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This book is dedicated to my loving parents Ian J. Johnson and Carolyn H. Johnson, who nurtured and supported my life and my career, and to my amazing children Ian, Erin, and Lauren, who have made my life worth living.

Preface

Bacterial pathogens have been purveyors of human disease and death throughout history, and their virulence factors play critical roles in the pathogen–host interactions that lead to this morbidity and mortality. Virulence factors can influence the ability of bacterial pathogens to enter human hosts, to grow and divide within different host niches, to cause host cell damage, and to evade the innate and adaptive host defense systems. Many of these factors are conserved between different genera and species, but each bacterial pathogen has its own unique "toolkit" of virulence factors that is essential for its survival and pathogenicity. A detailed knowledge of a pathogen's virulence toolkit is essential to understanding its disease-causing capabilities, and it may open up new anti-virulence therapeutic paradigms in the future that are geared toward treating pathogen-specific bacterial infections. These anti-virulence strategies would target the action of specific virulence factors and bypass the classical antibiotic routes that kill both pathogenic bacteria and beneficial human microbiota indiscriminately. Importantly, these antibody-based and small molecule-based approaches may provide critical diagnostic and therapeutic advantages in the rapidly approaching "post-antibiotic" age of increased levels of bacterial antibiotic resistance.

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About the Author

Douglas I. Johnson Received his B.S. degree in chemistry from Miami University in 1978 and his Ph.D. in biochemistry from Purdue University in 1983. His doctoral research with Dr. Ronald Somerville focused on the isolation of new mutants that affected the transcriptional regulation of amino acid biosynthesis in *E. coli*. Postdoctoral work with Dr. John Pringle at the University of Michigan centered on control of the yeast *Saccharomyces cerevisiae* cell cycle, with the initial discovery of the Cdc42 GTPase and its role in regulating the actin cytoskeleton. Dr. Johnson's research in the Department of Microbiology and Molecular Genetics at the University of Vermont extended the analysis of Cdc42 in *S. cerevisiae*, *Schizosaccharomyces pombe*, and the pathogenic yeast *Candida albicans*. In addition, anti-virulence studies with small molecule inhibitors of the budded-to-hyphal transition virulence determinant in *C. albicans* were undertaken. Dr. Johnson's primary teaching responsibilities are introductory microbiology and infectious disease and advanced clinical microbiology, which provided the impetus for writing this book.

Chapter 1
Bacterial Virulence Factors

What is a pathogen? What is a virulence factor? At one time, these were relatively straightforward questions to address. During the late nineteenth century, when Pasteur and Koch were developing the germ theory of disease, a pathogen was simply defined as a microorganism that was capable of causing disease in a host (only human hosts will be considered in this book). Pioneers in the burgeoning field of clinical microbiology showed that life-threatening diseases such as anthrax, diphtheria, tetanus, and tuberculosis were caused by single bacterial pathogens. These primary pathogens were capable of causing unique disease symptoms in a healthy host, as defined by the satisfactory fulfillment of Koch's Postulates. In addition, macromolecular factors (e.g., exotoxins, endotoxin, capsules) were shown to be responsible for inducing these specific disease symptoms. These so-called virulence factors were thought to be inherently present in pathogenic bacteria but not present in nonpathogenic bacteria.

While these simple cause-and-effect relationships were adequate early on to explain the pathogenicity of a number of major bacterial pathogens, it has become clear over the last 40 years that many infectious diseases are not due to the actions of a single primary pathogen and its virulence factors. Disease symptoms associated with the majority of bacterial infections are actually due to host tissue damage that results from either an overreaction or under-reaction of the host immune system to the pathogen. The concept that bacteria are either pathogenic or nonpathogenic ignores this essential interplay between a pathogen and its infected host and the ability of pathogens to modulate the host innate and adaptive immune responses to its advantage. Thus, the qualitative nature of pathogenicity and the quantitative measurement of virulence for a specific bacterial pathogen are only meaningful in the context of host–pathogen interactions and are not inherent qualities of pathogens versus nonpathogens (Casadevall and Pirofski 1999, 2001, 2014; Falkow 1997; Finlay and Falkow 1997; Kubori and Nagai 2016). As Casadevall and Pirofski wrote: "virulence is predicated on the variable nature and outcome of host-microbe interaction, rather than on either microbe- or host-based characteristics" (Casadevall and Pirofski 2001). This concept is no more evident than in the virulence of

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opportunistic pathogens, which are microorganisms that can cause disease in immunocompromised individuals but not in immunocompetent individuals. Not surprisingly, the appreciation of opportunistic pathogens and the central role of host–pathogen interactions in infectious disease coincided with the dramatic rise in the immunocompromised population, due in large part to chemotherapeutic immune suppression in organ transplantations and cancer treatments and in acquired immune suppression associated with HIV/AIDS. This appreciation has also led to a reevaluation of the concepts of pathogenicity, virulence, and virulence factors.

The truism that bacteria are only pathogens if they have a susceptible host, and the understanding of the importance of interplay between pathogens and host defense systems has led to a shift in the qualitative definition of pathogenicity. Instead of the ability to cause disease in a host, pathogenicity is now considered the ability to cause damage to a host (Casadevall and Pirofski 1999). Many bacterial pathogens have the ability to induce damage to host cells and tissues (i.e., changes in cellular homeostasis), but it is usually the response of the immune system to either the pathogen and/or the host damage that leads to the signs and symptoms of disease. This damage is either through a direct response to the presence of certain bacterial components termed pathogen-associated molecular patterns (PAMPs) by host pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), or through direct stimulation and/or suppression of innate and adaptive immune responses by bacterial virulence factors. This new definition of pathogenicity also allows for better insight into the immune system-dependent damage associated with opportunistic pathogens and their virulence factors.

With this reevaluation of pathogenicity also comes the need to reexamine the quantitation of virulence. Historically, virulence has been measured in many ways, but the most common was the ability of the pathogen to cause death of the infected host (i.e., an irreparable change in homeostasis). The lethal dose (number of microorganisms) required to kill half of infected hosts (LD_{50}) was a relatively simple endpoint measurement that could be used to compare the virulence of multiple pathogens against a common host. This absolute measurement was useful for primary pathogens that could induce host death, but it did not take into account alternative damage endpoints or the role of host defense systems in damage, especially those associated with opportunistic pathogens. In addition, LD_{50} measurements were subject to the genetic vagaries of the susceptible host utilized in the assay. Casadevall has recently proposed the more useful concept of pathogenic potential (PP) to quantify a pathogen's virulence (Casadevall 2017). PP takes into account (i) variable damage to the host, (ii) bacterial inoculum needed for host damage, (iii) level of mortality associated with an infection, (iv) disease communicability, (v) incubation times prior to disease symptoms, and (vi) toxicity of primary pathogens. PP promises to be an important new means to quantify the myriad of pathogenic properties that go into the measurement of a pathogen's virulence.

With revised concepts of pathogenicity and virulence, the categorization of bacterial factors that contribute to virulence has become muddied. Not only can a virulence factor be an inherent component of a pathogen that causes damage to host cells and/or tissues (e.g., exotoxins), but it also can be a molecule or structure (e.g.,

capsule, biofilm) that enables the pathogen to evade or modulate host defense systems to its replicative advantage. Included in this list of virulence factors are adherence factors that enhance the ability of a pathogen to resist host fluid flow, attach to specific target cells, and potentially invade those target cells. Invasion and survival within host cells is a special property of certain so-called intracellular pathogens (both obligate and facultative) that requires additional virulence factors not usually found in extracellular pathogens. Bacterial capsules and biofilms, which can protect pathogens from host defenses, such as phagocytosis or complement-mediated lysis, are critical to the ability of certain pathogens to disseminate from initial infection sites, often leading to life-threatening systemic diseases. Likewise, molecules that specifically modulate the expression or function of the host immune system, leading to a hyper- or hypo-immune response, would also be considered important virulence factors. Factors that enhance the metabolic capability of pathogens will not be addressed in this book except for factors that facilitate essential iron acquisition, an important virulence determinant for bacteria within the human host. Finally, transcriptomic and proteomic studies have clearly shown that the differential expression of bacterial virulence factors plays a critical role in the ability of a pathogen to induce host damage in response to both environmental and host signals.

Each of the abovementioned categories of virulence factors is discussed in this chapter, focusing on their general characteristics and functions. Subsequent chapters address specific bacterial pathogens and their associated virulence factors. Pathogens will initially be sorted by Gram-stain phenotype (Gram positive, Gram negative, no Gram stain), followed by an alphabetical sorting by genus. Within each chapter, a brief description of the pathogen's genome, morphology, speciation, and disease manifestations is presented, followed by a detailed listing of the known or hypothesized factors found within each virulence category. Non-exhaustive bibliographies accompany each chapter, allowing the reader to delve deeper into the specifics of each pathogen. The last chapter, which provided the initial impetus for writing this book, focuses on the emerging concept of devising antibacterial strategies based on counteracting specific virulence determinants, as opposed to the wholesale destruction of bacteria by antibiotics. This anti-virulence approach may help overcome the drastic worldwide rise in antibiotic-resistant pathogens by not targeting the destruction of the pathogen, which leads to reproductive selective pressure to become resistant to the antibiotic, but by blocking virulence mechanisms that may not be under selective pressure. Only by understanding the structure and function of virulence factors produced by specific pathogens can these new pathogen-specific therapeutic paradigms be envisioned and developed.

Adherence Factors (Adhesins)

Adhesins play essential roles in binding to host epithelial and endothelial cells, interactions with host mucosal layers and components of the extracellular matrix (ECM) that surround host cells, and in biofilm formation (Kline et al. 2009;

Pizarro-Cerda and Cossart 2006; Ringot-Destrez et al. 2017; Stones and Krachler 2015; Vengadesan and Narayana 2011). Intimate attachment to host cells and structures is crucial for protection from the shear forces associated with host fluid flow, especially in the gastrointestinal and urogenital tracts. These interactions are also critical for the initial colonization of host tissue and subsequent invasion of susceptible host cells. Binding can be non-specific to "sticky" host components, such as mucins, or it can be host cell specific. This specific binding is mediated through host cell receptors and is the key initial step in tissue tropism, leading to disease manifestations in different host locales. Beyond adherence, binding of adhesins to host cell receptors can also trigger intracellular signaling pathways that can lead to morphology changes, immune activation, and apoptosis. It is important to note, however, that adherence can be a double-edged sword: while binding to non-phagocytic cells can lead to advantageous colonization, binding to phagocytic cells, such as neutrophils, macrophage, and dendritic cells, can lead to phagocytosis and pathogen destruction.

Most pathogens express several different types of adhesins, but not all adhesins are expressed by all pathogenic species within a specific genus. The myriad of adhesins expressed on the bacterial cell surface makes it difficult to assign a specific virulence function to a specific adhesin in vivo. In addition, the structure and function of adhesins greatly depend on the structure of the pathogen's cell surface itself (i.e., Gram-positive vs. Gram-negative cell walls; capsule vs. non-capsule). The outer surface of Gram-positive bacteria contains a thick mesh-like peptidoglycan layer comprised of polymers of the polysaccharides *N*-acetylglucosamine and *N*-acetylmuramic acid that are extensively cross-linked via interpeptide bridges. As such, Gram-positive adhesins are usually attached directly to the outermost peptidoglycan layer (see sortases below), or if attached to the underlying cell membrane, they must be able to span the peptidoglycan layer. In contrast, the outer surface of Gram-negative bacteria is composed of a thin peptidoglycan layer that is separated by a periplasmic space from a surrounding outer membrane comprised of assorted lipids, lipoproteins, porins, and the tripartite lipopolysaccharide (LPS) layer. LPS is inserted into the outer membrane through a lipid A moiety, which is linked to polymers of complex core polysaccharides and O-antigen polysaccharides. These extended LPS structures are quite immunogenic and can be highly variable between pathogens, even within the same species. To help evade the host immune response, the LPS polysaccharides and lipid A of many pathogens can undergo phase and/or antigen variation (see Immune Evasion below). Based on these structures, Gram-negative adhesins can be LPS components themselves, or they can be attached to the outer membrane or to the underlying cell membrane and able to span the peptidoglycan layer, periplasmic space, and outer membrane. Not surprisingly, Gram-negative pathogens have evolved numerous protein secretion systems that facilitate the synthesis, transport, and assembly of adhesins across these different structural barriers (see Protein Secretion Systems below).

Certain major pathogens, such as *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Haemophilus influenzae,* contain a large capsule structure (glycocalyx, slime layer) surrounding their outer surfaces (Fig. 1.1). These self-produced

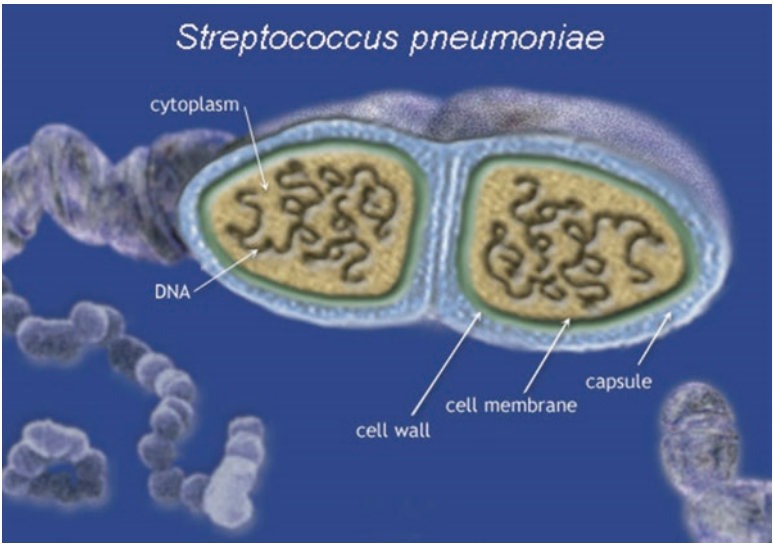


Fig. 1.1 *Streptococcus pneumoniae* capsule (Courtesy of the National Foundation for Infectious Diseases)

structures are quite variable in their lipid, polysaccharide, and protein composition. In addition to their primary function in blocking phagocytosis and complement-mediate lysis (see Immune Evasion below), capsules can act as adherence factors themselves, or they can sterically block the action of adherence factors such as LPS and adhesins.

Fimbrial Adhesins

Adhesins can be categorized structurally as either fimbrial or afimbrial. Fimbrial adhesins (fimbriae, pili) are long filamentous structures extending outward from the cell surface (Fig. 1.2). Fimbriae can have different morphologies, including the long thin P pili (pyelonephritis-associated pilus) and type I fimbriae, the bundled rope-like type IV fimbriae that are capable of retraction, and the thin aggregative curli fimbriae. The fimbrial rigid rods can contain a central channel (Fig. 1.2c–e, dark central thread), and end in a flexible, spring-like tip structure (Fig. 1.2d, e; arrow-heads) that contains the tip adhesin protein(s) needed for adherence. Gram-negative fimbriae are usually attached directly to the outer membrane or the cell membrane, whereas Gram-positive fimbriae, of which there are considerably fewer examples, are usually attached directly to the peptidoglycan layer.

Fimbriae are either homopolymeric or heteropolymeric protein structures that have complex assembly mechanisms (Fig. 1.3) (Kline et al. 2009; Pizarro-Cerda and Cossart 2006; Sauer et al. 2000). Multiple copies of the structural filament

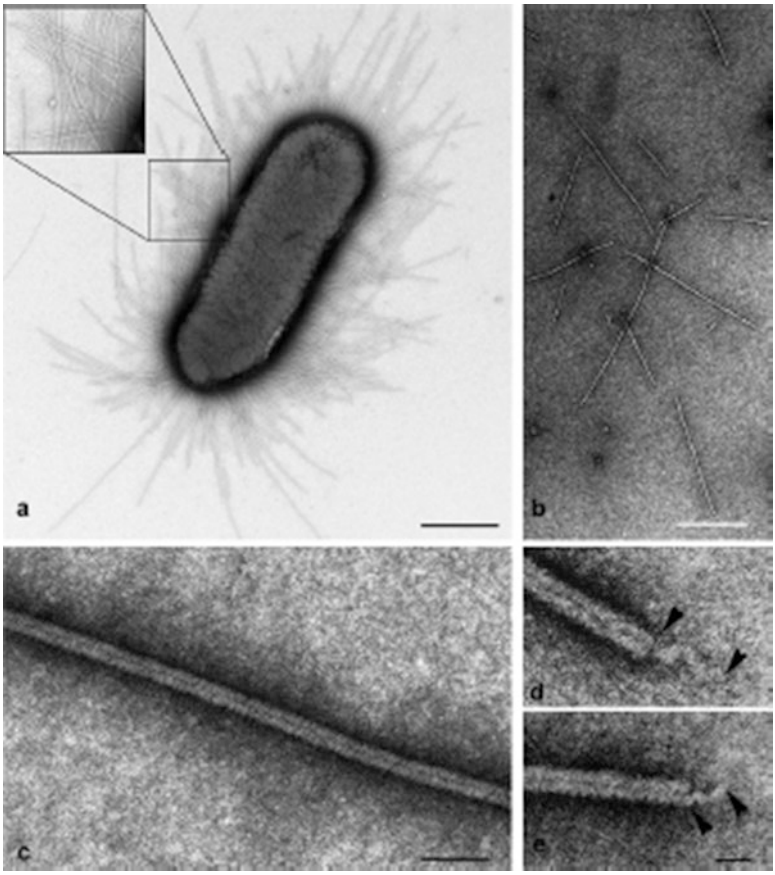


Fig. 1.2 *E. coli* P pili (From: Hahn et al. 2002)

protein (e.g., P pili PapA) are transported through the outer membrane and polymerized from their base. The adherence specificities of these fimbriae are primarily mediated by tip adhesins, which bind to different host cell surface receptors, such as the sugar moieties of glycolipids (PapG of P pili) and glycoproteins (FimH of type I fimbriae), the peptide domains within other cell surface proteins (PilC of type IV fimbriae), and mucin glycoproteins within the mucosal layer. The presence of specific receptors on host cell surfaces determines the binding and subsequent disease tropism of these fimbriae-containing pathogens. For instance, PapG binds to the α−D-galactopyranosyl-(1-4)−β−D−galactopyranoside sugar moiety present in surface glycolipids within upper urinary tract cells.

The chaperone–usher assembly pathway is used for the assembly of Gram-negative fimbriae found associated with gastrointestinal and urogenital pathogens, such as the P pili associated with uropathogenic *E. coli* (UPEC), type I fimbriae associated with UPEC and diffusely adherent *E. coli* (DAEC), and Afa/Dr adhesins associated with UPEC and DAEC (Fig. 1.3) (Busch et al. 2015; Sauer et al. 2000).

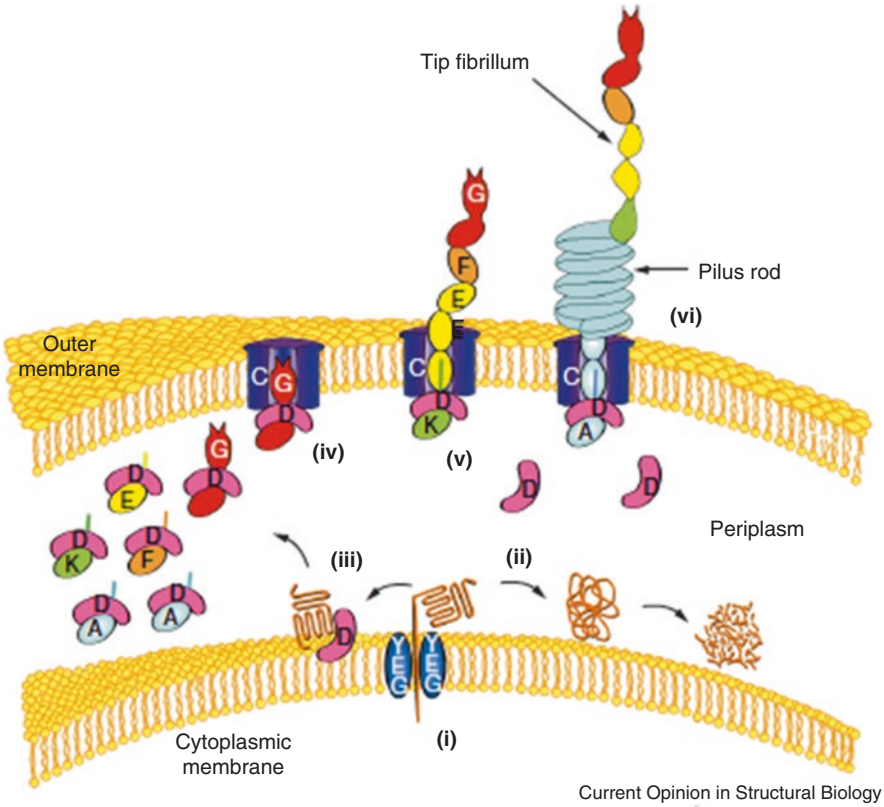


Fig. 1.3 Chaperone–usher P pili assembly (From: Sauer et al. 2000)

Since the ultimate site of fimbrial assembly is the outer membrane, chaperone proteins are needed to transport the filament and tip adhesin proteins through an usher transmembrane protein embedded in the outer membrane. Each fimbria has its own set of transport and assembly proteins (e.g., P pili Pap proteins, type I fimbriae Fim proteins; Fig. 1.3), which are usually encoded within gene clusters that are under transcriptional control. Unlike chaperone–usher fimbriae, type IV fimbriae are assembled within the inner cell membrane and extruded through the outer membrane. This assembly mechanism allows for fimbrial retraction/extension, which is important for the twitching motility exhibited by pathogens expressing type IV fimbriae as well as their adherence function (Giltner et al. 2012).

Fimbriae have been found in an increasing number of Gram-positive pathogens (Vengadesan and Narayana 2011). The paradigm for Gram-positive fimbriae is the Spa family of *C. diphtheriae*. These fimbriae are composed of only three subunits: the homopolymeric filament protein, the tip adhesin, and an accessory protein that binds to the shaft of the filament. Attachment of the fimbriae to the peptidoglycan cell wall is mediated through a sortase-based mechanism (Mazmanian et al. 1999; Ton-That and Schneewind 2003, 2004). Sortases are cysteine transpeptidases that

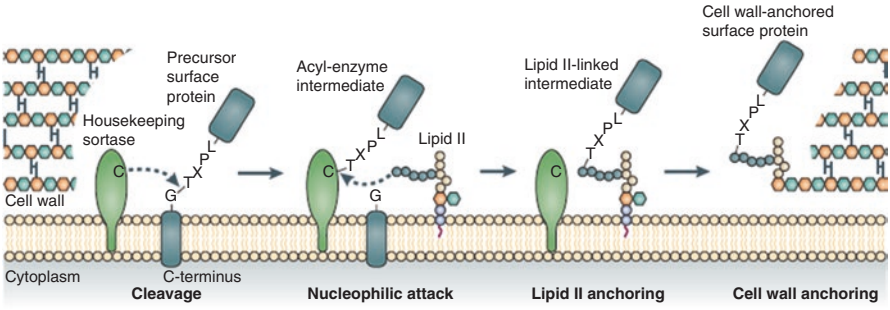


Fig. 1.4 Sortase mechanism (From: Hendrickx et al. 2011)

catalyze the covalent linkage of fimbriae, as well as other afimbrial adhesins, to the pentapeptide crossbridges found within the lipid II component of the peptidoglycan layer (Fig. 1.4) (Clancy et al. 2010; Hendrickx et al. 2011; Spirig et al. 2011). Sortases recognize proteins that have a carboxyl-terminal cell wall sorting signal (CWSS) containing the amino acids LPXTG or its variants. There are four major sortase classes with varying substrates and CWSSs: class A (SrtA in *S. aureus*) function as housekeeping sortases for many outer surface afimbrial adhesin proteins (see below), class B (SrtB) play a role in iron acquisition, class C (SrtC) are used for fimbriae attachment, and class D are utilized within spore-forming bacteria.

Afimbrial Adhesins

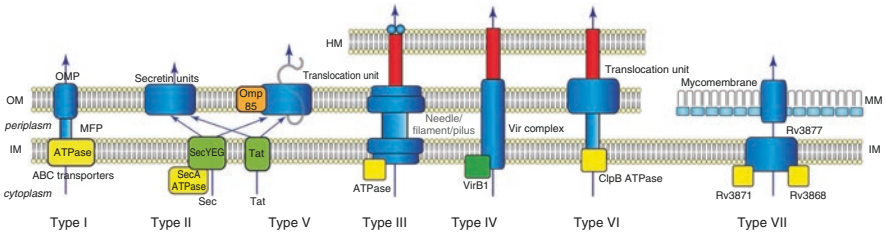
There are a plethora of afimbrial adhesins used by Gram-positive and Gram-negative pathogens. These proteins do not form filamentous structures and are attached either to the Gram-negative outer membrane or to the Gram-positive peptidoglycan layer. They usually do not extend a substantial distance away from the cell surface; hence, their binding can be sterically blocked by the presence of a capsule. Afimbrial adhesins have the differential capacity to bind to (i) specific host cell receptors, (ii) glycosylated mucins within the mucosal layer, (iii) ECM components, and/or (iv) soluble host proteins such as plasminogen, fibrinogen, and certain antibodies (IgA, IgG). Host cell receptors are usually glycosylated integral membrane proteins, including cadherins, integrins, selectins, and CD (cluster of differentiation) proteins such as CEACAMs (carcinoembryonic antigen-related cell adhesion molecules). While the mucosal layer is one of the first lines of defense against invading pathogens, some afimbrial adhesins as well as fimbriae and flagella can bind to the highly glycosylated mucin proteins within the host mucosal layer (Ringot-Destrez et al. 2017). This binding aids in the protection of the pathogen from host fluid flow stresses, but it does render the pathogen sensitive to expulsion via mucociliary escalators.

Gram-positive pathogens, such as *S. aureus* and *S. pyogenes*, utilize MSCRAMM (microbial surface components recognizing adhesive matrix molecules) proteins to bind to ECM components, such as type I and type IV collagens, elastin, fibronectin, proteoglycans, and laminin. MSCRAMMs are anchored to the peptidoglycan cell wall using a sortase-based mechanism and interact with ECM components through specific protein domains. Several MSCRAMMS can bind to multiple ECM components, such as fibronectin-binding proteins [FnBPs; (Henderson et al. 2011)], laminin-binding proteins [LnBPs; (Singh et al. 2012)], and collagen-binding proteins [CnBPs; (Singh et al. 2012)]. Fibronectin, laminin, and collagens can interact directly with host cell receptors, such as multiple integrin molecules, thereby linking the bound pathogen to the host cell through the bridging ECM component. These interactions can also stimulate various host cell processes, including actin cytoskeletal rearrangements, gene transcription, and immune responses (Finlay and Falkow 1997; Stones and Krachler 2015). *S. aureus* and other Gram-positive pathogens also express MSCRAMMs that can bind to soluble host proteins, such as fibrinogen and plasminogen, thereby affecting host coagulation pathways.

Protein Secretion Pathways

In addition to the sortase and chaperone–usher secretion mechanisms described above, Gram-positive and Gram-negative pathogens have developed additional mechanisms by which fimbrial and afimbrial adhesins can be transported and attached at the cell surface. These protein secretion pathways are also used to deliver bacterial proteins and toxins into the host environment or directly into host cells across one (inner cell membrane), two (Gram-negative outer membrane), and/or three (host membrane) hydrophobic phospholipid bilayers (Abby et al. 2016; Costa et al. 2015; Green and Mecsas 2016; Tseng et al. 2009). Both Gram-positive and Gram-negative pathogens can transport proteins into and across the inner cell membrane using either the SecYEG translocase machinery, which is part of the general secretion (SecDEFGY) pathway for unfolded proteins, or the twin arginine translocation (Tat) pathway for folded proteins. The Sec pathway is powered by ATP hydrolysis using the SecA ATPase. The translocated proteins can either remain in the inner membrane, which is mediated by a signal recognition particle (SRP)-specific signal sequence, or they can be transported into the periplasmic space or outside the cell using a SecB-specific signal sequence. However, secreted proteins in Gram-negative pathogens must transit through the outer membrane in order to reach the external environment. This added hydrophobic barrier necessitates the use of additional secretion systems.

There are six major protein secretion systems utilized by Gram-negative pathogens to bypass the outer membrane and host cell membranes (Fig. 1.5): type 1 secretion system (T1SS), type 2 secretion system (T2SS), type 3 secretion system (T3SS), type 4 secretion system (T4SS), type 5 secretion system (T5SS), and type 6 secretion system (T6SS). An additional type 7 secretion system (T7SS; ESX) has



Summary of known bacterial secretion systems. In this simplified view only the basics of each secretion system are sketched. HM: Host membrane; OM: outer membrane; IM: inner membrane; MM: mycomembrane; OMP: outer membrane protein; MFP: membrane fusion protein. ATPases and chaperones are shown in yellow.

Fig. 1.5 Protein secretion systems (From: Tseng et al. 2009)

been identified in *Mycobacterium* spp. and other Gram-positive pathogens that contain a heavily lipidated hydrophobic mycomembrane barrier beyond the peptidoglycan layer. It should be noted that not all Gram-negative pathogens express all six secretion systems and that the proteins secreted through these systems vary dramatically between genera (Abby et al. 2016).

T1SS uses ATP hydrolysis by ATP-binding cassette (ABC) transporters to passage proteins through a membrane fusion protein (MFP) linked to an outer membrane protein (OMP). This one-step process can secrete polypeptides up to 1000 amino acids and is Sec- and Tat-independent (Thomas et al. 2014). Examples of T1SS substrates include exotoxins (*B. pertussis* pertussis toxin PTx, adenylate cyclase toxin CyaA), RTX toxins [uropathogenic *E. coli* (UPEC) HlyA hemolysin, *V. cholerae* RtxA], adhesins (*S. enterica* SiiE), proteases (*P. aeruginosa* AprA), and heme-binding proteins (*P. aeruginosa* HasA).

T2SS uses a two-step process in which proteins transit the inner membrane in a Sec- or Tat-dependent process, and the secreted proteins fold in the periplasmic space prior to passage through an outer membrane secretin pore (Korotkov et al. 2012). T2SSs are structurally related to the machinery used to assemble type IV fimbriae. T2SSs are used for the transport of many exoproteins, including proteases, lipases, and phosphatases. Examples of T2SS substrates include *V. cholerae* cholera toxin, enterotoxigenic *E. coli* (ETEC) LT toxin, and the *P. aeruginosa* virulence factors ExoA (exotoxin A), PlcH (hemolytic phospholipase C), LasA (staphylolysin), LasB (pseudolysin elastase), PrpL (protease IV), AprA (aeruginolysin), ChiA (chitinase), and NanH (neuraminidase).

T3SS (Deng et al. 2017), T4SS (Kubori and Nagai 2016), and T6SS (Cianfanelli et al. 2016; Hachani et al. 2015) use one-step processes to directly inject bacterial proteins (effectors) into host cells. The assembly and function of these secretion systems involve a conserved set of proteins that create macromolecular structures spanning the bacterial inner and outer membranes and the host cell membrane. Each system secretes its own set of effectors, which vary dramatically between pathogens. Regardless, the primary function of all of these effectors is to manipulate host cell processes, including signal transduction pathways, actin cytoskeletal rearrange-

ments, intracellular vesicle transport and stability, and host immune responses. T3SS "injectisomes," which are structurally related to the flagellar apparatus, are used by many Gram-negative pathogens to deliver effectors into a wide variety of host cells. Actin dynamics, mitogen-activated protein kinase (MAPK) signaling, and nuclear factor-κB (NF-κB)-based inflammasome activation are modulated by enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), *P. aeruginosa*, and *V. cholerae* T3SS effectors. *Y. pestis* Yop effectors also affect these signaling pathways as well as facilitating intracellular persistence within macrophage. The intracellular pathogens *C. trachomatis, S. enterica*, and *S. flexneri* require T3SS effectors to invade and persist within the vacuolar system of host cells.

T4SS, which are structurally related to DNA conjugation systems, have the ability to transport DNA, DNA-protein complexes, and protein effectors across membranes. *N. gonorrhoeae* uses a T4SS to acquire virulence genes through horizontal gene transfer mechanisms. *L. pneumophila* uses the Icm/Dot T4SS to inject ~330 effector proteins that affect multiple host processes, including vesicle trafficking, autophagy, host protein synthesis, host inflammatory response, macrophage apoptosis, and host cell egress. *H. pylori* uses the Cag T4SS to insert effectors that modulate the host immune response. The contact-dependent T6SS uses a phage-tail-spike-like injectisome structure to deliver effectors not only to host cells but also to competitor bacterial species, thereby giving pathogens competitive advantages within certain host growth niches. T6SS effectors have been shown to function in adherence (*E. coli, C. jejuni, V. parahaemolyticus*), host cell invasion (*E. coli, C. jejuni, S. enterica, P. aeruginosa, Y. pseudotuberculosis*), actin dynamics (*E. coli, V. cholerae*), and host immune responses (*K. pneumoniae, V. parahaemolyticus*).

T5SS use a unique two-step process in which the substrates promote their own secretion; i.e., they contain their own β-barrel domain that forms a pore in the outer membrane with the help of the BAM β-barrel assembly machinery (Leyton et al. 2012). The first step for these so-called autotransporters utilizes the Sec-dependent machinery to translocate across the inner membrane. There are three subclasses of T5SS based on the second step of the secretion mechanisms. T5aSS substrates are single polypeptides that contain amino-terminal Sec-specific signal sequences and passenger domains and a carboxyl-terminal β-barrel domain, which forms the pore through which the passenger domain is translocated. Depending on the function of the passenger domain polypeptide, it can either be anchored in the outer membrane through the β-barrel domain (e.g., adhesins) or be secreted after cleavage from the β-barrel domain (e.g., toxins, exoenzymes). T5bSS (a.k.a., two-partner secretion; TPS) consists of two polypeptides: one polypeptide contains the β-barrel domain, and the other polypeptide is secreted. T5cSS [a.k.a., trimeric AT adhesin (TAA) system] consists of three polypeptides, which together form the β-barrel pore. Examples of T5SS effectors include adhesins (*B. pertussis* FHA, pertactin, and BapC, *E. coli* AIDA-I and Ag43, *H. influenzae* Hia, HWM1, and HWM2, *S. flexneri* IcsA, *Y. enterocolitica* YadA), proteases (*N. gonorrhoeae* and *N. meningitidis* IgA protease, *S. flexneri* SepA), and toxins (*H. pylori* VacA).

Host Cell Invasion and Growth

While entry of most pathogens into host cells results in their destruction, certain bacterial pathogens have the ability to actively invade and replicate within non-phagocytic and/or phagocytic cells. Obligate intracellular pathogens, such as *Chlamydia* spp. and *Rickettsia* spp., can only replicate inside host cells, primarily because they are metabolically crippled. Facultative intracellular pathogens, including enteroinvasive *E. coli* (EIEC)/*Shigella* spp., *Francisella* spp., *Legionella* spp., *Listeria* spp., *Mycobacterium* spp., *Neisseria* spp., *Nocardia* spp., *Salmonella* spp., and *Yersinia* spp., can replicate inside and outside host cells. The ability to invade non-phagocytic cells, such as mucosal epithelial cells or bloodstream endothelial cells, facilitates the bypass of host barriers and protects pathogens from host defense systems such as antibody-mediated opsonophagocytosis and complement-activated lysis. The ability to persist within phagocytic cells, which usually requires additional virulence determinants not found in extracellular pathogens, provides protection and also facilitates bloodstream and lymphatic dissemination of pathogens. These pathogens, which include *M. tuberculosis* and *L. pneumophila*, are passively phagocytized into macrophage using host cell machinery.

Invasion into non-phagocytic cells is usually accomplished through either a zipper mechanism or a trigger mechanism (Fig. 1.6) (Pizarro-Cerda and Cossart 2006;

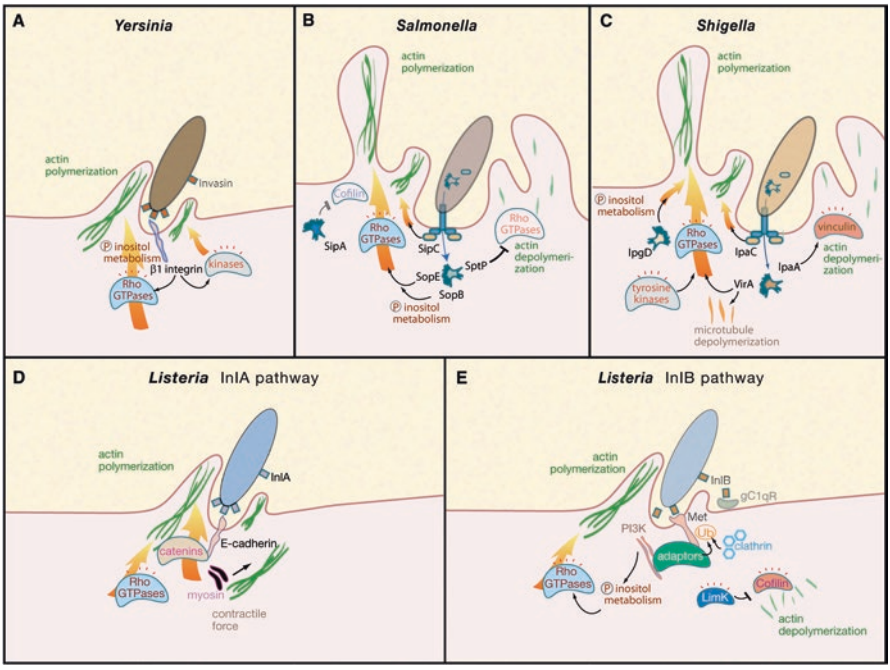


Fig. 1.6 Cell invasion mechanisms (From: Pizarro-Cerda and Cossart 2006)

Ribet and Cossart 2015). Pathogens such as *L. monocytogenes* and certain *Yersinia* species utilize a zipper mechanism. This mechanism depends on interactions between bacterial surface adhesin or invasin proteins and specific host cell receptors. Examples of these specific interactions include the *Y. enterocolitica* and *Y. pseudotuberculosis* Inv invasin protein interacting with host β_1-integrins, the *L. monocytogenes* InlA internalin protein interacting with E-cadherin, and the *L. monocytogenes* InlB protein interacting with the Met receptor tyrosine kinase hepatocyte growth factor receptor. These interactions activate Rho/Rac/Cdc42 GTPase-dependent signaling pathways that induce actin rearrangements (see Regulation of the Actin Cytoskeleton below), which lead to the host cellular membrane enveloping the pathogen. Pathogens such as *C. trachomatis,* EIEC/*Shigella, R. rickettsii*, and *S. enterica* utilize a trigger mechanism, whereas *L. pneumophila* uses a coiling phagocytosis mechanism. These mechanisms depend on bacterial effector proteins being inserted into host cells, usually via T3SSs, T4SSs, and/or T6SSs. Examples of effectors include the Sop proteins of *S. enterica*, the Ipa proteins of *S. flexneri*, and the Icm/Dot proteins of *L. pneumophila*. Some of these effectors can induce Rho/Rac/Cdc42 GTPase-dependent actin rearrangements that lead to membrane ruffling and micropinocytosis of pathogen cells.

Entry into host cells initially results in the pathogen residing within host membrane-derived vacuoles (non-phagocytic cells) or phagosomes (phagocytic cells). Intracellular pathogens can survive and multiply either by creating a replication-permissive environment within these pathogen-containing vacuoles (PCVs) or by escaping the compartment and replicating within the nutrient-rich cytoplasm (Creasey and Isberg 2014; Fredlund and Enninga 2014; Herweg et al. 2015; Poirier and Av-Gay 2015; Ribet and Cossart 2015). In either case, the pathogen must negate the inherent cellular defense mechanisms in order to replicate. *L. pneumophila*, *M. tuberculosis*, *S. enterica*, and *C. trachomatis* remain within PCVs and modify their environments to protect themselves. In order to survive in phagocytic cells, *L. pneumophila* and *M. tuberculosis* secrete effector proteins that inhibit membrane-trafficking Rab and Arf GTPases, thereby blocking the fusion of phagosomes to early endosomes and to degradative lysosomes (Stein et al. 2012). Additional effector proteins are used to modify the *Legionella*-containing vacuoles (LCV) and *Mycobacterium*-containing vacuoles (MCV) to promote nutrient acquisition and replication competence. *S. enterica* actually promotes the fusion of phagosomes to early endosomes but blocks subsequent lysosome fusion, thereby creating a unique replication niche within *Salmonella*-containing vacuoles (SCV). *L. pneumophila, M. tuberculosis*, and *Y. pestis*, but not *S. enterica*, can also block the maturation of phagolysosomes by inhibiting their vacuolar H+-ATPase-dependent acidification, which is necessary for the activation of host degradative hydrolases and the production of bacteriocidal reactive oxygen species.

L. monocytogenes, EIEC/*Shigella* spp., and *R. rickettsii* are able to escape from endomembrane compartments and replicate within the host cytosol. EIEC/*Shigella* spp. secrete numerous Ipa effectors through a T3SS that mediate membrane rupture and vacuolar escape. *L. monocytogenes* and *R. rickettsii* secrete hydrolytic enzymes to rupture the vacuolar membranes. *L. monocytogenes* produces listeriolysin O

(LLO) and phospholipases PI-PLC and PC-PLC, whereas *R. rickettsii* use the TlyC hemolysin and PLD phospholipase D. To survive within the host cytosol, intracellular pathogens must block innate cellular defense mechanisms, including nuclear factor-κB (NF-κB)-based inflammasome activation, autophagy, apoptosis, and pyroptosis (Bhavsar et al. 2007; Jorgensen et al. 2017; Lopez de Armentia et al. 2016). The host NF-κB transcription factor induces the expression of multiple defense gene products, including pro-inflammatory cytokines, such as TNF-α, IL-1, IL-6, and IL-8, and anti-apoptotic factors. The NF-κB pathway is induced following detection of pathogen-associated molecular patterns (PAMPs) by host pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs). Intracellular pathogens use multiple mechanisms to block the NF-κB pathway. For instance, *Y. pestis* and EIEC/*Shigella* spp. effectors block the ubiquitin-mediated proteolytic degradation of the NF-κB inhibitor IκB, thereby maintaining NF-κB in an inhibited state within the cytosol. In addition, certain effectors can block MAPK-dependent nuclear signaling pathways that inhibit histone phosphorylation and transcription of NF-κB-dependent transcripts (see Immune Evasion below).

Autophagy is a mechanism used to maintain host cell homeostasis and increase nutrient availability through the lysosome-dependent degradation of macromolecules. PAMP recognition by PRRs can trigger xenophagy, which is the autophagic destruction of intracellular pathogens (Gomes and Dikic 2014; Lopez de Armentia et al. 2016; Miller and Celli 2016; Pareja and Colombo 2013; Sherwood and Roy 2016; Winchell et al. 2016). Not surprisingly, numerous intracellular pathogens have developed mechanisms to avoid xenophagy. For example, *F. tularensis* O-antigen and capsular polysaccharides can block PRR detection of PAMPs, thereby blocking xenophagy activation and ubiquitin-mediated autophagosome capture. In addition, several host autophagy-related genes (ATGs) are downregulated during *F. tularensis* infection. *S. flexneri* and *L. monocytogenes* escape xenophagic recognition by blocking the binding of certain ATGs (e.g., Atg5) to bacterial proteins involved in co-opting the host actin machinery. *S. flexneri* VirG and *L. monocytogenes* ActA function as inducers of WASP/Arp2/3-dependent actin polymerization, leading to the attachment of actin tails that mediate bacterial movement within and between neighboring host cells. These proteins are also recognized by the xenophagic machinery, which can be blocked by masking the VirG and ActA recognition/binding sites by secreted bacterial effectors. The use of actin-based tails by *S. flexneri*, *L. monocytogenes*, and *R. rickettsii* for intracellular movement also plays a role in spreading and eluding host defense systems (Colonne et al. 2016; Ireton 2013; Lamason and Welch 2016). When actin-propelled pathogens encounter the host cell membrane, a pathogen-containing protrusion is generated, which is subsequently engulfed by neighboring cells. This process allows spreading of these pathogens from cell to cell without leaving the cytoplasmic environment, thereby providing protection from extracellular innate and adaptive defense systems.

Caspase-dependent programmed cell death (PCD) can have profound effects on intracellular pathogens (Friedrich et al. 2017; Jorgensen et al. 2017; Stewart and Cookson 2016). While certain pathogens induce PCD to evade host innate immune

defenses, especially those associated with phagocytic cells, apoptosis (non-lytic PCD) and pyroptosis (lytic PCD) are used by infected host cells to protect against the spread of intracellular pathogens. It is important to note that intracellular pathogens vary in their ability to induce and/or repress PCD pathways, depending on their lifestyle needs. Induction of apoptosis can either be through cell-extrinsic stimuli, such as tumor necrosis factor (TNF) proteins binding to death receptors, or through cell-intrinsic stimuli associated with DNA or mitochondrial damage that leads to the release of mitochondrial cytochrome *c* molecules. Both pathways mediate the activation of host caspase (cysteine protease) cascades that result in host cell protein degradation and subsequent apoptosis. Intracellular pathogens need to inhibit apoptosis to create replication-protected environments inside infected cells, but they also may need to induce apoptosis to promote cell egress and dissemination to deeper tissues. Bacterial effector proteins can affect apoptosis at multiple different steps. For instance, *Y. pestis*, *S. enterica*, and *C. trachomatis* secrete effectors that block the initial extrinsic signal recognition. *M. tuberculosis*, *L. pneumophila*, *S. flexneri*, and *S. enterica* can modulate the host NF−κB transcription pathway, which can have both pro-apoptotic and anti-apoptotic effects. The cytochrome *c*-dependent intrinsic pathway also can be modulated by effectors from these pathogens, primarily through the regulation of host Bcl-2 family proteins. Interestingly, *F. tularensis* and *N. gonorrhoeae* secrete effectors that block caspase activation by increasing the expression of host inhibitor of apoptosis protein (IAP) family members. Caspase-dependent inflammatory pyroptosis can be induced by bacterial molecules and structures, such as LPS, flagella, exotoxins, and components of secretion systems. Pyroptosis is associated with host cell lysis mediated by caspase cleavage of the gasdermin family of pore-forming proteins (e.g., gasdermin G). Antigenic modification of LPS and/or downregulation of flagellin synthesis are effective means for intracellular pathogens to evade pyroptosis activation.

Iron Acquisition Pathways

Arguably, the most important aspect of the intracellular (and extracellular) growth of bacterial pathogens is the ability to acquire needed nutrients. The general metabolic needs of pathogens will not be discussed in this book, but the ability to acquire iron within the human host is absolutely essential. As a biochemical cofactor and component of the energy-generating electron transport system, iron is essential for almost all life (spirochetes *Borrelia burgdorferi* and *Treponema pallidum* do not require iron as enzyme cofactors). Therefore, there is a fierce competition for iron between bacterial pathogens and host cells. Pathogens must scavenge tightly bound ferric (Fe^{+3}) iron from host transferrin, lactoferrin, and heme-containing proteins, such as hemoglobin–haptoglobin complexes and hemopexin, and then transport it across the bacterial cell wall and reduce it to ferrous (Fe^{+2}) iron to be used inside their cells.

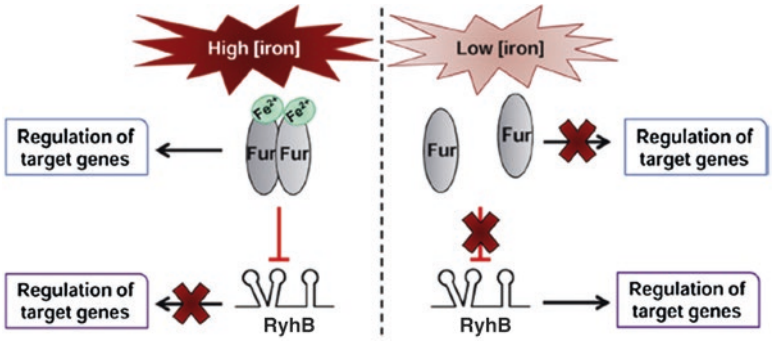


Fig. 1.7 Fe^{+2}–Fur regulation (From: Porcheron and Dozois 2015)

Not surprisingly, there are numerous mechanisms by which pathogens acquire iron in the environment and within human hosts (Sheldon et al. 2016). Many Gram-negative pathogens have the ability to directly import ferric and ferrous ions through specific FeoAB permease systems. Pathogens also can directly bind to and uptake iron-containing proteins such as heme-containing proteins, transferrin, and lactoferrin. Gram-positive pathogens, such as *S. aureus*, use Isd (iron-regulated surface determinant) and related genes to import iron across the cell wall and dissociate it from heme-containing proteins. Certain Gram-negative pathogens express specific families of receptor proteins, such as *N. gonorrhoeae* (TbpA, TbpB, LbpA, LbpB, HmbR, HpuA, HpuB) and *H. influenzae* (HgpA, HgpB, HgpC, HhuA, Tbp1, Tbp2, HitA, HxuA, HemR, Hup), that either reside in the bacterial outer membrane and directly bind to host transferrin and lactoferrin or are secreted as small hemophore proteins that bind to heme-containing proteins. These pathogens have developed intricate pathways, such as TonB-dependent transporters and ABC transporters, by which they import the iron-containing proteins across the outer membrane, dissociate the ferric iron, and reduce it to ferrous iron for use. Alternatively, both Gram-positive and Gram-negative pathogens can steal ferric iron using secreted small organic molecules called siderophores. Siderophores have varied molecular structures that can coordinate iron, but all bind to Fe^{+3} with greater affinity than host proteins. Examples of secreted siderophores include *S. enterica* (and other enteric bacteria) enterobactin and salmochelin, *Y. pestis* yersiniabactin, *S. aureus* staphyloferrins, *M. tuberculosis* mycobactin, and *P. aeruginosa* pyochelin and pyoverdine. Some pathogens not only can utilize their own siderophores but also can usurp other species' siderophores (xenosiderophores), increasing their iron fitness within hosts.

Control of iron acquisition and storage systems is tightly regulated by the concentration of iron within the bacterial cell (Porcheron and Dozois 2015). Iron concentrations can also affect the expression of other virulence factors such as exotoxins and invasins. The primary regulatory mechanism for Gram-negative and Gram-positive pathogens is through the ferric uptake regulator (Fur) protein (Fig. 1.7). High levels of intracellular Fe^{+2} can bind to Fur, which activates its binding to Fur-binding domains within target gene promoter regions, leading to the

transcriptional repression of iron acquisition genes. The Fe^{+2}-Fur complex also represses the synthesis of the small regulatory noncoding RNA RhyB, which can translationally regulate the expression of certain iron-responsive mRNAs. Low Fe^{+2} concentrations leave Fur in an unbound state, which leads to transcriptional derepression of RhyB, siderophore genes, and other iron-responsive genes. Certain Gram-positive pathogens such as *C. diphtheriae* (DtxR) and *M. tuberculosis* (IdeR) use structurally distinct but functionally conserved iron regulatory proteins (Merchant and Spatafora 2014).

Dissemination Throughout the Host

Most bacterial pathogens are blocked by innate defense barriers, such as skin, mucosal layers, and extracellular matrix, leading to localized infections within host tissue. However, some pathogens have the capability of breaching these barriers, leading to systemic infections throughout the host. These pathogens secrete exoenzymes, so-called spreading factors, that can specifically degrade components of these barriers. These exoenzymes include collagenases, elastases, hyaluronidases, mucinases, and sialidases (neuraminidases). In addition, numerous pathogens can secrete effectors and exotoxins that affect transcellular permeability between epithelial and endothelial cells.

Within the respiratory, gastrointestinal, and urogenital tracts, epithelial cells are covered by a mucous layer comprised predominantly of gel-forming mucin glycoproteins and glycolipids produced by goblet cells. Mucinases are a general class of hydrolytic enzymes that can disrupt components of the mucous layer. A major sugar component of the mucous layer and the ECM is hyaluronate (hyaluronic acid). Hyaluronidases, which include hyaluronate lyases, hyaluronoglucosaminidases, and hyaluronoglucuronidases, can be expressed in Gram-negative and Gram-positive pathogens, but only Gram-positive hyaluronidases are secreted enzymes that play a role in virulence (Gram-negative enzymes are localized in the periplasmic space) (Hynes and Walton 2000). Examples of Gram-positive hyaluronidases include *S. pyogenes* HylA, *S. aureus* HysA, and *C. perfringens* mu (μ) toxin. Mucin glycoconjugates contain high amounts of terminal sialic acid sugars, which can be cleaved by bacterial sialidases (Kim et al. 2011; Lewis and Lewis 2012). This cleavage produces free sialic acid that can be used nutritionally by pathogens and also can uncover binding sites on the outside of host cells that can be exploited by pathogens and their toxins for adherence or biofilm formation. For instance, *V. cholerae* NanH sialidase can uncover binding sites for cholera toxin, and *C. perfringens* NanH, NanI, and NanJ sialidases uncover binding sites for alpha toxin, a major contributor to gas gangrene. Other sialidase-producing pathogens, which have the ability to disseminate from their initial infection sites, include the respiratory pathogens *P. aeruginosa*, *S. pneumoniae*, and *H. influenzae*, and the gastrointestinal pathogens *B. fragilis* and *S. enterica*.

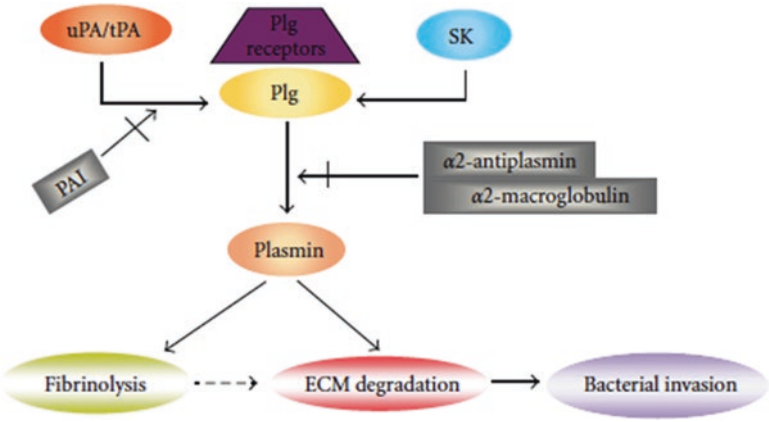


Fig. 1.8 Regulation of host plasmin (From: Bhattacharya et al. 2012)

As mentioned above (see Afimbrial Adhesins), components of the ECM can be binding partners for many bacterial adhesins. The major host function of the ECM, however, is to provide structural support for host connective tissues and to serve as a defensive barrier. To breach this barrier, disseminating pathogens must be able to disrupt interstitial and basement membrane ECM components, including collagens, elastins, fibrillins, fibronectins, and laminins. Collagens are the major glycoprotein components of connective tissues and the ~28 human collagen isoforms can form different three-dimensional structures within interstitial and basement membrane ECM (Singh et al. 2012). Certain pathogens, including *B. cereus*, *C. perfringens*, *P. aeruginosa*, *V. vulnificus*, and *V. parahaemolyticus*, secrete soluble metalloproteinases and/or serine proteases that can degrade collagen (collagenases), elastin (elastases), fibronectin, and laminin glycoproteins. In addition, numerous host matrix metalloproteinases (MMPs) and other proteases can degrade ECM components, especially under inflammatory conditions, with the most notable protease being plasmin.

The major function of host plasmin is to degrade fibrin (fibrinolysis), which controls blood coagulation and tissue repair (Fig. 1.8). Plasmin also can degrade several ECM components, including fibronectin and laminin, as well as proteolytically activate host MMPs and convert procollagenases into active collagenases. The circulating plasminogen (Plg) zymogen is converted into active plasmin protease by host tissue-type Plg activator (tPA) and urokinase Plg activator (uPA). To maintain appropriate levels of active plasmin, these PAs are regulated by the Plg activator inhibitors (PAI) PAI-1, PAI-2, α2-antiplasmin, and α2-macroglobulin. The inhibitory activity of PAI-1 and PAI-2 is turned on by binding to host vitronectin (Vn). The PAI-1/Vn complex inactivates tPA and uPA, causing a decrease in plasmin protease levels. Numerous bacterial pathogens can affect plasmin levels either by producing their own PAs, by producing Plg receptors (PlgR) that localize bound Plg to bacterial cell surfaces, or by modulating the synthesis and regulation of host PAs and PAIs (Bergmann and Hammerschmidt 2007; Bhattacharya et al. 2012; Lähteenmäki et al. 2001). The *S. aureus* staphylokinase, *S. pyogenes* streptokinase,

and *Y. pestis* Pla proteins can function as PAs on bound or free host Plg, leading to increased levels of host plasmin. *S. pyogenes*, *B. burgdorferi*, and *H. pylori* contain outer surface Plg receptors that can bind to Plg, thereby enhancing their ability to generate localized plasmin levels that can breach the ECM. *P. aeruginosa*, *S. aureus*, and *B. subtilis* can degrade host PAI-1, and the Gram-negative omptin proteins *Y. pestis* Pla, *S. enterica* PgtE, and *K. pneumoniae* Kop can degrade the PAI-1/Vn complex, leading to increased amounts of plasmin (Haiko et al. 2009; Korhonen 2015). Pla also can inhibit the α2-antiplasmin PAI, again increasing plasmin levels.

Paracellular permeability between adjacent epithelial cells (and endothelial cells) and between cells and the ECM is mediated by tight junctions (TJs), adherens junctions (AJs), and focal adhesions (FAs). TJs act as permeability barriers by closely juxtaposing adjacent cell membranes at the apical and lateral sides of polarized cells, such as intestinal epithelial cells. The interactions of host occludin and claudin transmembrane proteins with cytosolic zonulins (ZO-1, ZO-2) activate PLC/PKC (phospholipase C/protein kinase C) signaling, which regulates actin cytoskeleton reorganization. Numerous intestinal pathogens can disrupt TJs, leading to increased paracellular permeability and pathogen dissemination (Doran et al. 2013; Eichner et al. 2017). *V. cholerae* HA/P protease can cleave occludins, and Zot (zonula occludens toxin) can mimic host ZO-1 regulation of actin cytoskeleton reorganization. The EHEC and EPEC effectors EspF, EspG, and Map affect occludin and ZO-1 function, as do *C. difficile* toxin A and toxin B. In addition, *H. pylori* CagA affects multiple host signaling pathways that impact TJ permeability.

AJs form basally to TJs and are needed for TJ stability. AJ transmembrane cadherin proteins connect adjacent cells and interface with cytosolic catenins and the host actin cytoskeletal network through a submembrane plaque complex. FAs transmembrane integrin proteins are connected to components of the ECM, most notably fibronectin, as well as the actin cytoskeleton. Within the AJ and TA submembrane plaque complexes, numerous cell-specific anchor proteins interface with signaling Rho/Rac/Cdc42 family GTPases and actin-associated proteins, thereby linking these structures to the host cell actin cytoskeleton. Therefore, disrupting Rho/Rac/Cdc42 GTPase signaling or other plaque–actin complexes would result in changes in cell morphologies (rounding up) and increases in paracellular permeability. Not surprisingly, many pathogens secrete exotoxins that can modulate Rho/Rac/Cdc42 GTPases and the host actin cytoskeleton (see Toxins below). In addition, *L. monocytogenes* InlA protein interacts with host E-cadherin, facilitating *L. monocytogenes* host cell invasion and bypass of the intestinal barrier.

Damage to Host Cells

Many bacterial pathogens secrete polypeptides that can have deleterious effects on host cell structures and processes. These so-called exotoxins, which are the most potent virulence factors studied to date, can affect host cells at the initial site of bacterial infection or can affect cells at distal sites within the host. The extracellular

nature of these toxins allows them to cause damage and disease independent of the producing pathogen actually growing within the host. Exotoxins either can act at host cell surfaces by disrupting cell membrane structures or can act on intracellular targets to block essential cell processes, such as translation, signal transduction, intracellular trafficking, and actin cytoskeletal rearrangements. Each intracellular exotoxin has its own unique method of entering host cells, usually through receptor-mediated endocytosis or bacterial secretion systems (i.e., T3SS, T4SS, T6SS). Most exotoxins are single polypeptides with specific catalytic activities, although some exotoxins can form multi-polypeptide complexes, such as A-B exotoxins, that aid in cell entry and/or function.

Cytolytic Exotoxins

These cytolytic polypeptides can disrupt host cell membranes, causing defects in ion homeostasis that eventually lead to influx of H_2O, cell swelling, and lysis. Included in this broad category of exotoxins are pore-forming toxins (PFTs) and membranolytic toxins. PFTs are classified as either α-PFTs or β-PFTs, depending on whether their membrane-spanning pore-forming domains are composed of α-helices or β-barrels (Peraro and van der Goot 2016). Each has its own specific pore-forming mechanism and permeability limits, such that some only affect potassium or calcium ion homeostasis, while others allow large molecules or polypeptides to egress. Most of these toxins have specific host cell receptors (e.g., cholesterol, glycolipids, GPI-anchored proteins) that play a role in dictating disease tropisms. Examples of α-PFTs include *E. coli* colicins, *S. enterica* cytolysin A (ClyA/HylE), and *B. cereus* Nhe toxin and hemolysin BL. Examples of β-PFTs include *S. aureus* leukocidins, α-hemolysin, and γ-hemolysin, *C. perfringens* α-, δ- and ε-hemolysins and CPE enterotoxin, and the cholesterol-dependent cytolysins (CDCs) *C. perfringens* perfringolysin, *S. pneumoniae* pneumolysin, *S. pyogenes* streptolysin O, and *L. monocytogenes* listeriolysin O (disrupts phagosome membranes). Membranolytic exotoxins include phospholipases and sphingomyelinases, which hydrolyze glycerophospholipids and sphingophospholipids (sphingomyelin), respectively (Flores-Díaz et al. 2016). This hydrolysis disrupts phospholipid bilayers in host cell membranes and intracellular organellar membranes. Examples of membranolytic exotoxins include *C. perfringens* α-toxin, the major effector of gas gangrene, *S. aureus* β-toxin, *P. aeruginosa* PlcH and ExoU, and *L. monocytogenes* PI-PLC (disrupts phagosome membranes).

Intracellular Exotoxins

Unlike cytolytic toxins that have direct access to their host targets, intracellular exotoxins must enter host cells to exert their effects on targets or processes. Many of these exotoxins are composed of multiple polypeptide subunits (so-called A-B

exotoxins) that have catalytic activity (A subunits) and cell binding/entry activity (B subunits). B subunits, which can function with single polypeptide stoichiometry (i.e., A-B) or multiple polypeptide stoichiometry (i.e., A-B_5), recognize specific host cell receptors that are usually either transmembrane proteins or glycolipids. The A subunit alone or the A-B subunits together then enter host cells through endocytosis. Inside endocytic vacuoles, A-B subunits dissociate, and the released catalytic A subunit traffics to its intracellular location. Many of these exotoxins are encoded on mobile genetic elements, such as plasmids (*B. anthracis* anthrax toxin, *C. tetani* tetanus toxin) and bacteriophage (*C. botulinum* botulinum toxin, *V. cholerae* cholera toxin, *C. diphtheriae* diphtheria toxin), raising the possibility of horizontal gene transfer of these toxin genes.

The varied catalytic activities of A subunits, most of which affect the posttranslational modification of host polypeptides, dictate the type of cellular damage and disease symptoms observed. The predominant catalytic activity observed is ADP-ribosylation of host proteins. These so-called bacterial ADP-ribosyltransferase toxins (bARTTs) attach an ADP-ribose moiety derived from NAD to specific Arg, Asn, Thr, Cys, or Gln amino acids within target proteins, thereby inactivating the target protein (Table 1.1) (Simon et al. 2014). bARTTs can be classified either by their subunit stoichiometry or by their intracellular target. They can either be single polypeptide A-B toxins (e.g., *C. diphtheriae* diphtheria toxin, *P. aeruginosa* exotoxin A) or multiple polypeptide A-B_5 toxins (e.g., *V. cholerae* cholera toxin, *B. pertussis* pertussis toxin). Intracellular targets include translation elongation factor 2 (eEF2) (e.g., diphtheria toxin, exotoxin A), heterotrimeric Gα subunits (e.g., cholera toxin, pertussis toxin), G-actin monomers (e.g., *C. botulinum* C2 toxin, *C. perfringens* iota toxin, *C. difficile* CDT), and Rho/Rac/Cdc42 GTPases (e.g., *C. botulinum* C3bot, *B. cereus* C3cer). Other A subunit catalytic activities include Zn-dependent proteases that block intracellular vesicle trafficking of neurotransmitters (e.g., *C. botulinum* botulinum toxin, *C. tetani* tetanus toxin), RNA *N*-glycosidases that remove an adenine residue from the 28S rRNA of the 60S ribosome (e.g., *S. dysenteriae* Shiga toxin, EHEC Shiga-like Stx-1, Stx-2 toxins), calmodulin-dependent adenylate cyclase (e.g., *B. anthracis* edema factor), and glucosylation of Rho/Rac/Cdc42 GTPases (e.g., *C. difficile* TcdA and TcdB) and eEF1A (e.g., *L. pneumophila* Lgt1, 2, 3) (Jank et al. 2015).

Regulation of the Actin Cytoskeleton

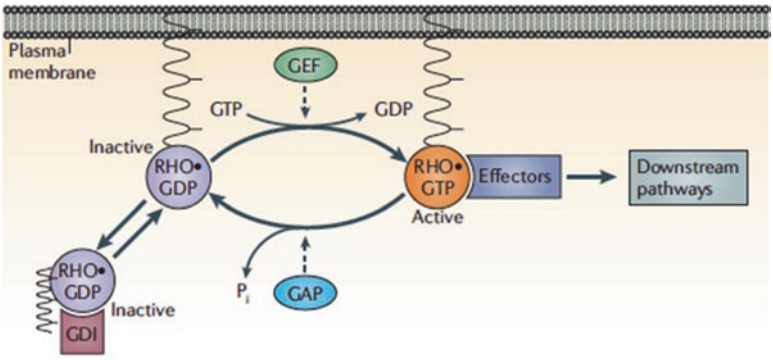
As mentioned above, the actin cytoskeletal network plays a central role in regulating host cell morphology, signal transduction pathways, and cell–cell junctions. Therefore, it is not surprising that many bacterial exotoxins and secreted effector proteins can affect proteins involved in regulating actin polymerization, thereby enhancing cell invasion, dissemination, and immune evasion. Some exotoxins can directly modify the structural G-actin monomers (e.g., *C. botulinum* C2 toxin, *C. perfringens* iota toxin, *C. difficile* CDT, *V. cholerae* RTX toxin), thereby blocking or

Table 1.1 bARTT subfamilies (Adapted from: Simon et al. 2014)

Toxin	Bacterium	Eukaryotic substrate	Cellular receptor or delivery
DT-like toxins			
DT	*Corynebacterium diphtheriae*	eEF2	HB-EGF
PE	*Pseudomonas aeruginosa*	eEF2	LRP1
ChxA[a]	*Vibrio cholerae*	eEF2	LRP1
CT-like toxins			
CT	*V. cholerae*	$G\alpha_s$	Ganglioside: GM1
Heat-labile enterotoxin	*Escherichia coli* (ETEC)	$G\alpha_s$	Ganglioside: various
PT	*Bordetella pertussis*	$G\alpha_i$	Ganglioside: various
C2-like binary toxins			
C2 toxin	*Clostridium botulinum*	G-actin	*N*-linked carbohydrates
Iota toxin	*Clostridium perfringens*	G-actin	LSR
CDT	*Clostridium difficile*	G-actin	LSR
CST	*Clostridium spiroforme*	G-actin	LSR
VIP	*Bacillus cereus*	G-actin	Unknown
SpvB[a]	*Salmonella* spp.	G-actin	Type-III secreted
AexT[a]	*Aeromonas hydrophila*	G-actin	Type-III secreted
Photox[a]	*Photorhabdus luminescens*	G-actin	Type-VI secreted (putative)
C3-like toxins			
C3bot	*C. botulinum*	RHOA, RHOB and RHOC	NA
C3Stau/EDIN	*Staphylococcus aureus*	RHOA, RHOB, RHOC and RHOE	NA
C3cer	*B. cereus*	RHOA, RHOB and RHOC	NA
ExoS	*P. aeruginosa*	RAS, ERM proteins and vimentin	Type-III secreted
ExoT	*P. aeruginosa*	Crkl and Crkll	Type-III secreted
HopU1[a]	*Pseudomonas syringae*	GRP7	Type-III secreted
SpyA[a]	*Streptococcus pyogenes*	Vimentin and actin	NA
Novel toxins			
TT[a]	*Salmonella enterica* subsp. *enterica* serovar Typhi	Unknown	Ganglioside: various, GD2 preferred
PTC3[a]	*P. luminescens*	G-actin	Secreted
PTC5[a]	*P. luminescens*	RHOA, RHOB and RHOC	Secreted

AexT A. hydrophila exoenzyme T, *bARTT* bacterial ADP-ribosyltransferase toxin, *CDT C. difficile* transferase, *ChxA* cholix toxin, *Crk* CT10 regulator of kinase, *CST C. spiroforme* toxin, *CT* cholera toxin, *DT* diphtheria toxin, *eEF2* eukaryotic elongation factor 2, *ERM* ezrin—radixin—moesi, *ETEC* enterotoxigenic, *Exo* exoenxyme, *GRP7* glycine-rich RNA-binding protein 7, *HB-EGF* heparin-binding epidermal growth factor, *LRP1* low-density lipoprotein receptor-related protein 1, *LSR* lipolysis-stimulated lipoprotein receptor, *NA* not applicable, *PE Pseudomonas aeruginosa* exotoxin A, *photox Photorhabdus* toxin, *PT* pertussis toxin, *VIP* vegetative insecticidal protein

[a]Toxins that were initially identified using bioinformatic approaches



Regulation of the RHO-family GTPase cycle. RHO-family proteins are inactive when bound to GDP and active when bound to GTP. Guanine nucleotide dissociation inhibitors (GDIs) prevent nucleotide exchange and keep the GTPases in the cytosol. Guanine nucleotide exchange factors (GEFs) activate RHO-family GTPases by inducing GDP–GTP exchange. In the active, GTP-bound form, RHO-family proteins interact with and regulate the activity of endogenous effectors. GTPase-activating proteins (GAPs) inactivate RHO-family proteins by facilitating GTP hydrolysis. RHO-family proteins are isoprenylated, which allows membrane binding. Figure is modified from REF. 17 © (2005) Macmillan Publishers Ltd. All rights reserved.

Fig. 1.9 Regulation of Rho/Rac/Cdc42 GTPases (From: Aktories 2011)

enhancing actin polymerization (Aktories et al. 2011). Others can alter signaling pathways that control actin polymerization. The primary signaling proteins that regulate actin polymerization are the Rho/Rac/Cdc42 family of small GTPases. These GTPases and their cognate regulatory proteins, which include guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs), are targets of numerous exotoxins and secreted effectors (Fig. 1.9) (Aktories 2011; Popoff 2014). For instance, *C. botulinum* C3bot toxin and *B. cereus* C3cer toxin ADP-ribosylate Rho GTPases, leading to their inactivation and subsequent actin depolymerization. Multiple *Clostridium* spp. toxins can glucosylate Rho, Rac, and Cdc42, including *C. difficile* TcdA and TcdB and *C. sordellii* TcsL, again leading to actin depolymerization. *B. pertussis* dermonecrotic toxin (DNT) can deamidate key Gln residues involved in GTPase activity, leading to enhanced actin polymerization, which also has detrimental effects inside the host cell. Many enteric pathogens, including *S. enterica*, *E. coli*, and *Y. pestis*, can inject effector proteins through T3SSs into host cells that mimic the action of Rho/Rac/Cdc42 regulatory proteins. For instance, *S. enterica* SopE and SopE2, EIEC/*Shigella* IpgB1, and *E. coli* EspT mimic Rho/Rac/Cdc42 GEFs, activating the GTPase to a GTP-bound state, which triggers the actin polymerization needed for membrane ruffling and cell invasion (see Host cell invasion above). Rho GAPs also can be targets of inhibitory effectors, including *Y. pestis* YopE and *P. aeruginosa* ExoS and ExoT, which block GTPase activity and lead to hyperactive GTPases and increased actin polymerization.

Endotoxin and Superantigen Hyperstimulation of Host Immune System

Although the abovementioned exotoxins and secreted effectors can elicit substantial deleterious effects on host cells, the most egregious cell damage is done by the host immune system itself. This damage is predominantly the consequence of inappropriate activation of inflammatory responses, either by Gram-negative endotoxins or by specific effectors that induce the synthesis of pro-inflammatory cytokines such as TNF−α, IL-1, IL-6, and IL-8. Endotoxin, which is only produced by Gram-negative pathogens, is composed of the outer membrane LPS that is released when Gram-negative bacteria are lysed (antibiotic treatment of certain Gram-negative infections is contraindicated due to the increased release of endotoxin from lysed cells). Unlike exotoxins, the effects of endotoxin are indirect, causing inappropriate activation or overstimulation of inflammatory responses that damage host cells. The lipid A moiety, which is the toxic component of the LPS, mediates the binding of LPS to host LPS-binding protein (LBP), and the LPS-LBP complex binds to CD14 and TLR4 proteins on macrophage cell surfaces (Schumann 2011). This binding can lead to the increased synthesis and release of large amounts of pro-inflammatory cytokines, which can lead to inappropriate activation of the host complement cascade and coagulation pathways. This hyperstimulation dramatically increases the levels of inflammation, which can lead to the potentially fatal effects of septic shock, hypotension, endothelial and epithelial cell damage, and organ failure.

Pyrogenic toxin superantigens (PTSAgs) are a family of potent immunostimulatory exotoxins that are predominantly produced by the Gram-positive pathogens *S. aureus* and *S. pyogenes* (Spaulding et al. 2013). There are ~19 *S. aureus* and ~11 *S. pyogenes* PTSAgs, including *S. aureus* toxic shock syndrome toxin (TSST), *S. aureus* emetic enterotoxins (SEs; major causes of food poisoning), and *S. pyogenes* toxic shock syndrome toxin. PTSAgs induce non-specific, massive T-cell proliferation (up to 20 % of all T cells) and "trick" T cells into releasing enormous amounts of pro-inflammatory cytokines, resulting in a "cytokine storm" (Tisoncik et al. 2012). This cytokine storm is responsible for severe life-threatening symptoms, including cell damage, capillary leakage, hypotension, shock, respiratory distress, and multi-organ failure.

Evasion of the Host Immune System

Bacterial pathogens can use many mechanisms to evade host innate and adaptive immune defenses (Finlay and McFadden 2006). Key processes within the innate defenses include phagocytosis, inflammation, and complement-mediated lysis. As mentioned above, intracellular pathogens can evade these defenses by invading and growing within phagocytic and non-phagocytic cells, which also can enhance their ability to disseminate throughout the host, often within phagocytic cells. The

expression of a polysaccharide capsule is a major mechanism by which certain extracellular pathogens can block phagocytosis (Gasparini et al. 2015; Hyams et al. 2010). Bacterial capsules are usually composed of proteins and sugars that are normally found in host cells, such as poly-D-glutamic acid (*B. anthracis*) or hyaluronic acid (*S. pyogenes*), so they are not recognized as foreign by macrophage and neutrophils. In addition, the thick capsular structures associated with *N. meningitidis*, *H. influenzae*, and *S. pneumoniae* can sterically block antibody binding to cognate cell surface antigens and/or binding of antibody F_c domains to F_c receptors on phagocytic cells, leading to reductions in opsonophagocytosis and complement-mediated lysis (see below). *S. aureus* can block phagocytosis by co-opting components of the host coagulation system to form blood clots surrounding the cell. *S. aureus* expresses both secreted and cell wall-bound coagulase enzymes that activate prothrombin to thrombin, which proteolytically converts fibrinogen into fibrin blood clots (Peetermans et al. 2015).

Another steric hindrance to phagocytosis is pathogen growth within biofilms. Biofilms are communities of microbial cells that are attached to abiotic and/or biotic surfaces through a self-produced matrix of extracellular polymeric substances (EPS) containing sugars, proteins, and DNA. Bacterial fimbriae, especially curli fimbriae (see above), are key components in the attachment of bacterial cells to biofilm surfaces. Biofilms provide a protective environment for bacteria by shielding them from antibodies and phagocytic cells and by inducing high levels of resistant to antibiotics (Moser et al. 2017; Peters et al. 2012; Tande and Patel 2014). The presence of these biofilms not only provides a sheltered environment for the growth of bacterial pathogens such as *S. aureus*, *E. coli*, *P. aeruginosa*, and *S. enterica* but also enhances the seeding of these pathogens to disseminated sites within the host, leading to life-threatening systemic infections (Fig. 1.10) (Lebeaux et al. 2013). Localized biofilm infections within the human host are most often associated with the presence of indwelling medical devices such as catheters, IV tubes, ventilator tubes, implants, and prosthetic devices. These infections, many of which are recalcitrant to antibiotic treatment, affect ~2 million hospitalized patients each year in the USA, leading to ~100,000 deaths and ~$28–36 billion in extra costs.

Innate immune responses are triggered by the recognition of bacterial surface components, such as peptidoglycan, LPS, lipoproteins, fimbriae, and flagellin, by host immune cells. These components, termed pathogen-associated molecular patterns (PAMPs), are recognized by pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), located on the surface of host immune cells. This recognition leads to the activation of MAPK and NFκB signaling pathways that induce the expression of pro-inflammatory cytokines and chemokines. Host inflammatory responses are one of the most potent aspects of innate immunity, so it is not surprising that extracellular pathogens have developed multiple strategies to block these signaling pathways (Luo et al. 2015; McGuire and Arthur 2015; Rahman and McFadden 2006). For instance, *S. enterica* TlpA, *S. aureus* TirS, and *Y. pestis* YpTIR interfere with TLR signaling to downstream pathways. *B. anthracis* Lethal Factor (LF) toxin can proteolytically inactivate MAPKs, while *C. trachomatis* CPAF toxin can cleave NFκB, thereby blocking those signaling pathways.

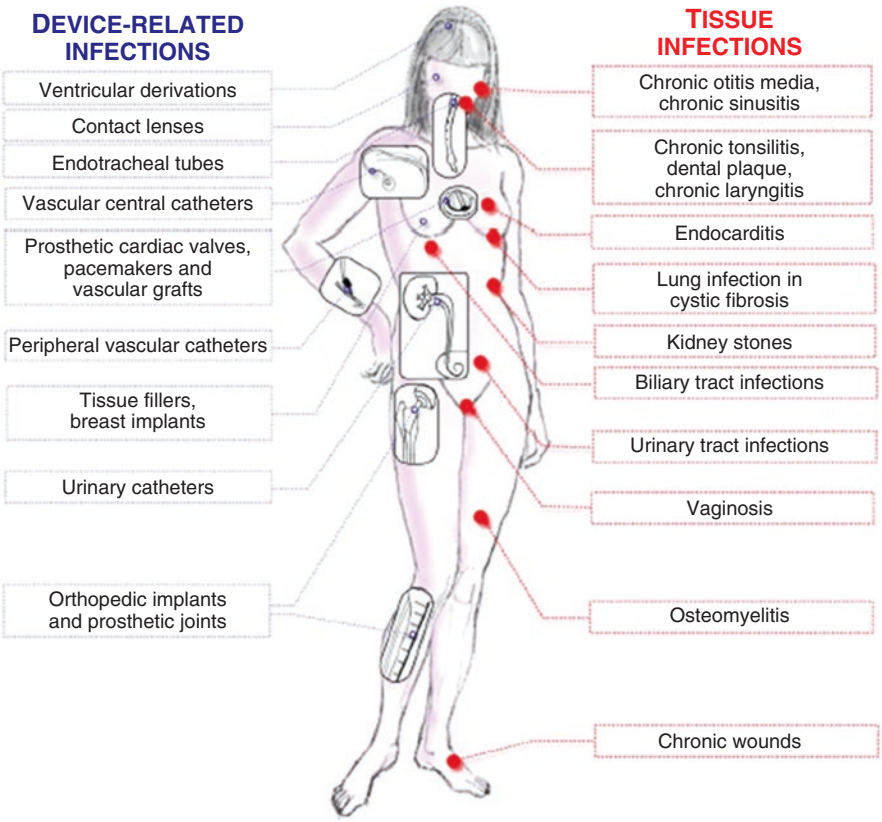


Fig. 1.10 Biofilms and human disease (From: Lebeaux et al. 2013)

Acetyltransferases from *V. parahaemolyticus* (VopA), *S. enterica* (AvrA), *Y. pestis* (YopJ/P), *and M. tuberculosis* (Eis) can modify MAPKs, blocking their phosphorylation-dependent activation. *S. flexneri* OspG, EPEC/EHEC NleH1 and NleH2, *S. enterica* SseL, *C. trachomatis* ChlsDub1, and *Y. pestis* YopJ/P can inhibit ubiquitin-mediated degradation of the host IκBα regulatory protein, which blocks the release of functional NFκB for transit into the nucleus.

Complement-mediated lysis is a major defense mechanism against bacterial pathogens, especially Gram-negative pathogens. Complement is a proteolytic cascade involving more than 30 different complement (C) proteins residing within the host circulatory system. Recognition of invading bacterial pathogens by one of the three complement pathways (classical, lectin, or alternative pathways) leads either to opsonization of the pathogen, enhancing their F_c-mediated phagocytosis by host phagocytes, or to direct lysis of the pathogen through the generation of membrane attack complex (MAC) pores in the bacterial membranes. Therefore, the ability of bacterial pathogens to block the activation of complement is considered a major virulence factor (Hallstrom and Riesbeck 2010; Pizza and Rappuoli 2015;

Rooijakkers and van Strijp 2007; Singh et al. 2010). Several pathogens have the ability to block F_c-mediated phagocytosis. For instance, *S. aureus* protein A and *S. pyogenes* SibA bind to the $F_c\gamma$ portion of IgG, thereby blocking opsonophagocytosis. *S. pyogenes* EndoS (endo-β-*N*-acetylglucosaminidase) removes carbohydrates from IgG, thereby blocking the binding to F_c receptors. *S. pyogenes* SpeB and IdeS are cysteine proteases that proteolytically inactivate IgG and other antibody molecules. Other factors inhibit the function of the C proteins directly. *S. aureus* SCIN proteins inhibit complement factor C3 convertase, which prevents the production of C3a, C3b, and C5a polypeptides, thereby blocking complement activation. Also, the *S. pyogenes* C5a peptidase ScpA proteolytically inactivates C5a.

Many pathogens display serum resistance, which is correlated to the ability to block complement-mediated lysis by co-opting host regulatory factors such as factor H (FH), factor H-like-1 (FHL-1), C4b-binding protein (C4BP), and vitronectin (Hallstrom and Riesbeck 2010; Rooijakkers and van Strijp 2007; Singh et al. 2010). These host factors can block different complement steps, which protects host cells from lysis. Vitronectin, a component of the ECM, blocks the formation of MACs in host membranes, whereas FH, FHL-1, and C4BP block the C protein proteolytic cascade. Bacterial pathogens can recruit these host proteins to their cell surfaces, thereby enhancing complement inhibition. For instance, *S. aureus* SdrE and Sbi can bind to FH, whereas *S. pyogenes* M protein can interact with FH, FHL-1, and C4BP. C4BP can also interact with *E. coli* OmpA and *B. pertussis* filamentous hemagglutinin (FHA). *N. meningitidis* has multiple FH and C4BP-binding proteins (PorA, Fhbp, PorB2, NspA, and NHBA) as does *B. burgdorferi* (OspA, OspE, CspA, CRASPs) (Madar et al. 2015), whereas *Y. pestis* only contains one FH and C4BP-binding protein (Ail). *H. influenzae* Hsf and protein E interact with vitronectin, which also plays a role in bacterial adherence (Hallstrom and Riesbeck 2010; Singh et al. 2010).

As mentioned above, certain intracellular pathogens block host programmed cell death (PCD) to facilitate their replicative lifestyles. However, many extracellular pathogens can induce apoptosis (non-lytic PCD) and/or pyroptosis (inflammatory lytic PCD) to evade host innate immune defenses, especially those associated with phagocytic cells (DeLeo 2004; Gao and Kwaik 2000; Grassme et al. 2001; Monack and Falkow 2000; Navarre and Zychlinsky 2000). These processes can be induced by a number of different virulence factors, including bacterial hemolysins, which disrupt host cell membrane integrity, or secreted exotoxins/effectors that disrupt host cell signaling pathways, predominantly MAPK and NF-κB pathways. For instance, *Y. pestis* YopJ/P blocks the translocation of NF-κB into the nucleus by blocking the degradation of IκBα, whereas *S. flexneri* IpaB and *S. enterica* SipB induce the caspase-dependent pyroptosis cascade. *P. aeruginosa* pyoverdin can induce apoptosis through the stimulation of reactive oxygen species (ROS) (Rada et al. 2011). *B. pertussis* FHA (filamentous hemagglutinin) and ACT (adenylate cyclase toxin) can induce apoptosis of macrophage and neutrophils (Melvin et al. 2014).

Beyond their phagocytic capabilities, neutrophils have the ability to excrete histone proteins and DNA to create neutrophil extracellular traps (NETs) that immobi-

lize invading pathogens (von Köckritz-Blickwede et al. 2016). Although initially observed in neutrophils, other phagocytic cells can produce similar structures. Several pathogens can counteract these NETs by secreting proteases and DNAases. *S. pyogenes* secretes multiple DNAases (SdaD2, Sda1, SdaB, SpdI1, and SpnA), whereas *S. aureus* only secretes the Nuc DNAase. In addition, *S. pyogenes* streptolysin O and *B. pertussis* ACT can suppress the formation of NETs, which is coupled to the suppression of the neutrophil oxidative burst.

Recognition of bacterial cell surface proteins or polysaccharides by host antibodies or complement proteins triggers multiple aspects of innate immunity. Therefore, it is not surprising that many pathogens have developed the ability to evade these defenses by altering the structural components of LPS, capsules, flagella, and/or fimbriae, through the process of phase or antigenic variation (Palmer et al. 2016; van der Woude and Baumler 2004). Whether the mechanism is through the on/off transcriptional regulation of the synthesis of cell surface moieties (phase variation) or the expression of antigenically distinct variations of the same molecule (antigenic variation), the end result is the presentation of new cell surface structures that are not immediately recognized by the host immune system. Examples of these changes include multiple capsule serotypes in *N. meningitidis* and *S. pneumoniae*, fimbriae/type IV pili alterations in *B. burgdorferi, E. coli, H. influenzae, N. meningitidis,* and *S. enterica*, LPS changes in *C. jejuni, H. pylori, N. meningitidis,* and *N. gonorrhoeae*, and flagella variations in *H. pylori, S. enterica,* and *C. jejuni*.

Regulation of Virulence Factor Expression

Beyond the antigenic and phase variation mentioned above, bacterial pathogens regulate the expression of their virulence factors in response to many different signals. These cues include environmental signals within the host milieu that affect the growth and persistence of pathogens, including iron availability, oxygen concentrations, temperature, osmolarity, and pH. Pathogens must also be able to respond to variations in the concentrations of host-specific defense molecules, including reactive oxygen and nitrogen species (ROS, RNS) and antimicrobial chemicals such as bile salts. In addition, pathogens growing within biofilms use small molecule autoinducers and quorum sensing (QS) pathways to alter their transcriptional patterns to adapt to conditions within the biofilm.

The predominant mode of virulence gene regulation is at the level of transcription initiation, utilizing either classic DNA-binding repressor and activator proteins [(including two-component regulatory systems (TCSs); see below], or alternative sigma (σ) factors that modulate RNA polymerase activity. For instance, *P. aeruginosa* expresses 4 TCSs, 434 transcription factors, 24 σ factors (8 % of genome), and 3 QS systems (Chatterjee et al. 2016). In addition, numerous examples of posttranscriptional regulation at the level of translation initiation by small noncoding RNAs (ncRNAs) have been identified recently. A well-studied example of transcriptional regulatory proteins is the ferric uptake regulator (Fur) superfamily of repressor pro-

teins (see Fig. 1.7; Iron acquisition pathways above) (Fillat 2014; Porcheron and Dozois 2015; Troxell and Hassan 2013). These regulatory proteins, which are ubiquitous in the bacterial world, control the expression of many different genes in response to metal ion concentrations, including iron (Fur), zinc (Zur), nickel (Nur), and manganese (Mur). The Fur repressor uses ferrous (Fe^{+2}) ion as a corepressor (Fur-Fe^{+2} complex) to bind to iron-regulated gene promoters and block transcription initiation in response to intracellular iron concentrations, an essential process for the virulence of many bacterial pathogens that compete with host cells for limited iron stores. Another important family of transcription regulators are the fumarate and nitrate reduction regulator (FNR) activators, which respond to O_2 availability through the oxygen-dependent conformational switch of Fe-S clusters (Green et al. 2014). These regulators are essential for the ability of facultative anaerobes such as *E. coli, S. enterica, K. pneumoniae,* and *Y. pestis* to persist in anaerobic and aerobic environments found in human hosts.

Sigma (σ) factors modulate the activity of RNA polymerase during transcription initiation (Feklistov et al. 2014). While the major housekeeping σ^{70} factor is found in almost all prokaryotes, alternative σ factors are used by *E. coli* and many other enteric pathogens to respond to general stress responses, including hyperosmolarity, acidic pH, oxidative stress, and lethal heat shock (Kazmierczak et al. 2005; Österberg et al. 2011). Proper and timely responses to these detrimental stresses play a key role in the persistence of many pathogens within the human host. The general stress response σ factor in Gram-negative enterics is σ^{s} (RpoS) (Hengge-Aronis 2002; Shen and Fang 2012), and it is σ^{B} (SigB) in *B. anthracis, M. tuberculosis*, and other Gram-positive pathogens (Paget 2015). Alternative σ factors also function in the specific transcription of virulence factors, such as sporulation genes (σF, σE, σG, σK) in *Bacillus* spp. (Fimlaid and Shen 2015), flagella biosynthesis (σ^{28}, FliA) in most motile pathogens (Helmann 1991), pyoverdine synthesis (PvdS) and pili formation (σ^{N}, RpoN) in *P. aeruginosa* (Miyazaki et al. 1995), and SPI-1- and SPI-2-encoded virulence factors in *S. enterica* (Kazmierczak et al. 2005).

The important role of small ncRNAs in the transcriptional and translational regulation of virulence gene expression has become more apparent in recent years (Bhatt et al. 2016; Cech and Steitz 2014; Fechter et al. 2014; Lebreton and Cossart 2017). These ncRNAs form complementary RNA–RNA complexes with the 5′-untranslated regions (5′-UTRs) of virulence genes and can act as riboswitches to regulate the stability of mRNA transcripts or the initiation of translation of these transcripts. Riboswitches can respond either to the levels of small metabolites, such as iron-responsive RhyB in *E. coli* (Porcheron and Dozois 2015), S-adenosylmethionine (SAM) riboswitches SreA and SreB in *L. monocytogenes* (Lebreton and Cossart 2017) and the QS regulator RNAIII in *S. aureus* (Fechter et al. 2014), or to environmental conditions, such as the PrfA thermosensor in *L. monocytogenes* (Lebreton and Cossart 2017). Small ncRNAs can also control the RNA-binding activity of other regulatory proteins, such as the CsrA/RsmA RNA-binding proteins of *Y. pestis* and other enteric pathogens (Heroven et al. 2012; Vakulskas et al. 2015).

Many of the environmental and host signals that control these different regulatory mechanisms are initially sensed by two component regulatory systems (TCSs)

(Beier and Gross 2006; West and Stock 2001; Zschiedrich et al. 2016). TCSs are some of the most abundant proteins expressed in bacterial pathogens, and they are intimately involved in various virulence mechanisms. TCSs are comprised of a sensor histidine kinase (HK) located at the bacterial cell surface and a response regulator (RR), which usually acts as a positive or negative transcription factor. Recognition of an environmental or host signal by the HK leads to its autophosphorylation on a histidine residue, followed by phosphorelay from the histidine residue to an aspartate residue in the RR, which in turn activates its transcriptional activity. Some well-studied TCSs (Table 1.2) include OmpR-EnvZ, which regulates osmolarity and porin synthesis in *S. enterica* and other enterics; *V. cholerae* ToxR-ToxS, which regulates the ToxT transcription factor that controls the expression of cholera toxin and toxin co-regulated pili (Tcp) in response to changes in pH, osmolarity, temperature, and bile salts; *B. pertussis* BvgA-BvsS, which regulates pertussis toxin expression and biofilm formation in response to temperature changes; *S. pyogenes* CovR-CovS, which regulates the expression of many virulence genes including streptolysin S (SLS) and streptolysin O (SLO); *M. tuberculosis* DovR-DovS, which responds to oxygen concentrations to regulate latency and resistance to nitric oxide gas (NO); and *S. aureus* AgrA-AgrC and *V. cholerae* LuxP-LuxQ, which control quorum sensing.

Quorum sensing (QS) is widely used by Gram-positive and Gram-negative pathogens to regulate virulence gene expression and biofilm formation in response to host signals and population densities (Castillo-Juarez et al. 2015; Dow 2017; Parker and Sperandio 2009; Papenfort and Bassler 2016; Hawver et al. 2016). Pathogens produce and secrete extracellular signaling molecules, termed autoinducers, that accumulate in the extracellular milieu. Increased levels of the autoinducers lead to cell-to-cell communication and regulation of multiple cellular responses in the pathogens. Gram-negative pathogens, such as *P. aeruginosa, V. cholerae,* and *L. pneumophila,* usually synthesize *N*-acyl L-homoserine lactones (AHLs) by LuxI-type synthases, which diffuse across cell membranes and interact with intracellular LuxR-type receptors to control virulence factor transcription. Gram-positive pathogens, such as *S. aureus, E. faecalis,* and *B. anthracis*, synthesize and secrete autoinducer oligopeptides (AIPs) that interact with TCSs to control QS and transcription. Regardless of the mechanism, the end result is the induction or repression of genes encoding many different virulence factors.

