophages and epithelial cells, although in epithelial cells the defect is not apparent during the initial replication phase [35, 64, 66, 67]. The SCV has been extensively studied, but little is known how *Salmonella* adapt to and/or

modify the cytosolic niche. It was shown that cytosolic *S. enterica* replicate to higher numbers than vacuolar bacteria in a polarized epithelial cell model, a phenotype termed *hyper replication* [68]. This study also showed that these two intracellular populations of bacteria are transcriptionally distinct. The intravacuolar bacteria are SPI2-induced, whereas the cytosolic bacteria are SPI1-induced and flagellated. An analysis of infected HeLa cells showed that although the majority of HeLa cells contain vacuolar *Salmonella,* these bacteria replicate rather inefficiently, threefold over 8 h, and cannot account for the net levels of intracellular replication in these cells. In contrast, while less than 20% of infected cells contained cytosolic bacteria, this population replicated 40-fold or more, so that epithelial cells were rapidly filled with bacteria. Thus, in epithelial cells, net replication of intracellular *Salmonella* is a reflection of both cytosolic and vacuolar replication [69]. Epithelial cells containing hyper-replicating *Salmonella* undergo inflammatory cell death and are ultimately extruded from monolayers, both *in vivo* and *in vitro*, and the invasion-primed *Salmonella* are released into the extracellular milieu [68]. This increased turnover of mucosal epithelium provides the host with an ideal defense mechanism against infection [70]. *Salmonella* possibly takes advantage of this process as a unique means of bacterial egress. Knodler *et al.* hypothesized that a vacuole maturation defect leads to the cytosolic release of a small, but significant, fraction of bacteria. The nutrient-rich cytosol supports a high bacterial replication rate and reprograms virulence gene expression toward invasion. The cytosolic load of bacteria is sensed by the host cell, leading to inflammatory cell death and extrusion, releasing the invasion-primed *Salmonella* into the lumen of the gastrointestinal and biliary tracts. Escape into the lumen allows *Salmonella* to infect secondary cells rapidly and may also contribute to host-to-host transmission. Thus, by subverting a host-dependent cell turnover event, *Salmonella* completes its infectious cycle [68].

Salmonella Metabolism Limits Possibilities for New Antimicrobials

Due to large metabolic flexibility of *Salmonella,* it is difficult to interrupt the metabolism of *Salmonella.* This passage explains why no new antibiotic can be developed. Becker *et al.* [27] obtained *in vivo* information for over 700 *S. enterica* enzymes from network analysis of mutant phenotypes, genome comparisons, and *Salmonella* proteomes from infected mice. More than 400 of these enzymes are nonessential for *Salmonella* virulence. Investigation of the essential enzymes showed that most of the characterized *Salmonella* metabolic enzymes are unsuitable as antimicrobial targets, as their inactivation failed to abolish *Salmonella* virulence. A total of 155 enzymes were identified as candidates for inhibition, 64 of them are conserved in a diverse set of major human pathogens, but almost all belong to metabolic pathways that are inhibited by current antibiotics or that have previously been considered for antimicrobial development. Eight newly identified candidates all have very high sequence identities to human enzymes with key roles in central metabolism, suggesting a direct risk for unwanted side effects. Taken together, these results suggest a shortage of new metabolic targets for broad-spectrum antibiotics [27].

Conclusions

Salmonella is an intracellular pathogen with an extraordinary metabolic versatility. In contrast to other pathogens, such as *M. tuberculosis* [71] or *C. jejuni* [43], *S. enterica* is less specialized for a specific host environment but rather a generalist. Therefore, *Salmonella* is able to use a broad spectrum of routes for central carbon metabolism. For example, glucose, the favored C-source during SCV colonization, can either be degraded by the glycolytic pathway or the KDPGP, with the former being preferentially used. The TCA cycle as the major biosynthetic origin of precursor and provider of reductive agents is complete and is supported by important anaplerotic reactions that lead the metabolic flux to the TCA cycle, mainly by the PEP carboxylase [5]. Furthermore, by the PPP, *Salmonella* can generate NADPH required for reductases in oxidative stress response. *Salmonella* is well equipped with these abilities to rapidly adapt to various environments during the passage through the host body.

The great repertoire of metabolic abilities makes it challenging to find targets for inhibition of metabolic functions during infection. It is known that absolutely essential enzymes are almost exclusively found in pathways relating to the biosynthesis of compounds that *Salmonella* cannot efficiently obtain from the host, or in central intermediary metabolism and energy production. Identified essential enzymes for inhibition represent already known antimicrobial targets, so that only few new classes of antibiotics have been approved in the past 30 years [27]. *Salmonella* efficiently modifies its unique vacuolar compartment for its benefits, a fact that complicates accession of inhibitors to *Salmonella.* Knowledge on the transport of nutrients to the SCV is limited, and research is currently focused on this issue. The SPI2-T3SS of *Salmonella* is responsible for translocation of over 30 virulence proteins into the host cell cytoplasm [10]. SifA is the most prominent effector in maintaining the integrity of the SCV and generates a network of tubular membrane compartments including SIF and recently identified further tubular compartment [72]. It is important to understand the precise function of these structures for the intracellular lifestyle of *Salmonella* in host cells. Future projects will focus on host–pathogen interactions, regarding the regulatory and immune response from the host during severe infections by *Salmonella*. In general, the link between metabolism and infection has to be further explored to improve therapeutic options against *Salmonella* [5].

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4
The Human Microbiome in Health and Disease

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Abstract

The human body is a complex assemblage of cells that form tissues, functional organs, and systems. This is the framework upon which complex bacterial communities are built. The body is colonized by a vast number of microbes, collectively referred to as the human microbiota. The analysis of the human microbiota is an exciting and rapidly expanding field of research. In the past decade, the biological relevance of the microbiota for human health has become very apparent. The interest generated has led to an explosion of research in the field but some of the progress has been impeded by some conflicting and inconclusive results. In this chapter, we attempt to convey both the intriguing potential of microbiota research and some challenges currently facing research in the area. We discuss the different techniques available for studying the microbiota, highlighting their advantages and disadvantages. We emphasize the importance of standardizing methods and analyses between centers, to distinguish true variation of the microbiota in unhealthy individuals from normal interpersonal variation. We report on recent literature describing the effect of diet and lifestyle on the microbiota and evaluate the potential role of the microbiota in a range of intestinal disorders. Attempts to manipulate the microbiota are detailed, such as administration of specific bacteria as probiotics, and wide-ranging perturbations of the microbiota by fecal transplantation.

Introduction

The human microbiota is among the most complex and diverse biological systems on the earth, traditionally believed to consist of 10 bacterial cells per human cell in the body, a figure which has recently been questioned [1]. The microbiome contains approximately 100 times the number of unique genes in the human genome [2] and is known to influence an array of biological and chemical processes in the body including, but not limited to energy balance [3], obesity [2, 4–6], type 1 and 2

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diabetes [7, 8], and cancer progression [9, 10]. A number of studies have revealed the role of microbiota in a wide variety of chronic and acute, localized and systemic conditions, and it has been therefore referred to as a *forgotten organ* [11]. The importance of the microbiota has been shown to be so crucial that systems biology should now take into account bacteria when assessing health and disease [12]. The microbiota plays a vital role in synthesis of some vitamins, including vitamins B and K2 and other bioactive metabolites [13]. These metabolites have functions ranging from inhibition of pathogens, metabolism of toxic compounds to modulation of host metabolism [14]. They possess the enzymatic capacity to digest complex polysaccharides that are otherwise indigestible by human beings [15]. The composition of the gut microbiota depends on a number of factors including age [16–18], individual genetics [19], diet [17, 20–22], lifestyle [23, 24], and geographical location [16, 22]. The diversity and complexity of these microbial populations varies greatly between individuals and by anatomical body site [25–27]. In fact, the bacterial communities vary more at different body sites on one individual than at the same site at a population level across different individuals [26]. The most comprehensively studied one of these bacterial populations is that of the gastrointestinal tract. The gut microbiota has been shown to be relatively plastic, which has enabled it to be successfully manipulated and examined in a number of animal and human studies. Yeasts, viruses, and archaea are also components of the human body, but are outside the remit of this chapter.

Methods for Characterizing the Microbiota

Until the 1990s, bacteriological culture was the main method for studying bacterial communities. The main advantage is that it is a cheap method, but it is labor intensive and gives an incomplete representation of the bacterial diversity as perhaps as little as 30% of the bacteria composing the gut microbiota have been cultured [28]. This figure should not be viewed as the upper limit of the culturable fraction of the microbial community; it merely reveals current deficiencies in our knowledge of the growth requirements of the remaining bacteria. It is probable that a far higher percentage could be grown, but appropriate conditions to grow the bacteria are yet to be developed or discovered. In the past 20 years, the advancement of culture-independent techniques has allowed for a deeper knowledge of microbiota composition [29]. There are a number of important considerations when embarking on culture-independent studies including cost, expertise in the laboratory, bioinformatic analysis abilities, and the biological questions being asked. Microbiota study can be complicated by the wide variety of techniques at the disposal of scientists. Sequencing technology has advanced hugely in the past decade or so [30] and there are now a vast array of different techniques available, the most commonly used ones are shown in Figure 4.1. Each technique has particular advantages and disadvantages, as outlined in Table 4.1.

The most extensively used next-generation technique is sequencing of the small subunit ribosomal RNA (16S rRNA) gene. This information processing gene is

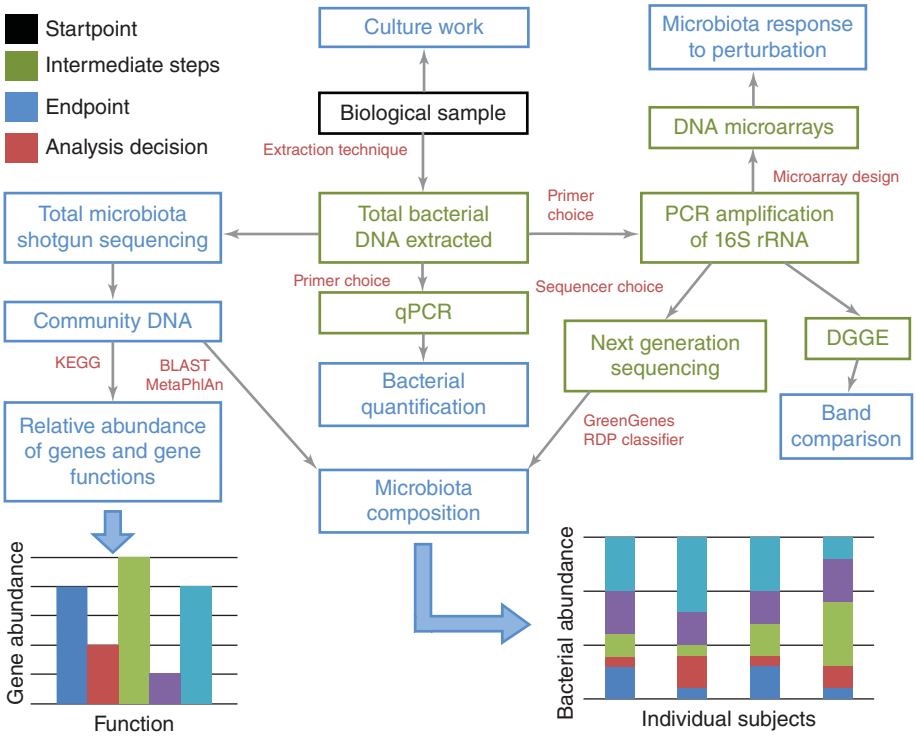

Figure 4.1 The wide variety of different choices available to characterize the microbiota. Each method has advantages and disadvantages. Each step separating the sample from the endpoint is a potential source of bias.

conserved in all bacteria and thus can give a representation of the entire bacterial diversity in a sample [29]. It has a combination of conserved sites, which can be used as annealing sites for polymerase chain reaction (PCR) primers, and nine variable regions that act as identifiers of the bacteria. There are numerous different primer sets available that amplify different parts of the 16S gene, and these can all affect and potentially bias results [31, 32]. This approach has other limitations also, because certain bacteria may not be amplified by the "universal" primers, leading to data that can underreport or even completely miss certain bacterial taxa [31–34]. Correct primer choice is thus essential to ensure maximum coverage of the bacterial diversity of any given sample, and standardization of primer sets across multiple studies would enable greater accuracy when comparing findings. Another issue with 16S rRNA is that it exists in variable copy number in different bacteria, meaning it is not truly quantitative, and the proportion of bacteria returned may be skewed by the differing number of 16S rRNA copies present in different bacterial genomes. The primary sequence platform of choice for 16S amplicons was originally pyrosequencing by the 454 platform, but in recent years this has been superseded

Table 4.1 Advantages and disadvantages of different characterization techniques.

Method	Core technology	Advantages	Disadvantages
Culture analysis	Isolation of specific bacteria on selective media	Cheap, semiquantitative	<30% culturable, labor intensive
Denaturing gradient gel electrophoresis (DGGE)	Separation of 16S PCR products by applying a denaturing gradient to a gel	Fast, semiquantitative, bands can excised for further analysis	No phylogenetic identification, PCR bias
Quantitative PCR (qPCR)	Measuring proportion of specific bacterial target using a fluorescent probe as sample undergoes PCR	Phylogenetic identification, quantitative, fast	PCR bias, specific/known targets only, low-level species difficult to distinguish
DNA microarrays	Fluorescent probe hybridizing with complementary sequences	Phylogenetic identification, semi-quantitative, fast	PCR bias, expensive, already known DNA sequences only
16S rRNA sequencing	Massive parallel sequencing of partial 16S rRNA amplicons (e.g., 454 pyrosequencing, Illumina®)	Phylogenetic identification, semiquantitative, fast, detect/identify unknown bacteria	PCR bias, expensive, labor intensive, computationally demanding
Shotgun sequencing from total DNA	Massive parallel sequencing of the whole genome (e.g., Illumina)	Phylogenetic identification, quantitative	Expensive, computationally demanding

by the advancement of Illumina technology, such as HiSeq and MiSeq, which utilize reversible-terminator sequencing by synthesis technology [35].

Another opportunity facilitated by next-generation sequencing is shotgun metagenomics. This eliminates the necessity for a PCR step, thus removing the inherent bias introduced by DNA amplification. The main barrier and disadvantage of this approach is that it remains computationally intensive. It has the advantage of sequencing genomic DNA from all bacteria present in the sample and can therefore be used to predict function of the microbiota through identification of metabolic capabilities [16]. It allows for complete genes and potentially genomes to be examined, allowing for the identification of specific genes and gene pathways of interest. This true metagenomic approach is currently the most powerful tool for analysis of the microbiota and can build a picture of community function [23, 36], rather than solely observing community composition, as provided by 16S-based

approaches. One large meta-analysis found that almost 40% of the genes from each individual are shared with at least half of the individuals of the cohort and identified 75 species common to >50% of individuals and 57 species common to >90% [12]. They attributed 99.1% of all genes in the human gene catalog to bacteria, with 0.1% of viral and eukaryotic origin, and the remainder being of archaeal origin. In addition, metagenomics may identify enriched genes and offer preliminary insights into relevant functional changes but may not provide a comprehensive systems-level understanding of the alteration and its prospective effect on the host–microbiome superorganism [27, 37, 38].

Studies have shown considerable variation in the microbiota profile of individuals depending on where in the gut the sample is taken from [27]. The gastric microbiome [39] is distinct and biopsy samples from the colon are also significantly different from those obtained from stool [40]. This means that depending on the site focused on, different populations are observed. This can be important especially when studying clinical outcomes, as much of the digestion, metabolism, inflammation, and other processes occur high in the gastrointestinal tract. Stool therefore may not always be the most appropriate sample for gauging microbial diversity and efficacy of clinical intervention to modulate the microbiota. However, fecal samples remain the most widely studied as stool is readily available and can be collected noninvasively, which facilitates repeated sampling for longitudinal studies. Subject recruitment for invasive sampling is inherently difficult, and it is challenging to recruit healthy controls for such studies. Therefore, this review will concentrate on data derived primarily from stool, for which sampling techniques vary. Processing stool within hours of voiding remains the gold standard but may often be unfeasible for large studies in which samples cannot be swiftly transferred to the laboratory [16, 24]. Comprehensive studies have investigated the optimal storage and extraction procedures for stool for microbiota analysis [41, 42]. It has been shown that certain taxa such as *Bacteroidetes* can be negatively affected by the freeze–thaw process [41] and the proportion of Gram-positive bacteria is significantly increased by the presence of a mechanical lysis step [42]. In summary, despite advances in technologies, each chemical, physical, and biological process between the sampling and generation of data from amplicons has the potential to introduce bias, thus affecting the results. Many laboratories have their own method of processing samples, and a greater streamlining and collaborative effort is needed among scientists to ensure that these factors are kept to a minimum to reduce the interproject variation currently introducing confusion and conflicting results [31, 41].

An effective method of studying the microbiota is to combine numerous smaller studies in a particular area into a large meta-analysis [43–45]. When performing meta-analysis of currently available literature, it is becoming apparent that it may be constructive to move away from traditional aggregation techniques. One alternative is to perform individual patient data meta-analysis (IPDMA) to collate the response of individuals to a treatment rather than taking the overall mean of a study [46, 47]. This allows for more precise analysis and examination of patients on an individual basis, which may enable a greater clarity. It enables the study of secondary outcomes and is not tied to the one metric studied in the original study. Collaboration between

groups is essential for this approach, and it would necessitate divulging of data on a scale far greater than generally occurs at present, but if possible, could increase the efficacy of these meta-analyses.

Diet and Geographical Factors

The human host and the microbiota combine to form a superorganism that determines an individual's response to a range of external influences (Figure 4.2). Diet has a role in the development of many gastrointestinal diseases, several of which are also associated with change in microbiota [2–4, 6, 17, 48]. However, a direct link between diet, microbiota, and disease pathogenesis has not been definitively shown for many of these diseases, even in animal models. Mice studies have shown huge variation in microbiota in response to perturbations in their diet [3]. However, a recent study has highlighted the pitfalls of using a mouse model to study the effect of diet on the microbiota of humans. It suggests that while the diet can account for 57–61% of the microbiota variance in mice, it may account for as little as 10% of the overall interpersonal variation in humans [49]. The human microbiome is far more complex and shares less than 5% of identical genes with the mouse microbiome. Laboratory mice are also genetically bred to have identical genomes, meaning the specific response to dietary intervention seen in mice may not transfer to a (relatively) genetically distinct human population. Another important concern is that the dietary interventions in murine studies to date are more extreme than those possible in humans and are performed under optimal conditions and with constant observation not possible in human studies; thus, careful consideration must be given to extrapolating evidence found in mice studies to conducting human trials. However, extreme short-term diets have been shown to have a pronounced effect on the human gut microbiota [50].

It has been shown that microbiota composition varies widely by geographic location [16, 22, 23], and much of the variation between populations appears to be diet and lifestyle-dependent. Populations in many developed countries in Europe and America consume a "Western" style diet, high in protein and fat, but relatively low in fruits and vegetables. The associated microbiota can be characterized by a high ratio of Firmicutes to Bacteroidetes [45], while in developing countries, such as Burkina Faso, this ratio is reversed [22]. This apparently reflects the microbial response to diet in these countries, which is typically low in fat and animal protein and rich in starch, fiber, and other plant-derived polysaccharides. inflammatory bowel disease (IBD) incidence is much higher in westernized countries. In a large systematic review of the literature [51], high dietary intakes of total fats, polyunsaturated fatty acids, omega-6 fatty acids, and meat were found to be positively associated with an increased risk of ulcerative colitis (UC) and Crohn's disease (CD). High fruit and fiber intake was found to decrease the risk of CD, with high vegetable intake decreasing the risk of UC. Dramatic distinctions are also noted between urban American populations, rural Malawians, and Venezuelans [16]. The most dramatic differences were between

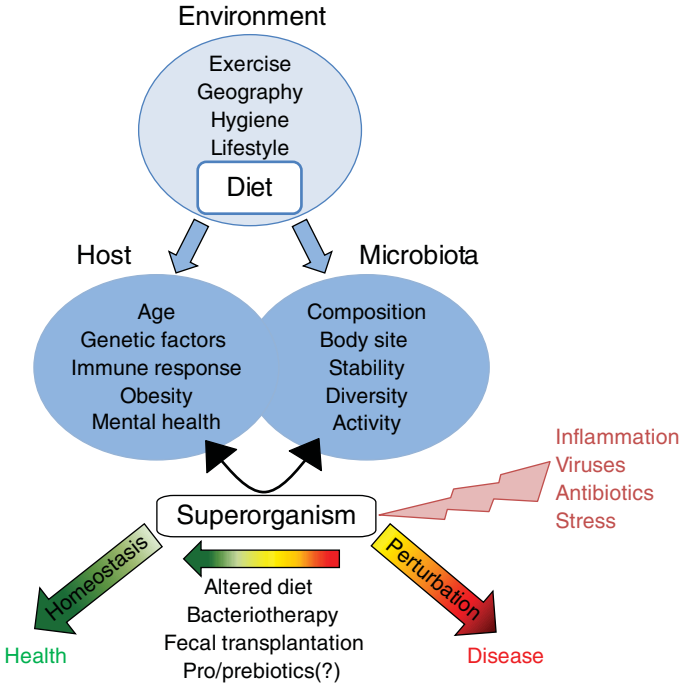

Figure 4.2 The complex interactions between host, microbiota, and environment. Both the host and the microbiota have a range of characteristics unique to an individual. The health status of the host–microbiota superorganism can be altered by a number of external environmental factors, perhaps most significantly by diet. Host factors and the composition of the microbiota determine an individual's response to stressors. These may or may not alter the homeostasis of the superorganism. A number of microbial therapeutics are currently under investigation to restore health, including bacteriotherapy, altered diet, and fecal transplantation. Probiotics and prebiotics are also candidate interventions, but efficacy for microbiota modulation is still unclear due to lack of mechanisms of action and inconsistency between studies.

the American cohort and the other two nationalities, reflecting the dramatically different lifestyle being led. In a related example, the Hadza tribe in Tanzania have a unique microbiota that is adapted to their primitive hunter–gatherer lifestyle [24]. They have a much higher overall microbial diversity compared to Western populations. They are enriched for previously unknown fiber-degrading microbes and rare "opportunistic" bacteria, while vastly depleted in "health-promoting" bacteria, such as the genus *Bifidobacterium*. Indeed, they are the only currently studied population with a reported absolute absence of *Bifidobacterium*. No infants were analyzed in this study, and with infants routinely being found to be dominated by *Bifidobacterium,* it would be interesting to examine what persists in the young in this cohort. Studies of the Hadza tribe and populations of other developing countries highlight an important question for microbiota research, namely whether or not particular

commensal bacteria can truly be termed either *beneficial* or *detrimental*. These findings are redefining our understanding of these commensals, demonstrating that specific taxa's benefits are context dependent, and function as part of a community rather than in isolation.

A study examining the subjects following consumption of a diet composed entirely of either plant or animal products for 4–5 days found that the microbiota activity mimicked the differences observed between herbivorous or carnivorous mammals, respectively [50], demonstrating trade-offs between carbohydrate and protein fermentation. Despite the two diets having similar calorific content, the meat-based diet led to significant weight loss in the subjects after 3 days. *Bilophila wadsworthia* is a sulfate-reducing bacterium whose production of H_2S is believed to inflame intestinal tissue [52]. In the subjects consuming the diet enriched in animal produce (meat and dairy), the proportion of *Bilophila* significantly increased and was positively correlated with dairy and saturated fat intake. This diet also increased bile acid concentrations and significantly increased the abundance of microbial DNA and mRNA encoding sulfite reductases, which provides further evidence for the hypothesis that diet may contribute to the development of IBDs. Fecal deoxycholic acid concentrations (DCA) increase significantly on a diet enriched in animal produce [20], and this secondary bile acid is the product of microbial metabolism. It has been shown that it can damage DNA and promote liver cancer [53]. Bile salt hydrolases also increase in abundance, which are prerequisites for DCA production. DCA elevation may contribute to the microbial disturbances observed as DCA severely inhibits the growth of many intestinal bacteria, including *Clostridium perfringens*, *Bacteroides fragilis*, lactobacilli, and bifidobacteria [54]. However, when consuming plant-based diets, plant viruses were recovered from stool, establishing that in general, viruses can transit the stomach and potentially colonize through consuming plant matter. The metabolic consequences to a high-protein and low-carbohydrate diet have been demonstrated in other studies [55], showing a linear correlation between carbohydrate consumption and short-chain fatty acids (SCFAs) concentrations. Butyrate is an important SCFA, which may prevent colorectal cancer [56, 57] and colitis [58]. A high-protein, low-carbohydrate diet delivers an increase in potentially harmful metabolites and a decrease in cancer-protective metabolites overall [20]. The metabolites in the study converged to similarity, despite disparate starting points, and show greater adaptation to diet than the actual microbiota community composition. These studies show that the microbiota can alter metabolite production in response to diet. This perhaps reflects a response to evolutionary pressures. Microbial communities that could appropriately shift their functional repertoire in response to a change in diet would have enhanced dietary flexibility, as food sources may have varied due to resource availability.

The Western diet is a very recent development on an evolutionary timescale, and the Paleolithic diet of tribes such as the Hadza may be more closely related to our ancestors' diet. Its significance remains unknown at present, but it is a fascinating example of a distinctly unique, apparently healthy, functioning microbiota. These findings indicate that low-carbohydrate consumption may be a metabolically

dangerous dietary choice, which may have long-term health implications [20, 49, 55]. The current trend toward high-protein, low-carbohydrate diets for weight loss and weight maintenance may be deleterious in the long term. The high levels of fat in these diets, which initially concerned nutritionists, may not be the most detrimental component, but may be dangerous due to the lack of important protective metabolites such as SCFA produced by carbohydrate digestion.

Functional Gastrointestinal Disorders

Many diseases are associated with alterations in the gut microbiota, including IBD [59]. IBD comprises a group of idiopathic, chronic, inflammatory intestinal disorders [45]. IBD are intermittent diseases with clinical manifestations that are unpredictable and unstable during relapse. CD and UC are two of the main components of IBD and vary in terms of location, inflamed areas, and histology [60], although clinical classification is difficult due to the overlapping pathological characteristics. Genetic susceptibility in chronic inflammation displays 50–60% concordance of monozygotic twins with CD [61]. However, this still leaves 40–50% of individuals with identical genetic make-up discordant for the disease. *Faecalibacterium prausnitzii* is an apparently protective bacterium with depleted microbiota abundance in several intestinal disorders, including colorectal cancer, UC, and in particular CD, in comparison to healthy controls. *Escherichia coli* and *F. prausnitzii* have been used to characterize IBD dysbiosis, and the ratio between the two can be useful to aid in IBD phenotype classification [62]. The potential role of the microbiota in IBD pathogenesis has been extensively reviewed elsewhere [51, 63].

Irritable bowel syndrome (IBS) is a chronic disease of unknown cause but one that is regarded as multifactorial, with genetic, neurobiological, and psychosocial elements. It may affect 10–20% of the population of developed countries and results in significantly reduced quality of life and reduced work productivity [64]. Animal and human data supports a role for the microbiota in the etiology of IBS [59, 65, 66], but the complexity of IBS means more specific mechanistic details currently remain beyond our understanding [67]. Onset or relapse may be triggered by external factors such as chemicals and viruses [68]. There is a difference in microbiota between IBS subjects and healthy controls [65, 66] with some apparently conflicting results [69], but the most common profile shows an increase in Firmicutes relative abundance and a reduction in Bacteroidetes proportions in IBS sufferers [70]. Over half the IBS subjects in that particular study had a high Firmicutes–Bacteroidetes ratio, with the remainder showing no detectable changes in the fecal microbiota. Different clinical subtypes of IBS have a distinct microbiota profile, with diarrhea-associated IBS having higher proportions of *Enterobacteriaceae* and a reduction in the proportion of *F. prausnitzii* compared to healthy controls [71]. The overall microbiota diversity is also lower in these patients. However, another subset of IBS patients was also found to have increased microbiota diversity [70], which may be due to increased bacterial load once inflammation has occurred, potentially exacerbating the condition. This study showed IBS microbiota populations group

together in clusters, some of which overlap with healthy controls. This means IBS microbiota profiles remain difficult to use as predictors of IBS susceptibility or disease activity. Interestingly, normal-like IBS patients had a higher incidence of depression relative to the control group, while IBS sufferers with an altered microbiota did not [70]. This builds on the theory that IBS, or subtypes of IBS, may be linked to processes in the brain such as stress, anxiety, and depression [72, 73].

Alteration of metabolite production by the commensal gut bacteria, particularly SCFA such as butyrate and acetate, has been examined for a possible role in IBS. Individuals with diarrhea predominant IBS have been shown to produce less overall SCFA than healthy controls do [74]. Sulfite-producing bacteria have already been mentioned in this review as a potential indicator of IBS pathogenesis [52] and an increase in levels of H_2S, H_2, and acetate in feces has been observed in the constipated subtype of IBS [75]. This production of sulfides is one part of an altered intestinal fermentation process and is accompanied by change in microbiota group composition. In a mouse model, this IBS subtype produces less butyrate than healthy controls, which is needed for the production of SCFA in the cecum [54]. Where the microbial signature is related to disease phenotype, it is difficult to establish whether the difference is due to an abnormal, diseased gut causing an alteration of the microbiota or if the microbiota drives the change in the diseased gut. Evidence exists from animal trials that either possibility could be true [76].

The role of viruses in the onset of IBS is largely based on observational studies but could involve the disruption of the mucosal barrier. Exposure of the gut epithelium to commensal bacteria results in an innate immune response, leading to a proliferation of commensal-specific and pathogen-specific T cells [77]. These long-lived T cells can then migrate and induce pathological inflammation. Moreover, because the mucosal immune system enables lymphocytes to migrate among different mucosal tissues, the resulting T cells generated at disparate body sites may migrate to the gut. This may explain the high relapse occurrence of IBS with respiratory and other diseases.

The effect of probiotics on IBS was assessed in a systematic review [78] that tentatively showed that probiotics appeared to be benefit to those suffering from IBS but the exact probiotic species inducing the benefit and indeed the magnitude of benefit remain uncertain. *Lactobacillus acidophilus* and *Lactobacillus paracasei* have both been reported to modulate pain and visceral hypersensitivity perception [79, 80]. It was previously hypothesized that systemic antibiotic treatment would exacerbate IBS [81, 82], but in a recent trial with a nonabsorbable antibiotic, rifaximin performed marginally better than placebo [83]. This type of antibiotic treatment may be an avenue to explore in future, perhaps in combination with better defined probiotic treatment.

Clearly, there is a plausible role for the microbiota in the etiology of IBS but the exact taxa that exert the most influence are not consistent across studies. This could be due to geographical disparity or differing experimental analyses. Heterogeneity of different subtypes of IBS results in conflicting reports of differences from healthy controls. Variation in methods used to assess the microbiota in different studies may also contribute to these apparently conflicting reports. Large studies of

phenotypically well-characterized IBS patients are required to narrow the bacteria of interest and remove background noise provided by normal intestinal variation between individuals.

A holistic approach is needed for any further research into IBS. A myriad of intertwined factors are clearly combining to result in individuals being affected by these conditions. The intestinal microbiota is believed to be stable over time in healthy adults [84, 85], with one study finding that 60% of the microbial strains in the gut are retained over the course of a 5-year sampling period [86]. With chronic diseases such as IBS, it would be interesting to sample individuals longitudinally to attempt to detect differences during periods of remission and relapse. Future studies should include detailed information on the mental state to determine the impact of stress, depression, and other psychological factors affecting the gut brain axis, assessing the influence of the brain in conjunction with microbiota-based fluxes. It would also be interesting to see if two distinct methods of disease progression exist, one bacterial driven and one psychologically driven. Finding a "smoking gun" bacterium appears an unlikely scenario with the vast amount of bacterial information available and the great numbers of altered bacterial taxa whose existence or absence is associated with varying degrees of confidence to IBS.

Manipulation of the Microbiota

The use of probiotics in foodstuffs to attempt to manipulate the microbiota is generally considered to be safe [87] and has become quite commonplace commercially. There is a debate about the efficacy of probiotics in treating diseases [88, 89], and whether the product does indeed contain the purported probiotic strain claimed. In a recent examination of five human and eight animal commercially available probiotics, it was found that label descriptions of organisms and concentrations accurately described the actual contents of only 2 of the 13 [90]. The difficulty in demonstrating clinically significant outcomes from consuming probiotics means great care is needed in regulating the use of the word probiotic and limiting its use to bacterial species with proven health benefits as concluded by a recent review by experts in the field [91].

A recent large-scale ($n = 2981$), multicenter study, examining antibiotic-associated diarrhea in the elderly failed to detect any clinical benefit of probiotic treatment [92]. Rates of antibiotic associated diarrhea are high in outpatients and can be as high as 30% [93]. A meta-analysis undertaken prior to the study [44] suggested that probiotic treatment was effective. Analysis of secondary outcomes including diarrhea severity, frequency of abdominal symptoms, length of hospital stay, and quality of life showed no evidence of a beneficial effect from consumption of probiotics. This meta-analysis and subsequent study identified that many probiotic studies are flawed or hampered by factors such as probiotic variation across studies, potentially poor study design, and poor quality of research. This again demonstrates the pressing necessity for better mechanistic understanding of probiotics and better regulation of the use of the word probiotic. Clinical guidelines

and outcomes need to be established and maintained, and until then, each probiotic trial needs to be assessed and examined for quality on a case-by-case basis [91].

One dynamic area of current research into is the treatment of intestinal disorders with fecal microbiota transplantation (FMT). *Clostridium difficile*-associated infection (CDI) is a particularly severe form of antibiotic-associated diarrhea, which is difficult to treat, and failure rates for antibiotic therapy are high [94]. FMT has been shown to be far more effective in treating CDI than traditional antibiotic treatment [95]. In a meta-study of 536 patients suffering from CDI, 467 (87%) experienced resolution of CDI [96]. Secondary side effects were IBS ($n = 1$), mild enteritis ($n = 3$), and suspected peritonitis ($n = 1$), leading the authors to declare FMT a safe and effective treatment for CDI. The route of administration is not standardized for FMT with the site of infusion appearing to influence efficacy. Diarrhea resolution rate is highest when FMT is administered in the cecum/ascending colon (93%). Standardization of stool, by identifying standard volunteer donors and freezing fecal material that is ready for administration to patients when it is needed is another method of improving efficacy of FMT [97]. Bacteriotherapy, the prospect of using keystone bacterial species to displace pathogenic organisms, is being examined in an attempt to refine FMT. It has been attempted in a mouse model of CDI [98], and in human trials [99, 100]. In a study of 31 patients comparing bacteriotherapy to FMT, 23 responded successfully to treatment: 16 of 23 (70%) receiving FMT and 7 of 8 (88%) receiving rectal bacteriotherapy of a mixture of known enteric bacteria in saline [101]. This approach addresses one of the primary concerns of FMT; the introduction of unknown, potentially pathogenic bacteria into a patient. FMT has been shown to replenish the levels of *Bacteroidetes* and *Firmicutes*, although their clinical importance in clearing CDI is unknown [102].

The levels of these taxa are important in IBD, and there is great interest in FMT as a potential therapeutic option for a range of intestinal disorders. Case reports suggest efficacy for UC in some cases [103]. In 2003, six patients with UC were treated by FMT once daily for 5 days, and a complete clinical, colonoscopic, and histological reversal of UC was demonstrated in all patients who previously had severe, relapsing UC [104]. A retrospective review was conducted with 62 UC patients who had undergone FMT over a 24-year period. This study reported a 91.9% response rate to FMT with 67.7% achieving complete clinical remission after FMT, 24.2% achieving partial remission, and only 8% nonresponders [105]. UC treatment by FMT is not as effective as it is for CDI treatment, and it does have a moderate-to-high success rate, but requires multiple treatments in many cases [106]. Data on CD treatment by FMT is limited to small case studies, and the findings suggest CD may not be amenable to FMT [103, 107]. FMT has also shown promise in metabolic diseases, significantly improving insulin sensitivity in obese males by increasing butyrate-producing bacteria [5]. These findings agree with previous data demonstrating the importance of microbiota on energy harvest [3, 6, 8, 48], but mechanisms are unknown.

The use of FMT to treat CDI is therefore well documented and generally regarded to be safe and efficient. The initial optimism that this may treat a range of gastrointestinal diseases has not yet been fulfilled, but with obesity and related metabolic diseases being strongly associated with gut microbiota, it follows that there may be

a role for FMT in alleviating some gastrointestinal symptoms. The resistance of CD to FMT displays the need for caution when proposing FMT as a potential therapeutic avenue for all gastrointestinal tract (GIT) disturbances. Despite preliminary screening of donors for pathogenic bacteria and viruses [97], it is very difficult to know the total bacterial load in these fecal samples. Therefore, caution with respect to donor identification remains necessary.

Conclusions

The area of microbiota research is dynamic and evolving quickly. Advancement of next-generation sequencing and increased analytical power of bioinformatic analysis has enabled vast amount of data to be generated in the past decade. Against this backdrop, experimental design and biological significance of studies remain as important as ever. Studies must consider clinical or exploratory significance of studies and refrain from a "sequence first, ask questions later" approach. Comparative analyses have revealed substantial variation in species and gene composition associated with a variety of disease states but at present these analyses fall short of providing a comprehensive appreciation of the impact of this disparity on the microbiota community and on the host. The current ambiguity of the role of the microbiota in IBS in particular is an example where the microbiota has been shown to be highly variable and further studies are necessary to underpin the exact role, if any, of microbiota in the disease. The advancement of FMT is promising for CDI and UC, but the specific biological mechanisms of the benefits remain elusive. Preparation of defined bacterial mixtures to redesign/replenish the gut microbiota would appear to be a safer method of treatment, so ongoing research on the most effective mixtures of bacteria is of great interest. It remains to be seen if one or a few different bacterial mixtures are effective in a widely diverse population with both significant genetic and microbial differences. The majority of geographical studies focus on dietary and lifestyle differences between the populations, and it would be interesting to investigate how human genetics affects the microbiota.

In conclusion, microbiota composition and function has been associated with a wide variety of disorders, but understanding of exact mechanisms remains elusive. The huge interest in the area means that stimulating progress is being made and in the coming years there will hopefully be major advances in our understanding of the processes at a mechanistic level.

