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Pilus biogenesis at the inner and outer membranes of Gram-negative bacteria

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Abstract

Pili are critical virulence factors of many Gram-negative pathogens. These surface structures provide bacteria with a link to their outside environments, allowing bacteria to interact with their hosts, other surfaces or with each other. They can even provide a conduit for the exchange of information. The surface chemistries and other biophysical properties of pili are uniquely adapted to the environmental challenges faced by bacteria. The assembly of these surface structures presents a molecular engineering challenge, which bacteria have solved in a variety of unique ways. In this review, we examine recent advances in our structural understanding of various Gram-negative pilus systems and discuss their functional implications.

Introduction

Bacteria assemble a variety of different non-flagellar surface organelles termed ‘pili’ or ‘fimbriae’. These structures are long, thin proteinaceous polymers that carry out diverse functions and are often critical virulence factors during disease processes[1]. Pilus assembly may be coordinated and catalysed by elaborate multi-protein complexes that either span both the bacterial inner and outer membrane (IM and OM) or by assembly systems localised in the OM only[2]. Moreover, the structures and functions of the resulting pili also vary and often reflect bacterial adaptations to unique environmental conditions. A common function of many types of pili is that they mediate attachment in some shape or form. Bacterial attachment may involve contact between the bacterium and host tissues during infection, between bacteria and other surfaces or between bacteria themselves[3]. In the case of conjugative pili, bacterial contact between donor and acceptor bacteria enables the transport of DNA from one cell to another, whereas contact mediated through other types of pili may lead to infection, bacterial motility, microcolony or biofilm formation[1,4-7]. This review will focus on the diverse structures and functions of the assembly machineries involved in the biogenesis of the five known types of pili/fimbriae in Gram-negative bacteria: the Chaperone-Usher (CU) pili, the type IV pili (T4P), the conjugative type IV secretion pili, the curli fibres, and the more recently described type V pili. In particular, we focus on recent structural progress that has enhanced our understanding of the molecular mechanisms leading to pilus assembly and how the biophysical properties of the assembled pili themselves uniquely contribute to their functions.

Chaperone-Usher Pili

Background

Chaperone-Usher (CU) pili are ubiquitously displayed appendages on the surface of many Gram-negative bacterial pathogens[8]. They are important virulence factors that facilitate host-pathogen interactions crucial for the establishment and persistence of an infection and for other key processes such as biofilm formation[1,5]. The role of CU pili in the pathogenesis of urinary tract infections (UTIs) caused by uropathogenic *Escherichia coli* (UPEC) has been particularly well documented[9,10]. UPEC elaborates two types of pili, type 1 and P pili[5], which will be used here as paradigms to highlight the structure and assembly of surface organelles assembled by the CU pathway. Type 1 and P pili have different functions in UPEC's targeting of the urinary tract, mediating interactions between the bladder and the kidney, respectively. However, they also cooperate in contributing to UPEC's ability to ascend from the bladder to the upper urinary tract during the course of an infection[11].

CU pilus biogenesis

Type 1 and P pili are assembled from distinct pilus subunits or 'pilins', encoded in the *fim* and *pap* operon, respectively[12]. They are organised into two subassemblies - a short, thin tip fibrillum mounted on a 1-2 µm long, helically wound rod[5] (FIG. 1a). Note that not all CU pili have the same architecture; some lack the tip fibrillum, while others lack a rod structure[13]. At their distal end, type 1 and P pili contain an adhesin protein (FimH or PapG for type 1 or P pili, respectively). These adhesins are composed of two domains, an N-terminal lectin domain which allows the bacterium to interact with specific host cell receptors, and a so-called "pilin" domain that engages with the next subunit in the polymer[14,15]. The assembly of a complete tip fibrillum requires the subsequent sequential addition of a single molecule of FimG and FimF for type 1 pili; and one molecule of PapF, 5-10 molecules of PapE and one molecule of PapK for P pili. The vast majority of the pilus length is formed by the rod, which is

composed of ~1000 copies of a single subunit (FimA or PapA for type 1 or P pili, respectively)[5] (FIG. 1a).

Individual CU pilus subunits are transported across the IM into the bacterial periplasm by the general secretory pathway (SecYEG machinery)[16]. In the periplasm, the pilus subunits are folded and stabilised by a dedicated periplasmic chaperone (FimC or PapD for type 1 or P pili, respectively) (FIG. 1a). The subunits are unstable on their own because they form incomplete immunoglobulin (Ig)-like folds, comprised of 6 β-strands, lacking the 7th C-terminal strand, which results in a hydrophobic groove that runs along the entire length of the pilin[14,17]. This groove contains five hydrophobic pockets, termed P1 to P5 pockets, which can be complemented by hydrophobic residues (termed P1 to P5 residues) present on a donor strand originating from either the chaperone before pilus assembly, or from another pilin subunit after pilus assembly. The process by which the periplasmic chaperone complements this groove by donating one of its own β-strands is termed donor-strand complementation (DSC)[14,17-19]. In the chaperone-subunit complex, the chaperone's donor-strand occupies the P1 to P4 pockets of the subunit's groove, leaving the P5 pocket free (see importance of a free P5 pocket below). Once engaged in a complex with the subunit, the chaperone then shuttles it to an OM-embedded nanomachine known as the usher (FimD or PapC for type 1 or P pili, respectively), where subunits are assembled into pili and through which the nascent pilus is secreted. Here, polymerisation occurs via a process called donor-strand exchange (DSE)[20-22]. During DSE, the chaperone's donor strand is replaced by the N-terminal extension (Nte) of the next subunit in assembly. Subunit's Ntes are 10-20 residue N-terminal sequences found in each subunit except for the tip adhesin. After DSE, the Nte provides the donor-strand for the previously assembled subunit, engaging the P1 to P5 pockets of the previously assembled subunit's groove via its corresponding P1 to P5 residues[20,23].

Pilus assembly at the usher

The process of pilus subunit polymerisation is the same for type 1 and P pili and occurs at the outer membrane (OM) usher. The usher itself is composed of a 24-stranded β-barrel pore spanning the OM, a periplasmic N-terminal domain (NTD), two periplasmic C-terminal domains (CTD1 and CTD2) and a plug domain, which maintains the integrity of the OM in the resting state[24,25] (FIG. 1b).

