**Review**

**Chaperone–usher pathways: diversity and pilus assembly mechanism**

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Up to eight different types of secretion systems, and several more subtypes, have been described in Gram-negative bacteria. Here, we focus on the diversity and assembly mechanism of one of the best-studied secretion systems, the widespread chaperone–usher pathway known to assemble and secrete adhesive surface structures, called pili or fimbriae, which play essential roles in targeting bacterial pathogens to the host.

**Keywords:** chaperone–usher; pilus biogenesis; host–pathogen interactions

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**1. INTRODUCTION**

Non-flagellar surface filaments were initially described in Gram-negative bacteria in the 1950s in *Escherichia coli* [1] and 5 years later the term ‘fimbriae’ was coined when their role in cell adhesion processes became evident [2]. The term ‘pilus’ [3] was later introduced referring to the same proteinaceous non-flagellar surface appendages, and therefore the terms fimbriae and pili can be used as synonyms. Before whole genomes became available, fimbriae or pili were classified in terms of their morphology as seen under the microscope and, if known, their function [4–6]. Yet this did not account for the phylogenetic relatedness or the genomic variability with respect to the number of components involved in secreting and building these fimbriae. Nowadays, the classification of fimbriae or pili is the result of a combination of genetics, biochemistry and structure that has led to a classification on the basis of the membrane-embedded assembly and secretion systems involved in their biogenesis (reviewed in Fronzes et al. [7]). This has led to the identification of four types of non-flagellar surface filaments produced by Gram-negative bacteria (reviewed in Fronzes et al. [7]), among which the so-called chaperone–usher (CU) pathway of pilus biogenesis is the most ubiquitous. We review here the mechanism of pilus assembly and secretion by these CU systems, highlighting recent mechanistic insights and also their diversity.

**2. CLASSIFICATION OF CHAPERONE–USHER PATHWAY**

The CU secretion systems are mostly grouped into gene clusters, some of them identified as operons, with a minimum of an usher-, a chaperone- and a fimbrial/pilus subunit-encoding gene [8]. The chaperone and usher proteins are the accessory proteins needed to assemble pilus subunits into a pilus and secrete the assembled pilus. These are relatively conserved. However, classification schemes for CU pathways based only on sequence homology between fimbrial subunits and/or between chaperones have a significant shortcoming: the CU pathway-encoding gene clusters or operons may vary in the number of chaperones, fimbrial subunits as well as of additional adhesin-encoding genes that group to distant branches in a phylogenetic tree and would therefore make any assignment ambiguous. However, there is always only one outer membrane (OM) usher present. As a consequence, Nuccio & Bäumler [8] proposed a classification scheme based on the usher protein. The fimbrial usher protein (FUP) family is distributed among the Proteobacteria, Cyanobacteria and *Deinococcus–Thermus* phyla [9]. The FUP is divided into six clades (table 1), designated α-, β-, γ-, κ-, π- and σ-fimbriae, each stemming from a common ancestor. The γ-fimbrial clade is further subdivided into four subclades, termed γ1-fimbriae, γ2-fimbriae, γ3-fimbriae and γ4-fimbriae. The α-, κ-, π- and σ-fimbrial clade names were assigned arbitrarily to recall a particular characteristic of the clade or a prominent member as follows: α-fimbriae, for alternate CU family; κ-fimbriae, for K88 (F4) fimbriae; π-fimbriae, for pyelonephritis-associated fimbriae (P fimbriae); and σ-fimbriae, for spore coat protein U from *Mycobacterium xanthus*. The β- and γ-fimbriae were assigned names alphabetically.

Another mode of classification of CU pathways is based on the chaperone structure, particularly on the length of a loop that connects their F1 and G1 strands [11]: the FGL (long F1–G1 loop) chaperone subfamily assemble non-fimbrial surface structures, while the FGS (short F1–G1 loop) chaperone subfamily assemble fimbrial filaments [11,12]. Within the FUP clades classification, the FGL chaperones CU systems fall into just one clade, the γ3-clade; however, the FGS chaperones CU systems fall into the β-, γ1-, γ2-, γ4-, κ- and π-clades.

