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Review

Reprint of "Biogenesis and adhesion of type 1 and P pili"☆,☆☆

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ABSTRACT

Background: Uropathogenic *Escherichia coli* (UPEC) cause urinary tract infections (UTIs) in approximately 50% of women. These bacteria use type 1 and P pili for host recognition and attachment. These pili are assembled by the chaperone-usher pathway of pilus biogenesis.
Scope of review: The review examines the biogenesis and adhesion of the UPEC type 1 and P pili. Particular emphasis is drawn to the role of the outer membrane usher protein. The structural properties of the complete pilus are also examined to highlight the strength and functionality of the final assembly.
Major conclusions: The usher orchestrates the sequential addition of pilus subunits in a defined order. This process follows a subunit-incorporation cycle which consists of four steps: recruitment at the usher N-terminal domain, donor-strand exchange with the previously assembled subunit, transfer to the usher C-terminal domains and translocation of the nascent pilus.
Adhesion by the type 1 and P pili is strengthened by the quaternary structure of their rod sections. The rod is endowed with spring-like properties which provide mechanical resistance against urine flow. The distal adhesins operate differently from one another, targeting receptors in a specific manner.
The biogenesis and adhesion of type 1 and P pili are being therapeutically targeted, and efforts to prevent pilus growth or adherence are described.
General significance: The combination of structural and biochemical study has led to the detailed mechanistic understanding of this membrane spanning nano-machine. This can now be exploited to design novel drugs able to inhibit virulence. This is vital in the present era of resurgent antibiotic resistance. This article is part of a Special Issue entitled Structural biochemistry and biophysics of membrane proteins.

1. Introduction: type 1 and P pili

Urinary tract infections (UTIs) affect an estimated 40–50% of women, and the primary causative agent of these is Uropathogenic *Escherichia coli* (UPEC) [1,2]. Key components of UPEC's ability to mediate urinary tract colonisation and infection are its type 1 and P pili [3–5]. These are adhesive appendages protruding from the bacteria which provide an effective anchoring mechanism, allowing UPEC to adhere to the specific receptors which they target. The role of type 1 and P pili in UPEC pathogenicity makes them a useful therapeutic target for a post-antibiotic era [1,6].

Type 1 and P pili (previously reviewed in [7–11]) are archetypal examples of chaperone-usher pili, offering a wealth of information into pathogenic bacterial adhesion, as well as into the successful manipulation *in vitro* of membrane protein machinery. The categorisation of type 1 and P pili as chaperone-usher pili reflects a relative simplicity in their construction — pili subunits are processed and assembled entirely by two proteins, a periplasmic chaperone and an outer membrane usher protein.

2. The pilus structure and its components

2.1. Overall architecture

The architecture of the type 1 and P pilus is shown in Fig. 1. The type 1 pilus is composed of subunits, sometimes termed 'pilins', encoded in the *fim* operon; the equivalent for the P pilus is the *pap* operon [12]. Each pilus protrudes 1–2 μm into the external milieu, and is composed of a tip section, and a rod. The pilus tip is the contact point with the host, because it contains at its distal end an adhesin protein (FimH/PapG), containing an N-terminal lectin domain (denoted $FimH_L$/$PapG_L$) and a C-terminal pilin domain (denoted $FimH_P$/$PapG_P$; see details below) [13,14]. The adhesin subunit is connected via linker

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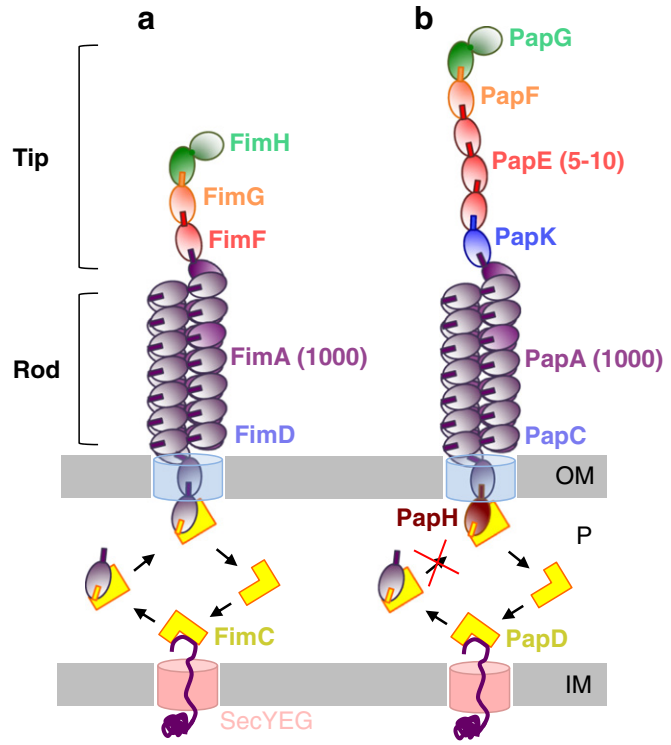

Fig. 1. Arrangement of subunits in type 1 and P pili. a) Type 1 pilus and b) P pilus global structure, showing subunits being located to the periplasm (P) via the Sec general secretory pathway across the inner membrane (IM), where they are folded and transported to the usher with the aid of a chaperone. Here, they undergo donor-strand exchange (DSE) before translocation beyond the outer membrane (OM) to form a pilus with a well regulated ordering of subunits, which may be divided into a 'tip' and a helically wound 'rod'.

subunits in a strictly defined order to the pilus rod: for the P pilus tip, one copy of PapG is followed by one copy of PapF, 5–10 copies of PapE, and one copy of PapK; for the type 1 pilus tip, one each of the FimG and FimF subunits connect one copy of FimH to the first rod subunit. In contrast to the limited numbers of distinct tip subunits, the rod is composed of circa a thousand copies of the same protein — FimA/PapA. The rod subunits wind in a super-helical spring-like quaternary structure, with 3.3 subunits per stack and a diameter of 7–8 nm, forming the bulk of the pilus length [15,16].

The pilus protrudes though the bacterial outer membrane by way of the usher, FimD/PapC, which spans the membrane and in the case of the P pilus is capped at the base by a termination subunit, PapH [17]. The type 1 counterpart for this has been suggested as FimI, although this has not been confirmed [18,19]. Addition of the termination subunit signals the end of growth, and is just one example of the tight regulation of chaperone-usher pilus biogenesis, ensuring a distribution of subunit resources amongst the hundreds of pili which surround the bacteria, to form an effective adhesive assembly.

2.2. The individual subunits in the periplasm

In order to reach its destination as part of a pilus, each unfolded pilin is first translocated through the inner membrane and into the periplasm by the general secretory pathway [20]. In the periplasm the disulphide bonds of the subunits are formed by the disulphide oxidoreductase DsbA. A two-domain 'L' shaped chaperone, FimC/PapD, then collects the subunit, binding mainly *via* its N-terminal domain, rejecting those disulphide misfolds for peptidic recycling [13,21]. The complete subunit folding processes have been examined in the type 1 rod subunit, FimA [21]. Upon binding, the chaperone directs the folding of the disulphide bonded subunit, accelerating it by at least four orders of magnitude when compared to unassisted folding. *In vivo*, the whole process of oxidation by DsbA, chaperone binding and folding of FimA is predicted to have a half-life of 2.1 s [21].

