Structural biology of the chaperone–usher pathway of pilus biogenesis

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Abstract | The chaperone–usher (CU) pathway of pilus biogenesis is the most widespread of the five pathways that assemble adhesive pili at the surface of Gram-negative bacteria. Recent progress in the study of the structural biology of the CU pathway has unravelled the molecular basis of chaperone function and elucidated the mechanisms of fibre assembly at the outer membrane, leading to a comprehensive description of each step in the biogenesis pathway. Other studies have provided the molecular basis of host recognition by CU pili. The knowledge that has been gathered about both the assembly of and host recognition by CU pili has been harnessed to design promising antibiotic compounds.

Chaperone–usher pilus
A bacterial cell surface appendage that is assembled by the chaperone–usher pathway of pilus biogenesis.

Curli
A type of fimbria that mediates binding to components of the extracellular matrix and is often implicated in biofilm formation.

Type IV pilus
An elongated, flexible appendage that extends from the surface of Gram-negative bacterial cells and is used for adhesion and for cell motility (twitching motility).

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Bacteria commonly express proteinaceous appendages on their outer surfaces. One class of extracellular polymers, known as pili or fimbriae, is used in attachment and invasion, biofilm formation, cell motility and transport of proteins and DNA across membranes. These non-flagellar appendages of Gram-negative bacteria can be categorized into five major classes on the basis of the biosynthetic pathway involved: chaperone–usher pili (CU pili), curli, type IV pili, the type III secretion needle and type IV secretion pili (reviewed in REF. 1). Of these five classes, the CU pili are the most extensively studied. CU pili are assembled at the outer membrane by two proteins, a periplasmic chaperone and an outer-membrane, pore-forming protein called the usher[2]. The chaperone facilitates folding of pilus subunits[3], prevents them from polymerizing in the periplasm and targets them to the usher[4,5]. The usher acts as an assembly platform, recruiting chaperone–subunit complexes from the periplasm, coordinating their assembly into a pilus and secreting that pilus through the usher pore. Investigations of the structure of CU pili have elucidated not only the mechanism of pilus assembly but also the functions of these pili in host–pathogen interactions. Here, we review the spectacular progress that has taken place over the past 10 years and has led to an overall understanding of the biogenesis and function of CU pili.

CU pilus morphology and structure

CU 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 ~12 kDa to ~20 kDa. CU organelles differ widely in complexity and morphology, ranging from thin, fibrillar fibres to thick rods composed of a helically wound cylinder and a distinct fibrillar tip. Phylogenetic analysis of usher sequences in all 189 known CU systems revealed 6 main clades: α-, β-, γ- (which is subdivided into γ1, γ2, γ3 and γ4), κ-, π- and σ-fimbriae[6]. This Review focuses on the rod-like or 'typical' fimbrial organelles, which are found in the α-, γ- and π-fimbrial clades. Among these, the P and type 1 pili (belonging to the π- and γ1 fimbrial clades, respectively) of uropathogenic *Escherichia coli* (UPEC) (BOX 1) have been the focus of extensive studies.

P pili consist of six different subunit types arranged into two distinct subassemblies, the tip fibrillum and the pilus rod. The flexible, distal tip fibrillum is ~2 nm in diameter and is composed of one copy of the PapG adhesin at the distal end, followed by one copy of the adaptor subunit PapF and 5–10 copies of the PapE subunit (FIG. 1). The long, rigid, 6.8 nm-wide pilus rod is composed of more than 1,000 copies of the PapA subunit[7] (FIG. 1), which are connected to the tip fibrillum by one copy of the adaptor subunit PapK and terminated at the proximal end (in the cell wall) by one copy of the termination subunit PapH. The PapA rod adopts a right-handed, one-start superhelical structure with a 2.5 nm pitch and 3.3 subunits per turn[8,9]. Type 1 pili display a similar but simplified architecture, with shorter tip fibrillae[10] consisting of one copy each of FimH (the adhesin), FimG and FimF (FIG. 1). The major rod subunit is FimA. No termination subunit has been identified in the type 1 pilus to date.

Pilus assembly

Chaperone–subunit interactions. Pilus subunits are taken up by their cognate periplasmic chaperones as soon as they exit the Sec machinery (which mediates general secretion)[11]. In the absence of the chaperone, the

Box 1 | The role of chaperone–usher pili in urinary tract infection

The urinary tract is a common site of bacterial infection in women, resulting in an estimated eight million outpatient visits yearly in the United States, with an estimated cost exceeding one billion US dollars. It is thought that acute urinary tract infections (UTIs) develop when bacteria from the faecal flora colonize the vaginal and periurethral areas and are subsequently introduced into the bladder by ascension through the urethra.

Uropathogenic *Escherichia coli* (UPEC) is the leading causative agent of UTIs and is responsible for up to 85% of community-acquired UTIs and 25% of nosocomial UTIs. Genetic, biochemical and cell biological approaches, together with a murine model of UTIs and a range of imaging techniques, have revealed an unexpectedly complex UPEC pathogenesis cycle that involves both intracellular and extracellular niches.

FimH-mediated binding to mannosylated receptors on the surface of urothelial cells is crucial for UPEC to cause bladder infection, as it mediates colonization and invasion of the superficial umbrella cells that line the luminal surface of the urothelium. After invasion, UPEC can be harboured in exocytic vesicles[59] or escape into the cytoplasm, where it can rapidly replicate into large biofilm-like aggregates (each containing 10^4–10^5 bacteria) known as intracellular bacterial communities (IBCs)[60–62]. Type 1 pili have been shown to assemble in IBCs, and several lines of evidence suggest that type 1 pili are required for the survival and proliferation of UPEC in superficial facet cells. As the IBC matures, the bacteria detach from the biomass and spread to neighbouring epithelial cells. Thus, the IBC pathway facilitates massive expansion of a bacterial population that invades superficial umbrella cells early in infection and then perpetuates the process. Repeated rounds of IBC formation in the acute stages of infection is a mechanism used by UPEC to subvert host defences and gain a crucial foothold.

