Molecular basis of usher pore gating in *Escherichia coli* pilus biogenesis

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Extracellular fibers called chaperone-usher pathway pili are critical virulence factors in a wide range of Gram-negative pathogenic bacteria that facilitate binding and invasion into host tissues and mediate biofilm formation. Chaperone-usher pathway ushers, which catalyze pilus assembly, contain five functional domains: a 24-stranded transmembrane β -barrel translocation domain (TD), a β -sandwich plug domain (PLUG), an N-terminal periplasmic domain, and two C-terminal periplasmic domains (CTD1 and 2). Pore gating occurs by a mechanism whereby the PLUG resides stably within the TD pore when the usher is inactive and then upon activation is translocated into the periplasmic space, where it functions in pilus assembly. Using antibiotic sensitivity and electrophysiology experiments, a single salt bridge was shown to function in maintaining the PLUG in the TD channel of the P pilus usher PapC, and a loop between the 12th and 13th beta strands of the TD (β 12–13 loop) was found to facilitate pore opening. Mutation of the β 12–13 loop resulted in a closed PapC pore, which was unable to efficiently mediate pilus assembly. Deletion of the PapH terminator/anchor resulted in increased OM permeability, suggesting a role for the proper anchoring of pili in retaining OM integrity. Further, we introduced cysteine residues in the PLUG and N-terminal periplasmic domains that resulted in a FimD usher with a greater propensity to exist in an open conformation, resulting in increased OM permeability but no loss in type 1 pilus assembly. These studies provide insights into the molecular basis of usher pore gating and its roles in pilus biogenesis and OM permeability.

outer membrane usher | macromolecular assembly | bacterial pathogenesis

The β -barrel membrane proteins of Gram-negative bacteria participate in a variety of outer membrane (OM) functions, including physiological maintenance, protein folding, transport, and organelle assembly (1–5). Some OM pore proteins are involved in protein secretion and assembly of virulence-associated surface-exposed appendages required for adherence to host surfaces (3, 5, 6). One such class of proteins is the ushers of the chaperone-usher pathway (CUP) found in various phyla of bacteria ranging from *Proteobacteria* to *Cyanobacteria* to *Deinococcus-Thermus* (7). Ushers are gated OM channels that function as molecular machines that convert subunit binding and folding energy into work to assemble highly stable macromolecular CUP fibers and facilitate their extrusion to the bacterial surface in Gram-negative bacteria. In uropathogenic *Escherichia coli* (UPEC), type 1 pili are critical in causing bladder infection (8), whereas P pili are important in pyelonephritis (9, 10). Type 1 and P pili are encoded by the *fim* and *pap* operons, respectively, and have been extensively used as model systems to elucidate the structural basis of chaperone-usher pilus assembly. We used both the type 1 and P pilus ushers in this study to elucidate fundamental insights into the gating mechanism of OM ushers.

P pilus assembly requires the function of a dedicated chaperone (PapD) (11, 12) and the usher (PapC) (3, 13). The P pilus comprises a flexible-tip fibrillum made up of minor pilins with the two-domain adhesin PapG at the distal end where it can

recognize Gal α 1–4Gal disaccharide-containing glycolipids found in the human kidney (14, 15). The P pilus tip is joined to a right-handed, helical pilus rod made up of PapA pilins (16, 17) and is anchored in the OM via the terminator/anchoring subunit PapH (18). Type 1 pili are composed of FimA subunits making up the pilus rod joined to the tip fibrillum, which contains the FimH tip adhesin joined to FimG and adapted to the FimA rod by FimF. FimC and FimD are the chaperone and usher, respectively.

