BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

pubs.acs.org/biochemistry

Functional Mapping of PilF and PilQ in the *Pseudomonas aeruginosa* Type IV Pilus System

Jason Koo,[†,‡] Tim Tang,[‡] Hanjeong Harvey,[§] Stephanie Tammam,[†,‡] Liliana Sampaleanu,[†] Lori L. Burrows,*[,§] and P. Lynne Howell*[,†,‡]

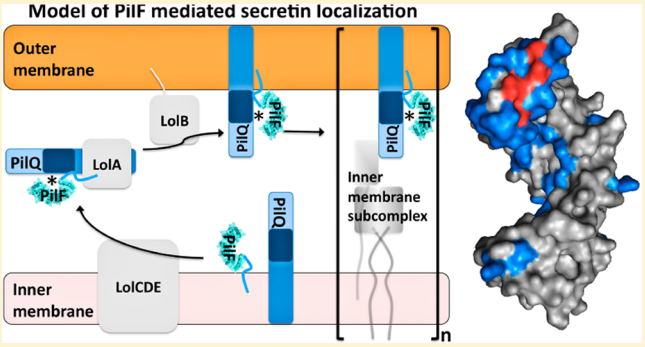
[†]Program in Molecular Structure and Function, Research Institute, Hospital for Sick Children, Toronto, ON, Canada

[‡]Department of Biochemistry, University of Toronto, Toronto, ON, Canada

[§]Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, ON, Canada

Supporting Information

ABSTRACT: *Pseudomonas aeruginosa* uses type IV pili (T4P) to interact with the environment and as key virulence factors when acting as an opportunistic pathogen. Assembly of the outer membrane PilQ secretin channel through which the pili are extruded is essential for pilus biogenesis. The *P. aeruginosa* T4P pilotin, PilF, is required for PilQ outer membrane localization and assembly into secretins and contains six tetratricopeptide (TPR) protein−protein interaction motifs, suggesting that the two proteins interact. In this study, we found that the first four TPR motifs of PilF are sufficient for PilQ outer membrane targeting, oligomerization, and function. Guided by our structure of PilF, site-directed mutagenesis of the protein surface revealed that a hydrophobic groove on the first TPR is required for PilF-mediated PilQ assembly. Deletion of individual domains within PilQ suggests that the N0, KH-like, or secretin domain, but not the C-terminus, interacts with PilF. Purified PilQ was found to pull down PilF from *Pseudomonas* cell lysates. Together, these data allow us to propose a model for PilF function in the T4P system. PilF interacts directly or indirectly with the PilQ monomer after translocation of both proteins through the inner membrane and acts as a co-chaperone with the Lol system to facilitate transit across the periplasm to the outer membrane. The mechanism of PilQ insertion and assembly, which appears to be independent of the Bam system, remains to be determined.



Type IV pili (T4P) are cell envelope-spanning biomolecular machines produced by bacteria and archaea. Extension, adhesion, and retraction of T4P mediate interactions between the organism and its environment.[1−7] In many pathogens, including *Pseudomonas aeruginosa*, T4P are important for virulence. An important step in T4P biogenesis is the formation of the outer membrane secretin channel that allows passage of T4P fibers out of the cell. The T4P secretin in *P. aeruginosa* is a dodecamer of PilQ subunits. PilF, the T4P pilotin, is required for localization of PilQ to the outer membrane and its multimerization.[8] While no structure of *P. aeruginosa* PilQ is yet available and only fragments of *Neisseria meningitidis* PilQ have been determined,[9] that of PilF [Protein Data Bank (PDB) entries 2HO1 and 2FI7] and that of its *N. meningitidis* ortholog, PilW (PDB entry 2VQ2), have been determined.[8,10,11] PilF and PilW each contain six tetratricopeptide (TPR) protein−protein interaction motifs, consistent with the hypothesis that PilF orthologs interact directly or indirectly with PilQ.

Pilotins from the type II and III secretion systems (T2SS and T3SS, respectively) have been structurally characterized, permitting the division of pilotins into three distinct structural classes:[12] Class 1 pilotins such as PilF and PilW of T4P systems are α-helical and contain TPRs. Class 2 pilotins associated with T3SS are composed primarily of β-strands. Class 3 pilotins associated with T2SS contain non-TPR α-helices. The pilotins from the *Erwinia chrysanthemi* T2SS (OutS), *Klebsiella oxytoca* T2SS (PulS), and *Shigella flexneri* T3SS (MxiM) each bind C-terminal to the membrane-embedded "secretin domain" of their corresponding secretin subunits.[13−15] In all three cases, the C-terminus undergoes a disorder-to-order transition upon pilotin binding. This mode of pilotin−secretin interaction does not appear to be absolutely conserved, as deletion of the equivalent C-terminal region of *Yersinia enterocolitica* YscC does not perturb T3SS function.[16] Furthermore, only some secretins require pilotins for full functionality.[17]

The way in which Class 1 TPR pilotins interact with their secretin subunits has yet to be characterized. TPR proteins contain multiple copies of an approximately 34-amino acid motif, which forms a superhelical fold that mediates protein−protein interactions in both prokaryotes and eukaryotes. Sequence conservation of the TPR motif is limited only to

Received: November 13, 2012
Revised: March 19, 2013
Published: April 2, 2013

   dx.doi.org/10.1021/bi3015345 | *Biochemistry* 2013, 52, 2914−2923

Table 1. Strains and Vectors Used in This Study

	strain or vector	characteristics	source or ref
strain			
	Escherichia coli BL21-CodonPlus(DE3)-RP	F^- *ompT hsdS*(r_B^- m_B^-) *dcm*$^+$ Tet^r *gal* λ(DE3) *endA* Hte [*argU proL* Cam^r]	Novagen
	P. aeruginosa mPAO1	wild-type strain	Jacobs et al.[47]
	mPAO1 *pilF*::Tn*5*	transposon insertion mutant of *pilF*	Jacobs et al.[47]
	mPAO1 *pilQ*::FRT	FRT scar insertion mutant at position 571 of *pilQ*	this work
vector			
	pET26b-$pilQ_{his}$	*pilQ* inserted into pET26b at the *Nde*I and *Xho*I restriction sites in frame with a C-terminal six-His purification tag; a silent mutation removes an internal *Xho*I site	this work
	pET28a-*pilF*	PAO1 *pilF* construct	Koo et al.[8]
	pUCP20Gm	shuttle vector with *Sma*I-flanked Gm cassette inserted into *Sca*I site within *bla*	Chiang and Burrows[2]
	pUCP20Gm-*pilF*	PAO1 *pilF* construct	Koo et al.[8]
	pUCP20Gm-*pilF* X#Y	PAO1 *pilF* construct with mutation of residue number # from amino acid X to Y	this work
	pUCP20Gm-$pilF_{his}$	PAO1 $pilF_{his}$ construct	this work
	pUCP20Gm-$pilF_{his}$ ΔL	PAO1 $pilF_{his}$ construct lacking residues 22−30 between the lipobox and TPR1	this work
	pUCP20Gm-$pilF_{his}$ ΔTPR#	PAO1 $pilF_{his}$ construct with deletion of TPR number #	this work
	pUCP20Gm-*pilQ*	PAO1 full-length *pilQ* construct inserted at the *Eco*R1 and *Hin*dIII sites	this work
	pUCP20Gm-*pilQ* ΔSS	PAO1 *pilQ* construct lacking residues 26−280	this work
	pUCP20Gm-*pilQ* ΔN01	PAO1 *pilQ* construct lacking residues 281−475	this work
	pUCP20Gm-*pilQ* ΔL	PAO1 *pilQ* construct lacking residues 477−517	this work
	pUCP20Gm-*pilQ* Δ#	PAO1 *pilQ* construct with C-terminal truncation starting at residue #	this work

residues that allow the motif to fold. Different surfaces along the superhelical fold can be used to bind the associated substrate(s). For example, the Hsp-organizing protein from *Saccharomyces cerevisiae* uses the central groove of the TPR superhelix to bind a protein substrate without regular secondary structure (PDB entry 1ELW).[18] Both the central groove and the convex surfaces of the *S. cerevisiae* mitochondrial fission TPR protein Fis1 are involved in binding the α-helical Caf4 protein (PDB entry 2PQR),[19] while multiple surfaces of *P. aeruginosa* PscG are involved in simultaneous binding of its partners, PscE and PscF (PDB entry 2UWJ).[20] TPR-containing proteins, such as *Homo sapiens* O-linked GlcNAc transferase, may be linked to additional, non-TPR domains (PDB entry 3PE4).[21] Given the variable modes with which TPR proteins and their partners interact, the structure of PilF alone is insufficient to identify the surfaces involved in its pilotin role.