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One contribution of 11 to a Theme Issue ‘Bacterial protein secretion comes of age’.
3. GENETIC DIVERSITY OF CHAPERONE–USHER PATHWAYS

The classification of the fimbrial gene clusters into FUP clades based on the usher sequences was shown to correlate with a core gene arrangement. The six established clades can be subdivided into four major clusters based on their gene organization and evolutionary relationship of their pilus subunit sequences. The first cluster is the $g_k$ cluster, containing the $g$, $k$ and $p$ clades. This cluster is characterized by a common pilus subunit homology domain (PFAM00419). Additionally, the $p$- and $k$-clades both share a core structure composed of genes encoding first a major subunit, then an usher, followed by a chaperone (MUC; table 1). The $\gamma$-clade does not share the same gene organization and this structure is different for each subclade: MCUT for $\gamma_1$, MCCU for $\gamma_2$, MCU or CUM for $\gamma_3$ and MCUT for $\gamma_4$ (T standing for the tip adhesin). The $\alpha$, $\beta$ or the $\sigma$-clades form their own individual clusters, with distinct gene organization: CMUT, MCU and MCUT, respectively. They also contain distinct fimbrial subunit homology domains: PFAM04449 for $\alpha$, PFAM06551 for $\beta$ and COG5430 for $\sigma$. This would suggest that the diversity in gene organization found within each clade never involved lateral exchange of subunit genes among these four major clusters, instead coevolving as complete clusters. In cases where there are divergences from the core gene structure, for example, when there is more than one chaperone within one fimbrial gene cluster, this could be explained by a probable gene duplication event, as on a tree based on chaperone sequence, when the chaperones encoded within the same gene cluster formed sister groups [8].

In addition to the variability in gene organization, sequencing of whole genomes has identified CU pathways which can be considered as hybrid: their gene clusters include sequences which code for components that are normally part of unrelated clusters encoding unrelated types of secretion systems. This is the case for the $g_4$-clade, which in some cases includes genes of the type Vb or two-partner secretion (TPS) systems either within the coding sequence for the CU pathway or flanking the CU gene cluster (table 1): in both Bordetella pertussis and Bordetella avium, the fim gene clusters are flanked upstream by FhaB (TpsA protein and adhesin) and downstream by a FhaC (TpsC

### Table 1. Classification of chaperone–usher systems according to Nuccio & Bäumler [8] into fimbrial usher protein (FUP) clusters, clades and subclades. One best representative of the core gene organization of each clade/subclade has been chosen and the typical host target tissues are listed, if known. For more details on adhesins and host receptor molecules, please refer to Nicastro et al. [10].

<table>
<thead>
<tr>
<th>FUP cluster</th>
<th>FUP clade</th>
<th>FUP subclade</th>
<th>operon structure</th>
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<td>$\alpha$</td>
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<td>E. coli cfaABCE</td>
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<td>B. cepacia Bamb1677-9</td>
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<td>V. parahaemolyticus csuABCDE</td>
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<td>$\gamma_1$</td>
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<td>E. coli smfACDHF</td>
<td>epithelial bladder, kidney cells</td>
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<td>Y. pestis cof1M,IA,1</td>
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<td></td>
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<td>abiotic surfaces, N/D</td>
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<td>B. pertussis/B. avium fhaBfim1ABC/hubC</td>
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<td>$\pi$</td>
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<td>B. cepacia Bamb_1523-6</td>
<td>kidney epithelial and blood cells</td>
</tr>
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Core components: M (major subunit) C (chaperone) U (usher) T (tip adhesin)

Additional components: SU (subunit) TpsB-like
and OM pore) protein. In *Pseudomonas fluorescens*, a complete TPS cluster is inserted within the CU gene cluster and in *Pseudomonas aeruginosa*, an orphan gene coding for an FhaC-like adhesin is found in the same location [8]. It is likely that whole-genome sequencing will eventually unveil more such hybrid pathways, involving other clades than the g4-clade and other secretion systems/pathways than TPS.