CUP pilus subunits are incomplete Ig-like folds missing their C-terminal β -strand. Through a process called donor strand complementation, the chaperone transiently completes the Ig fold of a subunit in the periplasm to form a binary complex, which facilitates the subunit's proper folding and stability and results in a subunit primed for assembly into a pilus fiber (19–21). Chaperone-subunit complexes are targeted to the OM usher, which catalyzes donor strand exchange (DSE), whereby the chaperone is displaced and the Ig fold of the polymerizing subunit is completed by its neighbor via an N-terminal extension in an ATP-independent manner (19, 22). The usher has five domains: an N-terminal periplasmic domain (NTD), a transmembrane β -barrel domain (TD), a β -sandwich plug domain (PLUG), and two periplasmic, β -sandwich C-terminal domains (CTD1 and 2) (3, 5). During the initiation of pilus assembly, a chaperone-adhesin is initially targeted to the usher NTD and then transferred from the NTD to the CTDs via catalytic dissociation by CTD2 (23).

Significance

Gram-negative bacteria use chaperone-usher pathway (CUP) pili to colonize host tissues and mediate biofilm formation. CUP ushers are outer membrane (OM) pore proteins that catalyze pilus assembly and mediate pilus extrusion in a gated fashion to maintain OM homeostasis. Using antibiotic sensitivity and electrophysiology experiments, specific residues of two ushers, FimD of type 1 pili and PapC of P pili, were identified as crucial for proper usher gating. Further, deletion of the P pilus anchoring/terminator subunit resulted in an open usher conformation, indicating this subunit may be a target for antivirulence compounds to potentiate antibiotic treatment. Identification of mechanisms that can be used to increase bacterial sensitivity to antibiotics is crucial for development of novel compounds to fight infections.

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X-ray crystal structures of the PapC and FimD ushers showed that the apo PapC and FimD pores are kidney-shaped β -barrels of 24 strands, the largest number of strands identified for a single OM polypeptide (3, 5, 24). The inner dimensions of the apo ushers are large enough to allow transport of folded protein domains (25 Å $\times$ 45 Å diameter) (3). Thus, a precise usher gating mechanism is needed to facilitate the extrusion of polymerized pilus fibers across the OM while maintaining OM integrity. In the apo usher, in the absence of a polymerizing and/or anchored pilus, the β -sandwich PLUG occupies the inactive pore. The PLUG seems to be kept stable in the pore via its interactions with residues of the β -barrel interior wall as well as the β 5–6-hairpin loop and an α -helix of the TD that caps the β 5–6-hairpin loop (3, 25). In the active form, as seen in the FimDCH structure, the FimD pore rearranges to a nearly circular pore of 32 Å diameter (5, 24) and the PLUG is relocated to the periplasm, where it forms a high-affinity, stable interaction with the NTD (23). The NTD/PLUG complex serves to further recruit chaperone-subunit complexes for repeated rounds of DSE pilus assembly (23).

We delineated the channel activities of the usher by focusing on two different ushers, the PapC usher of the P pilus system and the FimD usher of the type 1 pilus system. Using erythromycin (Erm) sensitivity assays, we identified a salt bridge interaction between R305 of the PLUG and E467 of the TD α -helix that functions to stabilize the PLUG in the PapC pore. Electrophysiology studies confirmed the destabilization of the PLUG in the R305A mutant. Pore gating and assembly functions of the usher were further decoupled by a pair of cysteine mutants, one in each of the NTD and PLUG domains of the FimD usher, which resulted in an open pore as assessed by Erm sensitivity in the absence of subunits while retaining pilus assembly function when the chaperone and subunits were coexpressed. We also discovered that a periplasmic loop between usher barrel β -strands 12 and 13 facilitates the removal of the PLUG, which is necessary for usher-catalyzed pilus biogenesis. Further, we demonstrated that proper anchoring of the mature pilus is necessary to retain the OM integrity. These results emphasize the importance of the coordinated, regulated expression and assembly of CUP pili. Collectively, these results demonstrate the cooperative roles that the usher plays in regulating pilus biogenesis and OM permeability.