Secretin subunits can be divided into a conserved C-terminal region, containing the secretin domain that is putatively embedded into the outer membrane, and a variable, system-specific N-terminal region.[22,23] Both regions may interact with other components of the system as well as with the substrates to be secreted or internalized. The N-terminal region contains multiple subdomains: (1) system- and species-specific elements at the extreme N-terminus, (2) a N0 domain resembling the TonB-dependent signaling receptor that may allow signal transduction between the inner membrane and outer membrane components of the system during secretion or uptake,[24,25] and (3) up to five heterogeneous nuclear ribonucleoprotein K homology (KH)-like domains (also named N1−N5) that may fulfill the DNA binding role in competence systems.[26] Secretins exhibit varying requirements for pilotins, accessory proteins, and cellular machineries for their proper outer membrane localization and assembly.[12,27]

Functional mapping of PilF's secretin assembly function has revealed a narrow hydrophobic groove on the first TPR motif as a putative PilQ interaction interface. Deletion mutagenesis also revealed that TPR2−TPR4 are required for secretin assembly; these motifs may contain additional functional surfaces that were not identified by site-directed mutagenesis or may be required to maintain the protein fold. Domain deletions of PilQ suggested that the N0, KH-like, or secretin domain, but not the C-terminus, is required for PilF-mediated secretin assembly, differentiating Class 1 pilotins from those previously characterized. Also, recombinant *P. aeruginosa* PilQ was expressed and purified and could pull down PilF from *P. aeruginosa* cell lysates, thus providing the first evidence of an interaction between the two proteins.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Vectors. Table 1 lists the bacterial strains and vectors used in this study. Antibiotic concentrations and introduction of the vectors into the bacterial strains were described previously.[8] The *pilQ*::FRT mutant was generated using the method outlined by Ayers et al.[28]

PilF Complementation Vector Generation and Mutagenesis. pUCP20Gm-$pilF_{his}$ was made using the pET28a-*pilF* vector generated previously.[8] QuikChange site-directed mutagenesis (Stratagene) was used to remove the stop codon between *pilF* and the downstream six-His tag in pET28a as well as to insert an *Sph*I restriction site after the six-His tag. The *Bam*HI and *Sph*I restriction sites were used to cleave and insert $pilF_{his}$ from pET28a-$pilF_{his}$ into pUCP20Gm. pUCP20Gm-$pilQ_{his}$ was generated by polymerase chain reaction amplification from mPAO1 genomic DNA with *Eco*RI and *Hin*dIII restriction sites at the N- and C-termini, respectively, for insertion into pUCP20Gm. The pET26b-$pilQ_{his}$ construct was generated in a similar manner but with *Nde*I and *Xho*I restriction sites at the N- and C-termini, respectively. An internal *Xho*I site was removed with a silent mutation to facilitate manipulation. Mutations of *pilF* and *pilQ* were made using either the QuikChange site-directed mutagenesis kit (Stratagene) or the "Round-the-horn" site-directed mutagenesis method.[29]

Assessment of Protein Expression by Western Blotting. Cell lysate preparations were made, after normalization by OD_{600}, by addition of equal volumes of 2× SDS−PAGE sample buffer with cell cultures grown overnight at 37 °C while being shaken at 200 rpm in Luria-Bertani (LB) broth.

dx.doi.org/10.1021/bi3015345 | *Biochemistry* 2013, 52, 2914−2923

Membrane fractionation samples were mixed at a 3:1 ratio with 4× SDS−PAGE sample buffer. Samples were separated by 12% SDS−PAGE and transferred to PVDF membranes. Purified polyclonal rabbit antibodies, generated with help from Lab Animal Services at The Hospital for Sick Children, to the Pil proteins were used to detect proteins of interest. Alkaline phosphatase conjugated to goat anti-rabbit IgG (Bio-Rad) was used as the secondary antibody. A BCIP (5-bromo-4-chloro-3′-indolylphosphate *p*-toluidine salt) and NBT (nitro-blue tetrazolium chloride) solution (BioShop) was used to develop the blots as described previously.[28]

Twitching Motility Assay. Twitching motility was assessed by stab inoculation of the cell strains into 1% LB agar plates. Uncoated Fisherbrand Petri dishes were incubated for a minimum of 24 h at 37 °C. Falcon tissue culture dishes treated by vacuum gas plasma were left at room temperature overnight followed by 7 h at 37 °C. The agar was removed, and cells adhering to the polystyrene plate were stained with 0.1% (w/v) crystal violet dissolved in water. The area of the twitching zone was measured using ImageJ (National Institutes of Health, Bethesda, MD) from a minimum of nine replicates.

Phage Sensitivity Assay. *P. aeruginosa* strains were tested for sensitivity to PO4 phage that target retractile T4P as previously described.[8] Three microliters of PO4 phage (approximately 10^8 plaque-forming units/mL) was spotted on an LB agar plate. The strain to be tested was then streaked through the drop. Sensitivity was defined as a lack of growth after contact with PO4 phage after overnight incubation at 37 °C.

PilQ Purification and Pull Down. To express full-length $PilQ_{his}$, pET26b-*pilQ*$_{his}$ was transformed into BL21(DE3). Overnight cultures (10 mL) were grown at 37 °C and used to inoculate 1 L of LB. The culture was grown to an OD_{600} of 0.4−0.5 and induced with isopropyl β-D-1-thiogalactopyranoside to a final concentration of 1 mM. Cultures were then grown at 37 °C overnight and harvested by centrifugation (7300*g* at 4 °C for 20 min) before fractionation using the method adapted from Schnaitman.[30,31] The cell pellet was resuspended in 26 mL of 30 mM Tris (pH 7.9), 20% (w/v) sucrose, 0.1 mg/mL lysozyme, 20 μg of RNase (Sigma-Aldrich), and 100 units of DNase (Fermentas) and incubated on ice for 20 min. Cells were pelleted by centrifugation (2790*g* at 4 °C for 20 min). The pellet was resuspended in 25 mL of buffer A [30 mM Tris (pH 8.0)]. To lyse the cells, the mixture was either sonicated for five cycles of 60 s pulses and then cooled for 60 s on ice or homogenized three times at 12000−15000 lb/in.^{2}. Cell debris was pelleted (2790*g* at 4 °C for 20 min). The soluble fraction containing cellular membranes was pelleted by centrifugation (90000*g* at 4 °C for 90 min). The pellet was resuspended in 26 mL of 25 mM Tris (pH 8.0), 1 mM $MgCl_2$, and 2% (v/v) Triton X-100 and vortexed intermittently for 30 min at room temperature to solubilize the inner membranes. Outer membranes were then collected by centrifugation (90000*g* at 4 °C for 90 min).

The outer membranes, containing $PilQ_{his}$, were resuspended in 10 mL of buffer A with 1% (w/v) Fos-Choline-12 (FC12, Anagrade Anatrace) overnight at 4 °C. The mixture was hand homogenized and insoluble debris pelleted by centrifugation (90000*g* at 4 °C for 90 min). The solubilized outer membranes were applied to a Ni-NTA resin column pre-equilibrated with buffer B [30 mM Tris (pH 8.0) with 0.1% (w/v) FC12]. To remove nonspecifically bound proteins, the column was washed with 50 mL of buffer B with 5 mM imidazole followed by 50 mL of buffer B with 20 mM imidazole. Bound proteins were eluted from the column with 15 mL of buffer B with 900 mM imidazole. Imidazole was removed by buffer exchange with a 50 kDa centrifugal filter using buffer B (Figure S1 of the Supporting Information).

