REVIEW ARTICLE

Surface organelles assembled by secretion systems of Gram-negative bacteria: diversity in structure and function

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Abstract

Gram-negative bacteria express a wide variety of organelles on their cell surface. These surface structures may be the end products of secretion systems, such as the hair-like fibers assembled by the chaperone/usher (CU) and type IV pilus pathways, which generally function in adhesion to surfaces and bacterial–bacterial and bacterial–host interactions. Alternatively, the surface organelles may be integral components of the secretion machinery itself, such as the needle complex and pilus extensions formed by the type III and type IV secretion systems, which function in the delivery of bacterial effectors inside host cells. Bacterial surface structures perform functions critical for pathogenesis and have evolved to withstand forces exerted by the external environment and cope with defenses mounted by the host immune system. Given their essential roles in pathogenesis and exposed nature, bacterial surface structures also make attractive targets for therapeutic intervention. This review will describe the structure and function of surface organelles assembled by four different Gram-negative bacterial secretion systems: the CU pathway, the type IV pilus pathway, and the type III and type IV secretion systems.

Introduction

Bacteria assemble a range of different proteinaceous appendages on their cell surface. These surface organelles are traditionally grouped into two categories: flagella, which are long, whip-like structures required for motility; or pili or fimbriae, which are thinner, hair-like structures required for adhesion. Bacterial surface structures distinct from flagella were first described in the late 1940s and early 1950s, made possible by the introduction of the electron microscope (Ottow, 1975). Duguid and co-workers first used the term 'fimbriae', Latin for thread or fiber, in 1955 to describe surface appendages that allowed *Escherichia coli* to bind and agglutinate erythrocytes (Duguid *et al.*, 1955). Brinton later used the term "pili", Latin for hair, to describe the nonflagellar surface structures expressed by *E. coli* (Brinton, 1959). In an important 1975 review, Ottow suggested that the term 'pili' be reserved for the F or conjugative pili involved in bacterial mating and that the term 'fimbriae' be used to describe surface fibers involved in adhesion (Ottow, 1975). However, this terminology scheme did not stick, and today, the terms 'pili' and 'fimbriae' are generally used interchangeably. We will use the term 'pili' in this review.

Various schemes have been proposed over the years to classify the different types of pili (Duguid *et al.*, 1966; Ottow, 1975; Orskov & Orskov, 1990; Low *et al.*, 1996; Thanassi *et al.*, 2007). Although most of these classification schemes are no longer commonly used, parts have entered the standard nomenclature, most prominently the term 'type 1 pili' to refer to surface fibers that mediate adhesion in a mannose-sensitive manner (Duguid *et al.*, 1966) and the term 'type IV pili' to refer to polarly localized appendages that mediate twitching motility (originally referred to by Ottow as Group 4) (Ottow, 1975). Unfortunately, this terminology causes confusion in that it has now become standard practice to refer to bacterial secretion systems by 'type' names. Thus, type I secretion is not related to type 1 pili, which are assembled by the chaperone/usher (CU) secretion system, and type IV pili are not related to type IV secretion, but instead are closely related to the type II secretion pathway (readers are referred to the companion review in this issue by Dalbey and Kuhn for an overview of bacterial secretion pathways).

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Organelles such as flagella and pili can be considered the functional end products of bacterial secretion systems; that is, they are assembled by dedicated secretion systems to function in motility or adherence. However, bacterial secretion systems also assemble surface structures that function as integral components of the secretion machinery itself. For example, some secretion systems assemble pilus-like structures to serve as extensions of the secretion machinery outside the bacterial envelope, providing conduits for the delivery of substrate proteins and in some cases enabling the transfer of effector proteins into the cytoplasm of target host cells. Such surface structures may also provide sensory feedback to the secretion systems to detect target cell contact or function as adhesins to allow docking to host cell receptors.

This review will focus on surface organelles assembled by secretion systems found in Gram-negative bacteria. We will provide overviews of the structure and function of two types of canonical adhesive pili: pili assembled by the CU pathway and type IV pili. We will also describe the type III and type IV secretion pathways and the surface structures assembled by these pathways for the delivery of bacterial molecules inside target cells. For the CU and type IV pilus systems, we will focus on the structures of the surface fibers and their roles during pathogenesis. For the type III secretion system, we will focus on the structure and function of the tip complex that caps the surface-exposed needle structure of the type III secretion machinery. Finally, for the type IV system, we will describe recent developments in understanding the structural organization of the secretion machinery and the structure and function of surface-exposed elements, including the conjugative pilus.

Pili assembled by the CU pathway

Introduction

The CU pathway is used by Gram-negative bacteria to assemble a large family of virulence-associated surface fibers (for recent reviews, see Nuccio & Bäumler, 2007; Waksman & Hultgren, 2009; Zav'yalov *et al.*, 2010). The CU pathway takes its name from the components of its secretion machinery, which consist of a dedicated periplasmic chaperone and an integral outer membrane (OM) assembly platform termed 'the usher'. The CU pathway assembles peritrichous surface fibers that range from thin, flexible filaments to rigid, rod-like pili or fimbriae. These structures generally function as adhesins, mediating bacterial attachment to and interactions with host tissues. As such, organelles assembled by the CU pathway often function as critical virulence factors. The structures of CU pili are optimized to withstand the various forces encountered during colonization of surfaces, and each pilus is adapted to its particular function in its particular niche. In addition to colonization of host surfaces, CU pili are important for bacterial–bacterial interactions, biofilm formation, and adhesion to abiotic surfaces. The mechanism of fiber assembly and secretion by the CU pathway has been subject to extensive study, and the CU pathway is one of the best understood bacterial secretion systems.

CU pathways are prevalent among enteric members of the γ-proteobacteria, including *E. coli*, *Salmonella*, and *Yersinia*, and are also found among members of the β-proteobacteria as well as in *Cyanobacteria* and *Deinococcus* (Yen *et al.*, 2002; Nuccio & Bäumler, 2007). A group of pili expressed primarily by enterotoxigenic *E. coli* (ETEC), exemplified by the CS1 and CFA/I pili, were thought to be evolutionarily distinct from the CU pathway and were termed 'the alternate CU pathway' (also known as class 5 fimbriae) (Sakellaris & Scott, 1998; Anantha *et al.*, 2004). However, recent genetic and structural studies have shown that these pili are in fact part of the canonical CU pathway family (Li *et al.*, 2007; Nuccio & Bäumler, 2007; Poole *et al.*, 2007). Genes for CU pathways are encoded on both chromosomal and plasmid locations and are clustered together with similar organization among different bacteria: a 5′ region containing regulatory genes and promoters and a single downstream operon containing the required structural and assembly components. In addition, multiple CU pathways may be present in a single bacterial genome, which presumably provides the ability to adhere to a variety of different receptors and surfaces (van der Velden *et al.*, 1998; Felek *et al.*, 2010; Korea *et al.*, 2010). Expression of CU gene clusters is typically highly regulated, subject to phase variation, and responsive to environmental cues (Chen & Elberg, 1977; van der Woude *et al.*, 1996; Blomfield, 2001). Regulatory cross-talk may occur among the multiple CU gene clusters present within a bacterium (Xia *et al.*, 2000). This cross-talk likely ensures that each bacterium only expresses a single pilus at a given time, thus controlling adhesive specificity.

Pili assembled by the CU pathway range from 2 to 10 nm in diameter and generally 1–3 μm in length. These pili can be divided into two general architectural classes. One class comprises rigid, rod-like fibers of larger diameter and the second class comprises thin, flexible fibers or amorphous structures. CU pathways can also be divided into two subfamilies based on conserved sequence differences present in the chaperones. Chaperones with a short loop connecting their F1 and G1 β-strands belong to the F1–G1 short (FGS) subfamily and chaperones with a long F1–G1 loop belong to the FGL subfamily (Hung *et al.*,

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1996; Zavialov *et al.*, 2007). Interestingly, FGL chaperones assemble only thin or amorphous pilus fibers composed of only one or two types of subunits. In contrast, FGS chaperones assemble both rod-like and thin pilus fibers, and the fibers are generally composed of multiple different types of subunits and may have composite architectures. The P and type 1 pili expressed by uropathogenic *E. coli* (UPEC) are prototypical rigid, hair-like pili belonging to the FGS subfamily, and the F1 capsule of *Yersinia pestis* is a prototypical thin, 'afimbrial' structure assembled by the FGL subfamily (Fig. 1). P pili bind to Galα(1–4)Gal moieties present in the globoseries of glycolipids on uroepithelial cells and are associated with the ability of UPEC to colonize the kidney and cause pyelonephritis (Bock *et al.*, 1985; Roberts *et al.*, 1994). Type 1 pili mediate binding to a number of host tissues in a mannose-sensitive manner. UPEC use type 1 pili to bind mannosylated glycoproteins present in the bladder, leading to bacterial invasion inside bladder epithelial cells and the development of cystitis (Abraham *et al.*, 1988; Mulvey *et al.*, 1998). The F1 capsule of *Y. pestis* forms a dense coating around the bacteria that, in contrast to the adhesive functions typically attributed to pili, acts as an 'antiadhesive' structure, preventing phagocytosis by macrophages and inhibiting internalization by respiratory tract epithelial cells (Du *et al.*, 2002; Liu *et al.*, 2006). The F1 capsule is also an important protective antigen of *Y. pestis* and has roles in transmission from the flea vector and in virulence (Titball & Williamson, 2001; Sebbane *et al.*, 2009; Weening *et al.*, 2011). The P and type 1 pili of UPEC and the *Y. pestis* F1 capsule will be used as examples in this section to highlight the structure and function of pili assembled by the CU pathway.

Structural details of CU pilus fibers

Many pili assembled by the CU pathway are composed of two distinct subassemblies: a rigid, helical rod that extends out from the cell surface and a distal tip structure that contains the adhesive activity. Type 1 and P pili exemplify this structural arrangement (Fig. 1b and c). Type 1 and P pilus rods measure 7–8 nm in diameter and are built from a linear homopolymer of over 1000 copies of the major pilin subunit (PapA for P pili, FimA for type 1 pili), wound into a 1-start, right-handed helix (Fig. 2) (Hahn *et al.*, 2002; Mu & Bullitt, 2006). The P pilus rod is terminated by the PapH minor pilin, which also plays a role in anchoring the pilus fiber in the OM (Baga *et al.*, 1987; Verger *et al.*, 2006). In contrast to the rigid helical rod, the pilus tip is a flexible, open helical fiber measuring 2 nm in diameter (Kuehn *et al.*, 1992; Hahn *et al.*, 2002). The P pilus tip fiber is approximately 40 nm long and composed mainly of PapE, which is present at approximately 5–10 copies per pilus. The P pilus adhesin PapG is present in single copy at the distal end of the tip fiber and is joined to PapE via the PapF adaptor subunit (Kuehn *et al.*, 1992; Jacob-Dubuisson *et al.*, 1993) (Fig. 2). Another adaptor subunit, PapK, links the tip fiber to the pilus rod (Jacob-Dubuisson *et al.*, 1993). Type 1 pili have shorter tip fibers compared to P pili (Fig. 1b and c)(Jones *et al.*, 1995; Hahn *et al.*, 2002). Type 1 tips measure 10–19 nm in length and contain a single copy of the FimH adhesin at the distal end, followed by the FimG and FimF adaptor subunits, which are generally present in single copy (Fig. 2) (Jones *et al.*, 1995; Hahn *et al.*, 2002; Le Trong *et al.*, 2010).

In contrast to type 1 and P pili, the F1 capsule is built from a single subunit protein, Caf1, which polymerizes

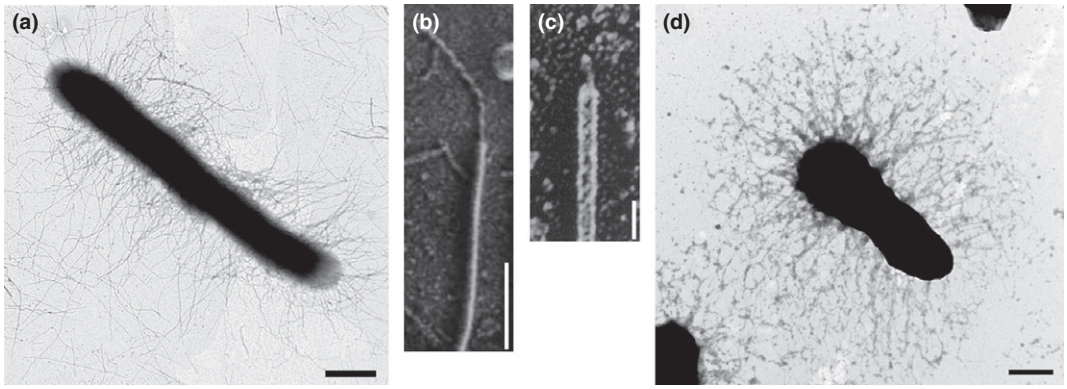

Fig. 1. Electron micrographs of pili assembled by the CU pathway. (a) An *Escherichia coli* bacterium expressing P pili. (b and c) High-resolution, freeze-etch images of individual *E. coli* P and type 1 pili, respectively, showing distal linear tip fibers and helical pilus rods. (d) A *Yersinia pestis* bacterium expressing F1 capsule. Scale bars = 500 nm (a), 100 nm (b), 20 nm (c), and 500 nm (d). Images in (a–d) reproduced with permission from (Kuehn *et al.*, 1992; Jones *et al.*, 1995; Runco *et al.*, 2008; Li *et al.*, 2010).

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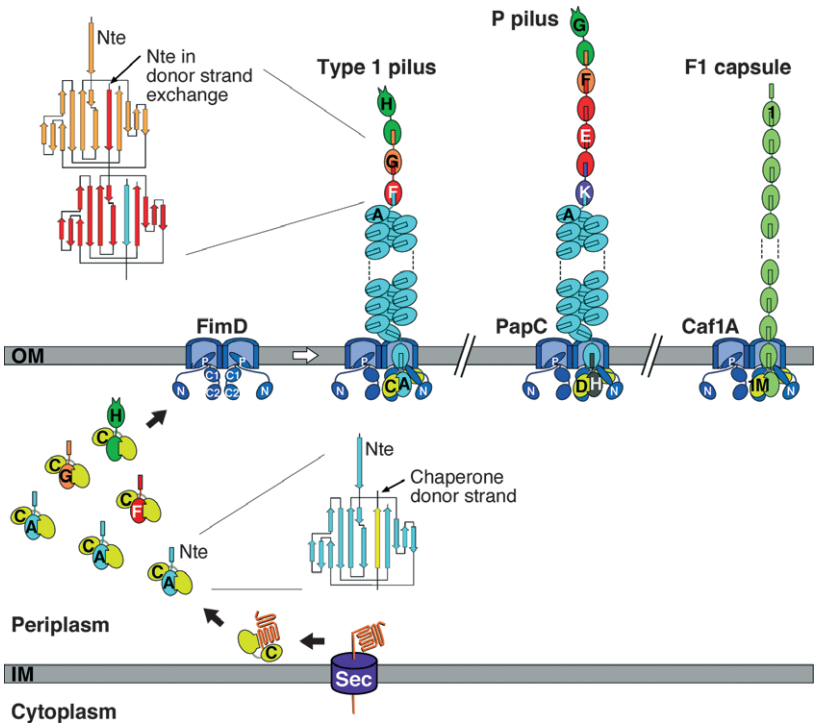

Fig. 2. Model for pilus biogenesis by the CU pathway. The assembly steps for *Escherichia coli* type 1 pili are shown, together with models for fully assembled *E. coli* type 1 and P pili, and the *Yersinia pestis* F1 capsule. The Fim, Pap, and Caf proteins are indicated by single letters (H, FimH; C, FimC; etc.). Pilus subunits enter the periplasm as unfolded polypeptides via the Sec pathway. The subunits fold upon interaction with the periplasmic chaperone, forming stable complexes via donor strand complementation. Assembly and secretion of the pilus fiber occurs at the OM usher, where chaperone–subunit interactions are replaced with subunit–subunit interactions via the donor strand exchange reaction. Topology diagrams are shown depicting the donor strand complementation and exchange reactions occurring in the periplasm and pilus fiber, respectively. The dimeric ushers are depicted with the plug domain that gates the channel shut (P) and the periplasmic N and C domains indicated. The N domain forms the initial binding site for chaperone–subunit complexes, and the C domains provide a second binding site for the assembling pilus fiber. Chaperone–adhesin complexes have highest affinity for the usher and initiate pilus assembly by binding to the usher N domain.

into an extended linear fiber comprising over a thousand subunits (Fig. 2) (Galyov *et al.*, 1990; Miller *et al.*, 1998; Zavialov *et al.*, 2003). Each F1 fiber is approximately 2 nm in diameter, with a flexible, open helical structure similar to the P and type 1 pilus tip fibers. Thus, although the F1 capsule and other so-called afimbrial structures may appear to lack distinct pilus-like architectures when viewed by electron microscopy (EM), these are in fact polymeric fibers similar to all CU pili. The individual F1 fibers likely coil randomly and aggregate together to form the dense, poorly defined coating typically attributed to the *Y. pestis* capsule (Chen & Elberg, 1977; Liu *et al.*, 2006; Runco *et al.*, 2008).

