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Architectures and biogenesis of non-flagellar protein appendages in Gram-negative bacteria

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Remi Fronzes, Han Remaut and Gabriel Waksman*

School of Crystallography, Institute of Structural and Molecular Biology, Birkbeck College/University College London, London, UK

Bacteria commonly expose non-flagellar proteinaceous appendages on their outer surfaces. These extracellular structures, called pili or fimbriae, are employed in attachment and invasion, biofilm formation, cell motility or protein and DNA transport across membranes. Over the past 15 years, the power of molecular and structural techniques has revolutionized our understanding of the biogenesis, structure, function and mode of action of these bacterial organelles. Here, we review the five known classes of Gram-negative non-flagellar appendages from a biosynthetic and structural point of view.

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Introduction

Anderson (1949) and Houwink (1949) were the first to notice the presence of non-flagellar appendages on the outer surface of bacteria. Early reports on bacterial appendages produced a confusing and varied nomenclature including ‘bristles’, ‘cilia’, ‘filaments’, ‘fimbriae’, ‘fibrillae’, ‘pili’ or ‘needles’ (Duguid and Anderson, 1967). Over the years, however, the terms ‘pili’ (Latin, hairs—hair-like structures) and ‘fimbriae’ (Latin, threads) have most frequently been used, whereas the terms ‘filaments’ and ‘needles’ now designate appendages associated with two subclasses of type III secretion apparatus (Cornelis, 2006). In the post-genomic era, it has now become clear that known Gram-negative non-flagellar appendages are part of five major classes based on their biosynthetic pathway: chaperone–usher (CU) pili, curli, type IV pili, type III secretion needle and type IV secretion pili (Figure 1).

CU pili/fimbriae

The CU pathway is responsible for the synthesis of linear multisubunit pili/fimbriae mainly found in Enterobacteriaceae and in a number of Pseudomonas, Haemophilus, Bordetella, Xylella, Burkholderia, Acinetobacter and Ralstonia species (Sauer et al., 2004). As more bacterial genomes are sequenced, it has become clear that CU pili form the most abundant group of bacterial cell surface appendages. CU pili often constitute important virulence factors, responsible for specific host attachment and/or the evasion of host responses (Wright et al., 2007; Zavialov et al., 2007).

Classification and nomenclature of CU fimbriae

An early hierarchical classification of CU systems based on conserved structural elements in the chaperones identified two distinct subgroups, FGL- and FGS-chaperone assembled pili (FGL and FGS standing for ‘F1G1-long’ and ‘F1G1-short’, respectively (see details below)), which correspond with the assembly of thin fibrillar and rod-like pili, respectively (Hung et al., 1996). However, recent phylogenetic analysis of usher sequences in all 189 known CU systems revealed that only the FGL-assembled organelles form a monophyletic group, with FGS-assembled organelles composed of several diverse phylogenetic clades (Nuccio and Baumler, 2007). CU systems are now divided into 6 major clades: α-, β-, γ- (subdivided into γ3, γ2, γ1 and γ0), κ-, π- and σ-fimbriae, based on common usher ancestry, and supported by similarities in operon structure and morphology of organelles within the separate clades (Nuccio and Baumler, 2007).

CU pili morphology and structure

CU-assembled polymers are formed by linear, unbranched polymers of several hundreds to thousands of pilus subunits, also called pilins, that range in size from ~12 to ~20 kDa. CU organelles differ widely in complexity and morphology, ranging from thin fibrillar material to thick fimbriae composed of a helically wound rod and a distinct fibrillar tip structure.

Fibrillar organelles are formed by CU systems belonging to the κ (Escherichia coli K88- and K99-related fimbriae) and γ3 (FGL-chaperone-assembled organelles) fimbrial clades (Nuccio and Baumler, 2007). γ3-Fimbriae are simple in architecture, containing just a single polymerizing subunit, possibly...
accompanied by a non-polymerizing subunit that caps the fibres at their distal end (Zavialov et al., 2007). At least in the case of E. coli Afa/Dr adhesins, this additional, non-polymerizing subunit serves as an invasin, inducing uptake of the bacteria by the host cell (Garcia et al., 1992). FGL or γ3 systems assemble into thin flexible fibres of ~2–3 nm thickness (often referred to as fibrillae) that frequently curl up into a capsule-like amorphous mass on the bacterial surface (Keller et al., 2002). κ-Fimbrial systems contain 3–5 pilus subunit types, which assemble into thin flexible fibrillae of 2–5 nm thickness that extend individually from the bacterial surface (Hahn et al., 2000; Nuccio and Baumler, 2007).