To prepare *P. aeruginosa* lysate for the pull down, a 10 mL overnight culture was used to inoculate 1 L of LB broth. The culture was then grown overnight at 37 °C and harvested by centrifugation (7300*g* at 4 °C for 25 min). The cells were resuspended in buffer B and lysed by sonication for five cycles of 60 s pulses and then cooled for 60 s on ice. Cell debris was spun down (2790*g* at 4 °C for 20 min). Approximately 3 mg of $PilQ_{his}$ was then mixed with 6 mL of Ni-NTA resin (Qiagen) pre-equilibrated in buffer B. Soluble cell lysate was applied to the $PilQ_{his}$-Ni-NTA resin on the column. The column was then washed with 50 mL of buffer B and 50 mL of buffer B with 25 mM imidazole. Absorbance at 280 nm was monitored to ensure the wash volumes were sufficient to remove unbound protein. Bound proteins were eluted with 15 mL of buffer B with 900 mM imidazole. The elution was concentrated 10-fold using a 10 kDa centrifugal filter and mixed at a 3:1 ratio with 4× SDS−PAGE sample buffer.

RESULTS

Importance of Individual Motifs within PilF. The minimal number of TPRs required to form a functional domain is three,[32] suggesting that only a subset of TPRs in proteins containing more than three repeats may be involved in partner binding, or that a TPR protein may bind to multiple partners. To identify the minimal functional region(s) of PilF, individual motifs were deleted from the pUCP20Gm-*pilF*$_{his}$ vector and the effects of the mutations were determined by complementation of a *pilF* mutant. T4P function was assayed using two techniques: (1) T4P-mediated movement of cells on a solid surface, called twitching motility,[33] and (2) sensitivity to PO4 bacteriophage, which target retractile T4P.[34−36] Several truncation mutants were made, including deletion of (1) residues 22−30, part of the linker between the lipidation site (C18) and the first TPR domain, which were not observed in the PilF structure and are predicted to be disordered; (2) individual TPRs; and (3) pairs of TPRs (Figure 1A). Deletion of the linker region, individual TPR5 or TPR6, or TPR5 and TPR6 together did not affect T4P-mediated twitching motility (Figure 1B) or sensitivity to bacteriophage (data not shown). Deletions affecting TPR1, -2, -3, or -4 resulted in loss of function. Therefore, TPR1−TPR4 are essential for PilF stability and/or function in PilQ assembly. Western blots for PilF showed that all mutant proteins except ΔTPR1,2 were expressed, and that the absence of twitching motility correlated with the lack of PilQ multimers (Figure 1C). As the PilF-specific polyclonal antibody cannot differentiate between a change in stability caused by the deletions within PilF and the loss of key epitope(s) required for antibody binding, several commercial antibodies against polyhistidine epitopes were tested for their ability to detect $PilF_{his}$ in *Pseudomonas* cell lysates. None produced a stronger signal than the PilF-specific polyclonal antibody (data not shown). Attempts to add a secondary six-His tag between the lipidation site at C18 and the TPR domain did not yield a stronger signal with the commercial polyhistidine antibodies (data not shown).

Functional Mapping of the PilF Surface. To map and identify functionally important surfaces of PilF, we used site-directed mutagenesis. Residues were selected on the basis of

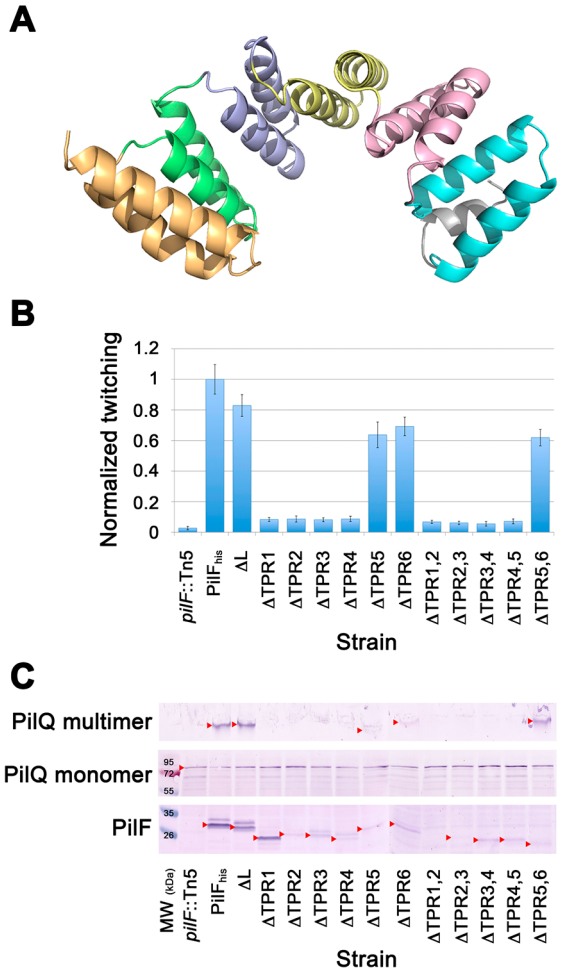


Figure 1. Mapping PilF by deletion mutagenesis. (A) Ribbon diagram of PilF showing the individual TPRs. TPR1−TPR6 are colored orange, green, purple, yellow, red, and blue, respectively. (B) Analysis of twitching motility of the TPR mutants. All values have been normalized to the wild-type complement $PilF_{his}$. (C) Western blots for PilF and PilQ for the TPR deletion mutants. The bands corresponding to PilF and PilQ are highlighted with red arrowheads.

several criteria: (1) the side chain was more than 10% exposed on the protein surface; (2) the residue was not required for TPR packing, i.e., was not part of the TPR consensus sequence; and (3) the residue was conserved across Pseudomonads. As such residues were primarily located on the concave surface of PilF, additional residues from the convex surface were selected using the first two criteria. The residues of interest are shown in Figure 2A. Residues were mutated to invert their chemical properties or to introduce a bulky side chain that could perturb a functional surface. Stable expression of mutant proteins and their effects on T4P functions and on PilQ assembly were then examined using *in vivo* complementation as described above (Figure 2B,C and Table 2). Although most mutations had no effect, alteration of residues A36, A39, L43, and G46 (which form a hydrophobic groove on TPR1 that is 18 Å long and one residue wide, with an accessible surface area of approximately 200 $Å^2$) to polar side chains resulted in a loss of PilQ outer membrane localization and/or multimerization and T4P function without significantly affecting protein folding (Figures S2−S6 of the Supporting Information). Decreasing the size of the side chain at position 43 from Leu to Ala also had a negative effect. Mutation of these residues did not significantly affect the overall fold or stability of PilF (see the Supporting Information). To define the extent of this functional surface, adjacent surface residues not involved in TPR packing were also mutated to Ala or Tyr (or Trp in the case of Y40). None of these mutations affected T4P function or secretin assembly (Figure 2B,C and Table 2). Together, the results suggest that a narrow, defined hydrophobic groove on TPR1 is important for PilF's secretin assembly role. Consistent with the results of motif deletion mapping, mutations in TPR5 and TPR6 or those outside the hydrophobic groove had no significant affect on T4P function.

