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Pilus biogenesis at the outer membrane of Gram-negative bacterial pathogens

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Pili belong to a broad class of bacterial surface structures that play a key role in infection and pathogenicity. The largest and best characterised pilus biogenesis system — the chaperone–usher pathway — is particularly remarkable in its ability to synthesise and display highly organised structures at the outer membrane without any input from endogenous energy sources. The past few years have heralded exciting new developments in our understanding of the structural biology and mechanism of pilus assembly, which are discussed in this review. Such knowledge will be particularly important in the future, as we approach an era of widespread resistance to common antibiotics and require new targets.

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Introduction

Gram-negative bacteria display a variety of proteinaceous structures on their surfaces. These include flagella, responsible for cell motility; injectosomes, thin needles that inject effector molecules into host cells; curli, involved in adhesion to surfaces and biofilm formation; and pili (from the Latin for hairs), a rather broad category that encompasses a wide variety of different structures and functions [1–5]. In pathogenic bacterial species, pili are often crucial virulence factors, mediating attachment to and infection of target cells, and involved in evasion of the host immune systems or biofilm formation. Because of this, they are considered attractive drug targets, and there is great interest in understanding their structures and the mechanism of assembly, in order to aid the drug discovery process [6,7].

A key challenge to all mechanisms of secretion by Gram-negative bacteria is the presence of two cell membranes, separated by a periplasmic space devoid of classical cellular energy sources such as ATP and the proton motive force. Two general mechanisms exist to overcome these obstacles. The first, as adopted by type IV pili, the (confusingly unrelated) type IV secretion pili, and the type III injectosome, makes use of large assembly machineries that span both membranes (see Figure 1a which provides an illustration of type IV pilus assembly as an example). Such machineries include inner membrane ATPases powering pilus assembly. In this case, pilus subunits often polymerise within the machinery, the growing pilus remaining confined within as it passes through the periplasm and outer membrane. A direct link to the inner membrane in this way is clearly necessary in cases where powered motility or active disassembly are required, or where substrates are transferred through the pili from the cytosol, as in conjugation or injection of effectors through the type III needle; however it is relatively expensive energetically and requires the synthesis of large protein complexes for function. The alternative, as used by the conserved chaperone/usher (CU) pathway and for the biogenesis of curli, appropriates the general secretion machinery of the cell to export subunits to the periplasm, and catalyses their subsequent assembly at the outer membrane with no additional energy input (Figure 1b). Such mechanisms harness the intrinsic folding energy of the proteins to power assembly, and generally require extrinsic factors such as chaperones to prevent premature polymerisation within the periplasm [8].

This review focuses on the latter category: pilus assembly at the outer membrane, and in particular recent advances in our understanding of the CU pathway.

Pili assembled by the CU pathway

The CU pathway is possibly the best-characterised bacterial pilus assembly system, with work in the past two decades on the archetypal P pilus (Pap) and type 1 pilus (Fim) systems of the uropathogenic *Escherichia coli* yielding a relatively detailed understanding of their structure and mechanism [8–10]. CU pili are non-covalent polymers of pilus subunits, approximately 7–8 nm wide and 2 μm long, attached at the base to the bacterial outer membrane. Electron micrographs of pili typically show two subassemblies: a long right-handed helical rod made of about a thousand copies of the major subunit (PapA in the P pilus and FimA in the type 1 pilus) followed by a terminal flexible tip comprising various minor subunits (PapK/PapE/PapF in the P pilus and FimF/FimG in the type 1 pilus) (Figures 1b and 2a,b) [11,12,13•]. The tip