4. ADHESINS AND PILUS–RECEPTOR INTERACTION

The first pilus protein to be identified as responsible for binding to host epithelial cells was FimH [13,14]. FimH forms part of the tip fibrillum in type I fimbriae and is the adhesive structure responsible for interaction with D-mannosylated proteins such as epithelial bladder and kidney cells [15] or uroplakins [16,17] (table 1). FimH was also the first structure to be determined, consisting of two subdomains: an N-terminal lectin domain containing the mannose-binding site, connected via a linker chain to a C-terminal pilin domain responsible for incorporating FimH into the fimbrial structure [18]. Type 1C and S fimbriae, which, like the type I fimbriae, belong to the γ1-clade, use as receptors the GalNAc-β1,4-Galβ moieties present in the globoseries of glycolipids (GbO3, GbO4 and GbO5) on the surface of kidney epithelial cells and erythrocytes. The tip adhesins of the α-clade (mainly enterotoxigenic *E. coli* (ETEC)) are known, but their receptor partners on epithelial intestinal cells remain elusive [15].

Most bacteria carry more than one CU system. Whole-genome sequencing of many strains of enterobacteria has indicated that the presence of multiple fimbrial gene clusters is the norm. In pathogenic bacteria, such as *P. aeruginosa*, five different so-called Cup (CU pathway) secretion systems (CupA–CupE) are described [10,19,20]. The apparent redundancy in such secretion machineries might reflect a high diversity in lectin domains, thereby ensuring attachment to a broader set of hosts.

While each adhesin expressed independently can promote adhesion of bacteria to a specific tissue, a sequential or synergistic expression of adhesins with diverse specificities could eventually determine the final host tissue destination of the bacteria. This was first proposed for uropathogenic *E. coli* cells (UPEC), with expression of first type I fimbriae and then P fimbriae [21], progressively targeting the bacterium from the bladder (type 1) to the kidney (P pilus). Determining how sequential expression of different lectins affects tissue tropism is a key aspect in understanding bacterial colonization. Similarly, the expression of the Cup and other pathways known to be involved in host colonization in *P. aeruginosa* seems to be dependent on the stage of biofilm formation [22,23], a process itself dependent on the production of fimbriae/pili.
The CU pathway pili are assembled into linear unbranched polymers consisting of several hundreds to thousands of pilus subunits (also known as pilins) that range in size from approximately 12 kDa to approximately 20 kDa. CU organelles differ widely in complexity and morphology, ranging from non-fimbrial 2–5 nm in diameter, flexible fibrillae (g3-clade: Dr; k-clade: F4), to rigid helical fimbrial shafts of up to 10 mm in diameter (a-clade: CS1; g1-clade: type1; g2-clade: F6; g4-clade: Mrk; p-clade: P) [24].

This review focuses on the type I and P pili of uropathogenic *E. coli*, or rod-like fimbrial organelles, which are members of the g1- and p-fimbrial clades, respectively, from which most of our current knowledge of the pilus assembly process has been derived (figure 1).

The P pilus is formed by six different subunits arranged into two distinct subassemblies: the tip fibrillum and the pilus rod (figure 1). The distal tip fibrillum is approximately 2 nm in diameter, is flexible and composed of one PapG adhesin at the distal end, followed by one adaptor subunit PapF and 5–10 copies of the PapE subunit (figure 1). More than 1000 copies of the PapA subunit form the long, rigid and 6.8 nm wide pilus rod. Both subassemblies are connected by the adaptor subunit PapK and the rod is terminated at the proximal end (in the cell wall) by the termination subunit PapH [25,26]. The PapA rod adopts a right-handed, one-start superhelical structure with a 25 Å pitch, a 7.54 Å rise per subunit and 3.3 subunits per turn. The filament has a maximum diameter of 82 Å and an axial channel 25 Å in diameter runs straight up the centre of the helical axis [27]. Type 1 pili are composed of four different subunit types (FimH, FimG, FimF and FimA). One copy of the distal adhesin FimH, followed by one copy each of FimG and FimF [28], forms a flexible tip fibrillum shorter than the Pap tip [29]. The tip fibrillum is attached to an extended rigid and helically wound rod of circa 1000 FimA subunits. No termination subunit has yet been characterized for the type 1 pilus.