Rod-like, or ‘typical’, fimbrial organelles are found in the α-, γ- and π-fimbrial clades, with UPEC P and type 1 pili (π- and γ1-fimbrial clades, respectively) and CS1 fimbriae in enterotoxigenic E. coli (ETEC; π-fimbrial clade) as prototypical examples. These CU systems typically assemble 7–10-nm-thick rigid pili with an often remarkably complex quaternary organization made up of multiple subunit types. P pili, for example, comprise no less than six different subunit types, arranged into two distinct subassemblies, a flexible distal tip fibrillum of ~2 nm diameter, displayed onto a long, rigid 6.8-nm-wide pilus rod (Figure 1) (Kuehn et al., 1992). The P pilus rod is formed by a homopolymer of >1000 copies of the PapA subunit. The PapA homopolymer organizes into a right-handed, one-start helical structure with a 2.5 nm pitch and 3.3 subunits per turn. The P pilus tip fibrillum contains a specialized adhesive subunit, PapG, present in a single copy located at the distal end of the pilus and displayed onto a short extended and flexible polymer of 5–10 PapE subunits. Two additional subunit types, PapF and PapK, serve as single-copy linker subunits between the tip adhesin and the PapE polymer and the PapF polymer and the PapA rod, respectively. Finally, the sixth pilus subunit, PapH, serves as terminator subunit, halting pilus growth at the basal end by arresting PapA polymerization (Verger et al., 2006). Type 1 pili (γ1-fimbrial clade) display a very similar architecture, but form shorter tip fibrillae (Figure 1) (Hahn et al., 2002). κ-Fimbrial organelles (alternate CU pathway) such as ETEC CS1 and CFA/I fimbriae lack a visible tip fibrillum all together and here the adhesive subunit is connected directly to the polymerizing major pilus subunit (Evans et al., 1979; Levine et al., 1984). No functional or structural information is available for α- and β-fimbriae: these families were defined entirely based on usher sequences and the fimbriae associated with these systems have not yet been observed.

**CU pilus assembly**

The CU biosynthetic pathway forms a terminal branch of the general secretory pathway and involves just two proprietary
assembly proteins: a specialized periplasmic chaperone and outer membrane assembly platform, called the usher (Sauer et al., 2004). Nascent pilus subunits cross the inner membrane via the Sec translocase from where they travel to the outer membrane usher as binary chaperone–subunit complexes. The chaperone aids subunit folding in the periplasm and maintains subunits in a polymerization-prone folding state (Barnhart et al., 2000; Sauer et al., 2002; Zavialov et al., 2003; Vetsch et al., 2004). The periplasmic chaperones are formed of two Ig-like domains that meet at a right angle (Holmgren and Vetsch, 1999). Cu pilus subunits are characterized by a non-canonical immunoglobulin fold, lacking the C-terminal β-strand (Figure 2) (Choudhury et al., 1999; Sauer et al., 1999) and by an unstructured N-terminal sequence termed ‘N-terminal extension (Nte)’. The missing strand leaves a deep groove that runs along the entire subunit structure. In the chaperone–subunit complexes, the chaperone inserts part of a β-strand (strand G1) in the subunit’s groove and thus complements in trans the incomplete fold of the pilus subunit, a process called donor-strand complementation (Figure 3A, top panel) (Choudhury et al., 1999; Sauer et al., 1999). During subunit polymerization, the complementing β-strand donated by the chaperone is replaced by the Nte peptide of a newly incoming subunit, a process called donor-strand exchange (DSE) (Figure 3A, top panel) (Sauer et al., 2002; Zavialov et al., 2003; Remaut et al., 2006).