Results

Screen of PapC PLUG Residues Involved in Pore Gating. Based on the crystal structure of the PapC TD/PLUG (3), residues were identified for mutagenesis to investigate the pore gating function of PapC. Given the close proximity of the PLUG domain to the β -barrel lumen and the luminal α -helix in the apo PapC structure (3) (Fig. 1A), we hypothesized that residues at these interfaces would be involved in positioning of the PLUG in the inactive usher. PLUG residues Q279, R303, and R305 contain charged side chains that extend from the PLUG domain toward a luminal TD β 5–6-hairpin loop (Q279) and a luminal α -helix (R303 and R305). P289 lies at the opposite side of the PLUG from Q279, R303, and R305 and forms a kink between two β -strands within the PLUG domain. D234, which resides within the β 5–6-hairpin loop, and E467, which extends from the base of the α -helix, both extend toward the PLUG domain. V322 is situated in the core of the PLUG domain facing the PLUG's own β -strands. Mutations were made in each of these residues to investigate their role in pore gating. Mutation of residue V322 was carried out to investigate any effects of internal changes to the PLUG domain β -strand packing on pore gating. In addition, deletions of the entire PapC PLUG domain, β 5–6-hairpin loop, and α -helix were constructed.

Gram-negative bacteria are normally resistant to the antibiotic Erm owing to their OM impermeability (26). Thus, an Erm sensitivity assay was used to detect changes in OM permeability and to test the impact of mutated residues on the pore

Fig. 1. Impact of PapC residues and loops on pore gating abilities of the usher. (A) Crystal structure of the inactive PapC usher monomer (TD/PLUG) viewed from the extracellular milieu. The PLUG domain (dark blue), is capped by the β 5–6-hairpin loop (yellow) and α -helix (green) (PDB ID code 2VQI). The TD β -barrel is orange and the residues mutated in our pore-gating screen are red. Erythromycin sensitivity profiles of PapC point mutants, loop and domain deletions were determined without (B) and with (C) the presence of polymerizing pili. Statistical analyses were performed using two-tailed Mann–Whitney test compared with WT; * P = 0.01, ** P = 0.003.

gating function of the PLUG domain. Expression of WT PapC from pTrc-PapC_{His6} in C600 cells caused an increase in Erm sensitivity [$\sim$ 0.5–0.6 cm inhibition zone radius (izr)], compared with the empty vector pTrc99a control ($\sim$ 0.2 cm izr), likely owing to spontaneous opening and closing of the PLUG domain that was observed in planar lipid bilayer electrophysiology studies (25). Most of the PapC point mutants had Erm inhibition zones similar to WT levels ($\sim$ 0.5–0.6 cm izr) (Fig. 1B). The most drastic effect on pore permeability was observed when the PLUG domain was deleted ($\sim$ 1.2 cm izr) (Fig. 1B). Deleting the α -helix and β 5–6-hairpin loop significantly increased pore permeability ($\sim$ 0.8–0.9 cm izr and $\sim$ 0.7 cm izr; P = 0.0048 and P = 0.013 by Mann–Whitney test, respectively) compared with the WT PapC, indicating their importance in stabilizing the PLUG domain in the closed position in the PapC β -barrel, as already suggested from electrophysiology (25). The R305A PLUG mutant had the most significant effect on pore gating of a single amino acid change ($\sim$ 0.8–0.9 cm izr), suggesting that this residue plays a critical role in stabilizing the PLUG within the TD pore. The effect of the R305A PapC point mutant is comparable to that of deleting the entire α -helix and slightly larger than deleting the β 5–6-hairpin (P = 0.053, Mann–Whitney test). Similar results were obtained with an R305P mutation. Mutation of a neighboring arginine residue, which also extends from the PLUG toward the TD, to alanine (R303A) did not cause any significant changes to Erm sensitivity (Fig. 1B), highlighting the specificity of residue R305 in regulating pore gating. Further, creating the double-mutant R305A+R303A did not further increase Erm sensitivity (Fig. 1B), indicating that R305 is a key residue involved in stability of the PLUG domain in the PapC pore. The OM protein expression levels of the PapC point mutants and deletion constructs are shown in Fig. S1.