Pilus subunits are translocated from the cytoplasm to the periplasm via the general secretory pathway SecYEG. Pilus subunits are unable to fold and unable to self-assemble at the cell surface on their own [30]. Two accessory proteins are needed: (i) a periplasmic chaperone essential in stabilization/folding of subunits, in avoiding premature subunit polymerization in the periplasm, and in targeting chaperone–subunit complexes to the other accessory protein, (ii) an OM assembly platform termed usher.

(a) **Donor-strand complementation**

The mechanism of stabilization of pilus subunits by periplasmic chaperones was first described for the PapD–PapK [31] and FimC–FimH [18] chaperone–subunit complexes. All pilus subunits exhibit an incomplete Ig-like fold, where the seventh C-terminal...
β-strand is missing. As a result, a deep hydrophobic groove is clearly visible on the subunit’s surface where the missing strand should be. Because of the missing secondary structure, pilus subunits are stable only when either bound to the chaperone or to an adjacent subunit within the nascent pilus. The chaperone provides in trans the missing secondary structure by inserting one of its own strands, strand G1, into the subunit’s groove in a process called donor-strand complementation (DSC; figure 2a). Periplasmic chaperones (approx. 25 kDa in mass) consist of two Ig-like domains forming a boomerang-like structure and it is the G strand of the N-terminal domain (NTD; or domain 1, hence G1) that provides the missing secondary structure [32,33]. By inserting into the subunit’s groove, the chaperone’s G1 strand reconstitutes the incomplete Ig-fold of the subunit, but, because it runs parallel to strand F, it does so atypically: in a regular Ig fold, strand G would run antiparallel to strand F. Strand G1 contains a motif of four alternating hydrophobic residues (termed P1–P4 residues), which, in chaperone–subunit complexes, interact with four sites/pockets (termed P1–P4 sites/pockets) in the subunit’s hydrophobic groove. All subunits’ hydrophobic grooves, except the pilus biogenesis terminator PapH [26], have an additional P5 site/pocket which is either never occupied by the chaperone’s strand or only partially occupied, depending on the length of the chaperone’s strand. This empty P5 pocket is crucial in pilus subunit polymerization, as will be detailed below (figure 2b).

(b) Donor-strand exchange: ‘zip-in–zip-out’ mechanism
The polymerization of pilus subunits at the usher occurs via a mechanism termed ‘donor-strand exchange’ (DSE) [34–36] (figure 2c). All CU pilus subunits contain a 10–20 residue-long N-terminal extension (Nte) peptide that is disordered in the chaperone–subunit complex and is not part of the subunit fold. Subunit polymerization occurs when the G1 β-strand of the chaperone complementing the subunit’s groove (termed ‘previously-assembled’ or ‘receiving’ or ‘acceptor’ subunit) is replaced by the Nte of the subunit next in assembly (termed ‘donating’ and ‘incoming’ subunit). As described before, the P1–P4 pockets in the acceptor subunit’s groove are occupied by the P1–P4 residues of the chaperone’s G1 strand in the chaperone–subunit complex (figure 2b), but after DSE, it is the P2–P5 pockets of the acceptor subunit’s groove that are occupied by the hydrophobic residues (termed P2–P5 residues) of the incoming subunit’s Nte. Residue P4 of the Nte of the acceptor pilus subunit is a Gly and is conserved among all pilus subunits. The P4 pocket in the subunit’s groove contains a bulk formed by an aromatic residue, and the small residue Glycine is the only residue able to adapt to this bulk: this ensures the proper registering of the Nte within the acceptor subunit’s groove. How is the G1 strand of the chaperone displaced by the Nte of the incoming subunit? The first step in the DSE reaction is the insertion of the P5 residue of the incoming Nte into the empty P5 pocket of the previously assembled or acceptor subunit, while the P1–P4 pockets remain occupied by the G1 strand of the chaperone [36,37]. Thus, the P5 pocket is the primary site of interaction for the incoming Nte. The identification (using non-denaturing mass spectrometry) of a transient ternary complex between the chaperone–subunit complex and the Nte of the incoming subunit during in vitro DSE reactions was crucial to the discovery of this mechanism [36]. Single-site mutagenesis of the P5, P4 and P3 residues to Ala in the Nte revealed a gradient of decreasing DSE efficiency moving away from the P5 initiation site, suggesting a ‘zip-in–zip-out’ mechanism, with the incoming Nte gradually displacing the chaperone G1 donor strand in a stepwise process from P5 to P1 [36]. Molecular dynamics simulations provided the first evidence for a zipper mechanism [38]. In the simulations, the chaperone donor strand was seen to unbind from the pilus subunit, residue by residue, in support of the ‘zip-in–zip-out’ hypothesis. Because the insertion and the subsequent zip-in of the incoming Nte occur in parallel to the acceptor subunit’s strand F, the resulting Nte-complemented Ig-fold of the acceptor subunit is now ‘canonical’. The topological transition occurring upon DSE (from non-canonical before DSE to canonical after DSE) is linked to conformational changes that result in the closing of the groove around the incoming Nte, creating a groove–Nte interaction that is one of the strongest ever documented [39]. Such a topological transition is thought to provide the energy driving the DSE reaction in one direction, that of pilus assembly.