The DSE reaction proceeds through a zip-in-zip-out mechanism, whereby the process of DSE is initiated by the insertion of a residue (the so-called ‘P5 residue’) located within the receiving subunit’s groove. This initial binding event is followed by the zipper-in of the incoming Nte within the receiving groove, whereas the G1 strand of the chaperone zips out (Remaut et al., 2006) (Figure 3A, bottom panel). As DSE progresses, the receiving subunit structure transitioned from a chaperone-stabilized high-energy semi-unfolded state to a lower energy folded state (Sauer et al., 2002; Zavialov et al., 2003). Remarkably, pilus biogenesis is an ATP-independent process. It is believed that it is the folding energy released upon DSE that drives subunit polymerization. Incorporation of the subunit PapH in the growing pilus terminates pilus biogenesis. This is because PapH is the only subunit deprived of a P5 pocket and, consequently, is unable to undergo DSE (Verger et al., 2006).

Subunit polymerization occurs at the OM usher. The usher recruits chaperone–subunit complexes to the outer membrane, catalyses their ordered polymerization and is responsible for pilus translocation to the outer surface (Nishiyama et al., 2008; Remaut et al., 2008). OM ushers are ~800 residue proteins comprised of four functional domains. The N-terminal ~125 residues form a distinct soluble periplasmic domain that binds chaperone–subunit complexes (Ng et al., 2004; Nishiyama et al., 2005). A second periplasmic domain is formed by the C-terminal ~150 residues. Though essential, the exact role of this domain is unclear. The central part of the protein gives rise to the usher translocation channel. This consists of a 24-stranded β-pore with an approximate inner diameter of 45 Å × 25 Å, compatible with passage of folded pilus subunits (Remaut et al., 2008) (Figure 3B). This β-barrel domain is interrupted at strands 6 and 7 by a soluble
Figure 3 Model of type 1 pilus assembly at the OM usher. The tip fibrillum of type 1 pili is composed of three single-copy subunits: FimH, the adhesin, and the FimG and FimF subunits. FimA is the major subunit of the type 1 pilus and is assembled immediately after FimF. In the type 1 pilus system, the chaperone is encoded by the *fimC* gene, whereas the usher is encoded by the *fimD* gene. Expression of the *fimC, fimD, fimF, fimG* and *fimH* genes results in the assembly of a FimD$_2$:C:F:G:H complex where FimH is in donor-strand exchange with FimG, itself in donor-strand exchange with FimF. FimF is also in donor-strand complementation with FimC. In that complex, two protomers of FimD are present (Remaut et al., 2008). (A) The donor-strand complementation and donor-strand exchange mechanisms. (Top left) The subunit in red is shown in donor-strand complementation with the chaperone G$_1$ strand in yellow. For clarity, only the F$_1$ and G$_1$ strands of the chaperone are shown. The A and F strands of the subunit are labelled. (Top right) The same subunit in red is shown in donor-strand exchange with the incoming subunit Nte (in cyan). For clarity, only the Nte peptide of that subunit is shown. (Bottom) Schematic representation of the concerted zip-in-zip-out donor-strand exchange mechanism. The diagrams show the F$_1$-G$_1$ β-hairpin (black) in the chaperone (yellow) and the A and F strands in the subunit (in red). The attacking Nte peptide is in cyan. (B) The type 1 FimD$_2$:C:F:G:H complex as derived by cryo-EM (Remaut et al., 2008) coloured blue, dark blue, yellow, red orange and green for FimD usher pore 1, 2, the FimC chaperone, FimF, FimG and the FimH adhesin, respectively. In addition, the model shows the tentative position (shaded in light grey) of an incoming FimC:A complex bound to usher 2’s N-terminal domain (FimD$_2$:C:A, coloured dark blue, yellow, cyan, respectively, and labelled N2, FimC and FimA, respectively). N1 indicates the N-terminal domain of usher 1. The dashed rectangle labelled ‘A’ refers to the region, the zoomed-in representation of which is shown in (A). (C) Schematic diagram of pilus assembly. The diagram shows the usher twinned pores (in blue and dark blue for usher 1 and 2, respectively), the ushers’ N-terminal domains (N1 and N2 for usher 1 and 2 in blue and dark blue, respectively), the FimH adhesion (H; in green), FimG (G; in orange), FimC:F (C1:F; in yellow and red) and an incoming FimC:A complex (C2:A; in yellow and cyan). The plug domain is in magenta. As usher 2 is not used for secretion, the plug (P) remains in place obstructing the usher 2 pore. In the activated usher 1, two alternative positions are proposed (P’ and P’’): the plug could either move sideways (P’) or be ejected from the pore (P’’). Note that the mechanism for pore activation or gating is unknown but is likely to be triggered by the binding of the chaperone–adhesin complex (Nishiyama et al., 2008; Remaut et al., 2008). For clarity, the C-terminal domains of usher 1 and 2 are not shown. At left, the FimD$_2$:C:F:G:H complex (as represented in B) recruits an incoming FimC:A complex through binding to the N-terminal domain of usher 2 (N2; step 1). The complex is brought within donor-strand exchange of FimF, resulting in the release of the FimF-bound chaperone (C1) and the dissociation of the N-terminal domain of usher 1 (N1) (middle panel; steps 2 and 3). N1 is now free to recruit another FimC:A complex (labelled C1A’; right panel, step 4), and bring the complex within proximity of the N2-bound FimC:A complex (step 5). Donor-strand exchange then releases N2 for recruitment of the next chaperone–subunit complex (step 6). Iteration of alternating binding to released usher N-terminal domains, followed by donor-strand exchange with the penultimate chaperone–subunit complex leads to stepwise growth of the pilus fibre (steps 1 through 6).
seeding nuclei (Gophna et al., 2001; Lundmark, 2005). Curli are also potent promoters of a proinflammatory response and can enhance hypotension and bleeding disorders during sepsis and septic shock by their absorption of contact-phase proteins and fibrinogen (Herwald et al., 1998).