One outcome of the acute pathogenic cascade can be the establishment of a chronic quiescent intracellular reservoir that can persist for months, protected from antibiotics and seemingly undetected by the host immune system, even after the acute infection is resolved and bacteria are no longer detectable in the urine[61,63]. This reservoir can later seed a recurrent infection, manifested by IBC formation, bacteriuria, extracellular colonization and inflammation.

The host has a number of defence mechanisms to prevent bacterial infiltration. Some of these defences are passive, such as urination, which represents a powerful force that bacteria must withstand in order to ascend the urethra and colonize the bladder. The type 1 pili-mediated binding to and invasion of the bladder epithelium by UPEC also potentiates the activation of Toll-like receptors (TLRs) such as TLR4, which recognizes bacterial lipopolysaccharide. Activation of TLRs induces a complex mixture of cytokines, which leads to inflammation and the infiltration of neutrophils[64–66] into the tissue to help eradicate the infection. Bladder colonization also triggers rapid exfoliation of the bladder epithelium as part of an innate defence to shed the infected cells out of the body through urination. Later, activation of the urothelial stem cell niche results in the regeneration of the bladder epithelium[49,67,68]. DNA microarray analyses indicate that FimH+ UPEC infection triggers genetic regulatory circuitries that are important in cell differentiation and proliferation, pro-inflammatory responses, apoptosis, stress responses, signal transduction, cell–cell contacts and metabolism[67,68]. Understanding these processes will be essential for the development of better therapeutics.

subunits cannot fold properly and form aggregates that are targeted to the DegP protease for degradation[3,12,13]. Thus, chaperones provide the scaffold onto and around which pilus subunits fold. The periplasmic chaperones are 'boomerang'-shaped, ~25 kDa proteins consisting of two immunoglobulin-like (Ig-like) domains[14,15]. The interactive surfaces that contact the pilus subunit are formed by two conserved basic residues located between the two Ig-like domains (specifically, Arg8 and Lys112, following Pap numbering)[16] and by the amino-terminal and seventh β-strands (strands A1 and G1, respectively) of the N-terminal domain of the chaperone (FIG. 2a,b). Mutations of Arg8 or Lys112 in the Pap system completely abrogate pilus biogenesis[15]. Pilus subunits are characterized by an incomplete Ig-like fold that lacks the C-terminal β-strand[17,18] (FIG. 2b). As a result, all subunits possess a large groove where the missing strand would normally be in a complete Ig fold. In chaperone–subunit complexes, a motif of four alternating hydrophobic residues on the chaperone G1 strand are inserted into this hydrophobic groove. These four residues are designated P1 to P4, and their corresponding acceptor sites in the subunit groove are known as the P1 to P4 pockets or sites (FIG. 2a). The interaction between the subunit groove and the chaperone G1 strand also includes the formation of hydrogen bonds between the main-chain atoms of the groove-flanking β-strands of the subunit and those of the chaperone G1 strand. Thus, the chaperone provides *in trans* (or 'donates') the secondary structural element that is missing from the pilus subunit, a process termed donor strand complementation (DSC)[17,18] (FIG. 2). By providing the structural information that is missing from the subunit Ig-like fold, the chaperone functions as a structural platform that catalyses the correct folding of the pilus subunits[3,13,19].

Depending on the length of the loop between the F1 and G1 strands (the F1G1 loop) of the chaperone, two structural scaffolds are observed[20]. In chaperone–subunit interactions that involve chaperones with short F1G1 loops, termed FGS chaperones, the G1 donor strand from the chaperone inserts between the A and F strands of the pilus subunit to form a continuous β-sheet[17,18] (FIG. 2a). In interactions that involve chaperones with long F1G1 loops, termed FGL chaperones, the A and F strands of the pilus subunit form the edge strands of the two opposing sheets in the Ig-like β-sandwich[21–23]. FGL chaperones contain an additional alternating hydrophobic residue on the G1 strand (known as P5, as it follows P4) that inserts intermittently into a corresponding P5 pocket in the subunit groove. In subunits that interact with FGS chaperones, this P5 pocket is never occupied by a chaperone residue, as the G1 strand is too short.

In all chaperone–subunit interactions, the donated chaperone G1 strand runs parallel to strand F in the subunit, resulting in an atypical Ig fold: in a typical Ig fold, the seventh strand (strand G) runs antiparallel to strand F. The consequence of this atypical fold complementation event is that the subunit is maintained in a polymerization-competent state, which is characterized by the accessibility of the P5 pocket to incoming subunits during pilus assembly and a partially flexible conformation in which a number of loops and secondary structural elements in the chaperone-bound subunit are disordered.

Subunit–subunit interactions. 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. During subunit polymerization, the complementing G1 β-strand donated by the chaperone is replaced by the Nte on the subunit of the incoming chaperone–subunit complex. This assembly reaction is termed donor strand exchange (DSE)[21,22,24] (FIG. 2c,d). Nte sequences are relatively conserved, as they contain a set of alternating hydrophobic residues that form an essential part of the subunit–subunit interaction. The P1 to P4 pockets in the subunit groove are occupied by the P1 to P4

Type III secretion needle
A needle-like secretion apparatus in Gram-negative bacteria that forms pores in host membranes and allows the injection of virulence factors from the bacterial cytoplasm into the cytosol of host cells.

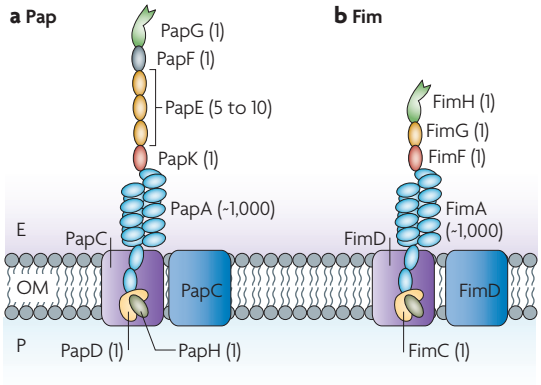


Figure 1 | **P and type I pili.** A schematic of P (part **a**) and type 1 (part **b**) pili, represented by the Pap and Fim systems, respectively. Numbers indicate the number of copies of each subunit in the pilus. The chaperones attached to the last subunit to be incorporated into each pilus are shown in yellow. P pili are terminated at the outer membrane (OM) by the termination subunit, PapH. No such subunit is known in the Fim system. The usher dimers are indicated in purple and blue. E, extracellular space; P, periplasm. Figure is modified, with permission, from REF. 35 © (2008) Elsevier.