Each subunit comprises an incomplete immunoglobulin (Ig-) like domain, an unstable 6 β-strand structure with an exposed hydrophobic groove resulting from the lack of a 7th β-strand. This groove contains hydrophobic pockets, "P1 to P5 pockets", which prove important to the subunits' binding. In addition to the Ig-like domain, each subunit has a flexible N-terminal extension (Nte) peptide of 10–20 residues. Once folded, the chaperone interacts with each subunit in a way which stabilises the incomplete Ig-like fold; it inserts its G1 β-strand into the subunit's groove (Fig. 2a) [13,22]. This interaction is termed donor-strand complementation (DSC), and it has enabled the purification and structural determination of numerous subunit-chaperone complexes where the monomeric subunits alone are unstable. A key component of the DSC is the insertion of four alternating hydrophobic residues, termed "P1 to P4" residues, from the G1 β-strand into the complementary subunit P1 to P4 pockets [13,22,23].

2.3. Incorporation of subunits into the pilus

The subunit is next transferred to the periplasmic N-terminal domain of the usher and incorporated into the pilus [24–26]. During the incorporation, the chaperone and its stabilising G1 β-strand are removed, replaced by the next subunit's Nte peptide which is ordered into a β-strand (Fig. 2b). Similar to the chaperone G1 β-strand, the Nte also contains alternating hydrophobic residues, termed "P2 to P5" residues, which become inserted into the P2 to P5 pockets. The mechanism of β-strand replacement is termed donor-strand exchange (DSE), and it provides a stabilising 7th β-strand to the pilus incorporated subunit and hence completes the Ig-like domain [22,27].

The product of donor-strand exchange, subunit bonded to subunit, is thermodynamically favoured over the chaperone–subunit interaction [28]. Indeed, the incoming subunit Nte lies anti-parallel to strand F of the receiving subunit, reconstituting the usual arrangement in Ig domains, whereas the chaperone strand was inserted in the opposite, less stable, arrangement in the chaperone-subunit complex (Fig. 2c) [29]. All subunits are therefore connected tightly to each other in the pilus via this same interaction, irrespective of a subunit's positioning within the tip or rod, or its positioning next to a neighbouring identical subunit (in the manner of FimA-FimA) or a non-identical neighbouring subunit (like FimG-FimF).

3. The role of the usher in pilus generation

The multi-domain usher has both periplasmic and outer membrane spanning regions. Aside from the aforementioned N-terminal domain (NTD), the usher carries two periplasmic domains at its C-terminus (CTD1 and CTD2). The transmembrane 24 strand β-barrel translocation domain contains a plug domain which in the resting state of the usher lies within the lumen of the usher [30,31], thereby sealing the membrane. This plug is transferred to a site proximal to the NTD in the periplasm once pilus formation has begun [32]. With the exception of the CTD1, each of these domains has been purified separately, allowing their structures and role in the translocation of the pilus to be examined [30,33–35]. The complete usher protein may also be co-purified with various subunits, providing transitional snapshots of the pilus in development. It is apparent how many roles the usher plays: it catalyses subunit polymerisation, it regulates the order in which the subunits pass, and it regulates passage at an individual subunit level.

3.1. The usher catalyses donor-strand exchange

3.1.1. Recruitment to NTD and subsequent handover to CTDs

The mechanism for pilus biogenesis is shown in Fig. 3. Formation of the type 1 pilus tip is the example chosen, due to the library of crystal structures available, although results from P pili and other chaperone–

