Structural and Functional Studies of the Pseudomonas aeruginosa Minor Pilin, PilE*

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Background: Type IVa pilus (T4aP) assembly is primed by minor pilins. Results: Non-core subunit PilE interacts with core minor pilins and is incorporated into pili; PilE is structurally similar to Neisseria PilX and PilV. Conclusion: PilE connects the priming complex and the major pilin. Significance: This function may be broadly conserved for non-core minor components in T4aP.

Many bacterial pathogens, including Pseudomonas aeruginosa, use type IVa pili (T4aP) for attachment and twitching motility. T4aP are composed primarily of major pilin subunits, which are repeatedly assembled and disassembled to mediate function. A group of pilin-like proteins, the minor pilins FimU and PilIVWXEN, prime pilus assembly and are incorporated into the pilus. We showed previously that minor pilin PilE depends on the putative priming subcomplex PilVWX and the non-pilin protein PilY1 for incorporation into pili, and that with FimU, PilE may couple the priming subcomplex to the major pilin PilA, allowing for efficient pili assembly. Here we provide further support for this model, showing interaction of PilE with other minor pilins and the major pilin. A 1.25 Å crystal structure of PilE∆1–28 shows a typical type IV pilin fold, demonstrating how it may be incorporated into the pilus. Despite limited sequence identity, PilE is structurally similar to Neisseria meningitidis minor pilins PilXNm and PilVNm recently suggested via characterization of mCherry fusions to modulate pilus assembly from within the periplasm. A P. aeruginosa PilE-mCherry fusion failed to complement twitching motility or piliation of a pilE mutant. However, in a retraction-deficient strain where surface piliation depends solely on PilE, the fusion construct restored some surface piliation. PilE-mCherry was present in sheared surface fractions, suggesting that it was incorporated into pili. Together, these data provide evidence that PilE, the sole P. aeruginosa equivalent of PilXNm and PilVNm, likely connects a priming subcomplex to the major pilin, promoting efficient assembly of T4aP.

Type IV pili (T4P)2 are long, thin, fibrous surface appendages found on Gram-negative and Gram-positive bacteria, as well as archaea (1, 2). They function in attachment, twitching motility, DNA uptake, electron transfer, and biofilm formation (1, 3). There are two main classes of T4P, type IVa and type IVb, distinguished by differences in their subunits and assembly machineries (1, 3, 4). The pili are composed of thousands of major pilin subunits, but minor (low abundance) pilin subunits are also present, potentially at the tip of the pili due to their role in priming of pili assembly (5–10).

Major pilin subunits are expressed as pre-pilins, which are processed by a bi-functional pre-pilin peptidase/N-methylase into assembly-competent mature subunits by removal of their type III signal sequence and methylation of the new N terminus (11–14). Although diverse in sequence, T4a major pilins share a conserved fold, consisting of an extended N-terminal α-helix connected to a four-stranded antiparallel β-sheet (6). The N-terminal α-helices, which form the hydrophobic inner core of an assembled pilus fiber, can be divided into two segments, α1-N and α1-C, with the highly conserved, hydrophobic α1-N segment retaining the monomers in the inner membrane prior to assembly. The globular C-terminal domains decorate the exterior of the pilus and typically contain a disulfide bond connecting the C terminus to the conserved β-sheet, forming a disulfide-bonded loop, also known as the D-region (3). Consistent with their incorporation into pili, the minor pilins are also processed by the pre-pilin peptidase and, based on the limited number of structures available, have architectures similar to major pilins (7, 9, 10, 15, 16).