The first step of pilus biogenesis is the recruitment of a chaperone-adhesin complex to the usher's NTD[26-28] (FIG. 1b, step 1). This step was captured crystallographically by a ternary complex of $FimD_{NTD}$-FimC-$FimH_P$ ($FimH_P$ refers to the FimH pilin domain), which shows that the interaction at the NTD occurs predominantly through the chaperone[29]. However, the affinity of the chaperone-adhesin complex to the NTD is higher than for any other chaperone-subunit complex suggesting that the adhesin also plays a role in the binding to the NTD[27,29]. Next, the chaperone-adhesin complex is transferred to the usher CTDs (FIG. 1b, step 2), allowing the NTD to be freed up to bind the next chaperone-subunit complex. This state of translocation was captured in the crystal structure of FimD-FimC-FimH[30]. At this stage the plug domain of the usher is displaced by FimH and moves into a position proximal to the NTD. In the next step of pilus biogenesis, another chaperone-subunit complex is recruited to the NTD (FIG. 1b, step 3). When this step is modeled onto the FimD-FimC-FimH structure, it becomes clear that the Nte of the incoming subunit is optimally positioned to undergo DSE with the previous subunit bound at the CTDs[30] (FIG. 1b, step 4). The process of DSE occurs by a zip-in-zip-out mechanism, whereby the P5 residue of the incoming subunit's Nte engages the empty P5 pocket of the previous subunit's groove. The chaperone's G1 strand (and thus the chaperone) is displaced as the P4, P3, P2 and P1 residues of the Nte progressively occupy their respective hydrophobic pockets in the previous subunit's groove[21,31,32]. Once displaced, the chaperone is released into the periplasm. DSE is followed by the translocation of the growing pilus through the usher pore and its

transfer to the usher's CTDs (FIG. 1b, step 5). The NTD-to-CTD transfer is driven by a differential affinity of the chaperone-subunit complex for the NTD (lower affinity) and CTDs (higher affinity)[33]. In addition, a favorable energetic "path or track" that forces the subunit within the pore lumen to rotate while it is being translocated out[34]. This subunit incorporation cycle is then repeated to add subunits to the base of the growing pilus (FIG. 1b, step 6). For the P pilus, stochastic incorporation of the termination subunit PapH terminates pilus biogenesis. This is because PapH does not have a P5 pocket and thus cannot undergo DSE with any other subunit[35]. It is not understood how type 1 pilus biogenesis stops as no such termination subunit has yet been identified.

CU pilus structure and function

Structure of the tip fibrillum

The overall architecture of type 1 and P pili consists of a short, thin tip fibrillum mounted on a much longer and thicker rod (FIG. 1a). The structure of the type 1 pilus tip fibrillum is known in isolation and in a complex with the usher and the two structures are very similar[34,36]. The structure of the FimH adhesin is also known pre- and post-transport. Indeed comparing the structure of FimD bound to FimC:FimH (representing FimH pre-transport[30]) with the structure of FimD bound to the tip fibrillum (representing FimH post-transport[34]), it is immediately apparent that the two domains of FimH undergo a major conformational change relative to one another: while the FimH pilin domain is aligned with the FimH lectin domain in its pre-transport conformation, it forms a 37.5° angle once extruded from the usher pore[34]. This conformational change is believed to prevent the tip fibrillum from slipping back into the usher. This hypothesis remains to be tested.

The host receptors targeted by FimH include D-mannosylated receptors such as the uroplakins of the bladder, whereas PapG targets galabiose-containing glycosphingolipids which are primarily located on the kidney epithelium[37-39].

Therefore, the receptor specificity of the type 1 and P pilus lectin domains may contribute to the observed tropism towards the bladder and/or the kidney during the course of an infection, respectively[40]. FimH can transition from a low-affinity to a high-affinity binding state depending on the tensile forces applied to the pilus[36,41-44]. This affinity modulation mechanism allows UPEC to adapt to the flow conditions experienced in their environment. In the absence of external forces, the adhesin-receptor interactions are relatively weak and thus allow bacteria to disseminate through the urinary tract, while in the presence of stronger forces such as those induced by urine flow; UPEC is able to resist being flushed out.

Structure of the pilus rod

The CU pilus rod also contributes to UPEC persistence in the urinary tract by dissipating hydrodynamic forces through the reversible uncoiling of its helical rod structure[45-48].

Recently, structures were determined for the type 1 pilus by solid state NMR[49] and for the P pilus to ~3.8 Å resolution by cryo-electron microscopy (cryo-EM)[50], showing a similar overall organisation. The structure of the P pilus in its coiled state shows a right-handed superhelix of 3.28 PapA subunits per turn, an axial rise of 7.7 Å and a helical pitch of 25.2 Å (FIG. 1c,d). Overall, P pili are ~81 Å in diameter with a hollow lumen of ~21 Å diameter. This atomic model of the P pilus explains the mechanism of rod uncoiling at the molecular level by revealing the details of an extensive inter-subunit interaction network[50]. As previously shown, the DSE interactions connecting adjacent subunits are strong hydrophobic interactions, which are topologically essential as they provide the fold-complementing interactions for each subunit in the polymer[20,21,51]. By contrast, the remainder of the subunit interaction network responsible for maintaining the helically wound quaternary structure of the pilus is not only composed of predominantly weak hydrophilic contacts but is also topologically non-essential as it is not involved in subunit folding

and stability[50]. These distinct sets of interactions explain how CU pili can gradually uncoil by first breaking the weaker hydrophilic interactions between the subunit stacks that form the pilus quaternary structure when an external force is applied, but retain the integrity of the polymer by virtue of the strong hydrophobic and topologically essential DSE interaction.

The pilus rod may also contribute functionally to pilus biogenesis itself. The bacterial periplasm is devoid of ATP[52], which raises the question of what drives CU pilus translocation. It has been suggested that random out- and back-sliding motions of subunits occur within the usher pore (Brownian motion), but the formation of the quaternary helical rod structure at the exit of the usher results in pilus extrusion by preventing backsliding[5]. This hypothesis remains to be tested.

Type IV pilus (T4P) system

Background

Type IV pili (T4P) are surface organelles present in many bacterial pathogens, where they act as important virulence factors responsible for causing human diseases[53]. These dynamic extracellular appendages range between 6-9 nm in diameter and can reach several micrometers in length. They are found in Gram-negative, Gram-positive and archaeal organisms[6,54,55] . In archaea, these systems are responsible for the assembly of the archaeal flagellum[56].

T4P functions vary widely and include adhesion to host cells and other surfaces, the formation of biofilms, bacterial aggregates or microcolonies, cellular invasion, electron transfer, phage and DNA uptake and twitching or gliding motility[1,6,57]. Most T4P can dynamically elongate and retract through the action of several cytoplasmic ATPases, providing the basis for their role in bacterial motility[58,59]. Furthermore, these appendages also display remarkable biomechanical properties and are able to exert and withstand forces in excess of 100 pN[60]. T4P can be classified into type IVa (T4aP) and type IVb (T4bP) subgroups based on features

of the major pilin protein and the organisation of the pilus genes[61]. This section will focus on recent structural progress in the T4aP systems and unless stated otherwise will use the *Pseudomonas aeruginosa* nomenclature.

T4P biogenesis

T4P biogenesis is closely related to the type II secretion system pathway, which translocates folded proteins from the periplasm of Gram-negative bacteria into the extracellular environment[6]. However, in the T4P system the principal substrates of translocation are the pilin subunits, which form the pilus filament (FIG. 2a).