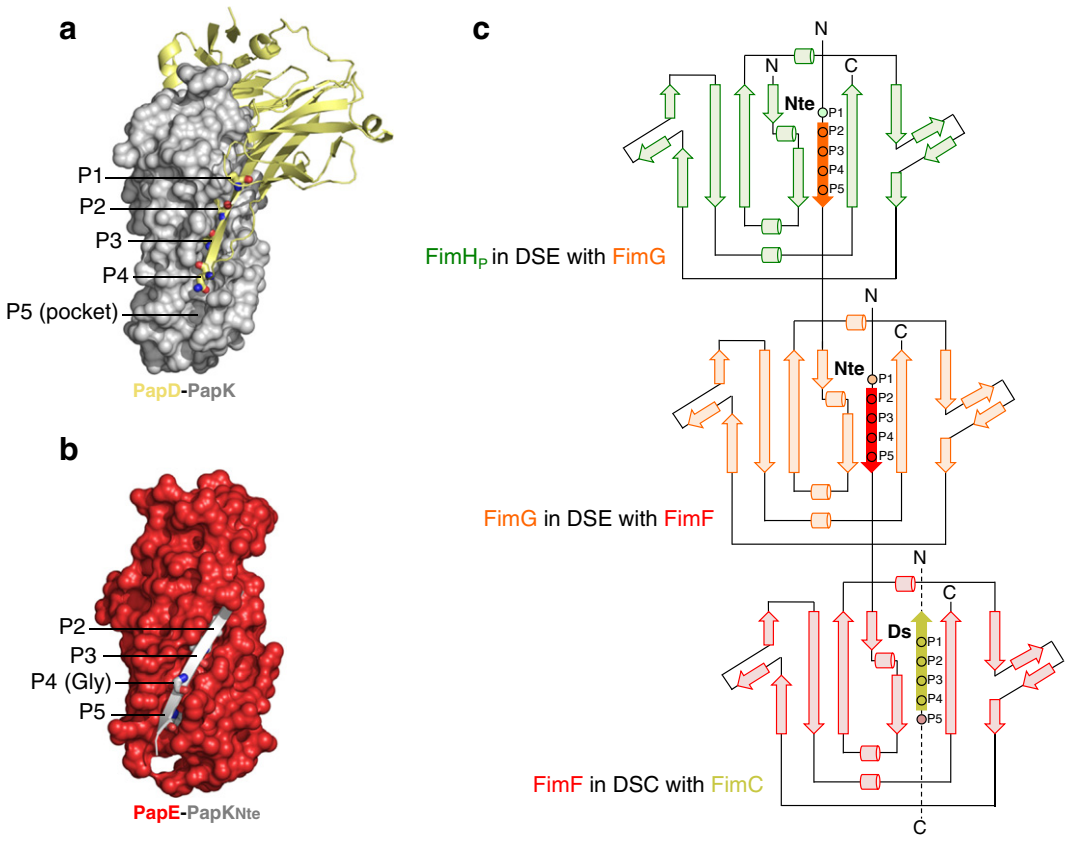

Fig. 2. Stabilisation of subunits by insertion of a β-strand. a) Subunits are C-terminally truncated Ig-folded proteins lacking the 7th strand, strand G. As a result of the missing strand, a large groove is created where the 7th strand should be. In the periplasm, subunits are stabilised by insertion of the chaperone G1 β-strand into the hydrophobic groove of the subunit. This is termed donor-strand complementation (DSC). An important component of this interaction is the insertion of the chaperone P1 to P4 residues into the P1 to P4 pockets of the subunit. The figure shows the insertion of the PapD residues (stick models; PapD displayed as yellow ribbon model), L107 (P1), I105 (P2) L103 (P3) and N101 (P4), into the pockets of PapK (grey surface representation) [22]. This arrangement leaves the PapK P5 pocket vacant. b) In the pilus, the N-terminal extension peptide (Nte) of each subunit fulfils the role of inserted β-strand, stabilising the hydrophobic groove of the preceding subunit. The example shown is the Nte of PapK (grey ribbon) inserting into PapE (red surface model). During donor-strand exchange (DSE) the Nte of the incoming subunit PapK fills the P5 pocket of PapE with its P5 residue, L9, before insertion of G7 (P4), F5 (P3), and V3 (P2) via a zip-in-zip-out mechanism (residues shown as stick models). The conserved G7 (P4) residue fits more successfully into the shallow P4 pocket than does N101 (P4) of PapD [27]. c) Topology diagram of a partially completed type 1 pilus as observed in the FimD-FimC-FimF-FimG-FimH crystal structure (Fig. 3 after step 8). The Ig-like folds of the pilin domain of FimH ($FimH_P$, green), FimG (orange) and FimF (red) can be seen. There is a key difference between orientation of the β-strand inserted during DSC and DSE; In DSC, the chaperone inserts its G1 β-strand parallel to the 6th stand (strand F) of the subunit. However, during DSE, the Nte is inserted in a more stable, anti-parallel, arrangement. This is key to the mechanism of pilus biogenesis. Note that in the DSC complemented FimF the P5 is vacant (red circle), whilst it is occupied after DSE in FimG and $FimH_P$.

usher systems are drawn upon. Subunits are sequentially added to a nascent pilus one by one, each going through a subunit incorporation cycle itself composed of defined sequential steps: i — recruitment at the usher NTD, ii — donor-strand exchange, iii — transfer to the usher CTDs accompanied by translocation through the usher barrel.

In the first step of pilus biogenesis, the chaperone-adhesin complex is recruited to the usher NTD (FimC-FimH is recruited to $FimD_{NTD}$ in Fig. 3, step 1) [24–26]. The interaction at the NTD can be observed through a ternary $FimD_{NTD}$-FimC-$FimH_P$ crystal structure, which shows a dominant $FimD_{NTD}$-FimC interaction surface [34]. The chaperone-adhesin affinity is higher than for recruitment of any of the other subunits however, reflecting an active role of both adhesin domains in the binding to the NTD [36–38]. Despite this affinity for the NTD, following recruitment, the chaperone-adhesin complex is handed over to the usher CTDs (Fig. 3 step 2). This binding mode at the CTDs was observed in the structure of FimD-FimC-FimH [32].

Exactly how FimC-FimH is transferred from the usher NTD to CTDs is unknown. Furthermore, the timing of displacement of the usher plug domain by $FimH_L$ in the FimD translocation pore remains to be observed. The isolated CTD2 is sufficient to displace a chaperone-subunit from the NTD, in studies carried out on the P pilus usher [33,36], and furthermore, the chaperone–subunit binding sites in both NTD and CTDs do overlap [32]. The glimpse of an observed quaternary complex between NTD-chaperone-subunit-CTD2 may offer a snapshot into an intermediate state, before the CTD fully displaces the NTD [36]. Future examinations of the domain transfer step should be conducted with the full-length usher, to complement studies already completed using individual usher domains [33,36]. This is key to identifying the mechanism of domain transfer. Use of a full-length usher may highlight the role of the pore in domain transfer, and show the timing of the domain movements relative to subunit translocation.

By solving the structure of FimD-FimC-FimH, Phan et al. answer the question of why the transfer of FimC-FimH, from the usher NTD to the CTDs, is a necessary step in the subunit recruitment cycle: such a transfer is required to free the NTD to recruit the next chaperone–subunit in assembly (FimC-FimG in Fig. 3 step 3). When positioned at the NTD, FimC-FimG is optimally positioned to bring about donor-strand exchange with FimC-FimH [32].

3.1.2. The usher domains' positioning enables a zip-in-zip-out DSE mechanism

Once FimC-FimH is positioned at the CTDs and FimC-FimG is at the NTD, they are ready to undergo donor-strand exchange (Fig. 3, step 4) [23,39,40]. In this configuration, the P5 residue of FimG's Nte is indeed positioned just above the P5 pocket of FimH. This positions the Nte peptide for invasion of the FimH's groove, and progressive occupation of its