The T4P system is evolutionarily related to the type II secretion (T2S) system, proposed to form a short pilus-like fiber in the periplasm that acts as a piston during the secretion of select exoproteins (17, 18). The T2S system has a set of four minor subunits, called the minor pseudopilins, which prime pseudopilus assembly (19). These four minor (pseudo)pilins are con-
served between T2S and T4aP systems, suggesting that they are core components of (pseudo)pilus assembly (20). In support of this idea, *Escherichia coli* K-12 T4aP core minor pilins can prime heterologous assembly of *Klebsiella oxytoca* pseudopilin and *vice versa* (21), and *Pseudomonas aeruginosa* T4aP assembly can be primed by either its minor pilins or its T2S minor pseudopilins (10).

Unlike *E. coli*, *P. aeruginosa* and *Neisseria* spp. T4aP systems include additional, non-core minor pilins (20). In *P. aeruginosa*, the core minor pilins are called FimU, PilV, PilW, and PilX, and are encoded in an operon with the large putative adhesin, PilY1 (22), and the non-core minor pilin, PilE (23). We showed (10) that PilVWX and PilY1 depend on one another for incorporation into pili, suggesting that they form a subcomplex and that this putative subcomplex was required for PilE to be recovered in the pilus fraction. We proposed that (among other functions) the minor pilins prime pilus assembly and are thus assembled into the pilus fiber (10).

Although *P. aeruginosa* has only one non-core minor pilin (PilE), *Neisseria meningitidis* has three, called ComP, PilXNm, and PilVNm. ComP is incorporated into T4aP of *Neisseria* and is required for competence (24, 25), binding directly to DNA via an electropositive region extending across the surface-exposed β-sheet region (16). The PilXNm protein (called PilLNg in *Neisseria gonorrhoeae*) is potentially orthologous to *P. aeruginosa* PilE, based on synteny (both genes are located at the 3′ mids used in this study are listed in Table 1. Bacterial strains and plasmids used in this study are listed in Table 1. Bacterial strains were stored at −80 °C in LB agar supplemented with 15% glycerol. *E. coli* strains were grown at 37 °C, unless otherwise stated, on LB agar containing ampicillin (100 µg/ml), kanamycin (50 µg/ml), gentamicin (15 µg/ml), or chloramphenicol (30 µg/ml), as appropriate. *P. aeruginosa* strains were grown at 37 °C on LB agar containing gentamicin (30 µg/ml).
**Structure and Function of Minor Pilin PilE**

**TABLE 1**

| Strains and plasmids used in this study | Description | Reference |
|----------------------------------------|-------------|-----------|
| E. coli strains                        |             |           |
| DH5a                                   |             | Invitrogen |
| TOP10                                  |             | Invitrogen |
| Origami B (DE3)                        |             | Novagen   |
| BTH101                                 |             | Euromedex |
| Strains and plasmids                   |             |           |
| **E. coli strains**                    |             |           |
| DH5a                                   | F’ Phi80d lacZ, ΔM15 (lacZYA-argF)U169 deoR recA1 endA1 hsdR17(k-m+1) | Invitrogen |
| TOP10                                  | F–mcrA Δ (mcr-hsdRMS-mcrBC) 880lacZAM15 ΔlacX74 recA1 araD139 Δ araE-leu2Δ7697 galU galK rpsL endA1 supG | Invitrogen |
| Origami B (DE3)                        | F’ ompT hsdR1 (c-mu Clsm) gal dcm lacY1 hsrC (DE3) gor522::Tn10 trxB (KanR, TetR) | Novagen |
| BTH101                                 | F’ , cya-99, araD139, galE15, galK16, rpsL1 (StrR), hsdR2, mcrA1, merR1 | Euromedex |
| **Plasmids**                           |             |           |
| pBADGr                                 | pMLMBad with aacC1 gene (gentamicin resistance) disrupting dfrB2 (trimethoprim resistance), arabinose inducible | 33 |
| pBADGriPE                              | PA01 pilE cloned in pBADGr, GmR | 7 |
| pBADGriPE mCherry                      | PA01 pilE cloned in pBADGr encoding a C-terminal mCherry fluorescent tag | This study |
| pUT18C                                 | pUC19-derived vector with T18 fragment (residues 225–399) of CyaA under control of lac promoter, MCS at 3’ end of T18 ORF, AmpR | 51 |
| pUT18C plipE                           | mature PA01 pilE cloned in pUT18C, AmpR | This study |
| pkT25                                  | pSU40-derived vector with T25 fragment (residues 1–224) of CyaA under control of lac promoter; MCS at 3’ end of T25 ORF, KanR | 51 |
| pkT25pilA                              | Mature PA01 pilA cloned in pkT25, KanR | 10 |
| pkT25fimU                              | Mature PA01 pilU cloned in pkT25, KanR | This study |
| pkT25pilV                              | Mature PA01 pilV cloned in pkT25, KanR | 10 |
| pkT25pilW                              | Mature PA01 pilW cloned in pkT25, KanR | 10 |
| pkT25pilX                              | Mature PA01 pilX cloned in pkT25, KanR | 10 |
| pT18zip                                | pT18 plasmid with 35-residue leucine zipper cloned into KpnI site, AmpR | 28 |
| pT25zip                                | pT25 plasmid with 35-residue leucine zipper cloned into KpnI site, CmR | 28 |
| pet151 pilE Δ1–28                     | PilE (Δ1–28 of mature protein) expression vector with N-terminal His6, V5 epitope tag | This study |