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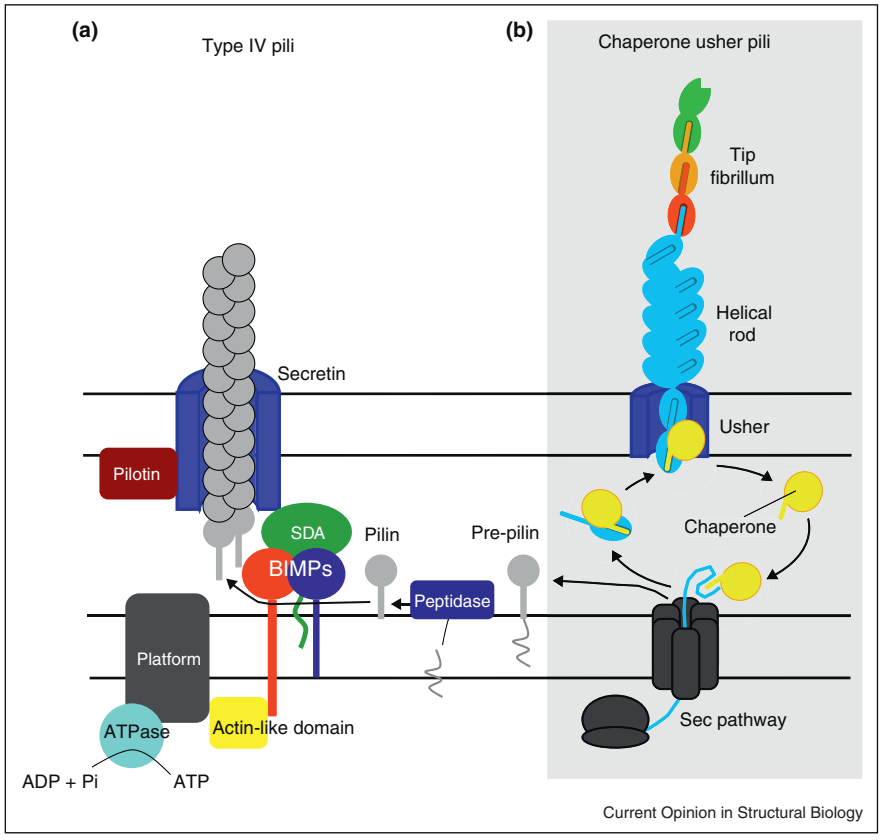
Figure 1



Pilus biogenesis systems assembled at the inner and outer membrane. **(a)** Schematic diagram of the assembly of type IV pili, illustrating ATP-driven assembly taking place at the inner membrane [4]. The type IV pilus secretion system spans both inner and outer membranes. The outer membrane complex consists of the secretin (blue) and the pilotin (brown) proteins, where the secretin forms the pilus exit pore. The inner membrane complex involves the platform protein (dark grey) which interacts with a cytoplasmic protein containing an actin-like domain (yellow), traffic ATPases (cyan) that provide energy for assembly or retraction of the pilus, and one or more BIMPs (bitopic inner membrane proteins, blue and red). The SDA protein (secretin dynamic-associated protein, green) is hypothesised to link the inner and outer membrane complexes. Once inserted into the inner membrane by the Sec machinery, the pre-pilin subunits (light grey) are processed by a specific pre-pilin peptidase (dark blue) before non-covalent polymerisation into a pilus. Although the assembly mechanism is still unclear, dark arrows indicate a hypothetical pathway. **(b)** The chaperone–usher pathway, illustrating self-powered assembly at the outer membrane. Individual pilus subunits are transported to the periplasm by the Sec pathway, where they are stabilised by a dedicated chaperone and targeted to the usher in the outer membrane for assembly. See main text for more details.

carries at its very top the critical receptor-binding subunit, or adhesin, which defines the bacterial tropism: the adhesin PapG (P pilus) recognises the Galα1–4Gal glycolipids of the kidney causing pyelonephritis whereas the adhesin FimH (type 1 pilus) binds to mannosylated receptor on the surface of the bladder epithelium causing cystitis. Recent work has shown that once associated at the tip, the adhesin conformation — and thus its affinity for the cognate sugar — is drastically modified and becomes regulated by the tensile strength applied to the pilus. The variation in the tip composition might be the result of a specific mechanical force sensor property that modulates and regulates the adhesin affinity [14••,15].