residues of the chaperone G1 strand (discussed above) in the chaperone–subunit complex, but after DSE it is the P2 to P5 pockets of the subunit groove that are occupied by the hydrophobic residues (termed P2–P5 residues) of the incoming subunit Nte (FIG. 2a,c,f). The P4 Gly residue in Nte sequences is strictly conserved (FIG. 2f). This residue lies on top of the P4 pocket in the subunit groove, which contains a bulk that is usually formed by an aromatic residue (FIG. 2e). Gly is the only residue that can accommodate this bulk, as any other amino acid would give rise to steric constraints that would destabilize the interaction between the groove and the Nte. Thus, the interaction of P4 Gly with the bulk of the P4 pocket ensures that the incoming Nte registers correctly in the accepting groove.

The DSE 'zip-in, zip-out' mechanism. The exchange reaction between the chaperone G1 strand and the Nte in the incoming subunit occurs through a concerted 'zip-in, zip-out' mechanism that is initiated by the insertion of the Nte P5 residue into the P5 pocket of the groove in the previously assembled subunit[22,25] (FIG. 3a). The identification of a ternary chaperone–subunit Nte complex during *in vitro* DSE reactions in *Salmonella* SafA subunits, using non-denaturing mass spectrometry, was crucial to the discovery of this mechanism[22]. The formation of the ternary complex was shown to depend on the accessibility of the P5 pocket in the accepting pilus subunit (FIG. 3b). As mentioned above, for pilus subunits that are assembled by FGS chaperones, the P5 pocket is not occupied by the chaperone G1 strand and is therefore freely accessible to the P5 residue of the Nte of an incoming pilus subunit. In systems such as the *Salmonella* Saf pili, which are assembled by FGL chaperones, the chaperone occupies the P5 site but does so in an equilibrium with a locally unbound, accessible state[22]. In the Saf system, mutating the chaperone G1 residue that occupies the P5 site to a residue with an improved steric fit to the pocket (an Ala to Phe substitution, in the case of the Saf system) shifts the equilibrium towards the closed state, thus suppressing formation of the ternary complex and drastically reducing the DSE rate. Similarly, decreasing the hydrophobic bulk of the P5 residue on the Nte of the incoming subunit negatively affects ternary complex formation and the DSE rate. Thus, the P5 pocket in a chaperone-bound subunit forms the primary acceptor site for the Nte of an incoming subunit. The interaction between the Nte (through its P5 residue) and the P5 pocket initiates DSE. Single-site mutagenesis of the P5, P4 and P3 residues in the Nte to Ala revealed a gradient of decreasing DSE efficiency moving away from the P5 initiation site, suggesting a 'zip-in, zip-out' mechanism, with the new Nte gradually displacing the chaperone G1 donor strand in a step-wise process from P5 to P1 (REF. 22) (FIG. 3a).

Termination of pilus biogenesis. Understanding the crucial role of the P5 pocket in DSE also helps us to understand the molecular basis of the termination of pilus biogenesis. In the P pilus system, termination occurs when the PapD–PapH chaperone–subunit complex is presented for assembly at the usher[8]. PapH undergoes DSE with the last PapA subunit of the pilus rod. However, PapH is unable to undergo DSE with any other subunits, as confirmed by *in vitro* experiments in which the PapD–PapH complex was refractory to challenge by Nte peptides derived from any of the Pap subunits[9]. This restriction is not due to a stronger chaperone–subunit interaction but has been ascribed instead to a single distinguishing structural feature: the absence of a P5 pocket in PapH[9] (FIG. 3b). The mechanism by which type 1 pilus biogenesis terminates is not known, as no homologue of PapH has been found in that system to date.

Subunit ordering. The location of PapG at the tip of the P pilus was first discovered by Lindberg *et al.*[26]. Later, high-resolution electron microscopy (EM) studies on various P pilus mutants elucidated the composite nature of the pilus and the fact that PapE constitutes the bulk of the tip fibrillum[7]. These studies were subsequently extended to deduce the order of the subunits in the pilus: PapF links the adhesin, PapG, to the tip fibrillum polymer, PapE, and PapK links the tip fibrillum to the pilus rod[27]. It was then shown that PapA and PapE are the only subunits in the pilus that have self-associating properties[28], consistent with the results from genetic and EM studies. The role of Ntes in specifying subunit–subunit interactions was first demonstrated by genetic and biochemical experiments[29] that used a series of N-terminally swapped subunits. PapF, which is required to link PapG to PapE[27], was proposed to have an Nte that fits into the PapG pilin groove. The Nte of PapF was swapped onto the pilin body of PapE to produce a fusion protein that could complement Δ*papF* mutants and link PapG to the PapE tip fibrillum[29]. Cells encoding a PapF that was missing the Nte behaved in a

Type IV secretion pilus
A pilus that is formed as part of the versatile secretion systems that are found in Gram-negative and Gram-positive bacteria. It can secrete a wide range of substrates, including protein–protein and protein–DNA complexes, and can directly target eukaryotic cells.