Pili agglutinate human erythrocytes owing to the ability of the tip adhesin, PapG, to bind to Gal α 1–4Gal on their surface, providing a measurement of the presence of functional pili. Despite causing an open PapC pore, the R305 point mutants did not negatively affect functional pilus biogenesis (Table S1), indicating

that the pore gating function of the PLUG domain is distinct from its involvement in the catalysis of pilus assembly. Importantly, the Erm sensitivity phenotypes of the PapC point mutants R305A, R305A+R303A, and R305P were reversed under pilus-inducing conditions, likely owing to mature/polymerizing pili blocking the PapC pore and preventing entry of the antibiotic Erm. However, the PapC PLUG deletion mutant remained Erm-sensitive, consistent with its inability to assemble pili. Interestingly, α -helix and β 5–6-hairpin loop deletion constructs remained Erm-sensitive even under pilus-inducing conditions (Fig. 1C). This may suggest that the α -helix and β 5–6-hairpin loop are involved in retaining pili on the cell surface or that their deletion may cause a change in the occluded shape/size of the transmembrane barrel such that Erm can still be transported even when a polymerizing pilus fiber is transiting the channel.

Electrophysiology Experiments Suggest a Possible Destabilization of the PLUG Domain in the R305A Mutant. As previously documented (25), the WT PapC channel is characterized by many short openings of various sizes, seen as downward spikes in (Fig. 2*A*). Few of these transitions have a conductance greater than 1 ns, and those that do are extremely transient (a few milliseconds). These small transitions have been interpreted as originating from the “jiggling” of the PLUG domain and/or the dynamic interference of the N- and C-terminal domains with the ion conduction pathways identified at the interface of the PLUG and the TD. However, in 7 out of 10 experiments performed on R305A PapC the channels displayed occasional large transitions that lasted for several seconds (3–30 s) (Fig. 2*B*), in addition to the openings of small conductance similar to those of the WT channel. These large openings have a conductance of ~4 ns, which is similar to the monomeric conductance of the PLUG-less PapC mutant (25), and thus could represent PLUG release. Just as was observed for the PLUG-less mutant (25), numerous flickers to lower conductance levels occur within the large openings,

Fig. 2. The R305C PapC channel displays frequent prolonged openings of large conductance. Electrophysiological recordings of the activity of PapC WT (*A*) and PapC R305A mutant (*B*) were obtained in 1 M KCl and 5 mM Hepes, pH 7.2, at -50 mV. The current level corresponding to closed channels is marked by C. Downward deflections represent opening events, as shown by the arrows. The segment of the R305A trace highlighted by a gray box is shown under the trace at an expanded time scale to illustrate a typical prolonged opening of ~ 4 ns.

Fig. 3. Salt bridge interaction between R305 and E467 residues is important in stability of PLUG in PapC pore. Erm sensitivity assays of PapC salt bridge mutants reveal that disrupting the residues involved in salt bridge interaction gives rise to a more open pore (R305A, R305A+R303A, E467A, and R305E) compared with WT PapC. The locations of the residues forming the salt bridge can effectively be switched to reverse the increased pore opening phenotype back to WT levels (R305E+E467R). Significance compared with WT: * $P < 0.05$, ** $P < 0.03$, *** $P < 0.005$, Mann-Whitney test.

probably representing a combination of the dynamic behavior of the released PLUG and the mobility of various other elements (loops or the β -barrel itself) within the conductance pathway. Therefore, although the behavior is not consistently observed in every sweep, the R305A channels seem to be more prone to spontaneously adopt a state in which the PLUG domain has been displaced and the pore is fully open for a significant amount of time.