Functional Mapping of PilQ. After defining a key surface on PilF involved in PilQ assembly and localization, we next sought to identify the region(s) on PilQ that might be critical for its correct insertion into the outer membrane. PilQ can be divided into several domains on the basis of sequence conservation, structural alignments with fragments of secretin subunits, and secondary structure predictions (Figure 3A). To characterize these domains *in vivo*, deletions were introduced into the pUCP20Gm-*pilQ* complementation vector and the resulting constructs introduced into a *pilQ* mutant (Figure 3B,D). Twitching motility (Figure 3E) and PO4 phage sensitivity (data not shown) were lost in the absence of the N0 and KH-like domains. Removal of the N-terminal system-specific domain significantly decreased, but did not abolish, twitching motility. Loss of the nonconserved linker region connecting the KH-like domain to the secretin domain had no effect on T4P function. Western blots showed that the mutant proteins were stably expressed and, with the exception of the N0 and KH-like domain deletion mutants, formed SDS- and heat-resistant multimers. The stability of mutant secretins in absence of either the system-specific domain or linker region was distinct from that of wild-type secretins, as a gradual return to the monomer form in SDS−PAGE sample buffer was noted over time (data not shown). No mutations in PilQ affected PilF expression (Figure 3D). The formation of secretin by PilQ therefore requires the more highly conserved N0 and KH-like domains but not the poorly conserved system-specific and linker regions.

Most Class 2 and Class 3 pilotins in T3SS and T2SS, respectively, interact with the secretin's distal C-terminus, following the secretin domain.[13−15] However, this mode of interaction is not universal.[16] Alignment of secretin sequences shows that those associated with Class 1 pilotins in T4P systems have much shorter C-terminal tails (Figure 3C). To determine if the C-terminus of PilQ is important for function, C-terminal residues were deleted. Four deletion mutants lacking the last two, four, six, or eight residues were generated and the resulting complementation constructs introduced into the *pilQ* mutant. Removal of residues from the distal C-terminus to residue 707 (i.e., the predicted boundary of the secretin domain) did not affect T4P function or secretin assembly (Figure 3D,E). A construct lacking residues 704−714, extending three residues into the predicted secretin domain, was unable to multimerize and was not functional (Figure 3D,E). These results suggest that the short C-terminal tail of PilQ is not required for interactions with PilF and differentiates the mode of Class 1 pilotin interaction from the other classes.

Characterization of a PilF−PilQ Interaction. While Class 1 pilotins are required for secretin function, it has not been established whether these TPR-type pilotins interact with the secretin. To determine if *P. aeruginosa* PilF and PilQ interact with one another and/or with other T4P proteins, pull down

dx.doi.org/10.1021/bi3015345 | *Biochemistry* 2013, 52, 2914−2923

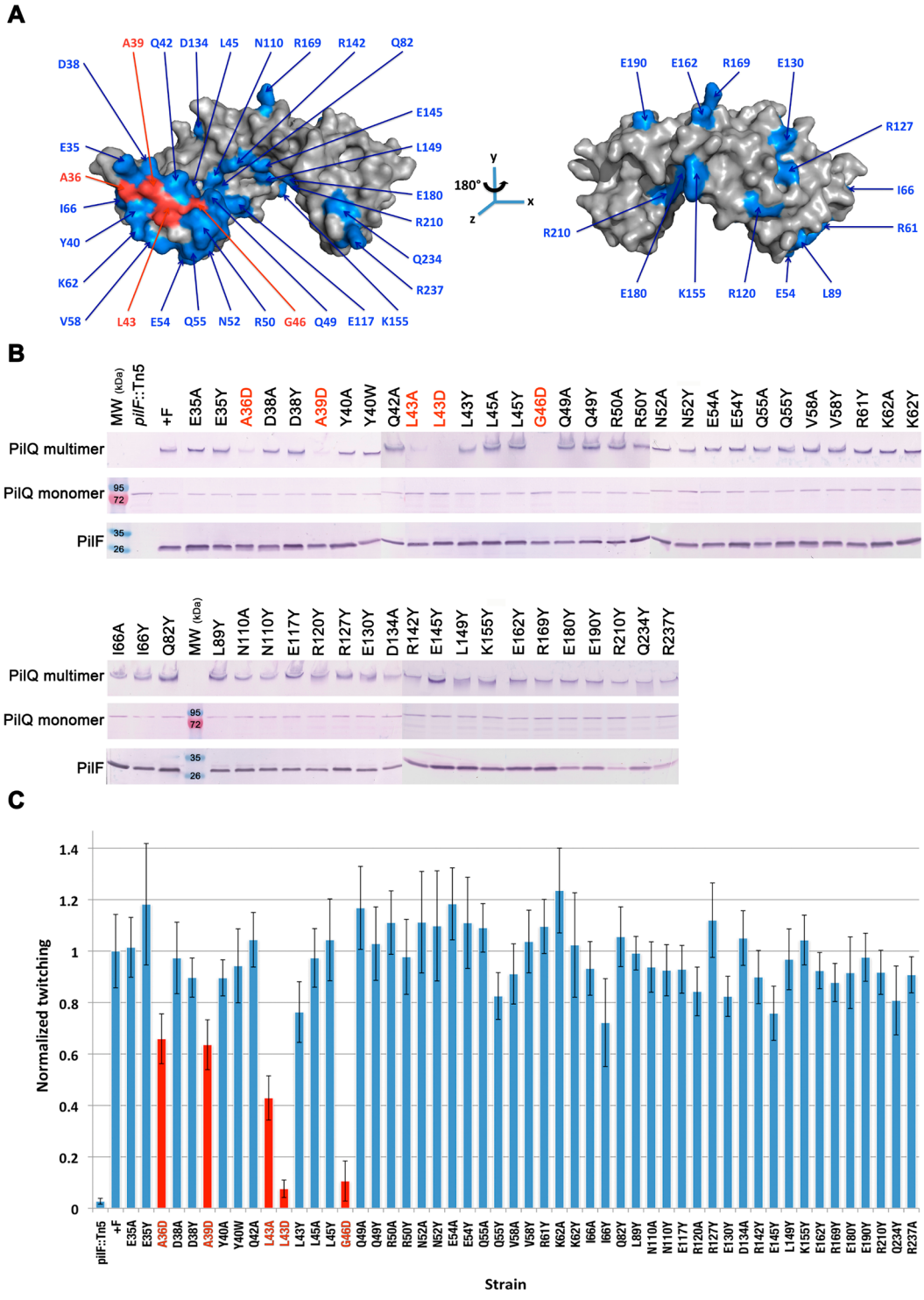


Figure 2. Mapping the surface of PilF by site-directed mutagenesis. (A) Surface representation of PilF showing the mutated residues. Residues that affect PilF function are colored red; all others are colored blue. (B) Western blots of PilF and PilQ in the *pilF*::Tn5 mutant, *pilF*::Tn5 complemented with wild-type PilF (+F), and *pilF*::Tn5 complemented with PilF point mutants. (C) Twitching motility of the mutants normalized to the wild-type complement +F.

assays were performed. $PilQ_{his}$ expressed and purified from *E. coli* was immobilized on Ni-NTA resin and used to capture interacting proteins from *P. aeruginosa* lysate. Western blots using antibodies to PilQ and PilF showed the expected presence of $PilQ_{his}$ but, more importantly, an enrichment of PilF in the elution fraction (Figure 4A). Enrichment for other T4P proteins (PilM, PilN, PilO, PilP, and PilA) was not detectable (data not shown). Soluble periplasmic fragments of PilQ, as described by Tammam et al.,[37] did not copurify PilF from mPAO1 lysates (data not shown). A control experiment showed that PilF does not bind nonspecifically to the Ni-NTA resin in the absence of $PilQ_{his}$ (Figure 4B). Similar pull down assays with the *pilF* mutant expressing either wild-type PilF or the Q42A, L43A, or G46D variant that has wild-type,

dx.doi.org/10.1021/bi3015345 | *Biochemistry* 2013, 52, 2914−2923

Table 2. Location of PilF Point Mutations and Their Effect on T4P-Mediated Phage Sensitivity