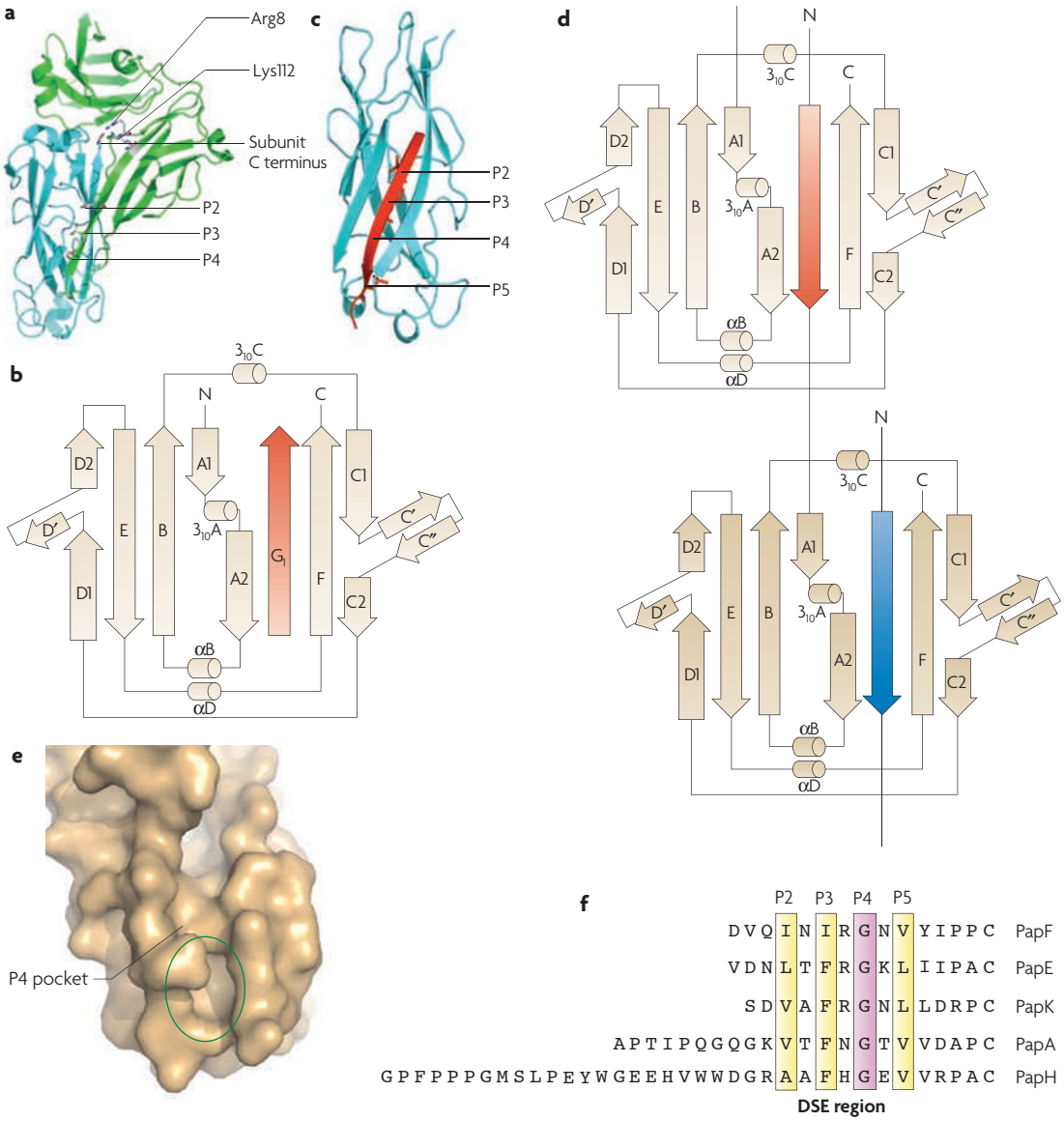


Figure 2 | **Donor strand complementation and donor strand exchange. a** | A ribbon diagram of the PapD–PapK complex structure. PapD and PapK are in green and cyan, respectively. The carboxyl terminus of PapK is shown as a ball-and-stick representation. The conserved chaperone residues that contact the subunit C terminus (Arg8 and Lys112) and the P2, P3 and P4 residues in the chaperone G1 β-strand are shown in the same representation. **b** | A topology diagram of PapK. Arrows and cylinders represent β-strands and α-helices, respectively. The C and amino termini are indicated. Note that in the chaperone–subunit complex structure, the N-terminal extension (Nte) is disordered and thus not represented in the topology diagram of the subunit prior to donor strand exchange (DSE). The red arrow represents the G1 β-strand donated by the chaperone. **c** | The structure of the pilus subunit after DSE. The subunit is shown in a ribbon diagram (cyan). The donor strand (or Nte) from the incoming subunit is shown in red. The P2, P3, P4 and P5 residues in the donor strand are shown in a ball-and-stick representation (red). **d** | A topological diagram of the subunits in the pilus. The representation is as in part **b**. The Nte (red) of the following subunit complements *in trans* the fold of the previously assembled subunit. **e** | A close-up of the P5 pocket (circled in green) and P4 pocket of PapA. **f** | A sequence alignment of the Ntes of all Pap subunits (except for PapG, which does not have an Nte). The alternating hydrophobic residue motif (DSE region) is highlighted in yellow, with the conserved Gly in magenta. Parts **b** and **d** are modified, with permission, from REF. 35 © (2008) Elsevier. Part **e** is reproduced, with permission, from REF. 31 © (2008) Elsevier.

similar manner to Δ*papF* mutants and were unable to incorporate PapG into the pilus. The results that were obtained using various combinations of N-terminally swapped and deleted subunits support the proposal that there is a role for Ntes in specifying subunit order in the pilus.

The role of the Nte–groove interaction in determining subunit ordering was confirmed by a study that examined the specificity of DSE in Pap subunit assembly[30]. An *in vitro* DSE assay was set up, in which all six chaperone–subunit complexes of the Pap system were incubated individually in the presence of peptides corresponding

