Concepts in Bacterial Virulence

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Concepts in Bacterial Virulence

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Concepts in Bacterial Virulence

Volume Editors

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Contents

Foreword

With the current volume of the Karger book series *Contributions to Microbiology*, we attempt to summarize some of the most important virulence mechanisms in bacterial infectious diseases. In many cases the disease pathway begins with the invasion of the host and ends with the outbreak of physiological responses that may lead to severe complications and ultimately death. Over the years it has been shown that the interplay between pathogenic bacteria and the host is complex and finely balanced. The ability of successful pathogens to survive in an immunologically hostile environment is provided by a large armamentarium of virulence mechanisms, which includes bacterial factors that evade, neutralize or counter the host defense systems, but also manipulate host homeostasis and normal cell functions. In order to give a comprehensive update, we were able to recruit some of the most eminent scientists in infectious diseases to give an overview of the most important recent findings in their fields. We hope that this volume provides a thought-provoking update on these important medical issues.

Lund, May 2004

Wayne Russell
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Fundamentals of Endotoxin Structure and Function

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In 1892, Richard Pfeiffer first defined endotoxin as a heat-stable toxic substance that was released upon disruption of microbial envelopes [1]. The toxicity is now known to be a consequence of the host inflammatory response, which appears to be optimally adapted for the clearance of most local infections. However, when severe infections become distributed systemically, the inflammatory response can lead to septic shock and death. Most of the early efforts to determine the signal transduction events that occur between the presentation of endotoxin to the myeloid cells of the immune system and the production of inflammatory cytokines have utilized lipopolysaccharide (LPS) from gram-negative bacteria [2]. The bioactive lipid A component of LPS is arguably the most potent of the substances that fit Pfeiffer's endotoxin definition, and lipid A has become synonymous with endotoxin. However, many other inflammatory mediators derived from bacteria can also be regarded as endotoxins, including peptidoglycan, the diacylglycerylcysteine moiety of bacterial lipoproteins, and bacterial nucleic acid signatures, to name only a few. The recent discovery that Toll-like receptor 4 (TLR4) is the lipid A inflammatory signal transducer has been followed by the identification of signal transducers for different inflammatory mediators [3, 4]. Coincident with these developments in endotoxin signaling has been the revelation that pathogenic gram-negative bacteria can modulate the structure of lipid A in order to evade detection by the host immune system. This article summarizes the recently elucidated pathways for the biosynthesis of lipid A in enteric bacteria, which provide a framework for understanding lipid A structure and function in all gram-negative bacteria. Readers are referred to the recent review of Raetz and Whitfield [5] for a more complete treatment of LPS structure and function that accounts for its diversity in more divergent organisms.

Overview of the Gram-Negative Cell Envelope

The cell envelope of gram-negative bacteria (fig. 1) consists of the inner membrane (IM), the peptidoglycan (murein) and the outer membrane (OM) [5]. The IM is a phospholipid bilayer, much like the plasma membrane of eukaryotic cells, and is permeable to lipophilic compounds. Numerous integral trans-membrane α-helical proteins and peripheral membrane proteins are primarily responsible for transport, cell signaling and metabolic functions [6]. The IM provides a topologically closed environment for the vectorial translocation of ions to generate a transmembrane electrochemical potential or proton-motive force that governs cellular energetics. Proteins synthesized with a cleavable amino-terminal signal peptide can be targeted for export across the IM [7]. The periplasm is the gelatinous material between the OM and the IM. It contains enzymes for nutrient breakdown as well as binding proteins to facilitate the transfer of nutrients across the IM. Additionally, the murein sacculus in the periplasmic space is composed of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) sugars that are cross-linked by short peptide bridges [8]. The highly reticulated murein layer plays a crucial role in maintaining the cell's characteristic shape and in countering the effects of osmotic pressure. The murein is bridged to the OM by the abundant covalently bound murein lipoprotein, while numerous low-abundance non-covalently-bound lipoproteins are anchored to the inner leaflet of the OM and a few are anchored to the outer leaflet of the IM.

The OM is unique to gram-negative bacteria, and its role is to serve as a protective structure. The lipid arrangements of the OM are highly asymmetric. While phospholipids [70–80% phosphatidylethanolamine (PtdEtn), 20–30% phosphatidylglycerol (PtdGro) and cardiolipin] occupy the inner leaflet, LPS molecules pack against one another in a tight architecture in the outer leaflet of the OM [9]. Due to the low fluidity of lipid A hydrocarbon chains and the strong lateral interactions between LPS molecules, the OM bilayer is impermeable to lipophilic compounds and, thus, serves as an important permeability barrier for gram-negative bacteria [10]. To allow uptake of essential nutrients, the OM is studded with trimeric β-barrel proteins, known as porins, which allow diffusion of solutes with a molecular weight below approximately 600 daltons. Additional β-barrel proteins in the OM are adapted for the uptake of particular nutrients that cannot gain access through porins, and a few OM β-barrel proteins function as enzymes [11]. One consequence of porins is that the OM is believed to lack any transmembrane electrochemical potential.

LPS is composed of three parts: the proximal, hydrophobic lipid A region, which anchors LPS to the outer leaflet of the OM, the distal, hydrophilic O-antigen repeats, which extend into the aqueous medium, and the interconnecting core oligosaccharide (fig. 2). The O-antigen and core sugars are not essential
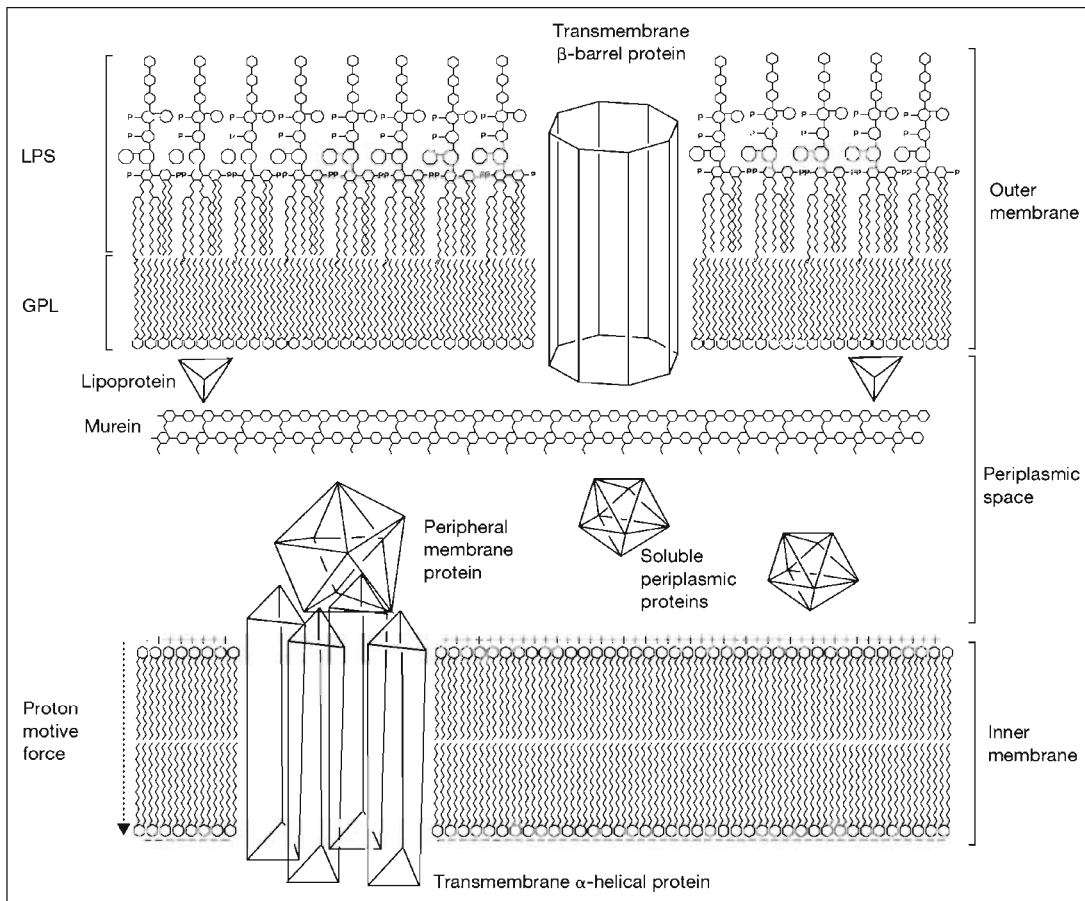
Fig. 1. Molecular organization of the gram-negative cell envelope. The OM is an asymmetric bilayer with an outer leaflet of LPS and an inner leaflet of glycerophospholipids (GPL). The integral OM proteins are exclusively transmembrane β-barrels. Lipoproteins anchored to the OM inner leaflet can link the OM to the murein exoskeleton. The energy-transducing IM is a phospholipid bilayer that supports the proton motive force and contains transmembrane α-helical proteins. The periplasmic space is the region between the IM and OM and contains numerous globular proteins.

for survival, but they provide bacterial resistance against various antimicrobial agents including detergents and the membrane attack complex of serum complement [12]. Wild-type cells that produce O-antigen are termed 'smooth' due to their glossy colony morphology, while those that lack O-antigen are termed 'rough'. The term LPS formally applies only to the molecule that contains the

O-antigen

Outer core

Inner core
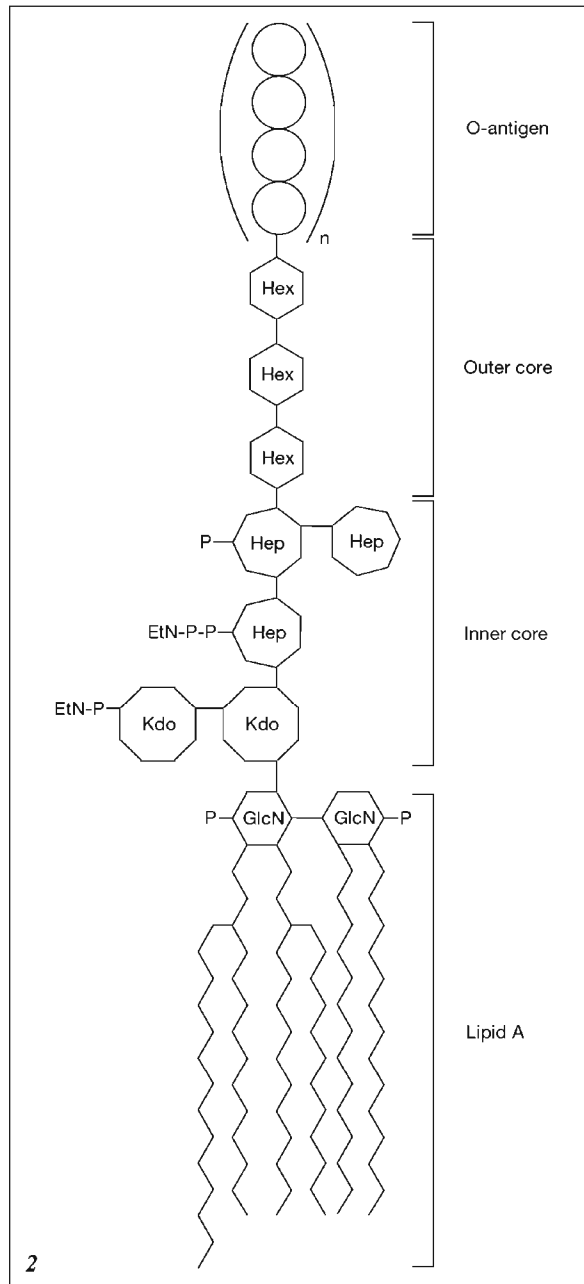
Lipid A

O-antigen polysaccharide, while molecules that lack O-antigen, as in the case of *Neisseria*, are more appropriately termed lipooligosaccharide or LOS. Lipid A is a target for the development of antibiotics and anti-inflammatory agents because it is both essential for survival and a potent inflammatory mediator.

TLR Signaling

When LPS is shed from the bacterial surface during infection, lipid A recognition in mammalian cells is mediated by the TLR4 signal transduction pathway [13, 14]. LPS is first recognized by the circulating acute phase LPS-binding protein (LBP), which then interacts with the glycosylphosphatidylinositol-anchored CD14 on the surface of myeloid cells. Subsequent interaction with TLR4 and its associated factor MD2 initiates a cascade of signaling pathways that, in turn, elicit the production of cationic antimicrobial peptides (CAMPs), a variety of cytokine and chemokine molecules, and the costimulatory molecules that are expressed on the surface of antigen-presenting cells and further signal the presence of an infection to the cells of the adaptive immune system [15]. Upon activation, TLR4 recruits to its intracellular Toll-interleukin receptor homology region (TIR), the adapter protein MyD88, which associates by a homotypic protein-protein interaction with its own TIR domain (fig. 3). Another homotypic protein-protein interaction between the death domains of MyD88 and the interleukin-1 receptor-associated kinase IRAK-1 initiates the autophosphorylation of IRAK-1, which then associates with a signal transduction way station known as tumor necrosis factor-α (TNF-α) receptor-associated factor-6 (TRAF-6). An ubiquitin-conjugating enzyme complex is bound to TRAF-6 along with the TAK-1 kinase complex, which is anchored by the TAB adapter proteins [3]. The pathway impinges on the master regulator of inflammation known as nuclear factor κB (NFκB), which activates transcription of inflammatory response genes. However, NFκB is normally sequestered in the cytoplasm in complex with its inhibitory subunit IκB. Proteolytic degradation of IκB enables NFκB to migrate into the nucleus and activate inflammatory gene

Fig. 2. Structural organization of LPS. The most highly conserved region of the LPS molecule is the lipid A domain, which is an acylated and phosphorylated disaccharide of glucosamine. Assembly of lipid A is contingent upon the addition of the two 8-carbon Kdo sugars, which are the only essential components of the inner core. The inner core normally includes three 7-carbon Hep sugars and can be modified by the addition of phosphate and pEtN substituents. Outer core sugars provide the acceptor for O-antigen ligation, but tend to be composed of hexose sugars that differ between species. The O-antigens represent the most highly species-variable component of the LPS molecule.
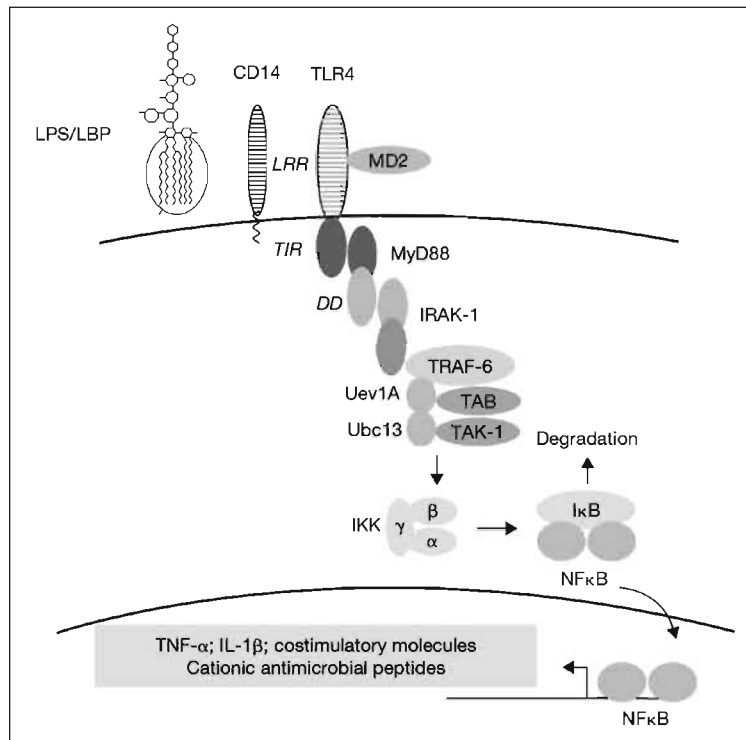
Fig. 3. TLR4 signal transduction pathway. LPS released from the surface of gram-negative bacteria is bound to the circulating LPS-binding protein (LBP) and delivered to the glycosylphosphatidylinositol-anchored CD14 on the surface of myeloid cells. The leucine-rich repeats (*LRR*) of CD14 are also shared with the extracellular domain of TLR4, which, in association with MD2, can transduce a signal to its intracellular *TIR*. *TIR-TIR* interactions with the adapter protein MyD88 promote interactions between the death domains *(DD)* of MyD88 and the interleukin-1 receptor-associated kinase IRAK-1. Autophosphorylation of IRAK-1 promotes an association with the TNF-α receptor-associated factor TRAF-6, which anchors both the kinase TAK-1, by its TAB adapter proteins, and the dimeric ubiquitin-conjugating enzyme complex composed of Uev1A and Ubc13. Subsequent phosphorylation events activate the trimeric IκB kinase complex IKK, which phosphorylates the NFκB inhibitory subunit IκB and targets it for proteolytic degradation. The master regulator of inflammatory response gene expression NFκB is then released and migrates into the nucleus where inflammatory response genes are transcriptionally activated.

expression. IκB is targeted for proteolysis upon phosphorylation catalyzed by the IκB kinase complex (IKK), which is itself phosphorylated by the TAK-1 kinase in a manner that depends on the ubiquitin-conjugating enzyme complex in association with TRAF-6. TAK-1 also phosphorylates mitogen-activated

protein kinases that impinge on the AP-1 transcription family members Jun and Fos, leading to further immune activation.

The response to LPS includes local inflammation, which is highly beneficial in providing antibacterial defenses. If infection persists, however, the subsequent systemic responses, including the overwhelming production of TNF-α and interleukin-1β by the host immune system, can lead to septic shock [16]. Efforts to understand the lipid A signal transduction pathway were largely motivated by a desire to develop endotoxin antagonists for the treatment of septic shock. The discovery that some bacteria can evade host immune defenses by modifying the structure of lipid A suggested that naturally occurring lipid A structures may function as potent endotoxin antagonists. The microbial pathways for the biosynthesis of lipid A and its derivatives have been elucidated recently and provide powerful tools for the investigation of endotoxin signaling, in addition to illustrating the pathogenic mechanisms utilized by gram-negative bacteria.

Re Endotoxin Biosynthesis

The recent completion of the Raetz pathway for lipid A biosynthesis [5] hinged on the serendipitous discovery of lipids X and Y in a conditional PtdGro-deficient *pgsA* mutant of *Escherichia coli* in 1979 [17]. Lipid X was subsequently shown to be a diacylglucosamine 1-phosphate bearing R-3-hydroxymyristoyl (3-OH-14:0) groups at positions 2 and 3, while lipid Y only differed from lipid X by the presence of a palmitoyl (16:0) group in acyloxyacyl linkage at position 2 [18, 19]. Around the same time, the determination of the correct chemical structure of lipid A [20] revealed possible biosynthetic routes for the production and utilization of lipids X and Y. The accumulation of these glucosamine-based phospholipids in the PtdGro-deficient mutant proved to be a consequence of a second unlinked conditional mutation in the gene *pgsB(lpxB)* [21, 22], which was later shown to encode the lipid A disaccharide synthase. LpxB generates the β-1′,6-glycosidic bond that is a characteristic feature of lipid A [23]. Both lipids X and Y could activate macrophages in a similar manner as lipid A [24], but only lipid X proved to be a substrate for LpxB [23], raising doubts about the physiological significance of lipid Y. Lipid A biosynthesis is now known to occur in four separate cellular compartments, namely, the cytoplasm, the cytoplasmic face of the IM, the periplasmic face of the IM, and in the OM (where the origin of lipid Y was recently found). Conceptually, it is helpful to recognize that lipid A and the core oligosaccharide are assembled together as a single unit starting in the cytoplasm and moving to the cytoplasmic face of the IM, but the subsequent lipid A modifications and en bloc ligation of O-antigen occur in the extracellular compartments.
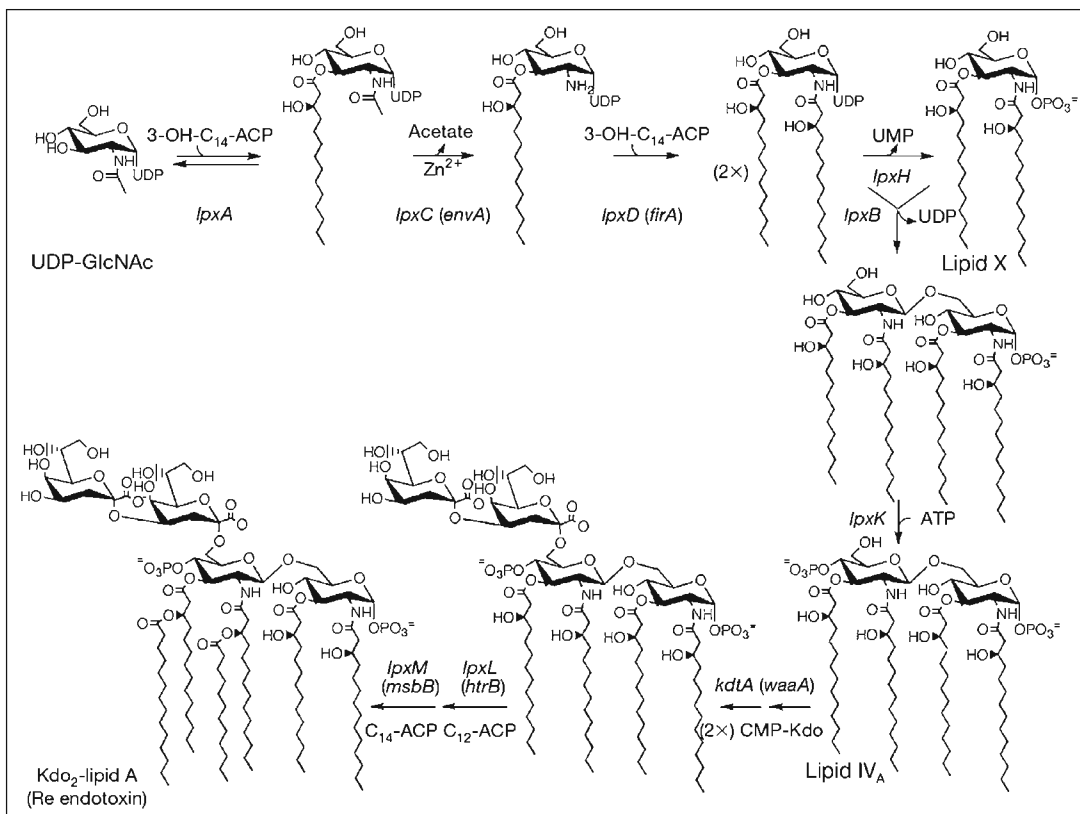
Fig. 4. The Raetz pathway for synthesis of Kdo$_2$-lipid A. LpxA catalyzes the addition of 3-OH-14:0 to position 3 of UDP-GlcNAc. LpxC then removes the acetamido group at position 2, which allows LpxD to add a second 3-OH-14:0 group. LpxH cleaves the nucleotide to generate lipid X, which is condensed with UDP-diacyl-GlcN to generate the disaccharide 1-phosphate. The 4'-kinase LpxK then generates lipid IV$_A$, which is converted into Kdo$_2$-lipid IV$_A$ by a bifunctional Kdo transferase KdtA. Kdo$_2$-lipid IV$_A$ is a substrate for the LpxL and LpxM acyltransferases, which generate the acyloxyacyl linkages at positions 2' and 3', respectively.

The molecular genetics and enzymology of the conserved steps of lipid A biosynthesis are best characterized in *E. coli*, as shown in figure 4. The Raetz pathway begins with the key precursor molecule UDP-GlcNAc, which is also the first substrate for peptidoglycan biosynthesis. The first enzyme in lipid A biosynthesis is a cytoplasmic acyltransferase LpxA, which selectively transfers thiolester-activated 3-OH-14:0 from acyl carrier protein (ACP) to the 3-OH of UDP-GlcNAc [25]. The crystal structure of LpxA revealed a homotrimeric

molecule that self-associates by a distinctive left-handed parallel β-helix motif [26]. *E. coli* LpxA is extraordinarily selective for 3-OH-14:0-ACP as the acyl donor substrate while the *Pseudomonas aeruginosa* LpxA prefers 3-OH-10:0-ACP. However, the specificity could be modulated by mutating certain key residues lining the active site cleft [27]. For example, the specificity for the G173M mutant of *E. coli* LpxA was shifted to 3-OH-10:0-ACP. In contrast, the specificity of *P. aeruginosa* LpxA could be extended to accommodate 3-OH-14:0-ACP in the corresponding M169G mutant. These findings demonstrated the existence of precise hydrocarbon rulers in LpxAs, which can explain variations in lipid A acylation that are observed between different organisms.

The acylation of UDP-GlcNAc by LpxA is thermodynamically unfavorable [25], and the first committed step in lipid A biosynthesis is the subsequent deacetylation catalyzed by LpxC (EnvA) [28, 29]. LpxC is a Zn^{2+}-dependent enzyme that is an established target for antibiotic development [30, 31]. The recent crystal and NMR structures of *Aquifex* LpxC revealed two slightly different models for the mechanism of catalysis [32, 33], but both include a critical role for Zn^{2+}. Most LpxC inhibitors are hydroxamate compounds that interact with the catalytic Zn^{2+} ion. Current challenges are aimed at the development of inhibitors with the ability to evade efflux pumps that provide resistance, particularly in pseudomonads [34, 35].

Following deacetylation, an N-linked 3-OH-14:0 moiety is incorporated from ACP by LpxD (FirA) to generate UDP-2,3-diacylglucosamine [36]. A highly selective pyrophosphatase LpxH then cleaves UDP-2,3-diacylglucosamine to form lipid X [37, 38]. Next the disaccharide synthase, LpxB, condenses UDP-2,3-diacylglucosamine and lipid X to generate the β-1',6-linkage found in all lipid A molecules [23]. The membrane-bound 4' kinase LpxK then phosphorylates the disaccharide 1-phosphate to produce lipid IV$_A$ [39, 40], which is an important pharmacological agent because it functions as an endotoxin antagonist in human cell lines [41, 42]. Next, two 3-deoxy-*D*-manno-2-octulosonic acid (Kdo) sugars are incorporated by a Kdo transferase, which is encoded by the *kdtA (waaA)* gene, using the labile nucleotide CMP-Kdo as the Kdo donor [43]. The final lipid A biosynthetic steps that occur on the cytoplasmic side of the IM depend on the prior addition of the Kdo sugars and involve the transfer of lauroyl (12:0) and myristoyl (14:0) groups from ACP to the distal glucosamine unit to produce acyloxyacyl linkages; the reactions are catalyzed at the 2'-position by LpxL (HtrB) and at the 3'-position by LpxM (MsbB), respectively [44–46]. Under conditions of cold growth at 12°C, LpxL is replaced by LpxP, which has a preference for palmitoleate ($16:1cis\Delta^9$) in ACP [47, 48]. The incorporation of an unsaturated acyl chain into lipid A likely increases membrane fluidity under cold growth conditions. Viable mutants that lack acyloxyacyl linkages in lipid A are attenuated for virulence and reveal the importance

of the lipid A acylation pattern in inflammation [49, 50]. All other enzymatic steps of the Raetz pathway, and those for the biosynthesis of CMP-Kdo, are essential for cell viability and, thus, provide potential targets for antibiotic development.

Assembly of LPS

Kdo_2-lipid A, also known as Re endotoxin, can be regarded as the simplest chemotype of LPS [5]. Completion of the core-Kdo_2-lipid A molecule involves the subsequent addition of core sugars to the nascent Kdo_2-lipid A anchored on the cytoplasmic side of the IM [51]. The two essential 8-carbon Kdo sugars are regarded as part of the inner core, which is normally extended to include three 7-carbon L-glycero-D-manno-heptose (Hep) sugars (fig. 2). Core oligosaccharide synthesis is contingent upon modification with phosphate at position 4 of the first Hep, which can be followed by the addition of phosphoethanolamine (pEtN) at the same position. Phosphate also normally occurs at position 4 of the second Hep, and pEtN modification at position 7 of the second Kdo can occur under Ca^{2+}-rich growth conditions [52]. The so-called 'deep-rough' mutants have defects in the inner core heptose sugars and are sensitive to detergents and hydrophobic antibiotics [53]. The outer core sugars are predominantly hexoses and exhibit a greater degree of structural diversity than is seen in the inner core and lipid A regions [54]. The outer core sugars provide the acceptor residue for O-antigen ligation.

The O-antigen is synthesized and anchored to a carrier lipid, undecaprenyl phosphate, in the IM. The remarkable diversity in O-antigen structures reflects the multitude of glycosyl transferases that utilize various sugars and create diverse glycosidic linkages, combined with the occasional presence of substoichiometric sugar modifications [5, 55]. However, biosynthesis of all O-antigens is initiated by the formation of a common diphosphate linkage between the first sugar and undecaprenyl phosphate. O-antigen units are then completed in the cytoplasm and transported to the periplasmic face of the IM by one of three distinct pathways termed Wzy-dependent, ATP binding cassette (ABC) transporter-dependent, and synthase-dependent. The most common of these is the Wzy-dependent pathway, which is characteristic of *E. coli* and is followed by polymerization of O-antigen units on the periplasmic face of the IM. Recent studies have implicated an essential ABC transporter MsbA in translocating the core-Kdo_2-lipid A molecule to the periplasmic side of the IM [56–60]. Core-Kdo_2-lipid A and polymerized O-antigens from the various pathways are then linked together by a common ligation mechanism at the periplasmic surface of the IM.

The completed LPS is transported for assembly in the OM by a poorly understood process. Interestingly, certain integral membrane proteins can passively

promote the translocation of phospholipids across the IM [61], but MsbA is required for the transport of both LPS and phospholipids to the OM [56, 57]. It has been known for more than 25 years that phospholipids freely exchange between the IM and OM, while LPS transport appears to be unidirectional [62, 63]. The mechanism by which LPS is translocated to the outer leaflet of the OM is unknown, but it may depend on the highly conserved OM protein OMP85 [64], which is also implicated in the assembly of OM proteins [65].

The OM Permeability Barrier

LPS contains phosphate and acidic sugars and is therefore negatively charged. In order to reduce the electrostatic repulsion between LPS molecules at the cell surface, the bacterial OM sequesters divalent cations, mainly Mg^{2+} [66, 67], which neutralize the negative charges and maintain the integrity of the OM. The presence of hydrogen-bond donors and acceptors in the lipid A molecule allows for additional lateral interactions that cannot occur between phospholipid molecules [67]. Moreover, the six or seven saturated acyl chains of lipid A serve to reduce the fluidity of the OM bilayer compared with the IM. The tight lateral interactions between LPS, combined with low membrane fluidity, provides a permeability barrier in the OM to lipophilic solutes and detergents [10].

Mechanism of Action of CAMPs

The requirement for Mg^{2+} ions to bridge LPS molecules at the cell surface is an Achilles' heel for the OM. Numerous CAMPs are produced in nature, but the main types produced by the immune system are the small α-helical proteolytic digestion products that are released from precursors known as the cathelicidins, and the disulfide-bonded β-sheet peptides known as the defensins. CAMPs can navigate through the OM by a nonporin pathway termed the 'self-promoted uptake pathway' [68]. They are initially unstructured in aqueous medium, and their initial electrostatic interactions with the bacterial surface serves to displace some Mg^{2+} ions. The reduced dielectric constant at the membrane interface induces dehydration of peptide bonds that become hydrogen-bonded in α-helical or β-sheet structures. The induced structure reveals the amphipathic nature of CAMPs, which may promote changes in phase and/or motion in the OM bilayer and, in turn, facilitates their translocation through the hydrocarbon layer. These peptides are then thought to target the IM bilayer and to produce a detergent-like disruption of permeability. Some possible consequences of IM permeation include the fatal depolarization of the transmembrane potential across the IM,

leakage of cytoplasmic contents, cell lysis and cell death. The actions of CAMPs are thought to selectively target bacterial membranes [69]. The outer leaflet of the bacterial IM is negatively charged because it contains anionic phospholipids, whereas eukaryotes tend to sequester anionic lipids internally. Moreover, cholesterol molecules, which are embedded only in the eukaryotic plasma membrane, could stabilize the lipid bilayer and, thus, reduce the activity of CAMPs.

Lipid A Modifications

Considering the importance of Mg^{2+} in maintaining the OM permeability barrier, it is not surprising that Mg^{2+} limitation can regulate the covalent structure of lipid A. Mg^{2+} limitation is also believed to signal the presence of an intracellular environment [70]. For example, in the phagocytic vacuoles of macrophages, the natural resistance-associated macrophage protein 1 (Nramp1) serves to pump divalent cations into the cytosol, thereby withholding Mg^{2+} required for bacterial growth [71]. Figure 5 outlines several covalent modifications of lipid A found under Mg^{2+}-limited growth conditions that have been characterized in *E. coli* and *Salmonella enterica* [72–75]. Three enzymes function to modify the acylation pattern of lipid A. LpxO is a hydroxylase that generates *S*-2-hydroxymyristate (2-OH-14:0) at position 3' [76]. PagP is a transacylase that incorporates a palmitate chain at position 2 [77], while PagL is a deacylase that removes the O-linked 3-OH-14:0 chain at position 3 [78]. Moreover, the phosphate groups at positions 1 and 4' of the lipid A disaccharide backbone can be modified with 4-amino-4-deoxy-*L*-arabinose (*L*-Ara4N) and/or pEtN, which serve to reduce the overall negative charge of lipid A [79, 80].

Roles in Counteracting CAMPs

Lipid A modifications provide a dual protective mechanism against CAMPs. First, substituting the phosphate groups on lipid A with *L*-Ara4N and pEtN could effectively weaken the electrostatic attraction between the negatively charged cell surface and CAMPs. In fact, the resultant neutralization of the negatively charged bacterial surface is associated with resistance to polymyxin B, a lipid A-binding cationic cyclic peptide antibiotic, in *E. coli* and *S. enterica* [81, 82]. Moreover, lipid A acylation may block the hydrophobic interaction between CAMPs and the membrane bilayer. Lipid A palmitoylation by PagP has been shown to provide bacterial resistance against CAMPs [83]. Possibly, the resultant hepta-acylated lipid A could further reduce OM fluidity and, thus, prevent CAMP insertion. The pattern of lipid A acylation is also
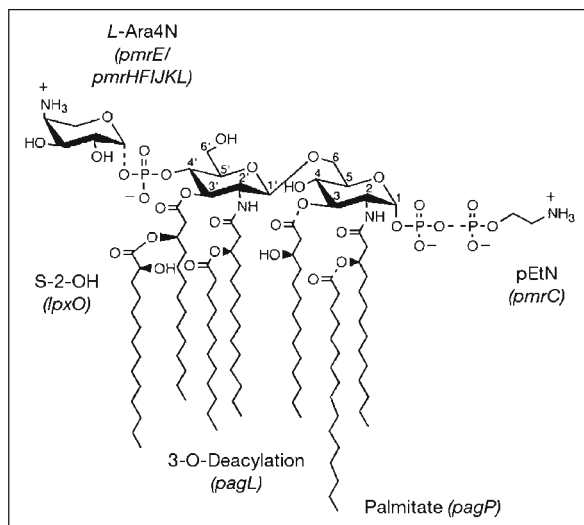
Fig. 5. Regulated covalent lipid A modifications. The conserved lipid A nucleus can be modified by the addition of _L_-Ara4N and pEtN to the phosphate groups, by the _S_-2-hydroxylation of the secondary myristoyl group at position 3′, by the removal of the 3-OH-14:0 group at position 3, and by the addition of a palmitate chain at position 2. Modifications to the acylation of lipid A are under the direct control of PhoP/PhoQ, while the phosphate modifications are controlled indirectly by PhoP/PhoQ through the downstream effectors PmrA/PmrB.

known to be critical in mediating its endotoxic activity through interactions with the TLR4 signal transduction pathway [50, 84]. Hepta-acylated lipid A bearing a palmitate chain can function as an endotoxin antagonist, which blocks the inflammatory effects of the hexa-acylated lipid A in human cell lines [85, 86]. Consequently, modifications to the acylation pattern of lipid A may, remarkably, block both direct interactions between CAMPs and the bacterial cell, and the induction of CAMP synthesis in the eukaryotic host. The enzymes responsible for S-2-hydroxylation and 3-O-deacylation are absent from _E. coli_ and their roles are less clear, but they may serve to stabilize lateral LPS interactions by introducing new hydrogen-bond donors [67].

## The PhoP/PhoQ and PmrA/PmrB Two-Component Regulatory Systems

Gram-negative bacteria use the PhoP/PhoQ two-component signal transduction pathway to respond to Mg^{2+}-limited environments that can be encountered during infection [87]. PhoQ is a membrane-bound sensor kinase that detects

Mg^{2+} and can phosphorylate and activate the transcriptional regulatory protein PhoP [88]. Mutants altered in the PhoP/PhoQ system display greatly reduced virulence. PhoP controls the expression of over 40 different genes, many of which are involved in Mg^{2+} transport and in lipid A modification. For example, transcription of *pagP*, *pagL* and *lpxO*, which are involved in the modification of lipid A acyl chains, are under the direct influence of PhoP/PhoQ [76–78].

The PmrA/PmrB two-component regulatory system is one of the downstream effectors of the PhoP/PhoQ system, and is required for the modification of lipid A with pEtN and L-Ara4N [80]. PmrA is the transcriptional response regulator and PmrB is the membrane-bound sensor kinase. While PmrA can be activated by PhoP/PhoQ via a mediating protein PmrD [89], the PmrA-induced genes can also be activated independently of PhoP/PhoQ by exposure of PmrB to Fe^{3+} or mild acidic conditions [90]. PmrA/PmrB activation has also been shown to repress PmrD expression [91], which thereby creates a negative feedback loop. Interestingly, CAMPs themselves have been reported to activate PhoP/PhoQ in *Salmonella* and PmrA/PmrB in *Pseudomonas* [92, 93].

L-Ara4N Cluster

PmrA/PmrB is only one of several clusters of *pmr* genes that were originally identified in polymyxin-resistant mutants of *E. coli* [94, 80]. The *pmrF (pbgP)* locus encodes an operon of 7 open reading frames *pmrHFIJKLM*, of which the first 6, together with the unlinked *pmrE (ugd)*, are required for *L*-Ara4N synthesis. The proposed biosynthesis and attachment of *L*-Ara4N to lipid A is shown in figure 6. The first step involves the conversion of UDP-glucose into UDP-glucuronic acid catalyzed by a dehydrogenase encoded by *pmrE*. Complex regulation of dehydrogenase gene expression reflects the fact that UDP-glucuronic acid is a precursor for both colanic acid-containing capsules and *L*-Ara4N [95]. Next, PmrI (ArnA) catalyzes the oxidative decarboxylation of UDP-glucuronic acid to generate a novel UDP-4-keto-pyranose intermediate [96]. PmrH (ArnB) then catalyzes a transamination reaction using glutamate as the amine donor to generate UDP-*L*-Ara4N [97]. The crystal structure of PmrH has verified that a pyridoxal phosphate cofactor contributes to the catalytic mechanism [98]. Interestingly, PmrI contains a second domain that formylates the 4-amine of UDP-*L*-Ara4N. The resultant UDP-*L*-Ara4-formyl-N is transferred by PmrF (ArnC) to the membrane-anchored undecaprenyl phosphate, forming undecaprenyl phosphate-*L*-Ara4-formyl-N [97]. The formylation step may drive forward the equilibrium of the transamination step, which is thermodynamically unfavorable. Formylation may also facilitate translocation across the IM by neutralizing positive charge. It is speculated that
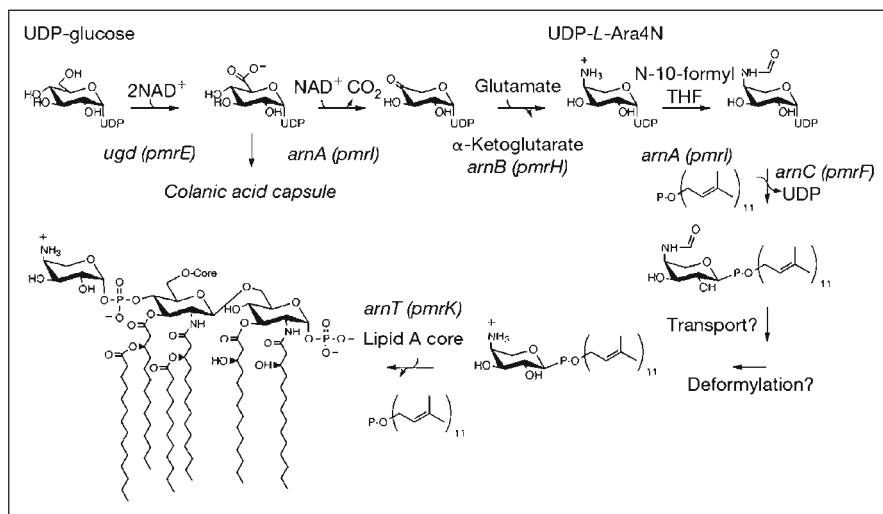
Fig. 6. Pathway for attachment of *L*-Ara4N to lipid A. The Ugd dehydrogenase converts UDP-glucose into UDP-glucuronic acid, which is a precursor for both colanic acid capsular polysaccharides and *L*-Ara4N. The first committed step of *L*-Ara4N biosynthesis is the ArnA-catalyzed oxidative decarboxylation, which generates a novel UDP-4-keto-pyranose intermediate. Transamination catalyzed by ArnB is followed by formylation due to a second catalytic domain in ArnA. Transfer of the formylated monosaccharide to undecaprenyl phosphate by ArnC is presumably followed by translocation to the periplasmic side of the IM for deformylation. Undecaprenyl phosphate *L*-Ara4N is the substrate for ArnT, which transfers *L*-Ara4N to the lipid A acceptor.

a putative transporter may be specific for the formylated compound and that deformylation may then occur at the periplasmic surface [97]. These steps would ensure the vectorial translocation of the lipid across the IM and avoid futile cycling. The necessity of the deformylation step is dictated by the fact that undecaprenyl phosphate-*L*-Ara4N is the substrate for PmrK (ArnT), which catalyzes the final transfer of *L*-Ara4N to lipid A at the periplasmic surface of the IM [99, 100]. Roles for the remaining *pmr* genes in the transport and periplasmic deformylation reactions are suspected, but remain to be established.

EptA

The putative pEtN adding enzyme EptA has recently been cloned from *E. coli* [101], and a homologous gene from *Neisseria* has been associated with the addition of pEtN to lipid A [102]. The EptA-encoding gene is the upstream
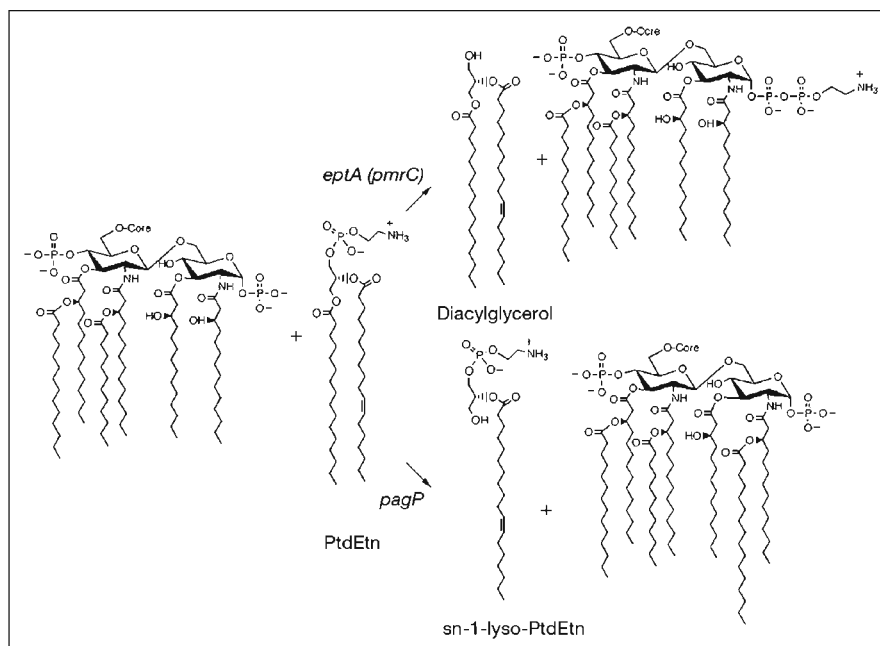
Fig. 7. Modification of lipid A with pEtN and palmitate. EptA at the periplasmic side of the IM uses PtdEtn as the pEtN donor to generate diacylglycerol and pEtN-modified lipid A. PagP also uses PtdEtn (or another glycerophospholipid) as the palmitoyl donor in the OM to generate sn-1-lyso-PtdEtn and lipid A modified by the addition of a palmitoyl group.

open reading frame that is part of the *pmrAB* operon, and is also known as *pmrC (pagB)* [103, 104]. PtdEtn is the reported pEtN donor (fig. 7) and several EptA homologues are likely responsible for pEtN addition to other cell envelope components including the inner core sugars of LPS. It is noteworthy that roughly one third of *E. coli* lipid A carries a diphosphate moiety instead of the monophosphate at position 1 [56], and that the putative phosphorylating enzyme shares with EptA the ability to generate a phosphodiester bond at the same position in lipid A.

PagP

PagP is encoded by a PhoP/PhoQ-activated gene and functions to transfer a palmitate chain from a phospholipid to the hydroxyl group of the N-linked 3-OH-14:0 chain on the proximal glucosamine unit of lipid A [77, 83]. PagP was the first enzyme of lipid A biosynthesis shown to be localized in the OM

[77]. Since thiolester-containing substrates are not available in the extracellular compartments, PagP uses a phospholipid as the palmitoyl donor instead (fig. 7). PagP appears to be responsible for the production of lipid Y as a side reaction in *lpxB* mutants. It was first identified in the salmonellae due to its role in providing resistance to CAMPs [83], and was subsequently purified from *E. coli* [77]. In addition to these enteric pathogens, PagP homologues are present in the respiratory pathogens *Legionella pneumophila* and *Bordetella bronchiseptica*, where PagP has been shown to be necessary for disease causation in animal models of infection [105, 106]. In *B. bronchiseptica*, PagP is controlled by a different two-component virulence signal transduction pathway known as BvgA/BvgS, and palmitoylation occurs at the O-linked 3-OH-14:0 chain on the distal glucosamine sugar [106]. PagP homologues are also found in *Yersinia*, *Photorhabdus* and *Erwinia* species, which adopt pathogenic lifestyles in animals, insects, and plants, respectively. Current efforts to understand the structure and function of PagP are aimed at developing a treatment for infections caused by this important group of pathogens. The structure and dynamics of PagP in detergent micelles have been determined by both NMR spectroscopy [107] and X-ray crystallography [Bishop and Privé, unpubl. data].

PagP is an 8-stranded antiparallel β-barrel preceded by an N-terminal amphipathic α-helix. The β-barrel is well defined in the structure while the extracellular loops are not. Unlike other β-barrel membrane proteins, proline residues at two sites between β-strands disrupt the continuity of hydrogen bonding in the outer leaflet half of the PagP β-barrel. These non-hydrogen-bonded regions are located between strands β-1 and β-2, generating a β-bulge, and between strands β-6 and β-7. The β-bulge is largely responsible for the highly dynamic nature of the extracellular loop L1 [107]. Additional features not seen in any other β-barrel membrane protein include a tilting of the PagP barrel axis by 30° with respect to the membrane normal and the presence of an interior hydrophobic pocket in the upper half of the β-barrel [Bishop and Privé, unpubl. data]. The hydrophobic pocket harbors a single detergent molecule and functions as a hydrocarbon ruler that allows the enzyme to distinguish palmitate from other acyl chains present in phospholipids. Mutation of Gly 88 lining the bottom of the hydrophobic pocket can modulate the acyl chain length specificity of PagP [Bishop and Privé, unpubl. data]. Internalization of phospholipid palmitoyl groups within the barrel interior likely occurs by lateral diffusion through the non-hydrogen-bonded regions between the β-strands in the upper half of the molecule.

Three putative catalytic residues were identified by site-directed mutagenesis and mapped to the extracellular loops L1 and L2, indicating that the active site is localized at the cell surface in the most dynamic region of the molecule [107]. The putative catalytic residues project their side chains toward the barrel

interior and are positioned above the hydrocarbon ruler [Bishop and Privé, unpubl. data]. The requirement of invariant His 33, Asp 76, and Ser 77 for catalysis might suggest that PagP utilizes an acyl-enzyme mechanism characteristic of known serine esterases. However, the putative active site residues are not organized into a catalytic triad that could enhance the nucleophilic character of Ser 77 [107]. The presence of two non-hydrogen-bonded regions that could provide simultaneous access for both substrates to the β-barrel interior raises the distinct possibility that PagP catalysis proceeds through the formation of a ternary complex. Such a mechanism could promote the direct transfer of the palmitoyl group from the phospholipid donor to the lipid A acceptor without the formation of an acyl-enzyme intermediate, but the detailed mechanism of PagP catalysis remains to be elucidated.

The clear alignment of the PagP active site with the OM outer leaflet creates an important topological problem for the enzyme. How does PagP access phospholipids if OM lipid asymmetry is maintained? Chelating agents such as EDTA can strip a fraction of LPS from the bacterial surface [108]. A large body of evidence indicates that EDTA promotes the migration of phospholipids into the OM outer leaflet [10]. Indeed, brief treatment of cells with EDTA rapidly induces lipid A palmitoylation through a process that is independent of both *pagP* gene regulation and de novo protein synthesis [Bishop, unpubl. data]. Lipid A palmitoylation induced by EDTA in vivo also requires functional MsbA [Bishop, unpubl. data], which is presumably needed to replenish phospholipids lost from the OM inner leaflet. These findings suggest that PagP may function to maintain the OM permeability barrier under Mg^{2+}-limited growth conditions, in addition to providing CAMP resistance and converting lipid A into an endotoxin antagonist.

LpxO

An Fe^{2+}/α-ketoglutarate-dependent dioxygenase homologue in *Salmonella* has recently been shown to catalyze the hydroxylation of lipid A and is expressed in a PhoP/PhoQ-dependent manner [76]. Under aerobic conditions, LpxO uses molecular oxygen to hydroxylate the 3' secondary acyl chain to generate 2-OH-14:0-modified lipid A (fig. 8). Homologues are found in other gram-negative bacteria that similarly incorporate S-2-OH groups into their lipid A. The function of S-2-hydroxylation is unknown, but the authors speculate that the action of leukocyte acyloxyacyl hydrolase, an enzyme that releases secondary acyl chains from the lipid A of phagocytosed bacteria, would release 2-OH-14:0, which is possibly converted into 2-OH-14:0-CoA, a known inhibitor of protein N-myristoylation needed for cell signaling functions.
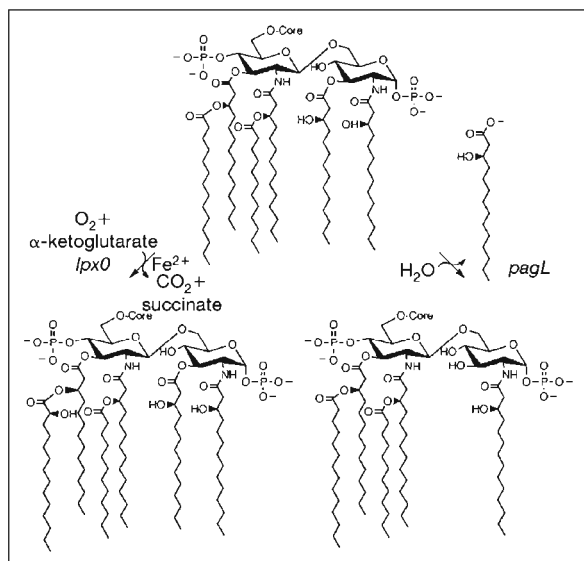
Fig. 8. S-2-hydroxylation and 3-O-deacylation of lipid A. LpxO is an IM Fe^{2+}/α-ketoglutarate-dependent dioxygenase homologue that uses molecular oxygen to incorporate a hydroxyl group into the secondary myristoyl group at position 3'. PagL is an OM lipase that removes the 3-OH-14:0 group at position 3.

S-2-hydroxylation may also function to provide an additional hydrogen-bond donor that could stabilize the lateral interactions between LPS molecules in the OM [67]. Given that S-2-hydroxylation is contingent upon lipid A acylation by LpxM, the LpxO reaction could occur on either side of the IM without interfering with the sequential steps of the Raetz pathway. However, LpxO is predicted to be anchored on the periplasmic face of the IM.

PagL and *Rhizobium* Lipid A

Lipid A 3-O-deacylase activity was observed in *Salmonella* during investigations of PagP in membranes from a PhoP-constitutive mutant [77]. The responsible enzyme was subsequently identified as the PagL gene product, which proved to be the second enzyme of lipid A metabolism that is located in the OM [78]. PagL functions to deacylate the O-linked 3-OH-14:0 chain at the proximal glucosamine unit of lipid A (fig. 8). By exposing the 3-OH group in lipid A, PagL may provide a new hydrogen-bond donor to stabilize the lateral interactions between LPS molecules in the OM [67]. Although a similar

reaction had been described in *Rhizobium leguminosarum* membranes [109], PagL homologues are only found in the various serovars of *Salmonella*.

Lipid A recovered from *Rhizobium* species is structurally quite different from *E. coli* lipid A, a fact that may reflect the symbiotic relationship between nitrogen-fixing rhizobia and leguminous plants, which normally mount an innate immune response to endotoxin. *Rhizobium* lipid A biosynthesis proceeds according to the Raetz pathway, but the molecule is subsequently remodeled by numerous modifying enzymes. Besides the absence of phosphate groups at positions 1 and 4' [110], due to the presence of specific phosphatases [111, 112], the distal glucosamine sugar exhibits a 27-OH-28:0 acyl chain as part of a characteristic acyloxyacyl moiety at position 2' and a galacturonic acid residue at position 4' [113, 114]. LpxQ is the third OM enzyme found to be involved in lipid A modification [115, 116], and catalyzes the oxidation of the proximal 1-dephospho sugar to generate an acylated 2-aminogluconate moiety.

Rhizobium lipid A serves to illustrate a fundamental point that is supported by functional genomics; namely, that the essential enzymes of the Raetz pathway are highly conserved in gram-negative bacteria and that the observed variations in lipid A structure are a consequence of the presence of additional modifying enzymes. Aside from variations in lipid A structure due to cytoplasmic ACP-dependent acyltransferases [117–119] and Kdo transferases [120, 121] with distinct substrate specificities, it appears that most modifying enzymes act on the lipid A nucleus in the extracytoplasmic compartments. These observations may reflect a need to avoid futile cycling and to maintain a sequential order of Raetz pathway reactions. These principles should faithfully guide future discoveries of new enzymes that are employed to generate novel lipid A structures in diverse organisms.

Perspectives

LPS structure and function are unique to gram-negative bacteria, but some intriguing parallels are seen with the cholesterol and glycosphingolipid-rich lipid rafts, and N-linked protein glycosylation pathways of eukaryotic cells. Both lipid A and eukaryotic glycolipids differ from phospholipids by the presence of hydrogen-bonded lateral interactions that tend to exclude phospholipids leading to the formation of detergent resistant lipid domains [67, 122]. Additionally, the undecaprenyl phosphate-dependent pathways for the synthesis and incorporation of O-antigens into the core-Kdo_2-lipid A molecule at the IM mirrors the dolichol phosphate-dependent pathway in the endoplasmic reticulum, where Glc_3-Man_9-$GlcNAc_2$ is incorporated into targeted protein Asn residues [123]. Finally, it now appears that many of the Raetz pathway enzymes

are conserved in the genomes of plants, perhaps reflecting the presence of lipid A-like molecules in plastids [5].

Lipid A and its regulated covalent modifications exhibit profound effects on bacterial and human physiology. Novel endotoxin antagonists and immune adjuvants have already been developed from modified lipid A structures [124, 125]. By revealing the biochemical details of lipid A structure and function we hope to understand its role in bacterial pathogenesis and to intervene with novel treatments for infection. However, we must remind ourselves that multiple molecular subtypes of lipid A are acting in concert in the bacterial cell. The need to unravel the interactions between individual lipid A modifications will provide fertile ground for future research.

Acknowledgments

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Bacterial Exotoxins

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Amongst the various mechanisms developed by pathogenic bacteria to cause disease, toxins play an important role, since they are responsible for the majority of symptoms and lesions during infection. Exotoxins act at a distance from the infectious site and can diffuse through the organism. While some cytotoxins can cause disruption of cells permitting the pathogens access to nutrients, other toxins are only active on specific cells, for example intestinal cells, neuronal cells, or leukocytes. This is achieved by the recognition of specific cell surface receptors. When bound to the receptor, toxins can unleash their toxic program at the cell membrane by interfering with signal transduction pathways, pore formation, or enzymatic activities towards membrane compounds. In contrast, other toxins enter the cytosol, and recognize and modify specific intracellular targets. According to the nature of the target and the type of modification, intracellular active toxins cause a dramatic alteration of cellular functions such as protein synthesis, cell homeostasis, cell cycle progression, vesicular traffic, and actin cytoskeletal rearrangements. Alternatively, invasive bacteria can directly inject toxins or virulence factors into target cells. This chapter is a comparative overview of the molecular mechanisms of the main bacterial exotoxins.

Toxins Active at the Cell Surface

Toxins Modulating Signal Transduction Pathways

Some enterotoxigenic *Escherichia coli* and other gram-negative enteropathogens *(Yersinia enterocolitica, Vibrio cholerae)* secrete heat-stable enterotoxins (STs) that can cause acute diarrhea in humans and animals. These toxins are small peptides which fall into two subgroups: methanol-soluble (STa or ST-I) and methanol-insoluble (STb or ST-II) toxins. Analysis of STs shows they possess a similar structure, containing 3 segments joined by 3 disulfide bridges. Ala13 in
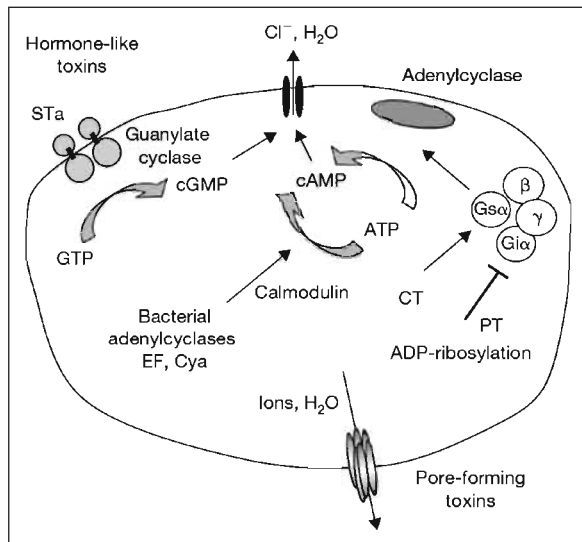
Fig. 1. Toxins that alter cell homeostasis. Some of the mechanisms used by bacteria to modify cell homeostasis are depicted. *E. coli* heat-stable enterotoxin (STa) binds to the extracellular domain of transmembrane guanylate cyclase, resulting in an increase in cyclic GMP, and secretion of Cl⁻ and H_2O. PFT inserted into the membrane cause leakage of ions and H_2O. CT and *E. coli* heat-labile toxins enter the cell cytosol and ADP-ribosylate the Gsα subunit of heterotrimeric G proteins, leading to a permanent active molecule by inhibition of its GTPase activity and subsequent stimulation of adenylcyclase. The resulting increase in cyclic AMP induces the secretion of Cl⁻ and H_2O. PT inactivates the inhibitory heterotrimeric G protein Giα, leading to a upregulation of adenylcyclase activity. Bacterial adenylcyclases, such as EF from anthrax toxin and *Bordetella* adenylcyclase (Cya), can also modulate cAMP levels in the cells.

the flexible central segment plays a key role in the toxin's activity. This residue is probably involved in the interaction of the toxin with its receptor. In the case of STa, the secreted protein encompasses 18–19 amino acids, including 6 cysteines, and is capable of forming 3 disulfide bridges to create a highly stable molecule. The carboxy-terminal segment of STs shares similarities with ionophores and is therefore expected to interact with metal ions. Enteroaggregative *E. coli* (EAggEC) strains also produce a heat-stable enterotoxin related to STa with similar pathological effects.

STa induces watery diarrhea without causing obvious histological morphological damage. The toxin binds to the extracellular domain of guanylate cyclase (GC-C) localized on the apical membrane of enterocytes. GC-C consists of 4 domains: an extracellular domain, a transmembrane segment, a kinase-like domain and an enzymatic domain, which catalyzes the formation of cyclic GMP (cGMP) (fig. 1). The kinase-like domain has an inhibitory effect on the catalytic

activity. Binding of STa to the extracellular domain of GC-C has been suggested to induce a conformational change in the protein kinase-like domain resulting in an uncontrolled increase of GC-C activity. Elevation of intracellular cGMP activates protein kinase II (cGKII), which in turn stimulates the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels. This results in a net fluid secretion through activation of apical Cl^- channels in parallel with the inhibition of coupled NaCl transporters. These findings have been confirmed in GC-C knockout mice, which have a lower intestinal GC-C activity and do not exhibit a secretory response to STa treatment [reviewed in 1].

STa was the first ligand found to bind GC-C and later studies demonstrated that the hormones guanylin and uroguanylin are the natural ligands for this receptor. These hormones have been shown to be involved in the regulation of fluid and electrolyte transport in many tissues. Guanylin and uroguanylin consist of 15 amino acids and are highly homologous to STa.

Toxins with Enzymatic Activity at the Cell Surface
That Alters Cell Signaling
Phospholipases
The first toxin that was recognized to possess an enzymatic activity was the *Clostridium perfringens* α-toxin. This protein is a zinc-dependent phospholipase C, which degrades phosphatidylcholine and sphingomyelin. Both in vitro and in vivo studies have shown that it has cytolytic, dermonecrotic, and hemolytic activities, and is lethal to animals at low doses. The toxin causes membrane damage to a variety of different human and animal cell types including platelets, leukocytes, and fibroblasts, as well as erythrocytes. It is the major toxin involved in gangrene, which is characterized by extensive local tissue destruction and necrosis progressing to profound shock and death. The secreted protein consists of 370 amino acids (43 kD), and contains 2 domains, an α-helical amino-terminal domain (residues 1–246) harboring the active site, and a β-sandwich carboxy-terminal domain (residues 256–370), which mediates membrane binding. The carboxy-terminal domain is structurally similar to eukaryotic calcium-binding C2 domains, which are involved in Ca^{2+}-dependent phospholipid binding. α-Toxin preferentially binds to phospholipids in the intact membrane, opening the active site of the toxin and resulting in cleavage of phospholipids [2]. In the activated state, the active site contains two tightly bound zinc ions and one loosely bound zinc ion and is accessible for substrate binding, whereas in the closed or inactive conformation, the active site is occluded and one zinc ion binding site is lost [2–4].

In addition to its lytic activity, α-toxin is also involved in intracellular signaling and the activation of endogenous metabolism cascades. Diacylglycerol and ceramide generated from limited hydrolysis of phospholipids and sphingomyelin,

respectively, activate endogenous phospholipases A_2, C and D, and protein kinase C. This in turn stimulates membrane phospholipases and initiates the arachidonic acid pathway leading to the production of proinflammatory molecules (prostaglandins, thromboxanes, and leukotrienes responsible for vasodilatation, bronchostriction), and platelet aggregation [4].