Curli structure and assembly
The genes involved in curli production are organized into two adjacent divergently transcribed operons, csgBAC/agfBAC and csgDEFG/agfDEFG in E. coli/Salmonella enterica, respectively (Hammar et al., 1995; Collinson et al., 1996). Curli biogenesis is proposed to follow an extracellular nucleation-precipitation pathway that involves aggregation of the secreted major subunit CsgA onto a membrane-associated minor subunit CsgB that exerts an effect as a nucleation factor (Figure 1) (Hammar et al., 1996; Chapman et al., 2002). Subunit secretion across the outer membrane is dependent on a pore-forming outer membrane lipoprotein (CsgG) (Robinson et al., 2006). Fibre assembly further requires two periplasmic accessory proteins (CsgE and CsgF), both of which can physically interact with the outer membrane partner CsgG (Robinson et al., 2006). The exact role of these periplasmic components is ill understood. CsgE appears to be required for the secretion of the major curli subunit CsgA, whereas CsgF influences the ability of secreted CsgA to aggregate onto the bacterial surface, suggesting a role in maturation or localization of the nucleator subunit, CsgB (Chapman et al., 2002; Gibson et al., 2007). Recent data in Salmonella suggest CsgC (AgfC) is also involved in the assembly process as a periplasmic factor, with CsgC knockout mutants influencing the structural features of aggregated CsgA (Gibson et al., 2007). CsgD does not form part of the assembly machinery but functions as a transcriptional regulator (Hammar et al., 1995).

Both curli subunits are ∼100 residues in size and each contains five repeating sequence motifs, including conserved glycines, glutamates and asparagines, commonly observed in amyloidogenic peptides. Computer modelling of CsgA and CsgB predicts a compact cross-β domain composed of five stacking β-hairpins (Figure 2) (White et al., 2001; Barnhart and Chapman, 2006). Curli are unbranched filaments likely formed by the edge-to-edge aggregation of these cross-β domains along the length of the fibre.