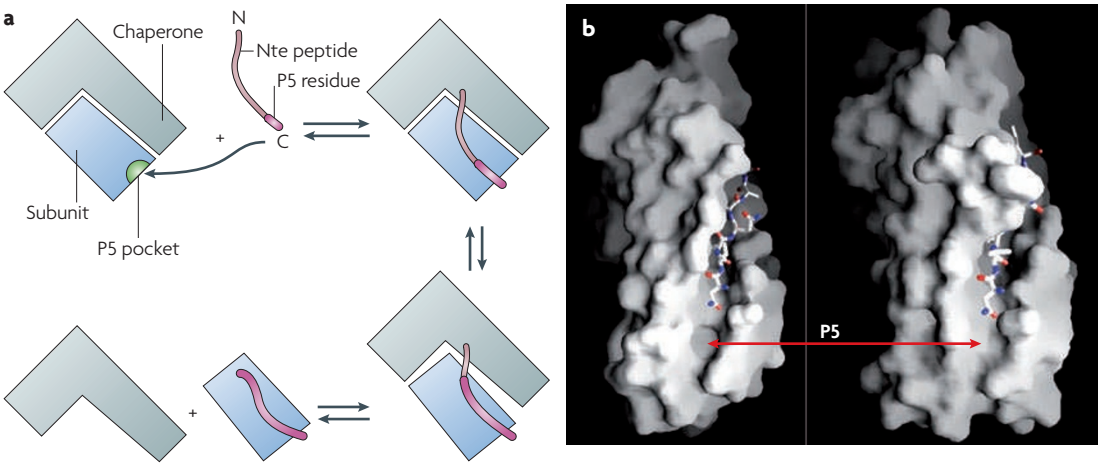


Figure 3 | **The concerted 'zip-in, zip-out' mechanism for donor strand exchange. a** | A schematic diagram for donor strand exchange (DSE). Progressive insertion of the P5, P4, P3 and P2 residues of the attacking amino-terminal extension (Nte) is shown in pink. **b** | The presence of a P5 pocket in PapK (left panel) and the absence of a corresponding pocket in PapH (right panel). All Pap subunits except PapH have a P5 pocket, which serves as an initiation site for the progressive zipping-in of the Nte of the subunit that comes next in assembly. As PapH does not have a P5 pocket, an Nte cannot challenge the PapD–PapH complex. Part **b** is reproduced, with permission, from *EMBO Reports* REF. 9 © (2006) Macmillan Publishers Ltd. All rights reserved.

to each of the five Pap subunit Ntes (PapG lacks an Nte, as it is positioned at the distal end of the pilus and acts only as a donor strand acceptor). The DSE reaction was followed by characterizing the time course of the disappearance of the chaperone–subunit complex by electrospray mass spectrometry. Of the 30 reactions examined, 7 mimicked the interactions that are known to occur in the pilus structure *in vivo* (referred to as cognate interactions) and the remaining 23 reactions involve non-cognate pairings. The results reveal a striking correlation between the apparent rate of DSE and the nature of the Nte (that is, whether is it cognate or non-cognate for the partner subunit), with the most rapid reactions uniformly occurring with cognate partners. The data suggest that the complementarity between each subunit groove and its cognate Nte plays a substantial part in determining subunit assembly. The range of reactivities between different chaperone and subunit Nte pairs is remarkable, given the structural homology between the subunit folds and the highly conserved Nte binding motif comprising the alternating P1 to P5 hydrophobic residues (FIG. 2f). Further studies identified the residues in the Ntes that are responsible for the specific groove–Nte interaction and found that the P5 residue and the residues immediately adjacent to it have key roles in determining DSE rates[30].

A comprehensive survey of the P5 pockets in all Pap subunits (except PapG) was recently published[31]. PapA and PapK both have well-defined P5 pockets (FIGS 2e,3b) and are known to undergo DSE at similar rates. In PapE, the A2–B loop is disordered and so the region around the P5 pocket is thought to be flexible. Remarkably, PapE is known to be a fast-exchanging pilus subunit and undergoes DSE at the highest rate among Pap subunits. By contrast, in PapH and PapF the P5 pocket is obstructed by residues in the A2–B loop (namely, Thr52 in PapH and Pro32 in PapF); PapH is unable to undergo DSE and PapF undergoes DSE at a slow rate. Thus, there seems to be a correlation between the level of accessibility and flexibility of the P5 pocket and the rate of DSE: the more accessible and flexible the region around the P5 pocket, the faster the exchange reaction. The P5 pockets in PapF and PapH were further investigated using molecular dynamics and site-directed mutagenesis. Although in both the PapH and PapF crystal structures the P5 pocket seems to be blocked, molecular dynamics simulations suggest that the P5 pocket of PapF becomes accessible intermittently, whereas that of PapH does not. These results, which were confirmed using site-directed mutagenesis followed by DSE experiments, provide an explanation for why PapF undergoes slow DSE and PapH no DSE at all.

Pilus assembly at the usher. *In vivo*, subunit polymerization occurs at the outer-membrane usher. The usher recruits binary chaperone–subunit complexes to the outer membrane, mediates their ordered polymerization and is responsible for translocation of the growing pilus to the outer surface[4,32–35]. Cryo-EM images of two-dimensional crystals of the UPEC P pilus usher PapC, reconstituted in *E. coli* lipids, show that PapC forms homodimeric channels. Complementation studies of PapC loss-of-function mutants subsequently established that these dimers constitute the functional unit for the usher[36,37]. However, although dimer formation is crucial for usher function *in vivo*, it might not be necessary for usher-mediated fibre formation *in vitro*[38]. Outer-membrane ushers are ~800 residue proteins that comprise four functional domains: an N-terminal, ~125-residue periplasmic domain, a C-terminal, ~170-residue periplasmic domain and a large, central, ~500-residue translocation pore domain that is interrupted by a conserved plug domain of ~110 residues[39–41] (FIG. 4a).

The N-terminal periplasmic domain binds chaperone–subunit complexes with high affinity[33,42]. The structures of the N-terminal domain of the FimD usher bound to

Molecular dynamics
A form of computer simulation that calculates the time-dependent behaviour of atoms and molecules, providing information about the motion of the atoms and the resultant conformational changes in the molecules over time or during an interaction.