Other bacterial phospholipases include phospholipase C from *Pseudomonas*, *Listeria*, and various *Clostridium* species, phospholipase A from *Helicobacter pylori*, phosphatidylinositol phospholipase C from *Bacillus*, *Clostridium*, and phospholipase D from *Corynebacterium*.

Bacteroides fragilis Enterotoxin

B. fragilis enterotoxin (BFT) induces morphological changes in cultured intestinal and renal cells, including cell rounding, increase in volume, and efface-ment of microvilli and apical junctional complexes. BFT has zinc-dependent protease activity, which has been shown to cleave the extracellular domain of E-cadherin, the primary protein of the zonula adherens. Experimental studies have led to the proposed two-step hypothesis, whereby the extracellular domain of E-cadherin is cleaved by BFT, followed by intracellular degradation by as yet unidentified protease(s). As a consequence, nuclear signaling and actin rearrange-ment occur, which leads to the production of proinflammatory cytokines, diminished epithelial barrier function, and activation of apical membrane ion transporters. These cytotoxic effects are reversible, since 2–3 days after toxin treatment cells appear normal [reviewed in 5].

Pore-Forming Toxins

So far more than 80 toxins have been identified that act by forming a trans-membrane pore in the target cell. The general mechanism of pore-forming toxins (PFT) is to bind to cell surface receptors where they then oligomerize. The insertion of the oligomer into the cell membrane results in the formation of a channel, which impairs the osmotic balance of the cell and causes cytolysis. Most of the PFTs are cytolytic and/or hemolytic and they have been classified into several families [for review see 6–8].

RTX toxins (repeats in toxin) are synthesized by many gram-negative pathogens *(Escherichia, Proteus, Pasteurella)*. Members of the RTX toxin fam-ily, including cytolytic toxins, metalloproteases and lipases, share a common gene organization and distinctive structural features. They are secreted by the type I secretion system which is mediated by the Sec machinery. At the carboxy-terminal end, RTX contains 10–40 repeats of glycine- and aspartate-rich nonapeptide domains. Most RTX toxins are posttranslationally activated by acylation. The prototype of this family is the α-hemolysin (110 kD) of *E. coli* and its target receptors on leukocytes have been identified as members of the β_2

integrin family. Insertion of α-hemolysin into the membrane, probably mediated by four predicted hydrophobic α-helices in the amino-terminal region, leads to the formation of a hydrophilic- and cation-selective pore of at least 1 nm in diameter [9]. A related family of hemolysins consists of streptolysin S and streptolysin S-like cytolysins expressed in streptococci.

Cholesterol-binding cytolysins are produced by a wide variety of bacterial species including *Streptococcus*, *Bacillus*, *Clostridium*, and *Listeria*. Perfringolysin O (PFO) is one of the best-studied toxins from this family. PFOs are secreted as water-soluble monomers, which contain 4 domains rich in β-strands. A short hydrophobic loop in domain 4 is involved in the binding to cholesterol [10]. After cholesterol binding, PFO undergoes a conformational change resulting in the unfolding of domain 3 α-helices and the formation of two amphipathic β-hairpins in each monomer. This leads to an association of neighboring monomers and the subsequent formation of a large β-barrel, which then inserts into the membrane forming the pore. In general, cholesterol-binding cytolysins form large pores (300 Å) containing about 50 monomers [11].

Staphylococcus aureus α-hemolysin, aerolysin and the binary staphylococcal leukocidins, such as LukF, are also synthesized as monomers consisting of a very hydrophilic sequence essentially arranged in β-sheets. Binding of monomers to an as yet unidentified cell receptor triggers the heptamerization of the toxin, which adopts a mushroom shape with cap, rim and stem domains. The amino-terminus detaches from the core monomer unmasking a small hydrophobic surface and assembles with the corresponding domains of the neighboring monomers to form the cap. In contrast to PFO, only one antiparallel β-hairpin loop of each monomer unfolds and contributes to the stem formation, which consists of 14-stranded β-barrels and results in pores with a small diameter (15–45 Å) [11, 12]. Aerolysin is secreted as an inactive precursor, which binds to a glycosylphosphatidylinositol (GPI)-anchored protein. The toxin is activated by cleavage of a carboxy-terminal peptide (40 amino acids) by soluble proteases (trypsin or chymotrypsin) or furin. The localization of the aerolysin receptor on lipid rafts probably facilitates toxin oligomerization [13]. *Clostridium septicum* α-toxin, which is responsible for gangrene, shares a similar mode of activation and pore formation with aerolysin [14].

The multicomponent leukocidins and γ-hemolysin from *S. aureus* also assemble in hexamers (1:1 stoichiometry), which form transmembrane pores [7]. One component (class S) is involved in the recognition of a cell surface receptor and allows the binding of the other component (class F). The β-toxin from *C. perfringens*, which is involved in necrotic enteritis, is related to *S. aureus* α- and γ-hemolysin, and triggers pore formation [15].

C. perfringens enterotoxin is a toxin that causes food poisoning via the specific binding of the enterotoxin to receptor(s) from the claudin family,

present on enterocytes. This complex is then able to associate with additional membrane proteins, including occludin, to form larger complexes. It has been suggested that these complexes form pores in the plasma membrane, which alters the permeability of the plasma membrane for small molecules and ultimately causes cell death by lysis or metabolic shut-down [16].

Superantigens

A particular class of bacterial toxins referred to as superantigens (entero-toxins, toxic shock syndrome toxins from *Staphylococcus* and *Streptococcus*) are characterized by their ability to bind both MHC class II molecules and T cell receptors. Unlike conventional antigens that are presented to the T cell receptor in complex with the MHC class II molecule, superantigens bind to the T cell receptors and MHC class II molecules outside the classical antigen-binding groove. This results in a massive antigen-independent proliferation of the targeted T lymphocytes, leading to the release of various cytokines and inflammatory factors [6].

Intracellularly Active Toxins

Inhibition of Protein Synthesis
Diphtheria Toxin – Inactivation of Elongation Factor 2

Corynebacterium diphtheriae is a human pathogen that normally colonizes the throat. The bacterium secretes a potent toxin, also known as diphtheria toxin (DT), which is one of the most extensively studied and well-understood bacterial toxins. Once DT has entered the bloodstream it can affect various organs, caus-ing serious complications such as nephritis and cardiac dysfunction associated with high mortality rates. DT is a single-chain protein of 58 kD encompassing three structural and functional domains: a carboxy-terminal domain rich in β-sheets (domain R), which binds to cell surface receptors, a central transloca-tion domain containing 9 α-helices (domain T), and the amino-terminal catalytic domain consisting of a mixture of α- and β-structures with a cleft forming the active site (domain C). The toxin is activated by proteolysis at a furin cleavage site located in an exposed loop between Cys186 and Cys201. The amino-terminal fragment corresponds to the catalytic domain and remains linked by a disulfide bridge to the rest of the molecule.

The receptor for DT has been identified as heparin-binding epidermal growth factor-like growth factor precursor which forms complexes with other membrane components, including CD9, heparin sulfate proteoglycans and inte-grins. Epidermal growth factors are synthesized as transmembrane proteins, which are subsequently cleaved close to the transmembrane segment to release
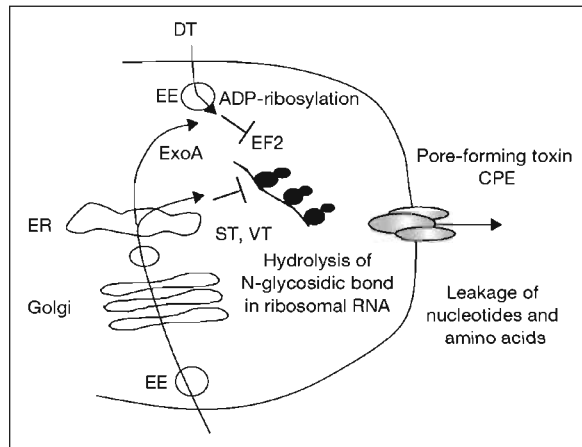
Fig. 2. Toxins that inhibit protein synthesis. DT enters the cytosol via the early endosomes (EE) and inactivates EF2 by ADP-ribosylation, which results in impaired protein synthesis. *Pseudomonas* exotoxin A (ExoA), Shiga toxin (ST) and *E. coli* verotoxin (VT) enter cells via the Golgi apparatus and ER. While ExoA inactivates EF2, ST and VT impair ribosomal RNA function by cleaving an N-glycosidic bond in the 60S subunit. PFT such as *C. perfringens* enterotoxin (CPE) inhibit protein synthesis by inducing leakage of nucleotides, amino acids, and other small molecules.

the active growth factor. Once bound to the receptor complex, DT is proteolytically cleaved by furin and internalized into cells by receptor-mediated endocytosis via clathrin-coated vesicles. DT is then transported to late endosomes and lysozomes where further degradation occurs. An acidification of the early endocytic vesicles (pH less than 6) triggers a conformational change in the T domain, to form a molten globule structure exposing hydrophobic sites (in particular TH5–7 and TH8–9) that insert into the membrane forming cation-selective channels. The amino-terminal fragment of DT is then translocated in an unfolded state from the endosomes into the cytosol where it inhibits protein synthesis by ADP-ribosylation of elongation factor 2 (EF2) [see details in 17–21].

The DT catalytic domain belongs to a family of mono-ADP ribosyltransferases, which bind to NAD and transfer the ADP-ribose group to a specific residue on the target protein. The active site is conserved among the bacterial ADP-ribosylating toxins. It consists of an α-helix bent over a β-strand, which forms the NAD-binding cavity that is flanked by two residues (His and Glu) that have a major role in catalytic activity. The ADP-ribosylation of diphtamide 715 by DT prevents the binding of EF2 to tRNA, resulting in the inhibition of protein synthesis (fig. 2).

Pseudomonas Exotoxin A – Inactivation of EF2

Pseudomonas exotoxin A (ExoA) is a 66-kD single-chain protein, which shares the same mechanism of action as DT. ExoA is the major virulence factor of the opportunistic pathogen *Pseudomonas aeruginosa*, which often infects immunocompromised patients. The toxin is synthesized as a precursor, containing an amino-terminal signal peptide that directs the polypeptide into the type II secretion pathway. The crystal structure reveals three distinct domains: an amino-terminal domain consisting of 17 antiparallel β-strands that recognizes the cell surface receptor, a central domain composed of 6 α-helices forming the translocation domain, and a carboxy-terminal domain containing the catalytic site. ExoA binds to lipoprotein-receptor-related protein (LRP), which is a multifunctional scavenger receptor that is expressed by many cell types. Upon binding to LRP, ExoA is internalized into the cell by receptor-mediated endocytosis. Inside the endosome, the toxin is cleaved by furin, which results in two fragments. The enzymatic domain is transported from the Golgi to the endoplasmic reticulum (ER), where it is then translocated to the cytosol. In the cytosol the enzymatic domain of ExoA catalyzes the ADP-ribosylation of EF2, resulting in an inhibition of protein synthesis and ultimately leading to cellular death [22, 23] (fig. 2).

Shiga Toxin – Inactivation of Ribosomal RNA

Another family of toxins consists of Shiga toxin, Shiga-like toxins, verotoxins, and verocytotoxins which are expressed by several enteric pathogens, including *Shigella dysenteriae* and enterohemorrhagic *E. coli*. This group of toxins plays an important role in the disease pathogenesis of a number of severe complications, such as hemorrhagic colitis and the hemolytic uremic syndrome.

Shiga toxins are composed of a catalytically active subunit (A subunit) and a receptor recognition subunit (B subunit). The B subunit that recognizes the cell surface receptor globotriosyl ceramide Gb3 consists of 5 B fragments that form a symmetrical ring-like structure in solution. The catalytic domain is located in the A subunit, which is activated by proteolytic cleavage leading to two fragments (A1 and A2) that are linked together by a disulfide bridge.

Several studies have previously shown that Shiga toxin enters the cell by the clathrin-dependent pathway and is then transported directly from early/recycling endosomes to the Golgi apparatus and then to the ER [24]. However, a clathrin-independent mechanism has also been described involving lipid rafts [25].

Activation of the catalytic domain probably occurs in the trans-Golgi network and/or in endosomes by the action of furin, and to a lesser extent by other cellular proteases. The A1 fragment is released into the cytosol and inactivates the 60S subunit of host cell ribosomes by cleaving the N-glycosidic bond of adenosine 4324 of the 28S ribosomal RNA of the 60S subunit. This induces

a dramatic inhibition of cellular protein synthesis (fig. 2). It has been reported that Shiga toxin and verotoxins also cause apoptosis characterized by DNA degradation and subsequent cell lysis by an independent mitochondrial pathway [26].

Alteration of Cell Homeostasis
Alteration of Heterotrimeric G Protein Signaling

Cholera Toxin. Cholera is a serious epidemic disease characterized by severe diarrhea and dehydratation, caused principally by the cholera toxin (CT). Other members of the CT family are the *E. coli* heat-labile enterotoxins LT-I and LT-II. The CT gene is localized to filamentous bacteriophage DNA and can be chromosomally integrated or replicated as a plasmid [27]. Similarly, the heat-labile enterotoxin genes are located on plasmids (LT-I) or are integrated into the chromosome (LT-II) [28]. CT and lethal toxin (LT) subunits are exported across the bacterial membrane by Sec proteins and assemble in the periplasm. In *V. cholerae*, CT is actively secreted through the outer membrane, while the release of LT-I depends on cell lysis [for a review, see 29].

Like Shiga toxin, CT and LTs consist of an A subunit (28 kD) and 5 B subunits (11 kD each) assembled in a pentamer (AB5 structure). The A subunit is proteolytically activated by a *V. cholerae* endopeptidase into two components A1 (approximately 22 kD) and A2 (approximately 5.5 kD) which remain linked by a disulfide bridge. The carboxy-terminal part of A2 extends through the central pore of the B pentamer and is linked noncovalently to the B subunits.

CT is internalized into noncoated vesicles after binding of the B subunits to ganglioside GM1, which is located at the epithelial cell surface. GM1 directs the toxin into lipid rafts from where it enters the Golgi via early and late endosomes in a Rab9-dependent pathway [30]. In the perinuclear region of the Golgi, the A subunit dissociates from the B subunits and enters the ER via coatomer I-coated vesicles. The carboxy-terminal sequence of the A2 fragment contains an ER retention sequence (KDEL), which recognizes the receptor Erd2p and directs the Golgi-ER trafficking of CT [31]. B subunits lacking an ER retention signal are also transported to the ER, via an unknown mechanism, and translocate into the cytosol via the Sec61 complex [32]. The A1 fragment is responsible for the enzymatic activities of the toxin in the presence of the membrane factor Arf. This activity includes NAD hydrolysis of ADP-ribose and nicotinamide, and transfer of ADP-ribose to Arg187 of the α-subunit of stimulatory protein (Gsα), leading to stimulation of adenylcyclase and elevated intracellular cAMP. The increased cAMP levels lead to an activation of protein kinase A, which subsequently phophorylates numerous substrates in the cell [33]. This results in an increase of Cl$^-$ secretion by intestinal crypt cells (fig. 1) and a decrease of NaCl-coupled absorption by villus cells.

Pertussis Toxin. Pertussis toxin (PT) is an important virulence factor of *Bordetella pertussis*, the causative agent of whooping cough in humans. PT is a hexameric protein consisting of an enzymatic A domain (subunit S1) and five binding B domains (subunits S2–S5). Whereas the 5 B subunits of CT are identical and arranged in a regular pentamer, the corresponding PT subunits are distinct (11–26 kD) and organized as an oligomer (S5-S2-S4-S3-S4). This structure forms a disc-like base upon which the pyramid-shaped enzymatic A subunit (S1) rests. The different B subunits form a pentameric domain in the center of the B oligomer, consisting of a ring of 30 antiparallel β-strands, which is surrounded by a barrel of five α-helices. The pore of the barrel is partially penetrated by the carboxy-terminus of S1.

Glycoproteins and glycolipids found on many types of eukaryotic cells have been shown to act as a receptor for the B oligomer of PT, seemingly via carbohydrate-recognizing domains on subunits S2 and S3. The interaction of the toxin with cells of the immune system leads to the induction of lymphocytosis, inhibition of macrophage migration, adjuvant activity, and T cell mitogenicity. The T cell mitogenic effect is mediated by the B oligomer and is thought to be independent of the S1 subunit of the toxin. PT possibly undergoes a retrograde transport to the ER to deliver S1 into the cytosol, although S1 does not contain an ER retention sequence [34].

Internalization of PT is mediated by endocytosis through coated pits, and seems to be routed to the late endosome and to the Golgi apparatus.

The S1 subunit of PT, which shares high homology with the enzymatic domains of CT and LT, catalyzes the ADP-ribosylation of the inhibitory α-subunits of the heterotrimeric GTP-binding proteins (G proteins) involved in a variety of signaling pathways. This results in the prevention of the α-subunit coupling with the corresponding β/γ-subunits, an increase of adenylcyclase activity, which is no longer negatively regulated, and the impairment of several second-messenger pathways including an increase in cAMP (fig. 1).

Adenylcyclase Activity
Bordetella Adenylcyclase
The adenylate cyclase toxin (Cya) of *B. pertussis*, the whooping cough agent, is a major virulence factor required for the early phases of lung colonization. Cya is a single-chain 177-kD protein consisting of two domains. The toxin is activated after posttranslational palmitoylation of the protein at Lys856 and Lys963. The enzymatic activity of Cya is located within the proximal 400 amino acids at the amino-terminus. The carboxy-terminal part, also referred to as the hemolysin domain, contains several glycine and aspartate-rich nonapeptide repeats that are related to those found in RTX toxins and represent the main Ca^{2+}-binding site of the protein. In addition to its intrinsic hemolytic

activity, this domain mediates the binding to and internalization of the toxin into eukaryotic cells. CyaA can penetrate a wide range of cell types, including erythrocytes and immune cells. In macrophages, neutrophils and dendritic cells, CyaA has been demonstrated to bind specifically $\alpha_M\beta_2$ integrin (CD11b/CD18) [35]. After internalization, possibly directly through the plasma membrane, Cya is cleaved and the catalytic domain is released into the cytosol, where it increases the cAMP levels in a calmodulin-dependent fashion (fig. 1). The toxin allows the pathogen to escape the host immune response by intoxicating neutrophils and macrophages, causing phagocyte impotence, and inducing apoptosis [36].

Anthrax Edema Toxin

Anthrax toxin is a tripartite toxin consisting of the protective antigen (PA), edema factor (EF), and lethal factor (LF). PA is the binding component, which permits the entry of either EF or LF into the cell. The combination PA and EF is termed anthrax edema toxin, while PA and LF is termed lethal toxin (a further description of LT can be found in the section Apoptosis below). The genes of the three components are localized on a large plasmid (pXO1) present in virulent *Bacillus anthracis* strains. The proteins are secreted by means of a signal peptide [reviewed in 37].

PA is secreted as an inactive protein (83 kD), which is activated after removal of a 20-kD amino-terminal peptide. The cleavage site contains the RKKR motif, which is sensitive to proteases such as trypsin or furin. The active protein (PA63) has four domains [38]: an amino-terminal domain (domain 1) that is relatively hydrophobic and which is involved in the binding of EF or LF, a heptamerization domain (domain 2) containing a large amphipathic flexible loop implicated in membrane insertion, a small domain of unknown function (domain 3), and a carboxy-terminal receptor-binding domain which is rich in β-strands (domain 4) [39, 40]. The cell surface receptor for PA has been identified as a membrane protein containing a von Willebrand factor A domain that is located in lipid rafts [41, 42]. Receptor-bound PA is activated at the cell surface and clusters in lipid rafts, resulting in the formation of PA63 heptamers that bind EF or LF. The complex is endocytosed and transported to endosomal compartments where the low pH induces a conformational change in the PA63 heptamers, leading to its insertion into the membrane and the formation of water-filled channels. The translocation of EF and LF into the cell occurs by different strategies. While LF is fully translocated into the cell cytoplasm, EF remains membrane bound, exposing its catalytic domains to the cytosolic compartment [43]. EF is an adenylcyclase, which is only active when associated with calmodulin (fig. 1). Ca^{2+}-bound calmodulin is much more efficient at activating EF than the Ca^{2+}-free form. The catalytic domain of EF is homologous with *B. pertussis* adenylcyclase, and contains the consensus ATP binding motif

(GxxxxGKS). The conversion of ATP by EF leads to an increase in intracellular cAMP levels. These effects are reversible and transient, since EF is instable in the cytosol. In human monocytes, EF enhances IL-6 production and inhibits LPS-dependent tumor necrosis factor (TNF) synthesis. It has been speculated that the main role of anthrax edema toxin is to impair the function of phagocytosing cells such as polymorphonuclear cells and macrophages, which may facilitate the early stages of bacterial infection [37].

Arrest of Cell Cycle

Cytolethal distending toxins (CDTs) belong to a recently discovered family of toxins, which cause irreversible cell cycle arrest and ultimately death of the target cells. CDT was first described in 1987 when certain strains of *E. coli* were found to cause cytopathic effects that were distinct from those induced by *E. coli* toxins such as LT, ST, verotoxin, and hemolysin. Cells that are sensitive to CDT first increase in size (3- to 5-fold), followed by a slowly developing cell distention, that finally leads to cell death. Apart from *E. coli*, CDTs are produced by a wide variety of gram-negative bacteria including *Shigella*, *Hemophilus ducreyi*, *Actinobacillus actinomycetemcomitans*, *H. pylori*, and *Campylobacter* [44]. In *E. coli*, it has been shown that CDT is encoded by three adjacent or slightly overlapping genes, *cdtA*, *cdtB*, and *cdtC*, all of which are required for the activity of the toxin. While CdtB contains the enzymatic activity, CdtA and CdtC are required for the translocation of CdtB into the target cell. Internalization of CDT from *H. ducreyi* occurs via endocytosis mediated by clathrin-coated pits. The toxin has been shown to traffic through the Golgi apparatus into the cytosol and the nucleus. The proposed mechanisms of action of CDTs are not yet fully elucidated; however, it has been reported that the toxin blocks cells in the G2 phase of the cell cycle by preventing dephosphorylation of the inactive form of cdc2. In addition CdtBs possess DNase I activity that causes double-strand DNA breaks (fig. 3) [45].

Apoptosis

Vacuolating Cytotoxin

The vacuolating cytotoxin (VacA) is one of the most important virulence factors produced by *H. pylori*, a causative agent of severe gastric diseases such as ulcers and cancer. VacA has been shown to induce large cytoplasmic vacuoles in cultured cells and apoptosis in gastric epithelial and parietal cells. Cleavage of the secreted VacA protein (95 kD) results in an amino-terminal 34- to 37-kD (p37) and a carboxy-terminal 58-kD (p58) fragment that remain associated with each other. The p58 fragment mediates VacA monomer binding to the target cell via a GPI-anchored protein, which leads to VacA oligomerization in the membrane and the formation of anion-selective channels that release bicarbonate, chloride
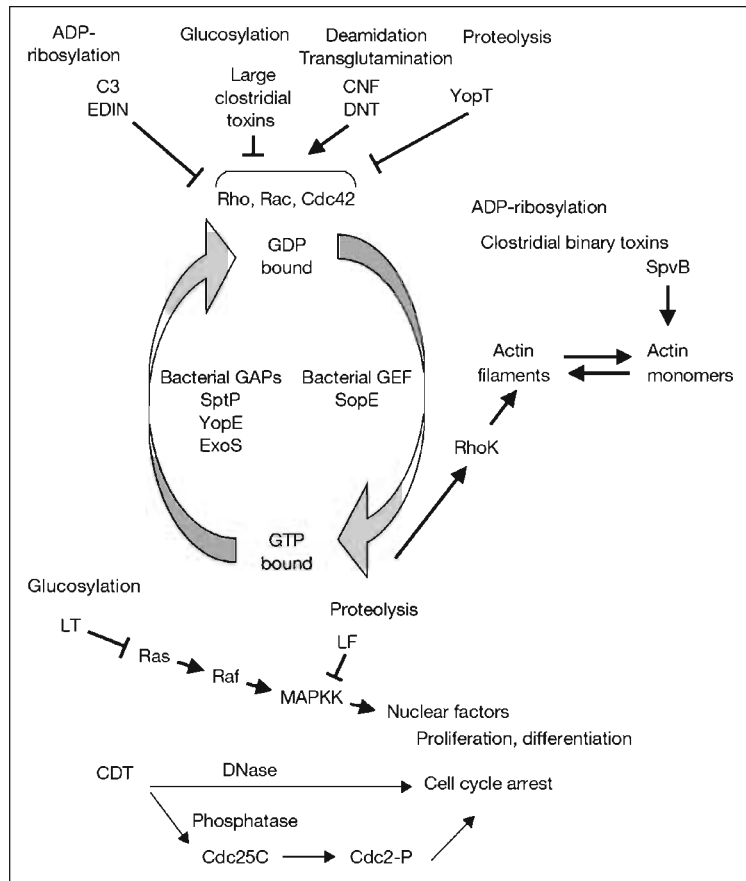
Fig. 3. Bacterial toxins that modify intracellular signaling, actin cytoskeleton rearrangement and cell cycle progression. Clostridial binary toxins and other toxins injected by the type III secretion system (SpvB) depolymerize actin filaments by ADP-ribosylation of actin monomers. While the large clostridial toxins and C3 inactivate Rho-GTPases and YopT impairs the translocation of Rho-GTPases to the membrane, CNF and DNT induce an activation of Rho-GTPases. In contrast, SopE and YopE activate Rho-GTPases via a GEF activity, or inactivate these molecules through a GAP activity, respectively. These factors are involved in the coordinated remodeling of the actin cytoskeleton permitting the bacterial invasion and the subsequent restitution of the normal cell architecture after bacterial entry. *C. sordellii* LT and anthrax LT (LF) downregulate the Ras signaling pathway by glucosylation of Ras molecules (LT) or proteolysis of MAPK kinase, whose subsequent molecular mechanisms and cell effects are still unclear. CDT interfere with the cell cycle through DNase activity, which induces DNA damage and subsequent cell cycle arrest. CDT probably also acts on the regulation of cyclin-dependent kinase (Cdc2) by converting this molecule to its phosphorylated inactive form.

and urea from the cell cytosol [46, 47]. VacA toxin channels are then internalized and transported to the late endosomal compartments where they change the anion permeability, leading to an enhancement of the vacuolar ATPase proton pump activity [40, 41]. It has also been reported that the p34 fragment of VacA targets mitochondria leading to the release of cytochrome c, activation of caspase 3 and cell apoptosis [48].

Anthrax Lethal Toxin

B. anthracis LT is a zinc metalloprotease that causes hyperinflammatory conditions in macrophages, the release of reactive oxygen intermediates, and secretion of proinflammatory cytokines, such as TNF-α and interleukin-1β [49]. LF (90 kD) is composed of 4 domains. As discussed for EF, domain 1 (amino acids 1–254) consisting of a 12-helix bundle, is involved in the interaction with PA. Interestingly, the structure of domain 2 is similar to that of the catalytic domain of *Bacillus cereus* VIP2 (vegetative insecticidal protein) and *C. perfringens* iota toxin (see below). However, LF is devoid of ADP-ribosylating activity. Domain 3 forms a small helical bundle, which is required for the substrate recognition and domain 4 (residues 552–776), consisting of a nine-helix bundle packed against a four-stranded β-sheet, contains the metalloprotease active site (HExxH). Analysis of the crystal structure revealed that domains 2, 3 and 4 form a long deep groove that holds the 16-residue amino-terminal tail of mitogen-activated protein kinase kinase 2 (MAPKK-2) [50].

Subsequent studies have shown that MAPKK-2 is not the only target for LF, since MAPKK-1 to 7 (except MAPKK-5) are also cleaved and inactivated by this enzyme [51, 52]. In macrophages, LF also inhibits the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPKs pathways (fig. 3). While high concentrations of LF cause cell necrosis, low concentrations (200 ng/ml) induce apoptosis in macrophages. However, in order to trigger apoptosis, cells have to be activated, for instance by LPS or other inflammatory mediators. Apoptosis of activated macrophages was found to be dependent on p38 inactivation, however, the mechanism is not fully elucidated [53].

Alteration of Vesicular Traffic, Blockade of Neuroexocytosis,
Clostridial Neurotoxins

The mode of action of botulinum (BoNT) and tetanus (TeTx) neurotoxins consists of four steps: binding, internalization, translocation and intracellular activation [see also reviews 54–58]. BoNT and TeTx recognize specific receptors on unmyelinated areas of the presynaptic membrane. The precise identity of neurotoxin receptors has still to be determined; however, gangliosides from the G_{1b} series and synaptic vesicle-associated proteins known as synaptotagmins (a family of membrane-trafficking proteins) seem to be involved [59].
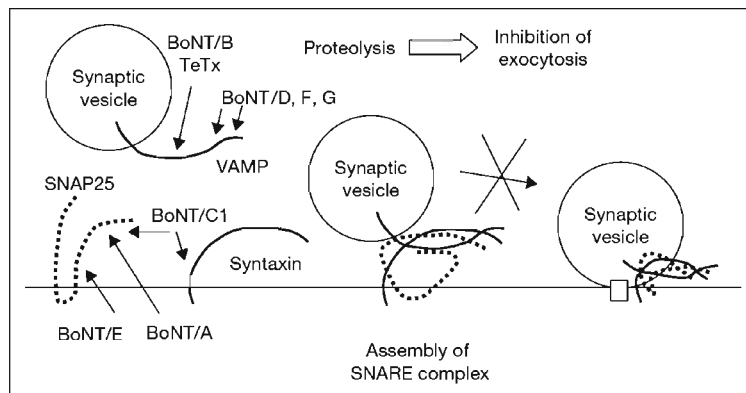
Fig. 4. Toxins that interfere with vesicular traffic. BoNTs and TeTx are zinc-dependent proteases, which cleave SNARE proteins (VAMP, SNAP25 and syntaxin) and result in SNARE complexes with a reduced stability. This prevents synaptic vesicles from fusing with the presynaptic membrane.

Neurotoxin bound to its receptor is internalized by receptor-mediated endocytosis. An essential difference between BoNTs and TeTxs is that the BoNTs are directly endocytosed in clathrin-coated vesicles, resulting in a translocation of the light chain into the cytosol. In the peripheral nervous system, the BoNT light chain blocks the release of acetylcholine at the neuromuscular junctions, leading to a flaccid paralysis. In contrast, TeTx is sorted to the fast axonal retrograde transport route, and delivered to the motoneurons, which are located in the spinal cord. TeTx enters inhibitory interneurons probably via coated vesicles, permitting the delivery of light chain into the cytosol where it inhibits the release of glycine and GABA.

The light chains of clostridial neurotoxins contain a conserved zinc-dependent proteolytic site (His-Glu-x-x-His) with endopeptidase activity [60, 61]. It has been shown that the different neurotoxins preferentially target proteins belonging to the SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors) family, comprising the three membrane-associated proteins VAMP/synaptobrevin, SNAP-25, and syntaxin. While TeTx, BoNT/B, D, F and G cleave VAMP/synaptobrevin and BoNT/A and E cleave SNAP25, BoNT/C1 utilizes both SNAP25 and syntaxin as substrates (fig. 4). Each neurotoxin recognizes its substrate at specific binding sites termed SNARE motifs (two in VAMP and syntaxin, and four in SNAP25), resulting in a cleavage pattern which is characteristic for each toxin. It should be noted that TeTx and BoNT/B cleave VAMP at the same site. While SNARE proteins are unstructured in solution, when they lie parallel to the membrane surface, they assemble in a ternary complex (SNARE complex) consisting of four tightly packed α-helices.

The SNARE complex is able to recruit a number of soluble cytosolic proteins such as NSF (N-ethymaleimide-sensitive factor) and SNAPs (soluble NSF accessory proteins). The resulting 20S SNARE complex has been recognized as essential in vesicle targeting and fusion. It has been shown that this complex is rapidly disassembled by NSF-dependent hydrolysis of ATP. Assembly and disassembly of SNARE proteins within the complex are thought to be essential in the exocytosis process. Importantly, clostridial neurotoxins can only cleave SNARE proteins when they are disassembled. The cleavage of SNARE proteins by clostridial neurotoxins results in a reduction of SNARE complex stability and impaired neurotransmitter release. Even though VAMP, SNAP25 and syntaxin have different physiological properties at neuromuscular junctions, all clostridial neurotoxins cause similar symptoms. However, the intensity and duration of neurotransmission inhibition vary depending on the neurotoxin [56, 57].

Alteration of Actin Cytoskeleton and Small G Protein Signaling
Toxins Active on Actin

Actin ADP-Ribosylating Toxins. Actin ADP-ribosylating toxins are binary toxins which share a common structure, composed of two individual proteins, a binding/translocation component and an enzyme component, which are nonlinked and assemble on the target cell. So far three families have been identified. The iota family, which encompasses iota toxin, produced by *C. perfringens* type E, *Clostridium spiroforme* toxin and an ADP-ribosyltransferase synthesized by some strains of *Clostridium difficile*. The second family (C2 family) contains the C2 toxins expressed by *Clostridium botulinum* type C and D, which have been shown to cause necrotizing enteritis and diarrhea. The third family concerns the insecticidal binary toxins or VIP produced by *B. cereus* and *Bacillus thuringiensis* [62].

The binding component binds to the surface of the target cell and is essential for the import of the toxin into the cell. For this, the binding component has to be activated by protease cleavage. In solution, the binding components of iota and C2 toxins (Ib and C2-II, respectively) can be processed by trypsin or α-chymotrypsin. However, unprocessed Ib and C2-II can also bind to the cell surface receptor, but do not mediate the entry of the enzymatic component. The processed binding component recognizes specific cell membrane receptors, heptamerizes and forms small ion-permeable channels that trap the enzymatic component into endocytic vesicles. The enzymatic component is subsequently translocated into the cytosol [63–66].

The enzymatic component catalyzes the ADP-ribosylation of actin monomers at Arg177 but not of polymerized F-actin, since Arg177 is located in the actin-actin binding site. The cumbersome ADP-ribose at the actin-binding site prevents the nucleation and polymerization of ADP-ribosylated actin

monomers. Moreover, ADP-ribosylated actin acts as a capping protein, it binds to the barbed end of the actin filament and inhibits the further addition of unmodified actin monomers. Actin filaments depolymerize at the pointed end and the released actin monomers are immediately ADP-ribosylated (fig. 3). In addition, ADP-ribosylation inhibits the intrinsic ATPase activity of actin. Cell microinjection of ADP-ribosylated actin monomers induces the same effect as C2 or iota toxin. This results in a complete disassembly of the actin filament and accumulation of actin monomers [67, 68]. While the microtubules are unaffected, the intermediate filaments are disorganized. As a consequence cells become rounded, detach from the surface, and die [reviewed in 68, 69]. Studies with epithelial and endothelial cells have shown that clostridial ADP-ribosylating toxins alter the tight and adherens junctions resulting in a loss of cell barrier function [70, 71]. While toxins of the iota family modify all actin isoforms, including cellular and muscular isoforms, C2 toxins only interact with cytoplasmic and smooth muscle γ-actin. Substrates for VIP have not yet been reported.

Type III Secretion System-Dependent ADP-Ribosylating Toxins. Nontyphoid *Salmonella* strains that are commonly associated with severe systemic infections carry a large plasmid harboring *spv* genes, which are required for bacterial growth in macrophages and monocytes. Among the four-gene operon *(spvABCD)*, it has been demonstrated that the *spvB* gene, encoding a 65.6-kD protein, is essential for the virulence phenotype. Based on database searches it has been proposed that SpvB has two functional domains, an amino-terminal domain related to the insecticidal toxin Tcal from *Photorhabdus luminescens* with an as yet unknown mechanism of action, and a carboxy-terminal domain that shares homology with the ADP-ribosylating part of iota, C2 and VIP. Recombinant SpvB ADP-ribosylates nonmuscle actin and microinjection of SpvB into CHO cells causes a breakdown of actin filaments (fig. 3). In vivo studies have shown that SpvB is crucial for the virulence in mice while a mutant strain lacking the *spvB* gene shows marked attenuation of virulence [72]. Evidence has been provided demonstrating that SpvB is injected into host cells by a type III secretion system. Once bacteria have entered epithelial cells and macrophages, SpvB is expressed after 6 h, and in infected macrophages SpvB-dependent cytotoxicity is evident after 10–12 h. Like SptP, SpvB reverses the actin cytoskeleton reorganization mediating bacterial entry, and permits the infected cells to regain their normal architecture after invasion. Another ADP-ribosyltransferase toxin that also targets actin and which is secreted into the target cell by a type III system has been found in *Aeromonas salmonicida* (AexT) [73].

Toxins Activating Small G Proteins

Enzymatic Modification of the GTPase Site. Some *E. coli* strains have been shown to produce cytotoxic necrotizing factors (CNFs). To date, two variants

termed CNF1 and CNF2 have been characterized. CNF1 is synthesized by strains mainly isolated from human urinary infections and neonatal meningitis, whereas CNF2 is produced by strains that infect animals [74].

Both factors are highly homologous at the amino acid level (86% identity) and are produced as single-chain proteins with a molecular weight of about 110 kD. CNFs are related to the dermonecrotic factor (DNT) from *Bordetella*, and homologous sequences to the *cnf1* gene have been found in the genomes of *Yersinia pestis* and *Yersinia pseudotuberculosis*. CNF toxins consist of three functional domains: an amino-terminal domain (amino acids 1–299), which is involved in the recognition of a cell surface receptor, a central domain (amino acids 299–720) containing two hydrophobic regions which have been proposed to translocate the toxin across the cell membrane, and a carboxy-terminal (720–1,014) catalytic domain. The carboxy-terminal domain of CNF1 has a novel protein fold as determined by crystal structure analysis. This unusual compact domain is formed by a central β-sandwich, that is composed of two mixed β-sheets, and surrounded by helices and extensive loop regions [75].

CNF1 catalyzes the deamidation of Gln63 in Rho and Gln61 in Rac and Cdc42 to glutamic acid. Gln63/Gln61 are located in the switch II region of the Rho protein. This region has an important function in the turn-off mechanism of RhoGTPases and is essential for GTP hydrolysis by this family of proteins [76, 77]. Thereby, CNF1 blocks the RhoGTPases in their active form linked to GTP. Studies with fibroblasts (Vero cells) have shown that CNF1 causes dense actin stress fibers and focal contact point formations, whereas in epithelial cells (Hep2) the formation of lamellipodia and filopodia predominates. In both cell types, CNF1 leads to cell spreading resulting from the increase in actin filament formation at the leading edge and anchorage of actomyosin filaments to focal contact points. This is followed by contraction of these filaments in a similar way to that seen in actin-based motility. These findings suggest that in epithelial cells CNF1 first activates Cdc42 and Rac followed by the activation of Rho, whereas in fibroblasts activation of Rho is predominant [78].

Activation of RhoGTPases by CNFs is only transient and it has been shown that deactivation of Rac correlates with an increase in the susceptibility of its deamidated form to ubiquitin/proteasome-mediated degradation. During the first phase of CNF intoxication, which corresponds to the activation of RhoGTPases, uroepithelial cells begin spreading followed by intense membrane ruffling. In the next phase of intoxication, lamellipodia are replaced by filopodia, cells become highly motile, and there is an alteration in cellular junction dynamics. This probably favors bacterial internalization, which requires coordinated RhoGTPase activation and inactivation for a maximal efficiency [79].

Type III Toxin-Activating RhoGTPases by Guanine Nucleotide Exchange Factor Activity. *Salmonella* enters the cell by a trigger mechanism that induces the

formation of large membrane ruffles, which engulf the bacteria. The subsequent rearrangements of the actin cytoskeleton and the plasma membrane are reminiscent of lamellipodia and filopodia responses stimulated by various agonists such as growth factors, hormones, or activated oncogenes. It has been demonstrated that Cdc42 and to a lesser extent Rac are involved in the *Salmonella*-dependent cytoskeletal rearrangements. These effects are mediated by SopE, which is delivered into the cell by a type III secretion system. Like guanine nucleotide exchange factors (GEFs), SopE activates Rac1, Rac2, Cdc42, RhoG, and also to a lesser extent RhoA by catalyzing the exchange of GDP for GTP [80]. Interestingly, SopE2, an isoform of SopE, interacts with Cdc42 but not with Rac1 [81].

SopE binds to the switch I and switch II regions of Cdc42 and promotes guanine nucleotide release. This mechanism is similar to that used by the eukaryotic Dbl-like exchange factor Tiam1 in complex with Rac1 (fig. 3). However, the catalytic domain of SopE has a different structure to that of Tiam1 and interacts with the switch regions via a GAGA motif [82]. SopE also acts as a GEF for Rab5 and mediates the recruitment of Rab5 in its GTP form to phagosomes containing *Salmonella*. This promotes the fusion of these phagosomes with early endosomes, preventing their transport to lysozomes and subsequent destruction [83]. In addition, activation of Cdc42 and Rac by SopE leads to stimulation of p21-activated kinase (PAK) and subsequent activation of JNK, the MAP kinase pathway and a number of transcriptional factors [80].

Toxins Inactivating Small G Proteins

ADP-Ribosylating C3 Exoenzyme. C. botulinum C3 exoenzyme belongs to the family of Rho-ADP-ribosylating toxins. Other C3-like ADP-ribosyltransferases have been identified in *S. aureus* and *B. cereus* and are termed EDIN (epithelial differentiation inhibitor) and *B. cereus* exoenzyme, respectively. It should be noted that genes encoding EDIN have a higher prevalence in *S. aureus* strains isolated from infection sites than in strains isolated from nasal carriers [84].

The C3-like exoenzymes ADP-ribosylate Asn41, which is located within the β-strand, align next to the switch I region of the Rho-GTPases [85]. However, the Asn41 residue is not accessible when Rho is associated with GDI (guanine nucleotide dissociation inhibitor), resulting in a protein that is resistant to C3 exoenzyme ADP-ribosylation. Studies have shown that ADP-ribosylation of Rho-Asn41 by C3 exoenzyme does not affect the activity of the protein [86, 87], but prevents Rho translocation to the membrane that is required for its activation and subsequent interaction with effector molecules [83]. While C3 exoenzyme recognizes RhoA, B and C, but not RhoE, EDIN ribosylates all four proteins. This results in the disassembly of actin filaments (fig. 3).

Glucosylating Toxins. Glucosylating toxins, also referred to as large clostridial toxins, are proteins with a molecular weight of approximately 250–300 kD. The family consists of *C. difficile* toxin A and B (ToxA, ToxB), *Clostridium sordellii* LT and hemorrhagic toxin, and *Clostridium novyi* α toxin (α-novyi). In *Clostridium* isolates that cause intestinal disease and myonecrosis, the toxins are considered to be the main virulence factors.

The glucosylating toxins are single chain proteins containing three functional domains. In ToxA and ToxB, the carboxy-terminal domains contain multiple repeated sequences and are involved in cell surface receptor recognition. A trisaccharide (Gal-α1–3Gal-β1–4GlcNac) has been found to be the motif recognized by ToxA. The central domain contains hydrophobic sequences that are thought to mediate the translocation of the toxin across the membrane and the enzymatic and cytotoxic activity (DxD motif) of the toxins is found at the amino-terminus. Sequence analysis has revealed that ToxB and LT are highly homologous (76% amino acid sequence identity) and are more distantly related to ToxA and α-novyi (48–60% identity) [88].

The large clostridial toxins enter cells by receptor-mediated endocytosis. The cytotoxic effects are blocked by endosomal and lysosomal acidification inhibitors (monensin, bafilomycin A1, ammonium chloride) and the inhibiting effects can be bypassed by an extracellular acidic pulse. This indicates that the large clostridial toxins translocate from early endosomes upon an acidification step. At low pH, ToxB and LT induce channel formation in cell membranes and artificial lipid bilayers, and show an increase in hydrophobicity [89, 90]. This is thought to involve a conformational change and insertion of the toxin into the membrane mediated by the hydrophobic segment of the central domain.

Large clostridial toxins catalyze the glucosylation of 21-kD G proteins using UDP-glucose as the sugar donor (with the exception of α-novyi that preferentially uses UDP-N-acetylglucosamine) (fig. 3). The toxins transfer the glucose or N-acetylglucosamine moiety to the acceptor amino acid Thr37 of Rho or Thr35 of Rac, Cdc42 and Ras proteins [91, 92]. Rho complexed to GDI is not a substrate for glucosylation, and modified Rho does not bind to GDI [93].

The conserved glucosylated Thr (Thr37/35) is located in switch I of Rho/Ras GTPases. Thr37/35 is involved in the coordination of Mg^{2+} and subsequently to the binding of the β and γ phosphates of GTP. The hydroxyl group of Thr37/35 is exposed at the surface of the molecule in its GDP-bound form, which is the only accessible substrate for glucosylating toxins. Crystal structure analysis of Ras modified by LT shows that glucosylation prevents the formation of the GTP conformation in the effector loop of Ras, which is required for the interaction with the effector Raf [94]. Similar results were found when RhoA glucosylation by ToxB was studied [91]. It has been shown that glucosylation of GTPase by the toxins reduces the intrinsic GTPase activity, completely

inhibits GTPase-activating protein (GAP)-stimulated GTP hydrolysis, and leads to accumulation of the GTP-bound form of Rho at the membrane [93, 95].

The modification of Rho proteins by the large clostridial toxins induces cell rounding, the loss of actin stress fibers, reorganization of cortical actin, and disruption of the intercellular junctions. ToxB and ToxA have been reported to trigger apoptosis as a consequence of Rho glucosylation. In addition to the effects on the cytoskeleton, the inactivation of Rho proteins impairs other cellular functions such as endocytosis, exocytosis, NADPH oxidase regulation, and transcriptional activation mediated by JNK and/or p38 [88].

Proteolytic Toxins. YopT is one of the six Yop effector proteins which are injected into host cells by the *Yersinia* type III secretion system. This protein inactivates Rho-GTPases leading to the disruption of actin filaments and the accumulation of inactive RhoA in the cytosol (fig. 3). Recently, it has been reported that YopT is a cysteine protease that cleaves prenylated Rho-GTPases near their carboxy-termini and results in the release of these proteins from the membrane [96].

Rho-GTPases Inactivating Toxins by GAP Activity. As discussed earlier, *Salmonella* enters nonphagocytic cells by delivering effector proteins, such as SopE, into the host cell cytosol by the type III secretion system that directly modulates host actin dynamics to facilitate bacterial uptake. Importantly, the infected cells quickly recover from the above-mentioned cytoskeletal rearrangements. It has been shown that the reversal of actin cytoskeleton rearrangements is promoted by SptP, another type III-secreted protein, which acts as a GAP for Cdc42 and Rac.

SptP is a modular molecule that consists of an amino-terminal domain that shares sequence similarity with YopE of *Yersinia* spp. and ExoS of *P. aeruginosa* and binds to Rac and Cdc42 but not Rho in the GTP-bound form. The carboxy-terminal domain is related to YopH and several eukaryotic tyrosine phosphatases [97]. Crystal structure analysis revealed that SptP binds Rac1 exclusively through an amino-terminal four-helix bundle domain that targets the nucleotide and both the switch I and switch II regions of the GTPase.

Interestingly, eukaryotic GAPs show a larger surface of interaction with Rho-GTPases than SptP. This outlines the minimal structure involved in the GAP activity and argues for a convergent evolution of eukaryotic and bacterial GAPs. SptP binding to Rac does not change the conformation of the carboxy-terminal domain (tyrosine phosphatase domain). It is possible that the GAP domain targets the tyrosine phosphatase to its relevant substrate(s) [98, 99] and it has been speculated that the tyrosine phosphatase activity of SptP is involved in the downregulation of the subsequent nuclear response to Cdc42 and Rac stimulation [100]. While SopE is rapidly degraded by the proteasome pathway, the

degradation kinetics of SptP is much slower, permitting the transient reorganization of the actin cytoskeleton involved during bacterial invasion [101].

YopE from *Yersinia*, ExoS and ExoT from *P. aeruginosa* are secreted into macrophages by the type III secretion system and display a GAP activity towards Rho-GTPases. Despite the fact that the amino acid sequences are not highly conserved among these proteins, the GAP domains of YopE and ExoS show a similar structure to that of SptP with a conserved Arg finger that is essential for activity [102]. These factors induce actin cytoskeleton disorganization and cell rounding, and support the antiphagocytic activity permitting the survival of the bacterium [103, 104]. In addition, ExoS exerts an ADP-ribosyltransferase activity towards several proteins including Ras [105].

Concluding Remarks

Whilst most of the bacterial toxins form pores that act on cell membranes, many of them have the ability to enter host cells and enzymatically modify intracellular targets. As discussed in the present review, while some toxins contain specific translocation domains that attach to the cell membrane forming small pores, others lack such domains and are directly injected into cells by a type III secretion system.

Over the last years, evidence has accumulated showing that many bacterial toxins interfere with physiological processes by modulating host effector systems. In contrast to the host, bacteria, however, are not able to regulate these cascades, since toxins often act in an uncontrolled manner. This may trigger a noxious amplification of the signal and lead to severe systemic complications from the infection. Among the numerous potential cellular targets, bacterial toxins have only selected some key physiological pathways, such as the inactivation of EF and ribosomal RNA, leading to the inhibition of protein synthesis, as well as interfering with cell homeostasis by stimulating the overproduction of secondary messengers. It is also interesting to note that even though the regulation of actin polymerization requires a large number of proteins, bacterial toxins only act on two essential targets, namely monomeric actin and Rho-GTPases.

Many toxins target the same host effector systems; however, the physiological effects can differ from species to species. For instance, *Clostridium* secretes actin-modifying exotoxins, which act at a distance from the bacterium and disrupt cell barriers and tissues, permitting massive bacterial colonization of necrotic tissues. In contrast, some bacteria use specific toxins at the site of infection which interfere with the cytoskeleton, facilitating the invasion into target cells or preventing phagocytosis.

Finally, the specificity of bacterial toxins makes these molecules highly attractive as potential therapeutic agents (for instance, botulinum neurotoxins and immunotoxins), valuable tools in cell biology and the vectorization of molecules into cells.

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Capsular Polysaccharides and Their Role in Virulence

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Bacterial pathogens exhibit a number of virulence factors that enable them to invade and colonize the tissues of host organisms. A number of these virulence factors are displayed on the cell surface and include adhesins that mediate attachment to host cells, toxins that may be secreted resulting in host tissue damage, and the possession of molecules that render them resistant to host antimicrobial defences. Capsular polysaccharide (CPS) has long been recognized as an important virulence determinant in isolates capable of causing infection in humans and animals [1]. CPS is found on the outermost surface of a wide range of the bacteria [2] and may be linked to the cell surface via covalent attachments to phospholipid or lipid A molecules [3]. In contrast, extracellular polysaccharide (EPS) molecules appear to be released onto the cell surface with no visible means of attachment. Such EPS can be loosely associated with the cell surface and easily sloughed off as slime.

CPS molecules are highly hydrated and typically constitute more than 95% water [4]. They are composed of repeating single monosaccharide units that are joined by glycosidic linkages. CPS may be homo- or heteropolymers and can be substituted with both organic molecules such as acetyl groups, and inorganic molecules such as phosphate. In addition, two monosaccharides may be joined in a number of configurations due to the presence of multiple hydroxyl groups within each monosaccharide that may be involved in the glycosidic linkage. Thus, CPS are a diverse range of molecules that can differ not only in their constituent monosaccharides but also in the manner in which they are joined. This diversity is illustrated in bacterial species such as *Escherichia coli* where over 80 distinct capsular serotypes have been described while in *Streptococcus pneumoniae*, there are over 90 capsular serotypes. The introduction of branches and substitution with organic or inorganic molecules to polysaccharide chains adds a further layer of structural complexity. However, chemically identical CPS may also be synthesised by

different bacterial species. The group B capsule of *Neisseria meningitidis*, a homopolymer of α2,8-linked N-acetylneuraminic acid (NeuNAc), is identical to the K1 antigen of *E. coli* [5], while the CPS of *Pasteurella multocida* type D is identical to the *E. coli* K5 capsule which comprises repeating disaccharides of glucuronic acid linked to N-acetylglucosamine [6]. The apparent conservation of particular CPS structures between taxonomically diverse genera of bacterial species raises intriguing questions regarding the evolution of capsule diversity and the acquisition of capsule biosynthesis genes.

Functions of Bacterial Capsules

As the polysaccharide capsule represents the outermost layer of the bacterial cell, it is not surprising that the capsule mediates interactions between the bacterium and its immediate environment. Accordingly, a number of functions has been ascribed to bacterial capsules. Each of these functions (resistance to desiccation, adherence, resistance to nonspecific host immunity, resistance to specific host immunity) is directly relevant to pathogenicity and as such contributes to the role of CPS as a virulence factor.

Resistance to Desiccation
As CPS are highly hydrated molecules that surround the cell surface, they may protect bacteria from the harmful effects of desiccation [7]. This property is probably most relevant in the transmission and survival of encapsulated bacteria in the environment demonstrated in the cases of isolates of *E. coli*, *Acinetobacter calcoaceticus* and *Erwinia stewartii*, which have been shown to be more resistant to desiccation than their isogenic acapsular mutants [8]. Furthermore, the capsule probably provides protection during transmission from host to host. In the case of *E. coli*, genes encoding enzymes for the biosynthesis of capsular colanic acid have been shown to be upregulated in response to desiccation [8]. While the mechanism of regulation is unclear, it is thought that external osmolarity is altered during desiccation, and it has been shown that expression of alginate EPS of *Pseudomonas aeruginosa* as well as expression of the Vi CPS of *Salmonella typhi*, which is essential for virulence, are increased in response to high osmolarity [9, 10].

Adherence
CPS may mediate adhesion of bacteria to surfaces (both biotic and abiotic) and to each other. Adhesion to abiotic surfaces may result in the establishment of biofilms and EPS-mediated interspecies co-aggregation within biofilms can enhance colonization of various ecological niches [11]. In addition, growth of

bacteria as a biofilm may offer some protection from phagocytic protozoa and present nutritional advantages, while it is thought that the presence of EPS acts as a permeability barrier against antimicrobial agents [12]. While adhesion to host tissues is undoubtedly a multifactorial process involving an array of bacterial surface components, CPS has been implicated in the adhesion of a number of human pathogens to host tissues. *Streptococcus pyogenes* or group A *Streptococcus* (GAS) is responsible for a range of clinical infections including skin infections, acute rheumatic fever, streptococcal pharyngitis, streptococcal toxic shock syndrome and necrotizing fasciitis [13, 14]. In the development of pharyngitis, colonization of the pharynx by streptococci not only represents a vital stage in the life cycle of GAS, but it is also likely that that the pharynx serves as a reservoir for infection from which GAS may be disseminated to other hosts as well as causing invasive infections such as necrotizing fasciitis. It has been demonstrated that the hyaluronic acid capsule of GAS binds to CD44 molecules on the surface of human keratinocytes, the predominant cell type in skin and the pharyngeal epithelium [15]. Once bound, bacterial contact with the epithelial surface induces lamellipodia formation on the surface of keratinocytes, which is not observed in an isogenic acapsular mutant [16]. Gram-negative pathogens such as *Salmonella* and *Shigella* spp. also induce lamellipodia formation following binding to host epithelial cells; however subsequent fusion of the lamellipodia entraps the bacteria, resulting in their internalization. GAS are inefficiently internalized as a consequence of the possession of their hyaluronic acid capsule. Furthermore, the binding of GAS to CD44 induces marked cytoskeletal rearrangements and cell signalling events leading to the opening of intercellular junctions, which is thought to promote tissue penetration by GAS [16]. Clearly this is not the case for all encapsulated pathogens, as the case of GAS involves molecular mimicry, with the CPS being identical to host hyaluronic acid. In other pathogens, initial attachment to host cells has been shown to be inhibited by encapsulation, as is the case for binding of *Klebsiella pneumoniae* to epithelial cell lines in vitro [17]. Paradoxically, encapsulated isolates of the same strain adhered better to a mucus-producing cell line than an acapsular mutant. These data suggest that in some cases the CPS may promote initial colonization of the mucus layer, while subsequent interaction with the underlying epithelial layer is reduced by the presence of a capsule, presumably due to the masking of bacterial components required for specific interaction with the epithelial surface. These observations support the notion that there is some form of co-ordinate regulation of capsule expression during the early stages of infection.

Resistance to Non-Specific Host Immunity

During invasive infections of humans and animals by encapsulated pathogens, interactions between the bacterial CPS and immune system of the

host play a critical role in determining the fate of the infection [18]. During an innate host response, the bacterial capsule may confer some resistance to complement-mediated killing. The main function of the complement system is the binding of host peptides to foreign organisms. Once bound, these are recognized by specific complement receptors on host phagocytes that facilitate opsonization and subsequent destruction. Thus, activation of the complement cascade involves an array of serum and cell surface proteins and three pathways of activation are recognized. In the classical pathway, an antibody response is generated, while the alternative pathway can be activated in the absence of specific antigen-antibody recognition. The mannan-binding lectin pathway recognizes surface polysaccharides and then activates the complement cascade [19]. These pathways generate C3 convertases that cleave C3 (the major complement component) to C3b, which can then bind to the cell surface. Factor C3b and its degradation product iC3b are the primary complement opsonins [20]. In the absence of specific antibody, CPS is thought to activate the alternative pathway in which C3b binds non-specifically to the bacterial surface. Bound C3b is then activated by interaction with factor B and forms the C3 convertase C3bBb, which binds to the bacterial surface along with further C3b. This complex termed $C3b_2Bb$ acts as the C5 convertase and promotes formation of the membrane attack complex (MAC), which can form pores in certain bacteria, causing their destruction.

CPS that contain NeuNAc are known to be poor activators of the alternative pathway [21, 22] and it is thought that this is because NeuNAc binds directly to factor H [21]. Bound factor H promotes the binding of factor I to C3b, forming iC3b, which breaks the amplification loop of the cascade, which in turn prevents formation of the MAC [23]. In such cases, the bacterial capsule usually acts in concert with other surface structures such as the O-antigen of lipopolysaccharide to confer resistance to complement-mediated killing [24]. Thus, a particular combination of surface structures can confer a high degree of resistance to the innate immune response. In the case of other encapsulated pathogens, it is thought that the presence of a CPS may actually provide a barrier to complement components by physically masking underlying surface structures that would normally be potent activators of the alternative pathway [24].

Finally, CPS may confer resistance to complement-mediated opsonophagocytosis. In the case of *Staphylococcus aureus*, the presence of a thick capsule has been shown to be antiphagocytic, as it interfered with recognition of cell-bound C3b and iC3b by phagocytic receptors [25]. Similar observations have been made in the case of *S. pneumoniae* where CPS also appears to block cell-bound C3b [26]. Furthermore, many CPS are highly negatively charged molecules and may also confer resistance to phagocytosis [1, 27, 28]. In addition to these direct interactions between CPS and components of the complement system, certain

CPS may modulate the host's immune system by stimulating the release of certain cytokines resulting in the disruption of the cell-mediated immune response [29]. One such example is the CPS of *K. pneumoniae*, which was shown to induce high levels of interleukin-10 (IL-10) in experimentally infected mice, in contrast to an acapsular mutant [30]. High levels of IL-10 inhibit gamma interferon-induced activation of macrophages, and therefore cell-mediated reactions such as delayed-type hypersensitivity, which are normally visible 24–48 h after infection.

Resistance to Specific Host Immunity

Although many CPS elicit a specific (antibody-mediated) immune response in the host, a certain small set of CPS are able to confer some resistance. Capsules such as those that contain NeuNAc, e.g. *E. coli* K1 and *N. meningitidis* serogroup B [31] in addition to the *E. coli* K5 polysaccharide which is identical to N-acetyl heparosan (precursor in heparin/heparan sulfate biosynthesis) [32], are poorly immunogenic. Infected individuals only mount a poor immune response to these antigens as a consequence of the structural similarities of these capsules to host polysaccharides encountered abundantly in the extracellular matrices [18, 33]. As a result, the expression of these capsules that mimic host structures provides protection against the specific arm of the host's immune response.

Polysaccharide Capsules of Pathogenic *E. coli*

A large number of capsule gene clusters, representing various capsular serotypes, have been identified and cloned from a number of gram-negative pathogens. In all cases, the capsule genes are clustered at a single locus allowing for the co-ordinate regulation of capsule gene expression. Each of the capsule serotypes appears to be represented within *E. coli*, and to date, *E. coli* capsules are amongst those most intensively studied. Thus capsule gene clusters of *E. coli* are regarded as a paradigm for capsule gene clusters in gram-negative bacteria.

As previously mentioned, over 80 different serologically and chemically distinct types of polysaccharide capsule have been described in *E. coli* [34]. Termed K antigens, these have been classified into four functional groups (table 1) based on a number of biochemical and genetic criteria [35]. Most pathogenic extra-intestinal *E. coli* express group 2 K antigens [2]. Group 2 CPS represent a heterogeneous group concerning composition, while in terms of structure and cell surface assembly they resemble the capsules of other gram-negative pathogens, *N. meningitidis* and *Haemophilus influenzae*.

Table 1. Classification of *E. coli* capsules [adapted from 34]

Characteristics	Group			
	1	2	3	4
Former K antigen group	IA	II	I/II or III	IB (O-antigen capsules)
Co-expressed with O serogroups	Limited range (O8, O9, O20, O101)	Many	Many	Often O8, O9 but sometimes none
Co-expressed with colanic acid	No	Yes	Yes	Yes
Thermostability	Yes	No	No	Yes
Terminal lipid moiety	Lipid A core in K_{LPS}; unknown for K antigen	α-Glycerophosphate	α-Glycerophosphate	Lipid A core in K_{LPS}; unknown for K antigen
Direction of chain growth	Reducing terminus	Non-reducing terminus	Non-reducing terminus?	Reducing terminus
Polymerization system	Wzy-dependent	Processive	Processive?	Wzy-dependent
Transplasma membrane export	Wzx (PST)	ABC-2 exporter	ABC-2 exporter?	Wzx (PST)
Elevated levels of CMP-Kdo synthetase	No	Yes	No	No
Genetic locus	*cps* near *his* and *rfb*	*kps* near *serA*	*kps* near *serA*	*rfb* near *his*
Thermoregulated (not expressed below 20°C)	No	Yes	No	No
Positively regulated by Rcs system	Yes	No	No	No
Model system	K30	K1, K5	K10, K54	K40, O111
Similar to	*Klebsiella, Erwinia*	*Neisseria, Haemophilus*	*Neisseria, Haemophilus*	Many genera

A model for assembly and attachment of group 2 capsules to the cell surface is shown in figure 1.