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5
Mechanisms of Dysbiosis in the Inflamed Gut

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Abstract

Sparked by the development of next-generation sequencing methods, we have come to appreciate that the human intestinal tract is home to a complex microbial ecosystem, termed microbiota. On the species level, the composition of the gut microbiota varies greatly between individuals and is influenced by numerous factors such as diet, immune status, and environmental circumstances. However, the overall composition of the microbiota, in particular, the dominance of obligate anaerobic bacteria over facultative bacteria is conserved between individuals. In contrast, episodes of intestinal inflammation are accompanied by a severe disruption of the normal bacterial community structure, termed dysbiosis. Inflammation-associated dysbiosis is frequently characterized by a bloom of facultative anaerobic bacteria of the phylum Proteobacteria, in particular, members of the Enterobacteriaceae family, and a relative depletion of obligate anaerobic members. While numerous groundbreaking studies have recorded the diversity of the gut microbiota, the molecular mechanisms dictating the interaction between the host and microbes residing in the gastrointestinal tract are a medically relevant topic that is still in its infancy. A mechanistic understanding of the complex relationship between the host and its microbiota will significantly contribute to the development of novel intervention strategies targeting the gut microbiota and dysbiosis-associated diseases. In this chapter, conceptual advances on molecular mechanisms that influence the structure of gut-associated microbial communities are discussed, with a focus on inflammatory diseases of the intestinal tract.

Introduction

The human gastrointestinal tract harbors a complex ecosystem comprised of several hundred microbial species. The vast majority of these microbes are found in the large intestine. These microbial communities contribute to a plethora of beneficial functions for the host, such as the proper development and homeostasis of

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the immune system, nutritional status, and by conferring colonization resistance to enteric pathogens (reviewed in [1–4]). At the same time, the presence of copious amounts of bacteria inside the body also represents a challenge to the host since these bacteria must be confined to the lumen to the intestine and poses a risk to the overall health of the host organism. For example, the microbiota plays an important role in the development of numerous diseases such as allergies, diabetes, obesity, cardiovascular disease, inflammatory bowel disease (IBD), infectious diseases of the gastrointestinal tract, and cancer. Nobel laureate Elie Metchnikoff, known mostly for his groundbreaking work on phagocytosis and innate immunity, evoked the concept of dysbiosis as a harmful alteration of the host–microbe relationship in the gastrointestinal tract in contrast to mutualism and symbiosis [5]. Originally, this idea was limited to bacteria and was often referred to dysbacteriosis. In the current literature, the concept of dysbiosis has been extended to include all domains of life and refers to quantitative changes in the microbial community structure or alterations in the metabolic activity of the individual members as well as entire microbial communities. The association of the gut microbiota and disease development has spawned a great interest in manipulating the interplay between the host and its microbiota as novel avenues for intervention strategies and drug targets.

Dysbiosis during Inflammatory Diseases of the Gastrointestinal Tract

Under homeostatic conditions, obligate anaerobic bacteria belonging to the classes Bacteroidia (phylum Bacteroidetes), Clostridia (phylum Firmicutes), and Actinobacteria dominate bacterial communities while facultative anaerobic members such as Enterobacteriaceae (phylum Proteobacteria) are minor constituents of the gut microbiota and account for less than 0.1% of the entire microbial population [6–8]. On occasion, members of other taxonomic groups may be present in low numbers, such as members of the phyla Fusobacteria, Tenericutes, Spirochaetes, Cyanobacteria, and Verrucomicrobia. The preponderance of obligate anaerobic bacteria over facultative anaerobes is a conserved characteristic of a normal gut microbiota [8–10].

In contrast, the onset of intestinal diseases is accompanied by a disruption of this normal community structure. One of the earliest studies investigating the composition of the entire gut microbiota during disease was performed in patients with IBD, a chronic, noninfectious inflammatory disease affecting the gastrointestinal tract (reviewed in [11, 12]). An increase in the relative abundance of Proteobacteria, in particular Enterobacteriaceae, was observed in a subset of patients with active disease [13, 14]. These initial findings were corroborated in subsequent studies (reviewed in [15, 16]). Similarly, necrotizing enterocolitis in preterm neonates results in a massive expansion of Enterobacteriaceae in the lumen concomitant with fulminant mucosal inflammation and secondary bacterial infiltration of the mucosa [17–19]. HIV-infected individuals, in particular during advanced HIV-infection (AIDS), commonly develop a chronic diarrhea termed idiopathic enteropathy, concurrent with an expansion of Alpha-, Beta-, and Gamma-proteobacteria [20].

In animal models of noninfectious colitis, experimental induction of mucosal inflammation begets an expansion of Proteobacteria, especially members of the Enterobacteriaceae family. Parasite-induced ileitis in a murine model of *Toxoplasma gondii* infection causes an outgrowth of commensal *Escherichia coli* (family Enterobacteriaceae) and other Proteobacteria in the intestinal lumen [21]. Moreover, infection with enteric pathogens such as *Citrobacter rodentium* or *Salmonella enterica* leads to dysbiosis of the large intestinal microbiota and a concomitant bloom of the bacterial pathogen [22–24]. While the etiology and induction of host responses is clearly different for each disease setting, one commonality is the elicitation of inflammatory responses in the mucosa, giving rise to the idea that inflammation acts as a selective force to shape the composition of the microbiota. Specifically, inflammatory conditions in the distal gut appear to favor the expansion of facultative anaerobic Proteobacteria such as Enterobacteriaceae, Pasteurellaceae, and Pseudomonadaceae, while under homeostatic conditions obligate anaerobes prevail. But what are the mechanisms that shape the overall composition of the microbiota during health and disease?

Nutrient Acquisition by Commensal Bacteria in the Normal Gut

Gut bacteria have evolved a great diversity of metabolic pathways to maximize growth rates and maintain colonization of the intestinal lumen in the face of fierce nutritional competition (reviewed in [25, 26]). In the absence of oxygen, most commensal microbes rely on fermentative pathways. Since amino acids and simple sugars are absorbed in the small intestine, dietary or mucus-derived complex carbohydrates serve as major carbon and energy source for most obligate anaerobic Bacteroidia, saccharolytic Clostridia, and Actinobacteria. Analyses of the genomic sequences of various obligate anaerobic commensals have revealed a surprisingly diverse ability of each organism to degrade various glycans [27, 28].

A well-characterized example of polysaccharide utilization is the *sus* starch utilization system in the Gram-negative commensal *Bacteroides thetaiotaomicron*, a prototypical member of the Bacteroidia class [26, 29]. Soluble starch chains are initially sequestered at the bacterial surface by a protein complex (SusDEF) and subsequently broken down to malto-oligosaccharides by the α-amylase SusG [30, 31]. Oligosaccharide fragments are actively transported across the outer membrane into the periplasm by the outer membrane protein SusC [32]. The energy for the active transport is provided by the TonB system (reviewed in [33]). The periplasmic amylase SusA and the glucosidase SusB further degrade malto-oligosaccharides [34], which are then actively imported against a concentration gradient. Sequestration of large polysaccharides and subsequent active transport mitigates the possibility of losing hydrolysis products to neighboring nutritional competitors. Interestingly, the genome of *B. thetaiotaomicron* (strain VPI-5482) is predicted to encode 172 distinct glycoside hydrolases, 106 paralogues of the transporter SusC and 57 paralogues of SusD, suggesting that up to 20% of the coding capacity of the genome is devoted to polysaccharide utilization [27].

Members of the Gram-positive genus *Bifidobacterium* (class Actinobacteria) utilize a great variety of plant-derived polysaccharides [35]. The genomic sequence of *Bifidobacterium longum* NCC2705 contains more than 40 glycoside hydrolases, most of which are membrane bound, and eight high-affinity MalEFG-type ATP-binding cassette (ABC) transporters for the uptake of structurally diverse oligosaccharides [28, 36]. Similarly, Gram-positive Clostridia utilize a vast array of glycoside hydrolases and ABC transporters to degrade glycans and import degradation products [37].

In contrast to obligate anaerobes, Enterobacteriaceae constitute only a small fraction of the entire gut microbiota. *E. coli,* a prototypical member of the Enterobacteriaceae, does not secrete glycoside hydrolases and relies on passive diffusion of oligo- and disaccharides through the outer membrane. The absence of diverse glycan degradation systems and reliance on cross-feeding of oligosaccharides by other microbes likely puts *E. coli* at a competitive disadvantage when growing on a limiting quantity of dietary or mucus-derived polysaccharides, providing a mechanistic explanation for the low abundance of Enterobacteriaceae in the healthy large intestine. Consistent with this idea, the ability to degrade a diverse array of glycans correlates with the relative abundance of any given microbe, regardless of the taxonomic grouping [38]. This finding supports the concept that in the healthy gut, the composition of the gut microbiota is largely determined by the availability of specific polysaccharides and the microbial ability to access this carbon and energy source.

Despite its low overall abundance in the large intestine, *E. coli* is highly prevalent and found in a large subset of the human population, suggesting that *E. coli* and other Enterobacteriaceae might occupy a unique niche: In healthy individuals, the large intestine is a fairly anaerobic environment as facultative anaerobic Enterobacteriaceae consume oxygen introduced by ingestion and traces of oxygen leaking from the mucosa into the lumen of the colon [39]. Small amounts of oxygen may support a population of *E. coli* in close proximity to the mucosa. Oxygen levels are likely a limiting factor for *E. coli* and other commensal Enterobacteriaceae since introduction of air into the intestine in patients undergoing ileostomy, a surgical opening of the gastrointestinal tract, results in a bloom of Enterobacteriaceae and dysbiosis [40]. One possibility to reconcile these observations with the apparent lack of sophisticated glycan degradation systems is that Enterobacteriaceae likely do not compete with obligate anaerobes for host- or diet-derived polysaccharides, but rely on other carbon sources. Respiration of oxygen diffusing from the tissue in conjunction with the utilization of nonfermentable carbon compounds would represent a limited, but unique metabolic niche for Enterobacteriaceae in the healthy gut. This conceptual framework may in part explain the greater abundance of obligate anaerobic bacteria over facultative anaerobic Enterobacteriaceae in the normal gut.

Nutritional Mechanisms for Dysbiosis in the Inflamed Gut

Intestinal inflammation is a stereotypic host response that involves activation of the epithelium and infiltration of the lamina propria with inflammatory cells to generate antimicrobial compounds and rid the tissue and mucosal surfaces of intruding

microbes [41]. Some antimicrobials interfere with bacterial growth by restricting uptake of trace minerals such as iron or zinc. For example, Enterobacteriaceae secrete enterobactin, an iron siderophore [42, 43]. Activation of epithelial cells by the pro-inflammatory cytokine results in the apical secretion of lipocalin-2 [44], which sequesters bacterial enterobactin, thus preventing iron uptake through this mechanism [45]. As most commensal Enterobacteriaceae are sensitive to the action of lipocalin-2, this mechanism is unlikely to be a major contributor to the bloom of this taxonomic group in the inflamed gut.

A second important arm of the host response to bacteria is the production of bactericidal reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Figure 5.1). Enzymatic sources of ROS include NADPH oxidase 1 (NOX1) and dual oxidase 2 (DUOX2) expressed by epithelial cells [49, 50] as well as phagocyte oxidase (PHOX), superoxide dismutase (SOD), and myeloperoxidase (MPO) expressed by neutrophils [51, 52]. Inducible nitric oxides synthase (iNOS), present in both epithelial cells and phagocytes, is the sole source for nitric oxide and all other RNS derivatives thereof [53]. These RNS and ROS can enter the lumen of the gut by passive diffusion or are directly released into this environment. Epithelial cells release ROS and RNS on the apical side, while neutrophils transmigrate across the epithelial layer and introduce ROS and RNS into the lumen as these cells undergo cell death. While the release of ROS and RNS creates an antagonistic environment for bacterial growth, the production of these antimicrobials has important side effects on the nutritional environment in the luminal space. iNOS-derived nitric oxide can react with superoxide radicals generated by host NADPH oxidases to yield the unstable RNS peroxynitrite ($ONOO^-$) [53]. Peroxynitrite is rearranged to nitrate (NO_3^-) in an isomerization reaction. Thus, the nitrate is generated in the inflamed gut lumen as a byproduct of the host response [47]. Similarly, ROS and RNS can oxidize endogenous organic sulfide compounds such as methionine or tertiary amines, such as trimethylamine, to the respective sulfoxide and amine *N*-oxide [54, 55]. Nitrate, various sulfoxides, and amine *N*-oxides can serve as alternative electron acceptors (reviewed in [56–58]). Consequently, host-derived electron acceptors, derived as byproducts of inflammatory responses, enable anaerobic respiration in the anoxic gut lumen (Figure 5.1) [47].

Due to the high standard redox potential of the nitrate/nitrite redox couple, nitrate is the energetically preferred electron acceptor in the absence of oxygen. The generation of nitrate in the inflamed gut would likely enhance the growth of any microbe capable of anaerobic nitrate respiration; however, the most consistent pattern recorded for dysbiosis is an outgrowth of Enterobacteriaceae. Curiously, among all taxonomic groupings of the gut microbiota, genes encoding putative or experimentally confirmed nitrate reductases are most abundant in the genome of Enterobacteriaceae [59]. In contrast, the genome of obligate anaerobic bacteria, such as Bacteroidia and Clostridia, are rarely predicted to encode putative nitrate reductases [59]. Thus, Enterobacteriaceae are more likely to benefit from the production of inflammation-derived electron acceptors such as nitrate since members of this taxonomic grouping are simply more likely to encode the enzymatic activities required to consume these compounds.

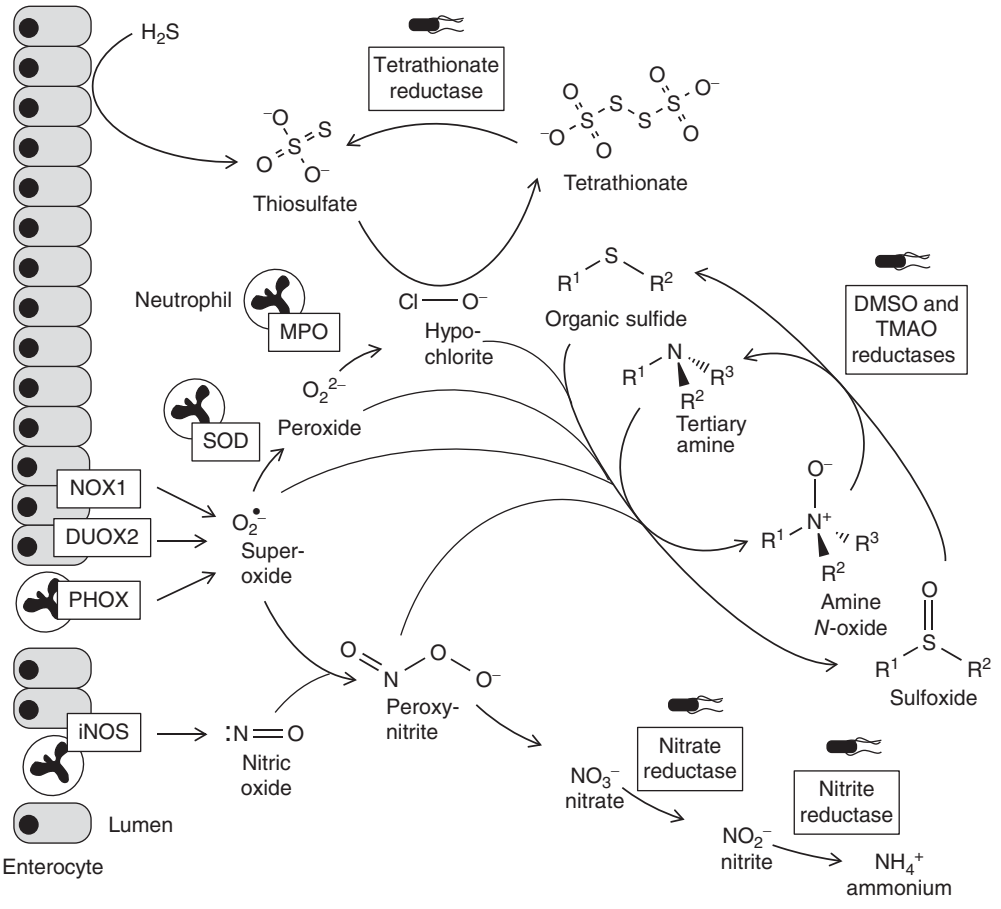

Figure 5.1 Generation of alternative electron acceptors as byproducts of the inflammatory response in the gut. Superoxide created by the host enzymes NADPH oxidase 1 (NOX1), dual oxidase 2 (DUOX2), and phagocyte oxidase (PHOX) is converted into peroxide by superoxide dismutase (SOD), which in return is used as a substrate for myeloperoxidase (MPO) to generate hypochlorite. Reactive oxygen species, in particular hypochlorite, oxidize endogenous thiosulfate to tetrathionate [46], a substrate for the *S. typhimurium* tetrathionate reductase. Nitric oxide produced by inducible nitric oxide synthase (iNOS) reacts with superoxide to form peroxynitrite. Peroxynitrite isomerizes to nitrate, which serves as the electron acceptor for bacterial nitrate respiration in the inflamed gut [47]. Breakdown of nitrate yields nitrite, which can be reduced by bacterial nitrite reductases. Organic sulfides and tertiary amines can be oxidized by reactive oxygen and nitrogen species to the respective sulfoxide or amine *N*-oxide. Structurally diverse sulfoxides or amine *N*-oxides can serve as terminal electron acceptors for Enterobacteriaceae [48].

Nitrate as well as various sulfoxides and amine *N*-oxides can serve as alternative electron acceptors for Enterobacteriaceae [60]. The *E. coli* K-12 genome encodes three distinct nitrate reductases (*narGHJI*, *narZYWV*, and *napFDAGHBC*), two dimethyl sulfoxide (DMSO) reductases (*dmsABC* and *ynfFGH*), and three trimethylamine N-oxide (TMAO) reductases (*torCAD*, *torYZ*, and *yedYZ*) [48]. The active site of all these reductases contains an essential molybdenum cofactor; mutants deficient for the biosynthesis of the molybdenum cofactor are thus lacking these anaerobic respiratory pathways [61]. In the absence of inflammation, anaerobic DMSO, TMAO, and nitrate respiration appears to be dispensable for colonization of the gastrointestinal tract [47, 62]. However, in genetic models of colitis and models of epithelial injury, experimentally introduced respiration-proficient *E. coli* wild-type strains outgrow the microbiota and efficiently colonize the gut lumen [47]. In contrast, isogenic mutants deficient for molybdenum cofactor biosynthesis or nitrate respiratory genes are recovered in substantially lower numbers, suggesting that anaerobic respiration, in particular nitrate respiration, only confers a growth advantage in the inflamed gut. Abolishment of RNS production by inhibition of iNOS nullifies the advantage conferred by nitrate respiration, indicating that the nitrate generated in the inflamed gut is host derived [47].

Oral administration of antibiotics alters the microbial community structure and concomitantly raises the inflammatory tone of the intestine [63, 64]. This moderate level of inflammation is sufficient for the production of RNS and host-derived nitrate [63, 65]. Anaerobic nitrate respiration contributes to the expansion of the *E. coli* population as a result of antimicrobial therapy [63, 65].

Another example of anaerobic respiration driving dysbiosis has been described in a murine model of dietary induced obesity [66]. *Bilophila wadsworthia*, a member of the Deltaproteobacteria, is scarce in the healthy intestine [67]. Administration of a diet high in milk fat resulted in augmented production of taurine-conjugated hepatic bile acids, such as taurocholic acid, accompanied by increased mucosal inflammation [66]. Through reduction of the sulfonate group, *B. wadsworthia* can utilize taurocholic acid as the terminal electron acceptor [68]. Increased availability of taurocholic acid is thought to enhance metabolic activity and fitness, thus fueling a bloom of *Bilophila* spp. in this model system [66].

Collectively, these studies provide strong support for the idea that changes in the nutritional environment can be critical drivers of dysbiosis during bouts of inflammation.

Inflammation-Driven Bloom of Enteric Pathogens in the Gut Lumen

The bloom of commensal Enterobacteriaceae during episodes of mucosal inflammation can be viewed as an accidental disruption of the intestinal ecosystem. However, it is tempting to speculate that the fitness advantage conferred by anaerobic respiration in the presence of inflammation is a strong selective force for the development of pathogenic species relying on this mechanism to interrupt the normal gut microbiota and create a nutritional environment that is well suited to the outgrowth of the enteric pathogen. For example, pathogenic *S. enterica*, a species closely related

to *E. coli*, encodes virulence factors that were acquired by the *Salmonella* lineage after divergence from the last common ancestor [69]. Of critical importance for the development of gastrointestinal disease are two distinct type three secretion systems (T3SSs) that are present in virtually all *S. enterica* serovars [70, 71]. The invasion-associated T3SS-1 mediates invasion of the intestinal epithelium [72] while a second type three secretion system (T3SS-2) enhances survival inside professional phagocytes in the mucosa [73, 74]. The most common nontyphoidal clinical isolate is serovar Typhimurium (*Salmonella typhimurium*) [75]. Invasion and replication of nontyphoidal *Salmonella* in the intestinal mucosa triggers a potent inflammatory response characterized by the influx of neutrophils into the affected tissues and transmigration into the gut lumen [70, 71]. Akin to the bloom of commensal *E. coli* during noninfectious colitis, iNOS-derived nitrate enhances growth of *S. typhimurium* in the lumen in the inflamed gut [76].

Since commensal Enterobacteriaceae can utilize the nitrate released during *Salmonella*-induced colitis [47, 63], reliance of the pathogen on this mechanism to foster its outgrowth is expected to put *S. typhimurium* in direct nutritional competition against commensal Enterobacteriaceae. Nevertheless, the outgrowth of commensal Enterobacteriaceae is suppressed during *Salmonella* colitis, and the predominant bacterial population in the gut is comprised of the enteric pathogen [23, 24]. Infection with *Salmonella* results in a prominent upregulation of the cytokine IL-22 [44, 77, 78]. IL-22 is a very potent activator of epithelial responses, which includes the release of lipocalin-2 into the intestinal lumen [44, 78]. Inhibition of enterobactin-mediated iron acquisition by lipocalin-2 restricts growth of commensal Enterobacteriaceae [78]. In the inflamed intestine, *S. typhimurium* secretes a glycosylated derivative of enterobactin, salmochelin [44]; lipocalin-2 does not sequester salmochelin, allowing *S. typhimurium* to subvert this host response aimed at limiting the acquisition of the micronutrient iron [44].

Another property that sets pathogenic *S. typhimurium* apart from its commensal competitors is the ability to reduce tetrathionate [79, 80]. This trait is the basis for tetrathionate broth, a method used to date to enrich for *Salmonella* species from complex clinical specimens. The relevance of tetrathionate respiration for the infection process has been recently investigated [46]. Commensal bacteria, in particular *Desulfovibrio* spp., reduce dietary sulfate to hydrogen sulfide, a cytotoxic compound. The intestinal epithelium detoxifies microbiota-derived hydrogen sulfide by oxidation to thiosulfate [81, 82] (Figure 5.1). During *Salmonella* gastroenteritis, transmigrating neutrophils introduce ROS into the gut lumen, thus further oxidizing thiosulfate to tetrathionate [46]. The generation of tetrathionate in the gut lumen enhances growth of the pathogen through anaerobic tetrathionate respiration, giving the pathogen a competitive edge over commensal bacteria [46, 83].

How Anaerobic Respiration Enhances Growth in the Inflamed Gut

While Enterobacteriaceae seem to be poorly equipped to compete with *Bacteroides* and *Clostridium* spp. for complex carbohydrates, numerous *in vitro* studies have

documented the versatile metabolism centered on the electron transport chain in *E. coli, S. typhimurium*, and other Enterobacteriaceae (reviewed in [56–58]). The anaerobic respiratory chain comprises three components: reduction equivalents travel via dehydrogenases (DHs) from electron–donor compounds to the quinone pool, and further to the respiratory enzymes to reduce the terminal electron acceptors, for example, tetrathionate or nitrate. Under anaerobic conditions *in vitro*, Enterobacteriaceae can utilize a diverse array of chemical compounds as exogenous electron donors, such as formate, hydrogen, lactate, and succinate. Curiously, these compounds are typical fermentation end products produced by the microbiota (Figure 5.2). For example, carbohydrate degradation by *Bacteroides* spp. yields fumarate, which is then utilized in a primitive electron transport chain (fumarate respiration) [25, 85, 86]. Succinate, the product of fumarate respiration, is excreted. Primary fermentation end products can be further metabolized as part of syntrophic metabolic networks. For example, the enzyme pyruvate formate lyase (formate acetyl transferase) in *Bacteroides* and *Clostridium* spp. catalyzes the formation of formate and Acetyl-CoA from pyruvate. Despite the high diversity in bacterial species and their metabolic functions, virtually all energetically favorable carbon sources in the normal gut are ultimately catabolized to fermentation end products, which are excreted into the gut lumen and accumulate to fairly high concentrations (1–10 mM) [87]. In this environment, the emergence of alternative electron acceptors could create a particular metabolic niche that can be exploited by commensal and pathogenic Enterobacteriaceae.

Reduction of an external electron acceptor can impact the metabolism on several levels to enhance growth (reviewed in [56–58]). Membrane-bound DH couple the oxidation of fermentation end products such as hydrogen or formate to the respiratory chain, contributing to the formation of a proton gradient across the cytoplasmic membrane. Proton translocation is achieved by scalar chemistry, that is, the proton consuming half-reaction is performed in the cytosol while a proton-generating half-reaction occurs in the periplasm, resulting in a net proton gradient. Alternatively, a subset of DH and reductases represent *bona fide* proton pumps. Examples include the NADH-DH I (NDH-I) or nitrate reductase (NarGHI). Thus, oxidation of electron donor compounds in the presence of inflammation-derived electron acceptors is predicted to enhance the growth by augmenting the proton motive force and ATP synthesis.

Additionally, alternative electron acceptors can contribute to maintaining cellular redox balance. The catabolism of simple sugars produces surplus reduction equivalents in the form of NADH. To maintain a favorable balance of NAD^+/NADH, excess NADH must be reoxidized. This can be achieved by reduction of endogenous compounds, such the conversion of pyruvate to lactate or acetaldehyde to ethanol as part of fermentative processes; however, this results in the loss of valuable metabolic intermediates that could otherwise be used for biosynthetic purposes. In the presence of exogenous electron acceptors, NADH conversion to NAD^+ is coupled to the electron transport chain by NADH-DH, alleviating the need to squander internal metabolic intermediates to maintain an appropriate cellular redox balance.

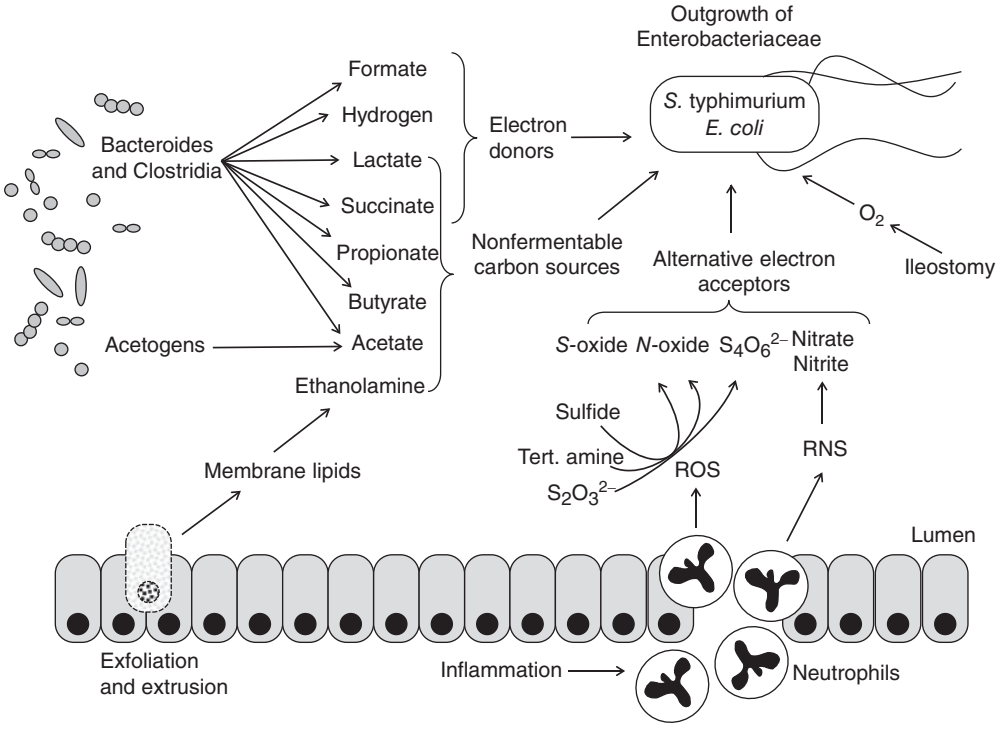

Figure 5.2 Metabolic environment conducive to the outgrowth of commensal and pathogenic Enterobacteriaceae in the inflamed gut. Production of reactive oxygen (ROS) and nitrogen (RNS) species yields oxidation produces that can serve as electron acceptors for Enterobacteriaceae (for details see Figure 5.1). Oxygen levels increase as a result of ileostomy, a surgical opening of the otherwise anaerobic intestinal lumen [40]. Primary fermenters in the normal gut microbiota break down complex glycans to formate, hydrogen, lactate, succinate, and short-chain fatty acids, including propionate, acetate, and butyrate. Acetate is also produced by acetogenesis from carbon dioxide and exogenous electron donors (reviewed in [25]). In the presence of alternative electron acceptors, these fermentation end products could be used as carbon and energy sources for Enterobacteriaceae. *S. typhimurium* utilizes ethanolamine, possibly derived from the membrane lipids of extruded or exfoliated enterocytes, in the presence of tetrathionate [84].

Moreover, coupling of energetically unfavorable reactions to the reduction of an external electron acceptor enables the breakdown of nonfermentable carbon sources. For example, enterocytes infected with *S. typhimurium* undergo cell death and are extruded from the epithelial lining in an attempt to rid the tissue of invading bacteria [88, 89]. The most abundant phospholipid in intestinal epithelial cells is phosphatidylethanolamine [90, 91], which is metabolized by the gut microbiota to ethanolamine. Ethanolamine is a poorly fermentable carbon source [92]. However, in the presence of respiratory electron acceptors, such as nitrate or tetrathionate, ethanolamine can be used as the sole carbon source for *S. typhimurium* under

anaerobic conditions [84, 92]. Emergence of tetrathionate during colitis allows *S. typhimurium* to access this nutrient source [84].

Under aerobic conditions, *E. coli* degrades fatty acids through the canonical β-oxidation pathway catalyzed by the FadABE complex, allowing growth on fatty acids as the sole carbon and energy source [93]. Recently, a distinct pathway operating under anaerobic conditions has been identified [94]. This homologous complex comprising the YfcY, YfcX, and YdiD proteins enables anaerobic fatty acid degradation but requires the presence of alternative electron acceptors, such as nitrate, while the FadABE complex is dispensable under these conditions [94]. Collectively, in the presence of electron acceptors, Enterobacteriaceae can circumvent nutritional competition with strict anaerobic members of the microbiota by exploiting nonfermentable compounds as carbon sources and electron donors for the respiratory chain. In contrast, most obligate anaerobic bacteria of the microbiota, such as Bacteroidia and Clostridia, lack sophisticated respiratory enzymes and metabolize diet-derived or mucus-derived carbohydrates solely through fermentative pathways.

Conclusions

Inflammatory diseases of the gastrointestinal are frequently associated with an imbalance of the microbiota structure, characterized by a bloom of Proteobacteria, in particular, Enterobacteriaceae. Dysbiosis is thought to exacerbate mucosal and systemic inflammation by mechanisms that are currently being investigated (reviewed in [4, 95]). The complexity of the interactions between the host and its microbiota as well as interactions between the different members of the gut microbiota poses a challenge for establishing causal relationships and pinpoint molecular mechanisms.

The emerging picture is that the local nutritional environment in the gut dictates the composition of the microbiota. Microbes have evolved numerous distinct strategies to efficiently colonize the gut lumen: obligate anaerobic bacteria rely on sophisticated glycan degradation systems, while facultative anaerobic Enterobacteriaceae require electron acceptors for optimal growth. In the healthy gut, the niche occupied by Enterobacteriaceae is limited due to the absence of electron acceptors besides residual oxygen emanating from the tissue. During inflammation, alternative electron acceptors such as nitrate are generated as byproducts of the oxidative burst and become available in the gut lumen, thus creating a peculiar nutritional niche. Due to their genetic capacity, that is, encoding various anaerobic respiratory enzymes, commensal and pathogenic Enterobacteriaceae (as well as other Proteobacteria) are uniquely suited to thrive in this habitat. Thus, inflammation-associated dysbiosis is a result of changes in the nutritional environment driven by the host response.

Conversely, this hypothesis does not exclude the likely possibility that dysbiosis indeed aggravates inflammatory responses, and these two concepts represent distinct but complementary processes. The identification of anaerobic respiration as a mechanism driving dysbiotic changes in the microbiota is a first critical step

toward novel treatment strategies affecting the microbiota with the intention of ameliorating infectious and noninfectious inflammatory disorders of the gut. Since the reduction of alternative electron acceptors is uniquely associated with disease, strategies targeting this metabolic pathway are predicted to selectively prevent the outgrowth of potentially harmful Enterobacteriaceae. Selective inhibition is predicted to restore a normal community structure without impeding growth of obligate anaerobic commensals, a stark advantage over traditional approaches relying on broad-spectrum antimicrobial therapy. Furthermore, compounds targeting the inflammation-associated metabolism of Enterobacteriaceae could become critical tools to disentangle the relationship between dysbiosis and host responses and shed light on mechanisms of microbiota-dependent exacerbation of mucosal inflammation.

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Strategies for Nutrient Acquisition by *Magnaporthe oryzae* during the Infection of Rice

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Abstract
Developing an understanding how microbial pathogens obtain nutrition from their hosts and how this process changes over the course of infection and varies between different pathogens may ultimately lead to the identification of novel control strategies. The following chapter details what is known of nutrient acquisition by the model fungal plant pathogen *Magnaporthe oryzae* during its infection of rice and highlights what directions future research could take.

Introduction

Rice blast disease is the most serious disease of cultivated rice and a significant constraint on worldwide rice production. As humankind depends on rice to provide almost a quarter of its calorific intake, rice blast is a significant factor in ensuring global food security [1, 2]. To cause disease, the rice blast fungus *Magnaporthe oryzae* undergoes a complex program of development that involves rapid physiological and morphogenetic responses to the differing environments that the fungus encounters during plant infection (reviewed by Fernandez and Wilson [3]). The main stages of pathogenic development are illustrated in Figure 6.1 (see also review by Wilson and Talbot [2]). Very briefly, *M. oryzae* infection of rice starts and ends with the release of spores from infected plants. These spores (or conidia) land on a healthy rice leaf to which they tightly adhere, germinate, and develop the infection structure known as the appressorium, which brings about host penetration. Fungal development up to the point of host penetration is strongly dependent on correct conditions of humidity and temperature and naturally takes place under conditions of low nutrient availability outside of the host. Upon entry to the plant, however, although the pathogen can now access rice tissue for its nutrient supply, it must act with some stealth, at least initially, so as not to elicit host defense. This stage of development therefore involves suppression of host defense (for review see [4]) and, perhaps also

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an avoidance of catabolic reactions that generate elicitors of host defense. Finally, during the necrotrophic growth phase, the fungus may aggressively degrade components of the plant, killing rice cells, and at the same time using acquired nutrients to exit the plant by building new spore-bearing structures to disseminate the disease to new plants. This chapter considers each phase of pathogenic development in turn and discusses what is known currently and what we might expect to be the nutritional status and the metabolic activities underlying each stage of fungal growth and development.

Adhesion and Germination

Adhesion is considered to occur using materials that are preformed in the spore [5] and germination rapidly follows this attachment. The surface of the plant is a low-nutrient environment and spores of the fungus contain sufficient reserves of carbohydrates and lipids to fuel germination and appressorium formation in the absence of any exogenous nutrients [6, 7]. Indeed, nutrient starvation (in particular nitrogen starvation) is considered to act as one of the triggers for infection structure formation [2, 8]. Which of the reserves, that are present in the spore, is important for germination is still unclear. Surprisingly, few germination mutants of *M. oryzae* have been reported in the literature and those that are known are delayed in germination, rather than being unable to germinate [9]. They are mostly mutants with regulatory defects where the downstream metabolic processes affected are undefined, or complex, such as a mutant lacking a probable calcium/calmodulin-dependent kinase [9], or cAMP (cyclic adenosine monophosphate)-dependent protein kinase A [10]. In the case of the *CPKA*-deletion mutant, subsequent analysis showed that degradation of both glycogen and lipid bodies are delayed in this strain [7], consistent with its delay in appressorium development.