Assembly of pili by the CU pathway: structure dictates assembly and function

The physical and functional attributes of pili assembled by the CU pathway can be understood through the structures of the subunit proteins and how they are assembled into the pilus fiber. Figure 2 provides an overview of pilus biogenesis by the CU pathway, as depicted for type 1 pili. See companion review by Dalbey and Kuhn in this issue for additional information on protein secretion mechanisms and recent reviews for detailed discussions of the CU assembly pathway (Waksman & Hultgren, 2009; Zav'yalov *et al.*, 2010). Newly synthesized pilus subunit proteins enter the periplasm via the Sec general secretory pathway (Driessen & Nouwen, 2008). Once in the periplasm, the subunits form stable complexes with the periplasmic chaperone. The chaperone enables proper folding of the pilus subunits, prevents premature subunit–subunit interactions, and maintains the subunits in an assembly competent state (Choudhury *et al.*, 1999; Sauer *et al.*, 1999, 2002; Zavialov *et al.*, 2003). Periplasmic chaperone–subunit complexes then must interact with the OM usher for release of the chaperone, assembly of subunits into the pilus fiber, and secretion of the fiber to the cell

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surface through the usher channel (Remaut *et al.*, 2008; Phan *et al.*, 2011). The usher acts as a pilus assembly catalyst, accelerating the rate of subunit incorporation into the pilus fiber (Nishiyama *et al.*, 2008). The usher forms a dimeric, gated secretion complex in the outer membrane, although only one usher channel is used for secretion of the pilus fiber, and the function of the usher dimer remains to be determined (Remaut *et al.*, 2008). Recent structural information shows that the usher monomer contains two binding sites for chaperone–subunit complexes, provided by the usher's periplasmic N- and C-terminal domains (Fig. 2) (Phan *et al.*, 2011). These binding sites likely underlie the catalytic activity of the usher, by enabling optimal positioning of chaperone–subunit complexes to promote chaperone release and pilus assembly. Pili are assembled in a top–down order, with the adhesin incorporated first, followed by the rest of the pilus tip and finally the rod. The usher channel is only wide enough to allow secretion of a linear fiber of folded pilus subunits (Remaut *et al.*, 2008; Phan *et al.*, 2011). Therefore, the pilus rod is constrained to a linear fiber as it passes through the usher and only converts to its final helical form upon reaching the bacterial surface (Fig. 2).

All pilus subunits contain a single pilin domain, which is formed by an incomplete immunoglobulin (Ig)-like fold. Adhesin subunits contain an additional, N-terminal, receptor-binding, or adhesin domain in addition to the pilin domain (Fig. 3). The pilin domain lacks the seventh, C-terminal β-strand present in canonical Ig folds, and thus, pilus subunits are unable to complete their own structure (Choudhury *et al.*, 1999; Sauer *et al.*, 1999, 2002; Zavialov *et al.*, 2003). This missing strand results in a deep groove on the surface of the subunit, exposing its hydrophobic core. To complete their folds, pilins must rely on structural information provided by interaction with the chaperone in the periplasm or with neighboring subunits in the pilus fiber. In the periplasm, the chaperone donates a β-strand to complete the pilin domain Ig fold in a mechanism termed 'donor strand complementation' (Figs 2 and 3) (Choudhury *et al.*, 1999; Sauer *et al.*, 1999). In addition to the pilin domain, all pilus subunits (except the adhesin) contain a conserved N-terminal extension (Nte) (Fig. 2). During pilus assembly at the usher, the Nte from an incoming chaperone–subunit complex displaces the donated chaperone β-strand from the preceding subunit, completing the Ig fold of the preceding subunit by a mechanism termed 'donor strand exchange' (Sauer *et al.*, 2002; Zavialov *et al.*, 2003). Thus, the pilus fiber is built from an array of Ig folds, with each subunit noncovalently bound to its neighboring subunit by the donor strand exchange reaction (Fig. 2). This arrangement provides great mechanical strength to the pilus fiber, which is reflected by the property that subunit–subunit interactions in the pilus are resistant to dissociation by SDS unless heated (Henderson *et al.*, 2011). This mechanical strength is critical for the ability of the pili to maintain adhesion in the face of external forces such as the shear force encountered in the bladder from the flow of urine. The helical pilus rod also makes important contributions to the ability of pili to withstand shear forces and maintain adhesion. The helical pilus rod can convert or uncoil under stress to an extended linear fiber, in essence acting as a spring or shock absorber to extend the lifetime of pilus–receptor interactions (Bullitt & Makowski, 1995; Fallman *et al.*, 2005; Miller *et al.*, 2006).

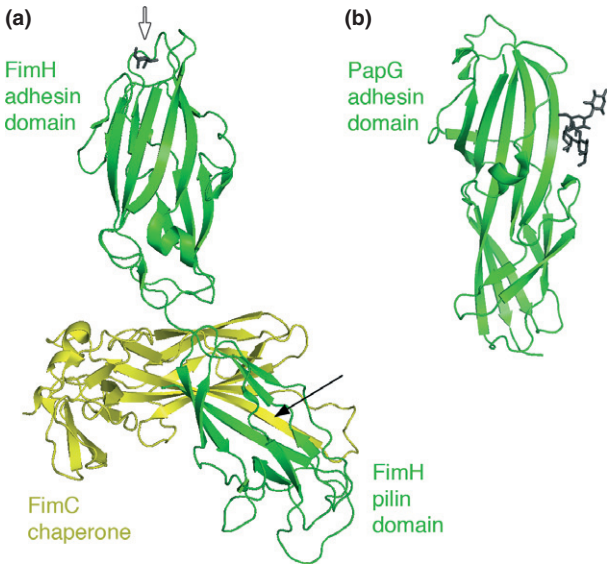

Fig. 3. Structures of the FimH and PapG adhesins. (a) Crystal structure of the FimCH complex with bound mannose (PDB ID: 1KLF; Hung *et al.*, 2002). The FimH adhesin and pilin domains are in green, with the bound mannose at the tip of the adhesin domain shown in dark gray stick representation (white arrow). The FimC chaperone is in yellow; the black arrow indicates the FimC β-strand engaged in donor strand complementation with the FimH pilin domain. (b) Crystal structure of the PapG adhesin domain with bound globoside (PDB ID: 1J8R; Dodson *et al.*, 2001). The adhesin domain is in green, and the tetrasaccharide bound at the side of the adhesin is in dark gray. Images were generated using PyMOL.

Adhesion mediated by CU pili

Many pili assembled by the CU pathway have a single, tip-located subunit (the adhesin) that contains the receptor-binding activity. Such pili have been termed 'monoadhesive pili' and include the type 1 and P pili (Fig. 2) (Zav'yalov *et al.*, 2010). However, not all pili assembled by the CU pathway contain a distinct tip structure or tip-located adhesin. For these pili, the structural subunit that builds the pilus fiber may also contain

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receptor-binding sites, and thus, the entire pilus fiber may function in adhesion. These polyadhesive pili are generally built from only 1–2 subunits and are exemplified by FGL pili such as the F1 capsule (Fig. 2) and the Afa/Dr family of adhesins (Zav'yalov *et al.*, 2010). Structural and binding studies of the polyadhesive pili have revealed binding sites along the exposed surfaces of each pilus subunit (Anderson *et al.*, 2004; Pettigrew *et al.*, 2004; Korotkova *et al.*, 2006).

Structures have been solved for a number of adhesins from monoadhesive pili (Choudhury *et al.*, 1999; Dodson *et al.*, 2001; Sung *et al.*, 2001; Hung *et al.*, 2002; Buts *et al.*, 2003; Merckel *et al.*, 2003; Li *et al.*, 2007). The adhesins are two domain proteins, with a C-terminal pilin domain and an N-terminal adhesin domain (Fig. 3). The pilin domain comprises an incomplete Ig-like fold, as found for all CU pilins, and mediates incorporation of the adhesin into the pilus fiber. Adhesin domains have Ig-like folds similar to the pilin domains, but the adhesin domains are complete (not lacking the terminal β-strand) and structurally distinct (Fig. 3). Duplication and fusion events of a pilin domain likely led to specialization of the adhesin domains for adherence. Despite their common architecture, the adhesins vary greatly in sequence and employ distinct receptor-binding mechanisms, reflecting their diverse functions within the host (Westerlund-Wikstrom & Korhonen, 2005). A comparison between the PapG and FimH adhesins is illustrative of these differences. The residues of PapG that interact with the glycolipid receptor lie in a shallow pocket on one side of the adhesin domain (Fig. 3b) (Dodson *et al.*, 2001; Sung *et al.*, 2001). P pili have a long, flexible tip fiber (Fig. 1b). The flexibility of the P pilus tip and side-on orientation of the PapG-binding site likely function to facilitate docking of the adhesin onto the globoside moiety of the glycolipid receptor, which is oriented parallel to the membrane surface (Dodson *et al.*, 2001). In contrast, the mannose-binding site of FimH is located in a deep pocket at the tip of the adhesin domain (Fig. 3a) (Choudhury *et al.*, 1999; Hung *et al.*, 2002). Type 1 pili have a shorter and less flexible tip fiber (Fig. 1c), placing the receptor-binding site directly at the distal-most tip of the pilus.

Work on the FimH adhesin has revealed additional mechanisms by which pili have adapted to promote adhesion under specific environmental conditions. FimH binding to mannose is enhanced by shear stress, such as encountered during bacterial colonization in the urinary tract under conditions of urine flow (Thomas *et al.*, 2002). The application of shear stress causes FimH to switch from a low-affinity to a high-affinity binding state. This greater affinity presumably allows UPEC to avoid being washed away by the flow of urine and may also provide a mechanism for the bacteria to discriminate between surface-located and soluble receptors, as binding to the latter will not result in force generation on the pilus and FimH will stay in the low-affinity state. The shear-enhanced binding of FimH is mediated by a catch–bond mechanism that involves allosteric activation of the FimH adhesin domain (Yakovenko *et al.*, 2008). Recent work has revealed the structural basis for the catch–bond behavior of FimH (Le Trong *et al.*, 2010). When incorporated into the type 1 pilus tip fiber, the pilin domain of FimH interacts with the adhesin domain to cause structural alterations of the adhesin that weaken the mannose-binding pocket. However, the application of force to the pilus fiber causes the FimH pilin and adhesin domains to separate, allowing the mannose-binding pocket to clamp tightly around the mannose ligand. Moreover, the structures of both the type 1 pilus tip fiber and the helical pilus rod appear to be designed to optimize the shear-enhanced behavior of FimH, and the flexibility of the pilus tip likely provides maximum opportunity for FimH to find its target receptors (Forero *et al.*, 2006; Aprikian *et al.*, 2011).

Pilus function during pathogenesis: more than just adhesion to host cells

Pili assembled by the CU pathway are critical virulence factors of pathogenic bacteria, as they initiate contact with host cells and mediate colonization of host cell surfaces. However, CU pili may play more complex roles than acting simply as adhesins, as they may modulate host cell signaling pathways, promote or inhibit bacterial invasion inside host cells, or function in bacterial–bacterial interactions such as biofilm formation. The contributions of type 1 pili during infection of the bladder by UPEC, leading to cystitis, are illustrative of the multifunctional nature of CU pili during pathogenesis.

Upon entering the urinary tract, *E. coli* use type 1 pili to bind to mannosylated receptors, such as the uroplakins that coat the luminal surface of the bladder, allowing the bacteria to colonize the bladder and avoid being washed out by the flow of urine (Mulvey *et al.*, 1998; Zhou *et al.*, 2001). Type 1 pili not only mediate binding of UPEC to the bladder surface, but also trigger host cell signaling pathways that lead to actin rearrangement in the urothelial cells and bacterial invasion inside the cells by a zipper-like mechanism (Mulvey *et al.*, 1998; Martinez *et al.*, 2000). In addition to the uroplakin receptors, bacterial uptake is facilitated by binding of the FimH adhesin at the tip of type 1 pili to β1 and α3 integrins on the host cells, and FimH alone is sufficient to trigger uptake into bladder epithelial cells (Martinez *et al.*, 2000; Eto *et al.*, 2007). Following invasion, UPEC replicate rapidly within the host cells and form intracellular biofilm-like commu-

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nities (Anderson *et al.*, 2003). Bacteria within these intracellular communities are protected from host innate immune responses and shielded from antibiotics. Type 1 pili are known to contribute to the formation of extracellular biofilms (Pratt & Kolter, 1998). Surprisingly, type 1 pili are also expressed by the intracellular bacteria and are required for the formation of the intracellular biofilm-like structures, separate from their function in host cell binding and invasion (Wright *et al.*, 2007). In agreement with this, Chen *et al.* (2009) identified residues in FimH that did not affect host cell binding, but which were required for the formation of intracellular bacterial communities. An *E. coli* strain expressing FimH mutated in these residues was defective for pathogenesis and rapidly cleared from the bladder, highlighting the requirement for functions of type 1 pili in addition to extracellular adhesion. Bladder urothelial cells respond to UPEC binding and invasion by exfoliating into the bladder lumen, a host-defense mechanism to wash out the colonizing bacteria (Mulvey *et al.*, 1998). However, UPEC counter this by fluxing out of the host cells and undergoing additional rounds of attachment to and invasion of neighboring cells, presumably mediated by type 1 pili as in the initial round of infection (Mulvey *et al.*, 2001; Justice *et al.*, 2004). During this process, the *E. coli* may gain access to the underlying bladder epithelium, which may lead to the formation of quiescent bacterial reservoirs from which recurrent infections can be seeded to begin the infection process anew (Mulvey *et al.*, 2001; Mysorekar & Hultgren, 2006). Therefore, type 1 pili have both extracellular and intracellular roles and function at multiple different points during UPEC pathogenesis in the urinary tract.

Type IV pili: dynamic and multifunctional surface fibers

Introduction

Type IV pili (T4P) are multifunctional surface structures that function in adhesion to host cells and other surfaces, formation of bacterial aggregates or microcolonies, biofilm formation, cellular invasion, electron transfer, DNA and phage uptake, and twitching or gliding motility (for recent reviews, see Burrows, 2005; Hansen & Forest, 2006; Craig and Li, 2008; Pelicic, 2008). Many of these functions are important during pathogenesis, and T4P are critical virulence factors. Some T4P are able to extend and retract dynamically, which provides the basis for their unique functions, including twitching motility – a form of bacterial movement on solid surfaces – and DNA uptake (Mattick, 2002; Burrows, 2005). T4P are widely distributed among Gram-negative bacteria, including *Pseudomonas aeruginosa* (Mattick *et al.*, 1996), *Neisseria gonorrhoeae* and *Neisseria meningitidis* (Merz & So, 2000), *E. coli* (Giron *et al.*, 1991), *Vibrio cholerae* (Taylor *et al.*, 1987), *Salmonella typhi* (Zhang *et al.*, 2000), *Moraxella bovis* (Marrs *et al.*, 1985), *Myxococcus xanthus* (Sun *et al.*, 2000), *Francisella tularensis* (Gil *et al.*, 2004), *Dichelobacter nodosus* (Han *et al.*, 2007), and the plasmid R64 thin pilus system (Yoshida *et al.*, 1998). Type IV pili have ancient evolutionary origins, as evidenced by the presence of homologous systems in Gram-positive bacteria and in archea, where they also assemble archeal flagella (Rakotoarivonina *et al.*, 2002; Peabody *et al.*, 2003; Varga *et al.*, 2006; Pelicic, 2008; Pohlschroder *et al.*, 2011).

T4P are thin, flexible fibers, 6–9 nm in diameter, and up to several micrometers in length. Despite their thin diameter, T4P have great mechanical strength and are capable of exerting and withstanding forces in excess of 100 pN (Maier *et al.*, 2002). The pili may be present on the bacterial surface as individual fibers or interact laterally to form bundles (see Fig. 6). T4P can be categorized into type IVa and IVb subgroups based on characteristics of the major pilin protein and the organization and composition of the pilus genes (Pelicic, 2008). The *pil* T4P systems of *P. aeruginosa* and *Neisseria* spp. are prototype members of the type IVa subgroup, and the bundle-forming pili (BFP) of enteropathogenic *E. coli* (EPEC) and the toxin-coregulated pili (TCP) of *V. cholerae* are prototype members of the type IVb subgroup. These pili are all critical virulence factors and mediate colonization of host surfaces, either through direct adhesion to host cell receptors or by promoting bacterial–bacterial interactions that lead to the formation of bacterial microcolonies and biofilms. These well-studied pili will be used as examples throughout this section.