In the first step of T4P biogenesis, the prepilin subunits are inserted into the IM by the SecYEG machinery, where their cytoplasmically exposed N-terminal signal peptides are cleaved, and the newly formed N-terminal phenylalanine is methylated by the prepilin peptidase PilD[62,63]. At this point, the conserved and hydrophobic N-terminal α-helix of the pilin subunit spans the IM and the C-terminal globular domain is exposed to the periplasm[1] (FIG. 2a). During pilus elongation, mature pilins are extracted from the IM and incorporated into the base of a growing pilus by the action of the T4P biogenesis machinery.

The T4P biogenesis machinery is composed of several sub-complexes, which are all required to form a functional system[64] (FIG. 2a). The OM secretin sub-complex includes PilQ, a multimeric OM pore protein through which the growing pilus is assembled and disassembled[65-68], and the pilotin protein PilF[69] (FIG. 2a-c). In *Neisseria gonorrhoeae* and *Myxococcus xanthus*, TsaP is also associated with this subcomplex and may function to anchor the secretin into the peptidoglycan cell wall through its peptidoglycan-binding LysM motif[70] (FIG. 2a-b). However, deletion of the putative TsaP homologue in *P. aeruginosa* had no effect on T4P expression or function[67]. The motor sub-complex is composed of the IM 'platform' protein PilC and the cytoplasmic ATPases, PilB and PilT, responsible for pilus elongation and retraction, respectively[71-73] (FIG. 2a,b). The so-called alignment sub-complex,

composed of PilM, PilN, PilO and PilP, bridges the secretin and the motor subcomplexes[74-78] (FIG. 2a,b). Recently, it was shown that this is not simply a static link between the two subcomplexes, but that their dynamic interactions contribute functionally to pilus retraction[79]. The interaction between the alignment and secretin subcomplexes occurs through the interaction of PilP and PilQ[75,80], whereas multiple interactions contribute to the bridging of the alignment and the motor subcomplexes, including the interaction of PilM with PilN[77] and PilC with the cytoplasmic ATPases[72]. The final structural component completing the T4P system is the helical pilus filament, composed of major and minor pilin subunits and adhesin molecules[81,82] (FIG. 2d).

T4P structure and function

Structure of T4P machinery

The purification of intact and fully assembled complexes often presents challenges in structural endeavors of large multi-protein assemblies. However, this limitation may be overcome by studying multi-component complexes by cryo-electron tomography (cryo-ET), which enables their visualisation inside their cellular environment (*in situ*), albeit at very low resolution. This approach was taken to visualise both the non-piliated (closed) and piliated (open) states of the T4P systems of *Thermus thermophilus*[83] and of *Myxococcus xanthus*[84] at 32-45 and 30-40 Å resolution, respectively. Overall, the T4P pilus machinery of both organisms is composed of the same core components with some differences in the cytoplasmic ATPases and an absence of a PilP homologue in *T. thermophilus*. *T. thermophilus* has a much larger periplasmic space than *M. xanthus* or other Gram-negative bacteria and therefore the T4P machinery has to be much longer to span both membranes (~70 nm versus ~ 30 nm)[85,86]. In the T4P system, this difference in length is achieved predominantly through a much longer PilQ channel protein[66].

Figure 2b shows a hypothetical architectural model built by fitting existing

structures into the densities observed by the cryo-ET studies of the *M. xanthus* system[84]. The ring-forming components of the T4P machinery were modeled in a 12-fold stoichiometry as this provided the best fit with cryo-ET density observed[84], although other stoichiometries have also been proposed[64,75]. Changes that were observed in the piliated state of both systems include the pilus traversing the periplasm and extending into the extracellular space, the opening of the PilQ gate (there is an additional gate present in the longer *T. thermophilus* PilQ protein), and the presence of additional cytoplasmic density attributed to the elongating ATPase in both studies[83,84] (FIG. 2b). In addition, an upward translocation of the PilQ channel with a concurrent upward shift of the OM were also observed in *M. xanthus*[84] (FIG. 2b).

In addition to our growing understanding of the overall architecture of the T4P system, we are continuously learning more about its individual components. Recently, a ~7.4 Å cryo-EM structure of the *P. aeruginosa* secretin pore was determined[67] (FIG. 2c). The *P. aeruginosa* secretin consists of two N-terminal AMIN (amidase N-terminal) domains, an N0 domain, an N1 domain and the secretin pore domain. The cryo-EM structure resolved the secretin and N1 domains, while the presumably more flexible N0 and AMIN domains, were not resolved. This structure shows a closed central gate near the OM-periplasmic interface and a large channel ~80 Å wide at the periplasmic side, expanding to ~100 Å near the gate and constricting to ~68 Å at the extracellular lip region (narrowest point). These dimensions are consistent with the passage of a ~6 nm pilus. Interestingly, this structure suggests that the secretin channel is a homo 14-mer with C7 symmetry. Although, other secretin structures have been reported with other symmetries, including the type III secretion system secretin InvG from *Salmonella typhimurium* (C15)[87] and a type II secretion system secretin GspD from *E. coli* and *Vibrio cholerae* (C15)[88]. There are several possible explanations as to why a number of different secretin symmetries have been observed including that there might be differences

between secretins of different secretion systems (e.g. type II secretion system secretins *versus* T4P secretins) and that different symmetries might correspond to different dynamic states related to function[89].

Structure of the pilin and the T4P filament

The structure of the monomeric pilin subunits and the structure of their assembled state in the T4P filament have been the subject of numerous studies. Several pilin crystal structures have been determined and show a conserved lollipop-like structure, with an extended N-terminal α-helix and a globular C-terminal domain[81,90] (FIG. 2c,d). In the fully assembled T4aP the N-terminal α-helices pack together in the centre of the filament and the C-terminal globular domains are oriented towards the outside of the structure, ensuring that the D-region and the αβ-loop (regions of the pilin domain important for functional interactions) are solvent accessible[91,92] (FIG. 2c,d). The assembly of pilins (major and minor pilins) into a pilus filament is thought to be mediated by interactions between conserved hydrophobic residues in the pilin's N-terminal α-helix[91] (FIG. 2d). In addition, a conserved glutamate at position 5 (Glu5) of the pilin subunit is required for pilin assembly and is thought to form a salt bridge with the N-terminal amine of its neighbouring subunit[93,94]. Recently, an atomic model of the *N. meningitidis* T4aP was built by fitting a 1.44 Å pilin crystal structure (PilE) into a ~6 Å cryo-EM volume[92]. This resolution allowed the positioning of individual pilin subunits and secondary structure elements, however cannot resolve side chain conformations. Nevertheless, Glu5 was found in a position consistent with the proposed salt bridge in the context of the pilus filament. This interaction may promote incorporation of pilin subunits into the growing pilus structure from the IM[91-93]. Interestingly, this structure also revealed a loss of α-helical order in the N-terminal helical region of PilE (between the helix-breaking residues Gly14 and Pro22), which may allow the filament to undergo spring-like extensions in response to external force[92] (FIG. 2d).