Type IV pili
Type IV pili are pilin polymers produced by many Gram-negative bacteria, including major human pathogens such as Neisseria gonorrhoeae, N. meningitidis, Pseudomonas aeruginosa, Vibrio cholerae, S. enterica, Legionella pneumophila and enteropathogenic E. coli (EPEC). Type IV pili are long (1–4 μm) and flexible filaments of 5–8 nm in diameter that can resist stress forces greater than 100 pN (Merz et al., 2000; Maier et al., 2002). Some bacteria such as V. cholerae and EPEC express bundled type IV pili (Craig et al., 2003; Ramboarina et al., 2005). These pili are essential for a large number of processes determining bacterial virulence, including auto-aggregation, adhesion, twitching motility, biofilm formation or cellular invasion (Craig et al., 2004; Burrows, 2005).

Classification of the pilins and maturation: type IVa and IVb pilins
Type IV pili are produced by rather evolutionarily divergent Gram-negative bacteria. However, these proteins have been grouped in the same class because they share common sequence features and are post-translationally modified in the same manner. Indeed, they are all produced as precursors that possess a basic N-terminal leader sequence. Then signal peptide removal and N-methylation are performed by a single bi-functional endopeptidase first identified in P. aeruginosa (PilD), which cleaves between an invariant glycine residue and a phenylalanine (or rarely methionine) (Nunn and Lory, 1991). All the type IV pili contain a 30 amino-acid hydrophobic N terminus with an invariant glutamic acid residue at the fifth position of the mature polypeptide. In addition, the C-terminal part of the pilin contains two conserved cystein residues. Type IVa and IVb pilins differ by the length of the signal peptide: type IVa pilin has a short 5–6 leader peptide, whereas type IVb pilins contain a longer one (15–30 amino acids).

Pilus structure
For clarity, we chose to only present in this review the latest full-length type IVa N. gonorrhoeae GC and N-terminally truncated type IVb V. cholerae TcpA structures as representative examples of their respective families (Craig et al., 2003, 2006).

Type IVa and IVb pilins share conserved structural features: a conserved structural core (Figure 2 in grey) flanked by a conserved extended N-terminal α-helix (Figure 2 in blue), which is itself flanked by two variable regions commonly called αβ-loop and D-region (in orange and green in Figure 2, respectively). The fold of the core domain is different in type IVa and IVb pilins: whereas the GC pilin displays four β-strands that are directly inter-connected, the TcpA pilin shows a more complex connectivity and contains a fifth β-strand. The two variable regions, α/β and D, are exposed on both sides of the molecule. The α/β region shows great structural variations among the type IV pilins. In addition, O-glycosylation and phosphorylation occurs in the GC pilin.

The structural model of N. gonorrhoeae GC pilus recently proposed by Craig et al. was deduced from a 12.5-Å resolution cryo-EM density map of the native pilus (Figure 2). In this model, the pilus has an outer diameter of 60 Å and a narrow central channel between 6 and 11 Å. The crystal structure of a single native GC pilin was docked into the EM map. The α-helices of the pilin interact tightly with each other in a three helices bundle to form the hydrophobic core of the pilus within the filament. The globular heads are pointing outwards exposing the variable α/β and D-regions to the surface of the pilus. In addition, the α/β and D-regions are also involved in pilin–pilin interactions within the filament. The overall type IVb pilin architecture is very similar to that of type IVa pilin (Craig et al., 2003).

Assembly–disassembly
Unlike curli and CU pili, type IV pili assembly requires a large assembly machinery that is ATP driven and spans both inner and outer membranes. Quite remarkably, and unique among the different bacterial cell surface filaments, type IV pilin assembly can be reversed and the formed pilin retracted.
Several bacterial processes such as twitching motility (Merz et al., 2000) or DNA uptake (Aas et al., 2002) rely specifically on this pilus disassembly mechanism. The assembly–disassembly mechanism requires the presence of conserved ATPases called PilB/F (P. aeruginosa/N. gonorrhoeae nomenclature) and PilT (Figure 1) (Turner et al., 1993). The PilB/F ATPase would be required for pilus growth, whereas PilT would catalyse a rapid depolymerization mechanism (1500 subunits/s) (Burrows, 2005). These two ATPases are homologous to the ‘traffic ATPases’ found in type II and IV secretion systems. In the Aquiflexaeicilus PilT structure (Satyshur et al., 2007), it was proposed that a 15 Å movement of the C-terminal domain observed upon nucleotide hydrolysis and γ-phosphate release would be sufficient to provide the mechanical force necessary to extract pilin subunits from the pilus during disassembly.