(c) Termination of pilus biogenesis
Verger et al. [26] showed that PapH was the terminator subunit regulating the pilus length. The authors ruled out as explanation a stronger PapD–PapH interaction preventing a displacement of the G1 strand of the chaperone via ‘zip-in–zip-out’ mechanism by a further subunit. Instead, the structure of the PapD–PapH complex revealed the absence of a P5 pocket that a previous study identified as the initiator site for DSE [36]. PapH functions not only as a terminator of pilus biogenesis, but also it anchors the pilus to the OM [25]. This is because the usher barrel can only accommodate the passage of subunits and not chaperone–subunit complexes (see §5e). No homologue of PapH has yet been found for the type I pilus system, and thus the mechanism for controlling type I pilus length is unclear.

(d) Subunit ordering
Immuno-electronmicroscopy (EM) studies resolved the order by which the P pilus subunits are arranged, the PapG adhesin being at the tip followed by PapF, PapE and PapK [40,41]. These four subunits form the flexible tip fibrillum [42], followed by the helical rod formed by a multimer of PapA subunits. PapF is required for the correct linkage of the adhesin at the distal end of the tip fibrillum, and PapK regulates the length of the tip fibrillum and joins it to the pilus rod [42]. Only PapE, present in several copies in the tip fibrillum, and PapA, present in more than 1000 copies in the rod, were shown to have self-associating properties in the periplasm [43]. PapF is an adaptor subunit essential in the display of PapG at the tip of

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the fibrillum, and PapK has a dual role as a terminator of the tip fibrillum and an initiator of rod assembly [42]. After elucidation of the DSC and DSE processes, awareness of the essential role of the Ntes in them led to a study of their role in the specific order of assembly. Deletion of the entire Nte of the adaptor subunit PapF impeded the incorporation of PapG into the pilus, while swapping of the Ntes of PapE and PapF led to PapF being unable to play its adaptor role [44]. These results suggest both that the N-terminal extension of PapE does not fit into the hydrophobic groove of the PapG pilin body and that the pilin body of PapF is not required in interactions between PapF and PapG [44]. Similar Nte swapping experiments were performed with PapE and PapK, where the Nte of PapF was fused to PapE (NtePapE and the Nte of PapE to PapK (NtePapK): the sole presence of the Nte allowed an interaction of NtePapE with PapG and the incoming PapK was still able to undergo DSE, being able to assemble a pilus in a PapF deletion mutant (PapFΔ). In the same fashion, NtePapK was able to complement the groove of PapF and pilus assembly was observed in a PapE− strain [42,44]. Another study examining the specificity of DSE in the Psp system confirmed the Nte–groove interaction as a determining factor in subunit ordering [45]. In an in vitro DSE assay, all six chaperone–subunit complexes were incubated individually in the presence of the Ntes of each of the five Pap subunits (except PapG, the adhesin located at the distal end of the pilus which acts only as an Nte acceptor). The dissociation rate of the chaperone–subunit complex owing to DSE was followed for each of the 30 combinations by electrospray mass spectrometry. The fastest DSE rate occurred uniformly with the cognate partners, suggesting a complementarity of Ntes and cognate hydrophobic grooves. The same study [45] and a subsequent one [46] ascribe a decisive role in subunit ordering to the P5 site and immediately adjacent residues.