Salt Bridge Between the Usher PLUG and α -Helix Is Important for Stable Positioning of the PLUG in the PapC Pore. In the PapC crystal structure, the PLUG residue R305 forms a salt bridge with residue E467, which extends from the base of the α -helix toward the PLUG domain (Fig. 1A). An E467A mutation increased Erm sensitivity equivalent to R305A or deletion of the α -helix, suggesting that much of the α -helix's contribution to PLUG's positioning in the pore is mediated through this salt bridge (Fig. 3). Consistent with this hypothesis, mutation of R305 to the acidic residue glutamate similarly increased Erm sensitivity ($\sim 0.8\text{--}0.9\text{ cm izr}$), suggesting that the observed changes in pore gating are likely due to charge-charge interactions. We thus switched the locations of the charged residues by creating the R305E+E467R double mutant. This double mutant restored Erm sensitivity to WT levels ($\sim 0.5\text{--}0.6\text{ cm izr}$) (Fig. 3), revealing that reestablishment of the charge-charge interaction between these two residues restores stability of the PLUG in the closed usher state.

Usher Periplasmic Loop β 12–13 Is Involved in Pore Gating and Pilus Assembly. In the crystal structure of the FimD usher in complex with the FimCH chaperone-adhesin complex, the usher periplasmic loop residues S421 and L422, found between FimD's β -barrel strands 12 and 13, stack against the PLUG domain, suggesting that translocation of the PLUG to the periplasm may be stabilized by these interactions. To test this hypothesis, we replaced the mature PapC β 12–13 loop residues 430–433 with a glycine and expressed the mutant PapC from the arabinose-inducible plasmid pNH305. This resulted in a decreased Erv

part to preventing the dissociation of pili from the cell surface and leaving behind an activated, open pore (23).

Discussion

Protein secretion across the OM of Gram-negative bacteria is a critical step in bacterial pathogenesis. We investigated PapC and FimD in P and type 1 CUP pilus biogenesis, respectively, to better understand the mechanisms used by Gram-negative bacteria to assemble pili while regulating pore gating. The transmembrane β -barrel domains of PapC and FimD are composed of 24 β -strands surrounding a large central pore that is gated by a PLUG domain, as determined by crystal structures and planar lipid bilayer electrophysiology studies (3, 5, 25). The PapC central channel is $45\text{\AA} \times 25\text{\AA}$ in diameter and thus would potentially allow passage of molecules, such as antibiotics, from the extracellular milieu across the OM. Thus, strict pore gating is critical for bacterial homeostasis. Other β -barrel proteins that shuttle constituents across or into the outer membrane use the energy of ATP or the membrane potential across the inner membrane to regulate a gated channel, for instance the TonB-dependent translocators (29), or as in the case of the recently solved BamaA, seem to facilitate insertion of proteins into the outer membrane by opening/closing of the BamaA β -barrel (30). In CUP ushers, OM integrity is maintained via the existence of a β -sandwich PLUG domain, which resides in the transmembrane channel in the absence of pilus assembly (Fig. 7). The PLUG is also needed for catalysis of pilus assembly, working in concert with the NTD and CTD periplasmic domains of the usher (23, 25). Polymerization of subunits from chaperone-subunit complexes into a final pilus structure and extrusion of the fiber across the OM does not require either the energy of ATP or that of the proton-motive force (22), but rather seems to be the consequence of concerted binding events starting with the binding of a chaperone/adhesin complex to the usher (23, 31, 32).

Our structure-function analyses revealed that the PLUG is maintained in the pore via interactions that involve the α -helix and $\beta 5-6$ -hairpin loop. Furthermore, a specific PLUG residue mutation, R305A, interferes with PLUG stabilization as determined by planar lipid bilayer electrophysiology studies, where the mutant channel is characterized by an increased tendency to produce large and

Fig. 6. Importance of mature pili for OM integrity of Gram-negative bacteria. (A) Bacteria that express P pili without PapH have significantly increased Erm sensitivity compared with bacteria carrying full-length pili. No significant change was observed between the 12r of vector controls and WT PapH (Mann-Whitney test, $P > 0.5$) (B) Electron micrographs revealing the different pili morphologies in the presence and absence of PapH. WT pili length and abundance is observed when *papH* is expressed; in the absence of PapH, scarce, longer, and shedding pili are seen. Vector control shows the absence of P pili.