Structural details of T4P and pilin proteins

T4P fibers consist solely or predominantly of repeating subunits of the major pilin protein (Figs 4 and 5b) (Parge *et al.*, 1995; Craig *et al.*, 2003, 2006; Ramboarina *et al.*, 2005; Li *et al.*, 2008; Campos *et al.*, 2011). Type IVa and type IVb pili share a similar overall structural arrangement, but differences in the sizes of the subunits lead to different sized fibers, with the type IVa pili of *P. aeruginosa* and *N. gonorrhoeae* having diameters of 6 nm and the type IVb pili of *V. cholerae* and EPEC having diameters of 8–9 nm (Craig *et al.*, 2004, 2006; Ramboarina *et al.*, 2005). In addition, differences in the pilin subunits affect their packing in the pilus fiber, with models indicating that type IVa and type IVb pilins form right-handed or left-handed one-start helices, respectively (Campos *et al.*, 2011). The pilin subunits contain a conserved N-terminal prepilin leader sequence, which is cleaved by a dedicated prepilin peptidase to yield the mature pilin (Strom *et al.*,

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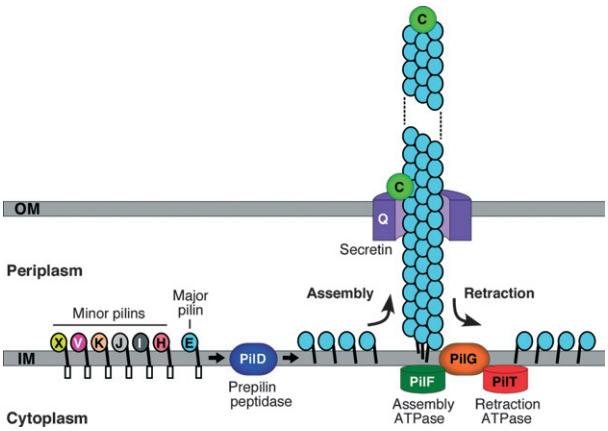

Fig. 4. Model for biogenesis of T4P by *N. meningitidis*. The major (PilE) and minor (PilH, I, J, K, V, and X) pilins are anchored in the inner membrane by their hydrophobic N termini, with the conserved prepilin leader sequence exposed to the cytoplasm and globular head domain exposed to the periplasm. The leader sequence is cleaved, and the mature pilins are *N*-methylated by the PilD prepilin peptidase. Processed pilin subunits are assembled into the pilus fiber from the periplasmic face of the inner membrane. PilF is the cytoplasmic ATPase that powers pilus assembly. The PilQ secretin provides the channel for secretion of the fiber across the outer membrane to the cell surface. Retraction of the pilus fiber is powered by the PilT cytoplasmic ATPase. During retraction, pilins are disassembled from the base of the pilus fiber and enter back into the inner membrane. The PilC protein functions as a tip-located adhesin and also localizes to the OM where it regulates pilus retraction.

1993). The leader sequence for type IVa pilins is short (< 10 residues) and positively charged. Cleavage of the leader sequence occurs following a conserved glycine, and the first amino acid of the mature pilin is phenylalanine (Hobbs & Mattick, 1993; Strom & Lory, 1993). In addition, a conserved glutamate is present at position +5 of the mature pilin, followed by a highly conserved hydrophobic region of approximately 25 residues (Strom & Lory, 1993; Craig *et al.*, 2003). The conserved glutamate is required for the assembly of pilin subunits (Pasloske *et al.*, 1989; Strom & Lory, 1991; Macdonald *et al.*, 1993). The remainder of the pilin sequence exhibits variability, except for the presence of two C-terminal cysteines, which define a disulfide-bonded antigenic loop or D-region (Fig. 5a) (Strom & Lory, 1993). Type IVb pilins are larger than type IVa pilins, have a longer leader sequence, typically 15–30 residues, and the first mature amino acid is not phenylalanine.

Structures have been solved for several type IV pilins (Parge *et al.*, 1995; Craig *et al.*, 2003, 2006; Hansen & Forest, 2006; Craig and Li, 2008). The pilins adopt an elongated, ladle-like shape, with the N-terminus forming an extended α-helix and the C-terminus folding into a globular domain in which the α-helix packs against an antiparallel β-sheet (Fig. 5a). The conserved hydrophobic region of the N-terminus forms the exposed 'handle' of the ladle. In assembly models for T4P, the hydrophobic N-terminal α-helices pack into the center of the pilus as a twisted three-helix bundle, forming a hydrophobic core that provides flexibility and tensile strength to the assembled fiber (Figs 4 and 5b) (Parge *et al.*, 1995; Craig *et al.*, 2003, 2006; Craig and Li, 2008). The C-terminal globular domains are oriented toward the outside of the fiber, providing specific subunit–subunit contacts and exposed surfaces that confer pilus function (Fig. 5b). Two regions of the pilin in particular, the D-region and a region connecting the N-terminal α-helix to the C-terminal β-sheet, termed 'the αβ-region', form prominent surface-exposed edges in the assembled pilus fiber and make important contributions to pilus interactions (Fig. 5) (Craig *et al.*, 2004). The surface of the pilus fiber is highly corrugated, with antigenically variable regions forming exposed ridges

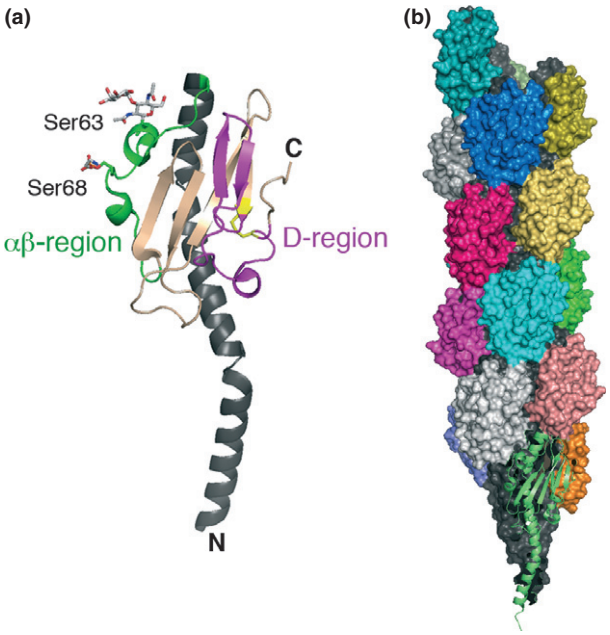

Fig. 5. Structures of the *N. gonorrhoeae* pilin and assembled T4P fiber. (a) Crystal structure of PilE (PDB ID: 2HI2; Craig *et al.*, 2006). The N-terminal α-helix is shown in dark gray, the αβ-region in green, and the D-region in magenta with the conserved disulfide bond in yellow. The phosphoethanolamine and disaccharide modifications to serine-68 and -63, respectively, in the αβ-region are shown in stick representation. (b) Atomic model of the T4P fiber based on the docking of the PilE structure into a cryoEM density map (PDB ID: 2HIL; Craig *et al.*, 2006). The pilin subunits are shown in surface representation, with their N-terminal α-helices in dark gray and surface-exposed C-terminal globular domains colored according to the individual pilin subunits. The bottom-most pilin is depicted in ribbon representation in light green. Images were generated using PyMOL.

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and conserved, functional residues lying shielded within deep grooves (Craig *et al.*, 2006). The presence of the grooves likely enhances the flexibility of the pilus fiber and, together with the exposed ridges, provides specific binding sites along the length of the pilus.

For some type IV pilus systems, the pilus subunits are subject to both phase and antigenic variation, which provide mechanisms to prevent or escape the development of a protective host immune response and allows for modulation of functional activity. The surface-exposed D-region is an important site for host immune system recognition and a hotspot of pilin antigenic variation (Craig *et al.*, 2004; Ramboarina *et al.*, 2005). Antigenic variation has been extensively studied in *Neisseria* spp., where variation is driven by recombination between the active, expressed pilin gene and multiple silent partial copies present in the chromosome (Haas & Meyer, 1986; Howell-Adams & Seifert, 1999). Type IV pilins may also undergo different types of post-translational modifications. These modifications are targeted to exposed regions of the pilus fiber and likely contribute to antigenic variation and immune evasion, and may also have important functional consequences as discussed later. Two serines in the exposed αβ-region of the *Neisseria* spp. PilE pilin are subject to varying O-linked modifications, with phosphocholine or phosphoethanolamine added to serine-68 and a disaccharide added to serine-63 (Fig. 5a) (Stimson *et al.*, 1996; Warren & Jennings, 2003; Hegge *et al.*, 2004). *Pseudomonas aeruginosa* pilins are subject to glycosylation with an O-linked trisaccharide at their C-terminus, which is surface-exposed in the pilus fiber and in close proximity to the functionally and antigenically important D-loop region (Castric *et al.*, 2001; Comer *et al.*, 2002).

In addition to the major pilin subunit, T4P systems encode a number of prepilin-like proteins, termed 'minor pilins' or 'pseudopilins', which contain the defining N-terminal prepilin leader sequence. These minor pilins are processed by the prepilin peptidase and may assemble into the pilus fiber as minor components that influence pilus assembly or function (Hobbs & Mattick, 1993; Mattick, 2002; Winther-Larsen *et al.*, 2005; Cisneros *et al.*, 2011). Pseudopilins are also present in type II secretion systems, where they form a pilus-like structure that is thought to aid secretion (Hobbs & Mattick, 1993; Mattick, 2002; Cisneros *et al.*, 2011). In *Neisseria* spp., the PilH, I, J, and K minor pilins are required for pilus biogenesis, as mutants lacking these proteins are nonpiliated (Winther-Larsen *et al.*, 2005; Carbonnelle *et al.*, 2006). In contrast, the PilX, PilV, and ComP minor pilins are not required for pilus assembly, but instead modify pilus function. The PilX minor pilin is required for T4P-mediated bacterial aggregation, and the PilV minor pilin influences the level of phosphocholine/phosphoethanolamine modification of the PilE major pilin and triggers host cell signaling events leading to the formation of membrane protrusions (Hegge *et al.*, 2004; Helaine *et al.*, 2007; Mikaty *et al.*, 2009). The ComP minor pilin acts to enhance DNA binding and uptake by T4P (Aas *et al.*, 2002). Proteins in addition to the minor pilins may also associate with T4P fibers to modulate pilus function or act as adhesins. For example, the PilC1 and PilC2 proteins of *Neisseria* spp. function as tip-located adhesins and also localize to the outer membrane to regulate pilus retraction (Nassif *et al.*, 1994; Rudel *et al.*, 1995a; Scheuerpflug *et al.*, 1999; Morand *et al.*, 2004).

Overview of the T4P assembly mechanism

The molecular details of T4P assembly are still not well understood. Biogenesis of T4P requires approximately 15 genes. For type IVA pili, these genes are found distributed on the chromosome in a scattered manner, but in defined clusters and conserved positions, such as for the *pil* genes of *P. aeruginosa* and *Neisseria* spp. (Mattick *et al.*, 1996; Tonjum & Koomey, 1997). For type IVB pili, the pilus genes are found clustered together at a single chromosomal or plasmid-based location, such as for the *tcp* and *bfp* genes of *V. cholerae* and EPEC, respectively (Sohel *et al.*, 1996; Stone *et al.*, 1996; Manning, 1997). Regulation of T4P biogenesis and function is complex and involves a number of signal transduction and chemosensory pathways, including quorum-sensing systems (Mattick, 2002). In *P. aeruginosa*, nearly 40 genes have been identified that affect T4P expression and function, with about half directly involved in pilus assembly and half contributing to the regulation of pilus expression (Mattick, 2002). Biogenesis of T4P occurs by a mechanism closely related to the type II secretion pathway (see companion review in this issue and Peabody *et al.*, 2003). This relationship is illustrated by the fact that T4P systems can function in the secretion of soluble proteins, and type II secretion systems can be induced to assemble fibers resembling T4P (Sauvonnet *et al.*, 2000; Durand *et al.*, 2003; Kirn *et al.*, 2003; Hager *et al.*, 2006; Han *et al.*, 2007). Moreover, in *P. aeruginosa*, which has both type II secretion and T4P systems, a single prepilin peptidase (PilD/XcpA) is shared between both systems, and the major T4P subunit (PilA) participates in secretion by the type II system (Lu *et al.*, 1997).

Figure 4 presents a model for T4P biogenesis, as depicted for the *N. meningitidis* Pil system. Newly synthesized prepilins (the PilE major pilin plus additional minor pilins) are inserted into the cytoplasmic membrane by the Sec system, with their prepilin sequence exposed to the cytoplasm, their hydrophobic N-terminal α-helix spanning the membrane, and their globular C-terminal

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domain exposed to the periplasm (Zhang & Donnenberg, 1996; Arts *et al.*, 2007; Francetic *et al.*, 2007). The prepilin leader sequence is cleaved, and the mature pilin *N*-methylated by the PilD prepilin peptidase (Strom & Lory, 1993; Zhang *et al.*, 1994). Mature pilins are then extracted from the cytoplasmic membrane and polymerized into the pilus fiber by the T4P assembly machinery (Fig. 4). Assembly of pilins into the pilus fiber is thought to be mediated by interactions between the conserved hydrophobic N-terminal α-helices. This also provides a mechanism for the incorporation of minor pilins, as these proteins contain the same conserved N-terminal sequence and structure (Craig *et al.*, 2006). Pilus assembly is energized by the PilF cytoplasmic ATPase, which belongs to the secretion superfamily of hexameric ATPases that also power type II and type IV secretion systems (Planet *et al.*, 2001; Peabody *et al.*, 2003; Crowther *et al.*, 2004; Satyshur *et al.*, 2007). In addition, the T4P assembly machinery includes the integral inner membrane protein PilG, for which the precise function remains to be determined, and requires several minor pilins (PilH-K) (Tonjum *et al.*, 1995; Crowther *et al.*, 2004; Winther-Larsen *et al.*, 2005; Carbonnelle *et al.*, 2006). The assembled T4P fiber crosses from the periplasm to the cell surface through a large, multimeric gated channel in the OM termed 'the secretin' (PilQ; Fig. 4), which is also present in type II and III secretion systems (Bitter *et al.*, 1998; Wolfgang *et al.*, 2000; Collins *et al.*, 2004; Reichow *et al.*, 2010).

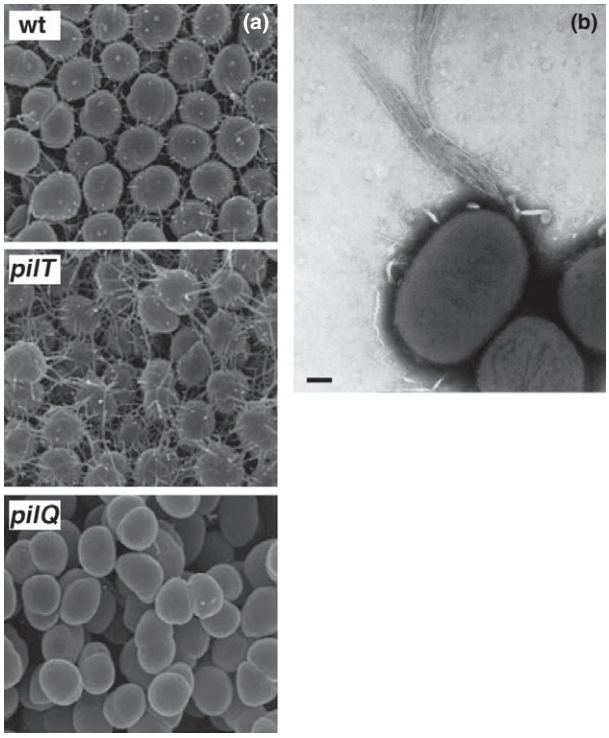

Fig. 6. Electron micrographs of bacteria expressing T4P. (a) Scanning EM images showing T4P expressed by wild-type, and *pilT* and *pilQ* mutant *Neisseria gonorrhoeae*. The *pilT* bacteria are hyperpiliated compared to wild-type, and the *pilQ* bacteria lack pili. (b) Micrograph of BFP expressed by EPEC showing a polar bundle of pili. Scale bar = 200 nm. Images in (a and b) reproduced with permission from (Wolfgang *et al.*, 2000) and (Stone *et al.*, 1996), respectively.