Towards a mechanism for T4P assembly

Recent progress in determining the low-resolution structures of the fully assembled T4P machinery *in situ* and of individual components and sub-complexes has led to models of T4P assembly. Based on the cryo-ET derived architectural model of the T4P machinery of *M. xanthus*, Chang and colleagues proposed a model for pilus assembly, whereby ATP hydrolysis by PilB (stabilised at the IM by PilM) results in a rotation of PilC, which in turn 'scoops' PilE subunits from the IM and incorporates them into a growing and upward translocating pilus[84]. This model is supported by the finding that PilB interacts with both PilM and PilC[95] and by the knowledge that the related archaeal flagellar motor is also comprised of a rotating component[56]. Furthermore, the recent structure of the ATPase region of PilB in complex with ATPγS from *T. thermophilus* also supports this model of ATPase-generated rotation of PilC[96]. However this model remains to be tested and confirmed.

Conjugative type IV secretion pili

Background

Bacterial conjugation in Gram-negative bacteria is the process whereby a donor cell exchanges genetic material, notably mobile DNA elements (plasmid DNA or the integrative conjugative element (ICE)), with a recipient cell after establishing initial contact through a conjugative pilus. Conjugation is the principal means by which horizontal gene transfer, a process crucial for the spread of antibiotic resistance genes among bacterial populations, is mediated[97]. Bacterial conjugation in Gram-negative bacteria involves two main structures: i) a versatile type IV secretion system (T4SS) responsible for assembling the conjugative pilus, and of secreting different types of cargos, ranging from ssDNA, proteins or protein-DNA complexes and ii) a dynamic conjugative pilus able to extend and retract[98,99]. Many types of conjugative pilus have been described[100], however this section will focus on the F pilus as it represents the best functionally and structurally characterised example.

F pilus structure and function

Structure of the TraA pilin

The recent cryo-EM structures of two members of the F pilus family (F and pED208) allowed the atomic model of TraA to be built *de novo* in the context of a fully assembled pilus[101]. The electron density of the TraA pilin structure encoded by the pED208 plasmid revealed an all α-helical structure, containing three α-helices (α1-3). The loop between α2 and α3 projects towards the lumen of the pilus whilst both N- and C-termini are located on the outside of the helical polymer[101] (for more details see FIG. 3c).

Structure of the helical filament

The cryo-EM structure of the F pilus (encoded by the pED208 plasmid) shows a filament with a diameter of 87 Å and an internal lumen of 28 Å in diameter[101]. The structure can either be described as a 5-start helical filament or as pentameric layers stacked on top of each other related by a 28.2° rotation with an axial rise of 12.1 Å (FIG. 3d). Interestingly, an additional unconnected electron density was observed in the vicinity of each TraA molecule. This density was consistent with the head group and acyl chain of a phospholipid, showing that the F pilus is built of a stoichiometric 1:1 complex between TraA and a phospholipid (FIG. 3c,d). Mass spectrometry analysis showed that the two main phosphatidylglycerol species present in the pilus are phosphatidylglycerol 32:1 and phosphatidylglycerol 34:1, the two most common phosphatidylglycerol species found in the bacterial cell membrane[101]. This phospholipid decoration of the pilus lumen dramatically alters its electrostatic potential. Without phosphatidylglycerol, the pilus lumen is overwhelming electropositive, whilst the addition of phosphatidylglycerol makes the channel moderately electronegative[101] (FIG. 3e). This feature presumably facilitates ssDNA transport within the pilus lumen. The presence of phospholipids within the structure

might also lower the energetic barrier for i- pilus extension and retraction from and back into the IM, thus facilitating re-insertion and extraction of pilus subunits in and from the membrane; ii- pilus insertion into the recipient cell membrane for efficient cargo delivery[101].

Conjugative pilus biogenesis

F pilins subunits (TraA) are synthesised as pro-pilin proteins with an unusual long leader peptide of approximately 50 residues[4,102]. The nascent protein is inserted into the IM by an ATP- and proton motive force (PMF)-dependent mechanism, which is independent of the SecYEG pathway[103]. Upon insertion, the leader peptide of the pro-pilin is cleaved by the peptidase LepB, resulting in a mature pilin with its N- and C-termini exposed to the periplasm, the α2 and α3 helices forming two hydrophobic transmembrane segments (TM) inserted in the IM, and the loop in between oriented towards the cytoplasm[102]. Next, TraX (also encoded by the F-plasmid) acetylates the N-terminus of TraA[104] (FIG. 3b).

Pilus assembly is carried out by the T4SS. Conjugative T4SSs consist generally of a core set of 12 different proteins, termed VirB1-VirB11 and VirD4[2] (FIG. 3a). VirB1 has glycosidase activity, does not form part of the secretion machinery itself but assists in breaking down the peptidoglycan layer between the IM and OMs. VirB2 (TraA in the F system) and VirB5 are the major and minor pilus subunits, respectively. VirB3, VirB6 and VirB8 are channel components in the IM, while VirB7, VirB9 and VirB10 are channel components in the OM. VirB10 spans the entire periplasm and is also inserted in the IM. Remarkably, three ATPases, VirB4, VirB11 and VirD4 are usually required for T4SS function, although VirB11 is sometimes absent[105]. Recently, an almost complete T4SS, encoded by the R388 plasmid, was purified and visualised using electron microscopy[106] (FIG. 3c). This 3.5 MDa complex is embedded in both the IM and OM and is composed of two large subcomplexes, the inner membrane complex (IMC) and the outer membrane “core” complex (OMC)

linked together by a stalk. The IMC contains multiple(s) of 12 copies of VirB3, VirB4, VirB5, VirB6, and VirB8 while the OMC contains 14 copies of VirB7, VirB9, and VirB10. The IMC also includes the 14 TM IM helices of VirB10. It is still unclear what functional role the symmetry mismatch between OMC and IMC might play in type IV secretion.

The OMC core complex is formed of two layers, one of which (termed "the O-layer") inserts in the OM[107,108]. Crystallographic analysis of the O-layer established that the channel through the OM is formed by a helical barrel, each VirB10 subunit contributing a helical hairpin[109]. The main features of the IMC are two periplasmic arches, an IM-embedded region/platform, and two barrel-like structures protruding within the cytoplasm[106]. Each barrel-like structure is formed by a VirB4 ATPase hexamer. This architecture of the T4SS is likely to be universal in Gram-negative bacteria, as suggested by the recent cryo-ET investigation of the *Legionella* T4SS apparatus[110].

It is not known how the T4SS apparatus assembles a conjugative pilus. However, the VirB4 ATPase is known to interact with VirB2 (pilin) and therefore might play a role in mediating the release of the pilin from the IM[111]. These data suggest a model whereby VirB4 catalyses the dislocation of the pilins into the T4SS by an ATP-dependent process. A second ATPase, VirB11, was shown to be important for pilus assembly[112], but no VirB11 homologue has been identified for the F-plasmid-encoded T4SS so far[113]. Therefore, the assembly of the F pilus might be powered solely by the activity of the F system's VirB4 homologue, with the help of some other T4SS IMC components. Perhaps, the two VirB4 ATPases can work in concert to extract and assemble VirB2/TraA-phosphatidylglycerol complexes from the IM into a pilus. A growing T4SS pilus polymerised from the IMC would enter the OMC, leading to its extrusion from the bacterial OM.