Identification and Characterization of New Virulence Factors

There is no question that the "omics" innovations of the last 30 years have transformed our knowledge of bacterial pathogens and their virulence factors (Bentley and Parkhill 2015; Jean Beltran et al. 2017; Land et al. 2015). With the advent of second-generation ("next-gen") and third-generation (single molecule) DNA sequencing protocols, the timescale to determine the whole genome sequence (WGS) of a bacterial pathogen has been reduced to hours and days. Beyond the

Table 1.2 TCS regulating bacterial virulence (Adapted from: Beier and Gross 2006)

Organism	TCS	Presumptive stimulus	Regulation of, or effect of inactivation
S. enterica	PhoP-PhoQ	Mg^{2+}/Ca^{2+}	Mg^{2+} uptake, modification of LPS, resistance to antimicrobial peptides, *pmrD*, transcriptional regulator genes *ssrB*, *hilA*, *slyA*, other virulence-related genes posttranscriptional regulation of SsrA
	PmrA-PmrB	Fe^{3+}	Lipid A modification
	RcsC-YojN-RcsB	Desiccation, osmotic shock, growth on solid surfaces; specific in vivo stimulus unknown	Colonic acid capsule synthesis, *ftsA*, *osmC*, motility and chemotaxis genes, *fhlDC*, *tviA*, *rprA*
	OmpR-EnvZ	Osmolarity	Porin genes, *ssrB-ssrA*, stationary phase acid response
	SsrB-SsrA	ND	SPI-2 TTSS and effector genes
	SirA-BarA	ND	*csrB*, *hilD*
Shigella flexneri	OmpR-EnvZ		Invasion genes
S. sonnei	CpxR-CpxA	pH?	Virulence regulator gene *virF*
Vibrio cholerae	ArcA-ArcB		Virulence regulator gene *toxT*
Helicobacter pylori	FlgR-FlgS	ND	Flagellar genes
	ArsR-ArsS	Low pH	Urease and other acid-resistance genes
Campylobacter jejuni	DccR-DccS	ND	Colonization defect
Legionella pneumophila	CpxR-CpxA	ND	*icmR* and other *icm-dot* genes, no effect on intracellular replication in amoeba and human macrophages
	LetA-LetS	ND	Growth defect in amoeba but not in human macrophages
Yersinia pseudotuberculosis	PhoP	ND	Virulence attenuation, reduced survival in macrophages
Pseudomonas aeruginosa	AlgR-FimS	ND	Alginate biosynthesis, twitching motility
	AlgB-KinB	ND	Alginate biosynthesis
	RocA1-RocS1 (SadR-SadS)	ND	Fimbrial genes, biofilm maturation
	PprB-PrpA	ND	Virulence genes and cell motility, QS signal production
	RtsM (RetS)	ND	TTSS and effector genes
Brucella abortus	BvrR-BvrS	ND	*omp* genes, virulence attenuation, reduced invasiveness in macrophages and HeLa cells

(continued)

Table 1.2 (continued)

Organism	TCS	Presumptive stimulus	Regulation of, or effect of inactivation
Neisseria meningitidis	MisR-MisS	ND	Composition of LOS inner core
B. pertussis	BvgA-BvgS	Temperature, redox state of quinones, SO_4^{2-}, nicotinic acid	Toxin and adhesin expression, biofilm formation
Listeria monocytogenes	DegU	ND	Virulence attenuation
	VirR-VirS	ND	Virulence attenuation
	AgrA-AgrC	ND	Virulence attenuation
	LisR-LisK	ND	Virulence attenuation
Mycobacterium tuberculosis	DevR-DevS	ND	Virulence attenuation
	MprA-MprB	ND	Virulence attenuation
	RegX3-SenX3	ND	Virulence attenuation
	PrrA-PrrB	ND	Intracellular growth defect during the early stages of macrophage infection
Streptococcus pneumoniae	CiaR-CiaH	ND	Virulence relevant gene *htrA*
	RR04-HK04	ND	Virulence genes *psaB*, *psaC*, *psaA*
	RR06-HK06	ND	Virulence gene *cbpA*
	RitR	ND	Iron homeostasis
	MicA-MicB	Oxygen?	Virulence attenuation
Streptococcus pyogenes	CsrR-CsrS (CovR-CovS)	Mg^{2+}	Capsule synthesis, virulence genes *ska*, *sagA*
Streptococcus agalactiae	CsrR-CsrS (CovR-CovS)	ND	Virulence attenuation
S. mutans	SMRR11-SMHK11	ND	Biofilm formation and acid resistance
Staphylococcus aureus	AgrA-AgrC	AIP	Regulatory RNA III
	SrrA-SsrB	Oxygen?	Exoprotein genes, RNA III
	SaeR-SaeS	ND	Exoprotein genes
	ArlR-ArlS	ND	Exoprotein genes
	LytR-LytS	ND	Holin-like genes *lrgA*, *lrgB*
Clostridium perfringens	VirR-VirS	ND	Toxin (*pfoA*, *cpb2*) and adhesion genes (*cna*)

ND not determined

relatively straightforward DNA-based speciation of pathogenic isolates, which has the potential to revolutionize the rapid clinical identification of disease-causing bacteria, genomic analyses coupled with metagenomics studies also have increased our knowledge of vital pathogen–host and pathogen–microbiota interactions within the host milieu. WGS analyses can rapidly identify putative virulence factors based on sequence homologies (as seen in the individual pathogen chapters herein), and virulence factor activity relationships (VFARs) can be used to compare the structures and activities of these newly identified virulence factors to known factors (Waseem

et al. 2017). However, transcriptomic and proteomic approaches are needed to determine the expression of these virulence factors in response to environmental and host signals. While the expression patterns of virulence factors within the host milieu is critical to understanding a pathogen's virulence potential, equally important is deciphering spatial and temporal protein–protein interactions (PPIs) between pathogen and host proteins using a myriad of proteomic approaches, including classical immunoprecipitation and cross-linking techniques, two-hybrid approaches, and immunoaffinity purification coupled to mass spectrometry (IP-MS) methodologies. MS technologies are also used to study the various different types of posttranslational modifications (PTMs), such as phosphorylation, adenylation, deamination, glycosylation, ADP-ribosylation, ubiquitination, and SUMOylation, that occur to both bacterial and host proteins during the infection process (Ribet and Cossart 2010). Data from these multi-omics approaches will not only provide valuable information about the virulence capabilities of bacterial pathogens but also will identify potential targets for novel anti-virulence strategies, which will be discussed in the final chapter. The ability to decipher the complete genomic, metabolomics, transcriptomic, and proteomic potential of bacterial pathogens in the context of pathogen–host interactions has never been greater, and all these approaches will need to be utilized to understand and exploit bacterial virulence factors in generating the new anti-virulence strategies so desperately needed in this age of antibiotic resistance.

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Part I
Gram-Positive Bacterial Pathogens

Chapter 2
Bacillus spp.

Genomics, Morphologies, and Growth Characteristics

- **Genomics:**
  - *Bacillus anthracis* – chromosome, 5,227,293 bp; 5508 predicted ORFs (Read et al. 2003):
    - pOX1 plasmid: 181,677 bp; 217 predicted ORFs
    - pOX2 plasmid: 94,829 bp; 113 predicted ORFs
  - *Bacillus cereus* – chromosome: 5,426,909 bp; 5366 predicted ORFs (Ivanova et al. 2003)
- **Cell morphology:**
  - Large, boxy rod-shaped cells; usually in single short chains or long chains (Fig. 2.1)
  - Endospore former; subterminal or central endospores that do not swell the cell
- **Gram stain:**
  - Gram positive; older cells tend to stain Gram negative
- **Growth:**
  - Obligate aerobes or facultative anaerobes; catalase positive
  - Ubiquitous environmental pathogens found primarily in soil; also in water, dust, agricultural products, and invertebrates; primarily exist in endospore form
  - Common laboratory contaminant
  - Most species are highly motile (except *Bacillus anthracis*) with peritrichous flagella – involved in biofilm formation (*B. cereus*)

D.I. Johnson, *Bacterial Pathogens and Their Virulence Factors*,
https://doi.org/10.1007/978-3-319-67651-7_2

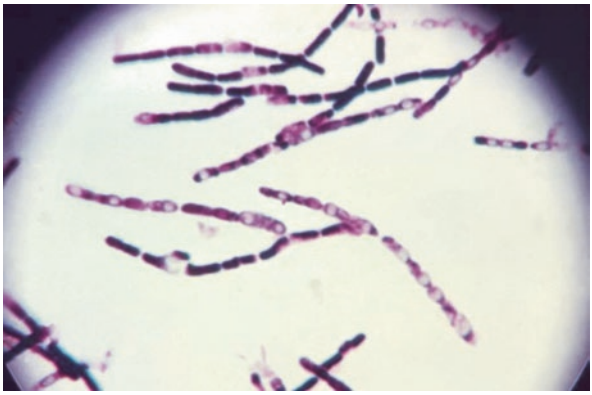

Fig. 2.1 *B. anthracis* cells (From: Public Health Image Library (PHIL) #9826)

- >300 species; most are rarely associated with human disease:
  - Two major human pathogens: *B. anthracis* and *B. cereus*

Disease States Associated with *Bacillus* spp.

- ***B. anthracis*:**
  - Anthrax – cutaneous (most common), inhalation (most lethal), and gastrointestinal (rare):
    - Cutaneous: endospores enter cuts or abrasions through direct contact with infected animal, wool, or animal hides; results in eschar lesion (Fig. 2.2)
    - Inhalation: endospores enter the lungs and germinate in lung phagocytes; bacteria enter bloodstream and lead to septic shock symptoms
    - Gastrointestinal: endospores are ingested usually in undercooked meat; leads to gastrointestinal pain and bleeding
- ***B. cereus*:**
  - Two forms of food-borne intoxications:
    - Short incubation time emetic intoxication:
      - Nausea and emesis (vomiting)
      - Associated with heat-stable emetic toxin (ETE) cereulide
    - Long incubation time diarrheal intoxication:
      - Abdominal cramps and diarrhea; non-emetic
      - Associated with heat-labile enterotoxin Nhe and/or hemolytic enterotoxin HBL
  - Ocular infections; endocarditis; musculoskeletal infections

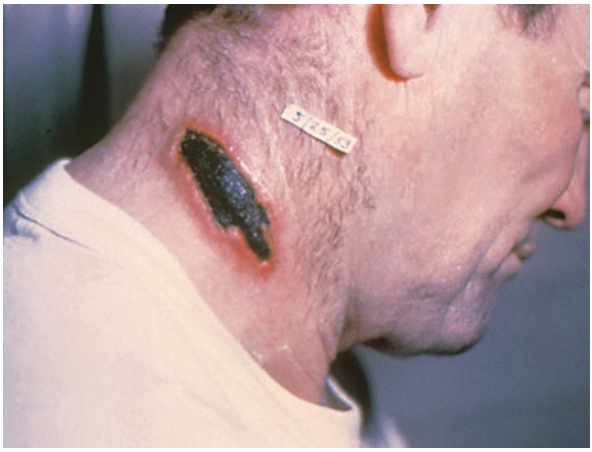

Fig. 2.2 Eschar lesion (From: PHIL #1934)

Virulence Factors

- ***B. anthracis*** (Mikesell et al. 1983):
  - Virulence plasmids pXO1 and pXO2 are essential for virulence
  - Adherence to host cells:
    - **BslA** (*Bacillus anthracis* S-layer protein A) (Kern and Schneewind 2008, 2010):
      - Major S-layer hydrophobic protein
      - Binds to host cells by interacting with host integrin α2β1 and complement component C1q
      - Negatively regulated by InhA protease (Tonry et al. 2012)
      - *bslA* gene encoded on the pXO1 virulence plasmid
    - **Capsule:**
      - Poly-γ-D-glutamic acid capsule
      - Antiphagocytic factor; used to evade host immune system
      - Encoded by *capBCADE* operon on the pXO2 virulence plasmid
    - **S-layer**:
      - ~24 proteins that self-assemble into a crystalline sheet surrounding the cell; may contain other adhesins besides BslA
      - Binds extracellular matrix (ECM) components
  - Growth in host cells:
    - Iron acquisition – required for growth in host:

- Siderophores (Cendrowski et al. 2004; Hotta et al. 2010; Wilson et al. 2010):
  - **Petrobactin**:
    - Catecholate iron siderophore; predominate siderophore secreted early in infection
    - Encoded by *asbAB* genes
  - **Bacillibactin**:
    - Catecholate iron siderophore; secreted late in infection
    - Encoded by *bacACEBF* operon
- Fe-heme scavengers (Segond et al. 2014):
  - **IlsA** (iron-regulated leucine rich surface protein type A):
    - Binds host heme, hemoglobin, and ferritin
    - Analogous to Isd (iron-regulated surface determinant) system in *Staphylococcus aureus*

- Biofilms: capable of forming biofilms, but unclear if biofilms of vegetative *B. anthracis* exist in the environment or in human hosts

– Damage to host cells:

- **Anthrax toxins** (Fig. 2.3) (Friebe et al. 2016; Liu et al. 2014; Prince 2003):
  - A-B exotoxins – one common B subunit with two different A subunits:

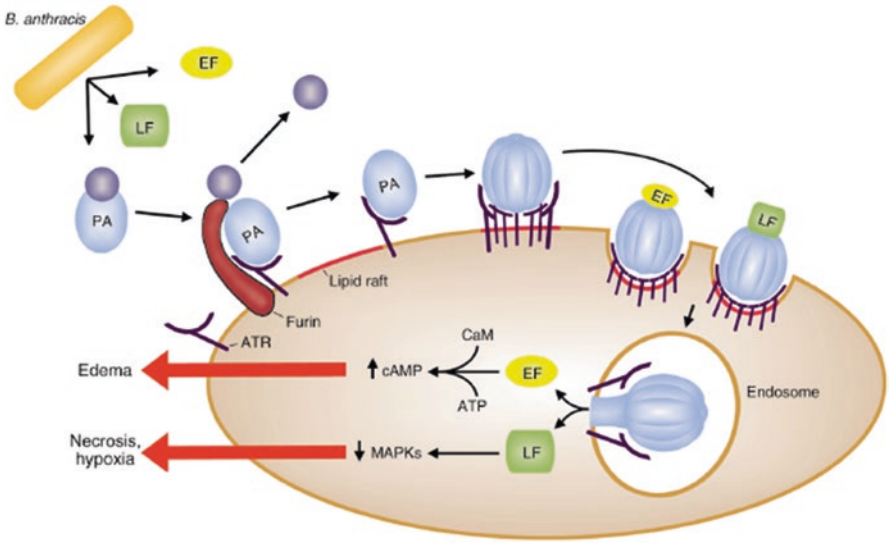

Fig. 2.3 Anthrax toxin effects (From: Prince 2003)

- B subunit:
  - **PA (protective antigen)**: forms homo-heptameric complex
  - Binds to host anthrax toxin receptor (ATR) and host capillary morphogenesis protein CMG2
  - Encoded by *pagA* gene on pOX1 virulence plasmid
- A subunits:
  - **EF (edema factor)**:
    - Calcium-/calmodulin-dependent adenylate cyclase; converts ATP to cAMP
    - Increases host intracellular cAMP levels; induces water and ion loss from cells and edema in surrounding tissues
    - Usually targets liver hepatocytes
  - **LF (lethal factor)**:
    - Zn metalloprotease
    - Cleaves host mitogen-activated protein kinases (MAPKs); loss of these signaling proteins leads to cell death
    - Usually targets cardiomyocytes and vascular smooth muscle cells
  - Encoded by *lef* (LF) and *cya* (EF) genes on pOX1 virulence plasmid

- **Anthrolysin O (ALO)** (Shannon et al. 2003):
  - Cholesterol-dependent cytolysin
  - Belongs to the β-pore-forming toxin (β-PFT) family of pore-forming toxins (Peraro and van der Goot 2016)
  - Lytic activity against host phagocytic cells
  - Encoded by *bas3109* gene

- ***B. cereus* –** most strains lack the pXO1 and pXO2 virulence plasmids:
  - Adherence to host cells: same as *B. anthracis*; capsule synthetic genes are encoded on the chromosome
  - Growth in host cells:
    - Iron acquisition: same as *B. anthracis*
    - Biofilms (Majed et al. 2016):
      - Excellent biofilm former in host and in environment
      - Plays key roles in persistence and ubiquitous distribution in the environment
      - Provides resistance to various stresses
      - Excellent adhesive capacity on biotic and abiotic substrates

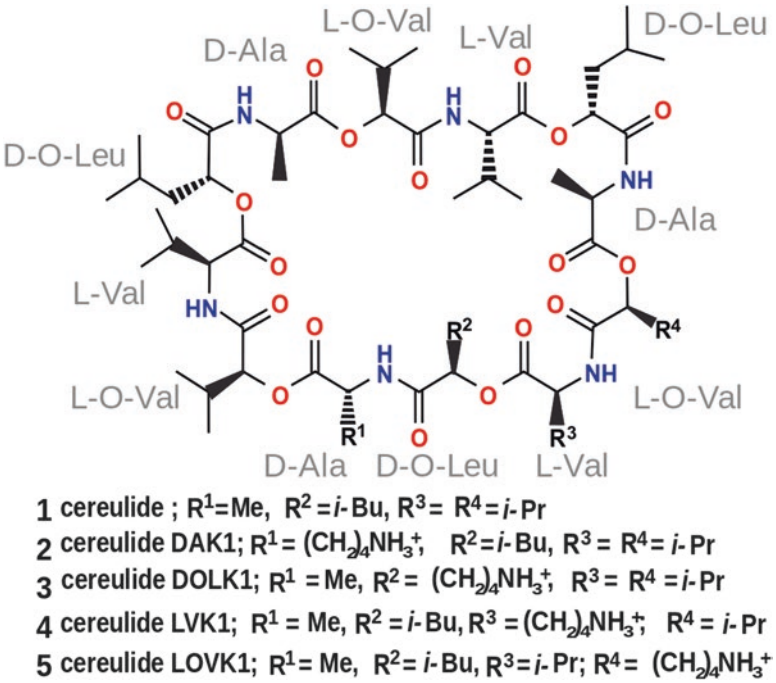

Fig. 2.4 Cereulide structure (From: Thieme E-journals)

– Host cell damage:

- A-B exotoxins: none; lack of pOX1 virulence plasmid
- **Cereulide** (Ehling-Schulz et al. 2015):
  - Heat-stable emetic toxin (ETE); associated with short incubation time emetic intoxication
  - Pore-forming cytolytic toxin
  - Hydrophobic cyclic dodecadepsipeptide (Fig. 2.4)
  - Encoded on a pOX-1-like plasmid
  - Synthesized by the nonribosomal cereulide synthetase (*ces*) complex (Ehling-Schulz et al. 2005)
- **Hemolysin BL (HBL)** (Ramarao and Sanchis 2013; Senesi and Ghelardi 2010):
  - Associated with long incubation time diarrheal intoxication
  - Tripartite toxin: B, L1, and L2 subunits; encoded by *hblA*, *hblC*, and *hblD* genes
  - Pore-forming cytolytic exotoxin; all three subunits are needed for toxicity

- **Nonhemolytic enterotoxin (Nhe)** (Senesi and Ghelardi 2010):
  - Associated with long incubation time diarrheal intoxication
  - Tripartite toxin: NheA, NheB, and NheC subunits; encoded by *nheA*, *nheB*, and *nheC* genes
  - Pore-forming cytolytic exotoxin; all three subunits are needed for toxicity
- **Hemolysin HlyI (cereolysin O)** (Ramarao and Sanchis 2013):
  - Homologous to *B. anthracis* anthrolysin O (ALO)
  - Heat-labile, cholesterol-dependent cytolysin; belongs to the β-PFT family of pore-forming toxins
  - Encoded by *hlyI* gene
- **Hemolysin HlyII** (Ramarao and Sanchis 2013):
  - Heat-labile, cholesterol-independent cytolysin; belongs to the β-PFT family of pore-forming toxins
  - Encoded by *hlyII* gene
- **Hemolysin HlyIII** (Ramarao and Sanchis 2013):
  - Heat-labile cholesterol-independent cytolysin
  - Encoded by *hlyIII* gene; least characterized *B. cereus* hemolysin
- **Hemolysin IV (cytotoxin K, CytK)** (Ramarao and Sanchis 2013; Senesi and Ghelardi 2010):
  - Cytolysin; belongs to the β-PFT family of pore-forming toxins
  - Encoded by *cytK* gene
- **Phosphatidylcholine-specific phospholipase C (PC-PLC)** (Pomerantsev et al. 2003):
  - Responsible for the hemolytic properties of *B. cereus*
  - Encoded by *plc* gene
- **Sphingomyelinase (SPH)** (Pomerantsev et al. 2003):
  - Responsible for hemolytic properties of *B. cereus*
  - Encoded by *sph* gene

Regulation of Virulence Factor Expression

- **Quorum sensing**:
  - **PlcR–PapR regulon** (Agaisse et al. 1999; Grenha et al. 2013):
    - **PlcR** – 34 kDa global transcriptional activator; helix-turn-helix (HTH) type regulator:
      - Inactive in *B. anthracis* due to nonsense mutation; hence, virulence factors activated in *B. cereus* are not expressed in *B. anthracis*
    - **PapR** – 48 aa peptide:
      - Secreted, reimported, and then truncated to a heptapeptide (PapR7)
      - Binding to PlcR activates its function
    - PlcR–PapR binds to PlcR-box palindromic DNA sequences upstream of target genes
    - At least 45 genes are under PlcR–PapR regulation: environmental sensors, enterotoxins, cytolytic exotoxins, and hemolysins – these virulence factors are not expressed in *B. anthracis*
- **Iron regulon:**
  - **Fur (Fe utilization repressor)** – Fe^{+2}-binding repressor protein:
    - Regulates all aspects of iron regulation
    - Fe^{+2}-binding activates Fur repressor activity
    - Fe^{+2}-Fur binds to Fur-box DNA sequences upstream of its negatively regulated target genes
    - Low Fe^{+2} concentrations lead to derepression of siderophore genes; also regulated by temperature, oxidative stress, and CO_2 atmosphere
- **Anthrax toxin synthesis –** *pagA*, *lef*, and *cya* regulon (*B. anthracis*):
  - **AtxA**: transcriptional activator (Dai et al. 1995; Fouet and Mock 2006; Uchida et al. 1993; Kolsto et al. 2009)
  - **PagR**: transcriptional repressor
  - Encoded on pXO1 pathogenicity island (PAI)
- **Capsule synthesis** – *capBCADE* operon (*B. anthracis*):
  - **AcpA** and **AcpB** (Fouet and Mock 2006):
    - Transcriptional activators; regulated by AtxA activator
    - Encoded on pXO2 pathogenicity island (PAI)

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Chapter 3
Clostridium spp.

Genomics, Morphologies, and Growth Characteristics

- **Genomics:**
  - *Clostridium botulinum* A strain Hall chromosome: 3,886,916 bp; 3650 predicted ORFs (Sebaihia et al. 2007)
  - *Clostridium difficile* chromosome: 4,290,252 bp; 3776 predicted ORFs (Sebaihia et al. 2006)
  - *Clostridium perfringens* chromosome: 3,031,430 bp; 2660 predicted ORFs (Shimizu et al. 2002)
  - *Clostridium septicum* chromosome: 3,266,706 bp; 3125 predicted ORFs (Benamar et al. 2016)
  - *Clostridium sordellii* chromosome: 3,571,992 bp; 3586 predicted ORFs (Scaria et al. 2015)
  - *Clostridium tetani* chromosome – 2,799,250 bp; 2372 predicted ORFs (Bruggemann et al. 2003):
    - Plasmid: 74,082 bp; 61 predicted ORFs; encodes the tetanus toxin
- **Cell morphology:**
  - Rod-shaped cells (Fig. 3.1)
  - Endospore formers: terminal or subterminal endospores; swollen sporangium gives cells a "drumstick" appearance
  - Flagella – most species have peritrichous flagella including *C. botulinum*, *C. difficile*, *C. septicum*, C. *sordellii*, and *C. tetani*:
    - *C. perfringens* does not have flagella
    - The role of Clostridia flagella in virulence is unclear; may play a role in binding to host mucus

D.I. Johnson, *Bacterial Pathogens and Their Virulence Factors*,
https://doi.org/10.1007/978-3-319-67651-7_3

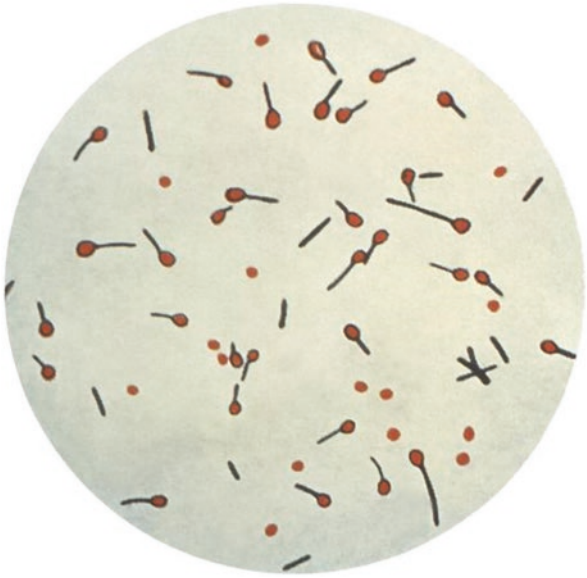


Fig. 3.1 *C. tetani* cells with terminal endospores (From PHIL # 14348)

- **Gram stain:**
  - Gram positive; older cells tend to stain Gram negative
- **Growth:**
  - Obligate anaerobes (vs. aerobic *Bacillus* spp.)
  - Ubiquitous environmental pathogens; found primarily in soil in endospore form
  - Biofilm formation (Pantaleon et al. 2014):
    - 71 *Clostridium* species can form either mono-species or multi-species biofilms
    - Nine species have the ability to form mono-species biofilms, including the pathogens *C. difficile* and *C. perfringens* (see below)
  - At least 100 species; most are not associated with human disease:
    - Notable human pathogens: *C. botulinum*, *C. difficile*, *C. perfringens*, *C. septicum*, *C. sordellii*, and *C. tetani*

Disease States Associated with *Clostridium* spp.

- Most of the disease symptomology associated with *Clostridium* infections is due to the action of secreted exotoxins
- *C. botulinum* (Shukla and Sharma 2005):

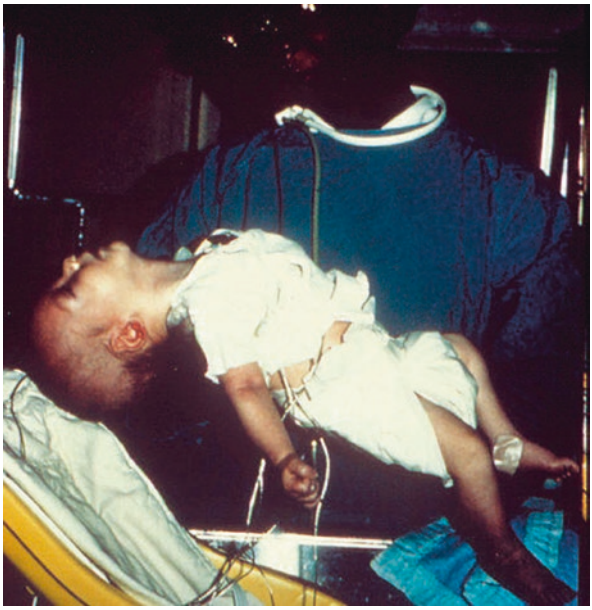


Fig. 3.2 Infant botulism; "floppy baby" syndrome (From: PHIL #1935)

- Botulism; flaccid paralysis
- Four clinical categories of botulism:
  - Food-borne intoxication: ingestion of foods contaminated with the botulinum toxin (BoNT)
  - Inhalation: endospores are inhaled
  - Infant: endospores are ingested (Fig. 3.2)
  - Wound: endospores are in wound
  - Endospores germinate within the host and begin to grow vegetatively, producing BoNT and other virulence factors
- BoNT acts locally by blocking the release of stimulatory acetylcholine (ACh) neurotransmitters at cholinergic synapses in the peripheral nervous system (see mechanism of action below):
  - Leads to an inability of muscles to contract – flaccid paralysis (Fig. 3.2)
- Initial symptoms are autonomic nervous system dysfunctions – weakness, dizziness, reduced salivation, nausea, vomiting, and abdominal pain
- Subsequent neurologic symptoms – blurred vision, paralysis of facial and throat muscles, leading to inability to swallow and speech difficulties
- Progressive paralysis ultimately results in weakening of respiratory and visceral muscles – respiratory paralysis usually is the cause of death
- Four groups of *C. botulinum* strains (Brunt et al. 2016):
  - Groups I–IV; based on disease association, germination signals, and the BoNTs produced (see below)

- *C. difficile* (Rupnik et al. 2009):
  - Antibiotic-associated diarrhea; pseudomembranous colitis:
    - Antibiotic treatment depletes the normal microbiota of GI tract, removing the barrier effect – allows ingested *C. difficile* spores to germinate and the resulting vegetative cells to gain a growth advantage (Theriot and Young 2015)
  - *C. difficile* endospores in healthcare environments are ingested; able to pass through the acidic stomach environment
  - Endospores germinate in the small intestine; vegetative cells colonize the small intestine and produce toxin A (TcdA) and toxin B (TcdB):
    - Toxins inhibit cell signaling pathways, resulting in the induction of pro-inflammatory cytokines and chemokines – leads to neutrophil infiltration and pseudomembranous colitis
- *C. perfringens* (Rood 1998):
  - Soft tissue infections: cellulitis, fasciitis; usually does not involve infection of the host musculature
  - Myonecrosis (gas gangrene) – also caused by *C. septicum* and *C. sordellii*:
    - Entry of *C. perfringens* type A vegetative cells or endospores into traumatic or surgical wounds
    - Endospore germination results in growth of vegetative cells and production of exotoxins
    - Leads to muscle necrosis with gas: intense pain, high fever, pus, subcutaneous gas bubbles, skin discoloration, and foul odor (Fig. 3.3)
    - Life-threatening rapid progression – leads to shock, renal failure, and death
  - Food poisoning:
    - Very common; third highest incidence behind *Salmonella enterica* and *Staphylococcus aureus*
    - Associated with ingestion of vegetative cells or endospores; often found in contaminated or undercooked meat products
    - Seven to 15 h after ingestion, vegetative cells sporulate, resulting in the production of the sporulation-associated CPE enterotoxin (see below)
    - CPE induces water loss into the intestinal lumen, resulting in crampy diarrhea and foamy, foul-smelling stools
  - Enteritis necroticans:
    - Associated with high-protein meat meals, predominantly undercooked pork, which allow *C. perfringens* type C vegetative cells in the contaminated meat to rapidly grow

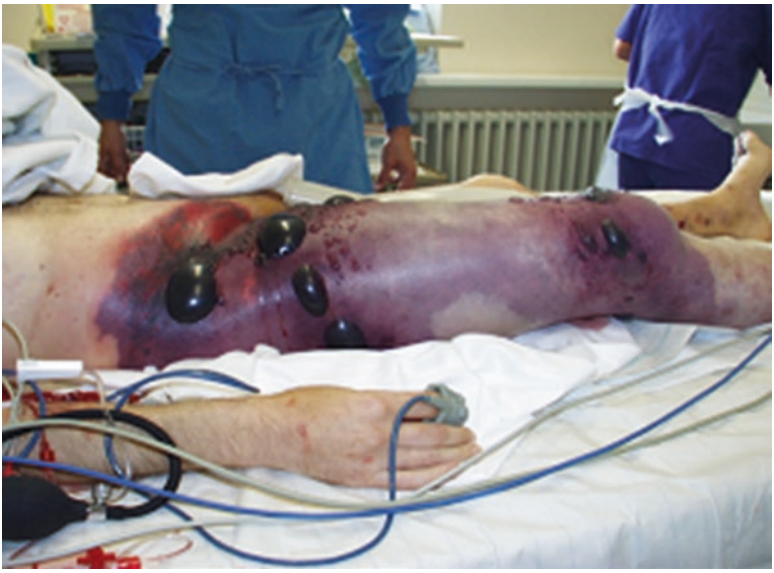


Fig. 3.3 Gas gangrene (From: Schropfer et al. 2008)

  - Vegetative cells produce the lethal, necrotizing beta (β) toxin (see below) – induces hemorrhagic necrosis and intestinal villi destruction
  - Leads to vomiting, diarrhea, severe abdominal pain, and bloody stools
  - Disease is also associated with ingestion of sweet potatoes – contain a heat resistant trypsin inhibitor that protects β toxin from trypsin-dependent inactivation in the intestinal tract

- *C. septicum*:
  - Myonecrosis (gas gangrene) – similar symptoms as *C. perfringens*:
    - Can be associated with tissue damage done by colorectal malignancies (Gnerlich et al. 2011; Hermsen et al. 2008)
  - Rare cause of meningoencephalitis/cerebritis or brain abscesses; associated with high morbidity and mortality (Macha et al. 2016):
    - May be the result of *C. septicum* leaving the GI tract, possibly through damage done by malignancies, and disseminating to the CNS
- *C. sordellii* (Aldape et al. 2006; Aronoff 2013):
  - Infections can occur following trauma, childbirth, routine gynecological procedures, and medically induced abortions; rare but almost 100% fatal
  - Fatal toxic shock syndrome: afebrile, nausea, vomiting, diarrhea, severe abdominal pain, and hypotension
  - Also can cause soft tissue infections and myonecrosis (gas gangrene)

Fig. 3.4 Tetanus spastic paralysis (From: Painting by Sir Charles Bell – 1809)

- *C. tetani* (Aronoff 2013):
  - Tetanus; spastic paralysis (Fig. 3.4)
  - Most cases result from contamination of wounds with endospores, which germinate into vegetative cells and release tetanus toxin (TeNT) through cell lysis
  - Unlike BoNT, TeNT enters peripheral nerves but is transported to the central nervous system, where it enters inhibitory motor neurons and blocks the release of inhibitory neurotransmitters glycine and gamma-aminobutyric acid (GABA)
  - Early symptoms include pharyngitis, difficulty swallowing and breathing, chest pain, and disorientation
  - Eventually leads to intense, painful spasms and muscle rigidity, including neck rigidity, jaw spasms (lockjaw), facial muscle paralysis (risus sardonicus), trunk and limb rigidity (opisthotonos), and respiratory failure (cause of death)

Virulence Factors

- *C. botulinum*:
  - Adherence to host cells: no known functional adhesins
  - Growth in host milieu:
    - Endospore germination (Brunt et al. 2016):

  - Essential for vegetative growth, and hence, disease in the host
  - Germinant signals bind to germinant (*ger*) receptors within the endospore inner membrane
  - Binding triggers release of Ca^{2+}-dipicolinic acid (DPA) and its exchange for water, which serves to rehydrate the core and induce vegetative cell outgrowth
- Germinants – amino acids L-alanine, L-cysteine, L-methionine, L-serine, L-phenylalanine, L-threonine, and glycine; L-lactate can help germination:
  - Varies between strains and groups I–IV
- Germinant receptors: GerA, GerB, and GerC; various alleles are present in groups I–IV

– Damage to host cells:

- Exotoxins:
  - **Botulinolysin** (Popoff 2014):
    - Member of the clostridial family of cholesterol-dependent pore-forming cytolysins (CDCs) – perfringolysin O, septicolysin, sordellilysin, and tetanolysin
    - Pore formation disrupts host cell membranes and ion homeostasis
  - **C2 toxin** (Aktories and Barth 2004):
    - A-B exotoxin:
      - A subunit (C2I): ADP-ribosyltransferase activity
      - B subunit (C2II): binding and translocation domains
    - ADP-ribosylates monomeric G-actin on Arg177; blocks the polymerization of host actin cytoskeleton
  - **C3bot1** and **C3bot2 toxins** (Wilde and Aktories 2001):
    - Members of bARTT family of C3 ADP-ribosyltransferases; similar to *P. aeruginosa* ExoS and T
    - ADP-ribosylates the RhoA, RhoB, and RhoC GTPases
    - Blocks the GEF-dependent activation of the GTPases; locks the GTPases in an inactive GDP-bound state (complexed with GDI)
    - Blocks Rho signaling and the actin cytoskeleton; disrupts tight junctions between epithelial cells and between endothelial cells
  - **Botulinum NeuroToxins (BoNT, Botox®)** (Montecucco and Molgo 2005; Swaminathan 2011):
    - Extremely potent neuroparalytic toxins:

  - Inhibit the ability of muscles to contract, resulting in flaccid paralysis
  - Death is usually due to respiratory arrest
- Eight distinct protein sequences – BoNT/A–H; 40 different subtypes:
  - Can use MALDI-TOF/MS diagnostically to analyze BoNT variants (Kalb et al. 2015)
  - Encoded on the chromosome, plasmids, and bacteriophage (Hill et al. 2015)
- BoNT production varies between the four groups of *C. botulinum* strains:
  - Group I produces A, B, F, and H
  - Group II produces B, E, and F
  - Group III produces C and D
  - Group IV produces G
  - BoNT/A, B, E, and F cause most of the human intoxications:
    - Order of toxicity: F > C > A > D > B
- A-B exotoxins:
  - Synthesized as a single polypeptide; posttranslationally cleaved to form two subunits held together by disulfide bonds:
    - A subunit (light chain): heat-labile Zn metalloprotease
    - B subunit (heavy chain): binding and translocation domains
- Mechanism of action – acts locally at neuromuscular cholinergic synapses in the peripheral nervous system:
  - B subunit binds host cell receptors:
    - Polysialoganglioside (PSG) receptor on presynaptic membranes
    - Synaptotagmin and/or SV2 protein receptors; binding facilitates entry into the lumen of synaptic vesicles
  - A subunit translocates into cytosol, where it cleaves SNARE complex proteins of the synaptic fusion complex using its Zn protease activity:
    - Different specificities for SNARE proteins:
      - BoNT/B, D, F, and G: cleave synaptobrevin-2 (VAMP-2)
      - BoNT/C: cleave syntaxin 1A
      - BoNT/A, C, and E: cleave SNAP-25

  - Cleavage of SNARE proteins inhibits synaptic vesicle fusion to the cell membrane – blocks release of activating neurotransmitter acetylcholine (ACh)

- *C. difficile* [(Janoir 2016) and reference therein]:
  - Adherence to host cells:
    - Fimbrial adhesins: type IV pili machinery is expressed in vivo, but evidence for a role in adherence is lacking
    - Afimbrial adhesins:
      - **CWP** (clostridial wall proteins) – family of 29 surface-associate proteins:
        - **SlpA**: binds to intestinal mucosa and the ECM proteins collagen type I, thrombospondin, and vitronectin
        - **Cwp84**: cysteine protease; involved in the proteolytic activation of SlpA; may also degrade ECM proteins fibronectin, vitronectin, and laminin
        - **Cwp66**: adhesin; binding to host cells is induced by heat shock
        - **CwpV**: adhesin; undergoes auto-proteolytic maturation; its auto-aggregative properties may play a role in biofilm formation
      - MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) – bind to ECM proteins including fibronectin, laminin, and collagen:
        - **Fbp68 (FbpA)**: binds to fibronectin
        - **CbpA**: binds to collagen
      - **CD0873**: lipoprotein; binds to Caco-2 human epithelial colorectal adenocarcinoma cells
      - **GroEL**: heat shock protein; anti-GroEL antibodies block adherence
  - Dissemination within the host:
    - **Zmp1**: protease; cleaves host heat shock protein HSP90b, IgA2 antibodies, fibrinogen, and fibronectin – disrupts fibronectin network
    - *C. difficile* is capable of producing the hydrolytic enzymes chondroitin-4-sulphatase, hyaluronidase, heparinase, and collagenase; unclear if they function as spreading factors or in nutrient acquisition (Seddon et al. 1990)
  - Growth in host milieu:
    - Endospore germination – essential for vegetative growth, and hence, disease in the host (see *C. botulinum* above):
      - Co-germinants: taurocholic acid (bile acid) and glycine
      - Germinant receptor: CspC (Francis et al. 2013)

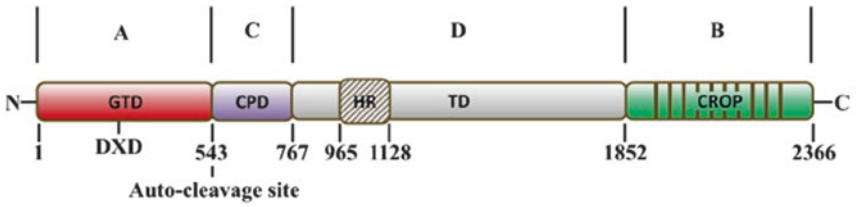
  - CspBA: serine protease that cleaves the cortex hydrolase SleC, leading to core rehydration

- Iron acquisition (Ho and Ellermeier 2015):
  - Siderophores: no biosynthetic enzymes are found in genome annotation
  - **Feo1,2,3**: Fe^{+2} ion transport; expression of Feo1, three is regulated by the Fe^{+2}-Fur regulatory system

- Biofilm formation – capable of forming mono-species and multi-species biofilms on abiotic and biotic surfaces (Đapa et al. 2013):
  - Unclear if biofilms play a role in virulence or persistence in vivo
  - Regulated by the Luxs/AI-2 quorum-sensing system

– Damage to host cells:

- Exotoxins:
  - ***C. difficile* binary toxin (CDT)** (Sundriyal et al. 2009):
    - ADP-ribosylates G-actin; same structure and activity as *C. botulinum* C2 toxin and *C. perfringens* iota (ι) toxin
  - **Toxin A (TcdA)** and **toxin B (TcdB)** (Chen et al. 2015; Jank et al. 2015):
    - Glucosyltransferase activity
    - Member of large clostridial cytotoxin (LCC) family; toxin B is ~1,000 more potent than toxin A
    - *tcdA* and *tcdB* genes are encoded on pathogenicity island PaLoc along with regulatory proteins (Hundsberger et al. 1997; Janoir 2016):
      - TcdR: alternative sigma factor – activates *tcdA* and *tcdB* expression
      - TcdE: putative holin – excellular release of toxin
      - TcdC: putative negative regulator of TcdA and TcdB synthesis
    - Each encodes a multi-domain polypeptide (Fig. 3.5):
      - A domain: glucosyltransferase domain (GTD)
      - B domain: receptor-binding domain
      - C domain: cysteine protease; auto-proteolytic activity
      - D domain: translocation domain
    - Mechanism of action (Fig. 3.6):
      - Internalized by endocytosis; endosome acidification results in translocation across membrane
      - C domain-dependent auto-cleavage releases the GTD



The ABCD model of *Clostridium difficile* toxins (toxin B as example). A domain, containing the DXD motif, is located at the *N* terminus (GTD, red, amino acids 1–543) and harbors the glucosyltransferase activity. The *C* terminus is characterized as receptor binding domain (green, B domain), consisting of combined repeat oligopepides (CROPs). The cysteine protease domain (CPD, purple, amino acids 544–767) is involved in the auto-cleavage process of the toxins. The middle part of TcdB is the translocation domain (TD, gray, D domain), within which there is a short hydrophobic region (HR, oblique line, amino acids 956–1128). The TD is considered to be involved in pore formation, conformational changes and the delivery of the GTD and CPD.

Fig. 3.5 *C. difficile* toxin B structure (From: Chen et al. 2015)

  - GTD transfers glucose molecule from UDP-glucose to host Rho/Rac/Cdc42 GTPases:
    - Glucose molecule is attached to conserved Thr amino acid with the switch 1 region of the GTPase
    - Modification blocks the interactions of the GTPase with downstream effectors
    - Leads to disruption of the actin cytoskeleton; loss of cell-cell contacts
    - Induces release of pro-inflammatory cytokines and chemokines, leading to inflammatory responses
- Evasion of host immune system:
  - Naturally resistant to host lysozyme – probably due to N-deacetylation of the GlcNAc in the peptidoglycan structure (Peltier et al. 2011)

- *C. perfringens* – most commonly isolated species (Rood 1998):
  - Adherence to host cells:
    - **NanI, NanJ,** and **NanH** (Li et al. 2011):
      - Sialidases (neuraminidases)
      - Cleave sialic acid-containing glycoproteins and gangliosides that are in mucin and on the surface of host cells
      - Increases adherence to host cells and extracellular matrix components
    - **Cna** (Jost et al. 2006):
      - Collagen I-binding protein; plasmid encoded

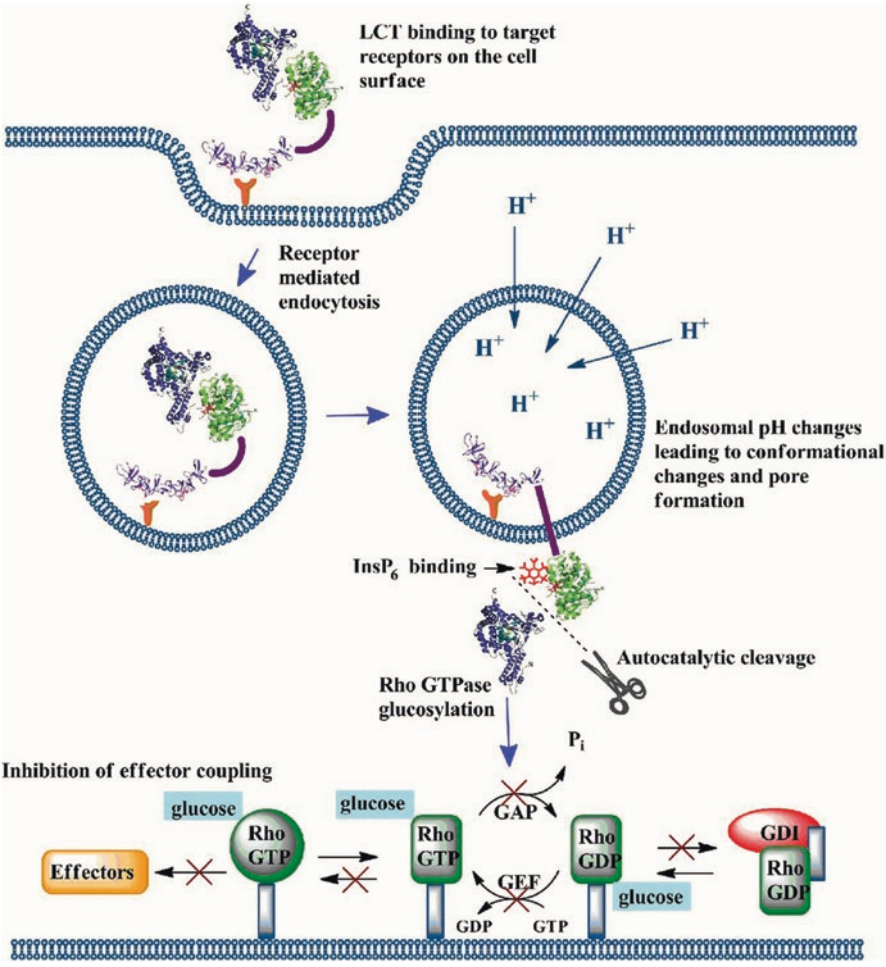


Fig. 3.6 Toxin internalization (From: Davies et al. 2011)

- Growth in host milieu:
  - Endospore germination: essential for vegetative growth, and hence, *C. perfringens* food poisoning (see *C. botulinum* above)
    - Germinants: L-alanine, L-valine, L-asparagine, and KCl (either alone or mixed), Ca^{2+}-DPA, Na^{+}, and inorganic phosphate (P_i)
    - Germinant receptors: GerKA, GerKB, GerKC, and GerAA transmembrane proteins; respond with different efficacies to different germinants (Paredes-Sabja et al. 2008)
    - Unlike *C. botulinum* and *C. difficile*, Ca^{2+}-DPA does not directly induce cortex hydration and hydrolysis in *C. perfringens*

- Iron acquisition – essential for growth; able to grow in vitro on hemoglobin, $FeCl_3$:
  - Genome annotation indicates the presence of one Fe^{+2} acquisition system, two heme acquisition systems, three siderophore-mediated iron acquisition systems, and one ferric citrate iron acquisition system
  - **FeoAB**: Fe^{+2} acquisition system (Awad et al. 2016)
  - **Cht**: heme acquisition; cell surface proteins ChtD and ChtE are involved in heme binding (Choo et al. 2016)
- Biofilms: capable of forming mono-species and multi-species biofilms on abiotic and biotic surfaces

– Damage to host cells:

- Exotoxins [see (Popoff 2014) and references therein]
- Many *C. perfringens* exotoxins are encoded on plasmids – CPE, CPB2, BEC, beta (β) toxin, epsilon (ε) toxin, and iota (ι) toxin (Freedman et al. 2015):
  - **Perfringolysin O [PFO; theta (θ) toxin]** (Tweten 1988a, b):
    - Member of the clostridial family of cholesterol-dependent pore-forming cytolysins (CDCs) – botulinolysin, septicolysin, sordellilysin, and tetanolysin
    - Forms large pore complexes (up to 50 PFO molecules) in cholesterol-containing membranes – disrupts host cell membranes and ion permeability
  - ***Clostridium perfringens*** **enterotoxin (CPE)** (Mitchell and Koval 2010):
    - Heat-labile cytolysin; encode on both the chromosome (70% of food poisoning cases) and plasmid (30%)
    - Causes both food-borne illness and non-food-borne GI symptoms
    - Produced when *C. perfringens* sporulates
    - Member of the aerolysin family of heptameric β-PFT pore-forming toxins; same activity as epsilon toxin (see below); disrupts membrane permeability
    - Binds to claudins – family of host membrane proteins; major components of intercellular tight junctions
  - Four major lethal exotoxins – alpha (α), beta (β), epsilon (ε), and iota (ι):
    - Basis for classification of *C. perfringens* strain types A–E:
      - Type A: only produces α toxin; most clinically relevant
      - Type B: produces α, β, and ε toxins
      - Type C: produces α and β toxins

  - Type D: produces α and ε toxins
  - Type E: produces α and ι toxins

- **Alpha (α) toxin** (Oda et al. 2015; Sakurai et al. 2004):
  - Lecithinase (phospholipase C) and sphingomyelinase activities; Zn metalloenzyme
  - Most important exotoxin associated with myonecrosis (gas gangrene)
  - Cytolytic toxin: removes charged head groups of membrane phospholipids; lyses host cells causing tissue necrosis and massive hemolysis
  - Also induces cell signaling processes leading to edema and cell death
- **Beta (β) toxin** (Shatursky et al. 2000):
  - Forms ion channels/pores in intestinal epithelial cell membranes
  - Belongs to *Staphylococcus aureus* alpha-toxin family; also delta (δ) toxin below
  - Produces necrotic lesions on intestinal mucosa; progresses to enteritis necroticans (see above)
- **Epsilon (ε) toxin (ETX)** (Alves et al. 2014; Popoff 2011):
  - Heptameric pore-forming β-PFT; disrupts membrane permeability and ion flow, same aerolysin family as *C. difficile* CPE
  - Produced as inactive pro-toxin; activated by trypsin cleavage
  - Binds to the host membrane protein HAVCR1 (hepatitis A virus cellular receptor 1) in vitro; unknown binding in vivo
  - Increases vascular permeability – edema in the lungs and kidneys
  - Can cross the blood–brain barrier and accumulate in the brain; can cause deadly neuronal damage
  - Extremely potent, can produce rapidly fatal enterotoxemia
  - May be associated with multiple sclerosis (MS) patients
- **Iota (ι) toxin** (Sakurai et al. 2003):
  - Dermonecrotic A-B exotoxin
  - B subunit (Ib): required for internalization
  - A subunit (Ia): ADP-ribosylation activity; modifies host G-actin; similar function as *C. botulinum* C2 toxin (see above)

– Minor toxins:

- **Beta2 toxin**: CPB2; same activity as beta toxin (see above) but not structurally homologous; role in human disease is unclear (van Asten et al. 2010)

- **BEC (binary enterotoxin of *C. perfringens*)**: similar activity to iota toxin; found in strains that don't produce CPE (Yonogi et al. 2014)
- **Delta (δ) toxin** (Seike et al. 2016):
  - Hemolysin; same family as beta toxin (see above)
  - Can induce caspase-independent rapid cell death
- **Kappa (κ) toxin** (Matsushita et al. 1994):
  - Extracellular Zn-dependent collagenase
  - Destroys host collagen; enhances dissemination of cells and toxins
  - Has necrotizing activity
- **Lambda (λ) toxin**: Zn metalloprotease (Jin et al. 1996)
- **mu (μ) toxin** – ColA protein (Matsushita and Okabe 2001):
  - Extracellular hyaluronidase; has necrotizing activity
- **nu (ν) toxin**: deoxyribonuclease (DNAase); hemolytic activity

- *C. septicum*:
  - Adherence to host cells: no known functional adhesins
  - Dissemination in host – swarming motility may play a role:
    - **Beta (β) toxin**: DNAase; spreading factor
    - **Gamma (γ) toxin**: hyaluronidase; spreading factor
    - **Sialidase** (neuraminidase): breaks down host mucin
  - Damage to host cells:
    - Exotoxins:
      - **Septicolysin**: member of the clostridial family of cholesterol-dependent pore-forming cytolysins (CDCs) – botulinolysin, perfringolysin O, sordellilysin, and tetanolysin (Popoff 2014)
        - Disrupts host cell membranes
      - **Alpha (α) toxin** (Knapp et al. 2010):
        - Heptameric pore-forming β-PFT; same aerolysin family as *C. perfringens* CPE and epsilon toxin
        - Binds to host GPI-anchored proteins
        - Pores allow Ca^{2+} influx, leading to programmed necrosis
      - **Delta (δ) toxin**: oxygen-labile hemolysin
- *C. sordellii* (Aldape et al. 2006):
  - Adherence to host cells: no known functional adhesins
  - Dissemination in host:

- **NanS** (Aldape et al. 2007):
  - Sialidase (neuraminidase)
  - Involved in the life-threatening leukemoid reaction (LR) by inducing the growth of HL60 promyelocytic leukemia cells
- **Spreading factors**: genome annotation suggests the existence of collagenase, hyaluronidase, and urease genes (Scaria et al. 2015)

– Growth in host cells:

- Endospore germination – essential for vegetative growth, and hence, disease in the host (see *C. botulinum* above):
  - Co-germinants: small amino acid (L-alanine or glycine), basic amino acid, aromatic amino acid, and bicarbonate; enhanced germination at acidic pH (Ramirez and Abel-Santos 2010)
  - Germinant receptor: unknown
- Iron acquisition – required:
  - **FeoAB**: Fe^{+2} – acquisition system (Awad et al. 2016)

– Damage to host cells:

- Exotoxins:
  - **Sordellilysin**: member of the clostridial family of cholesterol-dependent pore-forming cytolysins (CDCs) – botulinolysin, perfringolysin O, septicolysin, and tetanolysin (Popoff 2014)
    - Disrupts host cell membranes
  - **Lethal toxin (LT, TcsL)**: member of the LCC family of glucosyltransferases; analogous to *C. difficile* toxin A and toxin B (Chen et al. 2015; Jank et al. 2015)
  - **Hemorrhagic toxin (HT, TcsH)**: member of the LCC family of glucosyltransferases; analogous to *C. difficile* toxin A and toxin B (Chen et al. 2015; Jank et al. 2015)
  - **Phospholipase C**: same activity as *C. perfringens* alpha (α) toxin

- *C. tetani*:

  – Adherence to host cells: no known functional adhesins

  – Damage to host cells:

  - Exotoxins:
    - **Tetanolysin**: member of the clostridial family of cholesterol-dependent pore-forming cytolysins (CDCs) – botulinolysin, perfringolysin O, septicolysin, and sordellilysin (Popoff 2014)
      - Disrupts host cell membranes

- **Tetanus toxin (TeNT; tetanospasmin)** (Grumelli et al. 2005):
  - Heat-labile and oxygen-labile Zn metalloprotease; plasmid encoded
  - A-B exotoxin: single polypeptide; only one isoform (unlike eight BoNT isoforms)
  - Cleaved into light chain (A subunit) and heavy chain (B subunit); A subunit has Zn protease activity similar to BoNT A subunits
  - Mechanism of action – final target; inhibitory interneurons in central nervous system:
    - TeNT initially binds to peripheral nerve terminals
    - Unlike BoNT, which acts locally, TeNT is transported to the spinal cord inside motoneuron axons; accumulation within the spinal cord eventually leads to *trans*-synaptic movement into inhibitory interneurons
    - B subunit binds polysialoganglioside (PSG) and synaptic vesicle glycoprotein 2 (SV2A, SV2B) receptors on the surface of inhibitory interneurons
    - Acidification within interneuron endosomes causes release of the A subunit into the cytosol
    - A subunit cleaves the v-SNARE synaptobrevin II, part of the synaptic vesicle fusion machinery
    - Results in blockage of release of the inhibitory neurotransmitters glycine and gamma-aminobutyric acid (GABA)
    - Subsequent loss of inhibitory control of motor neuron activity results in rigid spastic paralysis, eventually followed by respiratory or heart failure (cause of death):
      - Spasms are due to opposing muscles inappropriately contracting simultaneously
    - Mortality rate is very high without treatment or vaccine

Regulation of Virulence Factor Expression

- *C. botulinum* sporulation and neurotoxin production (Cooksley et al. 2010):
  - Regulated by AgrB-D quorum-sensing systems (two in *C. botulinum*); similar to Agr system from *Staphylococcus aureus*:
    - **AgrD**: encodes autoinducer peptide (AIP)
    - **AgrB**: encodes transmembrane protein that modifies and secretes AgrD product
    - Likely to interact with a two-component phosphorelay system (TCS), as yet uncharacterized

- *C. perfringens* sporulation and toxin production (Chen et al. 2014):
  - Expression of the PFO, CPE, α toxin, β toxin, and κ toxins is induced in response to host cell interactions and metabolic cues
  - The different toxins that are present in the four *C. perfringens* type A–E strains (see above) are regulated by two-component phosphorelay systems (TCSs) and quorum-sensing systems
  - **VirS/VirR-VR-RNA** – two-component phosphorelay system (TCSs); ~18 TCSs in *C. perfringens*:
    - **VirS**: sensor kinase; senses the AgrD autoinducer peptide made by Agr system; leads to VirS autophosphorylation and subsequent transfer of phosphate to VirR
    - **VirR** – response regulator:
      - Activated VirR directly regulates five genes, including *pfoA* (PFO) and the VR-RNA regulatory RNA molecule, by binding to VirR boxes upstream of transcription start sites
    - **VR-RNA (*vrr*)**: regulatory RNA molecule; may be analogous to RNA III of *Staphylococcus aureus*; may act as a riboswitch to regulate translation of mRNAs
    - **Agr** system: quorum-sensing system (see *C. botulinum* above)
    - Sporulation-associated CPE expression is also regulated by quorum sensing and TCSs
  - **LuxS/AI-2**: quorum-sensing system; regulates α toxin, β toxin, and θ toxin production (Ohtani et al. 2002)

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Chapter 4
Corynebacterium spp.

Genomics, Morphologies, and Growth Characteristics

- **Genomics:**
  - *Corynebacterium diphtheriae*: chromosome 2,488,635 bp; 2320 predicted ORFs (Cerdeno-Tarraga et al. 2003)
- **Cell morphology:**
  - Rod-shaped cells; irregular, club-shaped (*Coryne*), or V-shaped arrangements (Fig. 4.1)
    - Can undergo snapping movements after cell division – cause cells to look like Chinese letters or palisades
  - Club shape due to metachromatic polyphosphate granules at ends of cells
- **Gram stain:**
  - Gram positive
- **Growth:**
  - Aerobes; catalase positive
  - Found in soil, water, and plants; nonpathogenic species are normal microbiota of the skin and most mucous membranes
  - >120 species; *C. diphtheriae* is only a primary pathogen (due to toxin; see below):
    - *C. amicolatum*, *C. striatum*, *C. urealyticum*, *C. ulcerans*, *C. xerosis*, *C. tuberculosis*, and *C. jeikeium* are opportunistic pathogens of immunosuppressed patients

D.I. Johnson, *Bacterial Pathogens and Their Virulence Factors*,
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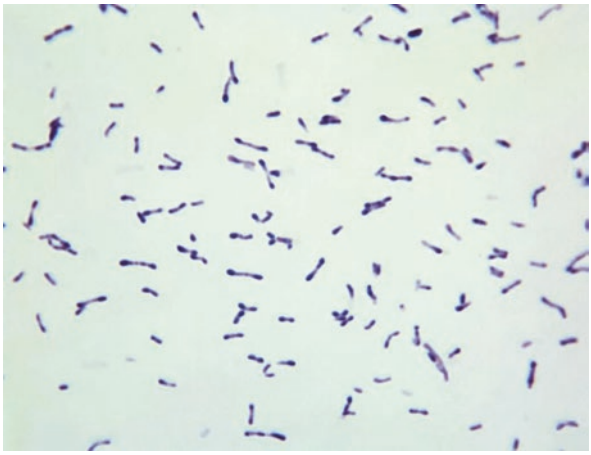

Fig. 4.1 *C. diphtheriae* cells (From: PHIL #12163)

- *C. jeikeium*: "group JK"; lipophilic multidrug-resistant nosocomial pathogen; frequently associated with bone marrow transplant patients and intravascular devices

Disease States Associated with *Corynebacterium diphtheriae*

- Diphtheria – pharyngeal and cutaneous infections:
  - Infection of tissues of the upper respiratory tract
  - Symptoms: mild fever, chills, sore throat, cough ("diphtheritic croup"), and difficulty swallowing and breathing; laryngeal diphtheria can lead to a swollen neck and throat ("bull neck")
  - Hallmark symptom is the presence of a dense, gray pseudomembrane structure composed of dead cells and fibrin matrix (Fig. 4.2)
  - Systemic infections can include toxic myocarditis (most likely the cause of death), cardiac arrhythmias, urinary tract infections, and cranial and peripheral nerve palsies and can also form biofilms on indwelling medical devices
  - Person-to-person airborne transmission, usually through coughs or sneezes, and direct contact with infected persons or fomites

Virulence Factors

- Adherence to host cells (Rogers et al. 2011):
  - **SpaA-, SpaD-, and SpaH-type pili** (Ton-That and Schneewind 2003, 2004):
    - Three antigenically distinct pilus structures

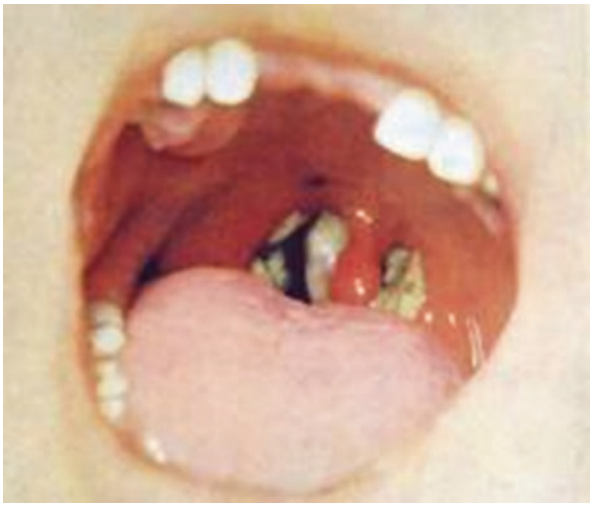

Fig. 4.2 *C. diphtheriae* pseudomembrane (From: http://medical-dictionary.thefreedictionary.com)

- Directly attached to the cell wall using sortases; common theme with Gram-positive cells (Mazmanian et al. 1999):
  - Transpeptidase enzymes that directly link pilin proteins to amino acids within the peptidoglycan cell wall
  - *C. diphtheriae* has five class C pilin-specific sortases: SrtA, SrtB, SrtC, SrtD, and SrtE
- SpaA-type pili topology (Fig. 4.3):
  - **SpaA**: major pilin; forms the shaft of the pilus; **SpaD** and **SpaH** are the major pilins for the other two pili
  - **SpaB** is located along the shaft and at the base of the pilus; **SpaE** and **SpaG** for the other two pili
  - **SpaC** is located at the tip of the pilus; **SpaF** and **SpaI** for the other two pili
  - SpaB and SpaC play the major role in adhering to host tissue (Mandlik et al. 2007)
- Essential for host cell binding specificity:
  - SpaA type: binds to pharyngeal epithelial cells
  - SpaD type: binds to the lung and laryngeal epithelial cells
  - SpaH type: binds to the lung and laryngeal epithelial cells

– Cell wall:

- **CdiLAM**: lipoarabinomannan (LAM) component of cell wall; binds to cultured HEp-2 epidermoid larynx carcinoma cells (Moreira et al. 2008)
- **DIP1281**: cell surface protein; binds to human pharyngeal cells (Ott et al. 2010)

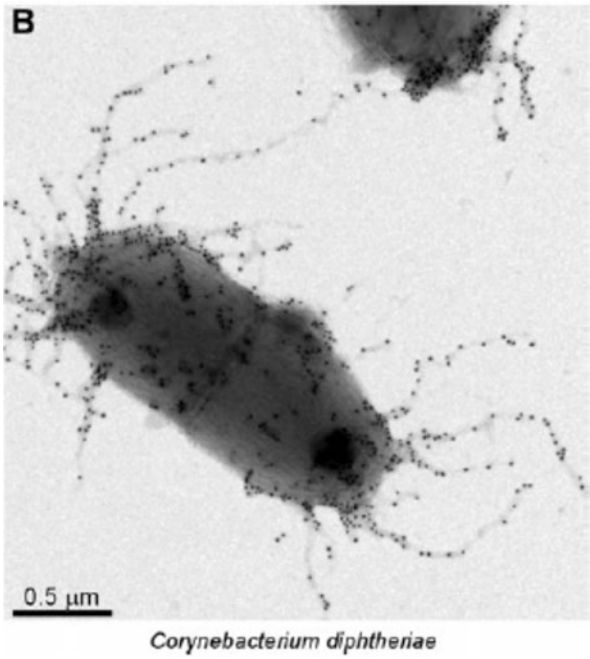

Fig. 4.3 Spa-type pili (From: Rogers et al. 2011)

- Growth in human host:
  - Iron acquisition:
    - Siderophore:
      - **Corynebactin** (Russell et al. 1984):
        - Synthesized (*ciuE*) and uptaken (*ciuA-D*) by the *ciuA-G* gene cluster (Kunkle and Schmitt 2005)
        - Regulated by the Fe-dependent DtxR repressor (see Regulation, below)
    - Hemin acquisition (Burgos and Schmitt 2016):
      - **HmuTUV** and **HtaA and HtaB**:
        - Acquire and transport hemin, hemoglobin (Hb), and hemoglobin–haptoglobin (Hb–Hp) complexes
        - In the cytosol, the HmuO heme oxygenase releases the heme-associated iron
        - Expression of *humO* is repressed in high-iron environments by Fe^{+2}-bound DtxR
        - Heme activates transcription through the **ChrSA** (predominant) and **HrrSA** two-component phosphorelay systems

- **ChtA and ChtC** (Allen and Schmitt 2015):
  - Bind hemin, hemoglobin (Hb), and hemoglobin–haptoglobin (Hb–Hp) complexes

– Biofilms: capable of forming biofilms on indwelling medical devices (Gomes et al. 2009)

- Damage to host cells:
  - **Diphtheria toxin (DT; DTx)** (Simon et al. 2014):
    - A–B exotoxin; one of the most widely studied bacterial protein toxins
    - Encoded by single *tox* gene; found on a lysogenic β-phage bacteriophage
    - Belongs to the family of bacterial ADP-ribosyltransferase toxins (bARTTs):
      - Catalyzes the transfer of ADP-ribose from nicotinamide adenine dinucleotide (NAD) to an amino acid in the target protein, thereby inactivating the protein
    - DT targets a modified histidine amino acid called diphthamide (Van Ness et al. 1980) in eukaryotic translation elongation factor-2 (eEF-2), thereby inhibiting protein translation and leading to cell death
    - Multi-step process for toxin processing, entry into host cells, and activation in host cytosol (Murphy 2011):
      - DT protein is proteolytically cleaved into two polypeptides:
        - A subunit: 21 kDa catalytic domain
        - B subunit – 41 kDa transmembrane and receptor binding domain:
        - B subunit binds to heparin-binding epidermal growth factor (hb-EGF) precursor in host cell membranes
      - After internalization in clathrin-coated endosomal vesicles, acidification of the vesicles results in refolding of the B subunit transmembrane domain, leading to the formation of pores in the vesicles
      - The A subunit dissociates from the B subunit and translocates through the pores into the host cytosol, where it uses NAD to ADP-ribosylate eEF-2

Regulation of Virulence Factor Expression

- Regulation of DT production:
  - **DtxR** (Schmitt and Holmes 1991):
    - Repressor protein; homologue of *Mycobacterium tuberculosis* IdeR repressor
    - Encoded by chromosomal *dtxR* gene; not on *tox* β-phage

- Regulation by Fe^{+2} concentrations:
  - Under high Fe^{+2} levels (outside host): DtxR is activated by Fe^{+2} binding – represses toxin production
  - Under low Fe^{+2} levels (in host): DtxR is inactive – induces toxin production
  - Resultant host cell death releasing Fe^{+2}, where it can be scavenged by siderophores (see above), which are also regulated by DtxR

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Chapter 5
Enterococcus spp.

Genomics, Morphologies, and Growth Characteristics

- **Genomics** (Palmer et al. 2014):
  - *Enterococcus faecalis* V583 (vancomycin-resistant clinical isolate) chromosome: 3,218,031 bp; 3182 predicted ORFs (Paulsen et al. 2003)
  - *Enterococcus faecium* TX16 chromosome: 2,698,137 bp; 2703 predicted ORFs (Qin et al. 2012)
- **Cell morphology:**
  - Cocci: diplococci or short chains (Fig. 5.1); difficult to distinguish from *Streptococcus*
- **Gram stain:**
  - Gram positive
- **Growth** (Ramsey et al. 2014):
  - Facultative anaerobes; catalase negative
  - Can be alpha hemolytic (predominate) or gamma hemolytic on BAP; formerly classified as Group D *Streptococcus*
  - Can grow in harsh environments – high temperatures (up to 45 °C), low and high pH, and high salt (6.5%) concentrations:
    - Resistant to detergents, ethanol, bile salts (bile esculin), heavy metals, and azide
    - Can be resistant to a wide variety of antibiotics, including penicillins, cephalosporins, aminoglycosides, linezolid, daptomycin, licosamides, and the glycopeptide vancomycin (vancomycin-resistant enterococci, VRE) (Arias and Murray 2012; Hollenbeck and Rice 2012):
      - *E. faecium* displays more antibiotic resistance than *E. faecalis*

D.I. Johnson, *Bacterial Pathogens and Their Virulence Factors*,
https://doi.org/10.1007/978-3-319-67651-7_5

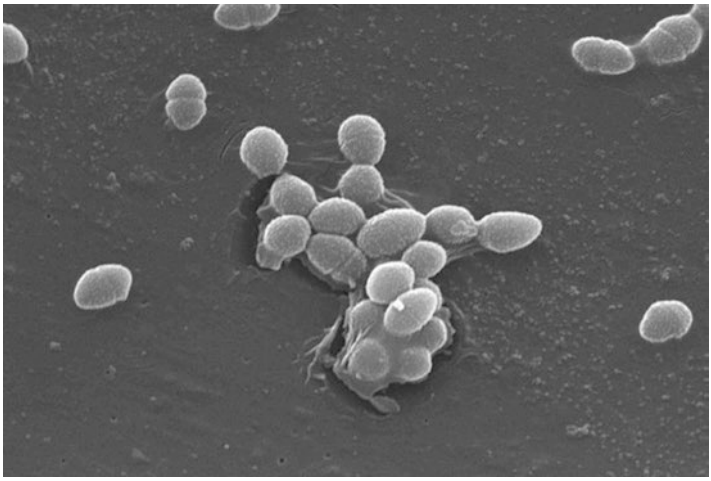

Fig. 5.1 *E. faecalis* cells (From: PHIL #258)

    - Diverse metabolism: can use glucose, fructose, arabinose, glycerol, lactate, malate, citrate, arginine, and keto acids as energy sources; can be used to classify different species
- Normal microbiota of mammals, reptiles, birds, and insects:
    - Human gastrointestinal tract, oral cavity, and vagina
- Found in soil, water, and food as fecal contaminant
- Biofilm former (see below)
- >40 species: *E. faecalis* (90–95%) and *E. faecium* (5–10%) are the predominant gastrointestinal tract commensals

Disease States Associated with *Enterococcus* spp. (Higuita and Huycke 2014)

- Opportunistic pathogens – member of the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp.) family of antibiotic-resistant nosocomial pathogens:
    - Transmission: person to person, person to medical device, or through contaminated water or food
    - Community-acquired (CA) and hospital-acquired (HA; nosocomial) infections
    - Immunocompromised individuals are increasingly susceptible

- Many of these disease states are associated with biofilm formation on host tissues and indwelling medical devices (Dunny et al. 2014)
- Ascending urinary tract infections (UTIs) and catheter-associated urinary tract infections (CAUTIs):
  - Lower urinary tract infections – frequently seen in older men
  - Upper urinary tract infections – can lead to bacteremia; endocarditis
  - Most likely acquired in healthcare environments; ~15% of ICU-acquired UTIs
  - CAUTIs: commonly associated with biofilm formation
- Bacteremia: second leading cause of hospital-acquired bacteremia; high risk of death with immunocompromised patients
- Peritonitis: infection of the abdominal lining; found with liver cirrhosis or in patients receiving chronic peritoneal dialysis
- Infective endocarditis – natural and prosthetic heart valve infections; third most common cause:
  - *E. faecalis* is more common that *E. faecium*
  - Cause 5–15% of total cases with 9–15% mortality rate
  - Enterococci bacteremia leads to biofilm plaque formation on heart valves; initial source of the enterococci is the GI or GU tract
  - Difficult to treat due to multidrug resistance
- Postsurgery intra-abdominal or pelvic infections: enterococci are isolated from these areas, but it is unclear if they play a causative role in the infection

Virulence Factors

- Adherence to host cells (Hancock et al. 2014; Hendrickx et al. 2009b):
  - Fimbrial adhesins (Fig. 5.2):
    - Gram-positive pili: polymers of LPxTG-containing surface proteins that are covalently attached to cell wall of peptidoglycan by the sortase transpeptidase enzymes (Clancy et al. 2010); structurally different from Gram-negative pili
    - **EbpABC pili** (Nallapareddy et al. 2006):
      - *E. faecalis* endocarditis and biofilm-associated pili
      - EbpC: major pilin subunit; EbpA is located at the tip of the EbpC polymer and EbpB is located at the pilus base
      - Involved in biofilm-dependent UTIs and endocarditis (Singh et al. 2007)
      - Binds ECM components fibrinogen and collagen (Montealegre et al. 2015)

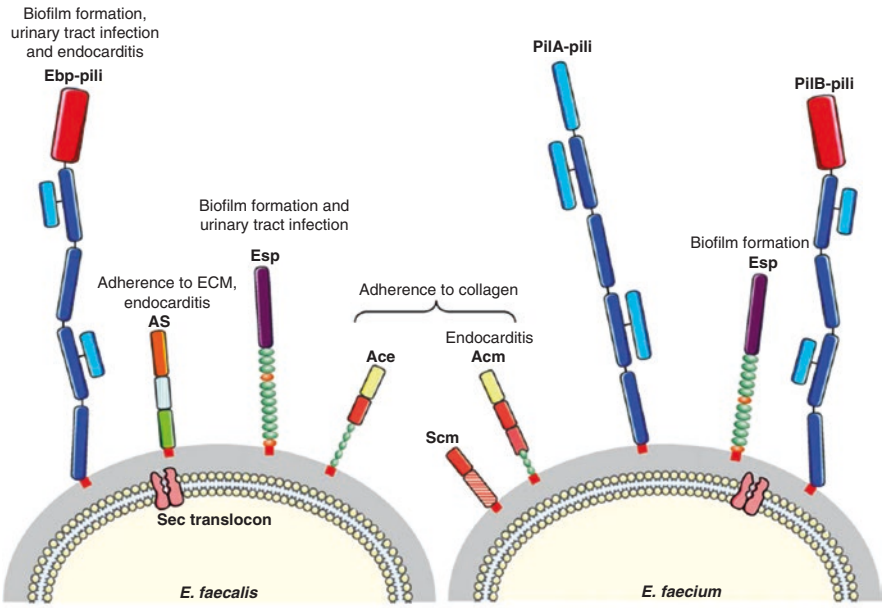

Fig. 5.2 *E. faecalis* and *E. faecium* adhesins (From Hendrickx et al. 2009b)

- **PilA and PilB pili** (Hendrickx et al. 2008):
  - *E. faecium* pili
  - PilA and PilB – major pilin subunits:
    - PilF: minor pilin subunit associated with PilA pili (Hendrickx et al. 2010)
  - Expressed at 37 °C but not 21 °C; enriched in HA-*E. faecium* isolates; suggests an, as yet unknown, function in virulence

– Afimbrial adhesins (Garsin et al. 2014; Hendrickx et al. 2009b):

- LPxTG-containing surface proteins: covalently attached to cell wall of peptidoglycan by sortase transpeptidase enzymes (see above)
- **Esp** (Heikens et al. 2011; Toledo-Arana et al. 2001):
  - *E. faecium* and *E. faecalis*
  - Plays a role in biofilm-associated endocarditis and UTIs
- **Asc1 and Asc10** (Waters et al. 2004):
  - *E. faecium*; belong to class of *E. faecium* aggregation substance (AS) proteins
  - Binds to lipoteichoic acid – enables conjugative DNA transfer between enterococci

- Functions in aggregation and binding to renal tubule cells, epithelial cells, enterocytes, and macrophage – induced by pheromone peptides
- May also bind ECM components fibrin, fibronectin, thrombospondin, vitronectin, and collagen type I (Hendrickx et al. 2009b)

- **SgrA** (Hendrickx et al. 2009a):
  - *E. faecium*
  - Binds to ECM components fibrinogen and nidogen (entactin)
  - Functions in biofilm formation on indwelling medical devices
- **LPxTG MSCRAMMs** (microbial surface component recognizing adhesive matrix molecules):
  - Recognize components of the host ECM; 15–17 predicted MSCRAMMs in *E. faecalis* and *E. faecium*
  - **Ace** (Rich et al. 1999):
    - *E. faecalis*
    - Binds to collagen IV, collagen I, laminin, and dentin (Nallapareddy et al. 2000):
      - Collagen binding is enhanced by GelE-dependent proteolysis
    - Functionally analogous to *Staphylococcus aureus* Cna protein
  - **Acm** (Nallapareddy et al. 2003):
    - *E. faecium*
    - Binds to collagen I and collagen IV
    - Functionally analogous to *Staphylococcus aureus* Cna protein
  - **Scm** (Sillanpaa et al. 2008):
    - *E. faecium*
    - Binds to collagen IV and fibrinogen
  - **EcbA** (Hendrickx et al. 2009a):
    - *E. faecium*
    - Binds to collagen V and fibronectin

- Growth in human host:
  - Iron acquisition:
    - Iron uptake mechanisms found in *E. faecalis* V583 annotated genome (Paulsen et al. 2003); no experimental evidence for a role in iron acquisition:
      - **FeuA and FatB**: Fe-chelator ABC transporters
      - **FeoA and FeoB**: ferrous iron uptake
      - Regulated by the Fe^{+2}-Fur repressor (ferric uptake regulator)

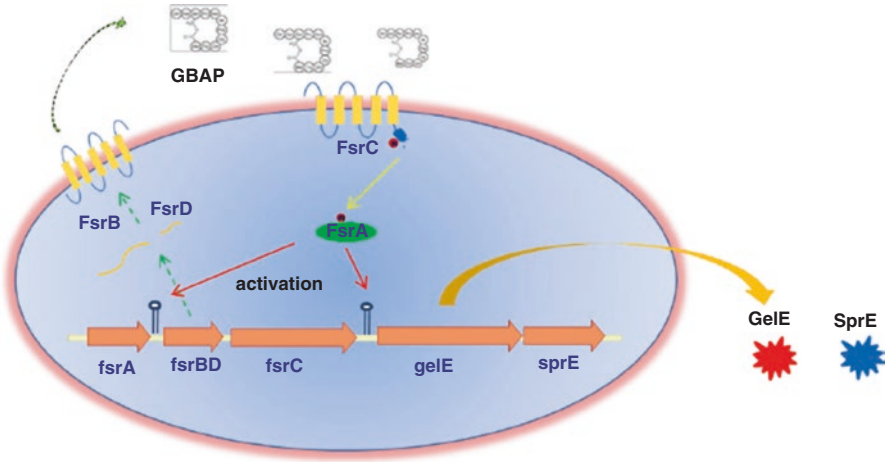

Fig. 5.3 Fsr quorum sensing (From: DebRoy et al. 2014)

- Biofilm formation (Dunny et al. 2014):
  - Essential for virulence
  - Biofilms can form on host tissues and indwelling medical devices
  - Many of the abovementioned adhesins are important for biofilm formation
  - Regulated by the Fsr quorum-sensing system (DebRoy et al. 2014; Dunny et al. 2014)
    - **FsrABCD** – two-component phosphorelay system (Fig. 5.3), analogous to *Staphylococcus aureus agrABCD* quorum-sensing system:
      - Autoregulates its own expression and regulates expression of Ace adhesin, GelE gelatinase, and SprE protease (see below) (Pinkston et al. 2011; Qin et al. 2000; Shankar et al. 2012)
      - FsrA: response regulator transcription factor; phosphorylated by FsrC
      - FsrC: sensor histidine kinase; senses extracellular levels of FsrD peptide
      - FsrD: encodes gelatinase biosynthesis-activating pheromone (GBAP) quorum-sensing peptide (Nakayama et al. 2006)
      - FsrB: cysteine protease; processes FsrD peptide
- **GelE** (Mäkinen et al. 1989):
  - *E. faecalis* gelatinase exoenzyme
  - Important for virulence, biofilm formation, and dissemination
  - Zinc matrix metalloproteinase (MMP) family

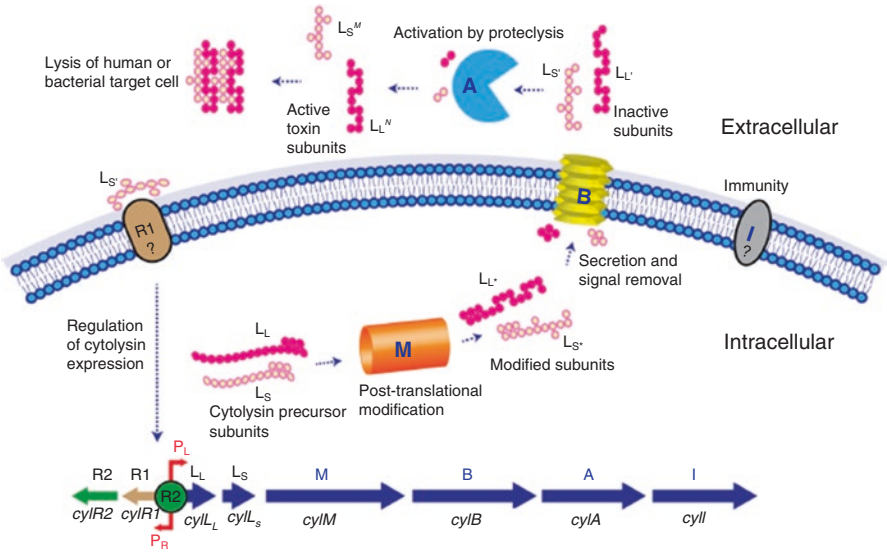

Fig. 5.4 Cytolysin processing (From: Garsin et al. 2014)

- Can degrade host proteins and ECM components, such as collagen, fibrin, and hemoglobin
- Can degrade complement proteins C3a and C3b – blocks opsonization and complement-mediated lysis mediated by membrane attack complex (MAC) formation (Park et al. 2007)
- Expression is regulated by Fsr quorum-sensing system (see above)

– **SprE** (Qin et al. 2000):

- *E. faecalis* serine protease exoenzyme; important for virulence; unknown substrates
- Expression is regulated by Fsr quorum-sensing system (see above)

– **Hyaluronidase** (Kayaoglu and Orstavik 2004):

- *E. faecalis* spreading factor
- Cleaves ECM component hyaluronan (hyaluronic acid); facilitates dissemination

• Damage to host cells:

– **Cytolysin** (hemolysin) (Fig. 5.4) (Booth et al. 1996; Garsin et al. 2014):

- Lyses RBCs, neutrophils, and macrophage; effective against Gram-positive bacteria (bacteriocin)
- Two peptide subunits – $CylL_L$ and $CylL_S$; both are required for lytic activity:
  - Proteolytically cleaved and secreted by CylB
  - Extracellularly cleaved and activated by CylA (Segarra et al. 1991)

- Regulated by the CylR1/CylR2 two-component phosphorelay system (Coburn et al. 2004; Haas et al. 2002)

- Evasion of host immune system:
  - **Capsule** (Thurlow et al. 2009b):
    - *E. faecalis*
    - Four serotypes (A, B, C, or D) based on immunological detection:
      - Only serotypes C and D actually express capsule polysaccharides (Thurlow et al. 2009a)
      - Serotypes A and B are against lipoteichoic acid, not capsule
    - Antiphagocytic: prevents detection of complement protein C3 on the surface of *E. faecalis*
    - Prevents detection of lipoteichoic acid by the host immune system
  - **GelE gelatinase**: blocks complement-mediated lysis (see above)
  - **Mn-superoxide dismutase, catalase, and peroxidases**: protect against host ROS (Ramsey et al. 2014)

Regulation of Virulence Factor Expression (DebRoy et al. 2014)

- **TCSs** – two-component phosphorelay systems:
  - *E. faecalis*: at least 15 TCSs
  - Respond to multiple host extracellular signals, including quorum sensing (Fsr system, see above), antimicrobials, bile salts, serum, and ethanolamine utilization
- **SlyA** (Michaux et al. 2011):
  - Member of MarR family of transcription factors
  - *SlyA* deletion mutants are more virulent, unlike the deletion mutants of other MarR family transcription factors, suggesting that SlyA may repress the synthesis of as yet unknown virulence factors
  - May play a role in resistance to bile salts
- **EbpR** (Bourgogne et al. 2007):
  - Member of the AtxA/Mga family of transcriptional factors
  - Positive activator: regulates the levels of the Ebp pili genes *ebpABC*, which functions in biofilm formation and adherence

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Chapter 6
Listeria spp.

Genomics, Morphologies, and Growth Characteristics

- **Genomics:**
  - *Listeria monocytogenes* chromosome (serovar 1/2a): 2,944,528 bp; 2853 predicted ORFs (Glaser et al. 2001)
- **Cell morphology:**
  - Rod-shaped cells; sometimes in short chains (Fig. 6.1)
  - Non-endospore former
  - Peritrichous flagella at 23 °C (outside host) but not at 37 °C (inside host)
- **Gram stain:**
  - Gram positive
- **Growth:**
  - Facultative anaerobes; catalase positive
  - Ubiquitous in nature: soil, vegetation, and animal feces
  - Cold enrichment: ability to grow at 4 °C
    - Major problem in food safety with contaminated food products (Donnelly 2001)
  - At least 17 species: *Listeria monocytogenes* is primary human pathogen:
    - Serotypes 1/2a, 1/2b, and 4b – ~90% of human isolates

D.I. Johnson, *Bacterial Pathogens and Their Virulence Factors*,
https://doi.org/10.1007/978-3-319-67651-7_6

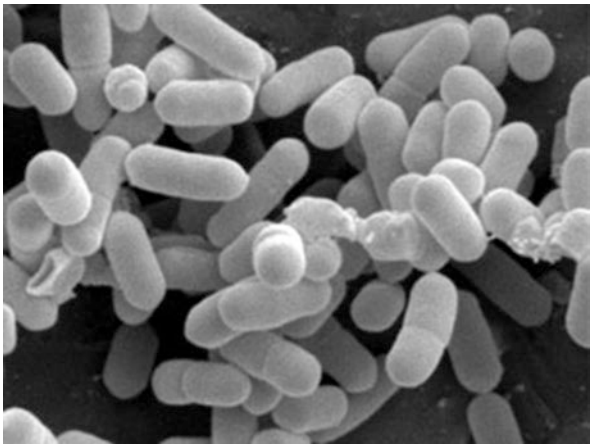

Fig. 6.1 *L. monocytogenes* cells (From: http://listeria-monocytogenes.com)

Disease States Associated with *Listeria monocytogenes*

- Listeriosis – symptoms can vary from mild to serious sepsis and meningitis in newborns, elderly, and immunocompromised individuals:
  - Uncommon but can be serious; 23–70% mortality rate with immunocompromised individuals
  - Immunocompetent individuals: usually asymptomatic or mild flu-like symptoms
  - Immunocompromised individuals, pregnant women, neonates, and elderly:
    - Gastrointestinal symptoms can progress to meningitis and/or meningoencephalitis, usually accompanied by septicemia
    - Infection of fetus is common; can lead to spontaneous abortion, stillbirth, or delivery of acutely ill neonate
  - Multiple disease states are due to the ability of *L. monocytogenes* to cross multiple tissue barriers – intestinal barrier, blood–brain barrier, and maternofetal barrier (Cossart 2011)
  - Transmission – usually enters the human host through contaminated food or water:
    - For example, hot dogs, deli meats, unpasteurized milk, soft-ripened cheeses, raw poultry, raw meats, ice cream, raw vegetables, raw and smoked fish, and cantaloupe

Virulence Factors

- Facultative intracellular pathogen
- Adherence to host cells:

- *L. monocytogenes* adheres to intestinal epithelial cells located at the tip of intestinal villi.
- **Ami** (Asanoa et al. 2012):
  - Autolysin amidase
  - Interacts with host glycosaminoglycans
- **FbpA** (Osanai et al. 2013):
  - Fibronectin-binding protein
- **InlJ** (Lindén et al. 2008):
  - Internalin
  - Binds to host MUC2; major component of intestinal mucus
  - InlB and InlC also bind to MUC2
- **Lap** (Burkholder and Bhunia 2010):
  - *Listeria* adhesion protein
  - Interacts with host cell stress response protein Hsp60; functions in adherence and entry into intestinal epithelial cells
- **LapB** (Reis et al. 2010):
  - Sortase-anchored surface adhesion
  - Upregulated during infection
- **Vip** (Cabanes et al. 2005):
  - Surface adhesion
  - Binds to endoplasmic reticulum (ER) resident chaperone Gp96

- Translocation across intestinal epithelium – leads to systemic dissemination:
  - **InlA** (Pizarro-Cerda et al. 2012; Ribet and Cossart 2015):
    - Internalin surface protein; one of 27 internalins found within the *L. monocytogenes* genome; plays a role in crossing the intestinal and placental barriers; also in invading host cells (see below)
    - Binds to host cell receptor E-cadherin – key component of host adherens junctions (Mengaud et al. 1996):
      - Expressed primarily in epithelial cells, delimiting *L. monocytogenes* tissue tropism
      - Mediates invasion and translocation across epithelial cells at the tips of intestinal villi by triggering host signaling pathways inducing endocytosis and actin cytoskeletal rearrangements
      - Mediates paracellular movement between mucus-producing goblet cells and neighboring enterocytes
  - **InlB**:
    - Internalin – mediates crossing the placental barrier along with InlA; also functions in invading host cells (see below)

- Binds to hepatocyte growth factor (HGF) receptor Met, the product of the *c-met* proto-oncogene (Shen et al. 2000)
- Met is expressed in almost all host cells; enhances *L. monocytogenes* tissue tropism
- Met: receptor tyrosine kinase; InlB binding mimics the binding of HGF to Met, thereby triggering receptor tyrosine kinase signaling pathways that recruit the host actin cytoskeletal network to the site of invasion (Niemann et al. 2007)

- Host cell invasion and growth – non-phagocytic cells; multi-step process:
  - Uses "zipper" mechanism – *L. monocytogenes* proteins interact with host cell surface proteins to trigger host cell signaling pathways that lead to actin cytoskeletal rearrangements at the cell periphery and clathrin-mediated endocytosis (Fig. 6.2):
    - Internalin proteins InlA and InlB: interact with E-cadherin and Met, respectively (see above) (Pizarro-Cerda et al. 2012)
    - **Auto** (Cabanes et al. 2004):
      - Cell wall-anchored autolysin
      - Needed for invasion and virulence

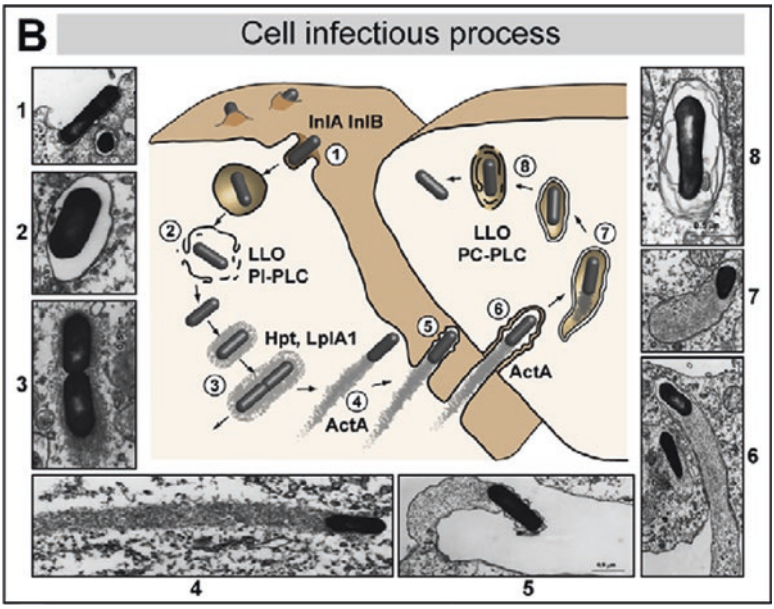

Fig. 6.2 Invasion mechanism (From (Cossart and Toledo-Arana 2008))

- **GtcA** (Faith et al. 2009):
  - Glycosylates cell wall teichoic acids
  - Needed for invasion
- **LpeA** (Reglier-Poupet et al. 2003):
  - Membrane lipoprotein
  - Involved in invasion but not adherence

– Once inside a host phagocytic vacuole, *L. monocytogenes* is capable of surviving reactive oxygen species (ROS) by producing **catalase** and **superoxide dismutase**
– Phagocytic vacuole is disrupted by several membranolytic proteins:

- **Listeriolysin O (LLO)** (Hernandez-Flores and Vivanco-Cid 2015; Seveau 2014):
  - Major virulence factor
  - Cholesterol-dependent, pore-forming cytolytic (CDC) and membranolytic toxin; induced by low pH environment in vacuole
  - Essential for the disruption of phagocytic vacuoles in phagocytic and non-phagocytic cells; also functions in internalization of *L. monocytogenes*
  - Cytolytic for red blood cells, macrophage, and other host cells
  - Encoded by *hly* gene within the *Listeria* pathogenicity island-1 (LPI-1)
  - Rod-like structure with four functional domains (D1–D4)
  - Major target of host innate and adaptive immune responses
  - Enters cells through TLR-4-dependent and TLR-4-independent pathways
  - Affects multiple cell signaling pathways (Fig. 6.3):
    – Induce pro-apoptotic signals
    – Induce pro-inflammatory cytokines
    – Induce stress response to unfolded proteins
    – Induce an increase in cytosolic Ca^{2+} levels
- **Phosphatidylinositol-specific phospholipase C (PI-PLC)**:
  - Plays a role in disrupting the phagosome membrane
  - Encoded by *plcA* within LPI-1
- **Phosphatidylcholine-specific phospholipase C (PC-PLC)**:
  - Activated by metalloprotease Mpl
  - Plays a role in disrupting the phagosome membrane
  - Encoded by *plcB* within LPI-1

– Rapid cellular multiplication within the cytosol:

- **Hpt** (Chico-Calero et al. 2002):
  - Permease
  - Mimics host glucose-6-phosphate transporter (G6PT), to steal G6P from host cell cytosol

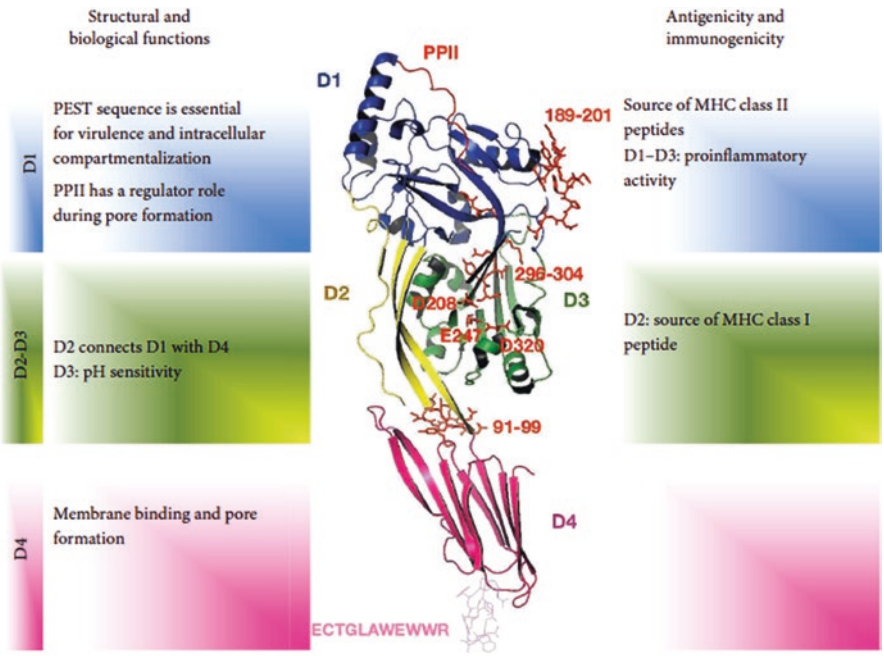

Fig. 6.3 Listeriolysin O functional domains (From Hernandez-Flores and Vivanco-Cid 2015)

- **LplA1** (O'Riordan et al. 2003):
  - Lipoate-protein ligase
  - Affects essential lipoyl modification of host pyruvate dehydrogenase

– Cells are capable of co-opting the host actin cytoskeletal network to move within the cytosol and move from host cell to host cell directly without leaving the cellular environs – thereby evading the host immune system:

- **ActA** (de Souza Santos and Orth 2015; Kocks et al. 1992):
  - Cell surface protein that mediates host actin polymerization, forming actin comets (actin tails) attached to the cell periphery
  - Similar activity to RickA of *Rickettsia* spp.
  - Expressed at one end of the *L. monocytogenes* cell
  - Mimics the action of host Wiskott–Aldrich syndrome protein (WASP)
  - Phosphorylated by CK2 (casein kinase 2), which induces ActA binding to the Arp2/3 complex
  - Activated Arp2/3 complex then nucleates host cell actin polymerization, creating the actin comets

– At the host cell periphery, *L. monocytogenes* can protrude within pseudopod-like structures (listeriopods) to invade neighboring cells:

- **InlC internalin** is involved in this step (Rajabian et al. 2009).