Type IV pilus retraction and twitching motility

A distinctive feature of T4P is the ability of these fibers to dynamically extend and retract (Mattick, 2002; Nudleman & Kaiser, 2004; Burrows, 2005). In systems capable of pilus retraction, this is powered by a second cytoplasmic hexameric ATPase, the disassembly ATPase PilT (Fig. 4) (Satyshur *et al.*, 2007; Misic *et al.*, 2010). Mutant bacteria lacking PilT are typically hyperpiliated, because extension of the pilus fiber driven by the PilF assembly ATPase still occurs, but is no longer countered by pilus retraction (Fig. 6a). Available data suggest that PilT acts to disassemble pilin subunits from the base of the pilus fiber, with the liberated subunits entering back into the cytoplasmic membrane via their hydrophobic N termini (Fig. 4) (Morand *et al.*, 2004). Thus, pilus extension and retraction appears to be a balance between pilin polymerization and depolymerization events occurring at the cytoplasmic membrane, caused by the action of opposing cytosolic ATPases (Burrows, 2005). Moreover, experimental evidence suggests that the primary function of a number of T4P machinery proteins is to stabilize the assembled pilus fiber or act as a counter to pilus retraction, rather than functioning directly in pilus assembly (Carbonnelle *et al.*, 2006). The recent structure of the *P. aeruginosa* PilY1 protein provides interesting insights into possible mechanisms of the regulation of pilus retraction (Orans *et al.*, 2010). PilY1 is homologous to the *Neisseria* spp. PilC proteins, is associated with the pilus fiber, and functions similar to PilC in counteracting PilT-mediated retraction (Heiniger *et al.*, 2010). The PilY1 structure revealed a modified β-propeller fold containing an EF-hand-like calcium binding site. A PilY1 mutation that prevented calcium binding, or removal of calcium using a chelator, caused loss of surface pili, whereas a mutation that mimicked a bound calcium ion caused hyperpiliation (Orans *et al.*, 2010). Thus, PilY1 appears to use calcium sensing as a means to regulate pilus extension and retraction.

Pilus extension and retraction enables a form of movement on surfaces termed 'twitching motility', after the jerky motion produced (Mattick, 2002). Individual T4P fibers extend out from the bacteria, adhere to a surface, and then retract, pulling the bacteria toward the site of adhesion (Skerker & Berg, 2001). The retraction of T4P

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generates extremely high forces, and PilT may be the strongest biological motor characterized to date (Biais *et al.*, 2008). Measurement of *N. gonorrhoeae* T4P retraction forces using optical tweezers revealed that a single pilus fiber exerts forces of 50–100 pN, and an analysis of pilus fibers acting cooperatively in bundles revealed retraction forces in the nanonewton range (Maier *et al.*, 2002; Biais *et al.*, 2008). Twitching motility mediates bacterial–bacterial interactions and the formation of bacterial communities and biofilms (O'Toole & Kolter, 1998; Merz *et al.*, 2000; Mattick, 2002). Type IV pilus-mediated twitching motility was recently shown to allow *P. aeruginosa* to orient perpendicular to a surface ('walk upright'), facilitating surface exploration and influencing biofilm formation (Gibiansky *et al.*, 2010). Pilus retraction is also required for functions such as DNA and phage uptake. Twitching motility performs essential functions during pathogenesis, as T4P assembled by *pilT* mutant bacteria are competent for adhesion but attenuated for host cell interactions and in host infection models (Bieber *et al.*, 1998; Comolli *et al.*, 1999; Merz *et al.*, 1999; Pujol *et al.*, 1999). Pilus retraction allows intimate adherence of bacteria to host cells and the formation of three-dimensional microcolonies on the host cell surface (Pujol *et al.*, 1999; Jude & Taylor, 2011). In addition, the forces exerted by pilus retraction appear to be an important component in the elicitation of host cell responses during bacterial colonization (Merz *et al.*, 1999; Howie *et al.*, 2005; Dietrich *et al.*, 2011).

Adhesion mediated by T4P: receptor binding and pilus bundling

T4P function as adhesins, and the ability to mediate adhesion to different surfaces, to other bacteria, and to DNA is critical for the various functions of T4P. Despite this central role, adhesion mechanisms of T4P are still not well understood. For most T4P, adhesion appears to be mediated by the major pilin subunit, and exposed surfaces such as the D- and αβ-regions are important sites for receptor binding (Lee *et al.*, 1994; Xu *et al.*, 2004; Hansen & Forest, 2006). *Pseudomonas aeruginosa* T4P have been shown to bind to β-GalNAc(1–4)βGal moieties present in asialo-GM1 glycolipids expressed by epithelial cells, with binding mediated by the D-region of the C-terminal globular pilin domain (Lee *et al.*, 1994; Hazes *et al.*, 2000). However, the *in vivo* relevance of binding to asialo-GM1 remains to be established, as clinical *P. aeruginosa* isolates do not appear to use this receptor for adhesion to host cells (Schroeder *et al.*, 2001). Moreover, Heiniger and co-workers recently provided evidence that the pilus-associated PilY1 protein may function as the *P. aeruginosa* T4P adhesin rather than the main pilin subunit (Heiniger *et al.*, 2010). The use of a separate, tip-located protein as the pilus adhesin is best characterized for the homologous PilC1 and PilC2 proteins expressed by *N. meningitidis* and *N. gonorrhoeae* (Nassif *et al.*, 1994; Rudel et al., 1995a, b). *Neisseria* spp. use their T4P for colonization of human mucosal surfaces. PilC localizes to the pilus tip, and purified PilC has been shown to bind various human epithelial and endothelial cells (Scheuerpflug *et al.*, 1999). The role of PilC is still controversial, however, as a direct requirement for PilC in host cell binding by *Neisseria* spp. T4P has not been demonstrated, and there are conflicting reports on the host cell receptor for the *Neisseria* spp. T4P. CD46 has been identified as a pilus receptor, and transgenic mice expressing human CD46 are susceptible to infection by piliated *N. meningitidis* (Johansson *et al.*, 2003). In contrast, other studies have not been able to demonstrate a role for CD46 in cell culture binding experiments (Tobiason & Seifert, 2001; Kirchner *et al.*, 2005). Understanding the function of PilC is further complicated by the fact that, as discussed previously, it also localizes to the OM and has been shown to promote pilus stability by counteracting PilT-mediated pilus retraction by an unknown mechanism (Morand *et al.*, 2004).

While T4P mediate the initial attachment of bacteria to host cells, the primary function of some T4P may be less in the binding to specific host cell receptors and more in the formation of subsequent bacterial–bacterial and bacterial–host interactions, leading to intimate host cell contact and the generation of bacterial microcolonies. Analysis of high vs. low adhesive strains of *N. meningitidis* revealed that high-level adhesion to host cells is mediated by bundling of T4P and the formation of bacterial aggregates, rather than increased adhesion to a host cell receptor. Thus, highly adhesive strains formed aggregates whereas less adhesive strains adhered as individual bacteria (Marceau *et al.*, 1995). Similarly, the BFP of EPEC not only function in the initial attachment of bacteria to the intestinal epithelium, but also are required for bacterial autoaggregation and the formation of three-dimensional microcolonies, termed 'localized adherence' (Giron *et al.*, 1991; Donnenberg *et al.*, 1992; Hyland *et al.*, 2008). Furthermore, retraction of BFP allows intimate contact of EPEC with the host epithelium and facilitates the delivery of virulence factors into host cells via the EPEC type III secretion system (Zahavi *et al.*, 2011).

T4P-driven bacterial aggregation is mediated by the association of pilus fibers and pilus bundling. These pilus–pilus interactions appear to be formed by specific contacts between grooves and ridges along the length of the pilus fibers. The TCP of *V. cholerae* are required for bacterial colonization of the intestine, and pilus-mediated autoaggregation is a critical component of this process

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(Tacket *et al.*, 1998; Kirn *et al.*, 2000). Recent structural studies of *V. cholerae* TCP revealed that deep cavities are present along the surface of the fiber, produced by a loose packing of the surface-oriented C-terminal globular pilin domains (Li *et al.*, 2008; Lim *et al.*, 2010). Combined with mutagenesis studies that identified specific residues required for pilus-mediated aggregation, these structures suggest that pilus–pilus interactions are mediated by packing of charged residues in D-region bulges on one pilus fiber into cavities on another fiber (Kirn *et al.*, 2000; Li *et al.*, 2008; Lim *et al.*, 2010). The cavities and grooves along the surface of the pilus fiber may also function in DNA binding and uptake by T4P, as modeling of the *N. gonorrhoeae* pilus fiber showed that some grooves are lined with positive charges that would facilitate interaction with negatively charged DNA (Craig *et al.*, 2006).

Minor pilins may also influence or mediate pilus–pilus interactions. In *N. meningitidis*, the minor prepilin-like protein PilX is required for T4P-driven bacterial aggregation and microcolony formation (Helaine *et al.*, 2005, 2007). PilX is not required for pilus assembly, but incorporates along the length of the fiber. PilX adopts a typical pilin structure, with a hydrophobic N-terminal α-helix and globular C-terminal domain. Modeling and mutagenesis experiments identified the PilX D-region, which would be surface-exposed in the pilus fiber, as required for *N. meningitidis* aggregation. Furthermore, bacteria expressing PilX could not form aggregates with bacteria lacking PilX, suggesting that PilX–PilX interactions between pilus fibers lead to bacterial aggregation (Helaine *et al.*, 2005). Thus, the incorporation of minor pilins with distinct binding surfaces may confer specific functionality to T4P.

The many roles of T4P during pathogenesis: the example of *N. meningitidis*

Studies on the pathogenesis of *N. meningitidis* have revealed the multifunctional contributions of T4P during host–pathogen interactions. *Neisseria meningitidis* is a causative agent of septicemia and cerebrospinal meningitis and an important human pathogen. *Neisseria meningitidis* colonizes epithelial surfaces of the human nasopharynx. T4P function as adhesins to mediate the initial contact of *N. meningitidis* with epithelial cells, possibly via the PilC minor tip protein (Nassif *et al.*, 1994). Following binding, the bacteria replicate on the epithelial cell surface and form tight, three-dimensional clusters or microcolonies. Microcolony formation requires pilus–pilus interactions mediated by the PilX minor pilin (Helaine *et al.*, 2005, 2007). As discussed later, binding via T4P also triggers host cell signaling pathways leading to cortical plaque formation and rearrangement of host cell actin (Merz *et al.*, 1999; Merz & So, 2000). T4P retraction allows intimate contact of the bacteria with the host cell surface, facilitating stable colonization mediated by other adhesins such as the OM opacity proteins (Virji *et al.*, 1993; Pujol *et al.*, 1999). Bacteria that colonize the nasopharynx may then invade across the epithelium to gain access to the blood stream, resulting in septicemia and ultimately meningitis if the bacteria are able to cross the blood–brain barrier. A recent study suggests that, in addition to mediating colonization, T4P play a central role in the dissemination step as well (Chamot-Rooke *et al.*, 2011). *Neisseria meningitidis* proliferating in association with host cells were found to upregulate the transferase that adds the phosphoglycerol post-translational modification to the PilE major pilin. The phosphoglycerol modification introduces a negative charge into a positively charged patch of the αβ-region of the pilin, which is thought to be important for pilus–pilus interactions (Craig *et al.*, 2006). The phosphoglycerol modification also sterically alters the surface of the pilus fiber. Thus, the increased levels of modified pilin will disrupt bacterial aggregation and promote detachment of bacteria from the microcolonies to facilitate dissemination (Chamot-Rooke *et al.*, 2011). In agreement with this model, mutant *N. meningitidis*, unable to modify their pilin, showed decreased release from host cell–associated microcolonies and decreased transmigration across epithelial cell monolayers (Chamot-Rooke *et al.*, 2011).

Once in the bloodstream, *N. meningitidis* must adhere to brain endothelial cells and cross the blood–brain barrier to cause meningitis. Adhesion to the endothelial cells is mediated by T4P, and PilC plays a role in this process (Pron *et al.*, 1997; Mairey *et al.*, 2006). Similar to colonization of the nasopharynx, the bacteria multiply rapidly on the endothelial cell surface and form large microcolonies. *Neisseria meningitidis* growing on the endothelial cells trigger the formation of filopodia-like protrusions. Generation of these host cell protrusions is dependent on T4P and on the PilV minor prepilin-like protein (Mikaty *et al.*, 2009). PilV-dependent interactions with the endothelial cell surface result in cortical plaque formation, which drives localized actin polymerization and generation of the cellular protrusions. These protrusions protect the bacteria from shear forces caused by blood flow, possibly by providing more host surface area for bacterial contact (Mairey *et al.*, 2006; Mikaty *et al.*, 2009). A similar mechanism may also provide resistance to shear forces in the nasopharynx, resulting from mucus flow and actions such as coughing and swallowing. In addition to formation of the cellular protrusions, host cell signals generated by *N. meningitidis* adhesion also appear to facilitate invasion of the bacteria across the blood–brain barrier. Adhesion via T4P triggers recruitment of host

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proteins involved in the formation of intercellular junctions to sites of bacterial attachment (Coureuil *et al.*, 2009). T4P, but not PilT-mediated pilus retraction, is required for this process. Recent work by Coureuil *et al.* (2010) demonstrated that the *N. meningitidis*-induced signaling occurs through the β2-adrenonergic receptor/β-arrestin pathway, which activates the Src tyrosine kinase to trigger actin polymerization and recruits host junctional proteins to sites of bacterial colonization. This depletes the junctional proteins from their normal positions at endothelial cell–cell interfaces and opens gaps to facilitate invasion of *N. meningitidis* across the blood–brain barrier (Coureuil *et al.*, 2009). Thus, T4P provide a continuum of functions during host–pathogen interactions, from initial host cell contact, to colonization and remodeling of the host cell surface, to subsequent invasion across host barriers and dissemination to new sites of infection.

Type III secretion needle tip complexes of bacterial pathogens: key virulence structures at the host–pathogen interface

Introduction

Type III secretion systems (T3SS) are encoded by a large number of Gram-negative bacteria. Members of the T3SS superfamily are responsible for the export and assembly of two distinct types of surface organelles: flagella and needles (the latter have been called needle complexes or injectisomes) (Blocker *et al.*, 2003; Macnab, 2004; Cornelis, 2006; Galan & Wolf-Watz, 2006) (Fig. 7). Flagella are used for bacterial motility, while needles are used for delivery of bacterial proteins into the membranes or cytosolic compartment of host eukaryotic cells. Assembly of T3SS needles and flagella is coordinately regulated by environmental signals. They are assembled in hierarchical fashion and made up of 20–30 proteins. T3SSs contain three main structural components: a set of core transmembrane proteins, a basal body, and surface-exposed flagella or needle. The core transmembrane proteins are highly conserved between the two systems, while the basal body and flagella/needle components are more divergent at the level of primary sequence and three-dimensional structure, reflecting their distinct functions. For recent reviews of work on the structures of T3SS core transmembrane proteins, basal bodies, and needles, see (Blocker *et al.*, 2008; Marlovits & Stebbins, 2010; Worrall *et al.*, 2011).

A description of the mechanism of type III secretion and an overview of the structures of the basal bodies and surface appendages of flagellar and needle T3SSs can be found in the accompanying review in this issue by Dalbey and Kuhn. Flagella and needles are helical structures with a central channel of ~25 angstroms. Flagellin monomers travel up the channel and assemble at the tip of the growing filament. It is assumed that needles assemble in the same manner. Substrates are believed to travel through this channel in needles in a partially unfolded state prior to delivery to host cells. Needle length is controlled by a ruler mechanism (Cornelis *et al.*, 2006; Deane *et al.*, 2010). A major distinction between flagella and needles can be seen by the structures of their tips. Flagella contain a cap protein that is required for subunit assembly at the growing tip (Macnab, 2004). In contrast, needles are capped by a 'tip complex' (Mueller *et al.*, 2005, 2008). The tip complex is largely composed of a hydrophilic protein that multimerizes (Blocker *et al.*, 2008; Mueller *et al.*, 2008; Wang *et al.*, 2008; Sato & Frank, 2011). In some bacteria (e.g. EPEC), the tip protein polymerizes into a long sheath that covers the needle, forming a pilus-like extension or filament (Sekiya *et al.*, 2001; Garmendia *et al.*, 2005; Enninga & Rosenshine, 2009). The tip complex is proposed to have several major functions, including regulation of secretion, sensing of host signals, transmitting activating signals to the needle, acting as a platform for recruitment and insertion of hydrophobic 'translocator' proteins into host plasma membrane, and forming a conduit between the needle and the translocation channel (Blocker *et al.*, 2008; Mueller *et al.*, 2008; Wang *et al.*, 2008; Sato & Frank, 2011). In addition, several of the tip

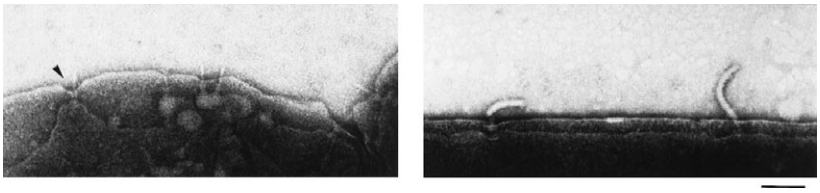

Fig. 7. Electron micrographs of osmotically shocked *Salmonella enterica* serovar Typhimurium bacteria. (left) Nonflagellated Δ*flhC S.* Typhimurium exhibits needle complexes on the bacterial envelope. Note the depression at the insertion point of the needle complex (closed arrow). (right) An *S.* Typhimurium *fliK* mutant exhibits flagellar polyhook basal bodies that span the inner and outer membranes. Scale bar = 100 nm. Reproduced with permission from (Kubori *et al.*, 1998).