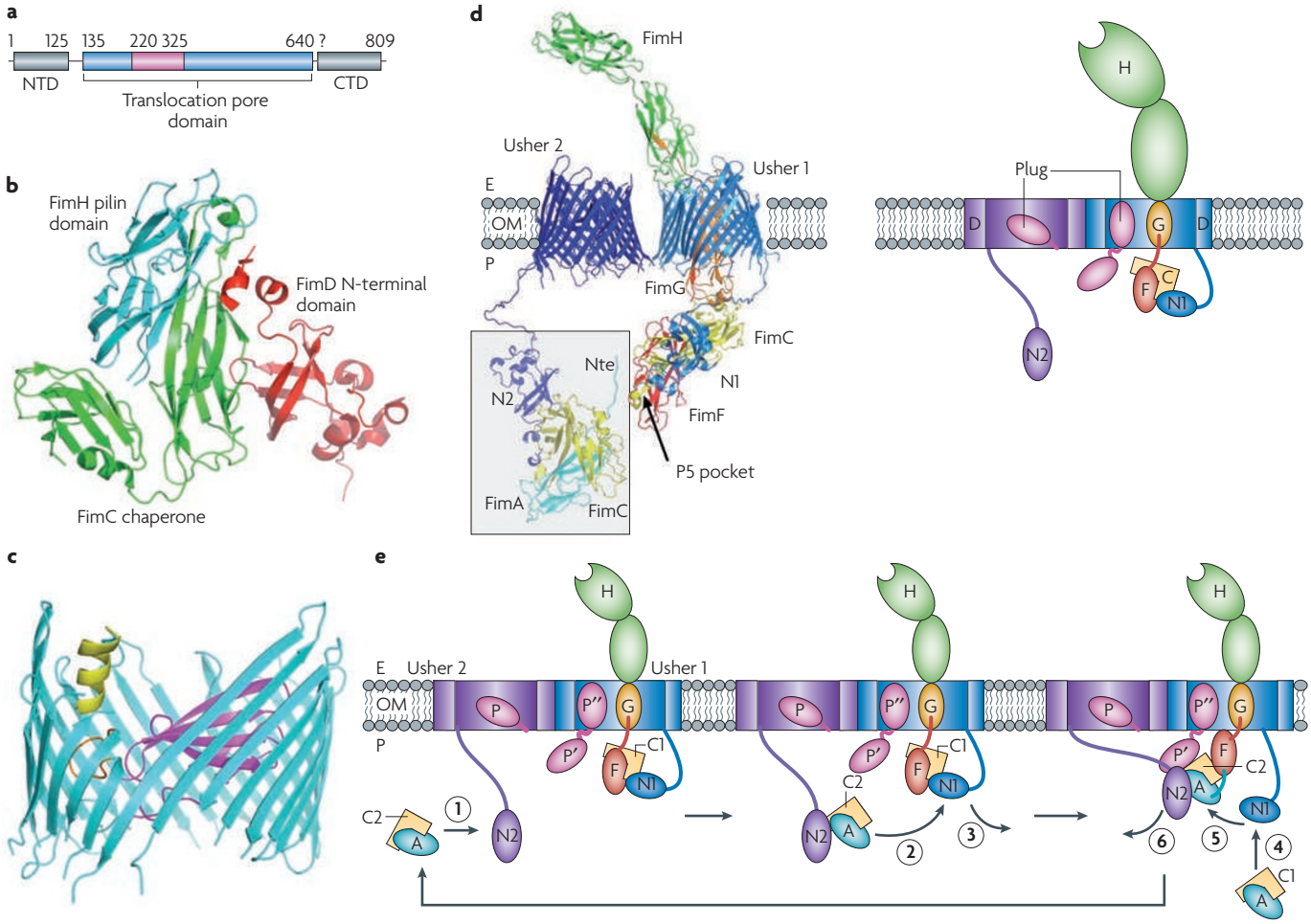


Figure 4 | **The structural biology of the usher. a** | The domain structure of PapC. The translocation pore and plug domains are shown in blue and magenta, respectively. The amino-terminal domain (NTD) and carboxy-terminal domain (CTD) are also indicated. **b** | The structure of the FimD N-terminal domain bound to the FimC–FimH complex. **c** | The structure of the translocation domain of the PapC usher, shown as a ribbon representation, with the barrel in cyan, the single helix in yellow, the trigger hairpin in orange and the plug domain in magenta. **d** | The cryo-electron microscopy structure of the $FimD_2$–FimH–FimG–FimF–FimC complex. The left panel shows a ribbon diagram of the structure. The two usher protomers are labelled usher 1 and usher 2.Their N-terminal domains are labelled N1 and N2, respectively. In the grey area, a model of FimC bound to FimA and N2 is shown. This model is oriented in such a way that the N-terminal extension (Nte) of FimA is within range for donor strand exchange with the FimF groove (that is, the P5 residue of FimA (not shown) is within interaction distance of the P5 pocket of FimF). The structure of the $FimD_2$–FimH–FimG–FimF–FimC complex is shown in the right panel in a schematic representation. The plug of usher 1 (the secretion pore) is shown in two different conformations: one in which it is still within the lumen of the pore but pushed to the side, and another in which it is pushed out into the periplasm (P). **e** | The subunit incorporation cycle, mediated by the twinned ushers. An incoming chaperone–subunit (FimC–FimA) complex is recruited by binding to N2 (step 1). Donor strand exchange with FimF causes the FimF-bound chaperone (C1) to be released, and N1 dissociates (steps 2 and 3) to recruit another FimC–FimA complex (step 4), adding it to the N2-bound FimC–FimA complex (step 5). Donor strand exchange then releases N2, which can recruit the next chaperone–subunit complex (step 6). Alternating binding to the released usher N-terminal domains and donor strand exchange with the next chaperone–subunit complex leads to stepwise growth of the pilus (steps 1–6). The plug domains are indicated as P in usher 2 and P′ and P″ in the alternative orientations shown for usher 1. C, FimC; E, extracellular space; F, FimF; G, FimG; H, FimH; OM, outer membrane. Parts **a**, **d** and **e** are modified, with permission, from REF. 35 © (2008) Elsevier.

the FimC–FimH chaperone–adhesin complex (FIG. 4b) and to the FimC–FimF chaperone–subunit complex were determined[33,43]. The main part of the interface between the usher N-terminal domain and both chaperone–subunit complexes is in the chaperone. However, a small part of the interface encompasses subunit-specific interactions, which are likely to explain the differences in affinity for the usher between chaperone–subunit complexes. Indeed, the usher exhibits decreasing affinity according to subunit order in the pilus assembly chain, a process that is believed to be at least in part responsible for subunit ordering[4,32,40].