Type V pili

Background

Type V pili are important virulence factors of *Porphyromonas gingivalis,* an oral pathogen associated with adhesion, co-aggregation and biofilm formation that can lead to gingivitis and severe periodontitis[114,115]. They are composed of a divergent superfamily of proteins (currently > 1800 unique members) found in the Bacteroidetes phylum, particularly in the class Bacterioidia[116]. In *P. gingivalis*, two morphologically distinct type V pilus types have been identified: major or long (0.3-1.6 μm)[117] and minor or short (80-120 nm)[118] pili. These major and minor pili are encoded in similar operons composed of genes coding for structural pilins forming the pilus stalk (major: FimA, minor: Mfa1), anchoring pilins (major: FimB, minor: Mfa2), minor subunits including tip pilins and other regulatory elements[115].

Type V pilus biogenesis and structure

Type V pilus subunits are expressed in the cytoplasm as lipoprotein precursors containing an N-terminal signal peptide and a consensus sequence called a 'lipobox'[119] (FIG. 4a,b). Pilin proteins are then transported to the periplasmic side of the IM by the SecYEG machinery, where a conserved cysteine residue in the lipobox becomes lipidated, followed by the cleavage of the signal peptide by a type II signal peptidase (FIG. 4a,b). From there, these pilins are presumably shuttled to the OM via a lipoprotein-sorting machinery/pathway[119]. The machinery responsible for pilus polymerisation at the OM remains to be identified (FIG. 4a). At the OM, a trypsin-like arginine or lysine-specific protease/proteinase, R- or K-gingipain (Rgp or Kgp), carries out a second required proteolytic step, prior to pilus polymerisation[119,120] (FIG. 4a-c). This cleavage event occurs after a conserved arginine or lysine residue, resulting in the removal of the first β-strand (A1) generating the mature protein (FIG. 4b). The naming of the strands follows the same nomenclature as used by Xu and colleagues. Each pilin is composed of an N-terminal domain (NTD) and a slightly

larger C-terminal domain (CTD)[116,121,122]. Crystal structures of *P. gingivalis* FimA (a type V pilus subunit not to be confused with the FimA of type 1 CU pili) superfamily stalk subunits revealed that the two C-terminal β-strands (A1' and A2') (of the CTD) can exist in an open or closed conformation, suggesting flexibility in the preceding G2-A1 loop region[116] (FIG. 4c). In the open conformation, a hydrophobic groove is exposed along the length of the CTD where the A1' strand would normally extend the β-sheet. Moreover, after the Rgp or Kgp-assisted removal of the A1 β-strand of the NTD at the OM, and with the A1' and A2' strands of the CTD in an open conformation, a hydrophobic groove is exposed that runs along the entire protein (NTD and CTD)[116] (FIG. 4c). It has been proposed that a strand-exchange mechanism whereby the hydrophobic groove is complemented by a strand from the neighbouring subunit allows pilus assembly[116,122]. Both the N- and C-terminal regions have been proposed to serve as the donor strand during this process[116,122]. However, cysteine crosslinking experiments support the idea that the next subunit's flexible C-terminal A1 'and A2' strands are involved in donor strand exchange[116]. Tip pilins that have been structurally characterised so far either lack the flexible A1' and A2' strands (e.g. Mfa4) or use them to interact with a fused C-terminal lectin domain (e.g. BovFim1C), and thus serve to cap the pilus structure[116,122]. Anchor pilins at the pilus base are also unique because they are not processed by Rgp or Kgp and retain their N-terminal lipid modification, thereby anchoring the pilus into the OM[116] (FIG. 4a).

Common 'threads' between different types of pili

Pili provide a means for bacteria to interact with and sense their environment. Although the individual subunits that make up a pilus, and their mode of biogenesis can be very different, several intriguing parallels can be drawn between unrelated pili. For instance, CU pili, T4P and type V pili all mediate bacterial adhesion[3,114]. The modular architecture of pili permits bacteria to display a variety of different binding modules at the tip or within their structures. Interestingly, lectin or lectin-like domains

are versatile carbohydrate-interacting domains found in many pilus types. Both CU and type V pili contain distal 'tip' or 'adhesin' subunits; these proteins are fusions of pilin and lectin domains that allow their successful incorporation into pili and confer adhesive properties, respectively[14,15,116]. One of the most fascinating properties of certain pili is their ability to 'stretch'. This biomechanical property is most pronounced in CU pili, which can extend to more than five times their original length by unstacking helically arranged subunits[45]. T4P can also 'stretch' when experiencing tensile force, by fully extending a stretch of residues in the N-terminal tail of the pilin subunit[92]. Therefore, T4P achieve their spring-like properties at the secondary structure level, whereas CU pili at the quaternary structure level. CU pili and type V pili are both assembled by proteins that only span the OM[1,115], whereas T4P and F/F-like (conjugative) pili are assembled from secretion systems that span both bacterial membranes[2]. Interestingly, CU pili and type V pili both utilise a strand-exchange mechanism for subunit polymerisation[17,20,116,122], although they are unrelated in terms of sequence and domain architecture. T4P and F/F-like pili share the fact that their subunits are extracted from the IM and then assembled into pili by components localised in the periplasm[1]. These double-membrane spanning systems are powered by ATP hydrolysis and require dedicated cytoplasmic ATPases for pilus assembly. This allows pili to dynamically extend and retract[1].

Conclusions and Outlook

Pili are critical virulence factors expressed by many pathogenic bacteria and are therefore important in human disease. In this review we have focused on structural progress, which has provided a wealth of information regarding the mechanisms of pilus assembly and the structures and properties of pilus filaments themselves. Crystal structures of individual components and subcomplexes have contributed significantly to our knowledge of pilus biogenesis and of pilus function. However, recent hardware and software advances in the field of cryo-EM, have made

challenging structural projects of large (often membrane-embedded) complexes or entire pilus assembly systems possible.

Currently, CU pili represent the best-characterised pilus system of Gram-negative bacteria. But the mechanisms of other pilus biogenesis systems are being elucidated. The recent models of the entire T4P assembly apparatus derived from cryo-ET experiments have provided an exciting glimpse of this very widespread bacterial surface structure. Future efforts will no doubt address remaining questions, such as the exact stoichiometry of the system. This, in combination with higher resolution structures of the assembly apparatus, will add to our understanding of what seems to be an intriguing assembly mechanism. The discovery that the F pilus is composed of a 1:1 stoichiometric protein-phospholipid complex adds another layer of complexity onto the biogenesis and function of conjugative pili. Future studies of the structure and function of the T4SS that assembles this type of pilus will hopefully reveal more about the mechanism of conjugative pilus biogenesis. Lastly, less is known about type V pili. However, recent progress has revealed their strand-exchange mechanism of polymerisation and future efforts will be focused on identifying all the players in their assembly pathway and understanding the mechanisms of these processes. The exciting developments in structural methods will undoubtedly continue shedding light onto these fascinating bacterial systems.