Each pilin subunit shares the same fold, an unstructured N-terminal extension (Nte) of 10–20 residues followed by a core domain of six-stranded β-sandwich topology (Figure 2c). In the adhesin, the Nte is replaced by a lectin domain containing the sugar-binding site. The core domain of all subunits exhibits an immunoglobin (Ig)-like fold but lacks the seventh strand of a canonical Ig-fold, resulting in a solvent exposed hydrophobic groove. In cells, each subunit is secreted into the periplasm through the general Sec machinery, where it is stabilised *in trans* by a dedicated periplasmic chaperone (PapD and FimC in P-pili and type 1 pili, respectively) — a 25 kDa protein consisting of two Ig-like domains arranged in an L-shape [8]. The chaperone stabilises the subunit by inserting one of its strands (the G1 strand) into the subunit's hydrophobic groove, an interaction called 'donor-strand complementation' (DSC) [16•,17•]. This prevents non-productive aggregation or proteolytic degradation of the

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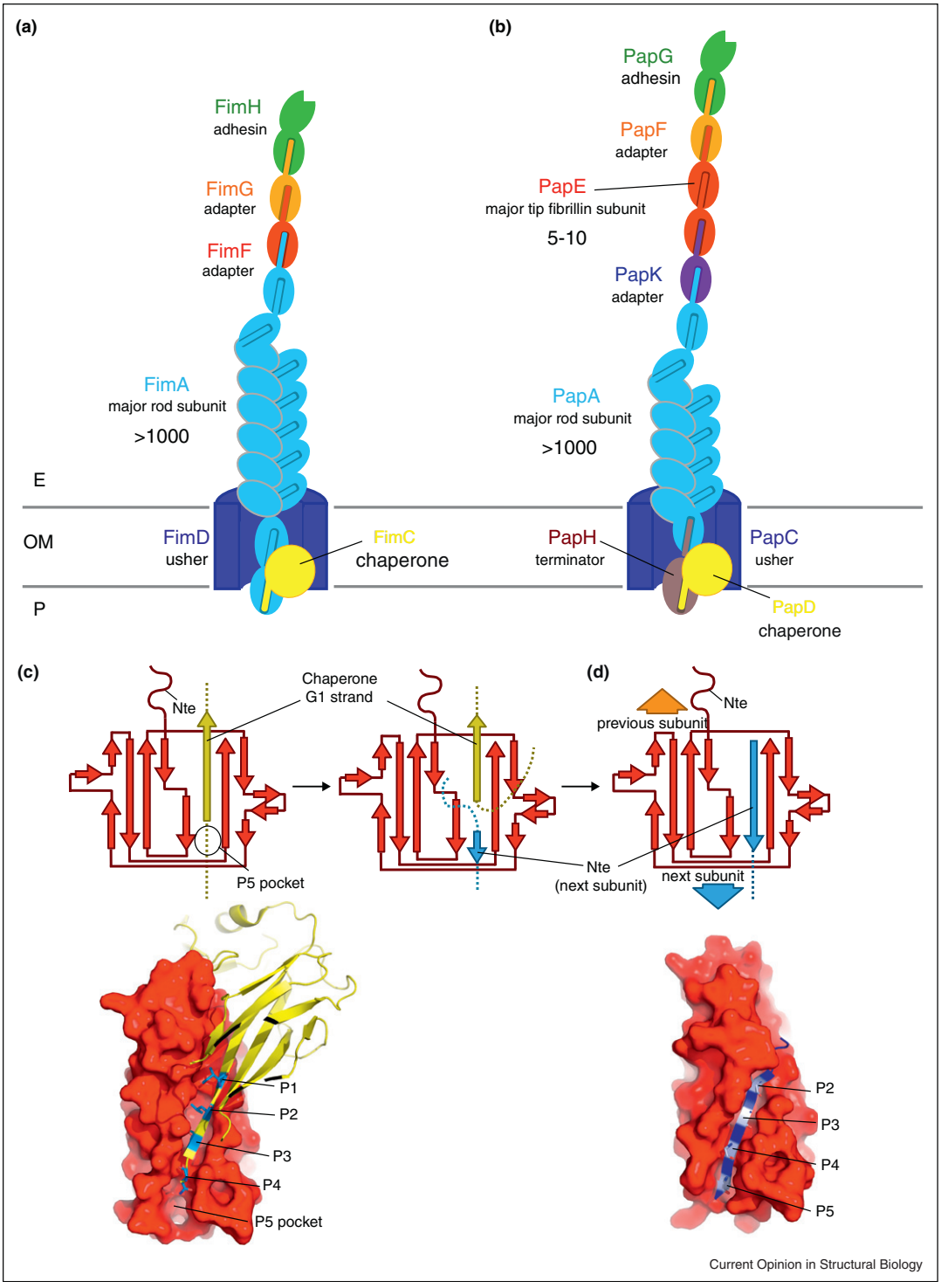
Figure 2