  - Phagocytosis by adjacent cells leads to the formation of a double-membrane secondary vacuole from which *Listeria* escapes, using LLO, PI-PLC, and PC-PLC as in the originally infected cell
  - This leads to new rounds of proliferation, actin-based motility, and intercellular spread

- Growth in human host:
  - Iron acquisition (Lechowicz and Krawczyk-Balska 2015):
    - Multiple mechanisms of Fe^{+3} scavenging; all are regulated by the classic Fur repressor
    - Do not synthesize siderophores; can import Fe^{+3}-hydroxamate xenosiderophores from other bacteria
    - **SvpA (Hbp1) and Hbp2**: heme uptake (Boreze et al. 2001)
    - **HupD, HupC, and HupG**: heme and hemoglobin uptake
    - Direct uptake of ferritin – unknown transporter
  - Biofilms: capable of forming biofilms on abiotic and biotic substrates
- Evasion of host immune system:
  - **BSH** (Dussurget et al. 2002):
    - Bile salt hydrolase
    - Cleaves bile salts; protects cells from antimicrobial activity of bile in the intestinal tract
  - **InlK** (Dortet et al. 2012):
    - Internalin-like protein
    - Blocks host cell autophagy, along with ActA
  - **OatA and PgdA** (Aubry et al. 2011):
    - Peptidoglycan deacetylases
    - Alter peptidoglycan layer – evades innate immunity response

Regulation of Virulence Factor Expression

- Many of the above virulence factors are encoded in *Listeria* pathogenicity island-1 (LPI-1) and transcriptionally activated by the master regulator PrfA:
  - **PrfA** (Heras et al. 2011):
    - Transcriptional activator
    - Belongs to the CAP (catabolite activator protein)/FNR family of transcriptional activators
    - Binds to promoter sequences (PrfA boxes) upstream of target genes

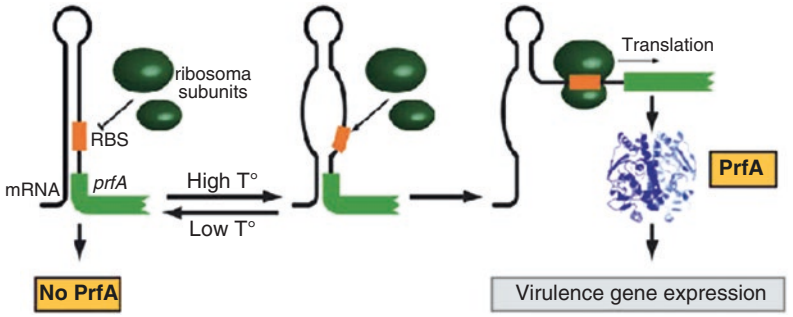

Fig. 6.4 Regulation of PrfA expression (From Cossart and Toledo-Arana 2008)

- Transcription and translation of PrfA are regulated:
  - Transcription of *prfA* is autoregulated and induced by stress signals and repressed by certain sugars
  - Translation of *prfA* mRNA is thermoregulated via a riboswitch (Fig. 6.4):
    - Induction at 37 °C within human host
    - At room temperature (~23 °C), the *prfA* mRNA assumes a conformation that prevents ribosomes from binding to the Shine-Dalgarno ribosome binding site (RBS) sequence – PrfA regulon is not activated
    - At 37 °C, the *prfA* mRNA conformational structure opens up, allowing binding of ribosomes and translation of PrfA – PrfA regulon is activated

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Chapter 7
Mycobacterium spp.

Genomics, Morphologies, and Growth Characteristics

- **Genomics**:
  - *Mycobacterium tuberculosis* strain H37Rv chromosome: 4,411,529 bp; 4,006 predicted ORFs (Cole et al. 1998; Camus et al. 2002)
  - *Mycobacterium leprae* chromosome: 3,268,203 bp; 1,604 predicted ORFs (Cole et al. 2001)
  - *Mycobacterium ulcerans* chromosome – 5,631,606-bp; 4,160 predicted ORFs:
    - Plasmid pMUM001: 174,155 bp; 81 predicted ORFs; contains the gene products needed to synthesize mycolactone (see below) (Stinear et al. 2007)
- **Cell morphology**:
  - Slender rod-shaped cells (Fig. 7.1):
    - Held together in parallel (serpentine) cords (Fig. 7.2) by cord factor (trehalose 6,6′-dimycolate; TDM); essential for virulence (see below)
  - Non-endospore former
  - No flagellar motility
- **Gram stain:**
  - Gram positive; acid-fast staining due to hydrophobic cell wall constituents (see below)
- **Growth:**
  - Obligate aerobes
  - Very slow growers: generation times from 12 to 24 h

D.I. Johnson, *Bacterial Pathogens and Their Virulence Factors*,
https://doi.org/10.1007/978-3-319-67651-7_7

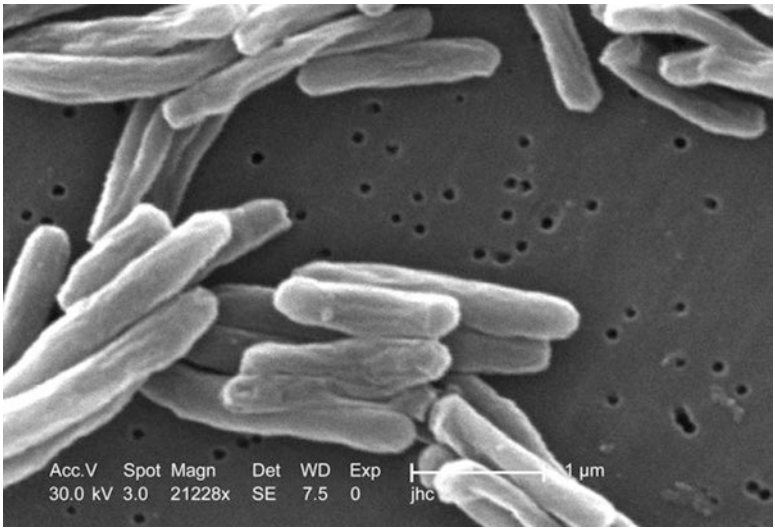

Fig. 7.1 *M. tuberculosis* cells (From: PHIL #8437)

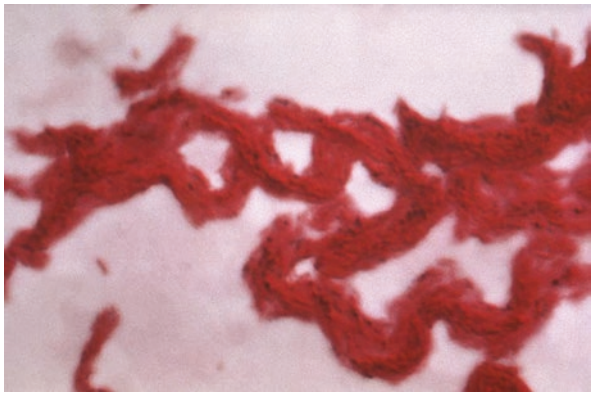

Fig. 7.2 *M. tuberculosis* serpentine cords (From: PHIL #14766)

- Over 120 species – most are nonpathogenic environmental microbes; three are major human pathogens:
  - *Mycobacterium tuberculosis*: facultative intracellular pathogen of macrophage (not free-living)
  - *Mycobacterium leprae*: obligate intracellular pathogen of Schwann cells and macrophage (not free-living)
  - *Mycobacterium ulcerans*: extracellular pathogen

Disease States Associated with *Mycobacterium* spp.

- *Mycobacterium tuberculosis*:
  - Tuberculosis (TB; consumption):
    - ~1/3 of the world population is infected with *M. tuberculosis*
    - ~90% have latent infections with no symptoms
  - Coevolved with humans; developed many mechanisms to evade and co-opt the host immune system (Cambier et al. 2014a)
  - Transmission – person-to-person via small aerosol droplets; highly contagious:
    - Small droplet size (two to three cells) and surface glycolipids (see below) facilitate the bypass of the antagonistic microbiota and microbicidal macrophage in the upper airway
    - This allows the cells to reach the axenic environment of the lower airway alveoli and to be phagocytosed into growth-permissive alveolar macrophage
  - Types of TB:
    - Primary active TB:
      - Initial symptoms – persistent cough, fever, fatigue; leads to difficulty in breathing, chest pain, wheezing, and coughing blood or sputum:
        - Symptoms are due to host immune responses and host cell damage
        - Death is usually due to massive lung damage leading to anoxia
      - Cells invade and survive inside *Mycobacterium*-containing vacuoles (MCV) within un-activated alveolar macrophages; occurs in 80–90% of cases; 90–95% of these infections are cleared by infected macrophage
      - Five to ten percent progress to primary TB with granuloma formation (tubercles):
        - Aggregates of infected macrophage containing live bacteria surrounded by host macrophage and neutrophils
        - Ghon complex: calcified tubercles observed in lung X-rays
      - Progressive tubercle expansion (spread of actively growing cells from dead macrophage to newly arriving macrophage) erodes lung air passages, causing TB symptoms
    - Latent TB:
      - Usual occurrence; most people remain infected without symptoms for life, unless their immune system is compromised
      - Persist in a dormant state within lung macrophage

- Recurrent TB:
  - Breakdown of tubercles releases actively growing cells – secondary TB
  - These cells can spread throughout the body – disseminated TB
    - Can spread to the bones, kidney, and brain
- Therefore, intracellular growth in alveolar macrophage and tubercle formation are essential for persistent infections in the lungs and host dissemination

- *Mycobacterium leprae* and *M. lepromatosis*:
  - Leprosy (Hansen's disease) (Britton and Lockwood 2004; Franco-Paredes and Rodriguez-Morales 2016):
    - Transmission: close contact, person-to-person (nasal aerosols), or through breaks in the skin; can be transmitted from infected nine-banded armadillos (zoonotic reservoir); not a highly contagious disease
    - Symptoms – skin and nervous system symptoms:
      - Usually asymptomatic for years
      - Infection can lead to skin granulomas, resulting in persistent, hypopigmented, disfiguring skin sores or bumps, scarring, and skin thickening
        - Affected skin is less sensitive to heat, pain, or touch than unaffected skin
      - Can infect and grow in Schwann cells of peripheral nerve endings:
        - Leads to nerve damage
        - Numbness or lack of sensation in extremities and progressive weakening of the muscles
    - *M. leprae* can epigenetically reprogram infected adult Schwann cells to facilitate host dissemination (Masaki et al. 2013)
    - *M. leprae* can also invade macrophage, dendritic cells, and endothelial cells (Barker 2006)
    - Symptoms are reflective of the state of the host immune system (Saini et al. 2017):
      - Tuberculoid leprosy: nonprogressive disease; patients have a strong T_H1-cell-mediated immune response and low levels of antibodies
      - Lepromatous leprosy: progressive tissue destruction; patients have a weak T-cell-mediated immune response and high antibody levels
  - Its status as an obligate intracellular pathogen is most likely due to coevolution with humans leading to a significant metabolic genome reduction, as compared to *M. tuberculosis*

- *Mycobacterium ulcerans* (Roltgen et al. 2012; Sarfo et al. 2016):
  - Buruli ulcer (Bairnsdale ulcer, Searls ulcer, Daintree ulcer):
    - Chronic debilitating disease: permanent disfigurement and disability; primarily seen in Africa, South America, and Western Pacific regions
    - Infection of the skin and subcutaneous tissues; primarily the arms and legs
    - Transmission: unknown but likely from aquatic insects, aquatic vegetation, or contaminated water through a break in the skin
    - Symptoms: start as painless, non-ulcerated nodules or plaques on the skin; progress to necrotic, ulcerated lesions; also can infect the bones

Virulence Factors

- *Mycobacterium tuberculosis*:
  - Facultative intracellular pathogen
  - *M. tuberculosis* does not produce exotoxins; these virulence determinants are not needed to facilitate intracellular growth
  - **Unique cell envelope** [(Abrahams and Besra 2016; Guenin-Mace et al. 2009; Kieser and Rubin 2014) and references therein]:
    - Found in all *Mycobacterium* spp. (Fig. 7.3)
    - Essential for environmental persistence, macrophage invasion, and intracellular growth
    - More than 200 proteins are needed to synthesize these structures; targets of multiple known antibiotics as well as potentially new antibiotics (Smith 2003)
    - Four basic layers: cross-linked peptidoglycan layer, highly branched arabinogalactan polysaccharide layer, long-chain mycolic acid layer, and capsule; interspersed glycolipids constitute the remainder of the structure
      - **Peptidoglycan layer**:
        - Basic cross-linked structure as in Gram-positive cells
        - Modified to contain additional glycine or serine residues, altered carboxylic acids, and N-glycolylmuramic acid residues
      - **Arabinogalactan layer**:
        - Contains arabinose and galactose sugars
        - Attached to the peptidoglycan through a N-acetylglucosamime (GlcNAc)-rhamnose linker unit
        - Galactose sugars are added to the linker unit, followed by arabinose sugars, which are the linkage to the mycolic acid layer

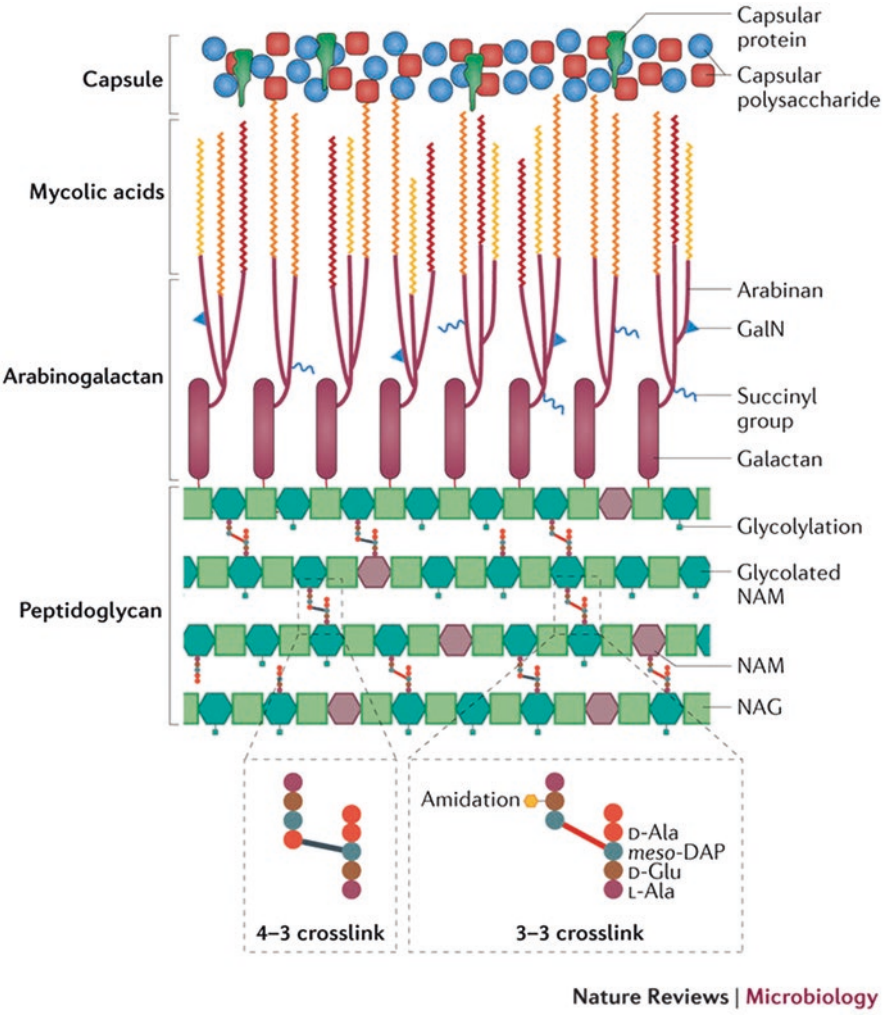

Fig. 7.3 Mycobacteria cell envelope (From Kieser and Rubin (2014))

- **Mycolic acid layer**:
  - Mycolate fatty acids linked to the arabinogalactan layer
  - Three forms: α-mycolates (70%), methoxy-mycolates (10–15%), and keto-mycolates (10–15%)
  - High lipid content results in increased hydrophobicity and resistance to harsh environmental conditions; cells are viable in aerosol drops for months
  - Also responsible for the difficulty in Gram staining, necessitating the acid-fast stain

- **Capsule** (Bornemann 2016; Schwebach et al. 2002):
  - Comprised primarily of α-glucan polymers similar to glycogen; also arabinose and mannose sugars, lipids, and proteins
  - Mediates immune evasion by blocking opsonophagocytosis
- **Glycolipids** (Briken et al. 2004):
  - Phosphatidyl-*myo*-inositol mannosides (PIMs), lipomannan (LM), and lipoarabinomannans (LAMs) interspersed between the three layers
  - Non-covalently anchored into the inner and outer membrane
  - Induce chemoattractant chemokines and pro-inflammatory cytokines in macrophages; inhibit phagosome maturation, apoptosis, and cytokine secretion
- **Surface glycolipids** (Cambier et al. 2014a; Cambier et al. 2014b):
  - Essential for invasion and intracellular growth; only made by pathogenic *Mycobacterium* spp.
  - PDIM (phthiocerol dimycocerosate), PGL (phenolic glycolipid), and others
  - PDIM masks the pathogen-associated molecular pattern (PAMP) molecules on the cell surface from being recognized by macrophage TLRs; used to evade microbicidal macrophage and microbiota in the upper airways
  - PGL induce macrophage chemokine/chemokine receptor CCL2/CCR2, which attracts growth-permissive macrophage in the lower airway and induces phagocytosis
    - Also prevents phagosomal acidification within macrophage

– Adherence to host cells (Govender et al. 2014):
  - Fimbrial adhesins:
    - **Type 4 pili**: produced but a role in adherence has not be shown
    - **Curli-like pili (MTP)** (Alteri et al. 2007):
      - Binds laminin; functions in aggregation and adhesion to macrophage
      - Expressed during adherence and infection
  - Afimbrial adhesins: most bind to ECM components fibronectin and laminin
    - **Heparin-binding hemagglutinin adhesin (HBHA)** (Pethe et al. 2002):
      - 28 kD protein; major immunogen
      - Involved in bacterial aggregation and binding to heparan sulfate-containing proteoglycans on host cell surfaces

  - Essential for colonization and host cell invasion
  - Also inhibits autophagy and induces caspase-dependent apoptosis (Zheng et al. 2017)

- **LBP** (Shimoji et al. 1999):
  - Binds to host laminin (Bornemann 2016)
- **Apa** (alanine-proline-rich antigen; Rv1860) (Ragas et al. 2007):
  - Cell surface glycoprotein adhesin
  - Binds human C-type lectin PSP-A and ECM fibronectin
- **Malate synthase** (**GlcB**; Rv1837c) (Kinhikar et al. 2006):
  - Binds to host laminin and fibronectin
- **GroEL** (Cpn60.2) (Hickey et al. 2010):
  - Heat shock chaperone
  - Plays a major role in adhesion to alveolar macrophage through an interaction with the CD43 glycoprotein

– Host cell invasion and growth:

  ○ *M. tuberculosis* produces virulence factors that induce apoptosis and necrosis once inside the infected macrophage; facilitates the spread of an infection beyond the original infected cell (Moraco and Kornfeld 2014)

  ○ **Cord factor** (Hunter et al. 2009):

    - Surface glycolipid trehalose 6,6′-dimycolate (TDM); most abundant and most toxic lipid produced by *M. tuberculosis*
    - Toxic to mammalian cells; induces necrotic caseating granulomas; associated with detached cord factor/lipid complexes
    - Inhibits phagosome–lysosome fusion and acidification of phagosomes; blocks macrophage killing; associated with cord factor remaining attached to the *Mycobacterium* cell surface
    - **Antigen 85A,B,C** (Puech et al. 2002):
      - Three homologous proteins
      - Mycolyltransferases: catalyze the production of trehalose dimycolate (cord factor) from trehalose monomycolate
      - Also binds to fibronectin; enhances macrophage phagocytosis

  ○ **PknG** (Walburger et al. 2004):

    - Cytosolic serine/threonine kinase; essential for growth in macrophage
    - Blocks phagosome–lysosome fusion; unknown substrate

- **ESAT-6** (early secreted antigenic target of 6 kDa)/**CFP-10** (culture filtrate protein of 10 kDa) (Renshaw et al. 2002):
  - Potent T-cell antigens; highly immunogenic; vaccine candidates
  - Co-expressed secreted proteins that form a 1:1 ESAT-6/CFP-10 pore complex
  - Recruits growth-permissive macrophages using host matrix metalloproteinase-9 (MMP9); enhances granuloma formation (Volkman et al. 2009)
  - Induces caspase-dependent apoptosis of infected macrophage, which is essential for granuloma expansion (Derrick and Morris 2007)
  - Inhibits autophagosome–lysosome fusion during macrophage autophagy (Zhang et al. 2012)
  - Co-expression is regulated by the PhoPR TCS (see below) (Broset et al. 2015)
  - Secreted through the ESX-1 *Mycobacterium*-specific T7SS
- **T7SSs** (Abdallah et al. 2007):
  - Five specialized secretion systems (ESX-1–ESX-5):
    - Found in *Mycobacterium* spp. (except *M. ulcerans*) and other Gram-positive bacteria
    - Needed to secrete proteins across the complex cell envelope
  - **ESX-1** – secretes **ESAT-6**, **CFP-10**, **EspA**, and **Rv3615c**:
    - These genes are deleted in the region of difference 1 (RD1) in the attenuated bacillus Calmette–Guérin strain used in BCG vaccine; suggests that they are essential for virulence
  - **ESX-2–ESX-5**: contain homologues of ESAT-6/CFP-10; ESX-3 is necessary for iron and zinc homeostasis
- **PanC/PanD** (Sambandamurthy et al. 2002):
  - Function in the synthesis of pantothenic acid (vitamin B5), which is essential for lipid biosynthesis and metabolism inside macrophage
- Iron acquisition – inside macrophage (Hameed et al. 2015):
  - Siderophores:
    - **Mycobactins:** produce both membrane-bound (extracellular vesicles, EVs) and soluble forms
  - Direct import of heme iron, lactoferrin, and transferrin
  - Regulated by **IdeR** (Gold et al. 2001):
    - Fe^{+2}-binding repressor
    - Homologous to DtxR from *Corynebacterium diphtheriae*

  - Fe^{+2} binding activates IdeR repressor activity
  - Low Fe^{+2} concentrations lead to transcriptional activation of mycobactin (*mbt*) genes and inactivation of iron storage genes

- **Phospholipases C** (Raynaud et al. 2002):
  - Four genes – *plc*A, B, C, and D
  - Deletion mutant phenotypes suggest that all four have phospholipase activity that is important for virulence
  - Expression is upregulated in macrophage
  - Virulence mechanism is unknown; possibly used to generate fatty acid nutrients for the cells

- Protection against intracellular stress conditions:
  - **HspX** (Desjardin et al. 2001):
    - ATP-independent chaperone; induced under hypoxic and reactive nitrogen stress in macrophage
    - Necessary for survival in low-oxygen environment of macrophage
  - **AhpC** (Pethe et al. 2002):
    - Alkyl hydroperoxide reductase activity
    - Protects against reactive oxygen species (ROS) and reactive nitrogen species (RNS) inside macrophage
  - **KatG** (Ng et al. 2004):
    - Catalase/peroxidase activity: degrades H_2O_2 produced from NADPH oxidase
    - Protects against reactive oxygen species (ROS) inside macrophage
  - **SodA; SodC** (Edwards et al. 2001):
    - Superoxide dismutases: metalloenzymes – SodA (Fe); SodC (Cu)
    - Converts superoxide anion to H_2O_2, protects against reactive oxygen species (ROS) inside macrophage

- Activation of host immune system:
  - **LpqH**, **LprA**, **LprG**, **LppX**, and **PstS1** (Drage et al. 2009; Prados-Rosales et al. 2011):
    - Lipoproteins
    - Agonists of host Toll-like receptor-2 (TLR-2); induce release of cytokines and chemokines from macrophage; LpqH can also induce macrophage autophagy
    - Interaction of lipoprotein-containing membrane vesicles (MVs) with alveolar macrophage elicits an intense inflammatory response; can lead to enhanced granuloma production and localized necrosis

- *Mycobacterium leprae*:
  - Obligate intracellular pathogen
  - Cell envelope, including cord factor, and ESX-1/ESAT-6 secretory system are similar to *M. tuberculosis*
  - Adherence to host cells:
    - **Phenolic glycolipid-1 (PGL-1)** (Ng et al. 2000):
      - Binds to α2-laminin in the basal lamina of Schwann cells; dictates *M. leprae* tropism for Schwann cells:
        - PGL-1/α2-laminin complex binds to extracellular α-dystroglycan (α-DG) protein; α-DG binds to transmembrane protein β-dystroglycan (β-DG), which in turn binds to dystrophin, a cytoskeletal protein that binds to host actin filaments
        - These interactions induce actin-dependent uptake into Schwann cells (Rambukkana et al. 1998)
      - PGL-1 also induces demyelination of nerve cells, which may aid in invasion of Schwann cells
    - **LBP21/Hlp** (Shimoji et al. 1999):
      - Binds to α2-laminin
  - Mechanisms for phagocytosis into macrophage and inhibition of phagosome–lysosome fusion are similar to *M. tuberculosis*
- *Mycobacterium ulcerans* – extracellular pathogen; toxigenic:
  - Damage to host cells
    - **Mycolactone** (Roltgen et al. 2012; Sarfo et al. 2016):
      - Major virulence factor of *M. ulcerans*
      - Polyketide: contains an invariant 12-membered lactone ring and two acyl side chains (Fig. 7.4)
      - Mycolactone biosynthetic genes (*mlsA1*, *mlsA2*, *mlsB*, *mup038*, *mup045*, *mup053*) are encoded on unique *M. ulcerans* plasmid pMUM001
      - Can be found postinfection within Buruli ulcer lesions and at other disseminated host sites
      - More toxic when it is found in association with extracellular vesicles (EVs) (Brown et al. 2015)
      - Two physiological targets:
        - Binds to Wiskott–Aldrich syndrome protein (WASP) and neural WASP (N-WASP) (Guenin-Mace et al. 2013)
          - Scaffold proteins that mediate ARP2/3-dependent actin polymerization

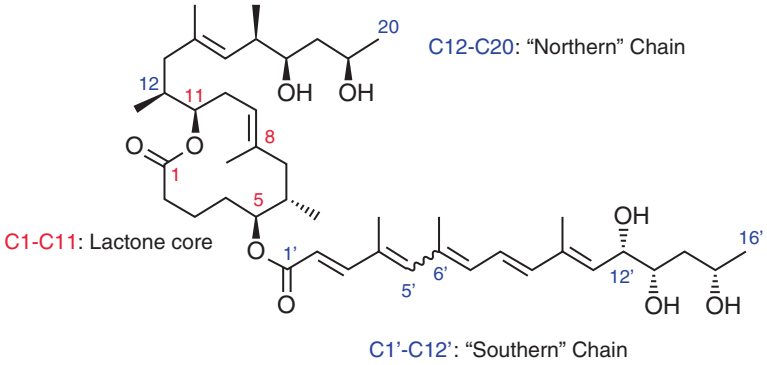

Fig. 7.4 Mycolactone structure (From: Sarfo et al. (2016))

- Mycolactone binding relieves WASP auto-inhibition, resulting in unregulated actin polymerization – loss of cell adhesion and induction of cell apoptosis
- Mechanism responsible for the tissue necrosis associated with Buruli ulcers

– Inhibits function of Sec61 translocon (Hall et al. 2014)

- Involved in the translocation of host proteins, including pro-inflammatory cytokines and N-linked glycosylated proteins, into the endoplasmic reticulum (ER)
- Sec61 inhibition leads to suppression of cytokine and chemokine production and their associated signaling pathways – immunosuppression
- Sec61 inhibition also blocks the production of membrane receptors and cell adhesion molecules

– ESX-1 secretion system that is essential for *M. tuberculosis* granuloma production (see above) is deleted in *M. ulcerans*; likely plays a role in the extracellular lifestyle of *M. ulcerans* (Stinear et al. 2007).

Regulation of Virulence Factor Expression

- *M. tuberculosis* latent infections:
  - ~1/3 of the world population have persistent infections in which *M. tuberculosis* is dormant within lung macrophage
  - To induce dormancy, the metabolism and replication of *M. tuberculosis* must be repressed; primarily controlled through transcriptional induction and repression

- **Alternative sigma (σ) factor** genes, *sigA*, *sigE*, *sigF*, and *sigH*, along with the **WhiB** family of transcription factors are expressed during infection, suggesting that they play a role in virulence and dormancy (Manganelli et al. 2004)

– Dormancy can be induced by O_2 depletion (hypoxia), the presence of nitric oxide gas (NO), nutrient starvation, and acid conditions
  - Hypoxia and NO induce the same set of 47 gene transcripts (Sherman et al. 2001)
  - Hypoxia and NO responses are induced early in infection through the DosR–DosS (DevR–DevS) and DosT two-component phosphorelay systems (TCSs) (Kendall et al. 2004; Roberts et al. 2004)
    - **DosR**:
      – Response regulator
      – Activates transcription of hypoxic-regulated genes
      – Phosphorylated by both DosS and DosT sensor kinases
    - **DosS; DosT**:
      – Sensor kinases; transfer phosphate to DosR, thereby activating its transcriptional activator function
      – Heme-containing kinases; regulated by the binding of Fe^{+2} and O_2
      – The kinases are active when in a deoxy-Fe^{+2} state – hypoxic conditions
        - DosT responds early to hypoxic conditions, and DosS responds later
      – The kinases are inactive when in a O_2-bound state
      – NO and CO can displace O_2 at the heme site; this activates the kinases and induces NO-responsive genes; explains why hypoxia and NO induce the same genes
  - NO-mediated killing of *M. tuberculosis* (Samanovic and Darwin 2016):
    - Occurs in macrophage; resistance to NO and other reactive nitrogen species (RNS) allows *M. tuberculosis* to maintain latency in macrophage
    - Mutant analysis indicates that the 26S proteasome is needed for NO resistance (Darwin et al. 2003)
    - **Pup-proteasome system (PPS)** (Samanovic and Darwin 2016):
      – Similar to eukaryotic proteasome that degrades ubiquitin-modified proteins
      – **Pup**:
        - Prokaryotic ubiquitin-like protein
        - Tags target proteins for proteolysis

  - Mutational loss of PPS causes increased synthesis of cytokinins, leading to sensitivity to RNS/NO and loss of cell viability in host cells

- **MprAB TCS** (Zahrt et al. 2003):
  - Needed for the establishment and persistence of infection
    - MprA – response regulator:
      - Transcription activator
      - Binds to MprA box upstream of regulated genes, such as EspR transcription factor
    - MprB – sensor kinase:
      - Regulates the expression of ESX-1 along with the ESAT-6 and EspA-secreted proteins (Cao et al. 2015)
      - Regulation is mediated through activation of the EspR transcription factor
  - PhoPR TCS also regulates ESAT-6 and EspA expression through EspR
- **PhoPR TCS** (Perez et al. 2001):
  - PhoR – sensor kinase:
    - Responds to Mg^{+2}, Cl^{-}, and pH within the macrophage
  - PhoP – response regulator:
    - Transcription activator
    - *PhoP* mutants have replication defects and loss of virulence; due to loss of ESAT-6 and EspA secretion

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Chapter 8
Propionibacterium spp.

Genomics, Morphologies, and Growth Characteristics

- **Genomics:**
  - *Propionibacterium acnes* chromosome: 2,560,265 bp; 2333 predicted ORFs (Brüggemann et al. 2004)
- **Cell morphology:**
  - Rod-shaped cells; "diphtheroids" (Fig. 8.1)
  - Similar to *Corynebacterium diphtheriae* in morphology and arrangement
- **Gram stain:**
  - Gram positive
- **Growth:**
  - Slow-growing aerotolerant anaerobes; prefers anaerobic environment at 37°C
  - Catalase positive; indole positive; produces propionic acid through fermentation
  - Skin commensal; also in the mouth, lung, urinary tract, gastrointestinal tract, and prostate:
    - Primarily colonizes sebaceous glands and hair follicles
    - Forms biofilms on indwelling medical devices through bacterial seeding
  - ~15 species; most are rarely associated with human disease
    - Other cutaneous skin commensals: *P. avidum, P. granulosum, P. lymphophilum,* and *P. propionicum* – role in disease states is unclear

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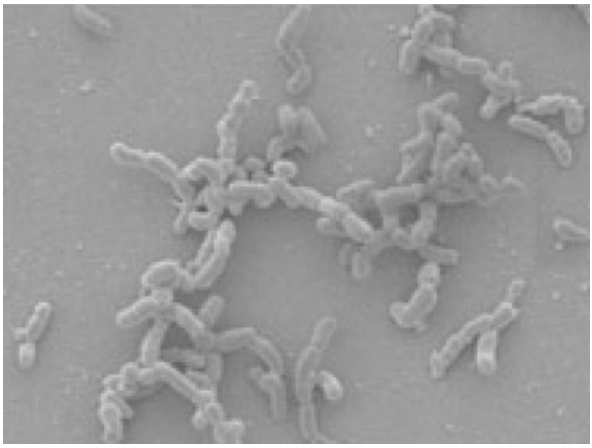

Fig. 8.1 *P. acnes* cells (From: Holmberg et al. (2009))

Disease States Associated with *Propionibacterium acnes*

- Opportunistic pathogen: associated with numerous inflammatory disease states (Achermann et al. 2014; Dessinioti and Katsambas 2010):
  - Induces innate immunity response mediated by Toll-like receptors TLR-2 and TLR-4 in macrophage and keratinocytes; triggered by peptidoglycan, lipoteichoic acids, and CAMP 1 binding (Lheure et al. 2016)
  - Pro-inflammatory cytokines (IL-1β, IL-8, and IL-12) and tumor necrosis factor alpha (TNF-α) are released:
    - Secretion of lipases, proteases, and fatty acids can also induce an inflammatory response
  - Induced keratinocyte growth results in the formation of whiteheads and blackheads
  - *P. acnes* activates classical and alternative complement pathways that produce neutrophil chemotactic factors, attracting neutrophils into sebaceous glands, leading to a purulent response
- Acne vulgaris: red inflamed lesions on the skin; most common symptom (Fig. 8.2):
  - Inflammation of sebaceous glands, where *P. acnes* resides
  - Excessive sebum production (triggered by hormones) provides nutrients for *P. acnes*
- Infections of indwelling medical devices, including breast implants, prosthetic joints, orthopedic devices, cardiovascular devices, and catheters (Achermann et al. 2014)

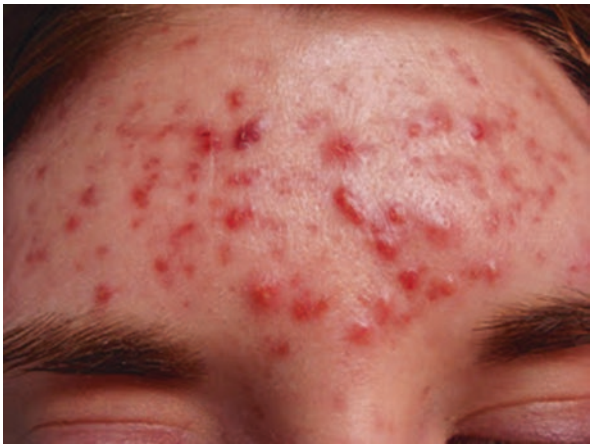

Fig. 8.2 Acne vulgaris (From: vgrd.blogspot.com)

- Associated with SAPHO (synovitis, acne, pustulosis, hyperostosis, osteitis) syndrome (Zimmermann and Curtis 2016):
  - Synovitis: inflammation of synovial joints
  - Synovitis: inflammation of synovial joints
  - Acne vulgaris: see above
  - Pustulosis: inflammation of the skin resulting in blisters and pustules
  - Hyperostosis: abnormal thickening of bone tissue
  - Osteitis: inflammation of the bone

Virulence Factors

- Many of these virulence factors are predicted by genomic sequencing (Brüggemann et al. 2004; Brzuszkiewicz et al. 2011):
- Adherence to host cells/tissues:
  - Nine putative adhesins
- Growth within the host milieu:
  - Iron acquisition:
    - **HtaA** (Allen and Schmitt 2009):
      - Heme acquisition cell surface protein
      - Major immunoreactive protein; similar to *Corynebacterium diphtheriae* HtaA
  - Biofilms form on biotic surfaces and abiotic indwelling medical devices through bacterial seeding – major medical and economic problem (Achermann et al. 2014; Bayston et al. 2007)

- Secreted degradative enzymes (Holland et al. 2010):
  - Two **triacylglycerol lipases**:
    - **GehA** – digests sebum (Miskin et al. 1997)
  - **Hyaluronate lyase**: degrades component of the extracellular matrix
  - At least 14 predicted **lipases/esterases**
  - Two **endoglycoceramidases**
  - Four **sialidases**
  - **Extracellular proteases**
- Damage to host cells:
  - **CAMP (Christie, Atkins, Munch-Petersen) factors** (Nakatsuji et al. 2011; Valanne et al. 2005):
    - Five pore-forming hemolysins
    - Similar to Group B Streptococci (*Streptococcus agalactiae*) CAMP factor
    - Binds to IgG and IgM molecules
    - Needs host sphingomyelinase for full activity
    - Leads to destruction of macrophage and keratinocytes
    - CAMP factor 2 is released in highest abundance (putative lysozyme)

Regulation of Virulence Factor Expression

- Regulation of protein secretion (Sec-signal recognition particle (Sec-SRP) and the twin-arginine translocation (Tat) systems are upregulated in growth phase-specific manner (Brzuszkiewicz et al. 2011):
  - Exponential growth increases secretion of certain virulence factors such as CAMP factors, endoglycoceramidases, lysophospholipase
  - Stationary phase increased secretion of hyaluronidase and adhesins

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Chapter 9
Staphylococcus spp.

Genomics, Morphologies, and Growth Characteristics

- **Genomics:** multiple *S. aureus* strains have been sequenced, including methicillin-resistant and methicillin-sensitive clinical isolates and community isolates (Holden et al. 2004):
  - *Staphylococcus aureus* strain MRSA252 (methicillin-resistant) chromosome: 2,902,619 bp; 2671 predicted ORFs
  - *Staphylococcus aureus* strain MSSA476 (methicillin-sensitive) chromosome: 2,799,802 bp; 2565 predicted ORFs
  - *Staphylococcus epidermidis* strain ATCC 12228 chromosome: 2,499,279 bp; 2419 predicted ORFs (Zhang et al. 2003)
  - *Staphylococcus saprophyticus* strain ATCC 15305 chromosome: 2,516,575 bp; 2446 predicted ORFs (Kuroda et al. 2005)
- **Cell morphology:**
  - Cocci: irregular "grape-like" clusters (Fig. 9.1)
- **Gram stain:**
  - Gram positive
- **Growth** (Ramsey et al. 2014):
  - Facultative anaerobes; catalase positive
  - Coagulase positive (*S. aureus*); most other *Staphylococcus* spp. are coagulase negative
  - Salt and desiccation tolerant; associated with colonization of the skin
  - Reservoirs: ~one-third of population are carriers; predominantly found on the skin, mucous membranes in the anterior nares (nose) and the respiratory tract, the gastrointestinal tract, and on vaginal mucous membranes; occasionally

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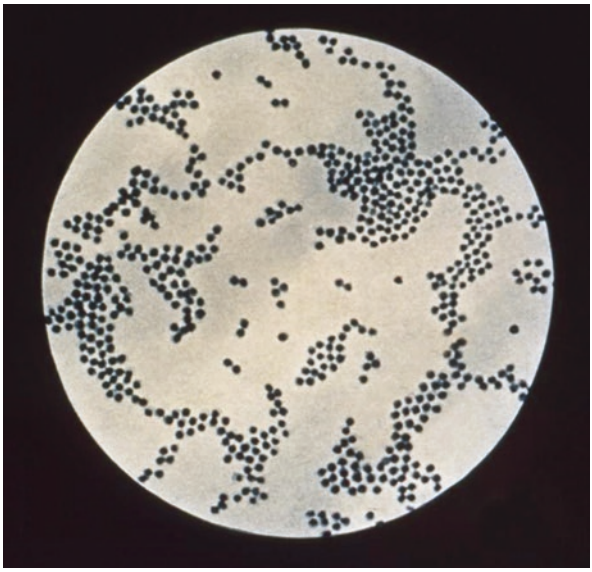

Fig. 9.1 *S. aureus* cells (From: CDC PHIL #5144)

found in contaminated soil and contaminated food products; antibiotic-resistant strains are often found in hospital environments
– Expert biofilm formers (see below)
– At least 40 species fall into 11 taxonomic groups, many of which are associated with specific mammalian species

Disease States Associated with *Staphylococcus* spp.

- Primary and opportunistic pathogens
- *S. aureus*: (Tong et al. 2015):
  - Member of the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp.) family of antibiotic-resistant nosocomial pathogens
  - Major cause of hospital-acquired (HA) and community-acquired (CA) infections; often associated with chronic, persistent antibiotic-resistant isolates, including small colony variants (SCVs) (Proctor et al. 2014)
  - Most common pathogen associated with bloodstream infections due to biofilm formation on indwelling medical devices
  - Localized purulent skin and soft tissue infections:
    - Abscesses
    - Folliculitis
    - Boils; furuncles; carbuncles
    - Impetigo

  - Systemic and/or invasive infections:
    - Staphylococcal scalded skin syndrome (SSSS; seen with neonates)
    - Staphylococcal toxic shock syndrome (STSS)
    - Bacteremia and septicemia (associated with biofilms on indwelling medical devices)
    - Infective endocarditis
    - Pneumonia (CA and HA; can become necrotizing)
    - Food poisoning (associated with enterotoxins)
    - Descending urinary tract infections (UTIs)
    - Septic arthritis
    - Osteomyelitis
    - Joint replacement surgical infections
  - Transmission: contact with purulent discharge from an infected surgical site or wound, contact with skin of an infected person, contact with fomites used by an infected person, ingestion of enterotoxin-containing food or water

- *S. epidermidis* (Becker et al. 2014):
  - Coagulase-negative Streptococci (CoNS); 50–80% of CoNS isolates
  - Normal microbiota of skin and mucous membranes
  - Opportunistic pathogen of immunocompromised population
  - Leading cause of nosocomial infections associated with biofilm formation on foreign indwelling medical devices; also *S. haemolyticus:*
    - Exit site and systemic infections; resultant bacteremia can lead to localized purulent inflammation, septicemia, infective endocarditis [including prosthetic valve infective endocarditis (PVIE)], prosthetic joint infection, and abscesses
  - Transmission: enters through wound or surgical breaks in the skin

- *S. saprophyticus* (Becker et al. 2014; Kline and Lewis 2016):
  - Coagulase-negative Streptococci (CoNS)
  - Predominantly colonize the human gastrointestinal tract, genitourinary tract, and rectum
  - Also can be found in the gastrointestinal tract of animals, leading to contamination of raw beef and pork, and in contaminated vegetables and cheeses (Hedman et al. 1990)
  - Ascending UTIs – second most frequent cause of uncomplicated UTIs:
    - Transmitted from the GI tract through the urethra and ascends to the upper GU tract
    - Symptoms: acute urethritis – burning sensation during urination, urge to frequently urinate, bloated feeling with sharp abdominal pain
    - Novobiocin susceptibility testing: only for coagulase-negative UTI source identification:

- *S. saprophyticus*: resistant
- *S. epidermidis*: sensitive

– Second most common cause of CA-UTIs; infection rate increases with sexual activity in women

Virulence Factors

- *S. aureus* virulence factors include a myriad of adherence proteins and exotoxins as well as multiple mechanisms used to evade the host immune system
- Adherence to host cells:
  – Afimbrial adhesins:
    - **MSCRAMMs** (microbial surface components recognizing adhesive matrix molecules):
      - Cell wall-anchored (CWA) proteins: attached to the cell wall by sortase transpeptidases (Foster et al. 2014):
        – **Sortases** (Bradshaw et al. 2015):
          - Found in most Gram-positive and some Gram-negative bacteria
          - Cysteine transpeptidase activity; six classes (class A–class F)
          - Catalyze the attachment of adhesins and other CWA proteins to the pentapeptide cross-bridges within the peptidoglycan structure
          - Recognize proteins that contain a C-terminal LPXTG-like cell wall sorting signal (CWSS)
      - **Cna** (Patti et al. 1992):
        – Collagen adhesin; binds collagen-rich tissues using "collagen hug" mechanism (Zong et al. 2005)
        – Not expressed in all *S. aureus* strains
        – Also binds to complement protein C1q; inhibits complement by blocking the binding of C1q to C1r90; used to evade host immune system (Kang et al. 2013)
      - **FnbpA, FnbpB** (Menzies 2003):
        – Binds ECM fibronectin, elastin, and fibrinogen.
        – Fibrinogen binding enhances blood clot formation (see below)
        – Also acts as an invasin through its interactions with fibronectin .and α5β1 integrin complex within host cell membranes (Foster et al. 2014)
        – Promotes biofilm formation
        – Most *S. aureus* strains express both FnbpA and FnbpB
        – Inactivated by **V8 protease (SspA)**; involved in transitioning between colonization and dissemination (McGavin et al. 1997)

- **ClfA, ClfB** (McDevitt et al. 1994; Ní Eidhin et al. 1998):
  - Cell wall-attached coagulases (clumping factors)
  - Two distinct genes, not allelic variants
  - ClfA bind to ECM fibrinogen at different sites; mediates platelet activation involved in blood clot formation (see below)
  - ClfB binds to keratin 10 and loricrin; plays a role in binding to desquamated nasal epithelial cells (Corrigan et al. 2009)
- **SdrC, SdrD, SdrE, Bbp** (bone sialoprotein-binding protein) (Foster and Höök 1998):
  - SdrC and SdrD play a role in binding to desquamated nasal epithelial cells (Corrigan et al. 2009)
  - SdrC binds to β-neurexin, a neuronal cell adhesion molecule; significance of this binding to virulence is unclear (Barbu et al. 2010)
  - Bbp binds fibronectin; interferes with coagulation (Vazquez et al. 2011)

- **IsdA** (Clarke et al. 2004):
  - Binds ECM fibrinogen and fibronectin
  - Also plays a role in iron acquisition (see below)
- **Eap/Map** (extracellular adherence protein/MHC analogous protein) (Harraghy et al. 2003):
  - Not a MSCRAMM
  - Binds ECM components fibronectin, fibrinogen, vitronectin, bone sialoprotein, and thrombospondin
  - Interacts with ICAM-1 (intercellular adhesion molecule-1); induces inflammatory response (see below)
- **EbpS** (Downer et al. 2002):
  - Integral membrane protein; not a MSCRAMM
  - Binds to ECM elastin
- **SasG** (Roche et al. 2003):
  - G5-E repeat cell wall-anchored adhesin
  - Functions in attachment to human desquamated nasal epithelial cells

- Dissemination in human host:
  - Bloodstream dissemination is essential for *S. aureus* systemic diseases, such as sepsis, infective endocarditis, osteomyelitis, pneumonia, deep tissue abscesses, and seeding of indwelling medical devices
  - Dissemination requires protection from the bloodstream innate immune system

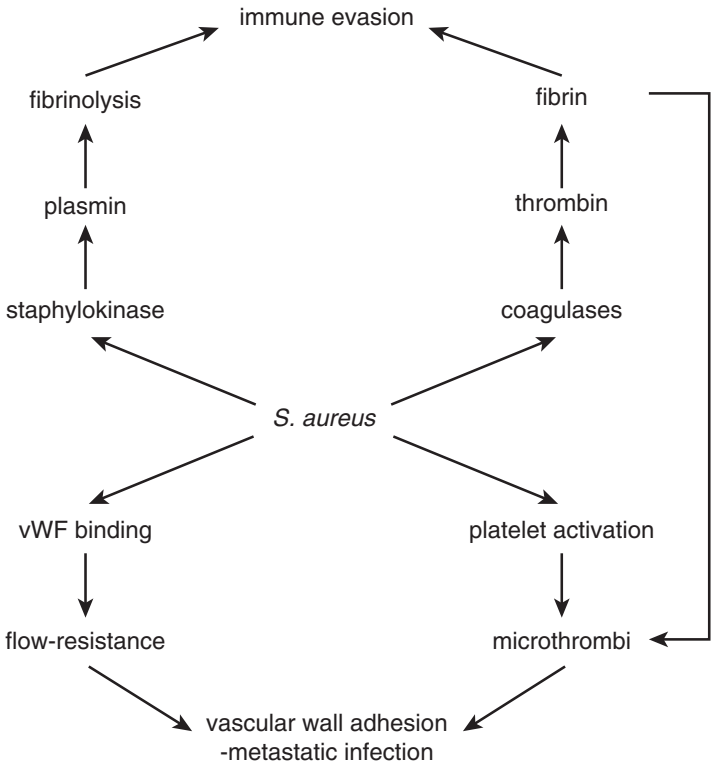

Fig. 9.2 *S. aureus* manipulation of host hemostatic system (From: Peetermans et al. (2015))

- For this protection, *S. aureus* co-opts the host hemostatic system by inducing the creation and dispersion of fibrin blood clots (Fig. 9.2)
- Coagulase-dependent blot clot formation: (McAdow et al. 2012; Peetermans et al. 2015; Thammavongsa et al. 2015) (Fig. 9.3):
  - **Coa (staphylocoagulase)**:
    - Secreted coagulase; binds host prothrombin
    - Coa binding, along with vWbp binding (see below), converts prothrombin into activated staphylothrombin protease complex
    - Staphylothrombin proteolytically converts host fibrinogen into a fibrin network (blood clot), which blocks opsonophagocytosis and bacterial killing
    - Staphylothrombin also induces the formation of microthrombi within the bloodstream and plays a role in abscess formation
  - **vWbp** (von Willebrand factor-binding protein):
    - Secreted coagulase; binds host prothrombin
    - vWbp binding activates prothrombin to staphylothrombin

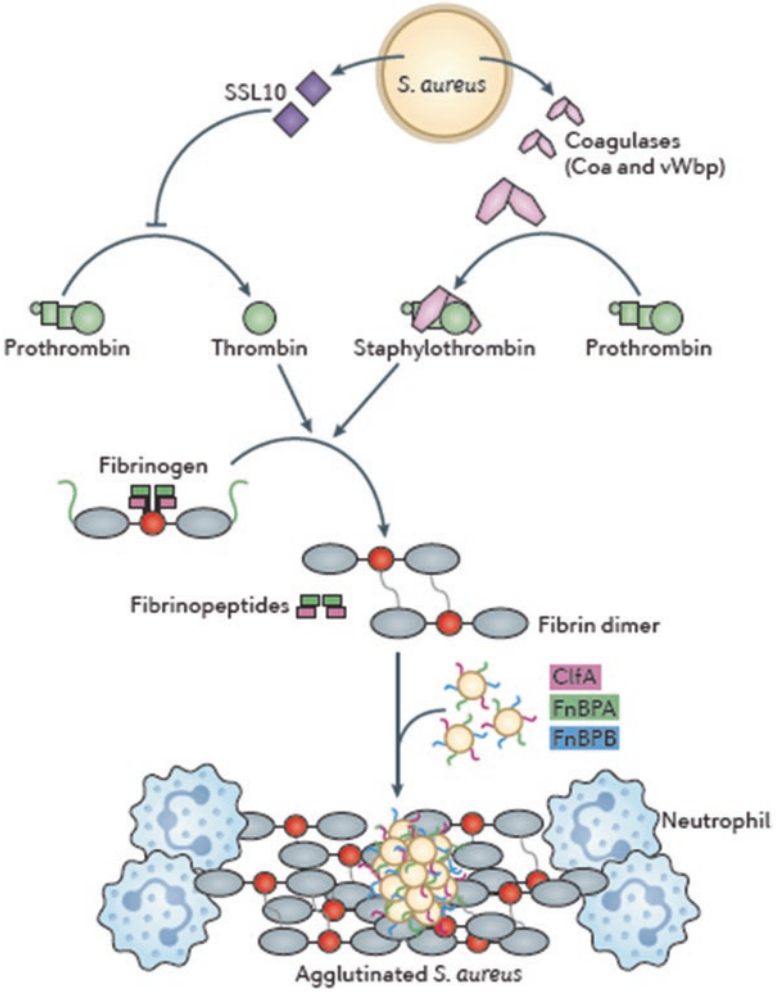

Fig. 9.3 *S. aureus* coagulation pathway (From. Thammavongsa et al. (2015))

- Also mediates the binding of *S. aureus* to damaged or activated vascular endothelium, which is needed to induce infectious endocarditis

- **ClfA, ClfB**:
  - Cell wall-attached coagulases (see above)
  - Plays a synergistic role with Coa and vWbp
  - Important in fibrin microthrombi formation; agglutinates *S. aureus* cells in blood clots
  - ClfA also binds complement regulator factor I (fI), blocking complement activation

- **FnbpA, FnbpB**:
  - Binds to ECM fibrinogen at different sites (see above)
  - Mediates platelet activation involved in blood clot formation

– Blood clot disruption:

- **Staphylokinase (fibrinolysin)** (Collen 1998):
  - Plasminogen activator (Pla)
  - Binds to host plasminogen, leading to its conversion into the fibrinolytic enzyme plasmin
  - Plasmin degrades fibrin blood clots, releasing *S. aureus* from the blood clot
- **Hyaluronate lyase (hyaluronidase)** (Makris et al. 2004):
  - Destroys ECM hyaluronic acid; spreading factor

– **EsxA, EsxB** (Burts et al. 2005):

- Secreted proteins required for virulence
- Part of T7SS analogous to *M. tuberculosis* ESAT-6 secretion system

• Growth in human host:

– Iron acquisition (Haley and Skaar 2012):

- Siderophores:
  - **Staphyloferrin A, staphyloferrin B**: also found in *S. epidermidis*
  - **HtsABC, SirABC**: transport siderophores into the cytoplasm
- Binding to host iron-containing proteins:
  - **Isd** (iron-regulated surface determinant) proteins: **IsdA-IsdH**
  - **IsdB**: binds host hemoglobin; removes heme from hemoglobin
  - **IsdH**: binds host hemoglobin/haptoglobin
  - **IsdA, IsdC**: heme-binding cell wall proteins; receive heme from IsdB, IsdH

– Biofilm formation essential for virulence (Götz 2002; Paharik and Horswill 2016):

- Staphylothrombin activation of fibrinogen (see above) that is accumulated on indwelling medical devices leads to fibrin deposition and biofilm formation
- Many of the CWA adhesins described above play an important role in biofilm formation
- **PIAs** (polysaccharide intercellular adhesins) (Cramton et al. 1996):

• Component of slime layer

- Sulfated polymers of β-1,6-linked N-acetylglucosamine [poly-*N*-acetylglucosamine (PNAG)]
- Essential for biofilm formation
- Encoded by the *icaABCD* operon

– **Lipases** (Rosenstein and Götz 2000):

  - Two secreted proteins in *S. aureus*
  - Dissolves host lipids; enhances colonization on the skin

- Damage to host cells:

– **Hemolysins/leukocidins** (Dinges et al. 2000; Otto 2014):

  - Cytolytic to red blood cells (RBCs), epithelial cells, and phagocytic cells
  - β-barrel pore-forming toxins (β-PFTs); most contain two subunits (except α-toxin); receptor-mediated cytolysis:

    - **α-hemolysin (α-toxin; Hla)** (Tomita and Kamio 1997):

      – Dermonecrotic and neurotoxic
      – Forms heptameric pores in epithelial cells and phagocytic cells
      – Essential virulence factor for diseases associated with epithelial cell interfaces, such as pneumonia
      – Binds to host ADAM-10, inducing its metalloprotease activity, which cleaves E-cadherin within intercellular junctions (Inoshima et al. 2011)

    - **γ-hemolysin (HlgA,B,C)** (Prevost et al. 1995):

      – High level of amino acid homology to PVL (see below)

        - HlgA, HlgC = LukS
        - HlgB = LukF

      – Destroys neutrophils, macrophage, RBCs

    - **LukA,B, HlgC,B, LukE,D** (Otto 2014):

      – Two component leukocidins
      – Destroy neutrophils, T cells, macrophage

    - **Panton-Valentine leukocidin (PVL; LukS,F)** (Prevost et al. 1995):

      – Destroys neutrophils, macrophage
      – Encoded on PVL bacteriophage; associated with CA-MRSA strains

– **β-hemolysin (β-toxin; Hlb)** (Doery et al. 1963):

  - Sphingomyelinase C activity
  - Cleaves host cell membrane sphingomyelin into phosphocholine and *N*-acylsphingosine (ceramide); results in cell lysis
  - Used in laboratory CAMP test to identify Group B Streptococci
  - *hlb* gene is disrupted in many virulent *S. aureus* cells

- **PSM (phenol-soluble modulins)** (Peschel and Otto 2013; Wang et al. 2007b):
  - Seven surfactant-like, 20–25 amino acid amphipathic peptides
  - **PSMα1 – α4; PSMβ1 – β2; PSM-mec**
  - Associated with increased expression in CA-MRSA infections
  - Cytotoxic to neutrophils, monocytes
  - **δ-hemolysin (δ-toxin; Hld)** (Mellor et al. 1988):
    - Homologous to PSMα peptides
    - Cytolytic effects on many host cells
- **PTSAgs (pyrogenic toxin superantigens)** (Spaulding et al. 2013):
  - Family of potent immunostimulatory exotoxins
  - Induce nonspecific, massive T-cell proliferation (up to 20% of all T cells):
    - Bind to TCR (T-cell receptor) Vβ variable chains on T cells and class II MHC (major histocompatibility complex) α- and β-chains on antigen-presenting cells (APCs); different than the normal antigen-specific TCR binding
    - Binding "tricks" T cells into releasing enormous amounts of host cytokines, resulting in a "cytokine storm"
    - Cytokine storm is responsible for severe symptoms – capillary leakage, hypotension, shock, respiratory distress, multi-organ failure, and death
  - 19 *S. aureus* PTSAgs:
    - **TSST-1 (toxic shock syndrome toxin-1)**
    - **SE** (staphylococci enterotoxin) serotypes: **SEA, SEB, SECn, SED, SEE, SEG, SEH, SEI, SEJ, SEK, SEL, SEM, SEN, SEO, SEP, SEQ, SER, SEU**
  - Food poisoning:
    - SE-induced gastroenteritis
    - Associated with the ingestion of one or more preformed SE present within *S. aureus*-contaminated food
    - Induces emesis (vomiting) but usually not fever; self-limiting
  - Toxic shock syndrome (TSS) (Schlievert et al. 1981):
    - Symptoms: fever (pyrogenic), diffuse macular rash, swollen lymph nodes, sore throat, vomiting, diarrhea, severe localized pain; leads to hypotension and shock
    - Associated with the use of high absorbency tampons
    - TSS occurs when large amounts of PTSAgs are secreted into the circulatory system:
      - Menstrual TSS: associated solely with the secretion of TSST-1, due to its unique ability to cross mucosal barriers

    - Non-menstrual TSS: associated with the expression of TSST-1 plus another SE (primarily SEB and SEC)

- **Exfoliative toxins (ET)** – "epidermolytic toxins" (Bukowski et al. 2010):
    - Staphylococcal scalded skin syndrome (SSSS):
        - Usually associated with neonates and infants
        - Initial symptoms: fever, lethargy, poor feeding
        - Later symptoms: erythematous rash, fluid-filled blisters, skin exfoliation leading to secondary infections
    - **ETA** (predominant serotype), **ETB**, **ETD**:
        - Serine proteases; cleave host desmoglein 1 (Dsg-1)
        - Dsg-1: desmosomal cadherin found in superficial skin layers
        - Cleavage results in a loss of keratinocyte cell-cell adherence, leading to the separation of superficial skin layers and SSSS

- Evasion of host immune system (Spaan et al. 2013; Thammavongsa et al. 2015):
    - Evasion of the host immune system is essential for bloodstream dissemination and systemic disease (see dissemination in human host, above)
    - **Capsule** (O'Riordan and Lee 2004):
        - Produced by over 90% of *S. aureus* strains
        - 11 capsular polysaccharide (CP) types; CP5 and CP8 predominate
        - Blocks opsonophagocytosis
    - **Nuc** (nuclease) (Berends et al. 2010; Thammavongsa et al. 2013):
        - DNAase activity; disrupts neutrophil NETs (neutrophil extracellular traps)
        - Converts NETs into deoxyadenosine (dAdo), which triggers caspase-mediated apoptosis (see below)
    - Blockage of neutrophil extravasation and chemotaxis:
        - **SSLs** (staphylococcal superantigen-like proteins) (Spaan et al. 2013):
            - Multiple alleles (SSL1–14) with different ligand specificities
            - Bind to many different neutrophil receptors, including TLRs:
                - For example, SSL5 blocks neutrophil rolling and extravasation on endothelial cells (Bestebroer et al. 2007)
            - Also block chemokine signaling
        - **CHIPS** (Chemotaxis Inhibitory Protein of Staphylococci): (de Haas et al. 2004):
            - Secreted protein
            - Binds to the complement protein C5a receptor
            - Blocks C5a-mediated neutrophil activation and chemotaxis

- Also binds formyl peptide receptor 1 (FPR1), blocking chemotaxis toward N-formyl-peptide (fMLP) chemoattractants

- **FLIPr; FLIPr-L** (FPR-like 1 inhibitory proteins) (Prat et al. 2009):
  - Bind and inhibit formyl peptide receptors (FPR1, FPR2)
  - FLIPr inhibits FPR1 and FPR2
  - FLIPr-L inhibits FPR2
  - Blocks chemotaxis toward N-formyl-peptide (fMLP) chemoattractants

- **Eap/Map** (extracellular adherence protein) (Chavakis et al. 2002):
  - Binds host ICAM-1 protein and ECM proteins
  - Inhibits neutrophil recruitment, diapedesis (extravasation), adhesion
  - Induces inflammation through binding to ICAM-1

- **Staphopain A, staphopain B** (Laarman et al. 2012; Smagur et al. 2009;1):
  - Secreted cysteine proteases
  - Staphopain A cleaves CXCR2 chemokine receptor; blocks neutrophil activation and migration toward CXCR2 chemokines
  - Staphopain B cleaves neutrophil CD11b and CD31 receptors; induces cell death

– Survival inside neutrophils:

- **Hmp** (Richardson et al. 2008):
  - Flavohemoglobin; detoxifies nitric oxide (NO)

- Detoxifying reactive oxygen species (ROS):
  - **SodA, SodM** (Karavolos et al. 2003):
    – Superoxide dismutase; inactivates superoxide radicals

- **KatA** (catalase) and **AhpC** (alkyl hydroperoxide reductase) (Cosgrove et al. 2007):
  – Scavenges and inactivates H_2O_2
  - **Staphyloxanthin** (Liu et al. 2005):
    – Golden carotenoid; gives *S. aureus* colonies their golden color (*aureus*)
    – Antioxidant activity; protects against H_2O_2 and hydroxyl radicals

- **AdsA** (adenosine synthase A) (Thammavongsa et al. 2011; Thammavongsa et al. 2013):
  - Cell wall protein
  - Dephosphorylates dATP, dADP, dAMP from disrupted NETs (see Nuc above), creating deoxyadenosine (dAdo) molecules
  - DAdo triggers anti-inflammatory signaling, blocks neutrophil respiratory burst, and induces neutrophil apoptosis

- Inhibition of complement-mediated lysis:
  - Multiple proteins block complement activation/function by binding antibodies (predominantly Ig, IgM) or by blocking complement protein C3 processing and/or function:
  - **SdrE** (Sharp et al. 2012):
    - Binds complement factor H (fH); blocks complement activation
  - **SpA** (staphylococcal **protein A**) (Falugi et al. 2013):
    - MSCRAMM cell wall-anchored protein, proteolytically released from the cell wall during cell growth
    - Binds Fcγ portion of IgG; blocks opsonophagocytosis
    - Binds and cross-links the Fab portion of V_H3 clan IgM and IgG; inhibits antibody development by inducing B-cell apoptosis
    - Also binds von Willebrand factor; functions as an adhesin
  - **Sbi** (staphylococcal binder of immunoglobulins) (Smith et al. 2011):
    - Can be secreted or bound to the cell wall:
      - Secreted form binds complement factor C3d and fH; blocks opsonophagocytosis through consumption of C3
      - Bound form binds to Fc portion of IgG (same as SpA); blocks opsonophagocytosis
  - **SCIN, SCIN-B, SCIN-C** (staphylococcal complement inhibitors) (Rooijakkers et al. 2005):
    - Inhibit complement factor C3 convertase; prevents production of C3a, C3b, and C5a
    - Blocks complement activation and complement-mediated phagocytosis
  - **Aureolysin (Aur)** (Laarman et al. 2011):
    - Secreted zinc-dependent metalloproteinase
    - Cleaves human antimicrobial peptide LL-37
    - Cleaves complement factor C3 to generate functionally active C3a and C3b; C3b is then degraded by complement factor I (fI) and fH, which blocks complement accumulation on the cell surface

- *S. epidermidis* (Becker et al. 2014; Otto 2009):
  - Opportunistic pathogen
  - The major virulence factor associated with *S. epidermidis* infections is the ability to form biofilms on abiotic and biotic surfaces
  - *S. epidermidis* biofilm formation involves many of the adherence factors associated with *S. aureus* infections (see above); however, most of the other *S. aureus* virulence factors are absent from *S. epidermidis*

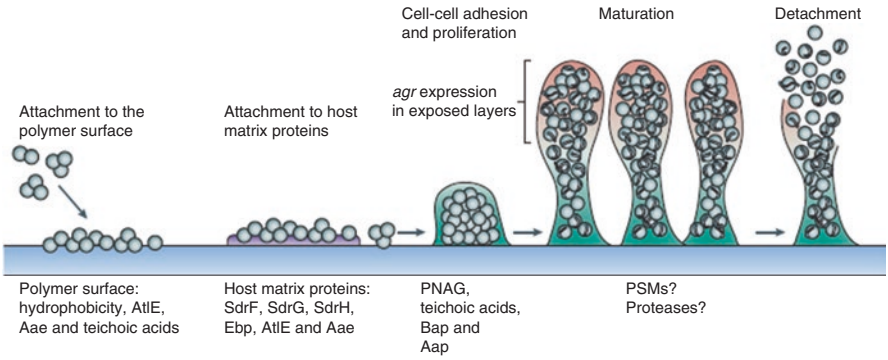

Fig. 9.4 *S. epidermidis* biofilm formation (From: Otto (2009))

- Biofilm formation (Fig. 9.4):
  - *S. epidermidis* attachment to foreign devices occurs during insertion of the device, most likely through endogenous bacteria from the skin or mucus membranes
  - These abiotic surfaces are quickly coated with platelets and host ECM components, such as fibronectin, fibrinogen, thrombospondin, collagen, von Willebrand factor, and vitronectin, which bind to *S. epidermidis* adherence factors:
  - **AtlE** (Heilmann et al. 1997):
    - Autolysin/adhesin; homologous to *S. aureus* Atl autolysin
    - Promotes the initial adherence, possibly through its catalytic hydrolysis of the peptidoglycan layer and release of eDNA, an important component of biofilms
    - Binds to host ECM vitronectin
    - Plays a role in internalization into endothelial cells
  - **Aae** (Heilmann et al. 2003):
    - Autolysin/adhesion: binds to fibrinogen, vitronectin, and fibronectin
  - **WTA** (wall teichoic acid) (Holland et al. 2011):
    - Charged components of the Gram-positive cell wall
    - Interact with abiotic surfaces through electrostatic interactions
    - Also binds to fibronectin
  - **ClpP** (Wang et al. 2007a):
    - Protease; degrades misfolded proteins
    - *clpP* mutant had reduced biofilm formation

- **Bhp** (Tormo et al. 2005):
  - Homologous to *S. aureus* Bap (biofilm associated protein) associated with bovine mastitis
  - Mediates biofilm formation in the absence of PIAs
- **GehD, GehC, GehSE1** (Bowden et al. 2002):
  - Extracellular lipases
  - Bind type I, II, and IV collagen
  - Function in skin colonization
- **Embp** (Christner et al. 2010):
  - Fibronectin-binding protein; distinct from *S. aureus* FnbpA,B
  - Also involved in intercellular adhesion and biofilm formation
- MSCRAMMS:
  - **SdrG/Fbe** (Hartford et al. 2001):
    - Fibrinogen-binding protein; similar to *S. aureus* ClfA
  - **SdrF, SdrH** (McCrea et al. 2000):
    - Binds to type I collagen
  - **SesC** (Shahrooei et al. 2009):
    - Fibrinogen-binding protein
- **PIA (PNAG)** (Heilmann et al. 1996):
  - Homologous to *S. aureus* PIA (PNAG)
  - Biosynthetic genes are encoded in *icaADBC* operon
- **PGA** (poly-γ-glutamic acid) (Kocianova et al. 2005):
  - Similar to PIA exopolymers
  - Protect against neutrophil phagocytosis and antimicrobial peptides (AMPs)
- **Aap** (Hussain et al. 1997):
  - G5-E repeat, cell wall-anchored adhesin
  - Homologous to *S. aureus* SasG
  - Induce PIA-independent biofilm formation
- **PSMs** (phenol-soluble modulins) (Mehlin et al. 1999):
  - Homologous to *S. aureus* PSMs
  - PSMα, PSMγ (δ toxin), PSMδ, PSMε, PSM-mec, PSMβ1, PSMβ2
  - PSMγ (δ toxin), PSMδ, PSMε: cytolytic activity
  - Induce pro-inflammatory response
  - May function in biofilm dispersal

- *S. saprophyticus* (Kline and Lewis 2016):
  - The most important virulence factors are involved in adherence and colonization within the genitourinary tract:
  - **Aas** (Hell et al. 1998):
    - Hemagglutinin, autolysin, and adhesin properties
    - Binds to fibronectin
  - **Ssp** (Gatermann et al. 1992):
    - Surface-associated lipase
    - Forms fimbriae-like surface appendages; its role in virulence is unclear
  - **UafA** (uro-adherence factor A) (Kuroda et al. 2005):
    - Cell wall-associated hemagglutinin
    - Binds to bladder epithelial cells
  - **UafB** (uro-adherence factor B) (King et al. 2011):
    - Serine-rich glycoprotein
    - Binds fibronectin, fibrinogen, and human bladder epithelial cells
  - **SdrI (**serine-aspartate-rich protein I) (Sakinc et al. 2006):
    - Binds collagen
    - Involved in acute UTI and persistent kidney infections
    - Homologous to S. aureus Sdr proteins
  - **Urease** (Gatermann et al. 1989):
    - Colonization and inflammation of the bladder and kidneys
    - Play a role in the formation of urinary stones; similar to those found in Proteus mirabilis UTIs
  - **Capsule** (Park et al. 2010):
    - Blocks complement-mediated opsonophagocytosis
  - **PSM** (phenol-soluble modulins):
    - Pro-inflammatory cytolytic toxins; similar to those found in *S. aureus* and *S. epidermidis*

Regulation of Virulence Factor Expression

- Virulence factor expression is regulated by several quorum-sensing systems, transcriptional activators and repressors, and alternative sigma (σ) factors
- Agr quorum-sensing (QS) system (Le and Otto 2015; Wang and Muir 2016):

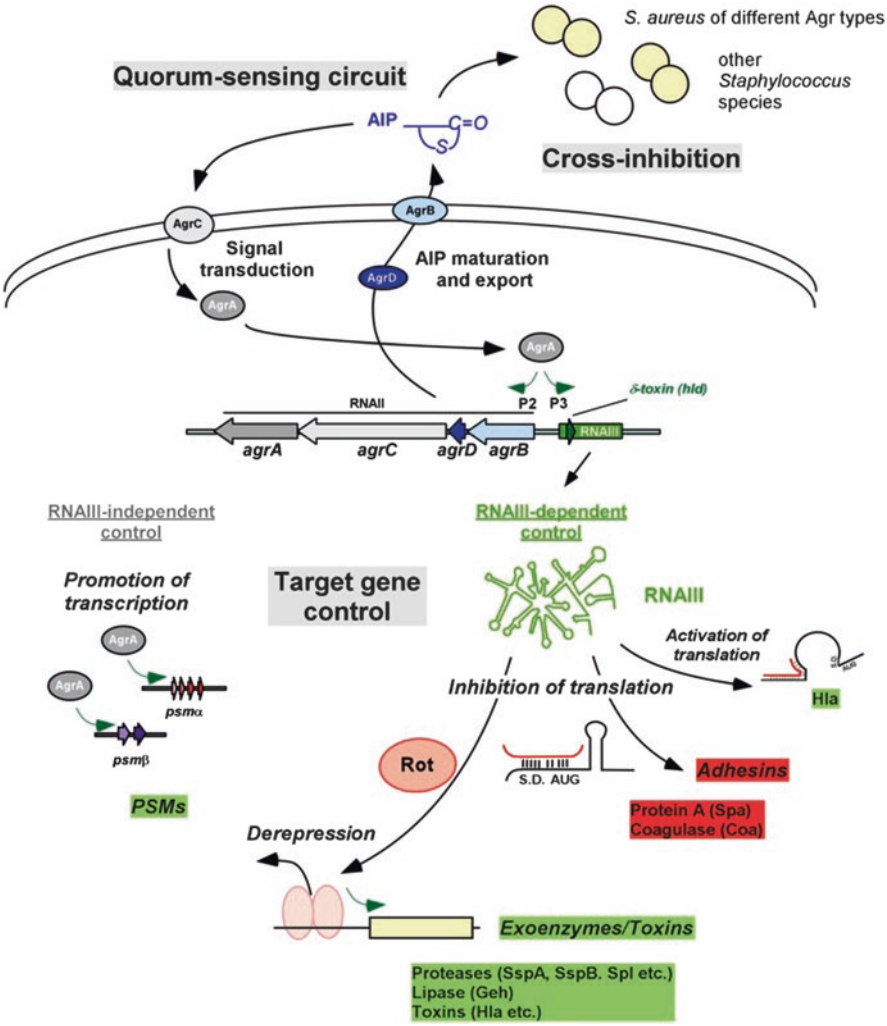

Fig. 9.5 *S. aureus* Agr QS system (From: Le and Otto (2015))

- Most important regulatory system for virulence gene expression in *S. aureus*
- Utilizes autoinducer peptides (AIPs) that interact with a two-component QS system
- Regulates the expression of the *agr* regulatory operon, virulence factors, and surface proteins needed for biofilm formation (Fig. 9.5)
- *agr* locus:
    - Two divergent transcriptional units – RNAII and RNAIII:
        - RNAII: mRNA expressing *agrB*, *agrD*, *agrC*, and *agrA* genes:

- AgrD: encodes precursor pro-AIPs autoinducers
- AgrB: processes and secretes AIPs outside cell
- Four autoinducers: AIP-I, AIP-II, AIP-III, and AIP-IV:
  - Differences are due to hypervariable regions in *agrD* and *agrC*.
  - Associated with different disease states:
    - I and II: most invasive diseases
    - IV: exfoliative toxins ETA, ETB (rare)
    - III: TSS (TSST-1 production)
- AgrC: sensor histidine kinase; phosphorylates AgrA:

  Activated by binding to AIP autoinducers
- AgrA: response regulator; transcription factor:
  - Upregulates the transcription of RNAII and RNAIII
  - Also directly upregulates the transcription of the PSM *psm*α and *psm*β operons

- RNAIII:
  - Regulatory RNA molecule; responsible for translational regulation of Agr target genes (Bronesky et al. 2016)
  - Also contains the *hld* gene for δ-toxin
  - Mechanism: affects the translation of Agr target mRNA by forming RNA duplexes with the 5′ untranslated regions of the target mRNA:
    - Activates the translation of α-toxin and the MgrA transcription repressor
    - Blocks the translation of protein A, LytM, Sbi, FnbpA, FnbpB, Coa coagulase, and other surface adhesins
    - Also blocks the translation of the Rot (repressor of toxins) transcription repressor:
      - Rot repression leads to upregulation of TSST-1, α-toxin, leukocidins, proteases, and lipases and downregulation of staphopains and surface proteins, blocking biofilm formation

- LuxS QS system:
  - Analogous to *Vibrio* spp. QS system
  - Uses the AI-2 autoinducer
  - Regulates capsule synthesis, biofilm formation, virulence factors, and antibiotic susceptibility

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Chapter 10
Streptococcus spp.

Genomics, Morphologies, and Growth Characteristics

- **Genomics** (Maruyama et al. 2016):
  - *Streptococcus pyogenes* M1 strain chromosome: 1,852,442 bp; 1752 predicted ORFs (Ferretti et al. 2001)
  - *Streptococcus pneumoniae* TIGR4 chromosome: 2,160,837 bp; 2236 predicted ORFs (Tettelin et al. 2001)
  - *Streptococcus agalactiae* 2603 V/R isolate chromosome: 2,160,267 bp; 2175 predicted ORFs (Tettelin et al. 2002)
- **Cell morphology:**
  - Cocci: chains of cells (*S. pyogenes*) (Fig. 10.1) or diplococci (*S. pneumoniae*)
- **Gram stain:**
  - Gram positive
- **Growth:**
  - Facultative anaerobes; catalase negative, oxidase negative
  - Reservoirs: normal animal microbiota
  - Excellent biofilm formers (see below)
  - Over 40 species: can be classified by hemolytic patterns, Lancefield antigens, M protein serotypes, and 16rRNA gene sequences
    - Hemolytic patterns:
      - Hemolysis of red blood cells (RBCs) on blood agar plate (BAP)
      - Alpha (α) hemolysis: partial "green" hemolysis; due to bacterial hydrogen peroxide oxidizing the hemoglobin (Fe^{+2}) to green methemoglobin (Fe^{+3})

D.I. Johnson, *Bacterial Pathogens and Their Virulence Factors*,
https://doi.org/10.1007/978-3-319-67651-7_10

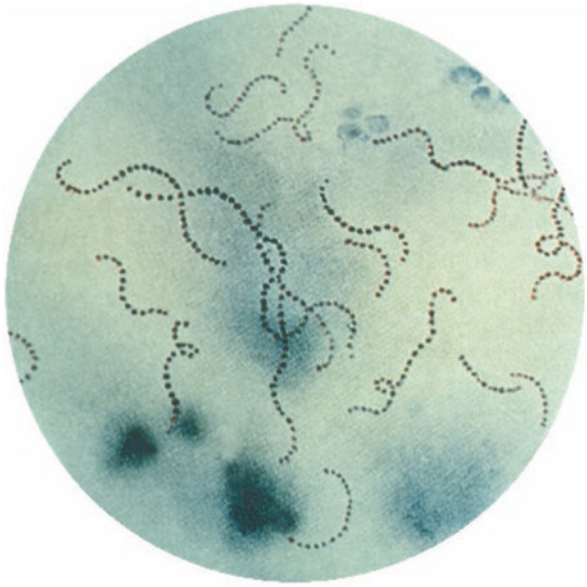

Fig. 10.1 *S. pyogenes* cells (From: PHIL #2109)

- Beta (β) hemolysis: complete hemolysis; due to streptolysin S, O (see below)
- Gamma (γ) hemolysis: no hemolysis

○ Lancefield antigens:

- Based on group-specific antigens or C substances
- Group A through H, K through V (no I, J)
- Groups A, B, C, E, F, G: polysaccharides antigens:
  - Group A carbohydrate (GAC): cell wall polysaccharide containing a polyrhamnose backbone with the immunodominant *N*-acetylglucosamine (GlcNAc) side chain; makes up 50% of cell wall by weight
  - Group B carbohydrate (GBC): cell wall multi-antennary structure containing four different oligosaccharides (rhamnose, galactose, N-acetylglucosamine, and glucitol)
- Groups D and N: teichoic acid antigens
- Group H: lipoteichoic acid antigens

○ M protein serotypes:

- M protein: pili-like adhesin (see below); encoded by *emm* gene
- Greater than 240 M protein serotypes; M1 is the most common *S. pyogenes* serotype

- 16rRNA gene sequences:
  - Six groupings: pyogenic (*S. pyogenes*, *S. agalactiae*), bovis, salivarius, mutans, mitis (*S. pneumoniae*), anginosus

Disease States Associated with *Streptococcus* spp.

- *S. pyogenes* – Group A, β-hemolytic *Streptococcus* (GAS) (Ferretti et al. 2016):
  - Human-adapted primary pathogen
  - Reservoirs: normal microbiota of the respiratory tract and skin
  - Transmission: airborne aerosol transmission; close contact person to person, usually with children who are carriers; contact with fomites contaminated by infected person; contamination of wounds, burns (skin breach)
  - ~700 million cases of mild, noninvasive infections/year worldwide
  - ~2 million cases progress to severe invasive infections; ~25% mortality
    - Predominantly due to M1 T1 serotype
  - Noninvasive purulent diseases – respiratory and skin diseases:
    - Pharyngitis (strep throat) (Fig. 10.2)
    - Scarlet fever (pharyngitis with a rash)
    - Impetigo (infection of the superficial keratin layer)
    - Erysipelas (infection of the superficial epidermis; lymphatic involvement)

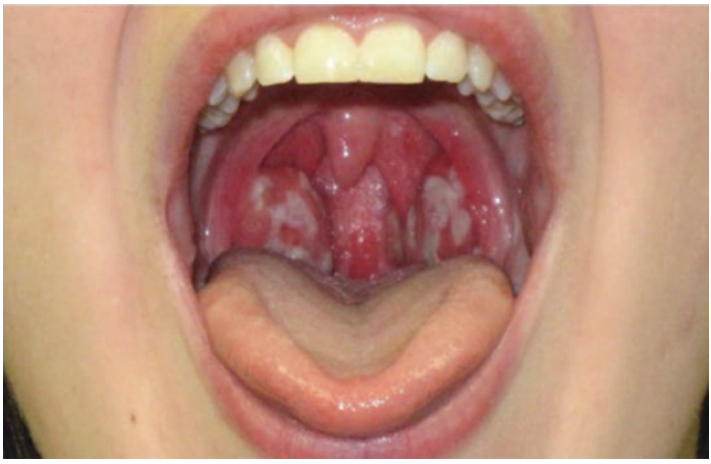

Fig. 10.2 GAS pharyngitis (From: Block (2014))

- Invasive non-purulent diseases:
  - Cellulitis (infection of subcutaneous tissue)
  - Necrotizing fasciitis (infection of deep subcutaneous tissues and muscle fascia)
  - Myonecrosis (infection of muscle)
  - Streptococcal toxic shock syndrome (STSS)
  - Bacteremia
  - Infectious endocarditis
  - Pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (PANDAS); children with obsessive-compulsive disorder (OCD) and/or tic disorders following a streptococcal infection
- Sequelae diseases:
  - Acute rheumatic fever (ARF), rheumatic heart disease (RHD), acute poststreptococcal glomerulonephritis (APSGN)
  - Due to host immunological reactions to GAS antigens
  - Usually the result of repeated untreated or undertreated infections
  - ARF (Cunningham 2016):
    - Molecular mimicry; antibodies directed against GAS GlcNAc and M proteins cross-react with myosin protein in heart valves; leads to inflammation and damage to heart valves
  - APSGN (Rodriguez-Iturbe and Haas 2016):
    - Due to inappropriate deposition of antigen-antibodies complexes in glomeruli of the kidney; leads to inflammation and kidney failure
    - Associated with antibodies against M proteins 1, 2, 4, 12, 47, 49, and 55; also associated with antibodies against nephritis-associated plasmin receptor (NAPlr; GAPDH) and SPE-B

- *Streptococcus agalactiae* – Group B, β-hemolytic *Streptococcus* (GBS):
  - Neonatal sepsis; meningitis; pneumoniae
  - Transmission: mother is colonized; infant is infected during birth
    - Risk factors: premature delivery, early membrane rupture
  - Low incidence but extremely serious
  - Reservoir: gastrointestinal tract, genitourinary tract
- *Streptococcus pneumoniae* (pneumococci) – α-hemolytic; no C substances identified (Feldman and Anderson 2016):
  - Reservoir: normal microbiota of upper respiratory tract; colonize the nasopharynx
  - Transmission: airborne transmission of aerosols (Fig. 10.3)
  - Inflammatory diseases
  - Can disseminate to ears (otitis media), sinuses (sinusitis), and bronchi (bronchitis)

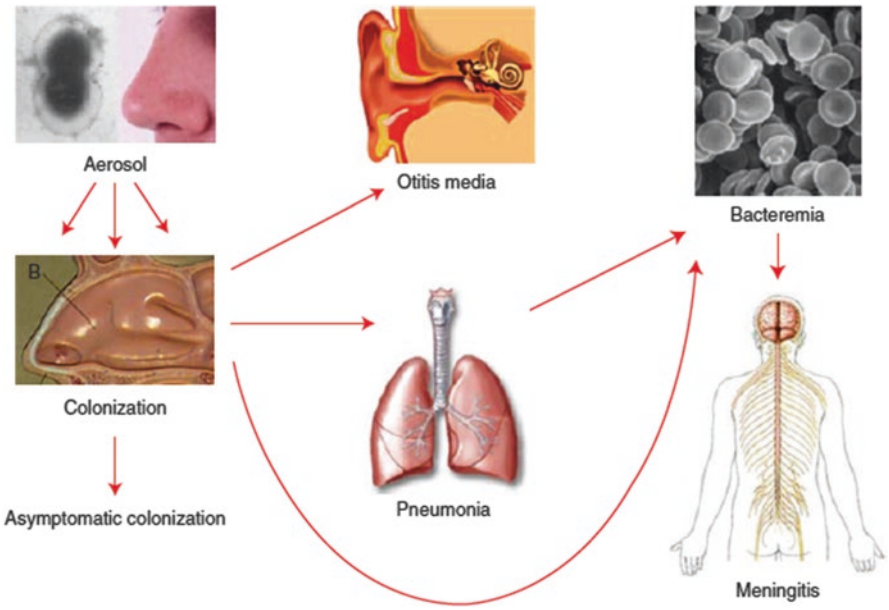

Fig. 10.3 Pneumococcal disease dissemination (From: Henriques-Normark and Tuomanen (2013))

- Invasive disease involves spread to lungs (pneumococcal pneumonia) and bloodstream (bacteremia/septicemia), which can lead to the meninges (meningitis)
- Pneumococcal pneumonia:
  - Most common community-acquired (CA) pneumonia
  - Abrupt, severe shaking chills; sustained high fever; productive cough; rust-colored sputum
- Bacteremia/septicemia: 25–30% of patients with pneumonia
- Meningitis: one of the leading causes of adult meningitis

Virulence Factors

- GAS (Wilkening and Federle 2017):
  - Adherence to host cells (Brouwer et al. 2016; Nobbs et al. 2009):
    - *Streptococcus* spp. can colonize multiple sites in the human host; due to the strain-specific expression of 100 s of fimbrial and afimbrial adhesins (for a comprehensive listing, see Brouwer et al. (2016), Nobbs et al. (2009))

- Biofilm formation plays a key role in adherence
- **Lipoteichoic acid (LTA)**:
  - Fatty acid domains of cell wall LTA interact weakly with fatty acid binding domains in host cell membranes
- Fimbrial adhesins:
  - **Pili** (Mora et al. 2005; Ros 2016):
    - Cell wall-anchored (CWA) long, flexible rods
    - **BP/FctA**: backbone pilin subunit
    - **AP1/Cpa; AP2/FctB**: accessory protein subunits; mediate the specific binding to host receptors
    - Mature pilus assembled via pilus-associated sortases SrtB/SrtC2
    - Assembled pilus is anchored to cell wall via SrtA sortase
    - FCTs – genomic regions that encode pili biosynthetic and regulatory genes:
      - Also encode adhesins that bind to fibronectin, collagen, and T antigens (FCT); FCT-1 to FCT-9
  - **M protein** (Fischetti 2016; Smeesters et al. 2010):
    - Major adherence factor to host cells; inhibits phagocytosis in the absence of opsonizing antibodies
    - Pili-like structure; alpha-helical coiled-coil dimer
    - Attached to the cell wall peptidoglycan by SrtA sortase; can extend up to 600 nm from the cell wall
    - >240 antigenic variants; used for classification (see above)
    - Variant-specific binding:
      - ECM components (Fn, collagen)
      - Complement regulatory proteins [factor H (fH), factor H-like-1 (FHL-1), C4 binding protein (C4BP)]
      - Immune components (T cell CD46, IgG, β 2-microglobulin); acts as a superantigen to induce T-cell proliferation (Påhlman et al. 2006)
      - Host serum proteins (fibrinogen, plasminogen, albumin)
      - Host proteins and polysaccharides [kininogen, glycosaminoglycans, sialic acid (mucin)]
- Afimbrial adhesins:
  - **MSCRAMMs** (microbial surface components recognizing adhesive matrix molecules):
    - Cell wall-anchored (CWA) adhesins: attached to the cell wall by sortase transpeptidases (Foster et al. 2014)
    - Bind to host ECM components fibronectin (Fn), fibrinogen, plasminogen, laminin, collagen, as well as Ig antibodies (Brouwer et al. 2016); attaches GAS to host cells via the ECM

- **Fibronectin-binding proteins (Fnbp)**:
  - Protein F1 (Sfb1/PrtF1); Protein F2 (PrtF2/FbaB/PFBP); FbaA; SfbX; SOF (serum opacity factor)/Sfb2
- **Fibrinogen-binding proteins**:
  - Protein F1; Protein F2; SOF/Sfb2, Mrp
- **Plasminogen-binding proteins**:
  - PAM, Prp, Epf
- **Laminin-binding proteins**:
  - Lsp, LBP
- **Collagen-binding proteins**:
  - Cpa/AP1, Slr, SpyAD
- **Immunoglobulin (Ig)-binding proteins**:
  - Protein H, Mrp, Arp, Sir

- **Scl1, Scl2** (Lukomski et al. 2017):
  - Collagen-like proteins: mimic triple-helical elongated protein structure of collagen
  - Bind to same host proteins as host collagen: cellular fibronectin, laminin, α2β1integrin, α11β1 integrin, lipoproteins, complement fH, complement factor H-related protein 1 (CFHR1)
- **Secreted adhesins**:
  - **SPE-B (streptococcal pyrogenic exotoxin B)** (Chiang-Ni and Wu 2008; Nelson et al. 2011):
    - mSpeB1, mSpeB2, mSpeB3 variants
    - May control adherence and cell invasion by binding to host laminin, $\alpha_5\beta_3$ integrin, $a_{2b}\beta_3$ integrin, thyroglobulin
    - Secreted cysteine protease – degrades host proteins:
      - ECM and serum components (fibronectin, vitronectin, plasminogen, kininogen), immunoglobulins (IgA, IgM, IgD, IgE, IgG), and complement component C3b
    - Degrades GAS surface adhesins:
      - M protein, protein F1, EndoS, SmeZ, Fba, streptokinase, protein H, Sda1

- Growth and dissemination in human host:
  - **HylA** (Hynes et al. 2000):
    - Hyaluronate lyase (hyaluronidase); breaks down hyaluronic acid in the ECM; allows spread of pathogen; may also provide nutrients
    - Bacteriophage-encoded hyaluronate lyases: may play a role in allowing bacteriophage containing virulence factors to penetrate the hyaluronic acid capsule and bind to cell receptors
    - Encapsulated GAS strains do not express the HylA hyaluronidase, while nonencapsulated strains do express it (Wessels 2016a)
  - **Streptokinase (Ska)** (Nolan et al. 2013):
    - Plasminogen activator; induces the host fibrinolytic pathways, releasing bacterial cells from fibrin blood clots and disrupting the ECM through the activation of matrix metalloproteinases
    - Allows bacterial spread and invasion of tissues
    - Also associated with postinfection acute glomerulonephritis sequelae (Nordstrand et al. 1998)
  - **GRAB** (protein G-related α2M-binding protein) (Rasmussen et al. 1999):
    - Binds the human protease inhibitor α2-macroglobulin (α2M); protects against host proteolysis
  - Iron acquisition:
    - Binding to host iron-containing proteins:
      - **Sia** (streptococcal iron acquisition) system:
        - Obtain heme from host hemoproteins; similar to *S. aureus* Isd system
  - Biofilm formation (Fiedler et al. 2015; Young et al. 2016):
    - Essential for virulence; EPS composed of DNA, protein, sugars
    - Able to form on abiotic and biotic surfaces
    - M proteins, capsule, and pili, along with the AspA and Scl1 proteins, play major roles in biofilm formation
- Damage to host cells:
  - **Streptolysin S (SLS)** (Molloy et al. 2011):
    - Oxygen-stable, membrane-disrupting cytolysin
      - SLS accumulation in membranes leads to the generation of transmembrane pores; results in osmotic lysis
      - Broad spectrum; cytolytic to many host cells, including leukocytes and red blood cells
      - Resultant destruction of cells and tissue is the major virulence mechanism for GAS

- Multifunctional 30-aa highly modified polypeptide
  - Member of family of thiazole/oxazole-modified microcins (TOMMs); related to family of class 1 bacteriocins
  - *sagABCDEFGHI* operon (Nizet et al. 2000):
    - *sagA*: structural gene for SLS
    - *sagB,C,D*: modifying enzymes
    - *sagG,H,I*: ABC transporter
  - Contains multiple modified Cys, Ser, and Thr amino acids
    - SLS modification: heterocyclic formation
      - Catalyzed by cyclodehydratase (SagC) and dehydrogenase (SagB)
      - Ser residues converted to oxazole
      - Cys residues converted to thiazole
      - Thr residues converted to methyloxazole
      - Modifications result in more rigid structure that is essential for bioactivity of membrane insertion/disruption

- **Streptolysin O (SLO)** (Peraro and van der Goot 2016; Tweten 2005):
  - Oxygen labile, pore-forming cytolysin; mechanistically different from SLS:
    - Member of the cholesterol-dependent cytolysin (CDC) family; homologous to *S. pneumoniae* pneumolysin (Peraro and van der Goot 2016)
    - Lytic to cells containing cholesterol in their cell membranes
    - X-ray crystal structure is similar to *C. perfringens* perfringolysin O (Feil et al. 2014)
  - Induces apoptosis in phagocytes
  - Toxic to heart and other tissues
  - Plays a role in the entry of streptococcal NAD-glycohydrolase into host cells; depletes the host stores of NAD (O'Seaghdha and Wessels 2013)
- **SlaA** (Nagiec et al. 2004):
  - Secreted phospholipase A; disrupts host cell membranes
  - *slaA* mutants have a decreased ability to adhere to host cells
- **SPE-B (exotoxin B)** (see above):
  - Protease; degrades host proteins including ECM components, IgG, cytokines, etc.