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proteins have been demonstrated to be the targets of immunoprotective antibodies (Mueller *et al.*, 2008; Sato & Frank, 2011).

This section will focus on the structures of T3SS tip complexes and highlight recent studies in three areas: (1) the role of tip complexes as assembly platforms for translocators to form channels in host plasma membranes; (2) the function of tip complexes as conduits between the needle and channel; and (3) the role of tip complexes as protective antigens. This section will concentrate on the well-characterized tip proteins in *Yersinia* (LcrV), *Pseudomonas* (PcrV), *Shigella* (IpaD), and *Salmonella* (SipD) species. We will attempt to integrate structural data of the tip complexes with studies of surface localization, antibody neutralization, and protective antibody epitope mapping (for a comprehensive recent review of the tip protein family, see Sato & Frank, 2011).

The end starts at the beginning: detection of organized LcrV protein on the bacterial surface

LcrV was identified as a protective antigen secreted by *Y. pestis* in 1956 and, at that time, it was called the V antigen (Burrows & Bacon, 1956). Forty-three years later, the first evidence that LcrV is located in an organized manner on the surface of *Y. pestis* and *Yersinia pseudotuberculosis* was obtained by microscopy (Fields *et al.*, 1999; Pettersson *et al.*, 1999). Immunofluorescence microscopy showed that LcrV was present in 'punctate zones' or 'clusters' on the surface of *Y. pseudotuberculosis* and *Y. pestis* (Fields *et al.*, 1999; Pettersson *et al.*, 1999). A functional T3SS was shown to be required for this surface localization (Fields *et al.*, 1999; Pettersson *et al.*, 1999). The number of clusters per bacteria was estimated at 40–80 (Pettersson *et al.*, 1999). Immunogold labeling in conjunction with EM gave a similar result, that is, clusters of immunogold particles were found at discrete areas of the bacterial surface (Pettersson *et al.*, 1999). Antibodies specific for LcrV reduced effector protein translocation into epithelial cells infected with *Y. pseudotuberculosis*, providing the first direct evidence that the surface-localized protein had a critical function in effector translocation (Pettersson *et al.*, 1999). In addition, LcrV released from the bacterial surface has been suggested to function as an immunomodulator (Brubaker, 2003).

Organized localization of tip proteins on bacterial surfaces has been detected in studies with *Shigella* (Espina *et al.*, 2006; Sani *et al.*, 2007), *Salmonella enterica* (Lara-Tejero & Galan, 2009), and *P. aeruginosa* (Lee *et al.*, 2010; Sato *et al.*, 2011). The sheath forming tip protein of EPEC, EspA, was shown to form a fibrillar structure extending from the bacterial surface to the host cell plasma membrane (Knutton *et al.*, 1998). Even earlier studies suggested that the *Shigella* tip protein IpaD was on the bacterial surface, but no evidence was obtained for an organized structure (Turbyfill *et al.*, 1998).

Structure of LcrV and other tip proteins

The *Y. pestis* LcrV protein lacking its N-terminal 22 amino acids was purified, and a crystal structure determined (Derewenda *et al.*, 2004). In addition to lacking N-terminal residues, Lys40, Asp41, and Lys42 were converted to Ala, and Cys237 was changed to Ser to facilitate purification and crystallization of the protein. Although structural information for several N-(23–27) and C-(323–326) terminal residues and two internal loops (49–63 and 260–275) was not obtained, the visible electron maps allowed determination of the overall LcrV structure (PDB ID: 1R6F). LcrV has an overall dumbbell shape (see Fig. 8), with the 'handle' comprising two helices (α-7 and α-12) that form a coiled coil (Derewenda *et al.*, 2004). The LcrV N-terminus forms a globular domain at one end of the handle. A second globular domain that is formed by the region between α-7 and α-12 in LcrV is found at the other end of the handle. There is overall low sequence similarity among tip proteins, but the structural determination of IpaD (PDB ID: 2J0O; Johnson *et al.*, 2007), BipD from *Burkholderia* (PD 2IZP; Erskine *et al.*, 2006; Johnson *et al.*, 2007), and EspA (PD ID code 1XOU; Yip *et al.*, 2005) revealed an overall structurally similar dumbbell shape with long central coiled coils. The globular domains among the family members vary in size, likely reflecting species-specific molecular functions. In IpaB and BipD, the N-terminal domain is proposed to function as an intramolecular chaperone for assembly of the subunits into the tip complex (Johnson *et al.*, 2007).

Structure of the needle and tip complex

T3SS basal bodies and needles were first isolated and characterized in *Salmonella* (Fig. 7) (Kubori *et al.*, 1998). The needle is formed by polymerization of ~100–150 copies of ~ 9 kDa monomers into a helical structure. The 9.5 kDa protein YscF forms needles in *Yersinia* (Hoiczyk & Blobel, 2001). In *Shigella*, there are 5.5 subunits of MxiH per turn, with a 4.6 Å axial rise per subunit (Cordes *et al.*, 2003). The helical structure is slightly different in *Salmonella* as this needle contains 6.3 subunits of PrgI per turn (Galkin *et al.*, 2010). X-ray crystal structures of several different needle subunits reveal a common fold for the central regions of these proteins: two α-helices connected by a short loop fold into a coiled coil (Blocker *et al.*, 2008). The N- and C termini of the subunits are disordered in the structures, suggesting they are connected to the coiled-coil fold by flexible regions (Blocker *et al.*, 2008). Mutational

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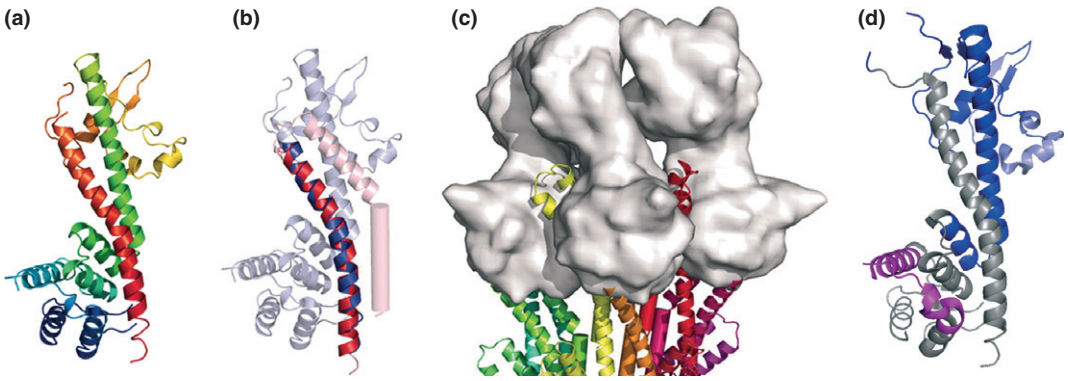

Fig. 8. Structures of LcrV and the putative tip complex. (a) Ribbon diagram of LcrV (Derewenda *et al.*, 2004) colored N (blue) to C (red) termini. (b) Overlay of the C-terminal helices of MxiH (red, residues 45–75) and LcrV (blue, residues 287–317), with all but the overlaid region made transparent to aid visualization. (c) Model of an LcrV tip complex (surface representation, gray) docked onto the tip of a T3SS needle. Reprinted with permission from (Deane *et al.*, 2006). (d) Locations of protective epitopes in LcrV. Ribbon diagram of LcrV structure (PDB ID: 1R6F; Derewenda *et al.*, 2004) shown in the same orientation as in (a). Conformational epitope recognized by protective monoclonal antibody 7.3 (amino acids 135–275) (Hill *et al.*, 1997; Hill *et al.*, 2009) is shown in dark blue. Linear epitope recognized by protective monoclonal antibody BA5 (residues 196–225) (Eisele & Anderson, 2009; Quenee *et al.*, 2010) is shown in light blue. Epitopes recognized by nonprotective AH1 monoclonal antibody (Eisele & Anderson, 2009), corresponding to amino acids 76–105 of LcrV (D. Anderson, pers. commun.), are shown in magenta. Image was generated using PyMOL.

analysis of needle subunits has yielded evidence supporting a role for the needle in sensing host contact and regulating secretion (Kenjale *et al.*, 2005; Davis *et al.*, 2010; Martinez-Argudo & Blocker, 2010).

Fitting of the MxiH structure into the 16 Å resolution EM density map of the *Shigella* needle yielded a model for the assembled appendage (Deane *et al.*, 2006; Blocker *et al.*, 2008). The loop connecting the two helices is exposed on the surface of the needle, the shorter N-terminal helix lines the channel and the longer C-terminal helix makes extensive subunit–subunit contacts. Small deletions at the C-terminus of IpaD prevent its association with needles (Espina *et al.*, 2006), an observation which is consistent with a requirement for the C-terminal helix in needle–tip complex interactions.

Scanning transmission EM (STEM) analysis of needles sheared from the surface of *Yersinia enterocolitica* revealed a unique structure at the distal end (Fig. 9) (Mueller *et al.*, 2005). Needles from an *lcrV* mutant of *Yersinia* lacked this tip complex, and antibodies specific for LcrV bound to the tip complex (Mueller *et al.*, 2005). Immunogold labeling and TEM were used to show that IpaD localizes to the tip of *Shigella* needles (Espina *et al.*, 2006; Sani *et al.*, 2007). Modeling of PcrV and AcrV of *Aeromonas* on the structure of LcrV revealed that these three molecules have different sized globular domains. Analysis by STEM of complexes formed from the hybrids of LcrV with PcrV or AcrV at the tips of *Yersinia* needles allowed determination that the N-terminal globular domain of LcrV is proximal to the needle, while the central globular domain is distal (Broz *et al.*, 2007). The structure of the tip complex produced by averaging the electron densities from images obtained by STEM was used as a framework to estimate that five LcrV monomers are present (Broz *et al.*, 2007). The pentameric structure arrived at has radial symmetry and a central channel (Broz *et al.*, 2007). The hybrid LcrV proteins were also exploited to investigate the role of the globular N-terminal domain for function (Broz *et al.*, 2007). Results showed that a hybrid LcrV protein containing the smaller PcrV N-terminal domain was defective for lysis of red blood cells and for insertion of the translocator YopB into red blood cell membranes (Broz *et al.*, 2007). These results suggest that the needle proximal N-terminal domain of LcrV plays a key role in the insertion of the translocation channel into host membranes.

Structural similarity between the C-terminal helix of MxiH and the C-terminal helix of tip proteins such as LcrV suggested a model for the molecular interactions that allow assembly of the complex at the tip of the needle (Deane *et al.*, 2006; Blocker *et al.*, 2008). In the model, the C-terminal helix of an LcrV subunit would replace the C-terminal helix of a needle subunit, resulting in a homo-pentamer complex with helical symmetry and a channel of ~25 Å (Fig. 8). As discussed earlier, Broz *et al.* (2007) arrived at an LcrV tip structure with radial symmetry. Thus, the two models for the structure of the LcrV tip complex differ with respect to radial (Broz *et al.*, 2007) vs. helical (Deane *et al.*, 2006) symmetry, but arrive at the same overall role for the coiled-coil fold in the assembly, the pentameric structure, the orientation of globular domains, and the presence of a channel. Interest-

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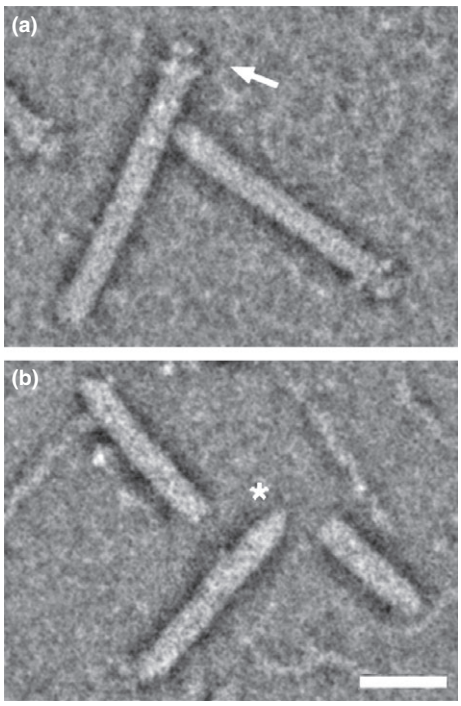

Fig. 9. STEM images of negatively stained T3SS needles. (a) Characteristic tip complexes (arrow), comprising a head, a neck, and a base, of wild-type needles isolated from *Yersinia enterocolitica* grown in secretion-permissive conditions. (b) Needles formed by *lcrV* mutant bacteria. The needles of *lcrV* mutant bacteria are distinctly pointed at one end (asterisk). Scale bar = 20 nm. Reprinted with permission from (Mueller *et al.*, 2005).

ingly, in both models, the central globular domain would be oriented distal from the needle, which has implications for the functions of neutralizing antibodies that recognize epitopes in this domain.

Determination of the crystal structure of IpaD allowed building of a model of the *Shigella* tip complex in which five monomers are tightly packed (Johnson *et al.*, 2007). This structure lacks a central channel of sufficient size to allow the passage of unfolded proteins and therefore has been suggested to represent a 'closed conformation' of the tip complex (Johnson *et al.*, 2007; Blocker *et al.*, 2008).

Evidence for the association of translocators with the tip complex prior to host cell contact

It is thought that a major function of the tip complex is to act as an assembly platform for the hydrophobic translocators prior to their insertion into the host cell plasma membrane (Mueller *et al.*, 2008; Mattei *et al.*, 2011; Sato & Frank, 2011). Following the nomenclature of Mattei and colleagues (Mattei *et al.*, 2011), the translocators fall into two classes, the so-called major (YopB, IpaB, SipB, and PopB) and minor (YopD, IpaC, SipC, and PopD) translocators of *Yersinia*, *Shigella*, *Salmonella,* and *Pseudomonas*, respectively. Very little structural detail on either class of translocator is available owing to their hydrophobicity and disordered character (Mattei *et al.*, 2011). Evidence suggests that once inserted into the host plasma membrane, SipB and IpaB adopt a hairpin structure, with the N and C termini exposed at the surface, and the two transmembrane domains (TMDs) spanning the lipid bilayer (McGhie *et al.*, 2002; Hume *et al.*, 2003).

YopB and YopD have not been detected at the tips of *Yersinia* needles, although YopD is reported to co-purify in small amounts with needle preparations (Mueller *et al.*, 2005). Similarly, SipB and SipC were not detected on the surface of *Salmonella* prior to host cell contact (Lara-Tejero & Galan, 2009). In contrast, IpaB has been shown to localize to the tip of *Shigella* needles (Johnson *et al.*, 2007; Olive *et al.*, 2007; Veenendaal *et al.*, 2007). IpaD is required for localization of IpaB to the needle tip (Olive *et al.*, 2007; Veenendaal *et al.*, 2007). Quantification of the relative amounts of IpaD and IpaB at needle tips suggest that one subunit of IpaB may associate with four subunits of IpaD (Johnson *et al.*, 2007; Veenendaal *et al.*, 2007). This observation leads to modeling of a modified complex in which four subunits of IpaD assemble at the tip via interactions with MxiH and adjacent IpaD proteins, continuing the helical structure of the needle. The fifth position of the complex is occupied by IpaB, interacting with MxiH below and laterally with staggered IpaD subunits (Johnson *et al.*, 2007). The presence of two predicted α-helices which could form a coiled-coil structure in IpaB has led to the predication that IpaB would have the same overall structure as IpaD and that the C-terminal helix would be important for its association with the complex. Consistent with this idea is the observation that small deletions at the C-terminus of IpaB prevent its association with needles (Roehrich *et al.*, 2010; Shen *et al.*, 2010).