The spore contains a variety of endogenous reserves, which are likely used for germination in a nutrient-free environment. These include glycogen, trehalose, polyols (such as glycerol, erythritol, and mannitol), and lipid bodies. Additionally, there is a possibility to degrade its own endomembrane system and other cellular constituents by autophagy. As germination is rapid, we might expect readily metabolized reserves such as glycogen or trehalose to be used first. However, although glycogen reserves are abundant in ungerminated conidia, they are not rapidly used during germination [11]. Additionally, glycogen breakdown is dispensable for germination because mutants lacking the sole amyloglucosidase and glycogen phosphorylase enzymes show delayed glycogen breakdown, but normal postpenetration development [11]. Trehalose reserves in the spore are degraded upon germination, but their breakdown is not essential for the process, because mutants lacking trehalase enzymes show normal germination rates [12, 13]. As *ICL1* deleted mutants lacking isocitrate lyase show delayed germination, we can assume that lipid reserves are used early during spore germination and metabolized through the glyoxylate cycle to provide much of the glucose required for anabolic processes underlying germination, such as cell

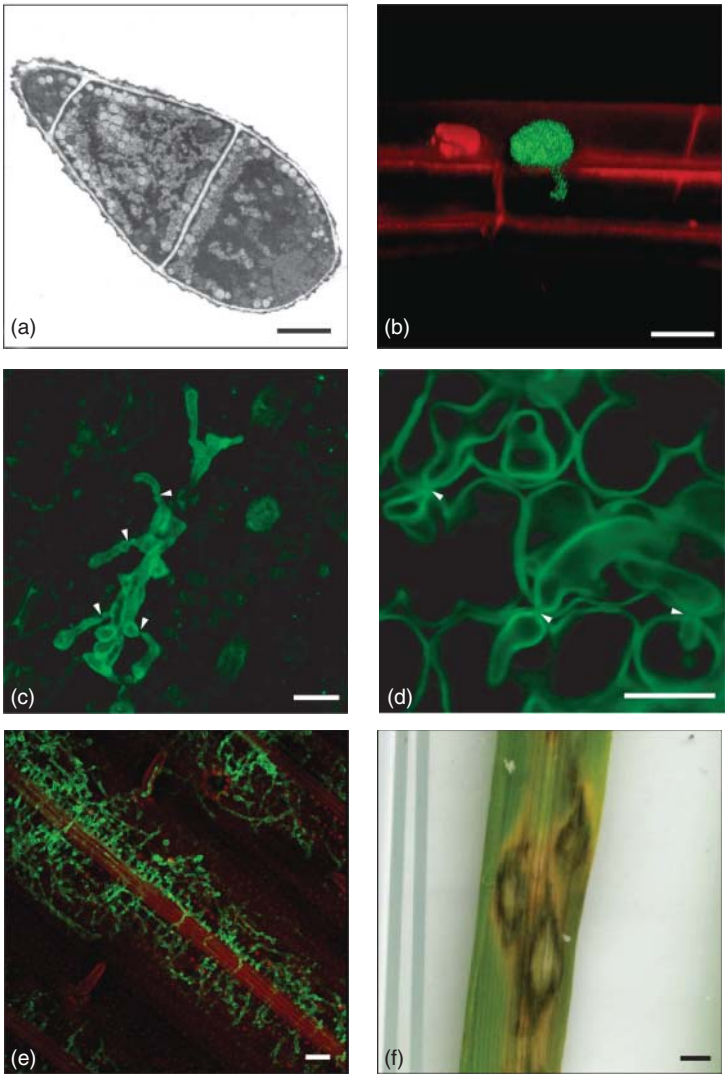

Figure 6.1 Developmental changes in *Magnaporthe oryzae* during infection of rice. (a) Electron micrograph of *M. oryzae* Guy11 spores, which infect rice via an appressorium, shown in (b), laser scanning confocal microscopy, where rice cell walls are stained red with propidium iodide, and green fluorescence denotes the appressorium and invasive hypha. Panels (c) and (d) show cell-to-cell movement of aniline blue stained hyphae of *M. oryzae* during the colonization of tissue. After the biotrophic phase of infection has finished, colonization continues as hyphae grow extracellularly as shown in (e), where red signal is derived from propidium iodide fluorescence, staining plant cell walls, and green fluorescence is from WGA-Alexa 488 stained fungal cell walls. *M. oryzae* compatible infection on leaves is characterized by (f) lozenge-shaped lesions. Scale bars represent (a) 2.5 μm, (b, c, e) 10 μm, (d) 5 μm, and (f) 2.5 mm. White arrowheads show cell-to-cell crossing points.

wall biogenesis [14, 15]. The majority of ubiquitin pathway genes are also induced during germination and proteasome inhibitors can completely block the process in the first 24-h time period, although the fungus can eventually germinate following longer incubation times [16]. A mutant lacking polyubiquitin shows reduced germination and subsequent disease-related development; therefore, polyubiquitination is required for normal germination [16]. These data suggest that ubiquitin-mediated targeted protein turnover contributes significantly to meeting the carbon and nitrogen demands of the germinating spore [16]. Autophagy contributes to germination because mutants deleted for *ATG1,* which encodes serine/threonine protein kinase essential for autophagy, have slightly delayed germination [9], but the contribution of autophagy at this stage is not as great as it is to appressorium development as we will discuss in the next section.

Taken together, what we know of germination so far indicates the operation of a failsafe mechanism by which germination uses a preferred energy reserve but when this is blocked then an alternative is used instead. Obviously, any mutant that produces spores that are unable to germinate would be effectively incapable of causing disease in the field, and therefore, given the fact that spore germination is a fungal-specific developmental program, defining this process in detail is attractive from the point of view of identification of novel targets for control of rice blast disease and cereal pathogens more generally.

Appressorium Formation

In terms of the research carried out to date, appressorium formation is the best characterized stage of pathogenic development in *M. oryzae.* A number of different studies and approaches have shown that autophagy is an essential process in providing the energy required to drive the formation of a functional appressorium [17–19]. Therefore, we know that recycling of the spore contents provides the energy to form an appressorium and to allow turgor-mediated plant penetration. Upon maturation of the appressorium, it is readily apparent that the spore collapses and the spore contents have been consumed [19]. Concomitant with the demise of the spore, glycerol accumulates in the appressorium to drive turgor-mediated penetration of the leaf cuticle [20]. The source of glycerol is likely to be from triacyl glycerol lipase degradation of lipid reserves, based on multiple lines of evidence [7, 21, 22]. Additionally, vacuolar recycling of proteins via the Spm1 protease is critical for appressorium maturation [23]. Acetyl CoA generated through fatty acid degradation and recycling of proteins can be channeled into gluconeogenesis and then to formation of polysaccharide components of cell walls and can also provide precursors for the synthesis of melanin and other secondary metabolites as well as key amino acids [14]. A mutant lacking the Mgd1 NAD(+)-dependent glutamate dehydrogenase, which plays a key role in the catabolism of amino acids, produced reduced numbers of appressoria and was additionally compromised in its ability to proliferate in the plant, producing fewer smaller lesions than the wild type [24], and indicating that recycling of protein is important for later stages of development as well as appressorium formation.

TOR signaling, which plays a positive role in regulating the import of nutrients and as a negative regulator of autophagy in *Saccharomyces cerevisiae* [25], has recently been implicated in controlling the response of *M. oryzae* to nutrient availability [26, 27]. The RNA-binding protein Rbp35 of the rice blast fungus has been implicated in target of rapamycin (TOR) signaling by its influence on the polyadenylation of specific transcripts, encoding TOR-related factors such as the negative regulator of TOR signaling Tap42), the FKBP (FK-506 binding protein) peptidyl-prolyl cis-trans isomerase and the Asd4 a transcription factor [26, 27]. Asd4 plays a critical role by suppressing the transcription of genes encoding enzymes for glutamine utilization, thereby influencing intracellular levels of glutamine [27]. Loss of this transcription factor leads to increased intracellular glutamine and untimely activation of TOR signaling during appressorium formation with, among other consequences, repression of transcription of *ATG8,* which encodes a ubiquitin-like protein essential for autophagy [19, 27]. Some of the spore contents, such as lipid bodies, may initially be translocated to the appressorium, but ultimately these are degraded allowing full turgor pressure to be generated and for the structure to become fully functional, thereby allowing penetration to take place [7]. Amino acid uptake does not seem to be an active process during appressorium formation, at least based on transcriptomics data [28].

In summary, appressorium formation has been the subject of the majority of research into the molecular genetic basis of the pathogenicity of *M. oryzae* and we already have a very detailed picture of the nutritional basis for this stage of development, which relies predominantly on autophagic recycling of the spore contents to the incipient appressorium.

Penetration

Appressorium-mediated penetration is considered to occur by purely mechanical means as *M. oryzae* can penetrate artificial surfaces, and studies to date have found no role for secreted cutinases in degrading the cuticle layer prior to penetration [29, 30]. A role for the Cut2 cutinase in the signaling that triggers the preceding step, appressorium formation, has been found [29]. Based on *in vitro* growth experiments, it is clear that until the fungus has actually penetrated the plant it can support itself nutritionally from its own reserves. However, this does not completely preclude the possibility that it may scavenge some nutrients from the rice leaf during prepenetrative growth, although there is no evidence that this occurs.

Growth in Planta: The Biotrophic Growth Phase

The study of the growth of the fungus *in planta* has been the subject of several exciting recent breakthroughs in our understanding of how *Magnaporthe* species cause disease. We now know that the fungus initially grows with thin hyphae resembling the germ tubes that emerge from germinating spores. After a short elongation, these

primary infection hyphae then take the form of what are known as bulbous invasive hyphae, which are surrounded by plant plasma membrane, forming the a specialized extra-invasive hyphal membrane (EIHM). Entry to adjacent cells has been reported to occur through plasmodesmata [31] using hyphae similar to the primary infectious hyphae. After initial formation of bulbous infection hyphae in each cell, a structure is seen with which the secretion of effector proteins is associated [31]. The location of this structure and its association with the secretion of effector proteins have led to this being known as the biotrophic interfacial complex (BIC).

A central player in coordinating the carbon and nitrogen assimilative activities of the cell is trehalose-6-phosphate synthase encoded by *TPS1* [13, 32]. The importance of this factor lies in its role as a sensor of glucose-6-phosphate (G6P), rather than its enzymatic role in the synthesis of trehalose [13]. In the presence of high levels of G6P, the Tps1 protein can directly bind NADPH, thereby influencing transcriptional repressors that bind NADP [33]. These repressors are considered, based on their influence on the activity of key transcriptional regulators, to control the production of enzymes appropriate to the prevailing nutritional status [33]. When glucose is plentiful, such as may be the case during biotrophic growth, Tps1 activates G6P-dehydrogenase activity and carbon is channeled into the phosphogluconate pathway resulting in the production of precursors of nucleotides and aromatic amino acids, as well as NADPH production, which can be used in reactions requiring reducing equivalents. Meanwhile, during the formation of the appressorium, discussed earlier, the balance of metabolic activity is catabolic rather than anabolic. Loss of Tps1 leads to an inability to proliferate in the plant, due to loss of nitrogen and carbon catabolite repression (CCR) leading to, among other effects, an inappropriate early activation of plant cell wall degrading enzymes (CWDEs), which presumably triggers host defense [32]. In this way, Tps1 not only has profound effects on the cells metabolic activity but may contribute to the necessary stealthy behavior during biotrophic growth and evasion of host defense [32].

The transcription of genes, whose products are required for utilization of quinic acid, is induced during the later stages of appressorium formation [28]. Together with the observation of significant quinic acid levels in infected leaves, this suggests that the fungus may actively divert metabolism of the plant, such that quinic acid is formed in preference to defensive compounds derived from phenylalanine [34]. This could be achieved by, for example, suppression of the shikimate pathway enzyme dehydroquinate dehydratase-shikimate dehydrogenase allowing precursors of quinic acid to accumulate, thereby both suppressing plant defense and providing a useful carbon source for the pathogen during the biotrophic growth phase [34].

As mentioned above, we can anticipate that upon entry to the plant, the fungus will initially proceed with some caution in its degradation of components of the plant cell. Damage to plant cell walls is, indeed, not apparent during biotrophic growth [31], and additionally, the transcripts of genes encoding most of the predicted CWDEs present in the pathogen's genome are conspicuously absent at this stage [35]. Some nutritionally relevant CWDE-encoding genes are clearly expressed, however. Transcripts strongly induced at 36 hpi include potentially carbohydrate-active enzymes of glycosyl hydrolase (GH) families 3 (a transcript corresponding to

a known β-glucosidase MoCel3A, which has been shown to release glucose from both cellulosic and hemicellulosic polysaccharides), a GH family 5 cellulase (which could potential hydrolyze 1-4-β-D-glucosidic linkages in cellulose and β-D-glucans) and a potential xylosidase/arabinosidase of the GH family 43 [35]. Among the most upregulated genes at 36 hpi are two chitinases, whose role is obviously not nutritional and which are likely to degrade potential defense-inducing chitin fragments [36]. The transcription of nine proteolytic enzyme-encoding genes is also induced at 36 hpi [35]. These might conceivably play a role in suppression of host defense rather than nutrition because, as mentioned above, amino acid uptake does not seem to be an active process during early infection based on transcriptomics data [28]. Additionally, auxotrophs for several amino acids do not survive beyond an early stage of infection, suggesting that peptides derived from the plant do not significantly contribute to the amino acid supply required for protein synthesis in the pathogen [37]. Of the most highly upregulated genes, one is predicted to encode an aspartyl peptidase with similarity to a peptidase known to be induced by carbon starvation in *Podospora anserina* [38]. Clearly, there is scope to more precisely define how the fungus derives its nutrition within the plant at this stage. It is considered that secretion during biotrophic growth focuses on small peptides, which actively suppress host defense or which mask the presence of the fungus by removal of potential proteinaceous elicitors of defense (reviewed by Zhang and Xu [39]). Taken together, what we know of the actions of the pathogen at this stage suggests that it derives the bulk of its nutrition by uptake of soluble sugars present in the apoplast. Therefore, transporters for uptake of sugars can be expected to play a key role in carbon assimilation at this stage. *M. oryzae* has many genes potentially encoding members of the sugar transporter family, and several of these are transcriptionally activated during appressorium formation [28]. Assigning a substrate to members of this family is often problematic due to the paucity of functional studies of sugar transporters in filamentous fungi. A likely sugar alcohol transporter encoding gene is, for example, transcriptionally expressed at later stages of infection (48 and 72 hpi [40]) and this would fit with the accumulation of sugar alcohols (notably mannitol) that occurs during infection [34, 41].

Amino acid metabolism within the plant is also of interest. Of those studied, wild-type strains of *M. oryzae* are able to grow on minimal media without amino acid supplementation and therefore are prototrophic for all amino acids. Leaves analyzed at an early time point after infection with the bacterial rice pathogen *Xanthomonas oryzae* contained only detectable amounts of serine, tyrosine, alanine, and aspartic acid among the amino acids [42]. Valine, proline, and phenylalanine were also found to be at a low level at 24 hpi with *M. oryzae* but increased in concentration as the infection progressed [34]. As available amino acids may be limited within the plant during biotrophic growth, auxotrophs for some key amino acids may not be able to survive in plants. A case in point is methionine, because mutants lacking *MoMET6* and *MoSTR3,* which encode cobalamin-independent methionine synthase and cystathionine *β*/lyase, respectively, are nonpathogenic [37, 43]. Compounds capable of selectively inhibiting such fungal amino acid biosynthetic enzymes, which are required for growth in planta, might provide

effective plant protectants [43]. Histidine and lysine biosynthesis have also been shown to be required for successful infection [44, 45].

In a similar manner to the case for certain amino acid biosynthetic pathways discussed above, purine biosynthesis is essential for the ability of the fungus to survive in planta, indicating that plant-derived purines are not available to the fungus [46].

Major clues to the nutritional basis for successful interaction of *M. oryzae* with rice (and two other hosts) have come from metabolic analyses [34]. These data suggest that sucrose and aspartate increase in abundance in infected tissues even at 48 hpi and suggest that the pathogen is able to influence their transport and that they may represent major sources of carbon and nitrogen for the pathogen at this early stage of infection [34]. As discussed earlier in the context of quinic acid accumulation, metabolomics data also strongly supports the view that the fungus actively subverts the plants metabolism for its own needs [34] and future research can be expected to bring exciting new insights into this metabolic reprogramming and exactly how this is achieved.

Growth in Planta: Necrotrophic Growth Phase

After 72 hpi, a time point at which the first visible signs of disease become apparent, and perhaps shortly after the biotrophic to necrotrophic switch has occurred, *Magnaporthe oryzae* induces the expression of several nutritionally-relevant genes [47–49]. These include genes whose products likely function in cellulose and arabinoxylan degradation [49]. These polysaccharides, together with mixed-linked β-glucans, represent the major components of the cell wall of cereals [50]. Although individually those xylanases tested so far are dispensable for virulence [51, 52], simultaneous silencing of multiple xylanases led to a reduced ability to spread within plant tissue, indicating that degradation of arabinoxylan occurs presumably for nutrition as well as to facilitate the movement of the pathogen between rice cells during the later stages of infection [53]. Gene silencing is an interesting approach here as although suppression of gene transcription is incomplete, this approach may more closely mimic pharmacological interventions where, as with silencing, residual activity of target enzymes remains. Therefore, gene silencing technologies might prove a useful means of target validation for crop protection in future. The most abundant plant polysaccharide is cellulose and cellulolytic activity resulting from synergistic activity of endoglucanases, cellobiohydrolases (GH families 6 and 7), and β-glucosidases could provide the fungus with a means to derive nutrition from cellulose. Based on its genome sequence *M. oryzae* can potentially produce three GH family 6 and six GH family 7 enzymes [54–56]. Several of these genes are induced during the later stages of infection, and silencing all of them led to a reduction in the ability to spread within the plant, although not as drastic as the reduction observed with strains silenced for xylanases [55]. Although this could indicate that the degradation of xylan plays a more important role in successful compatible interaction, the degree of silencing achieved with current technologies is highly variable

making such comparison between strains is problematic. Compared to cellulose, hemicelluloses such as arabinoxylan may be more accessible and readily utilized by the fungus for nutrition. Hemicellulose-degrading enzymes including a mixture of a xyloglucanase from *Aspergillus oryzae* and a xylanase and a 1,3-1,4-β-glucanase from *M. oryzae* have been shown to induce cell wall loosening in wheat coleoptiles [57] indicating that hemicellulose contributes to cell wall strength and that these enzymes may be important for proliferation during the necrotrophic growth phase by weakening the cell wall as well as to provide carbon for the pathogen in the form of glucose and xylose as well as smaller amounts of mannose, galactose, rhamnose, and arabinose. Arabinose and xylose utilization may depend on the product of the *PDR1* gene, which is active against both of these substrates [58] and shows strong expression during growth *in planta* [40]. Arabinose and xylose do not appear to be an important carbon source for *Magnaporthe* during infection, however, as strains lacking D-xylose kinase cannot grow on either sugar but retain full virulence [59]. Xylose, glucose, fructose, mannose, sucrose, cellobiose, and cellulose are good substrates for growth or the fungus in culture, whereas starch and pectin are less good and rhamnose, arabinose, and galactose were found to be poorly used [60]. These observations of growth in culture might reflect the preferred carbon sources in the plant.

Metabolite profile has provided insights into how the fungus may source its carbon and nitrogen following the switch to necrotrophy [34]. Glutamate, glucose, fructose, and mannitol increase markedly in concentration during later stages of infection. Conversion of plant sucrose to glucose and fructose by invertase and then sequestration by the fungus in a form unavailable to the plant such as mannitol (or other sugar alcohols) would create a sink for these sugars in the infected tissue [34]. Recent transcriptomics data supports such a view because transcript abundance for a predicted polyol transporter increases as infection progresses [40].

The importance of pectin degradation in the disease cycle of *M. oryzae* has been tested by construction of a mutant lacking *MGD1,* which encodes an endo-polygalacturonase and is the only predicted protein in the standard lab strain 70-15 with this particular enzymatic activity [61]. This study found the *MGD1* deleted strain to be unaffected in virulence suggesting that degradation of pectin is of minor importance in allowing the fungus to complete its disease cycle [61]. Consistent with these results, it is known that the pectin content of grasses is typically low [62].

In summary, the research conducted to date on the *M. oryzae*-rice pathosystem has offered some clues as to the nature of the CWDEs employed by the fungus during necrotrophic growth. To some degree, new technologies have overcome the difficulties of working with gene families with redundant members. We can anticipate that future studies will more precisely define the basis of fungal nutrition at this growth stage and more accurately pinpoint what activities are activated on the switch to necrotrophy and whether the switch itself is dependent on nutrient availability. Additionally, determination of whether toxins are required to kill the plant at this stage and, if so, what the metabolic pathways are required to generate precursors of the toxins would warrant further investigations.

Growth in Planta: Sporulation

Although less is known about this developmental stage than those preceding it, it might well be that the autophagy-driven sporulation observed in cultures [63] also occurs when the fungus exits the plant. Upon exhaustion of plant-derived nutrients, it would make a lot of sense energetically for the fungus to sacrifice the biomass it has accumulated in planta to build new survival structures that permit the pathogen to spread. Sporulation in many fungal species is considered a response to nutrient deprivation. In cultures of *M. oryzae,* however, the fungus best sporulates on rich media; therefore, it might be that in the plant nutrient availability is one of many factors determining the decision to sporulate. A diurnal cycle of spore formation and release has been observed in the field [64]; therefore, sporulation may also be linked to light-dark cycles or to the metabolic status of the plant. Sporulation will also be marked by the laying down in the maturing spores of the nutrient reserves and possibly also in an inactive state the enzymes required for the consumption of these reserves during germination. In a similar manner to germination, a more precise definition of the metabolism specific to this stage is attractive from the point of view of identifying novel fungal specific targets for disease control.

Life Outside of the Rice Plant

The vast majority of research into the rice blast fungus has quite naturally focused on its interaction with rice; however, no consideration of the nutrition basis of disease would be complete without acknowledging the importance of the ability of the fungus to utilize a wide array of carbon and nitrogen sources [65] and some speculation as to the significance of this for the survival of the fungus in the field. The ability of *M. oryzae* to use alternative sources of nutrition may be due to the need to survive outside of the rice growing season, for example, by overwintering on diseased crop stubble or by survival in infected rice seed [66]. Species of *Magnaporthe* can also infect other grasses and weeds, which might act as alternate hosts [67], and they can also infect other plant tissues including necks, roots [68], which would represent different challenges nutritionally to leaves [69]. These overwintering strategies and alternate hosts and tissues may therefore require the activation of metabolic processes outside of those used in infection of healthy rice leaves.

Conclusion

Having considered the major stages of pathogenic development, it is clear that the nutritional status at each stage and the special circumstances of interaction with its host necessitate major metabolic reprogramming at each stage of development. Certain predictions regarding the metabolic shifts that accompany fungal development are obvious and many clues already exist as outlined here. Prepenetration growth, for

example, is characterized by a lack of external nutrients and there would be no sense to the fungus to secrete enzymes to scavenge nutrients that are not present. What is commonly referred to as CCR, whereby enzymes required for utilizing nonpreferred carbon sources are produced only in the absence of preferred carbon sources (especially glucose), would make little sense energetically for the fungus in its prepenetration phase when it is programmed to rely entirely on its own internal reserves. Meanwhile CCR would make more sense during the biotrophic growth phase when soluble sugars from the plant are likely to be accessible and especially considering that other catabolic processes might raise host defense. Finally, the switch to necrotrophy may well be triggered by a change in the availability of key nutrients and be accompanied by induction of enzymes acting on a broader range of plant-derived substrates. Later, we can anticipate that the fungus will also use the own reserves within its accumulated biomass to drive sporulation by autophagy, as has been observed to occur in axenic growth and in this context nutritional status may be a key factor in initiation of metabolic reprogramming.

In general, the analysis of mutants lacking single carbohydrate active enzymes displays no clear phenotypes [51, 52]. One explanation is that a cocktail of enzymes is generally required to break down carbohydrates, such as cellulose, and there is evidence of functional redundancy with many families of carbohydrate active enzymes [70]. One approach to overcome this difficulty is to attempt to identify factors that control the production of these enzymes as has been achieved in *Cochliobolus carbonum* [71]. A key goal for future research will therefore be to identify key regulators. A major point of regulation exists at the transcriptional level and because genes required for catabolism of a particular carbohydrate are generally transcriptionally activated together, it is possible to identify transcription factors responsible for the temporally coordinated expression of these gene families. In *M. oryzae* several transcription factors condition the ability to survive in the plant, for example, the Mig1 transcription factor [72], and mutants lacking these transcription factors could be useful as tools to identify downstream targets and subsets of genes whose products play a critical role in controlling the metabolic program necessary for the biotrophic and necrotrophic growth phases.

The ease with which we can now perform transcriptome analysis is likely soon to give us ever more detailed clues as to the metabolic reprogramming that underlies the complex infection cycle of the rice blast fungus. Major goals for future research should therefore be to precisely define what triggers the switch to necrotrophy and the decision to sporulate and to define the metabolic programs at each stage of development and how these changes are orchestrated.

One attraction to the identification of carbohydrate active enzymes as key factors for the survival of the plant pathogen in planta is that the search for potential inhibitors is greatly aided by the existence of established assays for such enzymes. The reverse genetics approach has been widely applied and has greatly aided in the identification of factors required to cause disease in plant pathogens; however, it is likely that the dominance of this approach might also have led us to underestimate the potential of carbohydrate active enzymes as drug targets. As next-generation sequencing becomes more and more accessible, it might be that these technologies

can be combined with forward genetic approaches to define complex metabolic processes requiring several genetic components. Proof of concept may also require improved technologies, such as CRISPR-Cas9 genome editing to inactivate several genes in a single strain, thereby enabling robust gene family analysis. Understanding precisely how the fungus supports its growth within the host is an exciting prospect for the coming years, and we can anticipate that in the next decade we will begin to reap the rewards of our research efforts in fundamental biology fungal plant pathogens by the development of novel control measures for this devastating disease.

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Part Two
New Inhibitors and Targets of Infectious Diseases

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7
Outer Membrane Proteins as Potential Anti-infective Drug Targets in *Mannheimia haemolytica*

*Robert L. Davies**

Abstract

Mannheimia haemolytica is considered to be the most important bacterial pathogen associated with BRD (bovine respiratory disease) and is responsible for substantial economic losses to the livestock industries. Antibiotics play a primary role in the treatment and control of pneumonic pasteurellosis and BRD; they are used both therapeutically and metaphylactically. The antibiotics ceftiofur, enrofloxacin, danofloxacin, tulathromycin, tilmicosin, florfenicol, chlortetracycline, and oxytetracycline are commonly used in the treatment and control of pneumonic pasteurellosis and BRD. However, the emergence and spread of antibiotic resistance in *M. haemolytica* and other BRD pathogens is becoming an increasing problem. A further worrying trend in the development of antibiotic resistance in *M. haemolytica* is the transfer of multiple resistance determinants by integrative conjugative elements (ICEs). Therefore, there is an urgent need to design and develop new antibacterial agents against this economically important pathogen. The identification of new antibiotics is likely to involve a combination of cell- and target-based approaches. Novel drug targets do not need to be part of central metabolic processes and could include virulence genes. It will be important that the development of new antibiotics should also involve appropriate *in vivo* screening because *in vivo* growth has a profound effect on gene transcription and will affect the expression of potential drug targets. The outer membrane of Gram-negative bacteria contains in excess of 100 proteins with a wide range of functions ranging from outer membrane biogenesis and integrity to drug efflux, iron transport, and adherence; the outer membrane is at the interface between the bacterium and host, and many of these proteins play key roles in host–pathogen interactions. Therefore, many of these proteins represent potential drug targets that could be the focus for future research aimed at the design and development of new drugs to combat *M. haemolytica* and the other pathogens responsible for BRD.

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Introduction

Bovine respiratory disease (BRD) complex is a multifactorial condition of cattle that involves interactions between different bacterial and viral pathogens and causes significant economic losses to the livestock industries worldwide [1–3]. The principal viruses associated with BRD include bovine respiratory syncytial virus (BRSV), bovine herpes virus-1 (BHV-1), bovine parainfluenzavirus-3 (BPIV-3), and bovine viral diarrhea virus (BVDV) [3–5]. Bacterial species involved in BRD include *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni* (previously *Haemophilus somnus*), *Arcanobacterium pyogenes, Mycoplasma bovis,* and, most recently, *Bibersteinia trehalosi* (previously *Pasteurella trehalosi*) [1–3, 6]. These viral and bacterial pathogens have the potential to cause disease individually but complex and poorly understood interactions involving viruses and bacteria, and the host respiratory tract, are most often involved in BRD. All of these bacterial species are present within the cattle population as normal commensals of the upper respiratory tract but, as a consequence of stress or prior viral infection, are able to proliferate and cause disease [1, 3].

M. haemolytica is considered to be the most important bacterial pathogen associated with BRD [1, 6, 7]. The bacterium causes a fibrinous and necrotizing pneumonia, known as pneumonic pasteurellosis (or as shipping or transit fever), in stressed, immunocompromised animals [8]. *M. haemolytica* is associated with an acute to subacute fibrinous pleuropneumonia to distinguish it from the subacute to chronic bronchopneumonia caused by *P. multocida* [9]. *M. haemolytica* is estimated to cause losses to the US cattle industry alone in excess of $1 billion per annum [6, 10, 11]. Despite advances in chemotherapy, vaccination, and animal management, BRD continues to be a major problem affecting the cattle industry.

Mannheimia haemolytica

M. haemolytica is a weakly hemolytic, Gram-negative coccobacillus that is a commensal of the upper respiratory tract of healthy cattle, sheep, and other ruminants [8, 12]. The bacterium is a member of the *Pasteurellaceae* and comprises 12 capsular serotypes (A1, A2, A5, A6, A7, A8, A9, A12, A13, A14, A16, and A17) [13]. Serotype A2 isolates are most frequently recovered from the nasopharynx and tonsils of healthy cattle and appear to represent the dominant carrier phenotype [6, 8, 14–16]. Conversely, serotype A1 isolates are associated most frequently with pneumonic lesions of diseased cattle [6, 8, 14, 16]. Thus, serotype A1 is considered to be pathogenic to cattle, whereas serotype A2 is considered to be nonpathogenic [15, 16]. However, serotype A6 isolates are being increasingly associated with cases of bovine pneumonic pasteurellosis in Europe, North America, and Japan [6, 13, 17, 18] and probably have equal, or very similar, pathogenicity to isolates of serotype A1. Indeed, bovine serotype A6 isolates of *M. haemolytica* are genetically indistinguishable from

those of serotype A1 and most likely represent strains that have undergone a capsular switch from serotype A1 to A6 [19].

Although serotypes A1 and A2 colonize the upper respiratory tracts of healthy cattle and sheep, there are distinct differences in the association of these serotypes with diseased animals [19, 20]. A1 is the predominant serotype associated with bovine pneumonic pasteurellosis [8, 13], whereas A2 is the predominant serotype associated with ovine pneumonic pasteurellosis [12, 13]. However, population genetic analysis has further shown that distinct subpopulations representing isolates of serotypes A1, A2, and A6 are associated with disease in cattle and sheep[19]. Thus, bovine serotype A1/A6 isolates are genetically distinct from ovine isolates of the same serotypes and bovine and ovine serotype A2 isolates are similarly distinct. Major differences exist in the outer membrane protein (OMP) profiles of bovine- and ovine-specific A1/A6 and A2 isolates, suggesting that certain OMPs are involved in host specificity and virulence [19, 20]. This is discussed in further detail in the following section (OMPs).

Pathogenesis of Pneumonic Pasteurellosis

The sequence of events leading to infection by *M. haemolytica* is well defined [8, 11, 13] although the molecular basis of pathogenesis and the roles of individual virulence factors in infection are less well understood. As a commensal organism, *M. haemolytica* colonizes the nasopharynx and, in particular, the tonsils of healthy cattle in a symbiotic relationship [11, 13]. As discussed, these colonizing bacteria are predominantly of the A2 serotype, but small numbers of serotype A1 (or A6) bacteria are also likely to be present [8]. However, as a consequence of management- or environment-related stress and/or viral infection, the balance between harmless serotype A2 and pathogenic serotype A1 bacteria is disrupted and there follows an explosive and selective proliferation and colonization of serotype A1 bacteria within the upper respiratory tract [7, 8, 11]. The specific bacterial and host factors that are responsible for this shift from serotype A2 to A1 dominance are not understood although several studies have attempted to understand the mechanisms involved [13]. Extensive colonization of the upper respiratory tract by serotype A1 *M. haemolytica* subsequently leads to infection of the lung alveoli by inhalation of infected aerosol droplets and sloughed cells [7, 8, 11]. Once within the alveoli, the bacteria initiate an inflammatory response leading to the acute fibronecrotizing pleuropneumonia characteristic of pneumonic pasteurellosis (Figure 7.1) [7, 11]. The mechanisms by which *M. haemolytica* colonizes its host and eventually causes pneumonia, including the immune mechanisms of the host, are poorly understood [21]. However, *M. haemolytica* possesses an array of virulence factors that are required to cause disease, and these represent potential antimicrobial drug targets and vaccine antigens.

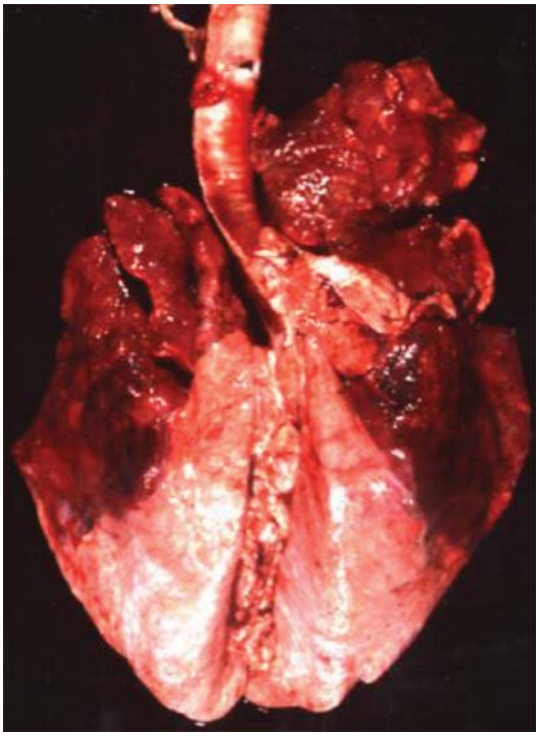

Figure 7.1 Lung of calf after experimental challenge with *M. haemolytica* showing pneumonic lesions.

Virulence Factors of *M. haemolytica*

The virulence factors of *M. haemolytica* include various colonization factors, toxins, enzymes, and OMPs that have been described in detail in various reviews [1, 7, 11, 13, 22, 23]. These virulence factors allow *M. haemolytica* to adhere to and colonize the mucosal surfaces of the respiratory tract, acquire nutrients (especially iron), and evade the host immune response. Together, these processes enable *M. haemolytica* to survive, grow, and colonize the mucosal surfaces of the upper respiratory tract and, under predisposing circumstances, lead to colonization of, and damage to, the lower respiratory tract. The virulence factors of *M. haemolytica* can be broadly classified either as (i) secreted toxins and extracellular enzymes or as (ii) surface proteins and carbohydrates.

Secreted Toxins and Extracellular Enzymes

Leukotoxin

M. haemolytica secretes a 102–105 kDa leukotoxin (LktA) that is generally considered to be the most significant virulence factor of the bacterium [7, 22, 23]. LktA

of *M. haemolytica* allows the pathogen to evade the phagocytic activity of immune cells and plays a major role in generating the extensive inflammatory response and associated lung damage that is characteristic of pneumonic pasteurellosis. LktA is a calcium-dependent cytotoxin that is a member of the RTX (repeats in toxin) family of toxins. It is a heat-labile protein that has been identified in all serotypes of *M. haemolytica* [24]. LktA is both species- and cell-type-specific since it exhibits activity against ruminant lymphoid cells only [25–27]. This specificity is linked to the binding of LktA to the transmembrane receptor CD18, a subunit of the β_2-integrin family, which, together with CD11a, forms the lymphocyte-function-associated antigen 1 (LFA-1) [28, 29]. However, the cell-type and species-specific effects of RTX toxins are actually due to subsequent host-signaling events rather than toxin binding *per se* [7, 30]. The effect of LktA on host cells is dose dependent. At high concentrations, LktA creates pores in the host cell membranes and this leads to K+ and Ca+ efflux, osmotic swelling, and eventual cell lysis [31]. However, at sublytic concentrations, LktA activates the cells to produce inflammatory mediators (such as TNF-α (tumor necrosis factor), IL-1β (interleukin), and IL-8) and causes apoptosis [7, 22]. The *lktA* gene has a complex mosaic structure and has evolved by a series of horizontal DNA transfer and recombination events that appears to reflect transmission of strains between cattle and sheep [17]. The *lktA* structural gene is part of a four-gene operon *lktCABD: lktC* encodes an activation protein required for posttranslational acylation of LktA, *lktA* encodes the structural protein, and *lktB* and *lktD* encode membrane transport proteins [32–34]. However, secretion of LktA also involves the TolC protein that forms an ion-permeable channel across the outer membrane [7]. The *lktCABD* operon also has a mosaic structure due to recombinational exchange although this has affected the transport genes, especially *lktD,* less than *lktA* [35]. The immune response to LktA plays an important role in disease resistance [13] although immunity against *M. haemolytica* requires both LktA-neutralizing antibodies and antibodies against cell-surface antigens [36].

Proteases

A number of secreted proteases have been associated with *M. haemolytica* including a sialoglycoprotease, a neuraminidase, and immunoglobulin proteases. Sialoglycoprotease activity is associated with all serotypes of *M. haemolytica* [37] and is thought to enhance adherence to host cell surfaces [22]. Antisialoglycoprotease antibodies have been detected in the sera of infected calves [38], but the precise role of this protein in pathogenesis has not been determined. All serotypes of *M. haemolytica* produce neuraminidase activity [39, 40], which is thought to play a role in colonization of the upper respiratory tract [41]. Other respiratory tract pathogens such as *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Pseudomonas aeruginosa* produce neuraminidases that cleave α-2,3-linked sialic acid from glycoconjugates. Since mucosal surfaces are heavily sialylated, it is possible that *M. haemolytica* neuraminidase exposes potential bacterial receptors, thereby promoting adherence, biofilm formation, and colonization [23]. Several respiratory tract pathogens, such as *H. influenzae* and *Actinobacillus pleuropneumonia*, produce proteases that cleave secretory antibodies into Fab and Fc fragments [22]. In cattle, IgA predominates in

the upper respiratory tract, whereas IgG is the primary secretory antibody in the lower respiratory tract. *M. haemolytica* produces an IgG1-specific protease in partially purified culture supernatants [42] that may play a role in cleaving IgG in the lower respiratory tract. However, analysis of *M. haemolytica* genome sequence data suggests that *M. haemolytica* also possesses cell-associated IgA protease activity that could be involved in cleaving IgA in the upper respiratory tract [22].

Surface Proteins and Carbohydrates

Capsule

M. haemolytica is surrounded by a polysaccharide capsule and variation in the chemical composition and structure of this capsule is responsible for the serotypic diversity described above. The chemical structures of the A1, A2, and A7 capsules have been determined [43–45]. Interestingly, the A2 *M. haemolytica* capsule has the same sialic (or colominic) acid structure as that of the serogroup B capsule of *Neisseria meningitidis* and the K1 capsule of *Escherichia coli* [43] and is nonimmunogenic. The capsule of *M. haemolytica* is thought to contribute to pathogenesis in several ways; it is involved in adherence to the mucosal surface of the lower respiratory tract [46], evasion of phagocytosis by neutrophils [47], and resistance to complement-mediated killing [47]. Although vaccination of cattle with purified capsular polysaccharide promotes the production of anticapsular antibodies, a positive correlation between anticapsular antibody and protection has not been established [23].