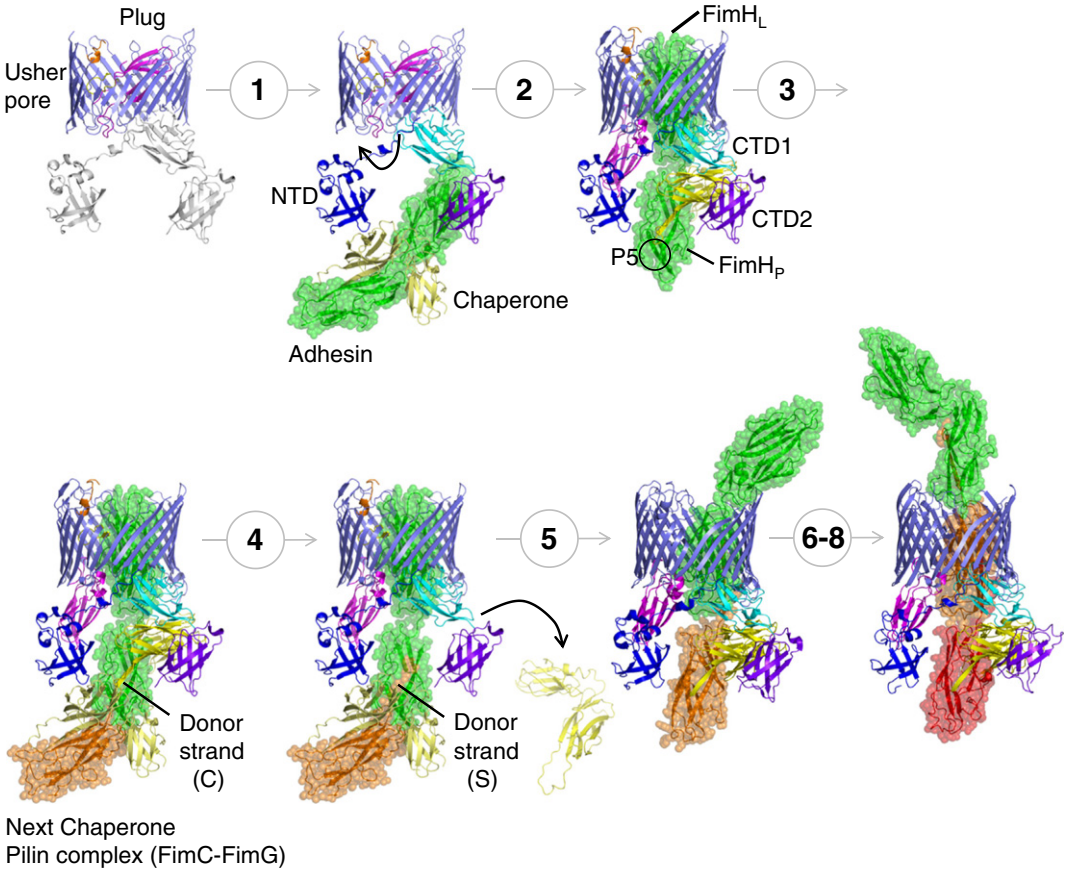

Fig. 3. Mechanism of type 1 pilus growth: Initially, the FimD usher pore (coloured indigo, PDB ID: 3OHN) transverses the bacterial outer membrane, a plug domain (coloured violet) maintains the membrane integrity. The positioning of the other FimD domains, N-terminal domain (NTD), C-terminal domains 1 (CTD1) and 2 (CTD2), coloured in grey, prior to translocation are unknown. Step 1: the chaperone–adhesin complex, FimC-FimH, coloured yellow-green, is recruited to the NTD of FimD (coloured blue). Step 2: by an unknown mechanism, the plug relocates to a position proximal to the NTD, and FimC-FimH relocates to the FimD CTDs (CTD1 in cyan; CTD2 in purple), where the FimH pilin domain, FimH$_P$, interacts with the CTDs, whilst the FimH lectin domain, FimH$_L$, fills the usher pore (PDB ID: 3RFZ). Step 3: the next chaperone-subunit complex, FimC-FimG, coloured yellow-orange, is recruited to the FimD NTD. At this stage, the FimG Nte peptide is positioned towards the FimH hydrophobic groove, which is currently still stabilised by FimC. Step 4: donor-strand exchange occurs, the FimG Nte peptide inserts into the FimH hydrophobic groove, displacing the formally FimH-bound FimC for recycling. Step 5: FimG transfers to the FimD CTDs, and FimH translocates upwards within the pore. Steps 6 to 8: the cycle repeats; FimC-FimF, coloured yellow-red, is recruited to the FimD NTD, undergoes DSE with FimG and transfers to the FimD CTDs, completing type 1 pilus tip assembly (PDB ID: 4J3O), prior to incorporation of many FimA subunits via the same mechanism.

P5 to P2 pockets. The chaperone on FimH is thus effectively displaced from the subunit, dissociates from the CTDs, and is returned to the periplasm to aid another subunit. In this manner, donor-strand exchange occurs rapidly, catalytically triggered by the optimal positioning of all reaction partners.

Once the FimH chaperone is displaced from the CTDs, FimH is translocated, and FimC-FimG is transferred to the CTDs (Fig. 3 step 5). The cycle required for addition of one pilus subunit is now complete, and it repeats for the next subunit; FimC-FimF is recruited at the newly freed NTD, inserts its Nte into FimG, and transfers to the CTDs (Fig. 3 steps 6–8). This completes the type 1 pilus tip complex, FimD-FimC-FimF-FimG-FimH, the crystal structure of which has been determined [41]. At each stage blocking of the NTD by an unbound chaperone is prevented by an allosteric two-proline lock mechanism [42].

The role of the usher in catalysing the donor-strand exchange mechanism can be dissected. For some subunits of the type 1 and P pili, it is possible to study donor-strand exchange without the usher present; a limited number of the chaperone–subunit complexes will spontaneously undergo reasonable time scale donor-strand exchange *in vitro*, and the reaction can also be observed by adding Nte peptides to chaperone–subunit complexes [43]. However, *in vitro* donor-strand exchange in the presence of the usher enables the depletion of a chaperone–subunit complex hundreds of times faster compared to having no usher present [44]. The role of the usher therefore, in the positioning of the subunits to allow their DSE, appears significant.

3.2. The usher regulates the order of subunits

Assembly order is important for the functioning of the pilus; the adhesin (the very tip subunit) must protrude towards its host saccharide targets, and the rod composition must be relatively homogeneous in order that the quaternary structure remains strong. The evidence points to the usher and the groove/Nte interaction playing vital roles in subunit regulation through a combination of affinity competition, combined with different rates of subunit donor-strand exchange, and differences in periplasmic concentrations of subunits [45].

The usher NTD has variable affinity for chaperone–subunit complexes. A simple trend of decreased affinity for consecutive subunits would regulate the order of incorporation. To some extent this is the case. The adhesin FimH/PapG is recruited first as it has the strongest affinity for the usher NTD (see Tables 1 and 2), and is possibly advantaged over other subunits by being the only subunit with a two domain structure [36,46].

For the P pilus, subunits added after the adhesin bind the usher NTD more weakly; indeed, Volkan et al. were unable to detect binding for most subunits, and suggest that the plug, relocated to its new position proximal to the NTD after translocation has started, is required to

Table 1
Affinity (K_D) and donor-strand exchange rates (k_{DSE}) of type 1 pilus subunits. The K_D of binding for various FimC-FimX pairs with FimD NTD was determined via fluorescence titration and taken from ref. [38]. The k_{DSE} for various FimD-FimC-FimX complexes + FimC-FimG/F/A (denoted G, F, or A respectively) were determined by quenched fluorescence assay and taken from ref. [45] at 20 °C, with the exception of FimC-FimA values, which were determined in ref. [44], at 23 °C, with a chromatographic method. Cognate k_{DSE} values are separated from non-cognate values for clarity. Both K_D and cognate k_{DSE} values are colour-coded via a traffic light system: green indicates strong binding or fast rate, amber intermediate, and red indicates weak binding or slow rate. N/D are undetermined values.

FimX	K_D FimC–FimX for FimD NTD (μM)	k_{DSE} FimD–FimC–FimX with cognate FimC–FimG/F/A (min^{-1})	k_{DSE} FimD–FimC–FimX with non-cognate FimC–FimG/F/A (min^{-1})
FimH	0.9	G: 171	F: 58 A: 0.0024
FimG	27	F: 3	G: 0.24 A: 0.0048
FimF	6.6	A: 0.03	G: 0.33 F: 0.36
FimA	29	A: 960	G: N/D F: N/D
		* G/F/A indicates the respective chaperone–subunit pair FimC–FimG/F/A	

bolster the affinity for recruitment at the NTD [33]. A weak correlation between ordering and NTD/plug affinity can be ascertained [33]. However, it is unlikely to be strong enough to be the only determining factor of ordering.

For the type 1 pilus, subunits added after the adhesin also bind the NTD more weakly, although these, in conjunction with a chaperone, have low micromolar affinity for the usher NTD, and stable interactions of chaperone–subunit complexes with the usher NTD alone have been observed [37,38]. The order of affinity to the usher does not match the subunit ordering observed, and the third subunit, FimF, trumps the second's, FimG's, binding affinity. However, the final subunit to be added, the rod subunit FimA, has the weakest affinity of all for the usher [38].

The correlation between affinity for the usher and subunit order therefore appears imperfect. Once bound to the usher NTD however, the rate of the next step in the pilus biogenesis, that of donor-strand exchange, is not equal across subunits, and helps support the observed ordering. This rate dependence may be unrelated to the usher, and rather intrinsic to the subunits themselves. A comprehensive donor-strand exchange rate study of Pap subunits showed that measured rates of non-catalysed donor-strand exchange are nearly always faster for cognate (neighbouring) pairs, than for non-cognate (non-neighbouring) pairs of subunits [43]. This is due to the complementary design of the interacting grooves and Ntes, particularly the P5 residue and surrounding ± 1 residue in the Nte of the NTD-bound subunit, sterically matching the CTDs-bound subunit's P5 pocket [47]. P5 pockets themselves have variable flexibility and openness, leading to differences in rate level. However the cognate pairing rule is rarely broken, which may ensure that subunit ordering remains correct [43,48].