Genetic Organization and Regulation of *E. coli* Group 2 Capsule Gene Clusters
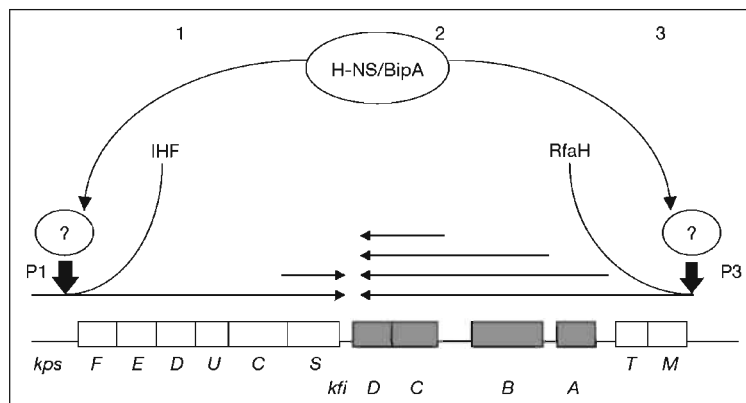
A number of group 2 capsule gene clusters have been cloned, and analysis has revealed that they have a conserved modular genetic organization, consisting
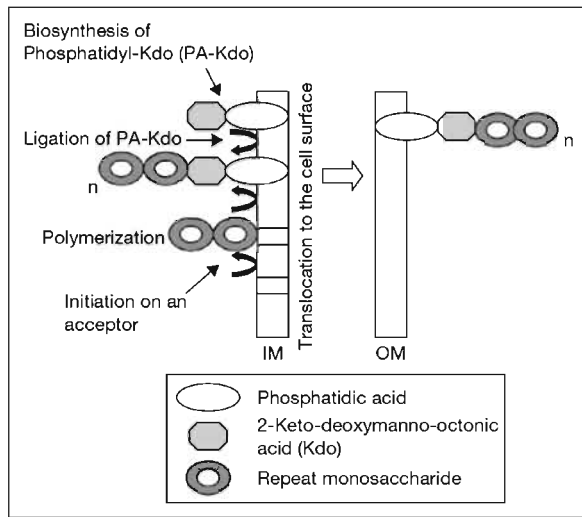
Fig. 1. A model for the assembly of group 2 capsules. Polysaccharide is polymerized at the non-reducing terminus and subsequently ligated to phosphatidyl-Kdo (PA-Kdo) at the reducing end prior to export across the cytoplasmic membrane. The presence of PA-Kdo may act as a motif for the export proteins as structurally diverse group 2 polysaccharides are all exported via the same conserved export proteins. IM = Inner membrane; OM = outer membrane.

of three functional regions (fig. 2). Furthermore it appears that this modular organization is applicable to capsule gene clusters of other bacteria [2]. Gene expression is achieved following transcription from two convergent promoters P1 and P3 which flank regions 1 and 3, respectively. Regions 1 and 3 are conserved amongst group 2 gene clusters and encode proteins necessary for the transport of the polysaccharide from its site of synthesis to the cell surface. Region 2 is serotype specific and encodes the enzymes responsible for biosynthesis (where necessary) and polymerization of the individual monosaccharides that comprise the particular polysaccharide. The size of this region is variable; however, size is thought to reflect the complexity of the polysaccharide, as region 2 in isolates that produce CPS with complex structures often encodes a larger number of open reading frames [36].

Region 1 comprises 6 genes *kpsFEDUCS* organized in a single transcriptional unit (fig. 2) that encode proteins involved in transport of the polysaccharide. A single *E. coli* σ^{70} promoter (P1) has been mapped 225 bp upstream of *kpsF* and transcription from P1 generates an 8.0-kb polycistronic transcript that is subsequently processed to generate a stable 1.3-kb *kpsS*-specific transcript [37]. This may facilitate the differential expression of KpsS, which may influence the attachment of phosphatidyl-Kdo (2-keto-3-deoxymanno-octonic acid) to nascent

Fig. 2. Genetic organization and regulation of *E. coli* group 2 capsule gene clusters. In this example, the gene cluster of *E. coli* K5 is shown. The numbers at the top refer to the three functional regions; the serotype-specific region, region 2, is shaded. P1 and P3 represent the region 1 and 3 promoters, respectively, and the straight arrows denote the major transcripts.

polysaccharide and regulate its entry into the export machinery. An intragenic Rho-dependent transcriptional terminator has also been identified within *kpsF*. This may play a role in regulating transcription by preventing synthesis of untranslated region 1 transcripts under conditions of physiological stress [38].

Region 3 of the gene cluster contains two genes *kpsM* and *kpsT* organized in a single transcriptional unit [2, 39]. The promoter (P3), which has a typical *E. coli* σ70 –10 consensus sequence but no –35 motif, has been mapped 741 bp upstream of the initiation codon of *kpsM*. No consensus binding sites for other alternative σ-factors or other DNA-binding proteins have been identified [40]. However, region 3 is subject to control by an antitermination process, conferred by RfaH and *ops* elements. A *cis*-acting regulatory sequence termed *ops*, which is essential for the function of RfaH has been identified 33 bp upstream of the initiation codon of *kpsM* [40]. The *ops* element, with the sequence GGCGGTAC, is contained within a larger regulatory element of 39 bp termed JUMPstart (just upstream from many polysaccharide-associated gene starts) [41]. RfaH is known to regulate a number of gene operons in *E. coli* including the hemolysin operon and the gene clusters for LPS core and O-antigen biosynthesis [42, 43]. In addition, RfaH is a homolog of NusG, an essential transcription elongation factor that is necessary for Rho-dependent transcription termination and bacteriophage λ-N-mediated antitermination. RfaH is thought to act as a transcriptional elongation factor that allows transcription to proceed over long distances. As such, mutations in *rfaH* give rise to increased transcription polarity throughout RfaH-regulated operons without disrupting initiation from operon promoters [42].

To act, *ops* elements must be located on the nascent mRNA transcript, where they recruit RfaH, and perhaps other proteins, promoting transcription elongation. It is thought that the JUMPstart sequence on the mRNA molecule may permit the formation of stem-loop structures at the 5′ end, which mediate the interaction with RfaH [41]. A mutation in *rfaH* or deletion of the JUMPstart sequence has been shown to abolish capsule production in *E. coli* K1 and K5 [40] and serves to confirm the role of RfaH in the regulation of group 2 capsule gene clusters.

The genetic organization of region 2 is serotype specific and differs among group 2 K antigens. In the case of K5, region 2 comprises 4 genes *kfiABCD* [44] while K1 comprises 6 genes *neuDBACES* [39]. In each case, transcription of region 2 proceeds in the same direction as that of region 3, which is important in the RfaH-mediated regulation of region 2 expression [40].

A feature of group 2 capsule gene expression pertinent to pathogenicity is that transcription from both P1 and P3 is temperature regulated, enabling capsule expression at 37°C but not at 18°C [37, 45]. Temperature regulation is in part achieved via the action of the global regulatory protein H-NS (histone-like nucleoid-associated protein), since *hns* mutants show comparable levels of transcription from P1 at both 18 and 37°C, albeit lower than those usually seen in a wild-type strain at 37°C, indicating that H-NS is required for maximal transcription at 37°C as well as repression at 18°C [46]. This situation is analogous to the H-NS-mediated thermoregulation of the *virB* promoter in *Shigella flexneri*. In this system however, activation of the *virB* promoter has an absolute requirement for the AraC-like protein VirF [47]. It is not yet clear whether an AraC-like transcriptional activator is involved in modulating transcription from P1 and P3.

Mutations in *bipA* also result in increased transcription at 18°C and reduced transcription at 37°C [44]. Although this phenomenon mirrors the effect of mutations in *hns*, the phenotype of a *bipA* mutant cannot be explained by a loss of H-NS function as this is unaffected in a *bipA* mutant. BipA was first described as a tyrosine-phosphorylating protein in enteropathogenic *E. coli* (EPEC) [48]. EPEC *bipA* mutants are unable to trigger cytoskeletal rearrangements in host cells, are hypersensitive to BPI (bactericidal/permeability-increasing protein) protein and demonstrate increased flagella expression and motility [48]. Furthermore, BipA is a GTPase with similarity to the TetO resistance protein and elongation factor G (EF-G), both of which interact with ribosomes. These data have led to the suggestion that BipA may represent a novel class of regulators that interact directly with the ribosome by regulating translation elongation [48]. It is therefore likely that BipA does not regulate P1 and P3 directly, but that regulation is achieved via interaction with other proteins that do modulate transcription of P1 and P3. This hypothesis is currently under investigation in our laboratory.

At 37°C, the mechanism of temperature regulation is further complicated by the interaction of integration host factor (IHF) with P1. IHF is required for

optimal capsule gene expression and IHF binding sites have been identified that flank P1 [40]. IHF usually acts to facilitate the activity of other regulatory proteins [49] and as such it is likely that IHF also acts in concert with an as yet unidentified regulatory protein or proteins that act to control transcription from regions 1 and 3 at 37°C. However, the lack of IHF binding sites in the region 3 promoter [40] demonstrates that the requirement for IHF is not absolute.

In summary, the regulation of *E. coli* group 2 capsules is complex, involving a number of overlapping regulatory circuits. However, of relevance to pathogenicity and virulence, there are still many unanswered questions. How are changes in temperature, such as those concomitant with entry into a susceptible host, sensed and transduced to induce changes in gene expression? How is capsule gene expression regulated in response to attachment and interaction with host cells? While it is known that encapsulation is an important virulence factor, understanding the regulation of capsule expression during the stages of infection still represents an interesting challenge. One further important area for future research is the understanding of the export of CPS onto the bacterial cell surface. Such understanding will lend itself to the design of chemotherapeutic agents targeted to selectively disrupt capsule export and therefore combat infections caused by encapsulated bacteria.

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Fimbriae, Pili, Flagella and Bacterial Virulence

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Filamentous surface structures have been detailed on gram-negative enterobacteria since the introduction of electron microscopy [1]. The bacteria appeared to express two types of extruding appendages: wavy flagella exceeding the length of the bacterium itself, and more rigid but somewhat thinner 'fimbriae' [2] or 'pili' [3]. Soon after the description of fimbriae and pili, it was realized that their expression correlated with the ability of the bacteria to bind to cells from potential host organisms. Fimbriated and piliated bacteria agglutinated erythrocytes in a fashion resembling classical hemagglutination and adhered to host epithelial cells [2, 4–6]. Moreover, for some strains bacteria-induced hemagglutination was inhibited by the addition of the monosaccharide mannose. This suggested that mannose is used as a receptor for adherence and that the free mannose functions as a hapten. For other bacteria-erythrocyte reactions hemagglutination was not inhibited by mannose implying another receptor selectivity in the binding reaction [7–9].

Since the initial notion that fimbriae or pili function as specific adhesive organelles that aid bacterial colonization of mucosal surfaces, a myriad of bacterial adhesive factors have been described, many of which have turned out to act as virulence factors and to have a fimbrial morphology. Thematically, therefore, the unique but often separate binding specificity expressed by the various fimbriae participates in determining host and mucosal tropism (fig. 1) [10, 11]. While such notions remain rather unchallenged, many recent observations imply additional functions for fimbriae. Distinct fimbriae are known to bind plasma proteins and to initiate intrinsic proteolytic cascades [12], whereas others are capable of activating Ca^{2+} influx and signal transduction cascades in host target cells [13]. In addition, fimbriae have been shown to act as invasion and motility factors, whereas bacterial flagella that typically mediate bacterial
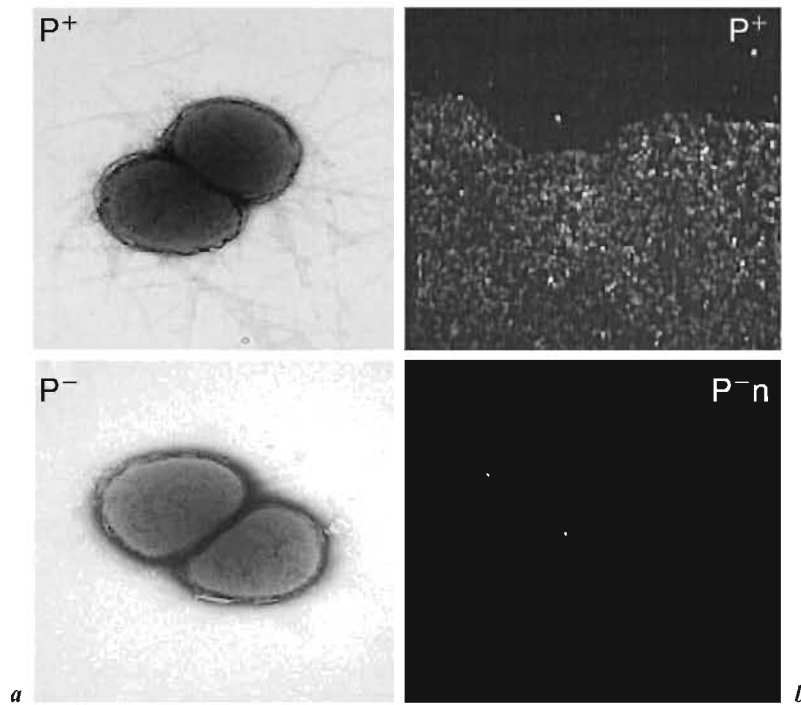
Fig. 1. a Transmission electron microscopy of type IV piliated (P^+) and nonpiliated (P^-) *N. gonorrhoeae.* ***b*** The adherence of fluorescently labelled P^+ and P^- bacteria to human cervical tissue sections is shown. The presence of type IV pili enables the bacteria to attach.

motility have also been ascribed functions in terms of bacterial adherence [14] and in the initiation of proinflammatory responses [15]. One purpose of this review is to highlight the more recently defined virulence-associated functions of fimbriae, pili (see The Role of Fimbriae in Pathogenesis of Mammalian Hosts as Illustrated through a Few Examples) and flagella (see Flagella as Virulence Factors) using a few illustrating examples, and to argue that these organelles have a role in the infection pathogenesis beyond the first step of surface colonization.

Classification and Biosynthesis of Fimbrial Adhesive Factors

The early notion of variation among fimbrial adhesive virulence factors brought the need for classification schemes [16]. However, the multitude by

which various bacterial organelles with different binding specificities started to emerge soon implied that typing approaches could become difficult based on a single characteristic, such as antigenicity or receptor specificity. Even for a single fimbrial type, the antigenic variation could be significant [17], and for many fimbriae no defined receptor structure was identified. Furthermore, not all adhesive factors appeared typically 'fimbrial' in morphology although they showed receptor-specific binding abilities [18, 19]. Finally, in selected cases even flagella are known to function as adhesive organelles [14].

While fimbriae and flagella can be defined as distinct structures, they share the need to create a polymer architecturally outside the ordinary bacterial anabolic machinery. This is also reflected in the complex organization of genes that are needed for either fimbrial or flagellar biosynthesis. The elucidation of the precise events involved in fimbrial biosynthesis by several laboratories has clearly formulated distinct fimbrial 'families' and assembly pathways that actually can define groups of fimbrial types. Thus, a given assembly pathway can be used as a gross classification criterion [20], within which fimbriae can be defined based on receptor specificity or antigenic variation.

Fimbriae Produced through the 'Chaperone/Usher' Pathway

The classical 'common' type-1 fimbriae that mediate mannose-sensitive hemagglutination, and the P-blood-group-antigen-binding P-fimbriae, or Pap pili, are produced through the so-called 'chaperone/usher' pathway [21]. The biogenesis and the basis for receptor recognition have been extensively studied for these two types of fimbriae. Therefore, the defined biogenesis and function of type 1 and Pap pili have functioned as guidelines when dissecting the molecular biology of fimbriae not only belonging to this class, and as a 'reference system' when dissecting other types of bacterial adhesins.

Yet, fimbriae that belong to this 'chaperone-usher' family come in several different variants, and are not only defined to *Escherichia coli*. Gene clusters that provide the fimbrial subunits, protein chaperones and outer membrane anchors for the fimbrial shaft, as well as specific fimbrial regulatory genes code for these fimbriae. Altogether nine 'biosynthetic' genes and two intrinsic fimbrial regulatory genes are included in the *E. coli pap* gene cluster responsible for the expression of P-fimbriae [21, 22]. The fimbrial constituents are translocated to the periplasm through the housekeeping Sec system, and are met by the fimbrial chaperones once translocated. Principally, the chaperones translocate fimbrial subunits to the usher which then initiates translocation and polymerization of the fimbrial subunits across the outer membrane. Thus, the fimbria elongates from the proximal end of its shaft.

Initially, these fimbriae were considered genuine homopolymers of the fimbrial protein subunit, the fimbrillin [23]. Furthermore, isolated fimbriae that

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appeared as one major protein species in Coomassie-blue-stained SDS polyacrylamide gels could agglutinate cells, implying that receptor recognition was closely associated with fimbrial subunits [24]. However, during the dissection of P-fimbrial biosynthesis it became evident that the formation of fimbria and the ability of fimbriae to mediate adhesion or hemagglutination (receptor recognition) could be separated [25, 26]. This showed that the P-fimbriae actually were composite fibers. The fimbrial fibers include at least two distinct functions: the constitution of a filament and the recognition of the receptor; these functions were separable [27]. Indeed, in addition to the major fimbrial subunit PapA, P-fimbrial filaments were found to contain minor subunits, including the PapE, PapF, PapK and PapG proteins located at the distal end of the fiber [21, 27]. The ability to bind the receptor resided in the PapG subunit, whereas other tip-located Pap proteins functioned as initiators of fimbrial polymerization and for adapting PapG to the fimbrial shaft. However, PapA alone forms the micrometer long shaft and hence substantially dominates preparations of isolated fimbriae [26]. This may explain why isolated fimbriae perform receptor recognition but require sensitive staining techniques to reveal minor components in gel analyses.

Not surprisingly, type 1 fimbriae are also composite fibers [22, 28–30], and may even include minor components scattered throughout the filament [31]. This could be due to a need to enhance fimbrial polymerization, and/or due to a need to include receptor-binding entities along the fimbrial shaft [20, 31]. That is to say, the addition of minor nucleator or lectin components along the shaft could increase the efficiency of polymerization, or the avidity of the receptor-recognizing potential of the fimbrial filament. Other fimbriae belonging to this class include the *E. coli* S-fimbriae recognizing sialyl galactosides and type 1C fimbriae [21, 22, 32].

Crystallographic studies have demonstrated that the periplasmic chaperone not only fulfils a transporting function for the respective pilus subunit proteins in the periplasmic space as initially thought [33]. The pilus subunit proteins have an incomplete immunoglobulin fold, due to the lack of the seventh β-strand creating a large hydrophobic groove in the pilus subunit protein. In pilus biogenesis this groove is transiently occupied by the G1 strand of the chaperone [34]. At the site of the usher, the chaperone G1 strand is replaced by the amino-terminal extension of the next subunit protein to become incorporated via a donor strand exchange mechanism [35]. During donor strand exchange, the subunit undergoes a topological transition that triggers the closure of the groove and seals the amino-terminal extension of the incoming subunit in place [36]. These findings help explain the ordered assembly of pili heteropolymers. A contributing factor to the ordered assembly is the different affinities that chaperone-subunit complexes have for the outer membrane usher

protein [37]. Outer-membrane PapC molecular ushers discriminately recognize periplasmic chaperone-pilus subunit complexes. That the initiating step in pilus assembly is an interaction between the adhesin, in complex with the chaperone and the outer membrane usher, explains why the adhesin ends up at the pilus tip [38]. Evidently, comparable complex strategies of assembly are also applied by other fimbria and fimbrial types, and reflected in the multitude of participating gene functions [20, 39].

The atomic structures of three minor fimbrial lectin subunits or lectin domains associated with the cognate receptor have been determined [34, 40, 41]. While these three lectin proteins do not share obvious sequence identity, they share a remarkably similar structural outline. The three proteins in question that, respectively, recognize mannosides (FimH of the type 1 fimbriae), Galα1 $\rightarrow$ 4Galβ (PapG of the P-fimbriae) and terminal N-acetyl-D-glucosamine (GafD or F17-G of the F17 fimbriae) share an immunoglobulin-like folding pattern forming an ellipsoid structure [41]. The receptor-binding pockets, however, seem to be somewhat differently positioned in relation to the superimposed imaginary core structure [41]. Thus, although all these fimbrial lectin proteins share the ability to bind a small carbohydrate epitope and to become integrated into the fimbrial filament, the lectin proteins apparently have not evolved just through modifications in one existing carbohydrate-binding pocket.

Fimbrial lectins are interesting candidate antigens for vaccine development. Due to the incomplete structural nature of the adhesin, vaccine trials have been conducted with adhesin-chaperone dimeric complexes. The FimH/FimC complex provided protection against uropathogenic $E.$ $coli$ in both a murine and a primate cystitis model [42, 43].

The CS1 Fimbrial Family

Fimbriae belonging to the class of the CS1 fimbrial family are assembled in a manner that phenotypically resembles the 'chaperone-usher' pathway [20]. The CS1 fimbria forms the prototype of this class that includes several antigenic variants, including the classical CFA/I fimbriae of enterotoxigenic $E.$ $coli$ (ETEC), and the type II pili of $Burkholderia$ $cepacia$ [44, 45]. The CS1 fimbrial subunit CooA is translocated to the periplasm through a Sec-dependent pathway, and then assisted by a protein CooB with chaperone-like function [20, 46, 47]. CooA is then fed to a larger transmembrane protein CooC concomitant with fimbrial polymerization. However, polymerization needs the presence of a minor fimbrial subunit protein CooD, which functions both as an initiator and the lectin subunit [45, 48].

The constituents of the transport and assembly machinery do not show apparent amino acid sequence homology to the P-fimbrial chaperone or usher components. In addition, the number of CS1-specific genes that participate in

fimbrial biogenesis as well as the number of specific fimbrial components tend to be more restricted within the CS1 family. Still, the number of genes involved may not be a definitive characteristic of a fimbrial class; N-acetyl-D-glucosamine-binding F17 fimbriae also need only four genes for their expression in *E. coli* K12 and yet show many characteristics of the P-fimbrial family [48, 49].

Type IV Pili

Type IV pili are multifunctional adhesive structures expressed by a number of diverse microorganisms, including *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa*, *Dichelobacter nodosus* and *Moraxella bovis* [50]. Related structures have also been identified in *Vibrio cholerae* (toxin-coregulated pili, Tcp) and enteropathogenic *E. coli* (bundle-forming pili, Bfp) [51, 52]. Type IV pili are typically 5–7 nm in diameter and can extend several micrometers in length (fig. 1). They share an unusual (amino-terminal) N-methyl phenylalanine, a high conservation of the amino-terminal 32 amino acids, and a proposed immunogenic carboxy-terminal disulfide-bound region. As with other types of fimbriae, type IV pili are composed primarily of a single protein subunit, termed pilin, which are arranged in a helical conformation with 5 subunits per turn. In addition, and somewhat unorthodoxal for prokaryotic structural proteins, type IV pili can be glycosylated and/or phosphorylated depending on the bacterial species [53–57]. Type IV pilus assembly is hypothesized to occur within the cytoplasmic membrane or periplasm. The assembly of pili requires a nucleotide-binding protein, a polytopic inner membrane protein, the prepilin peptidase, and a multimeric outer membrane protein that forms a pore in the outer membrane for pilus protrusion [58].

One most astonishing aspect of type IV pili is their ability to intimate their initial contact through pilus retraction. A core set of mechanisms, fiber assembly and extension, fiber adhesion, fiber disassembly and retraction, account for these functions. Genetic analysis has revealed multiple clusters of genes, scattered through the microbial genome coding for type IV pilus biogenesis genes, as well as for major pilin and minor pilin-like proteins. The fact that almost 40 genes have been identified in *P. aeruginosa* as essential for biogenesis and functionality of type IV pili evidently reflects the complexity both in pilus assembly and function [59, 60]. While bacterial fimbriae belonging to chaperone/usher or CS1 family may have evolved through a divergent evolutionary need to produce sticky, surface-located adhesive organelles [20], type IV pili may share evolutionary origins with filamentous bacteriophages [61], and with genes required for bacterial type II protein export and DNA uptake systems [62].

Type IV pili bind to a variety of surfaces, including both 'inert' nonbiological surfaces, to other bacteria, as well as to eukaryotic cells. In the case of type IV

pili, the tip of the pilus binds to specific receptors on mammalian epithelial cells as an initial engaging event. Pili attached to cells are always observed anchored to surfaces at their distal end, and broken pili also only attach via an end [63]. In *P. aeruginosa*, the above-mentioned carboxy-terminal disulfide-bonded region is exposed at the tip of the pilus and binds the carbohydrate moiety of the asialo-GM1 and asialo-GM2 glycosphingolipids on epithelial cells [64, 65].

Consequently, type IV pili of *Neisseria* are composed of a major pilus subunit PilE and several other pilus-associated proteins, which have different functions in pilus assembly and adhesion [66, 67]. One of these proteins is PilC, which is associated with the tip and the shaft of the pili [68] and the basal part in the outer membrane [69]. Adhesion of *Neisseria* to cells requires PilC, which appears to function as a tip adhesin, although it is also found in the cell membrane. The pili of *Neisseria* recognize and interact with the cell surface receptor complement regulator CD46 [70].

Fimbriae Produced through the Extracellular Nucleator Pathway: Curli Organelles

Many enterobacteria are capable of expressing elongated surface organelles, called AgfA fimbriae, with an 'aggregative' and chemically robust character [71, 72]. AgfA fibers appear not as straight but rather as twisted, curly structures and hence are referred to as 'curli' fimbriae [73]. Curli fibers of *E. coli* and *Salmonella enterica* sv Tyhpimurium are coded for by the *cfg* gene cluster. The cluster consists of two divergently transcribed units that include the *csgABC* and *csgDEFG* genes, respectively. Although curli fibers are coded for genetic elements comparable in size to the P-fimbrial *pap* operon [74, 75], the curli fiber polymerization process is apparently different. Interestingly, curli fibers show all the typical characteristics of amyloid fibers, such as the binding to the dye Congo red. However, unlike amyloid formation in human neurodegenerative disorders such as Alzheimer's disease, curli amyloids require a specific assembly machinery [76]. Thus, the CsgA and CsgB fimbrial subunits appear to be secreted out from the bacteria [72, 74], where after an interaction between the subunits in the extracellular compartment then leads to polymerization. The CsgA subunit occurs in excess in the isolated filament, whereas in vitro both the CsgB subunit [72] and the isolated CsgA subunit [76, 77] are capable of self-polymerization. Thus, as in analogy with type 1, P- and CS1 fimbriae the assembly of curli organelles also involves a nucleator component (CsgB), proteins with apparent chaperone functions (CsgE) [76], or a nucleator center (CsgG) [78]. As with type IV pili, curli fibers have a rather diverse spectrum of receptor targets. Curli fibers are reported to mediate binding to mouse small intestinal epithelial cells [73], in addition to various plasma and

extracellular matrix proteins [12, 71, 79, 80]. One reason for this promiscuity might reside in the participation of curli in the formation of biofilms [81, 82]. A more flexible binding specificity might be more efficient in collecting various organic molecules into the biofilm as compared to an organelle with a highly specific, but concomitantly more narrow receptor repertoire. Since the CsgD transcriptional regulator also affects bacterial production of cellulose an important role of curli might be to interact with cellulose fibrils in an extracellular matrix [83].

The Role of Fimbriae in Pathogenesis of Mammalian Hosts as Illustrated through a Few Examples

Chaperone/Usher Fimbriae and Urinary Tract Infection
Adhesion
E. coli is by far the most common causative agent of urinary tract infections (UTI) [84]. Consequently, the role of *E. coli* fimbriae in the infection pathogenesis of UTI has been given much attention, and has been used as a template for the analysis of other fimbrial structures [21, 85].

The ability to express certain types and sets of fimbriae seems overrepresented among urinary tract isolates of *E. coli*. The expression of type 1 fimbriae appears to be both an important colonization factor and a factor contributing to the persistence in the bladder epithelium [85]. However, the pattern of mannose binding by the protein FimH is somewhat different among commensal and UTI *E. coli*; UTI isolates seem capable of binding *D*-mannose whereas commensals seem to prefer trimannoside structures [86]. This difference in specificity resides in minute differences in the FimH fimbrial lectin molecule as coded for by separate alleles of *fimH*. Uroplakins, or rather mannosides contained on uroplakin, are believed to be the actual epithelial receptor in the urinary tract [84, 87]. Thus, it appears that the type 1 fimbria can be equipped with different variants of FimH, and that the receptor preferences expressed by FimH in turn steer the mucosal tropism of the bacterial even within a single host organism.

The P-fimbriae is another group of bacterial adhesins often expressed by UTI isolates of *E. coli*, in particular among strains causing upper UTI and urosepsis [84, 88]. P-fimbriae recognize the core Galα1 → 4Galβ entity contained in blood group antigen-carrying globoseries glycolipids [78]. Thereby, as the receptor is present on cells lining the human urinary tract, it provides an adhesion target for P-fimbriated bacteria ascending from the bladder to the ureter and further up into the kidney [84]. As with the FimH protein of type 1 pili, PapG possesses allelic polymorphism: the class I, II and III adhesins. Of these,

the class II G adhesin recognizes most members of Galα1 $\rightarrow$ 4Galβ-containing globoseries glycolipids and has been considered important for kidney infection in persons with a nonobstructed urinary tract [84, 89–91].

Beyond Adherence

Besides mediating adherence to the urinary tract epithelium, type 1 and P-fimbriae have been implicated in the later phases of infection, and in the generation of innate proinflammatory responses in the infected urinary tract epithelium. First, although cystitis-associated *E. coli* have generally been regarded as noninvasive bacteria, type 1 fimbriated *E. coli* have been observed to enter human bladder epithelial cell lines in vitro in a FimH-dependent manner [87]. Invasion could be mimicked by applying FimH-coated beads, and invasion was associated with host protein tyrosine phoshorylation and host actin cytoskeleton rearrangement [92]. This suggests that FimH alone, in analogy with *Yersinia* invasion factor Inv [93], can activate host signal transduction events that subsequently trigger actin cytoskeletal rearrangements in the host leading to bacterial uptake. Later it was observed in a mouse cystitis model that the bacteria were internalized into bladder epithelial cells and subsequently formed a biofilm-like mass [94]. Apart from type 1 fimbriated bacteria, uroplakin was also found in the biofilm. Thus, type 1 fimbriae appear multifunctional in the pathogenesis of UTI; they mediate initial adherence, invasion and seem to participate in the formation of an intracellular biofilm.

Many types of fimbriae, including type 1, type 1C and P-fimbriae have all been associated with the induction of proinflammatory responses in epithelial cells [95–97]. Type 1 fimbriated *E. coli* induce cytokine expression from both A498 kidney epithelial cells as well as in bladder cell lines [96, 98]. However, in bladder epithelial cells the majority of the IL-6 response seems to derive from lipopolysaccharide (LPS) signalling through the CD14-TLR4 pathway [98]. Still, type 1/*fimH*$^+$ fimbriae appear to be somewhat more potent inducers of IL-6 as compared to type 1/*fimH*$^-$ bacteria in LPS-hyporesponsive A498 cells. Likewise, type 1C fimbriae, also associated with cystitis, augment bacterial IL-8 release from A498 cells [95]. It is thus possible that bacterial attachment, the prerequisite for the infection in the first place, is also one cause for the symptoms of cystitis.

The mechanism by which P-fimbriae induces signal transduction casacades in kidney A498 cells appears complex, and differs from those mechanisms used by type 1 fimbriae [96, 99]. Binding of P-fimbriated bacteria causes a release of ceramide in the target cells concomitant with an activation of cytokine release [96]. Cytokines, such as TNF-α, also cause the release of ceramide from sphingomyelin, which eventually results in the activation of transcription factor NF-κB [100]. It has thus been suggested that ceramide
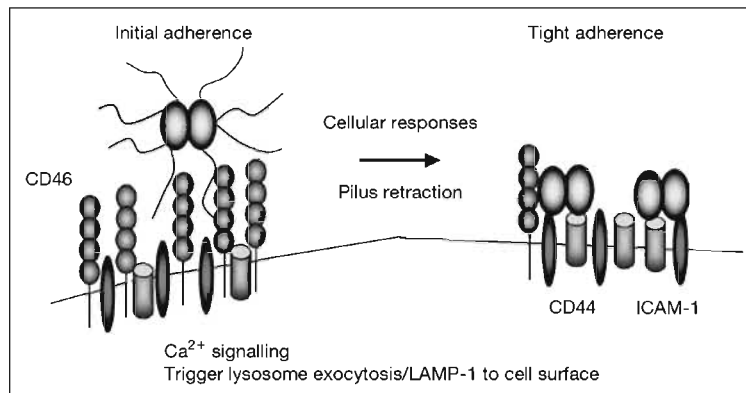
Fig. 2. Initial adherence of type IV piliated *Neisseria* involves initial contact with cell surface receptors followed by sophisticated cell signalling leading to tight adherence and invasion of host cells. Failure in the pilus retraction events and/or host cell signalling leads to lost or changed adherence patterns, and a loss of ability to enter and invade host cells.

release caused through attachment of P-fimbriated *E. coli* could induce nuclear responses as a result of ceramide release. Furthermore, the LPS-recognizing Toll-like receptor TLR4 has been implicated in P-fimbria-induced host responses [99]. Possibly, P-fimbriae can adapt both cermide- and TLR4-mediated signals to induce NF-κB nuclear translocation. Binding of P-fimbriated bacteria to A498 cells also caused an upregulation in the expression of TLR4 mRNA suggesting that one function of P-fimbria-mediated host cell responses might be to modify the surface of the host cell to better accommodate or promote the infection.

Type IV Pili in Sequential Attachment and Invasion
of Pathogenic Neisseria
Adhesion

The important initial interaction between pili of *Neisseria* and its host cell occurs through the receptor molecule CD46, a human cell surface protein involved in the regulation of complement activation. In cultured epithelial cells, binding of pili to CD46 is followed by release of Ca^{2+} from intracellular stores [13, 101]. This Ca^{2+} transient is sufficient to mediate exocytosis of a pool of the lysosomal/late endosomal vacuoles resulting in the increase of surface lysosomal components such as h-Lamp-1, and possibly other factors that could contribute to a tighter adherence of bacteria. During initial contact between bacteria and cells, pilus retraction exerts tensile forces upon the plasma membrane (fig. 2) [102]. The mechanical forces applied to the plasma membrane trigger actin polymerization accompanied by accumulation of phosphotyrosine-containing

proteins, which leads to the formation of compact microcolonies and so-called pilus-associated cortical plaques on the host cell [103, 104]. The cortical plaque structures are characterized by the accumulation of actin and actin-associated proteins, and trigger recruitment of transmembrane proteins such as CD44, ICAM-1, EGFR, and components of the cortical cytoskeleton, i.e. ezrin and cortactin, and contain tyrosine-phosphorylated host cell proteins beneath the microcolony [104, 105].

At later times after infection, bacteria disperse from the microcolonies, pili disappear, and individual diplococci become intimately associated with the host plasma membrane. Pilus loss, bacterial dispersal, and intimate adhesion are all blocked in a *pilT* mutant [106, 107]. The full set of rearrangements requires the expression of both type IV pili and PilT. Obviously, pilus retraction could account for elongation of microvilli towards the bacterial microcolony and bring the host cell and bacterial membrane into close contact [108]. For example *pilT* mutants of *N. gonorrhoeae* are unable to make intimate contact with or form attaching effacing lesions on epithelial cells [95]. *P. aeruginosa pilT* mutants are not infective in corneal tissue and exhibit reduced cytotoxicity to epithelial cells in culture [109–111].

To summarize, type IV pili do not only simply anchor the bacteria at the cell surface, they initiate a multistep adhesion cascade, which starts with a loose adherence and ends with the intimate attachment of bacteria [112]. Establishment of intimate attachment appears to require an intensive host-pathogen cross talk, and a complex sequence of bacteria-host cell interactions. Type IV pili also assist in the formation of biofilms [112] that may support further tissue colonization and protect the bacteria against antibodies and antibiotics.

Beyond Adhesion

In an experimental model system of *Neisseria* infection, using transgenic mice expressing human CD46, the crossing of the blood-brain barrier by bacteria occurred in CD46 mice but not in nontransgenic mice, indicating an important role for CD46 in meningococcal meningitis [113]. Intranasal infection of CD46 mice required piliated bacteria for the development of disease, supporting that CD46 facilitates pilus-dependent interactions at the epithelial mucosa.

Binding of Fimbrial Structures to Extracellular Components

Although a primary role of fimbriae indeed might be to mediate adhesion and subsequent events through binding to specific structures on host (epithelial) cells, it has recently become evident that fimbriae can also bind various connective tissue proteins, as well as plasma and serum proteins. Moreover,

binding to selected plasma components can induce subsequent intrinsic cascades leading to the activation of zymogen proteases and the release of biologically active host peptides [12, 80]. Such observations illustrate that fimbriae may contribute to the infection pathogenesis even after they have assisted adhesion and invasion.

The F17 fimbriae occur characteristically in *E. coli* isolates causing diarrhea and septicemia in newborn calves. F17 fimbriae mediate binding to the calf intestinal epithelium, which suggests a role for F17 fimbria in the intestinal colonization. In addition, the F17 fimbria is capable of binding plasminogen [114] and the extracellular matrix protein laminin [115]. Binding to laminin is inhibited by the receptor analogue N-acetyl-*D*-glucosamine, indicating that carbohydrate receptors on the extracellular matrix protein are recognized by the minor fimbrial lectin protein GafD [115]. The binding to plasminogen is not inhibited by the receptor analogue, but instead the binding leads to conversion of plasminogen to proteolytically active plasmin. Binding and activation of plasmin is not unique to F17 fimbriae. For instance, it has been shown that meningitis-associated S-fimbriae and *S. enterica* sv Typhimurium type 1 fimbriae as well as curli fibers are both plasminogen binders and activators [79, 114, 116, 117]. Such observations suggest that fimbriae may assist bacteria during tissue dissemination by directing them to extracellular matrix proteins, and by coating them with proteolytically active proteins that enable the bacteria to penetrate through the tissue. Indeed, enterobacteria capable of binding and activating plasminogen have been shown to degrade extracellular matrix proteins, and to penetrate reconstituted basement membranes in vitro [117].

Yet another aspect of binding to plasma proteins is illustrated by the ability of bacterial curli fimbriae to activate the contact phase pathway of the coagulation system, and thereby to induce proinflammatory reactions [12, 80]. Factor XI, factor XII, prokallikrein and H-kininogen are absorbed to curilated *E. coli* and *S. enterica* sv Typhimurium, but not to isogenic noncurliated mutants. Binding of the contact phase proteins by the curliated bacteria lead to a rapid release of vasodilatory bradykinin from kininogens and to prolonged clotting times of the infected plasma. While it is difficult to ascertain the biological function of such reactions, possibly they reflect an aspect of the innate line of defenses, such observations imply that a more massive encounter with curliated bacteria may contribute to the symptoms of septic shock [80].

Pili and Motility

The term twitching motility was used by Lautrop [118] in 1961 to describe flagella-independent spasmodic movements of bacteria. Twitching motility occurs in a wide range of bacteria, and has been well studied in *N. gonorrhoeae* and *P. aeruginosa*. It occurs on solid, wet surfaces and is mediated by type IV

pili. Twitching motility occurs by extension, tethering, and then retraction of type IV pili, which operate in a manner similar to a grapping hook, which has been shown by elegant studies in *N. gonorrhoeae* [102], *Myxococcus xanthus* [119], and *P. aeruginosa* [111].

Type IV pili serve as an initial bridge between bacteria and cells, and twitching motility allows bacteria to spread in the infected tissue. *P. aeruginosa* is an important pathogen, being the major cause of lung damage in patients suffering from cystic fibrosis as well as of opportunistic infections in immuno-compromised individuals, such as burn victims or patients undergoing chemotherapy. Twitching motility has been shown to be important for infection by *P. aeruginosa* as well as for biofilm formation, which appears to be involved in chronic infection [110, 112].

Type IV pili generate considerable force by retraction [102, 120]. For some pilus-dependent functions, the amount of force is critical, e.g. in host-cell responses and movement of bacteria through viscous mucous layers. Although *pilT* mutants adhere and colonize surfaces, *pilT* mutants are avirulent in many experimental model systems. PilT mutants are unable to retract their pili, leading to hyperpiliation and loss of twitching motility. It could be speculated that a signal could pass from the tip of the fiber to its base, by the propagation of a helix dislocation or a mechanical force such as tension, compression or flexion. The dislocation signal that reaches the base of the pilus could induce a beneficial movement response to the cell.

PilT, an ATPase associated with various cellular activities (AAA), seems to act as a molecular motor [121, 122]. Pilus retraction is thought to occur by filament disassembly mediated by PilT, a process that has been estimated to occur at around 1,000 pilin subunits per second. Genetic studies and structural data support the following molecular model. The cytoplasmic membrane has a reservoir of the prepilin subunits that are cleaved by PilD, the prepilin peptidase, and then polymerized into pili. In the model PilT is actively involved in the dissociation of a pilus. PilT is a member of the GspE family of hexameric AAAs, and one PilT unit could hydrolyze several (up to six) ATP molecules in the process of dissociating one pilin subunit. It is possible that epithelial cells sense the amount of force generated by pilus retraction and respond in a similar manner.

Phase Variation of Pilus Structures

As mentioned above, fimbriae and pili of the same type can be expressed as antigenic variants. For example, separate strains of UTI *E. coli* can express separate antigenic variants of the major fimbrial subunit protein [123], and a single strain can contain more than one P-fimbrial gene cluster [124]. Furthermore, as different P-fimbrial gene clusters may contain separate *papG* alleles [89], and

as P-fimbriae are subject to phase variation [125], the set-up provides *E. coli* with flexibility in terms of varying antigenicity and function of P-fimbriae. Still, for a given strain the repertoire is restricted to the number of fimbrial gene clusters contained, and hence usually narrow.

One extraordinary characteristic of the pathogenic *Neisseria* species is their enormous capability to vary their surface pili [17]. In this context, the changing in the antigenic structures of surface proteins is certainly an important immune escape mechanism [126]. Furthermore, the variation also modifies the function of these adhesions [127–130]. Small alterations on the primary structures of neisserial pilins cause changes in immunoreactivity, post-translational modification, adhesive function, and ability to form bundles of pili. As a consequence, the pathogens can selectively interact with certain cell types and thus occupy special niches in their host. Many pilin variants that promote strong adhesion to host cells also aggregate into laminar bundles, whereas variants that promote weaker adhesion tend to exist as single filaments [131, 132]. It is unclear how bundling promotes adhesion. Bundles could promote bacterial aggregation, increase receptor avidity by oligomerizing binding sites, or increase pilus stiffness. Bundles might also promote twitching motility by promoting coordinated fiber extensions and retraction processes that would be unfeasible with less-ordered structures.

Flagella as Virulence Factors

Like fimbriae, flagella are protein polymers, each flagellum consisting of thousands of flagellin monomers [14]. These filaments are connected to the cell surface through the 'hook' structure, and the basal structure that forms the rotation device and that traverses the bacterial cell wall. Consequently, flagella are complex structures and coded for by a large set of genes. While the primordial role of flagella is to ensure motility, either as swimming movement in liquid medium or as swarming on solid surfaces, these traits are also applied in bacterial virulence [14]. For example, flagella-mediated motility acts as a virulence function for *V. cholerae* [133], *Helicobacter pylori* [134] and for *Proteus mirabilis* [135]. The former two pathogens are noninvasive colonizers of the digestive tract. Evidently, these bacteria apply motility to gain contact with the intestinal or gastric mucosal cells, respectively, and thus to establish the infection. *P. mirabilis*, on the other hand, is believed to apply motility for ascending from the ureter to the bladder, and further up to kidney structures. For *V. cholerae* and *H. pylori*, the role of flagella as virulence factors is also supported through transcriptome analyses, which show an upregulation of motility genes in de facto infecting bacteria [136–138]. For *P. mirabilis*, the swarming state involves a

transition to a hyperflagellated state and an upregulation, the expression of selected virulence functions [135].

Besides mediating motility, flagella are in many instances known to adapt functions typically ascribed to fimbriae. The flagellar FliC and FliD proteins of the gram-positive anaerobe *Clostridium difficile*, a causative agent of pseudo-membranous colitis, have been shown to bind both to mouse catcall mucous and cultured cells [139]. Similarly, a nonflagellated *P. aeruginosa* mutant was shown to be attenuated in a mouse pneumonia infection model. In parallel flagellin was been shown to bind GM1, asialoGM1 and GD1 glycolipids in vitro [140]. For *S. enterica* sv Enteritidis, nonflagellated mutants are abrogated for their ability to adhere to gut epithelium and epithelial cells, and for their ability to invade host cells [141].

Whereas some bacteria, like *S. enterica* sv Typhimurium, can phase-variate between the expression of two alternative flagellar subunit proteins, others, like *Vibrio parahaemolyticus* and *Aeromonas* spp., apply two separate sets of flagella: polar and lateral sets [14]. The different flagellar sets expressed by *Aeromonas* primarily associate with a shift in motility, the lateral set being used for swarming. However, there is also evidence for different adhesive characters disposed by polar and lateral *Aeromonas* flagella [142]. Therefore, as for *P. mirabilis*, the switch to a swarming phenotype reflects a more fundamental alteration in the expression of the bacterial virulence potential.

As with fimbriae, flagella also activate host cell signal transduction cascades and inflammatory responses. At least in part, this response originates from the fact that flagella, like bacterial LPS, act as pattern molecules that are recognized by the host innate responses. While LPS is recognized by TLR4, flagellin from both gram-positive and gram-negative bacteria is recognized by TLR5 [15]. The interaction between flagellin and TLR5 signals via Myd88 to cause activation of inflammatory responses [143]. Both LPS and flagellin can cause tolerance in host cells, the cells becoming non- or hyporesponsive after prior exposure to the ligand. What is interesting in this context is that LPS and flagellin can cause cross-tolerance, at least in cell lines [144].

The flagellar assembly pathway is related to the contact-dependent, so-called type III protein secretion pathway that is applied by many pathogens, like *S. enterica* sv Typhimurium and *Yersiniae*, for the translocation of bacterial virulence protein into host cells [145]. In selected cases it has been observed that the flagellar basal body and hook structures in *S. enterica* sv Typhimurium can substitute for the transport function of virulence proteins [146, 147]. While this was observed against a background with the ordinary secretion machinery inactivated, it suggests that the flagellar protein secretion potential, normally reserved for flagellar components, also could be applied for more sinister purposes.

Concluding Remarks

The ability to express surface structures related to adhesiveness and motility appears to be a widespread ability among prokaryotes, reflecting the necessity of corresponding traits for microorganisms. In many cases these organelles mediate colonization and adhesion of the bacteria to their growth niche: the plant root or a vertebrate epithelium. However, the further investigations proceed from describing adhesion to resolving the biogenesis and host responses, the more complex the functions of the ad priori adhesive and motility organelles appear. Indeed, type IV pili are known not merely to function as passive adhesive fibers, but in addition as dynamic machines that participate in a surprising number of functions: adhesion to host cell surfaces, modulation target cell specificity, twitching motility, DNA transformation, and bacterial autoagglutination. Furthermore, fimbrial receptor recognition can actually represent the prelude to a much more elaborated host-parasite cross talk. This is illustrated by type 1 fimbria-mediated activation of host signal transduction cascades that result in concomitant bacterial internalization, or by P-fimbria that activates TLR4-mediated proinflammatory and causes increase in TLR4 expression to further amplify the process. Considering the impact of adhesion and motility in virulence, it is interesting to note that, at least in selected cases, the same protein-aceous extensions are being applied both for adhesion and motility. Perhaps it is the extendedness of the structure that makes it suitable for such purposes. It is important to mention that the host has evolved systems that recognize bacterial flagellae. It remains to be evaluated whether there are specific innate specific recognition systems for fimbriae or whether the ability of fimbriae to initiate proinflammatory responses in fact reflects attempts to eradicate bacterial colonization.

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Gram-Positive Adhesins

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In the process of bacterial infection, adhesion to host tissues represents an initial and essential step. Adhesion allows the pathogen to attach to and colonize specific sites of the body, thereby withstanding eradication through cleansing mechanisms such as excretion and peristalsis. Once attached to the target tissue, bacteria may either remain extracellular, multiply, and eventually spread into deeper tissue, or trigger their own uptake by host cells, resulting in an intracellular location that may allow the pathogen to persist or further spread within the cellular or subcellular compartment.

Bacterial surface components that mediate adherence are called adhesins. Among gram-positive pathogens, surface proteins represent the largest group of adhesins, although other factors such as polysaccharides and lipids may also display adhesive functions. Targets for these microbial adhesins are host molecules found on mucosal surfaces, skin, and wounds. Depending on the strength of this interaction, adhesins allow the pathogen to loosely associate with or intimately bind to specific cells or tissues. Most gram-positive pathogens express multiple adhesins that may bind to either the same or distinct target molecules. Multiple adhesins of one pathogen are likely to be involved in different stages of an infection, expressed under different environmentally determined conditions, and may display a redundant function. In the present article, adhesins of pathogenic gram-positive bacteria belonging to the genus *Streptococcus*, *Staphylococcus* and *Listeria*, as well as the most important host molecules targeted by these adhesins are reviewed.

The Extracellular Matrix – A Major Target for Pathogens

Many adhesins function by specifically recognizing and binding to various components found in the extracellular matrix (ECM) of the host. The ECM

forms the major structural support for cells and tissues and is responsible for maintaining the strength and elasticity of the body. Thus, it is ubiquitously present and frequently exposed in cases such as trauma and injury, a situation that renders its constituents ideal targets for many adhesins. The following section gives a short overview on the major ECM components, their structure and their basic function.
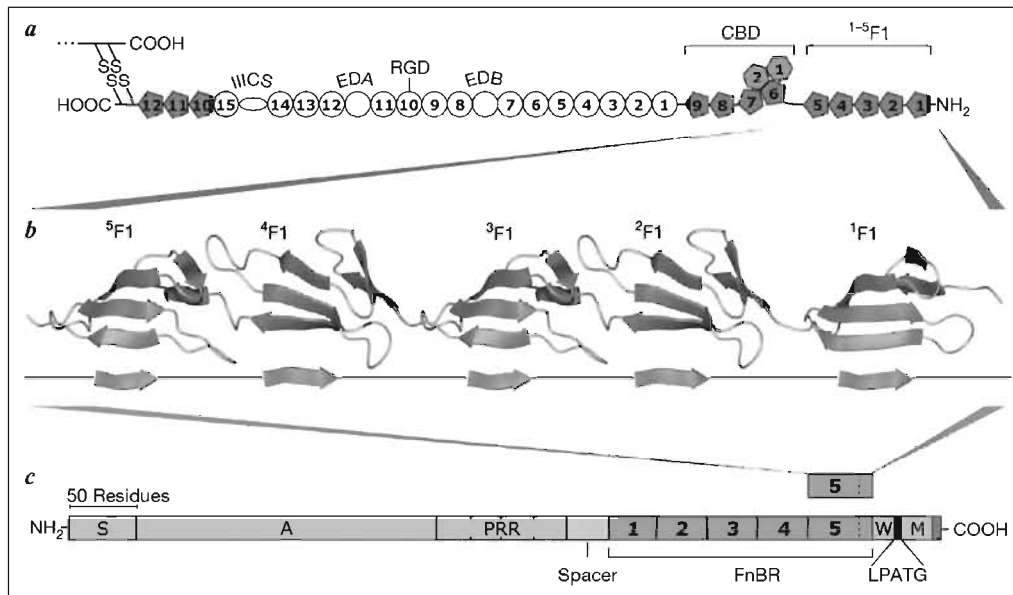
Collagens

Collagens are the most abundant proteins in the mammalian body and it is well recognized that collagens fulfill an important structural role in the ECM in a number of tissues. More than 25 distinct collagen types have been identified, in which identical or distinct α chains form a triple helix. Collagens can be divided into fibril-forming interstitial collagens (e.g. types I, II, III, V, and XI) and non-fibril-forming collagens such as type IV, VI, and X [1]. Type I collagen is found in tendons and muscle, while type II collagen is the major constituent of cartilage. The nonfibrillar type IV collagen is the major constituent of basement membranes, forming a network with laminins, nidogen, and sulfated proteoglycans. Collagen IV is composed of six chains ($\alpha 1-\alpha 6$) that form three basic sets of triple helical molecules. Collagens may interact with a variety of factors, including other matrix components such as fibronectin and laminin, as well as matrix metalloproteinases. The binding of collagen to cells is mediated by integrins, which constitute another group of receptors for collagens. Currently four collagen-binding integrins are known, $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{10}\beta_1$ and $\alpha_{11}\beta_1$, that mediate cellular binding and signalling. Bacterial binding to collagens, such as cartilage collagen and basement membrane collagen, represent important adhesion mechanisms among pathogens.

Fibronectin

Fibronectin, which exists both as a soluble protein in plasma and as a fibrillar polymer in the ECM, is a large glycoprotein involved in cell adhesion, migration, and differentiation. Fibronectin exists as a dimer composed of two 250-kD subunits which are carboxy-terminally linked via a pair of disulfide bonds [2]. Each subunit contains three distinct types of modules, the type I, II and III modules (fig. 1). Fibronectin efficiently binds to cell surfaces via numerous integrins, including the classic fibronectin-binding integrin, $\alpha_5\beta_1$ integrin. Integrin binding is mediated by an RGD sequence and also involves secondary sites on the fibronectin molecule. In addition to the interaction with integrins, fibronectin associates with heparin, collagen/gelatin, and fibrin. Heparin binding is governed by three domains that interact with heparan sulfate proteoglycans. Binding to collagen is mediated by type I repeats 6–9 and the two type II repeats. The two fibrin-binding sites are located at the carboxy- and

Fig. 1. a Modular structure of fibronectin (one subunit). Module types 1, 2 and 3 are symbolized by pentagons, hexagons, and circles, respectively. The amino-terminal domain ($^{1-5}$F1), the collagen-binding domain (CBD), alternatively spliced sites (curved labels) and the major integrin-binding site (RGD) are labelled. ***b*** The extended tandem β-zipper model of SfbI binding to $^{1-5}$F1. A model of the amino-terminal domain is shown on top. Short consecutive segments of a fibronectin-binding repeat form antiparallel β-strands on triple-stranded β-sheets of all five homologous F1 modules. ***c*** Molecular organization of SfbI from *S. pyogenes*. S = Signal peptide; A = nonhomologous region; PRR = proline-rich repeats; spacer = upstream fibronectin-binding region; 1–5 = Fn-binding repeats; W = cell wall spanning sequence; LPATG = cell wall anchor; M = membrane spanning region. (The tandem zipper model was kindly provided by Dr. Ulrich Schwarz-Linek, University of Oxford, UK.)

amino-terminal part of the molecule, the major site being formed by type I modules 4 and 5 located at the amino-terminal domain. Fibrin-binding and cross-linking to fibronectin via factor XIIIa is important in the generation of fibrin clots that form a provisional ECM network in the wound healing process. Fibronectin is an ideal target for many pathogens due to its wide presence in exudates, blood, wounds, as well as on the surface of cells.

Laminin

Laminin is a 900-kD glycoprotein and is a major component of the basement membrane. Its macromolecular structure is formed by assembly of three distinct

polypeptide chains, α, β, and γ [3]. Laminin functionally interacts with other components of basement membranes such as collagen IV and a variety of proteoglycans and ECM molecules. More than 10 different isoforms of laminin are known to be involved in cell proliferation and attachment, as well as in chemotaxis and angiogenesis. In the case of epithelial and endothelial injury, basement membrane components such as laminin are likely to be exposed and may serve as target structures for bacterial colonization of damaged tissue.

Elastin

Elastin is the major ECM protein of lung, skin and large arteries such as the aorta, imparting characteristics of extensibility and elastic recoil [4]. Elastin is formed by polymerization and cross-linking of its precursor tropoelastin. This process of ordered self-aggregation is called coacervation. Once deposited in tissues, polymeric elastin is not subject to turnover, but is able to sustain its mechanical resilience through millions of cycles of extension and recoil. Elastin consists of approximately 36 domains with alternating hydrophobic and cross-linking characteristics. The rubber-like mechanical properties result from the repetitive hydrophobic domains of tropoelastin that display an unstructured organization with higher entropy in the relaxed state, and a structured organization with lower entropy in the extended state. The major binding partners for tropoelastin are fibrillins, the main components of microfibrils which themselves may be attached to cells. Elastin serves as a target for pathogenic staphylococci, which use this molecule for adhesion to host tissue.

Vitronectin

Vitronectin is a multifunctional 75-kD glycoprotein present in blood and the ECM [5]. It binds collagen, plasminogen and the urokinase receptor, and stabilizes the inhibitory conformation of plasminogen activation inhibitor-1, thereby regulating the proteolytic degradation of the ECM. It further interacts with glycosaminoglycans via its carboxy-terminal part, and integrins of the α_V family via an RGD sequence located at the most amino-terminal part. Binding of the RGD sequence to integrins induces signalling cascades, and mediates attachment and spreading of cells on the matrix. Through its localization in the ECM and its binding to plasminogen activation inhibitor-1, vitronectin can potentially regulate the proteolytic degradation of this matrix. In addition, vitronectin binds to complement factors, heparin and thrombin-antithrombin III complexes, and therefore participates in the regulation of clot formation. The biological functions of vitronectin can be modulated by proteolytic enzymes, and exo- and ecto-protein kinases which are present in blood. Like fibronectin, vitronectin is an ideal target for adhesins of pathogens due to its presence in the ECM, in blood, and at sites of tissue injury.

Fibrinogen

Fibrinogen is a 340-kD plasma glycoprotein composed of six polypeptide chains, two Aα, two Bβ, and two γ chains that form a dimer. In the vascular system, fibrinogen mediates platelet adherence and aggregation at sites of trauma and injury, thereby acting as an important clotting factor [6]. Upon interaction with thrombin, subsequent stabilization of the fibrin clot is achieved by transglutaminase/factor XIIIa-mediated cross-linkage of the γ and α chains of fibrinogen. Binding to platelets is mediated through the interaction of fibrinogen with integrin $\alpha_{IIb}\beta_3$ on the platelet surface. In addition to its function in the coagulation system, fibrinogen also participates in inflammatory responses. Fibrinogen mediates leukocyte attachment to the vessel wall and transmigration through the endothelium. Fibrinogen binds to $\alpha_M\beta_2$ integrin on leukocytes and to $\alpha_M\beta_3$ integrin on macrophages, thereby regulating phagocytic clearance of fibrin clots during wound healing. Interaction with integrins is governed by two RGD sequences and other defined epitopes on the fibrinogen molecule. In addition, fibrinogen has the ability to bind a variety of factors such as fibronectin, collagen, and components of the fibrinolytic system, implicating this protein as a key factor in matrix organization, remodelling and wound repair. Many gram-positive pathogens have evolved distinct factors that specifically bind fibrinogen, evoking bacterial adhesion, aggregation, and evasion of phagocytosis.

Glycosaminoglycans

Glycosaminoglycans are polysaccharide chains covalently linked to a protein core to form proteoglycans. Being composed of distinct repeating disaccharide units, these molecules can be divided into different classes such as heparan sulfate, dermatan sulfate, and chondroitin sulfate. Glycosaminoglycans are present in the ECM of connective tissue but are also expressed on the surface of eukaryotic cells. Heparan sulfate [7] and dermatan sulfate [8] are ubiquitously found on the surface of cells and in the ECM and skin. Glycosaminoglycans function as stabilizers, cofactors and coreceptors of cytokines and chemokines, regulators of enzymatic activity, and signalling molecules in response to injury or infection. Glycosaminoglycans may mediate adherence and entry of pathogens including bacteria, viruses and parasites [8].

Streptococcal Adhesins

Streptococcus pyogenes

S. pyogenes, the group A *Streptococcus*, is an important human pathogen that causes localized infections of the respiratory tract and the skin, but also in

severe invasive diseases, such as sepsis and toxic shock-like syndrome. Severe nonsuppurative sequelae such as acute rheumatic fever and glomerulonephritis may follow primary group A streptococcal infection. *S. pyogenes* initiates infection by interacting specifically with host molecules present on mucosal surfaces or skin. A variety of different adhesins that either bind to identical or distinct target molecules are expressed by *S. pyogenes* (table 1). Among the large number of bacterial factors that bind to host molecules, only those for which adhesive properties were clearly demonstrated are herein termed adhesins.

S. pyogenes possesses at least nine distinct fibronectin-binding adhesins. Some of these occur in a large number of serotypes, such as SfbI protein or FBP54, whereas others such as M1 or M3 protein are exclusively expressed by M1 or M3 serotypes, respectively. Among all fibronectin-binding adhesins of *S. pyogenes*, SfbI protein and its allelic variant F1 are the most extensively studied. Identified in 1992, SfbI/F1 was shown to act as an adhesin on epithelial cells [9, 10]. SfbI protein has a modular architecture [11], and binds to fibronectin via two distinct domains [12, 13]. The carboxy-terminal repeat region and the adjacent nonrepetitive domain termed spacer 2 or UR synergistically bind to two distinct regions on the fibronectin molecule: the amino-terminal fibrin-binding fragment (harboring fibronectin F1 modules 1–5) and the gelatine/collagen-binding fragment (harboring F1 modules 6–9 and the two F2 modules) [14]. The carboxy-terminal repeat region of SfbI was demonstrated to be sufficient to mediate adherence to epithelial cells [14]. However, besides this activity, SfbI acts as a potent invasin that triggers internalization into eukaryotic cells [14–18]. SfbI mediates attachment to epithelial cells of the oral mucosa and the lung, but also to endothelial cells [18]. Binding to human cells was shown to be dependent on the presence of fibronectin-binding integrins [17], leading to the concept that fibronectin acts as a bridging molecule between bacteria and host cell integrins. Besides its potential to bind to cell surfaces, SfbI has the ability to recruit collagen via prebound fibronectin, a mechanism that enables the bacteria to form aggregates and renders the organism capable of colonizing collagen matrix [19]. The overall pathogenic potential of this protein is underlined by vaccination studies using recombinant SfbI that protected mice from lethal *S. pyogenes* infection [20]. Recently, the first three-dimensional structure for a bacterial fibronectin-binding peptide, the B3T peptide derived from the *Streptococcus dysgalactiae* FnBP, in complex with the $^1F1^2F1$ module of fibronectin was obtained. Based on this structural information, a compelling model for the interaction of the fibronectin-binding repeats of SfbI with the amino-terminal domain of fibronectin was developed, termed the tandem β-zipper model [21]. Short motifs within each of the carboxy-terminal repeats of SfbI were predicted to form antiparallel β strands along the five F1 modules in the amino-terminal domain of fibronectin (fig. 1), leading to binding affinities in the nanomolar

Table 1. Streptococcal adhesins

Adhesin	Ligand molecule	Target cells, tissue	Reference no.
S. pyogenes			
SfbI/F1	fibronectin, collagen	pharynx and lung epithelial cells, endothelial cells, collagen matrix	9–19, 21
F2/PFBP	fibronectin	n.d.	22, 23
FBP54	fibronectin	buccal epithelial cells	24
Fba	fibronectin	epithelial cells	26
FbaB	fibronectin	epithelial cells	27
Protein H	fibronectin, M proteins	epithelial cells	28, 29
M1 protein	fibronectin	epithelial cells	30–32
LTA	fibronectin, macrophage scavenger receptor	epithelial cells, macrophages	33, 34
M3 protein	type I and IV collagen, fibronectin	collagen matrix	35, 36
Cpa	type I collagen	n.d.	37
HA capsule	type I and IV collagen, CD44	collagen matrix, keratinocytes	35, 38
M6 protein	CD46	keratinocytes	39, 40
M proteins	glucosaminoglycans, M proteins	epithelial cells, fibroblast cells	29, 41, 42
Lbp	laminin	epithelial cells	43
SpeB	laminin, glycoproteins	n.d.	44
R28	n.d.	cervical epithelial cells	45
SclA/Scl1	n.d.	pharyngeal cells	46, 47
SclB/Scl2	n.d.	fibroblast cells	48, 49
S. agalactiae			
ScpB	fibronectin	pharynx and lung epithelial cells	50, 51
Lmb	laminin	n.d.	52
Alpha C protein	n.d.	cervical epithelial cells	53
S. pneumoniae			
SpsA/CbpA/PspC	SC, sIgA, factor H	pIgR-expressing cells	56–61
Phosphorylcholine	PAF receptor	endothelial and epithelial cells	62
PavA	fibronectin	n.d.	63, 64

n.d. = Not determined.

range. This is extremely important since high affinity binding is a prerequisite for bacterial attachment, a mechanism that has to withstand shear forces occurring on the mucosal surfaces or during the internalization process.