The pilus is likely to be assembled at the inner membrane from a mature pilin ‘pool’ that is inserted into the inner membrane through the N-terminal hydrophobic part of the α1-helix (Strom and Lory, 1987). The cytosolic ATPase PilB is thought to be recruited to the pilus base through an inner membrane-embedded protein (possibly PilC/G; Figure 1; Crowther et al., 2004). At the molecular level, the assembly–disassembly mechanism is far from elucidated, and present assembly–disassembly molecular models are highly speculative (see details in Craig et al., 2006).

PilQ forms a ~1 MDa dodecameric structure that forms the outer membrane pore that assists the pilus across the outer membrane (Figure 1) (Wolfgang et al., 2000; Collins et al., 2005). This protein is part of the outer membrane secretins family, which comprises secretins used in type II and III secretion or filamentous phage release mechanisms (Marlovits et al., 2004; Chami et al., 2005). PilQ interacts with the pilotin lipoprotein PilP (Hardie et al., 1996; Drake et al., 1997; Balasingham et al., 2007). The pilotin proteins have been shown to be important in the stabilization, the oligomerization and/or the outer membrane localization of many secretins.

Type III secretion-related appendage

Type III secretion-related appendages were first observed in S. typhimurium as needle-like surface structures responsible for bacterial entry into cultured epithelial cells and as a pilus-like structure in P. syringae and other plant pathogens (called the Hrp pilus) (Roine et al., 1997; Kubori et al., 1998). The assembly of these structures was dependent on systems now well established to form a flagellum-like secretion nanomachine known as the type III secretion system (T3SS). T3SS assembles a complex injectisome designed to secrete effector proteins across the bacterial and host cell envelopes (Cornelis, 2006). These secretory devices are found in a range of animal and plant pathogens including Enterobacteriaceae as well as Aeromonas, Burkholderia, Chlamydia, Chromobacterium, Pseudomonas, Raistonia, Vibrio and Xanthomonas species. So far, no adhesive role has been observed for T3SS appendages.

Needle structure and assembly

Injectisome assembly involves over 20 proteins (Cornelis, 2006). The injectisome basal structure forms a large cylindrical heterocomplex with two double rings that span the inner and outer membranes and that are linked by a hollow structure that crosses the intermediate periplasmic space (Figure 1) (Kubori et al., 1998). At its cytoplasmic side, the basal body is contacted by ATPases and accessory proteins responsible for driving and ordering protein secretion and filament assembly (Woestyne et al., 1994; Akeda and Galan, 2005; Muller et al., 2006). On the outer surface, the basal body assembles a hollow filamentous structure.

The basic extracellular structure, as first identified in the Salmonella SPI-1 T3SS (Kubori et al., 1998) and later observed in detail from purified Shigella injectisomes (Tamano et al., 2000; Blocker et al., 2001), is a short rigid hollow ‘needle’ of about 60 nm in length and inner diameter of 2–3 nm. The basic needle is composed of a homopolymer of 100–150 ~9 kDa subunits (PrgJ, MxiH, YscF and Escf in Salmonella, Shigella, Yersinia and EPEC, respectively). These subunits reveal a helix-loop-helix structure similar to the D0 portion of flagellin (Figure 2) (Deane et al., 2006). Similar to flagellin, needle subunits polymerize into a hollow superhelical structure. The distal end of the needle carries a distinct tip complex composed of the protein LcrV (Yersinia), one of the three translocators involved in pore formation in the target cytoplasmic membrane (Mueller et al., 2005). LcrV forms a dumbbell-shaped structure that has been modelled as a pentameric complex at the tip of the needle and thought of as providing the scaffolding base for the additional two translocator proteins, assembled upon contact with the target membrane (YopB and YopD in Yersinia) (Derewenda et al., 2004; Deane et al., 2006). In EPEC, this needle base is extended by a longer flexible ‘filament’ up to 600 nm in length (Knutton et al., 1998; Sekiya et al., 2001) and composed of an EspA homopolymer. In plant pathogens, the needles are replaced altogether by a long flexible ‘pilus’, the Hrp pilus (Roine et al., 1997; Li et al., 2002).