Components and interactions in the CU pathway. **(a)** Schematic diagram of the type 1 pili. **(b)** Schematic diagram of the P pilus. **(c)** Donor-strand complementation is shown both schematically (above) and from a crystal structure (below; PDB code 3JWN). The core domain of each subunit (red) forms an incomplete Ig-fold with the final β-strand missing, leaving a deep hydrophobic groove in the surface. This missing strand is provided *in trans* by the G1 chaperone (yellow), which occupies the P1–P4 pockets but leaves the P5 pocket free. **(d)** Donor-strand exchange, shown as for (c) (structure is from PDB code 3JWN). The Nte of the next subunit (blue) in assembly provides the missing strand to the Ig-fold of the subunit previously assembled, occupying pockets P2–P5. The intermediate structure illustrates the zip-in-zip-out mechanism of DSE starting with the insertion of the P5 residue of the incoming subunit's Nte into the P5 pocket of the receiving subunit's groove, followed by invasion of the receiving subunit's groove and zippering out of the chaperone's G1 strand.

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subunit in the periplasm. The chaperone G1 strand is characterised by a conserved motif of one small hydrophilic (P4) and three alternating bulky hydrophobic residues (P1–P3), which are inserted inside the corresponding P1–P4 subunit groove pockets (Figure 2c).

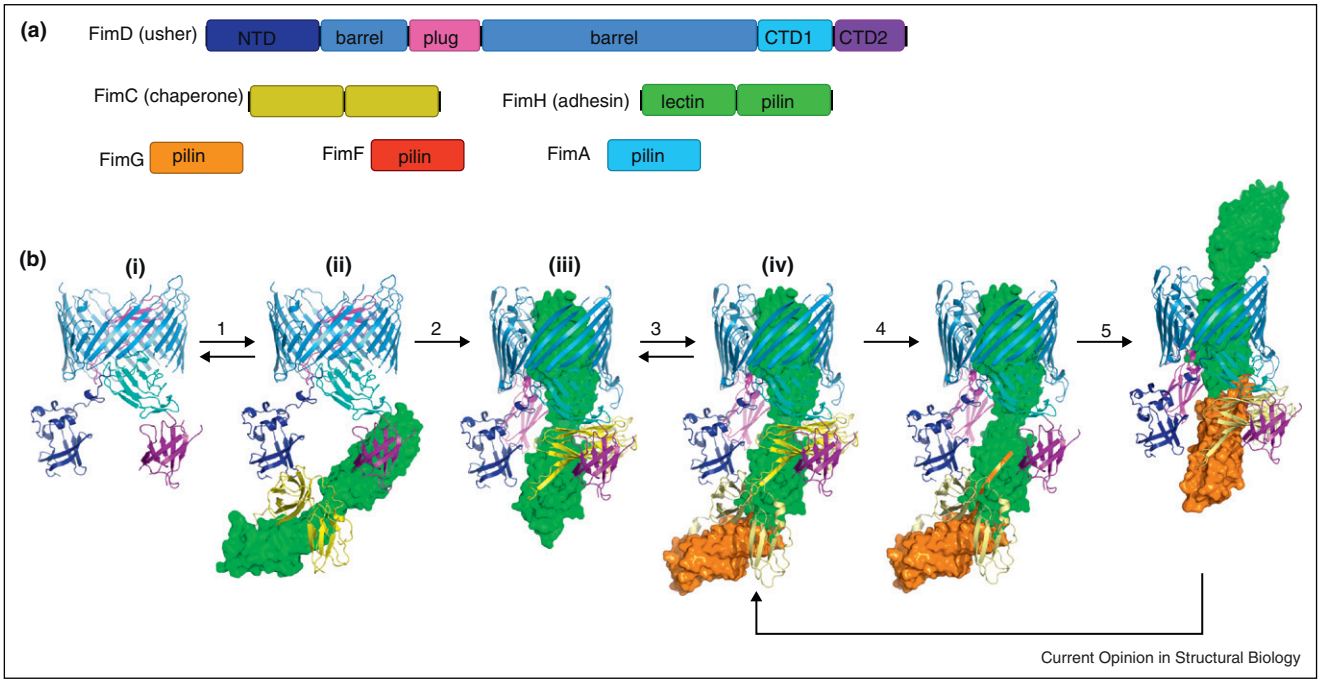
Pilus subunits polymerise using the same interaction groove as DSC. During polymerisation, the Nte of the incoming subunit replaces the chaperone G1 strand by a mechanism termed 'donor-strand exchange' (DSE) [18•,19•]. As in the case of the chaperone G1 strand, the Nte is characterised by a conserved motif of alternating hydrophobic residues, termed P2–P5, interacting with the corresponding subunit pockets (Figure 2d). Importantly, the subunit P5 pocket is not occupied in the chaperone-associated complex, which turns out to be mandatory for the initiation of DSE [20]. As demonstrated by competition experiments, real-time native mass spectrometry and molecular dynamic simulation, the Nte progressively replaces the G1 strand in a concerted manner, through a zip-in-zip-out mechanism, starting with the interaction of the incoming subunit Nte's P5 residue with the receiving subunit groove's P5 pocket, followed by progressive invasion of the receiving subunit's groove by the incoming subunit's Nte and the concomitant zippering out of the chaperone's G1 strand (Figure 2c and d) [21–24].