Lipopolysaccharide (LPS)

Lipopolysaccharide (LPS) is an important component of the outer membranes of all Gram-negative bacteria and comprises 10–25% of the dry weight of *M. haemolytica* [48]. The LPS molecule comprises the lipid A moiety, which is embedded within the outer membrane, the core-oligosaccharide region, and the polysaccharide side-chain (O-antigen) region. The LPS of *M. haemolytica* is unusual in that both rough and smooth forms occur in different isolates [20, 49]. Four core-oligosaccharide variants lacking O-antigen side chains have been identified in *M. haemolytica*, but each of these may, or may not, be associated with the same O-antigen type [20]. *M. haemolytica* presumably possesses the same basic lipooligosaccharide structure as mucosal pathogens of the genera *Neisseria* and *Haemophilus,* but some strains have acquired an O-antigen; *Campylobacter jejuni* has a similar LPS structure [50]. The association of only a single O-antigen type with the four core-oligosaccharides strongly suggests that the O-antigen biosynthesis genes have been acquired relatively recently by horizontal DNA transfer [20]. However, the distribution of the O-antigen among *M. haemolytica* serotypes is not random; serotype A1 and A6 isolates associated with bovine pneumonic pasteurellosis always possess the O-antigen, whereas serotype A2 isolates associated with ovine pneumonic pasteurellosis lack the O-antigen [20]. The role of smooth and rough LPS types of *M. haemolytica* in disease pathogenesis (e.g., in adherence and in providing resistance to the bactericidal effects of complement) is not well understood although the difference in LPS structure in isolates associated with bovine and ovine disease suggests that LPS has an important host-specific role in disease pathogenesis. The incidence and severity

of ovine pneumonic pasteurellosis was demonstrated to be greater after challenge with a rough strain compared to a smooth strain [51].

The majority of LPS-induced effects are mediated by the lipid A component [52]; lipid A induces endotoxic effects such as pyrexia and hypotensive shock. *M. haemolytica* LPS interacts with various bovine cell types and induces proinflammatory cytokine gene expression, which leads to neutrophil influx, inflammation, and damage to bovine pulmonary epithelial cells [7, 22]. It has also been demonstrated that *M. haemolytica* LPS forms high-molecular mass complexes with leukotoxin [53]. LPS enhances the biological effects of leukotoxin [54] and, together, the two molecules may act in synergy to cause tissue damage and inflammation *in vivo* [22].

Outer Membrane Proteins

The outer membrane of pathogenic Gram-negative bacteria is at the interface between pathogen and host and contains in excess of 100 proteins that play key roles in pathogenesis (Figure 7.2). However, the precise roles of individual OMPs in the pathogenesis of pneumonic pasteurellosis are poorly understood. Analysis of the OMP profiles of 184 *M. haemolytica* isolates demonstrated consistent differences between the profiles of bovine and ovine serotype A1, A2, and A6 isolates suggesting that OMPs play a role in host specificity [20]. In particular, specific molecular mass variants of OmpA were observed to be associated with bovine and ovine isolates [20]. It was subsequently demonstrated that these bovine and ovine OmpA variants (OmpA1 and OmpA2) differ in the amino acid sequences within hypervariable domains located at the distal ends of the four surface-exposed loops characteristic of this protein [55]. These findings suggested that OmpA is under strong selective pressure from the host and it was hypothesized that OmpA is involved in binding to specific host cell receptor molecules in cattle and sheep and plays a role in host adaptation [55]. It was subsequently confirmed that OmpA1 and OmpA2 possess immunologically distinct, surface-exposed epitopes that could potentially bind to different receptors in the bovine and ovine respiratory tracts [56]. In further developments, OmpA was shown to be involved in binding of *M. haemolytica* to bovine bronchial epithelial cells [57] and, more specifically, to fibronectin [58]. Other OMPs that are putatively involved in adherence of *M. haemolytica* to the host respiratory tract include a collagen-binding trimeric autotransporter, AhsA [59], the Lpp1 (PlpA) protein [57], a high-molecular-mass protein that is homologous to the Hia autotransporter protein of *H. influenza* [60] and a 68-kDa protein that is involved in adherence to tracheal epithelial cells [61].

Another group of OMPs that play important roles in pathogenesis are those proteins involved in iron uptake. *M. haemolytica* possesses a transferrin-binding receptor complex, which comprises two proteins, transferrin-binding proteins A and B (TbpA and TbpB), involved in acquisition of iron from host transferrin [62–64]. However, the pathogen also possesses two hemoglobin receptor proteins (HmbR1 and HmbR2) and a putative hemin receptor complex that are upregulated during growth under iron-limited conditions [65]. The bovine and ovine serotype A2 genomes encode the hemopexin acquisition operon *hxuCBA* [66] although only the *hxuA* gene is present in the serotype A1 genome [67].

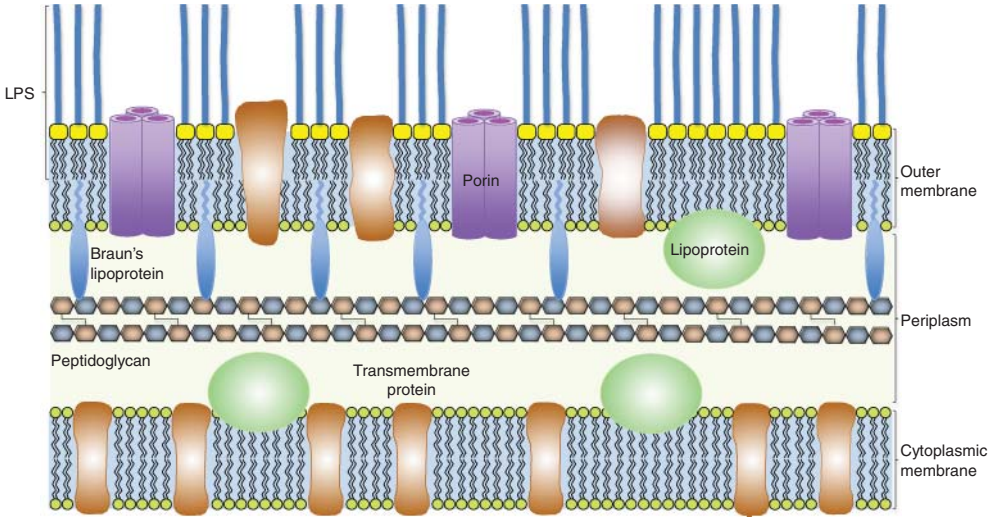

Figure 7.2 Generalized structure of Gram-negative cell envelope including the outer membrane, periplasm, and cytoplasmic membrane. The outer membrane comprises an outer leaflet of lipopolysaccharide (LPS), an inner leaflet of phospholipids, various transmembrane proteins which have characteristic β-barrel structures and include the porins, and lipoproteins.

Bioinformatic and immunoproteomic approaches were used to identify OMPs of *M. haemolytica* and identify potential vaccine candidates [68]. In this way, 132 immunoreactive proteins were identified and functions could be assigned to 55 of these but not all of these were OMPs. Although precise roles of relatively few OMPs in pathogenesis have been clearly demonstrated, many of these proteins have, nonetheless, been shown to be immunogenic in cattle [68–71]. Furthermore, not only are many of these OMPs immunogenic, but certain of them, such as the outer membrane lipoprotein PlpE [72, 73] and the autotransporter Ssa1 [69], provide protection in vaccinated cattle.

We have developed a bioinformatics workflow to allow the confident prediction of bacterial OMPs from genomic sequences [74]. Using this approach, we have confidently predicted 93 OMPs from the publicly available genomes of bovine and ovine serotype A1 and A2 isolates of *M. haemolytica* [75]. In this way, we have identified 15 proteins involved in outer membrane biogenesis and integrity, 28 proteins having transport and receptor functions (including the transferrin-binding receptor proteins TbpA and TbpB, the hemoglobin receptors HmbR1 and HmbR2, the heme-hemopexin proteins HxuA, HxuB, and HxuC, a hemin receptor protein, a hemin-uptake lipoprotein, the ferric hydroxamate receptors FhuA and FhuE, and various TonB-dependent receptor proteins), 6 proteins putatively associated with adherence, 11 proteins with enzymatic activity, 7 proteins having other functions, and 26 proteins of unknown function. Subsequent comparative sequence analysis of the genes encoding these predicted OMPs from the published bovine serotype A1/A2 and ovine serotype A2 genomes [66, 67] identified variation in key OMPs

(e.g., PlpE, Ssa, TbpB, OmpA, fHbp, Hsf, OmpW, FrpB), suggesting possible involvement in host specificity and virulence [75]. After separation of OMPs by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) (Figure 7.3) in conjunction with proteomic approaches, we have identified 55 OMPs expressed in bovine and ovine isolates of *M. haemolytica* grown under iron-replete growth conditions and a further 13 proteins expressed under iron-limited growth conditions.

Antibiotic Use against *M. haemolytica*

There are numerous commercial vaccines available against *M. haemolytica* but they provide limited protection, only seeming to be efficacious in approximately 50%

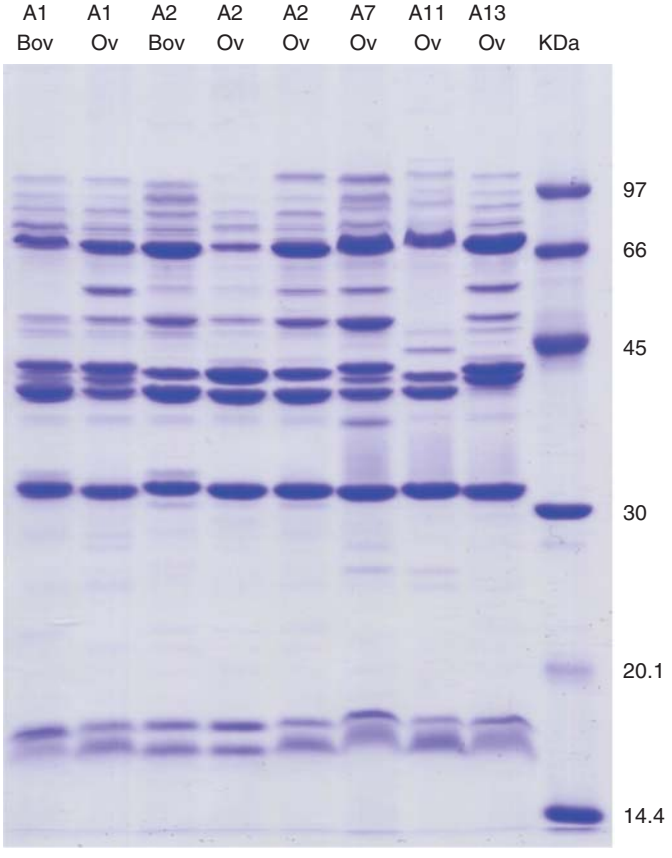

Figure 7.3 Outer membrane protein profiles of bovine (Bov) and ovine (Ov) *M. haemolytica* isolates of serotypes A1, A2, A7, A11, and A13. The bacteria were grown under iron-limited growth conditions and show enhanced expression of iron-regulated proteins in the upper molecular mass region.

of field studies [73, 76, 77]. Consequently, antibiotics play a primary role in the treatment and control of pneumonic pasteurellosis and BRD [78–80]. Antibiotics are used either therapeutically or metaphylactically for treatment and prevention, respectively. Therapeutic use refers to the administration of antibiotics to animals that are showing overt clinical signs of infection. Metaphylactic therapy involves the mass treatment of groups of animals considered to be at high risk of developing disease [81]. This procedure is very common in North America where high-risk cattle are commonly injected with an antibiotic on arrival at feedlots [79]. The agricultural sector is the largest user of antibiotics worldwide and the widespread use of these pharmaceuticals for growth promotion and metaphylaxis raises serious concerns regarding the development and spread of antibiotic resistance [79].

Antibiotics commonly used therapeutically and metaphylactically against *M. haemolytica* include ceftiofur, enrofloxacin, danofloxacin, tulathromycin, tilmicosin, florfenicol, chlortetracycline, and oxytetracycline [13, 80–82]. Ceftiofur is the primary β-lactam antimicrobial agent used in the treatment of BRD; it is a third-generation cephalosporin that is resistant to the β-lactamases produced by *M. haemolytica* [83]. β-Lactam antibiotics bind to penicillin-binding proteins thereby inhibiting cross-linking of peptidoglycan. Enrofloxacin and danofloxacin belong to the synthetic fluoroquinolone class of antibiotics. Fluoroquinolones prevent bacterial DNA from unwinding and duplicating by targeting DNA gyrase and topoisomerase IV [83]. Tulathromycin and tilmicosin are 15- and 16-membered ring compounds that belong to the macrolide class of antibiotics; tulathromycin is a semisynthetic derivative of the naturally occurring drug erythromycin, whereas tilmicosin is a derivative of the naturally occurring compound tylosin [84]. Two other macrolides, the 15-membered gamithromycin and the 16-membered tildipirosin, are also used in the treatment of BRD. Macrolide antibiotics block protein synthesis by binding within the peptide tunnel of the bacterial 50S ribosomal subunit [85]. Florfenicol is a member of the phenicol family of antibiotics; it is a fluorinated synthetic analog of thiamphenicol and chloramphenicol [78, 83]. The phenicols inhibit protein synthesis by binding to the 50S ribosomal subunit, leading to the inhibition of peptidyl transferase. The tetracyclines represent a large family of antibiotics characterized by their four-ring structures. Tetracyclines inhibit protein synthesis by binding to the 30S ribosomal unit; they block the attachment of charged aminoacyl-tRNA and prevent the introduction of new amino acids to the nascent peptide chain.

Antibiotic Resistance

Antibiotics are currently the most effective tool available for the control of respiratory infections in cattle [86]. Clearly, there are very serious economic and animal health concerns related to the consequences of the development of antimicrobial resistance in *M. haemolytica* and other BRD pathogens [15]. Unfortunately, the detection of emerging antibiotic resistance in *M. haemolytica,* and indeed in the

other BRD pathogens, has been hindered by the lack of annual routine surveillance programs [83]. The German Federal Office of Consumer Protection and Food Safety (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (BVL)) conducts one of the few national surveillance programs for antimicrobial susceptibility among BRD pathogens [87].

Although antibiotics have been effectively utilized to treat bacterial BRD infections, many *M. haemolytica* isolates are now resistant to penicillin, ampicillin, tetracycline, sulfonamides, and tilmicosin [13, 78]. In a study of antimicrobial susceptibilities of *M. haemolytica* and other BRD pathogens isolated from the lungs of beef cattle with pneumonia between 1994 and 2002, there were no significant changes in susceptibility of *M. haemolytica* to ceftiofur, enrofloxacin, or ampicillin, but significant declines in susceptibilities to erythromycin, florfenicol, and tilmicosin were observed [80]. A comprehensive study of antibiotic resistance in 416 *M. haemolytica* isolates recovered from cattle in two Canadian feedlots revealed very low levels (9.5%) of antibiotic resistance and even lower levels (1.2%) of multidrug resistance [15]. The antibiotics studied included ceftiofur, enrofloxacin, florfenicol, tulathromycin, tilmicosin, and oxytetracycline. However, the isolates investigated in this study were recovered from the nasopharynx of healthy cattle, either upon entry to or exit from the feedlots, and were dominated by serotype A2 (74.5%) but also included isolates of serotypes A1 (11.9%) and A6 (12.7%). Serotype A1 isolates accounted for a higher percentage of resistant isolates than observed in the general population but serotype A6 was not associated with the resistance phenotype. In a 10 year (2000–2009) study of antimicrobial susceptibility of bacteria associated with BRD in the United States and Canada, the *in vitro* susceptibility of 2977 *M. haemolytica* isolates to ceftiofur, penicillin, danofloxacin, enrofloxacin, florfenicol, tetracycline, tilmicosin, and tulathromycin was investigated [87]. Ceftiofur remained very active against all isolates and there was no change in penicillin but the percentage of *M. haemolytica* isolates that were susceptible to tilmicosin and tulathromycin declined over time; there were slight declines in susceptibility to danofloxacin, enrofloxacin, and florfenicol [87].

The susceptibility of 310 *M. haemolytica* isolates recovered from cattle with BRD in Japan between 2002 and 2010 to 16 antibiotics was investigated [18]. The proportion of isolates susceptible to all antibiotics decreased over this time period; in particular, there was a significant decrease in susceptibility to fluoroquinolones. Serotype A6 isolates have become prevalent in Japan since 2003, and a significantly higher proportion of serotype A6 isolates, compared to serotype A1 and A2 isolates, exhibited antibiotic resistance. In particular, 84 of 175 serotype A6 isolates were multidrug resistant, suggesting that antimicrobial selection pressure is a likely cause of the high prevalence of serotype A6 *M. haemolytica* in Japan. Antimicrobial multidrug resistance was surveyed retrospectively in *M. haemolytica* isolates recovered from BRD cases between 2009 and 2011 [88]. The antibiotics included ceftiofur, danofloxacin, enrofloxacin, florfenicol, oxytetracycline, spectinomycin, tilmicosin, and tulathromycin. No bacterial isolates were resistant to all six antimicrobials over the 3-year period of the study due primarily to a general lack of resistance to ceftiofur. Using resistance to three or more antimicrobials as the definition for multidrug

resistance, these authors demonstrated that isolates classified as multidrug resistant increased from 42% in 2009 to 46% in 2010 and 63% in 2011. The higher rates of multidrug resistance in isolates from lung tissue of diseased animals [88] compared to isolates from the nasal flora of healthy animals [15] suggests that disease status is driving the emergence of multidrug resistance since isolates from clinical cases are more likely to have had previous exposure to antibiotics.

Antibiotic resistance and other phenotypic characteristics were compared among *M. haemolytica* isolates recovered from healthy (49) and diseased (41) cattle in Canadian feedlots [14]. Resistance to at least one antibiotic occurred more frequently in *M. haemolytica* isolates associated with diseased cattle (37%) compared to those recovered from healthy cattle (2%). Notably, the resistant isolates were all of serotype A1 and the authors suggested that antimicrobial resistance provides an advantage to the dominant serotype implicated in BRD. However, the absence of antibiotic resistance among serotype A6 isolates recovered from cases of BRD did not fit with the hypothesis that antimicrobial therapy is driving resistance development in disease-related populations. A high overall rate of antibiotic resistance was also observed among BRD pathogens recovered from mortalities in Alberta, Texas, and Nebraska [79]. Seventy-two percent of *M. haemolytica* isolates demonstrated antibiotic resistance and 30% were resistant to more than seven antibiotic classes [79]. Significantly, the results confirm other reports [88–90] that multidrug resistance among BRD pathogens is on the increase. Another worrying finding was that up to seven antibiotic resistance determinants in *M. haemolytica* isolates were shown to be associated with integrated conjugated elements (ICEs); this represented the first detection of ICEs in *M. haemolytica* in American feedlots.

Resistance Mechanisms

Clearly, understanding the mechanisms responsible for the emergence and spread of antibiotic resistance in *M. haemolytica* and other BRD pathogens is hugely important for the future treatment and control of BRD. In the case of the quinolones, the primary mechanism of resistance is generally due to mutations in genes that encode for DNA gyrase (topoisomerase type II) and topoisomerase IV, namely, *gyrA*, *gyrB*, *parC*, and *parE* [91, 92]. These two enzymes are critical for DNA replication and both are targets for the quinolones. Mutations in the genes that affect cell permeability or drug export may also be involved. However, the precise mechanism of fluoroquinolone resistance in *M. haemolytica* is not known [93]. Florfenicol resistance in *Pasteurella* species is plasmid-mediated and the plasmid can replicate in different Gram-negative hosts [94–96]. The genetic basis of florfenicol resistance was investigated in a florfenicol-resistant *M. haemolytica* isolate and shown to be linked to a plasmid carrying the *floR* gene [78]. The macrolides are a particularly important class of antibiotics used in the treatment and prevention of BRD. Tilmicosin was introduced in 1992, tulathromycin in 2005, and tildipirosin and gamithromycin in 2011. The incidence of macrolide resistance remains relatively

low in *M. haemolytica* but three macrolide resistance determinants, *erm*(42), *msr*(E), and *mph*(E), have been discovered in isolates associated with BRD [85, 97]. The erythromycin resistance methyltransferase gene *erm*(42) confers resistance by encoding a monomethyltransferase that adds a single methyl group to 23S rRNA nucleotide A2058; the *msr*(E) gene encodes a macrolide efflux pump; and the *mph*(E) gene encodes a macrolide-inactivating phosphotransferase enzyme. Patterns of macrolide resistance in *M. haemolytica* fall into three distinct classes [97]. The first class involves monomethylation of 23S rRNA by the enzyme product of *erm*(42); the second class involves macrolide efflux and inactivation by the products of the *msr*(E) and *mph*(E) genes, which are arranged in tandem; the third class involves a high degree of resistance to a comprehensive range of macrolides due to possession of all three determinants, *erm*(42), *msr*(E), and *mph*(E). A multiplex PCR (polymerase chain reaction) assay has been developed to rapidly identify the three macrolide resistance determinants in *M. haemolytica* and *P. multocida* [98]. However, it has recently been shown that certain *M. haemolytica* and *P. multocida* isolates lack all three genes but are, nevertheless, resistant to a wide range of macrolides suggesting that other resistance mechanisms exist [84]. Resistance to β-lactam agents may be by alteration of penicillin-binding proteins or by enzymatic inactivation of the compounds by β-lactamase enzymes [99].

A worrying trend in the development of antibiotic resistance in *M. haemolytica* is the transfer of multiple resistance determinants by ICEs. These represent a diverse group of chromosome-borne mobile genetic elements that have recently been implicated in the horizontal transfer of antibiotic resistance genes [100]. These genetic elements harbor clusters of accessory genes that can include multidrug resistance cassettes that have been accumulated through recombination. A bovine *P. multocida* isolate was recently shown to harbor an ICE (ICE*Pmu1*) that carried 11 functional antibiotic resistance genes [101]. These included genes conferring resistance to streptomycin/spectinomycin (*aadA25*), streptomycin (*strA* and *strB*), gentamicin (*aadB*), kanamycin/neomycin (*aphA1*), tetracycline [*tetR-tet*(H)], chloramphenicol/florfenicol (*floR*), sulfonamides (*sul2*), tilmicosin/clindamycin [*erm*(42)], and tilmicosin/tulathromycin [*msr*(E)-*mph*(E)]. Crucially, certain *M. haemolytica* isolates have also been shown to possess ICEs that confer resistance for up to seven different antibiotic classes [79].

Discovery of New Antibacterial Agents

The development of widespread resistance to the principal antibiotics used in the treatment and control of *M. haemolytica* and other BRD pathogens would be economically devastating to the livestock industries. Therefore, there is an urgent need to develop new antibacterial agents. The problems associated with the development of new antimicrobials have been well documented in various reviews [102, 103]. There are essentially two main strategies for the development of new antibiotics: these include the discovery of antibiotics from natural sources and the development

of completely synthetic antibiotics. However, very few new antibiotics have emerged in the past decade highlighting the need for novel approaches to antibiotic discovery. Six major antibiotic targets have been identified and exploited, namely: ribosome assembly and action; cell wall stability and biosynthesis; DNA replication; DNA transcription; membrane integrity and biosynthesis; and folic acid biosynthesis [102]. The structural modification of known antimicrobial chemical scaffolds is one option for the generation of new antibiotics but this strategy is reaching its limits. The identification of antibiotic targets out with these six areas is an area of intense research activity.

Current antibiotics target central key metabolic processes that are essential for bacterial survival. However, an alternative approach is the development of antimicrobials that target bacterial virulence factors [104–106]. Virulence genes are involved in host–pathogen interactions but they are not usually essential for bacterial survival. Therefore, pathogenic bacteria are not subject to strong selection pressures driving resistance to antivirulence drugs [104, 105]. In addition, the specificity of antivirulence drugs does not lead to disruption of the host commensal microflora [104, 105]. Virulence determinants that may be targeted for this approach include adherence and colonization mechanisms, such as pili formation and type III secretion systems, invasion mechanisms, toxin production pathways, subversion of host defenses, biofilm formation, and quorum sensing. In Gram-negative bacteria such as *M. haemolytica*, components of the outer membrane are specifically involved in host–pathogen interactions, and potential targets for antivirulence drug development include LPS [102, 106] and a wide range of OMPs including those involved in outer membrane biogenesis and integrity, transport and receptor mechanisms, adherence, and colonization. In addition, specific targeting of efflux pumps could also be included within this list [102, 107].

It is evident that antibiotics have wide-ranging effects on bacterial transcriptomes, including the transcription of bacterial virulence factors; thus, the therapeutic effect of some antibiotics could be due to a combination of both growth and virulence inhibition [108]. There is certainly a growing body of evidence to suggest that antibiotics block virulence gene expression. For example, azithromycin affects quorum sensing and biofilm formation of *P. aeruginosa* [109] and polymyxin downregulates invasion and flagellar genes of *Salmonella typhimurium* [110]. However, very little is actually known about how antibiotics actually cause bacterial cell death [111, 112]. Antibiotic-mediated cell death begins with the drug-target interaction but is actually a complex process involving the transcription of hundreds of genes. Therefore, a better understanding of the sequence of events beginning with the binding of a bactericidal drug to its target and ending in bacterial cell death is necessary for the development of new antibiotics and the improvement of existing antibacterial therapies [112].

The two main approaches used to identify new antibiotics involve the use of cell- and target-based screens. Cell-based screening methods were responsible for the discovery of most of the antibiotic classes during the golden era of antibiotic discovery in the middle of the last century [113, 114]. In contrast, the genome

revolution led to the development of target-based screens, but these approaches have been unsuccessful in the identification of novel antimicrobials in general [103]; indeed, this approach has not yielded a single clinically useful antibiotic [113, 115]. The principal advantage of cellular screens is that they permit the identification of compounds with complex and pleiotropic modes of action resulting in cell death. However, the inability to identify the biochemical target hinders efforts to optimize the compound on the basis of structure-activity relationships. The main advantage of target-based screens is that, in theory, they allow for the rational discovery and optimization of new antibacterial leads although this has clearly not happened in practice. There is now a realization that the way forward is to combine cell- and target-based approaches [111]. Indeed, target-based whole-cell screening approaches have been developed to overcome these limitations: they combine the specificity of target-based approaches with the practical advantages of whole-cell screens [116–120]. One of these studies has led to the discovery of the first novel class of bacterial gyrase inhibitor, kibdelomycin, since the 1950s [119].

The Importance of *In Vivo* Growth Conditions

An important issue related to cell- and target-based screening assays for testing new compounds is the limited predictive value of *in vitro* culture conditions because culture media are not representative of growth conditions that occur *in vivo* [113]. These authors identified five compounds with bactericidal activity against *Mycobacterium tuberculosis* using an *in vitro* cellular screen. However, despite favorable pharmacokinetic/pharmacodynamic indices, three of these compounds selected for further analysis were found to be ineffective in reducing lung bacterial loads in the tuberculosis mouse model. Therefore, the discovery of new antibiotics against bacterial pathogens, including *M. haemolytica,* should be based on an in-depth knowledge and understanding of bacterial metabolism and host–pathogen interactions within host tissues [113].

The development of genomic, proteomic, transcriptomic, and metabolomic technologies in recent years now allows the expression of bacterial gene products to be analyzed under various growth conditions including during *in vivo* growth within infected host tissue. Using such approaches, it has been demonstrated that transcription of genes encoding two putative hemoglobin receptors, *hmbR1* and *hmbR2,* was strongly upregulated in the lung tissue of calves 2 days after experimental infection with a bovine serotype A1 *M. haemolytica* isolate [65]. The relative mRNA transcript levels of *hmbR1* and *hmbR2* were very similar in infected lung tissue and under iron-limited growth conditions, indicating that *in vivo* growth conditions are reasonably well represented by growing *M. haemolytica* under iron-limited conditions. Under the latter growth conditions, there was a general increase in the transcription of iron transporters and a decrease in the transcription of genes encoding iron-containing proteins, such as iron-sulfur proteins involved in electron transport [65]. In a more recent study, *in vivo* gene expression

of *M. haemolytica* was assessed 6 days after infection of calves [121]. Twenty-seven genes had lower levels of expression *in vivo* and many of these were involved in energy metabolism, amino acid transport and metabolism, and cell envelope biogenesis; several virulence-associated genes including those encoding leukotoxin, a capsule biosynthetic enzyme, and the serotype-specific antigen, Ssa, also had reduced expression [121]. The majority of genes upregulated during *in vivo* growth encoded hypothetical proteins. In a subsequent study, the expression of the virulence genes *lktA*, *gcp*, and *tbpB* were shown to be higher after 6–12 h of infection compared to *in vitro* [122]. Clearly, *in vivo* growth has a profound influence on the transcriptome of *M. haemolytica.* Since antibiotics have wide-ranging effects on the bacterial transcriptome (discussed in the previous section), the antibiotic sensitivity profiles of bacteria grown *in vivo* are likely to be different to the profiles of bacteria grown *in vitro.*

Potential Outer Membrane Targets for Novel Antibiotics against *M. haemolytica*

The outer membrane of Gram-negative bacteria contains in excess of 100 proteins that are involved in basic bacterial functions, such as outer membrane biogenesis, as well as having key roles in host–pathogen interactions. Many of these proteins represent potential drug targets and these can be classified into the following categories.

Proteins involved in LPS biosynthesis

Considerable efforts have been made to identify novel antimicrobial drugs that interfere with the biosynthetic pathways of LPS [102, 123, 124]. In particular, efforts have been focused on the identification of inhibitors of lipid A and ketodeoxyoctulosonate (KDO) biosynthesis. Enzymes involved in the biosynthesis of these components represent ideal drug targets because they are both essential and absent from eukaryotic cells. For example, LpxC is a zinc-dependent amidase involved in lipid A biosynthesis and inhibitors of this enzyme represent potentially new antibacterial agents [125, 126]. Since these are key components of the basic structure of LPS, such inhibitors would have bactericidal activity. Other efforts are being directed at biosynthesis of heptose units, which, like KDO, form part of the core-oligosaccharide region of LPS [102]. The first documented inhibitor of the ADP-heptose pathway was described by De Leon *et al.* [127] but *in vivo* studies subsequently failed to demonstrate drug activity, again highlighting the importance of *in vivo* studies. Efforts are also being made to identify antagonists of the interactions between LPS and host cell receptors [123]; such drugs would be classified as being of the antivirulence type. Clearly, similar research efforts could be directed at *M. haemolytica* and other pathogens associated with BRD in the future.

Efflux Pumps

Efflux pumps represent a significant barrier to antibiotic activity and have been the target for drug discovery since the early 2000s, in the main for *P. aeruginosa* [102, 107]. There are five families of efflux pumps but the resistance-nodulation division (RND) family is often associated with antibiotic resistance in Gram-negative bacteria [102]. Efflux pumps may confer resistance to a single antibiotic type (such as the Tet pumps that confer resistance to just tetracycline antibiotics) or to multiple antibiotic types (such as AcrB pumps). RND pumps represent a tripartite system comprising an inner membrane transport protein (AcrB) linked to an outer membrane transport protein (TolC) by a periplasmic intermediate (AcrA) (Figure 7.4) [107]. Mechanisms for inhibiting of RND pumps include blocking the outer membrane channel, introducing molecules that compete with the inner membrane protein pump, removal of the energy source for the pump and physically altering the pump itself. Although a number of potential efflux pump inhibitors have been identified, none have been approved for treatment [102, 128]. We have identified TolC in the outer membranes of seven isolates of *M. haemolytica* representing different serotypes using proteomic approaches [75], and it is reasonable to assume that this protein forms part of an active RND pump. Therefore, efflux pump inhibition could represent a strategy for treatment and control of *M. haemolytica* and other BRD pathogens.

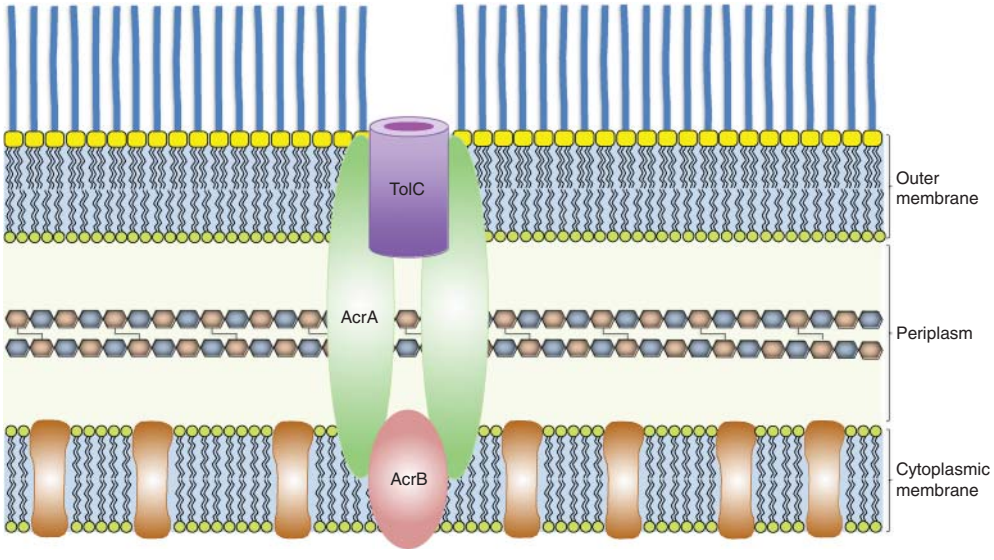

Figure 7.4 Schematic representation of the AcrAB–TolC efflux pump. AcrB represents the cytoplasmic membrane-spanning protein responsible for actively pumping antibiotics across the cell envelope; TolC represents the outer membrane channel through which the antibiotics are pumped; and AcrA represents the periplasmic adapter protein linking AcrB and TolC.

Outer Membrane Biogenesis Proteins

The BAM (beta-barrel assembly machinery) complex of Gram-negative bacteria is essential for the incorporation of integral β-barrel proteins into the outer membrane [129, 130]. It is required for the correct folding and insertion into the outer membrane of almost all integral OMPs identified to date. The BAM complex comprises five proteins, BamA to BamE (Figure 7.5). BamA and BamD are universally present in all Gram-negative bacteria and are essential for *E. coli* viability. β-barrel assembly is an essential process and the BAM complex (BamA and BamD in particular) is an attractive target for the development of new antibiotics; interfering with the functioning of the complex will inhibit the assembly of all integral OMPs. Recently, a small BamD-binding peptide has been shown to inhibit BamA and OmpA assembly [131]. It was suggested that molecules mimicking the BamD-binding peptide should interfere with OMP assembly and that it should be possible to develop antibiotics that selectively target the Bam complex. We have identified BamA in the outer membranes of seven isolates of *M. haemolytica* representing different serotypes using proteomic approaches [75]. Therefore, the development of antibiotics targeting the Bam complex of *M. haemolytic* would appear to be a realistic opportunity.

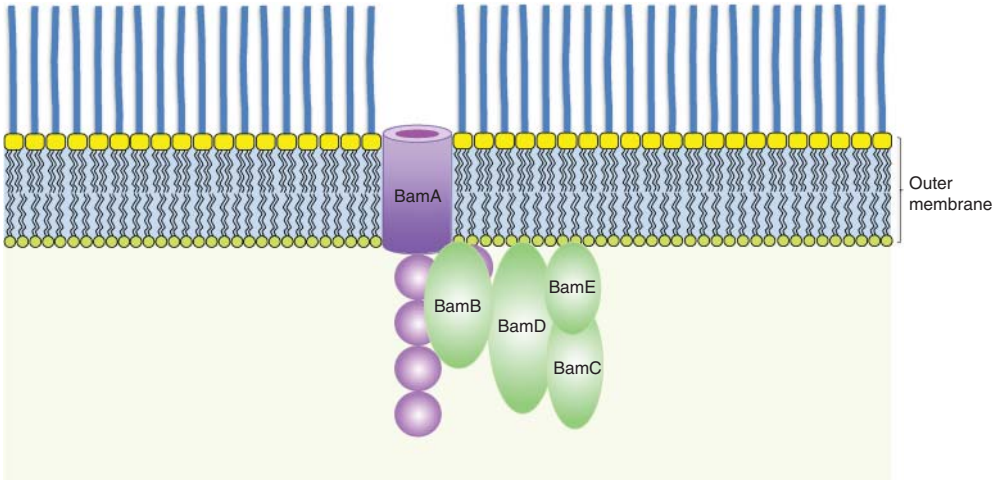

Figure 7.5 Schematic representation of generalized BAM complex, which is involved in folding and insertion of integral OMPs into the outer membrane. BamA is an integral membrane protein consisting of a transmembrane β-barrel domain and five polypeptide-transport-associated (POTRA) domains, which is required for insertion of proteins into the outer membrane; BamB to E are accessory lipoproteins localized in the inner leaflet of the outer membrane. BamA and BamD are essential proteins and inhibition of BamD may lead to interference with OMP assembly (128). Unfolded OMP precursors are transported across the cytoplasmic membrane by the Sec machinery and, in the periplasm, interact with the chaperone proteins SurA, Skp, and DegP (not shown).

Proteins Involved in Adherence

M. haemolytica possesses a number of OMPs putatively involved in adherence including OmpA, a collagen-binding trimeric autotransporter AhsA, Lpp1 (PlpA), a high-molecular-mass protein that is homologous to the Hia autotransporter protein of *H. influenza* and a 68-kDa protein that is involved in adherence to tracheal epithelial cells; these have all been discussed in the previous sections. The publicly available *M. haemolytica* genomes also contain the genes encoding type IV pili [66, 67] although expression of pili has not been definitively confirmed in this bacterium. New antibiotics targeting these proteins would fall under the antivirulence category. Interfering with bacterial adherence represents a useful strategy in combating infection because of its importance during the early stages of pathogenesis. Strategies aimed at inhibiting adherence due to pili can broadly be divided into two types: the first involves precluding pathogen binding to host cells by blocking the adhesive properties of pili; the second involves interrupting pili assembly. The same principles could apply to other putative adhesins. Chaperone-usher systems are necessary for the assembly of pili as well as other adhesive organelles in a wide range of pathogens. Therefore, inhibitors of the chaperone-usher system could offer a broad-range therapeutic approach. Pilicides are a class of pilus inhibitors that target chaperone function and inhibit pilus biogenesis [132–134]. Similar approaches could be used to target known adherence proteins of *M. haemolytica.*

Proteins Involved in Iron Uptake and Other TonB-Dependent Proteins

Iron is an essential element for bacteria (as it is for all living organisms) because it is a component of key metabolic enzymes such as iron-sulfur enzymes that are involved in the electron transport chain. However, iron is in short supply within the host because it is either compartmentalized within host cells (especially erythrocytes) or it is complexed with the high-affinity proteins transferrin and lactoferrin. Consequently, pathogenic bacteria have evolved numerous iron-acquisition systems with which to acquire iron from their hosts. Iron-acquisition systems can be classified as belonging to either the siderophore-mediated or direct-uptake types. *M. haemolytica* possesses a number of OMPs involved in the direct uptake of iron and these have been described above. Bacterial iron-acquisition systems (both of the siderophore-mediated and direct-uptake types) involve outer membrane receptor and transport proteins that derive their energy from the proton motive force (PMF) generated across the cytoplasmic membrane. The energy of the PMF is transferred to the outer membrane receptor/transport proteins by the TonB transduction system, which comprises three proteins, TonB, ExbB, and ExbD.