Although IpaB has been detected at the *Shigella* needle tip in the absence of host cell contact, (Johnson *et al.*, 2007; Veenendaal *et al.*, 2007; Roehrich *et al.*, 2010; Shen *et al.*, 2010), there is evidence that sensing of host bile salts regulates localization of IpaB to the tip complex (Olive *et al.*, 2007; Stensrud *et al.*, 2008; Dickenson *et al.*, 2010). Deoxycholate, a main component of bile salts, can bind to IpaD and induce a conformational change, which is important for the recruitment of IpaB (Dickenson *et al.*, 2010). In addition, the recruitment of IpaC to needle tips is stimulated by lipids such as cholesterol and sphingomyelin (Epler *et al.*, 2009). As IpaB binds to cholesterol (Hayward *et al.*, 2005; Lafont and van der Goot, 2005), it has been suggested that lipid binding to IpaB triggers a conformation change leading to the recruitment

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of IpaC and insertion of the translocation channel into the host plasma membrane (Epler *et al.*, 2009).

Evidence for direct contact between the tip complex and the translocon inserted in the host membrane

What evidence is there to support the concept that the tip complex maintains contact with the translocators inserted in the host plasma membrane, effectively forming a conduit for delivery of effectors? Several indirect pieces of evidence are worth discussing.

EspA filaments have been observed to form a direct link between EPEC and the host cell (Knutton *et al.*, 1998). However, to date, there is no direct evidence that EspA filaments are anchored to the host cell membrane via interactions with the translocators EspD or EspB.

Another interesting observation is that the tip protein SipD and the translocators SipB and SipC of *Salmonella* are required for a mode of bacterial–host cell contact that requires the T3SS (Lara-Tejero & Galan, 2009; Misselwitz *et al.*, 2011). This mechanism of bacterial binding to epithelial cells was detected using strains lacking expression of effectors (so-called effectorless mutants). The use of effectorless mutants is important to prevent the subsequent membrane ruffling and invasion by *Salmonella* that is normally triggered by effectors. Misselwitz *et al.* (2011) obtained evidence that the Sip-dependent binding mechanism, referred to as 'docking' was irreversible, while binding mediated by type 1 pili was reversible. It was suggested that docking represents an intermediate step of translocation with a dual role: delivery of effectors and maintenance of the bacteria at the site of ruffling (Lara-Tejero & Galan, 2009; Misselwitz *et al.*, 2011). Docking may physically represent binding of the tip complex to the translocon channel, although additional studies will be required to confirm this possibility.

Evidence that effectors located on the surface of bacteria can be translocated via a T3SS into host cells has recently been published (Akopyan *et al.*, 2011). The authors argue that translocation of surface-localized effectors occurs by a different pathway from the standard 'conduit' model (Akopyan *et al.*, 2011). However, it has not been ruled out that the connection between the tip complex and the translocation channel is loose enough to allow the access of extracellular effectors.

Tip proteins as targets of protective antibodies

Anti-tip protein antibodies have been shown to inhibit translocation function in T3SSs for LcrV (Pettersson *et al.*, 1999), PcrV (Sawa *et al.*, 1999), IpaD (Espina *et al.*, 2006; Sani *et al.*, 2007), and SipD (Desin *et al.*, 2010). Antibody binding could directly interfere with tip protein function by blocking interaction with other subunits, with translocator proteins or host receptors. Opsonization is another mechanism by which antibodies that bind tip proteins could mediate host protection.

The mechanism of neutralizing polyclonal anti-LcrV antibodies was investigated using cytotoxicity in *Yersinia*-infected macrophages as a read-out of effector YopJ translocation (Weeks *et al.*, 2002). Results showed that F(ab′)2 was as effective as whole antibody in inhibiting cytotoxicity (Weeks *et al.*, 2002). However, F(ab′)2 was ineffective in promoting phagocytosis (Weeks *et al.*, 2002). Cowan *et al.* (2005) measured YopH translocation into *Yersinia*-infected macrophages and showed that blocking Fc receptor function abrogated the neutralizing activity of anti-LcrV. On the basis of these results, it was suggested that Fc receptor-mediated phagocytosis is important for antibody neutralization of LcrV function (Cowan *et al.*, 2005). A major protective epitope in LcrV is conformational and localizes to amino acids 135–275 (Hill *et al.*, 1997; Vernazza *et al.*, 2009). The region containing this epitope corresponds to α-helix 7 and the needle distal globular domain (Fig. 8d). A protective monoclonal antibody (7.3) which recognizes this conformational epitope (Hill *et al.*, 1997, 2009) has been shown to promote phagocytosis and to reduce cytotoxicity in *Yersinia*-infected macrophages (Weeks *et al.*, 2002; Noel *et al.*, 2009). A second protective monoclonal antibody (BA5) that binds a linear epitope in LcrV (residues 196–225) promotes phagocytosis and reduces cytotoxicity in *Yersinia*-infected macrophages (Eisele & Anderson, 2009; Quenee *et al.*, 2010). The epitope recognized by BA5 corresponds to part of α-helix 8, all of α-helix 9 and α-helix 10, and part of β-strand 4, all within the needle distal globular domain (Fig. 8d). Interestingly, a nonprotective monoclonal antibody (AH1) was shown to inhibit cytotoxicity in *Yersinia*-infected macrophages but it did not promote phagocytosis (Eisele & Anderson, 2009). The linear epitope recognized by AH1 corresponds to residues 76–105 of LcrV (D. Anderson, pers. commun.). This epitope maps to the needle proximal globular domain (Fig. 8d). Taken together, these results suggest that anti-LcrV antibodies must both neutralize effector translocation and promote phagocytosis to be fully protective (Eisele & Anderson, 2009). According to the model of the LcrV tip complex (Fig. 8c), the epitopes recognized by 7.3 and BA5 should be exposed at the tips of the needles and oriented toward the center of the channel in an open complex (Fig. 8d). Such antibodies could simultaneously neutralize LcrV function and opsonize the bacteria. In contrast, the AH1 antibody, by binding to the laterally exposed needle proximal globular domain (Fig. 8d), may

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inhibit tip complex formation and therefore would not opsonize the bacterial surface to promote phagocytosis.

A protective Mab specific for PcrV, Mab166, recognizes a conformational epitope between amino acids 144–257 (Sawa *et al.*, 1999; Frank *et al.*, 2002). This corresponds to the globular domain distal from the needle and is analogous to the domain recognized by protective anti-LcrV antibodies. Interestingly, F(ab′)2 of Mab166 has been shown to be protective against *Pseudomonas* in animal infection models (Frank *et al.*, 2002; Sato & Frank, 2011), indicating that Fc receptor function may be differentially required for antibody-mediated protection against *Pseudomonas* vs. *Yersinia*.

Goure *et al.* (2005) showed that polyclonal anti-LcrV or anti-PcrV antibodies could prevent contact-mediated hemolysis of red blood cells infected with *Yersinia* or *Pseudomonas*, respectively. In addition, insertion of translocator proteins into red blood cell membranes was reduced in the presence of antibody, suggesting that insertion of the translocon pore was disrupted by neutralizing antibody (Goure *et al.*, 2005). Polyclonal antibodies to IpaD inhibited contact-dependent hemolysis of red blood cells and invasion of *Shigella* into human epithelial cells (Espina *et al.*, 2006; Sani *et al.*, 2007). Epitope-mapping studies suggested that the neutralizing antibodies recognized epitopes in the N-terminal 120 residues of IpaD (Espina *et al.*, 2006). This region corresponds to an epitope in LcrV that is neutralizing *in vitro* but is not protective *in vivo* (Eisele & Anderson, 2009). Desin and colleagues showed that antibodies against the SPI-1 needle tip protein (SipD) inhibit *Salmonella* invasion (Desin *et al.*, 2010). Epitope-mapping studies have not been reported for SipD.

Translocator proteins as targets of protective or neutralizing antibodies

Evidence that translocator proteins can be the targets of protective and/or neutralizing antibodies is extremely limited. In fact, most studies suggest that translocator proteins are not effective protective antigens (Andrews *et al.*, 1999; Sawa *et al.*, 1999). An exception is that YopD was shown to be a protective antigen against *Y. pestis* lacking the F1 capsule (Andrews *et al.*, 1999). Polyclonal antibodies specific for a complex of YopD and YopB were shown to be protective against $F1^-$ *Y. pestis* (Ivanov *et al.*, 2008). Anti-YopD/B antibodies do not appear to inhibit Yop translocation into macrophages (J.B.B., unpublished data) and only weakly promote phagocytosis (Noel *et al.*, 2009), and therefore, the mechanism of protection is unknown. Taken together, these results suggest that the translocator proteins are generally not accessible to neutralizing or opsonizing antibodies in the context of wild-type pathogens. Of note, vaccination of mice with recombinant YscF, the subunit of the polymeric T3SS needle, has been shown to confer protection against *Y. pestis* infection (Matson *et al.*, 2005; Swietnicki *et al.*, 2005). Anti-YscF antibodies may mediate protection by opsonization.

Given the evidence for stable association of IpaB with the *Shigella* tip complex (Johnson *et al.*, 2007; Olive *et al.*, 2007; Veenendaal *et al.*, 2007), it can be predicated that antibodies specific for IpaB should be neutralizing. In fact, a monoclonal antibody specific for IpaB was shown to partially neutralize *Shigella flexneri* T3SS function in an *in vitro* invasion assay (Mills *et al.*, 1988). This antibody, 2F1, was reported to recognize an epitope in the N-terminal region of IpaB (Mills *et al.*, 1988). Additional studies are needed to confirm and extend the observation that IpaB function can be neutralized by antibody binding.

The type IV secretion system machinery and conjugative pilus

Introduction

The type IV secretion systems (T4SSs) are a phylogenetically and functionally diverse group of translocation systems (Baron & Coombes, 2007; Alvarez-Martinez & Christie, 2009; Llosa *et al.*, 2009; Backert & Clyne, 2011; Fischer, 2011; Nagai & Kubori, 2011). These systems, present in nearly all bacterial and some archaeal species, translocate DNA and monomeric and multimeric protein substrates to target cells. T4SSs are composed of a few conserved machine subunits and variable numbers of other components from distinct ancestral lineages. These machine adaptations confer specialized functions exploited by bacterial pathogens and symbionts for the successful colonization of particular host niches (Baron & Coombes, 2007; Alvarez-Martinez & Christie, 2009; Llosa *et al.*, 2009; Backert & Clyne, 2011; Fischer, 2011; Nagai & Kubori, 2011).

T4SSs can be classified into functional groups as (1) conjugation, (2) effector translocator, and (3) DNA uptake/release systems (Cascales & Christie, 2003). The conjugation machines translocate single-stranded DNA substrates within and across many bacterial species by a mechanism dependent on direct target cell contact (Alvarez-Martinez & Christie, 2009; Juhas *et al.*, 2009). The effector translocators deliver protein substrates directly to eukaryotic target cells generally also by a contact-dependent mechanism; these systems are central to infection processes of a number of Gram-negative bacterial pathogens including *Helicobacter pylori*, *Brucella* spp., *Bartonella* spp., *Rickettsial* spp., and *Legionella pneumophila* (Baron & Coombes, 2007; Alvarez-Martinez & Christie,

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2009; Llosa *et al.*, 2009; Backert & Clyne, 2011; Fischer, 2011; Nagai & Kubori, 2011). The DNA uptake/release systems translocate DNA substrates across the cell envelope independently of target cell contact, as represented by the *N. gonorrhoeae* GGI system, which exports chromosomal DNA, and the *H. pylori* ComB system, which imports exogenous DNA (Karnholz *et al.*, 2006; Ramsey *et al.*, 2011).

Despite variability in subunit composition and function, the T4SSs likely adopt similar channel structures to convey substrates across the cell envelope. Consequently, recent structural findings for paradigmatic conjugation systems are probably thematic for T4SSs functioning in Gram-negative species. The *Agrobacterium tumefaciens* VirB/VirD4 T4SS is one of the best-characterized T4SSs and here will serve as a reference point for discussion of machine architecture and function. The *A. tumefaciens* system comprises 11 VirB proteins synthesized from the *virB* operon and the VirD4 subunit from the separate *virD* operon. These subunits coordinate the assembly of two structures, the envelope-spanning translocation channel and the extracellular conjugative pilus. Secretion substrates are recruited to the VirD4 substrate receptor, also termed the type IV coupling protein (T4CP), which sits at the entrance to the translocation channel. The channel spans the entire cell envelope and mediates substrate delivery to the cell surface in one step, reminiscent of the action of T3SSs (Christie, 2004; Alvarez-Martinez & Christie, 2009; Juhas *et al.*, 2009).

The conjugative pilus is composed of the VirB2 pilin subunit forming the pilus fiber and VirB5, a pilus tip adhesin (Backert, 2000; Aly & Baron, 2007). All 11 VirB subunits are required for the assembly of the conjugative pilus (Fullner *et al.*, 1996). At this time, it is unknown whether the assembly of conjugative pili initiates from a platform of VirB subunits at the inner or outer membranes. If the former, pilus biogenesis might resemble the assembly pathway described for type IV pili (Fig. 4); these pili are unrelated to conjugative pili but instead cluster phylogenetically with type II secretion systems (Craig *et al.*, 2006; Craig and Li, 2008; Ayers *et al.*, 2010). If the latter, the assembly pathway could be more reminiscent of the CU pathway mediating assembly of P or type I fimbriae (Fig. 2).

Structural details of T4SS components

Structures of several T4SS subunits and subassemblies have been solved by X-ray crystallography, NMR, or cryo-electron microscopy (CryoEM) (Yeo *et al.*, 2000; Gomis-Ruth *et al.*, 2001; Terradot *et al.*, 2005; Bailey *et al.*, 2006; Hare *et al.*, 2006; Bayliss *et al.*, 2007; Chandran *et al.*, 2009; Fronzes et al., 2009b; Durand *et al.*, 2011). A structure of the conjugative pilus encoded by the *E. coli* F plasmid T4SS also was solved by CryoEM and single-particle methods (Wang *et al.*, 2009). These data allow for modeling of some features of the T4SS machines and general comparisons with other Gram-negative cell channels and surface fibers. Complementary biochemical findings from the use of a ChIP-based, formaldehyde (FA) crosslinking assay (termed 'transfer DNA immunoprecipitation' or 'TrIP') established that several of the VirB/VirD4 subunits (VirD4 VirB11, VirB6, VirB8, VirB2, and VirB9) form close contacts with the translocating DNA substrate; these subunits are provisionally assigned as channel constituents (Atmakuri *et al.*, 2004; Cascales and Christie, 2004b; Jakubowski *et al.*, 2004, 2005). Here, we review recent advances in the structure–function studies that formulate our current understanding of how T4SSs assemble and translocate substrates across the Gram-negative cell envelope. The reader is referred to several excellent reviews on other details of type IV secretion systems pertaining to biological diversity, regulation, and contributions to virulence (Baron & Coombes, 2007; Alvarez-Martinez & Christie, 2009; Fronzes et al., 2009a, b; Llosa *et al.*, 2009; Frost & Koraimann, 2010; Backert & Clyne, 2011; Nagai & Kubori, 2011).

Energetics of T4SS assembly/function: the inner-membrane-associated ATPases

The Gram-negative T4SSs typically possess two or three ATPases that each multimerize as dimers or ring-shaped hexamers. The ATPase complexes also physically and functionally interact with each other to form a higher-order, energy-generating factory to drive T4SS machine biogenesis and substrate translocation.

All conjugation systems and most effector translocators rely on VirD4 ATPases for substrate transfer. The VirD4 ATPases are members of the FtsK/SpoIIIE ATPase family (Gomis-Ruth *et al.*, 2004). Most possess an N-terminal TMD and a large C-terminal cytoplasmic domain (Alvarez-Martinez & Christie, 2009). The C-terminal domain of TrwB from the plasmid R388 conjugation system possesses two domains, a nucleotide-binding domain (NBD) similar to RecA and DNA ring helicases and an all α-domain that exhibits structural similarity to site-specific recombinase, XerD of the λ integrase family. Six protomers of TrwB form a homohexameric ring that is 110 Å in diameter and 90 Å in height, with a 20 Å wide channel that constricts to 8 Å at the cytoplasmic pole (Fig. 10a) (Gomis-Ruth *et al.*, 2001; Gomis-Ruth *et al.*, 2002). TrwB ATP hydrolysis is stimulated by DNA binding, and TrwB also undergoes conformational changes upon binding of DNA, consistent with a motor protein function during secretion (Tato *et al.*, 2005).