The cognate rate ordering is also a feature of the type 1 pilus donor-strand exchange [45], as measured in a study where pure detergent-solubilised preparations of the wild type usher, together with purified chaperone–subunit complexes were used, thereby reflecting more effectively the *in vivo* situation. The catalysed rate constants show that subunits that are normally observed to be bound together in the pilus react more quickly at the usher than those which are not.

However, a limitation is identified; the variation in rate between cognate and non-cognate reactions is not enough. For example, FimH selects FimG only marginally faster than it does FimF [45]. In addition, FimF binds the usher more tightly than FimG [38], so it has more opportunity to undergo donor-strand exchange with FimH than FimG has. As a result, given identical concentrations of FimG and FimF, we would expect to see a substantial number of pili with a FimH-FimF order, rather than the observed FimH-FimG order, at the pilus tip. Whilst there is some suggestion that a small number of these are observed in *E. coli* strain W3110, a 10-fold excess of FimG concentration over FimF would be required to obtain the FimH-FimG order at the levels seen in pilus tips [16,45]. This implies that periplasmic concentrations of subunits at a given time must play a role. The concentrations of chaperone-subunit complexes within the periplasm, however, remain to be determined.

Whilst the tip subunits are being incorporated, the rod subunit FimA waits in the periplasm, due to its low affinity with the usher, and the slow rate of its donor-strand exchange with any of the tip subunits. However, once the first FimA subunit is taken on, addition of more FimA subunits is a quick task, with the rate of FimA–FimA polymerisation recorded as the fastest of all [44,45].

Similarly in the P pilus, the rod subunit PapA displays low affinity for the usher. Once the growing pilus has reached a length of approximately 1 μm, termination occurs with addition of PapH. This is a subunit that not only is unable to undergo DSE itself, due to the absence of a P5 pocket, but furthermore is unable to transfer from the NTD to the CTDs, as it has no binding affinity for the latter — the only Pap subunit with this feature [17,33]. Thus on two counts, one due directly to the role of the usher, and the other due to the composition of the subunit, polymerisation is terminated. Incorporation of PapH is concentration-dependent, over-expression of its gene leading to shorter pili than wild-type expression, and gene knock-out leading to pili being very long and often detached [17,49]. This suggests that incorporation of PapH is stochastic and that therefore, there is a defined mechanism controlling pilus length other than concentration.

Table 2
Affinity (K_D) and donor-strand exchange rates (k_{DSE}) of P pilus subunits. The K_D of binding for various PapD-PapX pairs with PapC NTD or PapC Plug domains (column "NTD | Plug") was determined via biolayer interferometry and taken from ref. [33]. The pseudo-first order k_{DSE} apparent for reactions between various PapD-PapX complexes and the Nte peptides derived from the various PapF/E/K/A/H subunits were determined by mass spectrometry and taken from ref. [43]. Cognate DSE rate values are separated from non-cognate values for clarity. In reporting k_{DSE} constants, a single letter is used to indicate the Ntes of the subunits used to challenge the PapD–PapX complex, for example 'F' indicates the Nte peptide of PapF. Both K_D and cognate k_{DSE} values are colour-coded via a traffic light system: green indicates strong binding or fast rate, amber intermediate, and red indicates weak binding or slow rate. Note: k_{DSE} values reported in this table are not directly comparable to those in Table 1: Table 1 reports usher-mediated k_{DSE}, whereas k_{DSE} rate constants in this table were obtained in the absence of the usher. N/D are undetermined values, N/A are unavailable values.

PapX	K_D PapD–PapX for PapC NTD | Plug (nM)	k_{DSE} PapD–PapX with cognate Nte (10^{-3} h^{-1})	k_{DSE} PapD–PapX with non-cognate Nte (10^{-3} h^{-1})	
PapG	3.2 | 51	F: 5.5	E: <0.7 A: 0.7	K: <0.7 H: <0.7
PapF	N/D	E: 9.4	F: 2.7 A: 2.8	K: 4.7 H: 2.5
PapE	1190 | 33.8	E: 190	F: 66 A: 40	H: 49
PapE final	1190 | 33.8	K: 211	F: 66 A: 40	H: 49
PapK	No binding | 17.9	A: 54	F: <1.6 K: 1.6	E: <1.6 H: 27
PapA	No binding | 30.2	A: 46	F: 2.9 K: 9.0	E: 3.3
PapA final	No binding | 30.2	H: 23	F: 2.9 K: 9.0	E: 3.3
PapH	No binding | 16.5 (No binding of CTD2)	N/A	N/A	
		*F/E/K/A/H indicates the respective Nte peptide of PapF/E/K/A/H		

3.3. The usher accommodates the passage of pilus subunits

Crystal structures of the usher pore have been determined in three states: prior to translocation (FimD/PapC translocation domain), at the initiation stage when the first subunit FimH inserts into the FimD pore (FimD-FimC-FimH), and during the elongation stage when the tip complex (FimD-FimC-FimF-FimG-FimH) is assembled [30,32,41]. Comparison of these structures shows that the usher is flexible and actively facilitates passage of protein subunits (Fig. 4).

Prior to translocation, in the FimD translocation domain structure, the usher pore is blocked with the plug domain [30,32]. This keeps the conductivity through the pore low, although transient movements make the barrier function imperfectly [50]. The shape of the pore is elliptical at this stage, with dimensions 100 Å^2 smaller than those it increases to upon insertion of a subunit [32]. At the point of the adhesin FimH recruitment and pore insertion, the pore is circularised to accommodate $FimH_L$. In the FimD-FimC-FimH structure, the plug is relocated to the position proximal to the NTD [32].

The circular pore is also evident in the FimD-FimC-FimF-FimG-FimH structure, where FimG – an archetypal (albeit trans-complemented) Ig-domain subunit – lies in the pore. This allows us to compare the translocation of an Ig-folded subunit (FimG) with that of $FimH_L$ which has a lectin fold, as well as suggesting that since all subunits following FimG share its fold, the derived translocation mechanism may apply to all later subunits [41].

Interactions with the pore lumen residues position each translocating subunit at the very centre of the pore. This is because the lumen residues form diametrically opposed binding sites, thus maintaining the subunit's position in the middle [41]. These binding sites are not the same for each subunit that is accommodated by the pore [41].

Upon translocation, each subunit undergoes a translation of 53 Å and a rotation about 110–120° (Fig. 5a). This translation/rotation is facilitated by lumen residues which form a low-energy pathway of exactly the same translational/rotational amplitude, obliging subunits to translate and rotate accordingly. The low energy pathway, shown by simulation of the transport of FimG through the usher (Fig. 5b) guides FimG with minimal energetic expense, and as mentioned above, the same is most likely the case for all later subunits. This is not so, however, for the adhesin; there is no low energy pathway to be seen, and the computationally-derived affinity of $FimH_L$ for the usher pore is much higher than that of FimG. Thus, the formation of the FimD-FimC-FimH initiation complex represents a pause step or a check point before pilus biogenesis can proceed further. What frees $FimH_L$ to translocate within the pore, escaping the grasp of pore lumen residues, remains unclear.