Since iron is so important to pathogenic bacteria, targeting iron acquisition would seem an attractive and logical strategy. Indeed, a review of iron acquisition as an antimicrobial target highlights successful inhibitors of siderophore biosynthesis enzymes [135]. However, the multiplicity and redundancy of iron acquisition systems in many bacterial species including *M. haemolytica* makes it difficult to identify a specific cellular target. On the other hand, all of the iron-acquisition

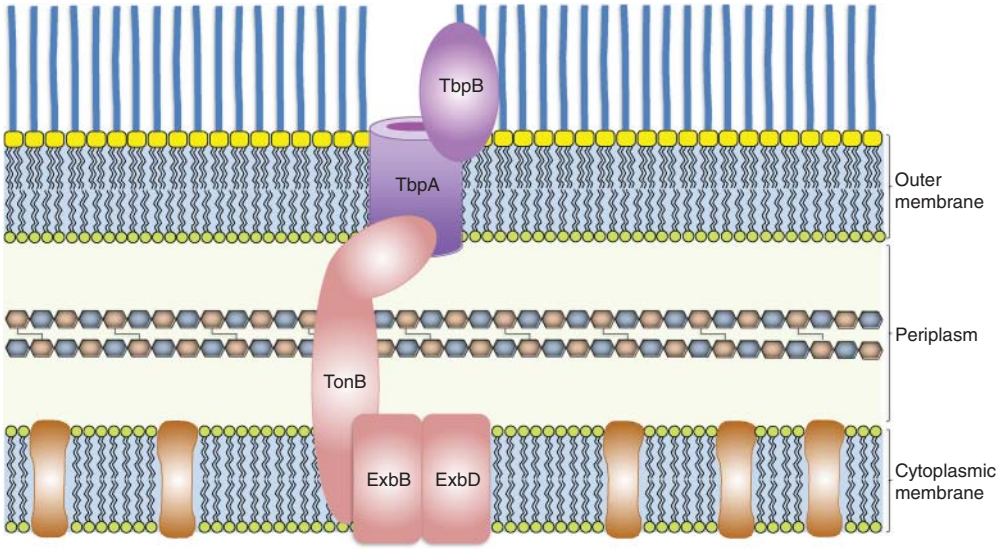

Figure 7.6 Schematic representation of the iron-uptake transferrin receptor complex TbpAB and the TonB energy-transduction system comprising TonB, ExbB, and ExbD. Transport of essential iron across the outer membrane via the TonB-dependent TbpA protein, and other iron-transport proteins, could be blocked by inhibiting the TonB system.

systems obtain energy via the TonB energy transduction system and this would seem a logical target (Figure 7.6). Using a whole-cell growth-based high-throughput screen of 149 243 compounds, 16 compounds were identified that arrested the growth of uropathogenic *E. coli* under iron-limited growth conditions [136]. TonB was subsequently demonstrated to be the target for two of these compounds. Clearly, the development of antibiotics that specifically uncouple the TonB energy-transduction system from the TonB-dependent iron-uptake proteins of the outer membrane would likely represent a major advance in the fight against not only *M. haemolytica* but Gram-negative pathogens in general.

Conclusion

M. haemolytica is considered to be the most important bacterial pathogen associated with BRD and is responsible for substantial economic losses to the livestock industries. The emergence and spread of antibiotic resistance in *M. haemolytica* and other BRD pathogens is becoming an increasing problem and there is an urgent requirement for the identification and development of new antibacterial agents against this economically important pathogen. The identification of new antimicrobials is likely to involve a combination of cell- and target-based approaches

and novel drug targets could include virulence genes as well as genes involved in central metabolic processes. *In vivo* screening approaches will be important in the identification of new targets because *in vivo* growth has a profound effect on gene transcription and will affect the expression of potential drug targets. The Gram-negative outer membrane comprises a large number of proteins with wide-ranging functions but many play key roles in host–pathogen interactions. OMPs represent potential drug targets that could be the focus for future research aimed at the design and development of new drugs to combat *M. haemolytica* as well as other pathogens involved in BRD.

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8
Identification of Anti-infective Compounds Using Amoebae

*Christopher F. Harrison and Hubert Hilbi**

Abstract
Drug-resistant bacterial pathogens represent an ever-increasing public health problem and call for the identification of novel anti-infective compounds. Environmental amoebae share many similarities with mammalian immune system phagocytes and therefore represent versatile and robust cellular models for screening and characterizing novel antibiotic, anti-virulence, or immune-boosting compounds. Using the amoeba *Acanthamoeba castellanii*, we developed robust fluorescence-based growth assays for the vacuolar pathogens *Legionella pneumophila* and *Mycobacterium marinum*. In screens for anti-virulence compounds, we discovered a hitherto unrealized antibiotic property for the β-lactone palmostatin M. Taken together, our studies revealed the utility of amoebae-based drug screening systems for the identification of novel anti-infective compounds.

Introduction

Following the ground-breaking work of Alexander Fleming and the initial discovery of antibiotics [1], the period of 1950s and 1960s were considered somewhat of a "golden age" of antibiotic development. During this time, over half of the antibiotic families in use today were discovered, developed, and brought to market. Unfortunately, this age is well and truly over – the number of patients with drug or multidrug resistant bacterial infections are rapidly increasing, while the "pipeline" of new antibacterials in development is shrinking year by year [2]. There is thus a need to develop new antibiotics, preferably those with novel chemical scaffolds as a means to minimize the onset of drug resistance.

Conventional drug development relies heavily on the use of high-throughput compound screening (HTS), a process that has been optimized to the point that many thousands of compounds can be screened per day. However, attempts to use this approach in antibiotic development have been troubled at best – huge numbers of HTS campaigns against essential bacterial targets have led to few or

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no lead compounds [3]. This approach relies on the identification of an essential target protein, followed by development of an *in vitro* screen to find inhibiting compounds. Unfortunately, the process of developing an *in vitro* hit into one that works on live bacteria has been so far fraught with difficulty. Better luck has been seen with the screening of compounds against live bacteria, as compounds found in this way are already known to inhibit bacterial growth. However, the next step, determining the mechanism of action, can then be very challenging [4].

Anti-virulence Compounds as an Alternative to Antibiotics

The ability of a compound to either kill or inhibit the growth of bacteria is, of course, one of the primary attributes required when developing a new antibiotic. However, this approach also carries a disadvantage in that selection pressure for development of drug-resistance against these compounds is extremely high. This selection pressure to develop antibiotic resistance can be observed in patients, such as those with chronic *Staphylococcus aureus* infections [5]. Given their fast replication times, stunning genetic diversity, and ability to exchange fitness-enhancing genetic elements, bacteria have a major advantage in the arms race with medicine.

A better method then would be to target the ability of bacteria to infect and attack the host, rather than targeting replication directly. These "anti-virulence" compounds would be aimed at specific mechanisms that promote pathogenicity such as binding to target cells, uptake processes, signaling and vesicle trafficking pathways, secretion and mode of action of toxins, or intracellular metabolism (Figure 8.1). Thus, anti-virulence compounds may reduce evolutionary pressure to develop drug resistance. A further advantage would be that by selectively targeting virulent bacteria, the normal host microbiota would remain relatively unaffected. Given that host biota are surprisingly important to general health [6, 7], and can change drastically in response to antibiotic treatment [8], this advantage should not be discounted.

The development of compounds that target bacterial virulence, rather than replication, has already begun. One approach involves neutralizing toxins released by the bacteria, usually using injectable antibodies. Examples here include an antibody that targets botulinum toxin [9], or Urtoxazumab, which binds to Shiga-like toxin 2 and has reached clinical trials [10]. While this strategy can be very effective, it has disadvantages, particularly in dealing with intracellular pathogens such as *Mycobacterium tuberculosis* or *Legionella pneumophila*. As these pathogens inject their proteins directly into host cells, there is no stage at which toxins and effector proteins can be intercepted by antibodies.

To avoid this problem, scientists have turned toward targeting the virulence systems of intracellular bacteria themselves, a process that can be direct or indirect. Compounds exemplifying the direct approach are salicylidene derivatives, several of which have been shown to inhibit the type III secretion system (T3SS) of *Salmonella enterica* Typhimurium, preventing uptake of the bacteria by the host and subsequent intracellular replication [11]. Structurally similar compounds

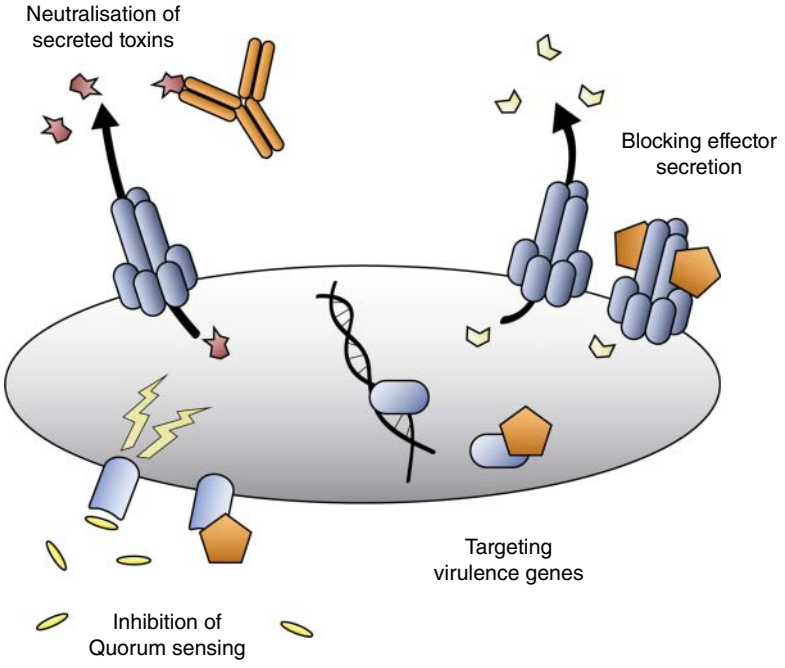

Figure 8.1 Potential targets of antivirulence compounds. Several antivirulence schemes can be exploited to restrict pathogens. Antibodies can bind to and neutralize secreted bacterial toxins. Small-molecule compounds can target bacterial secretion systems, preventing the injection of effector proteins, or modulate quorum sensing systems, thus blocking bacterial colonization and virulence. Lastly, compounds can be designed to bind to transcriptional regulators of virulence genes, thus inhibiting their expression.

have been reported to inhibit the T3SS of *Yersinia pseudotuberculosis* [12] and pathogenic *Escherichia coli* [13].

More indirect processes can also be targeted, preventing the expression of virulence-associated genes. As an example: Virstatin, a small-molecule inhibitor of the ToxT transcription regulator, is able to prevent the expression of several *Vibrio cholerae* virulence factors, preventing disease in mouse models [14]. Interference with bacterial quorum sensing systems can have similar effects – furanone compounds from algae have been shown to inhibit a number of bacterial quorum-sensing dependent processes [15]. Further chemical modification of these compounds has led to derivatives, which are able to assist the clearance of pulmonary *Pseudomonas aeruginosa* infection in mice [16]. Other compounds have been shown to block quorum sensing in *V. cholerae* [17], or enterohemorrhagic *E. coli* (EHEC) [18], thus preventing the expression of numerous bacterial virulence factors.

Targeting virulence-associated pathways is a complex approach, especially when compared to the simplicity of searching for compounds that prevent replication. This approach requires either knowledge of the process to be modulated (which can

then be assessed via an *in vitro* system) or a method to analyze the interaction of the bacteria with its host cell. Here, we discuss the second option, with a focus on the use of amoebae as model host systems.

Amoebae as Model Host Systems in Compound Screening

Amoebae are single-celled eukaryotes and can be considered one of the major predators of the microscopic world. Amoebae will actively track down bacteria, utilizing phagocytosis to engulf and deliver bacteria to lysosomal compartments for digestion. While simple, amoebae contain many of the systems associated with specialized cells of higher eukaryotes, including chemotaxis, cell–cell signaling, and phagocytic uptake – as such they have been heavily utilized as models in fields such as cAMP (cyclic adenosine monophosphate) signaling and cytoskeletal rearrangement [19–21].

Beyond this, amoebae have been employed in a number of screening processes. Amoebae infections can be deadly, so studies to determine amoebicidal compounds have been conducted, analogous to screens searching for novel antibiotics against bacteria. High-throughput screens targeting *Naegleria fowleri*, the causative agent of primary amoebic meningoencephalitis, or *Entamoeba histolytica*, the causative agent of the diarrheal disease amebiasis, have led to the successful discovery of several new therapeutics [22, 23].

Slightly more out-of-the-box thinking has been performed as well, such as in the use of the social amoeba *Dictyostelium discoideum* as a model for emetic compounds (which induce a vomit reflex) [24]. As these emetic or taste aversive compounds have a pronounced effect on *D. discoideum* chemotaxis, this feature has been utilized as a counter-screen for estimating bitterness of taste [25].

Amoebae have not yet been employed as a screening model for host–pathogen interactions. The relative simplicity and robustness of amoebae, combined with certain similarities to the roles of human immune phagocytes such as macrophages or neutrophils, imply that they may represent a potential model system. To determine the plausibility of this idea, we set out to examine the infection of *Acanthamoeba castellanii* with the intracellular, amoebae-resistant pathogens *L. pneumophila* and *Mycobacterium marinum*.

The Amoeba-Resistant Pathogens *L. pneumophila* and *M. marinum*

Environmental microorganisms are commonly found residing within biofilms: bacterial communities comprising large numbers of cells held together via adhesion molecules and secreted matrix compounds. Biofilms are ubiquitous in the environment and contain a range of bacterial species, often in communication via quorum sensing molecules [26]. These biofilms can be "grazed" by amoeboid or ciliate predators, with a concomitant reduction in the bacterial population.

A. castellanii is one of the most commonly found environmental protozoa, having been isolated from diverse environments including the soil [27], freshwater lakes and

rivers [28], roadside puddles [29], the atmosphere [30], ocean sediments [31], frozen lakes [32], and even the Antarctic [33]. Moreover, these amoebae are phagocytic feeders, grazing on the bacteria present in environmental biofilms.

Legionella species are ubiquitous, Gram-negative bacteria, which resist degradation by amoebae and other protozoa [34, 35]. Upon inhalation, the pathogenic bacteria can cause a severe pneumonia termed Legionnaires' disease. Studies of floating biofilms collected from a range of natural and human-made sources indicated that *Legionella* species are present in almost all cases. Many biofilms also contain *A. castellanii*, in some cases already infected by *Legionella* spp. [36]. In fact, while *L. pneumophila* has been shown to reside and survive within biofilms, efficient bacterial replication appears to require the presence of protozoan grazers such as *Acanthamoeba* or *Hartmannella* species [37].

Legionella utilizes a complex series of processes to infect host amoebae, almost all of which require a set of genes termed *icm*/*dot* [38, 39]. Together these genes encode the components of a type IV secretion system (T4SS), a transport apparatus that can inject a plethora of so-called "effector proteins" into the host cell. The Icm/Dot apparatus and its associated effector proteins are a versatile system for altering host processes, as the same proteins are required for uptake [40] and efficient intracellular growth within both protozoans and human macrophages [41–46]. Within these phagocytes, the pathogen forms an endoplasmic reticulum (ER)-derived replicative compartment termed "*Legionella*-containing vacuole" (LCV), which interacts with the endosomal, retrograde, and secretory vesicle trafficking pathways, but does not fuse with bactericidal lysosomes [47, 48]. The similarity of the infection process between amoebae and macrophages in turn implies that *A. castellanii* can be utilized as a model system for bacteria–macrophage interactions [49–51].

Another group of intracellular pathogens are the members of the genus *Mycobacterium*, the most notorious of which, *M. tuberculosis*, causes the respiratory ailment tuberculosis and is responsible for over a million deaths each year [52]. Pathogenic *Mycobacterium* species are able to manipulate host cell processes such as phagosome maturation [53], membrane trafficking [54], and apoptosis [55], allowing them to successfully replicate within a distinct "*Mycobacterium*-containing vacuole" (MCV) in macrophages, their erstwhile killers. Much of this manipulation occurs, as with *Legionella*, through the secretion of effector proteins into the host cell, in this case using several ESX type 7 secretion systems (T7SSs) [56].

As the pathogen is highly infectious and virulent, *M. tuberculosis* is classified as requiring biosafety three level control for all experimentation, which raises the cost of any research such as compound screening. To avoid this, we therefore chose to employ *M. marinum*, the closest genetic relative of *M. tuberculosis*, which also belongs to the slow growing group of mycobacteria and causes tuberculosis-like pathology in fish and frogs [57]. As *M. marinum* grows optimally at 30 °C, it is generally only capable of causing superficial skin lesions in humans, and thus can serve as a less dangerous model for *M. tuberculosis* infection.

As with *Legionella*, *Mycobacterium* spp. are capable of infecting and replicating within amoebae. It has been shown that at least 26 separate species are capable of

surviving and replicating within *Acanthamoeba polyphaga* [58], and *Mycobacterium avium* can reside within *A. castellanii*, protected from external antimicrobials [59]. As such, a screening system comprising *M. marinum* and *A. castellanii* also held promise. We therefore set out to develop new screening systems for the intracellular pathogens *L. pneumophila* and *M. marinum*.

Development of Novel Amoebae-Based Screening Systems

Successful use of a model system for drug screening requires that it be fast, reproducible, inexpensive, and amenable to high-throughput analysis. At the beginning of our studies, various methods had been developed to examine the properties of intracellular *L. pneumophila* [60–64]. Unfortunately, the majority of these approaches were not suitable for screening large numbers of compounds, due to factors such as high degree of complexity, lengthy setup and assay times, or high costs. A further disadvantage of numerous models was the requirement for charcoal agar-based growth media for *L. pneumophila*. Charcoal is included to absorb toxic chemicals produced during media preparation or excreted by the bacteria, but can also, naturally, absorb the compounds being assayed.

We therefore developed a system whereby *A. castellanii* amoebae seeded into 96-well plates were infected with green fluorescent protein (GFP)-producing *L. pneumophila* (Figure 8.2). The progress of intracellular replication could then be followed via the increase in fluorescence [65]. Careful choice of growth media minimized fluorescence and growth of the amoebae throughout the assay, simultaneously preventing the extracellular growth of the bacteria. Bacterial replication proceeded along a typical path: lag phase, followed by replication, after which all host amoebae were dead and so bacterial number and fluorescence remained steady. Our comparison of compound efficacy was based on the fluorescence levels at the start of the steady-state phase (approximately 24 h after infection) as this yielded the most reproducible results.

Initial testing of this system using antibiotics indicated that it was highly reproducible, with a median Z-factor of 0.692 (95% confidence intervals of 0.04). The Z-factor is a common statistical measure of the error and allows separation of positive and negative control results. Values greater than 0.5 are considered satisfactory, while the value of 0.692 compares well to other assays utilizing host–pathogen systems [66–68]. One drawback of the developed method was the presence of a noticeable "edge effect," a significant reduction in the reproducibility of replication in the outermost wells of the 96-well plate, which was unfortunately enhanced by the longer timescales required for intracellular replication. Various alterations in the procedure were attempted with limited success, and eventually, the final protocol simply ignored the outer wells for assay purposes. In the future, further optimization would be required to scale this process to the 384-well plate format for high-throughput screening.

Simultaneously, our collaborators developed a fluorescence-based assay to follow the replication of *M. marinum* in *A. castellanii* [69]. As with the *Legionella* assay, replication of the bacteria could be followed by an increase in fluorescence

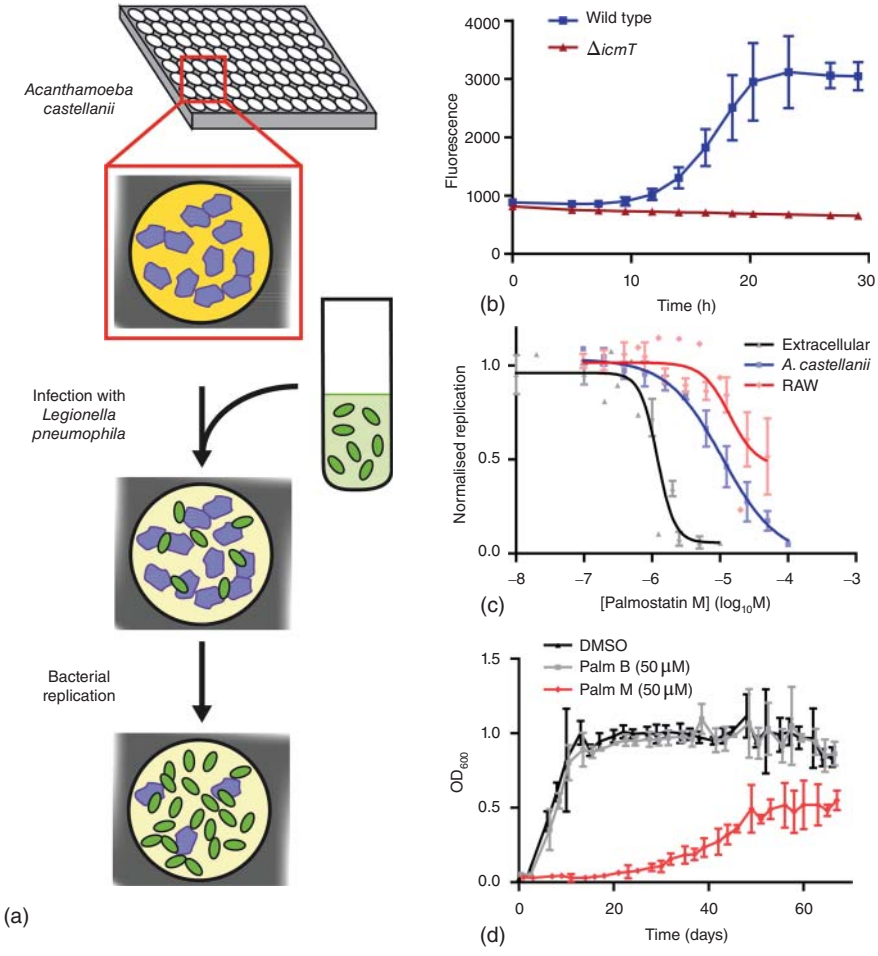

Figure 8.2 Setup and results of amoebae screens. (a) Overview of the fluorescence-based *L. pneumophila*/*A. castellanii* growth assay. Ninety-six-well plates seeded with *A. castellanii* are infected with GFP-producing *L. pneumophila*. The increase in GFP fluorescence is monitored, indicating the progress of intracellular replication. (b) Fluorescence growth curve of wild-type *L. pneumophila* and the replication-defective Δ*icmT* mutant strain within *A. castellanii* indicating the typical phases (delay-growth-stationary) over 30 h. (c) Dose-response curves of *L. pneumophila* treated with the β-lactone palmostatin M both in broth (extracellular growth) and within *A. castellanii* or RAW 264.7 macrophages (intracellular growth). Graph indicates mean and standard deviation (SD) from more than 10 separate experiments. (d) Palmostatin M also demonstrated significant inhibition of *M. tuberculosis* extracellular growth over a long timescale (60 days). Graph indicates mean and SD of triplicate assays. Figure adapted from [65].

over time. One major difference, however, was that during the course of the experiment *M. marinum* replication was continuous, with no stationary-phase observed. Thus, this system examined the effect of compounds on bacterial growth rate, rather than on total number, and thus utilized measurements across the entire experiment rather than at a single point. Assay robustness was excellent, with an overall Z-factor score of 0.74. Initial experiments with the *M. marinum* assay allowed accurate calculation of IC_{50} values for a number of typical anti-mycobacterial compounds, and these values compared well to commonly accepted measurements.

Palmostatin M – A Novel Antibacterial Compound Specific for *Legionella* and *Mycobacterium* Species

In an attempt to identify compounds that might boost host-cell immunity against pathogens, initial testing with the *Legionella* screening assay was conducted on a smaller set of eukaryote-targeting compounds [65]. This screening process was supplemented by assays for host cell toxicity, and those compounds that caused cell death were ignored in further studies. Nontoxic compounds included taxol and nocodazole (which target microtubule polymerization), latrunculin B (an actin polymerization inhibitor), brefeldin A, dynasore, and retro-1 (which interfere with vesicle trafficking), as well as palmostatin M and B (inhibitors of Ras GTPase signaling). Of these, only latrunculin B, dynasore and palmostatin M caused significant reduction in either intra- or extracellular growth of *L. pneumophila* at 10 μM concentration (Figure 8.2).

The strong inhibition of bacterial growth by the β-lactone palmostatin M was interesting, as this compound was believed to be a specific inhibitor of the eukaryotic Ras depalmitoylase enzymes APT1 and APT2, which block Ras GTPase depalmitoylation activity and in turn alter localization and activity of the small GTPase [70, 71] (Figure 8.3). The efficacy of the compound in inhibiting extracellular replication of *L. pneumophila*, as well as the fact that the bacteria do not produce Ras GTPase, indicated that palmostatin M may be causing off-target effects on bacterial (serine or cysteine) hydrolases with a similar active site. Thus, palmostatin M might covalently modify and inhibit the bacterial target(s) like the eukaryotic hydrolases APT1 and APT2.

Interestingly, while palmostatin M inhibited replication of *L. pneumophila* within *A. castellanii* and RAW 264.7 macrophages (Figure 8.2), the structurally similar β-lactone palmostatin B was ineffective, suggesting that palmostatin M had a highly specific, if off-target, effect. A number of *L. pneumophila* deletion mutant strains were tested for their response to palmostatin M, including knockouts of sigma factors, proteasome components, or up to 18% of the *Legionella* genome, but no apparent differences in susceptibility could be determined. Thus, the actual target of palmostatin M remains unidentified.

Further testing examined the species specificity of palmostatin M. To our surprise, we found that the compound was extremely effective at preventing the replication of numerous *Legionella* species, as well as *M. marinum* and *M. tuberculosis* [65]

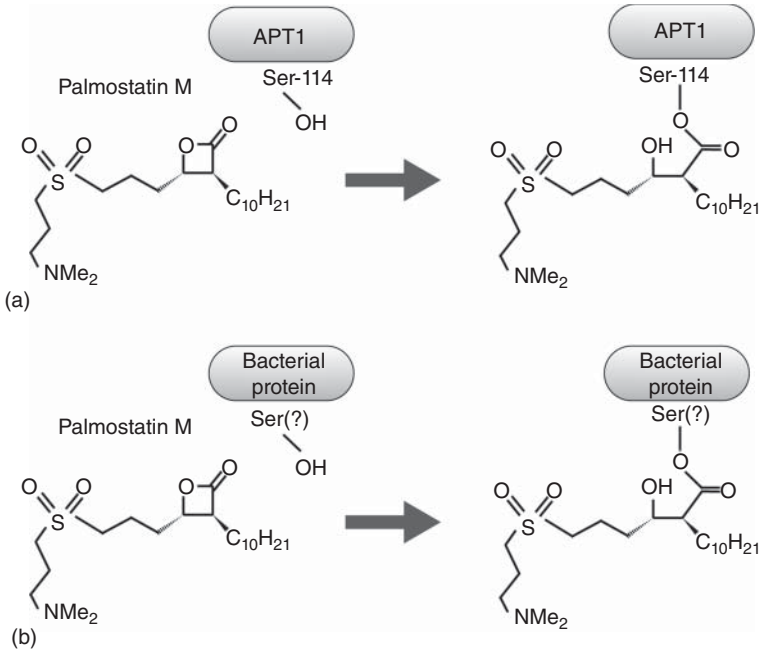

Figure 8.3 Hypothetical mode of action of the β-lactone palmostatin M. (a) The (probable) interaction of the β-lactone palmostatin M with the active site of the Ras depalmitoylase APT1, leading to covalent modification and inhibition of the enzyme [70, 71]. (b) The hypothetical interaction of palmostatin M with a *Legionella* or *Mycobacterium* target, presumably being modified with a similar mechanism as APT1. The closely related β-lactone palmostatin B had no effect, and thus, likely does not modify a bacterial target.

(Figure 8.2). By contrast, palmostatin B again did not have an effect, and no other bacterial species were affected by palmostatin M, suggesting that the genera *Legionella* and *Mycobacterium* produce an as yet unidentified target protein with common structural determinants.

The unique chemical scaffold of palmostatin M, coupled with its surprising selectivity for *Legionella* and *Mycobacterium* species suggested that it may represent a precursor and lead compound to a novel class of antibiotics. Accordingly, efforts are currently underway to identify the target of this compound, using a combination of randomized mutagenesis and sequencing to determine protective mutations. In summary, our studies, while not succeeding in identifying the expected antivirulence properties of these compounds, did lead to the discovery of a novel antibiotic scaffold.

Conclusion

To overcome the problem of (multiple) drug resistant bacterial pathogens, new antibiotics or antivirulence compounds are urgently needed. Antivirulence

compounds are aimed at specific mechanisms that promote bacterial pathogenicity and pathogen-host cell interactions, such as binding to target cells, uptake processes, signaling and vesicle trafficking pathways, secretion and mode of action of toxins, or intracellular metabolism. Due to their similarities to human immune phagocytes and ease of use, amoebae represent a novel, versatile, and powerful model system to screen and develop antivirulence compounds. Initial studies indicated that screening for antibacterial compounds can be performed using representatives of two pathogenic bacterial genera, *Legionella* and *Mycobacterium*. To this end, GFP-producing bacteria were used to infect *A. castellanii* on a 96-well plate scale, and bacterial growth was followed by fluorescence. In the course of these experiments, a novel antibiotic property for the β-lactone compound palmostatin M was discovered. In contrast, the closely related β-lactone compound palmostatin B was ineffective. Future development of this system may improve our ability to find new compounds targeting pathogenic bacteria, thus offsetting the rapid development of bacterial drug resistance.

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9
Stress Biology in Fungi and "Omic" Approaches as Suitable Tools for Analyzing Plant–Microbe Interactions

Stefan Jacob and Alexander Yemelin*

Abstract

Plant–microbe interaction is characterized by signal sensing and responses toward changing environments. Pathogens and hosts have to detect changing environmental situations quickly and adapt their physiological processes as soon as possible. The penetration of the host plant by phytopathogenic fungi is such a rigorous alteration of external stimuli. The internal environment of plant tissue and the plant defense mechanisms are extremely different in contrast to the conditions *ex planta*. The switching of local environmental conditions entails the fungus to identify this changing situation and to react in an adequate way enabling an effective and successful penetration and invasion of the host plant in order to complete the mostly complex life cycle. Signaling mechanisms and signaling pathways have been studied by a wide range of scientists for many years, but there is still a range of unknown processes, especially *in planta* activities. The postgenomic era comprises the "omic" technologies (genomics, transcriptomics, proteomics, and metabolomics) and empowers through their combination a deeper look towards the molecular basis of plant–microbe interactions. The major challenge therefore is how to combine these methodologies in order to understand signaling processes of pathogens, hosts, and their interaction. This chapter provides an overview of the major stress signaling systems in pathogenic fungi and modern technologies to study their physiological properties.

Introduction

Even within the best-studied phytopathogenic fungi, there is still a great challenge to learn more about how disease is established and propagated. The situation for phytopathogenic fungi in the course of plant–microbe interaction with regard to signaling pathways is of major interest for plant protection. In order to invade the host plant and during propagation *in planta,* the pathogen has to cope with

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various environmental stresses. Molecular events that constitute the general steps of host–pathogen interplay seem to involve ligand–receptor systems for pathogen recognition, signaling proteins, mitogen-activated protein kinase (MAPK) cascades for signal transduction or even the induction of signaling pathways in the plant that lead to plant defense responses. The main focus of this chapter is providing an overview of the molecular basis of the major stress signaling systems in pathogenic fungi and possibilities to study their physiological properties. The chapter therefore reviews the major signaling pathways responsible for stress adaptation of fungi and genome-wide approaches that have emerged in the postgenomic era, for example, comparative genomics and gene expression profiling experiments (including technologies such as RNA-seq, (phospho-) proteome, or metabolome analysis). The application of the "omic" technologies (genomics, transcripromics, proteomics, and metabolomics) represents a major opportunity to identify novel fungicide targets within the signaling systems of pathogenic fungi. One of the principal challenges today is how best to employ these technologies to identify which metabolic pathways and gene products are critical for disease establishment and progression and thus to increase the probability of finding new approaches to combat the pathogens.

Stress Signaling Pathways Are Important in Plant–Microbe Interactions

In the course of evolution, plant pathogens evolved numerous signaling cascades in order to get benefits for disease development and coping with environmental stresses during *in planta* growth. Well-known examples for such unsuitable conditions the pathogens have to combat is oxidative burst as a plant defense mechanism with local accumulation of H_2O_2 (reactive oxygen species, ROS; [1]) or exposure of the fungus to increasing concentrations of salts and osmolytes during *in planta* growth. Signaling pathways are therefore extremely important for the fungus and consequently responsible for transcriptional and physiological changes within the cell. External stimuli from the host plant or the environment give rise to expression of disease-associated genes, for example, genes responsible for formation of infection structures, genes involved in protection of the pathogen from the host, or even genes that are expressed to obtain nutrients from the host cell [2].

High Osmolarity

The regulation of cellular turgor in response to environmental changes is one of the most important developments for pathogenic fungi since they have to cope with rapid changing situations within their host. Upon invasion of the plant, the most apparent signal affecting cellular turgor is hyperosmotic stress, resulting in

the accumulation of high concentrations of compatible solutes within the cell, for example, glycerol, to maintain cellular homeostasis [3]. The adjustment of the turgor through the accumulation of these solutes within the cell prevents water loss due to osmosis. Responsible for the detection of and adaption to hyperosmotic stress in fungi is the high osmolarity glycerol (HOG) pathway [4]. The HOG pathway in *Saccharomyces cerevisiae* is well understood and consists of a sensory phosphorelay system and a MAPK cascade. High osmolarity in yeast is detected via the Sln1p-Ypd1p-Ssk1p phosphorelay system, thereby activating the MAPK cascade Ssk2p-Pbs2p-Hog1p, which initiates the physiological stress response. Sln1p is a hybrid histidine kinase (HK), Ypd1p a phosphotransfer protein, and Ssk1p a response regulator protein. The components of the MAPK cascade are the MAPKKK Ssk2p, the MAPKK Pbs2p, and the MAPK Hog1p [5]. In yeast the HOG pathway incorporates two independent signaling branches, Sln1-branch and Sho1-branch, which sense osmotic changes in different ways [6, 7]. The Sho1-branch appears to be dispensable for osmosensing, whereas the Sln1-branch is of high importance [7, 8]. Under normal environmental conditions, the membrane-HK Sln1p is constitutively phosphorylated. The phosphate is further transferred to Ypd1p and subsequently assigned to the response regulator Ssk1p [9]. If phosphorylated, the regulator Ssk1p is not capable of interacting with the MAPKKK Ssk2p and accordingly the MAPK module Ssk2p-Pbs2p-Hog1p remains inactive. Osmotic stress, however, inactivates and therefore dephosphorylates the sensor-HK Sln1p. Phosphate transfer cannot take place anymore and the cytoplasmic level of dephosphorylated Ssk1p regulator increases. Dephosphorylated Ssk1p is able to bind Ssk2p, thereby initiating the MAPK cascade Ssk2p-Pbs2p-Hog1p [10]. Activated Hog1p has numerous targets within the cell, for example, the histone deacetylase Rpd3p [11], transcription factors of histone acetylases [12] or the protein kinases Rck1p and Rck2p [13]. In order to deactivate the signaling cascade, dephosphorylation of Hog1p is conferred by the phosphatases Ptc1p, Ptc2p, Ptc3p [14] or Ptp2p, and Ptp3p [15]. Since constitutive Hog1p activation is lethal to the fungus, this dephosphorylation of the MAPK is essential under physiological conditions.

In contrast to yeast, the HOG signaling cascade in filamentous fungi comprises more elements, particularly multiple hybrid HK working as sensor elements in the phosphorelay system. Whereas Sln1p is the sole HK in *S. cerevisiae*, phytopathogenic filamentous fungi as *Botryotinia fuckeliana* (anamorph: *Botrytis cinerea*) (17 HKs), *Mycosphaerella graminicola* (anamorph: *Zymoseptoria tritici*) (17 HKs), and *Magnaporthe oryzae* (anamorph: *Pyricularia oryzae*) (10 HKs) have multiple HK encoding genes, which can be classified into 11 groups [16]. HKs have major functions in filamentous fungi, but there have been only a few reports concerning functional analysis of hybrid HK genes in these pathogens. Entirely the group III HK, for example, MoHik1p [17] or the group VI HK, for example, MoSln1p of *M. oryzae* [18] was closely studied in order to get insights into the mode of action of the fungicide fludioxonil [19]. Currently, the rice blast fungus *M. oryzae* is the sole plant pathogen in which all of its HK were functionally characterized with respect to a plethora of all the diversity of their functions. Recently, it was found that the HKs MoHik1p and MoSln1p both were individually dispensable for

vitality and MoHik1p functions as a sugar sensor and MoSln1p as a salt sensor. Furthermore, MoHik5p and MoHik9p were assumed to be involved in hypoxia sensing via the HOG-signaling cascade [16].