strain or mutant	location	effect on phage sensitivity[a]
pilF::Tn5	not applicable	−
+F	not applicable	++
E35A	TPR1	++
E35Y	TPR1	++
A36D	TPR1	+
D38A	TPR1	++
D38Y	TPR1	++
A39D	TPR1	+
Y40A	TPR1	++
Y40W	TPR1	++
Q42A	TPR1	++
L43A	TPR1	+
L43D	TPR1	−
L43Y	TPR1	++
L45A	TPR1	++
L45Y	TPR1	++
G46D	TPR1	−
Q49A	TPR1	++
Q49Y	TPR1	++
R50A	TPR1	++
R50Y	TPR1	++
N52A	TPR1	++
N52Y	TPR1	++
E54A	TPR1	++
E54Y	TPR1	++
Q55A	TPR1	++
Q55Y	TPR1	++
V58A	TPR1	++
V58Y	TPR1	++
R61Y	TPR1	++
K62A	TPR1	++
K62Y	TPR1	++
I66A	TPR1	++
I66Y	TPR1	++
Q82Y	TPR2	++
L89Y	TPR2	++
N110A	TPR3	++
N110Y	TPR3	++
E117Y	TPR3	++
R120A	TPR3	++
R127Y	TPR3	++
E130Y	TPR3	++
D134A	TPR3	++
R142Y	TPR4	++
E145Y	TPR4	++
L149Y	TPR4	++
K155Y	TPR4	++
E162Y	TPR4	++
R169Y	TPR4	++
E180Y	TPR5	++
E190Y	TPR5	++
R210Y	TPR6	++
Q234Y	TPR6	++
R237A	TPR6	++

[a]The symbols ++, +, and − denote wild-type, reduced, and abolished sensitivity, respectively, to the PO4 bacteriophage targeting retractile T4P.

decreased, or no T4P activity (Figure 2), respectively, indicate no change in their ability to be copurified by $PilQ_{his}$ (Figure S7 of the Supporting Information).

The reciprocal pull down assay with $PilF_{his}$ has been unsuccessful because of the difficulty in solubilizing PilQ from *P. aeruginosa* membranes. *In vivo* and *in vitro* cross-linking experiments aimed at producing PilF−PilQ complexes produced higher-molecular weight species that could not be resolved by SDS−PAGE or did not correspond to a single molecule of PilF bound to a single molecule of PilQ (data not shown). Therefore, these data show that PilF binds to PilQ, either directly or indirectly via as yet unidentified components in the cell lysate.

DISCUSSION

In *P. aeruginosa*, PilF is the pilotin required for outer membrane localization and assembly of PilQ into secretins.[8] TPR1−TPR4 of PilF form the minimal region required for secretin assembly and function, while a narrow hydrophobic groove on the first TPR mediates secretin assembly. Small grooves of non-TPR pilotins have similarly been implicated in their function.[13,15,38] The limited size of the PilF groove and the retained ability of mutants to be copurified with PilQ suggest that additional surfaces are involved or that other proteins may be involved in mediating the interaction between PilF and PilQ. While mutation of residues surrounding the groove had little effect on secretin assembly, single-point mutations may have been insufficient to perturb the interaction. Regions not contiguous with the groove that have yet to be mapped could also form part of the interface.

The site-directed mutagenesis results presented here reveal distinct differences between *P. aeruginosa* PilF and its *N. meningitidis* homologue, PilW. In PilW, aside from the lipidation site, only residues C115 and C150 that form a disulfide bond linking TPR3 and TPR4 were found to be important for PilQ multimerization.[39] While mutation of either cysteine had no effect on PilW stability, the effects of the mutations on the protein fold were not tested. The equivalent residues in PilF, Y116 and V150, respectively, are not absolutely conserved in Pseudomonads. Furthermore, both C150 in PilW and V150 in PilF are buried and thus unavailable to bind protein partners. Mutations of N110 in PilF (N109 in PilW) and residues in the same region identified to be important for PilW function by Szeto et al.[39] had no effect on twitching motility or secretin assembly in *P. aeruginosa* and could represent specific adaptations that allow natural competence and cell adhesion unique to *N. meningitidis*. However, the detrimental effects of PilW point mutations in TPR3 and TPR4 are consistent with our TPR deletion study and suggest a role for this region in PilF function.

Our mutation mapping of PilQ suggests that (1) the system-specific domain is not essential for multimerization but facilitates T4P function, (2) the N0 and KH-like domains are important for the formation of secretin multimers, and (3) consistent with it being one of the least conserved portions of PilQ, the linker region is not required for function. Similar results have been reported in the mapping of the *K. oxytoca* secretin, PulD.[40−42] PulD has an N-terminal N0 domain, three KH-like domains, and a secretin domain at its C-terminus. Unlike PilQ, PulD is able to multimerize both *in vivo* and *in vitro* in the absence of its pilotin, PulS. N-Terminal PulD truncations revealed that the last KH-like domain and the secretin domain form the minimal region required for