Apart from interaction specificities between subunits, the usher also plays an important role in subunit ordering as will be discussed in §5e.

(c) The usher assembly platform: structure and function

OM ushers are approximately 800-residue proteins comprising four functional domains: an N-terminal, approximately 125-residue periplasmic domain; a C-terminal, approximately 170-residue periplasmic domain and a large, central, approximately 500-residue translocation pore domain that is interrupted by a conserved plug domain of approximately 110 residues [30,47,48] (figure 46). The first structure of a membrane-integral usher part to be solved was the full translocation pore domain of PapC [49], a 55 kDa fragment comprising residues 130–640 corresponding to the predicted OM translocation domain and the middle or plug domain [48,49]. The structure of PapC130–640 has a kidney shaped, 24-stranded β-barrel (residues 146–635), 45 Å in height and with outer and inner dimensions of 65 x 45 Å and 45 x 25 Å, respectively (figure 3).

The β-barrel closes in an end-to-end fashion, positioning the N- and C-termini on the periplasmic side of the membrane. The N- and C-terminal globular domains are thus juxtaposed and reside in the periplasm, consistent with their role in chaperone–subunit domain recruitment and adhesin-induced pore activation [30,47,49–52]. The plug domain (residues 257–332) is positioned laterally inside the β-barrel pore, occluding the luminal volume of the translocation pore, preventing passage of solutes or periplasmic proteins across the channel in its non-activated or apo form (figure 3). Very recently, the structure of full-length FimD usher was solved in complex with its cognate FimC–FimH substrate [50] (figure 4a). Like PapC, FimD contains a 24-stranded β-barrel (residues 139–665) translocation pore domain. However, in contrast to the PapC structure in its non-activated state, the plug domain (residues 241–324) in the FimD–FimC–FimH complex now resides in the periplasm, underneath the translocation domain and next to the NTD, and the shape of the translocation domain has dramatically changed. Compared with the apo-PapC or apo-FimD pore domain structure, the 24-strand β-barrel rearranges from an oval-shaped pore with a 52 x 28 Å diameter to a near circular pore 32 Å in diameter (figure 3). With the plug domain displaced into the periplasm, the open and circular 32 Å channel is occupied instead by the FimH lectin domain, providing the first view of a transporter caught in the act of substrate transport.

Previous work has established that the FimC–FimH complex displays the highest affinity for FimD [47,52] and is the only complex capable of inducing the conformational changes in FimD required for usher activation [53]. The FimC chaperone alone was unable to bind to FimD, FimC–FimA, FimC–FimG and FimC–FimF bound to the FimD usher with KD values of 176 nM (FimC–FimA), 670 nM (FimC–FimG) and 1.37 μM (FimC–FimF), 20- to 150-fold higher than FimC–FimH (KD 9.1 nM). The relatively high association constant of the FimC–FimH complex is probably critical for it to associate first to the usher ensuring the localization of FimH at the tip of the pilus, and the FimH-induced conformational change constitutes a crucial activation step that is required for subunit polymerization and translocation at the usher assembly platform [53,54]. The differences in KD between the other subunits might not affect their order of incorporation into the pilus, as these differences are relatively small. More important is the specificity of interaction between the receiving subunits' hydrophobic grooves and the incoming subunits' Ntes.