Another question that has remained unanswered is the source of the energy driving the translocation step of subunits within the pore. At least theoretically, Brownian motions within the pore coupled to conformational changes preventing backsliding should be sufficient to extrude the nascent pilus during each subunit-incorporation cycle. Provided that the subunit is free to move within the pore, it would then be able to move up or down freely, but would move up if the part of the pilus emerging from the pore were to undergo conformational changes preventing backsliding. Such conformational changes have been observed: i — FimH undergoes a striking conformational change (Fig. 6a) upon extrusion from the pore which would prevent it from backsliding and ii — the FimA polymer forms a superhelical rod of 3.3 subunits per turn [16] as it extrudes from the pore, also preventing the nascent pilus rod from backsliding.

An important consequence of the low-energy spiralling pathway that pore lumen residues form within the usher is that it obligates subunits to translate/rotate during translocation, thereby facilitating the transfer of subunits from the usher NTD to the usher CTDs. Indeed, the translation/rotation needed to transfer the subunits from NTD to CTDs is of the same amplitude as that imposed on subunits by the low-energy pathway. Thus, residues within the usher lumen have been optimised to facilitate one of the most crucial steps in usher-mediated pilus biogenesis, the handover of the subunits from one chaperone–subunit binding site to another.

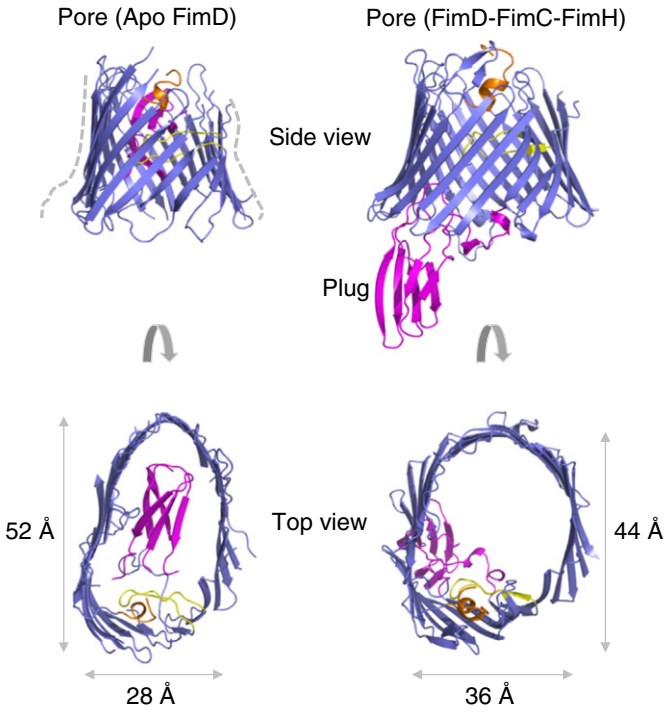

Fig. 4. Flexibility of the FimD usher pore, showing side (above) and top (below) views. The left hand side shows the pore in its resting/closed state prior to activation (PDB ID: 3OHN). The plug domain, the only α-helix of the FimD pore and β5-6 hairpin which are thought to help stabilise the plug in its closed form are shown in magenta, orange and yellow, respectively [30,32]. The right hand side shows the FimD pore whilst it contains the lectin domain $FimH_L$ (PDB ID: 3RFZ). The plug is relocated, and the pore cross-sectional area significantly increased to accommodate the translocating subunit.

3.4. Preventing growth of the pilus with pilicides

UPEC deficient in a type 1 or P pilus is significantly attenuated in its ability to mediate a UTI [3,5,51]. Can the exquisite knowledge of the molecular mechanism of usher-mediated pilus biogenesis be exploited to design ways to inhibit it?

Inhibitors have been designed to target the chaperone–subunit interaction [52] and the chaperone interaction with the usher NTD [53,54]. Inhibition of these interactions is intended to reduce or abolish the growth of pili, and as a result these inhibitors are termed 'pilicides'. Very recently, a new class of pilicides was discovered based on a screening of compounds able to prevent donor-strand exchange [55]. Presumably, these new compounds target the P5 pocket to inhibit the reaction. In practice these compounds will need to be administered to prevent UTI, not to cure an established infection, thereby limiting their use. However, it is well established that UTI are highly recurrent infections and that recurrence originates from the ability UPEC have to form intracellular biofilm-like colonies (IBCs) escaping both the immune system and antibiotic treatment, and lying dormant until they re-awaken to trigger another cycle of infection [56]. A pilicide would be extremely useful as a means of preventing recurrent UTIs.

4. The whole pilus combines to maximise adhesion

4.1. The adhesin and the point of adhesion

Recognition and adhesion of pili to host tissues is mediated by the adhesin subunits. In type 1 and P pili, their respective adhesins,

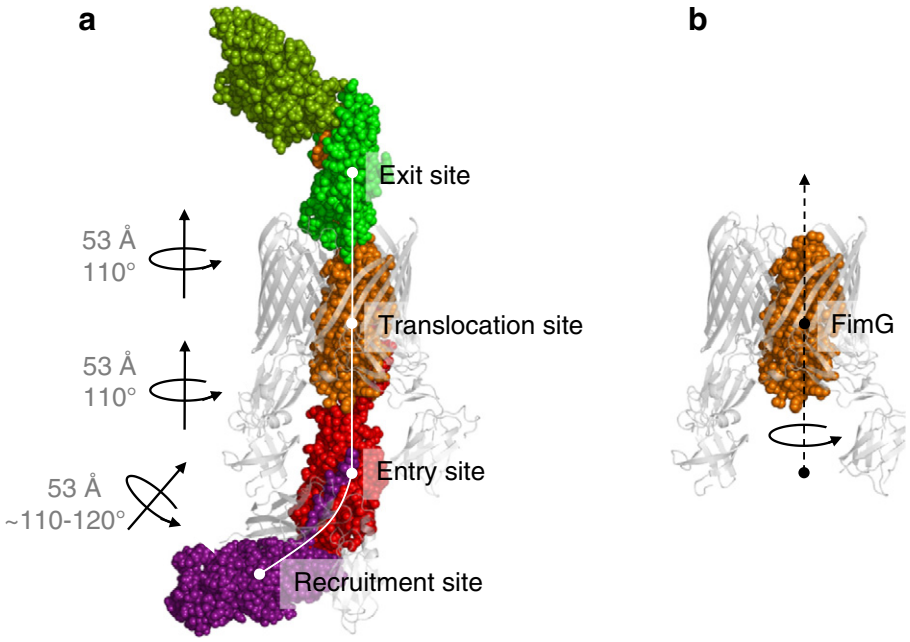

Fig. 5. The helical translocation path of pilus subunits. a) During each translocation step, a subunit is translated by 53 Å and rotated by 110°. b) Simulation of subunit transport (FimG) through the usher pore demonstrated that a low energy pathway, which winds around the inside of the usher pore, guides the helical motion.

FimH and PapG, are two domain proteins, with one pilin domain at the C-terminus used for polymerisation with the next subunit in assembly, and the other domain being a jelly-roll lectin domain specialised in receptor-recognition [57].