Curli (Text box)

Curli are extracellular functional amyloid fibres assembled by enteric bacteria. They play roles in biofilm formation and bacterial pathogenicity. The complex extracellular matrix formed in the biofilm, protects the bacterial community within from external stress conditions promoting colonisation and persistence. The reduced susceptibility of pathogenic bacteria to antibiotics and the host immune system is a major health concern[123,124]. Curli fibres are assembled by a dedicated secretion pathway, which begins with the transport of CsgA subunits to the periplasm through the SecYEG translocon in an unfolded state[125]. While in the periplasm, CsgA subunits can engage three possible routes: i) progress to the CsgG channel en route to the bacterial surface, ii) become proteolytic degraded, iii) remain in the periplasm where toxic fibres are neutralised by the action of CsgC binding[7]. At the OM, CsgA subunits first interact with the nonameric CsgE capping adaptor, before engaging with the CsgG diffusion channel (a 36-stranded and also nonameric β-barrel pore)[126,127]. When bound to CsgG, CsgE acts as a plug to the pre-constricted CsgG vestibule, but also as a secretion adaptor assisting CsgA subunits during their periplasmic transit[128]. When enclosed in the CsgG-CsgE cavity, which is sufficiently large to accommodate a CsgA subunit, an entropy gradient inside the channel promotes the diffusion of unfolded CsgA from the confined cage to the bacterial outer surface[7]. Once at the surface, CsgB controls the nucleation and polymerisation of CsgA molecules into curli fibres in a CsgF-dependent manner[129,130]. It is still unclear whether the incorporation of curli subunits occurs from the proximal or distal end of the curli fibre.

Figure legends

Figure 1: CU pilus architecture, biogenesis and structure.

a) Architecture of type 1 and P CU pili. Components that make up the CU pilus pathway are drawn schematically. Pilus subunits are transported across the IM by the SecYEG pathway. In the periplasm, a dedicated chaperone (FimC, PapD) helps pilus subunits to fold and stabilises their structure (see text). The chaperone-subunit complexes are then transported across the periplasm to an OM-embedded usher (FimD, PapC), where subunits are polymerised into the growing pilus. The usher is composed of a 24-stranded β-barrel pore, a plug domain, an N-terminal domain (NTD) and two C-terminal domains (CTD1 and

CTD2). The pilus fibre itself is composed of a thin tip fibrillum (FimH, FimG and FimF for type 1 pili; PapG, PapF, PapE and PapK for P pili) and a long rod structure (FimA, PapA). At the distal tip of the tip fibrillum, the adhesin (FimH, PapG) protein is responsible for host cell receptor interaction, whereas the rod endows CU pili with their spring-like biomechanical properties. The stochastic incorporation of PapH into the P pilus terminates pilus biogenesis (see text).

b) The mechanism of subunit translocation through the usher at the OM is illustrated with a series of crystal structures and modelled structures from the type 1 and P pilus systems. Proteins are coloured the same as in the schematic of panel **a**. Step 1: The chaperone-adhesin complex is recruited to and binds the NTD (modelled using Protein Data Bank (PDB) identifiers: 3BWU and 1QUN) of the usher (modelled using PDB: 3OHN and 3RFZ). Step 2: The plug domain relocates to a position next to the NTD and the chaperone-adhesin complex transfers to the CTDs. The adhesin's pilin domain interacts with the CTDs, while the adhesin's lectin domain traverses the usher pore (PDB: 3RFZ). Step 3: The next chaperone-subunit complex is recruited to the NTD and the subunit's Nte is positioned towards the adhesin's hydrophobic groove (still stabilised by the chaperone's donor strand) (modelled using PDB: 3RFZ and 3BWU). Step 4: Donor-strand exchange (DSE) occurs; the subunit's Nte replaces the chaperone's donor strand by a zip-in-zip-out mechanism and the chaperone is recycled (modelled using PDB: 3RFZ, 3BWU and 4XOE). Step 5: The chaperone-subunit complex previously bound to the NTD is transferred to the CTDs and the adhesin inside the usher's pore is translocated upwards (modelled using PDB: 3RFZ and 4J3O). Step 6: The cycle is repeated; another chaperone-subunit complex is recruited to the NTD, undergoes DSE, and transfers to the CTDs, causing the translocation of the previous subunit in the assembly (PDB: 4J3O).

c) Structural model of a CU pilus. The ~3.8 Å cryo-EM map (grey) of the P pilus rod (EM Data Bank (EMDB) identifier: EMD-3222) is positioned on top of the usher in the extracellular space. The molecular surface (purple) generated from the model of the P pilus (PDB: 5FLU) is fit on top of the map and linked to the molecular surface of the tip fibrillum as determined in the crystal structure of FimD-FimC-FimF-FimG-FimH (PDB: 4J3O). This structural model is a hybrid between structures of the type 1 and P systems, but nevertheless provides a visual picture of an entire CU pilus. Inset: ribbon diagram of the P pilus rod structure highlighting one PapA molecule (blue) amongst its neighbouring subunits (grey).

d) **i)** The molecular surface of the P pilus rod (PDB: 5FLU) with the subunits coloured in shades of blue and green. Boxed, the central subunit is 'subunit 0' and the

preceding and succeeding subunit are -1 and +1, respectively. **ii)** Rotated view of the boxed region showing ribbon diagram of three adjacent PapA subunits in the P pilus rod highlighting the mechanism of DSE.

IM, inner membrane; OM, outer membrane; PG, peptidoglycan.

Figure 2: T4P architecture, biogenesis and structure.

a) Components that make up the T4P system are drawn schematically. Pilus subunits (pilins) are first inserted into the IM by the SecYEG machinery and then incorporated into the base of the growing pilus. The T4P machinery is composed of the OM secretin complex, composed of the secretin PilQ and the associated peptidoglycan-interacting protein TsaP (non-essential in *Pseudomonas*), the alignment complex, composed of PilM, PilN, PilO and PilP, and the IM motor complex, composed of PilC and the cytoplasmic hexameric ATPases PilB and PilT. ATP hydrolysis by PilB and PilT provides the energy for pilus elongation and retraction, respectively, and is thought to involve a mechanism of PilC rotation.