Protein F2 or PFBP are homologous but distinct fibronectin-binding proteins, found in most isolates of *S. pyogenes* lacking the *sfbI/prtF1* gene [22, 23]. Like SfbI, protein F2 possesses two binding domains that interact with fibronectin.

Among the genes encoding fibronectin-binding proteins, the gene for FBP54 is the most abundant and found in all *S. pyogenes* isolates [24]. Although lacking the classical membrane anchor motif of gram-positive surface proteins, it appears to be localized on the streptococcal surface by a distinct mechanism [25], thereby acting as an adhesin for buccal epithelial cells but not for HEp2 cells [24]. These data also indicate that distinct fibronectin-binding factors may target different cell types and have a substantial effect on cell tropism.

Two other recently discovered fibronectin-binding proteins are Fba and FbaB [26, 27]. The *fba* gene was found in 5 serotypes of *S. pyogenes* including M types 1 and 49. An Fba mutant showed diminished adhesion to HEp2 cells, suggesting that this protein has adhesive properties [26]. However, it should be noted that the FbaB protein was only found in serotype M3/M18 *S. pyogenes* isolates and appears to be genetically most closely related to protein F2 [27].

Protein H, a member of the M protein family, binds to fibronectin in a unique manner [28]. Unlike the proteins described so far that mainly interact with the type I or type II module containing domains of fibronectin, protein H binds to the type III modules. In addition, protein H was shown to mediate streptococcal aggregation through a so-called AHP sequence that also promoted adhesion to epithelial cells [29].

M1 protein, another member of the M protein family, was demonstrated to bind fibronectin [30], and M1-specific antibodies efficiently blocked adherence to HeLa cells. Moreover, an M1-deficient mutant showed reduced adherence and invasion, indicating that M1 protein acts as an adhesin and invasin in serotype M1 *S. pyogenes* strains [31]. Importantly, as in the case of SfbI protein, $\alpha_5\beta_1$ integrins are the terminal receptor proteins on the cellular surface [32].

Lipoteichoic acid (LTA) was suggested to interact with fibronectin or hydrophobic residues on the cellular surface. It was defined as a first step adhesin, mediating low affinity and reversible binding to the ligand, whereas protein adhesins with high affinity binding to the ligand were termed second step adhesins [reviewed in 33]. At least one other cellular receptor exists for LTA: the type I macrophage scavenger receptor which exhibits a broad range of binding specificity [34].

Recent findings identified M3 protein as an important adhesin that binds to soluble type I and type IV collagen as well as to the native collagen matrix
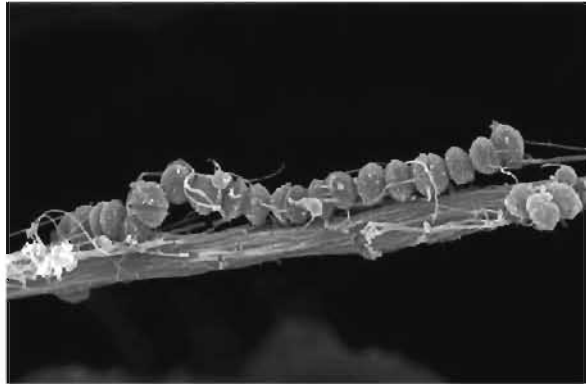
Fig. 2. Serotype M3 *S. pyogenes* adhering to collagen type I fibers via the M3 protein. (The scanning electron-microscopic image was kindly provided by Dr. Manfred Rohde, GBF, Braunschweig, Germany.)

[35] (fig. 2). The amino-terminal variable but M3-specific region of M3 protein is essential for collagen binding, explaining why other M proteins lack this function. Besides attaching bacteria directly to collagen matrix, aggregation of soluble collagen on the bacterial surface leads to formation of large bacterial aggregates that facilitate the colonization process [35]. The only other collagen-binding protein of *S. pyogenes* described so far is Cpa, which was identified in the M49 serotype and was suggested to mediate attachment to immobilized type I collagen [37].

In highly encapsulated M18 streptococci, collagen-binding activity and adhesive properties are mediated by the hyaluronic acid (HA) capsule. The assumption that M3 protein and streptococcal HA could indeed mediate adherence to the collagenous matrix was demonstrated ex vivo on native collagen fibers and in vivo by using a skin infection mouse model [35]. Apart from binding to collagen, HA interacts with human CD44 on the surface of keratinocytes, acting as an adhesin for the major cell type of the human pharyngeal epithelium and external skin [38]. This finding was of particular importance since former studies suggested an inhibitory role of HA in streptococcal cell attachment. The current concept, however, is that HA may act as an adhesin itself but may also mask binding interactions of other streptococcal surface molecules, depending on the type of the M serotype or tissue [38].

Another target receptor present on the surface of keratinocytes is CD46, the membrane cofactor protein which is bound by M6 protein [39]. The carboxy-terminal region of M6 protein as well as the short consensus domains 3 and 4 of CD46 were shown to be crucial for M6/CD46-mediated keratinocyte

attachment [40]. Although structurally closely related, M proteins represent a heterogeneous group of adhesins with respect to their ligands or target cells [41]. In contrast to the binding properties displayed by individual M proteins, such as the fibronectin-binding activity of M1 protein or the collagen-binding of M3 protein, homophilic interactions of M protein [29] and interactions with glycosaminoglycans [42] represent common adherence mechanisms among several types of M proteins. This is underlined by the finding that interactions with several types of glycosaminoglycans such as dermatan sulfate and heparan sulfate are predominantly, although not exclusively, mediated via the conserved carboxy-terminal part of the M proteins [42].

Laminin, another constituent of the ECM, also represents a target for *S. pyogenes*. Two laminin-binding proteins are known, Lbp that has adhesive properties for epithelial cells [43], and SpeB, the secreted cysteine protease which also displays glycoprotein-binding activity [44]. Whether SpeB indeed mediates adherence to host cells remains to be determined.

Within *S. pyogenes*, three adhesins have been identified of which the ligand molecules are still unknown. R28, a highly repetitive surface protein related to the *Streptococcus agalactiae* surface proteins Rib and α, binds to cervical epithelial cells [45], and two distinct collagen-like proteins termed SclA/Scl1 and SclB/Scl2 were shown to bind to pharyngeal and fibroblast cells, respectively [46–49]. Since the *scl* genes appear to be prevalent in all *S. pyogenes* serotypes, are differentially regulated, and display adhesive function, precise functional analysis of these potentially important factors will be helpful to understand their role in the infection process.

During recent years a large number of *S. pyogenes* adhesins have been identified and considerable progress has been made by analyzing the molecular mechanisms underlying the process of bacterial attachment to host cells and tissue. Future challenges will be to elucidate the three-dimensional structure of receptor/ligand complexes that will lead to a better understanding of the molecular nature of these interactions, and the development and use of appropriate in vivo and ex vivo models for studying the role of the adhesins in the infection process. The emerging number of available knockout cell lines and mice will serve as helpful tools, defining a promising interdisciplinary cutting edge between mouse genomics and infection biology.

S. agalactiae

S. agalactiae, the group B streptococcus, is a gram-positive commensal of the human vagina, but also the major cause of neonatal sepsis and meningitis. *S. agalactiae* may also cause serious infections in immunocompromised adults. Compared to *S. pyogenes*, the number of adhesins identified so far is relatively small (table 1). The host molecules known to be targeted by *S. agalactiae* are

fibronectin [50, 51], laminin [52, 53], and cytokeratin 8 [54]. The only known fibronectin-binding factor of group B streptococci is C5a peptidase (ScpB), a large serine protease that is secreted but also attached to the streptococcal surface. Purified recombinant ScpB was demonstrated to bind to immobilized fibronectin [51], as well as to HEp2 and A549 cells [50].

Lmb, a surface-associated lipoprotein belonging to the LraI family of proteins, was shown to mediate attachment of group B streptococci to laminin [52]. Whether Lmb indeed acts as an adhesin remains to be determined. Other data suggest a direct role for the alpha C protein in adherence to cervical epithelial cell [53]. The alpha C protein is the prototype for a family of long tandem repeat-containing surface proteins that also include R28 of *S. pyogenes* and Esp of *Enterococcus faecalis*. The cellular receptor for alpha C protein is, as in the case of R28, still unknown. The molecular nature of another streptococcal adhesin that binds to cytokeratin 8 [54], a molecule potentially important for colonization of keratinized epithelium or damaged cells, also remains to be identified.

Streptococcus pneumoniae

S. pneumoniae, the pneumococcus, is a natural colonizer of the nasopharyngeal epithelium and has the ability to penetrate the epithelial barrier, to translocate into deeper tissue, where it can cause severe infections such as pneumonia, meningitis and sepsis. Although binding to laminin, type IV collagen, and vitronectin was described over a decade ago [55], only three adhesins that bind to other target molecules have been identified in this streptococcal species.

To date, the best-studied adhesin of *S. pneumoniae* is SpsA, also named CbpA or PspC [56–58]. SpsA binds to human secretory IgA [56, 59], mediates adherence to activated human cells [57], and uses the human polymeric immunoglobulin receptor as a terminal receptor on the surface of host cells for adherence and translocation [60]. In addition to these properties, SpsA is a protective antigen that also binds to factor H [58, 61], suggesting a multifunctional role for this adhesin.

Attachment of pneumococci to activated cells was also shown to be mediated through phosphorylcholine on the bacterial surface, employing the platelet-activating factor (PAF) receptor as a target molecule on the cellular surface [62]. PAF receptor-mediated adherence was found to be coupled to invasion of epithelial and endothelial cells, suggesting a direct role for this interaction in subcellular spreading of the pathogen [62].

Among the various ECM molecules, fibronectin is one of the target molecules used by pneumococci for attachment [63]. The binding site of pneumococci was suggested to be located within the carboxy-terminal portion of fibronectin. Immobilized rather than soluble fibronectin was shown to be bound by this bacterial species, discriminating this binding factor from most of the

fibronectin-binding proteins found in *S. pyogenes* or *Staphylococcus aureus*, which efficiently bind to soluble fibronectin as well. PavA, a surface-associated pneumococcal protein, was identified as receptor for immobilized fibronectin [64]. It displays high similarity to FBP54, its orthologue found in *S. pyogenes*. Evident data demonstrate that PavA is essential for virulence [64]; however, its precise role in mediating cell or tissue adherence remains to be defined.

Staphylococcal Adhesins

S. aureus is an important opportunistic pathogen of humans and animals. The spectrum of diseases ranges from superficial skin infection to serious infections such as endocarditis, septic arthritis, and community-acquired and nosocomial sepsis. Besides this, *S. aureus* is a major cause of infections originating from catheters and implanted synthetic medical devices.

Many *S. aureus* isolates have the ability to bind fibronectin. Most strains express FnbpA and FnbpB (table 2), two related fibronectin-binding proteins encoded by closely linked genes [65–67]. These two proteins were shown to bind soluble and immobilized fibronectin via their carboxy-terminal repeat region, whereas FnbpA was also shown to bind fibrinogen via its amino-terminal A domain [68]. In vitro infection experiments employing distinct cell types as well as isogenic *S. aureus* strains either expressing or lacking one or both Fnbps revealed that fibronectin-coated devices, human epithelial cells, endothelial cells, and T lymphocytes are targets for Fnbp-mediated adhesion [69–73]. As in the case for the *S. pyogenes* fibronectin-binding proteins SfbI/F1 and M1, the underlying mechanism for this interaction was shown to be the use of fibronectin as a bridging molecule between the bacteria and host cell integrins such as $\alpha_5\beta_1$ integrin [71, 74, 75]. Consequently, *S. aureus* Fnbps may act as invasins governing the uptake of staphylococci by human epithelial and endothelial cells [74–78]. Analogous to the fibronectin-binding repeat region of SfbI from *S. pyo-genes*, the domain in fibronectin which is recognized by the Fnbp repeat region is located at the amino-terminus of the molecule, being composed of five F1 modules [79–82]. The interacting Fnbp repeat region was suggested to be unfolded, undergoing a conformational shift upon interaction with the F1 modules of fibronectin [83, 84]. Based on recent NMR-based structural data, FnbpA contains 11 fibronectin-binding repeat segments, each of which can potentially bind sequential F1 modules, most likely through the tandem β-zipper mechanism that has also been suggested for SfbI protein [21] (fig. 1). Altogether, these findings provide substantial insight into the molecular mechanisms of fibronectin-mediated adherence of pathogenic cocci. Whether Fnbps of *S. aureus* are also able to recruit collagen via prebound fibronectin remains to be determined.

Table 2. *S. aureus* adhesins

Adhesin	Ligand molecule	Target cells, tissue	Reference no.
FnbpA	fibronectin, fibrinogen	epithelial cells, endothelial cells, mammary glands, T lymphocytes	65–75
FnbpB	fibronectin	epithelial cells, endothelial cells, mammary glands	67, 69–75
Ebh	fibronectin	?	85
Cna	collagen	cartilage	86–94
ClfA	fibrinogen	thrombi, implanted biomaterial	95–100
ClfB	fibrinogen, cytokeratin	thrombi, implanted biomaterial, keratinocytes, nasal epithelial cells	101–102
SasG	?	nasal epithelial cells	103, 105
Pls	?	nasal epithelial cells	104, 105
Bbp	bone sialoprotein	bone tissue	106
Spa	vWF	damaged endothelium	108
vWbp	vWF	?	109
Map/Eap	fibronectin, fibrinogen, vitronectin, bone sialo-protein, thrombospondin, collagen, osteopontin ICAM-1	epithelial cells, fibroblast cells	110–116
Emp	fibronectin, fibrinogen, vitronectin, collagen	?	117
EbpS	elastin	?	118–120
PIA	?	biofilm formation, cell-cell adhesion	121
Capsule	?	epithelial cells, endothelial cells	122

Another fibronectin-binding protein of *S. aureus* is Ebh, a large 1.1-megadalton surface-associated protein that has been shown to bind soluble and immobilized fibronectin [85]. The role of Ebh in cell adherence is, however, still undefined.

Cna, the collagen-binding factor of S. *aureus* is an important adhesin which mediates attachment to collagen substrates and collagenous tissues [86, 87]. In addition to this, Cna is able to mediate adherence to cartilage, a potentially important mechanism during septic arthritis [88, 89] and/or osteomyelitis [90]. The ligand-binding domain of Cna was identified to be located on a 168-amino-acid-long segment within the amino-terminal A domain of the protein

[91]. A synthetic peptide mimicking a subdomain of this segment inhibited collagen binding to the bacteria and identified the critical residues for collagen binding [92]. Structural resolution of the binding domain revealed a trench-shaped organization of the binding module that was predicted to accommodate the collagen triple helix [93]. Interestingly, collagen binding to S. aureus cells is inhibited by capsule expression, suggesting a masking role for the surface polysaccharide [94]. This is in contrast to the collagen-binding characteristics observed in S. pyogenes where HA capsule expression does not inhibit but enhances collagen binding of S. pyogenes by directly binding to collagen [35].

S. aureus expresses two adhesins that mediate binding to fibrinogen, ClfA and ClfB. ClfA enables S. aureus to adhere to fibrinogen-containing substrates such as plasma clots and to clump in the presence of fibrinogen, giving this protein its name: clumping factor [95]. ClfA is a potentially important virulence factor since ClfA negative mutant staphylococci showed reduced virulence in a rat endocarditis model [96]. The ligand binding domain of ClfA was mapped to a 329-amino acid segment within the amino-terminal A domain [97]. ClfA recognizes the carboxy-terminus of the γ chain of fibrinogen, a region also recognized by the $\alpha_{IIb}\beta_3$ integrin on platelets, and thus inhibits platelet aggregation [98]. Analogous to the integrin/fibrinogen interaction, ClfA-mediated fibrinogen binding is affected by Ca^{2+} [99]. The structural basis for this interaction was found by analyzing the crystal structure of the fibrinogen-binding domain. A variant of the immunoglobulin (IgG) fold, a structure found in IgG, was defined to mediate adhesion, placing ClfA into the IgG fold group of adhesins [100].

ClfB, the second fibrinogen-binding clumping factor and adhesin of S. aureus, has an overall organization similar to ClfA [101]. However, in contrast to ClfA, ClfB binds to the α and β chains of fibrinogen. Another characteristic of ClfB is its ability to bind cytokeratin 10 via the amino-terminal A domain [102]. It was shown to promote adherence to human keratinocytes and desquamated nasal epithelial cells, suggesting that this adhesin plays an important role in nasal colonization [102].

SasG, a recently identified surface protein of S. aureus [103], and Pls, a surface protein of methicillin-resistant S. aureus [104], also promote adherence to desquamated nasal epithelial cells [105]; their receptor on the cellular surface is, however, still unknown.

Bone sialoprotein (BSP) is bound by Bbp, a surface protein of S. aureus [106]. BSP is present in high concentrations in newly formed bone tissue, the osteoid, and thus suspected to be of relevance in osteomyelitis, an infection mostly affecting the osteoid. Bbp, like ClfA and ClfB, belongs to the Sdr family of surface proteins, characterized by the presence of carboxy-terminal serine-apartic acid dipeptide repeats [107].

S. aureus has the ability to adhere to von Willebrand factor (vWF), a multimeric glycoprotein present at damaged endothelial sites. Two proteins have been identified that mediate binding of *S. aureus* to human vWF: staphylococcal protein A (Spa) and vWbp [108, 109]. Binding to soluble or immobilized vWF may not only be responsible for *S. aureus* endovascular adherence but also increase the risk of disturbed hemostasis and vascular thrombosis, both symptoms observed during severe *S. aureus* infection.

A surface-associated protein with broad matrix protein binding specificity was identified in 1993 [110], and subsequently characterized as Map or Eap protein [111, 112]. Map/Eap was shown to bind fibrinogen, fibronectin, thrombospondin, vitronectin, bone sialoprotein, osteopontin and collagen, and occurs as a secreted but also surface-associated protein [110–112]. Map/Eap was demonstrated to mediate adherence to cultured epithelial and endothelial cells [113, 114], and appears to enhance staphylococcal internalization into eukaryotic cells [115]. Furthermore, due to its binding ability towards ICAM-1 and the resulting impairment of leukocyte recruitment, Map/Eap plays a role as anti-inflammatory immune modulator [116].

Emp, another surface-associated protein of *S. aureus*, binds to fibronectin, fibrinogen, vitronectin and collagen [117]. Like Map/Eap, Emp lacks the carboxyterminally located LPXTG membrane anchor motif present in several gram-positive adhesins, but is found on the surface of *S. aureus* cells where it may display adhesive function.

The ECM component elastin is a target for EbpS, an elastin-binding protein [118]. The elastin-binding domain was localized within the amino-terminal portion of the transmembrane molecule, encompassing 21 amino acid residues shown to be exposed on the surface of intact *S. aureus* cells [119, 120]. As for Ebh, vWbp, and Emp, its role in mediating cell adherence remains to be investigated.

In addition to the various protein adhesins, *S. aureus* expresses polysaccharides that display an adhesive function: PIA, the polysaccharide intercellular adhesin, is required for biofilm formation and cell-to-cell adhesion [121]. Capsular polysaccharide of serotype 5 or 8, most frequently found to be expressed by *S. aureus* isolated from human infections, binds to monocytes as well as to epithelial and endothelial cells, demonstrating adhesive properties for the *S. aureus* capsule [122].

Other Gram-Positive Adhesins

Listeria monocytogenes is a gram-positive food-borne human pathogen that causes listeriosis, a severe invasive infection during which bacteria are disseminated to the fetoplacental unit and the central nervous system. Although

the overall number of cases of listeriosis is low, the severity of infection is high and the factors responsible for host cell interaction and spreading are well studied. *L. monocytogenes* expresses two important invasins, internalin A and B (InlA, InlB), that also mediate adhesion to host cells. The cellular receptor for InlA was shown to be human E-cadherin [123], a cell surface adhesion molecule contributing to cell cohesion via homophilic dimerization and formation of adherens junctions. Interestingly, the species specificity of listeriosis arises from a single amino acid variation in E-cadherins of distinct species: the presence of a proline residue at position 16 in human E-cadherin was demonstrated to be crucial for cell interaction, explaining the finding that mouse and rat E-cadherin harboring a glutamic acid residue at that position was not susceptible for listeriosis [124, 125]. Different mammalian cell lines have varying susceptibilities to InlA and InlB. The human intestinal epithelial cell line Caco-2 and the hepatocyte HepG2 cells are targets for InlA and InlB. Interaction with monkey kidney Vero cells, mouse hepatocytes, and human endothelial cells is mediated via InlB [125]. Three receptor molecules have been identified for InlB [reviewed in 126]. InlB binds to HGF-R or Met, a receptor tyrosine kinase that acts as a receptor for hepatocyte growth factor [127], to gC1q-R or p32, a receptor of the complement component C1q [128], and to proteoglycans [129].

Recent work has demonstrated that autolysins of gram-positive pathogens may also display adhesive properties. The first autolysin shown to act as an adhesin was AtlE of *Staphylococcus epidermidis*, a commensal of the skin and an opportunistic pathogen [130]. AtlE was suggested to play a role in the attachment to polystyrene surfaces and to vitronectin, thereby contributing to biofilm formation of *S. epidermidis* on implanted polymers. Aas, an orthologous autolysin of *Staphylococcus saprophyticus*, mediates adhesion and binds to fibronectin [131]. The third autolysin found to mediate bacterial attachment was Ami of *L. monocytogenes* [132]. Adhesive properties were localized within the noncatalytic carboxy-terminal cell wall-anchoring domain, composed of so-called GW modules, short dipeptide repeats containing the amino acid residues glycine and tryptophane [133]. Linkage of GW modules to LTA, as well as to glycosaminoglycans, anchor GW module-containing proteins to the surface of gram-positive bacteria [134]. GW modules are found within all adhesive autolysins described herein, but also in eight other listerial proteins including InlB [134]. Thus, to define the adhesive properties of the yet uncharacterized GW module-containing proteins will be a future goal. Interestingly, Cwp66 of *Clostridium difficile*, the first identified adhesin of this gram-positive spore-forming pathogen belonging to the genus clostridia, exhibits homology to the catalytic domain of CwlB, the autolysin of *Bacillus subtilis* [135]. In contrast to the above-described adhesive autolysins, Cwp66 lacks repetitive GW modules

but may be linked to the gram-positive cell wall via an alternative mechanism, explaining its surface localization.

It is important to mention that a variety of adhesins, colonization and cross-linking factors have been identified and characterized in commensal gram-positive bacteria such as oral streptococci, enterococi, and staphylococci. Since these adhesins were not the subject of this chapter, the reader should be referred to these reviews [136–140] summarizing the adhesive mechanisms of commensal organisms that may also play an important role as opportunistic human pathogens in the susceptible host.

Concluding Remarks

Among bacterial virulence factors adhesins represent an important group. Many gram-positive pathogens express adhesins with a broad specificity, as well as adhesins that recognize particular target molecules such as collagen or fibronectin. These proteins have evolved in distinct gram-positive and gram-negative pathogens via convergent mechanisms. Adhesins very often function synergistically and are highly specific factors that are a prerequisite for infection which subsequently governs the interplay between the microbe and the host. In particular cases, they may even have a direct impact on the phenotype of a disease such as septic arthritis in case of the *S. aureus* collagen-binding adhesin Cna [88, 89] or in autoimmune reaction based on the M3 protein of *S. pyogenes* [35].

Further characterization of the concerted function of multiple adhesins is a hallmark in understanding the initiation and progress of infection caused by a particular pathogen. Defining the target molecules in the adhesion process will help to understand individual host susceptibilities, and will link recent data on molecular interactions with epidemiological data collected over a whole century. The growing knowledge in the field of molecular mechanisms of pathogen adhesion will open up new perspectives in prevention and treatment strategies. Rational drug design based on the availability of structural data on receptor/ligand complexes, fine-tuned vaccination approaches based on minimal functional domains, and identification of new vaccine candidates are the challenging perspectives of future research in this field.

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# Microbial Pathogenesis and Biofilm Development

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Microbial infections constitute a major cause of premature death in large parts of the world, and for several years we have seen an alarming tendency towards increasing problems of controlling such infections by antibiotic treatments. It is hoped that an improved understanding of the infectious cycles of different microorganisms will eventually lead to improved treatments. Several bacteria have evolved specific strategies for virulent colonization of humans in addition to their otherwise harmless establishment as environmental inhabitants. In many such cases biofilm development seems to play a highly significant role in connection with chronic infections [1].

Bacterial growth on surfaces depends on several factors [2]. In nature, surfaces are probably often conditioned with a thin film of organic molecules, which may serve as attractants for bacterial chemotactic systems and which subsequently permit bacterial growth to occur. In laboratory model systems the growth of the surface-associated bacteria is supported by the nutrient supply in the moving or standing liquid. A benchmark of biofilm formation by several organisms in vitro is the development of three-dimensional structures that have been termed 'maturation', which is thought to be mediated by a differentiation process. Maturation into late stages of biofilm development resulting in stable and robust structures may require the formation of a matrix of extracellular polymeric substances (EPS), which are most often assumed to consist of polysaccharides. A recent striking finding is that DNA released from biofilm cells may be important as an initial matrix former [3]. At later times other EPS molecules may add to the shape and quality of the mature biofilm structure. Figure 1 summarizes the principle steps involved in the development of microbial biofilms.
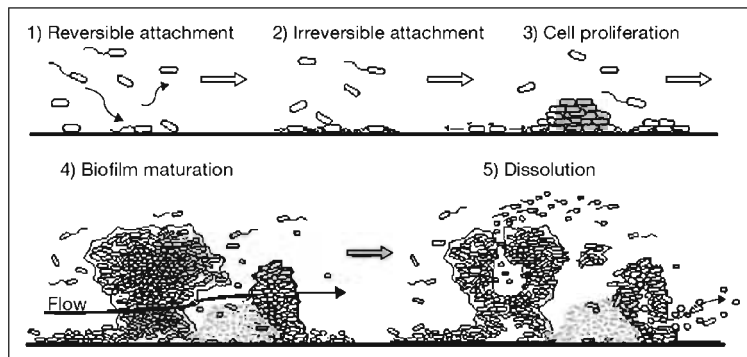
Fig. 1. The biofilm development cycle. Biofilm development is depicted as a general scheme involving attachment to the surface, formation of a tight association between bacterial cells and a surface, growth and intercellular adhesion allowing microcolony formation, maturation including EPS matrix development, and local dissolution leading to release of bacteria, which may eventually restart the cycle.

How do bacteria know that they are located in a biofilm? There is no doubt that cell density is an important factor that distinguishes the usually dilute suspensions of planktonic cells in water from the very cell-dense surface communities found where organic matter is abundant. One answer to the question therefore is: very high cell density. Another characteristic of biofilms and other types of surface-associated communities is the prevalence of internally heterogeneous environments and microenvironments, often generated and maintained by the presence of EPS. For the biofilm-associated bacteria this scenario is recognized as gradients of nutrients and stress factors. For planktonic cells such gradients rarely play a role. It is often argued that attachment to surfaces is the most important feature, and that surface-induced gene expression is therefore one of the key determinants of biofilm development. It should be remembered, however, that cellular contact with the substratum in a biofilm is a transient phenomenon (but most likely important for early gene activation), which is quickly converted to a state where essentially all bacterial cells are located far above the surface in microcolonies or in EPS-embedded 'mushrooms'. In these entities it is difficult to imagine any bacterial sensing of the surface association as a physical signal.

Thus, it seems that biofilm-associated bacteria must respond to the (1) very high cell density and (2) to the various positive and negative gradients. If it is assumed that bacterial evolution is mainly connected to the dominant life form of these organisms, and that bacteria in natural environments almost exclusively live an active proliferating life associated with surfaces (in biofilms), it is to be

expected that evolution has provided bacteria with properties that allow adaptation to life under high cell density conditions in environments with nutrient and antagonist gradients.

This leaves the following issues as the major common themes for biofilm investigations related to the microbial capacity to develop mature, heterogeneously structured surface-associated communities: How are the specific structural features in a biofilm created and maintained? Which functions are involved in the adaptation to high cell densities and nutrient gradients? How do biofilm bacteria evolve, and what are the major selective forces?

In the following we will present an overview of the current understanding of microbial biofilm development and its clinical relevance in relation to two examples of gram-negative pathogens, *Escherichia coli* and *Pseudomonas aeruginosa*, for which the biofilm lifestyle seems to be relevant during the course of infection.

E. coli

As the dominant facultative anaerobe of the normal human intestinal flora, *E. coli* remains harmlessly confined to the intestinal lumen. However, highly adapted clones have evolved the ability to cause a broad spectrum of diseases ranging from urinary tract infection (UTI) and diarrhea to sepsis and meningitis [4]. Many of these infections are initiated by bacterial colonization of mucosal surfaces of the genitourinary, gastrointestinal or respiratory tracts. Successful establishment in the host depends on the ability to overcome host defenses and shear forces present at most of these surfaces. Since biofilm formation has also been suggested to be an ancient bacterial survival strategy [5], it seems possible that at least a fraction of pathogenic *E. coli* clones have conserved or evolved the ability to enter a sessile lifestyle in multicellular biofilm communities in the host environment. Through investigations in recent years we now begin to realize that bacterial cell-cell interactions among *E. coli* cells on biotic and abiotic surfaces play a more significant role in pathogenicity than previously anticipated. It has therefore been of significant interest to clarify the mechanism(s) by which this organism colonizes surfaces and develops into substantial and robust biofilms.

In vitro Biofilm Development
Since *E. coli* K-12 has been the workhorse bacterium for molecular biologists for nearly 50 years, standard laboratory strains became model organisms used in an approach to assign a developmental program to *E. coli* biofilms formed in vitro. A simple genetic screen was implemented utilizing 96-well

microtiter dishes as abiotic substrates for biofilm development in vitro, allowing large-scale isolation of mutants attenuated in biofilm formation under static conditions.

Underlined by microscopic observations, the results of these initial studies were integrated in a developmental model for *E. coli* biofilm formation [6]. According to this model, *E. coli* K-12 utilizes flagella-mediated motility and type 1 pili to initiate early attachment processes. The major phase-variable outer membrane protein Ag43 was implicated in further development of microcolonies, and in agreement with the classical role ascribed to exopolysaccharides in stabilization of mature biofilms, the production of colanic acid was found to be required for the development of normal biofilm architecture in vitro.

In subsequent similar approaches, additional factors have been found to affect biofilm formation of *E. coli* on abiotic surfaces in conventional growth media; however, only the effects of a few of them have been studied in detail [7]. The intracellular localization of most of the proposed effector proteins such as the disulfide bond formation catalyzing DsbA or the acetate kinase AckA suggests an indirect influence, possibly by altering expression, assembly or function of already implicated surface appendages and outer membrane proteins. The importance of others such as the stress-response sigma factor RpoS or the stringent response proteins RelA and SpoT might simply indicate the requirement for metabolic pathways and stress responses within the heterogeneous biofilms that are less important during exponential growth in suspension.

Interestingly, the growth of *E. coli* K-12 biofilms in continuous hydrodynamic culture leads to the identification of biofilm-promoting factors, reflecting the reduced biofilm-forming capability of K-12 lab strains under these conditions. An *E. coli ompR234* mutant was isolated from the glass surface of a long-term continuous culture that was found to constitutively overexpress curli fimbriae [8]. The significantly improved biofilm formation phenotype was independent of flagella [9]. In 2001, Ghigo [10] discovered that conjugative plasmids enhance biofilm formation on submerged Pyrex slides under continuous flow when the expression of conjugative pili is derepressed. Mutant analysis demonstrated that at least for plasmid F, functional conjugative pili are indeed necessary to obtain the observed induction. In a subsequent study, evidence was provided that the promotion of biofilm formation in the presence of the conjugative transfer genes of plasmid F is independent of flagella, type 1 pili or Ag43 synthesis [11].

As the biofilm lifestyle is thought to be fundamentally different from bacterial life in mixed suspension, major differences in gene expression were expected to be encountered upon switching from planktonic to biofilm growth. This view was confirmed by an experimental approach that used random chromosomal insertions of a promoterless *lacZ* reporter gene [12]. A large fraction

(38%) of 885 fusions was differentially expressed in a curli-promoted static *E. coli* K-12 biofilm when compared to planktonic cells. However, a recent microarray analysis of a biofilm formed by a wild-type K-12 strain under continuous flow indicated a more modest impact on global gene expression [13]. The transcript level of only 5.4 and 13.6% of the 4,290 protein-encoding genes was found to be significantly different as compared to expression in either exponential or stationary planktonic culture, respectively. It is unclear whether these drastically different results in terms of changes in global gene expression can be ascribed to the different strain background and/or the experimental setup.

Due to the exclusive focus on K-12 strains in the vast majority of genetic studies, the relevance of the implicated factors for biofilm formation of non-domesticated *E. coli* isolates remains uncertain. For example, whereas the role of type I and curli fimbriae in the adherence of Shiga toxin-producing *E. coli* has been confirmed [14], a recent study suggests that the expression of colanic acid blocks adhesion of uropathogenic *E. coli* (UPEC) to inert abiotic surfaces [15]. Given the significantly elevated genome size of pathogenic *E. coli* as compared to K-12, determination of the diversity of molecular mechanisms used by the species *E. coli* in bacterial cell-cell interactions will necessitate the application of the already established molecular approaches at least to prototypic clinical *E. coli* isolates.

Gastrointestinal Biofilms

As a minority member of the normal flora of the large intestine in verte-brates, *E. coli* has to compete for nutrients with approximately 500 other indigenous species. In principle, successful coexistence can only be achieved by a growth rate that is at least equivalent to the washout rate from the intestine or by adherence to the intestinal epithelial cells [16]. Indeed, *E. coli* is capable of growing rapidly in intestinal mucus both in vivo and in vitro, whereas growth in luminal contents seems to be poor [17]. In addition, in situ hybridization experiments detected only separated single cells of commensal *E. coli* strains within the mucus layer but no bacterial cells associated with the epithelium [17, 18]. Thus, benign *E. coli* cells do not seem to be able to overcome the innate barriers that impede colonization in a healthy host and the natural lifestyle of these strains appears to be to reside and grow within the mucus layer almost exclusively as single cells.

In contrast, each highly adapted *E. coli* clone causing diarrheal disease has evolved efficient ways to penetrate the mucus layer and stably adhere to the underlying epithelial cells even at intestinal sites normally not colonized by *E. coli*, such as the small bowel mucosa [19]. As for other mucosal pathogens, surface colonization by diarrheagenic *E. coli* is a prerequisite to initiate disease.

Not surprisingly therefore, the most useful phenotypic assay for the diagnosis and differentiation of diarrheagenic *E. coli* pathotypes is an adherence assay using monolayers of epithelial HEp-2 cells. Strikingly, the adherence pattern of members of two major pathotypes of diarrheagenic *E. coli*, enteropathogenic (EPEC) and enteroaggregative (EAEC) *E. coli* involves – in addition to binding to eukaryotic cells – apparent strong interactions between bacterial cells leading to three-dimensional structures typically observed in bacterial biofilms. EPEC develop a characteristic localized adherence pattern appearing as microcolonies on the surface, whereas EAEC appear to aggregate both on the surface as well as more distantly from the epithelium in a characteristic stacked-brick config-uration [19]. Most importantly, similar biofilm-like adherence patterns have also been observed for both EPEC and EAEC in vivo.

While the adherence to epithelial cells has been extensively studied, little information is currently available about the factors that trigger bacterial cell-cell adherence or the relevance of the size of these cell aggregates for pathogenicity [20]. Although the plasmid-encoded bundle-forming pili (BFP) of EPEC have been suggested to mediate interbacterial interactions allowing formation of three-dimensional microcolonies on the surface of epithelia [21], BFP-expressing EPECs were found to bind to epithelial cells rather than to already formed microcolonies. Interestingly, BFP are subject to morphological changes from thin to thick pili as infection proceeds, resulting in loosening and dispersal of the aggregates [20]. A *bfpF* mutant that was found unable to undergo this morpho-logical change was significantly attenuated in virulence, indicating that formation and dispersal of microcolonies are both important for virulence.

Likewise, plasmid-encoded thin aggregative adherence fimbriae were found to mediate the adherence and aggregation pattern of EAEC strains in vivo and in vitro [19]. Interestingly, the aggregative adherence pattern also requires expres-sion of a secreted coat protein designated Aap (antiaggregation protein), which appears to promote dispersal of EAEC on the intestinal mucosa by forming a protein capsule on the bacterial surface. Mutations in *aap* lead to increased aggregation and significantly reduced mucus penetration in vitro, indicating that bacterial cell-cell adherence has to be tightly controlled in order to be advantageous in the intestinal environment [22].

Nevertheless, a large fraction of EPECs and EAECs lack BFP and aggrega-tive adherence fimbriae, respectively [19, 23]. Thus, *E. coli* clones seem to have evolved various divergent pathways to solve the same problem.

Intracellular Biofilm-Like Pods in UTI

The human urinary tract is usually a sterile system protected from the intestinal microflora by nonspecific resistance mechanisms that include phago-cytosis, endotoxin-induced shedding of bladder epithelial cells, and the flushing

effect of urine flow. However, UTIs are considered to be the most common bacterial infections [24], with UPEC remaining the predominantly isolated species [25]. Generally, UPECs are thought to migrate from the gastrointestinal tract to the periurethral area where they eventually enter the bladder via the urethra [26]. Further transport into the kidneys may even enable an invasion into the bloodstream.

Since intestinal *E. coli* clones are not equally able to survive within and colonize the urinary tract, UPECs are thought to be equipped with a variety of virulence factors including various adhesins of fimbrial nature such as curli, type 1 pili, P, S, and F1C fimbriae [27]. These surface appendages bind to specific host cell receptor molecules and facilitate attachment of bacteria to specific epithelial cells they encounter during their transit [28]. However, despite the clear importance of cell-surface interactions during the course of infection, bacterial cell aggregates typical for biofilm formation have not been demonstrated on epithelial cells in vivo.

Recent evidence suggests a novel role for biofilm-like cell-cell interactions during recurrent UTI. After artificial UTI infection of mice, Anderson et al. [29] observed large pod-like bacterial cell aggregates within superficial cells of dissected bladders whereas uninfected bladders appeared smooth. Bacteria within the pods had a uniform coccoid morphology, were interconnected by fibers and encased in a polysaccharide matrix. Although the presence of persistent *E. coli* in the bladder following acute UTI has been shown before, these large biofilm-like pods are observed after only 24 h of infection and represent a previously unrecognized intracellular microbial community and might play a role in the frequent recurrence of uncomplicated UTI (cystitis). However, the occurrence of these bacterial cell communities in human UTI has not yet been demonstrated.

Colonization of Indwelling Devices

For every artificial appliance placed in humans there is a corresponding microbial infection [30]. The crucial importance of biofilms associated with contamination of medical implant devices has been well established. Although *E. coli* has been found to adhere to implanted endotracheal tubes and contact lenses [6, 31], it is predominantly isolated from the surface of urinary catheters. Catheter-associated UTIs are indeed the most common among nosocomial infections. For example, 10–50% of patients experiencing short-term (<7 days) urinary catheterization [32], and virtually all patients undergoing long-term (>1 month) catheterization became infected [33].

During early stages of infection, *E. coli* is assumed to be present as a single species, whereas longer catheterization periods commonly lead to the formation of mixed communities of mainly gram-negative opportunistic pathogens,

including *P. aeruginosa*, *Proteus mirabilis*, and *Klebsiella pneumoniae* [34]. Such *E. coli*-dominated biofilms formed on the luminal surfaces can reach more than 400 μm in height, are usually embedded in a polysaccharide matrix [35], and can contain minerals such as hydroxyapatite and struvite that crystallize at the biofilm-urine interface as a result of the elevated pH achieved by bacterial urease activity. Although symptoms are seldom associated with the infection initially, ultimate blockage of the inner lumen of the catheter and/or ascent of bacteria to the bladder and kidney manifest severe consequences for the patient if left untreated.

Further support for a biofilm mode of growth after catheter colonization is derived from studies indicating that bacteria in these biofilms survive the urinary concentrations of antibiotics generated by standard treatment [36]. As a consequence, removal of the colonized device is the only efficient way to clear the infection. Given these complications generated by biofilms, several attempts have been made to prevent infection and bacterial colonization of catheters by incorporating conventional antibiotics or biocides such as silver oxide into the catheter material [34, 36]. Unfortunately, although the onset of bacteriuria could be delayed for several days with some catheter materials and treatments, most of these strategies were ineffective in preventing colonization [31].

A better insight into biofilm formation and ecology on catheters therefore appears to be required in order to identify more suitable and specific drug targets or to design more resistant catheters. It needs to be addressed whether initial colonization by *E. coli* supports a later establishment of other pathogens. Subsequent colonizers could attach to initial *E. coli* biofilms or benefit from provision of more suitable conditions in the local microenvironment such as changes of pH and nutrient supply. Interactions between different species during biofilm formation such as coaggregation might play an important role, as such phenomena have already been observed between lactobacilli and UPEC [37].

However, since standardized in vitro and in vivo models are crucial for obtaining any relevant information about virulence mechanisms, the lack of a nondestructive, longitudinal monitoring system is a major problem faced in indwelling-device-related biofilm research. A recently described mouse model of chronic biofilm infection that relies on biophotonic imaging of bioluminescent reporter bacteria constitutes an appealing approach to overcome this bottleneck [38].

P. aeruginosa

P. aeruginosa is an environmental microorganism found especially in freshwater and soil. In humans, *P. aeruginosa* may cause a wide range of infections.

The most prevalent and severe chronic lung infection in cystic fibrosis (CF) patients is caused by mucoid, biofilm-forming *P. aeruginosa*, which has become endemic in CF patients [1]. CF is the most common congenital, inherited disease among Caucasian populations with an incidence rate of 1:2,500–1:4,500. The pathology of the lung infection, however, is similar in severe chronic obstructive pulmonary disease, where the number of patients is much higher.

In vitro Biofilm Development

In contrast to the biofilm development for *E. coli*, which appears to be a case of relatively simple self-assembly processes in concert with surface association, *P. aeruginosa* is considered an example of a more elaborate biofilm developmental pathway involving several distinct steps of early and late maturation. Most of the work clarifying this developmental cycle has been performed with reference strains – PAO1, *P. aeruginosa* 14 and PAK – and so far it appears that at least these strains share the major features of the biofilm developmental cycle. In particular, the highly structured *P. aeruginosa* biofilms (comprising 'mushrooms', 'towers', voids and water channels) observed under some conditions have been a challenge to molecular geneticists, and below we will briefly summarize the current understanding of how the development progresses and is controlled.

It is first of all important to stress that structural biofilm development by *P. aeruginosa* appears to be conditional. The immediate environment is a key determinant of the eventual biofilm structure, illustrated by the finding that in flow chambers supplied with a citrate minimal medium *P. aeruginosa* forms a flat biofilm, while in flow chambers supplied with glucose minimal medium it forms a heterogeneous biofilm with mushroom-shaped multicellular structures [39]. In a series of investigations, it was shown that the formation of the flat *P. aeruginosa* biofilm occurs via initial growth of sessile bacteria forming microcolonies at the substratum, followed by expansive migration of the bacteria on the substratum, resulting in the formation of a flat biofilm [39]. Since biofilm formation by a *P. aeruginosa pilA* mutant (which is deficient in biogenesis of type IV pili) occurred without the expansive phase that results in discrete protruding microcolonies, it was suggested that the expansive migration of the bacteria on the substratum is type IV pili-driven, and that the shift may be induced by some sort of limitation arising in the initial microcolonies.

The formation of the mushroom-shaped structures in the heterogeneous glucose-grown *P. aeruginosa* biofilm was shown to occur in a sequential process involving a nonmotile bacterial subpopulation, which formed the initial microcolonies by growth in certain foci of the biofilm, and a migrating bacterial subpopulation, which initially formed a monolayer on the substratum, and subsequently formed the mushroom caps by climbing the microcolonies [40].

The nature of bacterial cell agglutinating factor(s) in very dynamic *P. aeruginosa* biofilms is not known at present. A role of alginate as a cell-to-cell interconnecting substance has been proposed previously [41], but recently it was concluded that alginate is not expressed at any significant level in such in vitro biofilms and therefore cannot be a key structural determinant under the defined conditions [42]. As we will see later, this situation is completely reversed in biofilms developing in some clinical cases, where alginate production appears to be essential for robust biofilm development. Some bacterial cell populations are apparently kept in the biofilm by substances that allow type IV pili-driven migration. Since twitching motility is powered by a mechanism involving extension, grip, and retraction of type IV pili [43], it is possible that type IV pili can play a role as cell-to-cell and cell-to-substratum interconnecting compounds. It has been reported that extracellular DNA may play a role as a cell-to-cell interconnecting substance in *P. aeruginosa* biofilms [3, 44], and interestingly there is evidence that type IV pili bind to DNA [45]. Yet, other bacterial cell-to-substratum and cell-to-cell connections keep the *pilA* mutant bacteria substratum-associated and agglutinated in the biofilms. Evidence is emerging that a novel type of fimbriae may function as adhesin in *P. aeruginosa* biofilms [46], and that certain exopolysaccharides may function as cell-to-cell interconnecting substances [Friedmann and Kolter, pers. commun.]. Such compounds could likely interconnect nonmigrating *P. aeruginosa* populations.

The apparent complexity of the biofilm developmental cycle of *P. aeruginosa* has stimulated the search for genetic regulatory activities, and the findings of Davies et al. [47] that quorum-sensing control seems to be essential for normal biofilm formation was in accord with the characteristics of the process. In light of the current knowledge about the above-described steps of biofilm development for this organism it is, however, important to emphasize that so far no specific target for quorum-sensing control has been identified as relevant for these particular processes. It therefore remains to be seen whether quorum sensing is regulating any of the described process features such as bacterial cell-cell adherence, colony climbing or population differentiation.

Chronic Lung Infections in CF

CF patients are intermittently colonized with nonmucoid *P. aeruginosa* strains for an average of 12 months before the infections become chronic, and the presence of mucoid strains and an antibody response is a sign of chronicity [48, 49]. The chronic *P. aeruginosa* lung infections in CF patients is responsible for most of the morbidity and mortality of these patients [50], and this state of the infection constitutes a lung-associated biofilm [51, 52]. The biofilm is characterized by the mucoid phenotype of *P. aeruginosa* producing an abundance of alginate [53]. In the conductive zone of the lungs the majority of the bacteria

stay inside the mucus and grow under anaerobic conditions using nitrate as electron acceptor [54]. Most of the bacteria are not located on the epithelial cells, but they induce an endobronchitis and endobronchiolitis without spreading to the blood or to other organs [54, 55]. In the respiratory zone of the airways, however, the environment is aerobic [56]. Foci of pneumonia in the alveolar tissue with extensive infiltration of polymorphonuclear leukocytes (PMNs) surround localized biofilms of *P. aeruginosa* which are situated within the alveoles and alveolar ducts [55, 57]. The location and organization of the bacteria in these biofilms are similar to those observed in mucoid colonies and in sputum from CF patients with microcolonies of mucoid *P. aeruginosa* [58]. High levels of antibodies are produced against alginate and other *P. aeruginosa* antigens, but elimination of the infections is not accomplished [59], and the resulting persistent immune-complex-mediated inflammation is the major cause of the lung tissue damage [59]. The biofilm mode of growth is resistant to the patients' defense mechanisms and to antibiotic treatment [59] and is the major reason for the persistence of the infection lasting for more than 30 years in some patients.

Adaptation of P. aeruginosa *to CF Lungs*

The CF lung is a stressful environment for *P. aeruginosa*, and, therefore, they have developed a range of survival strategies. When particles of >5 µm containing bacteria are inhaled, they are deposited in connection with the gel phase of the mucus on the airway surfaces in the relatively small conducting zone of the central airways, which are covered by ciliated epithelial cells and coordinated movements of these cilia beating in the sol phase (=epithelial lining fluid) remove the gel phase of the mucus towards the trachea [56]. The gel phase of the mucus is produced by submucosal glands and goblet cells. In normal persons the effect of the cilia's beating (also named the mucociliary escalator) removes the mucus towards the trachea in this way rapidly (60 µm/s) clearing the bacteria within 6 h [54, 60]. This clearance mechanism is the most important part of the noninflammatory defense mechanism of the respiratory tract. In CF patients, however, the basic defect of the CFTR protein leads to a reduced volume of the epithelial lining fluid [60], and the mucociliary clearance of the bacteria is therefore greatly reduced, leading to robust bacterial growth [54] and recruitment of the inflammatory defense mechanisms (PMNs) [59]. When particles of 2–5 µm containing bacteria are inhaled, they are deposited in the much larger peripheral respiratory zone of the lungs without mucus or cilia, and the major defense mechanism are the alveolar macrophages, which belong to the inflammatory defense mechanisms [56]. In accordance, bronchoalveolar lavage studies on CF infants have shown that recruitment of the inflammatory defense mechanisms (dominated by the phagocytic cells, PMNs and macrophages) takes place when aspirated microorganisms are colonizing the lower respiratory

tract [61]. When PMNs and macrophages engulf bacteria there is a metabolic burst in the phagosomes leading to a release of reactive oxygen species, some of which are leaked to the environment [62]. These oxygen radicals induce killing, DNA damage and mutations in the bacteria [62, 63].

Oxygen radicals produced by the inflammatory response (PMNs) induce mutations in e.g. the *mucA gene* leading to the alginate production, which is characteristic for *P. aeruginosa* biofilm infections in CF [64]. Alginate, on the other hand, is an oxygen radical scavenger [65] and provides mucoid *P. aeruginosa* with protection against further DNA damage compared to nonmucoid strains [66]. Alginate can also make the bacteria resistant to phagocytosis by PMNs and macrophages [67]. Alginate production of *P. aeruginosa* biofilms in CF lungs, therefore, seems to be the major mechanism of adaptation permitting mucoid strains to persist in the hostile environment of oxygen radicals originating from the phagocytic cells of the inflammatory defense mechanisms.

The lungs consist of the central conducting zone and the peripheral respiratory zone. When *P. aeruginosa* grow in the peripheral respiratory zone (niche), the growth condition is comparable to growth in an aerobic or microaerophilic incubation chamber (5–20% oxygen). The respiratory zone is the area of the lungs where the venous blood becomes oxygenated in the dense capillary network of the alveoles, thus providing continuous culture conditions with nutrient and oxygen from the blood [56]. The central conductive zone of the respiratory tract (the bronchi), on the other hand, where *P. aeruginosa* is located in sputum, is a completely different niche, since no oxygen is present in sputum [54]. Sputum consists mainly of dead PMNs and an abundance of released DNA [68] and leukocyte proteases [69] originating from PMNs in addition to mucus. In sputum the environment is anaerobic and the growth condition for *P. aeruginosa* is comparable to a batch culture in the stationary phase. There is not so much blood supply of the conducting zone compared with the respiratory zone [56] and the bacteria are located inside sputum and not at the epithelial surface [54]. Under these conditions *P. aeruginosa* may rely on anaerobic growth with NO_3^- as the electron acceptor [54].

In cases of infection with mucoid *P. aeruginosa* cells, which dominates chronic infections, a pronounced antibody response against the bacteria is observed in connection with deteriorating lung function and poor prognosis. In contrast, the few CF patients colonized only with nonmucoid *P. aeruginosa* have a low antibody response, and they maintain their lung function at the same nearly normal level similar to that of CF patients without chronic infection [70]. The persistent PMN inflammation around *P. aeruginosa* infection areas in the respiratory zone destroys the lung tissue of the infected foci of the lungs of the CF patients [71]. The alveolar macrophages in this zone [61], which migrate to the lymph nodes [56], are antigen-presenting cells, which are important for

initiating the antibody production of the B lymphocytes. Colonization of the conducting zone of the lungs, on the other hand, primarily leads to obstruction due to the abundance of mucus, and antibody production and lung tissue damage of the respiratory zone are normally not severe [54]. These observations suggest that severe respiratory failure in CF patients is caused by infection of the respiratory zone with mucoid *P. aeruginosa* located in biofilms [55, 57]. Pieces of these biofilms are visible in gram-stained smears of sputum from CF patients [58]. Although the mucoid phenotype of *P. aeruginosa* is characteristic for colonization of the respiratory zones in CF patients, nonmucoid variants of the same genotype are regularly present simultaneously in sputum [66]. The reason for this diversity has so far been obscure [58], but indications from in vitro investigations of stratified bacterial populations may be relevant for a better understanding of the phenotypical diversity of infectious *P. aeruginosa* populations in CF lungs [57, 72–74]. In a population of lung-associated mucoid *P. aeruginosa*, isogenic nonmucoid variants could represent a subpopulation of the original infecting cells (most likely not mucoid) occupying a niche in which mucoidy is not selectively favorable. Alternatively, the nonmucoid variants may be phenotypic revertants arising either as 'cheaters', benefiting from the alginate production of other bacteria within the biofilm, or as niche specialists in the anaerobic conductance zone. The fact that these variants seem to appear as individual bacteria outside the mucoid biofilm areas in sputum may indicate that they predominantly derive from the anaerobic zone.

Antibiotic Therapy

Bacteria growing in biofilms are often much more resistant to antibiotics than planktonic cells of the same isolate. Minimal inhibitory concentration and minimal bactericidal concentration may be increased 100- to 1,000-fold in old biofilms, whereas young biofilms are less resistant [75]. In contrast, planktonic bacteria released from such resistant biofilms are most often found to be as sensitive to antibiotics as the original planktonic cells [75]. Biofilm-induced resistance to antibiotics can be caused by several factors, such as slow growth, reduced oxygen concentrations at the base of the biofilm, penetration barriers e.g. binding of positive charges on the antibiotic molecules to the negatively charged alginate polymers, the presence of ß-lactamase from the bacteria which cleaves and/or traps β-lactam antibiotics and overexpression of efflux pumps [53, 76]. The increased resistance of biofilm bacteria usually results in the failure of antibacterial therapy with respect to eradication of the bacteria, but the antibiotic treatment regularly leads to temporary clinical improvement of the patient [53].

The development of traditional mechanisms of resistance to antibiotics occurs frequently in CF due to the intensive selective pressure provided by the

large amount of antibiotics used in these patients [53]. Mucoid and nonmucoid variants of the same strain are frequently simultaneously present in sputum but the nonmucoid variants are more resistant to antibiotics, possibly reflecting a higher antibiotic selection pressure outside the alginate biofilm [66]. The number of *P. aeruginosa* in sputum may be as high as 10^8-10^{10} CFU/ml. The high number of bacteria implies that mutations do occur in sputum. In addition, high frequencies (>30%) of hypermutable *P. aeruginosa* variants have been found in CF lung infection [77, 78], and the mutator strains (hypermutable strains) showing >20-fold higher mutation frequency than control strains [78] were also multiply resistant. The observations from *P. aeruginosa* strains from CF patients showed the occurrence of a high frequency of hypermutable *P. aeruginosa*, a high level of resistance to many antibiotics and, in the case of ciprofloxacin, several different mutations which increased over time [79]. In addition, mutations can be induced by means of oxygen radicals from PMNs, which in vitro leads to alginate production due to mutations in the *mucA* gene [64]. Furthermore, there is an antioxidant imbalance in the CF lung, which leads to oxygen radical damage [80]. Taken together, all these observations have led us to suggest that it is the chronic inflammation dominated by PMNs which induces a high level of mutations in *P. aeruginosa* in the CF lungs and that the resistant mutants are then selected by the heavy use of antibiotics. These conventional resistance mechanisms are then added to the physiological resistance caused by the biofilm mode of growth in the CF lung.

Perspectives

There is an increasing documentation concerning the importance of biofilms in connection with microbial infections – in particular in relation to persistent infections of opportunistic pathogens. The detailed investigation of several microbial biofilms has produced interesting information indicating that the multicellular life of bacteria may have its own genetic background that is controlled by bacterial interactions, which in some cases may resemble complex eukaryotic tissue development. One important question in relation to pathogenic bacteria is whether it is possible to extrapolate from these detailed in vitro observations and mechanisms to the conditions in the infected host. A word of caution is probably warranted: it is important to keep in mind that there is no indication of a consensus developmental program, and we therefore must resolve the individual biofilm pathways case by case. We also have strong indications that the in vitro biofilm conditions applied in the laboratory cannot be compared to those prevailing in the host, and it is therefore important to develop better model systems, if not performing the investigations in vivo. The genomic diversity of

bacteria is an additional complication; different isolates of the same species often behave quite differently from each other or when compared with reference strains or laboratory strains. We also have to keep in mind that simple molecular identification and characterization of various bacterial cell-cell interaction mechanisms only constitute the first step in an approach to interfere with cell-cell interactions necessary for virulence. Since the overall physical strength and resistance of biofilms to shear force presumably play a critical role in vivo, a better understanding of the binding forces exhibited by the individual implied molecular factors is required to identify realistic drug targets.

We now have some fundamental knowledge about the principles of bacterial life forms which seem to be important for a range of pathogens causing severe therapeutic problems in the clinic, and the technological and conceptual advances that have been made during the last 10 years of biofilm research should be applied with increased intensity in the investigations of infectious diseases. In particular, it will be important to establish the boundaries for our extrapolations from in vitro biofilm studies to the conditions prevailing in clinical cases, just as we must expand our investigation scenarios to encompass conditions which much better reflect what goes on in cases of suspected biofilm infections.

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Bacterial Peptidases

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Enzymes that catalyze the hydrolysis of peptide bonds are referred to as proteases or peptidases. They are widely distributed in nature, where a variety of biological functions and processes depend on their activity. Regardless of the complexity of the organism, peptidases in general are essential at every stage in the life of every individual cell, since all protein molecules produced must be proteolytically processed and eventually degraded. Therefore, it is not surprising that throughout cellular life forms, genes encoding proteases occur at a relatively high frequency, ranging from 1.15% (*Pirellula* sp.) to 6.06% *(Buchnera aphidicola)* of the total gene count, with the average being about 3%. Among bacterial species which are pathogenic for humans, the number of peptidases known and putatively functional ranges from 9–15 in small genomes, such as those of the *Mycoplasma* spp. (1.45–2.07% of the total gene count) to 98 (2.64%) and 121 (2.85%) in genomes such as *Pseudomonas aeruginosa* and *Escherichia coli*, respectively. Fortunately, only a small fraction of the expressed peptidases in any pathogen impose a direct or indirect deleterious effect on their human host and may therefore be considered a virulence factor. With respect to the number of protease genes, the record in the microbial world goes to *Bacillus cereus* [179 potentially functional peptidase genes out of a total of 5,243 genes (3.99%)]. In comparison, only three times more functional protease genes have been identified in *Homo sapiens* (489 + 143 out of 23,531, 2.7% of the total gene count).

Classification of Peptidases

Three major criteria are currently used to classify peptidases: (1) the reaction catalyzed, (2) the chemical nature of the catalytic site, and (3) the evolutionary relationship to other proteases, as revealed by the primary and/or tertiary structure of the protein.

Based on the reaction they catalyze, peptidases are divided into two classes, comprising the exopeptidases and endopeptidases. The exopeptidases act only near the ends of polypeptide chains. Those acting at a free amino-terminus to liberate a single amino acid residue, a dipeptide or a tripeptide are referred to as aminopeptidases, dipeptidyl-peptidases, and tripeptidyl-peptidases, respectively. On the other hand, exopeptidases that cleave a single residue or dipeptide from a free carboxy-terminus are called carboxypeptidases and dipeptidyl-dipeptidases, respectively. Other exopeptidases are specific for dipeptides (dipeptidases), or the removal of terminal residues, either carboxy- or amino-terminal, that are substituted, cyclized, or linked by isopeptide bonds. Isopeptide bonds are peptide linkages other than those joining an α-carboxyl to an α-amino group. This last group is collectively referred to as the omega peptidases and is of particular importance for prokaryotic organisms producing nascent proteins that start with N-formylmethionine at the beginning of their sequence, which needs to be removed.

In contrast to the exopeptidases, endopeptidases preferentially hydrolyze peptide bonds in the inner regions of peptide chains, away from the termini. Typically, the presence of free α-amino or α-carboxyl groups has a negative effect on the activity of these enzymes, but it must be kept in mind that it is not unusual for an endopeptidase to have both exo- and endopeptidase activity. A subset of the endopeptidases, with activity limited to oligopeptides or fairly short polypeptide chains, are referred to oligopeptidases.

According to the nature of their catalytic site, peptidases are divided into 6 types differing in their catalytic mechanism. The aspartic peptidases, sometimes incorrectly referred to as carboxypeptidases, have two aspartic acid residues involved in the catalytic process. The cysteine-type peptidases (incorrectly called thiol peptidases) have a cysteine residue in their active center. The metallopeptidases use a metal ion (commonly zinc) in their catalytic mechanism. The activity of the serine-type peptidases depends on an active serine residue, while threonine-type peptidases utilize a catalytic threonine. The last group constitutes a number of peptidases that cannot yet be assigned to any particular catalytic type. Among prokaryotic organisms, including pathogenic bacteria, peptidases of all 6 catalytic types are common, although the frequency of their appearance is often strongly disproportionate (see following sections).

A third way to classify peptidases is based on the evolutionary and structural relationship among enzymes, inferred from the comparison of amino acid sequences and/or tertiary structures. This method, introduced by Barrett et al. [2003], and currently implemented in the MEROPS database server (www. merops.ac.uk) [Rawlings et al., 2004], is a powerful tool, allowing the logical classification of all peptidases, since the structural similarities within a family of peptidases commonly reflect important similarities in catalytic mechanism and other properties. However, in some cases, the classification is not fully consistent with three-dimensional structural data, as observed for the structurally distinct astacins and adamolysins, englobed in the same family M12, or serralysins and matrixins, grouped into family M10. This classification may even extend to assigning the biological function of an enzyme for which only the encoding DNA sequence is known. Therefore, the classification system briefly described below will be used here to discuss bacterial peptidases.

The term 'family' is used to describe a group of peptidases in which each member shows an evolutionary relationship to at least one other, either throughout the whole sequence or at least in the part of the sequence responsible for catalytic activity. Each family is identified by an upper-case letter representing the catalytic type (A for aspartic type, C for cysteine type, M for metallo-type, S for serine type, T for threonine type, and U for unknown type), followed by a unique number. A family that contains deeply divergent groups is sometimes divided into subfamilies, identified by upper-case letters. Families are further clustered into clans. A clan contains all the present peptidases that have evolved from a single origin. It represents one or more families that show evidence of their evolutionary relationship, judged by similar tertiary structures, or when structures are not available, by the order of catalytic-site residues in the polypeptide chain and often by common sequence motifs around the catalytic residues. Each clan is identified by two letters, the first representing the catalytic type of the families included in the clan (with the letter 'P' being used for a clan containing families of more than one of the catalytic types: serine, threonine or cysteine).