Each adhesin targets a receptor abundant in a different part of the host. For the FimH adhesin, these are D-mannosylated receptors, such as the uroplakins of the bladder, and for PapG it is glycosphingolipids containing galabiose, primarily of the kidney epithelium (Fig. 6b and c) [58–60]. The Pap regulator PapB allows simultaneous expression of P pili and repression of type 1 pili, suggesting that there is competition between the pili expression. However, *in vivo* studies have also shown a cooperative effect between these two pili types when colonising the renal tubule [61,62].

The adhesins do appear to be adapted to their particular environment; FimH has a 'catch bond' which allows the adhered bacteria to withstand the high pressure urine flows of the bladder, whereas the P pilus has a 'slip bond', suitable for the more regular flows of the kidneys, which allows for maximum dissemination of bacteria [63–65].

The slip bond between PapG and the galabiose saccharide is a weaker interaction, at a maximum of 80 μM [66]. This is a bond whose lifetime decreases with increased force, and this bond can withstand forces in AFM experiments up to 49 pN [67]. The interaction is weak enough to allow rolling behaviour of bacteria for quick spreading, but strong enough to initiate destacking of the PapA rod superhelical structure. The shallow receptor binding surface of PapG lies on the side of the lectin domain, varying amongst three isoforms; PapG II is primarily the

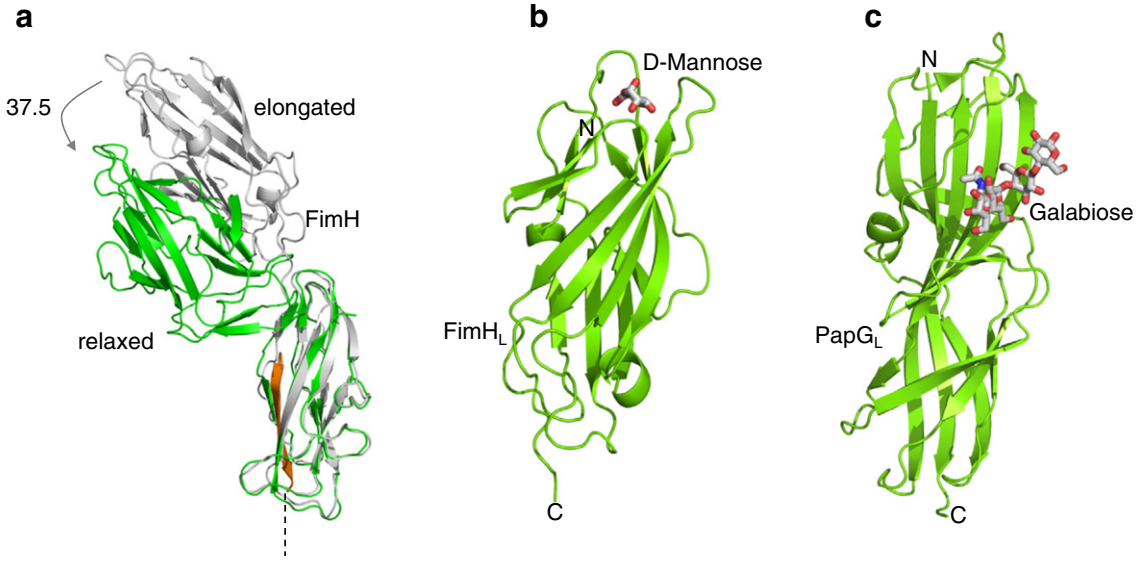

Fig. 6. The binding modes of FimH and PapG adhesins. a) FimH is a two domain adhesin whose domains may be in two orientations, elongated (coloured grey) before secretion when bound to the chaperone FimC (omitted for clarity) and relaxed (coloured in green) after secretion into the extracellular milieu. The elongated orientation accompanies a tightening of the mannose binding pocket, thought to be the physical basis behind the catch bond. b) In the FimH lectin domain, $FimH_L$, the mannose-binding pocket is proximal to the tip. c) In the PapG lectin domain, $PapG_L$, the galabiose target binds to a site distal to the tip. This, and the longer, more flexible tip of the P pilus may cause UPEC to bind via a side on approach.

isoform concerning human pyelonephritis, and binds a globoside moiety [14,68]. This lateral binding has been viewed in receptor bound crystal structures, prompting the suggestion of a "side on" approach of UPEC when utilising this pilus, by employing the long, flexible P pilus tip in a bending motion of which the shorter type 1 pilus would be incapable [14].

The FimH catch bond binding interaction appears more sophisticated, and is certainly stronger than its PapG homologue — the K_D for certain α-D-mannopyranosides reaches 7 nM [69]. A deep negatively charged binding pocket is located at the very tip of the adhesin [13]. A 'weak' or a 'strong' receptor-binding state has been observed for FimH, depending on the level and speed of force exerted upon it; the receptor initially binds weakly, in a state that would be ruptured by a 60 pN force and, similar to a finger trap toy [70], transitions to the strong binding state by a fast sustained pulling force, only rupturing at 140–180 pN of applied force [63]. This transition will help the bacterium weather the shear generated by urine flows during urination, as the host attempts to dislodge the bacterium from its receptors in the bladder [71].

The region connecting the two FimH domains has significant flexibility: indeed, these two domains are seen in two different conformations, "elongated" when FimH is bound to the chaperone (the long axis of the two domains are aligned) or "relaxed" after it is translocated (the long axis of two domains make a 142° angle) [13,41]. An 'elongated' conformation is also seen when FimH has inserted inside the pore, allowing passage through the snug usher channel [32]. It is likely that a stretching force will pull the domains apart into the elongated conformation and that the elongated *versus* relaxed states of the two domains relative to one another (shown in Fig. 6a) would be associated with the 'strong' verses 'weak' (respectively) binding conformations of FimH [70,72]. Moreover, mutation of residues at the domain connecting interface, which reduces flexibility of the two domains, renders the adhesin unable to switch between strong and weak binding [63,73].

A model has been proposed linking the stretching apart of the two FimH domains to the activation of the catch bond [63,74,75]. Here, activation is the culmination of a conformational change starting in the interdomain region, and involving much of the FimH lectin domain. The FimH-mannose bound crystal structure shows the twisting of the β-sandwich fold which enables the mannose binding pocket to decrease in dimension, clamping around the mannose receptor [70]. Further mutation has identified residues of three significant lectin domain regions (β5 helix, an α-helix switch and a pocket zipper), which promote (or prevent) allosteric conformational change into the high affinity state [76].

The purpose of this complicated activation is postulated to be discrimination between free floating mannose moieties which the pilus encounters, which are useless to the bacteria for adherence purposes, and those mannoses which are anchored to the host, and thus allow a strong foothold for the bacteria on the host bladder epithelium. Only the latter can promote catch bond activation, and hence once adhered to the host in a high affinity state, the potential for detachment by passing mannose inhibitors is reduced [77].

UPEC bacteria which express a FimH stuck in the high affinity adhesion state, unable to switch to the low affinity state, are less successful in invasion, highlighting the interesting result that a stronger interaction does not always ensure the survival of the bacterium [78]; it is the flexibility to adapt to the particular situation which is more important.

4.2. The rod structure

Whilst not directly involved in receptor-binding, a significant contribution to the mechanism of bacterial adherence is nevertheless due to the rod, and in particular its quaternary structure. Although not adhesive by itself, the rod is involved in the strength of adherence because it provides spring-like properties to the pilus that spreads evenly the impact of urine flow forces across the entire pilus, not just the adhesin [79].