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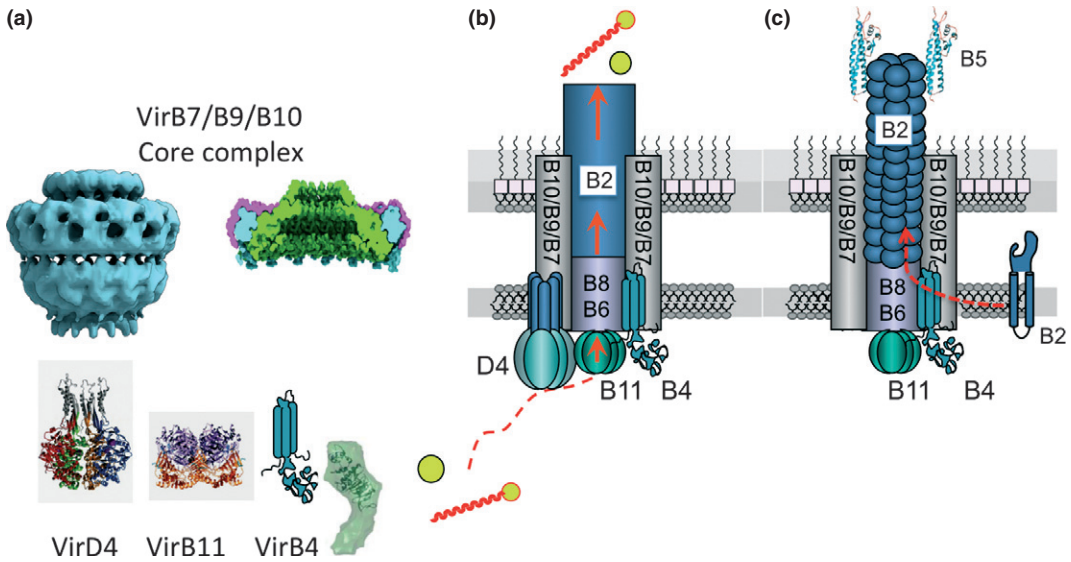

Fig. 10. T4SS structures and mechanisms of action of the T4SS ATPases in substrate transfer and machine biogenesis. (a) Upper: CryoEM (left) and X-ray (right) structures of the VirB7/VirB9/VirB10 core complex from pKM101. Lower: X-ray structure of the VirD4-like TrwB hexamer from plasmid R388; the N-terminal TMD was modeled onto the X-ray structure of the TrwB soluble domain. X-ray structure of *Brucella suis* VirB11 hexamer. Model of VirB4 dimer (left, schematic of predicted VirB4 topology; right, SAXS model of a *L. pneumophila* VirB4 monomer superimposed with the VirB4 C-terminus from *A. tumefaciens* atomic model in ribbon representation). (b) Model depicting substrate docking with the VirD4 T4CP, delivery to VirB11, passage through a channel composed of VirB6 and VirB8 with a contribution by VirB4, and a VirB2 conduit. (c) Model depicting the contributions of VirB4 and VirB11 to dislocation of VirB2 pilin from the inner membrane to build the conjugative pilus. Red arrows denote the substrate translocation route (b) and VirB2 pilin dislocation and polymerization (c). Structures are reprinted with permission from TrwB (Gomis-Ruth *et al.*, 2001); VirB11 (Yeo *et al.*, 2000); VirB4 (Durand *et al.*, 2011); VirB7/VirB10/VirB10 core complex CryoEM (Fronzes et al., 2009a, b), and X-ray crystallography (Chandran *et al.*, 2009); VIrB5 (Yeo *et al.*, 2003).

The mechanism by which T4CPs engage with cognate secretion substrates has been elucidated only for the TraD T4CP of the *E. coli* F plasmid. TraD carries a C-terminal domain extending from its NBD. This C-terminal domain was shown to form a specific interaction with TraM, a component of the relaxosome responsible for nicking the F plasmid at its origin of transfer sequence (*oriT*) (Lu & Frost, 2005; Lu *et al.*, 2008). The TraD–TraM interaction thus forms the basis for recognition of F plasmid substrate by the F T4SS (Lu *et al.*, 2008). Although other T4CPs lack a C-terminal TraM-binding domain, they bind relaxases, other relaxosome components, or protein effectors. Studies suggest that the T4CP interacts with an unstructured C-terminal domain of protein substrates bearing clusters of positively charged or hydrophobic amino acids (Atmakuri *et al.*, 2003; Nagai *et al.*, 2005; Vergunst *et al.*, 2005). Some T4CPs also recognize internal translocation motifs shown to be required for the translocation of protein substrates through some T4SSs (Schulein *et al.*, 2005; Lang *et al.*, 2010).

Results of the FA-crosslinking studies suggest the *A. tumefaciens* VirD4 T4CP delivers secretion substrates to the VirB11 ATPase (Fig. 10b) (Cascales and Christie, 2004b). VirB11 ATPases are members of a large family of structurally conserved 'secretion ATPases', also associated with the types II, III, and VI secretion systems [see companion review in this issue by Dalbey and Kuhn (Savvides *et al.*, 2003; Savvides, 2007)]. Like the other members of this superfamily, VirB11 subunits assemble as double-stacked homohexameric rings formed respectively by N- and C-terminal domains. The VirB11 hexamer is ~ 110 Å in diameter and ~ 70 Å in height with a central chamber of ~ 50 Å in diameter (Fig. 10a) (Yeo *et al.*, 2000; Hare *et al.*, 2006). VirB11 subunits undergo profound structural changes upon binding of ATP, as shown by a combination of X-ray crystallography and analytical ultracentrifugation (Savvides *et al.*, 2003). In the absence of nucleotide, the N-terminal domains exhibit rigid-body conformations, and nucleotide binding 'locks' the hexamer into a symmetric and compact structure. It was proposed that VirB11 subunits use the mechanical leverage generated by nucleotide-dependent conformational changes to drive substrate export or T4SS machine assembly (Savvides *et al.*, 2003). The VirB11 ATPases might use the energy of ATP hydrolysis to unfold protein substrates prior to translocation, reminiscent of unfoldase/chaperone functions described for similar hexameric ATPases associated with type III secretion systems (Akeda & Galan, 2005).

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VirB4 ATPases also participate in substrate translocation across the inner membrane (Atmakuri *et al.*, 2004; Cascales and Christie, 2004a, b). These are large (> 70 kDa) ATPases associated with all known T4SSs (Alvarez-Martinez & Christie, 2009). They display considerable variation with respect to the number of predicted TMDs, and those characterized fractionate as soluble, membrane-peripheral, or membrane-integral proteins. By small-angle X-ray scattering, low-resolution structural models were presented for a membrane-extracted dimeric form of pKM101 TraB and a soluble monomeric form of LvhB4 from *L. pneumophila* (Fig. 10a) (Durand *et al.*, 2011). These structures do not yet inform us how these ATPases function, but VirB4 homologs form contacts with multiple channel subunits, including the VirD4 and VirB11 ATPases, VirB3, and a complex composed of the VirB7, VirB9, and VirB10 proteins, which as described later serves as a structural scaffold for the VirB/VirD4 T4SS (Atmakuri *et al.*, 2004; Yuan *et al.*, 2005; Mossey *et al.*, 2010). *Agrobacterium tumefaciens* VirB4 is required for the delivery of substrates to the inner membrane channel subunits VirB6 and VirB8, although underlying mechanistic details of this reaction step are currently unknown (Fig. 10b) (Atmakuri *et al.*, 2004; Cascales and Christie, 2004a, b).

Recently, evidence was presented that *A. tumefaciens* VirB4 modulates the structural state of membrane-integrated VirB2 pilin by a mechanism dependent on ATP binding/hydrolysis (Kerr & Christie, 2010). VirB4 catalyzes structural changes resulting in the release of membrane-integrated VirB2 to the periplasm (Kerr & Christie, 2010), and VirB4 also is required for the formation of VirB2 complexes with the minor pilin subunit VirB5 (Yuan *et al.*, 2005). These findings support a proposal that VirB4 functions as a pilin dislocase, catalyzing extraction of pilin monomers from the inner membrane for incorporation into the secretion channel and the conjugative pilus (Fig. 10c). In the F plasmid T4SS, VirB4-like TraC is the only ATPase required for production of the F pilus (Lawley *et al.*, 2003). F pili undergo cycles of polymerization and depolymerization (Clarke *et al.*, 2008), suggesting a role for TraC in the regulation of both processes.

The inner membrane translocase

Reminiscent of SecA ATPase (see companion review), the T4SS ATPases are envisioned to coordinate the docking of secretion substrates with the cognate T4SS channel. Although the inner membrane translocase is not structurally defined, it most likely is composed of a polytopic VirB6-like subunit and TMDs contributed by bitopic VirB8- and VirB10-like subunits as well as other small membrane proteins, for example, VirB3. Polytopic membrane proteins are common constituents of macromolecular translocation channels, for example, SecY of the general secretory pathway (see companion review). Consistent with a direct role in substrate transfer, *A. tumefaciens* VirB6 forms FA-crosslinkable contacts with translocating DNA substrates, and crosslinking is dependent on the presence of the VirD4, VirB4, and VirB11 ATPases (Atmakuri *et al.*, 2004; Cascales and Christie, 2004b; Jakubowski *et al.*, 2004). VirB6 also contributes to the assembly and stabilization of other putative components of the inner membrane translocase, including VirB3 and a VirB7/VirB9/VirB10 core complex described later (Fig. 10b and c) (Hapfelmeier *et al.*, 2000; Jakubowski *et al.*, 2003).

The periplasmic- and OM-spanning channel: a T4SS core complex

In *A. tumefaciens*, early studies showed that OM-associated VirB7 lipoprotein and VirB9 interact to form a stable dimer, which in turn interacts with and stabilizes bitopic VirB10 (Anderson *et al.*, 1996; Fernandez *et al.*, 1996; Spudich *et al.*, 1996; Baron *et al.*, 1997). This VirB7/VirB9/VirB10 complex also exerts stabilizing effects on several other VirB channel subunits, suggesting that this complex forms an envelope-spanning structural scaffold early during machine biogenesis. Recently, a core complex composed of these subunits was visualized by CryoEM, and a portion was further solved by X-ray crystallography.

The pKM101 core complex, composed of 14 copies of each of the VirB7-like TraN, VirB9-like TraO, and VirB10-like TraF subunits, is a 1.05 MDa structure, 185 Å wide and 185 Å long, with the potential of spanning the entire Gram-negative bacterial cell envelope (Fig. 10a) (Fronzes *et al.*, 2009b). It is composed of two layers (I- and O-layers) that form a double-walled ring-like structure. The I-layer, composed of the N-terminal domains of TraO and TraF, is anchored in the inner membrane and is opened at the base by a 55-Å diameter hole. The O-layer, composed of TraN and the C-terminal domains of TraO and TraF, has a main body and a narrower cap on the outermost side of the complex that is inserted in the outer membrane. A narrow hole (10 Å) exists in the cap, resulting in a channel extending through the entire cylindrical structure. This channel could allow for substrate passage or exchange of small molecule signals between the cytoplasm and the extracellular milieu (Fronzes et al., 2009b).

A crystal structure of the entire O-layer reveals that VirB10 forms the OM channel (Fig. 10b) (Chandran *et al.*, 2009). Specifically, 14 copies of a VirB10 domain

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comprising two α-helices separated by a loop, termed 'the antennae projection' or 'AP', form the OM channel. These findings are remarkable; first, because the insertion of α-helices into the OM of Gram-negative bacteria is highly unusual; most OM channel proteins adopt a β-barrel structure. Second, VirB10 subunits are unique among described bacterial proteins in spanning the entire cell envelope; the N-terminal TM domain crosses the inner membrane, a Pro-rich region forms an extended structure across the periplasm, and the C-terminal α-helical AP forms an OM pore (Chandran *et al.*, 2009; Jakubowski *et al.*, 2009).

This overall topology lends itself well to a structural contribution of VirB10 to T4SS channel assembly. The 14 N-terminal TMDs of VirB10 form a ~55 Å diameter ring that is predicted to encircle the inner membrane translocase composed of the three ATPases (VirD4, VirB4, and VirB11), VirB3, VirB6, and the N-terminal domain of VirB8 (Christie, 2009; Fronzes et al., 2009a). Extending across the periplasm, the central chamber of the core complex is approximately 100 Å in diameter, sufficiently large to house VirB subunits including the VirB8 C-terminal domain, the pilin subunits VirB2 and VirB5, and the N-terminal domain of VirB9 (this domain was not part of the O-layer X-ray structure). The VirB2 and VirB9 channel components form close contacts with translocating substrate as shown by FA-crosslinking studies (Cascales and Christie, 2004b).

The OM pore includes but is probably not limited to the VirB10 AP. In the pKM101 crystal structure, the 14 copies of the TraF AP form a pore with a hole of 32 Å (Chandran *et al.*, 2009). It is possible that translocating substrates exit the OM directly through the AP pore. However, large deletions of the VirB10 AP do not abolish substrate transfer, and VirB10 does not form crosslinkable contacts with DNA substrate (Cascales and Christie, 2004a, b; Jakubowski *et al.*, 2009). These observations suggest the AP pore might encircle another protein complex configured as the translocation channel. In the FA-crosslinking studies, VirB2 pilin was crosslinked to the DNA substrate, raising the possibility that a pilus or pseudopilus structure extending across the OM forms a conduit for translocating substrate (Cascales and Christie, 2004b).

Structural variations of the core complex

The core complex and associated inner membrane translocase composed of VirB6 through VirB10 subunits are probably universal structural features of T4SSs of Gram-negative bacteria. However, in many systems, these core subunits possess novel domains that confer structural variability and probably modulate surface interactions with target cells. For example, some VirB6 subunits possess large central or C-terminal extensions predicted to reside in the periplasm or extracellularly (Fig. 11a). Such proteins often exceed 60 kDa, more than twice the molecular size of *A. tumefaciens* VirB6 (Alvarez-Martinez & Christie, 2009). Studies showed that these C-terminal extensions are important for entry exclusion, a property that prevents redundant transfer of conjugative elements to donor cells (Audette *et al.*, 2007; Marrero & Waldor, 2007). The entry exclusion phenotype is attributed to the projection of the C-terminal extension across the donor and recipient cell outer membranes and the formation of specific contacts with an entry exclusion (Eex) protein in the inner membrane of the recipient cell (Fig. 11a) (Audette *et al.*, 2007; Marrero & Waldor, 2007). These so-called extended-VirB6 subunits therefore not only function as channel subunits in the donor cell, they also specify an extracellular restriction system preventing the transmission of mobile DNA elements among donor cell populations.

Recently, two variant forms of the VirB7 lipoprotein were structurally characterized and found to contain N0 domains. An NMR solution structure of a VirB7-like lipoprotein in a *Xanthomonas citri* T4SS showed that the N-terminus forms contacts with a VirB9 homolog similar to contacts identified for the pKM101 VirB7 and VirB9 homologs. However, a large C-terminal globular domain (residues 52–133) adopts a structure similar to N0 domains of proteins from other systems involved in OM transport (Fig. 11b) (Souza *et al.*, 2011). In the *X. citri* lipoprotein, the N0 domains mediate the formation of VirB7–VirB7 homooligomers, with a result that a tetradodecamer modeled from the pKM101 core complex has an extra layer of structure surrounding it (Souza *et al.*, 2011). DotD, a lipoprotein associated with the *L. pneumophila* Dot/Icm T4BSS (Nakano *et al.*, 2010), also has an N0 domain structure in its C-terminus (Fig. 11b). In considering a role for the DotD N0 domain, the authors noted that similar structural folds recently were identified in N-terminal domains of secretins from the types II (GpsD) and III (EscC) secretion systems, and the T4P (PilQ) assembly system, as well in subunits of cell-puncturing organelles including bacteriophage subunits (gf27, gp44) and a T6SS (VgrG) (Reichow *et al.* 2010; Korotkov *et al.*, 2009; Spreter *et al.*, 2009). CryoEM and biochemical studies supplied evidence that the N0 domain of the *V. cholerae* secretin GspD forms a ring within the periplasm that engages with the cholera toxin substrate. Structural similarities between DotD and GspD led to the suggestion that DotD might similarly form a ring in the periplasmic portion of the *L. pneumophila* Dot/Icm core complex to regulate substrate trafficking through this T4SS (Nakano *et al.*, 2010).