For the purpose of this review it is worth introducing a fourth classification of bacterial peptidases according to their role in pathogenicity. Pathogenicity, which is a term synonymous with virulence, is generally delineated as the ability of a bacterium to cause infection. Virulence factors represent either bacterial products or a strategy that contributes to virulence, which entails the pathogen to colonize the host, evade host defense mechanisms, facilitate dissemination, and cause host damage [Isenberg, 1988; Mekalanos, 1992]. In many respects, proteolytic enzymes produced by several pathogenic bacterial species fit into the category of virulence factors since they are directly involved in one or more of the processes listed above. Taking into account the numbers of peptidases produced by bacteria, relatively few can be considered sensu stricto as virulence factors. In

this chapter we refer to peptidases, which preferentially target host proteins as 'primary virulence factors'. Many other peptidases are indirectly involved in pathogenicity, since they are indispensable for the expression of virulence factors per se. Such proteinases we call 'auxiliary virulence factors'. Finally, many other peptidases have well defined housekeeping functions. They do not harm the host either directly or indirectly, but are needed to withstand the stress of living in a hostile environment. We name them 'bystander virulence factors'.

Aspartic Peptidases

The MEROPS database currently (March 24, 2004) contains a total of 19,682 peptidase-related sequences and aspartic peptidases represent 6.3% of all peptidases, compared with 19.8% for cysteine, 30.2% for metallo-, 35.0% for serine, and 4.1% for threonine peptidases. The aspartic peptidases are subdivided into six clans. Two clans (clans AC and AF) contain enzymes present only in the major domain of living organisms made up by bacteria. Bacterial peptidases also constitute a separate family within clan AD. They are represented by three archetypal enzymes: lipoprotein signal peptidase (LspA) often referred to as signal peptidase II (SPase II), a type IV prepilin peptidase and omptin.

SPase II participates in prolipoprotein translocation through the cytoplasmic membrane of both gram-negative and gram-positive bacteria. With the exception of only three bacterial species, including *Mycoplasma penetrans*, *Mycoplasma gallisepticum* and onion yellows phytoplasma, the gene encoding a potentially functional protein has been found in all other species for which there is a completely sequenced genome (total 94). SPase II is a good example of a nonessential housekeeping enzyme, which, in the case of some pathogens, can contribute to their virulence. Apparently in *Listeria monocytogenes*, a gram-positive facultative intracellular human pathogen, temporally regulated expression of surface lipoproteins is critical for efficient phagosomal escape of *L. monocytogenes*. Mutants deficient in SPase II activity stayed entrapped inside the phagosomes of infected macrophages and have severely attenuated virulence [Reglier-Poupet et al., 2003].

The gene encoding a potentially functional homologue of the type IV prepilin peptidase is strongly conserved amongst bacteria (clan AD, subfamily 24A), although not to the same degree as SPase II. The enzyme cleaves, among other substrates, the leader sequence from type 4 prepilins or prepilin-like proteins secreted by a wide range of bacterial species. Its activity is required for a variety of functions, including type 4 pilus formation, secretion of toxins and other enzymes through the type II protein secretion system in gram-negative bacteria, gene transfer and biofilm formation. In many regards,

prepilin peptidase can be considered a housekeeping enzyme, but it contributes to the expression of well-defined virulence factors in several pathogenic species. In enteropathogenic *E. coli*, assembly of the type IV fimbriae known as the bundle-forming pilus (BFP) is dependent on the activity of the prepilin peptidase encoded by the *bfpP* gene [Anantha et al., 2000]. Biogenesis of BFP is required for autoaggregation and localized adherence to host cells and enteropathogenic *E. coli* mutants deficient in these surface appendages are nonvirulent in orally challenged human volunteers. Similarly, a knockout of the prepilin peptidase gene *(pilD)* in *Legionella pneumophila* greatly impaired the ability of the bacterium to grow within amoebae and human macrophage-like U937 cells [Liles et al., 1999]. The mutant showed strongly attenuated virulence in animal models due to the malfunction of the prepilin peptidase-dependent type II secretion system operating inside the phagocytes [Rossier et al., 2004]. In the case of *Vibrio cholerae*, functioning of the extracellular protein secretion apparatus encoded by the *eps* gene is strongly dependent on prepilin peptidase activity. Deletion of the peptidase gene resulted in a dramatic decrease in cholera toxin secretion and abolished surface expression of the type 4 pilus responsible for mannose-sensitive hemagglutination [Marsh and Taylor, 1998].

In contrast to SPase II and the prepilin peptidase, which are good examples of auxiliary virulence factors, the plasminogen activating surface peptidase, Pla, of the plague bacterium *Yersinia pestis* is a paradigm for the primary virulence factor. The Pla surface peptidase resembles mammalian plasminogen activators in function and converts plasminogen to plasmin by limited proteolysis. At the same time, the Pla peptidase inactivates α_2-antiplasmin, a potent inhibitor of plasmin [Kukkonen et al., 2001], facilitating unrestrained activity of this broad-spectrum peptidase that in turn degrades fibrin and noncollagenous proteins of the extracellular matrix and activates latent procollagenases. This causes local damage of the connective tissue and enables the highly efficient spread of *Y. pestis* from a subcutaneous site, where the pathogen is introduced by a vector bite, into the circulation [Sodeinde et al., 1992]. In addition, independent of proteolytic activity, the Pla peptidase mediates *Y. pestis* adhesion to basement membrane and invasion into human endothelial cells, which may also contribute to dissemination of the bacterium in the host [Lahteenmaki et al., 2001].

The Pla peptidase shares significant amino acid sequence identity (about 50%) with the *E. coli* integral outer membrane peptidases, OmpT and OmpP, referred to as omptins. Since some serine protease inhibitors weakly affect OmpT activity and site-directed mutagenesis studies appeared to implicate Ser99 and His212 as the active site residues [Kramer et al., 2000], the omptins have been classified as novel serine proteases (family S18) [Rawlings and Barrett, 1994]. However, the crystal structure of OmpT [Vandeputte-Rutten et al., 2001] followed

by structure-guided site-directed mutagenesis [Kramer et al., 2001] proved that OmpT activity depends on the Asp83-Asp85 and Asp210-His212 residues. These residues are strictly conserved in all OmpT homologues described to date, including PgtE of the *Salmonella* sp., peptidase SpoA of *Shigella flexneri*, putative peptidases of *Rhizobium loti*, a new species of legume root nodule bacteria, plant pathogens of the *Erwinia* sp. and *Agrobacterium tumefaciens*, and of course OmpP and the Pla peptidase. It is assumed that these peptidases have a conserved fold, consisting of a 10-stranded antiparallel β-barrel that protrudes far from the lipid bilayer into the extracellular space with the catalytic site located in a groove at the extracellular top of the vase-shaped β-barrel. Interestingly, activity of omptins is critically dependent on a specific interaction with lipid A of the LPS molecule [Kukkonen et al., 2004].

Omptins other than the Pla peptidase are typical housekeeping enzymes with their function/s not yet entirely understood. Nevertheless, they also seem to be implicated directly or indirectly in bacterial pathogenicity [Stathopoulos, 1998]. The presence of the *ompT* gene in clinical isolates of *E. coli* has been associated with complicated urinary tract disease [Webb and Lundigran, 1996], a notion supported by the observation that OmpT cleaves protamine, a highly basic antimicrobial peptide that is excreted by epithelial cells of the urinary tract [Stumpe et al., 1998]. Similarly, PtgE expression by *Salmonella enterica* may promote resistance to innate immunity by proteolytic inactivation of α-helical cationic antimicrobial peptides. On the other hand, SopA from *S. flexneri*, the causative agent of bacillary dysentery, cleaves the endogenous autotransporter IcsA, which has an essential role in the formation of actin tails in host cells, and therefore SopA might be indirectly involved in the actin-based motility inside infected cells [Egile et al., 1997; Shere et al., 1997].

Among omptins only the Pla peptidase is a potent plasminogen activator. Interestingly, however, OmpT can be easily converted into the plasminogen activator by subtle mutations at surface-exposed loops. Such conversion may represent an interesting example of the evolution of a potent virulence factor from a housekeeping protein [Kukkonen et al., 2001]. In the case of PgtE from *S. enterica*, the O-antigen of LPS sterically prevents recognition of large-molecular-weight substrates, rendering plasminogen activator activity cryptic in this enteropathogen. The O-antigen repeats also prevent plasminogen activation by the Pla peptidase and, in this context, it is now clear why *Y. pestis* lost the genetic locus involved in O-antigen synthesis [Kukkonen et al., 2004].

Collectively, it is apparent that the proteolytic activity of omptins contributes to virulence in a variety of ways. Their contribution ranges from bacterial defense and plasmin-mediated tissue infiltration to motility inside infected cells. Fortunately, they are produced by only a limited number of gram-negative bacteria which are pathogenic for plants and animals.

Cysteine Peptidases

The MEROPS database contains 3,897 cysteine-peptidase-related sequences (19.8% of the total sequences), which are divided into five phylogenetically related clans of proteins (CA, CD, CE, CF, and CH) and several families which are provisionally without a clan assignment. Bacterial peptidases are scattered among all of the clans except clan CH. It is a paradox, however, that although the bacterially derived cysteine peptidases, streptopain (SpeB) of *Streptococcus pyogenes* and clostripain from *Clostridium perfringens* were among the first proteolytic enzymes ever characterized, cysteine peptidases are underrepresented in prokaryotic organisms and show limited variation. Just one family (family C40) encompasses more than one third of the total cysteine peptidase count in prokaryotes (about 640 sequences). These enzymes are exemplified by dipeptidyl-peptidase VI from *Bacillus sphaericus* and murein endopeptidases (LytE and LytF) from *Bacillus subtilis* and represent typical housekeeping peptidases. Biochemically characterized enzymes have N-acetylmuramoyl-*L*-alanine amidase activity [Kuroda and Seikiguchi et al., 1991; Moriyama et al., 1996; Yamamoto et al., 2003] and are involved in a peptidoglycan turnover. They are widespread among both gram-positive and gram-negative bacteria and genes encoding from 1 to 6 functional homologous are present in at least 70 bacterial species with completely sequenced genomes (out of 94). No association with virulence has been reported for this group of peptidases.

Sortases (Family C60)

Peptidases comprising the C60 family constitute a functionally and structurally related group of proteins expressed by all gram-positive species of bacteria. The prototypical enzyme, referred to as sortase A (SrtA), was first described in *Staphylococcus aureus* as an enzyme that is anchored in the plasma membrane and is responsible for covalent tethering of protein A to the cell wall [Mazmanian et al., 1999]. It is now known that SrtA attaches a range of important surface proteins to the peptidoglycan component of *S. aureus* and many other gram-positive bacteria, including virulence-related microbial surface components recognizing adhesive matrix molecules (MSCRAMs). Substrates for SrtA are easily recognized by a carboxy-terminally located sorting signal made up by an LPXTG amino acid sequential motif, where X is any amino acid, followed by a hydrophobic domain composed of about 20 amino acid residues and a tail of positively charged residues. The hydrophobic domain and charged residues hinder polypeptide chain translocation through the plasma membrane, facilitating recognition of the LPXTG motif by SrtA. In a two-step transpeptidation reaction, sortase cleaves the LPXTG motif between the threonine and

glycine residues and covalently attaches a polypeptide chain, via the carboxy-terminal threonine, to the amino group of the pentaglycine crossbridge, thus tethering the protein to the cell wall. Although the structure of peptidoglycan crossbridging shows large variability in gram-positive bacteria, the mechanism of surface protein attachment is strictly conserved.

A comparative genome analysis indicated that gram-positive bacteria frequently encode more than one sortase (up to 7 paralogues) and an even larger number of potential substrates (up to 40 per genome) with their characteristic LPXTG-type cell wall sorting motif or derivatives thereof [Comfort and Clubb, 2004]. In contrast, a single gene coding for a sortase and only one potential substrate have been identified thus far in only five gram-negative bacterial species. The sortases can be partitioned into 6 distinct subfamilies (5 in gram-positive and 1 in gram-negative bacteria) based on amino acid sequence. Members of each subfamily are suggested to recognize a discrete variation of the sorting motif [Comfort and Clubb, 2004]. In the bacterial species with more than one sortase, usually the SrtA-like molecule is responsible for tethering of most cell wall proteins in an organism, while additional sortase(s) have more specialized functions. For example, in the case of *S. aureus*, sortase B (SrtB) recognizes and anchors a protein known as IsdD, which is involved in heme iron transport [Mazmanian et al., 2002, 2003]. This protein contains the NPQTN motif instead of the classical LPXTG sorting sequence exploited by SrtA, but otherwise the catalyzed reaction is identical. Also a protein, referred to as SvpA, which is anchored to peptidoglycan by SrtB of *L. monocytogenes* has the sorting motif, NAKNT, which is divergent from the one used by SrtA [Bierne et al., 2004]. As in *S. aureus*, the genes encoding SrtB and its target, SvpA, are part of the same locus. In *S. aureus*, *isd* genes are regulated by iron and encode factors for hemoglobin binding and the passage of iron, in the form of a heme group, to the cytoplasm [Mazmanian et al., 2002].

Some of the six sortase genes encoded in the genome of *Corynebacterium diphtheriae* are required for biogenesis of the pilus. Assembly of the fimbriae involves the cleavage of pilin precursors at the classical sorting signal (LPLTG), or at an LAFTG motif, by two different sortases, which then further catalyze amide bond cross-linking of adjacent subunits or tethering to peptidoglycan [Ton-That and Schneewind, 2003]. This covalent attachment of adjacent pilin subunits has probably evolved in many gram-positive bacteria, since sortase genes in close association with pilin subunit genes with sorting signals were found in enterococci, streptococci, *Actinomyces* spp., and *C. perfringens*.

The NMR structure in solution of SrtA [Ilangovan et al., 2001] and the crystal structure of SrtB [Zong et al., 2004] from *S. aureus* are available, revealing an eight-stranded β-barrel core structure with a helical subdomain at the amino-terminal end, which is unique among peptidases. The topology of the β-barrel is

identical in both enzymes with the critical cysteine residue (Cys184 and Cys223 in SrtB and SrtA, respectively) located at the tip of the β7 strand. Initially, it was predicted that Cys184 and His120 of SrtA form a thiolate-imidazolium ion pair for catalysis [Ton-That et al., 2002] as in the papain cysteine peptidases. However, pKa measurements for SrtB Cys184 and His120 residues refuted the involvement of the His residue in the transpeptidation reaction [Connolly et al., 2003]. From the crystal structure of SrtB and conservation of the Arg233 (Arg197 in SrtA) residue it is apparent that a unique Cys-Arg catalytic dyad constitutes the foundation of the catalytic machinery of sortases.

By exposing anchored proteins and polymeric structures such as fimbriae, the cell wall envelope of gram-positive bacteria can be considered to be a surface organelle maintaining contact between the microbe and its environment. It is now apparent that the assembly of these surface appendages is dependent on sortases. In this regard, sortases can be considered to be house-keeping enzymes. However, they are responsible for surface expression of acknowledged virulence factors, which mediate adherence to host tissues, host cell invasion, iron acquisition, and provide protection from assault by the formidable forces of the innate and acquired immune system. Therefore, sortases can be considered to be the classical example of an auxiliary virulence factor. Indeed, it was shown that sortase knockouts in various pathogenic bacteria, including *S. aureus*, *Streptococcus mutans*, *L. monocytogenes*, *Streptococcus gordonii*, and *Streptococcus pneumoniae*, have significantly attenuated virulence when tested in several different animal models. In this way sortase(s) are a very good target for the development of therapeutic inhibitors to fight gram-positive infections.

Family C66: IdeS Peptidase (MAC Protein)

A streptococcal protein (Mac) has been identified as a group A *Streptococcus* (GAS)-secreted protein of 35 kD with homology to the α-subunit of Mac-1, a leukocyte β2 integrin. Mac binds to CD16 (FcγRIIIB) on the surface of human polymorphonuclear leukocytes and inhibits opsonophagocytosis and production of reactive oxygen species, which resulted in significantly decreased pathogen killing [Lei et al., 2001]. Later, the MAC protein was shown to be identical to the IdeS peptidase (IgG-degrading enzyme of *S. pyogenes*) [von Pawel-Rammingen et al., 2002a, b], a previously unrecognized cysteine peptidase of *S. pyogenes*. The IdeS peptidase is an extremely specific enzyme, which exclusively cleaves the heavy chain of IgG at the Gly237 residue in the hinge region. The enzyme is active in human plasma and its ability to interfere with Fc-mediated phagocytic killing has been demonstrated in a variety of bactericidal assays. These data collectively show that the IdeS protease contributes to evasion of the adaptive immune system by GAS by cleaving opsonizing IgG antibodies at the bacterial surface [von Pawel-Rammingen and Bjorck, 2003]. There is, however, a debate as to whether

the proteolytic activity of IdeS (MAC protein) is absolutely necessary for inter-
ference with phagocytosis, which may only be dependent on molecular mimicry
and the presence of the Arg-Gly-Asp amino acid motif in IdeS, which is involved
in the interaction of the enzyme with the human integrins, $\alpha_v\beta_3$ and $\alpha_{II}\beta_3$ [Lei
et al., 2002; von Pawel-Rammingen and Bjorck, 2003].

The occurrence of orthologues of the IdeS peptidase is limited to a very
small subset of the streptococci. In GAS, the enzyme occurs in two allelic
variants among GAS serotypes, where the amino acid sequences of the variants
differ from each other by about 15%. The only three homologues of the IdeS
peptidase identified thus far are in the genome of *Streptococcus equi* (two
genes) and in *Streptococcus suis*. One enzyme from *S. equi* was expressed and
the recombinant protein was shown to possess the same activity as the IdeS
peptidase [Lei et al., 2003]. A distant homologue was also identified in the
genome of *Treponema denticola*. The recombinant protein was expressed in
E. coli and shown to have a nonspecific, general peptidase activity [Potempa,
unpubl. data].

The activity of IdeS depends on a thiolate-imidazolium ion pair formed by
Cys94 and His262, which act as the active-site residues as in the papain-like
peptidases. These residues are conserved not only in the enzymes from *S. equi*
and *S. suis*, but also in the *T. denticola* homologue. However, the amino acid
sequence is unique and the crystal structure of the IdeS peptidase needs to be
solved to delineate the relationship of the enzyme to other cysteine peptidases.

Based on the present cumulative knowledge, it is apparent that the IdeS
peptidase evolved to a primary virulence factor. It is also a good example of the
possibility that bacteria may contain more peptidases than predicted from
sequence alignments.

Clan CA

All clan CA peptidases have a common fold motif, consisting of an amino-
terminal domain that is mostly α-helical and a carboxy-terminal domain fea-
turing an antiparallel β-sheet, with the Cys and His catalytic residues forming
a thiolate-imidazolium dyad. However, it is also the most divergent and popu-
lous clan of the cysteine peptidases. The clan is divided into 12 families, of
which bacterial peptidases are found only in 6. Two of these families encom-
pass exclusively bacterial enzymes that have apparently evolved as important
virulence factors.

Family C1: The Papain Family

It is an evolutionary paradox that this major family of cysteine peptidases,
exemplified by papain and mammalian cysteine cathepsins and encompassing
more than 720 sequences, has only few representatives in bacteria. All together,

only 47 homologues of papain have been identified, including 22 bacterial species with a completely sequenced bacterial genome. In this context, it is interesting to note that two *Mycoplasma* species, *M. gallisepticum* and *M. penetrans*, carry three and two copies of a gene encoding a potentially active papain homologue, respectively. However, among the genus *Mycoplasma*, these two species are the richest with regard to their peptidase gene count.

Papain homologues occur predominantly in gram-positive species, the major representative being aminopeptidase C. This enzyme from *Lactococci* spp. has been thoroughly characterized [Vesanto et al., 1994; Fenster et al., 1997], and is also present in pathogens, but there are no reports that this peptidase or its homologues are involved in any aspect of bacterial pathogenicity.

Family C2: The Calpain Family

The protein fold of the peptidase unit for members of this family resembles that of papain. In mammals they are represented by calcium-regulated ubiquitous enzymes, but thus far only five highly diverged homologues have been identified in prokaryotes. The recombinant enzyme from *Porphyromonas gingivalis*, Tpr peptidase, was characterized as a general endopeptidase which also cleaves the bacterial collagenase peptide substrate. However, the enzyme has no collagenolytic activity [Bourgeau et al., 1992] and there is no indication that the Tpr peptidase is associated with the virulence of this major periodontopathogen.

Family C10: The Streptopain (SpeB) Family

The streptococcal cysteine peptidase was isolated and characterized in 1945 and was the second proteolytic enzyme after clostripain to be isolated from a prokaryote [Elliott, 1945]. For some time the identity of the peptidase was mistaken for the streptococcal pyrogenic toxin termed SpeB (streptococcus pyrogenic exotoxin B). The confusion ended when the entire genomes of several strains of GAS were sequenced, showing that SpeB and streptopain are the same protein. For historical reasons, however, streptopain is still very often referred to as SpeB. The enzyme occurs in two variants, which differ only in a single amino acid residue, glycine or serine, at position 164 from the amino-terminus of the mature enzyme. Most strains of *S. pyogenes* that are associated with severe invasive diseases express a Gly variant and therefore present an integrin-binding Arg-Gly-Asp motif at the surface-exposed loop. It was suggested that the ability of streptopain to bind integrins may be linked to the pathogenicity of these strains [Stockbauer et al., 1999].

Despite a lack of significant sequence similarity, the crystal structure clearly indicates that streptopain belongs to the papain clan (superfamily) of cysteine peptidases. The mature peptidase portion has the two-domain fold

characteristic of other papain-like enzymes, with an amino-terminal domain composed largely of α-helices and a carboxy-terminal domain based on a four-stranded antiparallel β-sheet, with the catalytic dyad in the same topological orientation as in actinidin, a close relative to papain. In contrast to the peptidase domain, the profragment of streptopain has a unique fold. While an extended strand of the prosegment runs the full length of the active site cleft in a direction opposite to that of a natural substrate, thus blocking the major specificity pocket in the papain-like peptidase, in prostreptopain the inactivation mechanism relies on displacement of the catalytically essential histidine residue by a loop inserted into the active site [Kagawa et al., 2000].

For more than 50 years, streptopain was recognized as a unique cysteine peptidase unrelated to papain or any other known peptidase. The first homologue of streptopain was identified in *P. gingivalis*, a bacterium involved in the pathogenesis of human periodontal disease [Madden et al., 1995], then another one from the same microorganism was purified and characterized [Nelson et al., 1999]. This peptidase, referred to as periodontain, shows a strong preference for the degradation of unfolded polypeptide chains, with the human plasma proteinase inhibitor, α_1-antitrypsin, being an important exception. This major inhibitor of human neutrophil elastase is very efficiently inactivated by cleavage in the reactive site loop [Nelson et al., 1998]. Locally, this may lead to a loss of control of neutrophil peptidases and contribute to connective tissue damage. On the other hand, any direct role of periodontain in *P. gingivalis* pathogenicity is obscure. The enzyme, together with its homologue, is probably involved in generating nutrients in the form of short peptides which are an indispensable source of carbon and energy for this asaccharolytic microorganism.

The MEROPS database lists only three streptopain homologues, two in *P. gingivalis* and one in the genome of *Bacteroides thetaiotaomicron*. However, closer analysis of partially finished bacterial genome sequences revealed that genes encoding potentially active streptopain-like peptidases are more widely spread. Three different homologues were found in the genome of *Prevotella intermedia*, two in *Prevotella ruminicola*, and one in each of *Tannerella forsythensis* and *Bacteroides fragilis*. These genes encode either secreted or intracellular proteins. Significantly, the potentially secreted enzymes carry profragments with significant similarity to the proregion of streptopain. In the context of streptopain, which is very likely to be a virulence factor, it would be very interesting to elucidate the role of these streptopain homologues from other bacterial species.

Streptopain is an outstanding example of a primary virulence factor with a very broad spectrum of activity. The list of pathogenetically relevant, biologically important proteins processed, activated, or otherwise altered by the enzyme is impressive. In vitro, streptopain cleaves the human interleukin-1β (IL-1β)

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precursor to form bioactive IL-1β [Kapur et al., 1993a], processes the monocytic cell urokinase receptor [Wolf et al., 1994] and degrades human fibronectin and vitronectin, two abundant extracellular matrix proteins engaged in maintaining host tissue integrity [Kapur et al., 1993b]. In addition, streptopain activates latent human matrix metallopeptidases (MMPs), a process hypothesized to participate in the extensive soft tissue destruction observed in some patients with invasive streptococcal disease [Burns et al., 1996].

Streptopain is able to cleave IgG molecules at the hinge region of the γ-chain, generating two Fab fragments and one Fc fragment [Collin and Olsen, 2000]. Interestingly, although streptopain can also cleave antigen-bound IgG, it does not affect antibodies bound to the bacterial surface through the Fc region [Eriksson and Norgren, 2003]. In this way, streptopain's ability to cleave off the Fc part of antigen-bound IgG contributes to the ability of GAS strains to escape opsonophagocytosis, while not interfering with the formation of a host-like coat of IgG immobilized on the bacterial surface through the Fc portion. This mechanism may significantly reinforce the defenses of *S. pyogenes* against attack by the adaptive immune response. In addition to streptopain, this deterrence system consists of (1) cell-wall-anchored surface proteins of the so-called M protein family, which binds IgG 'upside down' through the Fc fragment [Berge et al., 1997]; (2) a secreted, highly specific endoglycosidase (EndoS) that targets conserved N-linked oligosaccharides on IgG [Collin and Olsen, 2000], and (3) the uniquely IgG-specific endopeptidase, IdeS (see family C66). Taken together, this system is very effective in protecting *S. pyogenes* against opsonin-dependent uptake and killing by professional phagocytes [Collin et al., 2002].

Streptopain also seems to play a key role in shielding *S. pyogenes* from the innate immune system. The enzyme induces release of dermatan sulfate from the extracellular matrix resulting in the inactivation of antibacterial peptides [Schmidtchen et al., 2001] or directly eliminates the bactericidal potential of these peptides by degrading them [Schmidtchen et al., 2002]. Finally, and possibly the most important role of streptopain in the pathogenicity of *S. pyogenes* is the ability of streptopain to directly release the potent peptide hormone, bradykinin, from high-molecular-weight kininogen. This release is not under the control of the host system [Herwald et al., 1996]. Bradykinin released by bacterial pathogens has been shown to contribute to the dissemination of infection [Sakata et al., 1996] and symptoms of sepsis and septic shock [Herwald et al., 1998, 2003; Tapper and Herwald, 2000]. Studies conducted with animal models confirmed the significant pathogenic potential of streptopain. The purified enzyme is lethal to mice [Gerlach et al., 1983] and can cause myocardial necrosis when injected into rabbits, apparently due to its fibrinolytic activity [Kellner and Robertson, 1954]. Moreover, active immunization of mice with the purified streptopain elicits a protective response in a model of invasive

disease, while mice injected with lethal doses of *S. pyogenes* were cured by a single injection of streptopain-specific inhibitor [Bjorck et al., 1989]. Furthermore, experiments using a rat model of lung infection show that streptopain acts synergistically with either the streptococcal cell wall antigen or streptolysin O to augment lung injury [Shanley et al., 1996]. This observation is especially intriguing in the context of the recent discovery that streptolysin O is the functional equivalent of the type III secretion system in gram-positive bacteria [Madden et al., 2001] and invites speculation that in some circumstances streptopain may enter the host cell and act as an intracellular virulence factor.

Taking into account the results of in vitro and ex vivo experiments, it is somewhat perplexing that the importance of streptopain as an indispensable virulence factor in vivo is still questioned. In one study, the importance of streptopain for the virulence of *S. pyogenes* has been demonstrated in a mouse model using isogenic strains with the streptopain gene inactivated by genetic manipulation [Lukomski et al., 1997]. In the follow-up in vivo investigation, it was shown that streptopain helps *S. pyogenes* to resist phagocytosis [Lukomski et al., 1998], contributes to soft tissue pathology, including necrosis, and is required for efficient systemic dissemination of the organism from the initial site of skin inoculation [Lukomski et al., 1999]. In stark contrast, in a well-designed and executed study, Ashbaugh and Wessels [2001] proved that genetic inactivation of the streptopain gene did not significantly attenuate murine invasive infection, either after intraperitoneal or subcutaneous challenge. Also, in a model of necrotizing fasciitis, a streptopain mutant organism was found to be as effective in causing tissue damage, as the wild-type control strain [Ashbaugh et al., 1998]. These results are in keeping with the clinical observation of an inverse correlation between disease severity and streptopain production in vitro by genetically related M1T1 GAS isolates associated with invasive infection [Kansal et al., 2000]. This paradox may be explained, at least partially, by the ability of streptopain to proteolytically remodel *S. pyogenes* surface proteins. Although this process is considered advantageous for bacteria [Rasmussen and Bjorck, 2002], two studies have suggested that the overexpression of streptopain results in nonspecific degradation of the antiphagocytic protein M and solubilizing of the C5a peptidase [Berge and Bjorck, 1995; Raeder et al., 1998]. Together with degradation of secreted key virulence factors, such as superantigens (streptococcal pyrogenic exotoxins) [Kansal et al., 2003], excessive production of streptopain may therefore decrease the pathogenicity of *S. pyogenes*. This hypothesis is further corroborated by the observation that streptopain-negative isolates have a survival advantage in vivo [Reader et al., 2000] and the recent discovery that invasive M1T1 GAS undergoes a stable phase shift to a phenotype expressing no streptopain, but instead a full repertoire of secreted

proteins, which are apparently degraded by active streptopain [Aziz et al., 2004]. This phenotypic phase shift may be related to the marked resurgence of severe, invasive and potentially fatal GAS infection, including the necrotizing fasciitis and streptococcal toxic syndrome observed during the last 20 years.

The role of streptopain in GAS virulence confirms the ancient maxim that even for a bacterial pathogen too much of a 'good thing' can be bad. Indeed, *S. pyogenes* has developed its own system to regulate proteolytic activity and protect its surface-associated array of key virulence factors. Firstly, expression of streptopain is regulated at the transcriptional level [Heath et al., 1999]; secondly, streptopain is produced as an inactive zymogen, which undergoes an autocatalytic, multistep activation process assisted by the bacterial surface [Liu and Elliott, 1965a, b; Collin and Olsen, 2000; Chen et al., 2003], and thirdly, in vivo, the pathogen can coat its surface with the broad spectrum peptidase inhibitor, α_2-macroglobulin (α_2M) immobilized through interaction with the peptidoglycan-anchored protein, G-related α_2M-binding protein (GRAB). Bound to GRAB, α_2M protects protein M, and possibly other surface proteins, from being cleaved by streptopain [Rasmussen et al., 1999]. In this context, it is very interesting to note that *S. pyogenes* retains some of the streptopain enzyme displays associated with the bacterial cell surface, where the enzyme displays laminin-binding activity [Hytonen et al., 2001]. Taking into account the mechanism of peptidase inhibition by α_2M, it is tempting to speculate that the immobilized form of streptopain preserves proteolytic activity even in the presence of a high concentration of this inhibitor. Such a feature may be particularly useful in soft tissue infections where the experimental and epidemiological evidence strongly implies that streptopain plays a critical role in promoting infection [Svensson et al., 2000].

Family C47: The Staphopain Family

At present, this family is limited to the *Staphylococcus* genus. Staphopain occurs in two variants, apparently reflecting the duplication of an ancestral gene. *S. aureus* expresses both variants, referred to as staphopain A and staphopain B, which share about 47% identity at the amino acid sequence level of the mature enzymes. The single staphopain of *Staphylococcus epidermidis* is related to staphopain A (75% identity) [Dubin et al., 2001; Oleksy et al., 2004]. On the other hand, a gene encoding a close relative of staphopain B has been cloned from *Staphylococcus warneri*, while a cysteine peptidase similar to the staphopains was purified from the growth medium of *Staphylococcus simulans* biovar *staphylolyticus* [Donham et al., 1988; Neumann et al., 1993].

Both staphopains are processed from large precursors, but so far only the crystal structure of the mature staphopains is available [Hofmann et al., 1993; Filipek et al., 2003]. Remarkably, despite the low sequence similarity to

papain-like peptidases, the tertiary structure of the staphopains resembles the overall fold of papain.

The reciprocal relationship present between the staphopains apparent at the amino acid sequence level is also mirrored at the genetic level. The staphopain A gene *(scpA)* occurs in a bicistronic operon (scpA), in which it is followed by a gene (scpB) encoding a staphopain A-specific inhibitor. On the other hand, the staphopain B gene *(sspB)* is part of the tricistronic operon *sspABC*, where *sspA* and *sspC* encode the V8 protease and an inhibitor specific for staphopain B, respectively [Rzychon et al., 2003a, b]. The staphopain inhibitors, ScpB and SspC, termed staphostatins, have similar folds and apparently the same mechanism of target peptidase inhibition although they share less than 20% sequence identity [Rzychon et al., 2003a, b, Dubin et al., 2003]. Nevertheless, they are uniquely specific; ScpB affects only staphopain A activity, while SspC exclusively inhibits staphopain B, without any cross-reactivity. In some cases, the reactivity of the inhibitor does not extend to the orthologous enzyme from other staphylococcal species [Dubin et al., 2004]. Apparently, evolution has hand-tailored these inhibitors to control the activity of the coexpressed enzyme. Interestingly, staphopains are secreted, while staphostatins are intracellular proteins, suggesting that they function as so-called threshold inhibitors protecting cytoplasmic proteins from any prematurely folded peptidases [Rzychon et al., 2003a, b]. The genetic assembly of peptidase and inhibitor genes in cotranscribed, cotranslated units provides the means for very efficient elimination of active staphopain from the cytoplasm.

The extracellular activity of *S. aureus* is also the subject of multilevel control. All secreted peptidases, including both staphopains are coordinately regulated at the transcriptional level by an accessory gene regulator operon *(agr)* in a cell density-dependent manner [Janzon et al., 1989]. This regulation is fine tuned by direct, strong repression of the transcription of the *stpAB* and *sspABC* operons by SarA, the product of the staphylococcal accessory regulator *(sar)* locus [Chan and Foster, 1998; Lindsay and Foster, 1999; Ziebandt et al., 2001]. Additionally, this regulatory system is indirectly affected by the alternative sigma factor σ^B [Ziebandt et al., 2001] and probably by several SarA-like transcriptional factors. Collectively, this highly complex network of gene regulation assures the precisely coordinated synthesis of extracellular proteins, including staphopains and other peptidases.

In the case of the proteinases, the regulation of their activity does not stop at the transcriptional level. Aureolysin, the V8 peptidase (glutamylendopeptidase I) and the staphopains are secreted as proenzyme forms and activated in a cascade-like manner. It is well documented that aureolysin activates the zymogen of the V8 peptidase, which in turn cleaves pro-staphopain B [Drapeau, 1978; Rice at al., 2001]. Indeed, pro-staphopain B can be expressed in the zymogen form in *E. coli*

and activated in vitro by the V8 peptidase (J. Potempa, unpubl. data). In contrast, the means by which pro-staphopain A processing/activation occurs is obscure and nothing is known as to whether this pro-enzyme is inactive or which proteinase is responsible for its processing.

Tight regulation of staphopain expression, together with that of other acknowledged virulence factors, including toxins and adhesins, may be considered as indirect evidence of their importance for the survival of *S. aureus* in vivo. This association has revitalized interest in staphylococcal extracellular peptidases as markers of pathogenicity, a subject which has been neglected for many years. Unfortunately, the results of recent investigations using animal models of staphylococcal infection are contradictory and confusing. Firstly, it was shown that a mutant strain deficient in the V8 peptidase was severely attenuated in virulence in mouse abscess, bacteremia and wound infection models [Coulter et al., 1998]. However, the reduced virulence of this mutant was apparently due to a polar effect on the expression of the *sspB* gene encoding staphopain B, located downstream of the V8 peptidase gene *(sspA)* in the same operon [Rice et al., 2001]. Indeed, this assumption was confirmed using a *S. aureus* strain with the staphopain B gene eliminated by means of genetic manipulation [Shaw et al., 2004]. In this study it was shown that only the *sspB* gene knockout strain, but not the metalloproteinase (aureolysin) and staphopain A-deficient mutants were attenuated in the skin abscess model. However, these results were not confirmed in a model of septic arthritis in mice. The inactivation of any of the peptidase genes did not affect the frequency or severity of joint disease, indicating that, at least in this model, staphopain B does not act as virulence factor [Calander et al., 2004].

Taken together, the role of staphopains in the physiology and virulence of staphylococci is obscure, but stringent conservation of the *stpA* and *sspB* genes among *S. aureus* strains, as well as preservation of the *stpA*-like gene among coagulase-negative staphylococcal species, implies that their function is important for staphylococcus survival in vivo. Amongst the bacterial proteinases, staphopains are unique with regard to their secretion as zymogens and activation by limited proteolysis. In this respect they resemble streptopain from *S. pyogenes*. In addition, for an as yet not understood reason they are tightly regulated both at the transcriptional and posttranslational levels. At the protein level their activity is released in a cascade pathway unique among bacterial species and then is further controlled by highly specific inhibitors.

Family C39: Bacteriocin-Processing Peptidase

Bacteriocins are antimicrobial peptides produced by microorganisms belonging to different bacterial taxonomic branches and used by microorganisms for biological warfare and communications [Eijsink et al., 2002]. One type of

these peptides is posttranslationally modified (class I lantibiotics), while a second type does not contain modified amino acids (class II nonlantibiotic bacteriocins). Both classes are ribosomally synthesized in the precursor form. In most nonlantibiotic peptides and some lantibiotic peptides, the amino-terminal extensions are composed of a very characteristic leader sequence termed the double-glycine-type leader, which is cleaved after the second glycine, concomitant with export carried out by members of a specific family of dedicated ATP-binding cassette (ABC) transporters. The amino-terminal domain of these transporters, absent in other ABC transporters, contains conserved cysteine and histidine residues operating as the catalytic dyad. Also, other residues, including the glutamate and aspartate residues which participate in peptide bond hydrolysis by papain-like peptidases, are strictly conserved in this portion of the molecule, which apparently has a canonical fold characteristic of papain [Havarstein et al., 1995]. The peptidase domain, together with a central hydrophobic integral membrane domain and a carboxy-terminal cytoplasmic ATP-binding domain, constitutes the dedicated transport machinery which recognizes substrates and removes leader peptides while translocating them across the cytoplasmic membrane. In addition to bacteriocins, the ABC transporters are used to translocate peptide pheromones [Michiels et al., 2001].

Bacteriocin-processing peptidases are widespread amongst both gram-positive and gram-negative bacteria and constitute the second most numerous family of cysteine peptidases in prokaryotes (after family C40). None has been implicated as a virulence factor. On the contrary, as peptidases which are indispensable for the maturation of bacteriocins, they can be utilized in expanding applications using bacteriocins as natural food preservatives [Riley and Wertz, 2002].

Family C51: D-Alanyl-Glycyl Endopeptidase

Representatives of this family have thus far only been found in the three bacterial species, *S. aureus*, *S. epidermidis*, and *S. pyogenes*. The enzymes are phage-derived and can degrade the cell wall envelope. Autolysins LytN and LytA from *S. aureus* possess a *D*-alanyl-glycyl endopeptidase as well as N-acetylmuramyl-*L*-alanyl amidase activity, which is contained within the amino-terminal portion of the polypeptide chain [Navarre et al., 1999]. None of these autolysins has been implicated in virulence. Conversely, it has been suggested that they may be used to counter antibiotic-resistant staphylococcal infections [Fischetti, 2003].

Family C58: The YopT Peptidase Family

Bacterial pathogens share common strategies to infect and colonize animal and plant host [Staskawicz et al., 2001]. One system, widespread among gram-negative pathogens, referred to as the type III secretion system [Cheng and

Schneewind, 2000; Cornelis and Van Gijsegem, 2000] directly delivers different classes of proteins to the host. These proteins, now collectively termed type III effectors, mimic, suppress, interfere, or modulate host defense signaling pathways. Their sole function is to enhance pathogen survival, proliferation and dissemination and therefore may be considered to be primary virulence factors. The structural scaffold to dispense type III effectors is conserved but 'delivered goods' are custom designed to serve the particular needs of a given pathogen. This is exemplified by the YopT peptidase [Cornelis, 2002] and its homologues from *Yersinia* spp. and plant pathogens, including *Pseudomonas syringae* [Axtell et al., 2003], which, despite sharing the same fold and catalytic mechanism, target a different set of substrates inside host cells. In addition to the YopT peptidase onthologues, an overlapping set of pathogens has adopted a cysteine peptidase with a different fold and evolutionary origin (clan CE) [Orth, 2002] as the type III effectors.

The YopT peptidase is one of six proteins called Yop effectors (YopH, YopE, YopJ/YopP, YopO/YpkA, YopM, and YopT) injected into the host cell by the *Yersinia* type III secretion system [Juris et al., 2002]. They function in concert to thwart the host immune system. YopT itself exerts a cytotoxic effect in mammalian cells when delivered by the type III secretion system [Iriarte and Cornelis, 1998]. This effect is due to proteolytic cleavage of posttranslationally modified Rho GTPases by the YopT peptidase [Shao et al., 2002]. Apparently the YopT peptidase specifically recognizes prenylated Rho GTPases and executes a proteolytic cleavage near their carboxy-termini [Shao et al., 2003b]. This leads to the loss of the carboxy-terminal lipid modification on these GTPases, resulting in their release from the membrane and irreversible inactivation. Globally, this causes a disruption of the actin cytoskeleton, exerting a powerful antiphagocytic effect and thus protecting the pathogen from being killed by phagocytes.

AvrPphB is an avirulence (Avr) protein from the plant pathogen *P. syringae* that can trigger a disease resistance response in a number of host plants. The crystal structure revealed that the topology of the catalytic triad (Cys-His-Asp), together with other structural features, resembles that for papain-like peptidases, particularly staphopain [Zhu et al., 2004]. AvrPphB has a very stringent substrate specificity and apparently exerts only a single proteolytic cleavage in the *Arabidopsis* serine/threonine kinase PBS1 [Shao et al., 2003a]. It is suggested that the cleavage product is recognized by RPS5, a member of the class of R proteins that have a predicted nucleotide-binding site and leucine-rich repeats. In a resistant host these molecular events induce a hypersensitive response.

The *avr* genes of the YopT family are common amongst plant pathogens as well as symbiotic plant bacteria and multiple Avr proteins are found in a single

Pseudomonas strain. They all function as specific peptidases targeting different substrates in the plant host or possibly cleaving the same substrates at different positions, generating signals detected by distinct R proteins. It is speculated that the large number of YopT-like proteins found in plant pathogens may reflect coevolutionary pressures in which the evolution of a new R protein in the host that detects the cleavage products of a given peptidase selects for a pathogen with new protease variants [Axtell and Staskawicz, 2003; Zhu et al., 2004].

Clan CD

This clan was recognized based on a conserved sequential motive His-Gly-spacer-Ala-Cys encompassing the catalytic His-Cys dyad present in caspases, peptidases involved in apoptosis and cytokine activation (family 14), gingipains (family 25), plant and animal legumains, processing proteinases (family 13), bacterial clostripain (family 11), and separase, a proteinase required for sister chromatid separation during anaphase (family 50) [Chen et al., 1998]. The additional common feature of all these enzymes is a substrate specificity dominated by a specific P1 residue recognition, which is asparagine (legumain), lysine (Kgp), arginine (Rgp, clostripain, and separase), or aspartic acid (caspases). Although crystal structures are only available for caspases and one gingipain, it is expected that representatives of other families in the clan will also have a similar fold. The hallmark of this fold is a six-stranded parallel β-sheet in the middle of the molecule sandwiched by three α-helices on each side [Eichinger et al., 1999]. Out of the five CD clan families known so far, three are found in bacteria.

Family C11: The Clostripain Family

Clostripain was identified and partially purified in 1937 from the culture filtrate of *Clostridium histolyticum*. The enzyme was then characterized as a cysteine peptidase that is strictly specific for Arg-Xaa (Xaa stands for any amino acid) peptidyl bonds. The mature, active clostripain is a noncovalent heterodimer derived from an inactive precursor through the autocatalytic removal of a 9-residue linker peptide [Witte et al., 1996, 1994]. At least 16 clostripain onthologues homologues were identified in microbial genomes, most of them in *Clostridium* spp. [Labrou and Rigden, 2004]. None of them was ever implicated as a virulence factor in clostridial infections. On the contrary, clostripain is a very useful enzyme in technology, both in sequence analysis and in enzymatic peptide synthesis [Gunther et al., 2000].

Family C13: The Legumain Family

Mammalian asparaginyl endopeptidase (AEP) or legumain is a recently identified lysosomal cysteine peptidase belonging to clan CD. To date it has been

shown to be involved in antigen presentation within main-histocompatibility-complex (MHC) class II-positive cells and in proprotein processing [Shirahama-Noda et al., 2003; Manoury et al., 1998; Sarandeses et al., 2003]. Genes encoding potentially active legumain homologues have thus far only been found in a few bacterial species, including *Caulobacter crescentus*, *P. aeruginosa*, *Pseudomonas putida*, *P. syringae*, *Xanthomonas axonopodis*, and *Xanthomonas campestris*. Their function awaits elucidation.

Family C14: The Caspase Family

Caspases are important players in the programmed cell death of multi-cellular organisms ranging from humans to sponges [Wiens et al., 2003]. Comparative genomic studies have provided evidence which indicates that the eukaryotic apoptotic system emerged by acquisition of several central apoptotic effectors, including caspases, from α-protobacteria as a consequence of mitochondrial endosymbiosis [Koonin and Aravind, 2002]. Therefore, it is not surprising that homologues of caspases, referred to as paracaspases and meta-caspases [Aravind and Koonin, 2002], are abundant in diverse bacteria, particularly those with complex development, such as *Streptomyces*, *Anabaena*, *Mesorhizobium*, *Myxococcus*, and α-protobacteria. The role of these ancient enzymes in bacterial physiology is obscure.

Family C25: The Gingipain Family

So far gingipains have only been found in *P. gingivalis*, the major pathogen of adult onset periodontal disease. They are represented by the products of three genetic loci conserved amongst clinical and laboratory strains of *P. gingivalis*, one *(kgp)* encoding a lysine-Xaa peptide bond-specific endopeptidase (gingipain K, Kgp) and two others, *rgpA* and *rgpB*, which are arginine-Xaa-specific enzymes (Arg-gingipains, Rgps) [Curtis et al., 1999; Potempa et al., 1995]. The nascent translation products of gingipain genes undergo complex proteolytic processing and posttranslational modifications [Veith et al., 2002]. In the case of Kgp and RgpA, initial polypeptide chain fragmentation is necessary for assembly of a noncovalent complex composed of the catalytic, hemoglobin-binding and hemagglutination/adhesin domains [Potempa et al., 2003]. This complex is either anchored to the outer membrane through a glucan moiety attached to the carboxy-terminus of the domain derived from the carboxy-terminal portion of the nascent product, or released into the growth media in the nonglycated form. RgpB lacks the additional hemoglobin-binding and adhesin domains, but still undergoes complex modification consisting of the autoproteolytic removal of the profragment and either truncation at the carboxy-terminus (the secreted form of the enzyme) [Mikolajczyk et al., 2003] or glycosylation at the carboxy-terminus, the latter allowing RgpB to form an association with the cell envelope

[Veith et al., 2002]. Collectively, gingipain activity constitutes at least 85% of the general proteolytic activity produced by *P. gingivalis* [Potempa et al., 1997].

In every respect, gingipains can be considered to be primary virulence factors for *P. gingivalis*-dependent initiation and/or progression of periodontal disease. As peptidases, they target a large set of disease-relevant substrates which can be directly associated with the clinical hallmarks of the disease [Potempa et al., 2000]. Due to the large number of substrates it targets, gingipain activity is also thought to provide this asaccharolytic organism with nutrients. However, gingipains are certainly broad spectrum peptidases. Actually, in many cases they act with the precision and sophistication of the tailored host peptidases, mimicking their function. The best example of how *P. gingivalis* can manipulate the host is the use of the gingipains to affect the major proteolytic cascades of coagulation, complement activation, fibrinolysis and kinin generation [Imamura et al., 2003].

The coagulation cascade is targeted at several levels by Rgps, which convert factor X, factor IX, protein C and prothrombin to active peptidases by limited proteolysis, thus mimicking the action of host enzymes [Imamura et al., 1997, 2001a, b; Hosotaki et al., 1999]. In the case of factor X activation, this functional mimicry additionally involves enhancement of the Rgp-converting activity in the presence of phospholipids and Ca^{2+}, two critical cofactors of the normal coagulation cascade [Imamura et al., 1997]. The factor X activation is very efficient, with the catalytic potency in some cases matching that of natural activators. In this context it is worth emphasizing that gingipains are not controlled by host inhibitors, in stark contrast to the clotting factors. In vivo, at periodontal disease sites, the procoagulant activity of Rgps is apparently negated by the fibrinogen degradation carried out by Kgp [Scott et al., 1993; Imamura et al., 1995a, b], which contributes to a bleeding tendency, a hallmark of the disease, which correlates positively with the presence of *P. gingivalis* at discrete periodontal pockets. Collectively, the interaction of gingipains with the coagulation cascade leads to local, uncontrolled release of thrombin, an enzyme with a multitude of diverse biological activities, including the stimulation of prostaglandin, IL-1 and platelet-activating factor release by endothelial cells and macrophages. These mediators are considered predominant factors in the tissue destruction process in periodontal disease.

Another trademark of periodontitis is the increased flow of gingival fluid from periodontal pockets. This symptom can be directly associated with the unrivalled (compared to other bacterial proteases) ability of gingipains to release bradykinin. Physiologically, this potent mediator is released from high-molecular-weight kininogen by plasma kallikrein, which in turn is generated from prokallikrein by activated Hageman factor (factor XIIa). Rgps shortcut this cascade by activation of plasma prekallikrein, with kinetics, which are

better than those observed in prekallikrein activation by factor XIIa [Imamura et al., 1994]. In addition, Rgps working in concert with Kgp, can release bradykinin directly from high-molecular-weight kininogen [Imamura et al., 1995a, b]. Bradykinin exerts powerful biological activities and is responsible for pain and local extravasation at the site of infection/inflammation leading to edema, which underlies the mechanism of generation of gingival crevicular fluid.

The main targets for gingipains amongst factors of the complement cascade seem to be the proteins C3 and C5, but the mode of action on these factors is different. While C3 is destroyed, thus disabling the bactericidal and opsonizing ability of activated complement, the functional chemoattractant, C5a, is released from C5 by the action of the gingipains [Wingrove et al., 1992; Discipio et al., 1996]. In addition, gingipains can enhance the chemotactic activity of IL-8 [Mikolajczyk-Pawlinska et al., 1998]. Cumulatively, this gingipain-mediated generation of potent chemoattractants may lead to excessive neutrophil accumulation at periodontal sites, another clinical sign of active disease.

A large set of cell surface proteins and receptors, including the LPS receptor (CD14) [Sugawara et al., 2000; Tada et al., 2002], the C5a receptor (CD58) [Jagels et al., 1996], the IL-6 receptor (IL-6R) [Oleksy et al., 2002], and ICAM-1 [Tada et al., 2003] are targeted by the gingipains. Although the cleavage of these proteins may significantly contribute to *P. gingivalis*-induced pathological changes in the periodontium, activation of protease-activated receptors (PARs) deserves special emphasis. PARs mediate cellular responses to a variety of extracellular serine peptidases [Ossovskaya and Bunnett, 2004]. The four known PARs constitute a subgroup of the family of seven-transmembrane domain G protein-coupled receptors and activate intracellular signaling pathways typical for this family of receptors. Activation of PARs involves proteolytic cleavage of the extracellular domain, resulting in formation of a new amino-terminus, which acts as a tethered ligand. PAR-1, PAR-3, and PAR-4 are relatively selective for activation by thrombin whereas PAR-2 is activated by a variety of proteases, including trypsin and tryptase [Gabazza et al., 2004]. Rgps specifically activate intracellular signaling pathways through cleavage of PAR-2 on neutrophils [Lourbakos et al., 1998], PAR-1 and PAR-4 on platelets [Lourbakos et al., 2001b], and PAR-1 and PAR-2 on human oral epithelial cells [Lourbakos et al., 2001a] with efficiency matching that for the endogenous agonists. Collectively, hijacking of the PAR-dependent signaling pathways illustrates the ability of the gingipains to carry out functional mimicry, which contributes to potentiation of local inflammatory responses and can be directly linked to bone resorption, the most profound clinical sign of advanced periodontal disease.

The list of proteins cleaved by gingipains discussed above is far from complete. A more complete set includes *P. gingivalis* extracellular proteins, as well as many other host proteins, such as hemoglobin and heme/iron-binding proteins, cytokines, bactericidal peptides, host peptidase inhibitors, proteins of the extracellular matrix, latent matrix metalloproteinases, and epithelial junctional proteins. The significance of these protein cleavages for periodontal disease pathogenicity is often speculative, but there is no doubt that gingipains carry out an extremely diverse set of interactions with the host. Consistently, strains with the gingipain genes disabled by genetic manipulation have severely decreased virulence [O'Brien-Simpson et al., 2001] and the pathogeneicity of *P. gingivalis* can be supressed in vivo by gingipain-specific inhibitors [Curtis et al., 2002]. Finally, immunization with the gingipains as antigens has protective effects, as observed in animal models of *P. gingivalis* infection [Gibson and Genco, 2001; Gibson et al., 2004; Rajapakse et al., 2002].

Clan CE

This clan contains five families recognized thus far, three are found exclusively in viruses, one is unique for bacteria (family C55) and one is widespread among cellular organisms, except the archae (family C48). The archetypal enzyme of clan CE is the cysteine peptidase from adenovirus, adenain. Although adenain has a unique scaffold not seen in cysteine peptidases outside clan CE, the active site contains a Cys-His-Glu triplet and an oxyanion hole in an arrangement similar to that in papain [McGrath et al., 2003; Ding et al., 1996]. In this respect, the CE clan peptidases represent a powerful example of convergent evolution at the molecular level.

Family C48: The Ulp1 Endopeptidase Family

In eukaryotic cells, the modification of proteins by a small ubiquitin-like modifier (SUMO) plays an important role in the function, compartmentalization, and stability of target proteins, contributing to the regulation of diverse processes [Muller et al., 2004; Melchior et al., 2003]. The covalent modification of proteins by SUMO-1 is reversible and is mediated by SUMO-specific proteases. These proteases are ubiquitous in eukaryota and are thought to have a dual function. They are responsible firstly for the initial processing of SUMO-1 by cleavage of the precursor peptide at the carboxyl-terminus of the protein, and secondly for the subsequent processing and cleavage of high molecular weight SUMO-1 conjugates, releasing SUMO-1 and reducing the conjugation status of the target proteins. Homologues of these peptidases have thus far only been found in a few gram-negative bacteria, including *Bradyrhizobium japonicum*, *Chlamydia muridarum*, *Chlamydia trachomatis*, *Mesorhizobium loti*, *P. syringae* and *X. campestris*. In the genomes of these organisms, representing animal and

plant pathogens and plant symbionts, up to 3 genes encoding potentially functional SUMO-specific peptidases are present, but their role in symbiosis or virulence has not been established. However, taking into account the importance of SUMO conjugation for the functioning of eukaryotic cells [Yeh et al., 2000], it is tempting to speculate that bacterial homologues of SUMO-specific peptidases are also active inside the host cell, subverting its function to benefit the pathogen, as in the case of the YopJ peptidases described below.

Family C55: The YopJ Peptidase Family

It is fascinating to note that amongst the type III secretion effectors, human and plant pathogens, as well as plant symbionts, have evolved two conserved families of cysteine peptidases with completely different folds. Both families mimic the proteolytic activity of eukaryotic proteins that are essential for the normal maintenance of host signaling. Members of the YopT family discussed earlier have a typical papain-like fold which has been crafted by pathogen evolution to yield a new, specific role in bacterial pathogenicity. The YopJ family described here apparently evolved using the scaffold of SUMO-specific peptidases (see above). Regardless of their differences in structure and specificity, both groups of enzymes target a limited number of intracellular substrates, specific cleavage of which subdues the host reaction to benefit the invading pathogen.

YopJ, one of the effector molecules injected into the host cell by *Y. pestis* was the first protein in this family recognized as a peptidase, based on a comparison of the predicted secondary structure of YopJ to that of the known structure of the adenovirus cysteine peptidase, which revealed significant similarity between these two proteins [Orth et al., 2000]. Indeed, the intact catalytic dyad of Cys-His is absolutely necessary for YopJ to exert biological activity in the host eukaryotic cell. Also, the ability of the YopJ homologue, AvrBsT (the effector molecule secreted via the type III pathway by *X. campestris* pathovar *campestris*), to trigger the hypersensitive response in plants, was shown to be dependent on the proteolytic activity of AvrBsT. In the case of YopJ, the activity was exerted by cleaving SUMO-1-conjugated proteins. Now, it has become clear that plant homologues of YopJ are also cysteine peptidases with SUMO substrate specificity, since it was shown that XopD, an *X. campestris* pathovar *vesicatoria* type III effector injected into plant cells, translocated to subnuclear foci and hydrolyzed SUMO-conjugated proteins in vivo [Hotson et al., 2003]. This indicates that SUMO protein deconjugation is a common strategy utilized by animal and plant pathogens to alter signal transduction. The SUMO-dependant pathway of intracellular signaling is very ancient and evolutionarily conserved in eukaryotic cells. So is its sensitivity to proteolytic interference by YopJ, which cleaves SUMO-conjugated proteins in yeast, resulting in a blockage of

the mitogen-activated protein kinase (MAPK) kinase-dependent pathway of signaling [Yoon et al., 2003]. The cleavage of SUMO conjugates in mammalian cells by *Yersinia* YopJ peptidase also blocks MAPK kinase [Collier-Hyams et al., 2002] paralyzing both the innate and adaptive immune responses. There are, however, some differences between the function of different YopJ peptidases, which apparently reflects adaptation to the specific lifestyle of a given pathogen. An AvrA protein from common, mild enteropathogen of humans, *S. enterica* serovar *typhimurium*, although 86% similar in amino acid sequence to YopJ, only inhibits NF-κB signaling and augments apoptosis in human epithelial cells, giving rise to speculation that AvrA may limit virulence in vertebrates in a manner analogous to the avirulence factors in plant [Collier-Hyams et al., 2002]. The lack of an *avrA* allele in strains of *Salmonella typhi* and *Salmonella paratyphi* [Prager et al., 2000], which evade epithelial defenses and results in severe systemic diseases seems to support this hypothesis.

In summary, in the case of animal pathogens, SUMO protein deconjugation interferes with the innate immune response by blocking cytokine production and inducing apoptosis in the infected cells. The infected host cell cannot respond to invaders because YopJ-like peptidases disrupt an essential posttranslational modification that is required for activation of mammalian MAPK and NF-κB pathways [Orth, 2002].

Clan CF

The crystal structures of two peptidases from this clan are known and they are clearly unique. As yet, only one family was distinguished (family C15).

Family C15: The Pyroglutamyl-Peptidase I Family

Pyroglutamyl-peptidases remove the amino terminal pyroglutamate (pGlu) residue from specific pyroglutamyl substrates [Cummins and O'Connor, 1998]. To date, three distinct forms of this enzyme have been identified, but only type I pyroglutamyl-peptidase is a cysteine peptidase with a unique fold. The active enzyme is apparently a homotetramer [Odagaki et al., 1999]. Both in mammals and prokaryotes, it is located in the cytoplasm and displays a broad pyroglutamyl substrate specificity. Genes encoding pyroglutamyl-peptidase I occur in several, mostly gram-positive bacterial species, but there are no reports that this enzyme activity may be related to virulence.

Metallopeptidases

Metallopeptidases are hydrolases in which the nucleophilic attack on a peptide bond is carried out by a water molecule activated by a divalent metal

cation, which is usually zinc, but examples where cobalt, manganese or nickel are used have been reported. The metal ion is usually immobilized by three amino acid ligands, His, Glu, or Asp. In addition to the metal ligands, at least one other residue is involved in catalytic hydrolysis of the peptide bond exercising the functions of a general base in catalytic solvent polarization. In many cases this residue is a glutamate.

At present the MEROPS database allocates metallopeptidases to 15 clans recognized by the type and number of metal ions required for catalysis and, within these broad groups, by the sequential arrangement of the metal ligands and the catalytic residue. Within clans, separate families are distinguished based on structural similarities. The most divergent and densely populated clan is MA featuring the zincins, in which the water nucleophile is bound by a single zinc ion ligated to two His residues in a sequential motif of His-Glu-Xaa-Xaa-His, in which Glu is the general base and Xaa stands for any amino acid. Depending on the third Zn ligand, which is either a Glu or His/Asp located downstream of the Zn-binding motif, clan MA is divided into two subclans, MA(E) and MA(M) [Gomis-Ruth, 2003], respectively. These subclans putatively represent separate evolutionary lines of metallopeptidases after a very ancient divergence within clan MA. Also, peptidases grouped into clan MM utilize the His-Glu-Xaa-Xaa-His motif and use an Asp residue to ligate zinc, but they are structurally unrelated to clan MA enzymes. The other well-defined and characteristic sequential motifs involved in zinc chelation include His-Xaa-Xaa-Glu and His (clan MC), His-Xaa-Xaa-Glu-His and Glu (clan ME), His-Xaa-Glu-Xaa-His with the third ligand unidentified (clan MK), His-Xaa-Xaa-Xaa-Asp and His-Xaa-His (clan MO) and His-Ser-His-Pro-(Xaa_9)-Asp (clan MP).

In contrast to the limited occurrence of aspartic and cysteine peptidases amongst bacteria, metallopeptidases are widespread and they have representatives in 50 out of the 52 distinguished families of this class of enzymes. Even more interestingly, three metallopeptidases, including the FtsH protease [clan MA(E), family M41], methionyl aminopeptidase (clan MG, family M24), and homologues of sialoglycoprotease from *Mannheimia (Pasteurella) haemolytica* (Clan MK, family M22) are the only peptidases of any catalytic class which are absolutely conserved among bacterial species. Apparently, this trio features essential house-keeping enzymes and, therefore, a perfect target for the development of inhibitors, which, by blocking the activity of these peptidases, should arrest or kill most bacteria. Methionyl aminopeptidase I is an especially attractive target since the reaction it catalyzes, i.e. removal of the formylated amino-terminal methionine residue from newly synthesized polypeptide chains, is unique to bacteria. Therefore, one would expect that specific inhibitor of the methionyl aminopeptidase should exert no side effects on eukaryotic organisms, thus resembling the action of classical antibiotics. Unfortunately, however, the

mammalian homologues of methionyl aminopeptidase are also susceptible to bacterial enzyme inhibitors. Collectively, the promise of effective new drugs and the obstacles with regard to cross-reactivity has fuelled intense interest in the detailed investigation of this family of peptidases, which are of known tertiary structure, have a characterized mechanism of catalysis and are subject to inhibition by an array of different compounds [Bradshaw et al., 1998; Bazan et al., 1994; Douangamath et al., 2004; Oefner et al., 2003; Hu et al., 2004; Towbin et al., 2003; Copik et al., 2003; Klein et al., 2003; Li et al., 2004].