dx.doi.org/10.1021/bi3015345 | *Biochemistry* 2013, 52, 2914−2923

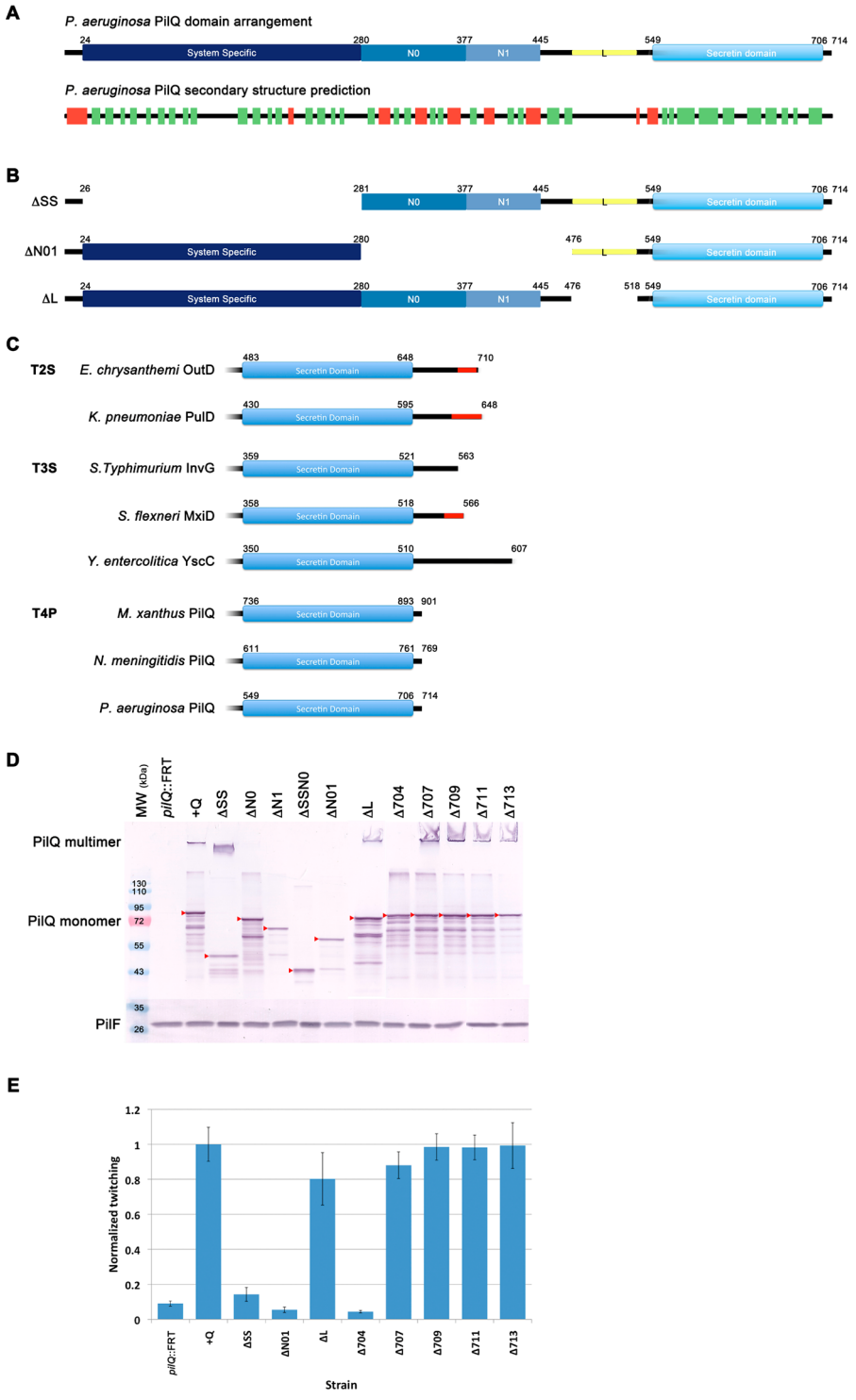


Figure 3. Functional mapping of PilQ. (A) Schematics of full-length PilQ. The domains of PilQ are shown in the top figure, and the predicted secondary structure is shown in the bottom figure with α-helices in red boxes, β-strands in green boxes, and regions without regular secondary structure colored black. (B) Schematics showing domain deletion mutants in which ΔSS represents the deletion of the system-specific periplasmic domain, ΔNO1 represents the deletion of the N0 and N1 KH-like domains, and ΔL represents the deletion of the linker region connecting N1 to the secretin domain. (C) Schematics showing C-terminal tails following the secretin domains of secretin subunits from T2SS, T3SS, and T4P systems. Regions highlighted in red have been shown to interact with their cognate pilotin. (D) Western blots for PilF and PilQ in the *pilQ*::FRT mutant, *pilQ*::FRT complemented with wild-type PilQ (+Q), domain deletions of PilQ, and C-terminal truncations of PilQ. The bands corresponding to PilQ and the PilQ mutants are highlighted with red arrowheads. (E) Analysis of twitching motility of the PilQ mutants where twitching has been normalized to +Q.

multimerization, consistent with our finding that a significant portion of the secretin N-terminus is dispensable for its assembly. Instead, these additional regions of PilQ could be involved in gating of the secretin in the absence of the pilus and/or form a stable conduit through the periplasm and peptidoglycan for the pilus.

dx.doi.org/10.1021/bi3015345 | *Biochemistry* 2013, 52, 2914−2923

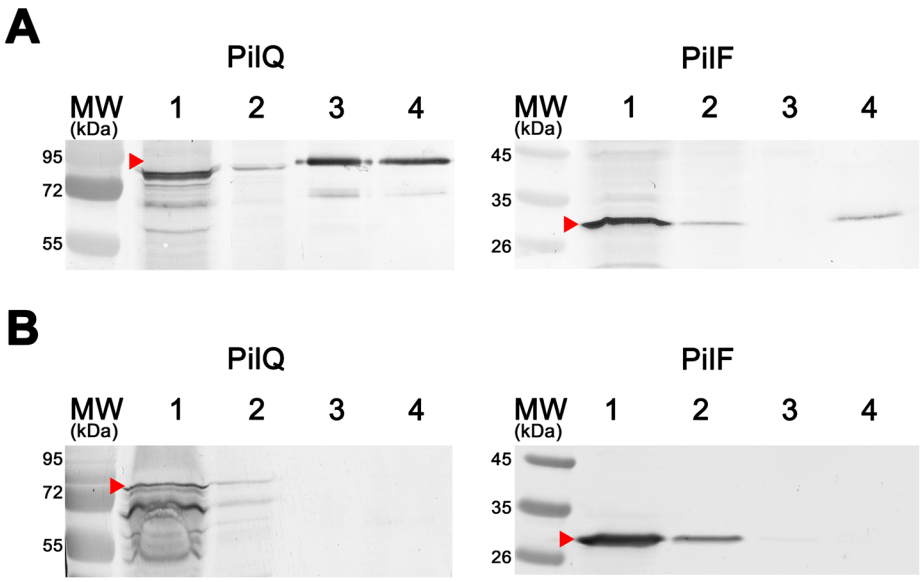


Figure 4. $PilQ_{his}$ pulldown of PilF from *P. aeruginosa* cell lysate. Western blots for PilF and PilQ pull down with $PilQ_{his}$ (A) loaded on the N-NTA column and (B) in the absence of $PilQ_{his}$. For each blot, lane 1 is the cell lysate flow-through, lane 2 the first wash fraction, lane 3 the second wash fraction, and lane 4 the elution of proteins bound on the Ni-NTA. The bands corresponding to PilF and PilQ ($PilQ_{his}$ in panel A and native PilQ in panel B) are highlighted with red arrowheads.

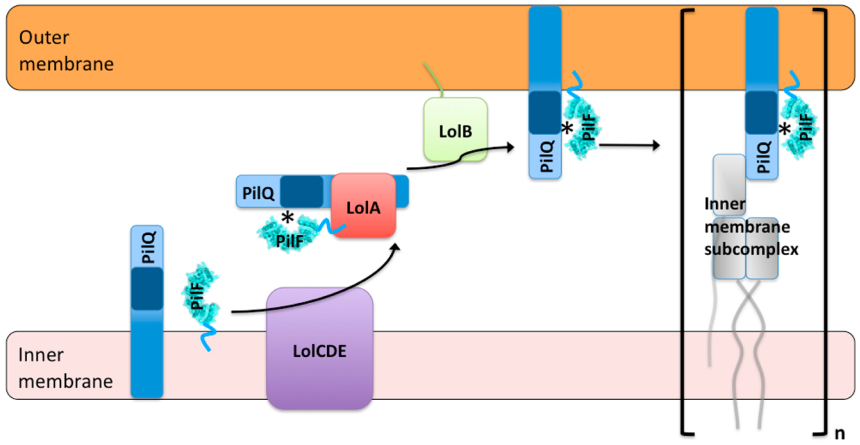


Figure 5. Proposed role of PilF in T4P assembly. PilF and PilQ interact directly or indirectly, as represented by the asteriks, at the inner membrane and are cotransported to the outer membrane by the Lol system. The N0 and N1 regions of PilQ that have been implicated in PilF-mediated secretin assembly are colored dark blue. PilQ then oligomerizes to form secretins and interacts with the inner membrane subcomplex to form functional T4P. The uncharacterized stoichiometry within and between the membrane subcomplexes is represented by "n".

In contrast to T2SS and T3SS secretins, the C-terminal region following the secretin domain of PilQ is dispensable for T4P function and therefore unlikely to be the site of PilF–PilQ interaction. Given the results of PilQ deletion mapping and the predicted proximity of PilF's critical TPR1 hydrophobic groove to the membrane–periplasm interface, a region within the N0, KH-like, or secretin domains likely contains either the PilF binding interface or the site that binds another protein to mediate the interaction between PilF and PilQ. Unlike that of secretins in many T2SS, PilQ assembly is not spontaneous but can be induced by a point mutation within the secretin domain.[43] Furthermore, the Bam cellular machinery that participates in the insertion of proteins into the outer membrane does not appear to be required for PilQ secretin formation and function.[27] The driving forces behind non-spontaneous PilQ assembly therefore remain unclear.

The copurification of PilF with PilQ indicates that the two proteins interact, but the use of cell lysates for this experiment does not exclude the possibility that another protein(s) mediates the interaction. While mutations of the hydrophobic groove on TPR1 of PilF affect T4P function, their copurification by PilQ indicates either that these residues mediate secretin assembly but not binding with PilQ or that a single point mutation is insufficient to completely abolish binding. The current assay does not allow the strength of the interaction to be quantified. While soluble fragments of PilQ can be copurified with the inner membrane T4P proteins,[44] no copurification was observed with the recombinant full-length protein. This is perhaps not surprising as the detergent present in the buffers required to maintain the solubility of full-length PilQ may interfere with or prevent protein binding. Purified PilF and PilQ did not copurify *in vitro*, nor could the complex be enriched by *in vivo* or *in vitro* cross-linking (data not shown).

dx.doi.org/10.1021/bi3015345 | *Biochemistry* 2013, 52, 2914–2923

The PilF−PilQ complex likely exists for a short time and is present at low frequency compared to what has been assembled into secretins and/or into T4P. Similar studies have also been unable to trap a soluble form of the PulS pilotin in complex with its associated PulD secretin subunit.[45] Combined, these results suggest that the interaction may be transient, occurs during protein folding, and/or is mediated by an additional protein partner(s). The additional partners could potentially include (but are not limited to) the inner membrane T4P proteins, cellular machinery involved in secretin formation, or a peptidoglycan-modifying protein that may be involved in making and/or maintaining a channel for the periplasmic region of the secretin.