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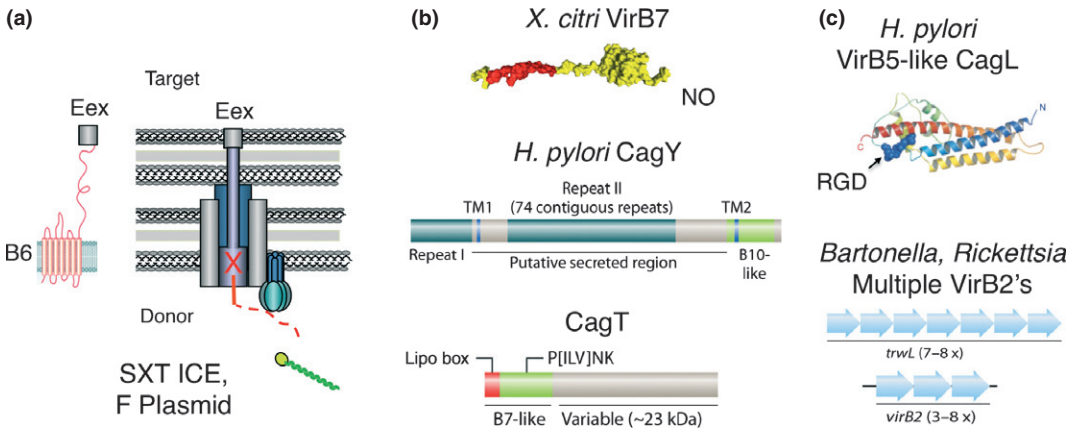

Fig. 11. Examples of T4SS subunit acquisitions of structural folds for the modulation of target cell interactions. (a) Some VirB6-like subunits possess C-terminal extensions implicated in surface exposure, for example, *Rickettsia* spp. VirB6 (not shown), or extension through the T4SS channel and the recipient cell to the inner membrane where contact is established with the entry exclusion protein Eex, for example, *V. cholerae* SXT TraG and F plasmid TraG, which prevents redundant transfer to donor cells. (b) Several T4SSs possess variant forms of the VirB7/VirB9/VirB10 core complex subunits. *Xanthomonas citri* VirB7XAC2622, a VirB7-like lipoprotein with an N-terminal (red) domain, implicated in binding VirB9XAC2620, and a C-terminal N0 domain (globular domain in yellow), which could mediate binding to target cells or substrate translocation through the T4SS. *Helicobacter pylori* VirB7-like CagT and VirB10-like CagY possess repeat domains implicated in forming a surface-variable sheath structure. (c) *Helicobacter pylori* VirB5-like CagL has an RGD motif implicated in binding of the β-integrin receptor on the host cell. *Bartonella* and *Rickettsia* spp. code for multiple copies of VirB2-like pilins, TrwL, and VirB2, respectively, implicated in forming surface-variable pilins for immunomodulation. The *X. citri* VirB7 X-ray structure is reprinted with permission from (Souza *et al.*, 2011). The modeled structure of VirB5-like CagL is reprinted with permission from (Backert *et al.*, 2008). The schematics in (b) and (c) are reprinted with permission from (Alvarez-Martinez & Christie, 2009).

A number of other T4SS surface components possess novel structural features. For example, in the *H. pylori* Cag T4SS, CagT and CagY are classified as VirB7- and VirB10-like, respectively, but both subunits are considerably larger than their VirB counterparts (Alvarez-Martinez & Christie, 2009) (Fig. 11b). Both localize extracellularly as a component of a large sheathed filament produced by the Cag T4SS, and both possess repeat regions that differ in size and composition among Cag systems of different *H. pylori* isolates. Similarly, with the *H. pylori* Cag, *Bartonella* Trw, and *Rickettsial* VirB/VirD4 T4SSs, multiple pilin genes exist often in tandem array, suggesting that variable pilus structures are elaborated through differential expression or intergenic recombination (Alvarez-Martinez & Christie, 2009) (Fig. 11c). In these systems, the production of surface-variable proteins or structures might serve to modulate pathogen–host cell interactions or contribute to immune evasion.

Pilin subunits and conjugative pili

Pilin subunits of T4SSs exhibit low overall levels of sequence identity. They are typically small (~5–10 kDa) proteins with an unusually long signal sequence and two predicted α-helical domains (Lawley *et al.*, 2003). Upon cleavage of the signal sequence, pilins are post-translationally modified. The N-terminus of TraA from the F-type T4SSs is N-acetylated, whereas two P-type pilins, TrbB from plasmid RP4 and VirB2 from *A. tumefaciens*, undergo novel cyclization reactions resulting in covalent linkage of their N and C termini (Eisenbrandt *et al.*, 1999; Kalkum *et al.*, 2002; Lawley *et al.*, 2003). The processed pilin subunits integrate into the cytoplasmic membrane via their hydrophobic α-helices; as mentioned earlier, the integrated pilins form a pool for incorporation into the translocation channel and pilus in response to an unknown signal.

VirB5 subunits also contribute to substrate transfer and pilus biogenesis. Early complementation studies supplied evidence that TraC, a VirB5 homolog from the pKM101 system, could be delivered by TraC-producing strains to neighboring *traC* mutants to restore transfer competence of the mutant strains (Winans & Walker, 1985). This finding led to a proposal that VirB5 subunits localize extracellularly, which has now been confirmed by EM studies showing that VirB5 localizes at the tip of the *A. tumefaciens* T pilus (Aly *et al.*, 2008). The structure of pKM101 TraC, solved by X-ray crystallography, comprises a 3-helix bundle flanked by a smaller globular part (Yeo *et al.*, 2003). TraC and another VirB5 homolog, CagL, of the *H. pylori* Cag PAI are structurally similar, and both are thought to mediate adherence to target cells. A solvent-exposed Arg–Gly–Asp (RGD) motif near the C-terminus of α-helix 1 is implicating in binding of target cell receptors (Figs 10c and 11c).

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The pili of Gram-negative bacterial conjugation systems are the best characterized of the surface structures elaborated by T4SSs (Lawley *et al.*, 2003; Clarke *et al.*, 2008; Wang *et al.*, 2009). The conjugative pili are composed of a VirB2-like pilin subunit and a VirB5-like minor pilin subunit attached at the pilus tip and/or base. The pili can be long (2–20 μm) and flexible with a diameter of 8 nm with a central lumen of 2 nm, as represented by the F-type pili, or short (< 1 μm) and rigid with a diameter of 8–12 nm, as represented by P-type pili. F-type pili enable *E. coli* donor cells to transfer DNA in liquid media, whereas P-type pili support efficient transfer only on solid surfaces. F-type pili are easily detected on the surfaces of F plasmid–carrying cells and undergo cycles of extension and retraction to promote the formation of conjugative junctions. P-type pili are rarely detected on donor cell surfaces and are instead sloughed from the cell surface where they mediate aggregation of donor and recipient cells through hydrophobic interactions.

F pili are tubular structures of ~ 8.5 nm in diameter with two different subunit packing arrangements (Wang *et al.*, 2009). One is a stack of pilin rings of C4 symmetry and the second is a one-start helical symmetry with an axial rise of ~ 3.5 Å per subunit and a pitch of ~ 12.2 Å. These two packing arrangements seem to coexist within the pilus structure. Interestingly, the central lumen of pilus is estimated at ~ 30 Å, large enough to accommodate ssDNA and unfolded protein(s), although whether the conjugative pilus serves as a conduit for T4SS substrates remains a subject of debate.

F pilus extension and retraction has been visualized on living cells by laser-scanning confocal microscopy (Clarke *et al.*, 2008). *Escherichia coli* donor cells appear to extend and retract their pili to sample the immediate surroundings. Extension involves the addition of pilin subunits to the cell–proximal base of the pilus. If the extended pilus establishes contact with a recipient cell, retraction generates a force sufficient to bring the cells together. As noted earlier, VirB4-like TraC is the only ATPase involved in F pilus dynamics, but at this time, there is little mechanistic understanding of how energy drives extension or retraction.

It is also presently unknown whether the conjugative pilus represents the outgrowth of a pilin fiber formed within the core complex or initiates polymerization on an outer membrane VirB platform. For most T4SSs, extracellular pili do not directly convey substrates as has been shown for the needle or pilus structures associated with the T3SSs. Rather, pili mediate formation of tight contacts or mating junctions between donor and target cell membranes. EM images of *E. coli* mating junctions show zones of tight membrane–membrane contacts extending sometimes along half the lengths of the mating cells (Dreiseikelmann, 1994; Samuels *et al.*, 2000). No pilus fibers connect these cells, supporting the notion that the primary function for such extracellular filaments is to promote the formation of tight membrane junctions.

Activating signals for type IV secretion

T4SSs are dynamic organelles that translocate substrates only upon the establishment of productive target cell contacts. Recent studies suggest that the T4SSs transduce signals bidirectionally to activate the translocation process. The activating signals within the cell include binding of substrate by the T4CP and stimulation of ATP hydrolysis among the ATPases. Conversely, activating signals at the cell exterior include contact with target cells or binding of bacteriophage to pilus receptors.

Activation of substrate translocation from the interior was shown to occur through an energy-mediated change in the conformation of VirB10. Upon sensing of ATP energy use by the VirD4 and VirB11 ATPases at the inner membrane, *A. tumefaciens* VirB10 undergoes a conformational change as shown by a difference in protease susceptibility in the unactivated and activated states (Cascales and Christie, 2004a). Energy activation of VirB10 in turn is required for the passage of DNA substrates through the distal portion of the translocation channel. A role for VirB10 in energy-dependent gating of the OM pore is suggested by the isolation of a mutation near the AP that 'locks' VirB10 in the energized conformation and allows for the release of secretion substrates to the cell surface independently of target cell contact (Banta *et al.*, 2011). It is also of interest to note that the ATPase activity of TrwB, the T4CP from the plasmid R388 conjugation system, is stimulated by binding of DNA and relaxosome components (Tato *et al.*, 2007). In a generalized model, therefore, the DNA substrate – T4CP docking reaction – might serve as a signal to activate the T4SS through the coupled stimulation of T4CP ATPase activity, VirB10 energization, and OM channel opening.

A recent study reported the interesting finding that the uptake of bacteriophage R17, whose receptor is the conjugative pilus encoded by the IncF plasmid R1, requires docking of the R1 relaxosome with the TraD T4CP (Lang *et al.*, 2011). Nicking activity of the R1 relaxase is also required, establishing that the relaxosome must be catalytically active. In this system, the TraD T4CP does not display detectable ATPase activity even when bound by relaxosome. Binding of bacteriophage R17 to the conjugative pilus stimulates TraD activity and, correspondingly, phage uptake. Activation of the R1 T4SS therefore requires both relaxosome docking at the T4CP and an external signal. Phage binding represents one activating external signal, and the authors suggest that another such signal is transduced during the conjugation in response

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to the formation of productive contacts with recipient cells (Lang *et al.*, 2011).

These findings advance early findings that F plasmid transfer requires a 'mating signal' propagated by recipient cells to donor cells (Lu & Frost, 2005). The nature of this mating signal remains unknown, but the recent work suggests it is transduced across the T4SS to the T4CP. Existence of such a mating signal also supports the notion that T4SSs are gated and activated to translocate substrates only upon formation of productive mating junctions. In other recent work on the *H. pylori* Cag T4SS, VirB5-like CagL was shown to bind and activate β_1-integrin receptors on mammalian epithelial cells through its RGD motif (Kwok *et al.*, 2007; Backert *et al.*, 2008; Tegtmeyer *et al.*, 2010; Conradi *et al.*, 2011). Integrin activation in turn activates the Cag T4SS, possibly by inducing channel opening to enable delivery of the CagA effector to the mammalian cell. Other T4SS subunits including CagI and CagY, as well as CagA itself, bind integrin receptors, suggesting that a complex network of protein–protein interactions at the *H. pylori*-mammalian cell interface is required for Cag T4SS activation and CagA translocation (Tegtmeyer *et al.*, 2011).

Concluding remarks

The diversity of surface organelles assembled by Gram-negative bacteria is remarkable and reflects the exquisite tuning of these appendages by evolutionary forces to perform specific tasks under specific environmental conditions. Pili and other surface structures assembled by bacterial secretion systems enable the bacteria to act at a distance, extending the functional reach of the bacteria while maintaining the protective integrity of the bacterial envelope. The surface organelles are engineered to withstand extracellular forces such as shear stress and enable the bacteria to maintain contact with their targets under adverse conditions. The ability to initiate contact at a distance provides a means for pathogenic bacteria to avoid detection or uptake by host cells. In addition, surface extensions of bacterial secretion systems, such as provided by the T3SS needle complex and T4SS pilus, allow the direct delivery of effector molecules to host cells, in contrast to the nonspecific release of virulence factors into the extracellular environment.

The different morphologies and functions of bacterial surface structures do not necessarily imply that the structures are unrelated or assembled by different secretion systems. This is perhaps best exemplified by the CU pathway, which assembles a variety of pili, ranging from rigid, rod-like fibers to amorphous, capsular structures. Despite their different architectures, all pili assembled by the CU pathway use the same structural building blocks, assembly mechanism and secretion machinery. Thus, attempts to group surface appendages by appearance alone will lead to inappropriate classifications. In contrast, secretion systems and assembly machineries are highly conserved and provide a more reliable means for classifying pili and other surface organelles.

The plasticity and adaptability of the surface structures provides a means for the bacteria to evade detection by the host immune system, through mechanisms including phase variable expression, antigenic variation, and post-translational modifications that alter the physical and functional attributes of the organelles. Unfortunately, these features also complicate the use of bacterial surface organelles as targets for vaccines and therapeutics. Nevertheless, an improved understanding of the structure and function of bacterial secretion systems and their surface-exposed appendages will facilitate the exploitation of these systems for antimicrobial development.

One promising approach is to target conserved, receptor-binding domains of pili for vaccination. Vaccination with the *E. coli* type 1 pilus adhesin FimH was found to be effective in protecting monkeys from colonization and infection of the urinary tract by UPEC (Langermann *et al.*, 2000). Interestingly, Tchesnokova and colleagues recently showed that the host antibody response may actually enhance binding of FimH to its ligands by stabilizing the adhesin's high-affinity binding state (Tchesnokova *et al.*, 2011). These findings suggest that an improved understanding of pilus adhesion mechanisms may allow tailoring of the antigen used for vaccination to provoke a more effective immune response. Receptor-binding sequences of T4P have also been targeted for vaccination. Kao and co-workers demonstrated that a peptide-based, consensus sequence vaccine targeting the receptor-binding domain of *P. aeruginosa* T4P could overcome antigenic variation and provide cross-protection against different *P. aeruginosa* strains (Kao *et al.*, 2007). Another approach for overcoming antigenic variation of T4P is to target conserved, functionally important minor pilin subunits, which are not subject to the high-level antigenic variation observed with the major pilins. The potential of such an approach was recently demonstrated for the PilX, PilV, and ComP minor pilins of the *N. meningitidis* T4P (Cehovin *et al.*, 2011). The targeting of conserved functional elements may also prove effective for vaccination against surface-exposed components of bacterial secretion machineries, such as described earlier for the *Y. pestis* LcrV and related proteins of the T3SS tip complexes.

An alternative approach to vaccination is to disrupt bacterial adhesion to host cells through the use of small molecule competitive inhibitors of pilus–receptor interactions. Examples include the use of galabiose- and mannose-based inhibitors to interfere with adhesion mediated by P and type 1 pili, respectively, with the goal of pre-

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venting UPEC colonization of the urinary tract (Salminen *et al.*, 2007; Wellens *et al.*, 2008). An additional approach is to develop small molecule inhibitors that disrupt the secretion and assembly machinery itself, thereby preventing the assembly of surface organelles. Almqvist and colleagues have advanced the development of a class of small molecules termed pilicides that prevent pilus assembly by the CU pathway (Pinkner *et al.*, 2006). Pilicides target the periplasmic chaperone and appear to disrupt pilus assembly by interfering with the binding of chaperone–subunit complexes to the OM usher assembly platform. High-throughput screens have been used to identify small molecules that inhibit protein secretion by T3SSs from a number of Gram-negative pathogens (Felise *et al.*, 2008; Keyser *et al.*, 2008). One such inhibitor also showed efficacy against type II secretion and T4P systems (Felise *et al.*, 2008). The inhibitor affected assembly of the T3SS needle complex and appears to act on the OM secretin protein, which is shared by the type III, type II, and T4P secretion pathways. Finally, interference with regulatory proteins is another approach to inhibit the assembly and function of bacterial surface structures. The small molecule virstatin prevents dimerization of the *V. cholerae* ToxT transcriptional activator and blocks expression of both cholera toxin and the toxin-coregulated T4P (Shakhnovich *et al.*, 2007). These examples highlight the potential for a new class of antibiotics that target virulence factor secretion systems and the assembly of virulence-associated surface structures rather than essential biological processes (Cegelski *et al.*, 2008). Such antibiotics should place less pressure on the bacteria than traditional antibiotics and should be less prone to the development of resistance mechanisms. Such antibiotics should also allow the selective targeting of pathogenic bacteria while sparing the normal bacterial flora.

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