Using the FtsH protease as a target to fight bacterial infection seems to be an even more challenging task than targeting the methionyl aminopeptidase I. FtsH is a member of the AAA superfamily (ATPases associated with diverse cellular activities), which includes proteins involved in a variety of cellular processes characterized by conserved regions which include an ATP-binding site and a metallopeptidase domain. These ATP-dependent proteases mediate the degradation of membrane proteins in bacteria, mitochondria and chloroplasts. They combine proteolytic and chaperone-like activities and thus form a membrane-integrated quality control system [Langer, 2000]. In bacteria, the FtsH peptidase is anchored to the cytoplasmic membrane with the catalytic domains exposed to the cytoplasm. In addition to being involved in quality control of integral membrane proteins, FtsH peptidase is involved in the posttranslational control of the activity of a variety of important transcription factors [Schumann, 1999]. In this way, FtsH peptidase is involved in the regulation of the stress response together with other chaperones with proteolytic activity, including serine peptidases such as ClpXP, ClpAP, HslUV and Lon [Hengge and Bukau, 2003; Wong and Houry, 2004]. However, unlike the serine peptidase chaperones, FtsH has never been implicated as an agent contributing to pathogenic fitness of a pathogen until recently, when it was shown that a S. aureus ftsH mutant was attenuated in a murine skin lesion model of pathogenicity [Lithgow et al., 2004].

The biological function of the sialoglycopeptidase in *M. (Pasteurella) haemolytica* has been investigated in some detail. The 35-kD enzyme isolated from the culture supernatant of this bacterium is active at neutral pH and is remarkably specific for O-sialoglycoproteins. It cleaves human erythrocyte glycophorin A, which is O-glycosylated at several positions, with a major site of cleavage at Arg31-Asp32, but does not cleave N-glycosylated proteins or nonglycosylated proteins [Abdullah et al., 1992]. The importance of the enzyme in the pathogenicity of bovine pneumonic pasteurellosis (shipment fever) caused by *M. (Pasteurella) haemolytica* is not clear, although the enzyme may interfere with cell-cell adhesion or with cytokine receptor binding through the cleavage of the cell surface O-sialoglycoproteins [Sutherland et al., 1992] during the development of the host immune response in the cattle lung. Also, the sialoglycopeptidase-mediated enhanced adhesion to bovine platelets may

initiate platelet aggregation and fibrin formation in alveolar tissue in pneumonic pasteurellosis [Nyarko et al., 1998].

Genes encoding potentially active homologues of the sialoglycopeptidase are conserved across all cellular forms of life, but their biological function is still a puzzle. The essentiality nature of this gene for some bacteria indicates that the enzyme has a very important biological function, but either we do not know its physiological substrate(s) or the protein carries out a function unrelated to proteolytic activity. At least in the case *Schizosaccharomyces pombe* the sialoglycopeptidase homologue has been shown to be involved in pro-protein processing [Ladds and Davey, 2000].

The large number of bacterial metallopeptidases excludes the possibility of a systematic description of each family of these peptidases in the context of their involvement in pathogenicity. It is interesting to note that a relatively large number of peptidase families in clans MA(E) (7 out of 16) and MA(M) (6 out of 12) have no counterparts in any other cellular form of life outside the (archae) bacterial kingdom. In addition to peptidases, which are strongly implicated as virulence factors, only members of families specific for bacteria are discussed below in more detail.

Family M4: Thermolysin Family

Thermolysin, an extracellular metallopeptidase isolated from *Bacillus thermoproteolyticus*, constitutes an archetype, not only of this family, but also for bacterial metallopeptidases in general. Enzymes homologous to thermolysin are expressed by several pathogens, including *L. monocytogenes, S. epidermidis, S. aureus, Enterococcus faecalis, C. perfringens, Helicobacter pylori, P. aeruginosa* and *V. cholerae*. Their involvement in pathogenicity is generally related to the broad substrate specificity of these peptidases, which can attack several physiologically important host proteins. A significant amount of data has been generated regarding the destructive function of pseudolysin from *P. aeruginosa*, an enzyme known for its strong elastinolytic activity [Wretlind and Wadstrom, 1977; Galloway, 1991]. This peptidase, also referred to as *P. aeruginosa* elastase, exerts its destructive action by direct degradation of several connective tissue proteins [Kessler et al., 1977; Heck et al., 1986; Galloway, 1991] and, indirectly, by inactivation of host proteinase inhibitors, including α_1-antitrypsin [Morihara et al., 1979]. Through its fibrinogenolytic and fibrinolytic activities, the elastase may disturb homeostasis and induce changes in the structure of the vascular wall, causing leakage of the plasma component, including cells into the extravascular tissue. This activity can potentially induce a hemorrhagic tendency and damage of infected tissue [Komori et al., 2001]. In lungs, the enzyme may degrade surfactant proteins SP-A and SP-D, which have important roles in the innate immune response.

This mechanism significantly contributes to the virulence mechanism in the pathogenesis of chronic *P. aeruginosa* infection [Mariencheck et al., 2003]. This data correlate well with the observation suggesting that the *P. aeruginosa* elastase is a potent inflammatory factor in a mouse model of diffuse panbronchiolitis [Yanagihara et al., 2003] and that the control of elastase release by *P. aeruginosa* may be beneficial for patients with diffuse panbronchiolitis. Also, pseudolysin seems to play an essential role in the initiation and/or maintenance of a corneal infection [Hobden, 2002].

The role of pseudolysin orthologues in other pathogenic bacteria is less well understood and requires further investigation. Nevertheless, aureolysin from *S. aureus* has been shown to contribute to connective tissue degradation by host peptidases through inactivation of host proteinase inhibitors [Potempa et al., 1986, 1991]. It may also assist in *S. aureus* dissemination by degradation of bacterial adhesins [McAleese et al., 2001]. A similar function is suggested for the hemagglutinin/peptidase of *V. cholerae*, which may be responsible for the detachment of these bacteria from cells through digestion of several putative adhesion receptors [Finkelstein et al., 1992]. On the other hand, the *L. pneumophila* Msp protease can significantly suppress antibacterial human phagocyte responses and contribute to the pathogenesis of Legionnaire's disease [Sahney et al., 2001]. A totally different mechanism seems to be utilized by the gelatinase (GelE) secreted by *E. faecalis*. This enzyme, which is also termed coccolysin, is implicated as a virulence factor by both epidemiological data and animal model studies and can apparently contribute to the dissemination of *E. faecalis* by fibrin degradation [Waters et al., 2003]. It is also possible that some of the manifestations of inflammatory conditions in the presence of *E. faecalis* are related to coccolysin-catalyzed inactivation of endothelin [Makinen and Makinen, 1994].

Family M6: Immune Inhibitor A Family

The name of this family, also known as thuringilysin family and belonging to the metzincin clan (MA(M)) [Gomis-Ruth, 2003], refers to the ability of proteins initially isolated from *Bacillus thuringiensis* to inactivate the antibacterial activity of insect hemolymph [Edlund et al., 1976]. It is now known that this protein is a metallopeptidase, exerting its insecticidal activity by proteolytic degradation of attacins and cecropins, two classes of antibacterial proteins in insects, and thus kills insect larvae [Dalhammar and Steiner, 1984; Lovgren et al., 1990]. This unique property contributes to the use of *B. thuringiensis* in biological pest control. Fortunately, this kind of peptidase, which is very effective in disabling the most important weapon of the host innate defense, is limited to insect pathogens. Nevertheless, several bacterial peptidases of different catalytic classes have been described to be able to inactivate human antibacterial peptides, once again indicating the importance of this activity in bacterial pathogenesis.

Family M9: Microbial Collagenase

By virtue of being able to degrade collagen, one of the major proteinaceous constituents of the connective tissue and extracellular matrix, bacterial peptidases with this activity are by default recognized as virulence factors [Harrington, 1996]. The members of this family are common among *Clostridium* spp., *Bacillus* spp., and *Vibrio* spp. Despite the potential ability to inflict extensive tissue damage and facilitate spreading of infection, the precise role of microbial collagenases in pathogenicity remains unclear.

Family M10

This family is divided into two subfamilies in MEROPS, though according to somewhat dubious criteria. Both belong to the metzincin clan [Gomis-Rüth, 2003], as well as those of the -equally cryptically subdivided- family 12. Subfamily 10A encompasses predominantly eukaryotic MMPs. Probable orthologues have been identified in the genomes of archaebacteria (*Methanosarcina acetivorans, Methanosarcina mazei* Göl, *Methanosarcina barkeri*), uncultured crenarchaeote, and bacteria (*Bacillus anthracis, Listeria innocua, L. monocytogenes, Leptospira interrogans,* and *S. pneumoniae*). In the latter cases, function as putative virulence factors or housekeeping enzymes remains to be assessed. According to MEROPS, subfamily 10A would further encompass a secreted 20-kD metallopeptidase toxin, *B. fragilis* toxin (BFT). The toxin also known as fragilysin is considered an important factor in the pathogenicity of infections with enterotoxigenic *B. fragilis* (ETBF), a recently identified enteric pathogen of children and adults. Fragilysin can directly damage human colonic mucosa [Riegler et al., 1999]. This effect is apparently dependent on cleavage of E-cadherin, the primary protein of the zonula adherens, leading to modification of epithelial cell morphology in vitro and resulting in increased fluid secretion into the intestine, which is clinically manifested as diarrhea [Wu et al., 1998; Sears, 2001]. Also, fragilysin contributes to intestinal mucosal inflammation by stimulation of the expression of the neutrophil chemoattractant cytokine, IL-8 [Sanfilippo et al., 2000]. According to another classification, fragilysin, together with three paralogues and an orthologue in the photosynthetic cyanobacterium *Nostoc punctiforme*, would constitute an independent family within the metzincins, though structurally probably related to MMPs [Gomis-Rüth, 2003].

Only bacterial peptidases are grouped in subfamily 10B, which are exemplified by the major metalloproteinase secreted by *Serratia marcescens*, termed serralysin. The other members of the subfamily include aeruginolysin, an alkaline protease from *P. aeruginosa*, mirabilysin (ZapA protease) from *Proteus mirabilis*, and several peptidases from *Erwinia* spp. Aeruginolysin seems to play a major role in the pathogenesis of eye infections by enhancing *P. aeruginosa* attachment to corneal epithelium [Pillar et al., 2000] and is a target for vaccine

development, and chemotherapy for bacterial eye infections. On the other hand, mirabilysin is considered to be an important virulence factor because it degrades host immunoglobulins, contributing to immune evasion during urinary tract infection [Walker et al., 1999; Almogren et al., 2003].

Family M26: IgA1-Specific Peptidase

Many of the important mucosal bacterial pathogens, including *Haemophilus influenzae, Neisseria gonorrhoeae, Neisseria meningitides, S. pneumoniae* and successful members of the human resident flora, such as *Streptococcus mitis, Streptococcus oralis, and Streptococcus sanguinis*, have developed peptidases exclusively specific for cleavage at the hinge region of IgA1. These peptidases apparently belong to three catalytic classes, but only enzymes belonging to the serine (family S6) and metallopeptidase (family M26) classes have been thoroughly characterized. The IgA1-metallopeptidases are produced by *Streptococcus* spp., with a significant exception being GAS *(S. pyogenes)*, while *Haemophilus* and *Neisseria* spp. produce serine-type IgA peptidases. Taken together, these peptidases are a striking example of convergent evolution to the same function by bacterial virulence factors [Kilian et al., 1996]. All these enzymes cleave peptide bonds at a P1 proline residue within the hinge region of IgA1, separating the antigen-binding Fab fragment from the Fc fragment. This mode of cleavage, which removes the Fc effector domain of the IgA1 molecule, not only eliminates the protective effect of the immunoglobulins, but can also serve to camouflage the bacteria with Fab fragments, which mask the epitopes recognized by intact, functional antibodies. Despite this narrow specificity, which is precisely aimed to not only disable the effector molecules of host immune system and to take advantage of them, the exact role of these enzymes in bacterial pathogenesis is still unclear. This is due to the lack of an appropriate animal model to test the contribution of these enzymes to pathogenicity, since they only cleave human, gorilla or chimpanzee IgA1 molecules [Reinholdt and Kilian, 1991].

In the context of convergent evolution it is worth mentioning the IgA specific metallopeptidase produced by *Clostridium ramosum* here (family M64) [Kosowska et al., 2002]. This enzyme has specificity for cleavage of both IgA1 and IgA2 molecules, which is a clear adaptation to the commensal lifestyle in the human gut, where both IgA isotypes are abundant.

Family M27: Tentoxilysin

Neurotoxins produced by several serotypes of *Clostridium botulinum* (BoNT type A–G) and *Clostridium tetanum* (TeNT) are the most potent natural toxins known to date. The toxins exert their biological effects at subfemtomolar

concentrations and they are released into the environment upon bacterial lysis as a single polypeptide chain of 150 kD. Proteolytic cleavage executed by host peptidases generates a two-chain, mature, active neurotoxin composed of a heavy chain (100 kD) and a light chain (50 kD) held together by a single disulfide bridge. The heavy chain is responsible for the specific binding of the toxin to presynaptic membranes and the translocation of the light chain into the neuron. The light chain is a very specific metallopeptidase with activity limited to a small subset of proteins, including VAMP/synaptobrevin, SNAP-25 and syntaxin, which play key roles in synaptic signal transduction [Schiavo et al., 1992a, b; Montecucco and Schiavo, 1994]. Cleavage of these proteins directly leads to the clinical manifestations of tetanus and botulism.

Cumulatively, tentoxilysins represent a very interesting example of the development of extremely specific and potent virulence factors. Fortunately, their occurrence is limited to a few *Clostridium* spp.

Family M34: Anthrax Lethal Factor

The anthrax toxin is one of the most lethal natural toxins. It is produced by *Bacillus anthracis* and spores of these bacteria are the active component of the most deadly bioweapon developed by mankind. The toxin is composed of three proteins, including protective antigen (PA), edema factor (EF) and lethal factor (LF). PA binds to specific cell surface receptors and, upon proteolytic activation by cell membrane-associated furin-like host peptidases, forms a membrane channel through which EF and LF enter the cell. LF is a unique multidomain metallopeptidase with a very narrow specificity to cleave the amino-terminus of mitogen-activated kinase kinases 1 and 2 (MMPKK1 and MMPKK2). The cleavage inactivates the signal transduction pathway dependent on these kinases. This signaling pathway plays a fundamental role in the overall intracellular signaling network, thus the overall signaling in the cell is compromised.

Family M56: BlaR1 Peptidase (S. aureus)

The BlaR1 peptidase from *S. aureus* is a metallopeptidase which cleaves a repressor (BlaI) of the synthesis of the β-lactamase enzyme BlaZ by this bacterium [Hackbarth and Chambers, 1993]. Thus, this peptidase controls antibiotic resistance by controlling the production of the β-lactamase. The BlaR1 peptidase orthologue, Mec R1, only found in methicillin resistant *S. aureus* (MRSA), controls the formation of the penicillin-binding protein 2a (PBP 2a) and thereby controls the resistance of the bacterium to methicillin [Hackbarth and Chambers, 1993; Brakstad and Maeland, 1997]. The BlaR1 molecule consists of two domains, an extracellular penicillin-binding domain and an integral-membrane zinc metallopeptidase domain [Zhang et al., 2001]. Upon penicillin binding, the BlaR1 peptidase autoactivates, then cleaves the repressor of β-lactamase

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synthesis, providing an interesting 'signal transduction' system which mediates this antibiotic resistance in the highly pathogenic staphylococcus species.

Family M66: StcE Protease

The StcE metallopeptidase, member of the cholorerilysins within the metzincin clan MA(M) [Gomis-Rüth, 2003] is produced by the enterohemorrhagic O157:H7 strain of *E. coli*, which causes diarrhea, hemorrhagic colitis, and the hemolytic uremic syndrome, specifically cleaves C1 inhibitor (also known as C1 esterase inhibitor). The peptidase is quite specific for C1 inhibitor and does not appear to cleave other proteins, although it has been shown to cause aggregation of cultured T cells, the significance of which is not completely understood [Lathem et al., 2002]. C1 inhibitor is known to control potent proinflammatory and procoagulant enzymes, and thus its inactivation by the bacterial peptidase is likely to cause proinflammatory effects which may be consistent with the disease outcomes caused by this strain of *E. coli*. Further experiments will be required to elucidate how critical this enzyme is to pathogenesis by this strain of the bacterium.

Family M73: Camelysin

Camelysin (casein-cleaving metalloprotease) is found on the surface of *B. cereus*, whose genome encodes a total of four paralogues. Possible orthologues have been identified in the genomes of *Oceanobacillus iheyensis* (five sequences) and *B. anthracis* (two sequences). Single sequences are further found in *B. thuringiensis, B. subtilis*, and *Bacillus halodurans* (Gomis-Rüth; personal communication). This bacterium is known to cause food poisoning and nosocomial diseases. Camelysins do not have a sequence consistent with metalloproteases, but the enzyme is active against a broad range of proteinaceous substrates and mass spectrometry analyses strongly indicate the association of a zinc ion with each enzyme molecule. Disruption of the gene for the enzyme causes a marked loss in the proteolytic activity of membranes from the bacterium and it is possible that the enzymatic activity plays a role in the pathogenic activity of the organism, although this remains to be firmly established [Grass et al., 2004].

Serine Peptidases

Peptidases which utilize a serine residue as the main catalytic residue are the biggest group of peptidases, making up 35% of the total peptidases listed in MEROPS. The serine peptidases are widespread across all organisms and are divided into 10 clans on the MEROPS database [SB, SC, SE, SF, SH, SJ, SK, SP, SR and S- (the last contains currently unassigned peptidases)]. Bacterial

proteases are present in all of these clans, except SH, SP and SR, which will therefore not be considered any further here.

By definition, this catalytic class contains a serine residue acting as the nucleophile during catalysis. Usually (as applies to enzymes in clans SB, SC and SK) the catalytic Ser residue combines with His and Asp residues to form the classical catalytic triad exemplified by chymotrypsin, the archetypal enzyme of the serine protease class. Variations on this do exist, for instance enzymes in clans SE and SJ use a Ser/Lys dyad to accomplish catalysis, while those in SF use either a Ser/Lys or a Ser/His dyad.

There are over 60 families represented within the serine-type catalytic class and many of these are subdivided into subfamilies. The sheer number of proteases in this catalytic class which are found in bacteria defies their being mentioned in any representative manner here. Thus the most interesting or well-characterized examples with direct relevance in pathogenicity were selected for presentation here.

Family S1B

The glutamyl endopeptidase I, better known as endoproteinase GluC or the V8 protease from *S. aureus*, is a member of the S1B family. The roles of this enzyme are somewhat related to pathogenicity (see section Family C47: The Staphopain Family above), but this enzyme is better known for its widespread biotechnological use as a specific protease in sequencing applications. Its structure has recently been solved [Prasad et al., 2004]. This family also contains the Spl peptidases, which have recently been identified as a new operon which is positively controlled by the Agr virulence regulator, indicating a possible role in pathogenesis by *S. aureus* [Reed et al., 2001].

Family S1C

An interesting group of peptidases is formed by members of the S1C family, which is required for growth at high temperatures by a number of organisms, such as *E. coli*. Some of these enzymes, generically termed protease Do (also referred to as DegP or HtrA), have been characterized as being associated with the virulence of *S. enterica* serovar *typhimurium*, *Yersinia enterocolitica* and *S. pyogenes*. DegP from *E. coli* has a fascinating dual function of acting as a chaperone and a peptidase, depending on the temperature of the environment. In the chaperone phase, a hydrophobic patch of amino acids plays the presumptive role of binding unfolded proteins and mediating their refolding. During chaperone operation, the active site for the peptidase is 'walled off', preventing substrate binding and catalysis. A change in the environmental conditions triggers the opening of the active site to substrates and allows catalysis. This fascinating mechanism allows the peptidase to process many different proteins needed for pathogenesis by the bacteria.

Family S1D

The family is entirely composed of the endoproteinase lysC and endoproteinase Arg-C, which have applications in the sequencing of proteins due to their high specificity for lysine and arginine amino acids at the cleavage point, respectively. An endoproteinase Arg-C orthologue from *P. aeruginosa* is thought to act as a virulence factor in corneal infections by this bacterium [Engel et al., 1998].

Family S6

The IgA1-specific serine endopeptidases which are found in *Neisseria* spp. and some *Haemophilus* spp. are typical members of the S6 family. In *N. gonorrhoeae*, the enzyme has been postulated to play a role in evading the host immune response by specifically cleaving IgA1 [Vitovski et al., 1999]. It has been suggested that the enzyme plays a role in bacterial invasion of host cells [Lin et al., 1997]. However, whether the IgA1-specific serine endopeptidase is a crucial virulence factor has yet to be determined [Johannsen et al., 1999].

Family S8A

This group of serine proteases contain enzymes generally referred to as subtilisin-like enzymes, named after the archetypal enzyme of the group. The family contains a large number of enzymes, most likely second only to family S1A which contains the mammalian chymotrypsin-like enzymes. The subtilisins and chymotrypsin-like enzymes are examples of convergent evolution, arriving at the same function and catalytic groups, but grafted onto very different scaffolds.

Perhaps the best-characterized virulence factor of this family is the C5a peptidase from group A and group B Streptococci, exemplified by the enzyme from *S. pyogenes*. As the name suggests, this enzyme cleaves the C5a component of complement, destroying its ability to act as a chemotaxin for polymorphonuclear leukocytes [Hill et al., 1988]. Recent studies suggest that this enzyme is also able to bind to fibronectin, which may be important in the binding and invasion of host cells by group B streptococci [Beckmann et al., 2002; Cheng et al., 2002b]. Recently, much effort has been invested into the development of C5a peptidase-based vaccines for the treatment of group A and B streptococcal infections [Shet et al., 2003; Cheng et al., 2002a].

Family S9B

Members of the family S9B are generally dipeptidyl peptidases, which cleave two amino acids at a time from the terminii of proteins. The bacterial peptidases in this subfamily are exemplified by the dipeptidyl aminopeptidase

IV, from organisms such as *P. gingivalis* [Banbula et al., 2000; Kumagai et al., 2000]. The enzyme is apparently important for the virulence of *P. gingivalis*, since bacteria lacking the protease or with a mutation in the catalytic domain have attenuated virulence [Kumagai et al., 2003].

Family S14 and S16

The S14 family is primarily composed of the endopeptidase Clp enzymes, originally discovered and characterized in *E. coli*. Endopeptidase Clp enzymes are rather similar to Lon proteases (S16 family) in that their activity as a peptidase is linked to the hydrolysis of ATP. The enzymes contain an ATP binding and catalysis domain and a distinct peptidase domain [Wang et al., 1997]. Some studies suggest that these enzymes are the functional equivalents of the proteasome complex found in all mammalian cells, which is crucial for the control of protein turnover in these cells. Interesting support for this hypothesis is provided by a recent study which suggests that the Clp enzyme is important for survival of bacteria which are in the stationary phase [Weichart et al., 2003]. The catalytic dyad of Lon proteases consists of Ser and Lys. The enzyme is normally induced under stress conditions [Botos et al., 2004], and animal studies suggest it is highly important *S. enterica* serovar *typhimurium* virulence [Takaya et al., 2003].

Conclusions

As is evidenced by the above review, which is by necessity not absolutely comprehensive, there is a wealth of information about bacterial peptidases. In many instances, however, knowledge is just starting to be accumulated about specific families or enzymes within families. Bacterial peptidases span a tremendous range of mechanisms, and frequently have surprising associations with additional domains which carry out separate functions. This adds a fascinating range to the potential activities of these enzymes. In many cases, the potential for inhibitors of the enzymes to be used as antibacterial agents will continue to drive the active and thriving research in this important field.

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Bacterial Invasins: Molecular Systems Dedicated to the Invasion of Host Tissues

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Bacterial pathogens have devised several strategies for their survival in the tissues of vertebrate hosts. Some of these strategies are common to a wide distribution of bacterial species, while others are quite specialized and unique to a particular pathogen. The term invasin has been traditionally assigned to virulence factors that specifically promote internalization of a bacterium by a host cell. This designation may also be collectively assigned to general virulence strategies required for host colonization. A typical pathogen must use one or a combination of mechanisms to colonize the host. Factors that promote colonization can be functionally quite diverse, from the release of a toxin into its surrounding environment, to the display of an individual surface ligand promoting receptor binding on a host cell. Many pathogens have evolved specialized macromolecular structures dedicated to the delivery of effector molecules directly into the cytosol of target cells. These injection systems bypass the requirement for association of a toxin with a target through diffusion, and therefore appear to be highly efficient pathogenic strategies. Collectively, these mechanisms allow the pathogen to manipulate molecular processes of host cells to promote adhesion, cytotoxicity, in some cases phagocytosis, and often general subversion of both the innate and adaptive immune systems. The end result is the establishment of an environmental niche in host tissues that will allow for the perpetuation of the bacterium. The secretion of polypeptides from the bacterial cytosol to targets in or beyond the cell wall envelope is a requirement common amongst most pathogenic strategies. Generalized secretion pathways are utilized or modified to accommodate these virulence strategies, resulting in specialized systems dedicated to the invasion of host tissues.

The Bacterial Cell Wall

The bacterial cell wall envelope provides the molecular scaffolding for the display of virulence factors and also provides the framework for the assembly of dedicated secretion systems. With few exceptions, bacteria may be categorized based on the composition and morphology of their cell wall. All bacteria contain an inner cytosolic compartment that is surrounded by a phospholipid membrane. Morphological distinctions become apparent beyond this primary barrier. In gram-positive bacteria, the inner membrane is surrounded by an elaborate peptidoglycan cell wall consisting of polymerized subunits of N-acetylmuramic acid-(β1–4)-N-acetylglucosamine. These glycan polymers are cross-linked via transpeptidation of murein peptides that are covalently attached to the D-lactyl groups on N-acetylmuramic acid. In general, the framework of the gram-positive cell wall is further supported by the inclusion of techoic acid, lipotechoic acid, or lipoglycan polymers, which may be covalently linked to the wall peptidoglycan or anchored to the outer leaflet of the inner membrane through lipid modification [1]. The gram-negative cell wall consists of a thin layer of peptidoglycan beyond the inner membrane. Outside of the peptidoglycan wall, a second phospholipid outer membrane is assembled. The outer leaflet of the outer membrane is composed of lipopolysaccharide (LPS or endotoxin), consisting of lipid A, an oligosaccharide core, and a distal O-antigenic polysaccharide [2]. The O-antigen is a key virulence determinant, often leading to the promotion of inflammation at the infection site. LPS can be described as the molecular signature of a particular pathogen, and several pathogens have devised schemes to alter their LPS structure, promoting serum resistance [3]. The outer membrane is also the destination of surface proteins required for adherence and for pore-forming protein complexes [4]. The double membrane arrangement of the gram-negative bacterium provides a compartment distinct from the cytoplasm called the periplasm. This compartment is typically rich in enzymatic factors required for adaptation to the extracellular environment, and proteins that influence the proper folding of secretion substrates [5]. Proteins destined for display on the surface of a gram-negative bacterium must therefore contain information that will provide for navigation through the inner membrane, the periplasmic space, and for insertion into the outer membrane. This contrasts with the navigation of a surface-displayed protein in a gram-positive bacterium, which need only contain information for secretion beyond the inner membrane. Gram-positive bacteria have devised a strategy for the display of proteins on their surface involving the covalent linkage between the polypeptide and the peptidoglycan itself, while the mechanisms for the secretion of factors beyond the cell wall are poorly understood.

Generalized Secretion Strategies

Proteins that are destined for localization in compartments outside of the bacterial cytosol must be translocated across the inner membrane. The Sec pathway is often required for this translocation process. This involves the delivery of a nascent signal-bearing precursor polypeptide to a specialized secretion apparatus (Sec translocase) in the inner membrane [6]. This process may be accomplished using either a posttranslational or cotranslational mechanism. The posttranslational secretion mechanism is thought to be utilized primarily for soluble proteins synthesized in the cytosol [7]. In the gram-negative bacterium *Escherichia coli*, newly translated polypeptide harboring an amino-terminal secretion signal is bound by a cytosolic chaperone SecB. This secretion signal consists of 18–30 hydrophobic residues that are preceded by a charged domain and succeeded by a signal peptidase cleavage site [6]. SecB delivers the signal-bearing precursor to the Sec translocase complex, composed of SecD, SecE, SecF, SecG, SecY, and YajC [7]. SecB is predicted to maintain the signal-bearing polypeptide in a secretion-competent conformation [8]. SecB associates with another factor SecA, which is a soluble translocation ATPase. SecA associated with signal-bearing precursor polypeptide binds the SecY translocase component, and the hydrolysis of ATP to ADP by SecA promotes delivery of short segments of the polypeptide into the translocation channel, consisting of SecE, SecG, and SecY [9]. The signal sequence is retained by the translocase until a signal peptidase cleaves the signal peptide at a particular site, allowing for the release of the mature polypeptide. Signal peptidases have been identified for the general secretion of soluble proteins (signal peptidase I), lipoproteins (signal peptidase II), and for virulence-associated prepilins (prepilin peptidase) for the assembly of type IV pili [10–12].

Cotranslational secretion of signal-bearing precursor polypeptides is in general thought to be associated with the proteins destined for insertion in the inner membrane after translocation [7]. The secretion mechanism involves the stalling of translation initiated through the binding of the bacterial signal recognition particle (SRP) to the signal sequence. SRP is a ribonucleoprotein complex consisting of the factor Ffh (P48) and a 4.5S ribonucleic acid [13]. Translation resumes when SRP binds to the inner membrane receptor FtsY and is displaced. The ribosome proceeds with translation and provides the force for the translocation of the nascent polypeptide through the Sec translocase [14]. Proteins destined for insertion into the inner membrane may be retained by two mechanisms during Sec-mediated translocation. Type I membrane proteins contain a noncleavable signal/anchor sequence that inserts into the membrane, whereas type II membrane proteins contain a downstream stop transfer/membrane anchor sequence that retains the mature polypeptide after cleavage [15, 16].

Outer membrane proteins often assume β-barrel structures prior to insertion. Proteins that are destined for insertion into the outer membrane or secreted beyond the outer membrane are often folded in the periplasm. DsbA and DsbC catalyze disulfide bond formation, and are both important in several of the gram-negative secretion systems [5]. In general, the Sec pathway is thought to be conserved among the gram-positive bacteria. *Bacillus subtillis* carries homologs of all of the Sec genes except SecB and SecG. It is assumed that gram-positive bacteria may utilize functional homologs of SecB for the chaperone-mediated delivery of secretion substrates to the Sec translocase [17].

A second general secretion system has been described recently. This system is called the twin-arginine translocation system or TAT pathway, named for the secretion signals conserved in the amino-termini of the substrates. The consensus amino-terminal secretion signal consists of a positively charged segment bordered by two consecutive arginine residues, a nonspecific amino acid, and two hydrophobic residues [18, 19]. This translocation system is likely devoted to the secretion of prefolded substrates and enzyme complexes in the cytoplasm that are required for general physiology and destined for localization in the periplasm [20]. Recent observations suggest that the TAT pathway may be associated with the secretion of virulence factors however, and examples of this requirement have been demonstrated in *E. coli* and *Pseudomonas aeruginosa* [21, 22]. In *E. coli*, the TAT translocation system is composed of TatA, TatB, TatC, and TatE, all integral membrane proteins. TatA, TatB, and TatC have been purified in complexes and are believed to be the structural components of the translocase [23, 24]. This relatively simple complex has been implicated in the secretion of Shiga toxin in enterohemorrhagic *E. coli* and was found to be required for toxin-mediated cytotoxicity of cultured cells [22]. The TAT system appears to be conserved among most bacteria, including gram-positive species, and future investigation may show this mechanism to be an important virulence determinant [25]. The general strategies required for protein secretion in bacteria are represented in figure 1.

Invasive Strategies of Gram-Positive Pathogens

Typical gram-positive pathogens have only a single barrier separating their cytoplasmic membrane from the extracellular environment. Secretion beyond or localization to the cell wall represent two general mechanisms employed to establish infections [1]. The secretion of toxins is a common feature, but the mechanistic process is poorly understood beyond the scope of Sec-mediated translocation. The display of virulence factors on the surface of the pathogen is a second common feature of the gram-positive pathogen. This mechanism
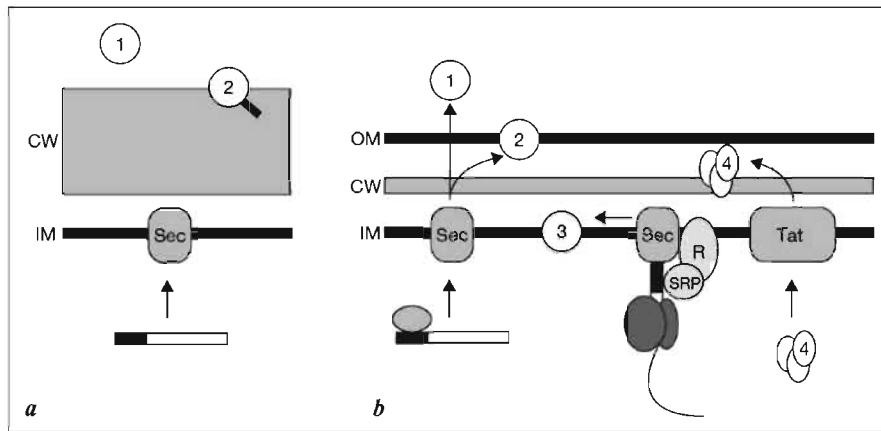
Fig. 1. General strategies involved in the secretion of bacterial proteins. *a* Gram-positive bacteria translocate signal-bearing precursor proteins through the inner membrane (IM) via the Sec pathway. Extracellular soluble proteins may passage through the peptidoglycan to diffuse in the extracellular space (1). Proteins destined for display on the surface of the bacterium (2) are often covalently linked to the cell wall peptidoglycan (CW). *b* Gram-negative bacteria transport signal-bearing soluble proteins to the periplasm via the Sec translocase. Proteins may be transported to the extracellular space by mechanisms requiring specialized secretion systems (1), or fold and insert into the outer membrane (OM) (2). Proteins destined for insertion in the inner membrane are bound by bacterial SRP at which time ribosomal synthesis is stalled. SRP binding to the membrane-bound receptor (R) promotes release of SRP from the precursor at the Sec translocase, where translation will resume, promoting insertion of the membrane protein (3). The Tat system is employed for the transport of prefolded substrates and enzyme complexes that localize in the periplasm (4).

involves the covalent linkage of secreted polypeptides to the peptidoglycan itself. This process is accomplished by a specialized membrane bound transpeptidase called sortase [26, 27]. The sortase mechanism was first characterized in *Staphlyococcus aureus*, a ubiquitous pathogen that causes a variety of human infections. Sortases have subsequently been identified in numerous gram-positive pathogens, including *Listeria monocytogenes*, *Streptococcus* spp., *Bacillus anthracis*, and others [28]. Staphylococcal protein A (Spa) is a surface protein that binds serum immunoglobulins to protect the bacterium from complement-mediated destruction. Spa is synthesized as a signal-bearing precursor that will promote its Sec-mediated translocation. Spa also contains a consensus carboxy-terminal sorting signal that consists of an LPXTG sequence motif followed by a hydrophobic stretch of 15–19 amino acids and a distal positively charged tail of 5–10 residues [29]. The hydrophobic/charged domain in the carboxy-terminus functions to retain Spa in the membrane after signal peptidase cleavage (P2
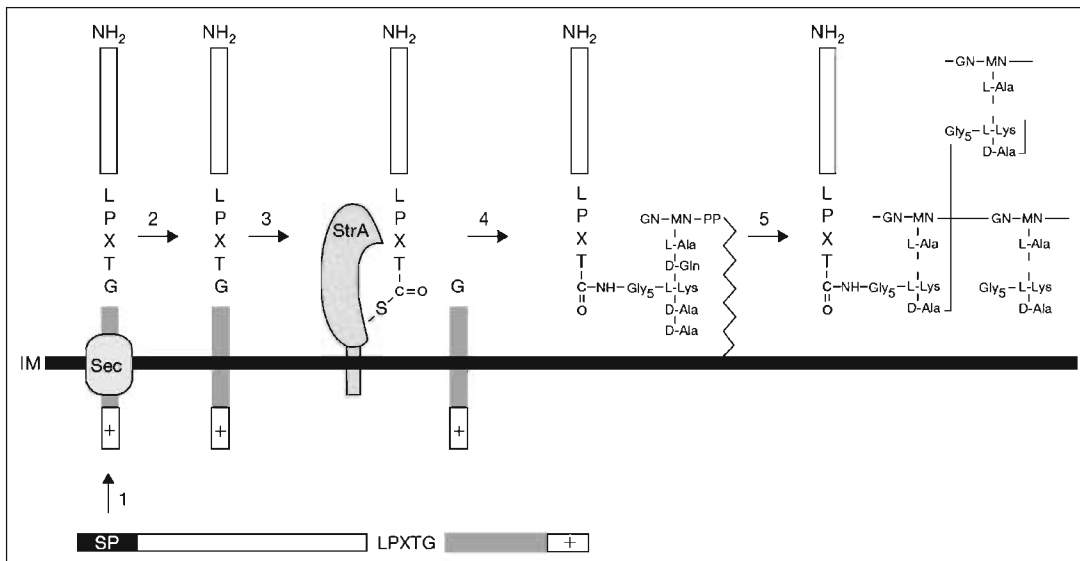
Fig. 2. The sorting reaction in *S. aureus*. P1 precursor protein substrates that harbor an amino-terminal signal peptide and a carboxy-terminal sorting signal are exported from the cytoplasm through the Sec translocase (1). The amino-terminal secretion signal is cleaved by signal peptidase generating a P2 precursor, which is retained in the plasma membrane by the carboxy-terminal sorting signal (2). Sortase (SrtA) catalyzes a cleavage reaction between the threonine and glycine residues of the LPXTG motif, generating a thioester enzyme intermediate (3). The acyl-enzyme intermediate is resolved through nucleophilic attack by a free amine group on lipid II, resulting in amide linkage of the sortase substrate to the pentaglycine cross-bridge (4). The mature surface protein is incorporated into the cell wall through a transglycosylation reaction (5). IM = Inner membrane.

precursor). Sortase catalyzes a transpeptidation reaction between the threonine and glycine residues of the LPXTG motif, where a proteolytic cleavage event links the threonyl carboxyl group to an active site cysteine, generating an acyl-enzyme intermediate through thioester linkage [30]. The carboxyl group of threonine is then amide linked to the amino group of the pentaglycine cross-bridge in the murein tetrapeptide segment of the lipid II cell wall precursor [31]. The reaction product is incorporated into new peptidoglycan polymers through transglycosylation, resulting in the mature cell wall-anchored Spa polypeptide. The general staphylococcal sorting reaction is represented in figure 2.

The identification of cell wall-anchored surface proteins based on carboxy-terminal sequence analysis has revealed the potential for numerous virulence-associated factors, including C5 peptidase in *Streptococcus pyogenes*, internalin A

in *L. monocytogenes*, and neuraminidase in *Streptococcus pneumoniae* [28]. Variations on the consensus sorting reaction have also emerged, which include multiple sortase enzymes encoded by the same organism that recognize alternate sorting signals, such as the recognition of the NPQTN consensus sorting signal by SrtB in *S. aureus* [32]. Further, evidence suggests that the expression of sortase enzymes is environmentally regulated, promoting the display of a particular set of surface proteins under specific conditions.

L. monocytogenes is a food- and water-borne pathogen that causes infections ranging from gastroenteritis to septicemia. It is an intracellular pathogen that employs a particularly interesting mechanism for migration through host tissue. The bacterium requires at least two surface factors for entry into cultured cells. Internalin A (InlA) and internalin B (InlB) each contain Sec-mediated amino-terminal signal sequences but are recruited to the bacterial surface by two different mechanisms [33]. *L. monocytogenes* harbors two sortase genes, *srtA* and *srtB*. SrtA is required for the cell wall anchoring of InlA, which contains a consensus LPXTG sorting signal. Deletion of the *srtA* locus results in a defect in invasiveness similar to that of an *inlA* mutant [34]. InlB contains carboxy-terminal repeat regions that promote a noncovalent interaction with lipoteichoic acids in the cell wall peptidoglycan [35]. InlA binds to the E-cadherin receptor on epithelial cells, while InlB interacts with the complement receptor gC1qR, glycosaminoglycans, and the tyrosine kinase receptor Met [36–38]. Activation of signal transduction cascades promotes phagocytosis and the bacterium is enveloped in a phagocytic vesicle. To combat the acidification of the phagocytic vacuole, the bacterium expresses the enzymes listeriolysin O (LLO) and phosphatidylinositol phospholipase C (PlcA), which are secreted and promote degradation of the vacuolar membrane [39, 40]. This event allows for escape from the phagocytic vacuole and promotes bacterial multiplication in the host cell cytoplasm. *Listeria* expresses another factor ActA, a membrane protein exposed on the bacterial surface. ActA recruits the host Arp 2/3 complex resulting in nucleation of actin filaments at the surface of the bacterium [41, 42]. ActA acts as a molecular mimic, functioning in a similar fashion to the WASP family of proteins. The WASP proteins are activated through binding of cellular GTPases, and conformational changes promote Arp 2/3 complex recruitment [43]. The assembly of an actin tail propels the bacterium through the cytoplasm, generating pseudopod-like extensions that promote phagocytosis by neighboring cells, resulting in the formation of a double membrane vacuole in the neighbor cell. In addition to the secretion of LLO and PlcA, phosphatidylcholine phospholipase C (PlcB) has been implemented in the escape of the bacterium from this specialized vacuole [44]. The intracellular growth cycle of the bacterium has been shown to result in localized tissue destruction with minimal exposure to components of the immune system.

Invasive Strategies of Gram-Negative Pathogens

Gram-negative pathogens have devised an array of mechanisms to promote colonization. Most of these strategies incorporate the modification of a generalized secretion pathway to either promote the display of a surface molecule for colonization, or deliver effector molecules beyond the bacterial envelope. Specialized secretion systems may be generally divided into two categories, those that promote the release of diffusible protein factors to the surrounding environment and systems that promote the delivery of effector proteins directly into the cytosol of target cells. There are currently five specialized secretion systems described for gram-negative bacteria (type I–V) that appear dedicated to virulence. Type I secretion incorporates a Sec-independent process to deliver toxin to the extracellular space without a periplasmic intermediate. Type II secretion, the main terminal branch of the general secretory pathway (GSP), represents a two-step translocation mechanism where factors secreted by the Sec pathway are transported by a protein complex that contains a characteristic outer membrane secretin. The type III secretion mechanism is a Sec-independent translocation process that involves the direct delivery of effector molecules from the bacterial cytoplasm to the cytosol of a target cell through a specialized channel or needle complex. Type IV secretion systems are similar to bacterial conjugational systems and harbor the ability to transfer proteins and/or nucleic acids into a target cell using either a one- or two-step translocation process. Type V secretion represents an alternate terminal branch of the GSP. Often referred to as the autotransporter mechanism, type V substrates are secreted by the Sec pathway and contain information in their carboxy-termini that promotes incorporation in the outer membrane and delivery of the amino-terminal domain outside of the cell. Each of these systems are discussed in detail below and figure 3 is a representation of the basic features associated with these secretion mechanisms.

Surface Proteins

The expression of a molecule on the surface of the bacterium, not unlike the display of surface proteins in gram-positive pathogens, represents a mechanism for colonization in some gram-negative bacteria. A prototypical example of this mechanism is the display of the factor invasin in *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*. The expression of *invA* in a noninvasive strain of *E. coli* results in the phagocytosis of the bacterium by cultured mammalian cells [45]. Invasin is a modular protein that harbors an amino-terminal outer membrane localization domain, as well as an extracellular carboxy-terminal domain that consists of repeats of an IgG-like fold, and an adhesive tip [46, 47]. It has been determined that invasin binds to β_1 integrin receptors localized on
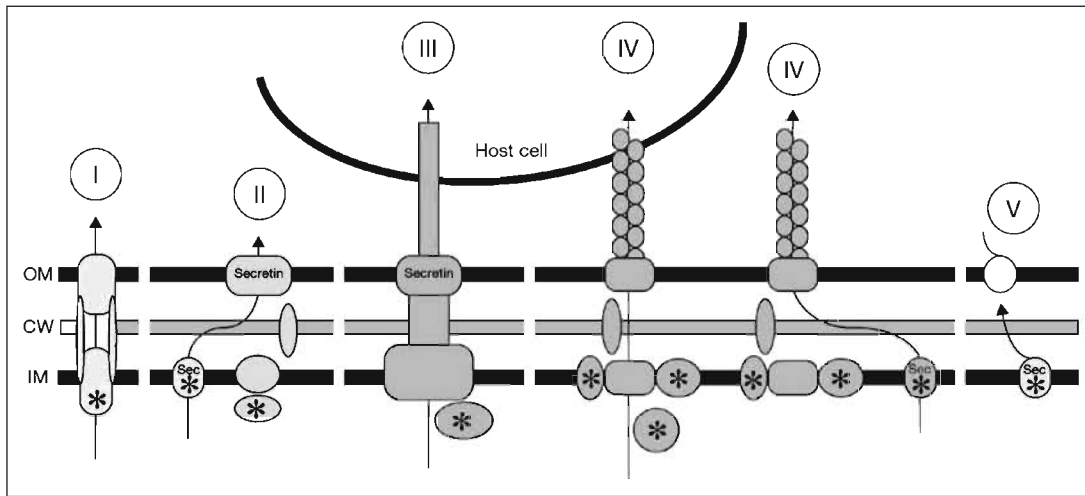
Fig. 3. Basic features of the five classes of gram-negative protein secretion systems.
(I) The type I mechanism involves a single, Sec-independent translocation event that incorporates an inner membrane (IM) ABC transporter for energy generation. (II) The type II mechanism represents a two-step translocation process, where signal-bearing precursor proteins are transported to the periplasm via the Sec pathway. Mature substrates are transported through an outer membrane (OM) secretin. (III) The type III mechanism incorporates a single translocation step to transport substrates from the bacterial cytoplasm into the cytosol of eukaryotic cells. The substrate is transported through a basal body complex, an outer membrane secretin, and a needle complex that penetrates the target cell membrane. (IV, left) The type IV secretion system is employed to transfer substrates into host cells. This process requires the assembly of a pilus structure at the outer membrane, a core assembly in the periplasm, and inner membrane-associated ATPases. (IV, right) Pathogens may also employ the type IV mechanism for secretion of diffusible toxins to the environment in a Sec-dependent manner. (V) Autotransporters are secreted by the type V pathway. A typical substrate is translocated to the periplasm by the Sec translocase. Insertion into the outer membrane promotes the secretion of the amino-terminal passenger domain. Autoproteolysis releases the diffusible mature protein. Localization of energy generating enzymes are indicated by *. CW = Cell wall peptidoglycan.

the apical surface of M cells, located amongst the follicle-associated epithelia and lymphoid follicles of the small intestine, commonly referred to as Peyer's patches [48, 49]. M cells sample contents of the intestinal lumen and transport particles contained in vesicles to the basolateral surface, which is rich in immune cells such as macrophages and polymorphonuclear leukocytes. The binding of invasin to M cells may therefore represent an early mechanism involved in the *Yersinia* infection process, as the bacteria have a specific tropism for lymphoid tissues. Numerous examples of adhesion factors have been identified, often associated with the protein subunits localized in the tip of pili or fimbriae.

Examples include the PapG adhesin of type I pili in *E. coli*, and the major pilin subunit PilA on the type IV pili of *P. aeruginosa* [50, 51].

Type I Secretion

The type I secretion mechanism involves the one-step translocation of a secretion substrate in a Sec-independent manner [52]. This mechanism is employed for the secretion of diffusible toxins into the extracellular space. The type I mechanism has been demonstrated for the secretion of α-hemolysin (HlyA) in *E. coli*, as well as for the secretion of *Bordetella pertussis* adenylate cyclase and *P. aeruginosa* protease [53]. In each instance, the toxin is recruited to a translocation complex that assembles upon association with the substrate. The type I secretion system is relatively simple in architecture, consisting of only three factors, each required for transport of the substrate. A characteristic feature of the system is the presence of an ATP-binding cassette (ABC) protein transporter [52, 54]. ABC transporters are inner membrane proteins that are found in a wide range of organisms, including gram-positive bacteria, lower eukaryotic, and mammalian cells, and are normally associated with the transport of small molecules. The secretion of HlyA has been extensively studied and the synthesis of the pro-hemolysin precursor protein (proA) requires a lipid modification for activation to mature HlyA [55]. The cytosolic factor HlyC as well as an acyl-carrier protein (ACP) are required for the myristoylation or palmitoylation of two lysine residues [56]. HlyC acts as an acyl-transferase for this process. Although this lipid modification step is required for the hemolytic activity of HlyA, this event is not required for the type I-dependent secretion of HlyA [57]. After modification, HlyA binds to the ABC transporter HlyB at the inner membrane [58]. The sequence information required for secretion of HlyA is contained in the poly-peptides carboxy-terminal 48 amino acids, and unlike signal sequences in Sec-mediated substrates, the signal sequence of type I substrates is not cleaved after translocation [59, 60]. The HlyB transporter associates with a second factor HlyD independent of substrate binding. The HlyD protein spans both the inner and outer membrane and trimerization of HlyD in the presence of HlyB bound to HlyA results in the recruitment of the outer membrane protein TolC [58]. Each subunit of the trimeric TolC contains an amino-terminal β-sheet domain that inserts into the outer membrane. A second carboxy-terminal α-helical domain extends deep into the periplasmic space and forms a barrier between the periplasm and the amino-terminal pore-like structure [61]. The binding of ATP to HlyB in the presence of HlyA may result in the specific recruitment of TolC by HlyD. The HlyB/HlyD/TolC complex then supports the HlyB mediated trans-location of HlyA through successive rounds of ATP hydrolysis [62], resulting in the delivery of HlyA to the extracellular space. Eleven tandem glycine-rich repeats (LXGGXGND) contained in the carboxy-terminus of HlyA are required

for calcium binding. Calcium-bound HlyA is competent for insertion in the host cell membrane, and results in pore-mediated leakage of the target cell [63].

Type II Secretion

The GSP represents the primary route for translocation of polypeptides to the extracellular space among gram-negative bacteria. The type II secretion mechanism represents the archetype for protein secretion in the GSP, and has been designated the main terminal branch. The type II pathway is associated with the secretion of virulence factors in several bacterial pathogens. Alternate GSP branches include the secretion of autotransporters (type V secretion), the chaperone/usher-mediated assembly of P or type I pili in *E. coli*, the assembly of type IV pili in *P. aeruginosa* and *Neisseria gonorrhea*, and the assembly of curli in *E. coli* [64]. The factors required for extrusion of filamentous bacterio-phage from the bacterial envelope also share conserved components with the GSP [65]. One common feature associated with all of these strategies is the requirement for the Sec-mediated translocation of secretion substrates to the periplasm. The type II secretion pathway therefore represents a two-step translocation process, incorporating distinct secretion reactions for translocation across the inner and outer membranes.

The type II-dependent secretion of pullulanase in *Klebsiella oxytoca* is a well-studied example of this secretion mechanism. Pullulanase (PulA) is a lipoprotein of the α-amylase family that enzymatically degrades the complex carbohydrate pullulan to maltotriose subunits, a substrate that may be transported into the bacterium [66]. The secretion of PulA requires the products of at least 25 genes, 14 of which are specifically involved in the translocation of PulA beyond the outer membrane [67]. After secretion to the periplasm through the Sec pathway, The PulA precursor is subjected to diacyl glyceride modification and cleaved by signal peptidase [68]. The lipid-modified PulA is retained in the outer leaflet of the inner membrane by an aspartyl residue located at the amino-terminus of the mature polypeptide. Factors required for the type II-dependent translocation step are localized within several compartments. A cytoplasmic ATPase (GspE) associates with the inner membrane through interaction with a second factor (GspL), an inner membrane protein that harbors a carboxy-terminal cytoplasmic domain [69, 70]. This interaction, coupled with ATP hydrolysis by GspE, may provide the energy required for PulA transport. Four additional integral membrane proteins GspC, GspF, GspM, and GspN are thought to assemble into a basal body complex, since the factors harbor carboxy-terminal domains that extend into the periplasm [71]. A characteristic feature of the type II apparatus is the requirement for periplasmic pseudopilin proteins. These factors, all harboring prepilin signal sequences, are secreted by the Sec pathway and processed by the inner membrane-associated prepilin peptidase GspO, which will also N-methlyate

the pseudopilin subunits [72]. Five pseudopilin factors, GspG, GspH, GspI, GspJ, and GspK, are processed in this manner, and assemble into a channel-like pilus structure linking components of the inner and outer membranes [73]. GspD is an outer membrane secretin required for the export of type II substrates. GspD is inserted into the outer membrane and assembles into a dodecameric channel-forming structure, a process that requires the outer membrane chaperone GspS [74]. Functional homologs of the GspD secretin are conserved amongst most of the alternate branches of the GSP and the GspD secretin is also conserved among the type III secretion systems (see below). The type II-mediated export of PulA may occur through its association with the basal body complex, with signal recognition most likely residing in the secondary or tertiary structure of the secretion substrate after folding in the periplasm. PulA is transported to the outer membrane secretin GspD and exported to the extracellular space. Similar type II secretion mechanisms have been identified for the release of exotoxin A, elastase, and phospholipase C in *P. aeruginosa* [71].

The secretion of AB-type holotoxins is also mediated by a type II-dependent process. This class of toxins includes the cholera toxin of *Vibrio cholerae*, *E. coli* enterotoxin, and the Shiga-like toxins of *E. coli* and *Shigella dysenteriae* [75]. Cholera toxin is composed of two separate polypeptides CtxA and CtxB. Sec-dependent secretion of the subunits to the periplasm results in proteolytic cleavage of signal peptides and the formation of an intramolecular disulfide bond in CtxA prior to cleavage. The CtxB subunits assemble into a pentameric ring structure and bind the carboxy-terminal domain of the CtxA subunit, generating the $CtxA_1$-$CtxB_5$ holotoxin [76]. Secretion of the holotoxin requires components of the *eps* gene cluster, which encodes several factors homologous to the type II secretion system in *Klebsiella* [77]. Export of the holotoxin will result in the binding of the $CtxB_5$ subunits to a G_{M1} ganglioside on the surface of intestinal epithelial cells [78]. Reduction of the disulfide in CtxA by host cytosolic thioredoxin promotes the release of the mature CtxA toxin from the $CtxB_5$ pentameric ring, where it will function to activate host cell adenlyate cyclase, resulting in the massive cellular fluid loss associated with the diarrhea in cholera disease [79].

Type III Secretion

The delivery of polypeptides from the bacterial cytoplasm directly into the cytosol of target host cells without the generation of an extracellular interme-diate is the hallmark feature of the type III secretion system [80]. Effector pro-teins that are translocated into host cells harbor enzymatic activities that manipulate cellular processes of the eukaryotic host, resulting in a variety of processes that culminate in perpetuation of the bacterium at the infection site [81]. The type III secretion mechanism was first characterized in pathogenic

Yersinia species, but has subsequently been identified and extensively studied in various pathogens including enteropathogenic *E. coli* (EPEC), *P. aeruginosa*, *Salmonella enterica* and *Shigella flexneri* [82]. Analysis of genetic information has revealed the potential for type III systems in several other gram-negative bacteria, and thus may represent a highly conserved pathogenic strategy [83].

Even though the process of injection of virulence factors by the type III pathway is a recently described phenomenon, the type III secretion apparatus appears both structurally and functionally similar to the basal body of the flagellar secretion system in gram-negative bacteria. In fact, the flagellar secretion system is now considered a type III pathway and recent observations suggest that the flagellar export system may also support the secretion of virulence factors [84]. Type III secretion systems most likely evolved from the flagellar machinery to support colonization in new nutrient-rich environments such as those found in higher eukaryotes. *Yersinia* species employ the type III pathway to maintain an extracellular lifestyle in the lymphoid tissues of their mammalian hosts and cause a variety of diseases ranging from bubonic plague in *Yersinia pestis* to acute enteritis in *Y. enterocolitica*. This is accomplished through the type III injection of effector proteins called Yops (*Yersinia* outer proteins) into host macrophages, resulting in prevention of phagocytosis and eventual apoptotic death of the host cell [85].

With the exception of the assembly of the secretion apparatus, the translocation of type III secretion substrates represents a Sec-independent process. The type III secretion system consists of three principle components, an inner membrane-associated basal body, an outer membrane secretin, and an extracellular needle complex. Assembly of a functional *Yersinia* type III apparatus requires the products of at least 21 *ysc* (*Yersinia* secretion) genes [86–88]. Eleven of these genes are conserved amongst other type III systems, including nine that are conserved with the flagellar basal body [89]. In general, the *Yersinia* type III secretion apparatus must be assembled in a similar fashion to the flagellar secretion system, where assembly of the basal body complex precedes any substrate delivery. The initiation of the assembly of the basal body complex in *Yersinia* likely begins with membrane insertion of the FliF homolog YscJ, after Sec-mediated translocation [86, 90, 91]. This event will allow the association of inner membrane proteins YscD, YscR, YscU, YscV and accessory factors to form the basal body complex. YscN is homologous to the FliI ATPase in flagellar secretion and contains the Walker boxes A and B, which are characteristic conserved ATP-binding domains [92]. YscN is predicted to provide the energy for the transport of type III secretion substrates and is required for Yop secretion. YscC is homologous to the GspD secretin involved in the transport of molecules in the type II pathway, and requires the outer membrane lipoprotein YscW for its localization and for the formation of the characteristic dodecameric rings in the outer membrane, resulting in outer membrane

channel formation [93, 94]. Accessory factor association between the basal body complex and the YscC secretin provides a conduit between the two components of type III secretion system, an assembly step that is not conserved with flagellar secretion. Secretion of the factors YscF, YscO, YscP, and YscX promote the assembly of the needle complex [95–97].

Although the type III needle complex remains to be isolated from *Yersinia*, needle complexes have been purified from *Salmonella*, *Shigella*, and *E. coli* [98–100]. The YscF protein has been determined to be the main component of the needle complex, where the protein multimerizes in a right-handed helical fashion. The YscF homolog MxiH of *Shigella* displays 5.6 subunits per turn, and is polymerized from the distal tip into a conduit as long as 50 nm with a width of 7 nm and a central tube of 2–3 nm [101, 102]. YscO and YscP are secreted by the basal complex. YscP has recently been suggested to participate in substrate recognition, as *yscP* mutant strains secrete an increased amount of the needle component YscF but fail to secrete Yop proteins in vitro. Mutations in the amino-terminus of YscU suppress the *yscP* mutant phenotype, reducing the amount of secreted YscF to wild-type levels and restoring the secretion of Yop proteins [95]. These results suggest that YscO, YscP, and YscU may control type III secretion at the level of substrate specificity, allowing for a switch between the secretion of structural components and the delivery of Yop substrates, similar to the switch between hook and filament proteins in the flagellar apparatus [89]. Assembly of the YscF needle complex would then represent the final step of assembly and provide a switch for the recognition of type III secretion substrates and the delivery of effector Yop proteins.

The hydrophobic nature of the YscF polymer has been predicted to provide a mechanism for the piercing of the host cell cytoplasm [96]. An alternate hypothesis suggests that three secretion substrates, YopB, YopD, and LcrV, each required for the translocation of Yop proteins, form a translocation pore in the host cell membrane allowing for subsequent delivery of effector proteins [80, 103–106].

Y. enterocolitica secretes 14 polypeptides via the type III pathway. One curious feature of each of these proteins is that they do not contain any amino acid sequences that would suggest the presence of a conserved type III secretion signal. Experiments performed using reporter proteins have revealed the presence of minimal secretion information contained in the amino-terminal 8–15 residues [107–110]. The nature of the minimal secretion information remains controversial. Scanning mutagenesis studies employed to determine the residues required for secretion of YopE revealed that no specific residues were necessary. Further, introduction of frameshift mutations in the minimal signal did not affect the secretion of reporter fusion constructs [107]. These results prompted the hypothesis that the minimal signal information is actually

contained in the mRNA rather than the protein. The *yop* mRNA might therefore recruit a translational complex to the type III apparatus promoting a cotranslational secretion mechanism.

The nature of the 5' mRNA/amino-terminal signal hypothesis has been highly contested however, and independent studies suggested that a mutant that generated multiple mutations in the mRNA sequence without affecting the amino acid sequence of the protein was indeed secreted [109]. This implied that the amino acid sequence, rather than the mRNA, contained the information required for type III secretion. Construction of a synthetic amphipathic amino-terminal signal that contained alternating serine and isoleucine residues between positions 2 and 9 of YopE also supported secretion [109]. Recent observations in the minimum secretion signals of YopE and YopQ have again resurrected the mRNA secretion signal argument, as it was discovered that minimum signals, such as the 1–10 positions of YopQ, do not tolerate frameshifts unless a downstream suppressor region of mRNA is included that contains codons 11–13 [110]. Further, single substitutions in codons 2 and 10 caused a defect in the secretion reporter fusions in the context of 10 but not 15 codons. Finally, multiple mutations in the wobble positions of *yopQ* 1–10 did not support the secretion of the reporter, again suggesting that mRNA rather than protein sequences initiate the transport of substrates via the type III pathway [110].

Beyond the context of the amino-terminal minimal secretion signal, experimental evidence suggests that type III substrates may require a second signal for their injection into the cytosol of host cells, and the presence of Syc (specific Yop chaperone) proteins may be required for the injection process [105, 111]. In general, Syc proteins are small acidic proteins that form dimers in the bacterial cytoplasm. Each chaperone appears to specifically bind a partner effector Yop protein in the cytoplasm and structures have been determined for secretion substrate/chaperone complexes [112]. Studies that examined the role of both the amino-terminal and chaperone-mediated secretion signals demonstrated that a defective secretion signal, when linked in context to the full length YopE protein, was secreted in an SycE-dependent manner, suggesting that the chaperone mediated secretion signal does not require the presence of a functional amino-terminal signal [113]. This prompted the hypothesis that Yop proteins harbor two independent secretion signals, the first required for initiation of the substrate into the type III pathway, and the second for injection into host cells.