The usher assembly complex: structure and mechanism

Pilus assembly *in vivo* is catalysed at the outer membrane by the usher, FimD [25••] (Figure 3; the Fim system is the best structurally characterised, and is used here as a model). FimD consists of five distinct domains (Figure 3a): an N-terminal domain (NTD) with a high affinity for chaperone:subunit complexes, thought to be their initial recruitment site [26,27•,28,29]; a β-barrel domain that spans the outer membrane and provides a pore through which the nascent pilus is threaded [30•]; a plug domain that blocks the usher pore when it is not in use, preventing leakage of solutes across the outer membrane [30•,31]; and two C-terminal domains (CTD1 and CTD2), which have recently been shown to form a second binding site for chaperone:subunit complexes [32,33••,34].

Advances in the past few years have provided a model for the entire cycle of pilus biogenesis (Figure 3b) [33••]. In 2008 the first crystal structure of the usher β-barrel domain, blocked by the plug, gave a snapshot of the usher in its inactive state (structure i in Figure 3b)

Figure 3



Structure and mechanism of the CU pathway. **(a)** Structural components and domain organisation of the Fim system, including the usher (FimD), the chaperone (FimC), and the four pilus components (FimH, FimG, FimF and FimA). **(b)** The current model for CU pilus assembly by the FimD usher, shown using known structures and with the same colour coding as in panel a. Images were created using the known structures of the FimD barrel and plug domains (structure i; PDB code 3OHN), the FimD NTD structure with bound FimC:FimH (PDB code 1ZE3), and the complete FimD structure with FimC:FimH bound (structure iii; PDB code 3RFZ). The steps are: (1) chaperone:adhesin binding; (2) initiation; (3) next chaperone:subunit recruitment at the NTD; (4) donor-strand exchange with concomitant release of the CTDs-bound chaperone and (5) transfer to the CTDs. These steps are described in more detail in the main text.

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[30•]. Pili are assembled one subunit at a time and pushed up through the usher pore as they form, so the first subunit to be incorporated must always be the adhesin (FimH) in order to ensure display of the lectin domain at the pilus tip — as required for function. Indeed, electron micrographs of Fim pili reveal FimH at the tip [13•], and *in vitro* usher-catalysed pilus assembly requires the FimC:FimH complex for initiation [25••]. On the basis of high affinity of the NTD for the FimC:FimH complex [27•,35], the first step of pilus biogenesis is likely to be the recruitment of FimC:FimH to the NTD (step 1, structure ii, in Figure 3b). In 2011, a ground-breaking structure of activated FimD in complex with FimC:FimH revealed that FimC:FimH localises to the CTDs, with the FimH lectin domain displacing the plug from the pore and inserting within the barrel's lumen (structure iii in Figure 3b) [33••]. This initiation step (step 2 in Figure 3b) must involve an elaborate choreography of domain movements that remain to be fully characterised. For example, the mechanism of plug displacement is unknown: electrophysiology experiments on the P pilus assembly usher suggest that the plug transiently flips out of the pore even in the absence of substrates [37]; so it is conceivable that the FimH lectin domain (while bound to the usher NTD as shown in structure ii in Figure 3b) inserts spontaneously during these brief time windows. Alternatively, binding of FimC:FimH to the usher NTD might trigger the movement of the plug.