The structure of FimD in the FimD–FimC–FimH complex reveals four periplasmic domains: the NTD, the plug and two C-terminal domains (CTDs), CTD1 and CTD2. Which roles do these periplasmic domains play? Earlier co-expression studies of FimD with FimC–FimH and subsequent trypsin digestion and pull-down assays isolated a C-terminal region of FimD bound to FimC–FimH [52]. The authors concluded that the CTDs are the probable site of interaction. Later studies, however, showed that the
usher’s NTD is also a binding site for chaperone–subunit complexes, including the chaperone–adhesin complex FimC–FimH \([47,51,55]\). The NTD of FimD on its own displayed the highest affinity towards the FimC–FimH complex \([47]\) and showed that residues 1–24 of the NTD specifically interact with...
both FimC and the subunit [55], FimH’s lectin domain being essential in the activation of the usher [53]. In the FimD–FimC–FimH complex structure, however, the NTD lies idle, making no interactions with FimC; the FimC–FimH complex instead is bound to two Ig-like domains formed at the usher C terminus, CTD1 and CTD2 (residues 666–750 and 751–834, respectively) [50]. The most extensive interaction with the FimC–FimH complex is with the usher’s CTD1 (figure 4a), which contacts the FimH lectin domain and FimC. CTD2 contacts primarily FimC. Removal of both CTDs, or CTD2 alone or point mutations in CTD1 abrogate pilus biogenesis [50,56]. Using electron paramagnetic resonance (EPR) spectroscopy, it was also demonstrated that subsequent subunits localize to the CTDs’ binding sites after undergoing DSE.

As there was now clear evidence that the usher’s NTD and the CTDs are both bona fide chaperone–subunit binding sites, the question arises whether these sites work in a parallel and coordinated or in a sequential manner. As the structure of the NTD of FimD (FimD<sub>N</sub>) bound to the FimC–FimF complex was available [57], both the structures of FimD<sub>N</sub>–FimC–FimF and FimD–FimC–FimH were superimposed (using FimD<sub>N</sub>) to investigate whether, in the FimD–FimC–FimH complex, both the NTD and the CTD binding sites would be able to accommodate two chaperone–subunit complexes at the same time, with the knowledge that the last incorporated chaperone–subunit complex remains bound to the usher CTDs [51,52]. This superimposition (figure 4c) demonstrated that, in fact, the NTD in the FimD–FimC–FimH complex is available for the recruitment of a chaperone–subunit complex without steric clashes with the FimC–FimH complex bound at the CTDs. Inactivation of the NTD by a bulky molecule severely disrupted further subunit incorporation in vitro, confirming the NTD of the usher as the recruitment site operating first in the pilus subunit incorporation cycle [47,51,55]. In all, the FimD–FimC–FimH structure together with previous structural and biochemical evidence on the function of the NTD of FimD allowed a pilus subunit incorporation cycle to be proposed where the usher is fully functional for pilus biogenesis as a monomer [50] (figure 5): the chaperone–subunit complex at the base of the growing pilus fibre resides at the usher’s CTDs; new subunits are recruited to the NTD and brought into ideal orientation to undergo DSE with the subunit bound at the CTDs (now the penultimate subunit, figure 5); upon DSE, the chaperone is displaced from the penultimate subunit and dissociates from the CTDs; to reset the assembly machinery for a new incorporation cycle, the chaperone–subunit complex dissociates from the NTD site and transfers to the CTDs’ site, concomitantly pushing the penultimate subunit into the translocation channel. According to this model, a rotational translation of the incoming chaperone–subunit complex must probably take place. However, how this ‘hand-over’ of the chaperone–subunit complex from the usher’s NTD to the CTDs occurs remains elusive.