The exact role of the usher C-terminal domain is unclear. Analysis of C-terminal truncation and substitution mutants of the P pilus usher, PapC, demonstrated that

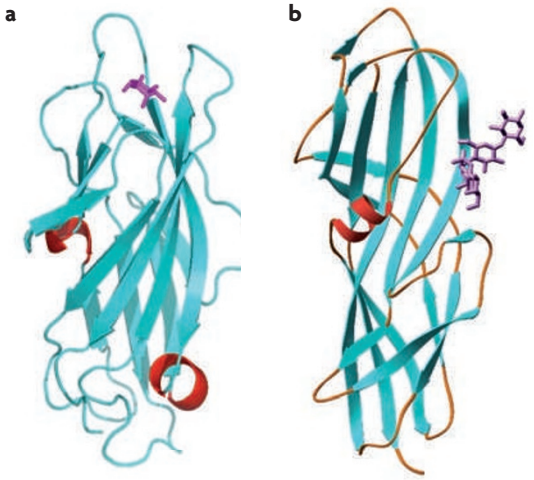


Figure 5 | **Receptor binding and pilus biogenesis inhibition. a** | The structure of FimH bound to α-D-mannose. FimH is shown as a ribbon representation, and α-D-mannose is shown as a ball-and-stick representation (magenta). **b** | The structure of the PapG receptor domain, in a ribbon representation, bound to galabiose, in a ball-and-stick representation.

the C terminus is required for proper binding of chaperone–subunit complexes to the usher and has an important role in the assembly of complete pili[37,38].

The usher translocation pore domain consists of a 24-strand β-pore with an approximate inner diameter of 45 × 25 Å. These dimensions are compatible with the passage of folded pilus subunits[35,38] (FIG. 4c). The β-barrel domain is interrupted at strands 6 and 7 by the plug domain, which blocks the channel. The plug domain is required for fibre assembly, as deletion of the plug region leads to the formation of an usher that is unable to assemble pili *in vitro*[38].

Differences in the protease susceptibility of the type 1 pilus usher, FimD, show that the usher undergoes a conformational change on binding the N-terminal domain of the tip adhesin, FimH[32,44,45]. This adhesin-induced conformational change constitutes a crucial activation step that is required for subunit polymerization and translocation by the usher[34,46]. Such an activation step must include repositioning of the plug domain, either moving it aside within the translocation pore or rotating it from the pore into the periplasm[35]. Complementation studies on non-functional *papC* mutants suggest that C-terminally truncated ushers fail to activate the translocation pore, implicating the C-terminal domain in usher activation events[37].

In the absence of the type 1 pilus rod subunit, FimA, pilus assembly stalls in a three-subunit intermediate comprising FimD bound to FimH, and the linker subunits FimG and FimF, with the last incorporated subunit, FimF, still bound to its periplasmic chaperone[46]. Structural analysis of the FimD–FimH–FimG–FimF–FimC assembly intermediate by cryo-EM reconstruction showed that, remarkably, only a single usher pore in the dimeric usher complex is used for secretion[35] (FIG. 4d). FimH extrudes from the pore, FimG resides in the pore and the FimC–FimF complex bound to the N-terminal domain of the usher resides on the periplasmic side, just under the secretion pore. In the isolated complex, the second usher pore lies idle, probably in its non-activated, closed form. The requirement for the second usher to make a functional assembly complex is believed to stem from the need for two chaperone–subunit recruitment sites: in the proposed model, the recruitment of new chaperone–subunit complexes to the assembly platform through their N-terminal domains alternates between the two usher protomers[35] (FIG. 4d,e). However, this model is not universally accepted as, at least *in vitro*, the ushers seem to function as monomers[38].

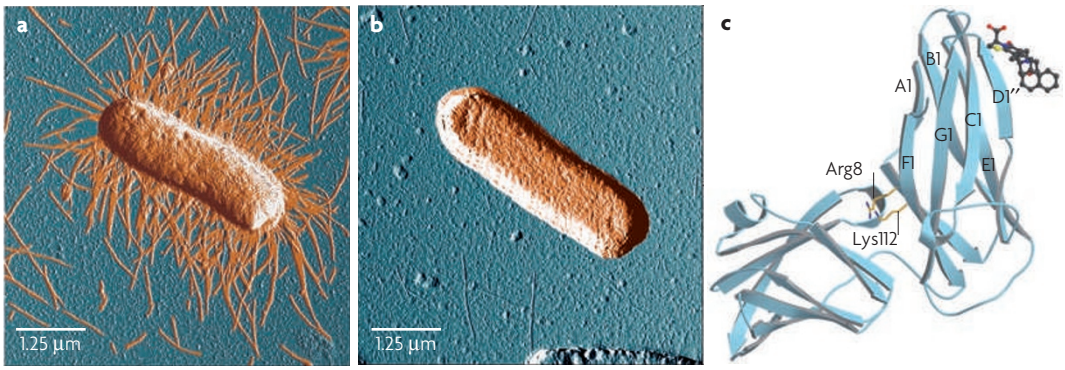
Subunit polymerization and fibre formation at the usher is a process that occurs independently of cellular energy[47]. In the absence of the usher, subunit polymerization is a spontaneous process that is driven by folding energy stored in the chaperone-bound subunits[24,48]. Chaperone-bound subunits are maintained in a high-energy folding intermediate that relaxes upon DSE with the Nte of a new incoming subunit[24]. However, this process is slow and occurs on the scale of hours to days. By contrast, pilus assembly *in vivo* occurs in a matter of minutes[47] and thus it has long been speculated that the usher acts as a polymerization catalyst. A recent study of UPEC type 1 pilus formation directly demonstrated that the usher does indeed increase the rate of subunit polymerization[34]. In the presence of the FimD usher, *in vitro* polymerization of FimA is two orders of magnitude faster and occurs in a processive manner, resulting in fewer, longer pili than usher-independent DSE. The observed catalytic function of the usher required pre-incubation with the FimC–FimH chaperone–adhesin complex, confirming the reported FimH-dependent activation step of FimD[32,37,45]. This step probably involves the gating of the usher secretion pore (discussed above).

As described above, *in vitro* DSE is a concerted process that starts with the insertion of the Nte of the incoming subunit into the P5 pocket of the subunit that is undergoing strand exchange. Interestingly, the orientation of the last-incorporated chaperone-bound subunit in the cryo-EM reconstruction of the type 1 pilus tip complex is such that its P5 pocket is exposed and points towards the chaperone–subunit recruitment site on the second usher (FIG. 4d). A mechanism of subunit polymerization at the usher was proposed, in which the spontaneous DSE process at the base of the fibre is catalysed by the effect of increased local concentration and the favourable orientation of the Nte and P5 pocket in the attacking and accepting subunits, respectively[35] (FIG. 4e).