- **SAgs (superantigen exotoxins)** (Proft and Fraser 2016; Spaulding et al. 2013):
  - Bind to TCR (T-cell receptor) Vβ variable chains on T cells and class II MHC (major histocompatibility complex) α- and β-chains on antigen-presenting cells (APCs); different than the normal antigen-specific TCR binding
  - Induce nonspecific, massive T-cell proliferation (up to 20% of all T cells)
  - Binding "tricks" T cells into releasing enormous amounts of host cytokines, resulting in a "cytokine storm"; same mechanism as *S. aureus* PTSAgs
  - **SPE-A (exotoxin A), SPE-C, SPE-G, SPE-H, SPE-I, SPE-J, SPE-K, SPE-L, SPE-M, SsA, SmeZ1,2**:
    - Streptococcal toxic shock syndrome (STSS) (McCormick et al. 2001):
      - Most often associated with SPE-A and SPE-C
      - Symptoms: fever (pyrogenic), diffuse macular rash, swollen lymph nodes, sore throat, vomiting, diarrhea, severe localized pain; leads to hypotension and shock
    - Scarlet fever (Wessels 2016b):
      - Rash along with a GAS infection, usually pharyngitis
      - Most often associated with SPE-A, SPE-C, and SSA

– Evasion of host immune system (Fig. 10.4) (Cole et al. 2011):
  - **Capsule** (Wessels 2016a):
    - Polymer of hyaluronic acid; does not stimulate antibody production due to similarity to host hyaluronic acid

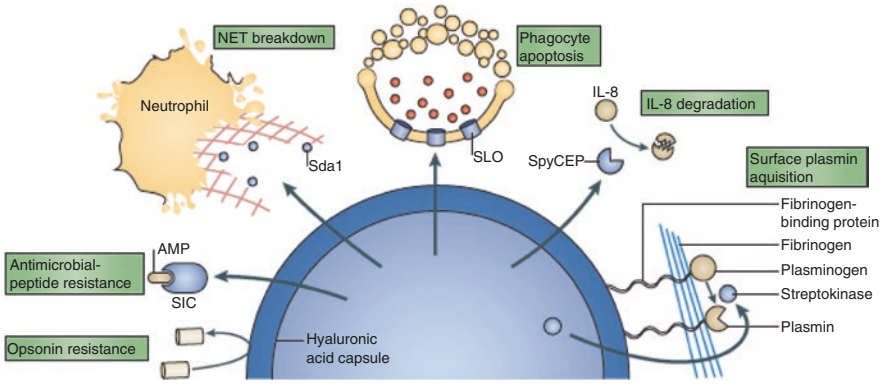

Fig. 10.4 Evasion of host neutrophils (From: Cole et al. (2011))

- Blocks opsonophagocytosis by interfering with leukocyte receptor binding to complement factors
- Acts as an adhesin by binding to host CD44 protein on cells

- **M protein** (see above):
  - Binds to complement regulatory proteins factor H (fH), factor H-like-1 (FHL-1), and C4 binding protein (C4BP) and to the Fc domain of IgA; blocks opsonophagocytosis
- **SibA** (Fagan et al. 2001):
  - Binds to the Fc and Fab region of IgA, IgG, and IgM; blocks opsonophagocytosis
- **DNAases** (streptodornases) (Walker et al. 2007):
  - Multiple different DNAases; destroy DNA-based neutrophil extracellular traps (NETs); allows escape from antimicrobial factors
  - **Sda1, SdaD2, SdaB, SpdI1, SpnA**
- **SodA** (McMillan et al. 2004):
  - Superoxide dismutase; detoxifies exogenous superoxide radicals produced by neutrophils
- **SIC** (streptococcal inhibitor of complement) (Fernie-King et al. 2001; Hynes and Sloan 2016):
  - Inhibits membrane attack complex (MAC) formation
  - Inhibits host antimicrobial proteins such as lysozyme, LL-37, defensins, and the chemokine MIG/CXCL9
- **IdeS** (Pawel-Rammingen et al. 2002):
  - Cysteine protease; similar to SpeB
  - Cleaves IgG; blocks opsonophagocytosis
- **Sib35** (Kawabata et al. 2002):
  - Binds IgG, IgA, and IgM
  - Shows similarity to IdeS; induces B-cell proliferation
- **C5a peptidase (ScpA)** (Cleary 1992):
  - Cleaves chemotactic complement factor C5a; blocks migration of neutrophils to the site of infection
- **IL-8 protease (SpyCEP)** (Zingaretti et al. 2010):
  - Serine protease; cleaves chemokine CXCL-8/IL-8 and all human CXC chemokines
  - Homologous to C5a peptidase from GAS

- **EndoS** (endo-β-*N*-acetylglucosaminidase) (Trastoy et al. 2014):
  - Removes carbohydrates from IgG; blocks binding to Fc receptors; blocks opsonophagocytosis
- **SsE** (Zhu et al. 2009):
  - Secreted esterase; similar to esterases that hydrolyze platelet-activating factor (PAF), which mediates chemotaxis
  - Diminishes the recruitment of neutrophils

- *S. agalactiae* (GBS) (Maisey et al. 2008):
  - Adherence to host cells:
    - Fimbrial adhesins:
      - **Pili** (Rosini et al. 2006):
        - Cell wall-anchored (CWA) long, flexible rods
        - Encoded in pili islands PI-1, PI-2a, and PI-2b; each island encodes three proteins
          - **Bkb**: backbone pilin subunit
          - **An1 (tip), An2 (base)**: accessory protein subunits; mediate the specific binding to host receptors
        - Mature pilus assembled via pilus-associated sortases SrtB/SrtC2
        - Assembled pilus is anchored to cell wall via SrtA sortase
    - Afimbrial adhesins:
      - **MSCRAMMs**:
        - **FbsA** (Ragunathan and Ponnuraj 2011):
          - Fibrinogen-binding protein
        - **FbsC (BsaB)** (Jiang and Wessels 2014):
          - Adhesin involved in biofilm formation
          - Binds fibronectin, laminin
      - **FbsB** (Devi and Ponnuraj 2010):
        - Anchorless fibrinogen-binding protein
        - Plays a role in epithelial and endothelial cell invasion
      - **Lmb** (Spellerberg et al. 1999):
        - Lipoprotein; binds host laminin; plays a role in epithelial and endothelial cell invasion
        - Homologous to GAS LBP

- **α-C protein (ACP)** (Baron et al. 2004):
  - Invasin: promotes host cell internalization by binding to surface glycosaminoglycan (GAG); mediated by Rho GTPase-dependent actin rearrangements

– Growth and dissemination in human host:
  - **HylB** (Mello et al. 2002):
    - Hyaluronate lyase (hyaluronidase); breaks down hyaluronic acid in the ECM; allows spread of pathogen; may also provide nutrients
  - Biofilm formation (Rosini and Margarit 2015):
    - Essential for virulence
    - Able to form on abiotic and biotic surfaces

– Damage to host cells:
  - **β-hemolysin/cytolysin** (Nizet 2002):
    - Oxygen-stable, pore-forming cytolysin; encoded by *cylE* gene
    - Cytolytic to lung epithelial cells, lung endothelial cells, brain endothelial cells, and macrophages
    - Induces caspase-dependent apoptosis
    - Induces a pro-inflammatory response; stimulates NO release
  - **CAMP (Christie, Atkins, Munch-Petersen) factor** (Lang and Palmer 2003):
    - Pore-forming hemolysin
    - Shows synergistic hemolytic effect with *S. aureus* β-toxin in CAMP test

– Evasion of host immune system:
  - **Capsule** (Ragunathan and Ponnuraj 2011):
    - Polymers of glucose, galactose, *N*-acetylglucosamine, and sialic acid
    - Ten serotypes: types 1a, 1b, 2, 3 (majority of neonate infections), 4, 5, 6, 7, 8, and 9
    - Blocks complement-mediated opsonophagocytosis by inhibiting binding of the activated complement factor C3b
  - **β protein (β-C protein)** (Areschoug et al. 2002):
    - Binds Fc domain of serum IgA and the complement regulator factor H (fH); prevents opsonophagocytosis
  - **C5a peptidase (ScpB)** (Cheng et al. 2002):
    - Cleaves chemotactic complement factor C5a; blocks migration of neutrophils to the site of infection
    - May function as an adhesin and fibronectin-binding protein

- **CspA** (Harris et al. 2003):
  - Serine protease; cleaves fibrinogen; resulting fibrils block opsonophagocytosis
- **BibA** (Santi et al. 2007):
  - Binds complement regulator C4 binding protein (C4BP); blocks phagocytosis
  - Also an MSCRAMM adhesin
- **SodA** (Poyart et al. 2001):
  - Superoxide dismutase; detoxifies exogenous superoxide radicals produced by neutrophils

- *S. pneumoniae* (pneumococci) (Fig. 10.5) (Henriques-Normark and Tuomanen 2013; Poll and Opal 2009):
  - Adherence to host cells:
    - *S. pneumoniae* must bypass at least three host barriers to adhere to epithelial cells in the nasopharynx and to escape the mucus layer in the lower respiratory tract (Mook-Kanamori et al. 2011):
      - Host mucus layer:
        - Pneumococcal capsule blocks binding to mucus sialic acid residues
        - Secretes exoglycosidases (NanA, NanB, BgaA, StrH) that deglycosylate mucus glycoconjugates; decreases mucus viscosity
          - **NanA, NanB** (Berry et al. 1996):
            - Neuraminidases; cleave terminal sialic acid from cell surface glycans such as mucin, glycolypids, and glycoproteins
          - **BgaA**: β-galactosidase (Dalia et al. 2010)
          - **StrH**: *N*-acetylglucosaminidase (Dalia et al. 2010)
        - **Pneumolysin (Ply)**:
          - Decreases mucociliary beating
          - Allows adherence and avoids clearance in lower respiratory tract
          - Major *S. pneumoniae* virulence factor (see below)
      - Host lysozyme:
        - ***N*-acetylglucosamine-deacetylase A (PdgA)** and ***O*-acetyltransferase (Adr)**: alter the peptidoglycan to make it resistant to lysozyme (Davis et al. 2008)
      - Host secretory IgA (s-IgA): (Fig. 10.4)
        - Secretes **s-IgA1 protease**: destroys s-IgA, which prevents removal by mucociliary escalator

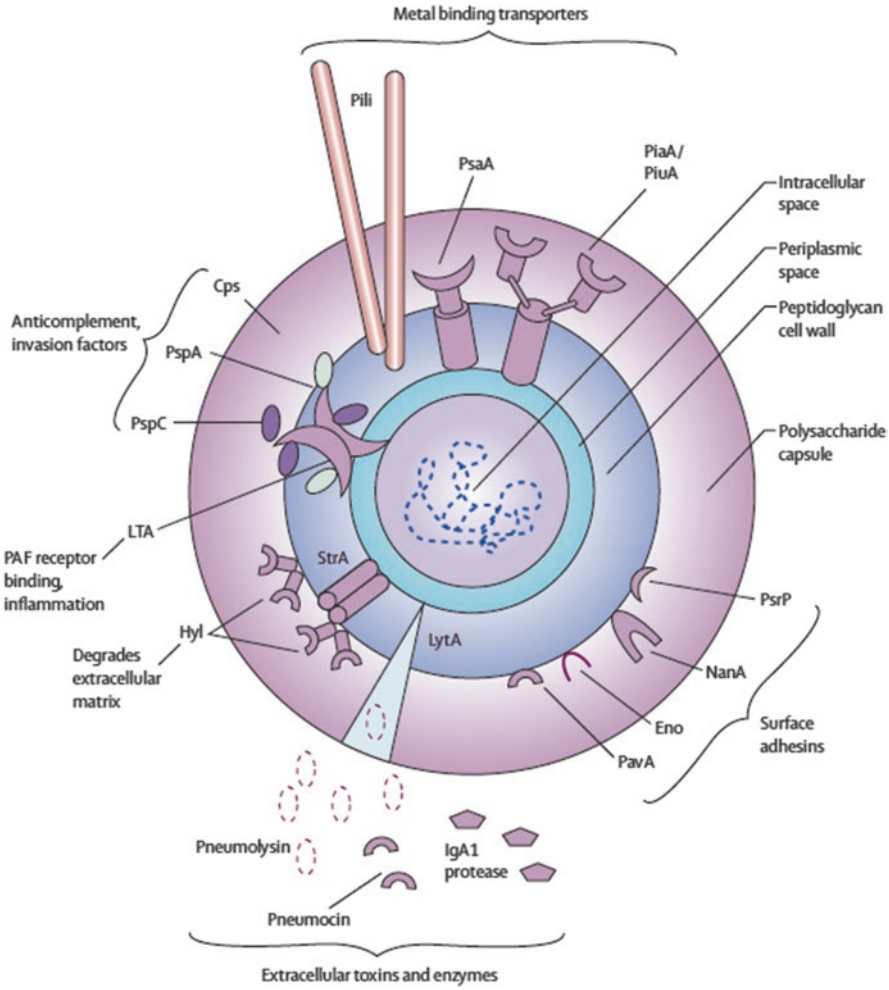

Fig. 10.5 *S. pneumoniae* virulence factors (From: Poll and Opal (2009))

- Fimbrial adhesins:
  - **Pili** (Bagnoli et al. 2008):
    - Cell wall anchored (CWA)
    - Binds fibronectin, laminin, collagen
    - Encoded in pili islands PI-1 and PI-2:
      - **PI-1**:
        - **RgaB**: backbone pilin subunit
        - **RgaA (tip); RgaC (base)**: accessory protein subunits

- **PI-2**:
  - **PitB**: single polypeptide
- Assembled pilus is anchored to cell wall via SrtA sortase

- Afimbrial adhesins:
  - **SpxB, Smi, MsrA, PlpA**:
    - Directly or indirectly facilitate the binding to polysaccharides, such as *N*-acetyl-D-galactosamine (GalNac), on the host cell surface
  - **Phosphorylcholine (PCho)**:
    - Present on the *S. pneumoniae* cell wall; *S. pneumoniae* has a nutritional requirement for choline
    - Binds platelet-activating factor receptor (PAFr) on epithelial cells; important for epithelial transmigration leading to dissemination
  - **CbpA/PspC** (Rosenow et al. 1997):
    - Choline-binding surface protein (CBP); 12 CBPs in *S. pneumoniae*
    - Attaches to PCho on the *S. pneumoniae* cell wall
    - Binds to polymeric immunoglobulin receptor (pIgR); promotes adherence and colonization of nasopharyngeal cells, as well as epithelial transmigration
    - Also binds S-IgA, complement fH, and complement component C3; involved in immune evasion
  - **CbpD, CbpE, CbpG, LytB, LytC** (Gosink et al. 2000):
    - Choline-binding proteins: attach to the phosphorylcholine present on the *S. pneumoniae* cell wall
    - Play a role in adhesion and colonization of the nasopharynx
  - **LytA** (Berry et al. 1989):
    - Autolysin; degrades peptidoglycan layer
    - Choline-binding surface protein
    - *lytA* mutants have reduced virulence
  - **PavA** (Holmes et al. 2001):
    - Fibronectin-binding protein; not a MSCRAMM
  - **PavB** (Jensch et al. 2010):
    - Fibronectin-binding protein; MSCRAMM
  - **PfbA** (Yamaguchi et al. 2008):
    - Binds to plasminogen and fibronectin; MSCRAMM

- Growth and dissemination in human host:
  - **HysA** (Li et al. 2000):
    - Hyaluronate lyase (hyaluronidase)
    - Breaks down hyaluronic acid in the ECM; allows spread of pathogen; may also provide nutrients
  - **Enolase (Eno)** (Bergmann et al. 2001):
    - Plasminogen-binding protein
    - Activated plasminogen-enolase-Gly3Ph-CbpE protein complex degrades E-cadherin in inter-epithelial adherens junctions, facilitating dissemination through pericellular migration
  - Iron acquisition:
    - Host hemoprotein binding proteins:
      - **Spbhp-37**: hemoglobin-binding protein (Romero-Espejel et al. 2016)
      - **Spbhp-22**: heme-binding protein (Romero-Espejel et al. 2013)
      - PiaABC, PiuABC, and PitABC – ABC transporters (Brown et al. 2001):
        - **PiaA**: lipoprotein; transports ferrichrome (Fch)
        - **PiuA**: lipoprotein; transport heme
        - **PitA**: lipoprotein; transports Fe^{3+} ions
  - Biofilm formation (Oggioni et al. 2006):
    - Essential for virulence
    - Able to form on abiotic and biotic surfaces
- Damage to host cells:
  - **Pneumolysin (Ply)** (Cockeran et al. 2002):
    - Pore-forming cytolysin; cytoplasmic, not secreted
    - Member of the cholesterol-dependent cytolysin (CDC) family; homologous to *S. pyogenes* streptolysin O (Peraro and van der Goot 2016):
      - Lytic to cells containing cholesterol in their cell membranes
    - Induces massive inflammatory response through activation of pro-inflammatory cytokine expression and pro-apoptotic pathways
    - Induces neuronal cell death in meningitis infections (Henriques-Normark and Tuomanen 2013)
    - Inhibits phagocytosis and the complement pathway
- Evasion of host immune system:
  - **Capsule** (Kadioglu et al. 2008):
    - Essential for bloodstream dissemination needed for bacteremia and meningitis; anti-phagocytic

- Very thick polysaccharide capsule; 200–400 nm thick
- Over 90 capsular types based on capsule polysaccharides:
  - Twenty-three types cause 88% of diseases
  - Capsule polysaccharides are the major components of pneumococcal vaccines; available for immunocompromised, elderly, infants
- Reduces complement deposition on bacterial surface
- Blocks ensnarement in neutrophil extracellular traps

○ **S-IgA protease (ZmpC)** (Oggioni et al. 2003):

- Cleaves S-IgA1 and matrix metalloproteinase 9 (MMP-9)

○ **PspA** (Ren et al. 2004):

- Choline-binding surface protein
- Blocks the accumulation of complement protein C3 on the cell surface; interferes with phagocytosis

○ **PspC (CbpA; SpsA)** (Dave et al. 2004):

- Choline-binding protein
- Binds complement proteins C3b, fH, C4BP; blocks opsonophagocytosis
- Binds laminin receptor (LR) on brain microvascular endothelial cells; this is the key binding interaction mediating *S. pneumoniae* meningitis; same receptor is targeted by adhesins of *Haemophilus influenzae* and *Neisseria meningitidis* (Orihuela et al. 2009)

○ **PhpA** (Zhang et al. 2001):

- Surface protein; degrades complement protein C3

○ **NanA, NanB, BgaA, StrH**:

- Exoglycosidases; decrease mucin viscosity (see above)
- Blocks the accumulation of complement C3 on the cell surface; interferes with phagocytosis

Regulation of Virulence Factor Expression

- GAS transcriptional regulation (Vega et al. 2016):
  - Thirty transcriptional regulators
  - Thirteen TCSs, including control of virulence regulators (CovR–CovS), quorum sensing (Rgg2–Rgg3), responses to host saliva (SalK–SalR), and response to host neutrophils (Ihk–Irr)
  - Transcriptional regulation of virulence factors is under complex, multifactor control, primarily involving the master regulator CovR–CovS:

- **CovR–CovS (CsrR–CsrS)** (Churchward 2007):
  - **CovS**: sensor histidine kinase; phosphorylates CovR
  - **CovR**: response regulator
    - Represses virulence gene expression
    - Regulates the expression of 15% (271 genes) of all chromosomal genes (Graham et al. 2002)
    - Directly represses the synthesis of the hyaluronic acid capsule, streptolysin S (SLS), streptolysin O (SLO), SPE-A superantigen, SPE-B cysteine protease, SpyCEP IL-8 protease, Ska streptokinase, Sda1 streptodornase DNAase, EndoS IgG modifying protein, and Fba fibronectin-binding protein
    - Indirectly represses G-related α2-macroglobulin-binding protein (GRAB) by repressing the RivR (Ralp4) regulator:
      - RivR also induces the expression of EndoS, M protein, and Fba
    - Represses the **Rov4** transcriptional regulator:
      - Rov4 induces the expression of protein F (Sfb1) and collagen-binding protein Cpa
      - Rov4 represses the expression of streptolysin S, SPE-B protease, and SPE-A superantigen
  - GAS M1 T1 strain – most abundant globally disseminated GAS strain; associated with invasive diseases, such as necrotizing fasciitis (Cole et al. 2011):
    - Contains a 7-bp frameshift mutation in *covS*, resulting in a truncated, inactive CovS protein (Sumby et al. 2006)
    - Inactivation of CovS blocks CovR transcriptional repression
    - Leads to upregulation of the abovementioned CovR-repressed virulence factors and upregulation of the abovementioned Riv4-dependent and Rov4-dependent transcripts
    - Hence, loss of CovR–CovS regulation results in a massive change in transcriptional regulation, leading to an enhancement of M1T1 strain virulence and increased incidence of invasive GAS diseases
- GBS contains a similar CsrR–CsrS (CovR–CovS) regulon controlling virulence factor expression (Jiang et al. 2005; Park et al. 2012)

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Part II
Gram-Negative Bacterial Pathogens

Chapter 11
Bacteroides spp.

Genomics, Morphologies, and Growth Characteristics

- **Genomics:**
  - *Bacteroides fragilis* chromosome: 5,277,274 bp; 4578 predicted ORFs (Kuwahara et al. 2004)
- **Cell morphology:**
  - Rod shaped (Fig. 11.1)
- **Gram stain:**
  - Gram negative
- **Growth:**
  - Anaerobes; catalase positive; relatively aerotolerant at nanomolar O_2 concentrations
  - Predominant mutualistic microbiota of human GI tract; helps break down food; produces nutrients and energy
  - >90 species: *Bacteroides fragilis* only significant human pathogenic species

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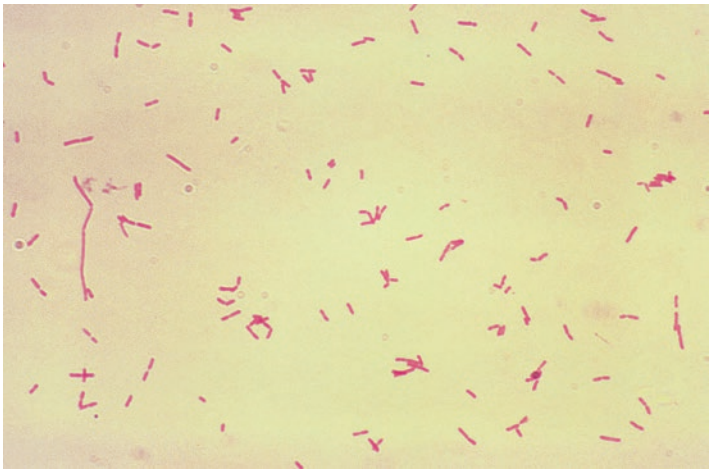

Fig. 11.1 *B. fragilis* cells (From: PHIL #2996)

Disease States Associated with *Bacteroides fragilis*

- Nonenterotoxigenic *B. fragilis* (NTBF):
  - Significant opportunistic pathogen
  - Perforations in colon can lead to formation of intra-abdominal abscess (Gibson et al. 1998):
    - Disruption of the intestinal wall through surgery, cancer, trauma, or disease can allow normal microbiota to infiltrate the abdominal cavity
    - Released aerobes cause tissue destruction, which reduces the redox potential of oxygenated tissue – favors anaerobic growth
    - *Bacteroides* and other anaerobes begin to replicate, leading to inflammation, diarrhea, and intra-abdominal abscesses – capsule polysaccharide PSA plays a role in binding to the abdominal wall and inducing pro-inflammatory cytokines (Gibson et al. 1998) (see below)
    - Dissemination outside the GI tract can lead to bacteremia and abscess formation occurring in the CNS (meningitis), head, neck, chest, abdomen, pelvis, skin, soft tissues
- Enterotoxigenic *B. fragilis* (ETBF) (Sears 2009):
  - Toxigenic primary pathogen – *Bacteroides fragilis* toxin (BFT); see below
  - Toxin-mediated acute diarrheal disease, especially in young children
  - Leads to acute and long-term inflammation, which is correlated with inflammation-induced colon neoplasia (Sears et al. 2014)

Virulence Factors

- Adherence to host cells:
  - **Capsule**:
    - Most complex bacterial polysaccharide capsule system
    - Eight different polysaccharides (PSA–PSH)
    - Function in adherence to peritoneal surfaces
    - PSA is sufficient to induce abscess formation outside colon:
      - Triggers TLR-2-dependent immune reactions; increases in anti-inflammatory cytokine IL-10 (Mazmanian et al. 2008)
  - **Lipooligosaccharide (LOS)**:
    - Lacks O antigen
    - Very low endotoxin activity (1000x less than *E. coli*)
  - **Fimbriae** (Oyston and Handley 1991):
    - May bind to host ECM components; role in virulence is unclear
- Growth within the host milieu:
  - Iron acquisition:
    - **FeoA, FeoB** (Veeranagouda et al. 2014):
      - Fe^{+2} ion transport; two-component system
    - **HupA** (Otto et al. 1996):
      - Heme binding
- Damage to host cells:
  - **ETBF toxin [*Bacteroides fragilis* toxin (BFT); fragilysin]:**
    - Not found in NTBF strains
    - Three isotypes: BFT-1, BFT-2 (most active), BFT-3; encoded by *bft* gene found on conjugative transposon in pathogenicity island (*Bf*PAI) (Franco et al. 1999; Moncrief et al. 1998)
    - Heat-labile Zn-dependent metalloprotease; similar to botulinum toxin and tetanus toxin
    - Synthesized as pre-protein that is cleaved by the fragipain (Fpn) cysteine protease to form the mature 20 kDa secreted toxin (Fig. 11.2) (Choi et al. 2016)
    - BFT mechanism of action (Sears et al. 2014):
      - Not cytolytic (Fig. 11.3)
      - Binds to unknown colonic epithelial cell (CEC) receptor
      - Induces the cleavage of E-cadherin, though not through direct proteolysis (Kharlampieva et al. 2015)

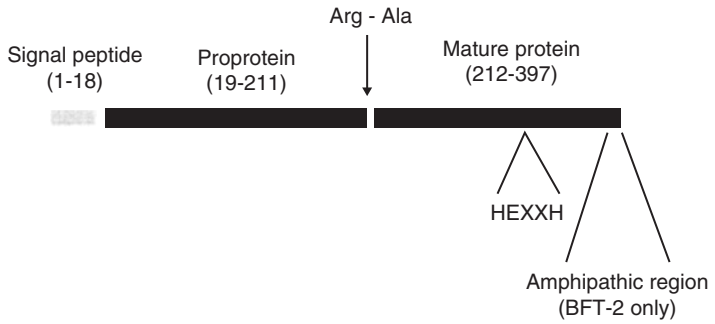

Fig. 11.2 BFT structure and processing (From: Sears 2001)

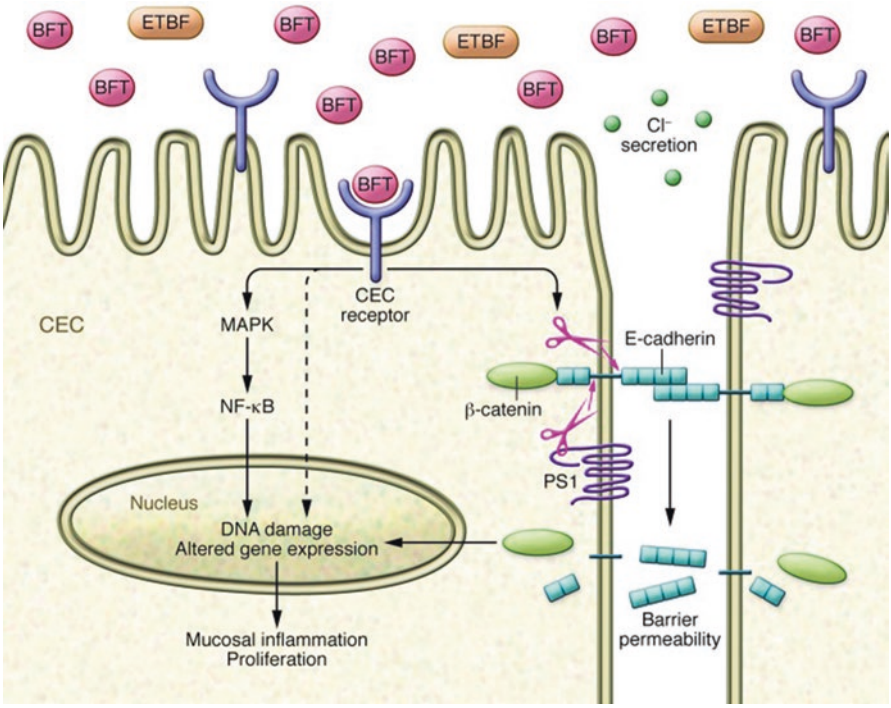

Fig. 11.3 BFT mechanism of action (From: Sears et al. 2014)

- Cleavage of E-cadherin results in increased adherens junction barrier permeability, resulting in Cl^- ion secretion and subsequent fluid loss leading to acute diarrhea
- Cleavage also releases β-catenin, which travels to the nucleus to induce Wnt nuclear signaling, induction of the oncogene c-Myc, and induction of spermine oxidase (SMO), which triggers ROS production and DNA damage (Sears et al. 2014)

    - In the cytosol, it activates MAPK and NF-kB signaling, resulting in the induction of pro-inflammatory cytokines, including IL-8

  - **Hemolysins**:
    - Ten hemolysins identified in annotated genome sequence; encode by *hlyA–hlyI* and *hlyIII* genes (Robertson et al. 2006)
    - Expression is repressed in O_2-rich environment and increased during infection via the iron-dependent Fur regulon, suggesting that they may play a role in virulence (Lobo et al. 2013)

- Evasion of host immune system:
  - **Bfp1, Bfp2, Bfp3, Bfp4** (Thornton et al. 2010):
    - C10 cysteine proteases
    - Structurally similar to *S. pyogenes* SpeB, which cleaves host proteins such as immunoglobulins, ECM components fibronectin and vitronectin, and cytokines (von Pawel-Rammingen and Bjorck 2003); it is unknown if Bfp proteases have the same activities

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Chapter 12
Bordetella spp.

Genomics, Morphologies, and Growth Characteristics

- **Genomics** (Parkhill et al. 2003):
  - *Bordetella pertussis* Tohama I chromosome: 4,086,186 bp; 3816 predicted ORFs
  - *Bordetella bronchiseptica* RB50 chromosome: 5,338,400 bp; 5007 predicted ORFs
  - *Bordetella parapertussis* 12,822 chromosome: 4,773,551 bp; 4004 predicted ORFs
- **Cell morphology:**
  - Very small coccobacilli; usually single cells or pairs (Fig. 12.1)
  - *B. bronchiseptica* is motile using flagella; *B. pertussis* is not – flagella genes are deleted
- **Gram stain:**
  - Gram negative
- **Growth:**
  - Obligate aerobes; oxidase positive
  - Fastidious slow growth; requires nicotinamide
  - Humans are only verified reservoir for *Bordetella pertussis*; no environmental reservoirs
  - Nine species: three primary mammalian pathogenic species
    - *Bordetella pertussis* – human pathogen
    - *Bordetella bronchiseptica* – occasional human pathogen; other mammals
    - *Bordetella parapertussis* – other mammals; dogs ("kennel cough")
  - Biofilm former – generally noninvasive

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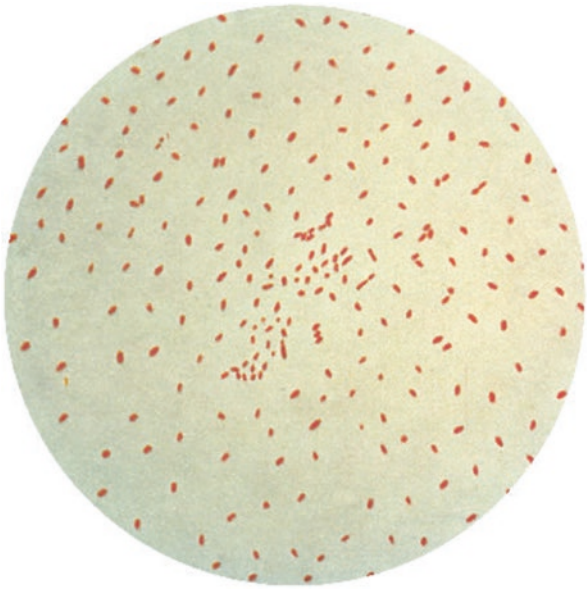

Fig. 12.1 *B. pertussis* cells (From: PHIL #2121)

Disease States Associated with *Bordetella pertussis*

- Pertussis; whooping cough:
  - Highly contagious respiratory disease
  - Transmitted person to person via respiratory aerosols
  - Three stages:
    - Catarrhal: initial infection in nasopharynx and trachea; symptoms like common cold
    - Paroxysmal (dry cough develops):
      - Increasing number of spasmodic coughing fits
      - Sharp intake of breath causes "whoop" sound
    - Convalescent: cessation of symptoms
    - Serious life-threatening infections can result from dissemination into lungs; usually in infants

Virulence Factors (Carbonetti 2016)

- Adherence to host cells: ciliated respiratory epithelial cells:
  - Fimbrial adhesins (Melvin et al. 2014):
    - **Filamentous hemagglutinin (FHA)** (Brown and Parker 1987; Tuomanen and Weiss 1985):
      - Adheres to ciliated respiratory cells and neutrophils
      - Binds to galactose residues on sulfated glycolipid (sulfatide)
      - Plays a role in immunomodulation and induces neutrophil and macrophage apoptosis (Inatsuka et al. 2005)
      - Encoded by FhaB structural protein; outer membrane secretion via FhaC
    - **Fimbriae (type I pili** (Livey et al. 1987; Mooi et al. 1990)):
      - **Fim2, Fim3**:
        - Structural proteins; produce serologically distinct fimbriae
      - **FimD**:
        - Cap protein; binds to heparin (sulfated sugar)
  - Afimbrial adhesins:
    - **Pertactin (PRN)** (Leininger et al. 1991):
      - Surface adhesin
      - Autotransporter family of outer membrane proteins
    - **BapC** (Riaz et al. 2015):
      - Surface adhesin
      - Autotransporter family of outer membrane proteins
  - **Lipooligosaccharide (LOS)**:
    - Lacks O antigen
    - Weak pro-inflammatory response
  - **Pertussis toxin**:
    - Functions with FHA in attachment to ciliated tracheal epithelial cells
    - Blocks ciliary movement

- Growth within the host milieu:
  - Iron acquisition:
    - Siderophores:
      - **Alcaligin** (Brickman and Armstrong 2007):
        - Biosynthesis and export functions are encoded by *alcABCDERS* genes and *fauA*, a TonB-dependent iron uptake receptor protein
        - Induced under iron starvation conditions
      - **Enterobactin xenosiderophore** (Beall and Sanden 1995):
        - Produced by other members of *Enterobacteriaceae*, not *B. pertussis*
        - **BfeA**: outer membrane receptor required for transport and utilization of ferric enterobactin
      - **Heme uptake** (Vanderpool and Armstrong 2001):
        - From host hemin and hemoglobin
        - Encoded by the *hurIR* and *bhuRSTUV* genes
  - Biofilms: can form on abiotic and biotic (respiratory mucosa) surfaces:
    - Occurs only in a Bvg^+ phase (see below)
    - c-di-GMP signaling may play a role (de Gouw et al. 2011)
- Damage to host cells:
  - Toxins: [for review article on toxins, see (do Vale et al. 2016)]
    - **Pertussis toxin (PTx** or **PT)** (Fig. 12.2) (Katada et al. 1983):
      - A-B exotoxin
      - Hexameric A-B_5 subunit structure; encoded by PtxA–E (subunits S1–S5, respectively)
      - PtxB-E (S2–S5): B subunit oligomer – binds to sialoglycoprotein receptors
      - PtxA (S1) (A subunit – catalytic domain):
        - ADP-ribosylates host Ga_i protein; inactivates signaling activity
        - Ga_i protein inhibits host adenylate cyclase (AC) activity; inactivation leads to rise in cAMP levels in host cells
        - Increased cAMP levels in alveolar neutrophils, among other effects, inhibits neutrophil chemotaxis, chemokine production, and neutrophil extracellular trap (NET) formation early in infection
    - **Adenylate cyclase toxin (ACT)** (Fig. 12.2) (Melvin et al. 2014):
      - Multifunctional protein; encoded by *cyaA* gene
      - N-terminal domain: secreted adenylate cyclase (AC); same calmodulin-dependent activity as *B. anthracis* edema factor (EF)

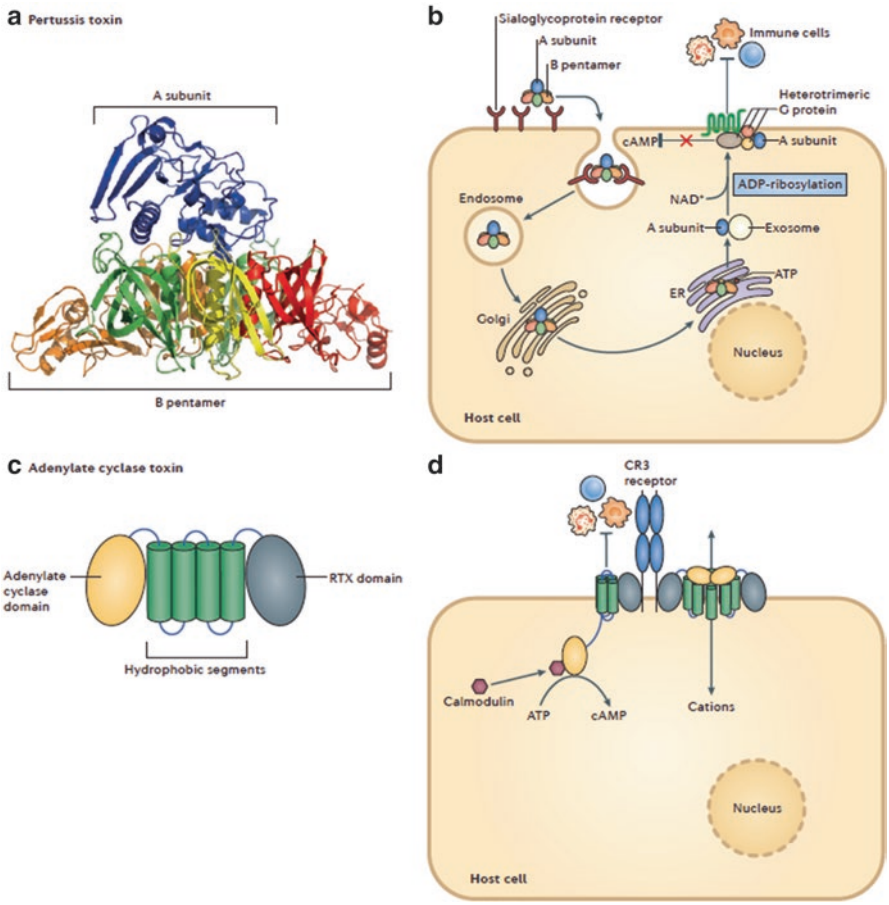

Fig. 12.2 Pertussis and adenylate cyclase toxin structures and mechanisms (From: Melvin et al. 2014)

- C-terminal domain: cell binding domain and pore-forming RTX (repeats in toxin) toxin with cytolytic activity
- Acts locally as major antagonist of neutrophils; inhibits neutrophil chemotaxis, phagocytosis, and reactive oxygen species (ROS) burst and induces apoptosis

○ **Tracheal cytotoxin (TCT)**: (Cookson et al. 1989)

- Disaccharide – tetrapeptide; not a protein toxin
- Released from *B. pertussis* cell wall
- Induces ROS; toxic to ciliated respiratory cells
- Induces pro-inflammatory cytokine release

- **Dermonecrotic toxin (Dnt; lethal toxin)** (Schmidt et al. 1999):
  - Four polypeptide subunits; heat labile toxin
  - Transglutaminase: constitutively activates Rho/Rac/Cdc42 GTPases
  - Cytotoxic effects; continuously transduces signals to downstream effectors
  - Causes inflammation and local necrosis
- **BteA** (Panina et al. 2005):
  - Cytotoxic factor; unknown mechanism of action
  - Secreted by type 3 secretion system (T3SS)

- Evasion of host immune system (Higgs et al. 2012):
  - Recruits host C4BP, C1INH proteins; blocks complement-mediated cell lysis
  - PT induces alveolar macrophage cell death (see above)
  - ACT and FHA induce macrophage apoptosis (see above)

Regulation of Virulence Factor Expression

- **BvgS-BvgA**:
  - Two-component phosphorelay system
  - Regulates biofilm formation and expression of virulence factors
  - Induced in vivo at 37 °C; Bvg^+ phase
  - BvgS: sensor histidine kinase; phosphorylates BvgA
  - BvgA: response regulator:
    - Transcriptional activator
    - Induces virulence-activated genes (*vags*):
      - Early *vags*: FHA, fimbriae
      - Late *vags*: PT, ACT toxins, pertactin, T3SS
    - Induces **BvgR** repressor – blocks virulence-repressed genes (*vrgs*)
- **RisA/RisK-RisS** (Coutte et al. 2016; de Gouw et al. 2011):
  - Two-component phosphorelay system
  - Regulates expression of virulence factors, chemotaxis and flagellar operons, and iron-regulated genes
  - RisS: sensor kinase
    - Inactive in *B. pertussis* (frameshift truncation), active in *B. bronchiseptica*
    - RisK may be sensor kinase in *B. pertussis* (Coutte et al. 2016)

- RisA: response regulator:
  - Transcriptional activator
  - Induces expression of virulence-repressed genes (*vrgs*); may antagonize BvgR repressor by unknown mechanism (Stenson et al. 2005)

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Chapter 13
Borrelia spp.

Genomics, Morphologies, and Growth Characteristics

- **Genomics:**
  - *Borrelia burgdorferi* chromosome: 910,725 bp; 853 predicted ORFs (Fraser et al. 1997)
  - *Borrelia burgdorferi* plasmids (Stewart et al. 2005):
    - Strain-specific numbers of linear and circular plasmids, several of which are required for virulence and the complex life cycle in ticks and mammals
    - ~9 linear plasmids: lp5, lp17, lp21, lp25, lp28 (1–4), lp36, lp38, lp54, and lp56
    - ~11 circular plasmids: cp9, cp26, and cp32 (1–9)
    - Encode most of the differentially expressed outer surface lipoproteins
- **Cell morphology:**
  - Spirochete – tightly coiled corkscrew shape (Fig. 13.1) (Charon et al. 2012):
    - Fourteen to sixty endoflagella (axial filaments):
      - Anchored at both ends of the cell and run lengthwise between the inner and outer membranes in the periplasmic space; enclosed in outer sheath
      - Causes twisting motion used for motility
  - Outer membrane:
    - No LPS (endotoxin); does not activate TLR4 (Radolf et al. 2012)
    - Contains diverse and unique assortment (>100) of lipoproteins and glycolipids, including cholesterol; major lipoprotein is OspA
    - Antigenic variation of lipoproteins is a major virulence factor

D.I. Johnson, *Bacterial Pathogens and Their Virulence Factors*,
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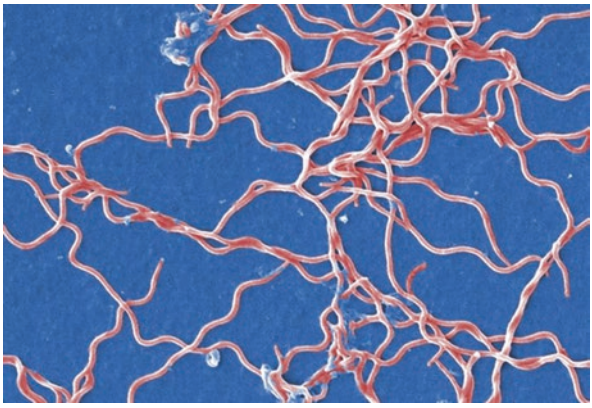

Fig. 13.1 *B. burgdorferi* cells (From: PHIL #13169)

- **Gram stain:**
  - Gram-negative-like: no LPS but outer membranes contain many diverse hydrophobic lipoproteins
- **Growth:**
  - Anaerobic or microaerophilic growth; slow grower; requires N-acetylglucosamine (NAG) for growth; auxotrophic for all amino acids, nucleic acids, and fatty acids
  - Reservoirs: wild and domestic animals such as white-footed field mice, rodents, deer, and birds
  - Fifty-two *Borrelia* species (Cutlera et al. 2017):
    - Twenty-one – Lyme disease group (*B. burgdorferi* sensu lato group)
    - Twenty-nine – relapsing fever group (*B. recurrentis*)
    - Two – nonconformist group

Disease States Associated with *Borrelia* spp.

- Lyme disease (borreliosis):
  - *Borrelia burgdorferi* and 20 other spp. (see above)
  - Zoonotic, vector-borne disease
  - Three-stage inflammatory disease:
    - Stage 1:
      - Early, localized symptoms
      - Erythema migrans – expanding red rash resembling a bull's-eye target (Fig. 13.2):
        - Seen in ~80 % of infected individuals, 7–10 days after bite
        - Can periodically reoccur

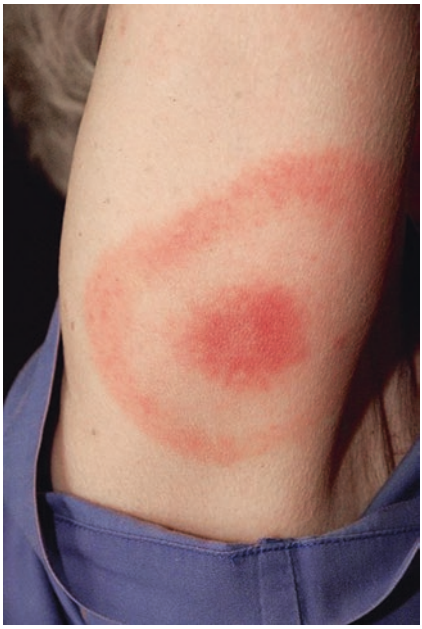

Fig. 13.2 Erythema migrans rash (From: PHIL #9875)

    - General malaise, flu-like symptoms, headache, chills, and fatigue
  - Stage 2:
    - Early, disseminated symptoms
    - Disseminates to the CNS, heart, skin, lymphatic system, and musculoskeletal system
    - CNS infections; can lead to neurological disease, facial tics, cardiac damage, meningitis, and Bell's palsy
  - Stage 3:
    - Late and chronic
    - Due to host immunological reactions
    - Can progress to inflammation, chronic arthritis, and neurological symptoms (similar to Alzheimer's disease or multiple sclerosis)
- Transmission – vector-borne disease:
  - Vector – deer tick *Ixodes scapularis*
  - Ticks feed on blood of reservoir animals containing *B. burgdorferi*
  - *B. burgdorferi* cells are then transmitted to humans (accidental host) during a blood meal; usually takes 1–2 days of tick attachment to transmit
  - *B. burgdorferi* cells are capable of invading many host tissues and disseminating through the bloodstream

- Relapsing fever:
  - Epidemic relapsing fever: louse-borne *Borrelia recurrentis*
  - Endemic relapsing fever: tick-borne *Borrelia* spp.
  - Acute infection: high fever, headache, and myalgia
  - Recurring febrile episodes

Virulence Factors

- *Borrelia burgdorferi*:
  - Adherence to host cells and extracellular matrix (ECM) (Brissette and Gaultney 2014):
  - *B. burgdorferi* is predominantly an extracellular pathogen, so adherence to host cells and tissues is essential for virulence:
    - **BBK332**, **RevA**, **RevB**, **BB0347**, **CspA**, and **CspZ**: fibronectin-binding proteins
    - **DbpA/B**, **Bgp**, and **BbhtrA**: glycosaminoglycan (GAGS)-binding proteins
    - **BmpA**, **ErpX**, **CspA**, and **CspZ**: laminin-binding proteins
    - **CspA** and **CspZ**: collagen-binding proteins
    - **P66** and **BB0172**: integrin-binding proteins
    - **CspA**, **CspZ**, and **ErpA/C/P**: complement regulator (fH, FHL-1)-binding proteins; block complement-mediated lysis
    - **CspA** and **CspZ**: capable of binding to multiple ECM components – fibronectin, laminin, collagen, plasminogen, and complement regulator proteins
- Dissemination and growth within the host (Radolf et al. 2012):
  - *B. burgdorferi* cells induce host matrix metalloproteinases (MMPs) that degrade ECM components
  - **BB0337**, **OspC**, **CspA**, **BBA70**, and **ErpA/C/P**:
    - Plasminogen-binding proteins
    - Enhances ECM destruction allowing dissemination
  - Iron acquisition: does not require iron for enzyme function; uses Mn-based enzymes (Posey and Gherardini 2000)
  - Biofilms: can form on abiotic surfaces in vitro (Sapi et al. 2012); unknown role in virulence but may play a role in environmental persistence
- Damage to host cells:
  - No known exotoxins
  - Induces host inflammatory responses; major mechanism for host cell and tissue damage; at least two mechanisms:

    - **Outer surface lipoproteins**: interact with host TLR-2/TLR-1 heterodimers on target cells; activate signal transduction pathway that leads to the production of pro-inflammatory cytokines (Radolf et al. 2012)
    - Phagocytosis by neutrophils and macrophage leads to internalization and destruction of *B. burgdorferi* cells:
        - Release of lipoproteins, peptidoglycan, and other cellular components leads to activation of intracellular pattern recognition receptors (PRRs) and induction of host inflammatory processes
        - Termed "phagosomal signaling" (Cervantes et al. 2014; Petnicki-Ocwieja and Kern 2014)

- Evasion of host immune system:
    - Antigen variation:
        - Major virulence determinant
        - Outer surface lipoproteins, such as VslE (Zhang and Norris 1998), undergo antigen variation to elude the host immune system
        - One of the reasons why vaccine development is problematic
    - Complement inhibition (Kelesidis 2014):
        - **OspA**, **OspE**, **CspA**, and **CRASPs**:
            - Outer surface proteins
            - Inhibit complement by binding host factors H, FHL-1, and C4b-binding protein (C4BP)
    - Block neutrophil function (Hartiala et al. 2008):
        - **OspB**:
            - Outer surface protein
            - Inhibits neutrophil phagocytosis and oxidative burst

Regulation of Virulence Factor Expression: Growth-Phase Regulation

- Multiple genes are transcriptionally repressed or derepressed during the tick phase and mammalian phase of the *B. burgdorferi* life cycle (Radolf et al. 2012):
    - Mammalian growth conditions – 35 °C; pH 7
    - Tick growth conditions – 23 °C, pH 8
- Involves two two-component phosphorelay systems (Fig. 13.3):
    - **Hk1-Rrp1** and **Hk2-Rrp2-RpoN-RpoS**.
    - Response regulator proteins Rrp1 and Rrp2 control the expression of alternative sigma factors in ticks vs. mammals

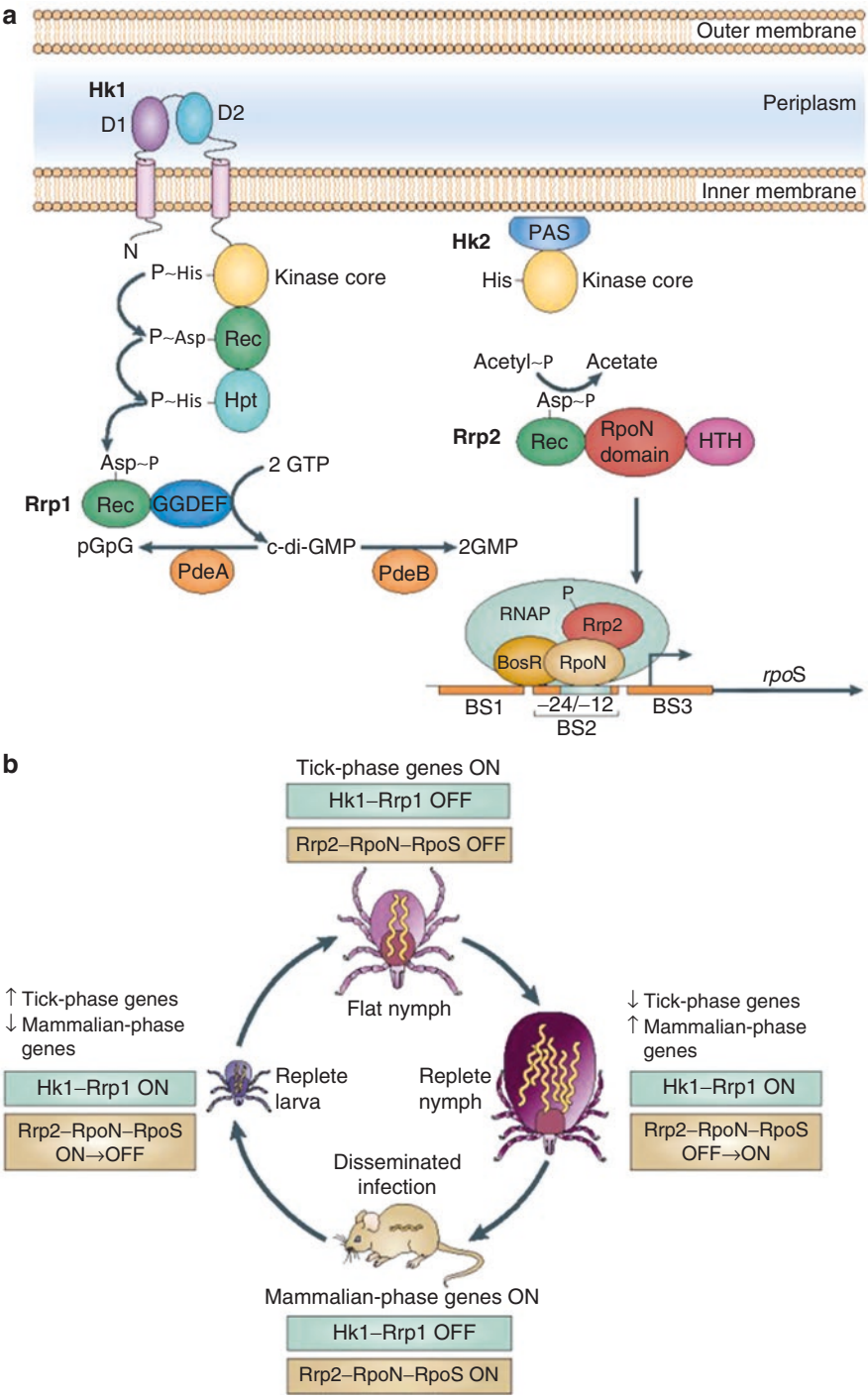

Fig. 13.3 Regulation of tick-phase and mammalian-phase genes (From: Radolf et al. 2012). (**a**) The histidine kinase 1 (Hk1)–response regulatory protein 1 (Rrp1) and alternative RNA polymerase σ-factor RpoS global regulatory systems. (**b**) Expression of the Hk1–Rrp1 and RpoS global regulatory systems during the B. burgdorferi life cycle

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Chapter 14
Campylobacter spp.

Genomics, Morphologies, and Growth Characteristics

- **Genomics:**
  - *Campylobacter jejuni* chromosome: 1,641,481 bp; 1,643 predicted ORFs (Gundogdu et al. 2007; Parkhill et al. 2000)
  - Plasmid pVir: 37,468 bp; 54 predicted ORFs (Bacon et al. 2002)
- **Cell morphology:**
  - Curved or spiral bacilli; "twisted bacteria" (Fig. 14.1)
  - Lipooligosaccharide (LOS, rough LPS) outer membrane:
    - Highly variable sialylated structures; plays a role in immune avoidance (Guerry et al. 2000)
    - Some structures resemble human neuronal gangliosides – molecular mimicry can lead to autoimmune reactions (Bowes et al. 2002; Yuki 2001)
      - For example, Guillain–Barré Syndrome occurs in ~1 out of 1,000 cases of campylobacteriosis (Godschalk et al. 2004)
  - Capsule (Karlyshev et al. 2005):
    - Highly variable polysaccharides
    - Plays a role in adherence to host cells and anti-phagocytosis immune evasion
  - Flagella:
    - Either monopolar or bipolar
    - Composed of FlaA (major) and FlaB (minor) flagellin subunits; undergo phase variation and antigen variation – evasion of host immune system
    - Contain significant levels of O-linked glycosylation; important for assembly and adherence to host intestinal epithelial cells

D.I. Johnson, *Bacterial Pathogens and Their Virulence Factors*,
https://doi.org/10.1007/978-3-319-67651-7_14

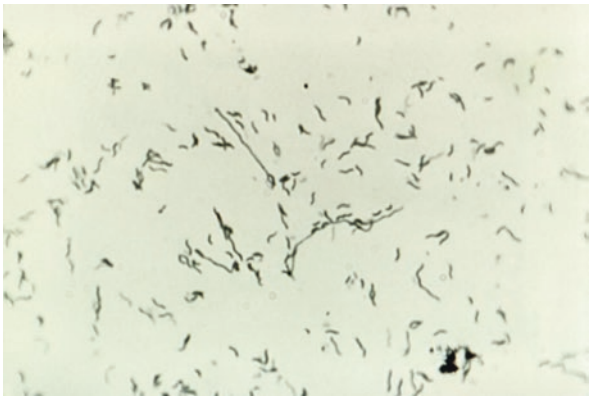

Fig. 14.1 *C. jejuni* cells with flagella (From: PHIL #6654)

- Flagellar movement through the mucus layer plays an essential role in colonization and virulence
- Flagellar type 3 secretion system (T3SS):
  - Functions in the secretion of bacterial effectors, such as FlaC (Song et al. 2004) and *Campylobacter* invasive antigen (Cia) proteins (Grant et al. 1993; Konkel et al. 2004) (see below)

- **Gram stain:**
  - Gram negative
- **Growth:**
  - Microaerophilic; catalase positive, oxidase positive
  - Capnophilic; prefers high CO_2 concentrations
  - Complex nutritional requirements (Hofreuter 2014):
    - Non-saccharolytic – lacks phosphofructokinase
    - Imports amino acids and keto acids for use in intermediary metabolism
  - Prefers growth at 42 °C over 37 °C – due to growth in poultry reservoir
  - Environmental reservoirs (Epps et al. 2013):
    - Zoonotic – wild and domestic animals
    - Predominantly chicken and other poultry
  - Biofilm formation (Gunther and Chen 2009):
    - Multiple abiotic surfaces; unclear if it plays a role in virulence or persistent infections
  - Sixteen species; 12 are associated with human disease:
    - 95% of human disease – *C. jejuni* and *C. coli*

Disease States Associated with *Campylobacter* spp.

- Campylobacteriosis:
  - Gastroenteritis; leading cause of bacterial gastroenteritis (5–11%) in the USA
  - Food-borne pathogen: ingestion via fecal–oral route; contaminated water, undercooked poultry, raw milk
  - Early symptoms: fever, severe abdominal cramping, diarrhea (loose, particularly foul smelling)
  - Later symptoms: bloody stools; bacillary dysentery

Virulence Factors (Bolton 2015; van Putten et al. 2009; Young et al. 2007)

- Adherence to host cells: jejunum and ileum epithelial cells
  - Fimbrial adhesins: none
  - Non-fimbrial adhesins:
    - **CadF** (Konkel et al. 1997)
      - Thirty-seven kDa outer membrane protein
      - Binds to ECM fibronectin
      - Binding to fibronectin activates integrin-β1-dependent host signaling pathways that lead to activation of the Cdc42 GTPase via the Vav-2 guanine nucleotide exchange factor (Fig. 14.2) (Krause-Gruszczynska et al. 2011)
      - Activated Cdc42 (and Rac1) stimulate actin polymerization and/or microtubule reorganization leading to the membrane ruffling needed for endocytosis via a trigger mechanism (Monteville et al. 2003)
    - **FlpA** (Larson et al. 2013):
      - Outer membrane lipoprotein; binds fibronectin
    - **JlpA** (Jin et al. 2003):
      - Surface lipoprotein; binds to host Hsp90
      - Induces host signaling pathways that lead to an inflammatory response
    - **PEB1 (CBF1), PEB2, PEB3, PEB4 (CBF2)** (Leon-Kempis Mdel et al. 2006; Pei and Blaser 1993):
      - Outer membrane protein adhesins
    - Outer membrane proteins (OMPs):
      - **MOMP (PorA)**: major outer membrane protein; putative porin and adhesion (Moser et al. 1997)

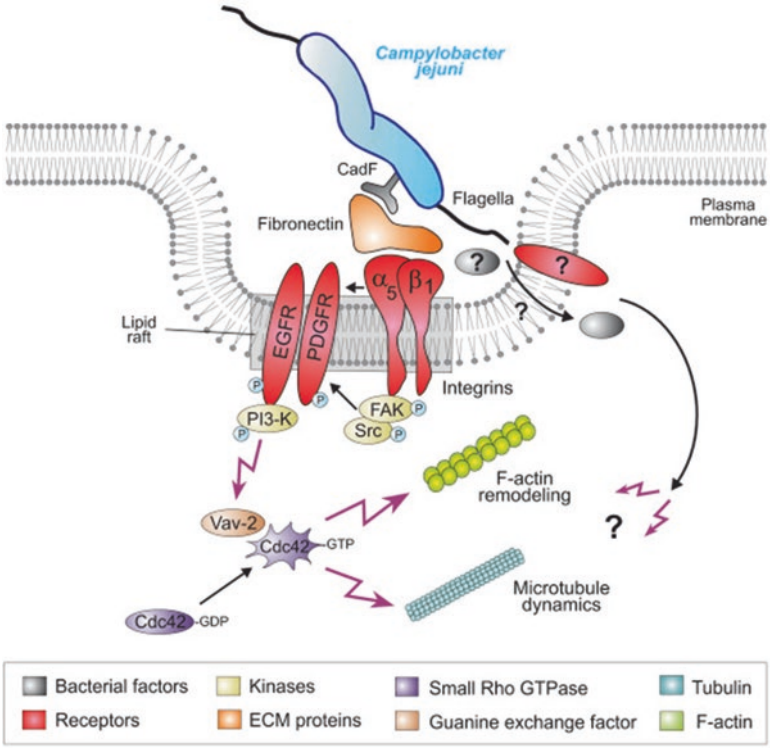

Fig. 14.2 CadF-induced cytoskeletal rearrangements (From Krause-Gruszczynska et al. 2011)

- Many OMPs are modified by N-linked glycosylation; first bacteria shown to have N-linked glycosylation system (Szymanski and Gaynor 2012; Wacker et al. 2002)
  - **Pgl system** (Nothaft and Szymanski 2010):
    - Modifies ~65 OMPs; essential for adherence
- **Flagella**: tip protein FliD binds to host glycosaminoglycans (Freitag et al. 2016)
- **Capsule** (see above)
- **LOS** (see above)

- Invasion of host cells and dissemination (O. Croinin and Backert 2012):
  - Colonizes the small intestine epithelial cells (microaerophilic environment)
    - **KatA** (catalase), **SodB** (superoxide dismutase): expression is induced during colonization in vivo; regulated by the **PerR** regulon (Palyada et al. 2009)

- Can transiently invade host cells; uses CadF-dependent activation of the actin cytoskeletal network (see above):
  - Can survive (but not grow) in *Campylobacter*-containing vacuoles (CCVs); leads to massive immune response and inflammation
- Disseminates either between (paracellular) or through (transcellular) epithelial cells (Backert et al. 2013):
  - **HtrA** (Boehm et al. 2015):
    - Serine protease; cleaves host E-cadherin
    - Enhances paracellular dissemination
    - Also involved as a potential chaperone affecting cell adherence and invasion
- **Type 4 secretion system (T4SS)** (Bacon et al. 2002):
  - Important for microtubule-based invasion
  - Encoded on the pVir plasmid
- **CiaA–CiaI** (Neal-McKinney and Konkel 2012):
  - *Campylobacter* invasive antigens; needed for maximal invasion
  - Secreted through flagellar T3SS into host cells
  - Involved in actin and microtubule rearrangements; precise mechanisms are unknown (Samuelson and Konkel 2013)

- Iron acquisition (Miller et al. 2009):
  - Under transcriptional regulation by the Fur repressor system (van Vliet et al. 1998)
  - **FeoA, FeoB**: Fe^{+2} iron transport (Naikare et al. 2006)
  - **Ferri-enterochelin/ferri-enterobactin** (exogenous siderophore)
    - **CfrA**: outer membrane porin (Carswell et al. 2008)
    - **CeuBCDE**: ATP transporter system
  - Hemin, hemoglobin, hemin–hemopexin, and hemoglobin–haptoglobin uptake:
    - **ChuA**: outer membrane receptor (Pickett et al. 1992)
    - **ChuBCD**: ABC transporter system
  - Ferri-transferrin, ferri-lactoferrin, and ferri-ovotransferrin uptake (Miller et al. 2008):
    - **CfbpA**: binding protein
    - **CfbpBC**: transport system

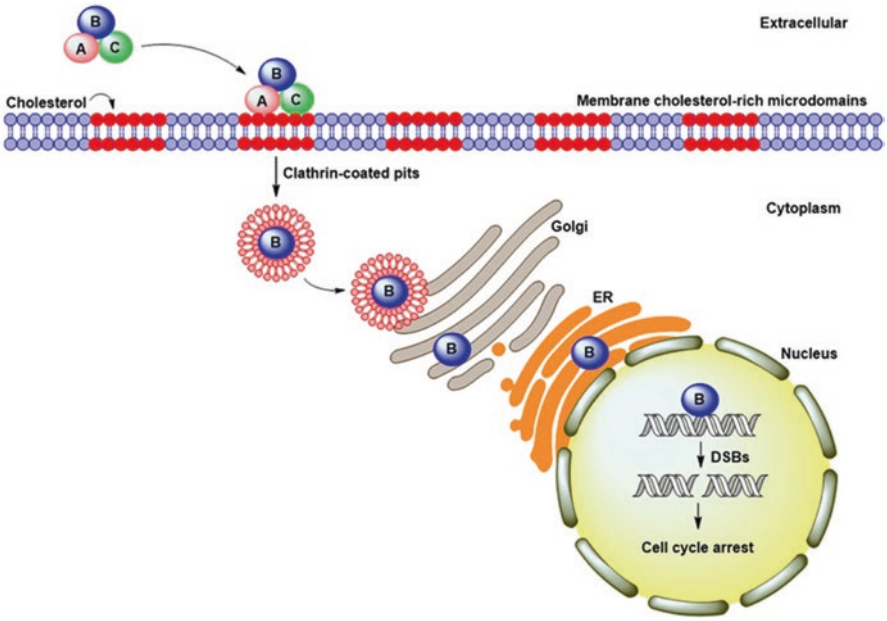

Fig. 14.3 CDT mechanism (From: Lai et al. 2016)

- Host cell damage:
  - **Cytolethal distending toxin (CDT)** (Lara-Tejero and Galan 2001, 2002):
    - A–B exotoxin: similar to CDT from *Haemophilus ducreyi* and *Salmonella* spp.
    - Causes DNA damage to chromosomal DNA (Fais et al. 2016)
      - Leads to host cell cycle arrest at DNA damage checkpoint; triggers p53-dependent apoptosis
    - Also leads to induction of pro-inflammatory cytokines
    - Three polypeptides (**CdtA, CdtB, and CdtC** (Fig. 14.3)):
      - CdtA and CdtC (B subunits):
        - Bind to clathrin-coated pits
      - CdtB (A subunit):
        - Translocates into the host nucleus and acts as a DNAase to induce double-strand breaks in the chromosomal DNA
  - **FspA** (Poly et al. 2007):
    - Induces apoptosis in cultured cells by unknown mechanism
    - Secreted through flagellar T3SS

Regulation of Virulence Factor Expression

- **CsrA** (Dugar et al. 2016):
  - Translational inhibitor
  - Blocks translation of FlaA flagellin mRNA
- **CprR–CprS** (Svensson et al. 2015):
  - Two component phosphorelay system
  - Regulates cell envelope components, HtrA and PEB4

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Chapter 15
Escherichia spp.

Genomics, Morphologies, and Growth Characteristics

- **Genomics:**
  - Detailed phylogeny is based on the sequence analysis of more than 180 *Escherichia coli* genomes (Kaas et al. 2012):
    - Core genome: 3,051 homology gene clusters (HGCs) present in 95% of all genomes; 1,702 HGCs in 100% of all genomes
    - Pan genome: 16,373 HGCs
    - Pathogen genomes can contain up to 1 Mb more DNA than commensal isolates; extra DNA is associated with the gain of virulence factors through horizontal gene transfer (HGT) mechanisms and the loss of virulence factors through mutational pathoadaptivity (Croxen et al. 2013)
  - *Escherichia coli* K-12 strain MG1655 chromosome: 4,639,221 bp; 4,288 predicted ORFs (Blattner et al. 1997)
  - *Escherichia coli* O157:H7 strain EDL933 chromosome: 5,547,323 bp; 5,498 predicted ORFs (Perna et al. 2001)
  - *Escherichia coli* O104:H4 *stx2*-positive strain 2011C-3493 chromosome: 5,273,097 bp; 4,963 predicted ORFs (Ahmed et al. 2012)
- **Cell morphology:**
  - Rod-shaped cells (Fig. 15.1)
  - Can be motile or nonmotile:
    - Peritrichous flagella (H antigens); more than 50 H variants (Zhou et al. 2015)
    - Flagellar motility plays an important role in certain pathotypes (UPEC); also important for biofilm formation

D.I. Johnson, *Bacterial Pathogens and Their Virulence Factors*,
https://doi.org/10.1007/978-3-319-67651-7_15

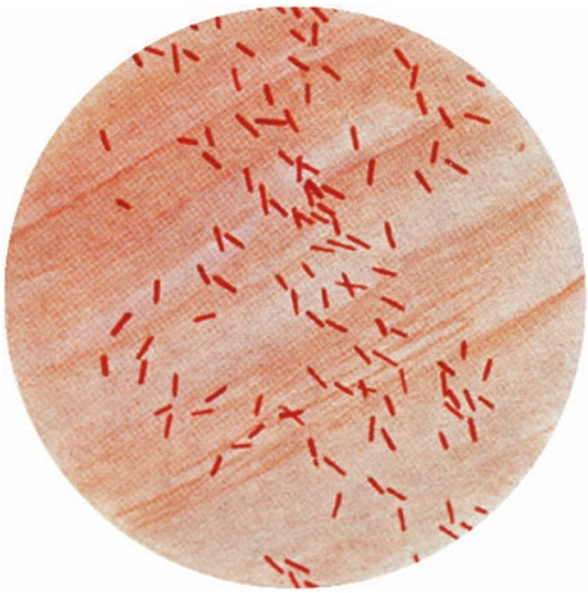


Fig. 15.1 *E. coli* O157:H7 cells (From: PHIL #2112)

- **Gram stain:**
  - Gram negative
- **Growth:**
  - Facultative anaerobes; oxidase negative
  - Reservoirs:
    - Part of the normal microbiota of the gastrointestinal tracts of humans and other warm-blooded animals
    - Fecal-contaminated food and water
  - Excellent biofilm formers (see below)
  - Eight species; *E. coli* is the major human pathogen
  - *E. coli* pathotype classification: based on disease states and virulence genes:
    - Diarrheagenic pathotypes:
      - AIEC: adherent invasive *E. coli*
      - DAEC: diffusely adherent *E. coli*
      - EAEC: enteroaggregative *E. coli*
      - EHEC: enterohemorrhagic *E. coli*; subset of Shiga toxin-producing *E. coli* (STEC)
      - EIEC: enteroinvasive *E. coli*; phylogenetically almost identical to *Shigella* spp.; uses same virulence mechanisms although EIEC shows reduced virulence and expression of virulence factors; discussed in more detail in Chap. 28 *Shigella* spp.
      - EPEC: enteropathogenic *E. coli*
      - ETEC: enterotoxigenic *E. coli*

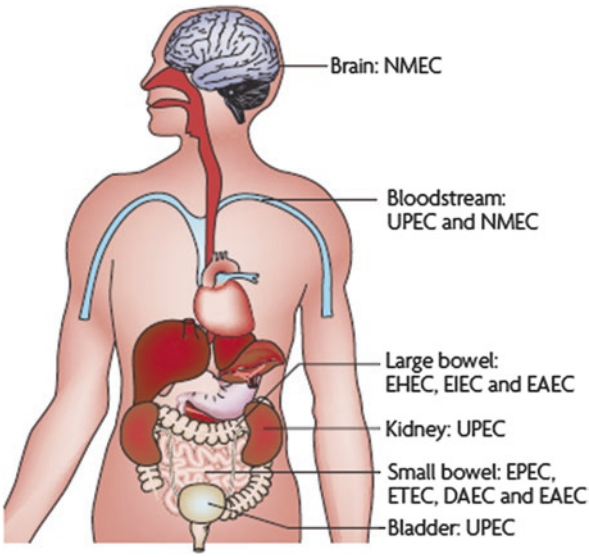


Fig. 15.2 *E. coli* infection sites (From: Croxen and Finlay 2010)

- Extraintestinal pathotypes; extraintestinal pathogenic *E. coli* (ExPEC):
  - UPEC: uropathogenic *E. coli*
  - NMEC: neonatal meningitis *E. coli*

Disease States Associated with *E. coli*

- Pathotypes are associated with disease states in different areas of the host (Fig. 15.2).
- Diarrheal diseases:
  - Either noninvasive, toxigenic (non-bloody diarrhea) infections or invasive, hemorrhagic (bloody diarrhea) infections
  - These are the major pathogens associated with the ~2–4 billion diarrheal episodes/year worldwide; diarrheal diseases are deadliest to infants and toddlers under the age of 5
  - Transmission: fecal–oral transmission from contaminated food and water; person to person (rare)
- Extraintestinal diseases:
  - Urinary tract infections (UTIs):
    - Ascending UTIs:
      - Endogenous source of UPEC; travels from host GI tract to urinary tract
      - Can lead to cystitis in the bladder and acute pyelonephritis in the kidneys

- Majority of hospital acquired (HA) UTIs
- Eighty to 90% of community-acquired (CA) UTIs
- Symptoms:
  - Uncomplicated: altered urination frequency, painful urination (dysuria)
  - Complicated: chills, fever, flank pain

– Neonatal meningitis:

- Endogenous source of NMEC; travels from GI tract to the meninges; crosses the blood–brain barrier (BBB)
- Most frequent cause of Gram-negative-associated meningitis in newborns; ~40% fatality

– Septicemia:

- Endogenous dissemination of UPEC and NMEC from the GI tract or UTIs
- Most common Gram-negative rod isolated from the blood
- Complication: endotoxic shock; mortality rate is high
- Neonatal sepsis:
  - *E. coli* (and *Streptococcus agalactiae*) are the most common causes
  - Associated with the K1 capsular polysaccharide antigen

Virulence Factors (Croxen and Finlay 2010; Croxen et al. 2013; Kaper et al. 2004)

- Virulence factors associated with most, if not all, pathogenic *E. coli* will be discussed first
- Each of the abovementioned pathotypes will be discussed separately, focusing on the unique virulence factors associated with each pathotype
- The primary discriminators for each pathotype are their repertoire of adherence factors and exotoxins
- It is important to note that with the 1,000s of different *E. coli* strains, serotypes, and pathotypes, not all of the virulence factors discussed below will be encoded or expressed in each strain
- Many of these virulence factors are encoded on plasmids and pathogenicity islands (PAIs)
- Archetypical *E. coli* virulence factors:

  – **LPS**:

  - Endotoxin activity: lipid A-dependent endotoxic shock

- O antigens:
  - More than 80 O serotypes
  - Immunostimulatory; trigger TLR4-dependent inflammatory reactions that lead to a cytokine storm and septic shock
- Also plays a role in serum resistance

– **Capsule** (Miajlovic and Smith 2014):

- Contains K polysaccharide antigens; more than 60 K serotypes have been identified; K1 is the predominant serotype associated with NMEC
- Found in NMEC, UPEC, EHEC, and EPEC
- Provides protection from phagocytosis, complement-mediated lysis, and microbicidal molecules – serum resistance
- Also important for biofilm formation

– **Iron acquisition** (Ma et al. 2009; Porcheron et al. 2013):

- Not all transporters and siderophores are expressed in each *E. coli* pathotype
- Fe^{+2} transporters:
  - FeoABC system: GTP-dependent transport across the inner membrane
  - ABC (ATP-binding cassette) transporters: transports Fe^{+2} and other solutes
- Fe^{+3} siderophores:
  - Can remove Fe^{+3} from host transferrin, lactoferrin, and ferritin
  - **Enterochelin (enterobactin)**:
    – Major siderophore in *E. coli*; almost all *E. coli* strains produce this siderophore
    – Cyclic polyester containing three DHBS (*N*-(2,3-dihydroxybenzoyl)-L-serine) residues
    – Internalized with the FepB chaperone via the FepA receptor and FepCDG ABC transporter
  - **Salmochelin**:
    – Enterochelin derivative; contains DHBS linked by a glucose residue; increases the hydrophilicity of enterochelin
    – Synthesis, excretion, and uptake are mediated by the IroBCDEN gene products and the FepCDG gene products
  - **Aerobactin**:
    – Uptake is via the IutA receptor and FhuBC ABC transporter

- **Yersiniabactin**:
  - Uptake is via the Psn/FyuA receptor and the YbtPQ ABC transporter

- Exogenous siderophores:
  - Ferrichrome (fungal siderophore): internalized via the FhuABCD system
- Ferric citrate transport:
  - Involves the FecABCDE transport system
- Heme uptake:
  - Secreted hemolysins and SPATE proteases (see below) can break down hemoglobin, hemopexin, and haptoglobin, releasing free heme
  - Heme uptake involves the Shu (EIEC/*Shigella*), ChuA (EHEC, UPEC), and Hma (UPEC) import systems
- Expression is regulated by Fe^{+2}-bound Fur repressor and RhyB sRNA molecules (Ma et al. 2009)

– **Biofilm formation** (Sharma et al. 2016):

- Capable of forming biofilms on biotic and abiotic surfaces, included catheters (UPEC and NMEC CA-UTIs) and other medical devices
- Type I fimbriae and curli fimbriae are the major initial adherence factors involved in biofilm formation
- Maturation of biofilms utilizes autotransporter proteins such as Ag43 (antigen 43), AidA, and TibA
- Major *E. coli* exopolysaccharides found within the biofilm include β-1,6-N-acetyl-D-glucosamine polymer (PGA), cellulose, and capsular colanic acid (negatively charged polymer of glucose, galactose, fucose, and glucuronic acid)
- LPS and capsules (UPEC, NMEC) play an important role in biofilm structure and cell-to-cell adherence
- Quorum sensing utilizes N-acyl-homoserine lactones (AHL) as signaling molecules along with the LuxIR-sensing system

- Diarrheagenic pathotypes (Clements et al. 2012; Gomes et al. 2016):

– **AIEC (adherent invasive *E. coli*):**

- Associated with patients with Crohn's disease (CD) and inflammatory bowel disease (IBD) (Mann and Saeed 2012)
- Adherence to host cells (Korea et al. 2011):
  - Adhere to and invade brush border cells in ileal epithelial cells; also invade macrophage
  - Flagella-mediated swimming motility is essential for approach and adherence

- Fimbrial adhesins:
  - **Type I fimbriae** (Barnich et al. 2003, 2007):
    - Chaperone–usher pathway (CUP) assembly mechanism
    - **FimA**: major structural subunit
    - **FimH**: tip adhesin
    - Binding is sensitive to D-mannose or methyl-α-D-mannopyranoside
    - Adhere to glycosylated CEACAM6 (carcinoembryonic antigen-related cell adhesion molecule 6), which is overexpressed in CD patients
  - **Lpf (long polar fimbriae)** (Chassaing et al. 2011):
    - CUP assembly mechanism
    - Interacts with M cells in Peyer's patches; can undergo transcytosis across the M cells; gains access to the submucosal lamina propria, and invades macrophage
- Host cell invasion:
  - **YfgL** (Rolhion et al. 2005):
    - Outer membrane lipoprotein
    - Important for the release of OMVs (outer membrane vesicles), which deliver bacterial effectors, such as OmpA, to host cells
  - **OmpA** (Rolhion et al. 2010):
    - Outer membrane invasion
    - Binds to host ER stress response chaperone Gp96
- Evasion of host immune system:
  - In CD patients, AIEC can survive within macrophage phagolysosomes without inducing cell death (Lapaquette et al. 2012):
    - Associated with defects in the host cell autophagy machinery in CD patients
    - Generates high levels of TNF-α, which is associated with chronic inflammation and granuloma formation

– **DAEC (diffusely adherent *E. coli*)** (Fig. 15.3) (Le Bouguenec and Servin 2006; Servin 2005):

- Associated with watery, non-bloody diarrhea; UTIs
- Characteristic diffuse adherence pattern over epithelial cells
- Some cells contain the adherence pathogenicity island "locus for enterocyte effacement" (LEE; see EHEC, EPEC below) – diffusely adhering enteropathogenic *E. coli* (DA-EPEC)
- Adherence to host cells (Korea et al. 2011):

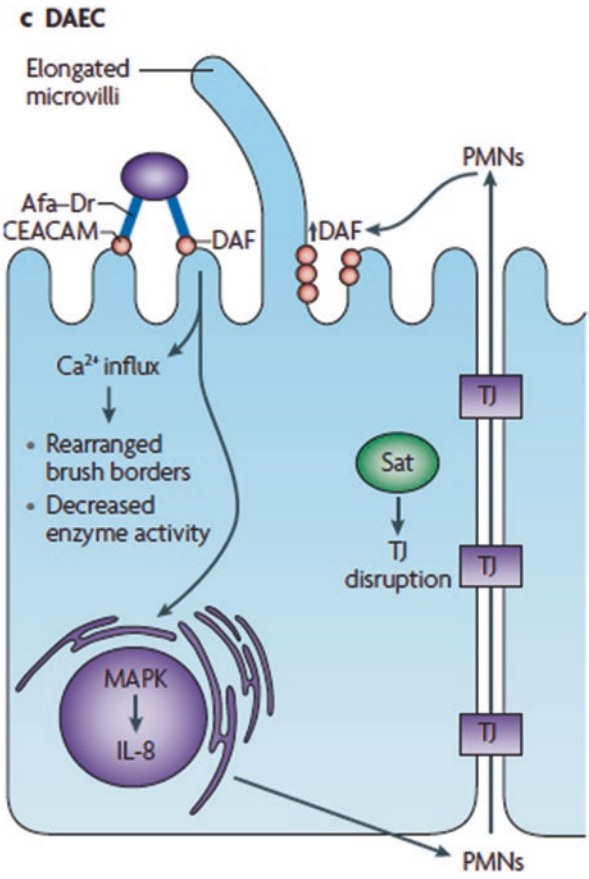


Fig. 15.3 DAEC virulence factors (From: Croxen and Finlay 2010)

- Fimbrial adhesins:
  - **Dr**:
    - Mannose-resistant adhesin
    - Bind to DAF (decay-accelerating factor; CD55); GPI-anchored protein present on apical side of polarized epithelial cells
    - Bind to type IV collagen
    - Bind to carcinoembryonic antigen-related cellular adhesion molecules (CEACAMs); GPI-anchored proteins
    - Binding leads to loss of brush border microvilli, internalization into epithelial cells (zipper mechanism), increased paracellular permeability, and activation of cell signaling pathways that lead to pro-inflammatory cytokine production
    - Encoded by *dra* operon

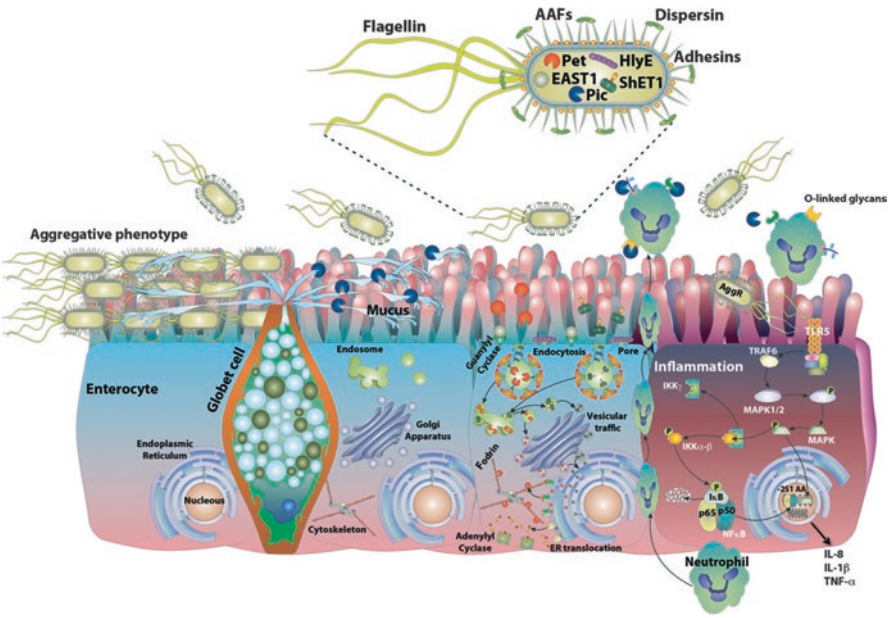


Fig. 15.4 EAEC virulence factors (From: Estrada-Garcia and Navarro-Garcia 2012)

- **F1845**:
  - Bind to DAF, CEACAMs
  - Encoded by *daa* operon
- **AAF-I, AAF-II, AAF-III**: unknown receptor(s)

- Afimbrial adhesins:
  - **AfaE-I, AfaE-II, AfaE-III, AfaE-V**:
    - Mannose-resistant adhesins
    - Bind to DAF; AfaE-III also binds to CEACAMs
  - **Dr-II**: binds to DAF
  - **NfaI**: binds to DAF

- Damage to host cells:
  - **Sat** (Guignot et al. 2007):
    - Serine protease; member of SPATE (serine protease autotransporters of *Enterobacteriaceae*) family of proteases (Pic of EAEC and EIEC/*Shigella*; EspC of EPEC; Sat of DAEC and UPEC; SigA and SepA of EIEC/*Shigella*; EspP of EHEC)
    - Disassembles tight junction proteins; increases permeability

- **EAEC (EAggEC) enteroaggregative *E. coli*** (Fig. 15.4) (Estrada-Garcia and Navarro-Garcia 2012):
  - Emerging pathogen; second most common traveler's diarrhea after ETEC
  - Symptoms: persistent, watery non-bloody diarrhea
  - Recent emergence of hemorrhagic Shiga toxin 2a (Stx-2a)-producing O104:H4 EAEC strains in Europe (Boisen et al. 2015; Navarro-Garcia 2014)
  - Adherence to host cells (Korea et al. 2011):
    - Aggregative "stacked brick" autoagglutination on epithelial cells; facilitates biofilm formation
    - Fimbrial adhesins:
      - **AAF-I–IV (aggregative adherence fimbriae)** (Nataro et al. 1992):
        - Dr family of adhesins; plasmid encoded
        - Bind to ECM fibronectin
        - Aggregation phenotype is counteracted by the dispersin protein (see below)
        - Important for biofilm formation
      - **Type IV pili** (Dudley et al. 2006):
        - Encoded on IncI1 plasmid; not found in all EAEC strains
      - **Hda** (Boisen et al. 2008):
        - Necessary for adherence in AAF-negative strains
    - **Dispersin** (Sheikh et al. 2002):
      - Anti-aggregation activity; counteracts AAF fimbriae; disperses EAEC cells
      - Non-covalently attached to bacterial cell membrane; forms a surface coat
      - Encoded by *aap* gene
    - **Pic** (Henderson et al. 1999):
      - Member of SPATE family of serine proteases (see DAEC Sat above)
      - Plasmid encoded
      - Mucinase; cleaves host mucin
      - Involved in biofilm formation and colonization of intestinal cells
  - Damage to host cells:
    - **EAST1** (Ménard and Dubreuil 2002):
      - Plasmid-encoded, heat-stable enterotoxin
      - Thirty-eight aa polypeptide; homologous to EPEC EAST1 and ETEC ST