The results of PilF and PilQ mutant characterization can be distilled into a working model of PilF function (Figure 5). The importance of the C18 lipidation site[8] and the membrane-targeting signal immediately following C18 suggest that PilF is initially transferred across the inner membrane by the Sec system and lipidated at C18. Bioinformatics analysis suggests that signal peptidase II would subsequently remove residues 1−17. PilQ is similarly predicted to be transported across the inner membrane where processing by signal peptidase I would remove the first 23 amino acids. The inner membrane localization of PilQ monomers in the absence of PilF[8] suggests they are initially retained there, although whether this occurs via the secretin domain or another region of PilQ is still unclear. The hydrophobic groove on TPR1 of PilF could then bind the N0, KH-like, or secretin domains of PilQ at the inner membrane or bind indirectly to PilQ through another protein. The outer membrane-targeting signal on PilF could then be recognized by the lipoprotein trafficking complex LolCDE, transferring the PilF−PilQ complex to the chaperone, LolA. If the PilF−PilQ−LolA complex were to pass through the periplasm and peptidoglycan, PilF and PilQ would be inserted into the outer membrane. LolB presumably inserts PilF, while PilQ either co-inserts spontaneously or requires other cellular machinery. The mechanism by which PilQ monomers assemble into a highly stable secretin is unclear, but the process does not appear to be spontaneous in T4P systems.[43] The inner membrane subcomplex of the T4P system[44,46] could then contact the PilQ−PilF complex at the outer membrane to align the two subcomplexes, allowing the pilus to pass through the outer membrane as it is polymerized. This greatly simplified model of T4P biogenesis both highlights the insights gained from our studies and indicates avenues that remain to be explored to understand T4P function. In particular, the stoichiometry and dynamics of the interactions within and across the outer and inner membrane subcomplexes during pilus assembly and disassembly remain to be determined.

■ ASSOCIATED CONTENT

Supporting Information

Mutants of the PilF hydrophobic groove on TPR1 (A36D, A39D, L43A, L43D, and G46D) compared to wild-type PilF by circular dichroism and the purification of full-length $PilQ_{his}$ and its ability to pull down PilF mutants. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*P.L.H.: Program in Molecular Structure and Function, The Hospital for Sick Children, 555 University Ave., Toronto, M5G 1X8 ON, Canada; phone, (416) 813-5378; fax, (416) 813-5379; e-mail, howell@sickkids.ca. L.L.B.: Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, ON, Canada; phone, (905) 525-9140, ext. 22029; fax, (905) 522-9033; e-mail, burrowl@mcmaster.ca.

Funding

This work was supported by Operating Grant MOP 93585 from the Canadian Institutes of Health Research (CIHR) to L.L.B. and P.L.H. J.K. and S.T. have been funded, in part, by graduate scholarships from CIHR and Cystic Fibrosis Canada, respectively. P.L.H. is the recipient of a Canada Research Chair.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

FC12, Fos-Choline 12; LB, Luria-Bertani; Ni-NTA, nickel-nitriloacetic acid; OD_{600}, optical density at 600 nm; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; SDS, sodium dodecyl sulfate; T2SS, type II secretion system; T3SS, type III secretion system; T4P, type IV pili; TPR, tetratricopeptide repeat.

■ REFERENCES

(1) Burrows, L. L. (2005) Weapons of mass retraction. *Mol. Microbiol. 57*, 878−888.

(2) Chiang, P., and Burrows, L. L. (2003) Biofilm formation by hyperpiliated mutants of *Pseudomonas aeruginosa*. *J. Bacteriol. 185*, 2374−2378.

(3) Mattick, J. S. (2002) Type IV pili and twitching motility. *Annu. Rev. Microbiol. 56*, 289−314.

(4) O'Toole, G. A., and Kolter, R. (1998) Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol. Microbiol. 30*, 295−304.

(5) Saiman, L., Ishimoto, K., Lory, S., and Prince, A. (1990) The effect of piliation and exoproduct expression on the adherence of *Pseudomonas aeruginosa* to respiratory epithelial monolayers. *J. Infect. Dis. 161*, 541−548.

(6) Semmler, A. B., Whitchurch, C. B., and Mattick, J. S. (1999) A re-examination of twitching motility in *Pseudomonas aeruginosa*. *Microbiology 145* (Part 10), 2863−2873.

(7) Whitchurch, C. B., and Mattick, J. S. (1994) Characterization of a gene, pilU, required for twitching motility but not phage sensitivity in *Pseudomonas aeruginosa*. *Mol. Microbiol. 13*, 1079−1091.

(8) Koo, J., Tammam, S., Ku, S. Y., Sampaleanu, L. M., Burrows, L. L., and Howell, P. L. (2008) PilF is an outer membrane lipoprotein required for multimerization and localization of the *Pseudomonas aeruginosa* Type IV pilus secretin. *J. Bacteriol. 190*, 6961−6969.

(9) Berry, J. L., Phelan, M. M., Collins, R. F., Adomavicius, T., Tonjum, T., Frye, S. A., Bird, L., Owens, R., Ford, R. C., Lian, L. Y., and Derrick, J. P. (2012) Structure and Assembly of a Trans-Periplasmic Channel for Type IV Pili in *Neisseria meningitidis*. *PLoS Pathog. 8*, e1002923.

(10) Kim, K., Oh, J., Han, D., Kim, E. E., Lee, B., and Kim, Y. (2006) Crystal structure of PilF: Functional implication in the type 4 pilus biogenesis in *Pseudomonas aeruginosa*. *Biochem. Biophys. Res. Commun. 340*, 1028−1038.

(11) Trindade, M. B., Job, V., Contreras-Martel, C., Pelicic, V., and Dessen, A. (2008) Structure of a widely conserved type IV pilus biogenesis factor that affects the stability of secretin multimers. *J. Mol. Biol. 378*, 1031−1039.

(12) Koo, J., Burrows, L. L., and Howell, P. L. (2012) Decoding the roles of pilotins and accessory proteins in secretin escort services. *FEMS Microbiol. Lett. 328*, 1−12.

(13) Gu, S., Rehman, S., Wang, X., Shevchik, V. E., and Pickersgill, R. W. (2012) Structural and functional insights into the pilotin-secretin complex of the type II secretion system. *PLoS Pathog. 8*, e1002531.