A type 1 or P pilus, stretched in an optical tweezers or AFM setup, exhibits three regions of elongation [80,81]; Region I is where the quaternary structure can weather the pulling force and, whilst the bonds between stacks within the superhelical architecture of the rod are stretched elastically, they are not broken. The force *versus* elongation curve is linear. In Region II, these bonds are broken. They do so from the distal end, uncoiling the pilus in a stepwise fashion, the outermost subunit, with fewer neighbours to distribute the force to, experiencing the greatest force [82–84]. In Region III, the linearised chain is stretched still further, burdening the donor-strand exchange interactions between subunits. Remarkably, however, the whole process is reversible; removal of the stretching force allows broken interactions between stacks to reform. The pilus can be repeatedly stretched and recoiled, as a spring, with no significant lasting damage to it [81,85].

The ability to break the inter-stack bonds in Region II and stretch the pilus more than five times its original length strengthens the adhered bacterial position, both at the single pilus level and where a number of pili are adhered simultaneously [80]. The pilus ability to stretch leads to a reduction in force experienced by the adhesin by as much as three fold [79]. A force of 30 pN in both the type 1 and P pili under steady state conditions leads to stack-to-stack Region II breakage [80,86]. The rate of stretching is crucial; type 1 pili are able to respond more quickly to an applied external force, which has led to speculation that this pilus, which mediates adhesion to the lower part of the urinary tract (the bladder) is more adapted to the conditions of more irregular but stronger urine flows found in this environment [86].

A fascinating correlation has been found between the activation of the catch bond, and the Region II stretching of the rod. For the FimH catch bond to be activated the force applied must be sufficiently fast [63]. It must also be at a reasonable level — greater than the 'critical' force required to activate it, but less than breakage force. Earlier, AFM correlated the activation force of the FimH catch bond to being within the Region II of the rod [87]. These processes appear coupled to one another so that if a mannose receptor appears bound to the cell and so activates the catch bond, this productive interaction is maintained by using the spring-like rod stretching.

Elastic protein networks are used in nature to engender strength in the presence of a deforming force. It is interesting to compare mechanical properties such as extensibility (the ability to extend), or resiliency (energy dissipated upon retraction). The type 1 pilus is more extensible than other well known elastic proteins – elastin, resilin, mussel byssal fibres, and viscid spider silk [65,88] – and has resiliency similar to that of mussel byssal fibres. These fibres are adhesive protrusions containing an adhesive stiff tip and extendable rod that keep mussels adhered to rocks. Extendable byssal fibres allow the sharing of an applied load, for example from sea currents, between multiple fibres, in addition to the reorientation of the adhesion bond along the vector line of the force applied, which minimises it. A macroscale homology therefore exists between pili and byssal fibres [65].

4.3. Strength of the donor-strand interaction

After stripping away the quaternary structure, a stretched P or type 1 pilus must rely upon the donor-strand interaction for maintaining adhesion. One of the strongest non-covalent interactions known in nature, this continues to prevent catastrophic breakage of the bacteria well into the Region III of the stretching profile. An empirical example of this strength is that it is not disrupted in SDS-PAGE, allowing subunit–subunit interactions to easily be viewed via this common laboratory method.

Aside from P and type 1 pili, other CU appendages may be connected solely by the donor-strand interaction. The Dr adhesin fimbriae form structures of primarily 2 nm width, which must reflect an end to end arrangement of subunits [89]. A non-helical arrangement will leave the pilus unable to benefit from subunit interactions between stacks. However, the strength of the subunit–subunit interaction is likely extremely

high. For example, in a similar system, the F1 capsular fibres of *Yersinia pestis*, the interaction T_m between subunits has been experimentally measured and found to be 87.5 °C [23,28]. The strength of association between subunits in the pilus is reflected by a large estimated dissociation enthalpy of 395 kJ/mol at 37 °C, a value significantly greater than that for the chaperone–subunit complex, 275 kJ/mol at 37 °C [28].

4.4. Rebuffing adhesion

UTIs cost the US $2–3 billion per annum. Antibiotics have become decreasingly effective. So as well as the pilus biogenesis prevention strategies described above, efforts are in place to generate drugs which disrupt the adhesive mechanism directly [90].

Mannosides target the actual adhesin-receptor interaction of type 1 pili, and include mannose based derivatives containing azobenzene, thiazoylamines and long chain alkyl groups [91–93]. Orally available diphenyl mannosides have proven effective in combatting established UTIs *in vivo* [94]. However, a number of developed mannosides have somewhat weak affinity for FimH, a consequence of the ingenuity of the catch bond mechanism [6]. The idea has therefore been mooted to beat FimH through allosteric anti-adhesives. These may target the interdomain region of FimH, or alternatively one of the other allosteric sites identified on $FimH_L$, such as the pocket zipper, β-bulge, or α-switch, which contribute to the catch bond mechanism [76].

The P pilus has also been targeted, with a mimic PapG receptor surface oligosaccharide expressed so as to inhibit PapG binding to its intended host receptor [95]. Natural oligosaccharide receptor mimics have been developed as a potential alternative to synthetic receptors, due to their inherent low toxicity [95], and glycoproteins from human milk have been reported to decrease hemagglutination [96]. Synthetic development of functionalised galabioside receptors has nevertheless continued, with results of decreased hemagglutination, and an IC_{50} value of 68 μM, which is competitive with the reported binding of the natural ligand [97].

The pilus rod, and the stretching which it provides, may be a future therapeutic target; the idea of preventing a pilus from stretching or recoiling, by inhibiting the stack-to-stack interactions within the PapA superhelical rod, has been presented and termed 'coilicide design'. Both purified PapD chaperone and attached anti-PapA antibodies affect the rod elasticity, proving that a material with affinity for the rod subunit may bring about this inhibition. A loss of the reversible rod elastic properties would render the adhesin–host interaction more vulnerable to urine flow [98,99].

Finally, whilst developing anti-adhesive drugs and pilicides, it should be considered that a combination of approaches might be the most effective in fighting UPEC. The antibiotic trimethoprim-sulfamethoxazole (TMP-SMZ) is a commonly prescribed prophylactic agent for UPEC sufferers, and as such is increasingly ineffective due to antibiotic resistance. However, in combination with a mannoside, its efficacy was reported to increase. The explanation suggested is that in combination, mannosides make UPEC less able to colonise the bladder epithelium cells, thereby concentrating them at the bladder lumen, a region where TMP-SMZ concentrates to inhibitory levels [90,94].

5. Concluding remarks

Type 1 and P pili are fascinating examples of bacterial nanomachines which have properties which would make engineers envious – for example type 1 pili are 1000 fold more extensible than high tensile steel [65,88]. Unfortunately, these are machines generating crucial virulence factors, thereby requiring our ingenuity in order to understand and repel them. To our credit, this is increasingly the case; the components can now be isolated, reformed *in vitro*, their rate of formation measured, and ultimately molecules designed which inhibit their mode of function. There are still some pieces to be put together; the mechanism of initiation, of the NTD-to-CTDs transfer, the energetics of pilus growth, as well as the much anticipated but not yet realised introduction of a pilicide or anti-adhesive drug into the therapeutic market. However the basics are in place for these developments to continue.

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