b) The architectural model of a T4aP system in the non-piliated (PDB: 3JC9) and piliated (PDB: 3JC8) state from *M. xanthus* (the PilQ AMIN domains have been removed for clarity). All components are coloured the same as in the schematic of panel **a**. This model was built by fitting existing structures of T4P components into electron density maps derived from cryo-ET experiments. TsaP has an N-terminal LysM motif that binds to peptidoglycan and a C-terminal domain connected via a flexible linker. PilN and PilO are structural homologues believed to form heterodimers *in vivo*; their extended helical regions form a coiled coil linking their periplasmically located globular domains to the IM via a transmembrane segment. PilN's N-terminal tail interacts with the cytoplasmically located PilM. PilP is a lipoprotein, which connects the IM-associated sub-complexes to PilQ (through interaction with PilQ's N0 domains). Differences between the non-piliated and piliated basal bodies include an additional density in a cytoplasmic disc region thought to be the elongating ATPase PilB, an upward shift of the OM and the OM pore complex (indicated by arrows) and the presence of a pilus fibre that extends from the IM stem, through PilQ and into the extracellular space. Since this architectural model was built, a new atomic model of the *N. meningitidis* PilE pilus fibre was determined by a combination of X-ray crystallography and cryo-EM (PDB: 5KUA). We have therefore updated the pilus structure in this architectural

model.

c) The ~6 Å cryo-EM map (grey) of the *N. menigitidis* T4aP (EMD-8287) is positioned on top of the ~7.4 Å cryo-EM map (grey) of the *P. aeruginosa* secretin PilQ (EMD-8297). This hybrid model provides a glimpse at the overall structure of these two essential T4P components. The remainder of the T4P machinery is drawn in cartoon representation as in panel **a**. Inset: ribbon diagram of the T4aP structure highlighting one PilE molecule (purple) amongst its neighbouring subunits (grey).

d) **i)** The structure of the *N. meningitidis* T4aP (PDB: 5KUA) can be thought of as a right-handed 1-start, right-handed 4-start or left-handed 3-start helix. The molecular surface of the T4aP is coloured to show the right-handed 4-start helix (also indicated by red arrows). **ii)** Ribbon diagram of the PilE monomer. The D-region and αβ-loop, important for pilus interactions, are coloured in orange and blue, respectively. The positions of residues involved in post-translational modifications (S63, S69), loss of helical order in the extended N-terminus (G14, P22) and pilus assembly and core stabilisation (E5) are indicated. **iii)** Zoomed-in view of the helical regions that stabilise the core of the pilus. Proposed salt-bridge interactions between E5 and the N-terminal amine group, and hydrogen bonds between E5 and T2 (between neighbouring pilins) are indicated with dotted lines.

IM, inner membrane; OM, outer membrane; PG, peptidoglycan.

Figure 3: F pilus architecture, biogenesis and structure

a) Architecture of the T4SS and F pilus. Components that make up the T4SS and F pilus are drawn schematically. The T4SS IM complex is composed of three ATPase (VirB4, VirB11 and VirD4), VirB3, VirB6 and VirB8. The OM complex is formed of VirB7, VirB9 and VirB10. VirB10 inserts in both the OM and IM. The conjugative F pilus, which protrudes from the surface of the T4SS to the extracellular environment, is composed of VirB2. The precise location of VirB5 is unclear and it is not indicated here.

b) The mechanism of TraA (VirB2 homologue in the F system) maturation in the IM and the assembly of a TraA-phospholipid complex into the F pilus is illustrated. TraA pro-pilin is transported to the IM by a PMF-dependent mechanism with the assistance of TraQ. The leader peptide from the pro-pilin is cleaved by the LepB peptidase followed by acetylation of the mature TraA pilin N-terminus by TraX. During TraA maturation within the IM, each molecule binds specifically to a

phospholipid from the phosphatidylglycerol family. It is still not fully understood how the TraA-phospholipid complexes are extracted from the IM by the T4SS. Once inside of the T4S apparatus the protein-phospholipid complex assembles into a helical filament.

c) A hybrid structural model of the conjugative pilus from *E. coli* showing the ~3.6 Å map of the F pilus from the pED208 plasmid (EMD-4042) positioned on top of the T4SS from the R388 plasmid (EMD-2567) in the extracellular space. Inset: the molecular model of the pilus subunit formed by a TraA-Phospholipid complex that was built from the F pilus electron density (PDB: 5LEG). The N-terminus of TraA extends out of the structure and is followed by a short helix, α1, which forms a two-helix bundle with the C-terminal end of α3. Helices α1 and α2 are separated by a loop that protrudes and folds back into the structure. The long α2 helix forms an extended two-helix bundle with the N-terminal part of α3 and is followed by a loop projecting to the pilus lumen. Therefore, α3 is able to interact with both α1 and α2. The phosphatidylglycerol head group is exposed to the pilus lumen whereas the acyl chain is buried in the pilus wall.

d) Overall architecture of the F pilus. i) Side view of the pilus in surface representation with each of the five helical strands individually coloured. ii) Each helical strand consists of 12.8 subunits of TraA-phospholipid per helical turn. iii) Two adjacent pentameric units are related by an axial rise of 12.1 Å and a 28.2° rotation.

e) Electrostatic potential of the pilus lumen calculated in the absence (left) and presence (right) of the phosphatidylglycerol phospholipid.

IM, inner membrane; OM, outer membrane; PG, peptidoglycan.

Figure 4: Type V pilus architecture, biogenesis and structure

a) A schematic overview of what is known about type V pilus biogenesis. Pilus subunits are expressed as lipoprotein precursors, targeted to the periplasmic side of the IM via the SecYEG machinery, a conserved N-terminal cysteine serves as a lipidation site and then the signal sequence is cleaved. Next, the pilin subunits are shuttled to the OM, presumably via a lipoprotein-sorting pathway through interaction with a lipoprotein chaperone. It is not known what factor(s) constitute the OM secretion apparatus or how subunit secretion occurs. A second proteolytic step mediated by Rgp or Kgp occurs, producing the mature pilin protein prior to pilus subunit polymerisation via a strand-exchange mechanism. Stalk subunits

(main structural subunits) are represented in shades of blue and green. Anchor pilins (shades of purple) do not undergo this proteolytic step and remain lipidated allowing these subunits to anchor the pilus to the OM, while tip subunits (shades of orange) presumably mediate pilus interactions with thus far unidentified ligand(s).

b) Proteolytic maturation of stalk pilins from the cytoplasm to the OM. Once on the periplasmic side of the IM, the pilin precursors become lipidated on a conserved N-terminal cysteine. Subsequently, the first proteolytic step occurs whereby the signal peptide (grey) is cleaved by a type II signal peptidase. The mature protein is generated by a second proteolytic step at the OM, where the first β-strand (A1) (yellow), and hence the lipid modification, is removed by Rgp or Kgp. This cleavage occurs after a conserved arginine or lysine residue. The nomenclature of β-strands follows that used by Xu and colleagues.

c) The strand-exchange mechanism of pilus assembly illustrated using topology diagrams. Each pilin is composed of an N-terminal domain (NTD) and a slightly larger C-terminal domain (CTD), each domain comprising a transthyretin-like fold containing seven core β-strands (arranged into two β-sheets). After Rgp/Kgp cleavage, and the switching of the A1' and A2' β-strands into an open conformation, a hydrophobic groove is exposed along the entire protein (NTD and CTD) (red dashed box). This hydrophobic groove can be complemented by the next subunit's A1' and A2' β-strands. The precise molecular mechanisms leading to this strand-exchange polymerisation are not known.