- Stimulates host guanylyl cyclase; increases cGMP levels; induces protein kinase G (PKG)-dependent fluid loss and diarrhea (see ETEC ST below for more details)

- **HlyE** (Chaudhuri et al. 2010):
  - Hemolysin E
  - Putative pore-forming cytolytic toxin; role in EAEC virulence is unclear

- **Pet (plasmid-encoded toxin)** (Navarro-Garcia et al. 1999):
  - Serine protease; member of SPATE family; plasmid encoded
  - Destroys α-fodrin (spectrin)
  - Disrupts actin cytoskeleton; results in cell rounding; exfoliation
  - Pet homologs: Sat, SigA

- **ShET1** (Fasano et al. 1995):
  - Chromosome (pathogenicity island) encoded
  - A-B_5 enterotoxin; same as EIEC/*Shigella* ShET enterotoxin
  - Increases cAMP and cGMP levels; leads to water loss and diarrhea

– **EHEC (enterohemorrhagic *E. coli*):**

- Subset of Shiga toxin-producing *E. coli* (STEC); most common serotype is O157:H7
- Symptoms: range from mild diarrhea to hemorrhagic colitis (bloody diarrhea)
- Complication: hemolytic uremic syndrome (HUS):
  - Shiga-like toxins (Stx1, Stx2) enter bloodstream and destroy red blood cells
  - Leads to kidney damage, vomiting, bloody diarrhea, and anemia
- Adherence to host cells (Croxen et al. 2013; Farfan and Torres 2012; McWilliams and Torres 2014):
  - Fimbrial adhesins:
    - EHEC encodes 14 potential fimbriae; 4 are unique to EHEC
    - Important roles in initial adherence, persistence, and tissue tropism
    - **LpfA (long polar fimbriae)**:
      - Primary adhesin
      - LpfA1 (predominant), LpfA2; similar to *Salmonella* Lpf
      - Binds to ECM components laminin, fibronectin, type IV collagen
    - **HCP (hemorrhagic coli pili)**:
      - Type IV pili
      - Binds to ECM laminin and fibronectin
      - Important role in biofilm formation

  - **Curli**:
    - Major adhesin in Lpf-negative cells
    - Major adhesin for biofilm formation and binding to vegetable leaves
    - Binds to ECM proteins laminin and fibronectin; also plasminogen and MHC class 1 proteins
  - **ECP (*E. coli* common pilus; Mat)**:
    - Unknown receptor
    - Important role in biofilm formation
  - **F9**: binds to ECM fibronectin
  - **ELF** (*E. coli* laminin-binding fimbriae): binds to ECM laminin
  - **SfpA** (sorbitol fermenting fimbriae protein A): unknown receptor
  - **Type I fimbriae**: important for biofilm formation; see AIEC above
- Afimbrial adhesins:
  - **EhaA, EhaB, EhaJ**:
    - Autotransporters (T5SS)
    - Bind to ECM components laminin, fibronectin, type IV collagen
    - Major role in biofilm formation
  - **EspP**:
    - Serine protease; member of SPATE family
    - Forms "rope-like" fiber structures; important for adherence and biofilm formation
    - Also cleaves coagulation factor V, pepsin, and complement factors C3/C3b, C5
    - Encoded on pO157 plasmid
  - **EibG (*E. coli* immunoglobulin-binding protein G)**:
    - Binds to IgG and IgA
    - Contributes to chain-like adhesion (CLA) pattern
  - **Efa-1/LifA (lymphostatin)**:
    - Large surface protein; unknown target(s)
- **A/E (attaching/effacing) lesions** (Schmidt 2010):
  - Efface (destroy) intestinal microvilli, generating attachment sites and actin "pedestals" (Fig. 15.5)
  - The major adherence mechanism for AHEC and APEC (see below); greater than 80% homology between protein components

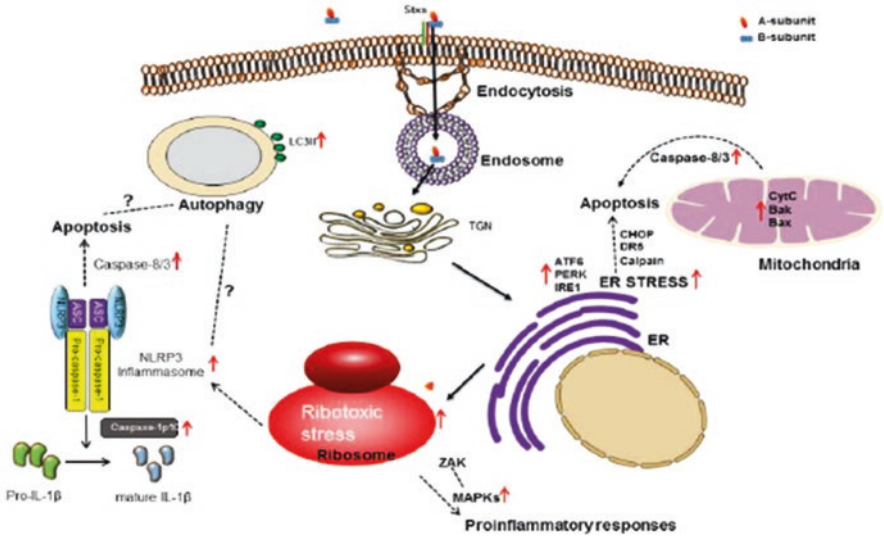


Fig. 15.5 Actin pedestals (From: Schmidt 2010)

- **LEE (locus of enterocyte effacement)**:
  - Chromosomal pathogenicity island; five operons containing ~41 genes; not found in nonpathogenic *E. coli*
  - Encodes the intimin–Tir adherence system (see below)
  - Encodes a type 3 secretion system (T3SS); translocates ~39 bacterial effectors into the host cytosol; many of these effectors have multiple effects within the host cell (Dean and Kenny 2009; Wong et al. 2011):
    - **EspA**: major subunit of filamentous needle structure
    - **EspB/D**: forms the translocon pore
    - **Paa**: required for A/E function
  - Effectors induce actin rearrangements, apoptosis, and suppress inflammation
- **Intimin–Tir adherence system** (Campellone and Leong 2003):
  - Promotes intimate attachment and colonization of intestinal epithelial cells following initial fimbrial adherence
  - **Intimin**:
    - 95 kDa protein
    - Anchored in outer membrane and peptidoglycan
    - Binds to bacteria-encoded translocated intimin receptor (Tir); strengthens bacteria–host attachment
    - Variations in the Tir-binding domains of intimin proteins from EHEC and EPEC mediate tissue tropism in the host intestine

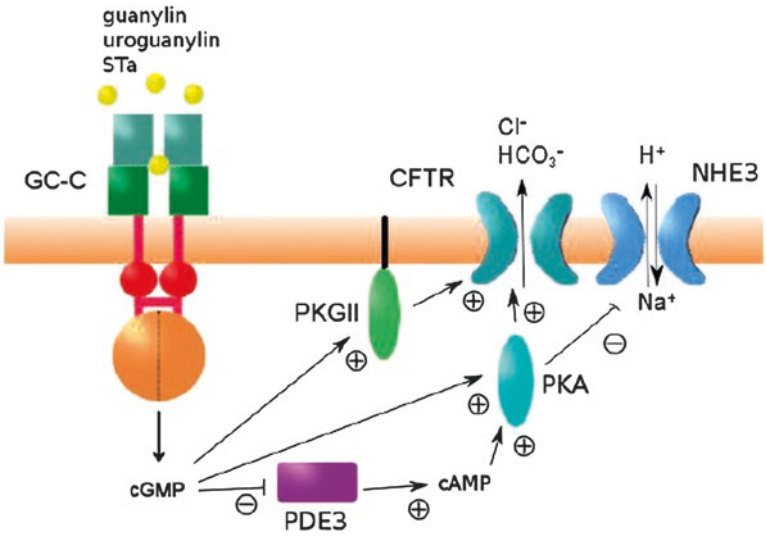


Fig. 15.6 EHEC vs. EPEC Tir signaling (From: Campellone and Leong 2003)

- **Tir**:
  - 57 kDa protein
  - Delivered to host cell cytoplasm through the T3SS
  - Displayed on the host cell surface; forms hairpin loop structure spanning the host cell membrane
  - Extracellular domain interacts with intimin
  - Intracellular domain interacts with host signaling proteins triggering N-WASP, Arp2/3-dependent actin rearrangements; leads to the formation of actin "pedestals" (Figs. 15.5 and 15.6)
  - EHEC and EPEC Tir differ in their initial host signaling interactions due to the presence of host cell tyrosine phosphorylation of EPEC Tir vs. the absence of EHEC Tir phosphorylation (EHEC Tir is lacking the essential tyrosine 474 residue) (Fig. 15.6):
    - EPEC Tir interacts with the SH2 phosphotyrosine-binding domain of the host Nck adaptor protein, which interfaces with WIP (WASP-interacting protein) and N-WASP
    - EHEC Tir interacts with host IRSp53 and IRTKS proteins, which link Tir to the bacterial effector protein **$EspF_U$** (Xs in Fig. 15.6); $EspF_u$ binds to N-WASP (de Groot et al. 2011)

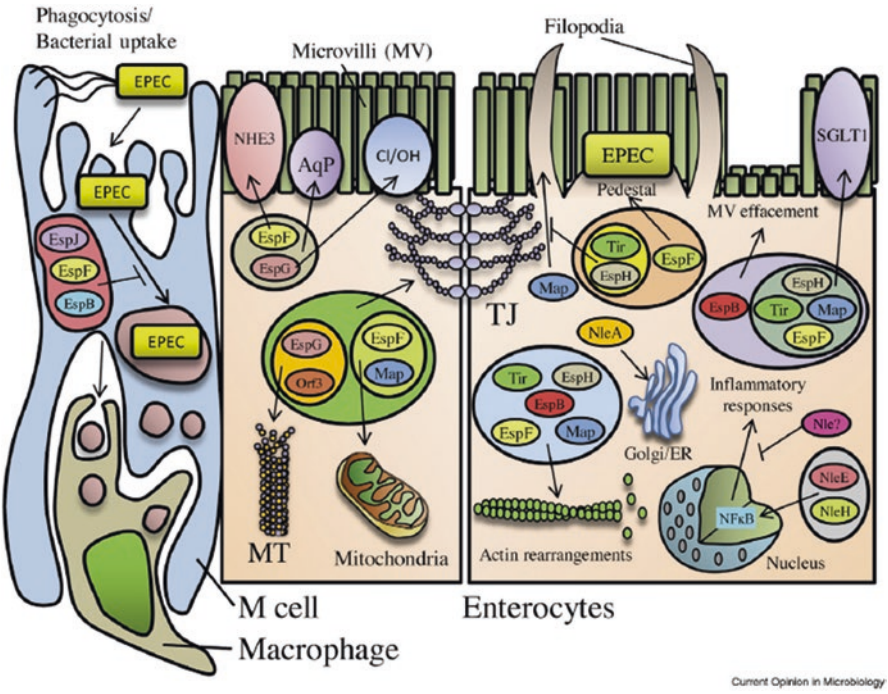


Fig. 15.7 Stx cellular effects (From: Lee et al. 2016)

- Damage to host cells:
  - **Shiga-like toxins (Stx1; Stx2)** (Bergan et al. 2012; Lee et al. 2016; Melton-Celsa 2014):
    - Induces ribotoxic stress response and ER stress response; induces inflammation, apoptosis, and autophagy (Fig. 15.7)
    - Lysogenic phage-encoded A-B_5 exotoxins:
      - **Stx1** (identical to EIEC/*Shigella* Shiga toxin Stx):
        - Three subtypes (Stx1a, Stx1c, Stx1d)
      - **Stx2** (~60% identical to Stx1):
        - Seven subtypes (Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, Stx2f, Stx2g)
        - Stx2 variants are more prevalent in colitis and HUS patients
    - A subunit:
      - Cleaved by furin or furin-like proteases into A1 and A2 subunits; held together by disulfide bond, which is disrupted within the host ER; A2 binds to B subunits; A1 enters the host cytosol

- A1 catalytic activity:
  - *N*-glycosidase activity; removes an adenine residue from the 28S rRNA of the 60S ribosome
  - Blocks binding to translation elongation factor 1 (EF1); inhibits host protein translation
- B subunits:
  - Forms homopentameric ring structure
  - Binds to glycosphingolipid globotriaosylceramide (Gb3; CD77) in cholesterol-containing lipid rafts on intestinal Paneth cells:
    - Gb3: ceramide lipid connected to [α-gal(1 → 4)-β-gal(1 → 4)-β-glc] trisaccharide (Lindberg et al. 1987)
- Released by phage-mediated lysis of EHEC cells (not secreted):
  - Antibiotic treatment can lead to EHEC cell death and release of toxin

- **EHEC-Hly or Ehx** (Bielaszewska et al. 2014):
  - Calcium-dependent, pore-forming, RTX (repeats in toxin) family hemolysin
  - Extracellular-free Hly targets and lyses host red blood cells, intestinal epithelial cells, and microvascular endothelial cells
  - Outer membrane vesicle (OMV)-associated Hly binds to host β_2 integrin proteins; leads to release of pro-inflammatory cytokines and caspase 9-dependent apoptosis
  - Encoded by *hlyA* gene; similar to UPEC α-hemolysin HlyA but with different host cell specificities
- **StcE** (Lathem et al. 2002):
  - Metalloprotease; cleaves host C1 esterase inhibitor (C1-INH):
    - C1-INH regulates the host complement cascade and coagulation
    - StcE cleavage disrupts complement activation; leads to tissue damage and decreases in platelet concentrations (thrombocytopenia)
  - Encoded on pO157 plasmid

- **EIEC: enteroinvasive *E. coli*** (see Chap. 28: *Shigella* spp.)
- **EPEC (enteropathogenic *E. coli*)** (Fig. 15.8) (Law et al. 2013; Singh and Aijaz 2016; Zhuang et al. 2017):
  - Subclassified into typical EPEC (tEPEC) and atypical EPEC (aEPEC):
    - tEPEC strains contain pEAF plasmid (encodes BFP; see below); aEPEC strains do not contain pEAF

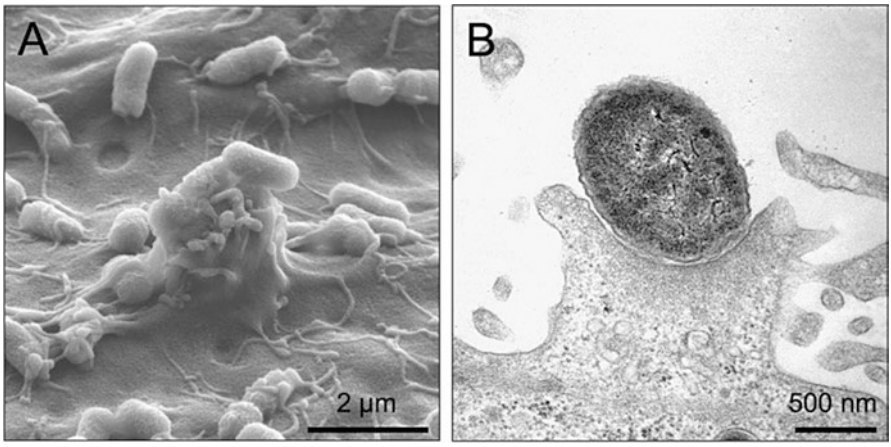


Fig. 15.8 EPEC virulence factors (From: Dean and Kenny 2009)

- Symptoms: watery non-bloody diarrhea; usually fatal to infants; caused by destruction of intestinal epithelial microvilli
- Adherence to host cells (Korea et al. 2011):
  - Fimbrial adhesins:
    - **BFP (bundle-forming pili)**:
      - Type IV fimbriae; encoded on large EAF plasmids
      - Leads to localized adherence (LA) pattern of attachment; three-dimensional microcolony formation is characteristic of tEPEC
    - **ECP (*E. coli* common pilus)**: see AHEC above
  - Afimbrial adhesin:
    - **LifA (lymphostatin)** unknown target(s):
      - Large surface protein
      - Required for intestinal colonization
    - **EspC**: homolog of EHEC EspP (see above)
  - Prototypical A/E (attaching/effacing) lesions: see EHEC above
- Damage to host cells:
  - T3SS effectors (Dean and Kenny 2009; Wong et al. 2011):
    - Many of these effectors have multiple effects within the host cell (see EHEC above)

- **EspF**:
  - Homologous to EHEC $EspF_U$ (see above)
  - Induces the internalization of aquaporins (AqP); affects water adsorption; leads to diarrhea
  - Inactivates (with Map, EspH, Tir) the Na^+-D-glucose co-transporter SGLT-1; also inhibits the Na^+/H^+-exchanger NHE3
  - N-terminal domain (with Map) induces mitochondria apoptosis pathway
  - C-terminal domain (with Tir, EspH, EspB, Map) disrupts host cell tight junction intestinal barriers through N-WASP-dependent actin rearrangements
- **EspG1/G2**:
  - Induces the internalization of aquaporins; affect water absorption; leads to diarrhea
  - Disrupts microtubules; leads to inhibition of Cl^- absorption due to loss of microtubule-dependent membrane localization of the DRA $Cl^-/HCO3^-$ exchanger
- **EspH**:
  - Functions in actin-based filopodia and pedestal formation
  - Blocks macrophage apoptosis
- **EspJ**:
  - Functions in colonization
  - Inhibits phagocytosis
- **Map**:
  - Affects Na+/H+ exchanger regulatory factor 2 (NHERF2) activity
  - Disrupts mitochondrial membrane potential; induces apoptosis
  - Acts as a guanine nucleotide exchange factor (GEF) for the Cdc42 GTPase; induces Cdc42-dependent filopodial formation
- **Paa**: see EHEC above
- **NleA (EspI)**:
  - Inhibits tight junction proteins ZO-1 and occludin; increases paracellular permeability
  - Inhibits protein secretion
- **NleE, NleH**:
  - Activate innate immune responses
  - Function in colonization

- Exotoxins:
  - EPEC does not produce Shiga toxins or other EHEC exotoxins
  - **CDT (cytolethal distending toxin)** (Lara-Tejero and Galan 2001, 2002):
    - Member of the tripartite family of CDTs; also found in *Haemophilus ducreyi*, *Campylobacter jejuni*, and *Salmonella enterica* serotype Typhi (only CdtB)
    - A-B exotoxin: three polypeptides **CdtA, CdtB,** and **CdtC**
      - CdtA and CdtC (B subunits):
        - Bind to clathrin-coated pits
      - CdtB (A subunit):
        - DNAase I: dsDNA break activity
        - Translocates into the host nucleus and induces double-strand breaks in the chromosomal DNA
        - DNA damage activates a cell cycle checkpoint; leads to G2 phase arrest (epithelial cells) or p53-dependent apoptosis (lymphocytes and monocytes) and induction of pro-inflammatory cytokines
  - **EAST1**: enterotoxin homologous to EAEC EAST1 and ETEC ST

– **ETEC: enterotoxigenic *E. coli*** (Fleckenstein et al. 2010; Gonzales-Siles and Sjöling 2016; Turner et al. 2006):
  - Major cause of traveler's diarrhea
  - Symptoms: mild to severe, watery non-bloody diarrhea; clinically indistinguishable from cholera
  - Adherence to host cells:
    - Fimbrial adhesins (Korea et al. 2011; Turner et al. 2006):
      - Colonization factors (CFs); reclassified as **CS (coli surface antigens)**
      - Not found in all strains; most are plasmid encoded
      - ~22 antigenically distinct proteins; different structural morphologies (fimbrial, fibrillary, helical, non-fimbrial):
        - Fimbrial (rigid rodlike structures): CFA/I, CS1, CS2, CS4, CS8, CS12, CS14, CS17, CS18, CS19, CS20, CS21
        - Fibrillary (thin, flexible structures): CS3, CS11, CS13
        - Helical: CS5, CS7
        - Non-fimbrial: CS6, CS10, CS15, CS22

- "Longus":
  - Type IV pilus; extends 40 μm from cell surface
  - Encoded by *lngA* gene

- Afimbrial adhesins:
  - **Tia** (Elsinghorst and Kopecko 1992):
    - 25 kDa outer membrane protein
    - Binds to host heparan sulfate proteoglycans
  - **TibA** (Cote et al. 2013; Elsinghorst and Weitz 1994):
    - 104 kDa autotransporter; outer membrane protein
    - Glycosylated by TibC; necessary for adherence and stability

- **EtpA** (Roy et al. 2009):
  - Flagellar-secreted tip adhesin
  - Binds to flagellin subunits
  - Degraded by **EatA** (Patel et al. 2004):
    - SPATE family of serine proteases, similar to EIEC/*Shigella* SepA
    - Modulates adherence; also LT secretion

- Damage to host cells:

- **LT** (Liang and Hajishengallis 2010; Spangler 1992):
  - Heat-labile enterotoxin; ~80% identical to cholera toxin
  - LT-I; LT-II (LT-IIa, LT-IIb, LT-IIc): antigenically distinct variants
  - A-B_5 toxin:
    - A subunit:
      - ADP-ribosyltransferase activity
      - Proteolytically cleaved to form A_1 and A_2 subunits
      - A_1 subunit ADP-ribosylates the $G\alpha_s$ subunit of the G_s heterotrimeric G protein
      - The inactivated $G\alpha_s$ subunit cannot hydrolyze its bound GTP – remains constitutively active
      - Results in constitutive activation of the downstream effector adenylate cyclase (AC), which converts ATP into cAMP:
        - Increases cAMP concentration more than 100-fold
      - cAMP binds to, and activates, protein kinase A (PKA), which phosphorylates and activates the cystic fibrosis transmembrane conductance regulator (CFTR):
        - CFTR: membrane ion channel protein; conducts Cl^- ions across epithelial cell membranes

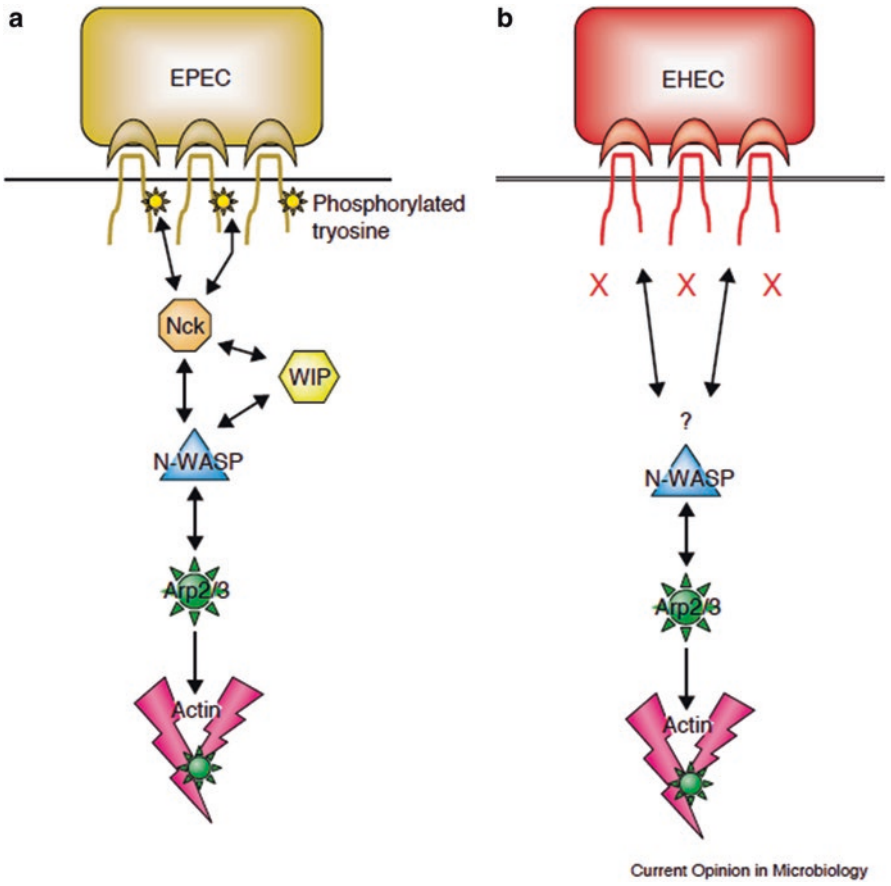


Fig. 15.9 ST mechanism of action (From: Weiglmeier et al. 2010)

- Cl^- release, as well as Na^+ entry into cells, causes a massive water efflux from intestinal epithelial cells – diarrhea

  - B subunits:

    - Forms pentameric ringlike structure; binds to GM1 gangliosides within lipid raft domains in the cell membrane of intestinal epithelial cells

- **ST** (Fig. 15.9) (Hughes et al. 1978; Weiglmeier et al. 2010):

  – Heat-stable enterotoxin
  – STa (ST-I):

    - Small peptides
    - ST-Ia (STp), 18 aa; ST-Ib (STh), 19 aa

  - Mimics the peptide hormones guanylin and uroguanylin; binds to the extracellular domain of guanylyl cyclase C (GC-C) receptors on the intestinal epithelium
  - Activation of guanylyl cyclase C leads to increases in cGMP levels:
    - cGMP activates cGMP-dependent protein kinase II (PKG-II); directly phosphorylates and activates CFTR
    - cGMP inhibits phosphodiesterase 3 (PDE3); leads to increases in cAMP levels, activation of PKA and CFTR, and inhibition of NHE3-dependent Na^+ re-absorption
  - End result is increases in H_2O efflux and diarrhea

- Extraintestinal pathotypes; extraintestinal pathogenic *E. coli* (ExPEC) (Dale and Woodford 2015; Vila et al. 2016):
  - **UPEC (uropathogenic *E. coli*):**
    - Most common cause of UTIs; initial cystitis can lead to pyelonephritis and septicemia
    - Adherence to host cells (Korea et al. 2011; Mulvey 2002):
      - Fimbrial adhesins:
        - UPEC strains encode many more fimbrial gene clusters than diarrheagenic strains; not all are expressed in every UPEC strain
        - **Dr adhesins**: see DAEC Dr adhesins above
        - **Type I fimbriae**; see AIEC type I fimbriae above:
          - CUP assembly mechanism
          - Functions in bladder colonization; also important for biofilm formation
          - **FimH** fimbrial tip adhesin:
            - Binds to uroplakin 1a (α-D-mannose-containing glycoprotein) and other uroplakin plaques on bladder epithelial cells; leads to invasion of uroepithelial cells
            - Also binds to ECM laminin, fibronectin, and type I and type IV collagen
        - **P fimbriae**:
          - CUP assembly mechanism
          - ~70% of UPEC have P pili; ~20% of GI tract *E. coli* have P pili
          - Encoded by *pap* genes
          - **PapA**: major structural subunit
          - **PapG** (fimbrial tip adhesin):
            - Binds to globotriaosylceramides GbO3, GbO4, and GbO5:
              - Digalactoside (Galα1–4Gal) core linked by a β-glucose: (Glc) residue to a ceramide group (P blood group antigen)

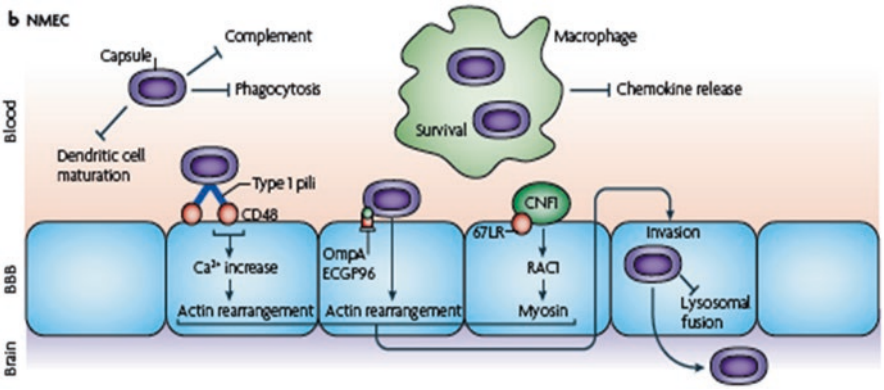


Fig. 15.10 UPEC virulence factors (From: Wiles et al. 2008)

- More associated with pyelonephritis and binding to renal epithelial cells

– **S fimbriae**:

- CUP assembly mechanism
- **SfaA**: major structural subunit
- **SfaS** (fimbrial tip adhesion):
  - Binds to sialic acids sugars on uroplakin proteins

– **Curli**: see EHEC Curli fimbriae above
– **F1C** (type IC fimbriae):

- Similar to S fimbriae; CUP assembly mechanism
- **FocA**: major structural subunit
- Binds to β-GalNac-1, 4β-Gal residues on glycosphingolipids; asialo-GM_1; asialo-GM_2

– **F9**: see EHEC F9 fimbriae above
– **Pix**: related to the P fimbriae (Lugering et al. 2003).
– **Yad; Ygi,** and **Yfc** (Spurbeck et al. 2011):

- CUP assembly mechanism
- More prevalent in UPEC than in commensal *E. coli*

- Invasion of superficial facet cells in bladder:
  - FimH binds to uroplakin Ia and IIIa receptors on facet cells
  - UPEC cells replicate and aggregate into intracellular bacterial communities (biofilm-like)
  - Can migrate into bladder lumen and ascend to kidneys; requires flagella genes to be turned on and pili genes to be turned off
- Damage to host cells (Fig. 15.10):
  - **CNF-1; CNF-2 (cytotoxic necrotizing factor)** (Flatau et al. 1997; Schmidt et al. 1997):

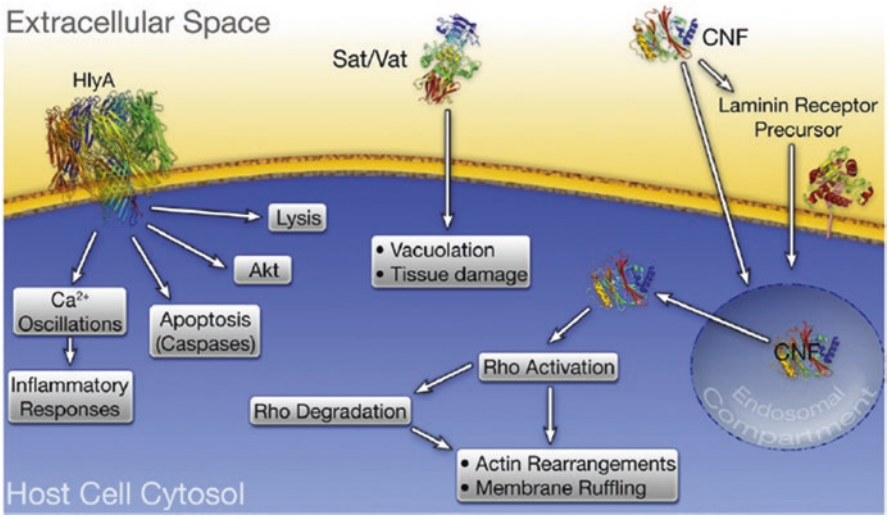


Fig. 15.11 NMEC virulence factors (From: Croxen and Finlay 2010)

  - 113 kDa transmembrane protein; homologous to *Yersinia* CNF_Y
  - Constitutively activates the Rho/Rac/Cdc42 GTPases through deamidation of the Glu61/63 residue
  - Induces actin rearrangements; cell apoptosis

- **HlyA**: see EHEC-Hly above (Ristow and Welch 2016)
- **Pic**: see EAEC Pic above
- **Sat/Vac**: see EAEC Pic above

- Evasion of innate immune system:

- **TcpC** (Snyder et al. 2013):

  - Blocks MyD88/TLR4-dependent cytokine production
  - Increases bacterial burden in the urinary tract

– **NMEC (neonatal meningitis *E. coli*)** (Fig. 15.11) (Croxen and Finlay 2010):

- Infection progression:

- Acquired perinatally from mother
- Intestinal colonization
- Bacteremic dissemination within macrophage in the bloodstream to the blood–brain barrier
- Invasion of brain microvascular endothelial cells (BMECs)
- Transmigrates through BMECs without multiplication into brain

- Adherence to host cells:

- Fimbrial adhesins:

- **Type I pili**; see above:
  - Binds to CD48; α3β1 integrins (Khan et al. 2007)
- **S fimbriae**: see UPEC above

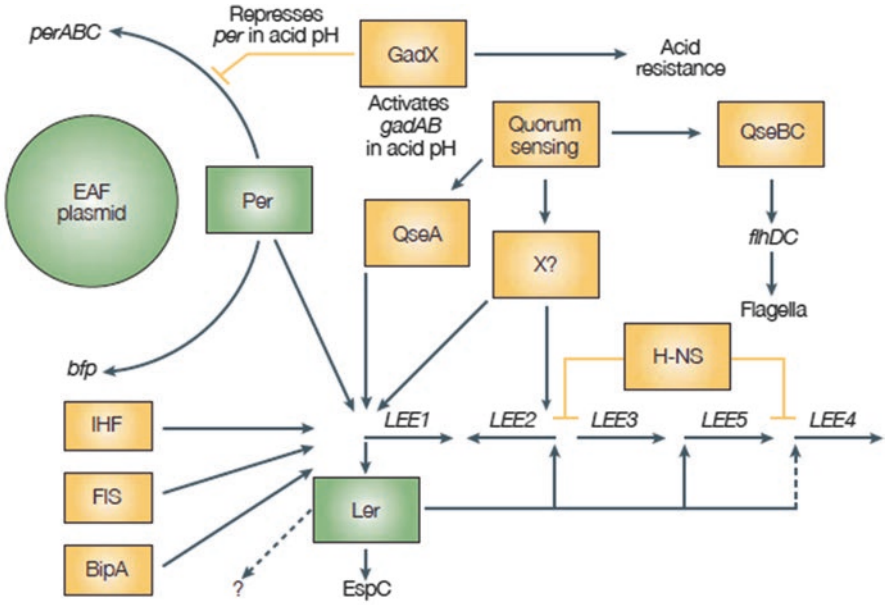
- Afimbrial adhesins:
  - **FdeC** (Nesta et al. 2012):
    - Similar to EPEC intimin
    - Binds to epithelial cells and collagen I, II, V, and VI
    - Functions in the kidney and bladder colonization; bacterial aggregation
  - **OmpA (outer membrane protein A)** (Prasadarao 2002):
    - Binds to ECGP96 receptor
    - Functions in internalization into BMECs
    - Binds complement C4-binding protein (C4BP); blocks membrane attack complex formation
  - **IbeA, B, C** (Huang et al. 2001):
    - Functions in internalization into BMECs

- Damage to host cells:
  - **CNF-1 (cytotoxic necrotizing factor-1)**; see UPEC CNF-1 above:
    - Functions in internalization into BMECs

- Evasion of host immune system:
  - **Capsule** (Kim et al. 1992):
    - Predominantly the K1 polysaccharide antigen
    - Homopolymer of polysialic acid
    - Protects NMEC from complement and phagocytosis in the bloodstream
    - Also blocks lysosome fusion during BMEC internalization

Regulation of Virulence Factor Expression (Fig. 15.12) (Kaper et al. 2004; Mellies and Lorenzen 2014)

- Pathogenic *E. coli* respond to a myriad of environmental and host signals to induce or repress the expression of virulence factors:
  - pH; acid tolerance: essential for transit through the stomach
  - Quorum sensing



Expression of virulence factors in pathogenic *E. coli* utilizes regulators that are present only in pathogenic strains as well as regulators present in all *E. coli* strains, commensals and pathogens. The attaching and effacing histopathology induced by EPEC and EHEC is encoded by the locus of enterocyte effacement (LEE) pathogenicity island, which contains five major polycistronic operons designated *LEE1–5*. Expression of the *LEE* genes is regulated by EPEC-specific regulators (depicted in green) and generic *E. coli* regulators (depicted in yellow). The first open reading frame of the *LEE1* operon encodes the LEE-encoded regulator, Ler, which positively regulates expression of other *LEE* operons by counteracting the repressive effects of H-NS[140,148]. Ler also regulates expression of the EspC enterotoxin that is produced by many EPEC strains and potentially other virulence factors. Expression of Ler is itself regulated by several factors, including IHF[149], FIS[150] and BipA[151], and quorum sensing through the QseA regulator[152]. Quorum sensing also regulates other factors that are potentially involved in virulence, such as flagella, through the QseBC two-component regulator[153]. In EPEC, but not EHEC, expression of Ler is positively regulated by the products of the *per* (plasmid-encoded regulator)[154] locus, which consists of three open reading frames, *perA*, *perB* and *perC*; PerA (BfpT) also regulates the *bfp* genes encoding a type IV pilus[155]. In acidic conditions, the *per* genes are repressed by GadX, which activates the *gadAB* genes involved in acid resistance[156]. This dual action of GadX could prevent premature expression of virulence factors in the stomach while enhancing survival of the organism until it reaches more alkaline conditions in the small intestine where expression of virulence factors is induced. Bip, Ig heavy chain binding protein; FIS, factor for inversion stimulation; IHF, integration host factor.

Fig. 15.12 Regulation of virulence factors (From: Kaper et al. 2004)

  - Carbohydrate availability, metabolism, and iron availability
  - Flagellar biosynthesis and motility: inactivated in biofilms and during attachment; activated during dissemination
  - Synthesis and T3SS secretion of adherence factors and toxins; LEE vs. non-LEE strains
- Use regulatory factors that are associated with virulence PAIs and plasmids [e.g., Ler (LEE encoded regulator), Per (plasmid-encoded regulator)] as well as those that are present in commensal strains

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Chapter 16
Francisella spp.

Genomics, Morphologies, and Growth Characteristics

- **Genomics:**
  - *Francisella tularensis* chromosome: 1,892,819 bp; 1804 predicted ORFs (Larsson et al. 2005)
- **Cell morphology:**
  - Small pleomorphic coccobacilli (Fig. 16.1)
- **Gram stain:**
  - Gram negative
- **Growth:**
  - Strict aerobes; oxidase negative
  - Fastidious growth: requires cysteine and iron
  - Found in many mammalian species, including rabbits, voles, mice, squirrels, muskrats, and beavers; macrophage are the usual host cell reservoir
  - Ten species with multiple subspecies: two major human pathogenic subspecies and several minor pathogenic species:
    - *F. tularensis* subsp. *tularensis* – Type A *Francisella*:
      - Highly virulent; associated with dry terrestrial reservoirs primarily in North America
    - *F. tularensis* subsp. *holarctica* – Type B *Francisella*:
      - Milder disease state; associated with aquatic reservoirs primarily in Europe and Asia

D.I. Johnson, *Bacterial Pathogens and Their Virulence Factors*,
https://doi.org/10.1007/978-3-319-67651-7_16

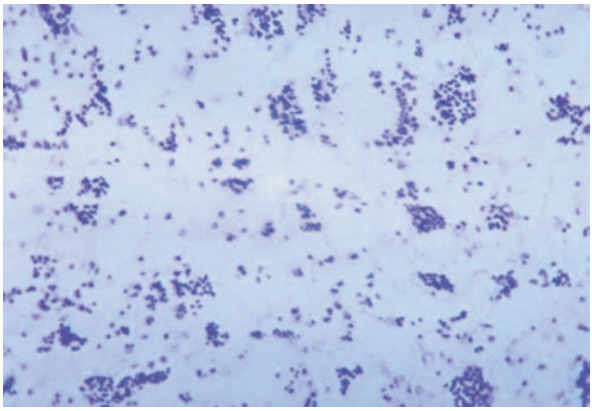

Fig. 16.1 *F. tularensis* cells (From: PHIL #2985)

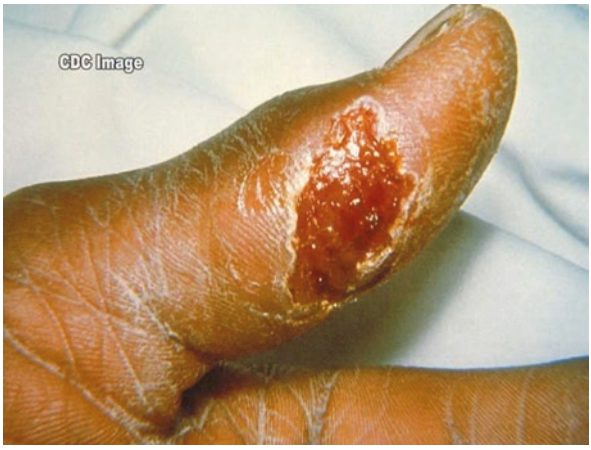

Fig. 16.2 Ulceration at bite site (From: CDC)

Disease States Associated with *Francisella tularensis*

- Tularemia ("rabbit fever"; "deer fly fever"):
  - Zoonotic disease
  - Facultative intracellular pathogen: can infect phagocytic and non-phagocytic cells
  - Transmission (not spread from person to person):
    - Vector transmission (bite of tick or deer fly):
      - Symptoms: ulceration at bite site (Fig. 16.2), sudden fever, chills, headaches, diarrhea, muscle aches, joint pain, dry cough, swollen lymph nodes

- Inhalation tularemia:
  - Usually from handling contaminated tissues or aerosolized urine or feces; risk for landscapers who kill carriers with lawnmowers
  - Causes a pneumonic form of tularemia
  - Can disseminate to the liver and spleen
  - Can lead to life-threatening "cytokine storm," particularly when inhaled
- Typhoidal tularemia: ingestion of contaminated food

– Potential bioweapon (McLendon et al. 2006):
- Tier 1 select agent
- Extremely virulent with low infectious dose; ~10 colony-forming units (CFU) can cause disease; 90–100% of infected patients get the disease; 30–60% of untreated infections can be fatal
- Easily disseminated via aerosolization; most deadly form

Virulence Factors (Jones et al. 2014):

- Facultative intracellular pathogen: essential for virulence
- Adherence to host cells:

– Bacterial adhesin–host receptor interactions vary depending on the opsonization state; opsonization increases internalization approximately tenfold (Fig. 16.3) (Moreau and Mann 2013)
– Unopsonized *F. tularensis* adherence:
- **Type IV pili (Tfp) system** (Salomonsson et al. 2011):
  - Essential for virulence and biofilm formation; not required for intracellular survival or replication
  - Encoded and assembled by *pil* gene products
  - Six structural pilin proteins: major pilin – **PilA (PilE1)**
  - Unknown human receptor
- Host mannose receptors (MR): primary target for unopsonized cells; unknown *Francisella* target protein
- **EF-Tu** (Barel et al. 2008)
  - Surface expressed *Francisella* elongation factor
  - Interacts with host surface-exposed nucleolin (SE-N)
- **FsaP** (Melillo et al. 2006)
  - Outer membrane protein; unknown human receptor
  - Increased expression in Type A cells

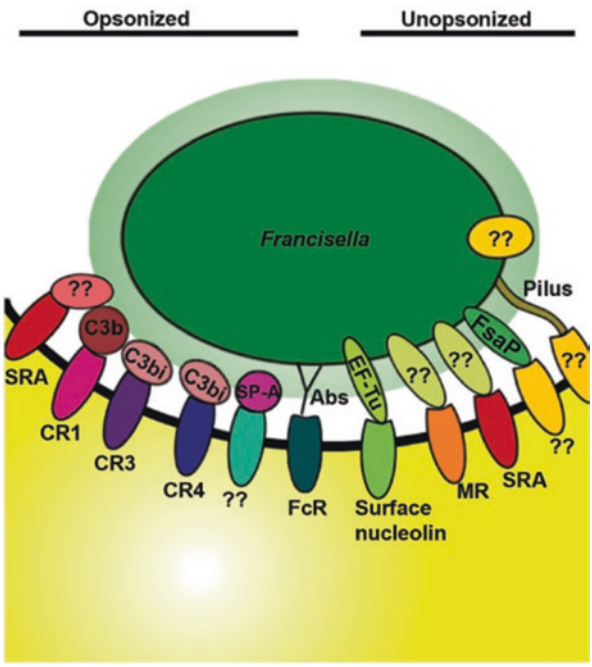

Fig. 16.3 *F. tularensis* adherence mechanisms (From: Moreau and Mann 2013)

- Opsonized *F. tularensis* adherence: type of opsonization affects binding:
  - Complement-based opsonization:
    - Targets LPS and capsule
    - Complement receptors (CR3, CR1, CR4) (Geier and Celli 2011)
      - Different host cell tropism; *F. tularensis* can cleave/inactivate complement protein C3b to C3bi, which mediates the receptor interactions
    - Scavenger receptor A (SRA): (Geier and Celli 2011)
      - Macrophage receptor; interacts with LPS
  - IgG antibody-based opsonization (Geier and Celli 2011):
    - Almost exclusively through interaction with Fcγ receptors (FcγR)

- Host cell invasion (Steiner et al. 2014):
  - Facultative intracellular pathogen; can infect phagocytic and non-phagocytic cells
  - Looping phagocytosis: generation of large asymmetrical pseudopod loops usually in areas of lipid rafts; depends on host actin cytoskeleton (Moreau and Mann 2013)

  - Once internalized, maturation of *Francisella*-containing phagosome (FCP) is blocked through acidification:
    - Prevents ROS respiratory burst by inhibiting NADPH oxidase maturation
    - Inhibits lysosome fusion, leading to rapid phagosomal escape and active cytosolic multiplication
  - Replication leads to host cell apoptosis and egress from the cell
  - Leads to induction of pro-inflammatory cytokines and chemokines, such as IFN-β and TNF-α, and a potentially lethal "cytokine storm"

- Growth within the host milieu:
  - Iron acquisition (Fortier et al. 1995):
    - Iron is essential for the virulence and intracellular growth of *F. tularensis*
    - Receptors for host transferrin, lactoferrin, heme, hemoglobin, or hemopexin are not found in the annotated genome
    - **Fe–siderophore** (Sullivan et al. 2006):
      - Polycarboxylate siderophore; rhizoferrin-like; synthesized by *fsl* genes
      - Regulated by Fe^{+2}-dependent Fur repressor (Ramakrishnan et al. 2008)
    - **Fe^{+2} iron uptake** (Perez and Ramakrishnan 2014):
      - Mediated by membrane-bound FupA and FeoB Fe^{+2} iron transporters
      - May play a role in heme uptake (Lindgren et al. 2015)
  - ***Francisella* pathogenicity island (FPI):**
    - Two copies in *F. tularensis*
    - Encodes many virulence factor genes; eight of which (IglE, IglC, IglI, IglJ, IglF, VgrF, PdpE, PdpA) are secreted upon macrophage infection
    - Some are components of a type 6 secretion system (T6SS), which is required for virulence

- Evasion of host immune system
  - Intracellular growth: see above
  - **LPS** (Hajjar et al. 2006)
    - Essential for full virulence
    - Different lipid A structure vs. other Gram-negative bacteria
    - Makes it unable to bind host lipid A binding protein (LBP) or toll-like receptor-4 (TLR-4); no endotoxin activity and no inflammatory response
  - **Capsule** (Apicella et al. 2010):
    - Type 4 capsule
    - Incorporates identical O-antigen polysaccharides as found in the LPS, but neither lipid A nor 2-keto-3-deoxyoctulsonic acid (KDO) is attached
    - Indicates that the capsule and the LPS are distinct structures

- Complement evasion (Jones et al. 2012)
  - Binds host plasmin; blocks antibody binding and complement activation
  - Binds host factor H; inhibits complement protein C3 activation, which blocks membrane attack complex (MAC) formation

Regulation of Virulence Factor Expression (Dai et al. 2010)

- Levels of virulence factors are transcriptionally regulated in response to changes in environmental and host stimuli during infection:
  - **MglA**:
    - Macrophage growth locus A; essential for virulence
    - Global positive regulator of >100 genes inside and outside of FPI; binds directly to RNA polymerase
    - Upregulated during macrophage infection
  - **SspA**:
    - High homology to MglA
    - Required for binding of MglA to RNA polymerase
  - **PmrA/KdpD**:
    - Two-component phosphorelay system
    - KpdD: sensor kinase; responds to unknown signal
    - PmrA: response regulator
      - Transcription activator
      - DNA-binding protein that interacts with MglA and SspA in activating virulence gene transcription
  - **FevR** (*Francisella* effector of virulence regulator):
    - Essential for replication of *Francisella* in macrophages
    - *fevR* mutants are trapped in phagosome
    - Does not bind DNA or RNA polymerase; acts upstream or parallel to MglA

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Chapter 17
Haemophilus spp.

Genomics, Morphologies, and Growth Characteristics

- **Genomics:**
  - *Haemophilus influenzae* chromosome: 1,830,140 bp; 736 predicted ORFs (Fleischmann et al. 1995)
- **Cell morphology:**
  - Very small pleomorphic coccobacilli (Fig. 17.1)
- **Gram stain:**
  - Gram negative
- **Growth:**
  - Aerotolerant anaerobes; catalase positive, oxidase positive:
    - In vitro growth requires CO_2-enriched environment
  - Very fastidious growth
  - *H. influenzae* requires X factor and V factor:
    - X factor: hemin; contains Fe^{3+} ion; released from lysed red blood cells (chocolate agar)
    - V factor: nicotinamide adenine dinucleotide (NAD)
  - Temperature sensitive:
    - Stocks must be stored at 37 °C for survival
  - Human commensal that colonizes mucous membranes of the upper respiratory tract, nasopharynx, mouth, vagina, and intestinal tract; opportunistic pathogen

D.I. Johnson, *Bacterial Pathogens and Their Virulence Factors*,
https://doi.org/10.1007/978-3-319-67651-7_17

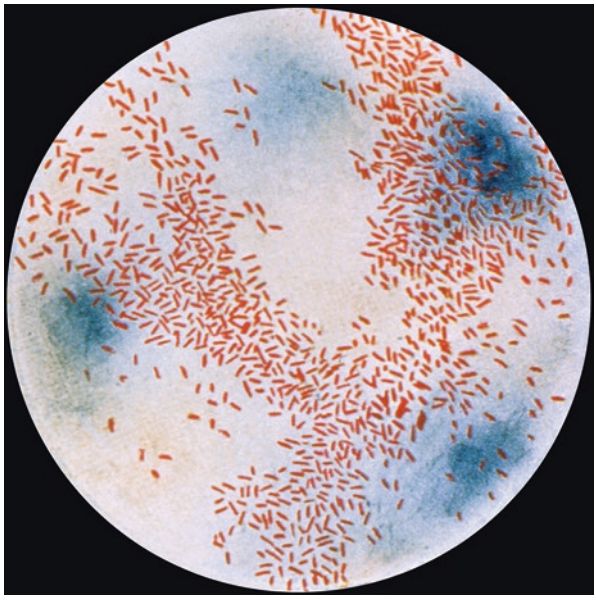

Fig. 17.1 *H. influenzae* cells (From: PHIL #1947)

- Eighteen species: one major human pathogenic species (*H. influenzae*) and several minor pathogenic species:
  - *Haemophilus influenzae* subtype *aegyptius*: acute, purulent conjunctivitis, Brazilian purpuric fever
  - *Haemophilus parainfluenzae*: opportunistic pathogen of chronic obstructive pulmonary disease (COPD) patients
  - *Haemophilus ducreyi*: causes inflammatory chancroid; sexually transmitted disease (Lewis and Mitjà 2016); contains a CDT DNAase that damages chromosomal DNA and causes a cell cycle arrest (Fais et al. 2016)
- *H. influenzae* strains are characterized (typed) by the presence or absence of polyribosylribitolphosphate (PRP) capsule (Fig. 17.2)
  - Encapsulated strains – typeable strains:
    - Serotypes a–f: based on antibodies against PRP capsule antigens
    - Serotype b (Hib): most common; caused serious systemic infections (meningitis) before Hib vaccine
    - PRP capsule antigens: primary antigenic constituent in polysaccharide and polysaccharide conjugate Hib vaccines
  - Nonencapsulated strains – non-typeable *H. influenza* (NTHi):
    - Normal serum is usually bacteriocidal for most NTHi strains but not encapsulated strains
    - Great heterogeneity between NTHi strains

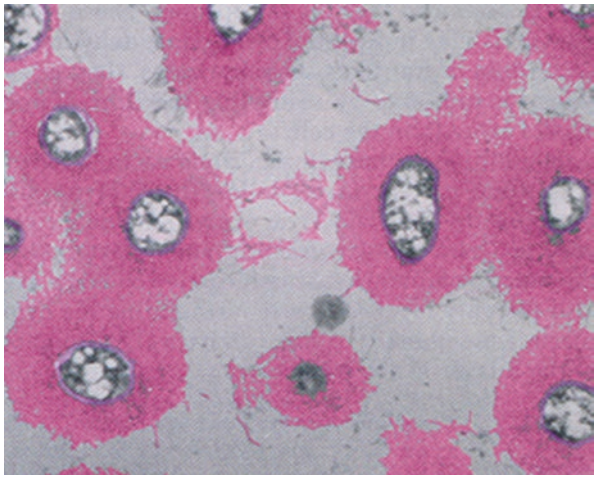

Fig. 17.2 *H. influenzae* capsule (From: balamoti.bol.ucla.edu)

Disease States Associated with *Haemophilus influenzae*

- Typeable strains (De Schutter et al. 2011):
  - Lung infections, epiglottitis, meningitis, septicemia, and community-acquired pneumonia
  - Transmitted person to person via respiratory aerosols
  - Primary cause of disseminated *H. influenzae* diseases
- NTHi strains (Duell et al. 2016; King 2012):
  - Cause majority of upper respiratory infections, pneumonia, sinusitis, conjunctivitis, and acute otitis media
  - Reemerging pathogen for COPD patients
  - Invasive to macrophage and epithelial cells in vitro; in vivo significance is unclear

Virulence Factors

- Adherence to host cells – respiratory and nasopharynx epithelial cells (Fig. 17.3):
  - Fimbrial adhesins:
    - **Pili**:
      - Found in typeable and non-typeable *H. influenzae*
      - Enhance adherence to mucosal cells in nasopharynx
      - Bind intercellular adhesion molecule 1 (ICAM1) and mucins (Kubiet et al. 2000)

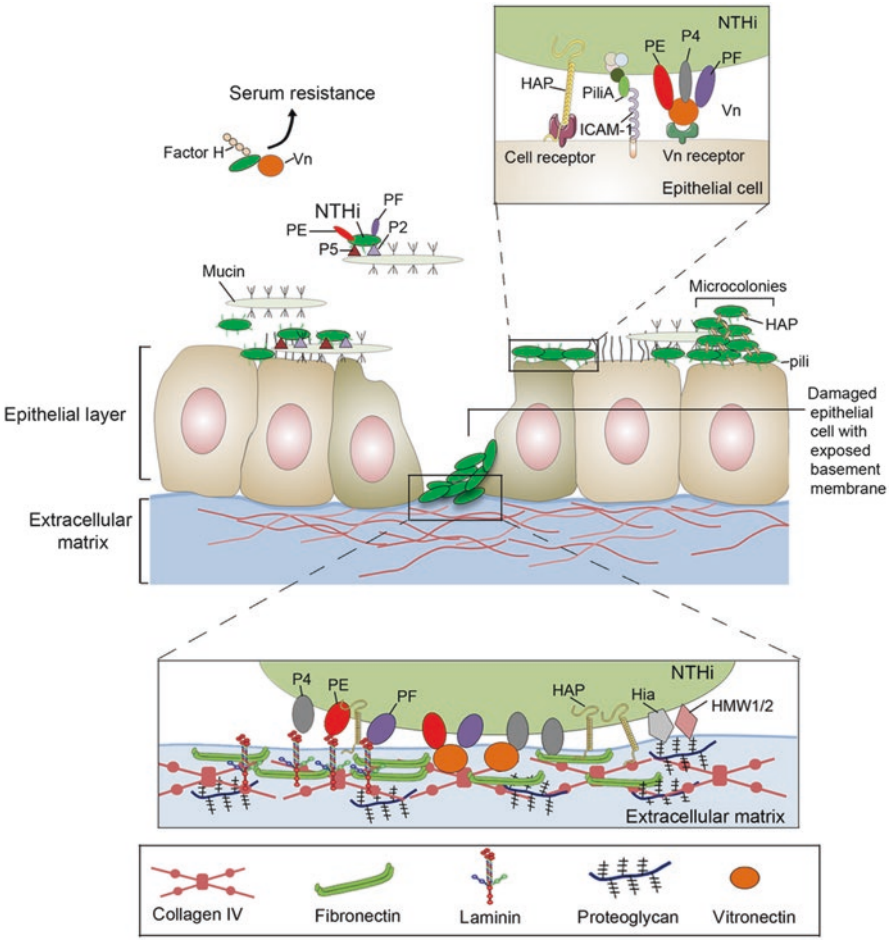

Fig. 17.3 *H. influenzae* NTHi adherence mechanisms (From: Duell et al. 2016)

- **HifA**: structural protein
- **PilA**: tip protein

– Afimbrial adhesins:

- **HMW1** and **HMW2** (St. Geme and Yeo 2009):
  - Only found in non-typeable *H. influenzae*
  - Mediate adherence to human respiratory and nasopharynx Epithelial cells; also bind proteoglycans in the ECM
  - Structurally similar to *B. pertussis* filamentous hemagglutinin (FHA)
  - **Hmw1A** and **Hmw2A** – structural proteins:

        - Secreted via T5SS two-partner secretion (TPS) system (St. Geme and Yeo 2009)
    - **Hia** (St. Geme and Cutter 2000):
        - Only found in non-typeable *H. influenzae*
        - T5SS autotransporter family of outer membrane proteins
    - **Hap** (Fink et al. 2002):
        - Only found in non-typeable *H. influenzae*
        - Bind to ECM components laminin, fibronectin, and collagen IV
    - **P2, P5, protein E,** and **protein F** (Finney et al. 2014):
        - Membrane proteins
        - Only found in non-typeable *H. influenzae*
        - P2 and P5 bind to mucins
        - Protein E and protein F bind to epithelial cells and extracellular matrix proteins
- Growth within the host milieu:
    - Iron acquisition (Hariadi et al. 2015):
        - Instead of using siderophores, acquires iron from host iron-binding proteins
        - **HgpA, HgpB,** and **HgpC** (Morton et al. 1999); **HhuA** (Maciver et al. 1996):
            - Hemoglobin- and hemoglobin-haptoglobin-binding proteins
        - **Tbp1** and **Tbp2** (Gray-Owen and Schryvers 1995); **HitA** (Sanders et al. 1994):
            - Transferrin-binding proteins
        - **HxuA, HemR,** and **Hup** (Pidcock et al. 1988):
            - Heme uptake from host hemin and hemoglobin
- Damage to host cells:
    - **Lipooligosaccharide (LOS)**:
        - LPS lacking extensive O-antigen polysaccharides
        - Essential for virulence
        - Elicits endotoxin pro-inflammatory response
- Evasion of host immune system:
    - **Polyribosylribitolphosphate (PRP) capsule**:

- Anti-phagocytic; more copies of capsule genes lead to greater resistance to phagocytosis and to complement-mediated cell lysis (Noel et al. 1996) (see below)
- Major role in bloodstream dissemination leading to meningitis
- ***cap b* locus** (Kroll and Moxon 1988):
  - Encodes the genes needed for the synthesis of type b capsule; capsule polysaccharide synthesis, modification, and export.
  - IS element-mediated recombination can lead to gene amplification and more copies of the locus; more PRP capsule is produced (Gilsdorf et al. 2004; Kroll et al. 1991).

– **IgA1 proteases** (Mulks et al. 1982):

- Three endopeptidases that cleave IgA antibodies in lung mucosa

– Phase variation:

- Modification of cell structures, such as LOS, adhesins, and pili that contribute to disease
- P2 and P5 proteins undergo antigenic drift (Duell et al. 2016)

– Complement-mediated lysis inhibition: (Hallstrom and Riesbeck 2010)

- Principle defense against *H. influenzae* infection
- Physical barriers (capsule, LOS) block activation and deposition of serum complement proteins, e.g., C4b; leads to blockage of membrane attack complex (MAC) formation
- Co-opt host complement regulators to block complement-mediated lysis
  - Adhesin proteins (protein E, protein F, and P4) and surface fibril Hsf bind host vitronectin (Hallstrom and Riesbeck 2010):
    – Binding enhances vitronectin's ability to block complement C5b-C9 proteins; inhibits MAC formation
- Bind to and activate host regulatory proteins C4BP, factor H, FHL-1
  – Binding blocks complement proteolysis cascade; inhibits MAC formation host cell invasion:
- Primarily NTHi strains; facultative intracellular pathogen
- Invade macrophage and lung epithelial cells

Regulation of Virulence Factor Expression

- Oxygen concentrations (Wong and Akerley 2012):
  – Must adapt to O_2-rich environment in the lungs and O_2-poor environment in inner ear and bloodstream when disseminating

- Lung environment – aerobic:
  - High in reactive oxygen species (ROS); need to scavenge for amino acids, nucleotides, and metal ions (Fe, Zn), which are in low concentrations
  - Increase LOS synthesis genes because LOS is damaged by ROS
  - Increase antioxidants, such as superoxide dismutase (SOD) and catalase, amino acid metabolism, ion transport
- Bloodstream environment – anaerobic:
  - Presence of macrophage and reactive nitrogen species (RNS)
  - Still need antioxidants and LOS synthesis genes expressed
- **ArcA–ArcB** – oxygen-sensing TCS:
  - ArcB: sensor kinase; transfers phosphate to ArcA
  - ArcA: response regulator
    - Transcriptional activator and repressor
    - Represses genes involved in aerobic respiration and de-represses genes involved in anaerobic respiration
    - Low oxygen conditions: ArcB phosphorylates ArcA in response to redox status of quinone electron carrier pool; phosphorylated ArcA transcriptionally activates or represses target genes
    - High oxygen conditions: oxidized quinones block ArcB activity – inactive in lungs
- **FNR** – transcription activator:
  - O_2 sensor; contains Fe–S center
  - O_2-binding inactivates Fe–S center and FNR activity
  - Active in anaerobic environments; needed to protect against RNS

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Chapter 18
Helicobacter spp.

Genomics, Morphologies, and Growth Characteristics

- **Genomics:**
  - *Helicobacter pylori* chromosome: 1,667,867 bp; 1,590 predicted ORFs (Tomb et al. 1997)
- **Cell morphology:**
  - Small, helical or curved, rod-shaped cells (Fig. 18.1)
  - Flagella: four to six lophotrichous flagella
    - Motility is essential for virulence (see below).
    - Two copolymerized flagellin proteins: FlaA and FlaB
  - LPS: has fucose-containing O-antigens
    - Mimics Lewis b-like blood group antigens
    - Plays a role in cell adherence (see below)
    - Can undergo phase variation to elude immune system (Salaun et al. 2004)
- **Gram stain:**
  - Gram negative
- **Growth:**
  - Microaerophilic; oxidase positive, catalase positive, urease positive (see below)
  - Normal reservoir: stomach and upper gastrointestinal tract
  - Biofilm formation: can form on abiotic surfaces and on gastric epithelial cells; under the regulation of the ArsR–ArsS TCS (Servetas et al. 2016; Stark et al. 1999)
  - ~23 species – classified as either gastric species or enterohepatic species:

D.I. Johnson, *Bacterial Pathogens and Their Virulence Factors*,
https://doi.org/10.1007/978-3-319-67651-7_18

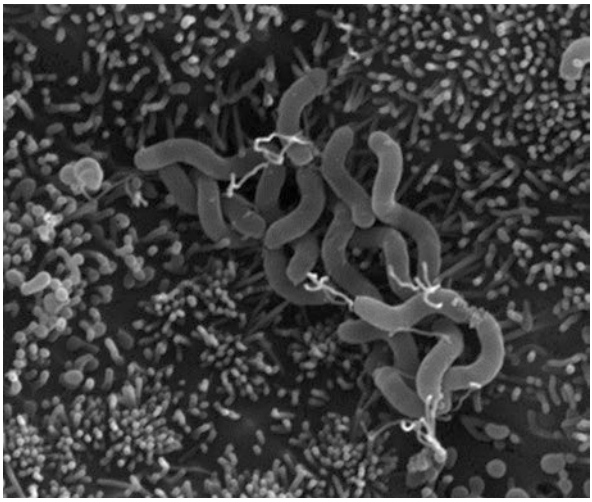

Fig. 18.1 *H. pylori* cells (From: scopeblog.stanford.edu; Photo by Shuman Tan and Lydia-Marie Joubert)

- *Helicobacter pylori* is the primary human pathogen
- Highly heterogeneous bacteria; probably due to coevolution with humans, its only ecological niche, and horizontal gene transfer events

Disease States Associated with *Helicobacter pylori* (Kao et al. 2016)

- Marshall and Warren: first to observe association between *H. pylori* and gastritis and peptic ulcers; Nobel Prize in 2005 (Marshall and Warren 1984)
- Transmission: usually acquired in childhood; person-to-person transmission or ingestion of fecal contaminated water or food
- Gastritis – stomach colonization with minor inflammation:
  - Abdominal pain, nausea, bloating, and belching
  - ~50% of world population is colonized; most widespread infection worldwide
  - ~80% of infected people are asymptomatic
- Peptic ulcers and duodenal ulcers:
  - Associated with high levels of inflammation caused by multiple virulence factors (see below)
  - *H. pylori* invade the gastric mucus layer using flagellar motility, attach to gastric epithelial cells, and induce inflammation that weakens the epithelial layer, leading in an efflux of acidic gastric juices into underlying tissues resulting in ulcers

- B-cell lymphoma of mucosal-associated lymphoid tissue (MALT lymphoma) and invasive gastric adenocarcinoma (third leading cause of cancer deaths worldwide):
  - *H. pylori* is the only recognized bacterial carcinogen
  - Incidence of gastric cancer varies geographically vs. ulcers and may be due to the presence of DupA virulence factor (Yamaoka 2010)
  - Increased levels of outer membrane proteins OipA, BabA, and SabA (see below) are more often seen with gastric cancer sera than with duodenal ulcer sera and may be a useful biomarker in the future (Su et al. 2016)
- Virulence varies geographically due, in part, to different forms and expression of the virulence factors CagA, VacA, and OipA; these virulence factors are co-expressed in virulent and carcinogenic strains (Yamaoka 2010)

Virulence Factors

- **Urease** (Mobley et al. 1995, 1988):
  - Essential for virulence
  - Dimeric Ni^{+2}-dependent urease
  - Encoded by *ureA and ureB* genes
  - *ureEFGH* genes encode urease activating proteins; *ureI* gene encodes an acid-dependent urea channel
  - Hydrolyzes urea found in abundance in the stomach into bicarbonate and ammonia:
    - $Urea + H^+ + 2H_2O \rightarrow HCO_3^- + 2(NH_4^+)$
  - Urea is also released from gastric epithelial cells via VacA-dependent pores
  - NH_4^+ molecules increase net pH in the stomach and disrupt tight junctions of gastric epithelial cells
  - Raising the pH also reduces the viscosity of mucus, which increases motility
- Host cell attachment:
  - Adhesins: attach to stomach epithelial cells (Kalali et al. 2014)
    - Hop family of outer membrane proteins (OMPs): **AlpA (HopC), AlpB (HopB), SabA (HopP), BabA (HopS), BabB (HopT), OipA (HopH), HopQ, and HopZ**
    - Hop expression can vary from patient to patient but is clonal within a patient
    - **OipA (HopH)** (Kwon and Graham 2000):
      - Necessary for virulence

- Induces the expression of pro-inflammatory cytokines, especially IL-8, similarly to CagA (see below)
- Regulation of expression: slipped-strand mispairing (CT dinucleotide repeats) in 5′-region of the *oipA* gene
  - OipA "on" – virulent; associated with more severe gastric diseases (duodenal ulcer and gastric cancer), neutrophil infiltration, and high IL-8 levels (Kudo et al. 2004)
  - OipA "off" – avirulent
- Secreted through *cag*PAI T4SS (see below)

- **BabA (HopS)–BabB (HopT)** (da Costa et al. 2015; Ilver et al. 1998):
  - Bind to fucose sugar residues on Lewis b antigens on the surface of gastric epithelial cells
- **SabA (HopP)** (Pang et al. 2014):
  - Binds to sialic acid sugar residues on Lewis b antigens on the surface of gastric epithelial cells.
  - X-ray crystal structure has been resolved
- **HopZ** (Peck et al. 1999):
  - Putative adhesin; *hopZ* knockout mutant has reduced binding to gastric epithelial cells
- **AlpA (HopC)–AlpB (HopB)** (Alm et al. 2000; Yonezawa et al. 2017):
  - Cell surface lipoproteins
  - Function in adherence and in biofilm formation
- **HP-NAP (*H. pylori* neutrophil-activating protein)** (Montecucco and de Bernard 2003):
  - Promotes attachment of neutrophils to endothelial cells and production of reactive oxygen species (ROS)
  - Induces inflammation that enhances nutrient availability for *H. pylori* growth
  - Encoded by *napA* gene
- **HpPrtC** (Zhao et al. 2017):
  - Collagenase
  - May function in ECM breakdown
- **HpHtrA** (Hoy et al. 2010):
  - Serine protease
  - Cleaves host E-cadherin at adherens junctions
  - May enhance paracellular dissemination beyond the gastric epithelium

  - **Flagella** (Huang et al. 2017):
    - Motility enables *H. pylori* penetration of the gastric mucus layer
    - essential for initial gastric colonization and subsequent virulence (Ottemann 2002)
    - Flagella propel *H. pylori* from stomach lumen (low pH) through the mucus layer and close to gastric epithelial cells (physiological pH)
    - Uses pH-sensitive sensors TlpA, TlpB, and TlpD for chemotaxis

- Iron and nickel acquisition – essential nutrients:
  - *H. pylori* is very sensitive to host levels of Fe and other metal cofactors such as Ni, Zn, and Cu (Haley and Gaddy 2015)
  - Does not use Fe^{+3} siderophores
  - Uses multiple outer membrane protein systems to directly import Fe from Fe-bound proteins such as hemoglobin, transferrin, heme, and lactoferrin (Haley and Gaddy 2015)
  - Under transcriptional regulation by Fe^{+2}-dependent Fur repressor (Gilbreath et al. 2013)
  - Nickel acquisition (Fischer et al. 2016; Cherrier et al. 2008):
    - Needed for essential urease activity
    - **NixA, NiuBDE, FrpB4, FrpB2, and FecA3**:
      - Ni^{+2} transporters
      - Under Ni^{+2}-dependent NikR transcriptional regulation; can act as a repressor or activator of transcription (Dosanjh and Michel 2006)

- Host cell damage:
  - *H. pylori* has the ability to induce host inflammatory processes in order to gain access to nutrients but also has the ability to block inflammation for survival in the host (Varga and Peek 2017)
  - **TlyA** (Javadi and Katzenmeier 2016):
    - Putative hemolysin; unknown mechanism
    - Putative RNA methyltransferase; unknown mechanism
  - **VacA** (Cover and Blanke 2005; Junaid et al. 2016):
    - Pore-forming exotoxin
    - Essential for colonization of the stomach
    - Induces multiple effects inside host cells (Fig. 18.2):
      - Adhesin: surface-bound VacA binds to host membrane sphingolipid, sphingomyelin (SM), within lipid rafts; likely to play a role in VacA internalization (Gupta et al. 2008)
      - Vacuolation: internalized VacA creates large vacuoles inside host cells (Leunk et al. 1988)

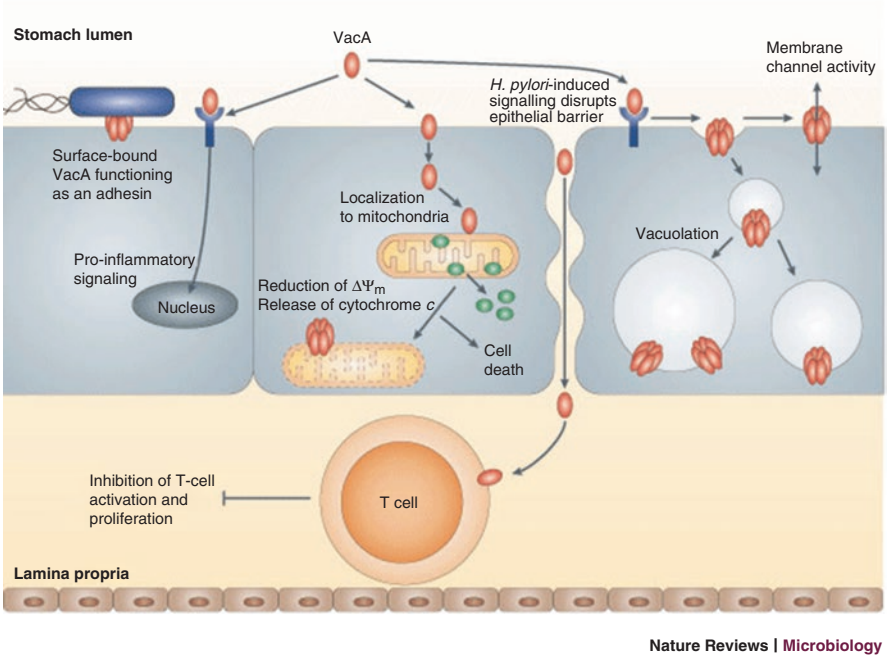

Fig. 18.2 VacA cellular effects (From: Cover and Blanke 2005)

- Membrane channels: secreted VacA forms anion-selective oligomeric channels in host cell endosomes and in the plasma membrane (Szabò et al. 1999)
- Alters mitochondrial membrane permeability: forms pores in mitochondria causing release of cytochrome *c*; leads to host cell apoptosis and programmed cell necrosis (de Bernard and Josenhans 2014; Galmiche et al. 2000)
- Activates several host cell-signaling pathways: leads to induction of pro-inflammatory responses (Nakayama et al. 2004)
- Disrupts gastric epithelial tight junction permeability: enhances paracellular dissemination of *H. pylori* outside the gastrointestinal epithelium (Papini et al. 1998)

– ***cag*-PAI pathogenicity island** (da Costa et al. 2015):

  - ~40 kB of chromosomal DNA
  - Encode 31 ORFs; most of which are components of the type 4 secretion system (T4SS):

    - Translocates bacterial effector proteins into host cells
    - **CagE** (Kutter et al. 2008):

      – ATPase; provides energy for effector translocation

    - **CagT** (Fischer et al. 2001):

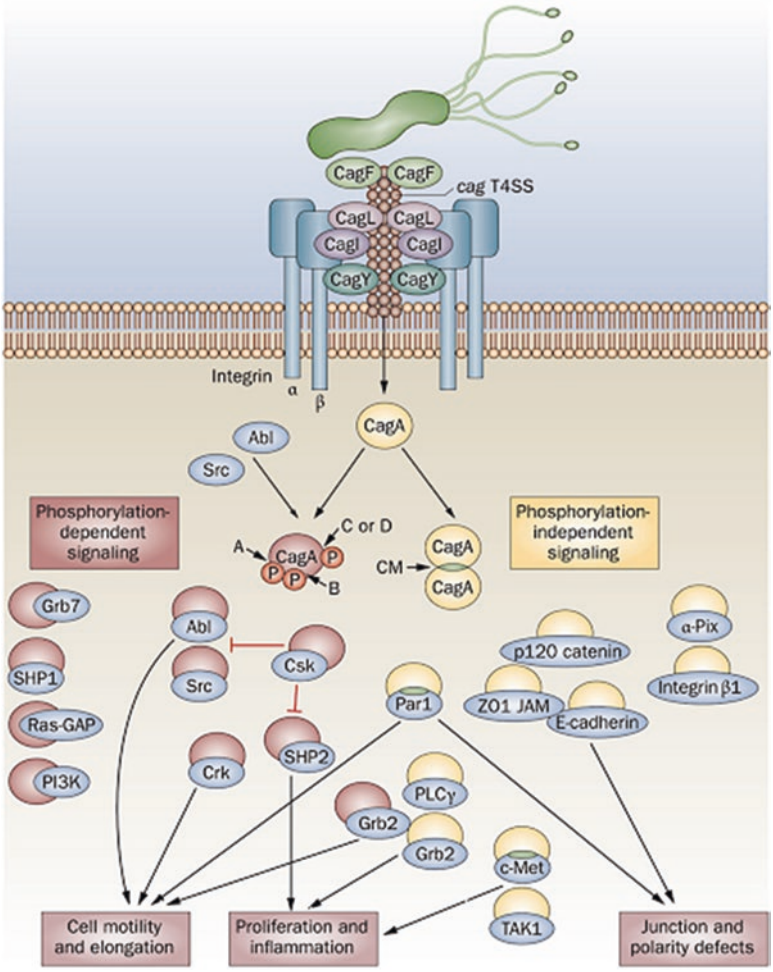

Fig. 18.3 CagA cellular effects (From: Yamaoka 2010)

  - Induces pro-inflammatory cytokine IL-8
- **CagM** (Fischer et al. 2001; Glocker et al. 1998):
  - Induces NF-kB-dependent signaling and IL-8 secretion
- **CagL** (Barden et al. 2013):
  - Localized at tip of T4SS
  - Binds to α5β1 integrin; induces IL-8 dependent inflammation

– **CagA** (Basso et al. 2010; da Costa et al. 2015; Backert and Blaser 2016) (Fig. 18.3):

- Bacteria-derived carcinogen; CagA-positive strains have a higher incidence of gastric carcinomas than CagA-negative strains
- Binds to ~20 host proteins in phosphorylation-dependent and phosphorylation-independent mechanisms (Fig. 18.3) (Bergé and Terradot 2017)
- Can disrupt tight junction permeability, enhancing paracellular dissemination of *H. pylori* outside the gastrointestinal epithelium
- Affects signaling pathways that lead to pro-inflammatory responses and host cell proliferation (oncogenesis)
- Three functional domains:
  - α5β1 integrin binding
  - Phosphatidylserine (PS) binding domain: attachment to host cell membrane
  - Phosphorylation domains: contain differing numbers of EPIYA amino acid repeats
    - Tyrosine (Y) in EPIYA is site of host Src and Abl tyrosine kinase-dependent phosphorylation

– **DupA** (Duodenal ulcer promoting gene A) (Abadi and Perez-Perez 2016; Hussein et al. 2010):

- Associated with ulcer formation and suppression of gastric cancer
- Induces secretion of pro-inflammatory cytokines (Lu et al. 2005)

• Evasion of host immune system (Mejías-Luque and Gerhard 2017):

– Phase variation:

- Alters virulence proteins, allowing cells to escape immune detection
- Can occur via gene switching through slipped-strand mispairing during chromosomal replication (Salaun et al. 2004)
- Seen with LPS sugars, outer membrane adhesins, and flagella

– **LPS**:

- Lipid A is under-acylated and under-phosphorylated vs. typical LPS
- One thousand-fold less active than typical endotoxin
- Lipid A is not recognized by TLR-4, so inflammatory processes are not induced
- Contains fucose and sialic acid Lewis blood group antigen-like sugars; molecular mimicry and phase variation of genes needed for the synthesis of these sugars (Bergman et al. 2006; Moran and Prendergast 2001)

– **Flagellin**:

- Modified N-terminal domain; not recognized by TLR-5
- Can undergo phase variation

- **Fucose-containing OMPs** (Bergman et al. 2006):
  - Activate anti-inflammatory cytokines
  - Can undergo phase variation
- **VacA**:
  - Inhibits T-cell proliferation by blocking G1/S cell cycle transition
  - Leads to localized immunosuppression (Gebert et al. 2003)
- **gGT** (Gong et al. 2010):
  - γ-glutamyltranspeptidase
  - Metabolizes extracellular glutathione leading to H_2O_2 production and inflammation
  - Induces apoptosis
  - Inhibits T-cell proliferation

Regulation of Virulence Factor Expression

- Multiple transcriptional regulatory circuits that respond to changes in host environment and metal ions (Danielli et al. 2010):
  - Fe^{+2}-dependent Fur regulation of iron-responsive genes (see above)
  - Ni^{+2}-dependent NikR regulation of nickel-response genes (see above)
  - Two-component phosphorelay systems (TCSs)
    - **ArsR–ArsS** (Goodwin et al. 2008; Servetas et al. 2016):
      - Acid response, SabA regulation, and biofilm formation
    - **CheA–CheY and FlgR–FlgS** (Niehus et al. 2004):
      - Flagellar motility
    - **CrdR–CrdS** (Waidner et al. 2005):
      - Copper resistance

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Chapter 19
Klebsiella spp.

Genomics, Morphologies, and Growth Characteristics

- **Genomics:**
  - *Klebsiella pneumoniae* strain MGH78578 chromosome: 5,315,120 bp; 4776 predicted ORFs (Lery et al. 2014)
- **Cell morphology:**
  - Rod-shaped cells (Fig. 18.1)
  - Nonmotile
  - Capsule: major virulence factor (Fig. 18.1; see below)
  - Lipopolysaccharide (LPS): immunostimulatory; associated with inflammation and endotoxic shock; also functions in immune evasion (see below)
- **Gram stain:**
  - Gram negative
- **Growth:**
  - Facultative anaerobes; oxidase negative
  - Urease positive; used to obtain nitrogen from host urea
  - Reservoirs: contaminated soil and surface water; human hosts (primary)
  - Can colonize human mucosal layers, including the gastrointestinal tract and the nasopharynx
  - Excellent biofilm formers (see below)
  - Six species and several subspecies: *K. pneumoniae* is the primary human pathogen; *K. oxytoca* is next most prevalent:
    - *K. pneumoniae* strains can be classified as either classical or hypervirulent (HV)

D.I. Johnson, *Bacterial Pathogens and Their Virulence Factors*,
https://doi.org/10.1007/978-3-319-67651-7_19

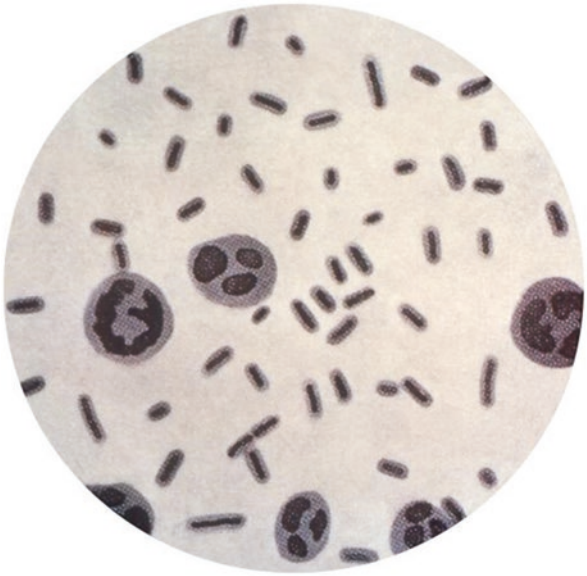

Fig. 18.1 *K. pneumoniae* cells (From: PHIL #14342)

- *K. pneumoniae* strains are serotyped by LPS (O) antigens and capsular (K) antigens:
  - 9 O antigen serotypes: O1 (most common)
  - 78 K antigen serotypes: K1–K78
- Member of the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus, Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa, Enterobacter* spp.) family of antibiotic-resistant nosocomial pathogens
- Significant increases worldwide in antibiotic-resistant strains and HV strains:
  - Antibiotic resistance – two major mechanisms:
    - Expression of carbapenemases; display resistance to all β-lactam antibiotics
    - Expression of extended spectrum β-lactamases (ESBLs); display resistance to cephalosporins and monobactams
    - Isolates can be found worldwide
    - Antibiotic resistance does not increase the virulence of these strains
  - HV strains (Paczosa and Mecsas 2016; Patel et al. 2014; Shon et al. 2013):
    - Predominantly O1:K1 (93%) and O1:K2 strains
    - Exhibit a hypermucoviscous phenotype on agar plates; due to the presence of a hypercapsule with increased production of capsule polysaccharide (CPS)

  - Also increased production and utilization of iron siderophores enterobactin, yersiniabactin, salmochelin, aerobactin
  - Isolates are found predominantly in Southeast Asia and Taiwan, although incidents are spreading worldwide

Disease States Associated with *Klebsiella pneumoniae*

- Inflammatory symptoms – correlated with either a classical or HV *K. pneumoniae* strain (Paczosa and Mecsas 2016):
  - Classical disease states:
    - Opportunistic infections of immunocompromised hosts; primarily nosocomial
    - pneumonia, urinary tract infections (UTIs), bacteremia, neonatal sepsis:
      - Pneumonia:
        - Community-acquired and hospital-acquired (HA); HA pneumonia isolates are much more likely to be antibiotic resistant
        - Cough, fever, leukocytosis, chest pain
        - "Currant jelly sputum": thick blood-tinged mucous
        - Transmission: primarily the aspiration of host's own nasopharyngeal microbiota; also associated with biofilms on contaminated medical devices (ventilators)
      - UTIs:
        - Ascending UTIs
        - Transmission: seeding from host's own GI tract or from contaminated urinary catheters [catheter-associated (CA) UTIs]
        - Painful urination (dysuria), increased frequency of voiding, and blood in urine (hematuria)
      - Bacteremia: either a primary infection within the bloodstream or a secondary infection due to dissemination from lungs or bladder
  - HV disease states:
    - Can occur in immunocompetent hosts
    - Primarily CA infections and systemic infections
    - Pyogenic liver abscesses and other organ abscesses, pneumonia, bacteremia, meningitis, soft tissue diseases, endophthalmitis

Virulence Factors (Paczosa and Mecsas 2016)

- Adherence to host cells:
  - Fimbrial adhesins:
    - **Type I fimbriae** (Struve et al. 2008):
      - Chaperone–usher assembly mechanism
      - Expressed in ~90% of isolates, including HV O1:K1 strains
      - **FimA**: major structural component
      - **FimH** (tip adhesin):
        - Binds to D-mannosylated glycoproteins (mannose-sensitive binding)
        - Can bind to mast cells; induces inflammation, chemokine production, and neutrophil infiltration
      - Expressed in the urinary tract but not in the lungs or GI tract; plays an important role in UTIs and biofilm formation in the bladder
      - Regulated by phase variation involving an invertible DNA promoter element upstream of the *fim* operon:
        - On orientation – transcription occurs
        - Off orientation – transcription is blocked
        - Orientation of promoter element is regulated by site-specific recombinases
    - **Type III fimbriae** (Murphy and Clegg 2012):
      - Chaperone–usher assembly mechanism
      - Expressed in almost all isolates, including HV O1:K1 strains
      - **MrkA**: major structural component; also adheres to abiotic surfaces in biofilms
      - **MrkD** (tip adhesin):
        - Binds to ECM types IV and V collagens
        - Does not bind mannose (mannose-resistant binding)
      - Not necessary for UTIs or in lung or GI tract infections
      - Primary fimbriae associated with biofilm formation on tissues and abiotic medical devices (VAP; ventilator-associated pneumonia)
      - Regulated by intracellular levels of cyclic di-GMP through the MrkHI proteins
    - **Kpc fimbriae** (Wu et al. 2010):
      - Expressed in HV O1:K1 strains
      - **KpcA**: major structural component
      - Important role in biofilm formation

- Regulated by phase variation similar to type I fimbriae

- **KPF-28 fimbriae**: (Di Martino et al. 1996)
  - Expressed in strains producing the CAZ-5/SHV-4 extended-spectrum β-lactamase
  - Encoded on the same R virulence plasmid that encodes the β-lactamase gene
  - May be involved in binding intestinal epithelial cells

– Non-fimbrial adhesins:

- **CF29K** (Darfeuille-Michaud et al. 1992):
  - Encoded on a R virulence plasmid that encodes the CAZ-1/TEM-5 extended-spectrum β-lactamase and the aerobactin siderophore associated with HV strains
  - Involved in binding microvilli of intestinal epithelial cells

- Growth in host milieu:

– Iron acquisition (Hsieh et al. 2008):

- Twelve iron uptake systems are encoded in the genome
- Fe^{+3} siderophores:
  - **Enterochelin (enterobactin)** (Tarkkanen et al. 1992):
    – Primary siderophore
    – Cyclic polyester containing three DHBS (*N*-(2,3-dihydroxybenzoyl)-L-serine) residues
    – Expressed in UTIs and lung infections
    – Neutralized by host lipocalin-2 (Bachman et al. 2012)
    – Fep–Ent system expressed in classical and HV strains
  - **Salmochelin** (Muller et al. 2009):
    – Enterochelin derivative; contains DHBS linked by a glucose residue; increases the hydrophilicity of enterochelin; protects against lipocalin-2 neutralization
    – Important for nasopharynx colonization
    – IroA system; rare in classical strains; predominantly expressed in HV clinical isolates (>90%)
  - **Aerobactin** (Nassif and Sansonetti 1986):
    – Citrate–hydroxamate structure
    – Major siderophore in lung infections
    – Iuc system; rare in classical strains; predominantly produced in HV clinical isolates (93–100%):
      - Always associated with hypercapsule formation

        - Encoded on the same large (180–220 kb) R virulence plasmid with the capsule-enhancing transcription factor RmpA (see below)

    - **Yersiniabactin** (Lawlor et al. 2007):

        - First identified in *Yersinia* spp.
        - Synthesized by a mixed nonribosomal peptide synthetase (NRPS)/polyketide synthase (PKS) mechanism
        - Expressed in lung infections
        - Blocked by host transferrin
        - Ybt system; rare in classical strains; predominantly produced in HV clinical isolates (~ 90%)

    - Fe^{+2} uptake: FeoABC system (Hsueh et al. 2013)
    - Hemoprotein uptake: HmuRSTUV hemophore system
    - ABC transporters:

        - Sit (Fe^{+2}), Kfu (Fe^{+3}), Fec (ferric citrate)
        - Kfu expression is associated with disseminated invasive infections in the liver and brain (Ma et al. 2005)

    - Hemoprotein uptake (HmuRSTUV hemophore system):

        - Expression is regulated by Fe^{+2}-bound Fur repressor and RhyB sRNA molecules

- **Biofilm formation** (Vuotto et al. 2014):

    - Able to form biofilms on biotic and abiotic surfaces:

        - Most often associated with urinary catheters, intravascular catheters, and ventilator tubing in hospital environments

    - Type I and type III fimbriae (major determinant), capsule, and LPS play important roles in biofilm formation
    - Strong correlation between biofilm formation and antibiotic resistance

- Evasion of host immune system (Fig. 18.2) (Li et al. 2014; Paczosa and Mecsas 2016):

    - **Capsule** (Cortés et al. 2002):

        - Major virulence factor of *K. pneumoniae*
        - Composed of strain-specific capsule polysaccharides (CPS) K antigens
        - Biosynthetic genes are encoded on the chromosomal *cps* operon (Shu et al. 2009)
        - Essential for immune evasion:

            - Blocks opsonophagocytosis and maturation of dendritic cells (DC)
            - Protects against neutrophil killing; allows dissemination of *K. pneumoniae* and systemic infections

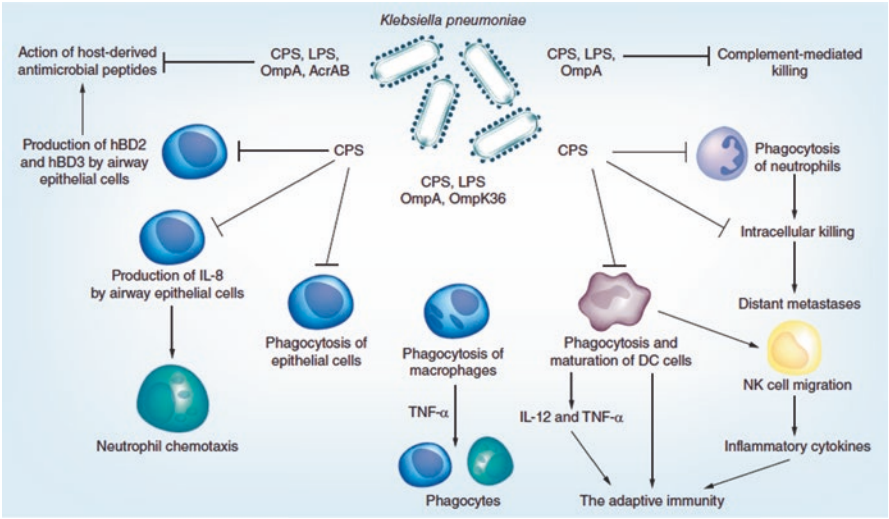

Fig. 18.2 Immune system evasion (From: Li et al. 2014)

- Blocks the action of antimicrobial peptides, such as β-defensins
- Blocks C3-dependent complement-mediated lysis
- Blocks NF-κB-dependent inflammatory responses, including reactive oxygen species (ROS), pro-inflammatory cytokines, and LPS recognition by TLR4

○ HV K1 and K2 serotypes express a hypercapsule; due to increased production of CPS:

- Increased expression of CPS is under transcriptional control
- **RmpA/RmpA2** (Hsu et al. 2011; Lai et al. 2003):
  - Transcriptional activators
  - RmpA is encoded on the chromosome and on a large (180–220 kb) R virulence plasmid (primary regulator)
  - RmpA2 is encoded on a large (180–220 kb) virulence plasmid
- **RcsA/RcsB** (Stout et al. 1991):
  - Low level expression; unstable due to proteolysis by Lon protease

– **LPS** (Llobet et al. 2015):

○ Can modify the lipid A moiety by addition of a 2-hydroxyacyl group; modified lipid A, which is found in isolates from lung tissue, abrogates the innate immunity inflammatory response; modification is regulated by the PhoP–PhoQ TCS

○ Lipid A modification also provides resistance to small cationic antimicrobial peptides (APs), such as β-defensins, colistin (polymyxin A), and

polymyxin B; colistin is one of the last effective treatments for multidrug-resistant *K. pneumoniae* infections
    - LPS also protects against complement-mediated lysis; segregates complement C3b protein away from the cell membrane, blocking MAC formation; associated with the O antigen-dependent smooth colony phenotype

– **OmpA** (Llobet et al. 2009; March et al. 2011):
    - Outer membrane protein
    - Inhibits pro-inflammatory cytokine production and provides resistance to β-defensins; only seen in the context of the whole cell (i.e., mutant analysis), not with purified protein

– **OmpK35; OmpK36** (Chen et al. 2010; Shin et al. 2012):
    - Outer membrane porins
    - Deletion mutants have reduced virulence; increased uptake into neutrophils
    - May function in blocking phagocytosis; also plays a role in antibiotic resistance

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Chapter 20
Legionella spp.