Y. enterocolitica transports a class of at least six factors into the cytosol of the host cell, each of which harbors an enzymatic function. All of these factors, which include YopE, YopH, YopM, YopO, YopP, and YopT, share sequence homology to proteins of eukaryotic origin, suggesting the pathogen evolved these strategies through intimate interaction with the host over time. Although all of the type III pathogens share a conserved mechanism for the delivery of these

effector proteins, the effector proteins themselves may or may not be conserved between pathogens. YopE is only cytotoxic to HeLa cells when injected via the type III pathway. It is a characteristic GTPase-activating protein (GAP) that acts upon the Rho family of eukaryotic GTPases. YopE inactivates RhoA, Rac1, and CDC42 by accelerating the conversion of GTP to GDP in these factors [114, 115]. This mechanism results in an inhibition of actin polymerization at the site of bacterial contact. YopH is a protein tyrosine phosphatase (PTPase) that is involved in the dephosphorylation of focal adhesions [116]. The amino-terminal domain of YopH appears to be a targeting domain that binds to p130^{Cas} and focal adhesion kinase (FAK) [117]. The carboxy-terminal domain harbors the phosphatase domain and acts specifically to dephosphorylate these substrates, resulting in an interruption of stress fiber formation [118]. YopM is required for virulence in mice and has been suggested to target to the nucleus and may influence transcription in the host cell, inhibiting inflammatory cytokine production [119, 120]. YopO is similar in sequence to RhoA kinase. YopO functions as an autophosphorylating serine threonine kinase that is activated in vitro through binding to actin [121]. The protein is believed to phosphorylate the Rho family of GTPases and enhance the inhibition of actin polymerization [122]. YopP acts as an inhibitor of IκB in the NF-κB pathway and also inhibits the MAP kinase pathway [123–125]. It has been reported that YopP is a cysteine protease that may function through a protein degradation pathway [126]. The cumulative effects of disrupting the NF-κB and MAP kinase pathways result in inhibition of the proinflammatory response, thus preventing the production of the cytokines TNF-α and IL-8 [123, 125]. YopP also induces apoptosis in macrophages, which is likely to be a cumulative result of the failure to activate the NF-κB signaling pathway and through the cleavage of Bid, a proapoptotic member of the Bcl-2 family [127]. YopT is also a cysteine protease that has been demonstrated to cleave RhoA, Rac1, and CDC42 at their carboxy-termini, sites that are prenylated for membrane anchoring [128]. Cleavage releases the factors from the membrane resulting in a defect in actin polymerization at the site of bacterial contact.

EPEC, a food- and water-borne pathogen that is a causative agent of human infantile diarrhea, uses the type III pathway to establish attaching and effacing lesions on intestinal epithelium [129, 130]. These bacteria inject a protein, translocated intimin receptor (Tir), which is subsequently displayed on the surface of the gastric epithelial cell [131]. The bacterial cell displays a ligand for this receptor on its outer membrane, called intimin. Interaction between the two factors results in tight binding between the bacterium and host cell. This event coupled with the cumulative effects of the type III injection of other factors will promote actin pedestal formation at the site of contact, allowing extracellular colonization and destruction of surrounding tissue.

S. enterica serovar spp. cause a variety of diseases in humans and animals, ranging from acute food poisoning and gastrointestinal inflammation to typhoid fever and septicemia. In general pathogenic *Salmonella* species are food- and water-borne pathogens that have a tropism for the intestinal epithelium. *S. enterica* serovar spp. harbor the genes encoding two separate type III secretion systems on their chromosome. The first system, designated *Salmonella* pathogenicity island 1 (SPI-1), is employed to invade nonphagocytic epithelial cells [132]. *Salmonella* uses the SPI-1 type III pathway to inject several effector molecules that leads to a massive reorganization of actin filaments promoting the formation of membrane ruffles and eventual phagocytosis. Similar to effector proteins in *Yersinia*, many *Salmonella* effectors target the signaling processes governing actin polymerization. SipA stabilizes F-actin through binding to the T-plastin protein [133]. SopE and SopE2 function as guanine nucleotide exchange factors (GEF) to activate Rac-1 and CDC42 [134, 135]. In order to promote recovery of the cytoskeleton, SptP is injected. SptP is a multi-functional enzyme that contains an amino-terminal YopE-like GAP domain and a carboxy-terminal YopH-like tyrosine phosphatase domain. SptP counteracts the enzymatic effects of SopE and SopE2 by downregulating Rac-1 and CDC42 [136, 137]. The second type III system located at SPI-2 appears to manipulate vesicular trafficking, allowing for perpetuation of the bacterium in a specialized vacuole, and is required for systemic infections [138].

Pathogenic *Shigella* species are typically water-borne pathogens that are a causative agent of bacillary dysentery, an infection of the colon. *Shigella* species utilize a type III secretion for the invasion of epithelial cells. *Shigella* are believed to enter epithelial cells from the basolateral surface. After engulfment by intestinal M cells and presentation to lymphoid macrophages, *Shigella* secretes an apoptotic factor IpaB which allows the bacterium to spread to adjacent cells [139]. *Shigella* also employs the type III pathway to promote phagocytosis by the epithelial cell through the cumulative effects of IpaA, IpaB, IpaC, and IpaD [140]. Unlike *Salmonella, Shigella* escape from acidified vesicles and reside in the host cell cytoplasm. An outer membrane protein IcsA nucleates actin polymerization through the binding to N-WASP and the formation of the Arp2/3 complex [141, 142]. Production of cytoskeletal filaments at the pole of the bacterium propels the organism into neighboring cells, similar to the process described for *L. monocytogenes*.

Type IV Secretion

The type IV secretion mechanism is employed for a wide range of functions in gram-negative bacteria. Several species utilize the type IV mechanism for interbacterial conjugative transfer of mobilized genetic elements. Pathogenic *Agrobacterium tumefaciens* employs the type IV system for the transfer of

tumorigenic DNA and protein into host plant cells, and several vertebrate pathogens such as *Brucella* spp., *B. pertussis*, *Helicobacter pylori*, and *Legionella pneumophila* use a modified type IV secretion system for the secretion of toxins or the delivery of effector proteins into the host cell [143, 144]. There is evidence to suggest that the secretion of substrates through the type IV apparatus requires a Sec-dependent translocation step, such as in the secretion of pertussis toxin; however, specialized systems such as those for *H. pylori* and *L. pneumophila* may bypass this requirement [145]. All type IV systems represent a modification of the conjugative transfer system found in strains of *E. coli*. In general, the mechanism involves the assembly of a secretion apparatus with a pilus-like projection that will provide intimate contact between the donor and recipient cell [146].

The transfer of DNA from bacterium to host in the pathogen *A. tumefaciens* represents the archetype for the type IV secretion pathway. Substrate translocation requires the products of the VirB-encoded system: VirB1–11 and VirD4 [147]. The mechanisms of VirB-mediated type IV transport have been extensively studied, and the system is currently employed as the general model for the type IV mechanism in animal pathogens [144]. Evidence suggests that the type IV apparatus is assembled to extract the major pilin subunit VirB2, a cyclic polypeptide, through the outer membrane [148]. The secretion and processing of the pilin subunits is a Sec-dependent process. VirB2 forms a pilus through multimerization and contains a second minor pilin subunit VirB5. Pilin subunits interact with an outer membrane lipoprotein VirB7 and are thought to assemble at the outer membrane [149]. VirB6 is an inner membrane protein that may provide the connection between components of the inner and outer membrane, as well as guide assembly of the periplasmic core [150]. VirB7 also interacts with VirB9, an outer membrane component, and VirB8, a muramidase localized in the periplasm that may provide for organization of the complex through wall peptidoglycan [149, 151]. Energy for the transport of type IV substrates is provided by the activities of three separate ATPases. The VirB4 dimer is localized in the inner membrane. A second inner membrane ATPase, hexameric VirB11 assembles into a ring structure and may provide a route for translocation of type IV substrates [152, 153]. VirB11 also interacts with the periplasmic core component VirB10. The third ATPase VirD4, also called 'coupling protein', is localized in the bacterial cytoplasm and is involved in substrate recognition [144].

The *ptl* system in *B. pertussis* represents an interesting link between the type II and type IV secretion pathways. *B. pertussis* is the causative agent of whooping cough, where pertussis toxin, an AB-type holotoxin, is the primary virulence determinant. Pertussis toxin is exported by the Ptl type IV secretion apparatus. The Ptl system appears functionally distinct from other type IV

secretion systems. Rather than supporting injection into host cells, the Ptl system exports pertussis toxin to the extracellular space, where the toxin is available to associate with the target cell membrane [145]. The mature enzyme acts as an ADP-ribosylating factor of G proteins in the host cell. Unlike the T-DNA translocation process, the PtxA and PtxB subunits are translocated to the periplasm, where they are processed and assembled into the $PtxA_1$-$PtxB_5$ holotoxin [154]. This protein complex then becomes a substrate for type IV-mediated export. Nine structural components of the Ptl system are homologous to the VirB system, where PtxA represents the major pilin subunit. The system also contains two membrane-associated ATPases, PtlC and PtlH, which are homologous to VirB4 and VirB11, respectively.

Recent discoveries have provided evidence that type IV secretion systems are competent in the delivery of effector proteins directly into the cytosol of host cells. *H. pylori* is a causative agent of several gastrointestinal syndromes ranging from peptic ulcers to MALT lymphoma and adenocarcinoma. Pathogenic strains of *H. pylori* harbor the CAG pathogenicity island which encodes a VirB-like type IV secretion system [155]. CagA is translocated by the type IV pathway into the host cell cytosol where it becomes tyrosine-phosphorylated and proteolytically processed to a carboxy-terminal phosphorylated fragment. The injection of CagA results in a change in the phosphorylation state of associated host cell factors, and is required for virulence [156]. *L. pneumophila* and *Brucella* species require type IV secretion systems for survival in intracellular vacuoles [157]. *L. pneumophila* is the causative agent of Legionnaire's disease, a severe respiratory pneumonia. *L. pneumophila* targets alveolar macrophages where it employs the Dot/Icm type IV secretion system for intracellular survival. The Dot/Icm transporter is more distantly related to the VirB system, but is homologous to the IncI conjugation system in *S. flexneri*, and is competent for conjugational transfer of DNA [158]. *L. pneumophila* bypasses destruction mediated by the endocytic pathway by creating an endoplasmic reticulum-like vacuole, presumably through the injection of effector molecules into the host cytosol [157]. RalF was the first factor identified to be an effector substrate. RalF is a guanine nucleotide exchange factor that functions to activate the ADP ribosylation factor (ARF) family of GTPases [159]. The factor has been localized on the surface of the *Legionella*-containing vacuole in a Dot/Icm-dependent manner, and is required for the early recruitment of ARF1, but is not required for intracellular survival of the bacterium. A second factor LidA has recently been identified to be exported in a Dot/Icm-dependent manner and localizes to the phagosomal surface [160]. It has been hypothesized that this factor may function as a gatekeeper for the premature release of other factors. It is not yet clear how *L. pneumophila* modulates the type IV pathway to support the export

of nucleic acid/protein hybrids or effector protein substrates under different environmental stimuli. The requirement for at least 24 genetic loci to promote intracellular survival suggests that the Dot/Icm transporter may be far more sophisticated than the VirB-like type IV systems [157].

Type V Secretion (Autotransporters)

The autotransporter pathway represents an alternate branch of the GSP that serves as a simplified mechanism for the translocation of substrates out of the cell. Analysis of various genomes suggests that the autotransporter mechanism is widely conserved across gram-negative bacteria [83]. Rather than a requirement for factors in the periplasm or outer membrane translocases, the autotransporter secretion substrate harbors information in its carboxy-terminus for insertion into the outer membrane, and for translocation of the amino-terminal domain out of the bacterium. Pathogenic species of *Neisseria* secrete Iga1 protease using the type V mechanism to promote survival in interstitial fluids. The activated enzyme, once exported will function to degrade secretory antibodies [161]. Iga1 protease is synthesized as a preproenzyme that harbors an amino-terminal signal sequence to initiate its translocation across the inner membrane through the Sec pathway. The signal sequence is cleaved by signal peptidase, and the carboxy-terminal domain folds into a β-barrel structure promoting insertion of the proenzyme in the outer membrane [162]. Insertion in the outer membrane generates a porin-like channel for the export of the amino-terminal passenger domain. Transport of the amino-terminal domain through the channel promotes an autoproteolysis event, cleaving the proenzyme between the N- and C-terminal domains at a proline residue [163]. This proteolytic event will result in the release of a diffusible active enzyme.

Examples of autotransport have also been described for the Hap adhesin of *Haemophilus influenzae*, the IcsA protein of *S. flexneri*, and the adhesin YadA of *Y. enterocolitica*. The IcsA autotransporter represents a modification of the type V pathway, where an outer membrane serine protease SopA is required for the cleavage of the IcsA passenger domain, which promotes proper actin cytoskeletal nucleation [164]. YadA in *Y. enterocolitica* may represent another modification of the type V pathway, where the amino-terminal passenger domain is delivered to the extracellular space, but is not cleaved from the carboxy-terminal β-barrel [165]. Further, YadA assembles into trimers in the outer membrane and the amino-terminal heads assume a lollipop-like structure extending from the narrow stalk domain. YadA is involved in the resistance to complement mediated lysis.

A second variation of the type V pathway involves a two-component secretory system, where the synthesis and secretion of an enzyme substrate requires a single outer membrane transporter for delivery to the extracellular

space. The ShlA hemolysin of *Serratia marcescens* is synthesized as a pro-enzyme that contains an amino-terminal signal sequence, promoting its Sec-dependent translocation to the periplasm [166]. The ShlB polypeptide also contains an amino-terminal signal sequence and is exported through the Sec pathway [167]. Both ShlA and ShlB are processed by a signal peptidase and fold into mature species. The ShlB protein folds into a β-barrel structure that inserts into the outer membrane. This event is required for the trans-location of the enzymatic substrate ShlA. Other examples of this modified type V pathway include the secretion of filamentous hemagglutinin (FHA) by *B. pertussis*, and the secretion of the HpmA hemolysin by *Proteus mirabilis* [168].

Concluding Remarks

Molecular mechanisms that promote bacterial colonization are seemingly countless, however the accumulation of an ever-increasing body of information has allowed for the detection of common themes in pathogenesis. Strategies employed for the translocation of protein from the bacterial cytoplasm to targets in or beyond the cell wall envelope represent prime examples of this common-ality. Not only are the mechanisms for protein secretion conserved across species, many seemingly distinct secretion mechanisms share common compo-nents. Although protein secretion mechanisms represent only a fraction of the virulence strategies employed by bacteria, several of these processes represent primary virulence determinants. It appears that several pathogens use a combi-nation of secretion mechanisms to establish infection, and the identification of mechanisms by analogy has allowed for rapid progress in the classification of a particular pathogen arsenal. This of course provides the potential for rapid biochemical characterization of secretion systems and their protein substrates, as well as development and application of therapeutic targets to cover a wide range of bacterial species.

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Bacterial Iron Transport Related to Virulence

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The Problem of Iron Supply

Under oxic conditions, iron occurs in the Fe^{3+} valence state and forms insoluble polymeric hydroxyl-aquo complexes. Therefore, all aerobically living organisms that contain iron in many cytosolic and membrane-bound redox proteins, in particular in respiratory chains, have developed means to solubilize Fe^{3+}. Bacteria and fungi synthesize iron-complexing compounds, designated siderophores, which are secreted, bind extracellular Fe^{3+}, and are transported as Fe^{3+} complexes via specific transport systems into the cells, where Fe^{3+} is released from the complexes, usually by reduction to Fe^{2+}, and then incorporated into heme, iron-sulfur proteins, and other forms of protein reaction centers.

Higher organisms synthesize heme, which is the most abundant form of iron-containing compounds. Only a small percentage of the heme occurs in free form; most of it is incorporated into hemoglobin and bound to hemopexin.

Important extracellular iron-binding proteins in higher organisms are transferrin and lactoferrin and intracellular ferritin. Transferrin is the predominant iron carrier that delivers iron to cells. The di-iron complex is taken up by transferrin receptors, and the iron is released in endosomes and then further metabolized. Lactoferrin is the predominant iron-binding protein in secretory fluids. Transferrin and lactoferrin bind Fe^{3+} so tightly that the free Fe^{3+} concentration in equilibrium with these proteins is in the order of 1 ion per liter. The extreme lack of iron inhibits growth of microorganisms. However, some bacteria synthesize transferrin and lactoferrin receptor proteins exposed at the bacterial cell surfaces, which remove the iron from transferrin and lactoferrin and transport iron across the outer membrane.

This short overview focuses on some prominent examples of iron supply systems formed by human pathogenic bacteria. The reader is referred to more comprehensive reviews on specific aspects [1-24].

Overview of Bacterial Iron Transport Systems

Transport across the Cytoplasmic Membrane

The design of Fe^{3+} transport systems across the cytoplasmic membrane is the same for gram-negative and gram-positive bacteria. The systems belong to the ATP-binding cassette (ABC) transporters, which consist of a binding protein, a permease, and an ATPase (fig. 1). The binding proteins of gram-negative bacteria are located in the periplasm. In gram-positive bacteria, the binding proteins are linked by a lipid of the murein-lipoprotein type (triacyl-glyceryl cysteine) to the outer surface of the cytoplasmic membrane. The permease consists of one or two proteins that are incorporated into the cytoplasmic membrane and translocate Fe^{3+}, Fe^{3+}-siderophores, or heme across the cytoplasmic membrane. The ATPase provides the energy derived from ATP binding and subsequent ATP hydrolysis [25].

Crystal structures have been determined for two Fe^{3+}-binding proteins, FbpA of *Neisseria gonorrhoeae* and hFbpA of *Haemophilus influenzae* [26], and for the ferrichrome-binding protein FhuD, which binds structurally related siderophores of the hydroxamate type and the antibiotic albomycin [16, 27]. The crystal structures of FbpA and hFbpA are similar, but differ from that of FhuD. The three proteins are composed of two globular domains; in FbpA and hFbpA, these domains are connected by a hinge region that permits closure of the globular domains upon binding of Fe^{3+} (like a Venus fly trap). In contrast, the two globular domains of FhuD are connected by a rigid, kinked α-helix that allows only a slight movement of the globular domains. The crystal structure of an entire ABC transporter, the vitamin B_{12} transporter of *Escherichia coli*, has recently been unraveled. The ABC transporter consists of the BtuC permease and associated BtuD ATPase [28], and the BtuF-binding protein [29]. Since the BtuF structure is similar to FhuD and the transmembrane topology of BtuC is comparable to that of FhuB [15] which transports ferrichrome across the cytoplasmic membrane [30], it is predicted that the structure of the vitamin B_{12} transport system is representative for the ferric siderophore and heme transport systems. BtuF can be positioned via salt bridges on top of the BtuC permease. BtuCD forms a translocation channel that is large enough to accommodate vitamin B_{12}. In the crystal, the channel is open to the periplasmic side and closed to the cytoplasmic side. BtuD controls opening of the BtuC channel. The two BtuD subunits located at the inner side of the

OM

PP

FhuD

ExbD
ExbB
TonB

FhuB

CM

FhuC

FhuC

TonB
ExbB
ExbD

a

b

ATP   ADP + P$_i$ ATP   ADP + P$_i$

1 *c*   Albomycin   Rifamycin CGP4832   *d*
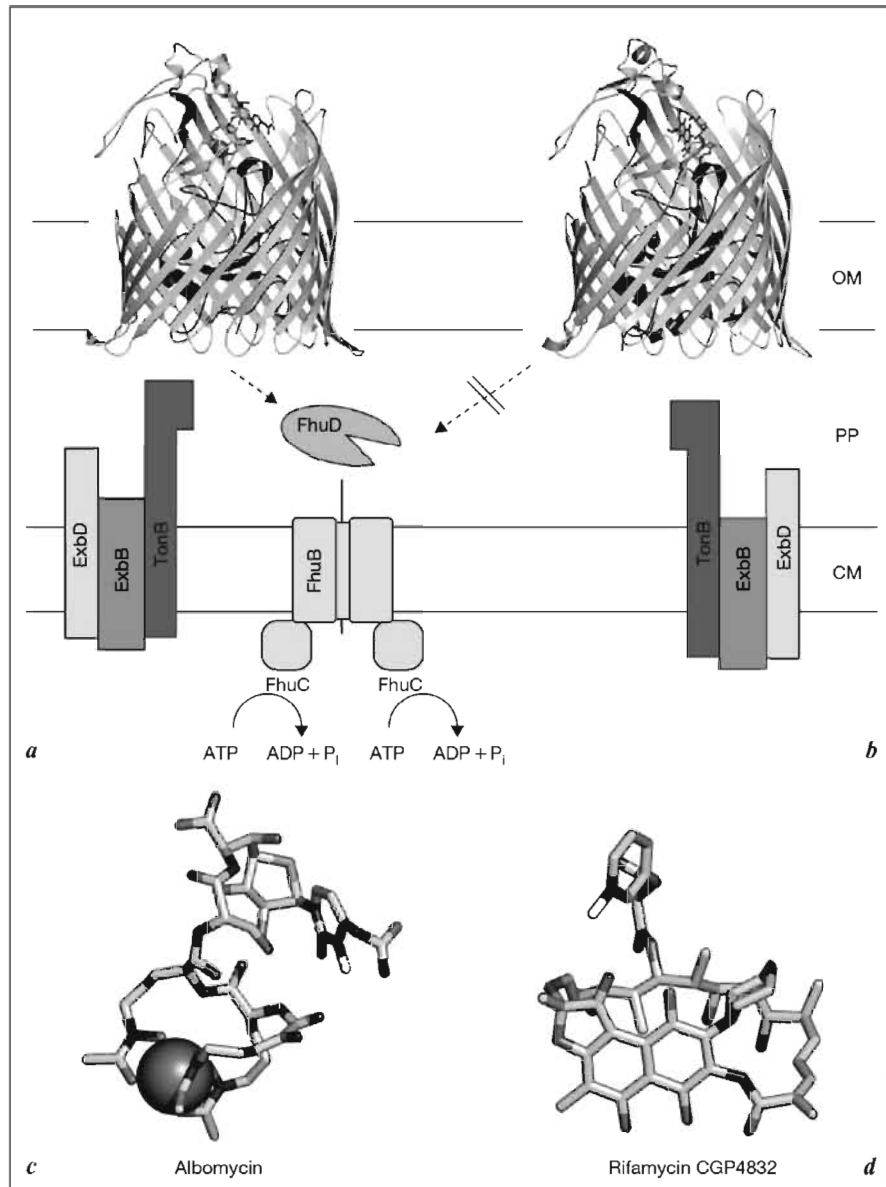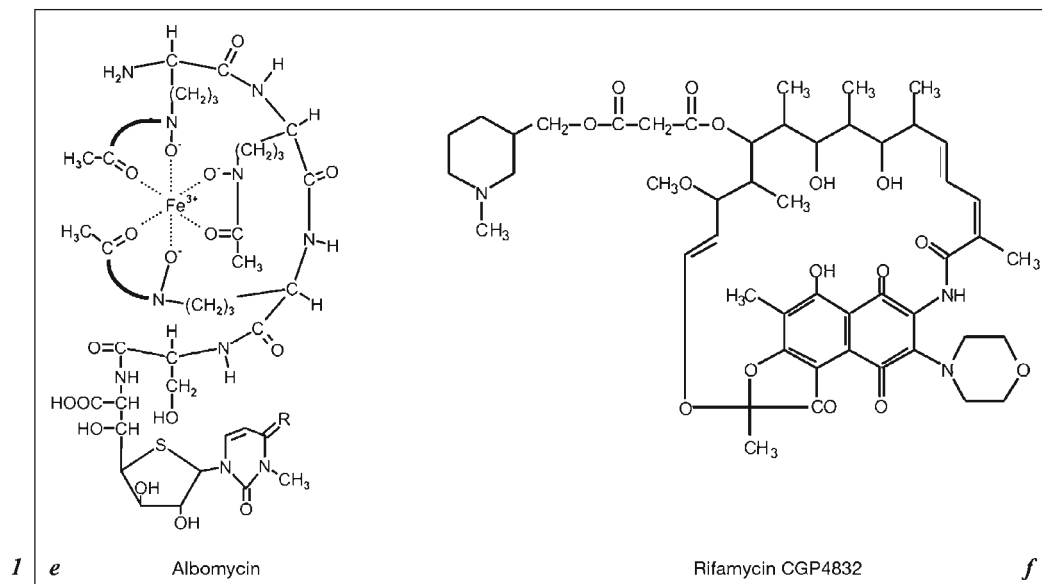
Fig. 1. Crystal structure of the FhuA outer membrane (OM) transport protein of *E. coli* with bound antibiotics albomycin (*a*) and rifamycin (*b*) CGP 4832, which are transported by FhuA. The structures of the antibiotics derived from the crystal structures (*c*, *d*) and the chemical formula (*e*, *f*) are shown. *a*, *b* The model illustrates the subcellular location of the proteins TonB, ExbB, and ExbD, which form the energy-transducing complex between the cytoplasmic membrane and the outer membrane, the transport proteins across the cytoplasmic membrane, and the interactions of the proteins. This protein arrangement is typical for all transport systems of gram-negative bacteria that transport Fe^{3+}, Fe^{3+}-siderophores, and heme. For further information, see the text. PP = Periplasm; CM = cytoplasmic membrane.

cytoplasmic membrane are in close contact to the two BtuC subunits. Binding of ATP moves the two BtuD subunits closer together. This might rearrange the two BtuC subunits such that the channel opens to the cytoplasmic side. BtuF loaded with vitamin B_{12} is bound to BtuC, delivers vitamin B_{12} to BtuC, and triggers ATP hydrolysis. The BtuD molecules move apart, which in turn closes the BtuC channel to the cytoplasmic side and opens it to the periplasmic side for the next round of vitamin B_{12} transport.

Transport across the Outer Membrane

Gram-negative bacteria contain an outer membrane that forms a permeability barrier for hydrophilic substrates above a certain molar mass, which in *E. coli* is 600 daltons [31]. The inner diameter of the porins through which the substrates diffuse across the outer membrane determines the substrate size. The Fe^{3+} siderophores usually have a molecular weight greater than 600 and cannot

diffuse with a sufficient rate through porins. In addition, their concentration is too low for diffusion to satisfy the growth requirement – in the order of 10^5 iron ions per cell per generation. The siderophores, heme, and the iron-binding proteins adsorb to outer membrane proteins, which not only serve as receptors but also function as transporters across the outer membrane. The iron compounds are thereby concentrated at the bacterial cell surface and are subsequently actively transported by an energy-consuming process across the outer membrane into the periplasm. There is no energy source in the outer membrane to drive active transport. Energy is provided by the cytoplasmic membrane through the proton motive force [32]. TonB, ExbB, and ExbD are the three known proteins that relay the energy from the cytoplasmic membrane into the outer membrane [33, 34]. These proteins are located in the cytoplasmic membrane and interact with each other, and TonB interacts with the outer membrane transport proteins. It is thought that these three proteins respond to the proton motive force of the cytoplasmic membrane (e.g., the proton gradient), react with a conformational change, and store the energy as potential energy. Upon interaction of energized TonB with the outer membrane transporters, the bound iron compounds are released from their binding sites and a channel is opened through which the iron compounds diffuse into the periplasm.

The crystal structures of three outer membrane iron transporters FhuA [35, 36], FepA [37], and FecA [38, 39], and the vitamin B_{12} transporter BtuB [40] provide a conceptual framework of how these transporters might function. The structures reveal a β-barrel composed of 22 antiparallel β-strands that form a channel. The channel is closed by a globular domain, which is designated as the cork, plug, or hatch. Binding of the substrates to the transporters occurs at a site well above the cell surface. Very strong binding occurs through approximately ten-amino acid side chains with a binding constant in the nanomolar range. Energy input is required to release the substrates from their binding sites and to move the cork so that a channel is formed through which the substrates gain access to the periplasm. The theory is that TonB transfers potential energy to the transporters, which alter their conformation to open a channel. TonB is deenergized, and the transporters close the channels after the iron compounds have passed through by diffusion. The genetically and biochemically identified sites of interaction between TonB and the transporters are located in the TonB box of the transporters and a region around residue 160 of TonB [41, 42]. The crystal structures and electron spin resonance determinations of nitroxide-substituted TonB box residues of BtuB demonstrate that the TonB box is exposed to the periplasm and moves upon binding of the substrates to the transporters [43]. The TonB box and the substrate-binding sites are far apart, which implies long-range structural transitions throughout the entire transporter. Transport across the outer membrane is mechanistically not coupled

to transport across the cytoplasmic membrane. The two membrane transport processes occur independently of each other.

Iron Transport Associated with Virulence

Iron-Controlled Bacterial Functions

Since iron is an essential element, but available only in growth-limiting concentrations, those bacteria that multiply in the human body express potent iron transport systems. The relationship of iron transport to virulence is usually not easy to establish since bacteria normally express several iron transport systems. Knocking out one system by mutation might not result in conversion of a pathogenic strain to a nonpathogenic strain since other iron transport systems take over the iron supply. For example, a pathogenic *E. coli* strain may transport Fe^{3+} by the siderophores aerobactin, enterobactin, salmochelin, citrate, ferrichrome, and heme, and Fe^{2+} via the *feo*-encoded transport system. *tonB*, *exbB*, and *exbD* are the only genes involved in all energy-coupled outer membrane iron transport systems of gram-negative bacteria. *tonB* mutants are impaired in virulence in various animal infection systems [44, 45]. However, some bacteria contain up to three *tonB* and *exbB*, *exbD* genes, which might participate in different iron uptake systems (see, for example, Iron Transport of *Vibrio cholerae* Related to Virulence). In addition, it is usually not known which iron transport system is important for proliferation at a specific infection site. Moreover, the iron limitation usually encountered in the human body could serve as an environmental signal that tells a bacterial strain its location in the human body. This could induce expression of genes required for multiplication, but might not be directly related to the iron supply. Therefore, different approaches are required to elucidate a relationship between iron transport and virulence. Such studies have involved knocking out a particular iron transport system and a genome-wide search for the expression of genes in vivo compared to the expression of genes in synthetic media under iron-deplete and iron-replete conditions. Such large-scale expression profiles usually reveal genes related to the iron supply. These genes encode proteins for siderophore biosynthesis and transport, heme transport, hemolysins, and toxins. The most prominent toxin is the diphtheria toxin, which is synthesized under iron-limiting conditions. Other iron-regulated toxins are the Shiga toxin of *Shigella* and *E. coli* strains, the hemolysins/cytolysins of *Serratia marcescens* and certain *E. coli* strains, exotoxin A of *Pseudomonas aeruginosa*, and the tetanus toxin of *Clostridium tetani*. By damaging cells, the toxins can mobilize intracellular iron sources and make them available to bacteria. *S. marcescens*, for example, colonizes the intestine of *Caenorhabditis elegans* and kills the nematode. *S. marcescens* mutants are

impaired in virulence when they carry a transposon in the hemolysin gene or in a siderophore biosynthesis gene [46].

Stress by Iron Surplus

Not only iron shortage, but also iron surplus can affect the outcome of a bacterial infection. Aerobic metabolism constantly creates hydrogen peroxide and superoxide radicals. If too much H_2O_2 is formed, it might not be completely destroyed by catalase and peroxidase. In the Haber-Weiss reaction, the oxygen radical reacts with H_2O_2 to form the highly reactive hydroxyl radical and hydroxyl anion. In the Fenton reaction, Fe^{2+} converts H_2O_2 to the hydroxyl radical and hydroxide anion. Fe^{3+} oxidizes the oxygen radical to oxygen. H_2O_2, the oxygen radicals, and the hydroxyl radicals damage DNA, lipids in membranes, and proteins. The lack of regulation of iron metabolism could, therefore, be deleterious to cells [47]. This has been demonstrated for E. coli, in which a mutation in the fur (iron uptake regulator) gene renders cells sensitive to oxygen. An additional mutation in the recA gene, which is involved in DNA repair, kills cells when they are cultivated under oxic conditions [48]. The surplus of reactive intracellular free iron might result from an uncontrolled import and the lack of intracellular iron storage proteins. Iron uptake is controlled by the fur gene in most gram-negative bacteria and certain gram-positive bacteria with a low GC content and by the dtxR gene in most (GC-rich) gram-positive bacteria. When the intracellular iron concentration reaches a certain level, the Fur and DtxR proteins are loaded with Fe^{2+} and repress transcription of genes encoding iron transport proteins and enzymes that synthesize siderophores [7].

Two types of iron storage proteins contribute to intracellular iron homeostasis in bacteria [22]. Ferritins are also found in eukaryotes, and heme-containing bacterioferritins are only found in bacteria. Both types are composed of 24 identical subunits that form an almost spherical shell into which more than 2,000 Fe^{3+} ions can be deposited. The FtnA ferritin of E. coli accumulates iron in the post-exponential growth phase in the presence of excess iron in the medium and supports subsequent growth under iron-deficient conditions. Helicobacter pylori and Campylobacter jejuni express a similar protein that stores iron and protects cells against oxygen damage. No physiological role has been ascribed to the Bfr bacterioferritin of E. coli, but a bfr mutant of P. aeruginosa is sensitive to peroxides.

Dps is another iron-binding protein that forms a shell, but with 12 subunits. Dps is probably less important for iron storage than for protecting DNA against the combined action of iron and H_2O_2.

Iron Transport of E. coli and Shigella Related to Virulence

Pathogenic E. coli strains express ten outer membrane proteins that transport ferric siderophores and heme (table 1). All the ferric hydroxamates (aerobactin,

Table 1. Iron transport systems of *E. coli*

Substrate	Outer membrane protein	Periplasmic protein	Cytoplasmic membrane proteins
Enterobactin	FepA	FepB	FepD[a], FepG[a], FepC[b]
Salmochelin	IroN	FepB	FepD[a], FepG[a], FepC[b]
Catecholates	Cir	FepB	FepD[a], FepG[a], FepC[b]
Catecholates	Fiu	FepB	FepD[a], FepG[a], FepC[b]
Ferrichrome	FhuA	FhuD	FhuB[a], FhuC[b]
Aerobactin	IutA	FhuD	FhuB[a], FhuC[b]
Coprogen	FhuE	FhuD	FhuB[a], FhuC[b]
Citrate	FecA	FecB	FecC[a], FecD[a], FecE[b]
Heme	ChuA	ChuT[c]	ChuU[a,c], ChuV[b,c]
Yersiniabactin[d]	FyuA	NI	YbtP, YbtP
Fe^{2+}			FeoB

[a]Transmembrane transport proteins in the cytoplasmic membrane.

[b]ATPase.

[c]Designations adapted from *S. dysenteriae* which is justified by the highly homologous *E. coli* and *Shigella* genomes. In *E. coli* K-12 ChuA alone is sufficient to support heme-dependent growth but the transport system in the cytoplasmic membrane may increase sensitivity to heme and rate of heme uptake.

[d]The transport system of yersiniabactin is encoded on pathogenicity islands which occur in various Enterobacteriaceae.

The nomenclature of reference 58 was used. For further details, see text and references 8 and 49–51. NI = Not identified.

ferrichrome, coprogen) for which specific transporters are found in the outer membrane are transported by the same transport system across the cytoplasmic membrane. The same holds true for the ferric catecholates, including ferric enterobactin and presumably ferric salmochelin, which are transported across the cytoplasmic membrane by the same system. It is not clear whether or to what extent the entire FepBCD transport system is involved in the ferric salmochelin transport. The heme transport system has been characterized in *Shigella dysenteriae* and its phylogenetic distribution in enteric bacteria has been determined [52]. The assignment of the heme genes to functions is based on the first functionally characterized heme transport system of *Yersinia enterocolitica* [53]. Heme and aerobactin transport, as well as TonB are required for virulence of the uropathogenic *E. coli* strain CFT073 in a mouse model of urinary tract infection [54]. In addition, *E. coli* strains isolated from patients with an intra-abdominal infection have been shown to secrete a protease, Hbp, that degrades hemoglobin. Hbp binds the released heme [55] and promotes the growth of *Bacteroides fragilis*, which is frequently

associated with *E. coli* in intra-abdominal infections. In a mouse infection model, Hbp contributes to the pathogenic synergy of these two organisms in abscess development. Heme transport systems are widely distributed among gram-positive and gram-negative bacteria [10, 11].

The Fe^{3+}-yersiniabactin transport system is frequently encoded on a 'high pathogenicity island', which occurs in several Enterobacteriaceae [56], but is also present in strains with less pathogenic potential [57]. The transport system of Fe^{3+}-yersiniabactin across the cytoplasmic membrane is interesting since the two permease proteins YbtP and YbtQ are each fused with the ATPase [58], as is found with human ABC export proteins. Subcutaneous infection by a *ybtP* mutant fails to cause disease in mice, a route that mimics *Yersinia pestis* transmission by fleas causing bubonic plague.

To date there has been no association reported between virulence and the ferric citrate transport system, in which FecB (binding protein), FecCD (permease), and FecE (ATPase) catalyze transport across the cytoplasmic membrane. A nearly identical transport system is located on a pathogenicity island of *Shigella flexneri* [59]. Coliform isolates of *E. coli* and *Klebsiella pneumoniae* from bovine inflammatory infections (mastitis) contain FecA, as evidenced by anti-FecA antibodies [60], and FecA is being considered as a vaccine component for the treatment of mastitis. A study of the regulation of the ferric citrate transport proteins uncovered a new type of transcription regulation. The inducer of the transcription of the transport genes binds to the FecA outer membrane protein and elicits a signal that is transmitted by FecA across the outer membrane to the FecR protein, which transmits the signal across the cytoplasmic membrane. In the cytoplasm, the FecI sigma factor is activated and directs the RNA polymerase specifically to the promoter of the *fec* transport genes upstream of *fecA* [61, 62].

Siderophores like ferrichrome and coprogen, which are not synthesized by *E. coli* or any other bacteria, but which are transported by many bacteria, including *E. coli,* might be used during coinfection with fungi that synthesize the siderophores or during bacterial growth outside the human body. The large variety of transport systems for ferric siderophores and heme found in *E. coli* and *Shigella* are typical for pathogenic bacteria. The systems are distributed among bacteria by horizontal gene transfer. For example, the aerobactin synthesis genes are found on plasmids in *E. coli* and *Salmonella*, on pathogenicity islands in *S. flexneri* and *Shigella sonnei*, and on the chromosome of *Shigella boydii* and certain *E. coli* strains [8, 63]. Another example is the recently discovered *iroN* gene, which was originally identified in *Salmonella enterica* and then shown to contribute to the uropathogenicity of *E. coli* isolates [64, 65]. *iroN* is encoded on a pathogenicity island on the chromosome [64] and on a transmissible plasmid [65]. In a mouse model of ascending urinary tract infection, IroN contributes to colonization of the bladder, kidneys, and urine [64].

In addition to the Fe^{3+} transport systems, *E. coli* also contains an Fe^{2+} transport system, which is encoded by the *feoAB* genes [23]. This transport system functions under anoxic conditions, as found in the colon and in biofilms.

Iron Transport of Salmonella Related to Virulence

S. enterica serovar Typhimurium has iron transport systems similar to those of *E. coli* and *Shigella*, but so far no heme or ferric citrate transport system has been described. However, a heme transport gene operon similar to that in *S. dysenteriae* is encoded on the *Salmonella typhimurium* genome. The known systems include those related to the outer membrane transporters FhuA, FepA, FoxA, Cir, and IroN. An additional transport system presumably transports iron, as was first demonstrated for the *sfuABC* iron transport system of *S. marcescens* [66] and then for the *fbpABC* system of *N. gonorrhoeae*, h*fbpABC* (hit*ABC*) of *H. influenzae* [18], and *yfuABC* of *Y. pestis* [67]. *sitA* encodes a putative periplasmic permease, *sitB* an ATPase, and *sitCD* a permease [68]. However, *sitABC* is not homologous to the *sfuABC*-type transport systems, but is homologous to *yfeABC* of *Y. pestis* and it transports Mn^{2+} with a much higher affinity than Fe^{2+}. The Sit system is widely distributed in all *S. enterica* serovars and is required for full virulence of *S. typhimurium* [69]; the Yfe system is essential for virulence of *Y. pestis* [70]. Iron transport systems are redundant, depending on the test system, since depleting one system may have no effect on bacterial virulence. The *S. enterica* genome also carries the *feoAB* genes, which encode an Fe^{2+} transport system. Single mutations of *sitA*, *feoB*, or *iucD* (Fe^{3+}-aerobactin transport) in *S. flexneri* do not impair the growth of these bacteria on a Henle cell monolayer; however, triple mutants do not form plaques [71].

A novel siderophore, designated salmochelin, was discovered only recently in *S. enterica* serovar Typhimurium LT2. The *iroB* gene product, encoded in the iron-regulated gene cluster *iroNEDCB*, shows sequence similarity to glycosyl transferases. This finding prompted a search for the function of IroB. Indeed, IroB was shown to encode an enzyme that glucosylates enterobactin at the 5′ position of the benzoyl ring, forming a C-C bond [106]. The published tentative structure carries the two glucosyl moieties inserted between two 2,3-dihydroxybenzoylserine residues [49]. In a *Salmonella* culture, salmochelin is more abundant and is more soluble than enterobactin. Therefore, it might be less able to elicit antibodies than enterobactin, which serves, bound to serum albumin, as a hapten. Transport of Fe^{3+}-salmochelin across the outer membrane is mediated by IroN and to a lesser extent by the FepA and Cir transporters.

Iron Transport of P. aeruginosa Related to Virulence

Pyoverdin and pyochelin are two well-studied siderophores that supply iron to *P. aeruginosa*. A number of indications show a relationship between

iron supply and virulence of *P. aeruginosa* in animal infection models: derepression of siderophore synthesis genes, synthesis of the siderophores pyoverdin and pyochelin and the related transport proteins, release of iron from the host iron-binding proteins transferrin and lactoferrin, and reduction of virulence of mutants deficient in synthesis of siderophores or Fe^{3+}-siderophore transport proteins. In addition, exotoxin A synthesis is controlled by the iron supply via the Fur repressor. A *tonB* mutant devoid of Fe^{3+} uptake via pyoverdin, pyochelin, and heme grows in the muscles and lungs of immunosuppressed mice, but does not kill the animals [72]. Pyoverdin- and pyochelin-negative double mutants multiply, but do not kill the mice; however, intranasal inoculation of wild-type bacteria results in multiplication and killing [73]. PvdS (see below) is an ECF sigma factor synthesized in chronic lung infections affiliated with cystic fibrosis and contributes to the synthesis of exotoxin A [74].

Complex regulatory devices underlie iron-mediated control of gene expression in *P. aeruginosa*. For example, iron-loaded Fur does not bind to the promoter of the *toxA* gene of exotoxin A, but acts via the *pvdS* gene product, which regulates 26 iron-repressible genes. *pvdS* encodes an ECF sigma factor of the FecI type (see Iron Transport of *E. coli* and *Shigella* Related to Virulence), and its synthesis is repressed by binding of Fe^{2+}-Fur to the *pvdS* promoter [75]. The activity of PvdS is controlled by pyoverdin secreted in the growth medium; pyoverdin (probably Fe^{3+}-pyoverdin) binds to the FpvA protein in the outer membrane. FpvA displays several functions: it acts as a signal receiver and as a signal transmitter across the outer membrane, and it transports Fe^{3+}-pyoverdin across the outer membrane. The signal is transmitted by the FpvR protein across the cytoplasmic membrane into the cytoplasm, where PvdS is converted into an active sigma factor. Since PvdS is active in mutants lacking FpvR and over-expression of FpvR inactivates PvdS, FpvR probably functions as an anti-sigma factor of PvdS [75]. PvdS directs the RNA polymerase to the promoter of the iron-repressible genes, including the pyoverdin synthesis genes. *fpvR* transcription is repressed by Fe^{2+}-Fur, as is transcription of a second ECF sigma factor gene, *fpvI*. FpvI synthesis is regulated like PvdS synthesis via Fe^{3+}-pyoverdin, FpvA, and FpvR, and controls synthesis of FpvA.

Heme uptake by *P. aeruginosa* is mediated by two systems, one of which is encoded by the *phuRSTUVW* genes (fig. 2) [76]. This system is very similar to the heme transport system of *Y. enterocolitica*. Heme is bound to the PfuR outer membrane protein that transports heme across the outer membrane. Further transport into the cytoplasm is achieved by an ABC transporter. The other heme transport system is similar to the heme transport system of *S. marcescens* and involves a hemophore that is secreted, releases heme from hemoglobin, and delivers it to the outer membrane transport protein (fig. 2).
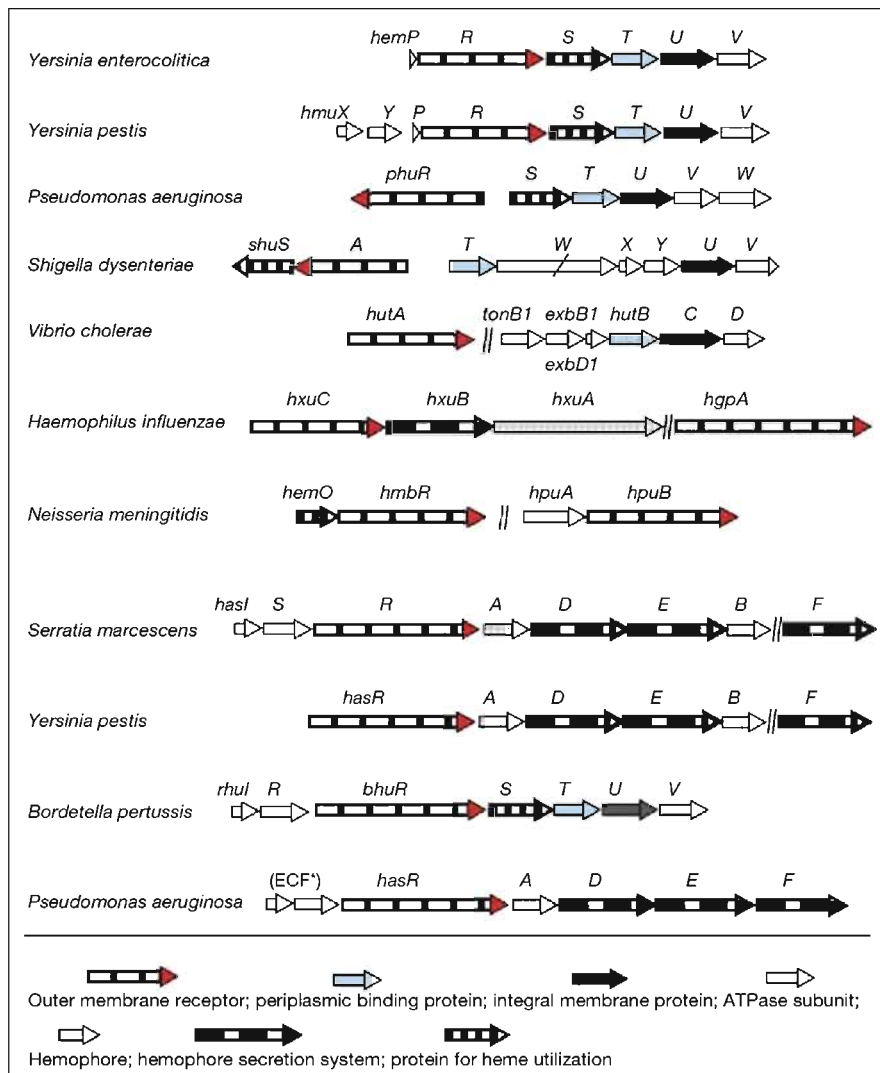
Fig. 2. Heme transport systems of gram-negative bacteria. The upper panel shows the transport genes and some promoters (P). In the lower panel, genes for hemophore synthesis, secretion, and regulation, and not the actual heme transport genes are shown for *S. marcescens*, *Y. pestis*, and *P. aeruginosa*. The HasA hemophores are secreted by the type I secretion mechanism catalyzed by the proteins HasD, HasE, and HasF. HasB is structurally and functionally a TonB-like protein. *hasI* and *hasS*, and *rhuI* and *rhuR* encode a transcription-signaling device of the FecIR type in which the I proteins represent extracytoplasmic membrane (ECF) sigma factors that receive signals from outside the cytoplasm and the R or S protein transfers the signals across the cytoplasmic membrane. In *Bordetella pertussis*, *rhuIR* regulates transcription of the *bhuRSTUV* heme transport genes [for further information, see 10, 62, 77].

In *S. marcescens*, regulation of heme transport gene transcription is mediated by a signaling device of the FecIRA type [77]. Heme-loaded hemophore binds to the HasR heme transporter and induces transcription of the *hasR* gene via HasI, which functions as an ECF sigma factor, and HasS, which acts as an anti-sigma factor. Since *P. aeruginosa* contains genes homologous to those in *S. marcescens* and arranged similarly, it is likely that the two Has regulatory systems function similarly.

Analysis of the genome of *P. aeruginosa* predicts nine additional regulatory devices of the FecIRA, HasISR, FpvA/Fpvl, FpvR, and PvdS type. These systems usually have the same gene arrangement as *fecIRA*, and the outer membrane proteins contain an extended amino-terminus, which in FecA interacts with FecR [12–14].

In addition to surface signaling elicited by the iron substrates, *P. aeruginosa* controls iron usage by a number of additional regulatory mechanisms. For example, pyochelin synthesis and uptake is repressed by Fe^{2+}-Fur, which binds to promoters of the synthesis and uptake genes. The regulatory protein PchR acts as a repressor in the absence of pyochelin and as an activator in the presence of pyochelin [78]. Regulation of ferric enterobactin usage is mediated by a two-component system consisting of the PfeS signal receiver and the PfeR response regulator. Ferric enterobactin in the periplasm binds to the PfeS sensor kinase, which is autophosphorylated and transfers the phosphate group to the receiver domain of PfeR. Phosphorylated PfeR functions as a transcription activator of the *pfeA* gene, which encodes the high-affinity PfeA outer membrane transporter [79]. In this iron transport system and in all the other iron transport systems studied in *P. aeruginosa*, the transported substrate induces synthesis of the cognate transport system. This is achieved by various mechanisms, but always results in the economic adaptation of the cells to the available iron source. If only iron depletion of the Fur protein would derepress gene transcription, many of the approximately 13 iron transport systems would be synthesized, even though only the one for the available iron source would be required.

Iron Transport of Vibrio cholerae *Related to Virulence*

Three heme transport systems have been identified in *V. cholerae*, represented by the outer membrane transporters HutA, HutR, and HasR [80]. A *hutA hutR* double mutant is impaired, but not completely unable to use hemin as an iron source. The triple mutant *hutA hutR hasR* is completely devoid of heme utilization. *V. cholerae* HasR is similar to the HasR proteins of *S. marcescens* and *P. aeruginosa*, which receive heme from the hemophore that releases heme from hemoglobin. In addition to the use of heme via transporters across the outer and cytoplasmic membranes, *V. cholerae* can use the iron complexes of

the siderophores vibriobactin, enterobactin, and ferrichrome [81]. The transporters are preferentially coupled to one of the two TonB proteins present in $V.$ $cholerae$ [82]. HasR, VctA, and IrgA, the latter two transport Fe^{3+}-enterobactin [83], are only coupled to TonB2, whereas HutA, HutR, ViuA (Fe^{3+}-vibriobactin transporter) and FhuA (ferrichrome transporter) can use TonB1 and TonB2 [80]. In an infant mouse model, the triple mutant competes with the wild-type strain, which indicated additional iron sources in vivo [80]. Analysis of gene transcription in the rabbit ileal loop model have revealed enhanced transcription of heme and Fe^{3+} transport genes and of the $feoAB$ genes, which encode an Fe^{2+}-transport system [84] that may have supplied the necessary iron.

Functions of Iron in Neisseria Related to Virulence

A $tonB$ mutant of $Neisseria$ $meningitidis$ does not actively transport iron and is unable to replicate within epithelial cells [85]. $N.$ $gonorrhoeae$ and $N.$ $meningitidis$ transport iron across the cytoplasmic membrane by an ABC transporter encoded by the $fbpABC$ genes [18], which are similar to the $sfuABC$ genes of $S.$ $marcescens$, the $hfbpABC$ ($hitABC$) of $H.$ $influenzae$, and the $yfABC$ genes of $Y.$ $pestis$ (see Iron Transport of $Salmonella$ Related to Virulence). No siderophore seems to be involved in iron transport. In $N.$ $gonorrhoeae$ and $H.$ $influenzae$, the iron might be delivered by the host transferrin and lactoferrin, which bind to highly specific outer membrane receptor proteins composed of two polypeptides: TbpA and TbpB for the transferrin receptor, and LbpA and LbpB for the lactoferrin receptor. The B components are lipoproteins and discriminate between iron-loaded and iron-unloaded transferrins and lactoferrins. The A components are similar to TonB-coupled ferric siderophore and heme transporters. TonB is not only required for the transport of iron across the outer membrane, but also for the release of Fe^{3+} from transferrin and lactoferrin [21]. The A and B components act in concert and interact with each other. Proteolytic degradation of TbpB is strongly influenced by coupling of TbpA to TonB. $N.$ $gonorrhoeae$ mutants that lack the transferrin receptor do not elicit symptoms of urethritis in human male volunteers [86].

Two hemoglobin receptors have been identified in $N.$ $meningitidis$: a two-component receptor designated HpuAB and a one-component receptor designated HpmR. No siderophores have been identified in $Neisseria$. However, $Neisseria$ can utilize Fe^{3+}-enterobactin taken up via a TonB-coupled transporter across the outer membrane and an ABC transporter across the cytoplasmic membrane [5, 87].

Iron Transport of Staphylococcus aureus Related to Virulence

In $S.$ $aureus$, several iron transport systems seem to operate. Ferrichrome is actively transported [88], and recently heme transport has been correlated with proteins (Isd) on the cell surface that are anchored to the murein by two sortases

[89]. *S. aureus* binds transferrin [90] and haptoglobin-hemoglobin [91]. In certain strains, slime production is enhanced by iron limitation [92]. Iron homoeostasis is regulated by the Fur repressor, whose synthesis is repressed by a homologous protein, PerR, which also regulates synthesis of the iron storage proteins ferritin and MrgA, a Dps homolog. PerR is required for full virulence of *S. aureus* in a murine skin abscess model [93]. The cell wall of *S. aureus* and *Staphylococcus epidermidis* contains the Tpn transferrin-binding protein, which is synthesized under iron-limiting growth conditions and elicits antibody formation in human serum and peritoneum upon staphylococcal infections [94]. The Tpn protein is the cell wall glyceraldehyde-3-phosphate dehydrogenase, which also binds plasmin [95]. It is assumed that the released iron is taken up into the cytoplasm by ABC transporters. Two such ABC transporters, encoded by the *sirABC* and *sstABCD genes*, have been partially characterized [96].

Fe3$^+$-Siderophores as Antibiotic Carriers

Multidrug resistance against currently used antibiotics forms an increasing problem in the treatment of bacterial diseases. One way out of the resistance dilemma is the development of new antibiotics. Since most antibiotics have been discovered during the decades of large-scale random screening, new strategies will have to be exploited. One possibility is the use of transport systems to transport antibiotics into cells. There are examples in which active transport, as opposed to diffusion, decreases the minimal inhibitory concentration (MIC) of an antibiotic more than 100-fold [97].

Antibiotics with Fe^{3+}-Hydroxamate Carriers

Most antibiotics diffuse into bacteria, and their rate of diffusion and their activity at the target sites determine their efficiency, as measured by the MIC. In gram-negative bacteria, the outer membrane forms an additional permeability barrier in addition to the cytoplasmic membrane, and renders gram-negative bacteria less sensitive to many antibiotics than gram-positive bacteria. However, if antibiotics are actively transported across the outer membrane, their MIC could be lower in gram-negative than in gram-positive bacteria because the antibiotics are accumulated in the periplasm and form a steep concentration gradient into the cytoplasm, thereby enhancing the diffusion rate, or the antibiotic might even be actively transported across the cytoplasmic membrane.

There are naturally occurring antibiotics that consist of an antibiotically active moiety and a siderophore carrier. The best-studied example is albomycin, which is composed of a trihydroxamate that binds Fe^{3+}, a peptide linker, and a thioribosyl pyrimidine moiety that inhibits tRNA^{Ser} synthetase [98]. Albomycin is

highly active toward gram-positive and gram-negative bacteria. The MIC against an *E. coli* strain is 200 times lower (0.05 μg/ml) than of ampicillin (12.5 μg/ml). The high specific activity comes from the active transport across the outer membrane and the cytoplasmic membrane into bacteria via the transport system of the structural analogue ferrichrome. The ferrichrome analogue serves as carrier of the antibiotically active thioribosyl pyrimidine group. After transport into the cytoplasm, iron is released from albomycin, and the thioribosyl pyrimidine group has to be cleaved from the carrier to be inhibitory. In *E. coli*, this is mainly achieved by peptidase N [1, 3, 97]. Mutants devoid of peptidase N activity are resistant to albomycin, and albomycin then serves as an iron carrier. Most of the thioribosyl pyrimidine moiety remains inside the cell, whereas the carrier is released into the culture medium. Albomycin is one of the very few antibiotics for which transport, intracellular activation, and target have all been characterized.

Albomycin has been cocrystallized with FhuA to determine whether it binds to the ferrichrome binding site of FhuA and where the bulky side chain is located in FhuA (fig. 1). The crystal structure reveals that the Fe^{3+}-hydroxamate portion of albomycin occupies the same site on FhuA and is bound by the same amino acid side chains as ferrichrome [99]. The thioribosyl pyrimidine moiety binds in the external pocket via five residues that are not involved in ferrichrome binding. The crystal structure also reveals the hitherto unknown conformation of albomycin and the conformation in the transport-competent form. Unexpectedly, albomycin assumes two conformations in the crystal – an extended and a compact conformation. Both conformations fit into the external cavity of FhuA and occupy seven different amino acid ligands. The solvent-exposed external cavity of FhuA is sufficiently large to accommodate the voluminous side chain of albomycin.

After transport across the outer membrane by FhuA, albomycin binds to FhuD in the periplasm. FhuD subsequently delivers albomycin to the permease in the cytoplasmic membrane. Cocrystals of FhuD with bound albomycin have been obtained in sufficient quality to determine the structure [100]. In contrast to FhuA, where albomycin sits inside the molecule, in FhuD albomycin is exposed to the surface of the protein. The thioribosyl moiety is not even seen in the crystal since it is not fixed to the protein and is thereby flexible. The fixation of albomycin at the surface of FhuD explains the broader substrate specificity of FhuD in contrast to FhuA since space is less restricted at the protein surface than within a protein.

Results of studies with albomycin demonstrate that the proteins involved in transport across the outer membrane and the cytoplasmic membrane tolerate substantial modifications of the substrate. The modular design of albomycin can be synthetically mimicked. Antibiotics that are ineffective because of poor entry

into the cells can be chemically linked to ferrichrome and then transported into cells as ferrichrome derivatives.

CGP 4832 is a semisynthetic rifamycin derivative with an activity against many gram-negative bacteria 200-fold higher than that of unmodified rifamycin [101]. The reason for the increased activity of CGP 4832 is its energy-coupled transport by FhuA across the outer membrane of $E.$ $coli$ [101]. The use of FhuA as transporter is surprising since CGP 4832 does not contain iron and has no structural resemblance to ferrichrome or any other hydroxamate. To obtain insights into how CGP 4832 is transported by FhuA, the crystal structure of FhuA loaded with CGP 4832 was determined [102]. CGP 4832 occupies in FhuA largely the same site as ferrichrome (fig. 1). Nine residues that bind CGP 4832 also bind ferrichrome. Of 16 amino acid residues that bind CGP 4832, 5 residues recognize those side chains of CGP 4832 in which it differs from unmodified rifamycin. Two additional amino acid residues specifically bind the unique CGP 4832 side chains, whereas the other residues bind to sites that CGP 4832 shares with rifamycin. The crystal structure reveals the conformation of CGP 4832, which demonstrates a completely different structure than that of ferrichrome. Unlike albomycin, CGP 4832 is not transported via FhuBCD across the cytoplasmic membrane [101]. Rather, its active transport across the outer membrane results in an elevated concentration in the periplasm, which facilitates diffusion across the cytoplasmic membrane. It is the active transport across the outer membrane that reduces the MIC 200-fold.

Salmycins have been isolated from $Streptomyces$ $violaceus$ 37290 (DSM 8286) and are highly active against staphylococci and streptococci (MIC 10 μg/ml). Salmycins consist of an Fe^{3+}-siderophore with a ferrioxamine group and an antibiotically active aminodisaccharide, which in salmycin B consists of a 2-ketoglucose linked to the 2-position of a 6-methylaminoheptopyranose [103]. It is assumed that the aminodisaccharide is released from the carrier by cleavage of the ester bond. Salmycins seem to inhibit protein synthesis by a yet unknown mechanism.

Ferrimycins are among the first sideromycins discovered [97]. The action of ferrimycins is antagonized by ferroxamine B, which competes for ferrimycin uptake. Ferrimycin inhibits incorporation of amino acids into proteins of $S.$ $aureus$ SG511. Ferrimycin is difficult to isolate and for this reason has recently been studied less than albomycin and salmycin.

Antibiotics with Fe^{3+}-Catecholate Carriers

Enterobactin is the most prominent catecholate siderophore with an extremely high Fe^{3+} stability constant. It consists of three dihydroxy benzoyl serine residues linked to a cyclic trimer by ester bonds. No natural Fe^{3+}-catecholates with antibiotic activity are known. However, chemically synthesized

catechol-substituted cephalosporins display MIC values below $1\,\mu g/ml$ [104, 105], particularly against gram-negative bacteria, including *P. aeruginosa*. Their antimicrobial activities can exceed the activity of the unsubstituted cephalosporins more than 100-fold. Their high activity is related to their active transport into the periplasm, where the target, the murein transpeptidase, is located. They are transported across the outer membrane by the Fe^{3+}-catecholate transport proteins Fiu and Cir [26]. Iron limitation increases the susceptibility of *E. coli* strains since low iron derepresses Fiu and Cir synthesis.

Resistance to Fe^{3+}-Siderophore Antibiotics

Resistant bacteria emerge on every nutrient agar plate containing antibiotics that are carried into the bacteria by active Fe^{3+}-siderophore transport systems. The higher the number of genes involved in a particular transport system, the higher the frequency of resistance. However, when two transport systems are used by an antibiotic, for example Cir and Fiu for the cephalosporin catecholates, the frequency of resistant mutants is low. Although the high resistance frequency seems to prevent development of such antibiotics as antibacterial drugs, the in vivo situation might be quite different. In cases where an iron transport system is important for the proliferation of the pathogenic bacteria, loss of the iron transport system is detrimental. Even when several iron transport systems exist and only one is inactivated by resistance to a particular antibiotic, the inactivated system might be the one that is essential for the bacteria to survive and multiply at the site of infection in the human host. Under these circumstances, it does not matter whether the number of bacteria is reduced by the antibiotic or by loss of the iron supply since under both conditions the immune defense system gains time to cope with the infection.

Concluding Remarks

Iron deficiency was also designated nutritional immunity which meant that growth inhibition by lack of iron prevents bacterial multiplication. Lack of growth or growth retardation gives the natural and the adaptive immunity system the chance to cope with an infection. Iron is the only nutrient for which an essential role in growth of many bacterial pathogens causing various diseases in humans and animals has been demonstrated. There are certainly many more nutrients which play a decisive role in extra- and intracellular multiplication of bacteria. However, it is difficult to identify these nutrients. Large-scale expression profiles of metabolic genes in bacteria isolated from human patients without further culturing and from animal models may indicate metabolic pathways from which the nutrients may be derived. From a purely scientific point of view

the iron supply systems are of great interest with regard to the various ways insoluble Fe^{3+} is complexed by siderophores, heme, transferrin, and lactoferrin and transported into the bacterial cells by distinct and very sophisticated mechanisms. For the avoidance of iron shortage and iron surplus the transport systems are regulated by various means, iron-dependent repression, downregulation by small RNAs, transcription enhancement by two-component systems, and transcription initiation by surface signaling. In the future, a detailed knowledge of iron uptake and intracellular iron metabolism may be applied to interfere with bacterial growth as a means to control bacterial diseases, and siderophore antibiotics (sideromycins) may be used when treatment with other antibiotics fails because of resistance.