IM, inner membrane; OM, outer membrane; PG, peptidoglycan.

Glossary

Pili: Long bacterial cell surface non-flagellar appendages, also referred to as fimbriae, present in a wide variety of Gram-negative (and Gram-positive) bacterial species involved in bacterial attachment, motility and horizontal gene transfer.

CU pilus section

Donor-strand complementation (DSC): Mechanism/process whereby the incomplete Ig-like fold of pilus subunits is completed and stabilised by a donor strand from the

dedicated periplasmic chaperone (FimC, PapD). This occurs in the periplasm.

Donor-strand exchange (DSE): Mechanism/process whereby the incomplete Ig-like fold of pilus subunits is completed and stabilised by a donor strand provided by an adjacent pilus subunit's N-terminal extension (Nte). This occurs once pilus subunits are assembled into the growing pilus by the usher.

Usher: Outer membrane (OM)-embedded protein composed of a 24-stranded stranded β-barrel pore domain, a periplasmic N-terminal domain (NTD), two periplasmic C-terminal domains (CTD1 and CTD2) and a plug domain. CU pilus assembly is catalysed and coordinated by this protein.

SecYEG translocon: An evolutionarily conserved membrane transporter located in the cytoplasmic membrane of bacteria and archaea and the membrane of the endoplasmic reticulum of eukaryotic cells. In bacteria, this machinery can transport proteins into the periplasm via a co- or post-translational transport mechanism and can insert membrane proteins into the inner membrane via opening of the lateral gate (IM).

Lectin domain: A versatile carbohydrate-binding domain found in many Gram-negative bacterial pili. This allows bacteria to attach to host tissues during infection.

Type IV pilus section

Type IV pilus (T4P): A widespread surface appendage and important virulence factor utilised by both Gram-negative and Gram-positive bacteria to enable bacterial attachment, biofilm formation and motility (twitching and gliding motility).

Secretin: Large, multimeric and gated OM pore-forming proteins found in T4P systems, type II and III secretion systems and in some filamentous bacteriophage extrusion systems.

AMIN (amidase N-terminal) domain: AMIN domains are widely distributed amongst bacterial peptidoglycan hydrolases and periplasmically located transporters and are thought to interact with the peptidoglycan cell wall. The presence of AMIN domains in secretins is thought to help anchor the T4P machinery into the cell wall.

Conjugative pilus section

Conjugation: The transfer of genetic material from a donor to a recipient bacterial cell. A mechanism of horizontal gene transfer.

Integrative and conjugative elements (ICE): Also called conjugative transposons, are a large family of chromosomally encoded mobile genetic elements, which encode a functional conjugative secretion system that mediates their excision, transfer and integration into a recipient cell.

Donor cell: Provides a conjugative genetic element, which is often a plasmid or an ICE that is eventually mobilised to a recipient cell at some point of the bacteria life cycle.

Recipient cell: Acquires a conjugative genetic element from the donor cell, which either gets incorporated into the bacterial chromosome (e.g. ICE) or remains in the bacterial cytoplasm (plasmid).

Type V pilus section

Gingipains: Trypsin-like cysteine proteases that cleave after arginine (Rgp) or lysine (Kgp) residues. Gingipains are important virulence factors produced by *P. gingivalis,* involved in a myriad of processes contributing to oral pathogenesis, including pilin maturation.

Curli section

Biofilm: Community of bacterial cells that form a dense surface-associated matrix of proteins, nucleic acids, and polysaccharides that provide a strong fitness advantage, such as an enhanced tolerance against antibiotics, and a reduced susceptibility to host immune responses and other physical and chemical stresses.

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Highlights:

30. This article describes the crystal structure of the FimD usher bound to a complex of FimC (chaperone) and FimH (adhesin). The FimH lectin domain is traversing through the usher pore.

34. This article describes the crystal structure of the FimD usher in the process of translocating the entire type 1 tip fibrillum (FimF-FimG-FimH). This structure also reveals the conformational changes required to prevent backsliding of the nascent pilus through the usher.

50. This study describes the atomic model of the P pilus built from a ~3.8 Å cryo-EM map and reveals the interaction network that forms the quaternary superhelical CU pilus structure. This is important for understanding how CU pili can reversibly uncoil in response to flow-induced forces.

67. This work describes the ~7.4 Å cryo-EM structure of the *P. aeruginosa* T4P secretin. This secretin is a homo 14-mer with C7 symmetry.

83. This work describes a cryo-ET study of the T4P system in *T. thermophilus.* The fully assembled T4P system is studied in the non-piliated (closed) and

piliated (open) state at resolutions of ~32-45 Å.

84. This article studies the T4P apparatus from *M. xanthus* by cryo-ET at a resolution of ~30-40 Å. The non-piliated (closed) and piliated (open) states were investigated and the individual protein components were localised in the cryo-ET map. The authors built an architectural model of the T4P system by fitting existing structures into their cryo-ET map.

92. In this article the atomic model of the *N. meningitidis* T4aP was built by fitting a 1.44 Å pilin crystal structure (PilE; major pilin in *N. meningitidis*) into a ~6 Å cryo-EM volume. This revealed how the N-terminal α-helical regions of PilE pack together in the core of the structure and describes a model of how T4P could stretch in response to tensile forces.

101. This study provides the first structural insight of a conjugative pilus determined by cryo-EM at a resolution of ~3.6 Å. The structure reveals a pilus built by a protein-phospholipid complex in a stoichiometric 1:1 ratio.

106. The first study to unveil the overall architecture of a type IV secretion system.

107. This paper describes the cryo-EM structure of the T4SS OM complex that inserts in both the OM and IM of Gram-negative bacteria.

109. This article describes the crystal structure of a ~0.6 MDa OM complex containing the entire O-layer.

116. The authors of this article determined 20 crystal structures of type V pilins; including tip, stalk and anchor pilins. This work shows the structural differences between tip, stalk and anchor pilins important for the assembly and function of type V pili and also describes the proteolytic processing. Furthermore, cysteine cross-linking experiments suggest that it is the C-terminal A1' and A2' strands that undergo donor-strand exchange during pilus assembly. (See Ref 122 for alternative mechanism proposed).

122. This article reveals the 1.9 Å crystal structure of Mfa4 (a type V pilin) and describes its proteolytic processing by Rgp/Kgp. The authors propose a strand-exchange mechanism for type V pilus assembly whereby an N-terminal strand complements a groove of the neighbouring subunit in assembly.

126. Together with reference 127, this study describes the atomic details of the OM secretion channel formed by the CsgG lipoprotein. The channel provides insights into how the secretion of curli subunits occurs in an unfolded state.