Genomics, Morphologies, and Growth Characteristics

- **Genomics:**
  - *Legionella pneumophila* chromosome: 3,397,754 bp; 2,943 predicted ORFs (Chien et al. 2004)
- **Cell morphology:**
  - Thin, rod-shaped cells; appear as coccobacilli in tissue (Fig. 20.1)
  - Atypical LPS:
    - Fifteen different O-chains
    - Lipid A: more hydrophobic and less endotoxic than typical LPS
  - Pili: long type IV; PilE subunits
  - Flagellum: monopolar; associated with increased virulence
- **Gram stain:**
  - Gram negative
- **Growth:**
  - Aerobes: catalase positive, oxidase positive
  - Prefers high temperatures: 32–45 °C
  - Requires cysteine, iron, low sodium (Na):
    - Lacks cysteine biosynthetic genes
    - Sodium (Na) sensitivity:
      - Na-sensitive cells – virulent
      - Na-resistant cells – avirulent

D.I. Johnson, *Bacterial Pathogens and Their Virulence Factors*,
https://doi.org/10.1007/978-3-319-67651-7_20

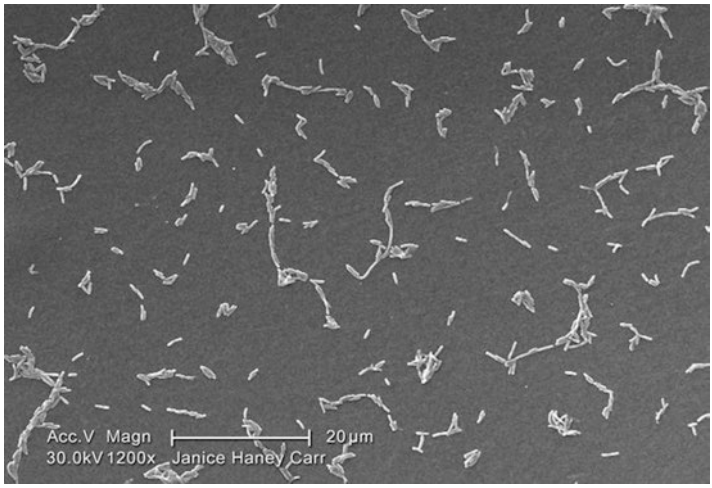

Fig. 20.1 *L. pneumophila* cells (From: PHIL #11101)

- Two growth phases:
  - Replicative (intracellular) phase: nonmotile, long filamentous rods
  - Active infective (transmissive) phase: motile, short rods; monopolar flagellum
- Normal reservoir: primarily aquatic environments; protozoans (amoeba) living in lakes and ponds:
  - Endosymbiotic relationship with freshwater amoeba (e.g., *Acanthamoeba castellanii*)
  - Same genes needed to grow in amoeba and human macrophage
  - Has access to DNA from a variety of bacterial, viral, and protozoan sources – "global mobilome" (Gomez-Valero and Buchrieser 2013)
  - Has the most eukaryotic-like genes of any prokaryote; acquired through horizon gene transfer (HGT)
- Sources of contaminated H_2O: biofilm formation on piping and other abiotic surfaces:
  - Premise plumbing: tap H_2O in schools, hospitals, and public and private housing
  - Cooling towers (40–60% tested)
  - Air-conditioning systems
  - Humidifiers
  - Hot tubs, spas, showers
  - Misting equipment for vegetables
- Excellent biofilm former: abiotic and biotic surfaces (lung epithelial cells)
- Fifty eight species with three subspecies and ~60 serotypes

Disease States Associated with *Legionella pneumophila*

- Legionnaire's disease (legionellosis) (Cunha et al. 2016):
  - Most diseases are caused by *L. pneumophila* strains Lp1 and Lp6
  - Opportunistic pathogen: "accidental pathogen" for humans
  - Lower respiratory tract infection: atypical pneumonia with flu-like symptoms, fever, chills, dry cough; also gastrointestinal and neurological symptoms
  - Predisposed in patients with underlying chronic obstructive pulmonary disease (COPD)
  - Facultative intracellular pathogen; primarily grows inside alveolar macrophage
  - Airborne transmission: aerosols from environmental sources
- Pontiac fever:
  - Non-pneumonia with flu-like symptoms
  - Usually self-limiting

Virulence Factors

- Adherence to host cells:
  - **Biofilm** formation facilitates binding to lung epithelial cells
  - **Atypical LPS** (see above): plays a role in adherence
  - Fimbrial adhesins:
    - **Type IV pili** (Stone and Abu Kwaik 1998):
      - Function in adherence and in biofilm formation
  - Afimbrial adhesins:
    - **Lcl** (Vandersmissen et al. 2010):
      - Collagen-like protein
      - Functions in adherence
    - **Mip (Macrophage infectivity potentiator)** (Kohler et al. 2003):
      - Binds ECM collagen
  - **PlaB** (Schunder et al. 2010):
    - Phospholipase B; outer membrane protein; important in infection
  - **Flagellum** (Heunera and Steinert 2003):
    - Functions in adhesion, host invasion, cell motility, and biofilm formation

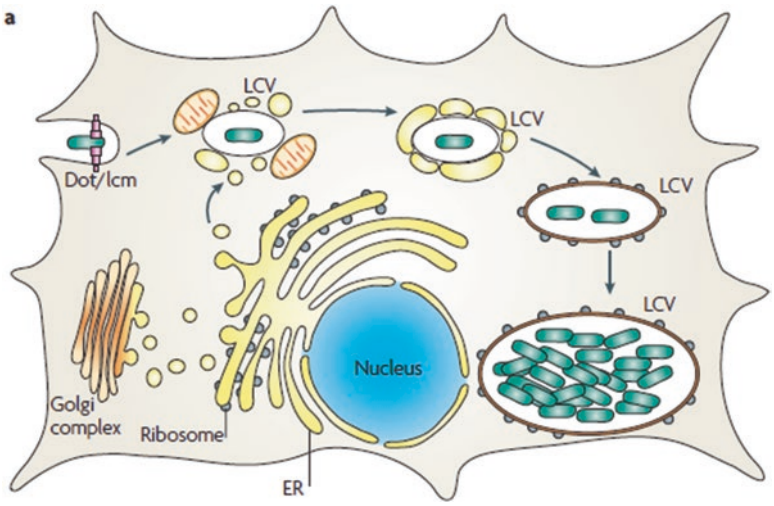

Fig. 20.2 *L. pneumophila* intracellular replication (From: Isberg et al. 2009)

- Host cell invasion and growth (Fig. 20.2) (Isberg et al. 2009):
  - Facultative intracellular pathogen; primarily alveolar macrophage
  - Coiling phagocytosis:
    - Macrophage pseudopodia engulf *L. pneumophila* in coiled vesicle
    - Recruits host phospholipids from endoplasmic reticulum (ER); becomes *Legionella*-containing vacuole (LCV)
    - LCV does not acidify; phagosome–lysosome fusion is inhibited
    - Outer membrane vesicles (OMVs): also inhibit phagosome–lysosome fusion (Shevchuk et al. 2011)
  - Intracellular replicative phase (Isaac and Isberg 2014):
    - Grows within LCV; lag time of ~6 h
    - Surface of LCV is covered with ER–ribosomes
    - Intracellular growth depends on bacterial effectors secreted by the Icm/Dot type 4 secretion system (T4SS) and the Lsp type 2 secretion system (T2SS)
  - **Icm/Dot T4SS** (intracellular multiplication/defective organelle trafficking):
    - **Icm/Dot translocated substrates (IDTS)** (Ge and Shao 2011; Isaac and Isberg 2014; Jo et al. 2013):
      - ~330 bacterial proteins injected into host cytoplasm
      - ~75 of these effectors are eukaryotic-like proteins
      - Mimic host posttranslational modifications: methylation, phosphorylation, prenylation, ubiquitination, glycosylation, etc.

- Affect multiple host processes: vesicle trafficking, autophagy, host protein synthesis, host inflammatory response, macrophage apoptosis, host cell egress:
  - Host vesicle trafficking:
    - Induces creation of LCV and fusion events between host ER and the LCV
    - Numerous effectors regulate host Rab and Arf GTPases localization to the LCV and act as GEFs, GAPs, and GDIs regulatory proteins for GTP/GDP cycling
  - Host cell autophagy: mammalian process used to capture and recycle cytosolic components; creates autophagosome that fuses with lysosome:
    - Triggered by nutrient starvation, hypoxia, stresses, and intracellular pathogens (xenophagy) such as *L. pneumophila*
    - **RavZ** (Choy et al. 2012; Horenkamp et al. 2015):
      - Blocks autophagosome formation; allows cytosolic growth
  - Host protein synthesis:
    - **Lgt1, Lgt2, Lgt3** (Belyi et al. 2008):
      - Inactivate host elongation factor EF-1A, thereby blocking host protein translation
      - Glucosyltransferases: add glucose residues to the EF-1A GTP-binding protein; inhibits its activity
    - **SidI, SidL**:
      - Inhibit EF-1A by unknown mechanisms
  - Block macrophage apoptosis and inflammatory response:
    - Induces activation of antiapoptotic genes downstream of NF-κB signaling pathway (Abu-Zant et al. 2007)
    - **SidF**:
      - Inactivates "pro-death" host genes BNIP3 and Bcl-rambo
    - **SdhA** (Creasey and Isberg 2012):
      - Maintains the membrane integrity of LCV; prevents *L. pneumophila* degradation products from leaving the LCV and inducing inflammatory cell death
  - Egress of *L. pneumophila* from host cells:
    - **LepA, LepB**:
      - GTPase-activating proteins (GAPs) for Rab1 GTPase
      - Inactivates Rab1

    - **LegG1**:
        - Guanine nucleotide exchange factor (GEF) for Ran GTPase
        - Activates Ran
  - **Kat1, Kat2** (Bandyopadhyay et al. 2003):
    - Catalase–peroxidases
    - Essential for intracellular growth
  - **RtxA** (Cirillo et al. 2001):
    - Cytolytic RTX exotoxin
    - Plays a role in adherence
- Iron acquisition (essential nutrient):
  - **FeoB** (Robey and Cianciotto 2002):
    - GTP-dependent Fe^{+2} transporter
  - **Legiobactin** (Cianciotto 2007):
    - Fe^{+3} siderophore
    - **LbtA, LbtB**: function in the synthesis and secretion of legiobactin
  - **Ccm** (cytochrome *c* maturation) system (Cianciotto et al. 2005):
    - Function in heme uptake
  - Regulated by the classic Fur repressor regulon

Regulation of Virulence Factor Expression

- Regulation of the synthesis of 100 s of Icm/Dot effectors (Fig. 20.3):
  - Three TCSs (Feldheim et al. 2016; Lucchetti-Miganeh et al. 2008):
    - **LetS–LetA**:
      - Indirect regulators of Icm/Dot effector expression
      - Mediated by **RsmY/RsmZ** and **CsrA** regulator (see below)
      - Activated in stationary phase by low amino acid levels and the (p)ppGpp alarmone
    - **PmrA–PmrB**:
      - Direct regulators of Icm/Dot effector expression
      - Unknown environmental signals

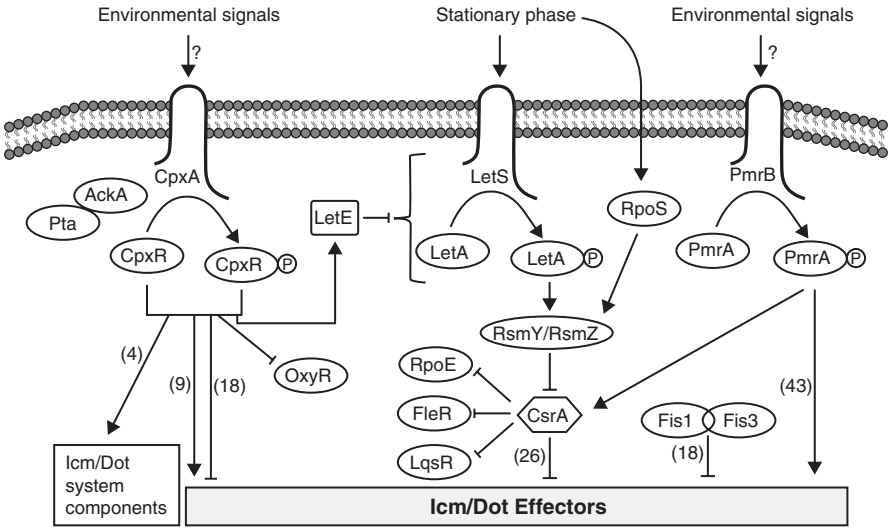

Fig. 20.3 *L. pneumophila* TCS (From Feldheim et al. 2016)

- **CpxA–CpxR**:
  - Direct regulators of Icm/Dot effector expression
  - Unknown environmental activators

- Regulation of bacterial protein translation:
  - **CsrA** (Molofsky and Swanson 2003):
    - One of three Csr RNA-binding proteins; found in many Gram-negative pathogens
    - Binds to mRNA sequences near the Shine–Dalgarno ribosome-binding site (RBS); inhibits ribosome binding, blocking translation
    - Inhibits LetE-dependent transmission phase genes
    - Activates intracellular growth (replicative) phase genes, primarily in the Icm/Dot system
    - When intracellular nutrients become limiting, increases in (p)ppGpp alarmone concentrations trigger differentiation to transmissive motile phase (see above) to aid in dissemination; mediated by the LetS–LetA TCS

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Chapter 21
Leptospira spp.

Genomics, Morphologies, and Growth Characteristics

- **Genomics** (Huang et al. 2014; Ren et al. 2003; Zuerner 1991):
  - *Leptospira interrogans* serovar Lai str. 56,601 chromosome CI: 4,332,241 bp; 4,360 predicted ORFs
  - *Leptospira interrogans* chromosome CII: 358,943 bp; 367 predicted ORFs
- **Cell morphology:**
  - Thin (0.1 μm), long (6–20 μm) spirochete:
    - Hooked ends – "question marks" (*interrogans*) (Fig. 21.1)
  - Two endoflagella (axial filaments):
    - Anchored at both ends of the cell and run lengthwise between the inner and outer membranes in the periplasmic space; enclosed in outer sheath
    - Causes twisting motion used for motility; important for dissemination in host
    - Endoflagella subunits (Lambert et al. 2012):
      - **FlaA1, FlaA2**: outer sheath
      - **FlaB1, FlaB2, FlaB3, FlaB4**: core polymer
- **Gram stain:**
  - Gram negative
- **Growth:**
  - Anaerobic or microaerophilic growth.
  - Slow grower (30–50 h doubling time); metabolically crippled:
    - No Krebs cycle or electron transport chain; gets ATP from glycolysis

D.I. Johnson, *Bacterial Pathogens and Their Virulence Factors*,
https://doi.org/10.1007/978-3-319-67651-7_21

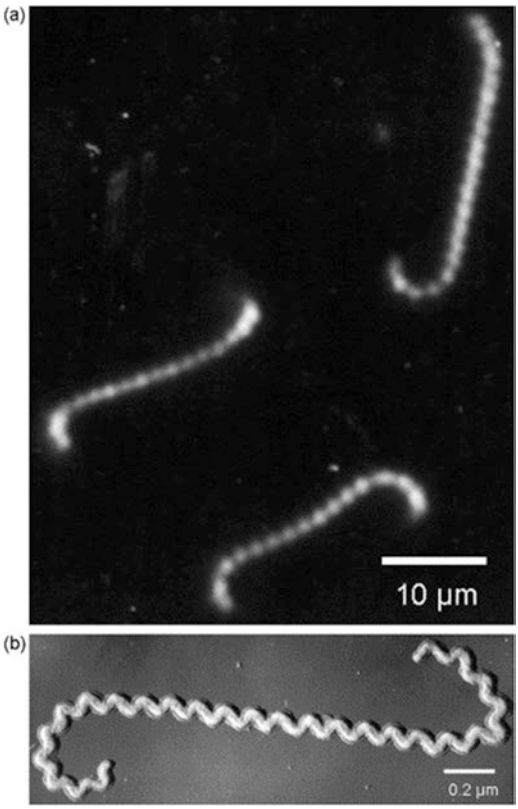

Fig. 21.1 *L. interrogans* cells (From: Adler and de la Pena Moctezuma 2010)

- No genes for synthesis of nucleotides, fatty acids, vitamins, cofactors, or amino acids; gets most of its nutrients through transport, not biosynthesis

– Reservoir: almost all-known mammals; humans are not reservoirs.
– At least 19 species: 13 are pathogenic and 6 are saprophytic; > 250 serovars:

- Leptospirosis: *L. interrogans* (predominant), *L. borgpetersenii*, *L. santarosai*, *L. noguchii*, *L. weilli*, *L. kirschneri*, *L. alexanderi*

Disease States Associated with *Leptospira interrogans* (Adler 2014; Picardeau 2017)

- Leptospirosis:
  – Most widespread zoonotic disease
  – Various clinical manifestations from mild flu-like symptoms to life-threatening kidney and liver failure

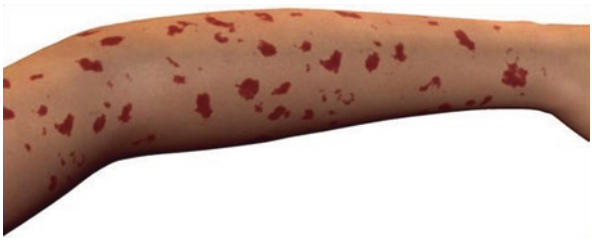

Fig. 21.2 *L. interrogans* rash (From: leptospirosis.com)

- Systemic disease: humans, dogs, cattle, swine
- Transmission: not person-to-person
  - Animals are renal (kidney) carriers; renal tubules are immunoprivileged sites
  - Transmitted through contact with urine from infected wild or domestic animals
  - Transmitted through contact with water (or floodwaters), soil, or food contaminated with the urine of infected animals:
    - Water sports enthusiasts are at significant risk due to spending extended time in contaminated water
  - Very rapid spread in bloodstream and tissues after infection
- Disease has two stages:
  - Stage 1 – anicteric leptospirosis; usually 7–12 days of incubation:
    - Rash (Fig. 21.2), fever, headache, muscle aches, vomiting, or diarrhea; patient may recover for a time but may become ill again
  - Stage 2 – icteric (jaundice) leptospirosis, Weil's disease:
    - More severe: affects multiple organ systems
    - Kidney or liver failure (jaundice), pulmonary damage, meningitis
    - Severe cases: pulmonary hemorrhages, platelet deficiency

Virulence Factors

- Adherence to host cells (Adler 2014):
  - Extracellular pathogen, so adherence to host cells is important for infection
  - Binds to renal tubular epithelial cells and ECM components
  - At least 30 putative adhesins; bind to multiple ECM components in vitro
  - However, none have been shown to be essential for virulence in vivo yet; probably due to extensive genetic redundancy

- Dissemination and growth within the host:
  - Capable of rapid dissemination and invasion of body tissues
  - **Endoflagella** and corkscrew motility: essential for dissemination and invasion
  - **KatE** (Eshghi et al. 2012):
    - Catalase
    - Protects against ROS inside macrophage
  - Iron acquisition:
    - **LipL41** (Lin et al. 2013):
      - Hemin-binding protein
    - **HbpA** (Lo et al. 2010; Sridhar et al. 2008):
      - Hemin-binding protein
      - Upregulated under low iron conditions by Fe^{+2}-regulated Fur repressor
    - **FecA** (Louvel et al. 2005):
      - Ferric citrate uptake
    - **FeoB** (Louvel et al. 2005):
      - Fe^{+2} uptake
- Biofilms (Ristow et al. 2008):
  - Capable of forming biofilms on the surface of water and abiotic and biotic substrates
  - May play a role in environmental persistence in aquatic environments and possibly in chronic persistence in human hosts
  - A number of outer membrane proteins are transcriptionally induced during biofilm formation (Iraola et al. 2016)
- Outer membrane stability:
  - **Loa22** (Ristow et al. 2007):
    - One of seven OmpA-like outer membrane proteins; second most abundant protein in *L. interrogans*
    - Probably functions in anchoring the outer membrane to the peptidoglycan layer
    - Expressed during infection and required for virulence in the hamster and guinea pig models of leptospirosis
- Host cell damage:
  - No known exotoxins
  - **LPS/endotoxin** (Haake and Zuckert 2015):

    - Low toxicity
    - Structurally different lipid A subunit; not recognized by host TLR-4
  - Outer membrane lipoproteins induce inflammatory responses:
    - **LipL32** (Hauk et al. 2008; Murray 2013):
      - Most abundant protein in *L. interrogans*; only found in pathogenic serovars
      - Binds to ECM laminin, collagen, and fibronectin
      - Recognized by TLR-2; activates signal transduction pathways that lead to the production of pro-inflammatory cytokines (Yang et al. 2006)
  - **Sph1, 2, 3, 4** (Zhang et al. 2008):
    - Putative cytolytic sphingomyelinases
    - Only Sph2 has been shown to have cytolytic activity
  - Hemolysins (Ren et al. 2003):
    - Four putative hemolysins
    - In vitro hemolytic activity
- Evasion of host immune system:
  - Inhibition of complement (Adler 2014; Castiblanco-Valencia et al. 2012):
    - **LenA, LenB**: bind to host complement regulatory protein factor H
    - **LcpA, lsa30**: bind to host complement regulatory protein C4BP
    - **LigA, LigB**: bind to host factor H and C4BP; expression is regulated by temperature and osmolarity (Haake and Zuckert 2015)

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Chapter 22
Neisseria spp.

Genomics, Morphologies, and Growth Characteristics

- **Genomics:**
  - *Neisseria gonorrhoeae* (*Ng*) NCCP11945 chromosome: 2,232,025 bp; 2,662 predicted ORFs (Chung et al. 2008); polyploid containing two to four genome equivalents (Tobiason and Seifert 2010)
  - *Neisseria meningitidis* (*Nm*) serogroup A chromosome: 2,184,406 bp; 2,121 predicted ORFs (Parkhill et al. 2000); polyploid containing three to five genome equivalents (Tobiason and Seifert 2010)
- **Cell morphology:**
  - Diplococci (small cells in Fig. 22.1) with flattened adjacent sides (Fig. 22.2):
    - *Ng*: gonococcus
    - *Nm*: meningococcus
  - Type IV pili (Tfp), present in *Nm* and *Ng* (Fig. 22.2):
    - Essential function in adherence (see below)
    - Responsible for twitching motility; due to attachment/retraction of pili
    - Important in transmission and colonization
  - Capsule, only present in *Nm*:
    - Responsible for the high level of virulence associated with *Nm*
    - Allows dissemination and survival within the bloodstream (see below)
  - Lipooligosaccharide (LOS) (Tong et al. 2002; Tsai 2001):
    - Atypical LPS; highly branched oligosaccharides
    - Undergoes antigenic variations that aid in immune evasion (see below)

D.I. Johnson, *Bacterial Pathogens and Their Virulence Factors*,
https://doi.org/10.1007/978-3-319-67651-7_22

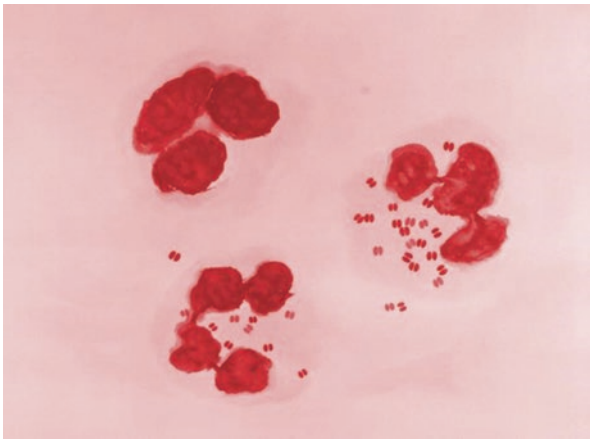

Fig. 22.1 *Ng* diplococci (From: PHIL #4086)

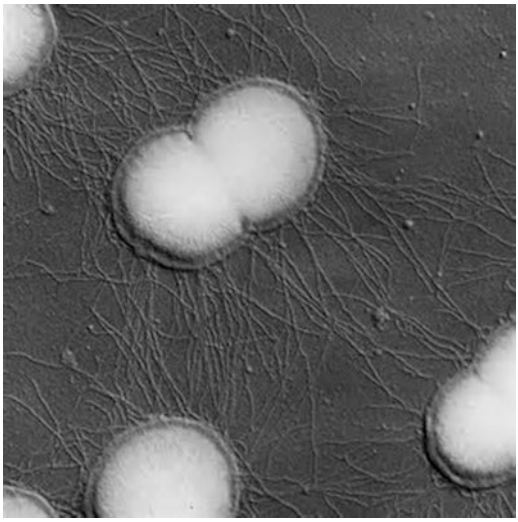

Fig. 22.2 *Nm* pili (From: medical-labs.net)

- **Gram stain:**
  - Gram negative
- **Growth:**
  - Obligate aerobes
  - Fastidious: requires hemoglobin, NAD (V factor), and iron [(found in chocolate agar plates (CAP)]
  - Capnophilic: requires 5–10% CO_2 in vitro
  - Temperature sensitive: clinical samples must be stored at 37 °C for survival
  - Fragile growth: susceptible to temperature changes, drying, UV light, and other environmental conditions; does not exist outside human host niches

- Biofilms:
  - *Ng* can form biofilms during infection
  - May play a role in persistence in women (Falsetta et al. 2011)
- At least 25 species; only *Ng* and *Nm* are human pathogens

Disease States Associated with *Neisseria* spp.

- Inflammatory diseases: very different clinical symptoms; due to differences in sites of colonization, portals of entry, adherence mechanisms, and virulence factors:
  - *Ng*:
    - Gonorrheal urethritis
    - Pelvic inflammatory disease (PID) in women
    - Sexually transmitted, primary pathogen of the urogenital tract
    - High prevalence in population; low mortality rate associated with infection
    - Emergence of antibiotic-resistant gonorrhea; one of top three urgent threats to health in the USA (CDC: Antibiotic Resistance Threats in the United States, 2013)
    - Infects epithelial cells of urethra; also throat, rectum, and conjunctiva mucosa (neonatal gonorrheal conjunctivitis)
    - Facultative intracellular pathogen; can invade epithelial cells, neutrophils, and macrophage
    - Male symptoms: purulent urethritis; usually self-limiting
    - Female symptoms: higher risk of infection; 20–80% of infected women are asymptomatic:
      - Purulent cervicitis, abdominal pain
      - Can induce inflammation in deeper tissue:
        - Progression into the fallopian tubes; can result in scarring, PID, tubal infertility, ectopic pregnancy, and chronic pelvic pain
    - Dissemination can lead to septic arthritis, dermatitis, and endocarditis
  - *Nm*:
    - Meningitis; septicemia (meningococcemia)
    - Opportunistic pathogen: asymptomatic commensal in the nasopharynx of 5–10% of healthy individuals (may be higher in different geographical niches)
    - Transmitted person-to-person by contact
    - Low prevalence in population; high mortality rate associated with infection – due to capsule-dependent dissemination and growth

- Low percentage of cells enter the bloodstream and disseminate to the central nervous system:
  - Capable of uncontrolled growth in cerebral spinal fluid (CSF)
  - Can cross the blood–brain barrier, leading to meningitis and potential neurological sequelae
- Epidemic meningitis: usually occurs in populations of military recruits or college students
- Bacteremia; septicemia (meningococcemia): septic shock can lead to purpura fulminans (high mortality) and petechiae from broken capillary blood vessels

Virulence Factors

- *Ng*:
  - No known exotoxins
  - Adherence to host cells (Hung and Christodoulides 2013):
    - Binds to microvilli of non-ciliated columnar epithelial cells
    - Fimbrial adhesin:
      - **Type IV pili (Tfp)** (Thanassi et al. 2012):
        - At least 23 Pil proteins are involved in pili synthesis and function
        - PilE (Hung and Christodoulides 2013):
          - Major structural protein
        - First step in attachment: Tfp binding to α1β1 or α2β1 integrins
        - Binding induces a tight attachment via interactions with host asialoglycoprotein receptor (ASPG-R); LOS also binds to ASPG-R on epithelial cell surface
        - Tfp may also bind in vivo to CD46 – transmembrane glycoprotein that binds to complement proteins C3b/C4b; interferes with complement-based lysis
        - Tfp undergoes antigenic variation and phase variation:
          - Provides protection against the host immune system
          - These variations are facilitated by the *Ng* polyploid genome
    - Non-fimbrial adhesins:
      - **Opa proteins** (Hauck and Meyer 2003; Hung and Christodoulides 2013):
        - At least ten proteins; differentially expressed
        - Second step in attachment; provides tight attachment to epithelial cells

- Eight-stranded β-barrel outer membrane proteins with four surface-exposed loops
- Two classes of Opa proteins:
  - **Opa_{HS}** proteins:
    - Bind heparan sulfate proteoglycans (HSPGs)
    - Bind ECM proteins vitronectin and fibronectin, which connect to host integrins
  - **Opa_{CEA}** proteins:
    - Bind carcinoembryonic antigen-related cellular adhesion molecules (CEACAMs)
- Also function as invasins (see below)
- Antigenic variation provides protection; facilitated by its polyploid genome

- **PorB** (Massari et al. 2003):
  - Porin; outer membrane, voltage-gated ion channel
  - *Nm* has two porins: PorA, PorB.1A, and PorB.1B alleles
  - Form 16-strand β-barrel fold structure
  - Functions in attachment and invasion into epithelial cells:
    - Prevents phagosome–lysosome fusion inside infected neutrophils
  - Also binds complement regulator C4BP (see *Nm* below)
  - Antigenic variation within and between strains
- **MafA** (Paruchuri et al. 1990):
  - Glycolipid-binding protein
  - Encoded on MGI (maf genomic islands) in *Ng* and *Nm* (Jamet et al. 2015); likely the result of horizontal gene transfer
  - Belongs to family of Maf adhesins in *Ng* and *Nm*

- Cell invasion:
  - Facultative intracellular pathogen
  - Invades epithelial cells and phagocytic cells
  - **Opa** proteins (Billker et al. 2002):
    - Binding to host CEACAMs triggers phosphorylation of host Src tyrosine kinases
    - Leads to actin-dependent phagocytosis through a zipper mechanism; depends on host Cdc42/Rac GTPases

- Inside neutrophils:
  - **Catalase** and **superoxide dismutase**: protects against the action of reactive oxygen species (ROS)
  - Polyploid genome allows for rapid DNA repair
  - Blocks activity of defensins and other antimicrobial peptides
  - Uses efflux pumps (Jerse et al. 2003):
    - Exports antimicrobial peptides, antibiotics, and detergents
    - Encoded by *mtrCDE* genes under the regulation of the *mtrR* gene

- Iron acquisition (Perkins-Balding et al. 2004):
  - Does not use siderophores
  - Direct uptake of host iron-bound proteins:
    - **TbpA and TbpB**: outer membrane transferrin receptor system
    - **LbpA and LbpB**: outer membrane lactoferrin receptor system
    - **HmbR**: outer membrane hemoglobin receptor protein
    - **HpuA and HpuB**: outer membrane receptor system for hemoglobin, methemoglobin, haptoglobin, and hemoglobin–haptoglobin complexes
  - Expression is regulated by Fe^{+2}-bound Fur repressor

- Host cell damage:
  - **LOS** (endotoxin activity leads to septic shock):
    - Triggers an intense inflammatory response that is responsible for the symptoms associated with gonorrhea:
      - *Ng* lipid A is optimally recognized by TLR-4, which activates the inflammation cascade
      - Subsequent lysis of phagocytes generates the purulent discharge associated with gonorrheal infections
      - Production of the cytokine tumor necrosis factor (TNF) is responsible for the PID complications

- Evasion of host immune system:
  - Can survive extracellularly in the presence of neutrophils and also inside neutrophils (see above)
  - **LOS** (Lewis and Ram 2014; Pizza and Rappuoli 2015):
    - Binds host sialic acid; forms microcapsule of sialylated LOS that blocks complement activation and phagocytosis by neutrophils – example of molecular mimicry
    - Phosphoethanolamine modification of lipid A increases *Ng* fitness during infection, blocks complement activation, and induces resistance to antimicrobial peptides (Hobbs et al. 2013; Lewis et al. 2013)

- Undergoes phase variation and antigenic variation; hinders antibody (Ab) recognition
- Released as "blebs"; acts as molecular decoys

- **IgA protease** (Almogren et al. 2003; Pohlner et al. 1987):
  - Cleave host respiratory immunoglobulin IgA1 and secretory IgA1 (S-IgA1)

- *Nm*:
  - Adherence to host cells (Virji 2009; Hung and Christodoulides 2013; Simonis and Schubert-Unkmeir 2016):
    - Binds to brain microvascular endothelial cells and bloodstream endothelial cells
    - Fimbrial adhesin:
      - **Type IV pili (Tfp)** (Fig. 22.3):
        - Essential for adherence of encapsulated *Nm*; non-fimbrial adhesins such as Opa and Opc are sterically blocked by capsule
        - Adherence is mediated by binding to host CD147, an immunoglobulin (Ig) superfamily receptor (Bernard et al. 2014)
        - Leads to the formation of microcolonies on endothelial cells
        - Tfp binding activates the β2-adrenergic receptor; leads to the generation of multi-protein complexes called cortical plaques within the membranes of endothelial cells:
          - Induces Cdc42 GTPase-dependent polymerization of the actin cytoskeleton; generates microvilli-like membrane protrusions that protect the attached cells from shear stress
        - Tfp binding enhances paracellular movement between the brain–endothelial cell junction, thereby allowing *Nm* to cross the blood–brain barrier:
          - Binding inappropriately recruits host Par3/Par6/PKCζ polarity complex (Coureuil et al. 2009) and adherens junctions proteins VE-cadherin and p120-catenin (Simonis and Schubert-Unkmeir 2016); leads to opening of cell–cell tight junctions
        - Similar signaling processes lead to loss of vascular integrity, resulting in purpuric lesions
    - Non-fimbrial adhesins (Fig. 22.3):
      - **NadA** (*Nm* adhesin A) (Comanducci et al. 2002):
        - Only found in some *Nm* strains
        - Trimeric surface protein; highly antigenic
        - Functions in adherence to epithelial cells through interactions with host β1 integrins

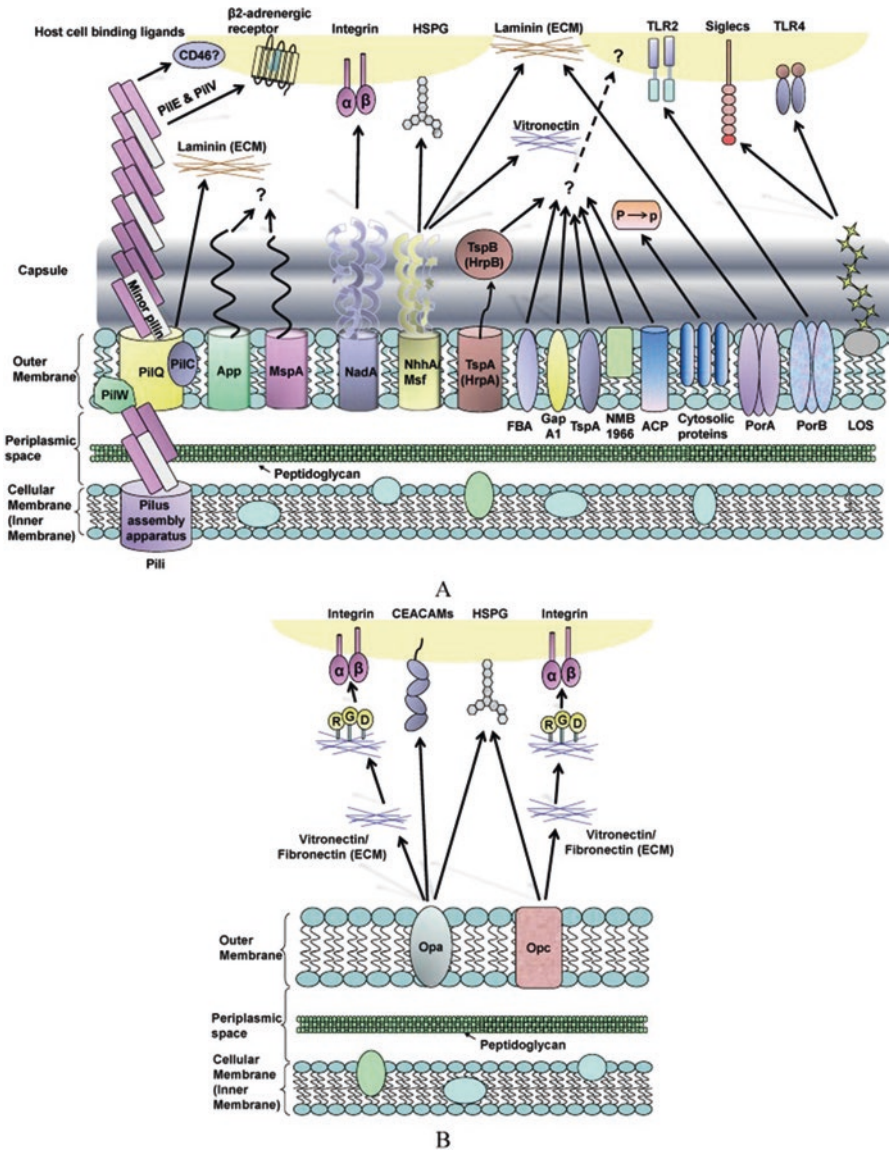

Fig. 22.3 *Nm* adherence factors (From: Hung and Christodoulides 2013)

  - Also functions in invasion of epithelial cells
  - Adherence is blocked by host Hsp90 heat shock protein (Montanari et al. 2012)

- **Opc** (Sa et al. 2010):
  - Only found in *Nm*; encoded by *opcA* gene
  - Ten-stranded β-barrel transmembrane protein

  - Functionally equivalent binding as the *Ng* Opa_{HS} proteins
  - Opc binds HSPGs; binding activates human acid sphingomyelinase (ASM):
    - ASM cleaves sphingomyelin in the outer leaflet of the host plasma membrane, resulting in increased levels of ceramide and ceramide-enriched platforms in the membrane
    - Opc-expressing *Nm* uses ceramide-enriched platforms for entry into brain endothelial cells (Simonis et al. 2014)

- **Opa** proteins:
  - Usually three to four proteins
  - Equivalent binding as the *Ng* Opa proteins (see above)

- **NhhA** (*Neisseria* hia/hsf homologue)/**Msf** (Scarselli et al. 2006):
  - Only found in *Nm*
  - Trimeric autotransporter adhesin
  - Similarity to Hsf and Hia adhesins of *H. influenzae*
  - Binds to epithelial cells and ECM components laminin and HSPGs
  - Blocks complement activation via binding to host vitronectin (Sjolinder et al. 2008)

- **MspA** (meningococcal serine protease A) (Turner et al. 2006; Khairalla et al. 2015):
  - Only found in *Nm*
  - Autotransporter protein with auto-proteolytic serine protease activity
  - Binds to mannose receptor and transferrin receptors on epithelial cells and dendritic cells
  - Internalized into host cell nucleus; cleaves histone H3
  - Induces caspase-dependent apoptosis

- **App** (adhesion and penetration protein) (Khairalla et al. 2015; Serruto et al. 2003):
  - Found in *Ng* and *Nm*
  - Contains auto-proteolytic serine protease activity; essential for function as adhesin
  - Binds to mannose receptor and transferrin receptors on epithelial cells and dendritic cells
  - Internalized into host cell nucleus; cleaves histone H3
  - Induces caspase-dependent apoptosis
  - Homologous to *H. influenzae* Hap

- **PorA, PorB** (Massari et al. 2003):
  - Porins: outer membrane, voltage-gated ion channels
  - PorB functions in attachment and invasion of epithelial and endothelial cells through binding to TLR-2 (Toussi et al. 2016)
  - PorA binds to ECM laminin and complement regulator C4BP (see below)
- **HrpA (TspA; T-cell stimulating protein A)** (Oldfield et al. 2007):
  - Only expressed in *Nm*
  - Part of two partner secretion (TPS) system (Jacob-Dubuisson et al. 2001)
  - HrpA undergoes **HrpB (TspB)**-dependent secretion; functions in pili-independent adherence to epithelial cells (Schielke et al. 2010)
- **ACP (adhesin complex protein)** (Hung et al. 2013)
  - Multiple versions found in *Nm* and *Ng*

– Host cell damage:
  - **Capsule** polysaccharides (Zughaier 2011):
    - Induces release of pro-inflammatory cytokines
    - Triggered by interactions with TLR-2 and TLR-4
  - **LOS**: septic shock (see above for *Ng*)
  - **NarE** (Valeri et al. 2015):
    - Bacterial ADP-ribosyltransferase (bARTT)
    - Induces cytoskeletal effects and apoptosis in epithelial cells; no known target
    - Undergoes auto-ADP-ribosylation to regulate its activity

– Evasion of host immune system (Gasparini et al. 2015; Lewis and Ram 2014; Pizza and Rappuoli 2015):
  - Most of these bacterial components undergo extensive antigenic variation and phase variation; important in the evasion of host immune system
  - **Capsule**:
    - Only found in *Nm*
    - Thirteen serogroups; based on capsule polysaccharides
    - A, B, C, Y, and W135: most important serogroups; vaccines are available against all of these serogroups
    - Blocks opsonophagocytosis and complement protein C4b accumulation
  - **LOS**: binds to complement regulatory protein factor H (fH); see *Ng* above
  - Multiple proteins block complement-mediated lysis: bind to complement regulatory proteins C4bp (C4-binding protein), fH, and vitronectin; blocks complement activation and opsonophagocytosis (Fig. 22.4):

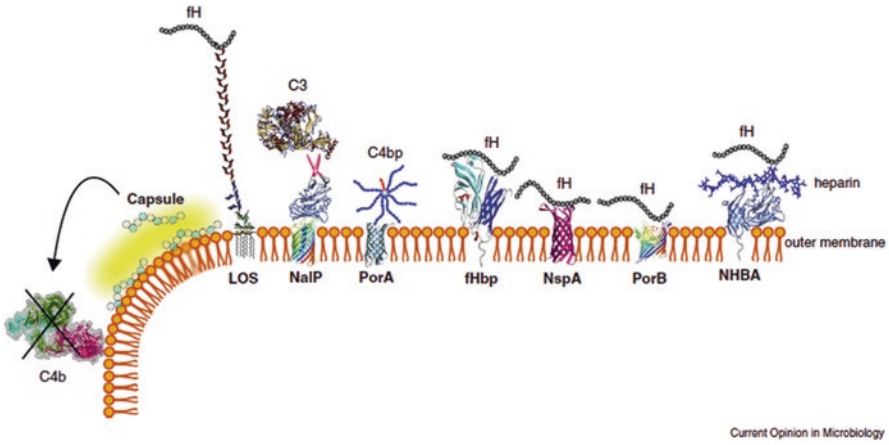

Fig. 22.4 Complement evasion mechanisms (From Pizza and Rappuoli 2015)

- **Fhbp (GNA1870), PorB2, and NspA** (Madico et al. 2006; Pizza and Rappuoli 2015):
  - Bind to complement regulatory protein fH
- **PorA and NspA** (Jarva et al. 2005):
  - Bind to host regulatory protein C4bp
- **NHBA** (neisserial heparin-binding antigen) (Serruto et al. 2010):
  - Binds heparin, which interacts with fH, C4bp, and vitronectin:
    - Heparin binding may block complement activation and increase serum resistance
  - NHBA is cleaved by **NalP**, resulting in the release of the C2 fragment; C2 induces increased endothelial permeability by inappropriately recruiting VE-cadherin (see Tfp section above) (Casellato et al. 2014)
- **NalP** (Del Tordello et al. 2014):
  - Serine protease autotransporter
  - Cleaves complement protein C3 and NHBA

Regulation of Virulence Factor Expression

- *Ng* and *Nm* survive in different niches within the human host – this is due, in part, to different transcriptional patterns (Claus et al. 2007; Echenique-Rivera et al. 2011; Schielke et al. 2010)

- *Nm* must adapt and survive within the host bloodstream:
  - When grown in ex vivo human blood for 90 min, 637 genes were differentially regulated; predominantly upregulated (Echenique-Rivera et al. 2011)
  - Regulated genes: iron acquisition and Fur regulation, sugar transporters, energy metabolism, amino acid biosynthesis, multiple adhesins, complement evasion proteins, and ROS detoxification survival genes
  - Similar results were obtained through proteomic analysis (Liu et al. 2016) and in a long-term in vitro colonization model (Hey et al. 2013)
- **PhoQ–PhoP (MisS-MisR)** (Jamet et al. 2009; Newcombe et al. 2003):
  - Two-component phosphorelay system (TCS); one of four TCSs expressed in *Nm*
  - Involved in regulating virulence, colonization, and the LOS inner core structure (Tzeng et al. 2004)
  - Only TCS that is upregulated during growth in the blood (Claus et al. 2007)
- Long-chain fatty acid resistance:
  - Long-chain fatty acids have antimicrobial activity; resistance promotes survival of *Ng* in urogenital tract (Schielke et al. 2010)
  - **FarR** (fatty acid resistance regulator):
    - Transcription repressor
    - ***Ng*FarR** represses ***farAB* operon**, which encodes a long-chain fatty acid efflux pump (Lee and Shafer 1999):
      - Repression depends on the MtrR regulatory protein (see above; cell invasion), which regulates *Ng*FarR expression (Lee et al. 2003)
      - Repression or inactivation of FarR leads to fatty acid resistance; promotes survival of *Ng* in the urogenital tract
    - ***Nm*FarR** represses the **NadA adhesin**, not the *farAB* operon:
      - Repression or inactivation of FarR leads to greater NadA-dependent adherence to, and invasion of, epithelial and endothelial cells
      - FarR activation reduces amount of NadA, which is highly immunogenic – enhances immune system evasion
- fHbp (factor H-binding protein) regulation (Loh et al. 2016):
  - Levels of fHbp are upregulated at high temperatures (37 °C and higher)
  - Regulation is through a RNA thermosensor at the level of translation, not transcription

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Chapter 23
Nocardia spp.

Genomics, Morphologies, and Growth Characteristics

- **Genomics:**
  - *Nocardia asteroides*: chromosome: 6,954,780 bp; 6106 predicted ORFs (Komaki et al. 2014)
  - *Nocardia cyriacigeorgica* GUH-2: chromosome 6,194,650 bp; 5405 predicted ORFs (Komaki et al. 2014)
  - *Nocardia brasiliensis*: chromosome: 9,436,348 bp; 8414 predicted ORFs (Vera-Cabrera et al. 2013)
- **Cell morphology:**
  - Filamentous hypha-like cells (Fig. 23.1)
- **Gram stain:**
  - Gram positive
  - "Beaded," partially acid-fast appearance; depends on the quantity of mycolic acid in the cell wall
- **Growth:**
  - Aerobes; catalase positive
  - Found primarily in soil and water
  - >80 species; most are rarely associated with human disease:
    - Antibiotic susceptibilities and molecular characteristics have allowed taxonomic differentiation of previous species into new complexes and clades (Brown-Elliott et al. 2006)

D.I. Johnson, *Bacterial Pathogens and Their Virulence Factors*,
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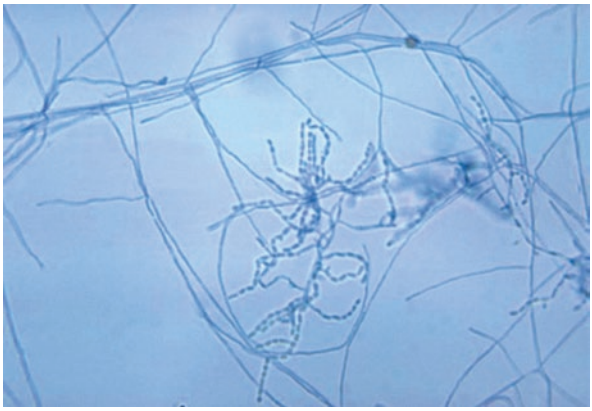

Fig. 23.1 *N. asteroides* cells (From: PHIL #4232)

Disease States Associated with *Nocardia* spp.

- Nocardiosis (Brown-Elliott et al. 2006; Wilson 2012):
  - Opportunistic pathogens and primary pathogens
  - Inhalation infections; cutaneous infections; catheter-related bacteremia
  - Pulmonary nocardiosis:
    - Opportunistic infection of immunosuppressed individuals
    - Predominantly *N. asteroides*; also *N. cyriacigeorgica*, *N. brasiliensis*
    - Transmission: inhalation of mycelial cells
    - Symptoms: persistent cough with purulent sputum, shortness of breath, chest pain, coughing up blood (hemoptysis), fever, night sweats, weight loss, and progressive fatigue
  - Extrapulmonary nocardiosis:
    - Hematogenous spread; CNS and cerebral nocardiosis are most common
    - Symptoms: abscess formation in the brain; headache, nausea, vomiting, neurological symptoms
  - Primary cutaneous nocardiosis:
    - Primary infection of immunocompetent individuals
    - Predominantly *N. brasiliensis*; also *N. asteroides*
    - Transmission: contact with contaminated soil
    - Produces skin abscesses and localized cellulitis; can also lead to mycetomas (Fig. 23.2)
    - Can disseminate to the lymph nodes, resulting in lymphocutaneous nocardiosis; similar to fungal sporotrichosis symptoms
  - Catheter-related bacteremia:
    - Rare but increasingly observed among immunocompromised patients
    - Associated with central venous catheter infections

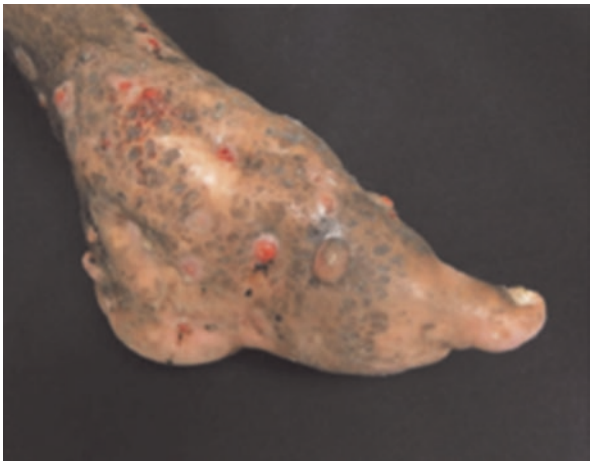

Fig. 23.2 Mycetoma (From: Vera-Cabrera et al. 2013)

Virulence Factors

- Many of the virulence factors listed below are predicted based on the genome sequencing and annotation of several *Nocardia* spp. (Komaki et al. 2014; Vera-Cabrera et al. 2013); experimental verification of many of their activities has yet to be determined
- **Biofilms** (Al Akhrass et al. 2011):
  - Excellent biofilm former on catheters
- Adherence to host cells and invasion:
  - Facultative intracellular pathogens (Vera-Cabrera et al. 2013)
  - **Invasin**:
    - Protein used to attach to host cells
    - Encoded by O3I_027570 gene
    - Similar to *Yersinia pestis* Inv
  - **Mce** proteins (mammalian cell entry):
    - 33 genes encoded in genome
    - Analogous to *M. tuberculosis* Mce proteins (Gioffre et al. 2005)
  - **KatN and KatG** (Vera-Cabrera et al. 1999):
    - Five catalase genes
    - Detoxify H_2O_2 in phagocytes
  - **SodA** (Alcendor et al. 1995):
    - Two superoxide dismutases
    - Destroys H_2O_2

  - **Cord factor** (Crowe et al. 1994):
    - Blocks phagosome–lysosome fusion in phagocytic cells
    - Induces granuloma formation in mice (Han et al. 1998)
    - Analogous to *Mycobacterium tuberculosis* cord factor
  - **Mycolic acids** (Beaman and Moring 1988; Nishiuchia et al. 1999):
    - Found in the cell wall; associated with partial acid-fast staining
    - Increases hydrophobicity of cells and provides resistance to harsh environmental conditions
    - Analogous to *Mycobacterium tuberculosis* mycolic acids
- Damage to host cells:
  - **Phospholipases C** (Vera-Cabrera et al. 2013):
    - Five potential PLC genes found in *N. brasiliensis* (not found in other *Nocardia* spp.)
    - Cytolytic activity; disrupts host cell membranes
    - Destroys host tissue; may play a role in cutaneous nocardiosis
  - **Hemolysins** (Barry and Beaman 2007):
    - Two genes identified
    - May be involved in the previously described ability of *Nocardia* spp. to induce caspase-dependent apoptosis in cultured human cell lines, possibly through activation of the host cell proteasome
- Iron acquisition:
  - Siderophores:
    - **Nocobactin** (Hoshino et al. 2011):
      - nbt-like siderophore
      - Similar to *M. tuberculosis* mycobactin
    - **Asterobactin** (Nemoto et al. 2002)

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Chapter 24
Proteus spp.

Genomics, Morphologies, and Growth Characteristics

- **Genomics:**
  - *Proteus mirabilis* chromosome: 4,099,900 bp; 3685 predicted ORFs (Pearson et al. 2008)
  - Plasmid pHI4320: 36,289 bp; 55 predicted ORFs
- **Cell morphology:**
  - Rod-shaped cells (Fig. 24.1)
  - Changes morphology to polyploid elongated swarmer cells on solid surfaces:
    - "Bull's-eye pattern" due to differentiation changes (Fig. 24.2)
- **Gram stain:**
  - Gram negative
- **Growth:**
  - Facultative anaerobes; catalase positive
  - Few peritrichous flagella in liquid; hyper-flagellation in swarmer cells on solid surfaces
  - Normal microbiota of human intestinal tract; also found in soil, water, hospitals, and long-term care facilities
  - Three pathogenic species:
    - *Proteus mirabilis*: ~80 % of infections
    - *Proteus vulgaris*
    - *Proteus penneri*

D.I. Johnson, *Bacterial Pathogens and Their Virulence Factors*,
https://doi.org/10.1007/978-3-319-67651-7_24

Fig. 24.1 *P. mirabilis* swarmer cells (From: slideshare.net)

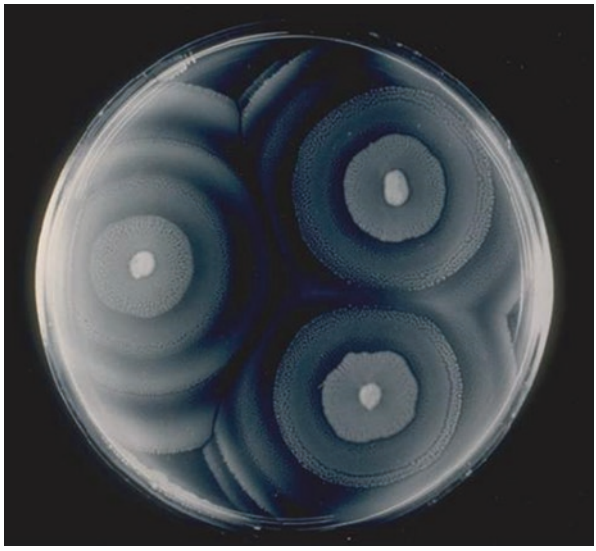

Fig. 24.2 Swarming motility on trypticase soy agar (From: PHIL #1046)

Disease States Associated with *Proteus mirabilis*

- Ascending urinary tract infections (UTIs) (Norsworthy and Pearson 2017):
  - Form urease-dependent crystalline biofilms on catheters
  - Catheters prevent clearance of *P. mirabilis* from the urethra, leading to catheter-associated urinary tract infections (CA-UTIs)
- Ascending bladder and kidney infections
- Infectious urinary stones, including bladder stones and kidney stones, which can lead to urosepsis (Bichler et al. 2002)

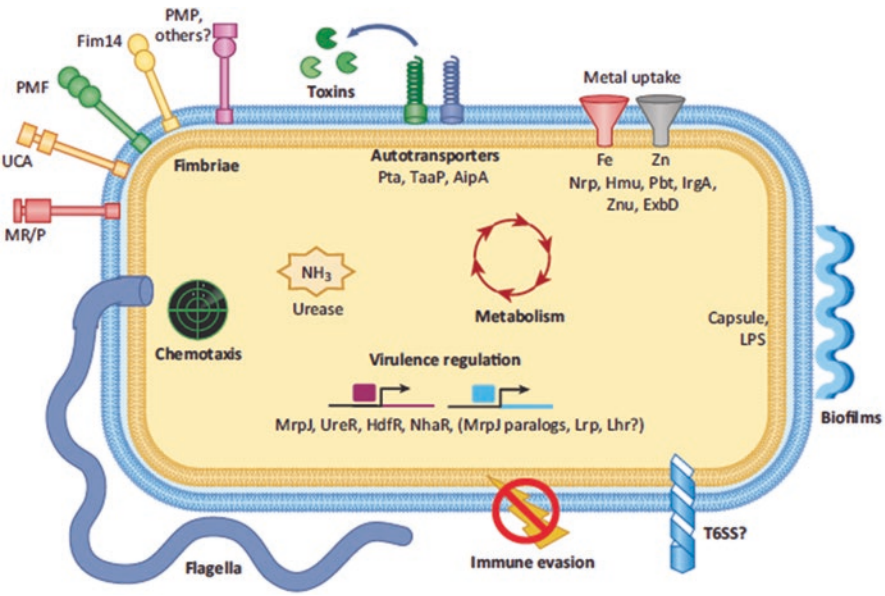

Fig. 24.3 Virulence mechanisms (From: Norsworthy and Pearson 2017)

Virulence Factors (Fig. 24.3) (Coker et al. 2000; Norsworthy and Pearson 2017)

- **Urease** (Mobley et al. 1995):
  - Essential for virulence
  - Trimeric Ni^{+2}-dependent urease:
    - Encoded by *ureA, ureB, ureC* genes (Island and Mobley 1995)
    - Hydrolyzes urea into bicarbonate and ammonia:
      - $Urea + H^+ + 2H_2O \rightarrow HCO_3^- + 2(NH_4^+)$
    - NH_4^+ molecules increase net pH; leads to precipitation of Mg^{2+} and Ca^{2+} ions
    - NH_4^+ binds with Mg^{2+} in urine to form struvite crystals $[(NH_4)(MgPO_4 \cdot 6H_2O)]$ (Bichler et al. 2002)
    - CO_2 binds with Ca^{2+} in urine to form carbonate apatite $[Ca_{10}(PO_4)_6CO_3]$ (Bichler et al. 2002)
    - Struvite and apatite crystalline biofilms block catheter flow; more recalcitrant biofilms than typical biofilms
    - Also plays a role in bladder and kidney infections
- Adherence to host cells/tissues:
  - Fimbrial adhesins:

- Fimbriae:
  - Chaperone usher assembly mechanism; 17 different operons
  - **MR/P** (mannose-resistant/*Proteus*-like) fimbriae:
    - Essential for the colonization of the urethra, kidney, and bladder (Coker et al. 2000)
    - Major contributor to biofilm formation (Rocha et al. 2007)
    - Structural subunit encoded by *mrpA* gene
  - **UCA** (uroepithelial cell adhesin) fimbriae (Pellegrino et al. 2013):
    - a.k.a. **NAF** (nonagglutinating fimbriae)
    - Functions in the colonization of the urinary tract via attachment to uroepithelial cells
    - Structural subunit encoded by *ucaA* gene
  - **PMF** (*P. mirabilis* fimbriae):
    - Functions in the colonization of the urethra, kidney, and bladder
    - Structural subunit encoded by *pmfA* gene
  - **PMP** (*P. mirabilis* P-like fimbriae) (Bijlsma et al. 1995):
    - May be involved in the colonization of the large intestine
    - Structural subunit encoded by *pmpA* gene
    - Homologous to Pap fimbriae of uropathogenic *E. coli* (UPEC)
  - **Fimbriae 8 (*fim8A*)**; **fimbriae 14 (*fim14A*)**: unknown functions; proteins found in surface protein preparations
- Afimbrial adhesins:
  - **AipA** (*a*dhesion and *i*nvasion mediated by the *Proteus a*utotransporter) and **TaaP** (*t*rimeric *a*utoagglutination *a*utotransporter of *Proteus*):
    - Bind to ECM collagen and laminin in vitro
- **Flagella**:
  - Functions in the dissemination of *P. mirabilis* from the urethra to the bladder and kidneys (Coker et al. 2000)
  - Essential for the colonization of the urethra and bladder epithelial cells
  - Essential for the invasion of cultured urinary tract cells in vitro; in vivo invasion is unclear (Schaffer and Pearson 2015)
  - Structural subunits encoded by *flaA* and *flaB* genes (Belas 1994); hybrid flagella may indicate antigen variation (Coker et al. 2000; Schaffer and Pearson 2015)

- Growth within the host milieu:
  - Iron acquisition (Himpsl et al. 2010):

    - Expression of iron acquisition pathways is induced in low-iron environments that exist in the urinary tract
    - Siderophores:
        - **Yersiniabactin related**:
            - Encoded by *nrp* genes
            - Synthesized by nonribosomal peptide synthetase (NRPS)
        - **Proteobactin**:
            - Encoded by *pbt* genes
            - NRPS-independent synthesis
    - Heme uptake:
        - Encoded by *hmu* genes
    - Fe^{+2} iron uptake:
        - Encoded by *sit* genes
    - Ferric citrate transport:
        - Facilitated by TonB-dependent receptor 3706
  - Catheter-associated biofilms; major medical problem (see above) (Stickler 2014)
- Damage to host cells:
  - **HpmA** (Swihart and Welch 1990):
    - Hemolysin
    - Cytotoxic to kidney epithelial cells
    - Encoded by *hpmA*
    - Transported and activated by HpmB (Coker et al. 2000)
  - **Pta (*Proteus* toxic agglutinin) toxin** (Alamuri and Mobley 2008):
    - Serine protease; remains attached to *Proteus* cell
    - Cytotoxic to host cells
    - Causes urinary tissue damage
  - **LPS endotoxin**
- Evasion of host immune system:
  - **IgA protease (ZapA)**:
    - Zinc metalloprotease
    - Cleaves both serum and secretory IgA1and IgA2 antibodies; also IgG antibodies (Senior et al. 1991)
    - Degrades β-defensin-1 and LL-37 antimicrobial proteins
    - Encoded by *zapA* gene (Wassif et al. 1995)

- **Flagellin** subunits undergo antigen variation (Coker et al. 2000).

Regulation of Virulence Factor Expression

- MrpJ regulon (Bode et al. 2015):
  - Master regulator of virulence factors
  - Regulates the transition between swarming cells and swimming cells
  - Numerous virulence factors are positively upregulated: MR/P fimbriae, Pta toxin, ZapA protease, LPS modification genes, and Type 6 secretion system (T6SS)
  - Oxygen limitation induces MrpJ regulon; upregulated during infection
  - **MrpJ**:
    - Transcription activator
    - Helix-turn-helix (HTH) protein
    - Induces transcription of MrpJ regulon genes by binding to MrpJ binding site upstream of target gene
  - Fifteen other MrpJ-type transcription factors regulate other fimbrial regulons
  - Also represses motility through the flagellin *flhDC* regulon [(Pearson and Mobley 2008); see below] and swarming through the *umoA* and *umoB* regulators; supports inverse relationship between fimbria-mediated attachment and motility/swarming
- FlhDC regulon (Belas 1994; Clemmer and Rather 2007):
  - Master regulatory of flagella biosynthesis and chemotaxis
  - Positively upregulates flagellar components and assembly machinery
  - Essential for swarming motility; increased FlaA flagellin synthesis in swarming cells
  - **$FlhD_4C_2$**:
    - Heterodimeric complex
    - Transcription activator; binds to DNA upstream of target genes
  - Transcriptionally repressed by MrpJ and RcsBCD phosphorelay RcsB repressor (Howery et al. 2016)
  - **Lon protease** negative regulates FlhDC by degrading FlhD (Clemmer and Rather 2008)
- Urease regulation (Schaffer and Pearson 2015):
  - Expression is induced in the urinary tract
  - **UreR**:
    - Transcriptional activator of *ureDABCEFG* operon
    - Induces urease expression in the presence of urea

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Chapter 25
Pseudomonas spp.

Genomics, Morphologies, and Growth Characteristics

- **Genomics**:
  - *Pseudomonas aeruginosa* chromosome: 6,264,403 bp; 5570 predicted ORFs (Stover et al. 2000)
- **Cell morphology**:
  - Rod-shaped cells (Fig. 25.1)
  - Flagellum: monotrichous (*P. aeruginosa*); other species can be lophotrichous:
    - Swimming motility
    - Important role in adherence; immunostimulatory
  - Type IV pili:
    - Several pili at the same pole as the flagellum
    - Essential function in adherence; immunostimulatory
    - Responsible for twitching motility; due to attachment/retraction of pili
  - Capsule:
    - Can form an alginate-based pseudocapsule during chronic infections (see below)
  - Lipopolysaccharide (LPS):
    - Associated with inflammation and endotoxic shock
    - Also functions in adherence (see below)
- **Gram stain**:
  - Gram negative

D.I. Johnson, *Bacterial Pathogens and Their Virulence Factors*,
https://doi.org/10.1007/978-3-319-67651-7_25

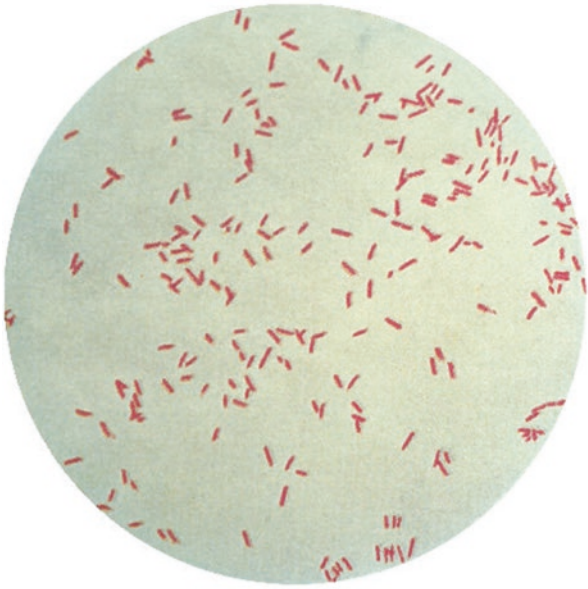

Fig. 25.1 *P. aeruginosa* cells (From: PHIL #2118)

- **Growth:**
  - Aerobes; catalase positive, oxidase positive; respiration (no fermentation)
  - Ubiquitous in environment; soil and water; transient microbiota in humans
  - Can infect animals, plants, and nematodes
  - Produces several pigments; can be useful for species identification
    - Pyocyanin: blue green
      - Redox-active metabolite; virulence factor
    - Pyoverdine: yellow green; fluorescent
      - Fe^{+3} siderophore (also pyochelin)
    - Pyorubin: red
    - Pyomelanin: brown black
  - Excellent biofilm formers: can tolerate poorly oxygenated atmospheres (see below)
  - >190 species; one major human pathogen – *Pseudomonas aeruginosa*

Disease States Associated with *Pseudomonas aeruginosa* (Gellatly and Hancock 2013)

- Opportunistic pathogen; third most common nosocomial infection

- Member of the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter* spp.) family of antibiotic-resistant nosocomial pathogens
  - Metabolically versatile with a large plastic genome; makes it a dangerous opportunistic pathogen
  - Multidrug resistance strains are present in healthcare environments
- Respiratory pneumonia: acute and chronic infections
  - Acute infections:
    - Associated with immunocompromised and COPD patients
    - Establishing acute infections (Fig. 25.2) (Hauser 2009):
      - ExoT (dual activity exotoxin, see below) aids in *P. aeruginosa* bypass of the lung epithelial mucosa
      - ExoU (phospholipase A_2) and ExoS (dual activity exotoxin, see below) block early inflammatory responses; later inflammatory responses are enhanced by ExoU-induced eicosanoid release; leads to host tissue damage
      - ExoS, ExoT, ExoU, and ExoY (adenylate cyclase) collectively block phagocytic activity; also disrupt epithelial and endothelial barriers
      - Disruption of barriers leads to bacteremia and septic shock; also leads to fluid influx into the lung, reducing levels of pulmonary surfactant and deceasing oxygenation
  - Chronic infections:
    - Adapts to the lung environment; grows as a biofilm; associated with cystic fibrosis (CF) patients and COPD patients
    - CF:
      - Result of a mutation in the CFTR (cystic fibrosis transmembrane conductance regulator) cAMP-dependent chloride channel; usually a loss-of-function misfolding mutation
      - CFTR is essential for proper mucus consistency in the lung
      - CFTR mutants have reduced water and Cl^- secretion:
        - Mucus becomes too viscous
        - Failure of mucociliary clearance mechanisms
        - Results in an intense chronic inflammatory response, leading to lung tissue damage and secondary bacterial infections
        - Also results in the loss of lung surfactant and decreases in O_2 tension, usually leading to death
  - *P. aeruginosa* expresses different virulence factors during acute infections vs. chronic infections (Fig. 25.3) (Smith et al. 2006; Sousa and Pereira 2014):

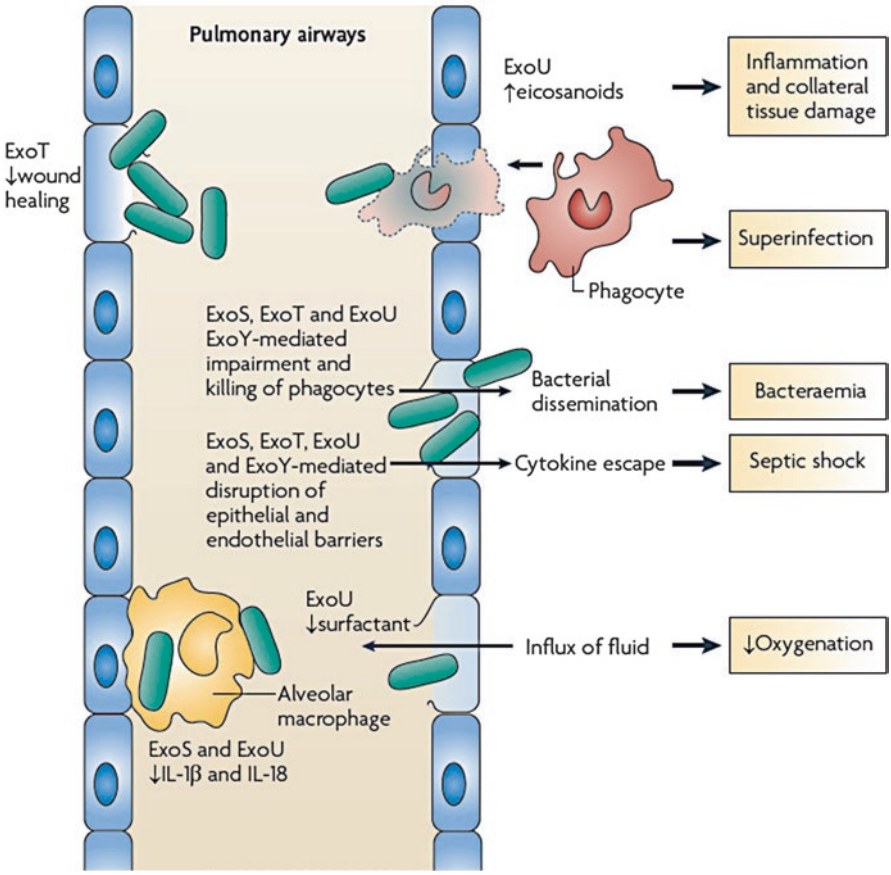

Fig. 25.2 Establishing acute pneumonia (From: Hauser 2009)

- Isolates from chronic infections lack flagella and pili and are found in biofilms
- Results from the increased production of an alginate pseudocapsule; the reduced expression and secretion of many virulence factors, including the PlcH hemolytic phospholipase; increases in small colony variants (SCVs); and increased antibiotic resistance
- Immune responses during chronic infections generate reactive oxygen species (ROS; *e.g.*, H_2O_2), which induce a hyper-mutator phenotype that generates mutations in many *P. aeruginosa* genes:
  - Flagellar synthesis mutations (*e.g.*, *fliC* mutant); decreases phagocytosis
  - Quorum-sensing mutations (*e.g.*, *lasR* mutant)
  - Induction of a mucoid phenotype; increased alginate synthesis through the inactivation of the MucA anti-sigma (σ) factor; enhances biofilm formation

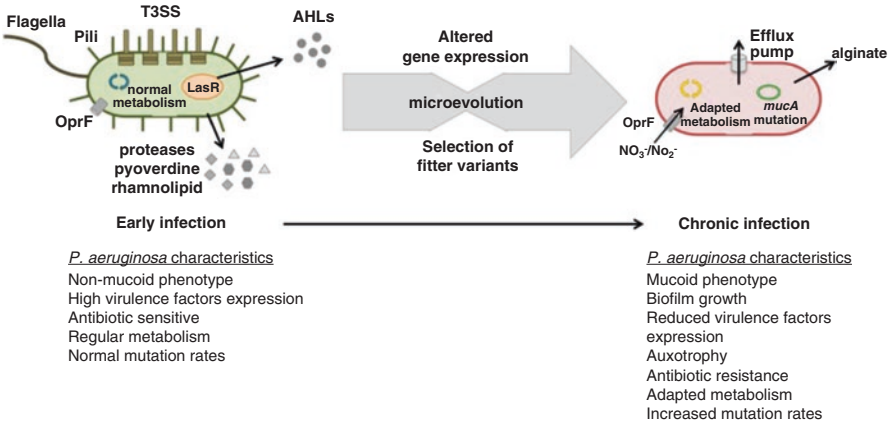

Fig. 25.3 Changes during chronic infection (From: Sousa and Pereira 2014)

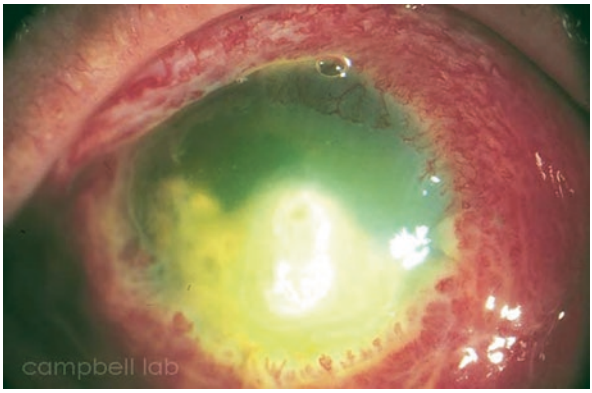

Fig. 25.4 *P. aeruginosa* corneal ulcer (From: Charles T. Campbell Laboratory)

  - Decrease in ciliary clearance
  - Decrease in innate immune clearance

- Soft tissue infections: burns, open wounds, surgery (nosocomial); the presence of blue–green pyocyanin in burn victim's wounds can lead to acute, rapid sepsis
- Eye (keratitis) infections (Fig. 25.4): corneal abrasions, contaminated eye solutions
- Biofilm-associated diseases:
  - Ventilator-associated pneumonia (VAP); second most common pathogen; can lead to sepsis
  - Urinary tract infections; associated with urinary catheters
- Otitis externa (swimmer's ear): primary pathogen
- Otitis media folliculitis: rash associated with hot tubs; primary pathogen

Virulence Factors

- Adherence to host cells:
  - **LPS:**
    - Binds to CFTR in host membrane, inducing binding and internalization
  - **Flagellum** (Adamo et al. 2004):
    - Binds to host cell surface asialo-GM1,2 gangliosides – generated when *P. aeruginosa* neuraminidase removes sialic acid residues from GM1,2 gangliosides
    - Binding activates Ca^{+2}-dependent pro-inflammatory responses
  - Fimbrial adhesins:
    - **Type IV pili** (Hahn 1997; Mikkelsen et al. 2011):
      - Type IVa pili: essential for twitching motility
      - Type IVb pili (Flp pili): major adherence factor; also necessary for biofilm formation
      - At least 37 proteins are involved in pili synthesis, function, and regulation; PilA is major Type IVa structural subunit
      - Binds the same asialo-GM1,2 gangliosides as flagella
      - Synthesis of pili is regulated by the PilR–PilS and PprA–PprB two-component phosphorelay systems (TCSs)
    - **CUP (chaperone–usher pathway) fimbriae** (Mikkelsen et al. 2011):
      - Five CUP fimbriae: CupA–CupE; each encoded by different gene clusters
      - Important for adherence and biofilm formation
      - Primarily regulated by the RocS1-RocA1-RocR TCS
  - Afimbrial adhesins:
    - **LecA (PA-IL)**, **LecB (PA-IIL)** (Grishin et al. 2015):
      - Lectins; bind glycoproteins on lung epithelial cells
      - LecA: binds to terminal *D*-galactose (highest affinity) and *N*-acetyl-*D*-galactosamine sugars on host glycoproteins
      - LecB: binds to terminal *L*-fucose (highest affinity), *D*-mannose, *L*-fucosylamine, *L*-galactose, *D*-arabinose, and *D*-fructose on host glycoproteins
      - Form homo-tetrameric structures that can bind four sugar molecules
      - Also play a role in biofilm formation, reduction of mucociliary beating, and exotoxin A-mediated epithelial barrier disruption
- Damage to host cells:

- **LPS**: endotoxin activity; triggers inflammatory reactions that lead to septic shock
- **Hydrogen cyanide (HCN)** (Pessi and Haas 2000):
  - Inhibits host cytochrome *c* oxidase within the electron transport chain
  - Produced by HCN synthase; encode by the *hcnABC* genes
  - Synthesized in low oxygen concentrations; regulated by the ANR anaerobic transcription regulator and by the LasI-LasR, RhlI–RhlR, and PQS quorum-sensing systems (see below)
- **Pyocyanin** (Rada et al. 2011):
  - Secreted, membrane-permeable heterocyclic member of the phenazine family
  - Redox-active metabolite; triggers the formation of superoxide and other reactive oxygen species (ROS) inside host epithelial cells
  - Oxidative stress affects multiple host functions, including ciliary function, epithelial cell growth, pro-inflammatory cytokine release, and neutrophil apoptosis
  - Also is bacteriocidal, giving *P. aeruginosa* a selective advantage in the lung
- **Rhamnolipids** (Maier and Soberon-Chavez 2000):
  - Rhamnose-containing glycolipids; produce mono- and di-rhamnolipids
  - Biological surfactants; can solubilize lung surfactant, facilitating its degradation during infection
- **T2SS** (Type 2 secretion system) and **T3SS** (Type 3 secretion system) (Filloux et al. 1998; Roy-Burman et al. 2001):
  - Secrete numerous bacterial effectors that act as virulence factors
  - T2SS effectors:
    - **ExoA (exotoxin A)** (Michalska and Wolf 2015):
      - A–B exotoxin
      - Adenosine diphosphate ribosyl transferase (ADPRT) activity
      - ADP-ribosylates host elongation factor eEF-2; same mechanism as diphtheria toxin
      - Blocks host cell translation; induces cell necrosis
    - **PlcH** (Ostroff et al. 1990):
      - Hemolytic phospholipase C
      - Degrades phosphatidylcholine and sphingomyelin in host membranes
      - Damages lung epithelial cells and degrades lung phospholipid surfactant (>80 % phosphatidylcholine)

- **LasA (staphylolysin), LasB (pseudolysin)** (Kessler et al. 1998; Spencer et al. 2010):
  - Zn metalloproteinases: LasA (protease); LasB (elastase)
  - LasA enhances the elastase activity of LasB; work synergistically to destroys host elastin within connective tissue
  - LasA has staphylolytic activity; targets peptidoglycan pentaglycine bridges; rapid lysis (Barequet et al. 2012)
- **Protease IV (PrpL)** (Engel et al. 1998; Malloy et al. 2005):
  - Serine protease
  - Can cleave Igs, complement proteins, fibrinogen, plasminogen, transferrin, lactoferrin, elastin, and lung surfactant
- **AprA (aeruginolysin)** (Zhang et al. 2014):
  - Secreted alkaline protease; belongs to family of Ca^{2+}-dependent repeats-in-toxin (RTX) exoproteases
  - Cleaves host fibrin, cytokines, Igs, and complement proteins
  - Cleaves *P. aeruginosa* flagella (along with LasB); abrogates host immune reaction against flagellin (Casilag et al. 2015)

- T3SS effectors (Fig. 25.5) (Engel and Balachandran 2009; Hauser 2009):
  - Four effectors; not expressed in all strains
  - **ExoS**: dual activity
    - N-terminus: GTPase-activating protein (GAP) activity (Goehring et al. 1999)
      - Stimulates the GTPase activity of Rho/Rac/Cdc42 GTPases
      - Leads to disruption of the actin cytoskeleton
      - Inhibits phagocytosis, cell migration, and cell division; induces apoptosis
    - C-terminus: adenosine diphosphate ribosyl transferase (ADPRT) activity
      - ADP-ribosylates and inactivates Ras, Rap, and Rab GTPases, disrupting many cell signaling pathways (Ganesan et al. 1999)
        - Activated by host 14-3-3 protein
      - Also ADP-ribosylates ezrin, radixin, moesin (ERM) proteins; disrupts actin cytoskeleton, blocks phagocytosis, and increases cell rounding
      - Can ADP-ribosylate many other host proteins (vimentin, cyclophilin A, IgG3, apolipoprotein A1, Rac1, Cdc42), but the role of these modifications is unclear

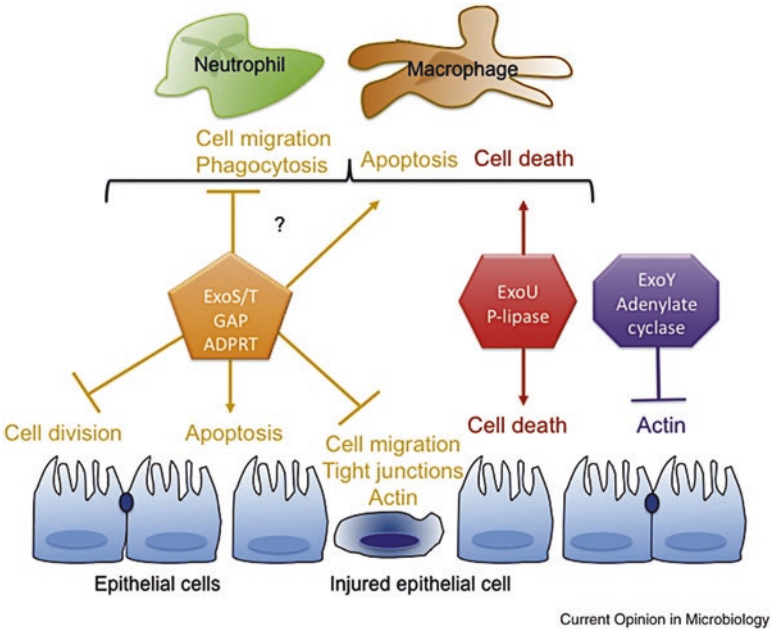

Fig. 25.5 T3SS effectors (From: Engel and Balachandran 2009)

- **ExoT**: dual activity
  - 76% identical to ExoS; lesser role in virulence than ExoS
  - Rho-GAP activity and substrates are the same as ExoS
  - ADPRT activity is the same but different set of substrates from ExoS:
    - ADP-ribosylates the Crk-I and Crk-II adaptor proteins; prevents binding to focal adhesion proteins p130cas and paxillin; blocks Rac GTPase signaling and cell division (Sun and Barbieri 2003)
- **ExoU** (Sato et al. 2003):
  - Phospholipase A_2 (PLA_2) activity
  - Rapid cytolytic host cell death
  - Induces inflammatory responses
- **ExoY** (Yahr et al. 1998):
  - Inherent adenylate cyclase; increases host cell cAMP concentrations
  - Disrupts actin cytoskeleton; increases endothelial permeability
  - Similar to *B. anthracis* edema factor (EF)

- **PASP (P. aeruginosa small protease)** (Tang et al. 2013):
  - Secreted protease
  - Induces corneal abrasions; plays a role in keratitis
  - Degrades collagens, complement C3, fibrinogen, antimicrobial peptide LL-37
- **LepA (large exoprotease A)** (Kida et al. 2008):
  - Secreted protease
  - Cleaves the tethered ligand of protease-activated receptors (PAR)-1, PAR-2, PAR-4
  - Leads to activation of NF–κB-dependent inflammation pathways

- Growth in host milieu
  - Iron acquisition (Cornelis 2010):
    - Fe^{+3} siderophores:
      - **Pyoverdine**:
        - Major peptide siderophore; multiple peptide variants
        - Ferribactin precursor is modified in periplasm to form pyoverdine
      - **Pyochelin**:
        - Minor peptide siderophore
      - Can utilize many xenosiderophores made by other bacteria
    - Direct uptake of host iron-bound proteins:
      - **Phu system**:
        - Binds directly to host hemoproteins, such as hemoglobin, hemopexin, and leghemoglobin
      - **Has system**:
        - HasAp: secreted hemophore; takes heme from host hemoproteins and brings it to the HasR receptor
    - Expression is regulated by Fe^{+2}-bound Fur repressor
  - **Pyocins** (Michel-Briand and Baysse 2002):
    - Bacteriocin
    - Cytolytic to other *Pseudomonas* species and a few other Gram-negative bacteria
    - May give *P. aeruginosa* an advantage in specific growth niches

- Evasion of host immune system:
  - **Biofilm formation** (Rybtke et al. 2015):

- Involved in medical device-related infections as well as chronic diseases such as CF pneumonia, wound infections, and otitis media
- Important for immune evasion and increased antibiotic resistance
- EPS matrix comprised of DNA, polysaccharides, and protein
- Major exopolysaccharides: **Pel, Psl**, and **alginate**
  - Synthesis is regulated by the RetS, LadS, and GacS–GacA TCSs
- Biofilm formation is regulated by the BfiS–BfiR (biofilm initiation), BfmS–BfmR (biofilm maturation), and MifS–MifR (microcolony formation) TCSs (Mikkelsen et al. 2011)

– **Alginate** (Ertesvag 2015; Whitfield et al. 2015):

- High molecular weight polymer of β-D-mannuronate linked to α-L-guluronate
- Forms a pseudocapsule structure, giving colonies a shiny mucoid appearance
- Essential for biofilm formation and persistence in the lung
- Protects against phagocytosis and antibody binding
- Tightly regulated synthesis; induced late in CF infection

Regulation of Virulence Factor Expression

- Highly complex process involving many transcription regulatory factors, two-component phosphorelay systems (TCSs), and quorum-sensing (QS) systems (Balasubramanian et al. 2013)

– 14 TCSs, 434 transcription factors; 24 σ factors (8 % of genome), 3 QS systems

- QS systems (Fig. 25.6) (Chatterjee et al. 2016):
  - Each QS system regulates a different subset of virulence factors as well as biofilm formation
  - **LasI-LasR system**:

– **3-oxo-C12-AHL** (*N*-(3-oxododecanoyl)-L-homoserine lactone)

- Diffusible autoinducer signaling molecule
- **LasI** directs the synthesis of 3-oxo-C12-AHL
- Binds to **LasR** to activate the transcription of multiple virulence factors (LasA, LasB, AprA, ExoA, phospholipases C and A_2, and hydrogen cyanide) as well as the T2SS

– Also affects host cell signaling and immune responses (Liu et al. 2015)

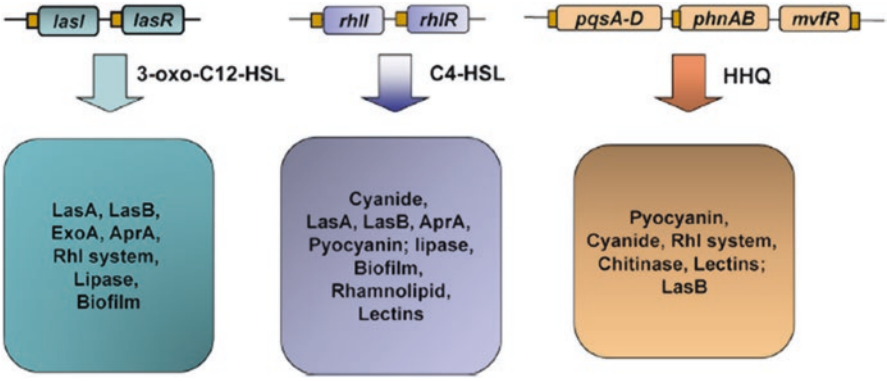

Fig. 25.6 QS systems (From: Chatterjee et al. 2016)

- **PQS (Pseudomonas quinolone signal) system**:
  - **PQS** (2-heptyl-3-hydroxy-4-(1H)-quinolone) and its precursor *HHQ* (2-heptyl-4(1*H*)-quinolone)
    - Diffusible autoinducer signaling molecules
    - **PqsA-E** proteins direct the synthesis of PQS and HHQ.
      - Production of PQS is induced by the LasI-LasR system and repressed by the RhlI–RhlR system
    - Bind to **MvfR (PqsR)** to activate the transcription of the **pqsA-E** and **phnAB** operons, and multiple virulence factors – pyocyanin, LecA and LecB lectins, rhamnolipids, neuraminidase (chitinase), hydrogen cyanide
  - Also affects host cell signaling and immune responses (Liu et al. 2015)
- **RhlI–RhlR system**:
  - **C4-AHL** (*N*-butanoyl-l-homoserine lactone)
    - Diffusible autoinducer signaling molecule
    - RhlI directs the synthesis of C4-AHL
    - Binds to RhlR to activate the transcription of multiple virulence factors – LasA, LasB, AprA, pyocyanin and other phenazines, Type IV pili, rhamnolipids, phospholipases C and A_2, hydrogen cyanide, and LecA and LecB lectins

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Chapter 26
Rickettsia spp.

Genomics, Morphologies, and Growth Characteristics

- **Genomics:**
  - *Rickettsia prowazekii* chromosome: 1,111,523 bp; 834 predicted ORFs (Andersson et al. 1998)
  - *Rickettsia rickettsii* chromosome: 1,257,710 bp; 1218 predicted ORFs (Ellison et al. 2008)
- **Cell morphology:**
  - Small coccobacilli (Fig. 26.1)
  - Surrounded by LPS and protein-containing S-layer
- **Gram stain:**
  - Gram negative
- **Growth:**
  - Aerobes
  - Obligate intracellular pathogens
  - Reservoirs: mammals and some arthropods
  - Can be transmitted by arthropods such as lice, ticks, fleas, and chiggers
  - Two groups of mammalian pathogenic species:
    - Typhus group (TG) – two species; cause typhus worldwide:
      - *Rickettsia prowazekii*: epidemic typhus fever
      - *Rickettsia typhi*: endemic murine typhus
    - Spotted fever group (SFG) – at least 20 species; cause spotted fevers in different geographic locations:
      - *Rickettsia rickettsii*: Rocky Mountain spotted fever (North America)

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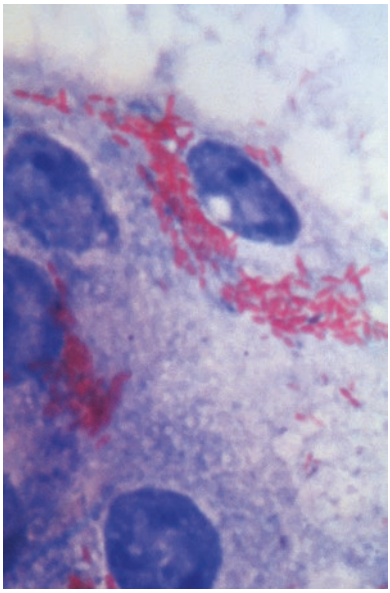

Fig. 26.1 R. rickettsii cells (From: PHIL #19028)

- *Rickettsia sibirica*: Siberian tick typhus
- *Rickettsia australis*: Queensland tick typhus
- *Rickettsia japonica*: Oriental spotted fever
- *Rickettsia africae*: African tick-bite fever
- *Rickettsia felis*: cat-flea typhus; emerging worldwide pathogen

Disease States Associated with *Rickettsia* spp.