Acknowledgments

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Pathogenicity Islands and Their Role in Bacterial Virulence and Survival

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Infections caused by bacterial pathogens are still a significant problem in modern medicine. Therefore, the identification of the factors that are related to the infections and the understanding of the processes involved in the evolution of pathogenic bacteria from their nonpathogenic progenitors is an important subject of research. It has long been known that acquisition of virulence determinants by horizontal gene transfer is one of the major driving forces in the emergence and evolution of new pathogens [reviewed in 1–4]. Furthermore, our knowledge of the organization of the bacterial genome has greatly increased within the last few years due to the availability of more than 120 completely sequenced eubacterial genomes, including those of almost all pathogenic bacteria, which has introduced a new area of pathogen research. It has become evident that the typical bacterial genome consists of a conserved 'core gene pool' comprising genes that encode essential structural features and fundamental metabolic pathways, and a 'flexible gene pool' that is more variable and encodes functions only advantageous under specific growth conditions. Core genes are characterized by a relatively homogenous G + C content and they are normally encoded in stable regions of the chromosome that are conserved in their organization in closely related species. In contrast, the flexible gene pool comprises variable regions of the chromosome and various mobile genetic elements such as plasmids, bacteriophages, IS elements and transposons, conjugative transposons, integrons and superintegrons that are transferred between different organisms by the means of natural transformation, transduction or conjugation. Many of the genes encoding toxins, adhesins, secretion systems, invasins or other virulence-associated factors have been found to be encoded by mobile genetic elements [overviews in 5, 6]. Furthermore, the analysis of the genomes

of closely related species has revealed that the conserved chromosomal backbone is interspersed with large regions that exhibit features of former mobile genetic elements that have been termed genomic islands (GEIs) [7, 8]. GEIs are broadly distributed and seem to be a common theme in most bacterial genomes. Originally, such elements were identified in uropathogenic *Escherichia coli* strains and were designated 'pathogenicity islands' (PAIs), because they encoded key virulence factors of these bacteria [9]. However, when regions with similar features were also found in nonpathogenic bacteria where they encoded other accessory functions, it was recognized that these elements are not limited to bacterial pathogens, but are present in most bacteria that have been analyzed. In this chapter, the role of GEIs in bacterial virulence and survival will be discussed.

The Concept of GEIs

Features of GEIs

A comparative analysis of microbial genome sequences has revealed that bacterial genomes can harbor variable and frequently significant amounts of foreign DNA [3]. The genome size of different variants of the same species or closely related species can vary by more than one megabase, which can be accounted for by the acquisition of large blocks of DNA such as plasmids, bacteriophages and GEIs, as well as by the acquisition of smaller pieces of foreign DNA that have been described as 'islets'. Generally, GEIs represent distinct pieces of DNA that have most of the following features in common suggesting that they originate from events of lateral gene transfer [10].

(1) GEIs are present in the genomes of many bacteria but absent from the genomes of closely related strains or species. (2) GEIs occupy relatively large regions of the chromosome and can cover between 10 and more than 100 kb, which may reflect the introduction of large pieces of DNA into a new host by horizontal gene transfer. Some strains also carry smaller pieces of DNA (1–10 kb) that have been termed 'genomic islets' in contrast to the larger islands. (3) GEIs differ in their G + C content and their codon usage from that of the conserved regions of the chromosome. (4) GEIs are often flanked by direct repeats that may have been generated during integration of GEI-specific regions into the host chromosome via site-specific recombination. (5) GEIs are frequently associated with tRNA loci. The 3' end of tRNA genes have been recognized as preferred target sites for the integration of foreign DNA [reviewed in 11]. (6) GEIs often possess functional or cryptic genes coding for factors that are involved in genetic mobility such as integrases, transposases, phage genes and origins of replication. Furthermore, GEIs normally do not represent homogenous elements but

rather are generated by multistep processes including DNA rearrangements via IS elements which is reflected by mosaic-like structures. (7) Some GEIs tend to be unstable DNA regions due to recombination between the flanking direct repeats, between IS elements or between other regions of homologous sequences. Generally, little is known about the mechanisms that have led to the acquisition of GEIs and there are only few examples of inter- or intracellular mobilization of GEIs [12–16].

GEIs are prevalently found in organisms that show frequent gene transfer by bacteriophages and plasmids which are regarded as possible precursors of GEIs [8]. However, GEIs have also been described in bacteria that exhibit natural competence such as *Helicobacter pylori*, *Neisseria gonorrhoeae* and *Streptococcus pneumoniae*, and that tend to introduce smaller pieces rather than large regions of foreign DNA into their genome [17–19].

GEIs Contribute to Bacterial Fitness

Besides selfish genes such as genes involved in recombination and transfer or modification of DNA, GEIs often carry determinants that are beneficial for their host bacterium in certain environments thereby increasing bacterial fitness and consequently survival. GEIs were divided into different subtypes reflecting their contribution to the respective microbial lifestyle [8] (table 1). GEIs that encode virulence traits were defined as 'pathogenicity islands' (PAIs). The original definition of GEIs was based on the characteristics of PAIs in pathogenic *E. coli*, but intensive studies of the genome structure of bacterial pathogens resulted in the identification of similar structures in many phylogenetically unrelated organisms including gram-negative as well as gram-positive bacteria (tables 2–3). Typical virulence factors encoded on PAIs include toxins, adhesins and fimbriae, factors involved in host cell entry, capsules, secretion systems and iron uptake systems. Based on the broad distribution of PAIs, it can be concluded that they have contributed significantly to the evolution of virulent variants. However, the still growing number of genome sequences has made it clear that GEIs are not restricted to pathogenic species. GEIs contributing to the adaptation to specific growth conditions or the interaction with a eukaryotic host organism have been described in environmental, commensal or symbiotic bacteria and have been designated 'symbiosis islands', 'ecological islands' or 'resistance islands', according to the respective encoded functions. Relatively well-studied examples of GEIs include the symbiosis island of *Mesorhizobium melioti* that carries genes required for nitrogen fixation, whereas GEIs such as the *mec* region enhance survival of staphylococci in hospitals where they have to face antimicrobial substances. Other islands encode enzymes involved in the degradation of phenolic compounds or for uptake and metabolism of certain carbohydrates (table 1). Finally, a recently described island in *Magnetospirillum*

Table 1. Examples of GEIs

Subtype of	Designation	Organism	Encoded functions	Reference
PAI	PAI II_{536}	*Escherichia coli*	P fimbriae, α-hemolysin	26
PAI	HPI	*Yersinia* spp.	Iron uptake	21
ECI	HPI	Fecal *Escherichia coli*, *Salmonella enterica* subgroups III + IV	Iron uptake	23, 24
ECI	CTn*scr94*	*Salmonella senftenberg*	Sucrose uptake and metabolism	13
ECI	*clc* element	*Pseudomonas putida*	Degradation of phenolic compounds	16
ECI	Magnetosome island	*Magnetospirillum gryphiswaldense*	Formation of magnetosomes	20
REI	STI1	*Salmonella enterica* DT104	Antibiotic resistance	76
REI	*mec* locus	*Staphylococcus aureus*	Antibiotic resistance	77
SYI		*Mesorhizobium melioti*	Nitrogen fixation	78
SYI		*Sinorhizobium fredii*	Type III secretion system	79

ECI = Ecological island; REI = resistance island; SYI = symbiosis island.

gryphiswaldense is required for the formation of magnetosomes and the characteristic magnetotactic phenotype of these bacteria [20]. Interestingly, some GEIs have been assigned to different subtypes depending on the habitat and genetic background of the respective bacterium. An example is the so-called 'high pathogenicity island' (HPI) that was originally found in derivatives of *Yersinia* spp. exhibiting increased virulence in mice [21]. As this island and the associated iron uptake system have been found in many pathogenic and nonpathogenic enterobacteria [22–24], HPI can be considered as a 'broad host range GEI'. Whereas it contributes to virulence in pathogenic variants and has therefore been defined as a PAI, it enhances the capability of fecal *E. coli*, *Klebsiella* spp. and nonpathogenic *Salmonella enterica* spp. to grow under iron-limiting conditions and has therefore been defined as an 'ecological island' in these strains.

Table 2. PAIs of pathogenic Enterobacteriaceae

Organism	Designation	Encoded traits	Size, kb	Junction	Integrase	Insertion site	Reference
Escherichia coli 536 (UPEC)	PAI I$_{536}$	α-Hemolysin, put. adhesions	75.8	DR 16 bp	CP4-like (cryptic?)	*selC*	26
Escherichia coli 536 (UPEC)	PAI II$_{536}$	α-Hemolysin, put. P fimbriae (Prf), adhesion	102	DR 18 bp	P4-like	*leuX*	26
Escherichia coli 536 (UPEC)	PAI III$_{536}$	S fimbriae (SfaI), *iro* siderophore system, hemoglobin protease	76.8	DR 46 bp	Sfx-like	*thrW*	26
Escherichia coli 536 (UPEC)	PAI V$_{536}$	K15 capsule	>75	DR 23 bp	P4-like	*pheV*	Unpublished
Escherichia coli J96 (UPEC)	PAI I$_{J96}$	α-Hemolysin, P fimbriae (Pap)	>170	?	?	*pheV*	32
Escherichia coli J96 (UPEC)	PAI II$_{J96}$	α-Hemolysin, P fimbriae (Prs), cytotoxic necrotizing factor 1 (CNF1)	110	DR 135 bp	P4-like	*pheU*	32
Escherichia coli CFT073 (UPEC)	PAI I$_{CFT073}$	α-Hemolysin, P fimbriae (Pap)	58	DR 9 bp	P4-like	*pheV*	29
Escherichia coli CFT073 (UPEC)	PAI II$_{CFT073}$	P fimbriae (Pap), iron acquisition	71	No DR	P4-like	*pheU*	29
Escherichia coli AL862	PAI$_{AL862}$	*afa*8 adhesin	61	DR 14 bp or DR 136 bp (imperfect)	P4-like P4-like	*pheU* *pheV*	80
Escherichia coli Ec222 (APEC)	VAT-PAI	Vat autotransporter	22	No	SfiI-like (truncated)	*thrW/yagU*	81
Escherichia coli C5	PAI I$_{C5}$	α-Hemolysin, P fimbriae (Prs), cytotoxic necrotizing factor 1 (CNF1), heat-resistant hemagglutinin	~100	DR 18 bp	?	*leuX*	82

Escherichia coli E2348/69 (EPEC)	EspC-PAI	Autotransporter/ enterotoxin	15.2	No DR	No	*ssrA*	83
Escherichia coli E2348/69 (EPEC)	LEE	Type III secretion, invasion	35	No DR	No	*selC*	84
Escherichia coli EDL933 (EHEC)	LEE	Type III secretion, invasion	43	No DR	CP4-like	*selC*	27
Escherichia coli RW1374 (STEC)	LEE	Type III secretion, invasion, parts of the *she* PAI (*S. flexneri* 2a)	>80	?	No	*pheV*	85
Escherichia coli RDEC-1 (REPEC)	LEE	Type III secretion, invasion, put. adhesion	?	No DR	P4-like	*pheU*	37
Escherichia coli 83/39 (REPEC)	LEE	Type III secretion, invasion, put. adhesin, enterotoxin	59.5	No DR	P4-like	*pheU*	61
Escherichia coli 84/110-1 (REPEC)	LEE	Type III secretion, invasion	~85	DR 23 bp (imperfect)	P4-like	*pheV*	61
Escherichia coli 135/12	EPEC Afa-PAI	Diffuse adherence adhesin	>11	?	P4-like	*pheV*	86
Escherichia coli (EHEC)	LPA	Serine protease (EspI), vitamin B_{12} receptor (BtuB), adhesion	33	No DR	CP4-like	*selC*	87
Escherichia coli 10407 (ETEC)	TPAI-1	Invasion	46	DR 25 bp	Yes	*selC*	88
Pathogenic Escherichia coli, nonpathogenic Salmonella	HPI (PAI IV$_{536}$)	Yersiniabactin synthesis, transport	31–43	No DR	P4-like	*asnT*	23, 24
Yersinia enterocolytica Ye8081	HPI	Yersiniabactin synthesis, transport	45	No DR	P4-like	*asnT*	21
Yersinia pseudotuberculosis	HPI	Yersiniabactin synthesis, transport	36	DR 17 bp	P4-like	*asnT, U, W*	12
Yersinia pestis	HPI (*pgm* locus)	Yersiniabactin synthesis, transport, hemin uptake	102	IS100 DR 17 bp	P4-like	*asnT*	89

Table 2. (continued)

Organism	Designation	Encoded traits	Size, kb	Junction	Integrase	Insertion site	Reference
Shigella flexneri	SHI-1 (*she*)	Enterotoxin (Set), protease (Pic)	46.6	DR 22 bp (imperfect)	P4-like	*pheV*	68
Shigella flexneri	SHI-2	Aerobactin synthesis, colicin V immunity	23–30		CP4-like	*selC*	90, 91
Shigella flexneri	SRL	Ferric dicitrate transport, antibiotic resistances	66	DR 14 bp	Yes	*serX*	92
Shigella flexneri	Shi-O	Genes involved in serotype conversion	11	No DR	Yes	*thrW*	93
Salmonella enterica sv Typhimurium	SPI-1	Type III secretion, invasion into epithelial cells, apoptosis	40	No DR	No	Between *fhlA/mutS*	41
Salmonella enterica	SPI-2	Type III secretion, invasion into monocytes	40	No DR	No	*valV*	43
Salmonella enterica	SPI-3	Invasion, survival in macrophages	17	No DR	No	*selC*	44
Salmonella enterica	SPI-4	Invasion, survival in monocytes	25	No DR	No	Putative tRNA gene	45
Salmonella enterica	SPI-5	SPI-1 effector protein (SopB)	7	No DR	No	*serT*	46, 94
Salmonella enterica sv Typhi	SPI-7	Vi exopolysaccharide production	134	DR 55 bp	Yes	*pheU*	47
Erwinia amylovora Ea321	*hrp* PAI	Type III secretion, effectors	~60	?	Yes	*pheV*	56

DR = Direct repeat; APEC = avian pathogenic *E. coli*; REPEC = rabbit enteropathogenic *E. coli*; ETEC = enterotoxigenic *E. coli*; LPA = locus of proteolysis activity; SRL = *Shigella* resistance locus; put. = putative; STEC = Shiga toxin-producing *E. coli*.

Table 3. Examples of PAIs of gram-positive bacteria

Organism	Designation	Encoded traits	Size, kb	Junction	Integrase	Insertion site	Reference
Staphylococcus aureus RN4282	SaPI1	Toxic shock syndrome toxin-1 (TSST-1)	15	DR 17 bp	Yes	Near *tyrB*	14
Staphylococcus aureus RN3984	SaPI2	Toxic shock syndrome toxin-1 (TSST-1)	?	?	?	Near *trp* gene cluster	14
Staphylococcus aureus COL	SaPI3	Enterotoxin serotypes B, K, Q	16	DR 17 bp	Yes	?	95
Staphylococcus aureus RF122	SaPIbov	Toxic shock syndrome toxin-1 (TSST-1), enterotoxin C	16	DR 74 bp	?	Intergenic	96
Staphylococcus aureus TY114	*etd* PAI	Exfoliative toxin D, glutamyl endopeptidase	15	DR 5 bp	No	Intergenic	97
Enterococcus faecalis	*Enterococcus faecalis* PAI	Cytolysin, surface protein (Esp), aggregation substance	~150	DR 10 bp	Yes	Intergenic	60
Clostridium difficile	Pathogenicity locus (PaLoc)	Enterotoxin (TcdA), cytotoxin (TcdB)	19	No DR	No	Intergenic	58
Streptococcus pneumoniae	PPI1	Iron uptake system	27	No DR	Recombinase	*yefA*	18
Pathogenic *Listeria*	LIPI-1	PrfA-dependent virulence gene cluster (phospholipases, listeriolysin, ActA)	9	No DR	No	Intergenic	57
Listeria ivanovii	LIPI-2	Internalins, sphingomyelinase C	22	No DR	No	Intergenic	57

PAIs Contribute to Virulence of Bacterial Pathogens

PAIs of Enterobacterial Pathogens

Most of the characterized GEIs so far have been found in members of the Enterobacteriaceae (table 2), which may in part be explained by the fact that this family has been intensively studied, but also indicates that PAIs have played a pivotal role in the evolution of enterobacterial pathogens. *E. coli* normally lives as a harmless commensal in the bowels of humans or animals, but some variants have the potential to cause gastrointestinal as well as extraintestinal infections [25]. Pathogenic *E. coli* can be linked to a variety of quite diverse symptoms that include enteric diseases that range from cholera-like diarrhea to severe dysentery and hemorrhagic colitis, cystitis or pyelonephritis, septicemia and meningitis. Based on their mode of pathogenesis, virulent *E. coli* have been classified into different pathotypes such as uropathogenic *E. coli* (UPEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), entero-toxigenic *E. coli* or enteroinvasive *E. coli* and the pathogenetically related *Shigella* species. They are characterized by the expression of specific virulence factors that enable them to exploit new niches in their host and to disrupt the normal host physiology. In pathogenic *E. coli* and *Shigella* spp., many of these key virulence factors are encoded on PAIs, which underlines their importance in the formation of the various pathotypes. The diversity of diseases that are associated with *E. coli* infections is also reflected by the structural and functional varieties in PAIs (see table 2, fig. 1). Whereas some PAIs are widely distributed among different enterobacterial species [e.g. HPI and the locus of enterocyte efface-ment (LEE)], others are closely related to a specific pathotype. Furthermore, most strains carry multiple PAIs that can cover more than 5% of the genome. For example, at least five PAIs (PAI I_{536} to PAI V_{536}) have been identified in the chromosome of the uropathogenic isolate *E. coli* 536 (table 2) [26].

Besides PAIs that have been identified on the basis of functional studies, a still increasing number of putative GEIs have been detected in the completely sequenced genomes of pathogenic *E. coli* and *Shigella flexneri*. However, it has yet to be investigated whether the encoded factors contribute to virulence or fit-ness of the respective pathogen [27–29]. Most PAIs of pathogenic *E. coli* exhibit a mosaic-like modular structure and although some PAIs show similarities in respect to the presence and linkage of certain virulence determinants, there is also a great variability in regard to size, organization and chromosomal local-ization even among strains of the same patho- or serotype [26, 30]. Interestingly, some tRNA genes seem to represent hot spots for the integration of foreign DNA including PAIs. The majority of PAIs in enterobacteria is linked to either *selC*, the gene for a selenocysteine-specific tRNA, or one of two genes for a phenylalanine-tRNA, *pheV* or *pheU*. Whereas the associated integrase genes
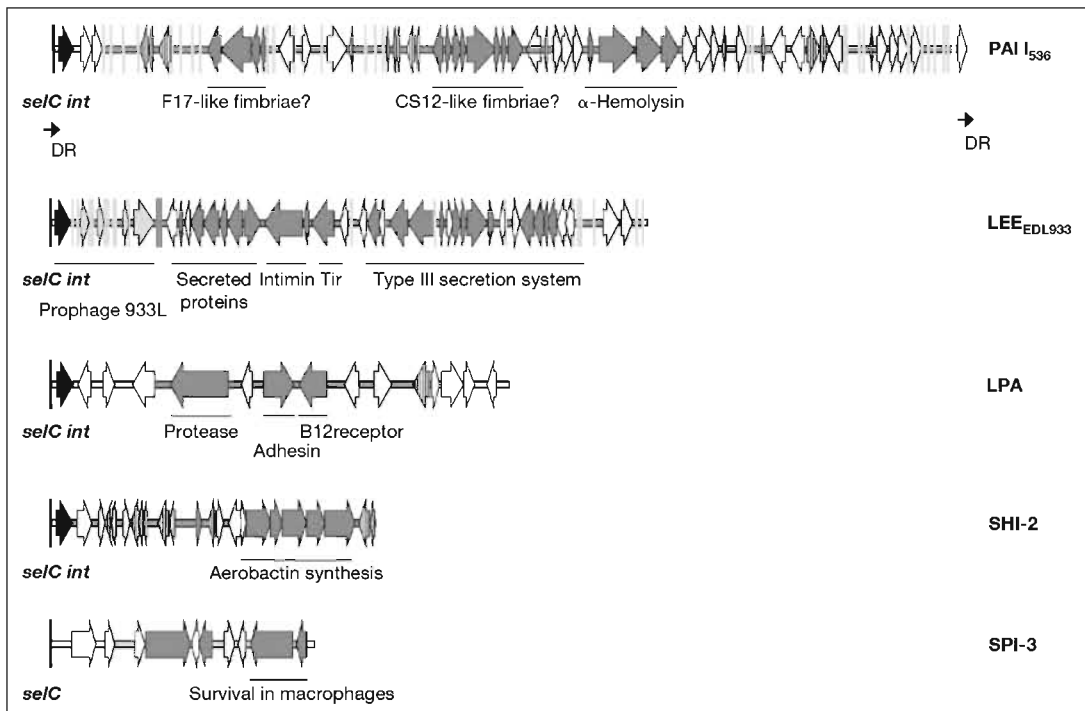
Fig. 1. Examples of *selC*-associated PAIs of Enterobacteriaceae. The organization of *selC*-associated PAIs is shown. Known or putative virulence genes are shown as gray arrows and their (predicted) function is given. ORFs with similarity to transposases are indicated by hatched arrows. Also shown are genes for a CP4-like integrase *(int)*. With the exception of SPI-3 from *S. enterica*, highly similar genes are present in all islands. Finally, direct repeat sequences in PAI I_{536} and the prophage sequence present in EPEC EDL933 are also shown.

are well conserved and seem to be specific for the linked tRNA gene, the further structural organization and nucleotide sequence of islands that are integrated into identical sites in their respective bacterial host are not necessarily closely related but rather encode functions that determine the lifestyle and pathotype of the bacterium (fig. 1).

The best-studied PAIs of *E. coli* belong to the LEE island and PAIs of uropathogenic strains. One important trait of UPEC isolates is the presence of adhesins that enables them to adhere to uroepithelial cells [31]. Besides type I fimbriae, PAIs often carry genes that are specific for P fimbriae that bind to the Gal α(1–4)Gal moieties of glycoproteins and S-fimbrial adhesions. Furthermore, UPEC produce the pore-forming toxin α-hemolysin, several iron uptake systems, as well as capsules that function as a protection against the host

defense. All these traits are encoded by PAIs that are similar to each other, but not identical in the different isolates [26, 30, 32]. Interestingly, adhesin and toxin gene clusters are often linked with each other, suggesting a coevolution of these factors [33].

The LEE island encodes the outer membrane adhesion protein intimin, a type III secretion system and several secreted effector proteins. One of these secreted proteins is Tir (translocated intimin receptor) that is inserted into the eukaryotic host cell membrane where it serves as a receptor for intimin to mediate binding of the bacterium to the host cell [34]. Strains carrying the LEE locus cause characteristic attaching and effacing lesions. When LEE is transferred to *E. coli* K-12, it exhibits the same phenotype which indicates the potential of LEE to transform a nonpathogenic strain into a more virulent variant [35]. The LEE island has been identified in *E. coli* isolates of humans and many animals as well as in *Citrobacter rodentium* [36]. Similar to PAI I_{536} of UPEC strain 536, the LEE locus is located next to *selC* in some EHEC and EPEC strains (fig. 1), but can also be associated with *pheV* or *pheU* in other isolates. When LEE sequences of EHEC, EPEC and the rabbit isolate RDEC-1 were compared, it became evident that the *esc* genes encoding the secretion apparatus were highly conserved, whereas the other genes were less similar than it would have been expected from clonal lineages. This may reflect the differences in interactions with the specific host but also suggests that the LEE locus has been acquired more than once during the evolution of *E. coli* [36, 37].

Similar to pathogenic *E. coli*, PAIs have played a fundamental role in the evolution of the genus *Salmonella*. Five PAIs (SPI-1 to SPI-5) have been identified in a range of serovars of *S. enterica* and were characterized in more detail. Furthermore, additional chromosomal regions that exhibit features of GEIs have been found in the genomic sequences of serovars Typhimurium and Typhi [38, 39]. SPI-1 is regarded as a very ancient island which was already introduced into the genome of a common ancestor of *S. enterica* and *Salmonella bongori* [40]. Consequently, it has become a stable part of the chromosome and lacks most of the typical traits of PAIs. Similar to the LEE locus, SPI-1 encodes a type III secretion system including the components of the secretion apparatus, effector proteins, specific chaperones, and virulence gene regulators [41]. SPI-1 mediates invasion of host cells and induction of macrophage apoptosis [reviewed in 42]. SPI-2 (located next to *valV*) encodes a second type III system that is required for systemic infections and replication within macrophages [43]. Similarly, SPI-3 and SPI-4 have also been shown to be involved in intra-macrophage survival [44, 45]. As for PAI I_{536} and LEE, SPI-3 is associated with *selC*, but seems to have lost the corresponding integrase gene (fig. 1). The *mgtBC* operon of SPI-3 is not only required for replication in macrophages, but also for in vitro growth under low Mg^{2+} conditions. SPI-5 encodes an effector

protein (SopB) that acts as a substrate for the SPI-1-encoded secretion apparatus. This is an example of a tight connection between different PAIs of one strain [46]. Finally, the so-called SPI-7 is only present in a subset of *S. enterica* isolates including *S. enterica* serovar Typhi CT18 that produce the Vi capsular polysaccharide. The corresponding genes reside on a 134-kb island that seems to have evolved from several independent insertion events and carries a region with similarity to the pilus genes of the conjugative plasmid R46 [47].

PAIs of Other Gram-Negative Pathogens

Besides in Enterobacteriaceae, PAIs are also present in the genomes of several other gram-negative bacterial pathogens and can contribute significantly to the virulence potential of their host bacterium. In the case of *Vibrio cholerae*, two PAIs have been described [48, 49]. The first *Vibrio* pathogenicity island VPI-1 is present in all epidemic and pandemic strains, but absent in most nonpathogenic strains. The 39.5-kb island encodes a type IV pilus, the toxin-coregulated pilus (TCP) that functions as an essential intestinal colonization factor in humans and animal models [50]. Besides its role as an adhesion factor, TCP also functions as the receptor for the cholera toxin encoding filamentous phage CTXφ [51]. Therefore, acquisition of VPI-1 seems to be a prerequisite for the emergence of highly pathogenic *V. cholerae* variants. Furthermore, VPI-1 is linked to cholera toxin production because *toxT*, the gene for a transcriptional activator of the AraC family, also resides on the island. ToxT is involved in both activation of the *tcp* gene cluster and the toxin genes. A second pathogenicity island, VPI-2, has recently been found to be present in the majority of toxigenic (CTXφ-positive) strains, but absent from nontoxigenic isolates [49]. VPI-2 encodes a neuramidase and a putative metabolic pathway for amino sugars. The role of these determinants for either virulence or fitness of *V. cholerae* has yet to be elucidated. Similarly, the impact of a putative pathogenicity island in the genome of *Legionella pneumophila* serogroup 1 Philadelphia-1 (LpPI-1) that carries genes for a type IV secretion system is still unclear [52]. In contrast, the role of the *cag* island in the virulence of *H. pylori* has been intensively studied. This island is only present in *H. pylori* strains that are associated with severe forms of gastroduodenal disease (type I strains) suggesting that acquisition of this region has been an important event in the evolution of more virulent forms of *H. pylori* [19]. Like LpPI-1, the *cag* island encodes a type IV secretion system that resembles other toxin secretion systems as well as transport systems that are required for transfer of DNA. It has been shown that CagA is delivered by the island-encoded secretion apparatus into host cells where it induces cellular growth changes that are specific for infections with type I strains of *H. pylori* [53].

Finally, PAIs have also been identified in animal and plant pathogens; however, they have not been as extensively studied as in human pathogens. In *Dichelobacter nodosus*, the causative agent of foot rot in sheep, two chromosomal regions with PAI-typical features have been described [54, 55]. However, their role in virulence is still unclear.

Similar to many enterobacterial pathogens of humans and animals, several gram-negative plant pathogens also use type III secretion systems to inject effector proteins into plant cells that induce a plant tissue defense line including programmed cell death. The corresponding genes have been designated *hrp* (hypersensitivity response) or *hrc* (hypersensitivity response and conserved) and form PAI-like regions that can be located either on the chromosome or on plasmids [reviewed in 56]. This reveals common themes in virulence of human, animal or plant pathogens.

PAIs in Gram-Positive Pathogens

Chromosomal regions with the typical features of PAIs in gram-negative bacteria are less frequently found in gram-positive pathogens. However, a few regions that exhibit some of the characteristics of PAIs have also been identified in gram-positive bacteria (table 3). For example, virulence gene clusters in *Listeria* spp. or *Clostridium difficile* are not flanked by direct repeats or linked to mobility genes, but have been described as PAI-like elements [57, 58]. In *S. pneumoniae*, the characterization of an iron uptake system that is required for full virulence revealed that the corresponding genes for an ABC transporter are linked to a recombinase gene in a 27-kb region designated as PPI1 [18]. Furthermore, GEIs seem to be crucial elements for genetic exchange in staphylococci. Besides the above-mentioned methicillin resistance islands, staphylococcal pathogenicity islands (SaPIs) contribute to horizontal transmission of resistance and toxin genes in *Staphylococcus aureus* [reviewed in 59]. PAIs of *S. aureus* share many of the criteria of PAIs of enterobacteria and are mobilizable by phage transduction (see also below). SaPIs of human as well as animal isolates have been distinguished by the different toxin types they encode such as superantigen toxins (e.g. toxic shock syndrome toxin), exotoxins and enterotoxins. Finally, a large chromosomal island has recently been identified in the genome of an *Enterococcus faecalis* isolate that caused an infectious outbreak [60]. This island encompasses more than 150 kb, is flanked by direct repeat sequences and exhibits a lower G + C content than the rest of the genome. It encodes several putative virulence factors including a cytolysin and a surface protein that contributes to the colonization of the bladder. A closer investigation of *E. faecalis* isolates revealed that structural variations of the island occur with relatively high frequencies thereby enabling strains harboring this island to modulate their virulence potential.

PAIs and Genome Plasticity

The relatively high genetic flexibility of a bacterium is thought to facilitate the access to new ecological niches and may represent an advantage over organisms with less flexible genomes. As already discussed, PAIs have contributed to the long-term evolution of many bacterial pathogens, but beyond that, they may also be involved in relatively recent changes within the genetic information of an organism thereby modulating the virulence potential of a strain.

Intact or rudimentary mobility genes on PAIs give evidence that they have been acquired by means of horizontal gene transfer such as transduction or conjugation; however, so far only little is known about the actual mechanisms that have been involved. It has been assumed that integration into the recipient's chromosome, at least in some cases, was mediated by site-specific recombination similar to the integration mechanism of several bacteriophages. This is supported by the findings that some island-encoded integrases still have the potential to carry out these reactions [61, 62]. Most PAIs have undergone modifications such as deletions and mutations within the direct repeat sequences or mobility genes (fig. 2). Often, these processes have resulted in a relatively stable integration of PAIs in the bacterial chromosome which has been designated as 'homing'. Examples of PAIs that have become locked in the chromosome are some of the islands specific to *S. enterica* (also see above). This may at least partly reflect the fact that the encoded traits have become indispensable for the host bacterium and are becoming part of the core chromosome.

In contrast to such presumably very ancient elements, other islands are still mobilizable and can be transferred from one bacterium to another, at least in laboratory settings. VPI-1 has been transmitted among *V. cholerae* strains by a transducing vibriophage, CP-T1 [15] and there is also one report that VPI-1 itself may correspond to a functional prophage [63]. Furthermore, almost identical regions to VPI have been found in the chromosome of some *Vibrio mimicus* isolates, which suggests a relatively recent gene transfer between these two species [64]. General transduction also plays a role for transfer of SaPI1 and related islands in *S. aureus*. Even though these PAIs are not self-transferable, they can be propagated by staphylococcal phages such as $\phi80\alpha$ and $\phi13$ [14] in a mechanism reminiscent of the relationship between the defective coliphage P4 and its helper phage P2 [65]. Besides mobilization by transduction, conjugation may have played a role for the acquisition of PAIs and it is not unlikely that PAIs may have been derived from conjugative plasmids. In the last few years a number of elements termed 'integrating conjugative elements (ICEs)' or 'conjugative transposons' have been reported to be normally integrated in the chromosome, but can excise in a precise manner to be subsequently transferred by close cell-to-cell contact [66, 67]. Similar to PAIs, these
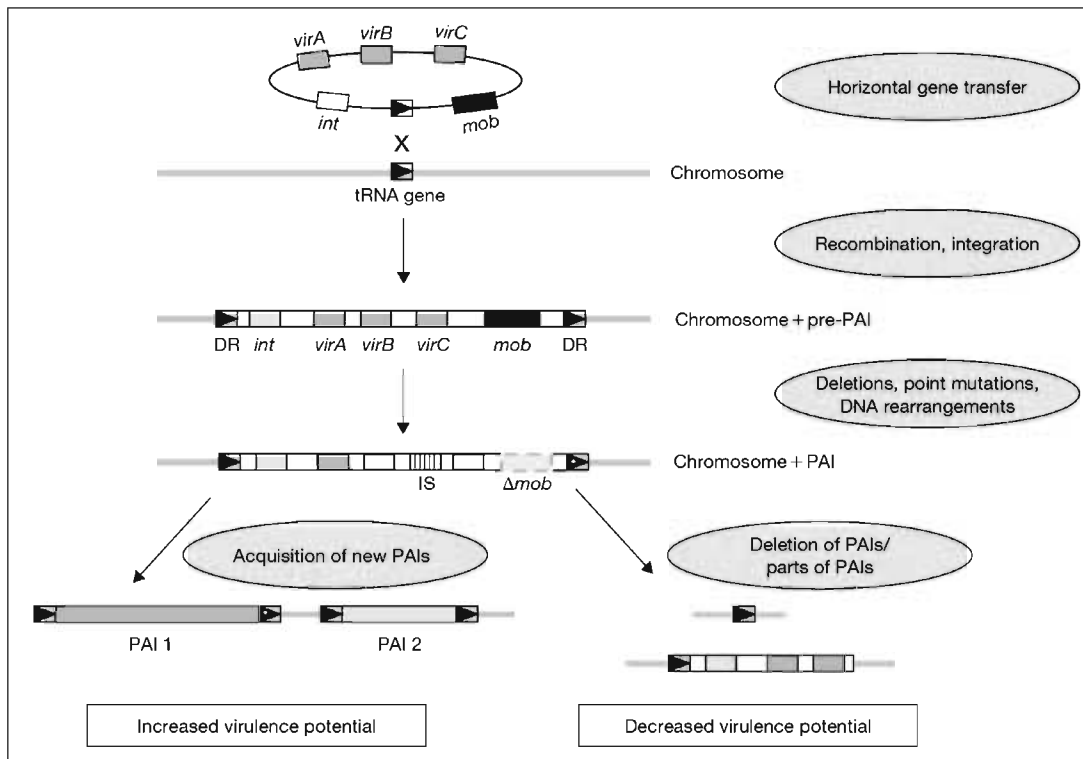
Fig. 2. PAIs and genome plasticity. Evolution of PAIs is based on acquisition of novel DNA by horizontal gene transfer followed by point mutations, recombination and deletion events that can render the PAI immobile. The virulence potential of a bacterium can subsequently be either increased by introduction of new PAIs or decreased by partly or complete deletions of PAIs. *int* = Integrase gene; *vir* = virulence-associated gene; *mob* = mobility genes; Δ*mob* = truncated mobility genes; DR = direct repeat sequences.

elements encode site-specific recombinases and lack the ability to replicate autonomously.

Finally, several islands seem no longer mobilizable, but have a tendency to delete from the chromosome either as a complete unit or in parts [12, 19, 61, 68, 69]. Precise deletion from the chromosome has been observed for PAIs of the UPEC isolate 536 and requires functional integrase genes [70] (our unpubl. results). Furthermore, an increase of deletion incidences was observed under certain environmental conditions [71]. It has been speculated that loss of virulence determinants may play a crucial role during the transition from an acute state of disease to chronic infections [8, 72]; therefore it will be interesting to further

investigate how the content of virulence genes can be modulated by environmental, bacterial or eukaryotic host factors.

Similarly, rearrangements or deletions within islands are often mediated by coresiding transposons or IS elements (fig. 2). This is especially true for *H. pylori* where an ongoing adaptation between bacterium and host based on IS*605* mediated DNA rearrangements within the *cag* island (see also PAIs of Other Gram-Negative Pathogens) has been described [reviewed in 73]. Full virulence of *H. pylori* depends on an intact *cag* island, whereas deletions within the island render the bacterium less pathogenic. The interaction of *H. pylori* with epithelial cells results in an elevated production of cytokines such as interleukin-8 (IL-8). This induction of IL-8 production correlates with the presence of a complete *cag* island, whereas *H. pylori* strains carrying only parts of the island induce IL-8 at significantly lower levels [74].

Conclusions

GEIs contribute to virulence and survival of pathogens in several ways. First, the acquisition of GEIs has been described as 'evolution in quantum leaps', because they often carry more than one virulence or fitness determinant [75]. These GEI-encoded factors enable the bacterium to colonize novel niches in the eukaryotic host and facilitate the adaptation to the respective environmental conditions. This increase of fitness gives an advantage over coresiding bacteria. Furthermore, the genome of many bacterial pathogens contains more than one GEI that encode important virulence factors, thereby determining the capability to cause disease. In addition, to ensure coordinated expression of virulence or virulence-related genes that are located on GEIs, a tight connection to regulatory networks of the bacterium has evolved, as well as a link of island-encoded regulators to genes encoded elsewhere in the genome. Finally, an ongoing mobilization and transfer of GEIs as well as reorganization, partial or complete deletion of existing GEIs affect long-term (macro-) as well as short-term (micro-) evolution of pathogenic bacteria.

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Horizontal and Vertical Gene Transfer: The Life History of Pathogens

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Viewpoints regarding the evolution of pathogenic bacteria have themselves evolved over the past two decades. Although it is perhaps extreme to suggest different teleological camps have been established, it is fair to say that opinions regarding the evolution of pathogens are varied, and the strength of different points of view have waxed and waned. Initially, many viewed pathogenic bacteria as being specialized, highly derived bacteria, which evolved complex and intimate associations with their hosts. In this way, special evolutionary mechanisms were perhaps responsible for the origin or persistence of pathogens. Gradually, a viewpoint that every microorganism was adapted to a particular niche was widely accepted, and pathogenicity represented just another bacterial lifestyle; therefore, no special evolutionary forces were at play. The evolution of well-studied pathogens could even be used as models for how other bacteria adapted to their environment.

Somewhat surprisingly, perhaps, data collected in the 'genomic era' have brought opinion back to the view that the evolution of pathogens indeed may encompass evolutionary paths typically not experienced by nonpathogenic bacteria. That is, the association of pathogens with particular hosts results in smaller effective population sizes, low genetic diversity, infrequent recombination and other factors influencing their evolution as dictated by their population genetics. As a result, pathogens would not serve as good models for the evolution of nonpathogenic bacteria that do not share these population genetic constraints. As discussed below, both viewpoints are perhaps true, when applied to the different stages of pathogen evolution. At the heart of the difference between the stages of pathogen evolution are the relative roles of gene acquisition via horizontal gene exchange versus gene loss (genome degradation). Rather than representing

different paths of pathogen creation or modification, these modes of genomic evolution likely represent a continuum or pathway along which a single lineage may travel.

Early Examples of Horizontal Gene Transfer

Horizontal gene transfer (HGT) is defined as the transfer of genetic material between bacterial cells uncoupled with cell division [1–3]. In contrast, vertical inheritance is the transmission of genetic material from mother cell to daughter cell during cell division. Most often, HGT refers to gene transfer across large phylogenetic distances (that is, between otherwise unrelated organisms), whereby genes are integrated into a replicon by illegitimate means. On occasion, HGT is used to denote allelic exchange among closely related bacterial strains where integration occurs via homologous recombination; herein that process will be referred to as 'recombination'.

Some of the earliest examples of HGT involved the transfer of antibiotic resistance genes [4], many times among pathogens, which were often facilitated by the localization of these genes on plasmids. Here, virulent strains of bacteria could acquire resistance to antibiotics at alarmingly high rates, ones inconsistent with the evolution of such a complex trait from preexisting genetic material via random point mutation. Further investigation revealed that the bacteria had obtained a gene conferring antibiotic resistance from another bacterium. This observation reinforced the idea that the strong selection imposed by the adoption of a pathogenic lifestyle allowed investigators to see otherwise rare evolutionary events, like horizontal gene exchange. As a result, this process of gene exchange was not considered to be a potent evolutionary force. A cogent model of bacterial evolution relied on the systematic periodic selection of random mutations arising in the population [5]. The exchange of genes among bacterial strains was not considered to be important until many years later [6–8], and the potential high rates of occurrence were not appreciated until rather recently [9, 10].

Pathogenicity Islands

The special role of HGT in pathogen evolution was reinforced as the sequences of bacterial genes became abundant [11]. Early analyses suggested that the genes encoding virulence functions in many pathogens were somehow different from other genes in the chromosome; differences often included changes in overall nucleotide composition (%GC), codon usage bias, association with mobile genetic elements, and association with tRNA genes (frequent sites of

bacteriophage integration). The term 'pathogenicity island' was coined to denote the distinct evolutionary histories reflected by these bits of genetic material, histories that were not shared with the remaining genes in the chromosomes [12–17]. Almost uniformly, genes encoding virulence factors mapped to pathogenicity islands, thus implicating HGT in virtually every step of pathogen evolution. Functions encoded by pathogenicity islands included those required for adhesion and invasion [12, 13], type III secretion systems for altering host cell metabolism [18–20], toxin production [21–23] and a host of metabolic capabilities including the acquisition of phosphate and iron at low concentrations [24].

Creating Pathogens by Gene Transfer

While the role of HGT in pathogen evolution was compelling, it is not the only route to pathogen creation. In some cases, pathogens are merely bacteria found in the wrong place at the wrong time. For example, *Legionella* persists in macrophages using mechanisms that evolved to allow it to passage through its more common *Entamoeba* host [25, 26]; this strategy may be common among pathogens [27, 28]. *Clostridium tetanus* is just a soil anaerobe delivered unexpectedly into the human body via a puncture wound [29]; certainly some of the more unexpected results of tetanus infection [e.g. autism-like symptoms, 30] are not considered traits resulting from strong selection for particular virulence functions. Even the well-characterized pathogen *Salmonella enterica* has been implicated in causing disease in nematodes, which may represent their primary host [31].

Yet other times, pathogens inhabit a somewhat different ecological niche than do their nonpathogenic ancestors. For example, the ancestor of *S. enterica* was likely an intestinal-dwelling bacterium which never invaded epithelial cells. Here, new physiological capabilities are required for the pathogen to succeed in its new environment, and acquisition of fully functional genes from other pathogens is an effective strategy in making this transition [32–35]. Analyses of many genomes have demonstrated that similar genes are found in diverse organisms, and that their evolutionary histories reflect frequent travel among genomes [1, 36–38]. As a whole, one may view pathogen evolution as the gain of genes via HGT coupled with the loss of genes (necessary from a population genetic point of view, as discussed below), which changes the ecological capabilities of a bacterial taxon (fig. 1).

The power of HGT in creating pathogens from nonpathogens is strikingly demonstrated in the examination of the complete genome sequences of four strains of *Escherichia coli*, including one benign laboratory strain [39], two pathogenic strains of *E. coli* [23, 40] and the phylogenetically very closely related strain *Shigella flexneri* [41] (despite being placed in a different genus, gene
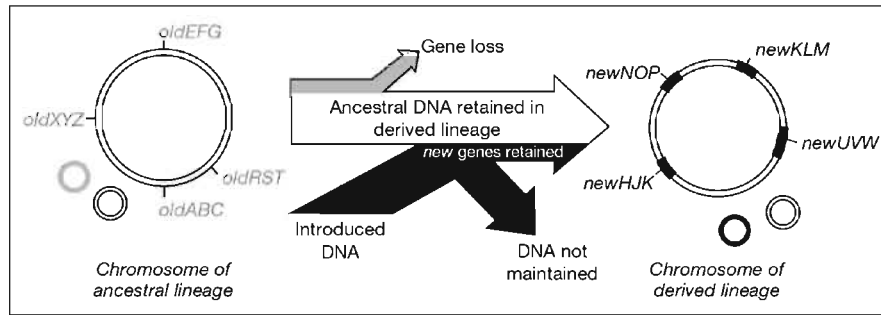
Fig. 1. The role of HGT in changing a bacterial species. Here, an ancestral taxon gains (black genes and arrows) and loses (gray genes and arrows) both chromosomal and episomal genes. Both classes of events alter the phenotypic capabilities of the bacterium, and both classes of events may increase the pathogenicity of the bacterium (see text).
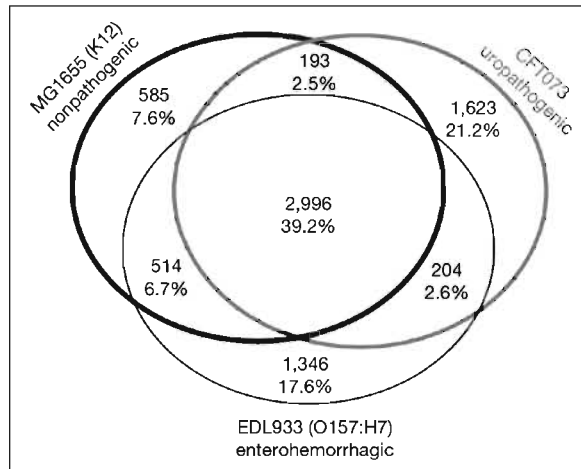
Fig. 2. Genetic differences between three completely sequenced isolates of *E. coli*: the nonpathogenic strain MG1655 (bold line), the uropathogenic strain CFT073 (gray line), and the enterohemorrhagic strain EDL933 (thin line). The number of genes shared among genomes, or unique to a genome, is shown in the appropriate location in the Venn diagram [after 40].

sequences group *Shigella* within the *E. coli* complex [42, 43]). Even though high sequence identity among genes shared among the four strains place them all in the same species [43], less than 40% of the collective gene pool among the three named strains of *E. coli* is shared (fig. 2), and nearly 47% of the genes are unique to one of the three taxa [40]. Each strain has numerous genes found only in that genome, the lion's share found in the two disparately pathogenic *E. coli* strains

(fig. 2). In contrast, the *Shigella* genome has many pseudogenes and prophages, but fewer unique genes, implicating large-scale gene transfer as a factor in its evolution to a lesser degree [41]. Indeed the role of gene loss (the *cadA* and *ompT* genes) in maintaining virulence has been noted in *Shigella* [44, 45]. Taken together, the variation among these strains shows that gene transfer can act quickly to introduce genes that allow for dramatic changes in lifestyle, but, as discussed further below, it is not the only route.

Generalized Lifestyle Alteration

Examination of variation among natural isolates of *E. coli* shows that huge dynamics in gene content are evident even among these nonpathogenic isolates [46–48]. Therefore, the lessons imparted by pathogenicity islands are extensible to the examination of other, nonpathogenic bacteria. If pathogenicity islands can allow for rapid adoption of the pathogenic lifestyle, one could posit that the introduction of other genes would allow for similarly effective invasion of nonpathogenic niches [32–34]. Examination of the genomes of numerous bacteria shows that nonpathogenic bacteria have a great deal of DNA that is 'atypical' with respect to the majority of genes in the genome and could have been introduced recently by HGT [49]. As seen in figure 3, genomes of both pathogenic and nonpathogenic bacteria show abundant signs of recent gene acquisition.

The methods employed to generate figure 3 rely on a simple premise: genes introduced into a genome from a donor chromosome all share one characteristic: they did not evolve for long periods of time in their current genomic context. Each organism experiences a unique set of 'directional' mutation pressures [50–52] which impart signature patterns of nucleotide composition [53–55], codon usage bias [56], nucleotide strand bias [57–59], dinucleotide signatures [60–62] and patterns detected by Markov chain models [63]. In effect, genes evolving in the same genome 'look alike' due to the mutational proclivities of the DNA polymerase, the composition of the nucleotide pools during replication, the nature and efficiencies of the DNA mismatch-repair systems, abundance of tRNA species and other factors. As a result, 'atypical' genes are often interpreted as having evolved in a different genomic context, their unusual features reflecting the different mutational pressures of their parental donor genome. Initially, these genes are readily detected as having unusual compositional patterns, but over time, these patterns are erased as genes evolve in their new genomic context [54, 55].

Alternatively, genes introduced by HGT can be detected since their relationship to homologues in other bacterial genomes will not be congruent with the

Pseudomonas aeruginosa
Escherichia coli
Mycobacterium tuberculosis
Bacillus halodurans
Vibrio cholerae
Bacillus subtilis
Synechocystis PCC6803
Deinococcus radiodurans
Xylella fastidiosa
Pasteurella multocida
Lactococcus lactis
Archaeoglobus fulgidus
Neisseria meningitidis Z2491
Neisseria meningitidis MC58
Halobacterium NRC-1
Thermotoga maritima
Mycobacterium leprae
Pyrococcus abyssi
Pyrococcus horikoshii
Methanobacterium thermoautotrophicum
Aeropyrum pernix
Campylobacter jejuni
Haemophilus influenzae
Helicobacter pylori 26695
Aquifex aeolicus
Thermoplasma acidophilum
Methanococcus jannaschii
Treponema pallidum
Borrelia burgdorferi
Rickettsia prowazekii
Mycoplasma pneumoniae
Ureaplasma urealyticum
Buchnera aphidicola
Mycoplasma genitalium

Typical genes | Atypical genes, with atypical phylogeny

0   1   2   3   4   5   6
Megabases of protein-coding DNA

Fig. 3. The amount of recently acquired DNA in 34 bacterial and archaeal genomes, as inferred from the identification of genes with atypical sequence features, including aberrant nucleotide composition, dinucleotide signatures and codon usage bias patterns; these atypical genes were confirmed as being horizontally transferred by performing a phylogenetic concordance test. (That is, the strongest matches to the gene in the database differed significantly from the set of strongest matches shown by other genes in the chromosome.)

relationships of other genes in the same genome. These 'phylogenetic' methods can include the direct comparison of dendrograms to identify genes with unusual branching patterns [64], or examine relationships en masse to detect genes with discordant relationships [65]. Each method has the ability to detect different sets of genes, since each relies upon different sets of criteria for the identification of potentially transferred genes [66–68]. A comparison of these 'parametric' methods of identifying genes potentially introduced by HGT with phylogenetic methods that identify genes unique to a lineage show that the two approaches are, for the most part, congruent in their predictions [68]. Therefore, it is fair to say that some lessons learned from the evolution of pathogens – that is, rapid adaptation can occur via HGT – are extensible to the evolution of nonpathogens.

Pathogens with Little Foreign DNA

Examination of figure 3 shows that many pathogens have little recently acquired DNA. This observation would seem to conflict with the conclusion that gene acquisition plays such a strong role in the evolution of pathogens. Yet there is good theoretical and empirical evidence that HGT would be of lesser importance in the evolution of virulent or host-restricted pathogens, or specialized bacterial symbionts, like those with small genomes (fig. 3). That is, one may consider pathogen evolution to be a two-step process. First, HGT allows the introduction of genes which allow adoption of the pathogenic lifestyle. Metabolic and physiological capabilities may be augmented, and pathogenicity islands will be detected in the genomes. However, as the pathogen adapts to its new role, HGT becomes both less important and less feasible, and further evolutionary change is accommodated by alteration of existing genes. As existing foreign genes ameliorate to their new genomic context [54], few genes will be detected as 'foreign' using the methods employed for generating figure 3; phylogenetic methods would still detect ancient transfer events, as evidenced by the facile detection of the transfer of the phenylalanine tRNA synthase into the ancestor of spirochetes from an archaeal donor [64].

Factors Reducing Rate of HGT in Pathogens

Three primary influences lead to the reduction in the rate of HGT into pathogen genomes. First, many symbionts and pathogens have a reduced exposure to the agents facilitating gene transfer: conjugation, transduction and transformation [69]. With a lower opportunity for exposure to foreign DNA, fewer foreign genes would be detected in the genomes of these relatively sheltered organisms. Second, fewer genes may be of utility to organisms that have adapted to a specialized environment. Here, the pathogen may have no use for the majority of foreign genes that are introduced into its genome, since few would offer functions of utility [70, 71]. Lastly, the changes in population structure coincident with specialization – lower population size and rare rates of recombination – raise the threshold for an effectively neutral mutation [70]. As a result, fewer genes, even those offering a potential benefit, would be retained; the benefits they confer would differ significantly from selective neutrality (that is, small benefits are unable to allow a gene to persist in the face of stochastic changes in gene frequency – termed random genetic drift – that dominate the fates of variant alleles at low population sizes; this effect whereby genes which would confer a benefit in a larger population cannot do so in a small population has been termed 'effective neutrality' [72, 73]), and the genes would be lost.

It is this same loss of population size that leads to genome decay in many pathogens, like *Mycobacterium leprae* [74] or *Rickettsia prowazekii* [75–77]. In these cases, the populations are insufficiently large to retain the genes present in the ancestral organism, and potentially deleterious mutations – those that eliminate gene function by producing pseudogenes – accumulate. That is, although the genes so mutated may have provided a serviceable function, the losses of the genes were insufficiently detrimental to prevent pseudogene formation; as a result, the mutations were effectively neutral, given the population size, structure and rate of recombination. The effects of such population bottle necks are evident in many pathogens just beginning this process of genome decay, including *Salmonella typhi* [78, 79] and *Mycobacterium tuberculosis* [80–83].

Correlated Genome Changes

In addition to pseudogene accumulation and the failure to retain genes introduced by HGT, the genomes of pathogens may experience other phenomena at abnormally high rates. Again, these events reflect a decreased ability for cells containing such deleterious rearrangements to be eliminated from the population, not an increased rate of their initial occurrence. For example, inversions that do not contain the origin or terminus of replication are rare [84]. However, such chromosome rearrangements are common in many bacterial genomes, including *Bordatella pertussis*, *Rickettsia*, and *Salmonella typhi* [85–87].

The increased numbers of inversions in *B. pertussis* (and, to a lesser extent, in *Bordatella parapertussis*) are thought to have resulted from an accumulation of transposable elements [88]. The IS elements provide sites of DNA identity in inverted orientation at which homologous recombination may act, thus creating an inversion [89]. Similar recombination between IS elements in direct orientation may lead to potentially large chromosome deletions, a phenomenon deduced to have occurred in the *Buchnera aphidicola* genome. A similar accumulation of IS insertions, especially of IS*1*, is seen in *Shigella* [41, 90]. In both cases, IS elements are not more prone to transpose in these genomes; rather, the strains carrying large numbers of IS elements are not removed from the population since the insertions are insufficiently detrimental.

Gene Loss during Pathogen Evolution

As noted above, several gene losses – at the *ompT* [45] and *cadA* [44] loci – were critical for the evolution of pathogenicity in *Shigella* [91]. Similarly, loss of genes – especially those involved in the production of surface antigens – was

important in the evolution of the highly virulent strains *B. pertussis* and *B. parapertussis* from the relatively broad host-range pathogen *Bordatella bronchiseptica* [88]. Common genes losses were detected [92] when comparing the smaller genomes of *M. leprae* and *Mycobacterium bovis* [92] to the larger genome of *M. tuberculosis*, suggesting that adaptation occurred via loss of function and not gain of functions by way of horizontally transferred genes; indeed no genes are found to be unique to *M. bovis* [92], unlike the situation with pathogenic *E. coli* [40].

These changes reflect more than just the inevitable loss of genes that are no longer under selection for function [93]. Rather, gene loss can be beneficial if the gene product interferes with the functions of the newly evolving pathogen, either by diverting metabolic flux along an unproductive pathway or by actively creating substances that attenuate its virulence. Alternatively, chromosomal deletions may be beneficial if the loss of DNA removes potentially problematic DNA sequences, like genetic parasites [93], or inverted DNA (as discussed above) that may interfere with chromosome replication and segregation [58, 94–97].

Gene Modification during Pathogen Evolution

Although gene gain and gene loss are effective means by which the character of a bacterial species may change, we have so far overlooked perhaps the most fundamental mode of bacterial evolution: gene alteration by mutational processes. Mutation has played a critical role in the origin or maintenance of pathogenicity in many organisms. For example, increased virulence of *B. pertussis* is due in part to an increased level of expression of the *ptxA* gene, facilitated by mutations which increased the strength of its promoter sites and binding sites for the BvgA regulatory protein [88]. Here, it was not gene gain that led to toxin production but an increased level of expression of a preexisting toxin gene.

A different kind of mutational processes, replication slippage, plays an important role in regulating the expression of antigenic loci in a stochastic fashion in both *Haemophilus influenzae* and *Neisseria* species [98–100]. Here, genes can be turned on or off at random via the addition of microsatellite repeats embedded within protein coding genes (allowing in-frame translation in only 1 of 3 slippage states), or genes may be attenuated in expression by the addition or subtraction of bases in its promoter region. Lastly, single point mutations can bring about enormous changes in virulence. *Yersinia pestis*, the causative agent of bubonic plague, is virtually indistinguishable from its parent strain *Yersinia pseudotuberculosis* [101]. This 'instant species' apparently has recently emerged by virtue of only a handful of genomic modifications [102, 103].
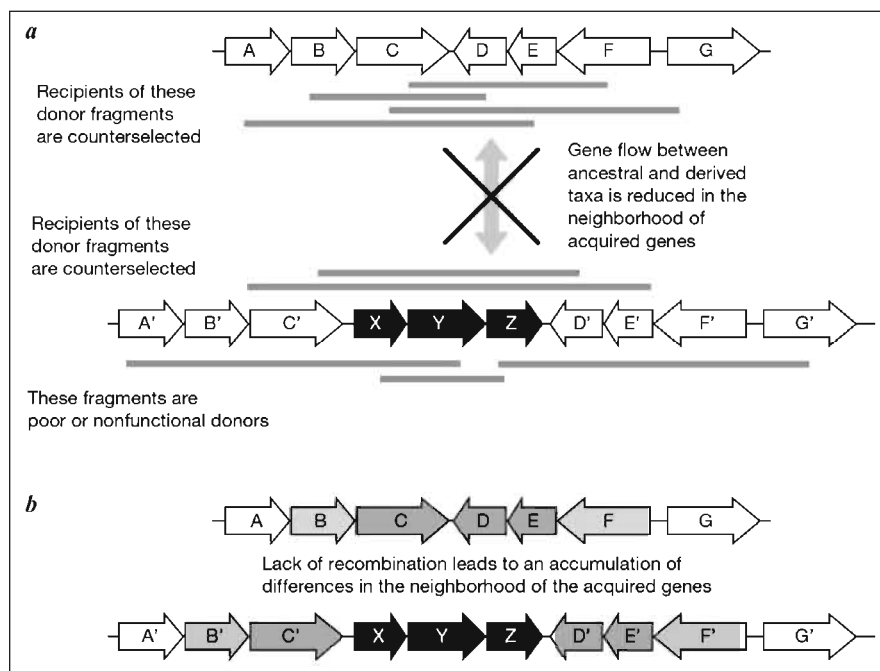
Fig. 4. The localized decrease in homologous recombination as catalyzed by an HGT event that leads to ecologically different lineages of bacteria. ***a*** After genes (depicted in black) are acquired by lateral transfer, homologous recombination in flanking genes decreases, both because recombination events leading to the addition or removal of the acquired genes are counterselected, or because fragments with ends within the acquired genes serve as poor donors. ***b*** Lower rates of recombination lead to increased sequence divergence (denoted by gray shading) at flanking genes due to their inability to participate in local periodic selection events [figure after 35; 110].

Interplay between HGT, Mutation and Recombination

Though seemingly distinct processes, genes introduced by HGT or modified in an adaptive way by mutational processes affect the process of intraspecific recombination (that is, gene exchange among closely related microorganisms) in a very particular way. While mutations and HGT events introduce potentially important genetic variation into a population, recombination among strains disseminates this genetic information among closely related strains. Among strains of a bacterial 'species' – defined as those which exchange genes at high frequency by homologous recombination [6] – strains can show dramatic differences in the environments they inhabit. This phenomenon has been shown for

Free-living, benign ancestor	*Escherichia coli*
Gain of pathogenicity islands ↓	
	Escherichia coli O157:H7
Broad host range pathogen	*Salmonella typhimurium*
	Bordatella brochiseptica
Host specialization ↓	
Virulent, narrow host range pathogen	*Salmonella typhi*
Population crash, loss of recombination ↓	
Virulent, host-dependent pathogen	*Mycobacterium tuberculosis*
	Bordatella pertussis
Massive gene loss, pseudogene accumulation, genome decay ↓	*Mycobacterium leprae*
Obligate, host-dependent pathogen	*Rickettsia*
	Mycoplasma

Fig. 5. The pathway to pathogenicity (see text). Steps in the evolution of pathogens are depicted on the left, and examples of bacteria exhibiting these properties are depicted on the right.

natural isolates of *E. coli* and other enteric bacteria [104–106], and is also evident in the strong genotypic and phenotypic differences between pathogenic and nonpathogenic strains of *E. coli* [40]. Therefore, differences that are adaptive for one strain may not be adaptive for other strains; as a result, recombination events which introduce genes into a nonadaptive strain background, or remove important genes that were not present in the donor taxa, will be counterselected [35]. In effect, the events which cause phenotypic differentiation among strains lead to genetic isolation of these strains (fig. 4); therefore, the processes of pathogenicity island acquisition may contribute to the lack of recombination among strains, therefore catalyzing subsequent genome reduction.

Genome Evolution and the Progression of Pathogenicity

As detailed above, pathogens may evolve through several distinct phases, each of which is characterized by different evolutionary mechanisms acting to shape the content and composition of their genomes. This process is outlined in figure 5. At the start, a presumably benign, free-living ancestor adopts a pathogenic lifestyle after acquiring virulence factors by HGT. Here, HGT acts as it does for many bacterial lineages in providing genetic modules for rapid and effective exploitation of a new environmental niche. At this point, populations of broad host-range pathogens may be similar to their nonpathogenic sisters in

terms of population size and structure. This stage may be typified by pathogenic strains of *E. coli*, for example.

However, host specialization (as seen in *S. typhi*, for example) leads to lower population sizes, lower rates of recombination, and eventual gene loss. *B. pertussis* shows an intermediate phenotype, whereby many genes have been lost, IS elements are accumulating, and pseudogenes are evident. *M. leprae* represents a genome in massive decay, wherein the pseudogenes almost outnumber functional genes. Eventually, this period of genome instability passes; pathogens with extremely small genomes (e.g., *Rickettsia* or *Mycoplasma*) remain as the result. In endosymbionts – which experience similar processes of genome decay [107, 108] – this period of stability can last for millions of years [109].

Conclusions

While the introduction of pathogenicity islands by HGT is considered a hallmark in the evolution of pathogenic bacteria, this process represents only one step in a multifaceted and complex evolutionary process. Some of the principles of pathogen evolution are widely applicable to the evolution of nonpathogen organisms (e.g., adaptation via the acquisition of foreign gene), while others are not.

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