Another open question related to the mechanism of FimD usher activation by the FimC:FimH complex is that, while the FimC:FimH complex is known to be recruited to the usher at the usher NTD, it is observed bound to the usher CTDs in the FimD:FimC:FimH structure. Since both sites (the NTD and the CTDs) are confirmed chaperone:subunit binding sites [27•,33••] and the NTD site is known to operate before the CTDs site in the subunit incorporation cycle [33••], one important question is the mechanism of FimC:FimH complex handover from the NTD to the CTDs [33••]. Such a handover must involve dissociation and rebinding of FimC:FimH, or an as-of-yet unidentified third binding site — potentially on the plug [31,32,36].

The crystal structure of the FimD:FimC:FimH complex also suggested a compelling mechanism for the subsequent subunit polymerisation reaction [33••]. Modelling the structure of the next chaperone:subunit complex (FimC:FimG) onto its binding site on the NTD (structure iv in Figure 3b) shows FimG perfectly situated to undergo DSE, with its Nte located right next to the binding groove of FimH. It is highly likely therefore that the usher's ability to catalyse DSE resides in the precise positioning and orientation of the two substrates: chaperone:subunit complexes are recruited by the NTD (step 3 in Figure 3b), whereupon they spontaneously undergo DSE, releasing the previously bound chaperone (step 4) and allowing transfer of the newly incorporated subunit to the CTDs (step 5). This latter step is analogous to handover of FimC:FimH during initiation and the same questions arise: while electron paramagnetic resonance (EPR) spectroscopy and NTD-blocking experiments confirm that handover does indeed take place [33••], the structure and kinetics of the process still require elucidation.

After FimG incorporation, a single copy of FimF is inserted at the base of the pilus followed by about 1000 copies of FimA (each new subunit incorporation follows steps 3–5 in Figure 3b). As the FimA subunits emerge from the extracellular side of the pore they fold into a right-handed helix of 3.3 subunits per turn, forming the rigid pilus rod and potentially providing a driving force for pilus export. In some CU systems, including Pap, a terminator subunit is incorporated once the pilus reaches its final length, which prevents further polymerisation as it lacks the P5 pocket required to initiate DSE [20]. However, no such subunit has been identified in the Fim system, and it is unclear how the length of type 1 pili is regulated.

Kinetics and energetics of assembly at the outer membrane

One of the remaining questions is how the usher is able to catalyse the specific ordering of pilus subunits. *In vitro* experiments have demonstrated a key role for the Nte in determining the specificity of subunit-subunit association, with different Ntes reacting preferentially with their cognate substrates [38,39•]. More detailed analysis has revealed a particularly crucial role for the P5 pocket and its surrounding residues [40,41], in keeping with its role in initiating DSE [22]. Subunit order is also governed by the rate at which the usher recruits the different chaperone:subunit complexes from the mixed periplasmic pool. This in turn is a product of the differential affinity of the usher NTD for the various subunits and of their relative concentrations within the periplasm [35,42]. However, it has yet to be established whether the usher has a more direct role in subunit ordering, that is, whether it specifically matches subunits according to their position in the final pilus.

Perhaps the most impressive feature of the CU pathway is its ability to direct efficient and well-ordered pilus subunit assembly without apparent external energy input. Isolated chaperone-subunit complexes can spontaneously polymerise into fibres *in vitro*, but at a very slow rate [25••,39•]. A number of structural, thermodynamic and computational studies have led to the conclusion that DSE causes the subunits to undergo conformational transition from a high-energy chaperone-associated intermediate to a tremendously stable, tightened and polymerised state [18•,19•,20,24,43–46]. In this way, the chaperone stores the folding energy of the subunit and uses it to power assembly. Furthermore, once the pilus

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has passed through the usher pore into the extracellular medium it no longer has access to chaperones and is therefore prevented from undergoing the reverse reaction. This kinetic trapping gives the fully formed pili an amazing durability despite their reliance on non-covalent interactions for integrity [8,43].

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