6. HYBRID CHAPERONE–USHER PATHWAYS

So far, the only hybrid CU pathway reasonably well characterized is the CupB pathway in P. aeruginosa PAO1 [22,23,58,59]. The cupB gene cluster codes for six proteins: a pilin subunit (CupB1), an usher (CupB3), two chaperones (CupB2 and CupB4), an adhesin (CupB6) and, remarkably, a protein (CupB5) sharing 44 per cent similarity with the TpsA4 protein identified in the PAO1 genome. TpsA proteins are adhesins normally transported to the cell surface by a cognate TpsB OM pore protein in what is called the type Vb or TPS system. However, no gene encoding a TpsB-like protein was found within the cupB operon and CupB5 can therefore be classified as an orphan TpsA-like protein within a CU cluster. In

Figure 5. Schematic of the proposed chaperone–subunit incorporation cycle. Initial targeting of the periplasmic chaperone–subunit complex (FimC–FimG) to the NTD of the outer membrane usher (FimD), followed by DSE between the previously assembled subunit (FimH) and the incoming subunit FimG via a ‘zip-in–zip-out’ mechanism, which releases the chaperone FimC from FimH. Subsequently, the incoming FimG–FimC’ complex is handed over from the NTD to the CTDs, and the nascent pilus is secreted.
silico analyses comparing CupB5 with other TpsA proteins showed sequences at the N-terminus that are characteristic of TpsA-like molecules and are known to interact with specific sequences in TpsB-like transporters called polypeptide transport-associated (POTRA) domains. The usher CupB3 transports and assembles CupB1, the pilin subunit, to the surface, as would be expected from an usher. However, transport of the TpsA-like adhesin CupB5 to the cell surface was also shown to be CupB3-dependent [58]. Sure enough, a POTRA domain was identified at the N-terminus of CupB3. Interestingly, this POTRA domain seems to coordinate transport of the CupB1 pilus subunit and the non-fimbrial adhesin CupB5. In a truncated version of cupB3 lacking the POTRA domain, CupB1 transport was impeded and CupB5 transport apparently not. However, when in addition to the POTRA-truncated version of cupB3 the cupB5 gene was interrupted, CupB1 transport was restored. This indicated that the POTRA domain is not essential for CupB1 transport, but might be a prerequisite for translocation of CupB5, which otherwise impedes correct CupB1 assembly. Moreover, the whole secretion process of both CupB1 and CupB5 is dependent—either directly or indirectly, this remains to be established—on the chaperone CupB4. So far, only the structure of CupB2 has been resolved and it is most likely the chaperone targeting CupB1 to the usher [60]. In conclusion, the most interesting feature of this system is that the usher is able to transport to the cell surface both fimbrial (CU pathway components) and non-fimbrial adhesins (TpsA-like CupB5). Mechanistic details as to how this is achieved remain to be described.

7. CONCLUSION

There has been much progress in recent years in the understanding of key aspects of secretion through the CU pathway: first, how the subunits are stabilized when secreted to the periplasm (DSC) and targeted to the assembly platform at the OM, the usher; second, how the subunits polymerize (DSE) and in what order; and third, how the usher is activated to a secretion competent form, catalyses DSE and how its various domains are involved in chaperone–subunit complex recruitment, subunit assembly and pilus secretion. Probably the most intriguing aspect to be resolved is how a single usher manages the hand-over of the chaperone–subunit complex from NTD to CTD, which has to involve a major relocation of its periplasmic domains at each assembly step. Thus, acquiring a dynamic view of the usher as it incorporates subunits to the nascent pilus will be a major research challenge for the next few years.

Hybrid secretion systems are fascinating, but their evolutionary significance remains to be elucidated. Do they confer a selective advantage to the bacterium? Do they borrow the best from both the CU and TPS ‘worlds’? Why would an usher be more advantageous as a means of secretion than a TpsB transporter? Why use the same transporter to secrete adhesins that are so different in structure? All these questions will no doubt drive future research in the field.

Unravelling the mechanisms of pilus biogenesis has already helped in efforts to inhibit biofilm formation and bacterial adhesion in uropathogenic E. coli [61]. The recent FimD–FimC–FimH structure provides a whole new cadre of targets for the next generation of pilus biogenesis inhibitors. This structure exemplifies the need for structural and mechanistic studies of bacterial secretion in order to design a new antimicrobial arsenal specifically targeting virulence factors and tame the resurgence of hospital-acquired antibiotic-resistant pathogens.

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