Reactive Oxygen Species (ROS)

Besides osmotic stress, ROS play a major role in plant–microbe interactions. Identification of a pathogen by the plant immediately triggers the oxidative burst reaction, which is characterized by H_2O_2 accumulation and necessary for further defense reactions. The specific role of ROS is still unclear [20]. Within the cell, ROS interfere with DNA, proteins, lipids, and carbohydrates. In the early stage of pathogen recognition, the rapid and transient production of ROS by plants is mainly caused by membrane-associated respiratory burst oxidase homologs, which are homologous to the mammalian $gp91^{phox}$ [21]. A primary effect of oxidative burst in the plant tissue is cell wall reinforcement, but ROS also function as diffusible second messengers, increase the synthesis of pathogen-related proteins and phytoalexins and induce the programmed cell death in neighboring cells [22]. Phytopathogenic fungi have to cope with ROS in different ways, since fungal microbe-plant crosstalk can be separated in two classes: the biotrophic and necrotrophic fungi, both of which have individually different infection strategies. Biotrophic fungi evolved mechanisms to inhibit or overcome the oxidative burst reaction, for example, through effector proteins. Necrotrophic fungi appear to increase the ROS accumulation to facilitate infection and colonization of the host plant [23]. However, several important plant pathogens developed a third infection strategy, termed hemibiotrophy [20]. Hemibiotrophism is characterized by an initial biotrophic phase that switches into a necrotrophic one during host infection. Duration of the biotrophic phase varies in different pathosystems [24]. Thus, the adaption and reaction of the pathogen to the plant oxidative burst appears to differ accordingly between these groups. The virulence of biotrophic and hemibiotrophic pathogens appears to depend on their ability to detoxify or quench ROS, for example, with mannitol [25]. Further detoxification mechanisms such as the glutathione system came up and there are some more enzymatic processes that facilitate ROS detoxification. The important enzyme for degradation of the superoxide is the superoxide dismutase, which serves the antioxidant role by conversion of O_2^- into H_2O_2. The latter can then be processed by catalases and peroxidases [26]. The ability of the pathogen *M. graminicola* to tolerate H_2O_2 during the different stages of its life cycle was reflected by *in vitro* experiments demonstrating that 5 mM H_2O_2 inhibited the development from 4-day-old *M. graminicola* cultures, whereas a 10-fold higher concentration of about 50 mM was required to inhibit the development from 16-day-old cultures [27]. Necrotrophic plant pathogens have a completely different behavior to cope with the plant defense. In order to successfully invade the host plant, necrotrophic fungi such as *B. cinerea* induce ROS accumulation within the plant tissue. During the infection process of the gray mold fungus, high levels of

H_2O_2 could be detected in all adjacent tissues indicating that a strong oxidative burst takes place [28]. Besides bearing toxic effects such as damage to membranes and macromolecules, ROS also represent important members for signal transduction acting as second messengers in differentiation processes. Thus, it is assumed that ROS are of particular relevance for *in planta* growth and differentiation of pathogenic fungi [29]. Molecular events of ROS signaling during plant–microbe interactions are still not well understood. In contrast to reports about the current understanding of the function of ROS in differentiation processes, the exact extra- and intracellular signaling mechanisms of these effects are still unknown [29]. In order to transport ROS information from extracellular space to the nucleus, several different modes of action could be achieved. Specific ligands and receptors might detect the stress signal and subsequently induce signaling cascades that regulate gene expression. Recent studies on the rice blast fungus *M. oryzae* indicate that two sensor HK, named MoHik5p and MoHik9p, appear to be involved in H_2O_2 sensing and hypoxia adaption via the HOG pathway [16]. In addition, ROS might modify the activity of transcription factors and furthermore proteins of various signaling cascades could be directly oxidized by ROS [20].

Plant Hormones (Phytoalexins)

Plant–microbe interactions are also characterized by the use of hormones by plants as defense reaction against pathogens. The production of low-molecular secondary metabolites with antimicrobial activity synthesized *de novo* after stress or pathogen recognition extend the plant defense mechanisms and these metabolites are collectively known as phytoalexins [30]. Phytoalexins are a heterogeneous group of molecules that show a broad biological activity toward a series of plant pathogens. However, biosynthesis of most phytoalexins, the regulation processes involved in their induction by pathogens, and the molecular mechanisms behind their antimicrobial activity are largely unknown. The major phytoalexin found in the model plant *Arabidopsis thaliana* is camalexin, which was named after a plant in the Brassicaceae family, *Camelina sativa*, from which it was first isolated [31]. Several steps of the biosynthesis pathway of camalexin have been characterized and it was long thought to be the only phytoalexin in *A. thaliana* until a second one, rapalexin A, has also been found in this species [32]. However, the antimicrobial activity of camalexin toward plant pathogenic fungi appears to be based on the induction of the fungal apoptotic programmed cell death. That was recently shown for *B. cinerea* since a transgenic strain with enhanced anti-apoptotic capacity is less susceptible to camalexin. The anti-apoptotic machinery thus would enable the fungus to continue infection and host invasion [33]. Exposure to camalexin furthermore induced the gene expression of the efflux ABC (ATP-binding cassette)-transporter BcAtrBp acting as a protective mechanism against the plant hormone [34]. A set of possible detoxification mechanisms have also been reported. *Rhizoctonia solani* inactivates camalexin by hydroxylation, *Sclerotinia sclerotiorum* transforms

camalexin by glucosylation, and *B. cinerea* detoxifies camalexin by conversion to 3-indolecarboxylic acid and other intermediates [35]. However, molecular components or signaling pathways involved in how the plant hormone stimulus is detected and transported within the pathogen cell remain unknown and have to be studied.

Technologies for Studying Signaling Pathways

The postgenomic era is upon us and enables genome-wide profiling with the "omics" technologies. Many technologies have been developed allowing the analysis of mRNA (transcriptomics), protein (proteomics), and metabolite (metabolomics) profiles. Data from these technologies will lead to identification of a large number of potential candidate genes encoding factors for stress-induced signaling mechanisms. These factors might have crucial functions either for invasive growth and development or for vegetative growth. However, these candidates have to be validated by gene deletion or gene silencing experiments. Even if deletion or silencing of the gene reveals a mutant showing a phenotype associated with the assumed stress signaling cascade, it must be further established whether the purified target protein is functional in *in vitro* test systems.

The Age of "Omics" – General Information

Having today the knowledge of plethora of fungal genomes and advanced technologies to study global mRNA, protein, and metabolite profiles, we have entered in the postgenomic era, which opens up new horizons in fungal biology. With the emergence of the so-called omic technologies (genomics, transcriptomics, proteomics, and metabolomics), we got the unique possibility to comprehensively characterize plant–pathogen interactions and pathogenesis-related processes at molecular level using this exceptional toolbox. "omics" encompass an increasingly wide range of fields, including genomics (the quantitative study of protein coding genes, regulatory elements, and noncoding sequences), transcriptomics (RNA and gene expression), proteomics (e.g., focusing on protein abundance), and metabolomics (secondary metabolites and metabolic networks). Taking into account the advances in the postgenomic era, this array of disciplines can be further extended and spans also the other fields such as phylogenomics (analysis involving genome data and evolutionary reconstructions, especially phylogenetics) and interactomics (molecular interaction networks). Figure 9.1 reflects the flow of the genetic information through distinct levels of "omics" approaches and their interplay . It also illustrates the increasing need of data integration generated by these "omics" technologies in order to uncover the genotype–phenotype interactions. The determination of functional properties within the functional genomics can be further supported by at least two commonly used strategies known as "forward" and "reverse" genetics.

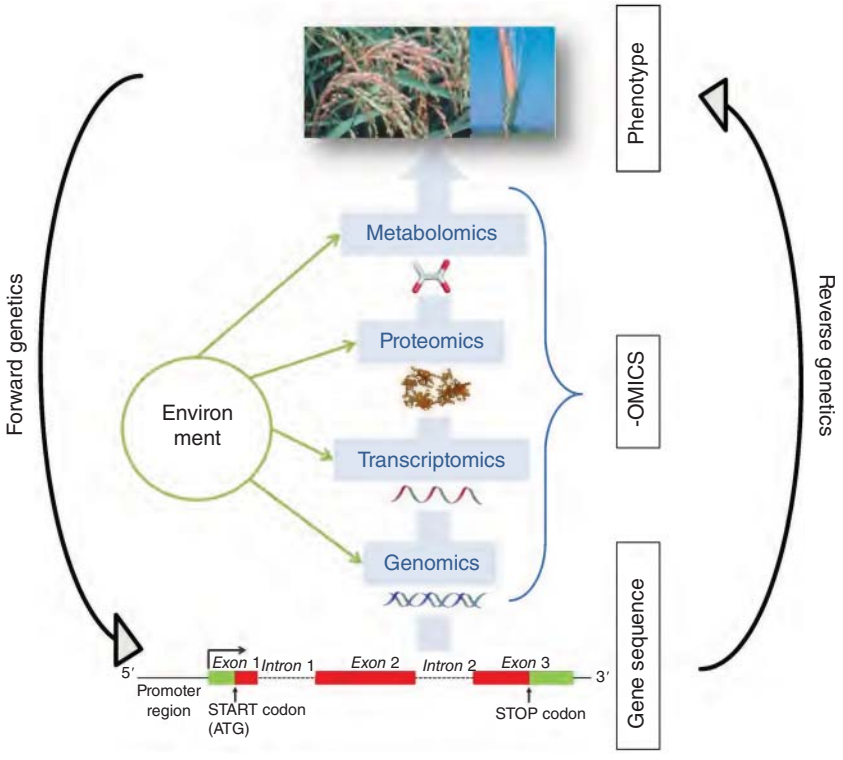

Figure 9.1 Flowchart of "omic" disciplines and their interplay in the postgenomic era.

A major goal of exploration of these data is determination of dynamical models that predict phenotypic traits and results, outlining crucial biomarkers and engendering important insights into the genetic underpinnings of the heritability of complex traits, which lead to a better comprehension of the role of genetics and genomics in sophisticated outcomes [36]. With the use and further development of these approaches, an improved understanding of the relationship between genomic variation and phenotypes derived from plant–pathogen interactions may be revealed.

In this chapter, we are going to focus on isolated few examples of "omic" technologies. There are already huge amount of data generated by these technologies and the future trend is increasing, taking into account the central role of second- and third-generation sequencing technologies, leading to an exponential rise in the amount of sequencing data available. This chapter provides an overview of transcriptomic, proteomic, and metabolomic approaches, and its application in plant–pathogen interactions within the scope of fungal plant pathology.

Retrospectively, in the postgenomic era, transcriptomics, proteomics, and metabolomics are increasingly important and allow us, projected on fungal biology, to obtain deeper insights into the complexity of the fungal physiology. Today, several

hundred fungal genomes are sequenced (supported by programs such as the Joint Genome Institute (JGI) or the Broad Institute) and other genomes follow at a fast pace, against the background of ever-decreasing costs of the sequencing pipeline infrastructure. However, this increasing trend is from the genomics sector alone and thus the steady supply with genomic data is by far not enough to understand the complex biological mechanisms associated with different fungal lifestyles. The development of "omic" technologies has made it possible to collect valuable data for functional characterization of genomes in high-throughput manner, aiming at the collective quantification of pools of biological molecules that translate into the structure, function, and dynamics of microbial life. However, the other side of the coin is the ever-growing challenge regarding the downstream data handling and analysis. This challenge is still the major bottleneck taking into account the mentioned exponential increase of the output data.

Transcriptomics

By definition, transcriptomics deals with the global analysis of the transcriptome and thus analyzes the expression pattern of gene sets at the RNA level in the spatiotemporal manner. At the same time, it is undoubtedly a key element in functional genomics, greatly contributing to our understanding of gene function. With the completion of the sequencing of over 200 fungal genomes (JGI (MycoCosm), the Broad Institute), the monitoring of global changes in gene expression is an increasingly attractive method for dissecting the molecular basis of fungal–plant interaction and pathogenesis (Figure 9.2).

Looking back in the history of the development of the expression profiling technologies, we can see the tremendous improvement in the expression profiling methodologies.

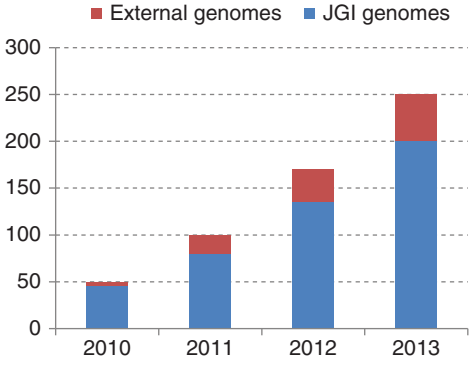

Figure 9.2 Growth of annotated genomes in MycoCosm. The genomes sequenced by JGI are shown in blue and those sequenced by others are shown in red.

The starting point of transcriptomics era was previously set by ESTs (expressed sequence tags) analyses [37]. But soon afterward this technology went through diversified advances, for example, the introduction of a number of sequencing- or hybridization-based methods, including MPSS (massively parallel signature sequencing) [38], SAGE (serial analysis of gene expression) [39], microarrays [40, 41], and RNA-seq [42–44]. All of these methods have by now been applied in order to expand our understanding of the processes that occur during developmental transitions, stress signaling, and plant–pathogen interactions, but also were crucial in identifying a number of differentially expressed genes whose functions during development were determined in subsequent studies. Without delving deeper into technical details and providing a detailed comparison between these approaches, because that would certainly be beyond the scope and purpose of this chapter, we would like to mainly focus on at least two expression profiling technologies that have found a wide application, giving a few examples of the successful usage of these methods in the recent publications: RNA-seq and microarray technology.

Today RNA-seq is perhaps the most used technique to address the gene expression profiling of fungi under different developmental stages [45]. Its highly qualitative dynamic range and resolution of transcript detection allows us to accurately and precisely determine the genomic attributes such as expression levels of specific genes, differential splicing, and allele-specific expression of transcripts leading to comprehensive dissection of many biological-related issues of the fungi. The discovery of these features is only limited to the RNA-seq methodology and are not achievable from previously widespread hybridization-based or tag sequence–based approaches. However, the increased specificity and sensitivity for enhanced detection of genes and the large amount of generated data derived from NGS (next-generation sequencing) platforms provide clear advantages as well as new challenges and issues [46].

Although the technology is maturing and RNA-seq is becoming more feasible for a wide range of applications, there are still several good reasons for using microarrays. Microarrays are relatively inexpensive, widely available, allowing many biological and technical replicates to be analyzed [47]. However, the emergence and a rapid development of NGS platforms, for example, Illumina sequencing machines such as the "HiSeq," have dramatically accelerated genome-wide studies of transcriptomes. The steadily decreasing cost of sequencing associated with RNA-seq, technical improvements resulting in ever greater yields (read length and data generation throughput), and not least the recent development of publicly available bioinformatic tools for RNA-seq data analysis have made RNA-seq an increasingly attractive method for studying fungal transcriptomes. Moreover, the steady improvements in RNA-seq library preparations, leading to a reduction in quantity of samples required and the possibility of sample multiplexing, allowing to share the sequencing capacity of a single-flow cell lane, have also greatly contributed to a reduction in sequencing costs. RNA-seq has several inherent advantages over microarray analysis. For instance, there are several limitations in the case of microarray, which make RNA-seq technology more favorable, including, for instance, the reliance upon existing knowledge about genome sequence and

high background levels owing to cross hybridization [48]. In contrast, RNA-seq is (at least conceptually) able to identify all transcripts and is therefore not reliant on a cDNA dataset or detailed annotation [47]. RNA-seq data provides an obvious means for updating and improvement of older genome annotation data, providing the ability to look at alternative gene spliced transcripts, posttranscriptional modifications, gene fusion, mutations/SNPs (single-nucleotide polymorphisms), and changes in gene expression, even those expressed at low levels. A disadvantage of RNA-seq is that it is less well established than microarray technology, produces much more data, and makes the data handling more complex. The application of RNA-seq technology leads therefore to a higher overhead in terms of informatics as well as hardware and data storage capacity [47].

Transcriptomics in Use

As for successful usage of RNA-seq to explore the molecular mechanisms of pathogenicity-associated processes in fungal pathogens, we want to give a brief overview of recent publications that underline the power of this technology. For instance, two transcriptome analysis using RNA-seq have been reported to improve gene annotations and to identify alternative splicing events [49, 50]. Sikhakolli *et al.* [51] used RNA-seq technology in order to perform a comparative expression analysis of fruiting body formation in *Fusarium graminearum* and *Fusarium verticillioides* analyzing six developmental stages, thus enriching our understanding of *Fusarium* biology and pathogenesis. The other advanced studies using RNA-seq were linked to investigations of *in planta* growth of the fungus and growth-associated developmental transitions. Thus, to understand plant–pathogen interactions comprehensively, it is valuable to monitor the gene expression profiles of both interacting organisms simultaneously in the same infected plant tissue, in natural infection conditions, and without any artificial treatments. For this purpose, in order to exploit the full potential of the RNA-seq technology and facilitate the study of signaling mechanisms underlying the plant–fungal interaction, the "Dual-RNA-seq" was established, aiming at the identification of putative key genes differently expressed *in planta* [52]. Thus, the simultaneous transcriptome analysis of plant and pathogen was performed in banana plants infected with *Fusarium oxysporum* f. sp. *cubense* [53] and rice with *M. oryzae* [54], aiming to uncover infection-responsive genes in the pathogen and host plant, separately. In the case of *M. oryzae,* upregulation of several hundred fungal transcripts encoding putative secreted proteins, glycosyl hydrolases, cutinases, and LysM domain-containing proteins was observed in infected leaves at 24 h postinoculation, which is the point when the primary infection hyphae penetrates leaf epidermal cells, suggesting that these candidates of fungal effector genes may play an important role in the initial infection processes [54]. On the part of the rice plant, pathogenesis-related and phytoalexin biosynthetic genes were upregulated. Furthermore, more drastic

changes in expression were observed in the incompatible interactions than in the compatible ones in both rice and blast fungus [54].

Using genome-wide approaches, including microarray analysis, a considerable progress has been made in understanding the nature of appressorium development and pathogenicity, for example, in *M. oryzae.* In 2005, the rice blast genome was published, along with the first global gene study on appressorium development using the first generation of the Agilent microarray [55]. Several reports have appeared since the Agilent microarray chip is used to examine *M. oryzae* under a variety of conditions and developmental stages. Donofrio *et al.* [56] for instance examined gene expression for mycelium under nitrogen starvation condition. Among these genes, a GATA family TF gene *NUT1* had a significantly increased transcript abundance in both nitrogen starvation condition and inside infected rice, suggesting *NUT1* is a global nitrogen regulator. Earlier it was shown that a hydrophobin encoding gene *MPG1,* which is necessary for infection-related development of *M. oryzae*, relies on *NUT1* for full expression [57].

In 2008, the Agilent microarrays were used again to perform a more in-depth analysis of appressorium development, trying to answer the question how this process is affected by the addition of cyclic adenosine monophosphate (cAMP) [58]. Oh *et al.* [58] looked at global gene expression profiles during spore germination and appressorium formation on both an inductive hydrophobic surface and in response to cAMP. Many fungi differentiate appressoria prior to invasion. The development of such structures involves a large number of genes, and mutations in many of them will arrest infection. Genome-wide transcript profiling has revealed that genes involved in amino acid degradation, lipid metabolism, secondary metabolism, and cellular transport are all differentially upregulated during appressorium formation in *M. oryzae*.

The other quite intriguing study was conducted using a transcriptome analysis based on microarray technology, aiming to analyze one of the critical steps of the *M. oryzae*-infection cycle, the conidiation. Measuring genome-wide gene expression profiles during conidiation in *M. oryzae* isolate KJ201, several hundred genes differentially expressed during conidiation were identified [59]. A subsequent comparative analysis of gene expression profiles during sporulation conditions between the wild-type strain and deletion mutant for *MoHOX2,* encoding a stage-specific transcriptional regulator, revealed a gene set of conidiation-related genes regulated by this homeobox transcription factor [60, 61]. *MoHOX2* seems to play a crucial role in *M. oryzae* conidiation process, as *ΔMohox2* mutants fail to produce conidia.

In general, as mentioned in the previous sections, the host specialization by pathogens is accompanied with a fine-tuned regulation of gene expression and a remarkable range of virulence factors. For instance, the fungal wheat pathogen *Z. tritici* (*M. graminicola*) represents a powerful model pathosystem for the discovery of genetic elements that underlie virulence and host specialization [62]. Recently, Kellner *et al*. [62] examined a gene expression profiling of *Z. tritici in planta* at the early stages of infection in order to elucidate candidate determinants of host specialization. This was achieved by comparing the *in planta* transcriptomes of the compatible host (wheat) and a noncompatible host (*Brachypodium distachyon*),

revealing infection regulatory programs common to both hosts and genes with striking wheat-specific expression.

In a subsequent study, a deep RNA sequencing was implemented combined with metabolomics to investigate the physiology of plant and pathogen within the infection cycle of *Z. tritici* [63]. This investigation resulted in more than 7000 wheat genes, over 3000 pathogen genes and more than 300 metabolites, which were differentially regulated throughout an asexual reproductive cycle of *Z. tritici* on wheat leaves. This study also provided an intriguing observation due to unequal contribution of individual fungal chromosomes to the overall gene expression changes. Furthermore, the transcriptional downregulation of putative host defense genes was detected in inoculated leaves. At the same time, considering the distinct infection phase-specific expression patterns, genes encoding effector proteins, and putative secondary metabolite clusters were identified (although displaying some degree of overlap in their functions concerning the virulence-related processes) [63].

Proteomics

Since 2000, many interesting reviews on the fundamentals, applications, advantages, and limitations of proteomics dealing with fungal pathogens have appeared [64–67]. Within the "omic" techniques, proteomics became nowadays a fundamental discipline in the postgenomic era. Some of the recent reviews, however, clearly illustrate a restricted application of the proteomics technology by the fungal phytopathogen community, at least in the past years, linking it to a lack of genome sequence information [65, 68]. Nevertheless, the use of the proteomic approaches increases rapidly. The advances in the genomic era largely contribute to this technique, allowing it to fulfill its real potential.

Proteomic analysis became today indeed an important method for deriving biological knowledge from genome sequences and helps us to close the existing gaps of functional genomics. Yet, the remaining challenge is still the assignment of function to fungal proteins, encoded either by *in silico* annotated or unannotated genes [69]. The continuous increase in the amount of fungal genomes published leads inevitably to the ever-growing potential and application of the high-throughput proteomic analysis of these organisms. While genomics sheds some light on the genetic potential of a regarded organism, proteomic analysis aims at making a statement on the relative amounts of proteins produced by this organism at a given point in time and at certain conditions [69].

A combination of high-throughput, quantitative proteomics, allied to transcriptomic sequencing, will undoubtedly reveal much about protein function in fungi [69]. The increasingly important interplay between the "omic" technologies allows us to perform screening and analysis of peptides and proteins at the subcellular level. They also highlight posttranslational modifications of proteins, for example, glycosylation, phosphorylation, acetylation, or cleavage, underpinning the relevance of

these posttranslational modifications for virulence. As with transcriptomics section, we want to limit ourselves to a few examples, which demonstrate the power and successful utilization of the proteome analysis approaches, which contribute to the knowledge of fungal pathogens biology. Thus, for a comprehensive review of fungal proteomics, the interested reader is referred to the intriguing publications Refs. 66, 69–71.

To date, most proteomic studies in plant pathogenic fungi have been limited to 1D and 2D electrophoresis analyses and have been focused on mycelium extracts [72, 73]. However, various powerful proteomic methods have been developed in the recent years in order to perform genome-wide analysis of differential protein expression, localization, and protein–protein interaction in fungi. The rapidly increasing advances of available proteomic resources and the ever frequently occurring tendency to integrate the large-scale genomics and proteomics data enable the elucidation of global networks and system biology studies of fungal plant pathogens. Many new proteomics techniques have been developed, including the second-generation MS (mass spectrometry) technologies for quantitative proteomics such as 2-DIGE (difference gel electrophoresis), an array of stable isotope labeling methods such as iTRAQ (isobaric tags for relative and absolute quantification, a multiplexed isobaric tagging technology), SILAC (stable isotope labeling by amino acids in cell culture) or ICAT (isotope-coded affinity tag), and finally label-free methods (based on peak integration, spectral counting) such as SELDI-TOF-MS (surface enhanced laser desorption/ionization time-of-flight mass spectrometry) or FTICR (Fourier transform ion cyclotron resonance), which are beginning to be widely applied to fungal proteomics research [74–79]. With the unprecedented depth of investigation and the breathtaking pace of proteomics development, there is today a tendency to combine multiple "omic" techniques aiming to interrogate system-level networks with the goal of analyzing the functions of proteins.

Along with precise absolute peptide quantification, the gel-free technologies relying on isotopic labeling approaches, such as iTRAQ, have made remarkable improvements in peptide resolution sensitivity and should therefore greatly contribute to identification of apoplastic proteins upon a response to attempted pathogen attack [80]. Recent literature, for instance, testifies the usefulness of this novel gel-free technique in protein identification and quantification in pathogenic fungi such as *B. cinerea* [81] and *Phytophthora infestans* [82].

However, all of the mentioned methodologies are often restricted only to a subset of the complete proteome or metabolome, due to a wide diversity in chemistry of the analyzed proteins and metabolites. On the contrary, transcriptomics allows us to analyze more or less, with the consideration of technical shortages due to sample preparation prior to sequencing, all transcripts produced from a genome. Nevertheless, proteomics and metabolomics analyses can and will contribute valuable insights into the dynamics of genome activity. In this context, it is worth to mention that transcript levels do not always correlate with protein expression levels, due to some occurrences on posttranscriptional level of regulation, unavoidably limiting the capture range of transcriptomics approaches [45].

Proteomics in Use

By definition, proteomics is the systematic analysis of the proteome, allowing qualitative and quantitative measurements of a subset of proteins that directly influence cellular biochemistry [64]. The application of proteomics technology makes it possible to analyze the cellular state or system changes during growth, development, and response to environmental factors. Thus, it represents, along with transcriptomics and metabolomics, a key technology for the investigation of highly complex and dynamic biological systems.

Proteomics aims at obtaining information on the entire compliment of proteins in an organism. Compared to transcriptomics, proteomics provides a more accurate quantification of active cellular processes, as the transcript abundances and its corresponding proteins are not always well correlated due to posttranscriptional regulation mechanisms [83].

Generally, independent of the choice of the method to be used in the context of the proteome analysis, the starting point is the isolation and purification of total proteins, which are then typically separated by liquid chromatography (LC) or by 2D electrophoresis gels (e.g., PAGE, polyacrylamide gel electrophoresis). The separated protein fractions are then digested and the resulting peptides are characterized using MS methods, such as MALDI-TOF (matrix-assisted laser desorption/ionization time of flight) and ESI-MS (electrospray ionization mass spectrometry). MS does not measure the mass of peptides, but instead their m/z ratios, which are then compared to predicted proteins from genome analysis. In the following, we give some interesting examples that emphasize the successful usage and the power of proteomic technology.

Several proteome studies investigating *F. graminearum* have been reported [84–90]. Most of these studies conducted on *F. graminearum* have focused mainly on the secretomes/exoproteomes, phosphoproteome, and impact of the mycotoxin deoxynivalenol (DON), providing a sufficient theoretical guidance in the light of disease management and control of mycotoxin production. Hence, comparative proteomic analyses identified secreted proteins, including cell wall degrading enzymes (CWDEs), during the *F. graminearum* infection process and subsequent secretome studies have revealed more potential novel effectors, more of which were specific and are likely to play critical roles in fungal virulence [86, 87, 91, 92]. Undoubtedly, these observations provide a useful foundation for future experimental studies to verify their function in pathogenesis. Additionally, phosphoproteomics of *F. graminearum* revealed important posttranslational modification processes such as phosphorylation and ubiquitination [88, 89].

Facilitated by the availability of the *M. oryzae* genome, proteomics have also started to uncover biochemical processes associated with infection in *M. oryzae.* Recently proteome profiling studies have been carried out to study the conidial and appressorium proteomes in this phytopathogenic fungus [93, 94]. Using LC-MS/MS (liquid chromatography tandem mass spectrometer) and spectral counting-based, label-free quantification approach, Franck *et al.* [93] aimed to determine the proteomic changes/perturbations occurring during germination and appressorium

formation in response to cAMP treatment. Consequently, these genome-wide proteomic studies shed light on the cellular processes associated with infection-related development, leading to the identification of proteins associated with a wide range of cellular activities including melanin biosynthesis, lipid metabolism, glycogen metabolism, and anti-oxidation. Furthermore, altered protein abundances during appressorium development revealed enzymes involved in cell wall biosynthesis and remodeling, changes of proteins associated with secondary metabolism, and an increased abundance of secreted proteins, providing important insights into the cellular processes involved in the early stages of rice blast disease [93].

Using the available *M. oryzae* genome additional proteomic studies have been employed to compare the proteome of wild-type conidia to those of a mutant defective in the conidial regulator *COM1* [95], and to analyze the identity of apoplastically secreted proteins during early infection [96, 97]. Moreover, proteomic studies contributed to the elucidation of metabolic perturbations in a trehalose synthesis deficient mutant *Δtps1* compared to wild-type, giving fresh insights into nutrient adaptation and the control of fungal development during infection, thereby providing insights into glucose metabolism in *M. oryzae* [98].

The example of *M. oryzae* also clearly underpins the relationship between recently achieved advances in fungal genomics and the improvement in the basic knowledge of biochemical pathways related to rice blast infection. Future challenges remain to be addressed, particularly referred to *in planta* investigations, where studies are starting to reveal how biochemical pathways might be involved in different fungal lifestyles [99, 100]. Both of these gene functional studies support earlier genome-enabled microarray work of Donofrio *et al.* [56], mentioned in transcriptomics section, which indicated early infection occurred under nitrogen starvation conditions.

Finally, many studies have combined heterogeneous types of genome-scaled data: Liu *et al.* [101] used genomics and proteomics to evaluate the lignocellulolytic potential of *Penicillium decumbens*, whereas Poulsen *et al.* [102] identified the organic acid response of *Aspergillus niger* by analyzing the transcriptome and extracellular metabolites, and Floudas *et al.* [103] integrated comparative genomics, secretomics, and transcriptomics to reconstruct the paleozoic origin of enzymatic lignin degradation using comparative analyses of 31 fungal genomes. Vitikainen *et al.* [104] performed a high-resolution array-based comparative genomic hybridization (CGH) experiment using the genome sequence of natural isolate *Trichoderma reesei* QM6a to identify in other *Trichoderma* strains genomic alterations that lead to the improvement of cellulase production. This CGH analysis identified dozens of mutations in each strain analyzed [104]. These studies have generated intuitive insights that rely on the integration/interplay of omic technologies, where each simple analysis on one type of "omic" data contributes to our understanding of biological systems as a whole. Despite several current challenges linked to cross-platform comparisons, the further improvement and optimization of computational infrastructure aiming at integrating the gathered information will certainly provide more efficient leads to drive functional genomics studies of fungi in future [105].

Metabolomics

By analogy with the other "omic" techniques, metabolomics is concerned with metabolome analysis and aims to make the nontargeted/global profiling of all metabolites in the regarded organism [106]. The predominant purpose of this approach is to provide an overall qualitative and/or quantitative view of an organism's metabolic profile. As such, the metabolome represents the ultimate response of a biological system to genetic and/or environmental changes [107]. As with proteomics and transcriptomics, the availability of data from fungal genome sequencing speeds up the discovery and characterization of new compounds and searching for genes encoding key enzymes involved in secondary metabolism in fungal pathogens [108]. Despite their huge potential to decipher many facets of fungal metabolism, only a couple of comprehensive studies have been conducted to date. Further comprehensive studies predominantly linked to the extended investigation of metabolic adaptations of pathogens to their host's chemistry are of great importance and still remain a major challenge in order to examine the mechanisms underlying the interactions between plants and phytopathogens. Such studies are only just beginning to appear and their popularity will undoubtedly increase in near future, because the data that can be obtained from metabolomics approaches is a logical complement to similar data from transcriptomics and proteomics experiments [109]. Thus, metabolomics can be viewed as a complementary technique to other functional genomics disciplines.

To address this challenge, several techniques were developed, which differ in their sensitivity and selectivity, but allow us to obtain a reproducible data, at relatively low cost [110]. It is important to understand that none of the currently available tools has the capacity to detect the complete set of metabolites in a given biological sample at once. For the main part, because of the heterogenic nature and chemistry of the metabolites, the analyses result in various classes of compounds. Among these techniques, it is worth to mention a few of high-throughput methods for quantitative metabolite identification, including MS coupled with GC (gas chromatography), LC or capillary electrophoresis, and NMR spectroscopy [111]. All of the listed methods represent to date the most widely applied metabolomics approaches in fungal biology and phytopathology.

To the present, most of the publications referring to metabolome profiling in fungal pathogens are focused on the examination and screening for secondary metabolites, rather than primary metabolites. For this purpose LC-MS is the tool of choice, as in the most cases using the other methods for screening of secondary metabolites are unsuited [67].

Metabolomics in Use

As mentioned above, metabolomics is the study of metabolism at the global level investigating a subset of metabolites in a biological system with a major goal to elucidate metabolic fingerprints of specific cellular processes. Along with other

"omic" technologies this rapidly developing discipline has important potential implications for understanding the fungal physiology. Especially the emergence of large-scale techniques facilitating the global metabolite profiling, also known as untargeted metabolomics, allows us to gain and deepen our understanding of fungal pathosystems by revealing and linking cellular pathways to biological mechanisms under certain defined conditions [112]. In the following, we want to briefly overview some valuable publications underpinning the progress made in this area in recent years. For a further overview, especially regarding technical comparison of the deployed methodologies and strategies, the following reviews are recommendable [111].

To date in fungi, LC-MS has been typically used to examine and screen for secondary metabolites rather than nontargeted scanning of primary metabolites [113]. Typically, the secondary metabolites are incompatible with GC-MS application as they are often too large, particularly after derivatization [67]. For instance, recently, comparative metabolomics approach based on ultra-high-performance liquid chromatography (UHPLC) coupled to high-resolution orbitrap MS was performed to determine metabolites differentially expressed in the *Aspergillus flavus* wild-type and *Δpks27* mutant strains. This differential analysis combined with accurate mass data from the orbitrap and ion trap multiple-stage MS allowed four metabolites (asparasone A (358 Da), an anthraquinone pigment, and related anthraquinones with masses of 316, 340, and 374 Da) to be identified that were produced only by the wild-type strain [114]. There has been a spate of recent publications favoring the use of LC/MS/MS for multi-mycotoxin analysis covering as many as 39 mycotoxins in a single analytical run [115]. Although this approach represents a good and useful example for Fusarium-toxin analysis, it is nevertheless still targeted and requires assumed knowledge of what mycotoxins might be expected. Tanaka *et al.* [116] established an LC/TOF-MS method for simultaneous determination of nine *Fusarium* mycotoxins (including nivalenol, DON, fusarenon X, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, HT-2 toxin, T-2 toxin, diacetoxyscirpenol, and zearalenone) and four *Aspergillus* mycotoxins (aflatoxins B1, B2, G1, and G2) in corn, wheat, cornflakes, and biscuits.

The application of NMR in metabolomics is on the contrary restricted only to a handful of reports having been so far published. For instance, 1H-nuclear NMR metabolomic approach has been recently employed for the exploration of nutritional content of the apoplastic fluid in wheat during infection by *M. graminicola* [117]. For this purpose, samples at different distinct time points of infection (06 = 6 days, 09 = 9 days, and 13 = 13 days after treatment) were collected, covering both the latent period of infection and the necrotrophic phase of interaction. While no significant changes in metabolite levels compared with the mock treatment were detected during the biotrophic phase, the metabolic analysis of the samples derived from apoplastic fluid collected during the necrotrophic phase identified a significant increase in many compounds including glucose, fructose, and many amino acids, which were all present in greater amounts. The presence of these essential growth nutrients marks therefore the onset of necrotrophic growth and

correlates with a measurable increase in *Septoria tritici* growth, which has been shown to occur at day 9 and 10 (review by Shetty *et al.*[27], Tan *et al.* [67], Keon *et al.* [117]).

Using GC-MS in order to dissect the metabolome of the wheat pathogen *Stagonospora nodorum*, the crucial role for the sugar alcohol mannitol and trehalose in the pycnidial development was observed [118, 119]. Furthermore, GC-MS profiling was applied in order to analyze the metabolome of *S. nodorum* during salt stress, leading to the observation that arabitol and glycerol are primarily involved in the osmotic stress response in this fungal pathogen [120].

An intriguing study was carried out by Parker *et al.* [121] using ESI-MS and GC-MS, aiming to examine the metabolome of susceptible host plant species (barley, rice, and *B. distachyon*) and to identify a common pattern of metabolite regulation within plant–pathogen interaction. Targeted metabolite profiling by GC-MS uncovered the modulation of a conserved set of metabolites. Overall, the results derived from this study were consistent with the hemibiotrophic growth habit of *M. oryzae* during plant tissue invasion [121]. They revealed the dynamic reprogramming of the host metabolism and suppression of host defenses during colonization, allowing the fungus to grow in the living plant cells.

Conclusion

The approaches to study plant–microbe interactions described in this chapter have focused on the current mainstream "omic" technologies such as transcriptomics, proteomics, and metabolomics. In summary, genomics has provided detailed genetic information about a variety of destructive phytopathogenic fungi. Improved genomic and functional annotations, detailed phenomics and enriched transcriptomics and finally the great advances in generation of proteomics and metabolomics data open up new possibilities for applied systems biology approaches. This given capability will lead to enhanced investigations of the regulatory networks essential to comprehend cellular functions and pathogenesis within a framework of plant–pathogen interactions. However, the growing scale of modern "omic" projects brings us to the fore computing challenges underpinning the increasing need to implement new and innovative ways to mine the resulting very large and complex data sets.

Maybe at this point, we want to refrain from giving any forecast how these "omic" technologies will develop in the immediate future. But relying on the present standpoint, we can see a clear tendency of transcriptomics led by the further development of NGS and third-generation sequencing technologies providing the big data output to ever-decreasing costs per sample. Proteomics will continue to be dominated by 2-DE. But with the emergence and accessibility of gel-free proteomic technologies coupled with their permanent technological improvements in their quantitative power (iTRAQ, SILAC, spectral counting, etc.) will lead to their increased usage. The metabolomics field will improve with the enhanced resolution

of mass spectrometers. However, NMR is also becoming more sensitive and will be increasingly applied in fungal pathogen studies.

This new generation of available technologies will greatly aid in compound identification, closing more and more of "remaining gaps." Exciting new information will be gained from these studies, including broadened knowledge on the molecular background of plant–microbe interactions, pathogenesis, and fungal colonization of plants [64].

The simultaneous measurement of transcripts, proteins, and metabolites from the same sample will promote future systems in biology studies, aiming to characterize and accurately predict the dynamic properties of the biological network that is under scrutiny [122]. Thus, there is today a clear need for data integration technology to maintain and manage the big data derived from "omic" technologies. The complexity of these datasets will further on prompt the use of computational biology and application of systems or network theory with the objective of understanding biological systems as a whole [123].