- Zoonotic diseases transmitted from mammalian reservoirs via arthropod vectors
- Infections lead to disseminated inflammation of the microvascular epithelium; usually caused by induction and release of many pro-inflammatory cytokines and chemokines (Sahni et al. 2013)
- *Rickettsia prowazekii* – typhus fever; "war fever":
  - Transmitted by body lice and fleas or from flying squirrel ectoparasites (North America reservoir)
  - Fever, chills, headache, and myalgia
  - Small, flat macular rash appears on the trunk and later spreads to extremities (Fig. 26.2)
  - Other symptoms can include rales and delirium
  - Can reoccur as a milder form of disease – recrudescent typhus or Brill–Zinsser disease

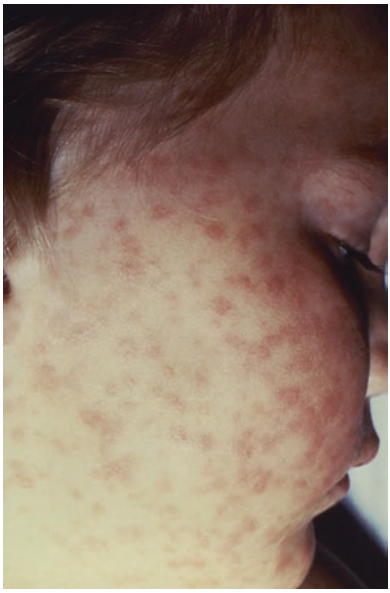

Fig. 26.2 Typhus fever rash (From: PHIL #14489)

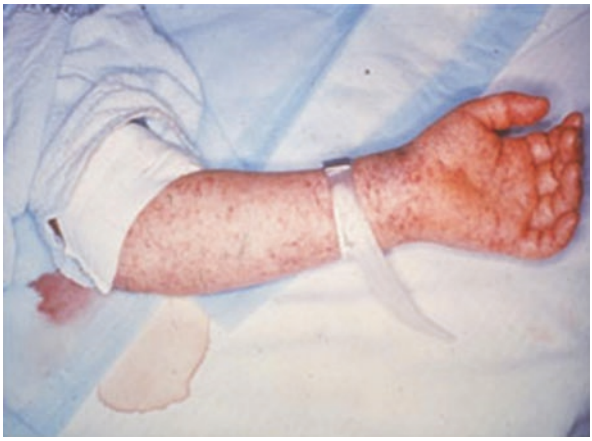

Fig. 26.3 Rocky Mountain spotted fever rash (From: CDC)

- *Rickettsia rickettsii* – Rocky Mountain spotted fever:
  - Transmitted by members of the *Ixodidae* tick family (hard ticks); also the natural reservoirs
  - Initial symptoms: fever, nausea, vomiting, severe headache, muscle pain, and lack of appetite
  - Later signs and symptoms: maculopapular rash (Fig. 26.3), petechial rash, diarrhea, and abdominal pain

- Can be a very severe long-term disease that requires hospitalization; blood vessel disruption in the respiratory system, CNS, gastrointestinal system, or kidneys

Virulence Factors (Sahni et al. 2013)

- Obligate intracellular pathogen
- Prefers to infect vascular endothelial cells in blood vessels, leading to multiple dysfunctions of the cardiovascular, respiratory, and neural endothelium
- Adherence to host cells/tissues:
  - **Sca** (surface cell antigen) (Balraj et al. 2009; Gillespie et al. 2015):
    - Family of surface proteins:
      - β-barrel autotransporter (T5SS) proteins
      - Seventeen members (**Sca0–Sca16**) in nine *Rickettsii* genomes
    - **rOmpA (Sca0)** (Li and Walker 1998):
      - Involved in adherence and invasion of host cells
    - **rOmpB (Sca5)** (Uchiyama et al. 2006):
      - Predominant component of S-layer
      - Interacts with host Ku70 subunit of DNA-dependent protein kinase in areas of host lipid rafts; interaction promotes actin cytoskeletal recruitment to the membrane (Martinez et al. 2005)
    - **Sca1, Sca2, and Sca4** (Chan et al. 2010; Gillespie et al. 2015):
      - Also play a role in adherence and actin polymerization (see below)
  - **Adr1 and Adr2** (Renesto et al. 2006; Vellaiswamy et al. 2011):
    - Potential adhesins
- Host cell invasion and growth:
  - Enter host cells through classic actin-dependent zipper mechanism of induced phagocytosis (Walker 1984)
  - Rapidly escape the phagosome to multiply in the cytosol of infected cells
  - *R. rickettsii* and other SFG pathogens can co-opt the host actin cytoskeleton for cell-to-cell motility, but *R. prowazekii* cannot; they are released through cell lysis (Heinzen et al. 1993)
  - Phagosome escape:
    - **PLD** (Renesto et al. 2003; Whitworth et al. 2005):
      - Phospholipase D activity

- Disrupts the phagosome membrane; major effector of phagosome escape

- **TlyC** (Whitworth et al. 2005):
  - Putative hemolysin C that has membranolytic activity
- **Pat1 and Pat2** (Rahman et al. 2013):
  - Phospholipase A_2 (PLA_2) activity
  - Pat1 is only found in TG pathogens; Pat2 is in TG and SFG pathogens

– Actin-based motility – only seen in SFG group:

- **RickA** (Gouin et al. 2004; Jeng et al. 2004):
  - Involved in promoting the nucleation of host actin polymerization
  - Works through the Cdc42 GTPase and the mammalian Arp2/3 complex
  - Used for cell-to-cell motility
  - Similar to mammalian Wiskott–Aldrich syndrome protein (WASP) and *Listeria monocytogenes* ActA
- **Sca2**: similar to mammalian formins; actin nucleators
- **Sca4**: binds host vinculin; anchors actin to the membrane
- **RpRalF** (Alix et al. 2012):
  - *R. prowazekii*-secreted effector
  - May have guanine nucleotide exchange factor (GEF) activity and play a role in actin nucleation
  - Probably secreted through Type 4 secretion system (T4SS) (Gillespie et al. 2015)

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Chapter 27
Salmonella spp.

Genomics, Morphologies, and Growth Characteristics

- **Genomics:**
  - *Salmonella enterica* serovar Typhimurium LT2 chromosome: 4,857,432 bp; 4,489 predicted ORFs (McClelland et al. 2001)
  - *Salmonella enterica* serovar Typhi CT18 chromosome: 4,809,037 bp; 4599 predicted ORFs (Parkhill et al. 2001)
- **Cell morphology:**
  - Rod-shaped cells (Fig. 27.1)
  - Flagella (peritrichous (Fig. 27.1)):
    - Swimming motility is essential for *Salmonella* cells to approach and attach to the intestinal epithelium
    - Immunostimulatory
  - Lipopolysaccharide (LPS): immunostimulatory; associated with inflammation and endotoxic shock
- **Gram stain:**
  - Gram negative
- **Growth:**
  - Facultative anaerobes: catalase positive, oxidase positive
  - Reservoirs: poultry, pet birds, reptiles pet turtles, livestock; contaminated soil and water
  - Excellent biofilm formers (see below)

D.I. Johnson, *Bacterial Pathogens and Their Virulence Factors*,
https://doi.org/10.1007/978-3-319-67651-7_27

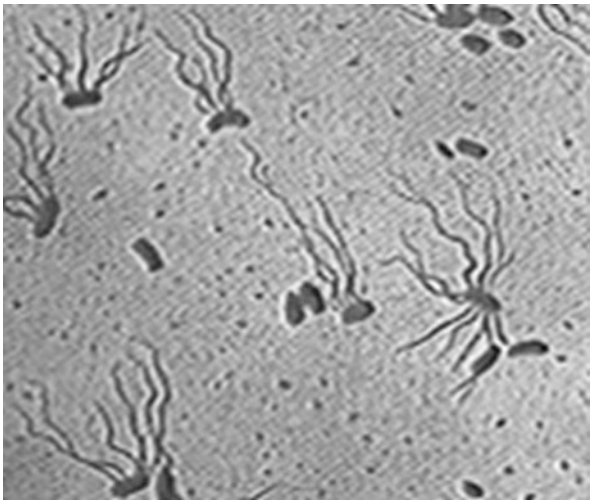


Fig. 27.1 *S. enterica* serovar Typhi cells (From: wikidoc.org)

- Two species, six subspecies:
  - *Salmonella enterica* subspecies: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), *indica* (VI)
    - ***S.* Enteritidis**: *Salmonella enterica* subspecies *enterica* serovar Enteritidis
    - ***S.* Typhimurium**: *Salmonella enterica* subspecies *enterica* serovar Typhimurium
    - ***S.* Typhi**: *Salmonella enterica* subspecies *enterica* serovar Typhi
    - ***S.* Paratyphi**: *Salmonella enterica* subspecies *enterica* serovar Paratyphi
  - *Salmonella bongori* (subspecies V; rare)
  - >2500 serotypes; 99% of clinical isolates belong to subspecies I *enterica*
    - Based on O antigens (LPS) and H antigens (flagella)
    - Also *S.* Typhi Vi antigens (capsule)

Disease States Associated with *Salmonella* spp. (Fabrega and Vila 2013; Wiedemann et al. 2014)

- Zoonotic pathogen: transmitted from animal reservoirs to humans
- Non-typhoidal salmonellosis:
  - Caused by strains other than *S.* Typhi
  - Foodborne salmonellosis:

- Enterocolitis: most common disease state
  - Primarily caused by *S.* Enteritidis; *S.* Typhimurium
  - Must ingest 10^6–10^8 cells for symptoms; appear 6–48 h post ingestion
  - Sources: contaminated chicken meat, contaminated eggs, direct contact with infected animals
  - Massive inflammatory symptoms: fever, diarrhea, non-bloody stools, abdominal cramps, nausea, vomiting
  - Self-limiting; can cause serious life-threatening illness (dehydration) in immunocompromised patients, elderly, and very young

– Invasive septicemia:

- Dissemination of *S.* Typhimurium in the bloodstream (bacteremia)
- Usually progresses to the liver and spleen; leads to massive inflammatory reaction

• Typhoidal salmonellosis:

– Paratyphoid fever: *S.* Paratyphi; less severe symptoms
– Typhoid fever: *S.* Typhi:

- Systemic disease: if untreated, fatal in 10–30% of cases
- Early symptoms (0–2 weeks):
  - Fever (often as high as 103–104 °F), rash, headache, weakness and fatigue, abdominal pain, diarrhea (rare; more common in children), or constipation
- Late symptoms (3–4 weeks):
  - High fever, diarrhea (“pea soup”), distended abdomen, extreme weight loss, delirium
- Complications:
  - Intestinal hemorrhage; usually not fatal
  - Intestinal perforation in distal ileum; frequently fatal
  - Encephalitis
  - Neuropsychiatric symptoms (“muttering delirium”)
  - Metastatic abscesses, cholecystitis, endocarditis, osteitis
- Dissemination:
  - Transcytosis through intestinal epithelial M cells and invasion into phagocytic cells, predominantly macrophage; protects against host immune system; doesn’t elicit an inflammatory reaction
  - Travel in macrophage to lymph nodes and other tissues (liver, spleen, bone marrow, gall bladder):
    – Persists in gall bladder (carrier state)
    – Forms biofilms on gall stones; protects against bile
  - Shed in bile back through intestinal epithelium

Virulence Factors

- It is important to note that with so many different *S. enterica* strains and serotypes, not all of the virulence factors discussed below will be encoded or expressed in each strain
- Many of these virulence factors are encoded on *Salmonella* pathogenicity islands (SPI); – 21 SPIs; SPI-1, SPI-2, SPI-3, SPI-4, SPI-5 encoded genes play the predominate role in virulence
- Adherence to host cells: intestinal epithelial M cells within Peyer's patches.
  - Fimbrial adhesins (Townsend et al. 2001; Wagner and Hensel 2011; Wiedemann et al. 2014):
    - At least 13 different fimbrial operons
    - *agf* (*csg*), *fim*, *pef*, *lpf*, *bcf*, *saf*, *stb*, *stc*, *std*, *stf*, *sth*, *sti*, and *stj*.
    - Not all are expressed in every *S. enterica* strain; most are not expressed in vitro, and it is unclear which are expressed in human hosts
    - Bind to host cells with different specificities
    - **Agf** (Collinson et al. 1996):
      - Curli fimbriae; thin aggregative fimbriae (Tafi); chaperone-usher assembly mechanism
      - Encoded by divergent *agfDEFG* and *agfBAC*; AgfA is primary structural subunit
      - Attach to intestinal microvilli; interact with extracellular matrix fibronectin and other glycoproteins
      - Important for biofilm formation; aggregation with other bacterial cells
    - **Type 1 fimbriae** (Korhonen et al. 1980):
      - Chaperone-usher assembly mechanism
      - Encoded by *fimAICDHF* operon
      - **FimA**: major structural subunit
      - **FimH**: tip adhesin; binds to host laminin through its terminal α-D-mannose residues; plays a role in T3SS-1 independent uptake into host cells
      - Can undergo phase variation for immune system evasion
    - **Lpf (long polar fimbriae)** (Bäumler et al. 1996b):
      - Chaperone-usher assembly mechanism
      - Encoded by the *lpfABCDE* operon
      - Attachment to intestinal Peyer's patches
      - Plays a role in biofilm formation
      - Can undergo phase variation for immune system evasion

- **Pef (plasmid-encoded fimbriae)** (Bäumler et al. 1996a):
  - Encoded by *pefABCD* operon on pSLT plasmid
  - Attachment to intestinal microvilli; interact with Lewis-X blood group carbohydrate
  - Plays a role in biofilm formation
  - Can undergo phase variation for immune system evasion
- **Std** (Chessa et al. 2009):
  - Chaperone-usher assembly mechanism
  - Binds to a host cell receptor containing terminal α(1-2) fucose residues
  - **StdA**: major pilin subunit
- **Type IV pili** (Wagner and Hensel 2011; Zhang et al. 2000):
  - Only found in *S.* Typhi
  - Required for twitching motility, adherence, and invasion
  - Encoded by *pil* operon on SPI-7

– Non-fimbrial adhesins:

- T5SS (type 5 secretion system): autotransporters
  - **MisL** (Dorsey et al. 2005):
    – Binds extracellular matrix fibronectin
    – Encoded on SPI-3
  - **SadA** (Raghunathan et al. 2011):
    – Trimeric adhesin
    – Important for adherence and biofilm formation
    – Does not bind extracellular matrix proteins; role in virulence is unclear
  - **ShdA** (Kingsley et al. 2002):
    – Binds extracellular matrix fibronectin and collagen I
    – Encoded on CS54 genomic island
- T1SS (type 1 secretion system):
  - **SiiE** (Gerlach et al. 2007):
    – Large secreted adhesin
    – Encoded on SPI-4; *siiABCDEF* operon encodes adhesin and T1SS
    – Adherence to epithelial cells; interacts with *N*-acetyl-glucosamine and α-2,3-linked sialic acid terminal residues on host proteins and sugars
    – Adherence is required for T3SS-1-dependent invasion

- **BapA** (Latasa et al. 2005):
  - Large secreted adhesin; contains numerous bacterial immunoglobulin-like domains
  - Important for colonization and biofilm formation
- **SivH** (Kingsley et al. 2003):
  - Involved in colonization and host cell invasion
  - Homologous to *Yersinia pseudotuberculosis* invasin and *E. coli* intimin
  - Encoded on CS54 genomic island
- **RatB** (Kingsley et al. 2003):
  - Involved in colonization
  - Encoded on CS54 genomic island

– LPS: role for the LPS in adherence is unclear
– Flagella: role for flagella in adherence is unclear

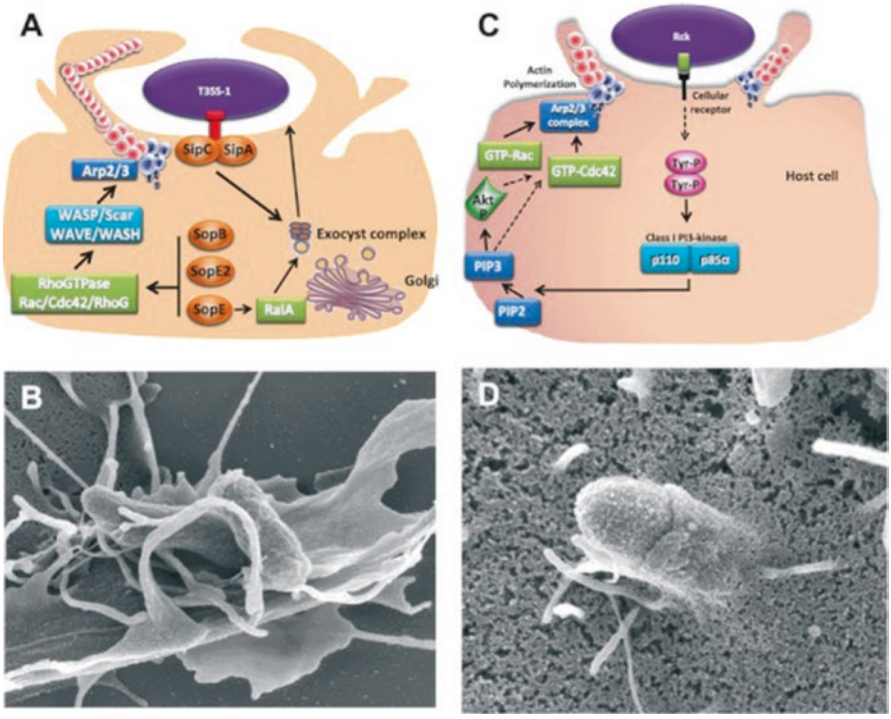


Fig. 27.2 *S. enterica* trigger and zipper mechanisms (From: Velge et al. 2012)

- Growth in host milieu (facultative intracellular pathogen):
  - Host cell invasion (Fig. 27.2):
    - Uses both trigger mechanism (Fig. 27.2a, b; host cell membrane ruffling) and zipper mechanism (Fig. 27.2c, d; receptor-mediated entry) for internalization (Velge et al. 2012)
    - Both mechanisms involve manipulation of the host actin cytoskeleton
    - Trigger mechanism (Galán 2001):
      - **T3SS-1** (type 3 secretion system-1) dependent
      - Injects at least 15 effectors into host cytoplasm
      - Disrupt the host actin cytoskeleton, leading to induction of the massive inflammatory processes associated with non-Typhi serovars
      - Encoded on Salmonella pathogenicity island 1 (SPI-1):
        - **SopA** (Zhang et al. 2006):
          - Ubiquitin ligase
          - Plays a role in inflammation and neutrophil transepithelial migration
        - **SopB (SigD)** (Zhou et al. 2001):
          - Inositol 3-phosphatase (IPase)
          - Activates SGEF (SH3-containing GEF) for RhoG (Patel and Galán 2006)
          - Trigger actin remodeling
          - Also induces signaling pathways that stimulate pro-inflammatory cytokine production
        - **SopD** (Bakowski et al. 2007):
          - Acts with SopB to induce inflammation and fluid secretion
        - **SopE, SopE2** (Bakshi et al. 2000; Hardt et al. 1998):
          - Guanine nucleotide exchange factor (GEF) for Cdc42 and Rac1 GTPases; ~70% identical
          - Stimulates GDP/GTP exchange; converts GTPase to an active, GTP-bound form
          - Induces Arp2/3-dependent actin remodeling
          - Also induces signaling pathways that stimulate pro-inflammatory cytokine production
        - **SptP** (Fu and Galán 1999; Kaniga et al. 1996):
          - N-terminus (GTPase-activating protein (GAP) for Cdc42, Rac1):
            - Converts GTPases back to an inactive GDP-bound form
            - Counteracts SopE, SopE2 function; allows actin cytoskeleton to recover; reverses inflammatory responses

- C-terminus (tyrosine phosphatase activity):
  - Reverses phosphorylation events that occur upon *S. enterica* invasion

– **SipA, SipC** (Myeni and Zhou 2010; Zhou et al. 1999):
- Part of the translocase machinery
- Binds to host F-actin
- Nucleates and promotes F-actin bundling; stabilizes actin filaments

– **SipB** (Hersh et al. 1999):
- Part of the translocase machinery
- Binds and activates caspase-1
- Induces inflammasome-dependent pyroptosis in macrophage
- Homologous to IpaB from *Shigella flexneri*

- Zipper mechanism (T3SS-1 independent):
  - **Rck** (Ho et al. 2010; Rosselin et al. 2010):
    – 19 kDa outer membrane protein (OMP); homologous to *S.* Typhimurium PagC and *Yersinia enterocolitica* Ail
    – Encoded on virulence plasmids
    – Binding to its host cell receptor induces cell signaling pathways that result in the activation of the Rac1 and Cdc42 GTPases
    – Leads to induction of Arp2/3-dependent actin polymerization
    – Also displays serum resistance through binding host complement factor H (fH)
  - **PagN** (Lambert and Smith 2009):
    – 26 kDa OMP invasin
    – Binds heparin sulfate proteoglycans

– Formation of replicative niche (Fig. 27.3):
- Internalized *Salmonella* cells manipulate the host endocytic trafficking machinery to block early endosome to late endosome maturation and subsequent lysosome fusion; results in the creation of a protected replicative environment within *Salmonella*-containing vacuoles (SCVs).
- Limits inflammation; activates pro-survival, anti-apoptotic mechanisms
- SCVs migration to the perinuclear space induces the formation of membrane tubules called *Salmonella*-induced filaments (SIFs); SIF formation signals replication to begin.
- **SopB** (see above):
  - IPase activity; secreted by T3SS-1
  - Blocks endosome maturation and lysosome fusion

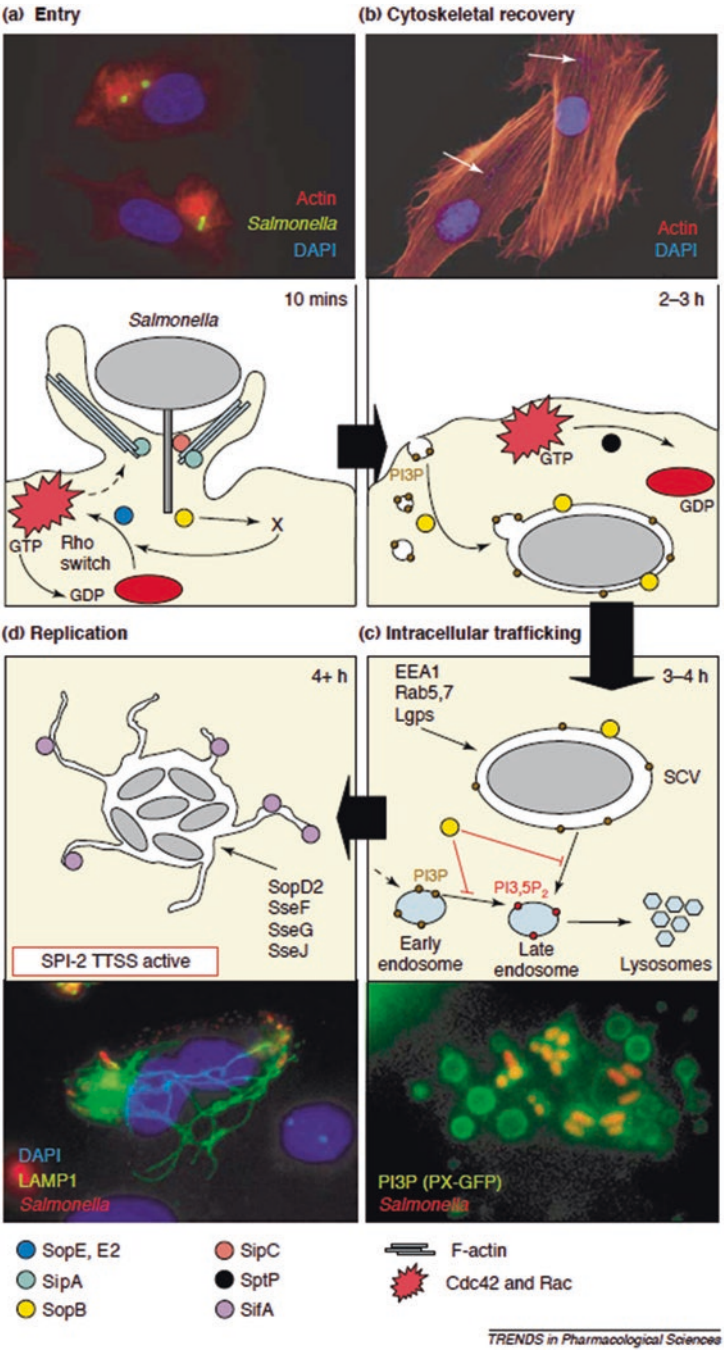


Fig. 27.3 *S. enterica* cell invasion and survival (From: Patel et al. 2005)

- **T3SS-2 (type 3 secretion system-2)**:
  - Encoded on SPI-2; only expressed after cell entry
  - Secretes more than 30 effectors from the SCV into the host cytosol
  - Essential for the integrity of the SCV
  - Responsible for SIF formation and replication within the SIF
  - **SseBCD** (Nikolaus et al. 2001):
    - Translocase machinery; secretes effectors into the host cytosol
  - **SifA** (Jackson et al. 2008):
    - Binds host SKIP (SifA and kinesin-interacting protein)
    - Blocks Rab9 GTPase function in endocytic trafficking
    - Promotes SIF formation; localizes to SCV and SIF
    - Stabilized by SipA binding (see above)
  - **SopD2** (Brumell et al. 2003):
    - 41% identical to SopD (see above)
    - Blocks endocytic trafficking by blocking Rab7 GTPase
  - **SsaB (SpiC)** (Uchiya et al. 1999):
    - Inactivates host Hook3 protein
    - Inhibits endocytic trafficking by blocking SCV-lysosome fusion
  - **SpvB** (Lesnick et al. 2001):
    - Adenosine diphosphate ribosyl transferase (ADPRT) activity
    - ADP-ribosylates actin; blocks vacuolar-associated actin polymerization (VAP) around the SCV
    - Triggers apoptosis of infected cells late in infection
    - Facilitates host dissemination associated with severe systemic infection
    - Part of the *spvRABCD* operon; located on pSLT virulence plasmids found in *S. enterica* serovar I strains
  - **SpvC** (Mazurkiewicz et al. 2008):
    - Phosphothreonine lyase activity; removes phosphate groups from host signaling proteins
    - Deactivates inflammatory signaling pathways (anti-inflammatory)
    - Can be secreted by T3SS-1 and T3SS-2
    - Located on pSLT virulence plasmids
  - **SspH2** (Miao et al. 2003):
    - Interacts with actin-remodeling proteins filamin and profilin
    - Plays a role in vacuole-associated actin polymerization (VAP) around the SCV

- **SseI** (Miao et al. 2003):
  - Interacts with actin-remodeling proteins filamin and profilin
  - Plays a role in VAP around the SCV
- **SseJ** (Ruiz-Albert et al. 2002):
  - Acyltransferase/lipase activity
  - Recruits active RhoA to the SCV; contributes to SIF formation
- **SseF, SseG** (Kuhle and Hensel 2002):
  - Interact with each other; localize to SCV and SIF
  - Function in SCV migration and microtubule reorganization necessary for SIF formation

– Iron acquisition:

- Fe^{+3} siderophores:
  - **Enterochelin (enterobactin)** (Pollack and Neilands 1970; Raymond et al. 2003):
    - Minor siderophore in *S. enterica*; major siderophore in *E. coli*
    - Cyclic polyester containing three DHBS (*N*-(2,3-dihydroxybenzoyl)-L-serine) residues
  - **Salmochelin** (Hantke et al. 2003; Muller et al. 2009):
    - Major siderophore
    - Enterochelin derivative; contains DHBS linked by a glucose residue; increases the hydrophilicity of enterochelin
    - Synthesis, excretion, and uptake are mediated by the *iroBCDEN* operon
- Expression is regulated by Fe^{+2}-bound Fur repressor and RhyB sRNA molecules

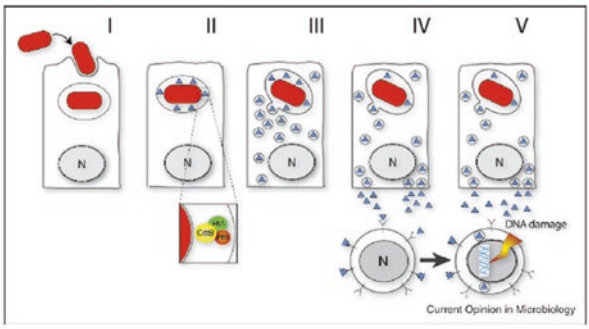
– **Biofilm formation** (Fabrega and Vila 2013; Wolska et al. 2016):

- Induced in stationary phase, reduced nutrient availability, aerobic conditions, low osmolarity, and low temperature
- Primarily depends on the secretion of cellulose exopolysaccharide and Agf curli fimbriae
- Also uses the BapA surface protein and the type I, Lpf, and Pef fimbriae
- *S.* Typhi forms biofilms on gall stones within the gall bladder; protects against antimicrobial effects of bile, leading to a carrier state

- Damage to host cells:

– **LPS** (Patel and McCormick 2014):

- Endotoxin activity
- Bacteremia triggers TLR4-dependent inflammatory reactions that lead to a cytokine storm and septic shock



Model for typhoid toxin delivery. After internalization (I) *S.* Typhi reaches a compartment where expression of *cdtB*, *pltA*, and *pltB* can take place (II). The CdtB/PltA/PltB complex is secreted from the bacteria into the lumen of the *S.* Typhi containing vacuole and it is recognized and packaged into transport carriers (III). The complex is then transported to the plasma membrane and secreted to the extracellular medium (IV) from where it can target susceptible non-infected cells (e.g. cells of the immune system) and induce DNA damage (V). Infected cells that do not express a receptor for the toxin would be resistant to the toxin and provide a safe haven for the bacteria (IV and V) (adapted from [13••]).

Fig. 27.4 Typhoid toxin delivery (From: Spano and Galan 2008)

- **Typhoid toxin** (Spano and Galan 2008; Spano et al. 2008):
  - Only found in *S.* Typhi and *S.* Paratyphi
  - **CdtB-PltA-PltB** (Fig. 27.4):
    - Forms a tripartite A_2B_5 complex (CdtB)(PltA)$(PltB)_5$ (Song et al. 2013)
    - Only toxigenic when all three subunits are bound together
    - Contains two separate biochemical activities
    - **CdtB**:
      - A subunit of the tripartite family of A-B cytolethal distending toxins (Cdts); also found in *Haemophilus ducreyi* and *Campylobacter jejuni*:
        - The usual receptor B subunits (CdtA, CdtC) are not encoded in *S.* Typhi
      - DNAase I: dsDNA break activity
      - DNA damage activates a cell cycle checkpoint; leads to G2 phase arrest (epithelial cells) or cell apoptosis (lymphocytes and monocytes)
      - Expression is only induced after cells are internalized into the SCV
      - CdtB must be exported from the SCV to the extracellular medium in order to invade and destroy other uninfected host cells
      - Travels through autocrine and paracrine pathways for host dissemination
        - PltA and PltB: needed for export from SCV and invasion of non-infected cells

- **PltA**:
  - Homologous to pertussis toxin ADP-ribosyltransferase A subunit
  - ADP-ribosylates $G\alpha_i$ protein; inhibits cell signaling activity
  - This activity is not needed for transport or activity of CdtB
- **PltB**:
  - Homologous to one subunit of the pertussis toxin B subunit
  - Forms a homopentameric polymer in host cell membranes
  - Binds to host *N*-acetylneuraminic acid (Neu5Ac) terminated glycan molecules (Deng et al. 2014)

– **ClyA (HlyE, SheA)** (Oscarsson et al. 2002; Wallace et al. 2000):

- Pore-forming cytolysin; found in *S.* Typhi and *S.* Paratyphi
- Encoded by SPI-18
- Homologues found in *E. coli and S. flexneri*

- Evasion of host immune system (Keestra-Gounder et al. 2015):
  – *S.* Typhi can evade the host innate immune system:
    - Ability to grow and disseminate within macrophage
      - **Mig-14; VirK** (Brodsky et al. 2005):
        – Important for survival within host macrophage
        – Blocks binding of CRAMP (cathelin-related antimicrobial peptide); high levels in macrophages
        – Provides resistance to the antimicrobial peptides polymyxin B and protegrin-1
      - Blocks recruitment of phagocyte NADPH oxidase to SCV (Vazquez-Torres et al. 2000)
    - **Vi capsular** polysaccharide antigen expression (Raffatellu et al. 2006):
      - Contains polymers of α-1,4 (2-deoxy)-2-*N*-acetylgalacturonic acid
      - Biosynthetic genes are located on SPI-7, which is absent in non-Typhi serovars
      - Blocks opsonophagocytosis and neutrophil infiltration
      - Increases resistance to complement-mediated lysis
    - Repression of flagella expression:
      - Lack of flagella blocks TLR5-dependent inflammation
      - Mediated by the TviA auxiliary regulatory protein; represses flagella and induces capsule formation (Winter et al. 2009)
      - TviA expression is autoregulated in conjunction with the RcsB response regulator of the RcsC-RcsD-RcsB TCS

- *S.* Typhimurium and *S.* Enteritidis cannot evade the host immune system:
  - Toll-like receptors (TLRs) recognize the PAMPs LPS (TLR4) and flagellin (TLR5); induce pro-inflammatory pathways
  - T3SS-1 secretes effectors that induce cytokine secretion; leads to neutrophil infiltration and the inflammatory symptoms associated with non-Typhi infections
  - Also functions to contain the localized infection, blocking dissemination

Regulation of Virulence Factor Expression

- Highly complex, interdependent transcriptional regulation involving many transcriptional regulatory factors, two-component phosphorelay systems (TCSs), and quorum-sensing (QS) systems (Fabrega and Vila 2013)
- Primarily associated with differential expression of the virulence factors encoded in SPI-1, SPI-2, and the pSLT virulence plasmid
- **HilA**:
  - Transcription activator
  - Encoded within SPI-1
  - Plays a major role in invasion:
    - *hilA* deletion phenotypes mimic SPI-1 deletion phenotypes
    - HilA activates the expression of all of the T3SS-1 genes encoded in SPI-1
    - Expression of HilA is affected by all the TCSs and the environmental signals encountered by *S. enterica* cells

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Chapter 28
Shigella spp.

Genomics, Morphologies, and Growth Characteristics

- **Genomics:**
  - *Shigella flexneri* serotype 2a chromosome: 4,607,203 bp; 4434 predicted ORFs (Jin et al. 2002):
    - pCP301 virulence plasmid: 221,618 kb; 267 predicted ORFs
      - Similar virulence plasmids (pINV) are found in all pathogenic *Shigella* spp. (The et al. 2016)
      - Contains ~31 kb pathogenicity island (SHI) that encodes ~34 genes that are necessary and sufficient for virulence, including the Mxi-Spa Type 3 secretion system (T3SS)
  - *Shigella dysenteriae* serotype 1 chromosome: 4,369,232 bp; 4557 predicted ORFs (Yang et al. (2005); genomic sequences for *S. sonnei* and *S. boydii* are also included)
- **Cell morphology:**
  - Rod-shaped cells (Fig. 28.1)
  - No flagellar motility
    - Flagellar genes are inactivated
    - Actin-based motility (ABM) is used within and between host cells (see below)
  - Lipopolysaccharide (LPS):
    - Immunostimulatory
    - Associated with inflammation and endotoxic shock

D.I. Johnson, *Bacterial Pathogens and Their Virulence Factors*,
https://doi.org/10.1007/978-3-319-67651-7_28

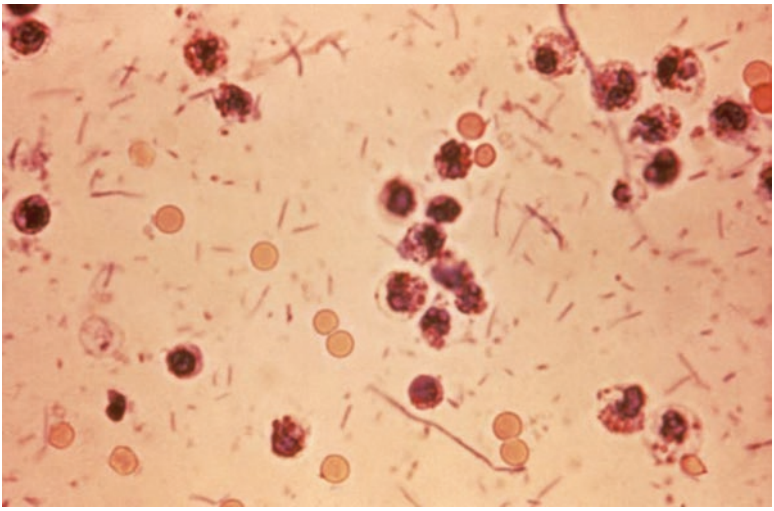

Fig. 28.1 *S. dysenteriae* cells in stool exudate (From: PHIL #6659)

- **Gram stain:**
  - Gram negative
- **Growth:**
  - Facultative anaerobes; oxidase negative
  - Found in fecal-contaminated food and water; endemic in tropical and subtropical regions
  - Four groups: phylogenetically almost identical to enteroinvasive *E. coli* (EIEC)
  - Sequence analyses indicate that pathogenic *Shigella* spp. evolved from noninvasive *E. coli* ancestor through the acquisition of virulence genes on plasmids and pathogenicity islands by horizontal gene transfer and the loss of antivirulence genes, such as porins, flagella, and fimbriae (Prosseda et al. 2012; Schroeder and Hilbi 2008)
    - *Shigella dysenteriae*: Group A (15 serotypes; based on LPS O-antigens)
    - *Shigella flexneri*: Group B (19 serotypes)
    - *Shigella boydii*: Group C (19 serotypes)
    - *Shigella sonnei*: Group D (1 serotype)

Disease States Associated with *Shigella* spp.

- Shigellosis (bacillary dysentery):
  - Human-adapted invasive disease; highly specific for primates
  - Transmission: fecal–oral route; contaminated food or water

- Invades and destroys intestinal epithelial cells (IECs) within the colon
- 5–15% of all diarrheal cases worldwide; ~150 million cases/year
- Localized infections; no dissemination throughout the host
- Highly infectious; ~10–100 cells infective dose; acid resistant, so can survive transit through the stomach
- Symptoms: inflammatory; moderate to severe diarrhea
    - Early symptoms: fever, watery diarrhea, cramping, abdominal pain
    - Later symptoms: bacillary dysentery – bloody, mucous-filled diarrhea
- *Shigella dysenteriae*:
    - Expresses Shiga toxin [serotypes 1, 4 (rare)]
    - Can lead to hemolytic uremic syndrome (HUS); uncommon in the USA
        - Lysis of red blood cells; low platelet count; kidney failure
        - Symptoms: vomiting, bloody diarrhea, lethargy, purpura, weakness
        - Leads to dehydration, anemia, and uremia
    - Can lead to inflammation-dependent neurological effects, such as seizures, paralysis, blindness
- *Shigella flexneri*:
    - Expresses ShET1 and ShET2 enterotoxins
    - Most common disease isolates outside the USA; 60% of the cases in developing nations
- *Shigella sonnei*:
    - Common infections in children <5 years old
    - Most common disease isolates in USA; 77% of cases in developed nations

Virulence Factors (Carayol and Tran Van Nhieu 2013; Mattock and Blocker 2017; Schroeder and Hilbi 2008)

- Facultative intracellular pathogen
- Adherence to host cells:
    - Fimbrial adhesins: none
        - Fimbriae genes are inactivated
        - Lack of fimbriae (and flagella) provide cells with the ability to evade the TLR-dependent host innate immune system
    - Non-fimbrial adhesins:
        - **IcsA (VirG)** (Brotcke Zumsteg et al. 2014):
            - Dual-function protein:
                - Adhesin activity

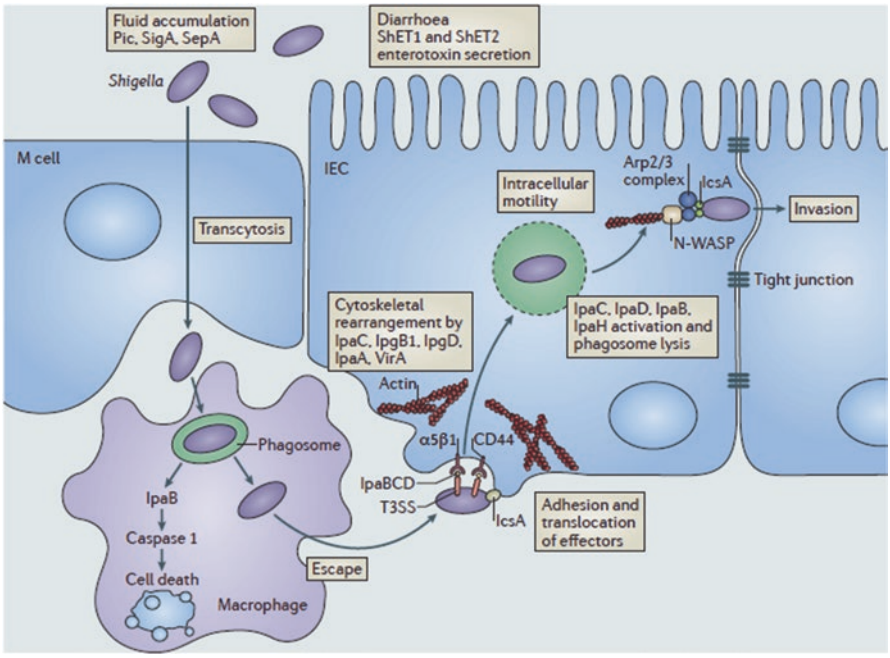

Fig. 28.2 Host cell invasion process (From: The et al. 2016)

  - Induced by the presence of bile salts
  - Helps Type 3 secretion system (T3SS)-based invasion
  - Nucleates actin-based motility (see below)

- **OspE1, OspE2** (Faherty et al. 2012b):
  - Outer membrane proteins
  - Interact with integrin-linked kinase (ILK) at focal adhesions
  - Induced by bile salts
  - Secreted through T3SS

- Host cell invasion: multistep process (Fig. 28.2):
  - Penetration of host mucus layer:
    - Expresses mucinases and neuraminidases (Haider et al. 1993); disruption of the mucosal layer can also lead to intestinal fluid accumulation.
    - **Pic** (Henderson et al. 1999):
      - Serine protease; mucinase activity
      - Auto-secreted protein; belongs to SPATE (serine protease autotransporters of *Enterobacteriaceae*) family (Dautin 2010)
      - Encoded in pathogenicity island SHI-1 (*she*) in *S. flexneri* 2A and *S. sonnei*; not found in *S. flexneri* 5A
      - Contributes to serum resistance by unknown mechanism

- **SepA (Shigella extracellular protein A)** (Benjelloun-Touimi et al. 1995):
  - SPATE protease; enterotoxic activity
  - Encoded on pINV virulence plasmid
- **EatA**:
  - Homologous to enterotoxigenic *E. coli* EatA
  - Predicted to degrade MUC2 mucin

– M cell transcytosis:

- Invasion and passage through M cells within intestinal Peyer's patches
- Stays within endosome for transit; little is known about the mechanism (Adam 2001)

– Macrophage phagocytosis and pyroptosis:

- After transcytosis across M cells, *Shigella* cells are phagocytosed by submucosal macrophage
- Rapidly lyse phagosomes and induce IpaB-dependent macrophage pyroptosis:
  - Due to binding and activation of caspase-1 by IpaB (see below)
  - Releases pro-inflammatory cytokines IL-1β and IL-18, resulting in the inflammatory response associated with shigellosis
- Macrophage pyroptosis releases *Shigella* into the submucosal space, where it can reinvade colonic IECs

– Reinvasion of IECs from basolateral side:

- Uses a trigger mechanism with the Mxi-Spa T3SS; similar to the *Salmonella enterica* mechanism:
  - Effector proteins promote actin cytoskeletal rearrangements, resulting in localized filopodia and lamellipodia formation (membrane ruffles)
  - Membrane ruffles mediate engulfment through macropinocytosis
- **Mxi-Spa T3SS** (Fig. 28.3):
  - **Mxi** (membrane excretion of Ipa) and **Spa** (surface presentation of antigens) genes:
    – Structural and assembly proteins of T3SS
    – **MxiH**: needle structural subunit
  - **IpaB, IpaC, IpaD**:
    – Translocator proteins
    – Localized to the T3SS tip complex in the presence of bile salts
    – Regulate the binding to filopodia on IECs and the secretion of effectors

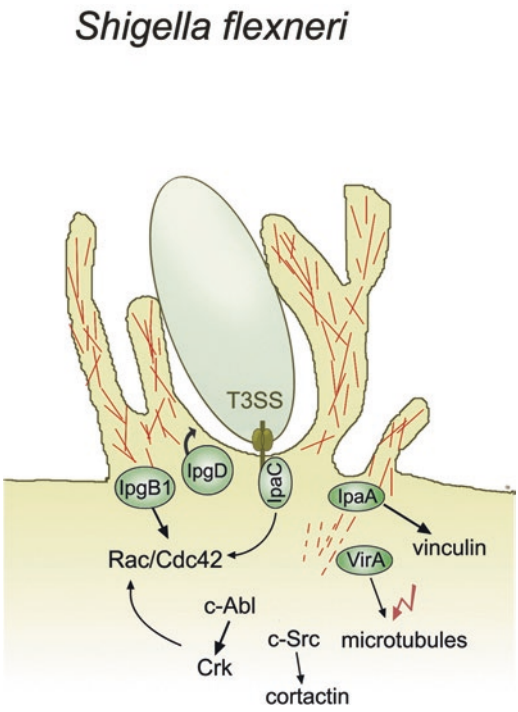

Fig. 28.3 T3SS effectors (From: Rottner et al. 2005)

- Complex binds to α5β1 integrin receptor within cholesterol-rich lipid rafts in IEC membranes
- **IpaB** (Picking and Picking 2016):
  - Multifunctional protein
  - Forms the pore complex (translocon) with IpaC that is necessary for effector secretion
  - Binds to cholesterol and host hyaluronic acid receptor CD44 within the IEC membrane; plays a role in recognition of IEC membrane
  - Binding and activation of cytosolic caspase-1 induce macrophage pyroptosis
  - Also promotes survival within infected IECs by inducing cell cycle arrest (see below)
  - Stabilized in the *Shigella* cytoplasm by forming a heterodimeric complex with the IpgC chaperone
- **IpaC** (Nhieu et al. 1999):
  - Forms the pore complex (translocon) with IpaB that is necessary for effector secretion

- Induces Arp2/Arp3-dependent actin polymerization through activation of a Cdc42 GTPase-dependent and Src kinase-dependent signaling cascade (Mounier et al. 2009)
- Major role in lysing phagosome (see below)

– **IpaD** (Espina et al. 2006):

- Forms a homopentameric structure
- Major component of tip complex
- Binds bile salts; induces invasion

- **IpaA** (Bourdet-Sicard et al. 1999):
  - Binds and activates host vinculin; reinforces actin cytoskeleton interactions with host integrins during the invasion process
  - Also induces actin depolymerization post-invasion
- **IpgD** (Niebuhr et al. 2002):
  - Inositol 4-phosphatase
  - Dephosphorylates phosphatidylinositol 4,5-bisphosphate [PtdIns-(4,5)P_2] to phosphatidylinositol 5-monophosphate [PtdIns-(5)P], facilitates uptake into host cells by disrupting cortical actin structures
  - Also blocks CXCL12-dependent T-cell migration
- **IpgB1, IpgB2** (Handa et al. 2007; Killackey et al. 2016):
  - IpgB1 binds to host ELMO protein:
    – Co-localizes with ELMO and Dock180 in membrane ruffles
    – IpgB1 stimulates membrane ruffling through RhoA–ELMO–Dock180 complex by acting as a guanine-nucleotide exchange factor (GEF) for Rac1 and Cdc42 GTPases
  - IpgB2 acts as a GEF for RhoA
- Following internalization, the *Shigella*-containing vacuole (SCV) is rapidly disrupted; allows for growth and replication in the IEC cytosol
  - **IpaB, IpaC, IpaD** (see above):
    – Form a vacuolytic pore within SCV membrane
    – IpaC plays the major role in this membrane disruption
    – IpaD induces B-cell apoptosis
  - **IpgD** (see above):
    – Recruits Rab11 GTPase to site of invasion; needed for subsequent SCV rupture and rapid escape from the SCV (Mellouk et al. 2014)

- Intracellular growth:
  - **OmpC; OmpF** (Bernardini et al. 1993):
    - Outer membrane porins
    - Form hydrophilic channels in membrane
    - Essential for virulence
    - Regulated by the EnvZ–OmpR two-component phosphorelay system (TCS) in response to osmolarity changes
  - *Shigella* spp. use multiple T3SS effectors to block host cell signaling pathways (primarily MAPK and NF–κB signaling) that promote inflammation and apoptosis in order to enable replication and survival within the infected IECs
    - Blocks secretion of pro-inflammatory cytokines by disrupting Golgi and ER–Golgi trafficking
  - **IpaB** (Iwai et al. 2007):
    - Interacts with the anaphase-promoting complex (APC) inhibitor MAD2L2 (mitotic arrest deficient 2-like protein 2; MAD2B)
    - Induces a G2/M phase cell cycle arrest; facilitates growth within IECs
  - **IpaH** (Ashida and Sasakawa 2015):
    - 12 *ipaH* genes; IpaH0722 encoded on the chromosome; IpaH9.8 encoded on the virulence plasmid
    - E3 ubiquitin ligase activity; auto-inhibition is released by substrate binding
    - Blocks NF–κB-dependent inflammation; promotes cell survival
    - Also induces caspase-1-dependent inflammasome activation and macrophage pyroptosis; enhances escape from macrophage
  - **IpaJ** (Burnaevskiy et al. 2013):
    - Cysteine protease and de-myristoylation activity
    - Cleaves the ARF1p and ARF2p GTPases at the N-terminal site of myristoylation
    - Leads to Golgi fragmentation
  - **OspB** (Ambrosi et al. 2015):
    - Activates MAPK pathways, inducing inflammation early in infection
    - May function with OspF in the nucleus to block MAPK-dependent inflammation late in infection
    - Blocks xenoautophagy
  - **OspC3** (Kobayashi et al. 2013):
    - Blocks activation of caspase-4

- Prevents heterodimerization of caspase-4-p19 and caspase-4-p10 subunits
- Inhibits pyroptosis within IECs

○ **OspF** (Li et al. 2007):

- Phosphothreonine lyase; removes the phosphate group from MAP kinases within the host nucleus
- Blocks MAPK activity and downstream NF–κB signaling; blocks inflammatory responses

○ **OspG** (Kim et al. 2005):

- Ser/Thr kinase activity
- Binds to E2~ubiquitin (E2~Ub) complex; inhibits TNFα-dependent degradation of IκBα signaling protein; blocks NFκB-dependent inflammation

○ **OspI** (Sanada et al. 2012):

- Deamidase activity
- Inactivates the E2 ubiquitin-conjugating enzyme Ubc13 by converting Gln-100 to Glu-100
- Blocks TRAF-6 ubiquitin ligase-dependent NF–κB signaling; blocks acute inflammation

○ **OspZ** (Newton et al. 2010):

- Predicted methyltransferase activity
- Blocks nuclear translocation of p65 subunit of NF–κB; blocks NFκB-dependent acute inflammation

– Intracellular motility and intercellular dissemination (Agaisse 2016):

○ Actin-based motility (ABM) propels cells through the cytoplasm and between adjacent IECs; same mechanism as *Listeria monocytogenes*
○ Intercellular spread requires the generation of cell protrusions at cell–cell junctions; mediated by host proteins (Valencia-Gallardo et al. 2015)
○ **IcsA (VirG)** (Brotcke Zumsteg et al. 2014; Egile et al. 1999):

- Autotransporter protein
- Polar localization in outer cell membrane; depends on IcsP and LPS; important for unidirectional ABM
- Mimics the Cdc42p GTPase by binding and autoactivating its effector, neural Wiskott–Aldrich syndrome protein (N-WASP)
- Induces ARP2-/ARP3-dependent actin polymerization

○ **IcsB** (Campbell-Valois et al. 2015; Campbell-Valois and Pontier 2016):

- Plays a role in autophagy escape:
  – Protects IcsA/VirG from ATG8-/LC3-dependent and Atg5-dependent xenoautophagy recognition during cell-to-cell dissemination

  - Blocks Atg5 binding to IcsA/VirG
- Functions with VirA to facilitate escape from LC3-containing vacuoles
- Binds cholesterol

- **VirA** (Campbell-Valois et al. 2015; Campbell-Valois and Pontier 2016):
  - GTPase-activating protein (GAP) for Rab GTPases
  - Blocks recruitment of autophagy protein ATG8/LC3 during cell-to-cell dissemination
  - Functions with IcsB to facilitate escape from LC3-containing vacuoles
  - Also induces the degradation of p53; blocks apoptosis

– Neutrophil recruitment (Schroeder and Hilbi 2008):
  - Growth of *Shigella* inside IECs induces IL-8 chemokine production
  - Leads to massive infiltration of neutrophils:
    - Kills *Shigella* and resolves infection; blocks dissemination to deeper tissues
    - Neutrophil elastase: degrades IpaB, IpaC, and IcsA; blocks phagosome destruction (Averhoff et al. 2008)
    - Neutrophils also contribute to the destruction of the intestinal epithelial layer – bacillary dysentery symptoms

- Iron acquisition (Wei and Murphy 2016):
  – Essential for intracellular growth
  – Fe^{+3} siderophores:
    - **Enterobactin**:
      - Not expressed in *S. flexneri* or *S. boydii* due to mutational inactivation
    - **Salmochelin**:
      - Only expressed in *S. dysenteriae*
    - **Aerobactin**:
      - Variants are encoded on SHI-2 and SHI-3
      - *IucABCD* operon: biosynthesis genes; not expressed in *S. dysenteriae*
    - **Ferric dicitrate**:
      - Uptake system
      - Encoded by *fec* genes encoded on SRL pathogenicity island
  – Heme uptake system:
    - **Shu system**:
      - Direct binding of heme or heme-containing proteins to ShuA receptor
      - Expressed in *S. dysenteriae* and *S. sonnei*

- Fe^{+2} uptake:
  - **Feo, Sit, Efe systems**:
    - Direct binding of Fe^{+2} to inner membrane receptors
- Expression is regulated by Fe^{+2}-bound Fur repressor and RhyB sRNA molecules

- Damage to host cells:
  - **LPS**:
    - Endotoxin activity
    - Bacteremia triggers TLR4-dependent inflammatory reactions that lead to a cytokine storm and septic shock
  - **Shiga toxin (Stx; verotoxin)** (Lee et al. 2016; Melton-Celsa 2014):
    - Only expressed in *Shigella dysenteriae* serotype 1 (serotype 4 rarely)
    - Homologues are expressed in enterohemorrhagic *E. coli* strains (Stx1a, Stx2a)
    - $A\text{-}B_5$ exotoxin:
      - Encoded by *stx* operon on lysogenic bacteriophage
      - A subunit:
        - Cleaved by furin or furin-like proteases into A1 and A2 subunits
        - Held together by disulfide bond, which is disrupted within the host ER; A1 enters the host cytosol
        - A1 has catalytic activity; A2 binds to B subunits
        - *N*-glycosidase catalytic activity:
          - Removes an adenine residue from the 28S rRNA of the 60S ribosome
          - Blocks binding to translation elongation factor 1 (EF1); inhibits host protein translation
          - Leads to ribotoxic stress response and ER stress response
          - Induces inflammation, apoptosis, autophagy (Fig. 28.4)
      - B subunits:
        - Forms homopentameric ring structure
        - Binds to glycosphingolipid globotriaosylceramide (Gb3; CD77) in cholesterol-containing lipid rafts
          - Gb3: ceramide lipid connected to [α-gal(1→4)-β-gal(1→4)-β-glc] trisaccharide (Lindberg et al. 1987)
        - Induces membrane invagination and internalization in endosomes

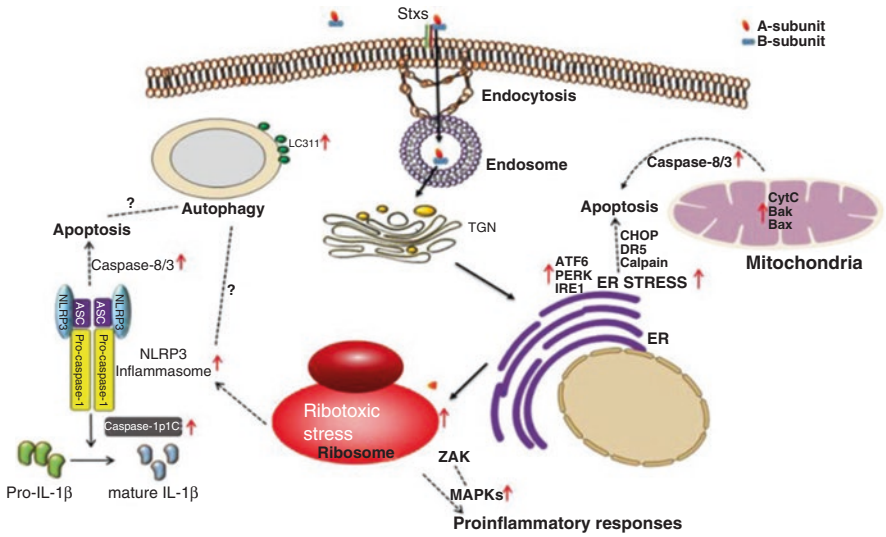

Fig. 28.4 Stx cellular effects (From: Lee et al. 2016)

– **ShET1** (Fasano et al. 1995):
  - Dimeric protein
  - Encoded by *set1A* and *set1B* genes within the SHI-1 pathogenicity island; only encoded in *S. flexneri*
  - Associated with watery diarrhea symptoms associated with *S. flexneri* infections (Faherty et al. 2012a)
– **ShET2** (Nataro et al. 1995):
  - Encoded by *ospD3* (*sen*) gene on virulence plasmid; only encoded in *S. flexneri*
  - Secreted by T3SS
  - Associated with watery diarrhea symptoms associated with *S. flexneri* infections (Faherty et al. 2012a)
– **SigA** (Al-Hasani et al. 2000, 2009):
  - SPATE protease
  - Cleaves host fodrin
  - Enterotoxic activity; plays a role in fluid accumulation
  - Encoded on SHI-1

Regulation of Virulence Factor Expression

- Regulation occurs in response to environmental factors within the human host; temperature, pH, osmolarity, iron concentrations (Fig. 28.5) (Di Martino et al. 2016; Picker and Wing 2016; Schroeder and Hilbi 2008)
  - **H-NS** (Picker and Wing 2016):
    - Histone-like nucleoid-structuring protein; H-NS paralogs **StpA, Sfh**
    - Binds to A-T-rich regions of DNA; oligomerizes along DNA, forming large H-NS:DNA complexes
    - Complexes silence the expression of virulence genes on the virulence plasmid under non-host conditions; <37°C; pH <7.4; low osmolarity

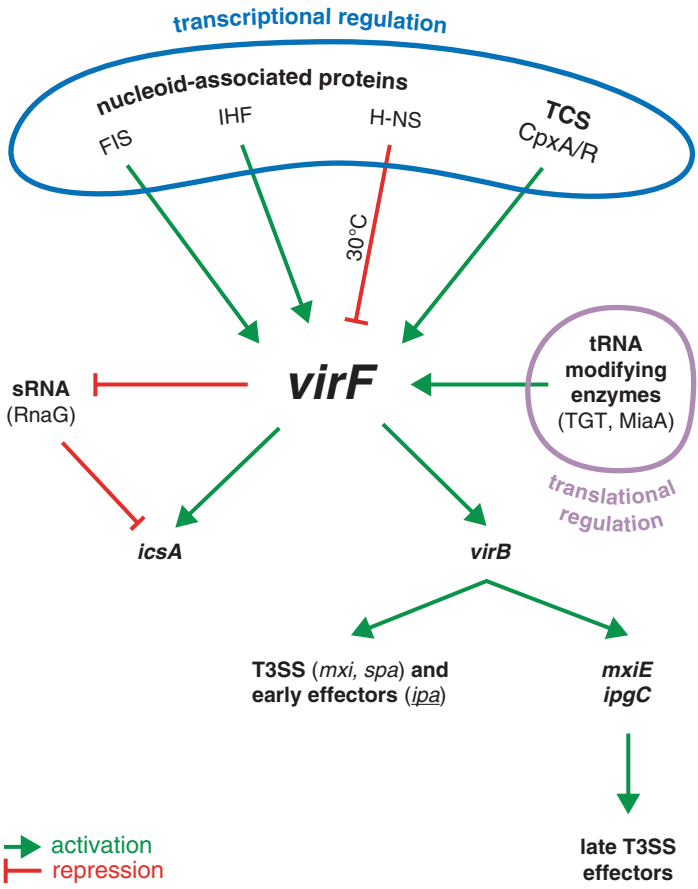

Fig. 28.5 Transcriptional regulation of virulence factors (From: Di Martino et al. 2016)

- At <37°C, H-NS:DNA complexes induce DNA bending that precludes RNA polymerase binding to promoter sequences; acts as a thermosensor
- At 37°C, DNA bending within the *virF* promoter is relaxed; allows RNA polymerase binding and triggers VirF expression; also involves the FIS and INH transcription factors

– **VirF** (Di Martino et al. 2016):

- Master regulator of virulence gene expression
- Member of the AraC family of transcription activators
- pH changes affect VirF expression; mediated by the CpxA-CpxR TCS
- Directly activates the transcription of the VirB anti-silencing factor as well as the IcsA actin nucleator (see above)

– **VirB**:

- Acts by transcriptional anti-silencing; relieves H-NS silencing, thereby leading to the transcriptional induction of >30 plasmid-encoded virulence genes
  - Induces the expression of an initial set of genes, including the T3SS and its effectors (Fig. 28.5)
  - Induces the expression of the MxiE transcription factor

– **MxiE**:

- Binds to IpgG co-activator
- Induction of the T3SS triggers MxiE-IpgG to transcriptionally activate the expression of a secondary set of T3SS effectors

– **sRNA riboregulators** (Fris and Murphy 2016):

- Function in rapid responses to changes in environmental conditions
- **CsrB-CsrC** (carbon metabolism); **RnaG** (temperature); **RyhB** (iron availability); **5′ UTR of ShuA** (ribothermometer for heme uptake)

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Chapter 29
Treponema spp.

Genomics, Morphologies, and Growth Characteristics

- **Genomics:**
  - *Treponema pallidum* chromosome: 1,138,006 bp; 1041 predicted ORFs (Fraser et al. 1998)
- **Cell morphology:**
  - Thin (0.2 μm), long (6–15 μm) spirochete (Fig. 29.1):
    - Tightly coiled corkscrew shape (Charon et al. 2012)
    - Three to six endoflagella (axial filaments):
      - Anchored at both ends of the cell and run lengthwise between the inner and outer membranes in the periplasmic space; enclosed in outer sheath
      - Causes twisting motion used for motility; important for dissemination in host
      - Endoflagella subunits (Champion et al. 1990; Lafond and Lukehart 2006):
        - FlaB1, FlaB2, and FlaB3 – flagellar core
        - FlaA subunit – outer sheath
  - Outer membrane – very fragile:
    - No LPS (endotoxin)
    - Not tightly associated with underlying peptidoglycan and cellular membrane
- **Gram stain:**
  - Gram negative: no LPS

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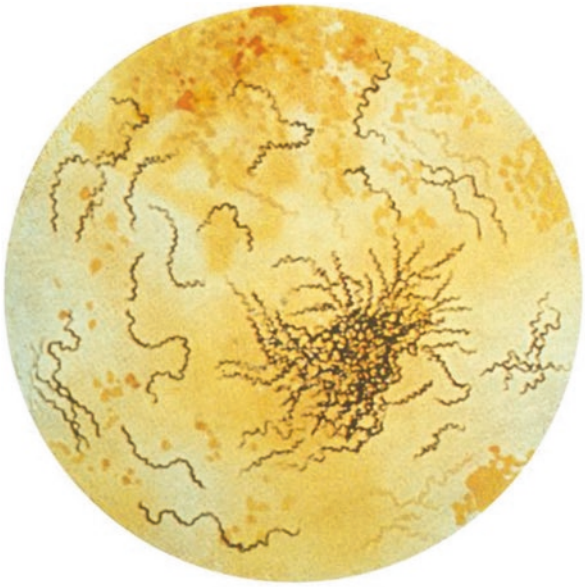

Fig. 29.1 *T. pallidum* cells; Levaditi stain (From: PHIL #2144)

- **Growth:**
  - Anaerobic or microaerophilic growth; no catalases or oxidases
  - Slow grower (30–50 h doubling time); metabolically crippled:
    - No Kreb's cycle or electron transport chain; gets ATP from glycolysis
    - No genes for synthesis of nucleotides, fatty acids, vitamins, cofactors, or amino acids; gets most of its nutrients through transport, not biosynthesis
  - Reservoir: humans (*T. pallidum*); survives poorly outside human host
  - At least 33 species – four *Treponema pallidum* subspecies and *T. denticola* are human pathogens:
    - *T. pallidum* subsp. *pallidum*: causes syphilis; sexually transmitted disease
    - *T. pallidum* subsp. *endemicum*: causes bejel (endemic syphilis); chronic disease of skin and tissues, usually found in arid environment
    - *T. pallidum* subsp. *carateum*: causes pinta; skin disease; usually found in Central America and South America
    - *T. pallidum* subsp. *pertenue*: causes yaws; non-sexually transmitted infection of skin, bones, and joints; usually found in the tropics
    - *T. denticola*: oral pathogen; periodontitis

Disease States Associated with *Treponema pallidum*

- Syphilis (Lafond and Lukehart 2006):
  - Sexually transmitted infection
  - Inflammatory disease of blood vessels and perivascular areas
  - Transmission requires direct skin or mucous membrane contact with infectious secretions from syphilitic lesions
  - Chronic disease: *T. pallidum* cells can persist for decades inside human hosts despite intense immune response
  - If untreated, disease has three stages:
    - Primary stage:
      - Localized infection
      - Sensitive to penicillins
      - Transmission – most often via genital contact; can be contracted in mouth, anal area, or other areas of skin:
        - Enters through breaches in the skin or penetrates mucous membranes
      - Multiplies quickly, disseminates through bloodstream from original infection site; can invade many tissue types
      - *T. pallidum* lipoproteins induce an inflammatory reaction in host blood vessels and capillary system at the original site of infection
      - Chancre appears 3–4 weeks postinfection (Fig. 29.2):
        - Ulcerated but typically non-purulent syphilitic lesion at the original site of infection
        - Usually not painful, so it can often be overlooked if it is located in areas that are not visible (e.g., anal or cervical area)
        - Usually spontaneously heals in 4–6 weeks
      - Also leads to regional lymphadenopathy

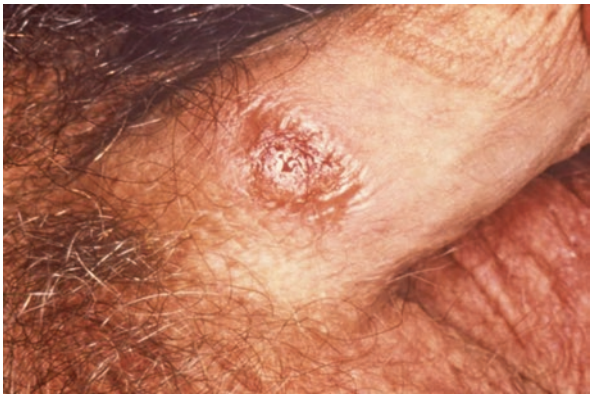

Fig. 29.2 Primary syphilis chancre (From PHIL #6758)

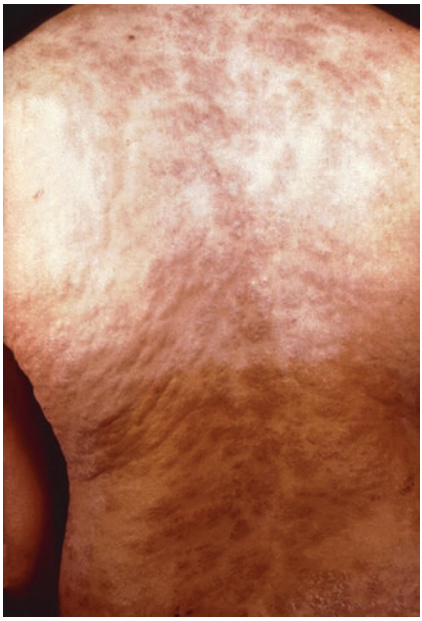

Fig. 29.3 Secondary syphilis lesions (From: PHIL #6756)

- Secondary stage:
  - Two to 20 weeks postinfection (if there is no treatment)
  - Sensitive to penicillins
  - Fever, sore throat, and generalized lymphadenopathy
  - Disseminated mucocutaneous rash: trunk, palms of the hands, and soles of the feet; crusting over (Fig. 29.3)
  - *Condylomata lata*:
    - Wart-like lesions on arms and genitals
    - ~5% of patients
  - Can lead to kidney damage and arthritis; due to deposition of antibody-antigen complexes
  - If there is no treatment; patient enters latency period:
    - After 4–10 years: ~70% of patients will display no progression of the disease
    - The other ~30% of the patients will go on to tertiary stage
- Tertiary stage:
  - Non-infectious period; symptoms are due to immunological reactions
  - Insensitive to penicillins
  - Gummas: necrotic granulomatous lesions mostly on skin and bones
  - Cardiovascular syphilis; aneurysms
  - Neurosyphilis: paralysis with dementia, seizures, blindness, meningitis, and other neurological symptoms

- Congenital syphilis:
  - *T. pallidum* cells passes through the placenta
  - Infants can be born with secondary or tertiary syphilis
- Chronic syphilis:
  - Can lead to spontaneous abortion, stillbirth, death of the neonate, or disease in the infant
  - *T. pallidum* cells can disseminate to immune-privileged sites and evade the immune system
  - Rare outer membrane proteins (Tpr proteins) are poorly antigenic; immunogenic proteins are usually periplasmic or attached to the inner membrane and only uncovered after *Treponema* cell death

Virulence Factors (Radolf et al. 2016)

- Adherence to host cells and extracellular matrix (ECM):
  - Extracellular pathogen, so adherence to host cells is important for infection
  - Afimbrial adhesins:
    - Bind to ECM components
    - **Tp0155, Tp0483, and Tp0136** (Brinkman et al. 2008; Cameron et al. 2004):
      - Fibronectin-binding proteins
    - **Tp0751** (Cameron 2003):
      - Laminin-binding protein
    - **BamA** (Desrosiers et al. 2011):
      - Outer membrane protein
      - Part of complex that inserts OMPs into the outer membrane
- Dissemination and growth within the host:
  - Capable of rapid dissemination and invasion of body tissues
  - Motility essential for dissemination and invasion
  - Induce host collagenase (MMP-1); breaks down ECM collagen barrier (Chung et al. 2002)
  - Iron acquisition: can bind to host transferrin and lactoferrin (Alderete et al. 1988)
- Host cell damage:
  - No known exotoxins
  - No LPS (endotoxin) but still induces host inflammatory responses: major mechanism for host cell and tissue damage

  - TpN15, TpN17, and TpN47 (Brightbill et al. 2006; Chung et al. 2002):
    - Lipoproteins recognized by TLR-2 on target cells
    - Activate signal transduction pathways that lead to the production of pro-inflammatory cytokines

- Evasion of host immune system:
  - Few integral outer membrane proteins; helps avoid antibody-based immune detection (Salazar et al. 2002)
  - **TprK** (Centurion-Lara et al. 2004):
    - Outer membrane protein undergoes extensive antigen variation; helps avoid immune detection

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Chapter 30
Vibrio spp.

Genomics, Morphologies, and Growth Characteristics

- **Genomics:**
  - *Vibrio cholerae* El Tor N16961 chromosomes (Heidelberg et al. 2000):
    - Chromosome 1: 2,961,151 bp; 2770 predicted ORFs; contains most of the genes involved in pathogenicity and essential cell functions
    - Chromosome 2: 1,072,914 bp; 1115 predicted ORFs
  - *Vibrio vulnificus* strain YJ016 chromosomes (Chen et al. 2003):
    - Chromosome 1: 3,354,505 bp; 3262 predicted ORFs; contains most of the genes involved in pathogenicity and essential cell functions
    - Chromosome 2: 1,857,073 bp; 1697 predicted ORFs
  - *Vibrio parahaemolyticus* strain RIMD2210633 chromosome (Makino et al. 2003):
    - Chromosome 1: 3,288,558 bp; 3080 predicted ORFs; contains most of the genes involved in pathogenicity and essential cell functions
    - Chromosome 2: 1,877,212 bp; 1752 predicted ORFs
- **Cell morphology:**
  - Curved rods (Fig. 30.1)
  - Flagellar motility:
    - Polar flagella with sheaths
    - Monopolar (*V. cholerae*), peritrichous, or lophotrichous
    - Essential for attachment, virulence, and biofilm formation (Watnick et al. 2001)

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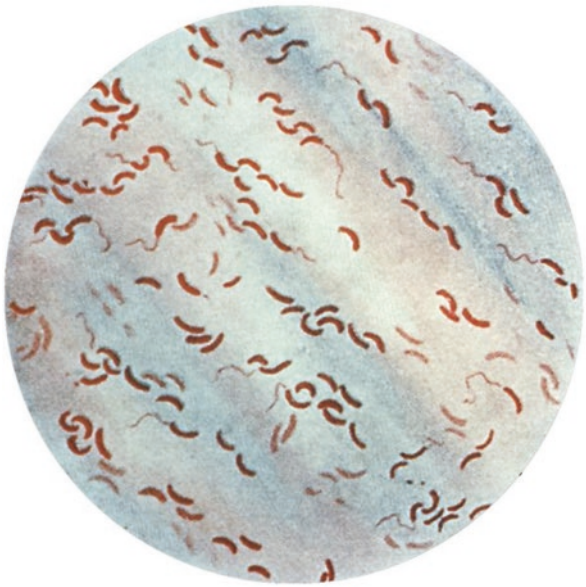

Fig. 30.1 *V. cholerae* cells (From: PHIL #5324)

  - LPS: endotoxin
    - Responsible for cytokine-induced shock associated with *Vibrio* infections
    - Modified by glycine addition; this changes the charge of the outer membrane, which affects immune recognition; modifications are regulated by the **VprA-VprB** TCS (Herrera et al. 2014)

- **Gram stain:**
  - Gram negative
- **Growth:**
  - Facultative anaerobes; oxidase positive
  - Halophilic marine microbes: typically found in seawater associated with contaminated water or seafood
  - Exists in the environment as either individual planktonic cells or colonized within biofilms on chitin-covered zooplankton, crustaceans, and/or phytoplankton
  - Biofilms (Teschler et al. 2015):
    - Essential for environmental persistence, dissemination, transmission
    - *Vibrio* polysaccharide (VPS): comprises 50% of the biofilm mass
    - Flagella and type IV pili function in adherence within biofilms (Utada et al. 2014)
  - Species: ~110 species; three major human pathogens; *V. cholerae, V. vulnificus, V. parahaemolyticus*

- *V. cholerae*: different serotypes are associated with cholera outbreaks
  - *V. cholerae* (O1): pandemic cholera:
    - Subgroups are based on LPS O-antigen serotypes – Inaba (A, B), Ogawa (A, C), Hikojima (A, B, C)
    - Biotypes: Classic and El Tor (twentieth century)
    - Phage types: >100 vibriophage
  - *V. cholerae* (O139): epidemic cholera in India, 1992
  - *V. cholerae* (non-O1/non-O139): much milder diarrhea; do not agglutinate with O1 antibodies
  - *V. cholerae* virulence evolution is primarily due to extensive horizontal gene transfer (HGT) mechanisms; binding to chitin on zooplankton can activate DNA uptake machinery:
    - All pathogenic *V. cholerae* strains have the CTXϕ bacteriophage and the pathogenicity islands VPI-1 and VPI-2:
      - CTXϕ lysogenic bacteriophage:
        - Encodes cholera toxin, Ace toxin, and Zot virulence factor
        - Differs among *Vibrio* serotypes
      - Vibrio pathogenicity island-1 (VPI-1) (Faruque and Mekalanos 2003):
        - Encodes the Tcp pilus: essential for host cell attachment; receptor for CTXϕ bacteriophage
        - Encodes the ToxT transcription factor and the TcpP-TcpH TCS (see below)
      - VPI-2 (Faruque and Mekalanos 2003):
        - Encodes NanH neuraminidase

Disease States Associated with *Vibrio* spp.

- *Vibrio cholerae*:
  - Cholera:
    - Greatest epidemic disease of the nineteenth century
    - Brought about great changes in public health and sanitation of drinking water
  - Noninvasive, toxigenic pathogen
  - Affects epithelial cells in the small intestine
  - Signs and symptoms: primarily associated with the action of cholera toxin

- Watery diarrhea:
  - "Rice water" stools
  - Clear, watery stool containing flecks of mucus and epithelial cells; fishy odor
- Very, rapid onset with duration of ~5 days
- In serious cases, patients can lose up to 1 l of water/h
- Without rigorous fluid replacement, death is due to dehydration, electrolyte loss, and metabolic acidosis

- *Vibrio vulnificus*:
  - Highly invasive pathogen
    - ~50% mortality rate
    - Responsible for ~95% of seafood-related deaths in the USA
  - Found associated with shrimp, fish, oysters, clams
  - Transmission: ingesting contaminated seafood (primarily raw oysters) or through open wounds exposed to saltwater
  - Signs and symptoms:
    - Seven to 16 h post-consumption
    - Fever, chills, nausea, hypotensive septic shock
    - Formation of secondary blister-like lesions on extremities; rapidly spreading necrosis (Fig. 30.2)

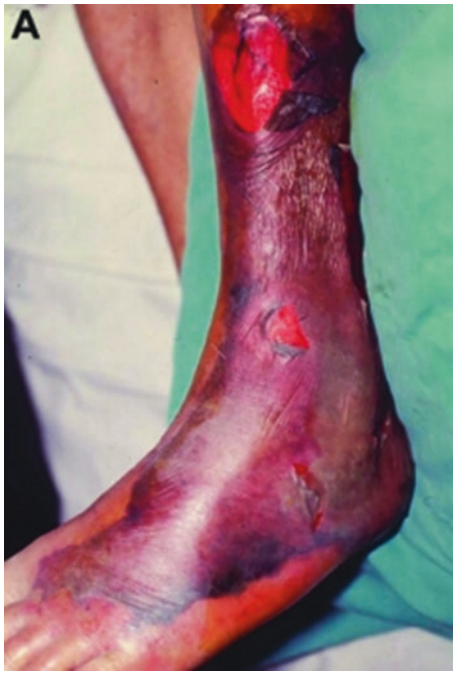

Fig. 30.2 *V. vulnificus* necrosis (From: Hsueh et al. 2004)

- *Vibrio parahaemolyticus*:
  - Acute seafood-borne gastroenteritis (vibriosis)
  - Invasive pathogen; primarily affecting epithelial cells in the colon
  - Transmission: ingesting contaminated seafood (primarily raw oysters)
  - Signs and symptoms:
    - Twenty-four hours post-consumption; relatively mild
    - Explosive, watery diarrhea
    - May be accompanied by nausea, vomiting, abdominal cramps

Virulence Factors

- *Vibrio cholerae*:
  - Colonization on chitin-covered zooplankton or phytoplankton:
    - Chitin:
      - Insoluble polymer of β1,4-linked N-acetylglucosamine (GlcNAc)
      - Second most abundant polysaccharide in nature
      - Major component of crustacean exoskeletons
      - *V. cholerae* and other *Vibrio* spp. can utilize chitin as a carbon, nitrogen, and energy source
      - In the presence of chitin, *V. cholerae* upregulates the synthesis of two secreted chitinases (ChiA-1, ChiA-2), a chitin porin (ChiP), and a chitin-regulated type IV pilus (ChiRP), which functions as an fimbrial adhesin in binding to chitin surfaces (Meibom et al. 2004)
      - Multiple chitin-binding proteins function in attachment:
        - ChiRP, MSHA, GpbA, FrhA
        - These proteins also function in adherence to human epithelial cells and play a role in biofilm formation (see below)
  - Colonization of human hosts:
    - Gains access to the small intestine either as planktonic cells or part of microcolonies or biofilm, which protects against acid environment within the stomach
    - **Flagella**: needed for movement through the host mucus layer
    - **Mucinases**: degrades mucin, ECM fibronectin

- Adherence to host cells (Almagro-Moreno et al. 2015):
  - Fimbrial adhesins:
    - **Maltose-sensitive hemagglutinin (MSHA)** (Utada et al. 2014):
      - Type IV pilus
      - Binds sialic acid sugars on host cells and chitin polymers
      - Similar functions as chitin-regulated pilus ChiRP (see above)
      - Mediates binding of *V. cholerae* to secretory immunoglobulin A (s-IgA); binding blocks penetration of mucus barriers and attachment to epithelial cells:
        - Repression of MSHA expression by the ToxT transcription factor (see below) protects against s-IgA (Hsiao et al. 2006).
      - Important for biofilm formation
    - **Tcp** (Toxin co-regulated pili) (Thelin and Taylor 1996):
      - Type IV pili
      - Acts as a receptor for CTXϕ bacteriophage
      - Also functions in protein secretion
      - Expression is co-regulated with cholera toxin genes by the ToxT transcription factor (see below)
  - Afimbrial adhesins:
    - **Mam7** (multivalent adhesion molecule 7) (Krachler and Orth 2011):
      - Binds fibronectin and membrane phosphatidic acid
      - Also found in *V. parahaemolyticus*
    - **OmpU** (Goo et al. 2006; Sperandio et al. 1995):
      - Fibronectin-binding protein
      - Found in all *Vibrio* spp.
    - **GpbA** (Bhowmick et al. 2008):
      - Binds to N-acetylglucosamine (GlcNAc), the monomeric sugar that comprises polymeric chitin, on zooplankton (Kirn et al. 2005)
      - Also interacts with GlcNAc residues within mucin surrounding human epithelial cells
      - Important for biofilm formation
      - Also found in *V. parahaemolyticus*
    - **FrhA** (Syed et al. 2009):
      - Flagella-regulated hemagglutinin A
      - Binds to N-acetylglucosamine (chitin) on zooplankton and human epithelial cells
      - Important for biofilm formation

- **AcfA-D** (accessory colonization factors) (Everiss et al. 1994; Hughes et al. 1995):
  - Chemotaxis regulatory proteins that are important for colonization
  - Part of the ToxT regulon (see below)
- **NanH**:
  - Neuraminidase
  - Binds host sialic acid residues on glycoproteins
  - Degrades host gangliosides to form GM1 gangliosides

– Dissemination in host:

- Disrupts tight junctions (TJs); increases paracellular permeability
- Affects TJ proteins occulin and zonulin (ZO-1), which regulate the host actin cytoskeleton
  - **HA/P** (hemagglutinin/protease):
    - Cleaves occludin but not ZO-1
  - **Zot** (zonula occludens toxin) (Di Pierro et al. 2001; Fasano 2011; Fasano et al. 1995):
    - Mimics host ZO-1; binds ZO-1 receptor
    - Activates PLC/PKC (phospholipase C/protein kinase C) signaling, which regulates actin cytoskeleton reorganization
  - **RtxA** (Satchell 2015):
    - Member of the MARTX (multifunctional-autoprocessing repeats-in-toxin) family of toxins
      - Unique toxins that encode their own secretion system for bacterial effectors; can be used in lieu of T3SS
      - Also found in *V. vulnificus* but not *V. parahaemolyticus*, which has a T3SS
      - Single polypeptide:
        - Secreted by T1SS
        - N-terminal glycine-rich repeat domain binds to host receptor and forms pores in the membrane
        - Cysteine protease domain (CPD): cleaves the MARTX toxin, releasing effector domain(s) into the host cell
        - At least ten different effector domains with different biochemical functions, e.g., adenylate cyclases, ADP-ribosyltransferases
        - Two to five domains are contained in each MARTX

- RtxA effector domains:
  - Actin cross-linking domain (ACD):
    - Covalently cross-links G-actin monomers; creates bond between lysine 50 and glutamic acid 270
    - Blocks actin polymerization
  - Rho inactivation domain (RID):
    - Peptidase
    - Localized to cell membrane
    - Proteolysis of, as yet unknown, target(s) (presumably Rho-GEFs) block the activation of Rho GTPase
  - Alpha/beta hydrolase (ABH):
    - Indirectly activates the Cdc42 GTPase by unknown mechanism
    - Antagonizes Rho GTPase
- Overall RtxA effect is the disruption of actin polymerization and the actin cytoskeleton; induces cell rounding and increases paracellular permeability

- Iron acquisition (Payne et al. 2016):
  - Fe^{+2} and Fe^{+3} are essential nutrients for *Vibrio* spp.
  - Multiple mechanisms for obtaining Fe from environment; reflects different environment niches
  - Fe^{+3} siderophores:
    - **Vibriobactin**:
      - *V. cholerae* catechol siderophore
      - Synthesized by nonribosomal peptide synthetase (NRPS) complex
    - Also, capable of using multiple xenosiderophores in the environment
  - Heme receptors: **HutA, HutR,** and **HasR**
  - Fe^{+2} transporters: **FeoABC**
  - Fe^{+3} transporters: **FbpABC**
  - All mechanisms are regulated by the Fe^{+2} – Fur repressor system
- Host cell damage:
  - Exotoxins:
    - **Cholera toxin (CT)** (Cassel and Pfeuffer 1978; Clemens et al. 2011):
      - Encoded on the CTXϕ bacteriophage
      - Part of ToxR regulon (see below)

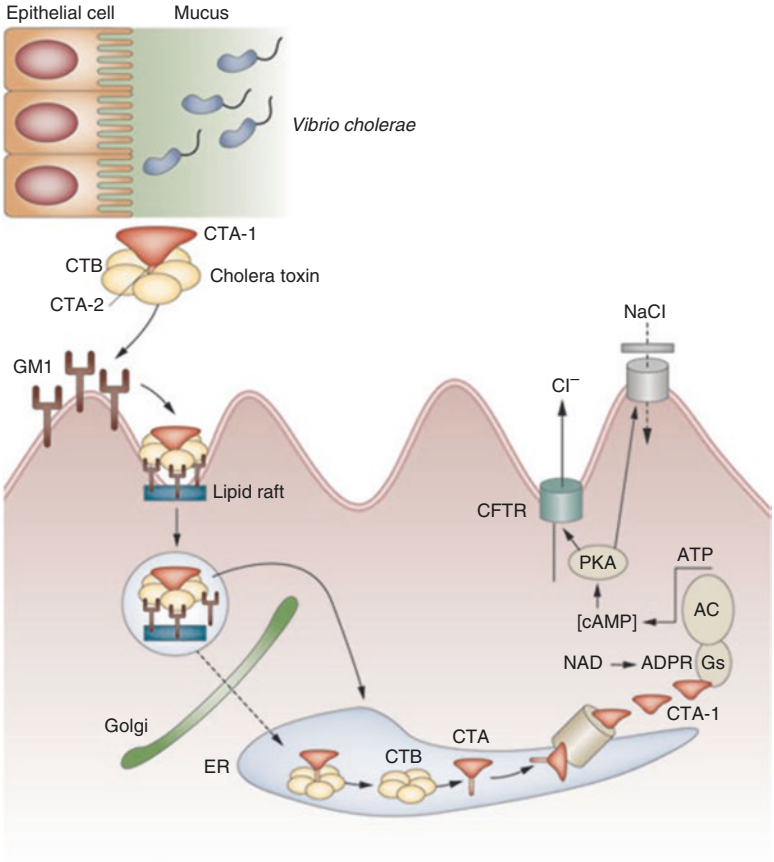

Fig. 30.3 Cholera toxin mechanism (From: Clemens et al. 2011)

- A-B exotoxin:
  - One A subunit (CTA): ADP-ribosyltransferase activity
  - Five B subunits (CTB): binding subunit; forms ringlike structure
- CTB ring binds to GM1 gangliosides within lipid raft domains in the cell membrane of intestinal epithelial cells in the small intestine (Fig. 30.3).
  - *V. cholerae* secretes neuraminidase (see above), which degrades host gangliosides to form GM1 gangliosides
- A–B complex is internalized by endocytosis and transits to the endoplasmic reticulum (ER)
- CTA is released from CTB within ER, where it dissociates into CTA-1 and CTA-2 subunits through the reduction of a disulfide bond
- CTA-1 subunit binds to ADP-ribosylation factor 6 (Arf6) within the ER; binding activates CTA-1 catalytic activity

  - CTA-1/Arf6 complex ADP-ribosylates the $G\alpha_s$ subunit of the heterotrimeric G protein
  - The inactivated $G\alpha_s$ subunit cannot hydrolyze its bound GTP – remains constitutively active
  - Results in constitutive activation of the downstream effector adenylate cyclase (AC), which converts ATP into cAMP:
    - Increases cAMP concentration more than 100-fold
  - cAMP binds to, and activates, protein kinase A (PKA), which phosphorylates and activates the cystic fibrosis transmembrane conductance regulator (CFTR):
    - CFTR: membrane ion channel protein; conducts Cl^- ions across epithelial cell membranes
  - Cl^- release, as well as Na^+ entry into cells, causes a massive water efflux from intestinal epithelial cells – diarrhea

- **Ace** (accessory cholera exotoxin) (Trucksis et al. 1993):
  - Stimulates Ca^{2+}-dependent Cl^- secretion
  - Encoded on CTXϕ bacteriophage
- **NAG-ST** (non-agglutinable heat stable enterotoxin) (Arita et al. 1986):
  - Only produced in non-O1 strains
  - Activates guanylyl cyclase; increases cGMP production (Chaudhuri et al. 1998)
  - cGMP activates protein kinase G (PKG), which also. phosphorylates and activates the CFTR ion channel
- **Cholix toxin (ChxA)** (Awasthi et al. 2013):
  - Only produced in non-O1/non-O139 strains
  - A-B exotoxin:
    - ADP-ribosylates translation elongation factor 2 (EF-2)
    - Similar to *Corynebacterium diphtheriae* diphtheria toxin
  - Blocks translation, resulting in cell death
- **Vibrio cholerae cytolysin (VCC)** (Khilwani and Chattopadhyay 2015):
  - β-barrel pore-forming toxin (β-PFT); similar to *S. aureus* α-hemolysin
  - Creates anion permeable pores; leads to cytolysis and vacuolation
    - Induces cholera symptoms without expression of the cholera toxin
  - Also induces apoptosis and pro-inflammatory cytokine production

- *Vibrio vulnificus* (Horseman and Surani 2011; Jones and Oliver 2009):
  - Acid resistant (*V. cholerae* is acid sensitive):
    - Neutralizes low-pH environments using lysine decarboxylase, which breaks down lysine to form cadaverine and CO_2 (Rhee et al. 2002)
    - **Lysine decarboxylase**:
      - Encoded by the *cadBA* operon
      - Upregulated in low-pH environments
    - Cadaverine is also a superoxide radical scavenger; connection between acid resistance and oxidative stress
  - Host cell adherence:
    - Fimbrial adhesin:
      - **Type IV pili** (Paranjpye and Strom 2005):
        - Essential for adherence to epithelial cells, biofilm formation and virulence
    - Afimbrial adhesins:
      - **IlpA** (immunogenic lipoprotein A) (Lee et al. 2010):
        - Adhesin and immunostimulant
      - **OmpU** (Goo et al. 2006; Sperandio et al. 1995):
        - Fibronectin-binding protein
        - Found in all *Vibrio* spp.
  - Host cell damage:
    - **RtxA1 (VvRtxA)** (Lee et al. 2007):
      - MARTX exotoxin (see *V. cholerae* RtxA above)
      - Primary virulence factor for *V. vulnificus*
      - Deletion mutants are avirulent
      - Expression is regulated by the HlyU transcription factor
    - **VvhA** (Kim et al. 1993; Wright and Morris 1991):
      - Cholesterol-dependent pore-forming hemolysin
      - Also releases iron from host hemoglobin
      - Deletion mutants are still cytotoxic; role in virulence is unclear
    - **VvpE** (Chang et al. 2005):
      - Metalloprotease: destroys type IV collagen
      - Activates procaspases in the apoptosis pathway
      - Deletion mutants are still cytotoxic; role in virulence is unclear

- Iron acquisition (Payne et al. 2016; Simpson and Oliver 1987):
  - Increased serum iron levels enhance *V. vulnificus* virulence
  - Fe^{+3} siderophores:
    - **Vulnibactin**:
      - *V. vulnificus* catechol siderophore
      - Synthesized by a nonribosomal peptide synthetase (NRPS) complex
    - Scavenges iron from transferrin and holotransferrin
    - Also, capable of scavenging multiple xenosiderophores in the environment
  - Heme receptors:
    - **HupA, HvtA**: transport hemin
    - **HupA**: transports hemoglobin and haptoglobin
  - All mechanisms are regulated by the Fe^{+2}–Fur repressor system
- Evasion of host immune system:
  - **Capsule** (Yoshida et al. 1985):
    - Blocks opsonophagocytosis and increases serum resistance
    - Essential for virulence and dissemination in the infected host

- *Vibrio parahaemolyticus* (Lovell 2017; Zhang and Orth 2013):
  - Adherence to host cells:
    - **MAM7**: see *V. cholerae* MAM7 above
    - **GbpA**: see *V. cholerae* GbpA above
  - Host cell invasion and growth:
    - Uses two T3SS: **T3SS1** (all *Vibrio* spp.) and **T3SS2** (*V. parahaemolyticus* clinical isolates); similar to *Yersinia* spp. Ysc T3SS
    - Three characterized T3SS1 effectors:
      - **VopQ (VepA)** (Burdette et al. 2009):
        - Functions in generating autophagic vesicles in host cells; protects against phagocytosis and apoptosis
        - Blocks inflammasome activation
        - Also induces MAPK signaling pathways
      - **VopS** (Yarbrough et al. 2009):
        - Transfers AMP molecule (AMPylation) onto Rho/Rac/Cdc42 GTPases; blocks interactions with downstream effectors
        - Disrupts actin cytoskeleton, resulting in cell rounding and increased paracellular movement
        - Blocks inflammasome activation

- **VPA0450** (Broberg et al. 2010):
  - Inositol polyphosphate-5-phosphatase
  - Blocks actin binding to host cell membrane

- Five characterized T3SS2 effectors: also found in non-O1/non-O139 *V. cholerae* strains that lack cholera toxin
  - **VopC** (Zhang et al. 2012):
    - Deamidates glutamine 61 on Rac and Cdc42 GTPases
    - Constitutively activates the GTPase, leading to actin cytoskeleton changes and invasion into non-phagocytic cells
  - **VopV** (Hiyoshi et al. 2011):
    - Binds and bundles F actin; role in invasion is unclear
  - **VopL** (Yu et al. 2011):
    - Actin nucleation factor
    - Induces stress fibers in host cells
  - **VopA (VopP)** (Trosky et al. 2004):
    - Acetylates MAPK kinases; unknown substrate in *V. parahaemolyticus*
    - Homologue of *Yersinia* spp. YopJ
    - Unclear which MAPK signaling pathways are affected by VopA/P (i.e., cell growth, differentiation, and/or cytokine induction)
  - **VopT** (Kodama et al. 2007):
    - ADP-ribosyltransferase
    - Modifies Ras GTPase, which controls multiple MAPK signaling pathways
- Iron acquisition (Payne et al. 2016):
  - Fe^{+3} siderophores:
    - **Vibrioferrin**:
      - Hydrophilic carboxylate siderophore
      - Unlike siderophore in other *Vibrio* spp. (Yamamoto et al. 1994)
    - Also, capable of using multiple xenosiderophores in the environment
    - Scavenges iron from transferrin and holotransferrin
  - All mechanisms are regulated by the Fe^{+2}–Fur repressor system

- Host cell damage:
  - **TDH** (thermostable direct hemolysin) (Yanagihara et al. 2010):
    - Pore-forming cytolysin
    - Cardiotoxicity and enterotoxicity
    - Activates intracellular Ca^{2+} release, which leads to activation of the CLCA Cl^- channel; water loss and diarrhea
    - Only found in *V. parahaemolyticus*
  - **TRH** (TDH-related hemolysin) (Ohnishi et al. 2011):
    - Heat labile pore-forming cytolysin
    - ~70% homologous with TDH
    - Only found in *V. parahaemolyticus*

Regulation of Virulence Factor Expression

- *V. cholerae* CT, Tcp, and AcfA expression (Skorupski and Taylor 1997):
  - Coordinately upregulated by **ToxT** transcription factor
  - ToxT expression responds to changes in pH, osmolarity, bile salts, temperature
    - Strain specific – El Tor strains are sensitive to high temperature
  - ToxT expression is co-regulated by two TCSs:
    - **ToxR-ToxS**: also regulates porin production
      - ToxS: sensor kinase; phosphorylates and activates ToxR
      - ToxR: response regulator
        - Transcription activator
        - Activates ToxT expression directly
        - Also, directly activates CT expression independent of ToxT
    - **TcpP-TcpH**:
      - TcpH: sensor kinase; phosphorylates and activates TcpP
      - TcpP: response regulator
        - Transcription activator
        - Activates ToxT expression directly
- Quorum sensing (Cámara et al. 2002):
  - Regulates virulence factor expression in *V. cholerae* and *V. vulnificus*
  - Two autoinducer peptides (**AI-2, CAI-1**) act through two TCSs (**CqsS, LuxP-LuxQ**) signaling cascades to induce virulence gene expression at low cell density and to repress virulence gene expression at high cell densities
  - Ultimately mediated through the ToxT regulatory protein

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Chapter 31
Yersinia spp.