Assembly of T3SS appendages is thought to follow a mechanism similar to that seen in flagella (He et al., 2004; Macnab, 2004). The components that form the base complex are assembled mostly in a Sec-dependent manner. Filament subunits and secretion substrates, however, lack classical N-terminal leader sequences and are secreted through the nascent secretion apparatus itself. In flagella, the extracellular hook and flagellar filament are formed by the secretion of subunits directly from the cytoplasm through the base complex and through the growing hollow filament (Macnab, 2004). In T3SS filaments, growth by subunit secretion at the distal end has been directly demonstrated in the case of the Hrp pilus and the EspA filament in P. syringae and EPEC injectisomes, respectively (Li et al., 2002; Crepin et al., 2005). The inner dimensions of the flagella and T3SS filaments argue that filament subunits and T3SS effectors traverse the structure in a largely extended, unfolded state.

In Yersinia, the length of the needle complex is linearly correlated with the primary structure of the YscP protein, therefore also called a ‘molecular ruler’ (Journet et al., 2003). A hypothetical mechanism has been proposed in which the molecular ruler resides inside the needle and allows secretion of needle subunits until the needle reaches the length of the unfolded, extended ruler protein. In the Salmonella SPI-1 T3SS, a different mechanism for needle length control has been suggested (Marlovits et al., 2006). Purified Salmonella injectisomes indicate the presence of an inner rod structure (possibly composed of a specialized subunit, PrgJ) inside the
basal body of the secretion system. In the proposed model, secretion of rod and needle subunits (PrgJ and PrgP, respectively; Figure 1) occurs simultaneously. Completion of the inner rod assembly signals a halt in secretion of rod and needle subunits, thereby controlling needle length (Marlovits et al, 2006).

**Type IV secretion pili**

Type IV secretion systems (T4SSs) are ancestrally derived from the mating pair formation cluster proteins involved in bacterial conjugation. T4SSs are also used by plant and human pathogens such as Agrobacterium tumefaciens, Bordetella pertussis, Legionella pneumophila and Helicobacter pylori to secrete virulence factors (DNA and/or proteins) into host cells. T4SS pili are thought to establish a stable and specific contact between cells before substrate transfer (Schroder and Lanka, 2005).

**Morphology**

T4SS pili are classified into two major groups: IncF-like pili (conjugative pili produced by Inc-F, -H, -T and -J systems) and IncP-like pili (conjugative pili produced by Inc-P, -N, -W systems) (Lawley et al, 2003). The IncE-like pili are long (2–20 μm) and flexible appendages with 8–9 nm in diameter; IncP-like pili are short (<1 μm) and rigid rods with 8–12 nm in diameter (Bradley, 1980). These different pilus types are assembled by two different classes of conjugative T4SSs: IncP-type and IncF-type T4SSs. IncP-type T4SSs are type IVa secretion systems. They are composed of VirB-like components (in reference to the A. tumefaciens VirB/D4 T4SS where 11 VirB proteins (VirB1-11) and 1 VirD protein (VirD4) are essential for T4SS structure, assembly and function (Figure 1)). IncF-type T4SSs are type IVb secretion systems: they not only contain VirB-like components but also additional proteins that do not have any counterpart in IncP-type T4SSs.

In the case of T4SSs that are not involved in bacterial conjugation, the morphology of the pili is rarely known or they cannot be classified into the subgroups defined above. For example, the A. tumefaciens T-pilus has a different morphology (T-pili are variable in length and flexible with 10 nm in diameter) than IncP-like and IncF-like pili even if the VirB/D4 T4SS organization is very similar to IncP conjugative T4SSs (Eisenbrandt et al, 1999). H. pylori produces 100–200 nm needle-like type IV secretion-related appendages at the tip of which CagA (a H. pylori T4SS substrate) can be found (Kwok et al, 2007).