(14) Okon, M., Moraes, T. F., Lario, P. I., Creagh, A. L., Haynes, C. A., Strynadka, N. C., and McIntosh, L. P. (2008) Structural characterization of the type-III pilot-secretin complex from *Shigella flexneri*. *Structure 16*, 1544−1554.
(15) Tosi, T., Nickerson, N. N., Mollica, L., Jensen, M. R., Blackledge, M., Baron, B., England, P., Pugsley, A. P., and Dessen, A. (2011) Pilotin-secretin recognition in the type II secretion system of *Klebsiella oxytoca*. *Mol. Microbiol. 82*, 1422−1432.
(16) Burghout, P., Beckers, F., de Wit, E., van Boxtel, R., Cornelis, G. R., Tommassen, J., and Koster, M. (2004) Role of the pilot protein YscW in the biogenesis of the YscC secretin in *Yersinia enterocolitica*. *J. Bacteriol. 186*, 5366−5375.
(17) Viarre, V., Cascales, E., Ball, G., Michel, G. P., Filloux, A., and Voulhoux, R. (2009) HxcQ liposecretin is self-piloted to the outer membrane by its N-terminal lipid anchor. *J. Biol. Chem. 284*, 33815−33823.
(18) Scheufler, C., Brinker, A., Bourenkov, G., Pegoraro, S., Moroder, L., Bartunik, H., Hartl, F. U., and Moarefi, I. (2000) Structure of TPR domain-peptide complexes: Critical elements in the assembly of the Hsp70-Hsp90 multichaperone machine. *Cell 101*, 199−210.
(19) Zhang, Y., and Chan, D. C. (2007) Structural basis for recruitment of mitochondrial fission complexes by Fis1. *Proc. Natl. Acad. Sci. U.S.A. 104*, 18526−18530.
(20) Quinaud, M., Ple, S., Job, V., Contreras-Martel, C., Simorre, J. P., Attree, I., and Dessen, A. (2007) Structure of the heterotrimeric complex that regulates type III secretion needle formation. *Proc. Natl. Acad. Sci. U.S.A. 104*, 7803−7808.
(21) Lazarus, M. B., Nam, Y., Jiang, J., Sliz, P., and Walker, S. (2011) Structure of human O-GlcNAc transferase and its complex with a peptide substrate. *Nature 469*, 564−567.
(22) Burkhardt, J., Vonck, J., and Averhoff, B. (2011) Structure and function of PilQ, a secretin of the DNA transporter from the thermophilic bacterium *Thermus thermophilus* HB27. *J. Biol. Chem. 286*, 9977−9984.
(23) Korotkov, K. V., Gonen, T., and Hol, W. G. (2011) Secretins: Dynamic channels for protein transport across membranes. *Trends Biochem. Sci. 36*, 433−443.
(24) Brillet, K., Journet, L., Celia, H., Paulus, L., Stahl, A., Pattus, F., and Cobessi, D. (2007) A β strand lock exchange for signal transduction in TonB-dependent transducers on the basis of a common structural motif. *Structure 15*, 1383−1391.
(25) Larsen, R. A., Thomas, M. G., and Postle, K. (1999) Protonmotive force, ExbB and ligand-bound FepA drive conformational changes in TonB. *Mol. Microbiol. 31*, 1809−1824.
(26) Tarry, M., Jaaskelainen, M., Paino, A., Tuominen, H., Ihalin, R., and Hogbom, M. (2011) The Extra-Membranous Domains of the Competence Protein HofQ Show DNA Binding, Flexibility and a Shared Fold with Type I KH Domains. *J. Mol. Biol. 409*, 642−653.
(27) Hoang, H. H., Nickerson, N. N., Lee, V. T., Kazimirova, A., Chami, M., Pugsley, A. P., and Lory, S. (2011) Outer membrane targeting of *Pseudomonas aeruginosa* proteins shows variable dependence on the components of Bam and Lol machineries. *MBio 2*, e00246-11.
(28) Ayers, M., Sampaleanu, L. M., Tammam, S., Koo, J., Harvey, H., Howell, P. L., and Burrows, L. L. (2009) PilM/N/O/P proteins form an inner membrane complex that affects the stability of the *Pseudomonas aeruginosa* type IV pilus secretin. *J. Mol. Biol. 394*, 128−142.
(29) OpenWetWare-contributors (2010) Round-the-horn site-directed mutagenesis, OpenWetWare. http://openwetware.org/index.php?title=%27Round-the-horn_site-directed_mutagenesis&oldid=458415.
(30) Schnaitman, C. A. (1971) Effect of ethylenediaminetetraacetic acid, Triton X-100, and lysozyme on the morphology and chemical composition of isolate cell walls of *Escherichia coli*. *J. Bacteriol. 108*, 553−563.
(31) Schnaitman, C. A. (1971) Solubilization of the cytoplasmic membrane of *Escherichia coli* by Triton X-100. *J. Bacteriol. 108*, 545−552.
(32) Allan, R. K., and Ratajczak, T. (2011) Versatile TPR domains accommodate different modes of target protein recognition and function. *Cell Stress Chaperones 16*, 353−367.
(33) Lautrop, H. (1961) *Bacterium anitratum* transferred to the genus *Cytophaga*. *Int. J. Syst. Evol. Microbiol. 11*, 107−108.
(34) Bradley, D. E. (1972) Shortening of *Pseudomonas aeruginosa* pili after RNA-phage adsorption. *J. Gen. Microbiol. 72*, 303−319.
(35) Bradley, D. E. (1972) Evidence for the retraction of *Pseudomonas aeruginosa* RNA phage pili. *Biochem. Biophys. Res. Commun. 47*, 142−149.
(36) Bradley, D. E. (1974) The adsorption of *Pseudomonas aeruginosa* pilus-dependent bacteriophages to a host mutant with nonretractile pili. *Virology 58*, 149−163.
(37) Tammam, S., Sampaleanu, L. M., Koo, J., Manoharan, K., Daubaras, M., Burrows, L. L., and Howell, P. L. (2013) PilMNOPQ from the *Pseudomonas aeruginosa* Type IV Pilus System form a Transenvelope Protein Interaction Network that Interacts with PilA. *J. Bacteriol.*,.
(38) Korotkov, K. V., and Hol, W. G. (2013) Crystal structure of the pilotin from the enterohemorrhagic *Escherichia coli* type II secretion system. *J. Struct. Biol.*, 10.1016/j.jsb.2013.02.013.
(39) Szeto, T. H., Dessen, A., and Pelicic, V. (2011) Structure/function analysis of *Neisseria meningitidis* PilW, a conserved protein playing multiple roles in type IV pilus biology. *Infect. Immun. 79*, 3028−3035.
(40) Guilvout, I., Chami, M., Berrier, C., Ghazi, A., Engel, A., Pugsley, A. P., and Bayan, N. (2008) In vitro multimerization and membrane insertion of bacterial outer membrane secretin PulD. *J. Mol. Biol. 382*, 13−23.
(41) Guilvout, I., Chami, M., Engel, A., Pugsley, A. P., and Bayan, N. (2006) Bacterial outer membrane secretin PulD assembles and inserts into the inner membrane in the absence of its pilotin. *EMBO J. 25*, 5241−5249.
(42) Guilvout, I., Nickerson, N. N., Chami, M., and Pugsley, A. P. (2011) Multimerization-defective variants of dodecameric secretin PulD. *Res. Microbiol. 162*, 180−190.
(43) Nickerson, N. N., Abby, S. S., Rocha, E. P., Chami, M., and Pugsley, A. P. (2012) A Single Amino Acid Substitution Changes the Self-Assembly Status of a Type IV Piliation Secretin. *J. Bacteriol. 194*, 4951−4958.
(44) Tammam, S., Sampaleanu, L. M., Koo, J., Sundaram, P., Ayers, M., Andrew Chong, P., Forman-Kay, J. D., Burrows, L. L., and Howell, P. L. (2011) Characterization of the PilN, PilO and PilP type IVa pilus subcomplex. *Mol. Microbiol. 82*, 1496−1514.
(45) Collin, S., Guilvout, I., Nickerson, N. N., and Pugsley, A. P. (2011) Sorting of an integral outer membrane protein via the lipoprotein-specific Lol pathway and a dedicated lipoprotein pilotin. *Mol. Microbiol. 80*, 655−665.
(46) Sampaleanu, L. M., Bonanno, J. B., Ayers, M., Koo, J., Tammam, S., Burley, S. K., Almo, S. C., Burrows, L. L., and Howell, P. L. (2009) Periplasmic domains of *Pseudomonas aeruginosa* PilN and PilO form a stable heterodimeric complex. *J. Mol. Biol. 394*, 143−159.
(47) Jacobs, M. A., Alwood, A., Thaipisuttikul, I., Spencer, D., Haugen, E., Ernst, S., Will, O., Kaul, R., Raymond, C., Levy, R., Chun-Rong, L., Guenthner, D., Bovee, D., Olson, M. V., and Manoil, C. (2003) Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. U.S.A. 100*, 14339−14344.