Adhesin–receptor interaction

Colonization of mucosal surfaces is a key event in bacterial pathogenesis. Bacterial attachment is often the result of the specific recognition of a pilus-associated adhesin by a host receptor; indeed, it has been shown that type 1 pili are essential for UPEC to cause cystitis[49–52]. Using scanning and high-resolution EM and a mouse cystitis model, it was demonstrated that adhesive, type 1-piliated UPEC can bind and invade host superficial facet cells.

Box 2 | **Chaperone–usher pili as antibacterial targets**



Because of their important role in bacterial virulence, chaperone–usher pili have received considerable attention in vaccine development programmes and in the search for new antibacterials. FimH has been shown to have efficacy as a vaccine in both murine[69] and primate models[70]. The pilus assembly processes of type 1 and P pili and ligand recognition by their tip adhesins[71] have also been targeted in the search for chemical antagonists of urinary tract infections[72–74]. For both adhesins, carbohydrate-derived molecules have been shown to act as competitive inhibitors for receptor binding. Alkyl- and aryl-α-D-mannosides bind FimH with nano- to micromolar affinities, block bacterial adhesion on uroepithelial cells and counteract internalization and biofilm formation[74,75]. *p*-Methoxy-phenyl derivatives of galabiose (galabiosides) form potent inhibitors of PapG, with low micromolar IC_{50} (half maximal inhibitory concentration) values[72].

In addition, compounds have been identified that block the pilus assembly process. A family of bicyclic 2-pyridones, termed pilicides, was rationally designed to bind the conserved basic residue pair that is present between the two domains in the chaperone (see the figure). The designed compounds inhibit mannose-sensitive haemagglutination and biofilm formation in uropathogenic *Escherichia coli* by suppressing type 1 and P pilus formation (see the figure, which shows bacteria that were not exposed (part **a**) and bacteria that were exposed (part **b**) to the pilicide). These compounds were shown by NMR titration experiments to bind the PapD chaperone with millimolar affinity[73,76]. The X-ray structure of a pilicide bound to the P pilus chaperone, PapD, revealed that the compound binds to a conserved hydrophobic patch on the F1, C1 and D1″ strands on the back of the amino-terminal domain of the chaperone (see the figure, part **c**, which shows PapD in a ribbon representation and the pilicide as a ball-and-stick representation). This surface is known to interact with the usher, and surface plasmon resonance experiments indeed confirmed that pilicides interfere with the binding of chaperone–subunit complexes to the usher[33,43,73]. Figure part **c** is reproduced, with permission, from REF. 74 © (2006) National Academy of Sciences.

This process is dependent on the FimH adhesin, which is located at the distal end of the pilus and binds mannose residues on host epithelial cells.

The structures of the adhesin domains of FimH from UPEC and enterotoxigenic *E. coli* (ETEC), PapG from UPEC, and GafD and F17-G in G- and F17-fimbriae from UPEC and ETEC, respectively, show that, despite their lack of sequence similarity, these adhesins have a similar β-barrel jelly-roll fold[53–55]. However, the receptor-binding sites in these adhesins differ markedly and occupy different parts of the structure. The structure of FimH bound to D-mannopyranoside[53] revealed that the mannose is buried in a deep, negatively charged pocket at the tip of the receptor-binding domain (FIG. 5a). Residues that are important for the interaction with mannotriose (Man(α1–3)[Man(α1–6)]Man) and D-mannose were confirmed by site-directed mutagenesis. Using these mutants in a mouse model, it was found that the monosaccharide α-D-mannose is the primary bladder cell receptor for type 1-piliated UPEC[53,55].

Conversely, when PapGII (one of the three PapG classes, each of which binds different globoseries glycolipids) binds to its receptor, the sugar is bound in a shallow binding pocket formed by three strands and a loop and located on the side of the molecule (FIG. 5b). This interaction is thought to be crucial for the ability of pyelonephritic strains of *E. coli* to cause kidney disease[56]. The analogous GafD and F17-G bind the terminal *N*-acetyl-D-glucosamine (GlcNAc) residues of glycoproteins. The structures of GafD and F17-G in complex with GlcNAc reveal a shallow sugar-binding site, also on the side of the molecule but in a location unrelated to that in PapG. Together, these structures underline the flexibility with which different bacterial strains can establish tropism in infection through the use of a common scaffold for receptor binding.

Conclusions

Gram-negative bacterial pathogens use the CU pathway to assemble pilin subunits into pili. Work on CU pathways over the past decade has used structural biology combined with a multidisciplinary approach to examine the details of protein–protein and protein–carbohydrate interactions in pilus biogenesis and their crucial functions in bacterial pathogenesis. The periplasmic chaperones that are required for pilus assembly were found not only to serve as folding templates for pilus subunits, but also to actively catalyse pilus biogenesis by stabilizing high-energy conformations in the subunits[3,19].

This work has provided new insights into one of the most basic questions in molecular biology: how do proteins fold into domains that can serve as building blocks for macromolecular assemblies? Current studies are integrating multiple disciplines, ranging from innovative translational research to providing snapshots of molecules caught in the act of triggering disease processes[57,58], to unveil unanticipated mechanisms and reshape models of bacterial infections (BOX 1). Ultimately, these efforts will lead to a better understanding of infection, hopefully sparking better therapies to treat chronic and recurrent infections (BOX 2) and generating models that can be used for the study of an array of human diseases. Work on the CU pathway is generating new insights into some of the most basic principles of protein folding and macromolecular assembly and is providing a paradigm for understanding infectious diseases and developing improved strategies for their treatment and prevention.

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DATABASES

Entrez Genome Project: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomeprj
Escherichia coli
UniProtKB: http://www.uniprot.org
DegP | FimA | FimC | FimD | FimE | FimG | FimH | PapA | PapC | PapD | PapE | PapF | PapG | PapH | PapK

FURTHER INFORMATION

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