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10
Targeting Plasmids: New Ways to Plasmid Curing

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Abstract
The dramatic increase of fatalities due to bacterial infectious diseases especially of nosocomial infections with multiresistant pathogens is truly alarming. Several national and international organizations call for transnational joint efforts to fight the spread of bacterial resistance. Among others the introduction of new antibiotics, but also the reduction of resistant bacteria in the environment could help to cope with this issue. One way how resistance genes are transmitted, even between different species, are plasmids. The first evidence that resistance plasmids are responsible for drug resistant epidemics dates back to the 1960s. By that time the idea of the "chemotherapy of bacterial plasmids" was conceived. In recent years, most likely due to the rising resistance problem and the ever-expanding scientific knowledge on plasmids, this idea attracted more and more researchers. This chapter recaps the methods that are used to cure bacterial cells and summarizes the approaches and possible curing agents that were introduced since 2000. In addition, concepts of possible applications of plasmid curing agents are discussed.

Introduction

Plasmids are small circular double-stranded DNA molecules that replicate independently from the chromosome. They are widespread not only in bacteria but also among other organisms such as *archaea* [1] and fungi [2]. Plasmids usually carry genes that give the host organism special abilities such as resistance to antibiotics, heavy metals, or pathogen factors [3]. Often, they can be naturally transferred to other bacteria, even of different species [4, 5]. This makes them able to spread easily and at a high pace. The additional DNA raises the maintenance costs for the cell and therefore only offers an advantage when the genes on the plasmid are beneficial under the conditions the bacteria live in. If this is not the case, the plasmid is usually

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lost again quickly. But the bacterial community as a whole will continue to carry the plasmid, as long as the transfer rate is higher than the loss rate [6].

Plasmid curing describes the induced loss of one or more plasmids of a plasmid-bearing strain, leading to a plasmid cured strain. Plasmid curing was originally mainly of interest for research. When studying a new strain, phenotypes can be classified as being chromosome or plasmid coded. For this, the strain has to be cured of its plasmids and then compared to the wild type strain that contains the plasmids [7]. Curing of a plasmid in research also often is necessary to prevent plasmid incompatibility, when developing a new strain in molecular biology. Cells can harbor more than one type of plasmid that coexist if the replication and partition systems are compatible. If plasmids are incompatible one will be lost by subsequent cell division [8].

As mentioned, antibiotic resistance is often conferred by plasmids. Most resistance plasmids code for more than one antibiotic resistance [3]. Curing these resistance plasmids would make the respective resistant pathogens susceptible to antibiotics again. This would mean that antibiotics that cannot be used anymore because of high overall resistance could be used again, restocking the available antibiotics to cure an infection without the need to develop new drugs. This is not a new concept. The "chemotherapy of bacterial plasmids" was suggested in 1979 by Hahn after epidemics of drug resistant shigellosis and typhoid fever caused thousands of fatalities in the 1960s and 1970s [9]. This idea has drawn more attention over the last years, as resistance is ever spreading among bacteria, and research for new antibiotics is sparse [10]. The WHO warned of the raising overall resistance in a global report on surveillance of antimicrobial resistance and called for a global action plan to mitigate antimicrobial resistance [11]. "One important finding of the report, which will serve as a baseline to measure future progress, is that there are many gaps in information on pathogens of major public health importance. In addition, surveillance of antibiotic resistance generally is neither coordinated nor harmonized, compromising the ability to assess and monitor the situation" [11].

Where it was possible, the WHO gave an overview of worldwide resistance to the main antibiotics for the respective strain. Due to the lack of coordinated surveillance, this was only possible for a few pathogens. The following graphs summarize their findings about distribution of resistance to fluoroquinolones in *Escherichia coli* (Figure 10.1), resistance to third-generation cephalosporins in *Klebsiella pneumoniae* (Figure 10.2) and resistance to β-lactam antibacterial drugs in *Staphylococcus aureus* (Figure 10.3).

While *E. coli* is part of the normal flora in the intestine in humans, it can also cause diseases. It is the most frequent cause of urinary tract, bloodstream, and foodborne infections. The infection is mostly caused by autoinfection, but a transmission between individuals, through animals or food is also possible. Fluoroquinolones are among the most widely used oral antibacterial drugs; however, plasmid mediated resistance against fluoroquinolones has been shown for clinical isolates [12–16]. While resistance to fluoroquinolones in *E. coli* (Figure 10.1) reaches the highest maximal percentage with over 90% in the Western Pacific Region (Australia, China,

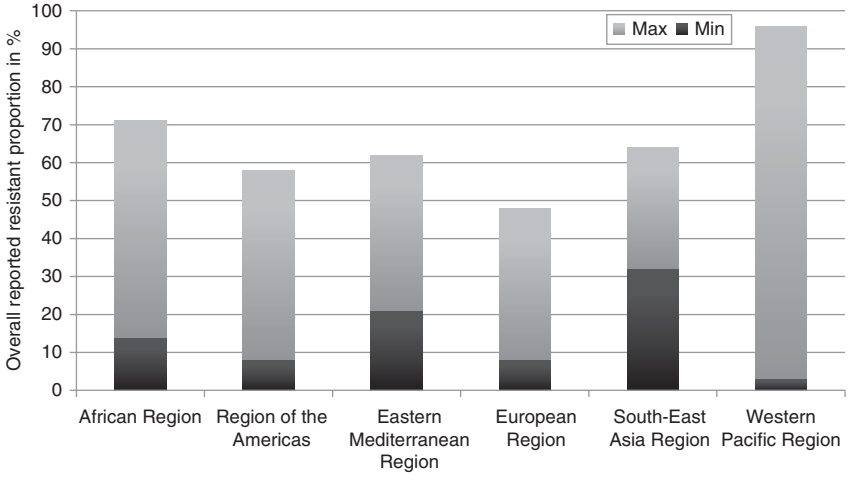


Figure 10.1 Resistance to fluoroquinolones in *E. coli*, national data. (Based on data from the WHO report [11].)

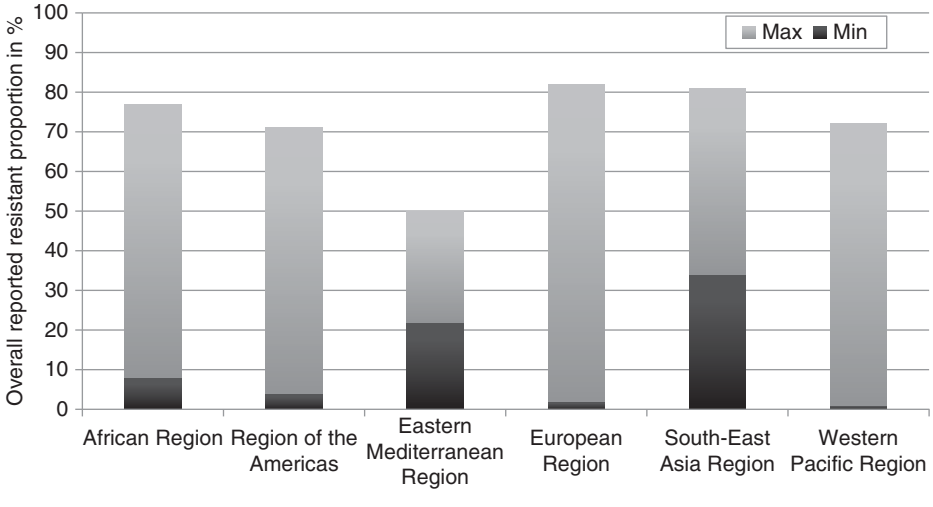


Figure 10.2 Resistance to third-generation cephalosporins in *K. pneumoniae*, national data. (Based on data from the WHO report [11].)

Japan, Malaysia, Philippines, Republic of Korea, Singapore, among others), the highest minimal percentage with over 30% is reached in the South-East Asia Region (Bangladesh, India, Thailand, among others). It should also be noted that the maximal percentage never falls below 45%. Resistance to quinolones may be indicative of resistance to one of the last available oral treatment options in some settings.

Klebsiella bacteria are common colonizers of the gut in humans. Infections with *K. pneumoniae* mostly appear in weakened patients and cause urinary and respiratory tract infections. Nosocomial outbreaks, especially in intensive care

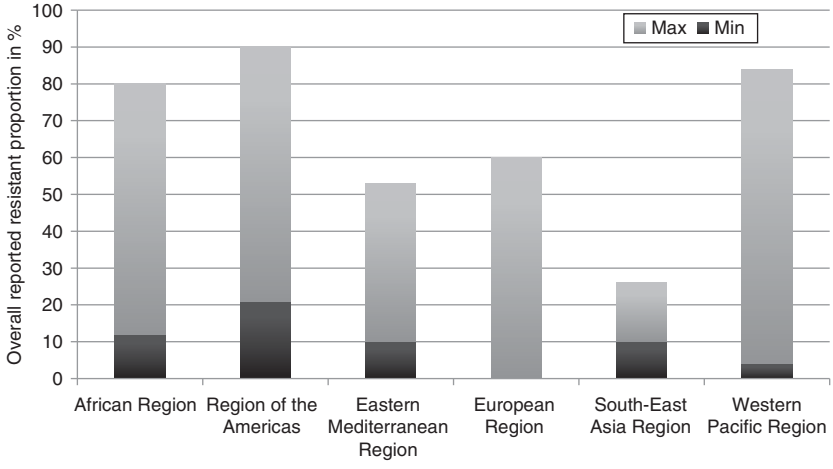


Figure 10.3 Resistance to β-lactam antibacterial drugs in *S. aureus* (i.e., methicillin-resistant *S. aureus*, MRSA), national data. (Based on data from the WHO report [11].)

units, are favored by the readily spread of the bacteria. The chromosome of *K. pneumoniae* carries a resistance gene that naturally renders penicillins with an extended spectrum ineffective, such as ampicillin and amoxicillin. Plasmid mediated resistance has been shown, especially by extended-spectrum-lactamases [17]. Third-generation cephalosporins have been the standard intravenous treatment for severe *Klebsiella* infections in hospitals. The resistance to third-generation cephalosporins in *K. pneumoniae* (Figure 10.2) shows the highest maximal value with around 80% for the African (all of Africa except Morocco, Tunisia, Libya, Egypt, Sudan, Eritrea, and Somalia), European (Benelux, France, Germany, Israel, Poland, Russian Federation, Spain, Turkey, United Kingdom of Great Britain and Northern Ireland, among others), and South-East Asia Region, followed by around 70% in the Region of the Americas (North and South America) and the Western Pacific Region. The highest minimal resistance with 20–30% is found in the Eastern Mediterranean Region (Afghanistan, Egypt, Islamic Republic of Iran, Iraq, Morocco, Pakistan, Saudi Arabia, among others) and the South-East Asia Region. The Eastern Mediterranean Region shows the lowest maximal resistance with 50%. The high proportion of cephalosporin resistance worldwide means that treatment for verified or suspected severe *K. pneumoniae* infections in many situations has to rely on carbapenems. These are usually not as readily available and involve higher costs and a risk of further expansion of carbapenem-resistant strains.

S. aureus can be part of the normal flora on the skin and in the nose, but is also one of the most important human pathogens. It can cause a variety of infections, such as of the skin, soft tissue, bone, bloodstream, and wounds. Some strains produce toxic factors that lead to toxic shock syndrome or food poisoning. Resistance to penicillin was already observed in the 1940s [18]. It is caused by a β-lactamase enzyme that inactivates the antibiotic. New drugs were designed to either inhibit the lactamase, which could be given in combination with the antibiotic, or the antibiotic itself was

designed to be resistant to the enzyme. Nowadays, there exist strains of *S. aureus* that have a different resistance mechanism and are therefore also resistant to the lactamase-stable antibiotics [3]. They are termed methicillin-resistant *Staphylococcus aureus* (MRSA). Plasmids can carry these resistance genes [19]. Resistance to *β*-lactam antibacterial drugs in *S. aureus* (Figure 10.3) reaches the highest maximal percentage in the African Region, the Region of the Americas, and the Western Pacific Region with over 80% resistance. The Region of the Americas shows the highest minimal resistance with around 20%. These high proportions of MRSA imply that *S. aureus* may require second-line antibiotics, which are more expensive and have stronger side effects, making monitoring during treatment necessary.

The spread of resistance genes is not only occurring within the community, but also seems to be a problem in individuals. Sommer *et al*. [20] functionally characterized the resistance reservoir in the microbial flora of healthy humans and found an immense diversity of resistance genes. They hypothesized that by the use of antibiotics, the natural microbiome of humans accumulates resistance genes. This could lead to future emergence of antibiotic resistance in human pathogens, as resistance plasmids are transferable between species. Through this mechanism, an infection could turn a susceptible bacterium into a resistant one during treatment, when the pathogens take up resistance plasmids from the individual microbiome.

Research Approaches for Plasmid Curing

In theory, antiplasmid agents can act in many different ways. A deterministic mathematical model of Volkova *et al*. [21] evaluated the possibilities to reduce the fraction of resistant enteric *E. coli* in its animate and nonanimate habitats within a beef feedlot's pen. This setting is transferrable to instances such as hospitals and long-term care facilities. It produced several possible modes of action. Among these was the direct curing of the plasmid, reducing the number of bacteria, and lowering the rate of plasmid transfer. This could all be achieved by drugs targeting plasmids (Figure 10.4) [21].

One approach could be to target plasmid replication (Figure 10.4A). The challenge for this would be to address various types of plasmids with just one drug, as there are three general replication mechanisms: theta type, strand displacement, and rolling circle [22, 23].

Replication is naturally regulated, most commonly by antisense RNA-based systems. The antisense RNA is expressed constitutive with a short half-life; therefore, its concentration is proportional to the plasmid copy number. There are multiple mechanisms by which the antisense RNA controls plasmid replication, for example, by inhibiting translation of the Rep protein. Incompatibility groups of plasmids are often defined by the mechanism of these antisense RNAs [22]. This mechanism could be used to cure strains of their plasmids.

Secondly, the plasmid-bearing strains could be killed directly or hindered in their growth by activating plasmid-coded toxin-antitoxin systems (Figure 10.4B).

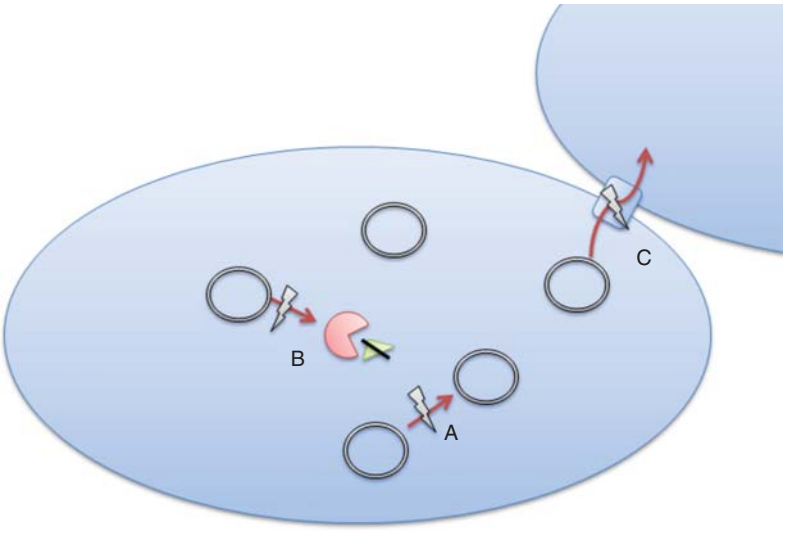


Figure 10.4 Possible sites of action for plasmid curing agents. (A) Inhibition of replication. (B) Activation of plasmid coded antitoxin–toxin systems. (C) Inhibition of conjugation.

Various studies showed that these systems are widespread among resistance plasmids, with some very common toxin-antitoxin systems such as MazF/MazE or RelA/RelB. An outstanding summary about toxin-antitoxin systems is given by the book *Prokaryotic Toxin-Antitoxins* edited by Gerdes [24]. Naturally, these systems are responsible for plasmid stability, especially in low-copy plasmids. They consist of a stable toxin and an unstable antitoxin that are both constitutively expressed. Upon plasmid loss, the antitoxin degrades and thus activates the toxin. The active toxin kills the plasmidless cell. Through this mechanism, resistance plasmids remain stable within a bacterial community even in a nonselective environment, as cells that lose the plasmid are killed off. An artificial activation of this system would lead to cell death in pathogens containing the resistance plasmids. Cells initially without the plasmid, that would not be affected by the toxin activator drug, would be nonresistant and therefore susceptible to the respective antibiotic. This approach is most suited for an *in situ* use, as it would act quickly, contrary to the other two approaches that most likely would need more time to cure a pathogenic strain of their resistance plasmids.

Lastly, the agent could inhibit plasmid conjugation (Figure 10.4C). This method would mainly inhibit the spread of resistance plasmids in a bacterial community rather than curing a resistant pathogen during an infection. As discussed in a paper by Lili *et al*. [6], plasmids could be lost within a strain population if conjugation is inhibited. Their model showed that persistence of a plasmid within a population relies on the exchange of plasmids between plasmid-bearing and plasmid-free cells. Without that exchange, the plasmid is likely to be lost over time.

Prerequisites for Clinical Use and Possible Application Methods

The idea of using plasmid curing in medicine has gotten more attention in the last years, as resistance to antibiotics is ever spreading among the world. It is believed that resistance is able to spread quickly because most resistance genes are plasmid coded. Plasmids can be transferred at a high rate, even between bacteria of different species. At the same time, research for new antibiotics has almost stopped. None of them would be a new class of antibiotics, and therefore resistance to these substances is expected to occur within few years [10, 25]. Developing a tool to eliminate resistance plasmids would make the old and by now often ineffective antibiotics powerful weapons against resistant pathogens again. In some cases, plasmids also encode virulence factors, for example, the pXO1 plasmid of *Bacillus anthracis* [26] or the tumor inducing (pTi) or root-inducing (pRi) plasmids of *Agrobacterium tumefaciens* [27]. Curing these strains therefore leads to a loss of virulence.

Using plasmid curing in medicine raises new demands toward the substances in question. Besides the usual requirements like the way of application, there are three points that seem to be especially hard to come by regarding plasmid curing. First, they need to be nontoxic to humans. Known substances often act nonspecific, making them more likely to be toxic. Another major problem seems to be the time required until there is a curing of plasmids observable. In most experiments so far, long incubation times or multiple treatment steps were necessary. For practical clinical use, time is valuable. Lastly, the overall curing rate should be as high as possible, because contrary to research, the goal in clinical treatment is not to select cured colonies among many uncured ones. Instead, the infected patient needs to be treated quick and with a high success rate, preferably without side effects.

The most intuitive way of applying an antiplasmid agent implies using the drug similar to antibiotics in case of an infection with multiresistant pathogens [9]. It would be administered as a combination of antibiotics and antiplasmid agent. The pathogens would lose their resistance plasmid due to the antiplasmid agent and therefore become susceptible to the antibiotics again or alternatively die directly by the antiplasmid agent. For this, the agent needs to act like a common drug, quick and over a broad range of plasmids and preferably pathogens. Furthermore, it needs to be applicable in the same way as the antibiotic partner without toxicity to humans.

An alternative and very different approach was described by Baquero *et al*. [28]. They propose the so called "eco-evo" strategy that means to prevent the spread and evolution of resistance or re-establishment of susceptible bacterial populations rather than just simply kill all bacteria. "Eco-evo" drugs or interventions do not necessarily cure individuals but certain environments/ecological niches such as hospitals or farms. In their review, they suggested among other ideas to generate a general selection pressure in larger settings, for example, hospitals, by distributing antiplasmid agents similar to disinfectants. This method would have two big advantages. First, the agent would not need to act as quick as when administered in patients. It just would need to shift the selection pressure in the bacterial community toward plasmid loss over a long period of time. Under this selection pressure, the plasmidless and nonresistant strains would be in advantage over the

ones with resistance plasmids. Secondly, the potential drug would not have to be nontoxic to human cells as drugs applied *in situ*, as it would be used like a general disinfectant for surfaces. This way of application calls for a cheap drug, of course, as it would be needed in larger quantities. Such a drug would decrease the reservoir of resistant pathogens in the population, which may be the cause for the seemingly ineffective measures in hospitals to get rid of resistant pathogens, as described in a paper by Smith *et al.* [29]. This ambitious approach does not only affect resistance but also could influence the whole microbial system [30].

Contrary to the aforementioned suggestion, the concentration of antibiotics in the environment in subinhibitory conditions is rising in the long term and might enhance the dissemination of resistance plasmids. Even if the existence of an environmental resistome seems to be an inexhaustible source for antibiotic resistance genes by itself [31, 32] it is evident that antibiotics in subinhibitory concentrations can promote bacterial resistance [33]. One recently published example is the laboratory analysis of plasmid transfer rates of a multidrug resistant plasmid with *E. coli* acting as a donor and *Pseudomonas aeruginosa* or mixed cultures as receptors by Kim *et al.* [34] showed higher plasmid transfer rates even at doses as low as 10 ppb of tetracycline.

Common Methods and Compounds in Use

Plasmid curing agents have been known since the 1960s. So far, none of them is used in medicine, but in research. Among the most common drugs are intercalating compounds such as acridine orange [35] or ethidium bromide [36], as well as detergents such as Sodium dodecyl sulfate (SDS) [37]. Trevors [38] reviewed the common methods and compounds that were used back in 1986. These methods usually do not have a high curing rate, but are still used today as they are easy to apply and act over a broad range of organisms. On the other hand, this broad range seems to be more or less due to the vague mode of action. Plasmid loss most likely is evoked by general cell stress, and mutations and other cell alterations besides the desired plasmid loss cannot be ruled out. Ethidium bromide, for example, is known to be mutagenic. Mutations in the chromosome can lead to a different phenotype, which interferes with the analysis of the plasmid. Furthermore, all of these substances are not suited for clinical use to cure patients, as they are toxic to humans. Curing rates of plasmids can also be increased by applying stress in other forms than chemical stress. There have been publications about the curing effect of electroporation [7], increased temperature [39], or carbon–phosphorous-limited conditions [40]. These experiments show that a medium curing rate can be achieved by stressing the cells. Under bad conditions, cells without the extra DNA to replicate and to maintain have a growth advantage, leading to plasmid loss within the population. This leads to another drawback of these methods: As the plasmid loss occurs spontaneously, cells have to be observed over a long range of time. This is not only time consuming in screening approaches but also makes these methods inappropriate in treating infections.

An already known nontoxic plasmid-curing substance seems to be ascorbic acid [41, 42]. It was tested *in vitro* against antibiotic resistant *S. aureus.* When only ascorbic acid was applied, the curing rate was over 50%, but not for all strains tested. In combination with antibiotics a 50–75% decrease in the minimal inhibitory concentration for the respective antibiotics could be observed. An important detail is that the cells only had to be incubated for 6 h with 1 mM ascorbic acid to observe this effect. Moreover, as ascorbic acid is not harmful to humans, it could be added to antibiotics to make them more effective. Although similar results were described by others [43–45], the mode of action does not seem to be understood.

Among other known plasmid-curing substances are antibiotics such as rifampicin [46], 4-quinolones [47] as well as novobiocin [48], or bleomycin [49]. To cure bacteria of plasmids, these substances are applied in sublethal doses, putting the cells under mild stress. It seems that the plasmids are more susceptible in contrast to chromosomes, maybe due to differences in DNA topology. Under these conditions, plasmid loss is favored [50]. Each antibiotic works only for a small range of strains, which is why these substances are not as often used as acridine orange, for example, when trying to cure a new strain.

Approaches and Techniques to Identify Antiplasmid Effects/Compounds since 2000

Plasmid Incompatibility

Plasmids replicate by various mechanisms. If two plasmids have the same replication mechanism, they compete with each other for replication and segregation into daughter cells. This is called plasmid incompatibility [51], and the less successful plasmid will be erased from the strain over time. The phenomenon was mainly a problem in molecular biology when transforming bacteria, but also offers the possibility to cure pathogens of their virulence or antibiotic resistance plasmids. Therefore, it gained more attention lately. Introducing a small incompatible plasmid or compound can generate plasmid-cured strains at a high rate, without inducing spontaneous mutations in the host chromosome.

The plasmid coded DNA transfer system of *A. tumefaciens* is used in constructing transgenic plants and fungi. For this, the bacteria have to be cured of the wild type plasmids first, which are very stable and hard to cure by common methods. The group of Uraji *et al*. [27] developed a novel curing method based on plasmid incompatibility for *A. tumefaciens*. The replication gene *repABC* of the pathogenic plasmids was cloned into a plasmid without an own replication mechanism. The plasmid further contained a *sacB* gene that produces a toxic substance under high sucrose conditions. To cure the cells of their tumor-inducing (pTi) or root-inducing (pRi) plasmids, they were first transformed with the constructed plasmid and in a second step cultivated with 10% sucrose to eliminate the curing plasmid. The efficiency was 32–99%, depending on the strain.

In a similar manner, Liu *et al*. [26] could construct a curing plasmid for *B. anthracis*. The virulent form of this bacterium harbors two large pathogenicity-related plasmids: pXO1, which encodes the anthrax toxin genes, and pXO2, which carries the genes responsible for capsule synthesis and degradation. Both plasmids are essential for full pathogenicity; elimination of either dramatically attenuates the virulence of *B. anthracis*. Common curing methods were not suited here, as they are not capable of specifically eliminating one plasmid while leaving the other one in the cell. Liu and colleagues used a putative replication origin of pXO1, cloned into a temperature-sensitive plasmid, to eliminate the large plasmid pXO1 from *B. anthracis* vaccine strain A16R and wild type strain A16. After eliminating the temperature-sensitive curing plasmid from the cells, they obtained a nonvirulent strain.

The main problem of these methods for a nonlaboratory use is the introduction of the curing plasmids to the cells. Taking up free plasmid DNA does not occur with a high enough likelihood under natural conditions. Until there is a way to overcome this problem, curing of plasmids via incompatibly plasmids will only be useful for research. On the other hand, the specific hindrance of plasmid replication rules out side effects such as mutagenesis of the chromosome and lowers the possibility for cytotoxicity.

Antisense RNAs control replication by regulating the expression of the target initiator protein. Various reviews on regulation of plasmid replication by antisense RNA were published in recent years, one by Brantl just recently [52–54]. The antisense RNA is coded on the plasmids and constitutively expressed. A change in plasmid copy number changes the level of antisense RNA proportionally. In most cases, the target is the mRNA encoding the replication initiator protein. Therefore, a rise in copy number raises the antisense RNA, which in turn lowers the level of the replication initiator protein, leading to less plasmid replication. This system could be used for plasmid curing, either through a drug that imitates the natural antisense-RNA or through a small vector that inserts the gene for the appropriate RNA.

The group around Hergenrother [55] tried to identify small substances that mimic the incompatibility effect of plasmids belonging to the IncB incompatibility group, which are widespread among bacteria. Here, the synthesis of the enzyme responsible for replication (RepA) is regulated by a RNA I at the translational level, as described above. The aminoglycoside antibiotic apramycin (Figure 10.5) was able to bind specifically to the respective mRNA region of RepA. Whole *E. coli* cells could

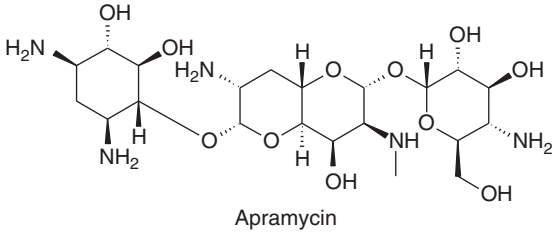


Figure 10.5 Structure of apramycin.

be cured from a resistance plasmid with almost 100% success rate at a concentration of 18 μg ml^{-1} apramycin, and over 70% with 12 μg ml^{-1}. Point mutations in the SL1 region of RepA mRNA showed high resistance to apramycin, indicating a specific mode of action. While this means that little side effects such as mutagenesis of the chromosome are expected, it also shows that mutants could easily become resistant to this substance.

Toxin-Antitoxin Systems

Toxin-antitoxin systems (or suicide modules) were first discovered on plasmids due to the fact that they seemed to mediate higher stability of the plasmids they are coded on [56, 57]. Some of them can also be found on chromosomal DNA, where they seem to be a part in stress response or cell death. They consist of a stable toxin and an unstable antitoxin, both expressed constitutionally. Upon plasmid loss, the unstable antitoxin is depleting quicker than the toxin. This leads to free active toxin, which kills the plasmid free cell. Plasmids bearing such toxin-antitoxin systems are generally hard to cure, as cured cells die to the remaining toxin in the cells. In recent years, this topic gained attention and next to a book giving a comprehensive overview [24] several reviews were issued [58, 59]. Especially, these systems are discussed as possible drug targets since a lot of clinical important plasmids with resistance genes do harbor such systems. They were identified in clinical isolates, such as *P. aeruginosa* and *S. aureus* [60], vancomycin-resistant Enterococci [61], and *Acinetobacter baumannii* [62]. Last but not least, the idea of using these systems to combat resistance was picked up in several synopsis too [63–66].

Schaufler *et al*. [67] had to examine various multiresistant strains of *E. coli* derived from clinical isolates and need to obtain plasmidless variants as a control. As the different resistance plasmids contained at least one toxin-antitoxin system each, constructing viable plasmid-cured variants was difficult. Curing attempts using ethidium bromide or acridine orange were unsuccessful. Therefore, they performed successfully a heat technique in which the strains were incubated at 45 °C for 24 h, followed by subcultivation at 37 °C. The researchers speculated that compared to ethidium bromide, these mild conditions enable the cells to adapt to changing conditions rather than dying due to excess stress, or that the toxins degenerate at higher temperatures.

A more specific way to cure such stable antitoxin–toxin plasmids was developed by Hale *et al*. [68]. They cloned key regions of the replicons and the postsegregational killing loci of plasmids of the F incompatibility group (IncF) of *E. coli* and the broad-host-range IncP-1 family into an unstable cloning vector carrying sacB. When transferred into cells containing plasmids with these replication mechanisms, the curing plasmid competes with the natural ones for replication, leading to loss of the resistance plasmid over several generations. SacB confers susceptibility to sucrose, which allows selection of clones lacking the curing plasmid in a second step. This approach results in highly efficient displacement and can be extended to other F-like plasmids or even different groups of plasmids.

The toxin-antitoxin system could be used in medicine to deal with pathogens, whose antibiotic resistances are coded on plasmids. The treatment would need to activate the toxin to kill the cells, for example, by inhibiting the expression of the toxin-antitoxin gene. With no new antitoxin being produced, free toxin would accumulate as the antitoxin would degrade quicker. Another possibility would be to prevent the forming of the toxin-antitoxin complex or disruption of toxin-antitoxin interaction. Cells without the plasmid would be unaffected by this substance, as they would not contain the toxin-antitoxin system. However, they would be susceptible to the antibiotics to which the plasmid-bearing cells are resistant. A combination of both drugs would therefore kill all cells, no matter if they carry the resistance plasmid or not. Substances that activate such a toxin-antitoxin system have yet to be discovered. Kolodkin-Gal *et al*. [69] reported the discovery of a symmetric linear pentapeptide that activates the MazF-MazE toxin-antitoxin system and called it EDF (extracellular death factor), but the results could not be reproduced in another laboratory since [70]. The quorum sensing molecule EDF is a small peptide, consisting of five amino acids (NNWNN), and is believed to associate with MazF, preventing inhibition by MazE. The activity of MazF was determined by a fluorometric assay, in which active MazF (0.25 μM) cleaves a labeled oligonucleotide. Adding MazE (0.025 μM) led to almost no activity of MazF. The inactivation by MazE was prevented by adding 7.5 μM (4.95 μg ml^{-1}) to 15 μM (9.9 μg ml^{-1}) EDF. This led to a restored signal of 70–80%, respectively [71]. In another set of experiments, they tested the ability of EDF to induce cell death by MazF activation. The concentration of EDF for inducing cell death ranged from 2.5 ng ml^{-1} [69] up to 15 μM (9.9 μg ml^{-1}) [72]. So far, EDF is the only example of a toxin antitoxin modulator [71]. The fluorometric assay used for the studies above was developed to characterize MazF and to set up a screening option for disruptors of the MazE/MazF complex in a high-throughput scale [73].

Inhibition of Conjugation

Transfer of plasmids by conjugation through close cell-cell contact is one of the main types of resistance gene distribution. Inhibiting the dispersal of resistance plasmids by conjugation is an approach worth following too, as it would slow down the overall occurrence of antibiotic resistance in the bacterial community. So far, most of the known substances act unspecific. Nalidixic acid, for example, inhibits DNA gyrase, which is important in DNA metabolism [74, 75]. Chlorpromazine triggers the inhibition of conjugation of plasmid F [76], possibly by general stress elevation [77]. Nitrofurans inhibit conjugation for plasmids of six different incompatibility groups by causing a general disruption of bacterial DNA [78].

Fernandez-Lopez *et al*. [75] developed an automated high-throughput conjugation assay that uses plasmid R388 and a laboratory strain of *E. coli* as a model system, and bioluminescence as a readout for conjugation activity. The plasmid was chosen because it contains a very simple and widespread conjugation system, which is similar to the system in *A. tumefaciens*, and the crystal structures of two key proteins (relaxase TrwC [79] and coupling protein TrwB [80]) are already

known, making modeling of protein-inhibitor cocomplexes possible. Furthermore, it contains a *lux* operon under a *lac* promoter. *lux* expression in conjugative donor cells is repressed by the *lac* repressor LacI carried in a coresident and nonmobilizable multicopy plasmid. Upon conjugation, only the plasmid R388 is transferred, leading to luminescence in the transconjugant cells. Because of that, the assay helps to distinguish between true conjugation inhibitors and substances that diminish conjugation due to perturbations in cell growth or bacterial physiology. They screened over 12 000 microbial extracts, finding that the unsaturated fatty acid dehydrocrepenynic acid (Figure 10.6, Minimal Inhibitory Concentration (MIC) 70 mM) and other polyunsaturated acids such as linoleic acid (Figure 10.6, MIC 400 μM) were effective. The inhibiting effect on conjugation could be validated in further experiments by Fernandez-Lopez *et al*. [75]. This assay could be applied to high-throughput approaches to identify potential conjugation inhibitors by screening of compound libraries.

The key protein of conjugation is relaxase, which is the common component in this process. It catalyzes the initial and terminal steps [4, 5, 81]. Lujan *et al*. [82] tried to identify relaxase-specific inhibitors while investigating the F TraI relaxase. *In vitro* they characterized a limited number of compounds and identified several nanomolar inhibitors having the same structural features: two phosphonate moieties separated by three or fewer atoms, for example, ETIDRO (Figure 10.7, etidronic acid) and CLODRO (Figure 10.7, clodronic acid). The most active compounds of the *in vitro* assay were tested if they were able to inhibit the DNA transfer in living bacterial cells too but at higher concentrations. ETIDRO, for example, showed an EC_{50} of 330 nM in transfer inhibition. Lujan *et al*. [82] identified the first small molecules that were able to inhibit this relaxase.

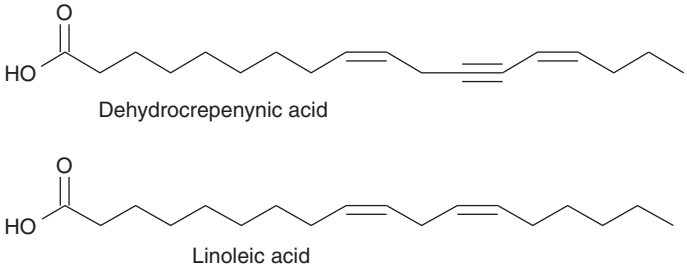


Figure 10.6 Structures of dehydrocrepenynic acid and linoleic acid.

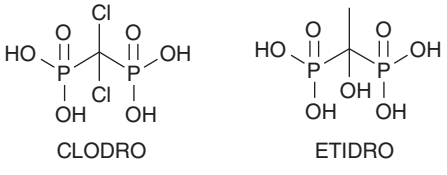


Figure 10.7 Structures of ETIDRO and CLODRO.

Inhibition of conjugation is also possible by bacteriophages [83]. Lin *et al*. [84] investigated the mechanism of inhibition by the phage M13 and found that occlusion of the conjugative pilus by the phage coat protein g3p is the main cause. The rate of conjugation was determined by mixing F+ cells expressing CFP (cyan fluorescent protein) with a large excess of F2 cells expressing eYFP (enhanced yellow fluorescent protein). Successful transfection could be observed by a change in the fluorescence signal. Addition of 3 nM of the soluble *N*-terminal domains of g3p to the culture media resulted in nearly complete inhibition of conjugation.

Ojala *et al*. [85] used the lytic bacteriophage PRD1, which can replicate in a wide range of gram-negative bacteria harboring conjugative drug resistance conferring plasmids, to investigate whether it can lower the transfection rate of the resistance plasmids. The phage binds to a conjugative plasmid encoded protein to enter the cell. Two antibiotic-resistant bacterial strains of *E. coli* K-12 were cultivather for 3 days. One of the strains contained a conjugative plasmid RP4 conferring resistance to several antibiotics of different classes, whereas the other strain was plasmid free but resistant to rifampicin due to a chromosomal mutation. The transfer of the RP4 plasmid would create a new multiresistant strain. Bacteria are resistant to phage infections if they are free of the plasmid or they harbor a conjugation-defective mutant. In the presence of the phage PRD1, the frequency of multidrug-resistant *E. coli* that formed via horizontal transfer was reduced by several orders of magnitude, even in the presence of an antibiotic against which the plasmid provided resistance. Most of the bacteria also lost their ability for conjugation.

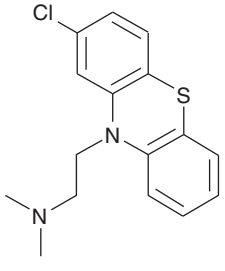
As described by Pearl *et al*. [86], phages could be used to fight persister cells, which are a big problem, for example, in infections with *Mycobacterium tuberculosis*. Bacterial persistence is often triggered by toxin-antitoxin modules [87]. They found that persistent *E. coli* are not protected from lytic infection with λ phage. Experiments showed that the expression of lytic genes is suppressed in infected persistent bacteria, but when they switch to normal growth, the infecting phage resumes the process of gene expression, which leads to cell lysis. Phages are also suited to treat cells in biofilms, which are, besides a high rate of persister cells, additionally hard to treat by many antimicrobial agents because of the thick extracellular matrix [88]. This would make phages a suitable treatment for resistant pathogens that are already hard to treat due to their growing behavior.

Miscellaneous

Often, the traditional plasmid curing agents such as SDS and acridine orange do not work on specific strains. Keyhani *et al*. [89] faced this problem when they tried to cure a resistance plasmid of a clinical isolate of *Enterococcus faecalis*. They successfully used sodium N-lauroylsarcosinate (sarkosyl; 0.02%) instead, leading to a low curing rate of 3%. A simultaneously tested *E. coli* strain did not show sensitivity against this reagent. Using the cured strain, they could show that the observed kanamycin and partially the tetracycline resistance were coded on the resistance plasmid.