Fig. 7. Structural view of the determinants of PLUG positioning in usher states. (A) A single salt bridge between R305 (blue surface) and E467 (orange surface) respectively emanating from the PLUG domain (marine cartoon) and β -barrel wall (orange cartoon) extending from underneath the α -helix (green) and above the β 5–6-hairpin loop (magenta) stabilizes the PLUG in the closed, apo usher state. Results were mapped onto the structure of the PapC translocation pore (PDB ID code 2VQI). (B) The β 12–13 loop (orange surface) along with the engineered cysteine mutants at position 114 (cyan surface) within the NTD (cyan cartoon) and position 281 (blue surface) within the PLUG domain promotes PLUG stability in the open, active usher state. Results were mapped onto the structure of the FimD usher in complex with the chaperone-adhesin FimCH (PDB ID code 3RFZ).

persistent openings. Residue R305 forms a salt bridge interaction with residue E467 (Fig. 7). Disruption of this salt bridge interaction promotes an open usher state, whereas flipping the residue identities across the two positions maintains electrostatic contact and the WT antibiotic sensitivity phenotype, confirming the critical nature of this salt bridge as part of a mechanism of pore gating. Salt bridges were also shown to be important factors in the gating of much smaller pores without a PLUG domain such as OmpC and OmpA (33, 34). Despite increased pore permeability of the R305A mutant, functional pilus biogenesis was not affected. This was also the result for the P281C + T114C double cysteine mutant of FimD. Although we were unable to verify that P281C formed a disulfide bond with T114C, the results imply that usher activity in pilus biogenesis is independent of the ability of the PLUG to be stably maintained in the usher pore, because this mutant is mostly in an open conformation as determined by Erm sensitivity.

The translocation of the PLUG domain to the periplasm seems to be stabilized by the periplasmic loop extending from β -strands 12 and 13, which stacks against the PLUG in the open conformation (Fig. 7) in the FimDCH structure and may help stabilize the PLUG in an orientation that binds to the NTD in the active usher. Mutating the β 12–13 loop significantly decreased the open conformation of the pore, resulting in Erm sensitivity levels equal to vector control levels, despite mutant PapC expression levels equal to the WT. The β 12–13 loop mutation also caused a significant decrease in functional pilus assembly, presumably owing to its inability to stably relocate the PLUG and open the pore. However, a low level of pilus assembly was still observed (Fig. 4 and Fig. S2) in the presence of chaperone-subunit complexes, indicating that the mutant usher is capable of carrying out the necessary conformational changes to open the pore and function in pilus biogenesis, albeit less efficiently.

We found that deletion of the terminator subunit, PapH, caused a significant increase in OM permeability, presumably owing to the shedding of pili from the cell surface and the NTD/PLUG domain interaction that leaves the usher in an open state. In the presence of WT pilus expression, Erm sensitivity is significantly lower. This finding suggests proper PapH-mediated pilus

termination is necessary to retain OM integrity. These results highlight the necessity of organized CUP operons to ensure strictly regulated expression of all subunits and suggest that compounds that could prevent this termination event could increase antibiotic efficacy and thus have therapeutic value.

Overall, our studies reveal mechanisms by which OM usher proteins regulate channel conductivity and pilus biogenesis. Results obtained in this study indicate important interactions needed for the usher to retain OM integrity while catalyzing efficient pilus biogenesis and extrusion to the cell surface.

Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions. Bacterial strains were grown in LB agar, Tryptic Soy Agar (TSA), or LB broth with antibiotics as appropriate. Detailed strain and plasmid information is provided in [Table S2](#).

Erm Sensitivity Assays. Erm sensitivity was determined using disk diffusion assays on agar plates using discs containing Erm (15 µg; BD Biologicals). [SI Materials and Methods](#) gives further details.

HA Titer Assays. HA titers for type 1 pili were carried out using cells after 2 × 24 h of 10 mL static bacterial growth with appropriate antibiotics as described previously. HA titers for P pili were performed on bacteria grown on TSA plates overnight as previously described (35).

Pili and protein expression/purification, electrophysiology, and pili assays were done as previously described (23, 25, 36). Details can be found in [SI Materials and Methods](#).

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