Genomics, Morphologies, and Growth Characteristics

- **Genomics:**
  - *Yersinia pestis* (*Ype*) chromosome: 4,653,728 bp; 4012 predicted ORFs (Parkhill et al. 2001):
    - pPst/pPCP1: 9612 bp; 9 predicted ORFs
    - pYV1/pCD1: 70,305 bp; 97 predicted ORFs; found in all pathogenic *Yersinia*
    - pFra/pMT1: 96,210 bp; 103 predicted ORFs
  - *Yersinia enterocolitica* (*Ye*) chromosome: 4,615,899 bp; 4037 predicted ORFs (Thomson et al. 2006)
  - *Yersinia pseudotuberculosis* (*Yp*) chromosome: 4,744,671 bp; 3974 predicted ORFs; ancestor to *Ype* and *Ye* (Chain et al. 2004)
- **Cell morphology:**
  - Rod-shaped coccobacilli (Fig. 31.1)
  - *Ype*: lipooligosaccharide (LOS, rough LPS) outer membrane; does not have O antigens – IS insertions inactivated O antigen synthesis genes
  - *Yp* and *Ye*: smooth LPS
  - *Ype* – F1 protein fibrillar capsule:
    - Comprised of Caf1 subunits
    - Synthesized in humans (37 °C) but not in fleas (27 °C)
    - Blocks phagocytosis (Du 2002)
    - Involved in forming biofilms in fleas but not humans – biofilm blocks swallowing in fleas, enhancing the spread of *Ype* through a flea bite

D.I. Johnson, *Bacterial Pathogens and Their Virulence Factors*,
https://doi.org/10.1007/978-3-319-67651-7_31

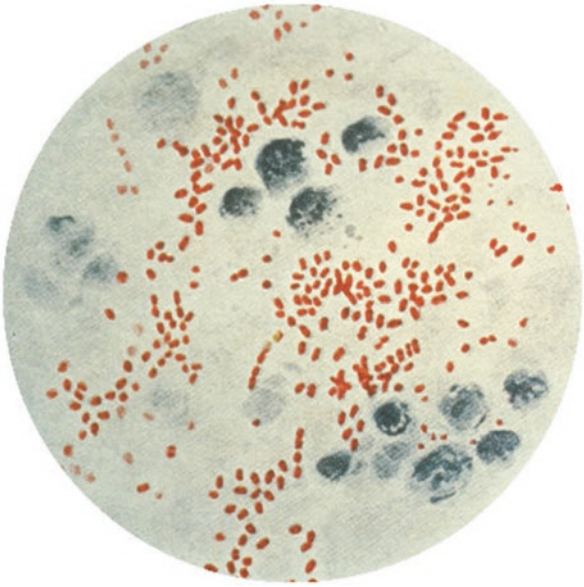


Fig. 31.1 *Y. pestis* cells (From: PHIL #2117)

  - Flagellar motility:
    - *Ye* and *Yp*: low-temperature (28 °C) motility – essential for virulence
    - *Ype*: nonmotile in host

- **Gram stain:**
  - Gram negative
- **Growth:**
  - Facultative anaerobes; catalase positive
  - Lactose negative, oxidase negative, urease negative, indole negative
  - Calcium-dependent growth at 37°C; affects the function of the type 3 secretion system (T3SS)
  - *Ye* and *Yp* are psychrophiles:
    - Can grow at 0°C
    - Major problem for food safety
    - Mediated through cold shock proteins (Csp) (Keto-Timonen et al. 2016)
  - Environmental reservoirs:
    - *Ype*: fleas on rodents; amplifying hosts – ground squirrels [prairie dogs (Fig. 31.2), chipmunks], mice ("wobbly"), rats
    - *Yp* and *Ye*: contaminated food and water

Fig. 31.2 Black-tailed prairie dog (From: Dean Biggins, courtesy USGS)

- 17 species – three cause human disease:
  - *Yersinia pestis* (*Ype*):
    - Bubonic plague; pneumonic plague
    - Evolved from *Yp* by horizontal gene transfer (HGT) and gene loss through insertion sequences (IS) mutations
  - *Yersinia enterocolitica* (*Ye*):
    - Enteropathogenic: food-borne disease
    - Six biogroups (1A, 1B, 2, 3, 4, 5) based on phenotypic characteristics
      - Biovar 1B is highly virulent
  - *Yersinia pseudotuberculosis* (*Yp*):
    - Enteropathogenic: food-borne disease
    - Subgroups based on O antigens

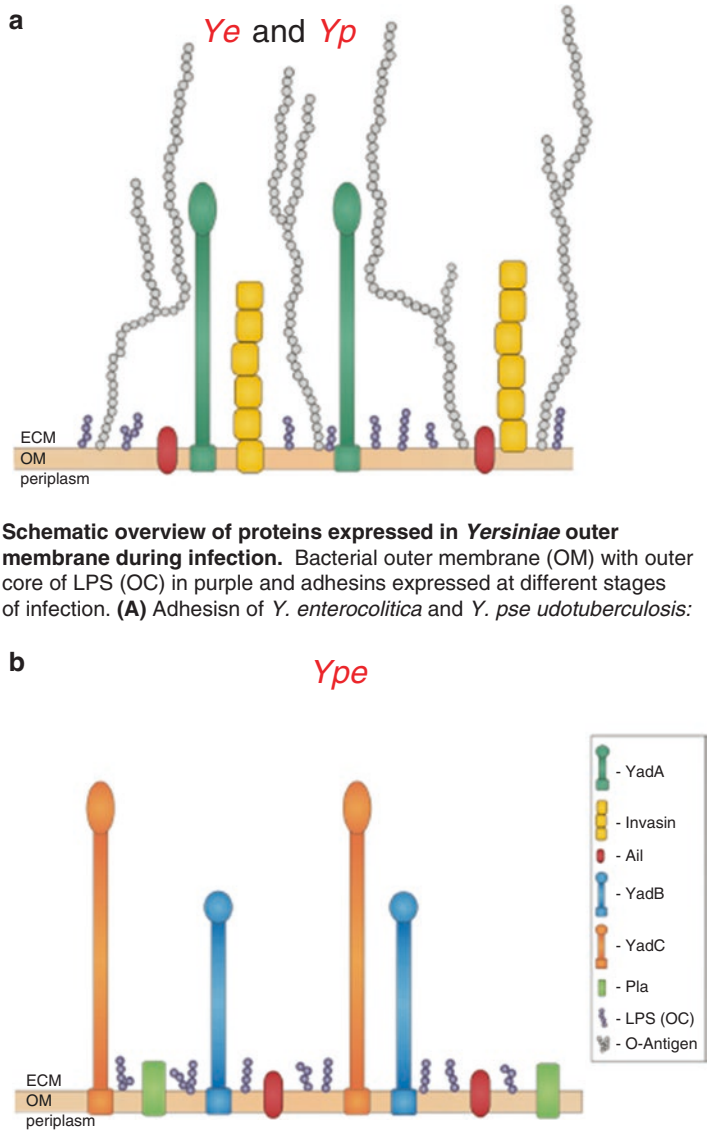
Disease States Associated with *Yersinia* spp.

- First stage:
  - Invasion of host cells
  - Facultative intracellular pathogens
- Second stage:
  - Dissemination, egress from cells, and extracellular growth
  - Virulence mechanisms are needed to avoid the immune system both intracellularly and extracellularly

- Plague (black death): *Ype*
  - Bubonic plague:
    - Urban plague: infection of rats in urban areas
    - Sylvatic plague: infection of prairie dogs, mice, etc.
    - Transmitted by bite of flea (endemic reservoir)
    - Following flea bite, *Ype* travels to lymph nodes inducing inflammatory lesions called buboes
    - Can also be transmitted by cutaneous exposure to infected animal
    - Early symptoms: high fever, buboes, headaches, and chills
    - Later symptoms: tissue necrosis ("black death"), endotoxic shock (usually fatal), and septicemia
    - Can disseminate to the spleen and liver (primary targets)
  - Primary septicemic plague:
    - Transmitted by flea bite
    - *Ype* does not travel to lymph nodes
    - Disseminates through the bloodstream
    - Symptoms: hypotension, delirium, seizures, and septic shock
  - Primary pneumonic plague (Pechous et al. 2016):
    - Person-to-person transmission through inhaled aerosols containing *Ype*
    - Symptoms: initially flu-like, fever, chills, coughing, and chest pain; leads to pneumonia
    - Dissemination leads to rapid multi-system failure and septic shock
  - If treated: ~15% mortality
  - If untreated, death comes quickly; average 8 days from first symptoms:
    - ~50 to 60% mortality for bubonic plague
    - ~100% mortality for septicemic plague and pneumonic plague
- Yersiniosis:
  - Enterocolitis associated with *Ye* and *Yp* (rare) infections
  - Zoonotic food-borne disease:
    - Most often contaminated pork, dairy products
    - Fecal contaminated water
  - Symptoms: diarrhea, abdominal pain, and fever; ranging from mild self-limiting diarrhea to acute lymphoid tissue infection
  - Usually afflicts children and infants
  - Septicemia complications are rare in healthy individuals
  - Mortality much higher in immunocompromised patients, due to systemic infection

Virulence Factors (Atkinson and Williams 2016)

- Adherence to host cells:
  - Adhesins (Ke et al. 2013; Mikula et al. 2012):
    - *Ype*, *Ye*, and *Yp* have different repertoires of adhesins (Fig. 31.3)
    - Encoded on chromosome and plasmids expressed under different pH and temperature conditions
    - Reflects different lifestyles – growth in flea (<30°C) vs. host cell invasion in human (37°C)
    - Psa and Ail: found in all three species
    - Fimbrial adhesins:
      - **Type IV pili**: only expressed in *Yp* (Collyn et al. 2002)
      - **Psa** (*Ype*,*Yp*)/**MyfF** (*Ye*):
        - 15 kDa fimbrial adhesin
        - Expressed at pH 5–6.7 (Psa, pH 6 antigen) and 37 °C (Lindler and Tall 1993)
        - Binds to glycosphingolipids in intestinal epithelial cells, through β1-linked galactosyl residues and lipoproteins (Makoveichuk et al. 2003)
        - Binds to phosphatidylcholine (PC) in alveolar epithelial cells and to lung surfactant
        - Plays a role in pneumonic plague (Galvan et al. 2007; Pakharukova et al. 2016)
        - Adheres to and agglutinates red blood cells (RBCs)
        - Has anti-phagocytic properties
    - Afimbrial adhesins:
      - **Ail** (attachment-invasion locus) (Kolodziejek et al. 2012):
        - *Ype* (some strains), *Ye*, and *Yp*
        - 17 kDa adhesin; four protein variants
        - Highly expressed at 37 °C in stationary phase; comprises 20–30% of the outer membrane proteins (OMPs) expressed
        - Binds ECM fibronectin and laminin
        - Adherence to epithelial cells
        - Blocks complement-mediated lysis through activating host factor H and C4BP (Bliska and Falkow 1992)
        - Promotes Yop effector delivery into phagocytic cells, epithelial cells (Felek and Krukonis 2009)
      - **YadA** (Muhlenkamp et al. 2015):
        - *Ye* and *Yp*
        - Trimeric type 5 autotransporter protein
        - Expressed at 37°C



Schematic overview of proteins expressed in *Yersiniae* outer membrane during infection. Bacterial outer membrane (OM) with outer core of LPS (OC) in purple and adhesins expressed at different stages of infection. **(A)** Adhesisn of *Y. enterocolitica* and *Y. pse udotuberculosis:* invasin in yellow, YadA in dark green, Ail in red, and O-Antigen in light grey; **(B)** Adhesins of *Y. pestis*: Pla in green, YadB in blue, YadC in orange, Ail in red. ECM stands for extracellular matrix. All the molecules are on approximately the same scale.

Fig. 31.3 *Yersinia* adhesins (From: Mikula et al. 2012)

  - Binds to ECM components (Schulze-Koops et al. 1992):
    - *Ye*YadA: prefers collagen, laminin
    - *Yp*YadA: prefers fibronectin
  - Adheres to epithelial cells, macrophage, and neutrophils
  - Blocks complement-mediated lysis by activating host factor H and C4BP
  - Pseudogene in *Ype* due to point mutation that causes a frame-shift:
    - *Ype* has YadB and YadC adhesin homologues

- **Inv** (invasin) (Young et al. 1990):
  - *Ye* and *Yp*
  - 92 kDa protein
  - Similar to enterohemorrhagic *E. coli* (EHEC) intimins
  - Expressed at 25°C and pH 8 prior to uptake into human host
  - Attachment and invasion into intestinal M cells in Peyer's patch and other non-phagocytic cells (Marra and Isberg 1997)
  - Binds host β1-integrins (receptors) on epithelial cells:
    - There is a high density of β1-integrin binding around the entire host cell surface; needed for attachment and internalization via "zipper" mechanism
    - Induces actin cytoskeletal rearrangements (Young et al. 1992)
  - Also induces host signaling pathways; increased expression of pro-inflammatory cytokines
  - Not made in *Ype* due to an IS element insertion mutation

- **YapC** (Felek et al. 2008):
  - *Ype* autotransporter adhesin; biofilm formation

- **YapE** (Lawrenz et al. 2009):
  - *Ype* autotransporter adhesion
  - Proteolytically activated by Pla

- **YapV, YapJ, YapK, and YapX** (Nair et al. 2015):
  - *Ype* and *Yp*; high sequence identity
  - Bind ECM components

- **Flagella**:
  - *Ye*
  - Secretes YplA; phospholipase essential for survival in Peyer's patch (McNally et al. 2007)

- Host cell invasion and growth:
  - Enter host cells through classic actin-dependent zipper mechanism of induced phagocytosis
  - Uses type 3 secretion system (T3SS) (see below)
  - *Ype*:
    - Invades epithelial cells, macrophage, and dendritic cells by phagocytosis
    - Can disseminate to lymph nodes inside macrophage and dendritic cells
    - Mediated by **Ail, YadB, YadC, and Pla**
    - **Pla** (Lahteenmaki et al. 2001):
      - Only found in *Ype*
      - Belongs to the omptin family of outer membrane proteases
      - Plasminogen activator
      - Essential for virulence
      - Encoded on virulence plasmid pPst/pPCP1
      - Adhesin:
        - Binds to epithelial cells and macrophage
        - Binds to host CD205 protein for internalization
      - Aspartic protease activity (Kukkonen et al. 2003):
        - Independent of adhesin activity
        - Sterically inhibited by LPS but not by LOS
        - Has many host targets; enhances invasion, dissemination, and survival
        - Degrades host ECM laminin
        - Activates host plasminogen; affects host coagulation and fibrinolytic pathways
        - Inactivates complement protein C3
        - Cleaves antimicrobial peptides
  - *Ye* and *Yp*:
    - Invade intestinal M cells in Peyer's patch (PP)
    - Can disseminate to lymph nodes and then liver, spleen
    - **Inv**: binds host β1-integrin receptors in M cells
  - **Type 3 secretion system (T3SS)** (Cornelis 2002):
    - Found in *Ype*, *Ye*, and *Ype*
    - Essential for virulence; greater than 10,000-fold loss of virulence in the absence of T3SS
    - Encoded on virulence plasmid pYV1/pCD1
    - Encode Yop (Yersinia outer membrane protein) effector proteins and Ysc T3SS system:

- **Ysc T3SS** system:
  - Comprised of 29 proteins that comprised the T3SS needle structure
  - **LcrV** (V factor) (Brubaker 2003):
    - Adhesin on tip of T3SS
    - Secreted LcrV induces IL-10 release
- **Yops** (Cornelis and Wolf-Watz 1997):
  - Translocator Yops (Yop B/D) (Håkansson et al. 1996):
    - Form pores in host membrane
  - Effector Yops:
    - Enter host cell
    - Affect cell signaling pathways that regulate the actin cytoskeleton; drives initial phagocytosis into the cell:
      - **YopE** (Pawel-Rammingen et al. 2000):
        - GTPase-activating protein (GAP) activity
        - Turns off multiple Rho GTPases
      - **YopT** (Iriarte and Cornelis 1998):
        - Cysteine protease
        - Inhibits RhoA GTPase
        - Disrupts actin cytoskeleton and inhibits phagocytosis post-egress
      - **YopO/YpkA** – dual functions (Barz et al. 2000):
        - Kinase activity:
          - Binding to actin turns on kinase activity
          - Phosphorylation inactivates cytoskeletal-assembly proteins
        - Guanine-nucleotide dissociation inhibitor (GDI):
          - Binds to and inhibits RhoA and Rac1 GTPases
          - Blocks actin polymerization
    - Affect inflammation and apoptosis:
      - **YopH** (Sauvonnet et al. 2002):
        - Tyrosine phosphatase
        - Blocks ROS production
        - Induces apoptosis in T cells

- **YopJ** (Mukherjee et al. 2006; Palmer et al. 1998):
  - Acetyltransferase
  - Acetylates MAPK kinases
  - Induces macrophage apoptosis
- **YopP** (Boland and Cornelis 1998; Orth et al. 2000):
  - Cysteine protease
  - Inhibits pro-inflammatory cytokine release
  - Activates macrophage apoptosis
  - Reduces neutrophil phagocytosis post-egress
- **YopM** (Chung et al. 2016; Ratner et al. 2016):
  - Inhibits YopE-mediated pyrin inflammasome activation
  - Also shown to have E3 ubiquitin ligase activity
  - Activates NOD-like receptor (NLR)-family pyrin domain-containing 3 (NLRP3) inflammasome
  - Induces host cell necrosis (Wei et al. 2016)
  - May reflect the ability of *Yersinia* to both activate and elude inflammation (Philip et al. 2016)

– Iron acquisition:

  - **Yersiniabactin (Ybt)** (Perry and Fetherston 2011; Perry et al. 1999):
    - Fe^{+3} siderophore
    - *Ype*, *Ye*, and *Yp*, essential for plague
    - Synthesized by a mixed nonribosomal peptide synthetase (NRPS)/polyketide synthase (PKS) mechanism
    - Repressed by Fe^{+2}–Fur repressor
    - Activated by YbtA–Ybt complex
  - **Hmu** (Thompson et al. 1999):
    - Acquires iron from hemin, hemoglobin, haptoglobin–hemoglobin, myoglobin, heme–hemopexin, and heme–albumin
  - **YfeABCD; YfuABC** (Bearden and Perry 1999; Gong et al. 2001):
    - ABC transport systems for iron and manganese

- Dissemination and egress from macrophage:
  - *Ype* systemic dissemination is inside dendritic cells and macrophage; can cause septicemic plague in bloodstream
  - In lymph nodes, *Ype* egresses from macrophage through apoptosis/necrosis of the macrophage
  - Replicates to high numbers extracellularly
  - Also replicates in micro-abscesses within infected tissues, leads to tissue necrosis

  - Activates anti-phagocytic factors (**Psa, F1 capsule, Yops**) while in initial macrophage; protects extracellular *Ype* cells from macrophage and neutrophils
  - **F1 protein (Caf1)** (Du 2002):
    - 16 kDa protein; encoded on pFra/pMT1 only found in *Ype*
    - Forms fibrillar capsule; blocks phagocytosis upon initial infection
    - Also produced inside macrophage to block phagocytosis upon egress
- Host cell damage – exotoxins:
  - **CNF_Y** (cytotoxic necrotizing factor *Yersinia*) (Schweer et al. 2013):
    - Only found in *Yp*
    - Activates Rho/Rac/Cdc42 GTPases through deamidation of the Glu63 residue
    - Increases Yop effector translocation
  - **Yst** (Delor et al. 1990):
    - Only found in *Ye*
    - Three variants
    - Heat-stable enterotoxin
    - Homologous to enterotoxigenic *E. coli* (ETEC) ST toxin
  - **YaxA and YaxB** (Wagner et al. 2013):
    - Only in *Ye*
    - Pore-forming toxin

Regulation of Virulence Factor Expression (Erhardt and Dersch 2015)

- As an enteric pathogen, *Yersinia* must respond to a myriad of environmental and host cues
- Utilizes combinations of protein and RNA regulators in complex pathways (Fig. 31.4)
- Invasion and motility are inversely regulated (Ellison et al. 2004; Fabrega and Vila 2012):
  - Yops, YadA, Ysc T3SS, and VirF/LcrF transcription factor are expressed at 37°C through a translational thermoswitch mechanism:
    - Similar thermoswitch mechanisms also regulate adaptation to oxidative stress and heat shock, induction of immune suppression, and metabolic processes (Nuss et al. 2017)

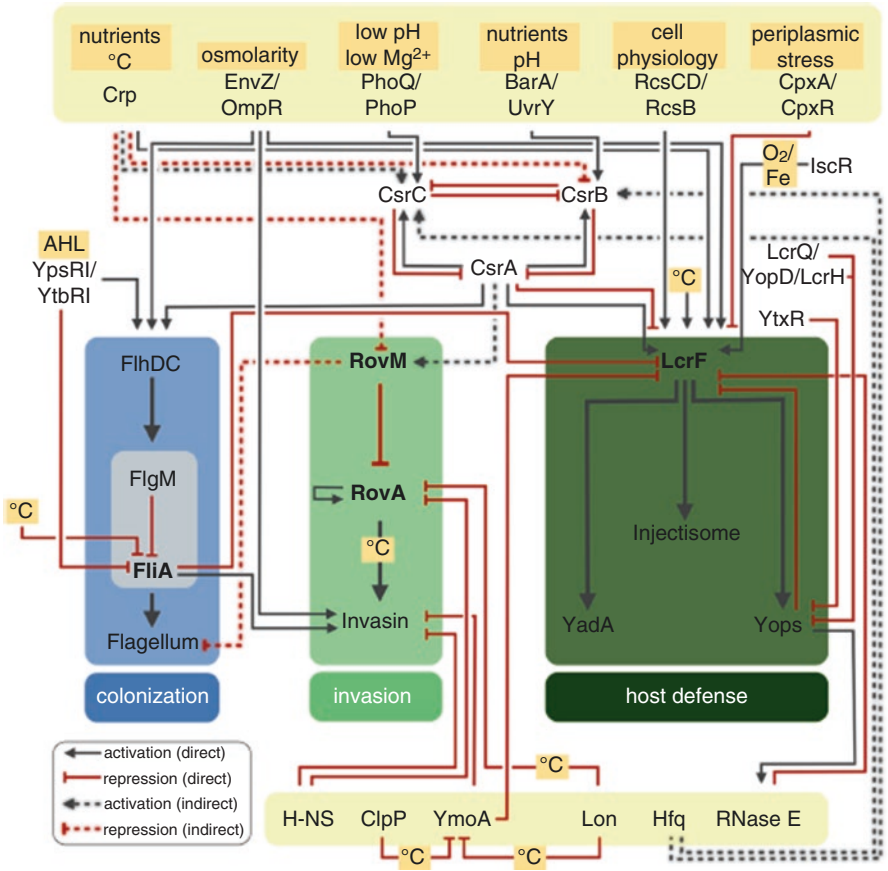


Fig. 31.4 Regulatory networks (From: Erhardt and Dersch 2015)

  - Inv, flagella genes: expressed at <30°C; repressed at 37°C:
    - **RovA**: transcription activator; expressed at <30°C; repressed at 37°C
    - **H-NS and YmoA**: repressors that compete with RovA binding at *inv* promoter
    - Transcription activated by RovA and repressed by H-NS/YmoA complex

- Carbon source regulation (Porcheron et al. 2016):
  - Mediated through cAMP + cAMP receptor protein (CRP)
  - Impinges on RovM–RovR regulon
  - Regulates Pla, T3SS, PhoP–PhoQ two-component phosphorelay system (TCS) and sRNA molecules CsrB and CsrA:
    - Csr regulon, etc. (Nuss et al. 2017; Porcheron et al. 2016):
      - Large numbers of RNA molecules function as riboregulators in many *Yersinia* regulatory networks

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Chapter 32
Chlamydia spp.

Genomics, Morphologies, and Growth Characteristics

- **Genomics:**
  - *Chlamydia trachomatis* chromosome: 1,042,512 bp; 887 predicted ORFs (Stephens et al. 1998)
  - *Chlamydia pneumoniae* chromosome: 1,230,230 bp; 1029 predicted ORFs (Kalman et al. 1999)
- **Cell morphology:**
  - Ovoid shape (Fig. 32.1)
  - Two developmental states with different morphologies:
    - Elementary body (E; EB): 0.3–0.4 μm
    - Reticulate body (R; RB): 1 μm
  - Cell wall:
    - Thick structure of LPS and a meshwork of cysteine-rich adhesin proteins, but no peptidoglycan layer
    - "chlamydial anomaly" (Liechti et al. 2014; Klockner et al. 2014)
      - There is no peptidoglycan (PG) layer surrounding the cell, but:
        - PG biosynthetic genes and penicillin-binding proteins (PBPs) genes are present in the genome and expressed
        - Penicillins block cytokinesis but are not cytolytic to *C. trachomatis* cells
      - Resolution: PG is found predominantly at cell division site but is not found around the periphery of the cell

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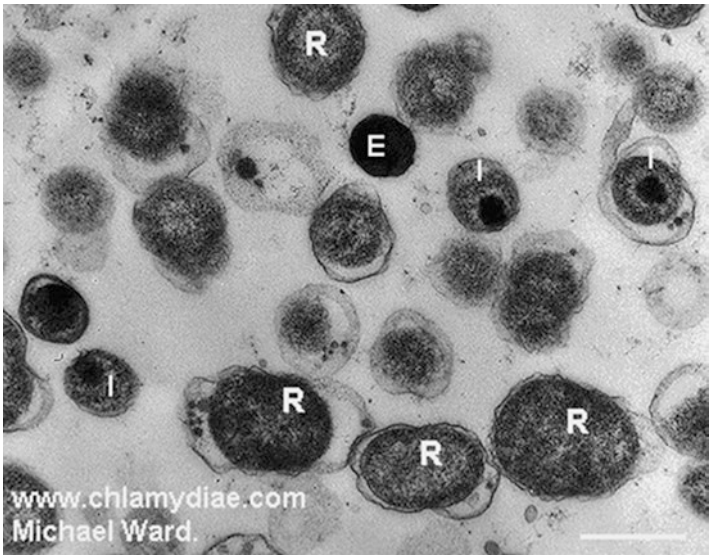

Fig. 32.1 *C. trachomatis* EB and RB cells in HeLa cells (From: Michael Ward, www.chlamydiae.com)

- **Gram stain:**
  - Gram-negative staining – contain LPS but no peptidoglycan around the cell
- **Growth:**
  - Obligate aerobes
  - Extremely temperature sensitive; clinical samples need to be refrigerated as soon as they are obtained
  - Obligate intracellular pathogen of humans and other vertebrates; also, can exist in aquatic environments:
    - Co-adapted to human host through "use it or lose it" evolution
    - Must get nutrients and ATP from infected host cells
  - Two developmental states with different morphologies: (Fig. 32.2).
    - Elementary body (EB):
      - Extracellular, metabolically inactive, non-replicating, infectious form
      - Resistant to environmental stresses
    - Reticulate body (RB):
      - Intracellular, metabolically active, replicating, noninfectious form
      - EB differentiate into RB and develop inside membrane-bound inclusions in host cytosol

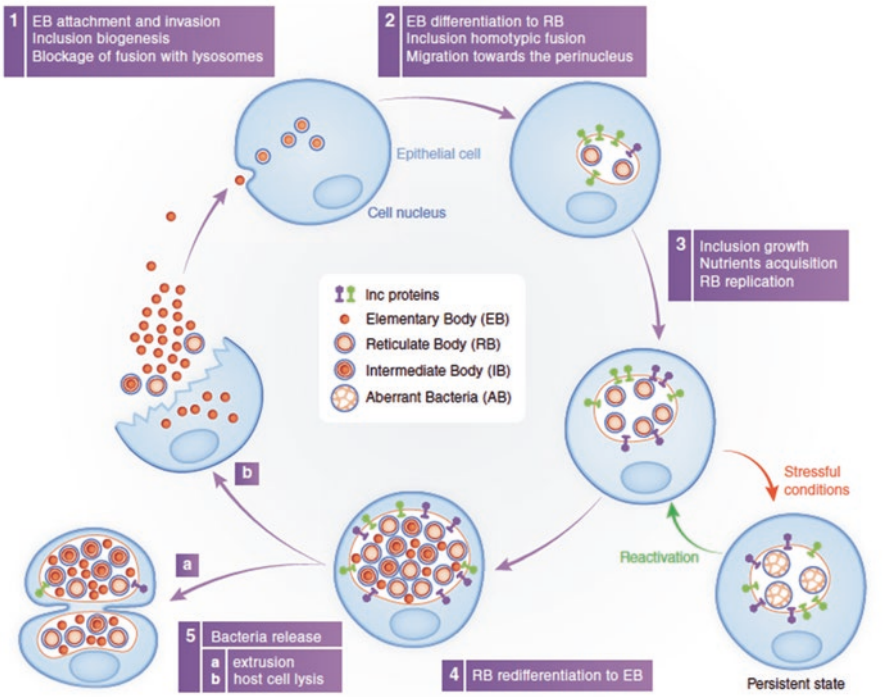

Fig. 32.2 *C. trachomatis* life cycle and developmental states (From Damiani et al. 2014)

- After cell division, RB re-differentiates back into EB, which are released through exocytosis or cell lysis
- Aberrant bodies (AB): large, abnormally shaped RB
  - Non-replicating, noninfectious form induced by stress, such as nutrient deprivation or penicillin treatment
  - Leads to chronic (persistent state) infection

– At least 11 species; two infect humans:
  - *Chlamydia trachomatis*: sexually transmitted pathogen; genital and ocular infections
  - *Chlamydia pneumoniae*: bronchitis, pharyngitis, community acquired pneumonia

Disease States Associated with *Chlamydia* spp.

- Inflammatory diseases: (Bastidas et al. 2013; Redgrove and McLaughlin 2014)
  - Associated with release of pro-inflammatory cytokines, such as interleukin1(IL-1), IL-6, and tumor necrosis factor α (TNFα)
  - Triggered by TLR2 and TLR4 recognition of LPS and cytosolic pattern recognition receptors (PRRs) recognizing peptidoglycan
- ***Chlamydia trachomatis:***
  - Ocular and genital infections
    - Serovars Ab, B, Ba, and C:
      - Trachoma eye infections; leading cause of preventable blindness worldwide
      - Transmitted via hands and fomites and through respiratory tract
      - Causes inflammation and scarring of cornea
      - Reduces the function of tear glands, leading to bacterial infections
      - Inclusion conjunctivitis: milder form of trachoma
    - Serovars D–K:
      - Sexually transmitted; leading cause of bacterial sexually transmitted disease in the USA
      - Nongonococcal urethritis, pelvic inflammatory disease
      - Mostly silent; ~75% of infected women and ~50% of infected men are asymptomatic
      - Males: urethritis with purulent discharge
      - Females – cervicitis, pelvic inflammatory disease (PID); can lead to tubal scarring, ectopic pregnancies, and infertility:
        - ~40% of infected woman get PID
        - ~20% of them become infertile; 9% will have ectopic pregnancy
    - Serovars L1, L2, and L3:
      - Sexually transmitted
      - Lymphogranuloma venereum; infection of the lymphatic system chancre on genitals; often overlooked
      - Lymphatic inflammation; buboes
      - Fever, headache, and myalgia
- ***Chlamydia pneumoniae:***
  - Responsible for 10–15% of community acquired pneumonia:
    - More common in elderly
    - Pharyngitis, bronchitis, and sinusitis

- Person-to-person transmission through small water droplets
- Also associated with asthma, bronchitis, coronary heart disease, and atherosclerosis, but a causative connection is unclear

Virulence Factors

- Adherence to host cells: (Frohlich et al. 2014)
  - Can invade most cells
  - Cell wall:
    - LPS + meshwork of cysteine-rich adhesin proteins
    - **MOMP**: major outer membrane protein
      - Functions as adhesin and as a β-barrel porin
      - Undergoes antigenic variation – immune system evasion
    - **OmcB and OmcA**:
      - Only found in EB
      - Cross-link MOMP to peptidoglycan
    - **Pmps**:
      - Nine polymorphic outer membrane proteins
      - **PmpA-I**: can undergo antigenic variation to evade the host immune system
- Host cell invasion: (Nans et al. 2015)
  - *C. trachomatis*:
    - Uses classic trigger mechanism
    - Type 3 secretion system (T3SS) in EB secretes effectors into the infected cell that affect the actin cytoskeleton, thereby inducing endocytosis
      - **TARP** (translocated actin-recruiting phosphoprotein): (Lane et al. 2008)
        - Activates Rac and Cdc42 GTPases that control actin polymerization
        - Acts as a scaffold for Rac guanine nucleotide exchange factors (GEFs)
      - **CT166**:
        - Cytotoxin
        - Glucosyltransferase activity
        - Inactivates Rac and Cdc42 GTPases
        - Analogous to *Clostridium difficile* toxin B (TcdB)

    - **CT694**:
        - Binds to AHNAK (desmoyokin) in host cell
        - Actin-binding protein

- *C. pneumoniae*:
    - Uses classic zipper mechanism
    - **Pmp21**:
        - Invasin protein
        - Binds to epidermal growth factor receptor (EGFR)
        - Induces cell signaling pathways

- Growth inside host cells: (Bastidas et al. 2013; Damiani et al. 2014)
    - Obligate intracellular pathogen
    - EB differentiate into RB that reside in membrane-bound inclusions within the host cytosol
    - Inclusions interact with the rough endoplasmic reticulum (rER), creates a "pathogen synapse"
        - Host nutrients (cholesterol, lipids, peptides, etc.) are obtained through inclusion interactions with host cell rER and Golgi
    - **Incs**:
        - Inclusion proteins
        - Inserted into inclusion membrane from RB through a T3SS
        - Interact with host cell proteins in rER and cytosol, such as Rab GTPases that regulate membrane trafficking in the host cell
            - e.g.**, CT229 Inc**. protein interacts with Rab4 GTPase
    - **CPAF** (*Chlamydia* protease-like activity factor): (Jorgensen et al. 2011; Zhong et al. 2001)
        - Serine protease activity
        - Recent reassessment of CPAF activities suggests that a number of its previously identified substrates are artifactual (Bavoil and Byrne 2014)
        - Does cleaves host proteins later in infection after inclusion breakdown; vimentin filaments and lamin-associated protein-1 (LAP1) (Snavely et al. 2014)

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Chapter 33
Mycoplasma spp.

Genomics, Morphologies, and Growth Characteristics

- **Genomics:**
  - *Mycoplasma pneumoniae* chromosome: 816,394 bp; 677 predicted ORFs (Dandekar et al. 2000; Himmelreich et al. 1996)
  - *Mycoplasma genitalium* chromosome: 580,076 bp; 470 predicted ORFs (Fraser et al. 1995)
- **Cell morphology:** (Fig. 33.1)
  - Pleomorphic – due to lack of cell wall:
    - *M. pneumoniae*: elongated cell with attachment organelle (AO) at the tip
    - *M. genitalium*: less elongated but still contains AO
  - Some of the smallest living microbes
  - No peptidoglycan; no Gram stain:
    - Lack peptidoglycan biosynthetic genes
    - Naturally resistant to cell wall disrupting antibiotics
  - Cell membranes contain cholesterol:
    - Essential growth factor; must get it from host
- **Gram stain:**
  - No Gram staining
- **Growth:**
  - Facultative anaerobes
  - Very small genome; lost the genes necessary for Kreb's cycle, the biosynthesis of amino acids, fatty acids, vitamins, and electron transport chain

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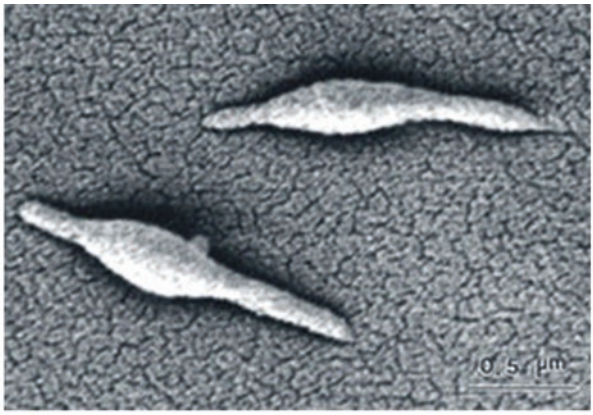

M. genitalium cells. From: http://www.strgen.org/images/about/corr-to-cell_hutchison.png

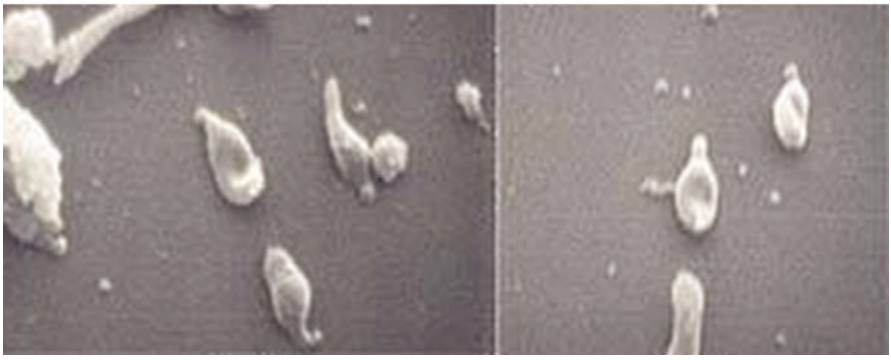

Fig. 33.1 *M. pneumoniae* cells (From: microbewiki.kenyon.edu)

- Grows very slowly; must get nutrients and ATP from infected host cells
- Specifically infects humans; does not exist as a free-living organism
- At least 104 species
  - *Mycoplasma pneumoniae*:
    - Respiratory tract infections
    - Type 1 and type 2 clinical isolates: based on P1 adhesin sequence differences
  - *Mycoplasma genitalium*:
    - Urogenital tract infections

Disease States Associated with *Mycoplasma* spp.

- *Mycoplasma pneumoniae* (Parrott et al. 2016; Waites et al. 2017):
  - Upper and lower respiratory tract infections
  - Community acquired pneumonia
  - Usually self-limiting and not fatal and depends on bacterial load
  - Person-to-person transmission through small aerosol droplets
    - Primary atypical pneumonia:
      - "Walking pneumonia"
      - Caused by host inflammatory responses
    - Lower respiratory tract infection:
      - Cough, fever, and headache
      - Can be a long convalescence; several weeks
      - Mainly affects children 5–9 years old; 8–15 % of all pneumonias in school-age children
  - Serious increases in worldwide antibiotic-resistant *M. pneumoniae* (Saraya et al. 2014)
- *Mycoplasma genitalium* (Taylor-Robinson and Jensen 2011):
  - Urogenital tract infections
  - Emerging sexually transmitted pathogen
  - Symptoms:
    - Men: nongonococcal urethritis
    - Women: urethritis, pelvic inflammatory disease, and cervicitis
      - Increased risk of preterm birth, spontaneous abortion, and infertility
  - Serious increases in worldwide antibiotic-resistant *M. genitalium* (Unemo and Jensen 2017)

Virulence Factors

- Adherence to host cells/tissues:
  - Primary colonization and virulence mechanism
  - Surface attachment to ciliated respiratory epithelial cells (*M. pneumoniae*) or urogenital epithelial cells (*M. genitalium*); binds to the base of ciliated cells
  - **Attachment organelle (AO)**:
    - Polar extension at the tip of filamentous cells
    - Essential for adherence to host cells

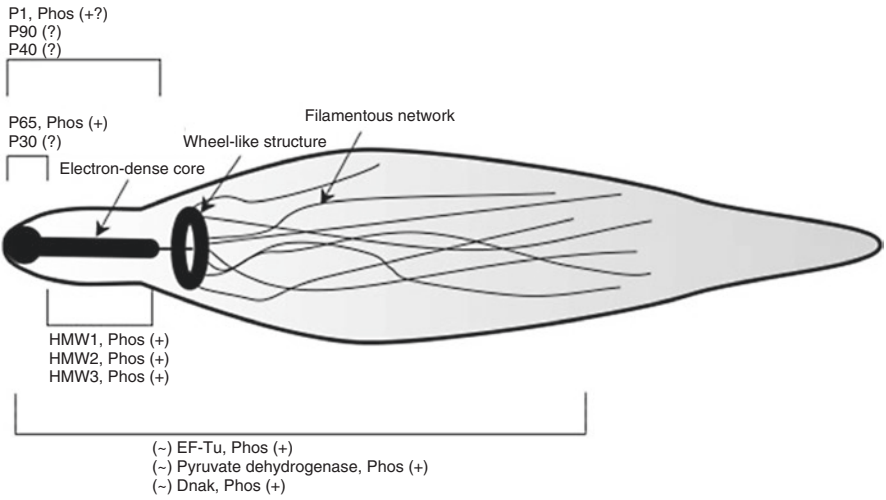

Fig. 33.2 AO proteins (From: Su et al. 2007)

- Large proteinaceous structure (~37 proteins) that has cytoskeletal qualities (Nakane et al. 2015); many of the proteins are phosphorylated (Su et al. 2007) (Fig. 33.2)
- Attaches to sialoglycoproteins and glycolipids on host cells
- Also involved in gliding motility and cell division (Balish 2014)

– Afimbrial adhesins:

- Interact with multiple cytadherence accessory proteins
- **P1** (*M. pneumoniae*); **MgPa** (*M. genitalium*) (Krause 1996, 1998):
  - Found around periphery of cell but concentrated in the AO
- **P30** (*M. pneumoniae*) (Baseman et al. 1987; Romero-Arroyo et al. 1999); **P32** (*M. genitalium*) (Relich and Balish 2011)
  - Restricted localization to the AO tip structure
- Cytadherence accessory proteins:
  - Responsible for AO structure and for P1 and P30 localization to AO
  - **P40, P90**: surface proteins (along with P1 and P30)
  - **HMW1, HMW2, HMW3, P24, P28, P41, P65, P200, TopJ**: internal proteins

– Biofilm formation (Simmons et al. 2013):

- Type 2 strains form more robust biofilms than type 1

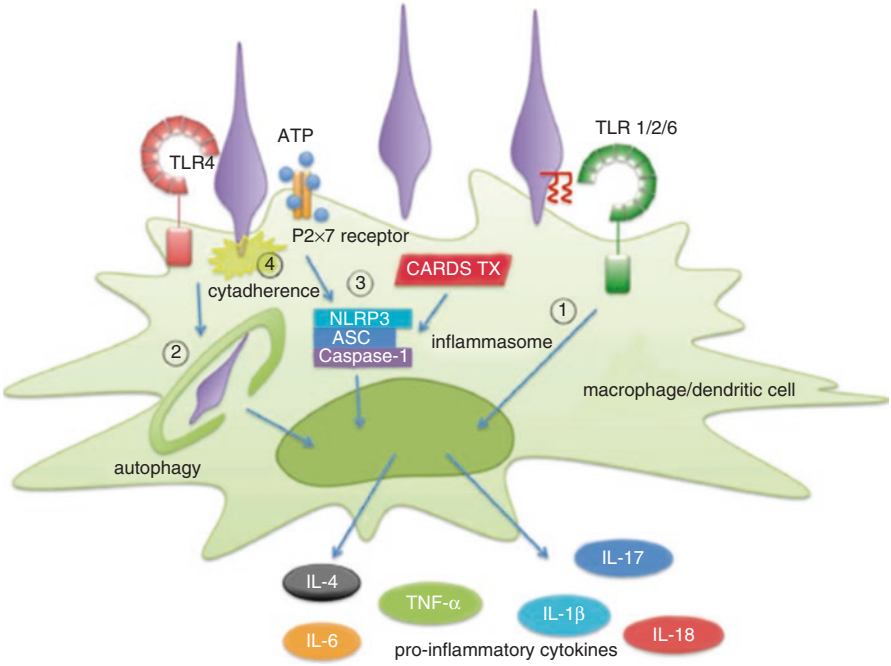

Fig. 33.3 Inflammation pathways (From Shimizu 2016)

- Damage to host cells:
  - Induce inflammatory responses (Shimizu 2016; Waites and Talkington 2004): (Fig. 33.3)
    - H_2O_2 and ROS production: induces oxidative stress and damages host cells
    - Membrane lipoproteins (Shimizu 2016):
      - Induce inflammatory response through interaction with host Toll-like receptor-2 (TLR-2)
      - Increases expression of pro-inflammatory cytokines (TNF-α, IL-1, IL-6) and chemokines; leads to neutrophil infiltration into lungs
  - **CARDS** (community-acquired respiratory distress syndrome) toxin (Kannan and Baseman 2006):
    - Induces host inflammatory response
    - ADP-ribosylation and vacuolating activity; similar to *Bordetella pertussis* pertussis toxin activity
    - ADP-ribosylates and activates the NOD-like receptor containing pyrin domain 3 (NLRP3) inflammasome (Bose et al. 2014)

- expression is increased during infection (Kannan et al. 2010); higher levels in type 2 strains, which may correlate to increased toxicity (Lluch-Senar et al. 2015)

- MyMpn database (http://mympn.crg.eu): online database on *Mycoplasma* – omics (Wodke et al. 2015)

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Chapter 34
Anti-Virulence Factor Therapeutics

There is little doubt that the startling increases in the prevalence and diversity of antibiotic-resistant bacterial pathogens, which are leading to the significant loss of effective antibiotics to treat bacterial infections, are one of the most, if not the most, critical threats to the health of the entire world population. "We risk being in a post-antibiotic world," said Thomas Frieden, former director of the US Centers for Disease Control and Prevention (Fortune, May 27, 2016). Dr. Frieden also stated that "It basically shows us that the end of the road isn't very far away for antibiotics – that we may be in a situation where we have patients in our intensive care units, or patients getting urinary-tract infections for which we do not have antibiotics" (Washington Post, May 27, 2016). These remarks were in reference to the first identification of a colistin-resistant *E. coli* isolate in the USA, which the study's authors state "heralds the emergence of a truly pan-drug resistant bacteria" (McGann et al. 2016). Former United Nations Secretary General Ban Ki-moon remarked: "Antimicrobial resistance poses 'a fundamental, long-term threat to human health, sustainable food production and development.' It is not that it may happen in the future. It is a very present reality – in all parts of the world, in developing and developed countries; in rural and urban areas; in hospitals; on farms and in communities, we are losing our ability to protect both people and animals from life-threatening infections" (SG/SM/18107-DEV/3241; 21 September 2016).

Beyond curbing the inappropriate prescription and use of antibiotics in the healthcare and agricultural fields, which is one of the major factors driving the rise of antibiotic resistance, new strategies must be explored that overcome or bypass the molecular mechanisms that are responsible for the resistance (Kostyanev et al. 2016; Nathan 2015). By their very nature, antibacterial antibiotics target essential functions within the bacterial cell, including metabolic processes, replicative processes (replication, transcription, translation), and structural components (cell wall, membranes), leading to inhibited growth (bacteriostatic) or death (bacteriocidal) of the bacterial cell (Blair et al. 2015; Geisinger and Isberg 2017; Holmes et al. 2016). Hence, antibiotic treatment imposes a significant selective pressure on the affected bacteria, leading not only to the loss of sensitive bacteria (the goal of antibiotic use)

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but also to the proliferation of mutant bacteria that are resistant (the unintended consequence). Bacteria can be intrinsically resistant to an antibiotic (i.e., lack the target or is impermeable to the antibiotic), or they can acquire resistance through mutations (i.e., changes in target, bypass of target process, or increase in efflux of antibiotic) or through the horizontal gene transfer (HGT) of mobile genetic elements that encode resistance genes (i.e., enzymes that inactivate the antibiotic). Since most antibiotics are actually produced by microbes themselves, they have been present in the environment for millions of years, as have the mechanisms that lead to resistance. Therefore, it is not uncommon to clinically observe resistance to a bacteriocidal antibiotic within a very short amount of time following introduction into the patient population. It is also important to keep in mind that although antibacterials usually target specific proteins or structures, these structures can be conserved in many different bacterial species, including the beneficial normal microbiota found in human hosts.

To circumvent antibiotic resistance, new (and old) therapeutics are being evaluated. These approaches include the use of specific bacteriophage (and their associated lysins) and structurally nanoengineered antimicrobial peptide polymers [SNAPPs; (Lam et al. 2016)] to disrupt the cell membranes of susceptible bacterial pathogens (Fernebro 2011; Gadakh and Aerschot 2015; Rios et al. 2016), the use of antibiotic adjuvants that can minimize resistance mechanisms and enhance the efficacy of current antibiotics (Fernebro 2011; Mühlen and Dersch 2016; Wright 2016), the generation of virulence factor-specific vaccines that enhance the host antibody responses (Fernebro 2011; Rios et al. 2016), and the use of photoexcited quantum dots or photoreactive dyes to either alter the redox state of the bacteria (Courtney et al. 2016) or generate reactive oxygen species (ROS)-based cell death (Taylor et al. 2002). In addition, new antibiotics are being identified by screening uncultivable microbes using iChip technology (Nichols et al. 2010) [e.g., teixobactin (Ling et al. 2015)] and target-based approaches [e.g., bedaquiline (Osborne 2013; Zumla et al. 2013)]. Also, semisynthetic chemical methodologies [i.e., new macrolides; (Seiple et al. 2016)] and metagenomic in silico approaches (Fortman and Mukhopadhyay 2016) are being used to generate new antibiotics. However, most of these approaches still rely on the ability of the antibiotic to kill or stop the growth of the bacterial pathogen.

Instead of attacking the growth and replication of bacterial pathogens, new anti-virulence (anti-infection) strategies target specific virulence traits without affecting the viability of the pathogen itself (Cegelski et al. 2008; Czaplewski et al. 2016; Dickey et al. 2017; Escaich 2010; Fernebro 2011; Gadakh and Aerschot 2015; Hauser et al. 2016; Mühlen and Dersch 2016; Rasko and Sperandio 2010). These approaches have the potential to significantly lessen the selective pressure driving antibiotic resistance while mitigating the effects on the normal microbiota and the associated clinical side effects. However, since these strategies do not result in the direct destruction of the bacterial pathogen, they may still persist within the host and eventually cause damage if not attacked by the host immune system. In addition, these so-called virulence blockers would only be effective against pathogens that express the specific targeted virulence factor. Therefore, anti-virulence factors may

find their most useful clinical effects in combination with other anti-virulence factors or with known antibiotics.

Previous chapters in this book have described the different classes of bacterial virulence factors and their expression in specific pathogens. This chapter will focus on anti-virulence strategies and molecules that are directed against these different classes, including adherence factors, secretion systems, exotoxins, immune evasion, biofilm formation, and quorum-sensing/two-component system (QS/TCS) regulatory networks. While this chapter is not designed to be an exhaustive compilation of specific anti-virulence therapeutics, informative examples of molecules that have been identified will be presented, some of which have been approved by the FDA and/or studied in clinical trials (Fig. 34.1) (Czaplewski et al. 2016; Dickey et al. 2017). The hope is that the reader will be informed by this material as well as stimulated to think about new strategies to combat this serious healthcare risk into the future.

Anti-adherence Factor Therapeutics

Attachment to host surfaces is often the first step in the infection process and, therefore, a critical target for anti-virulence strategies. In addition, bacterial adhesins play an important role in promoting biofilm formation, another key virulence trait of many bacterial pathogens. Major adherence factors include fimbrial adhesins (e.g., type I fimbriae; Curli fimbriae, type IV fimbriae; P pili, etc.), afimbrial adhesins (e.g., sortase-dependent MSCRAMMs, OMPs, adhesive autotransporters, etc.), and outer surface lipopolysaccharides. One strategy to block adherence is to interfere with the binding of bacterial adhesins to their cognate host receptors. This effect can be accomplished by using either derivatives of host receptors to compete for the binding to the adhesin, specific antibodies to block the adhesin binding sites, or small molecules that interfere with the binding. For instance, preclinical studies identified the mAb926 monoclonal antibody and the small molecule sugar biaryl mannoside 22, both of which can compete with the binding of host mannose residues to the FimH tip adhesin of type I fimbriae in UPEC (Jarvis et al. 2016; Kisiela et al. 2015). Also, the KP3-engineered antibody can interfere with the *K. pneumoniae* MrkA type III fimbriae subunit (Wang et al. 2016), and the SMITB14 monoclonal antibody can block the binding of *M. tuberculosis* outer surface lipoarabinomannan (Hamasur et al. 2004).

Another strategy is to inhibit the synthesis and/or assembly of fimbrial and afimbrial adhesins. Many fimbrial adhesins, including type I fimbriae and P pili, are secreted and assembled by the chaperone-usher pathway (CUP). Pilicides and curlicides inhibit the assembly of fimbriae/pili by targeting the CUP chaperone function. For instance, the pilicide bicyclic 2-pyridone 2c blocks the ability of the chaperone to transfer the structural fimbriae/pili subunits to the CUP usher component in both type I and P pili assembly (Pinkner et al. 2006). The antiparasitic drug nitazoxanide (NTZ) prevents the proper folding of the usher transmembrane β-barrel pore, which

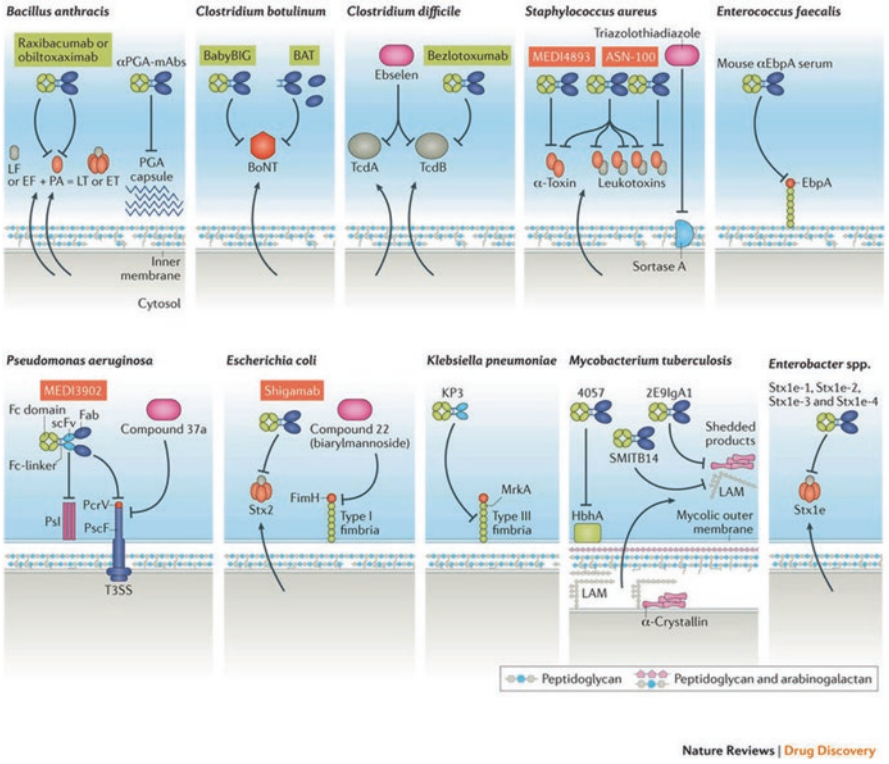

Fig. 34.1 Antivirulence agents targeting secreted and surface-exposed virulence factors. Overview of pathogenic secreted and surface-exposed virulence factors and inhibitors. Antivirulence agents in green or orange boxes are FDA approved or in clinical trials, respectively. Bacillus anthracis secretes lethal factor (LF) and oedema factor (EF), which interact with the common receptor-binding component, protective antigen (PA), to form lethal toxin (LT) or oedema toxin (ET). Raxibacumab and obiltoxaximab block the interaction of PA with the receptor. In Staphylococcus aureus infections, α-toxin and four members of the leukotoxin family have been targeted by antibodies: MEDI4893 binds α-toxin; ASN-1 binds α-toxin and three members of the S. aureus leukotoxin family; and ASN-2 targets LukGH, the only leukotoxin not recognized by ASN-1. The combined ASN-1 and ASN-2 mAb combination is called ASN-100. MEDI3902 is an engineered bispecific antibody that recognizes both Psl and PcrV. Fimbriae and pili are involved in cell adhesion, and these have been targeted by EbpA anti-sera (which blocks the EbpA pilus tip subunit in Enterococcus faecalis), Compound 22 (which inhibits the FimH component of type I fimbriae in Escherichia coli) and KP3 (which binds the MrkA shaft of type III fimbriae in Klebsiella pneumoniae). BabyBIG, botulism immune globulin intravenous; BAT, botulism antitoxin heptavalent; BoNT, botulinum neurotoxin; LAM, lipoarabinomannan; mAbs, monoclonal antibodies; PGA, poly-γ-D-glutamic acid; scFv, single-chain variable fragment; Stx, Shiga toxin; T3SS, type III secretion system

inhibits the type I and P pili from UPEC (Chahales et al. 2016). The curliside ring-fused 2-pyridone FN075 blocks the formation of Curli fimbriae, as well as Curli-dependent biofilms, by interfering with the polymerization of the CsgA structural subunit (Cegelski et al. 2009). Using a structure-based in silico screening approach,

the small molecule AL1 [*N*-(4-chloro-phenyl)-2-(5-[4-(pyrrolidine-1-sulfonyl)-phenyl]-[1,3,4]oxadiazol-2-yl-sulfanyl)-acetamide)] was identified by its ability to inhibit the polymerization of type I fimbriae structural subunits (Lo et al. 2014).

Afimbrial adhesins (MSCRAMMs) and fimbriae in Gram-positive pathogens are linked to the peptidoglycan layer through a sortase-dependent mechanism. Sortases are cysteine transpeptidases that catalyze the covalent linkage of the adhesin polypeptide to the pentapeptide cross-bridges found within the lipid II component of the peptidoglycan layer. Inhibition of sortase enzymatic or substrate-binding activity is predicted to hinder adherence of Gram-positive pathogens such as *S. aureus* and *S. pyogenes*, thus providing potential anti-virulence capabilities (Cascioferro et al. 2014). Using high-throughput screening and virtual in silico screening, numerous small organic molecules have been identified that block these activities. One of the most promising is molecule 6e ([3-(4-pyridinyl)-6-(2-sodiumsulfonatephenyl) [1,2,4]triazolo[3,4-b][1,3,4]thiadiazole]), which inhibits SrtA from *S. aureus* and *S. pyogenes* and is protective in BALB/c mice studies (Zhang et al. 2014). Other promising synthetic and plant natural small molecule inhibitors include DMMA [(Z)-3-(2,5-dimethoxyphenyl)-2-(4-methoxyphenyl) acrylonitrile)] (Oh et al. 2010), β-sitosterol-3-O-glucopyranoside, berberine chloride, curcumin, diihydro-β-carboline, and aryl(β-amino)ethyl ketones (AAEKs) (Cascioferro et al. 2014). Non-cleavable, substrate-derived peptidomimetic peptides also have irreversible, competitive inhibitory activity, including the peptidyl-diazomethane and peptidyl-chloromethane analogs Cbz (benzyloxycarbonyl)-Leu-Pro-Ala-Thr-CHN_2 and Cbz-Leu-Pro-Ala-Thr-CH_2Cl (Scott et al. 2002).

Anti-secretion System Therapeutics

Gram-negative bacterial pathogens use several different secretion systems to export exotoxins and effector polypeptides beyond their hydrophobic outer membranes into the environment or directly into host cells. T3SSs and T4SSs are usually only expressed in pathogens and are the predominant secretion systems used to insert effector proteins into host cells. T3SSs are used by ~25 Gram-negative pathogens including *E. coli*, *S. enterica*, EIEC/*Shigella*, *P. aeruginosa*, and *Y. pestis*, and T4SSs are used by *L. pneumophila* and *H. pylori* to insert effector proteins. Since the structural components for T3SSs and T4SSs are located outside the bacterial surface, they are prime candidates for anti-virulence strategies (Charro and Mota 2015; Duncan et al. 2012; Hauser et al. 2016; Johnson and Abramovitch 2017; Marshall and Finlay 2014; Mühlen and Dersch 2016). The synthesis, assembly, and secretory functions of these systems are complex, involving many different polypeptides. Therefore, to block these systems, one could target the regulation of the synthesis of the structural polypeptides, their assembly into a functioning "needle" complex, or their interaction with host cell receptors. In the Gram-positive acid-fast pathogen *M. tuberculosis*, T7SS (ESX) secretion systems play an essential role in virulence, and anticytolytic screens have recently identified benzothiophene

(BPT15) and benzyloxy benzylidene hydrazine (BBH7) compounds that block secretion in this important pathogen (Johnson and Abramovitch 2017; Rybniker et al. 2014).

Whole-cell-based high-throughput screens (HTS) have identified numerous natural and synthetic small molecules that can interfere with T3SSs. For instance, the *O*-acetyl salicylideneanilide clioxanide and *N*-hydroxybenzamidazole derivatives block the multiple adaptational response (Mar) transcription factor LcrF that regulates the T3SS in *Y. pseudotuberculosis* (Kauppi et al. 2003; Kim et al. 2009), and the fungal metabolite cytosporone B (Csn-B) inhibits the *S. enterica* T3SS by affecting the transcription of key regulator proteins (Li et al. 2013). Essential T3SS ATPases, such as *Y. pestis* YscN, provide the energy for translocation and are involved in dissociating effectors from their bacterial chaperones. Compounds 7086, 7832, and 7812, which were identified in an in silico approach for molecules that inhibit the catalytic domain of the YscN ATPase, could block translocation of a *Y. pestis* effector protein (Swietnicki et al. 2011). Secretin proteins, which form the pore within the T3SS basal body through which effectors are translocated, are effectively blocked by TTS29 and other 2-imino-5-arylidenethiazolidin-4-one analogs in *S. enterica*, *Y. enterocolitica*, and *P. aeruginosa* (Kline et al. 2008). Assembly of the needle complex is the target of the salicylidene acylhydrazide (SAH) compound INP0400 (Veenendaal et al. 2009). Numerous SAH compounds have been identified and seem to block various different aspects of T3SS function in EHEC, EIEC/*Shigella*, *S. enterica*, *Y. pseudotuberculosis*, and *C. trachomatis*, some of which can be reversed by the addition of iron (Charro and Mota 2015; Ernst and Peschel 2011; Marshall and Finlay 2014). Another target within the assembly process is the structural filament itself, and a target-based approach identified two 15-amino acid peptidomimetics, CoilA and CoilB, that block assembly of the EspA EPEC filament protein by mimicking its essential coiled-coil domain (Larzabal et al. 2010). One of the most promising strategies is using specific antibodies to block tip adhesin proteins, such as *P. aeruginosa* PcrV, that interact with host receptors in the cell membrane (Sawa et al. 2014). The multifunctional bispecific anti-PcrV/anti-Psl antibody MEDI3902 (a.k.a. BiS4αPa) is protective in mouse models of pulmonary infection and is undergoing testing in Phase II trials (Dickey et al. 2017; DiGiandomenico et al. 2014). Numerous natural products have been shown to be effective T3SS inhibitors, including the glycolipid caminoside B isolated from the marine sponge *Caminus sphaeroconia* (Linington et al. 2006) and the guadinomines and Aurodox produced by *Streptomyces* spp. (Iwatsuki et al. 2008; Kimura et al. 2011).

Anti-exotoxin Therapeutics

Exotoxins are secreted polypeptides that have been implicated in the pathogenesis of most medically relevant bacterial pathogens, including *B. anthracis*, *C. botulinum*, *C. tetani*, *C. diphtheriae*, EIEC/*Shigella*, *S. aureus*, *S. pyogenes*, and *V.*

cholerae. Anti-exotoxin therapeutics have focused on chemical inhibitors that either block the expression and secretion of exotoxins or affect the entry and trafficking of exotoxins into host cells, and on specific antibodies that neutralize the toxin once inside the human host (Dickey et al. 2017; Garland et al. 2017; Hauser et al. 2016; Johnson and Abramovitch 2017; Mühlen and Dersch 2016; Ruer et al. 2015). For instance, the small molecule virstatin blocks the dimerization of the *V. cholerae* ToxT transcription factor that regulates the expression of cholera toxin (Hung et al. 2005; Shakhnovich et al. 2007), and toxtazins A and B affect the expression of ToxT (Anthouard and DiRita 2013). Several of the T3SS inhibitors described above, such as compounds 7086, 7832, and 7812 and some SAHs, block the translocation of exotoxins into the host cytoplasm.

The B subunit of A-B exotoxins, such as anthrax toxin, botulinum toxin, and Shiga toxin, interacts with specific host cell surface molecules to promote attachment of the toxin and entry of the catalytic A subunit into susceptible host cells. This essential interaction is the target of small molecule inhibitors and antibody inhibitors (Dickey et al. 2017; Fernebro 2011; Nestorovich and Bezrukov 2014). Binding of the PA anthrax toxin B subunit to the host receptors ANTRX2/CMG2 and ANTRX1/TEM8 can be blocked by peptidomimetic peptides and small molecules (Basha et al. 2006; Rogers et al. 2012). Several monoclonal antibodies have been successfully used to block anthrax toxin binding to its receptors, including raxibacumab from GlaxoSmithKline and obiltoxaximab from Elusys Therapeutics, both of which are now FDA approved for use (Greig 2016; Migone et al. 2009; Tsai and Morris 2015). The B subunits of Shiga and Shiga-like toxins interact with Gb3 glycolipid receptors; however, attempts to block this interaction with Gb3 sugar mimics and the C-9 inhibitor of glucosylceramide synthase have had varying degrees of success (Kitov et al. 2000; Silberstein et al. 2011). Branched multivalent ligands can be used as GM1 receptor mimics for cholera toxin B subunits (Pickens et al. 2002). These receptor mimics, which are derived from *m*-nitrophenyl-α-D-galactoside (MNPG), also show efficacy against ETEC heat labile LT toxin, raising the possibility that they may be effective against both toxins in the human host.

Numerous antibody-based therapeutics have been successfully used to neutralize exotoxin activity, several of which are FDA approved for use (Fig. 34.1). Examples include BabyBIG and BAT antibodies directed against *C. botulinum* (botulinum toxin) [(Arnon et al. 2006); FDA approved], Shigamab antibodies directed against EHEC/*Shigella* Shiga and Shiga-like toxins [(Sauter et al. 2008); Phase I trials], bezlotoxumab antibodies directed against *C. difficile* TcdB toxin [(Lowy et al. 2010); FDA approved], and the MEDI4893 and AR-301 antibodies directed against *S. aureus* α-hemolysin [(Oganesyan et al. 2014); Phase II clinical trial]. Small molecule inhibitors of exotoxin activity have also been identified, including PT-8420 and PT-8541 that block lethal factor (LF) anthrax toxin activity (Jiao et al. 2010), compound 22 that blocks edema factor (EF) anthrax toxin activity (Chen et al. 2012), and ebselen, a small organoselenium compound that blocks *C. difficile* TcdA/TcdB cysteine protease domain activity (Bender et al. 2015).

Hemolytic pore-forming toxins (PFTs), such as *S. aureus* α-toxin and *S. pyogenes* streptolysin-O, bind to host red blood cell (RBC) membranes resulting in

cytolytic effects. A fascinating new strategy is to use hydrogel retaining toxin-absorbing nanosponges that are coated with RBC membranes to absorb and neutralize the toxins at infection sites away from their cellular targets (Hu et al. 2013; Wang et al. 2015). These nanosponges were effective in neutralizing α-toxin and in treating methicillin-resistant *S. aureus* (MRSA) infection in a mouse model (Wang et al. 2015). This hydrogel/RBC membrane hybrid material approach promises to be an effective weapon against PFTs in the future. In a similar approach, toxin-absorbing ion exchange resins, such as Synsorb 90 (Synsorb-CD), colestipol, and tolevamer, have been used to bind to the *C. difficile* TcdA and TcdB toxins. However, unsuccessful clinical trials have led to abandonment of these resins (Fernebro 2011).

Anti-immune Evasion (Immunomodulatory) Therapeutics

The ability to evade the host innate immune system is a characteristic of many bacterial pathogens. The major mechanism to avoid phagocytosis is the expression of an anti-phagocytic capsule. Beyond the vaccine-derived antibodies that are effective against *S. pneumoniae*, *N. meningitidis*, and *H. influenzae* (Hib) capsules, engineered monoclonal antibodies (11D, 4C, F26G3, F24F2, and F26G4) directed against the *B. anthracis* poly-γ-D-glutamic acid (PGA) polypeptide capsule are protective in preclinical mouse models of inhalation anthrax (Chen et al. 2011). An early step in the activation of host inflammatory processes is the recognition of bacterial pathogen-associated molecular patterns (PAMPs) by host pattern recognition receptors (PRRs), including the Toll-like receptors TLR4 (binds LPS), TLR1-TLR2 (binds lipoteichoic acid and lipoproteins), and TLR5 (binds flagellin). Thus, anti-virulence strategies that activate these TLRs or induce pro-inflammatory responses are under investigation, especially with pathogens that stimulate intense inflammatory reactions such as GI pathogens (Hennessy et al. 2010). Examples include eritoran, a synthetic lipodisaccharide TLR4 antagonist [(Mullarkey et al. 2003); Phase III trials] and Cadi-05, a polyTLR agonist [(Parkinson 2008); Phase III trials]. Also, in diseases associated with abnormal immune responses to GI microbiota, such as ulcerative colitis and Crohn's disease, immunosuppressive anti-inflammatory antibodies targeting the TNFα cytokine (infliximab) and the α4 integrin subunit of leukocyte adhesion molecules (natalizumab) are in Phase II clinical trials (Jiang et al. 2011).

The use of antimicrobial peptides [AMPs; a.k.a., host defense proteins (HDPs)] and bacteriocins has garnered significant interest (Fernebro 2011; Rios et al. 2016). AMPs are 10–50 amino acid cationic polypeptides that have pore-forming cytolytic activity, primarily through electrostatic attachment to anionic membrane structures (Hancock and Sahl 2006). Eukaryotic AMPs, such as the cathelicidin LL-37, β-defensins, and magainins, can have bacteriolytic and/or immunomodulatory mechanisms of action and are effective against bacterial biofilms (Batoni et al. 2016; Bowdish et al. 2005; Dawgul et al. 2014; Duplantier and van Hoek 2013). The defensin-mimetic PMX-30063 (brilacidin) is active against *S. aureus* skin infections

and is currently in Phase II trials for two types of inflammatory bowel disease (IBD) (www.polymedix.com). The fungal AMP, plectasin, and its improved derivative, NZ2114, are effective against MRSA and *S. pneumoniae* and act by targeting the bacterial cell wall precursor lipid II (Schneider et al. 2010). The synthetic AMPs pexiganan and omiganan are being developed for the topical treatment of diabetic foot ulcers and the prevention of catheter-related infections, respectively. While numerous host factors, including proteolytic degradation, binding to serum proteins, and binding to host cells (albeit with lower affinity), may affect the clinical usefulness of AMPs (Starr et al. 2016), new approaches combining two or more AMPs that have synergistic effects and/or adjusting pH, ionic strength, and specific ionic factors are being explored to optimize AMP activity (Walkenhorst 2016).

Bacteriocins are bacterially produced cationic AMPs that can block the growth of other related species (Riley and Wertz 2002). Both Gram-positive bacteriocins (e.g., nisins) and Gram-negative bacteriocins (e.g., colicins) have potential as anti-virulence factors. For instance, nisins and other lactic acid bacteria (LAB)-produced lantibiotics (class I bacteriocins) are already being used as preservatives in the food industry to combat food-borne pathogens (Huang et al. 2013) and are effective against MRSA and VRE (vancomycin-resistant *Enterococci*) (Brand et al. 2010). In addition, piscicolin 126 and diversin RV41 (class IIa bacteriocins) are effective against *Listeria monocytogenes* in mice (Lohans and Vederas 2012). Nisin Z can modulate the immune system by stimulating the expression of chemokines MCP-1, IL-8, and Gro-α, providing protection against *S. aureus*, *S. enterica*, and *E. coli* in mice models (Kindrachuk et al. 2012). This immune system modulation is similar to that seen with the host AMPs LL-37 and defensins.

Anti-biofilm Therapeutics

Biofilms account for ~80% of persistent bacterial infections in humans (National Institutes of Health) including antibiotic-resistant infections, many of which are associated with indwelling medical devices, such as catheters, IV tubes, ventilators, and prosthetic devices. Primary biofilm infections are localized at the site of the indwelling medical device or the initial colonization, whereas secondary biofilm infections can result from cells dispersing from the initial biofilm and migrating throughout the body. Current options for managing biofilm infections include surgically removing the biofilm and/or the contaminated indwelling device or using pulsed, low-dose antibiotic treatment with a combination of bacteriostatic drugs. Anti-virulence strategies to block biofilm-related diseases could involve creating an antibacterial surface by stopping initial attachment (primarily mediated by type I fimbriae and Curli fimbriae or MSCRAMMs; see above), disrupting the synthesis, maturation, and stability of the biofilm extracellular polymeric substances (EPS) matrix structure or blocking the quorum-sensing mechanisms used by biofilm-forming bacteria (Fig. 34.2) (Beloin et al. 2014; Bjarnsholt et al. 2013; de la Fuente-Nunez et al. 2013; Wu et al. 2015).

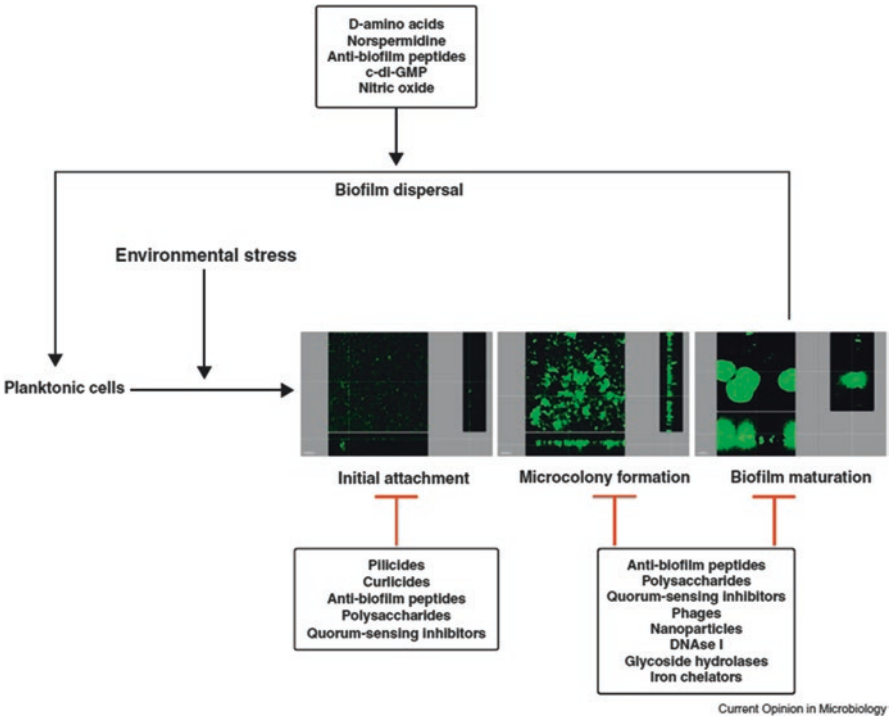

Fig. 34.2 Anti-biofilm strategies (From: de la Fuente-Nunez et al. 2013)

Creating an antibacterial surface that reduces the attachment and subsequent proliferation of bacteria can be accomplished by multiple strategies (Campoccia et al. 2013; Hasan et al. 2013). Surface modification through coating or impregnating central venous catheters and other indwelling devices with antimicrobial molecules, such as AMPs (de la Fuente-Nunez et al. 2016), chlorhexidine plus silver sulfadiazine (*Silvazine*), or nanoparticle silver (and other metal) ion coatings (Markowska et al. 2013), or with broad-spectrum antibiotics, such as minocycline plus rifampin, has had promising results in blocking catheter colonization but does not significantly reduce sepsis or mortality (Gominet et al. 2017; Lai et al. 2016; Miquel et al. 2016; Ramasamy and Lee 2016). Blocking the synthesis and/or assembly of bacterial fimbrial adherence factors has anti-virulence and anti-biofilm possibilities. As mentioned above, the curliside FN075 blocks Curli fimbriae-dependent biofilms (Cegelski et al. 2009). Also, *B. subtilis* biofilms can be inhibited by AA-861 (a benzoquinone derivative), which blocks the formation of TasA-dependent amyloid-like fibers needed for biofilm formation (Romero et al. 2013).

Once formed, the regulated dispersal of biofilms occurs naturally in response to environmental signals, with the subsequent bacterial dissemination leading to life-threatening secondary site infections. There are multiple potential anti-virulence mechanisms by which biofilm dispersal could be achieved (Guilhen et al. 2017).

Biofilms can be dispersed by enzymatically destroying the EPS matrix. Dispersin B (DspB) is a glycoside hydrolase produced by the oral pathogen *Aggregatibacter actinomycetemcomitans* that destroys glucosamine sugars in the EPS matrix (Ramasubbu et al. 2005). Dispersin B, along with other hydrolytic enzymes such as lysostaphin, alpha amylase, and serrapeptase, can increase the efficacy of antibiotics vancomycin and rifampin against MRSA biofilms (Hogan et al. 2017). Overexpression of the *P. aeruginosa* PslG protein, a periplasmic β-D-xylosidase that degrades the Psl polysaccharide in biofilms, can disperse preformed biofilms and has a synergistic effect with the antibiotic tobramycin (Yu et al. 2015). DNAases have also been shown to be effective in targeting the extracellular DNA (eDNA) found in *S. aureus* biofilms (Bhattacharya et al. 2015). The 2-aminoimidazoles, including oroidin, ageliferin, mauritiamine, and 2-aminoimidazole/triazole (2-AIT) conjugates, can disperse biofilms and increase the sensitivity of biofilm cells to typical antibiotics (Rogers et al. 2010). In addition, the QS autoinducer analogs *cis*-2-decenoic acid and furanones can disperse biofilms generated by multiple pathogens, including *P. aeruginosa*, *E. coli*, *K. pneumoniae*, *S. pyogenes*, and *S. aureus* (Davies and Marques 2009; Lonn-Stensrud et al. 2009).

Another strategy for blocking biofilm formation and/or enhancing dispersal is to alter the concentrations of the second messenger c-di-GMP (bis-(3′,5′)-cyclic diguanylic acid) (Antoniani et al. 2010; Guilhen et al. 2017; Ryjenkov et al. 2005; Tolker-Nielsen 2014). In Gram-negative pathogens, high levels of c-di-GMP activate the switch from motile planktonic cells to sessile cells in biofilms, primarily by inducing the synthesis of matrix polysaccharides, such as *P. aeruginosa* Pel and Psl, and by inhibiting motility. However, low levels of c-di-GMP induce biofilm dispersal. Diguanylate cyclases (DGCs) catalyze the synthesis of c-di-GMP, whereas phosphodiesterases (PDEs) break down the molecule. Therefore, inhibitors of DGCs or activators of PDEs would be good anti-virulence targets. For instance, nitric oxide gas (NO) triggers biofilm dispersal in *P. aeruginosa* by activating specific PDEs that break down c-di-GMP (Barraud et al. 2009). The *E. coli* c-di-GMP-binding biofilm dispersal mediator protein BdcA effectively binds and blocks c-di-GMP, leading to biofilm dispersal (Ma et al. 2011). The antimicrobial compound sulphathiazole was identified in a screen for inhibitors of the AdrA DGC and can inhibit c-di-GMP biosynthesis and prevent biofilm formation (Antoniani et al. 2010). Also, a catechol-containing sulfonohydrazide was identified in a screen for inhibitors of the PleD DGC (Fernicola et al. 2015).

Anti-QS Therapeutics

Quorum sensing (QS) is widely used by Gram-positive and Gram-negative pathogens to regulate virulence gene expression and biofilm formation in response to host signals and population densities (Castillo-Juarez et al. 2015; Dow 2017; Hawver et al. 2016; Papenfort and Bassler 2016; Parker and Sperandio 2009). Therefore, it is not surprising that anti-QS therapeutics, termed quorum quenching (QQ), is a

very active area of anti-virulence and anti-biofilm research (Fetzner 2015; Grandclément et al. 2016; Jiang and Li 2013; Kalia 2013; Rampioni et al. 2014; Saurav et al. 2017; Tay and Yew 2013; Wang and Ma 2014; Welsh and Blackwell 2016). Pathogens produce and secrete extracellular signaling molecules, termed autoinducers, that accumulate in the extracellular milieu. Gram-negative pathogens, such as *P. aeruginosa*, *V. cholerae*, *E. coli*, and *L. pneumophila*, synthesize *N*-acyl L-homoserine lactone (AHL) autoinducers by LuxI-type synthases, which diffuse across cell membranes and are sensed by intracellular LuxR-type receptors to control virulence factor transcription. Gram-positive pathogens, such as *S. aureus*, *E. faecalis*, and *B. anthracis*, synthesize and secrete autoinducing peptide (AIP) autoinducers that are sensed by TCSs. AI-1 and AI-2 autoinducers are active against both Gram-negative and Gram-positive bacteria. Therefore, QS inhibitors (QSIs) could target the biosynthesis and secretion of the autoinducer, the receptor sensing the autoinducer, or the autoinducer itself.

Biosynthesis of Gram-negative AHLs by LuxI-type synthases requires S-adenosyl methionine (SAM) and an acyl chain. Therefore, QSIs could block AHL synthase activity, SAM biosynthesis, or fatty acid biosynthesis (the source of acyl chains). For instance, inhibition of the LuxI-type synthase enzyme 50-methylthioadenosine/S-adenosylhomocysteine nucleosidase (MTAN) by the transition state SAM analogs MT-DADMe-Immucillin-A, EtT-DADMe-Immucillin-A, and BuT-DADMe-Immucillin-A can inhibit AI-2-based QS in *V. cholerae* and *E. coli* O157:H7 (Gutierrez et al. 2009). Also, S-adenosyl-homocysteine can block AHL synthesis in *P. aeruginosa* (Parsek et al. 1999). Recent high-throughput screens have identified several small molecule inhibitors of LuxI-type AHL synthases (Christensen et al. 2013) and the AHL analog J8–C8 (N-(3-oxocyclohex-1-enyl) octanamide from the rice pathogen *Burkholderia glumae*, which inhibits the TofI LuxI-like synthase (Chung et al. 2011). Gram-positive AIP biosynthesis inhibitors identified to date include the fungal cyclohexenone metabolite ambuic acid, which inhibits AgrB-dependent AIP maturation in *S. aureus* and *L. monocytogenes* (Nakayama et al. 2009).

Many small molecules, including many plant natural products, have been identified that competitively inhibit the binding of autoinducers to their cognate receptors. The *S. aureus* Agr QS system uses the AgrA–AgrC TCS to regulate the expression of a number of major virulence factors, including phenol-soluble modulins (PSMs), peptidases, and nucleases that are needed for biofilm dispersal. The small molecule inhibitor savirin [3-(4-propan-2-ylphenyl)sulfonyl-1H-triazolo[1,5-a]quinazolin-5-one] targets the AgrA response regulator and efficiently blocks Agr-regulated gene expression and protects against *S. aureus*-induced dermonecrosis without affecting the commensal *S. epidermidis* Agr system (Sully et al. 2014). Also, the N-acetylated trAIP-I D2A derivative of one of the four *S. aureus* AIPs, AIP-I, could inhibit all four Agr systems (Gordon et al. 2013; Lyon et al. 2002). *P. aeruginosa* has two major LuxR-LuxI-type QS systems, and multiple structural analogs of AHL autoinducers have been used successfully to competitively inhibit binding to LuxR-type receptors. For instance, E22 (a phenoxyacetyl homoserine lactone) and mBTL (meta-bromo-thiolactone) are active against the RhlR-RhlI pathway (Eibergen et al.

2015; O'Loughlin et al. 2013), whereas ITC-12 and ITC-13 (isothiocyanate-AHLs), C-30 (halogenated furanone), and 3-oxo-C12-(2-amino-cyclohexanone) act on the LasR–LasI pathway (Hentzer et al. 2003; Wu et al. 2004). The halogenated furanone (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone, isolated from the marine algae *Delisea pulchra*, blocks LuxR from binding DNA and disrupts QS in *E. coli*, *V. harveyi*, and *V. fischeri* (Defoirdt et al. 2007). Also, the cyclic peptide antagonist ZBz1-YAA5911 can block the Fsr QS system in *E. faecalis* (Nakayama et al. 2013). The small molecule, LED209 [N-phenyl-4-(3-phenylthioureido) benzenesulfonamide], inhibits the binding of AI-3 autoinducers to their cognate receptors, QseC-like sensor histidine kinases, thereby inhibiting QseC-mediated activation of *E. coli* virulence gene expression (see Anti-TCS Therapeutics below). Finally, the use of antibody-based QQ to block autoinducer binding to receptors has had recent success (Grandclément et al. 2016). Antibody RS2–1G9, raised against 4-methoxyphenyl amide AHL analogs, can inhibit QS signaling in *P. aeruginosa* (Kaufmann et al. 2006), whereas, antibody AP4-24H11, directed against AIP analogs, blocks the production of the QS-regulated α-hemolysin in *S. aureus* and protects mice from a *S. aureus* challenge (Park et al. 2007).

Four catalytic classes of QQ enzymes that degrade or modify AHLs have been identified in many QS and non-QS bacteria (Chen et al. 2013; Fetzner 2015; Grandclément et al. 2016). Lactonases, such as the prototypical *Bacillus* spp. AiiA (Dong et al. 2002), hydrolyze the homoserine lactone ring, whereas amidases/acylases, including *P. aeruginosa* PvdQ (Huang et al. 2003), hydrolyze AHLs at the amide bond. Reductases, such as the metagenome-derived BpiB09 (Bijtenhoorn et al. 2011), convert 3-oxo-substituted AHLs to inactive 3-hydroxyl-substituted AHLs, whereas cytochrome oxidases such as cytochrome P450 oxidase CYP102A1 from *Bacillus megaterium* (Chowdhary et al. 2007) oxidize acyl chains. In addition, mammalian paraoxonases (PON1, PON2, and PON3) can cleave AHLs (Draganov et al. 2005). It is important to note that simple sequestration of the autoinducer may accomplish the same goal as destruction. For instance, apolipoprotein B, which is a major structural protein of very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL), can sequester AIP-1, thereby blocking MRSA invasive infections (Peterson et al. 2008).

Anti-TCS Therapeutics

Many bacterial pathogens use two-component phosphorelay systems (TCSs) to sense environmental and host signals and respond to these cues by regulating the transcription of many different genes, including virulence genes (Beier and Gross 2006; West and Stock 2001; Zschiedrich et al. 2016). TCSs are comprised of a sensor histidine kinase (HK) located at the bacterial cell surface and a response regulator (RR), which usually acts as a positive or negative transcription factor. Recognition of an environmental or host signal by the HK leads to its autophosphorylation on a histidine residue, followed by phosphorelay from the histidine residue to an

aspartate residue in the RR, which in turn activates its transcriptional activity. Therefore, blocking TCS-dependent transcription of virulence factors would be a good target for anti-virulence strategies (Gotoh et al. 2010; Johnson and Abramovitch 2017; Mühlen and Dersch 2016; Worthington et al. 2013). This inhibition could be accomplished by either blocking the ability of the HK to sense the signal, inhibiting HK autophosphorylation, interfering with HK-RR interactions, or inhibiting the RR transcriptional activity. Some well-studied TCSs that have been targeted include WalK–WalR (a.k.a. YycG–YycF), which regulates Gram-positive cell wall metabolism; KinA–Spo0F, which regulates endospore development; the QS regulators *E. coli* QseC–QseB and *S. aureus* AgrA–AgrC; *M. tuberculosis* PhoP–PhoR, which responds to acidic pH; and *M. tuberculosis* DosR–DosS–DosT, which responds to oxygen concentrations to regulate latency and resistance to NO.

Several ATP-competitive molecules that inhibit the histidine kinase catalytic activity through binding to the ATP-binding domain have been identified. For example, walkmycin C inhibits the WalK (a.k.a., YycG) kinase from *B. subtilis*, *S. aureus*, and *S. mutans* (Eguchi et al. 2011; Okada et al. 2010; Wilke et al. 2015). Closantel and RWJ-49815 are two of several synthetic molecules that block the autophosphorylation activity of KinA and KinA-like sensor kinases in *S. aureus*, *S. pneumoniae*, and VRE (Stephenson and Hoch 2002). As mentioned above, LED209 blocks binding of AI-3 autoinducers to QseC and QseC-like sensor histidine kinases, thereby inhibiting QseC-mediated signaling in EHEC, *S. enterica*, and *F. tularensis*. Also, the small molecule savirin targets the AgrA response regulator and efficiently blocks Agr-regulated gene expression. Ethoxzolamide (ETZ; 6-ethoxy-1,3-benzathiazole-2-sulfonamide) was identified as an inhibitor of PhoP-PhoR signaling in *M. tuberculosis*, blocking T7SS (ESX-1) secretion and attenuating virulence in a mouse model (Johnson et al. 2015). Three molecules, artemisinin, HC102A, and HC103A, were shown to block the *M. tuberculosis* DosS and DosT sensor kinases, leading to inhibition of the DosR regulon and *M. tuberculosis* persistence (Zheng et al. 2017). Anti-virulence molecules targeting HK-RR interactions or RR transcriptional activity have not been identified to date.

Concluding Remarks

Identifying and characterizing the virulence factors of established and emerging bacterial pathogens, which this book has hopefully promoted, as well as understanding the host-pathogen interactions that drive disease manifestation, are the first steps in developing meaningful anti-virulence strategies. To facilitate these studies, multidisciplinary approaches will need to be utilized, including "-omics" (genomics, proteomics, transcriptomics, etc.) as well as genetic, biochemical, immunological, and structure-based rational design strategies. Since these strategies will be inherently specific for individual pathogens and virulence mechanisms, a therapeutic protocol combining established antibiotics and new anti-virulence factors may well need to be used to impede the increasing incidence of antibiotic resistance.

From the limited studies undertaken to date, it is nonetheless clear that anti-virulence strategies can have profound effects on bacterial pathogenesis in the laboratory. However, for this new anti-virulence paradigm to gather momentum and, more importantly, acquire increased funding from both government agencies and commercial biopharmaceutical companies, more research and clinical studies need to be advanced. Only a few anti-virulence agents have begun clinical trials, and even fewer have received FDA approval, most of which are antibody-based approaches directed against exotoxins (Dickey et al. 2017; Czaplewski et al. 2016). Since most anti-virulence molecules are being identified and characterized using in vitro and animal models, the next step of clinical trials is critical and must be aggressively fostered intellectually and financially. Only through successful clinical trials will potential new anti-virulence drugs be identified and, eventually, be available for use in the treatment of highly recalcitrant antibiotic-resistant bacterial infections.

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