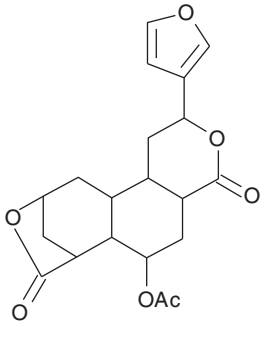
The group of Spengler [90] tested the effect of promethazine, trifluoperazine, and 9-aminoacridine on doxycycline-resistant enteric bacteria (*E. coli*, *Citrobacter freundii*, and *Enterobacter cloacae*). Phenothiazine derivatives are intercalating agents that result in relaxation of the supercoiled plasmids [91]. Spengler *et al*. [90] showed that the plasmid curing effects of these compounds were increased in the presence of a trifluoroketone proton pump inhibitor and hypothesized that the weak plasmid curing effect of some agents may be due to inefficient penetration of the membrane. In a review, they summarized the curing effects of heterocyclic compounds [92]. Phenothiazine tricyclic compounds and nonphenothiazine tricyclic compounds, to which ethidium bromide belongs, were tested on *E. coli* K12LE140. The most active agent of these groups was 2-chloro-10-(2-dimethylaminoaethyl)-phenothiazine (Figure 10.8), which has a plasmid curing activity of 90% and a MIC of 3.1 $\mu g\,ml^{-1}$.

The group of Shriram tested plant extracts on various multiresistant strains for plasmid curing effects [93–95]. For this, they extracted the plants using methanol, acetone, or water and incubated the pathogens with the extracts. Following the treatment, they counted the surviving colonies and tested them for plasmid loss. They found 8-epidiosbulbin E acetate (Figure 10.9, EEA) with a concentration of 25 $\mu g\,ml^{-1}$ isolated from *Dioscorea bulbifera L.* bulbs was curing the cells of their plasmids with up to 44% efficiency. The mode of action of the respective compounds was not investigated.



2-Chloro-10-(2-dimethylamino-aethyl)-phenothiazine

Figure 10.8 Structure of 2-chloro-10-(2-dimethylamino-aethyl)-phenothiazine.



8-Epidiosbulbin E acetate

Figure 10.9 Structure of 8-epidiosbulbin E acetate (EEA).

Conclusion

According to the WHO, antibiotic resistance is the biggest health threat worldwide. One must keep in mind that widespread antibiotic resistance would not only make treating infections harder but would also affect important practices such as immunosuppression in transplants, anticancer chemotherapy, and advanced surgery. Besides developing new antibiotics, preventing the spread of resistance plasmids and curing resistant strains could be the main tool to fight resistant and multiresistant pathogens. Since 2000, research groups around the world have shown that there is a multitude of approaches to cure bacteria from their plasmids. Most of plasmid curing research still aims at curing a resistant pathogen strain for research purposes. Curing rates do not need to be as high for that purpose compared to treatment of human infections, and it seems that it is not easy to obtain a quick acting antiplasmid drug that would be useful for acute treatment of resistant pathogens.

In areas with a high selection pressure toward antibiotic resistance, such as hospitals, a slow acting but long time active agent would offer the possibility to shift evolutionary pressure for the bacterial community toward plasmid loss [28]. As Smith *et al*. [29] stated, it might also be necessary to include long-term care units, as they act as a source for antibiotic-resistant bacteria. Without including the main ecological/human-made niches responsible for the increase in antibiotic-resistant bacteria, the rate of resistance will always be higher than the rate at which resistant bacteria are losing their resistance plasmids.

Even an efficient antiplasmid drug would not be able to get rid of all antibiotic resistance we face today, as resistance can be coded on the chromosome too. But it would impede the crosstalk between different strains: each of them would have to invent resistance on their own or acquire them plasmid-independently, making it much less likely to become a common problem in modern medicine. Confrontation with a multiresistant pathogen would only occur very rarely, without the danger of resistance spreading to other pathogens or common microbiota ("normal flora") as well.

The antibiotics introduced in the last six decades decreased the mortality due to infectious diseases enormous but nowadays the occurrence of resistant bacteria reverses this and the number of deaths are rising again. The suggestion to cure bacterial plasmids expressed by Hahn in 1979 [9] was neglected for decades, but today it could be one way how mankind could cope with multiresistant bacteria.

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11
Regulation of Secondary Metabolism in the Gray Mold Fungus *Botrytis cinerea*

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Abstract

Botrytis cinerea is responsible of the gray mold disease on a wide range of host plants. During the infection process, it produces unspecific phytotoxins (botrydial and botcinic acid) and other secondary metabolites that play a significant role in plant tissue colonization. Regulation of the biosynthesis of these bioactive compounds is therefore crucial for the outcome of fungus/plant interactions. Combining genetics and transcriptomic approaches, the role of different signaling cascades, transcription factors, and other regulators such as those belonging to the VELVET complex have recently been investigated. Overall, these studies suggest a strong link between the regulation of secondary metabolism and the light-dependent development in *B. cinerea*. Further knowledge of the complex regulatory network controlling secondary metabolism in *B. cinerea* is crucial to understand the interaction between *B. cinerea* and its different host plants and may help to define new strategies to control this crop-devastating fungus.

Botrytis cinerea, the Causal Agent of Gray Mold Disease

Botrytis cinerea Pers. Fr. causes severe pre- and postharvest crop losses on more than 200 host plants including important crops and is therefore one of the most studied phytopathogenic fungi [1]. This Ascomycete (Leotiomycetes class, Sclerotiniaceae family) is also named the gray mold agent because of the production of masses of melanized conidia on infected plants. Conidia allow *B. cinerea* to disperse widely on further plants while the ability to form sclerotia allows the fungus to survive in the soil for extended periods. These melanized survival structures can also be fertilized by microconidia from a strain of the opposing mating type to form fruiting bodies (apothecia) with sexual recombinants (ascospores) [2, 3].

B. cinerea is considered a typical necrotroph, inducing host cell death and feeding on dead tissues. Thanks to an arsenal of plant cell wall–degrading enzymes, to

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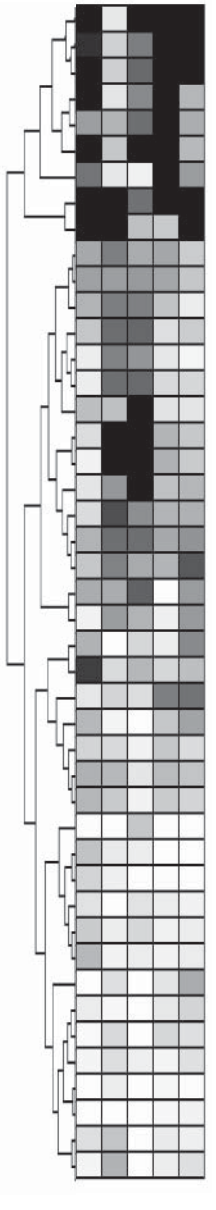

Key enzymes	**Known metabolites (in bold)** or predicted ones
BcSTC1/ BOT2	**Botrydial** (BOT) [13]
BcPKS6/ BOA6^{S}	**Botcinic acid, botcinins** (BOA) [14-15]
BcPKS9/ BOA9^{S}	**Botcinic acid, botcinins** (BOA) [14-15]
BcSTC4	Sesquiterpene [5]
BcPKS8^{S}	Reduced polyketide [16]
BcPKS13^{S}	**(Conidial) DHN melanin** [17-19]
BcABA1*	**Abscisic acid** [12, 20]
BcCHS1/ BPKS^{S}	**Pyrones, resorcylic acids, resorcinols** [21]
BcOAH^{S}	**Oxalic acid** [22]
BcPHS1^{S}	**Carotenoids and retinal** [17]
BcPKS18^{S}	Polyketide [16]
BcNRPS9	Peptide [23]
BcPKS19	Polyketide [16]
BcNRPS1^{S}	Peptide [23]
BcNRPS7	Ferrichrome siderophore [23]
BcPKS4	Reduced polyketide [16]
BcPKS5	Amino-acid containing polyketide [16]
BcPKS11	Reduced polyketide [16]
BcPKS12^{S}	**(Sclerotial) DHN melanin** [17, 19]
BcNRPS8	Peptide [23]
BcPKS20	Polyketide [16]
BcNRPS6^{S}	Coprogene siderophore [23]
BcPKS7	Amino-acid containing polyketide [16]
BcDTC1	Diterpene [5]
BcDMATS1^{S}	Alkaloid [5]
BcNRPS2^{S}	Ferrichrome siderophore [23]
BcPKS21^{S}	Polyketide [5]
BcSTC3	Sesquiterpene [5]
BcPKS15	Polyketide, probably orsellinic acid [24]
BcSTC2	Sesquiterpene [5]
BcNRPS3^{S}	Ferrichrome siderophore [23]
BcDMATS2^{S}	Alkaloid [5]
BcPKS3	Amino-acid containing polyketide [16]
BcPKS1^{S}	Reduced polyketide [16]
BcPAX1	Indole-diterpene [5]
BcPKS10^{S}	Reduced polyketide [16]
BcPKS16	Polyketide [16]
BcNRPS5^{S}	Peptide [23]
BcDTC2	Diterpene [5]
BcPKS14	Polyketide [16]
BcPKS17	Polyketide [16]
BcPKS2^{S}	Reduced polyketide [16]
BcDTC3	Diterpene [5]
BcNRPS4^{S}	Peptide [23]
BcSTC5	Sesquiterpene [5]

Figure 11.1 Repertoire of the predicted secondary metabolism key enzymes (KE) genes in *Botrytis cinerea* and their expression in different growth conditions. Conidial suspensions of the wild type strain B05.10 were incubated for 48 h on minimal medium (**M**), complete medium (**C**), grape Juice medium (**J**), grape berries (**G**; *Vitis vinifera*), or on bean leaves (**B**; *Phaseolus vulgaris*). Expressions (i.e., log2-normalized intensities) from NimbleGen array data were clustered and depicted by a color scale, where light gray represent weakly expressed genes and dark gray represent highly expressed genes. [S]Indicates that the KE is also present in the close species *Sclerotinia sclerotiorum* [5]. [*]The KE responsible for ABA synthesis remains unknown, so the gene encoding the BcABA1 P450 monooxygenase was included in the analysis [12].

phytotoxic small proteins and metabolites, and to the ability to encounter strong oxidative stress, the fungus can develop on many different host tissues under a wide range of environmental conditions [3–7]. *B. cinerea* is more destructive on mature or senescent tissues, but it can gain entry to these tissues at a much earlier stage and remain quiescent until the host physiology becomes favorable for its proliferation [3]. In some cases, the endophytic behavior of *B. cinerea* was clearly demonstrated [8, 9]. Finally, under specific environmental conditions in vineyards, *B. cinerea* may not cause gray mold but noble mold, which allows the production of sweet wines like Sauternes. In this case, the development of the fungus is limited to the outermost layers of grape epidermis [10]. In conclusion, *B. cinerea* encounters many different biotic and abiotic interactions and consequently modifies its metabolism and physiology to adapt to the altering environmental conditions.

Repertoire of Secondary Metabolites Produced by *B. cinerea*

Secondary metabolites (SMs) are expected to play an essential role in the versatile life of *B. cinerea* as described in the previous section. In the pregenomic era of this fungus, about eight families of SMs have been isolated from *in vitro* mycelium, but sequencing of the genome revealed a repertoire of gene clusters dedicated to the synthesis of approximately 40 different SMs [5, 11]. As in other fungi, these clusters include one (or two) gene(s) encoding the key enzyme (KE) responsible for synthesis of the raw product, genes encoding enzymes for further modifications and in some cases, genes encoding transporters and/or regulators. Among the 44 KEs (Figure 11.1) are many polyketide synthases (21 PKSs) [16, 21, 24], nonribosomal peptide synthetases (9 NRPSs) [23], and sesquiterpene cyclases (5 STCs) and diterpene cyclases (3 DTCs). Notably, only 21 of these KE-encoding genes are shared with the closest related genus *Sclerotinia* [5]. To date, only a small number of SMs have been identified and connected with the genetic make-up of *B. cinerea*. Among them are the predominant nonspecific toxins botrydial (BOT) and botcinic acid (BOA) (Figure 11.2) and other SMs that may be involved in plant/fungus interactions:

- *Botrydial (BOT)* and related compounds are sesquiterpenoids [26]. The discovery of the genetic basis for BOT biosynthesis included the identification of a cluster of coregulated genes [13, 27]. Deletion analyses of *bcbot1, bcbot3,*

bcbot4 encoding cytochrome P450 monooxygenases and *bcbot2/bcstc1* encoding a STC proved their requirements for BOT biosynthesis [13, 25] (I.G. Collado *et al*., unpublished). BOT would trigger plant cell death via induction of the hypersensitive response [28].

- *Botcinic acid (BOA)* and its derivatives (botcinins) are combined polyketides [29, 30]. The cluster of coregulated genes (*bcboa1-17*; Figure 11.2) includes two PKS-encoding genes essential for BOA biosynthesis: BcBOA6 mediates the formation of the tetraketide core, whereas BcBOA9 is responsible for the synthesis of the side chain [14, 15]. Analysis of mutants lacking the ability to produce BOT, BOA, or both toxins suggested a redundant role in plant tissue colonization [14]. Additionally, several botcinins would have antifungal activities [29, 30].
- *Abscisic acid (ABA)* is a plant hormone and belongs to the group of sesquiterpenoids [31]. While in higher plants, its biosynthesis is mediated via the carotenoid (CAR) pathway, *B. cinerea* synthesizes ABA directly from farnesyl diphosphate via different oxidative steps. The *B. cinerea* KE gene responsible for the first step of ABA biosynthesis remains unknown but a cluster of four genes (*bcaba1-4*) involved in the later steps was characterized [12, 20]. To date, no evidence exists that fungus-derived ABA influences fungus–host interactions (V. Siewers and P. Tudzynski, unpublished).
- *Oxalic acid (OA)* is a compound that is produced via the oxaloacetate acetylhydrolase (OAH) in *B. cinerea* and *Sclerotinia sclerotiorum*. Loss of OA formation affects virulence in closely related fungi in different ways: while it results in avirulent mutants in *S. sclerotiorum*, *bcoahA* mutants are still able to colonize the host tissue [22, 32, 33].

B. cinerea also produces several pigments that can act as protectants against UV and other exogenous stresses (oxidative stresses, desiccation):

- *1,8-Dihydroxynaphthalene (DHN)-melanin* is a polyketide derivative accumulating in the conidiophores, conidia, and sclerotia of *B. cinerea,* giving them their characteristic color [34, 35]. In contrast to other DHN-melanin forming fungi, *B. cinerea* possesses two developmentally regulated PKS (KE)-encoding genes (*bcpks12*, *bcpks13*), while the other melanogenic genes are present in single copies [17]. Recently, it was shown that BcPKS12 and BcPKS13 provide the precursors for sclerotial and conidial melanogenesis, respectively [18, 19].
- *Carotenoids (CARs)* are tetraterpenoids produced by plants and fungi to protect cells from free radicals and singlet oxygen [36]. A cluster of four light-induced genes (*bop2-bcphd1-bcphs1-bccao1*) was recently identified in *B. cinerea* [17] that shares similarity with the CAR gene cluster in *Fusarium fujikuroi* suggesting that *B. cinerea* produces CAR via a similar biosynthetic pathway than *F. fujikuroi* [37].
- *Bikaverin (BIK)*, a red pigment, is a well-known polyketide in some *Fusarium* species [38] but that is also produced by rare strains of *B. cinerea* due to a probable horizontal gene transfer [39, 40].

The repertoire of KEs (Figure 11.1) suggests that *B. cinerea* can also produce several siderophores [23] and many other SMs that remain to be chemically identified.

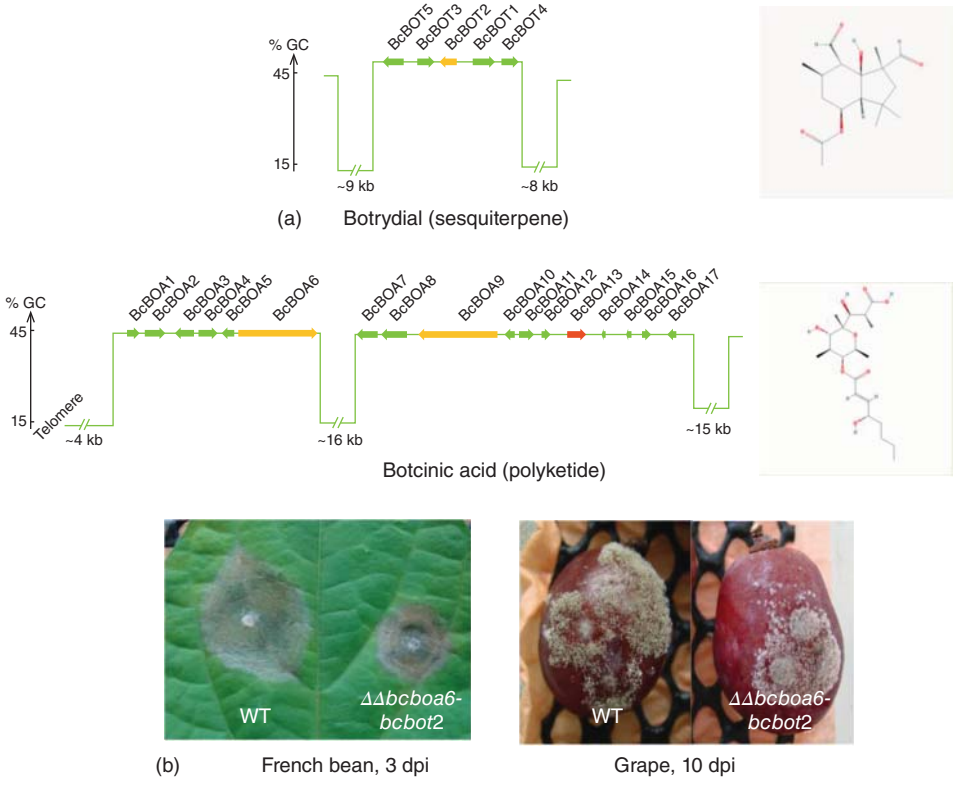

Figure 11.2 The two toxins botcinic acid (BOA) and botrydial (BOT) are produced from clusters of co-regulated genes and have a redundant role in virulence. (a) BOT and BOA clusters predicted in the wild strain B05.10 [13, 14] and surrounding AT-rich regions (J. van Kan *et al*., unpublished); chemical structures of BOT and BOA (PubChem). The KE for BOT synthesis is the Sesquiterpene cyclase (STC) encoded by *bcbot2/stc1*. Other co-regulated genes encode for P450 monooxygenases (*bcbot1, 3,* and *4*) also involved in BOT synthesis [12] (I. G. Collado *et al*., unpublished) and a putative acetyl transferase (*bcbot5*). Two KEs are required for BOA synthesis: the polyketide synthases (PKSs) *bcbot6/pks6* and *bcboa9/pks9* [14, 15]. Other co-regulated genes encode for putative monooxygenases (*bcboa2, 3, 4,* and *7*), dehydrogenases (*bcboa5* and *17*), a FAD-binding protein (*bcboa8*), a dehydratase (*bcboa16*), a thioesterase (*bcboa10*), a transferase (*bcboa11*), and unknown proteins (*bcboa12, 14,* and *16*). A pathway-specific $Zn(II)_2Cys_6$ transcription factor (TF) is encoded by *bcboa13* and a Nmr-A like regulator is putatively encoded by *bcboa1*. (b) While both single mutants Δ*bcboa6* and Δ*bcbot2*, impaired in BOA and BOT production, respectively, are not altered in virulence compared to the wild type (WT) strain (not shown), the double ΔΔ*bcboa6-bcbot2* mutant, unable to produce any toxin, causes significant smaller necrotic lesions on several host plants. Here, conidial suspensions were inoculated on leaves of *Phaseolus vulgaris* (French bean) and *Vitis vinifera* (grape) berries.

Importantly, the property to produce the mentioned SMs varies among *B. cinerea* wild strains. ABA and BIK are only formed by few strains [12, 40], and not all wild strains produce OA, BOT, and BOA [41, 42].

Expression of SM Genes during *In Vitro* and *In Planta* Conditions

In the past years, several genome-wide expression studies were designed to investigate the transcriptome of *B. cinerea* either during saprophytic growth on various culture media or during the infection process on different host plants. Figure 11.1 presents the expression patterns of the SM KE-encoding genes of the model strain B05.10 after 2 days of growth in three *in vitro* conditions *versus* two *in planta* conditions. The media used were minimal medium (**M**) [43], complete medium (**C**) [17], and grape juice medium (**J**) [44]. The *in planta* conditions are grape berries (**G**) [43] and bean leaves (**B**) [41] both at 48- h postinfection. Figure 11.1 highlights several features about the regulation of SM in *B. cinerea*:

- About half of the KE-encoding genes (mainly those on the top of the Figure 11.1) are highly expressed in at least one of the culture conditions. Among the tested media, the one made with grape juice stimulates the highest number of KE-encoding genes (16 genes highly expressed). This medium with a high sugar content (about 200 g l^{-1}), a low pH (around 4), and the presence of plant compounds was originally designed to mimic the conditions that the fungus encounters during infection of grape berries [44] and then chosen to compare the expression of SM genes in different regulation mutants (see Section 11.5 and Figure 11.3).
- In opposite, five of the KE-encoding genes (e.g., *bcpks2* or *bcdtc3*) are not expressed in any condition tested. Whether these genes are functional in *B. cinerea* remains to be elucidated.
- Finally, the clustering shows that BOT and BOA KE-encoding genes have similar expression patterns. This coregulation includes also the other *bcbot* and *bcboa* genes presented in Figure 11.2. These data indicate that the biosynthesis of both toxins is activated during plant tissue colonization and in grape juice medium but also in synthetic minimal medium. The moderate expression under *in vitro* conditions has allowed for the identification of the underlying biosynthetic pathways [45].

Overall, the transcriptomic data indicate that, on one hand, the SM genes belonging to one cluster are tightly coregulated [13, 14, 20] and, on the other hand, almost each cluster has its own expression pattern when we consider the different growth conditions. It is known from other fungi that the regulation of SM gene clusters occurs at several levels, that is, by pathway-specific transcription factors (TFs) that are usually encoded by a cluster gene, by broad domain TFs that integrate environmental signals such as the availability of carbon and nitrogen sources and the ambient pH, and by other global regulators that could affect gene expression via chromatin remodeling [46–48]. Recent advances regarding *B. cinerea* are presented in the following sections.

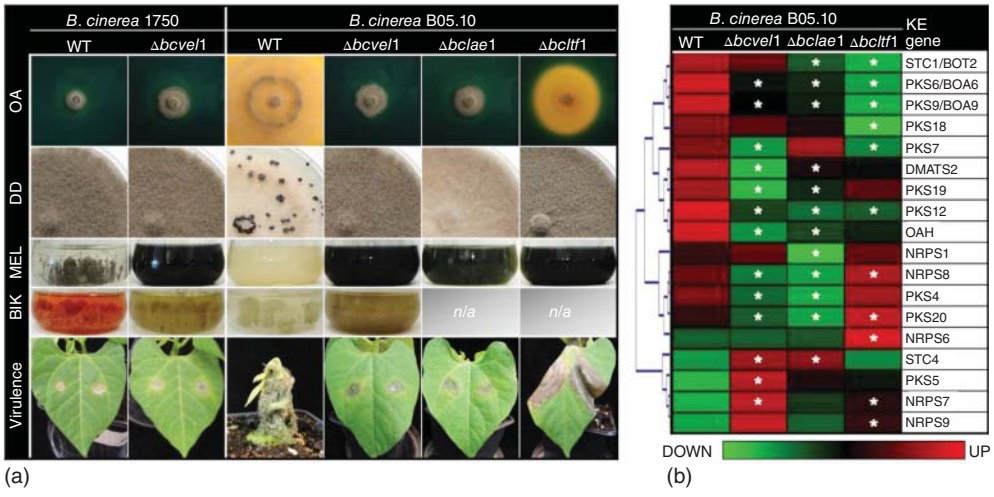

Figure 11.3 Members of the VELVET complex (BcVEL1, BcLAE1) and the light-responsive transcription factor BcLTF1 control light-dependent differentiation, secondary metabolism, and virulence. (a) Phenotypes of the deletion mutants in wild strain 1750 (BIK producer) and the standard recipient strain B05.10. OA – detection of oxalic formation by acidification of the culture medium 6 dpi (bluish green: pH >7, yellow pH <6). DD – differentiation phenotype 12 dpi in constant darkness; the WT:B05.10 produces sclerotia while the other strains produce conidia. MEL – accumulation of DHN melanin in liquid cultures 7 dpi (minimal medium with $NaNO_3$ as nitrogen source). BIK – accumulation of bikaverin in liquid cultures 7 dpi (minimal medium with NH_4NO_3 as nitrogen source). Virulence – lesion formation on primary leaves of *Phaseolus vulgaris* (French bean) 6 dpi. (b) Comparative gene expression studies of the mutants. Conidial suspensions of the wild type B05.10 and the three deletion mutants were incubated for 48 h on solid grape juice medium with cellophane overlays. Material from four biological replicates were used for hybridization of NimbleGen arrays. Statistical analyses revealed 18 KE-encoding genes that are differentially expressed in at least one mutant (*: fold change >2; p <0.05). Relative expression values of these genes (i.e., log2-normalized intensities scaled by gene) were clustered and depicted by color scale, in which shades of green and red represent under- and overexpressed genes, respectively. GEO accession GSE63021 and (A. Simon *et al.*, unpublished).

Pathway-Specific Regulation by Transcription Factors

Many fungal SM gene clusters contain a gene that encodes a Zn(II)2Cys6 zinc finger TF that specifically regulates the adjacent biosynthetic genes [48]. In *B. cinerea*, 40% of the SM clusters, including the BOA one, contain a gene encoding such a candidate TF [5, 14]. The function of the candidate *bcboa13* (Figure 11.2) was recently investigated by gene deletion and expression analysis. Results indicated that BcBOA13 regulates genes that are part of the BOA cluster but not other SM genes such as those of the BOT cluster, suggesting that BcBOA13 is indeed a BOA-specific TF (A. Porquier *et al.*, unpublished). Another pathway-specific TF identified in *B. cinerea* is the Zn(II)2Cys6 protein BcRUM1 (regulator of unknown metabolite).

Bcrum1 inactivation demonstrated that this TF is required for the co-expression of the four adjacent genes that are all possibly involved in the biosynthesis of an unknown metabolite during the early stages of the infection process [49].

As opposed to the BOA cluster, the predicted BOT cluster lacks a TF-encoding gene (Figure 11.2) [13]. Therefore, a yeast one-hybrid (YOH) approach was developed to identify regulators of the BOT genes [44]. The bidirectional promoter of *bcbot1/bcbot2* was used as bait for screening a library containing 396 out of 406 *B. cinerea* TFs, revealing a physical interaction of the BOT promoter with a Cys2His2 TF (BcYOH1). A global transcriptomic analysis of the Δ*bcyoh1* mutant further revealed that this TF is not specific to the BOT cluster but is rather involved in the regulation of a wide range of genes. The expression of 22 SM clusters including the BOT and BOA ones appeared BcYOH1 dependent. Moreover, the expression of genes involved in other processes such as carbohydrate metabolism, transport, virulence, or detoxification was also affected in the Δ*bcyoh1* mutant [44]. Further experiments are ongoing to identify a possible BOT-specific TF.

Concerted Regulation of Secondary Metabolism and Light-Dependent Development

A prominent global regulator of fungal SM is the putative methyltransferase LaeA (loss of aflR expression) that was first identified in *Aspergillus nidulans* as a regulator of SM gene clusters [50, 51]. Later, LaeA was shown to form a protein (VELVET) complex with the fungal regulatory proteins VeA and VelB that coordinates development and secondary metabolism in response to light in *A. nidulans* and other filamentous ascomycetes [52–54]. Light is also an important environmental cue for *B. cinerea* as it triggers the formation of macroconidia and represses the formation of sclerotia. Like *A. nidulans*, *B. cinerea* contains a VELVET complex composed of the orthologs BcVEL1 (VeA), BcVEL2 (VelB), and BcLAE1 (LaeA) whereby BcVEL1 functions as the bridging partner by interacting with both BcVEL2 and BcLAE1 [55]. General features of Δ*bcvel1* and Δ*bclae1* mutants are the "always conidia" phenotype (conidiation in light and darkness accompanied by the loss of sclerotia formation), increased production of conidial/BcPKS13-derived melanin, loss of OA formation, deregulation of several SM-related genes, and reduced virulence on bean and tomato (Figure 11.3) [41, 55]. The deletion of the VelB ortholog BcVEL2 results in a similar phenotype to that of Δ*bcvel1* regarding differentiation and virulence [56], which is in accordance with the interaction found between these two proteins. The outstanding role of BcVEL1 as the central part of the VELVET complex that links development and virulence is furthermore supported by the finding that mutations of this gene (single nucleotide polymorphisms causing stop codons) were identified in two wild strains (T4, 1750) showing the "always conidia" phenotype and reduced aggressiveness on the host [40, 41]. The latter strain (1750) belongs to the group of the BIK producers; and by targeted deletion of the whole *bcvel1* in this genetic background it was shown that the formation of the red pigment depends on the VELVET complex (Figure 11.3) [40].

Recently, the light-responsive TF1 (BcLTF1) was identified by a random mutagenesis approach. As deletion mutants of the VELVET complex, the Δ*bcltf1* mutant exhibits hyper-conidiation accompanied by increased production of conidial/BcPKS13-derived melanin and loss of sclerotia formation (Figure 11.3). However, in contrast to the other mutants, Δ*bcltf1* mutants massively suffer from oxidative stress and are therefore severely impaired in growth in the light. Microarray analyses were performed to compare the transcriptional responses to 60 min of light treatment between undifferentiated mycelia of the wild type and the mutant demonstrating that BcLTF1 is required for proper regulation of the majority of light-responsive genes and additionally for the regulation of SM-related genes in a light-independent manner [17]. However, the question remains open whether BcLTF1 directly affects SM by its function as a TF or whether the effect is indirect and possibly due to changes in primary metabolism and/or the cellular redox status.

For the direct comparison of the impacts of BcVEL1, BcLAE1, and BcLTF1 on gene expression, a microarray approach was performed using grape juice medium and the three deletion mutants (Figure 11.3). Even under this condition, the deletion of *bcltf1* affected the expression profile of 11 KE-encoding genes; six genes are underexpressed and five genes are overexpressed compared to the wild type. *Bcpks13* is also overexpressed in the mutant, but to a lesser extent than on complete medium [17]. This is because *bcpks13* is already strongly expressed in the wild type on grape juice medium compared to the complete medium (Figure 11.1). The deletions of both VELVET complex members affect the expression levels of several KE-encoding genes; the profiles were similar but not identical. Thus, *bcpks7* is underexpressed in Δ*bcvel1* and Δ*bcltf1* but not in Δ*bclae1*, and *bcnrps1* is underexpressed in Δ*bclae1* only [55] (J. Schumacher *et al*., unpublished).

BcWCL1 ("white collar"-like 1) is an example for a broad-domain TF that allows for the integration of light signals due to a blue light-sensing LOV (light-oxygen-voltage) domain. Deletion mutants exhibit hyper-conidiation as well but are not affected in OA formation and virulence in standard illumination conditions. Moreover, the mutants are partially "blind": expression of certain light-responsive genes is no longer induced by light [57]. Recent genome-wide expression analyses revealed that a couple of KE-encoding genes are differentially expressed in the mutant, for example, *bcpks13* (conidial melanin) is overexpressed while *bcphs1* (CARs and retinal) is not induced in response to light treatment in the mutant (J. Schumacher *et al*., unpublished).

The strong interdependence of SM and light-dependent development in *B. cinerea* is furthermore supported by identification of other mutants that are affected in both processes. For instance, the deletion of the bZIP-TF BcATF1 results in increased accumulation of aerial hyphae, reduced conidiation, loss of sclerotia formation, and increased production of BOA and BOT, which is accompanied by slightly increased colonization efficiencies on different host plants. Though BOA- and BOT-encoding genes are underexpressed in the mutant, no physical interactions between their promoters and BcATF1 could be established by yeast one-hybrid experiments, which suggests an indirect effect on SM-related gene expression [58].

Regulation of Secondary Metabolism by Conserved Signal Transduction Pathways

Another level of regulation of SM is mediated by conserved signal transduction pathways that comprise cAMP (cyclic adenosine monophosphate) and Ca^{2+} as second messengers and further include Ras superfamily proteins and mitogen-activated protein (MAP) kinases. These pathways may regulate broad domain and pathway-specific TFs. The key components of the pathways including the three MAP kinases BMP1 (FUS3/KSS1 ortholog), BMP3 (SLT2 ortholog) and BcSAK1 (HOG (high osmolarity glycerol) ortholog), the adenylate cyclase BAC and the protein kinase A (PKA) that are part of the cAMP cascade, and the calcineurin phosphatase (CN) as one of the main actors of the Ca^{2+} signaling pathway. Heterotrimeric G proteins that act as upstream elements have also been functionally studied in *B. cinerea* with regard to development, responses to different kinds of stresses as well as virulence [59, 60]. However, not much is known how these signaling pathways influence SM. The deletions of the three MAP kinases affect the production of melanin, which is accompanied by defects in proper differentiation of conidia and/or sclerotia [61]. A recent study revealed impact of the stress-activated MAP kinase BcSAK1 on the formation of the known phytotoxins: BOT, BOA, and its derivatives are produced in much lower quantities in axenic cultures of Δ*bcsak1* mutants compared to the wild type [62]. The same effect was found in deletion mutants of the G*α* subunit BCG1. As mutants of the cAMP pathway, that is, Δ*bac* and Δ*bcpka1* mutants are not impaired in the formation of toxins [63], a second downstream pathway of BCG1 has been hypothesized. This turned out to be the Ca^{2+}/CN pathway that is linked with the heterotrimeric G protein *via* a phospholipase C (BcPLC1) [64]. The inhibition of CN by cyclosporine A and the deletion of components of the latter pathway such as the CN regulator BcRCN1 and the CN-responsive TF BcCRZ1 resulted in decreased expression levels of BOT- and BOA-biosynthetic genes [27, 66]. More recently, the contribution of Ca^{2+}-dependent signal transduction to the regulation of the carotenogenic genes has been revealed; their expression levels are induced by Ca^{2+} addition, suggesting a link between Ca^{2+}- and light-/BcWCL1-dependent signaling (J. Schumacher *et al*., unpublished).

Role of the Chromatin Landscape in the Regulation of Secondary Metabolism?

Active TFs are not always sufficient to express SM genes as clusters can be embedded within transcriptionally silent heterochromatin. Chromatin-based regulation through histone acetylation and methylation has recently been shown to play a significant role in the regulation of SM clusters in *A. nidulans* and other model fungi [47, 48, 67]. In *B. cinerea*, the occurrence of such mechanisms has not yet been shown, but several relevant clues are already available.

Firstly, some of the clusters are in particular genomic environments. The whole genome sequence of *B. cinerea* was recently assembled into 16 core chromosomes

(J. van Kan *et al*., unpublished) and this revealed that six SM gene clusters including the BOA one are located in subtelomeric regions. *bcboa1* is even the closest gene to one of the telomeres of chromosome 1. Studies in other fungi have shown that these regions are more often subjected to chromatin-mediated regulation [67]. In addition, both BOT and BOA clusters are surrounded by several kb of AT-rich regions (>80%; Figure 11.2) [14] (J. van Kan *et al*., unpublished). In the case of the BOA cluster, a 16 kb-stretch of AT-rich sequence is even present inside the cluster between *bcboa6* and *bcboa7*. Again, such AT-rich regions may correspond to heterochromatin structure and may be under the control of histone modifiers as demonstrated for effector genes that are specifically expressed during infection in *Leptosphaeria maculans* [68]. The fact that the clusters encoding the two main phytotoxins of *B. cinerea* share similar AT-rich surrounding regions raises questions about the importance of the role of these genomic features in chromatin structure and gene expression.

Secondly, the characterization of several regulators in *B. cinerea* revealed a possible involvement of chromatin-mediated regulation. The mode of action of the global regulator LaeA mentioned above remains enigmatic, but they are some evidences *in A. nidulans* that it counteracts some silencing heterochromatic marks [69].

Another example is the transcriptional regulator BcREG1 that is required for conidiogenesis, production of BOA and BOT, and colonization of the host tissues [70]. The mode of action of BcREG1 remains obscure, but a recent study on the ortholog SGE1 in *Fusarium fujikuroi* suggests that these regulators may act on the chromatin level [71].

Finally, the mutation of *bcspt3*, encoding a component of the multisubunit SAGA (Spt-Ada-Gcn5-acetyltransferase) complex, resulted in an altered brownish/yellowish pigmentation suggesting that the disruption of the SAGA complex may affect the repertoire of expressed SM genes in *B. cinerea* [72]. The SAGA complex is required to activate transcription of a subset of RNA polymerase II-dependent genes by modifying the histones, especially in response to environmental stresses [73, 74].

Genetic studies on histone modifiers are currently in progress to investigate the role of chromatin modifications (methylation and acetylation) in the regulation of secondary metabolism in *B. cinerea* (J. Schumacher *et al*., unpublished; A. Porquier *et al*, unpublished).

Concluding Remarks

Although many actors of the regulation of the secondary metabolism in *B. cinerea* have been identified in the past years, important studies remain to be conducted to understand the complex regulatory network from the upstream environmental signals to the downstream regulators that directly interact with SM biosynthetic gene clusters to allow the production of bioactive compounds. This knowledge is crucial to understand the development and virulence of *B. cinerea* and may provide useful start points for the identification of new biological targets to control

this crop-devastating fungus. This knowledge could also provide approaches to "wake up" silent SM clusters during *in vitro* growth and discover new bioactive compounds. Possible approaches include genetic engineering (e.g., overexpression of pathway-specific TFs, modification of the chromatin landscape) and co-culturing with other microorganisms [47, 74]. In addition to their activities toward plants, new SMs isolated from *B. cinerea* could have interesting toxic activities toward other organisms (fungi, oomycetes, insects, bacteria, nematodes, etc.) that can be exploited in biotechnology. Indeed, as more fungal genome sequences are becoming available and more SM biosynthetic genes are being identified, fungi re-emerge as important resources for new therapeutic and agrochemical agents [75, 76].

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