**Pilus structure**

Fibre X-ray diffraction and electron microscopy studies of the F-pilus showed that these pili are cylindrical filaments with an external diameter of 8 nm and an internal lumen of 2 nm in diameter. These filaments exhibit a 5-fold helical symmetry (Marvin and Folkhard, 1986).

In A. tumefaciens, it has been shown that VirB5 and VirB7 are minor T-pilus components in addition to the major structural pilin VirB2 (Schmidt-Eisenlohr et al, 1999; Lai and Kado, 2000; Sagulenko et al, 2001b). These proteins have homologues in other pathogenic bacteria such as Brucella suis or Bartonella henselae (called VirB5/VirB7 in both species) and also in conjugative plasmids such as pKM101 (TraC/TraN), RP4 (TraF/TraH), R388 (Twi1/TwiH) or F factor (TraE/TraV). The atomic structure of VirB5 homologue of pKM101 conjugative plasmid (called TraC) is composed of a three long helices bundle flanked by a smaller globular part (Figure 2) (Ye et al, 2003). It has been suggested that VirB5 homologues may have adhesive functions (Schmidt-Eisenlohr et al, 1999; Ye et al, 2003).

**Pilus assembly**

Little is known concerning the assembly of the type IV secretion pili. Before being assembled in the pilus, the pre-pilin undergoes several conserved steps of maturation that can be categorized into two classes.

After signal peptide removal, most of the IncF-like pilins are inserted into the inner membrane and N-acetylated. In the case of the F-pilus, the pilin TraA is N-acetylated by TraX (Moore et al, 1993). TraQ is an inner membrane chaperone-like protein, which is essential for the correct insertion and accumulation of the pilin in the inner membrane (Lu et al, 2002).

The IncP-like pilins such as conjugative RP4 TrbC and A. tumefaciens VirB2 are cyclic peptides (Eisenbrandt et al, 1999). In TrbC, the pre-pilin is 145 residues long and undergoes a first maturation event where its last 27 amino acids are cleaved by an unidentified endopeptidase. Then, the 36 amino-acid N-terminal signal peptide is removed by LepB. Finally, the plasmid-encoded TraF is responsible for TrbC cyclization (Kalkum et al, 2002).

Pilus assembly is clearly dependent on the integrity of the T4SS. T4SSs, where each component of the T4SS has been mutated, fail to assemble a pilus (Fullner et al, 1996; Haase et al, 1995). Pilus production and substrate transfer functions in T4SSs are clearly distinct: mutations in some T4SS components or pilins have been shown to uncouple pilus biogenesis and substrate transfer (Eisenbrandt et al, 1999; Sagulenko et al, 2001a; Jakubowski et al, 2004).

**Concluding remarks**

Gram-negative bacteria assemble a wide range of extracellular appendages on their outer surfaces, which they use to interact with their environment. Functionally, non-flagellar appendages appear to have specialized roles either in adhesion or in transport. CU pilii, type IV pili or curli help pathogenic bacteria to specifically recognize and adhere or even invade their target cells and are not involved in transport. T3SS appendages are used as transport devices and have no described adhesive properties, whereas T4SS pili could be the only pili to have dual roles. However, non-flagellar appendages can be viewed as functionally complementary. Indeed, bacteria using secretion systems to inject virulence factors generally rely on adhesive molecules such as CU pili, type IV pili or other adhesive proteins (e.g. autotransporter and TPS adhesins) to specifically target their host cells (see for example, B. pertussis (Jacob-Dubuisson et al, 1994). Bacteria have developed sophisticated ways to select, attach and infect their target cells. Adhesive and secretion pili are key protagonists during these events. Further progress in structural biology of bacterial pili will help to understand how they are assembled and how they are involved in pathogenicity. This will open avenues for interference with their function as virulence factors, or for their use as display and/or delivery tools in future nanobiotechnology applications.
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