**Bacterial Adenylyl Cyclase Two-hybrid Assay**—A bacterial adenylyl cyclase two-hybrid assay (28) was used to test for protein–protein interactions between PilE and other pilins. The DNA sequence encoding full-length, mature *P. aeruginosa* PA01 PilE was PCR-amplified using the forward and reverse primers: 5’–GGTCTAGATCTACGGTGGAAATGGAAGTGTGTTGGT–3’ and 5’–CATGGATCCATCGCCACGCGTCACTCGT–3’, respectively, followed by restriction digestion with XbaI and KpnI for directional cloning into pUT18C for a T18 N-terminally tagged protein. Similarly, full-length mature FimU was N-terminally tagged with T25 by PCR amplifying *fimU* with the forward and reverse primers: 5’–GGTCTAGATCTACGGTGGAAATGGAAGTGTGTTGGT–3’ and 5’–CATGGATCCATCGCCACGCGTCACTCGT–3’, respectively, followed by digestion with XbaI and EcoRI for cloning into pKT25. Plasmids were confirmed by sequencing. pUT18C pilE was co-transformed with pKT25-fimU or pKT25-pilA/V/W/X (10) into *E. coli* BTH101 for interaction experiments. Briefly, a single colony from each transformation was grown in LB supplemented with kanamycin (50 μg/ml) and ampicillin (100 μg/ml) overnight at 30 °C, followed by subculturing into fresh medium with antibiotics and 1 μl isopropyl-1-thio-β-D-galactopyranoside for induction. Cells were grown to an *A*600 of 0.6 and spot plated in triplicate on LB agar + X-gal and MacConkey agar + maltose and incubated at 30 °C for 24 h. A T18- and T25-tagged leucine zipper expressed from pT18-zip and pT25-zip (28) was used as a positive control. The experiment was performed in triplicate, and representative images were taken.

**Protein Expression and Purification**—PA01 pilE encoding N-terminally truncated mature PilEΔ1–28 was PCR-amplified using forward primer 5’–CCACTTCCCCCTCAACCCG–3’ and reverse primer 5’–CACTCCGCTCAACCG–3’ and transformed into TOP10 cells for propagation. The correct construction was confirmed by sequencing. Cells were harvested by centrifugation at 3,200 × *g*, and the pellet was resuspended in lysis buffer (20 mM Tris, pH 8, 500 mM NaCl, and 0.1% lauryl-dimethylamine oxide) with 1× benzamidine. Cells were lysed by three passages through a French press, and after centrifugation to remove cell debris, the clarified lysate was applied on an AKTA FPLC system to a 5-ml Ni HiTrap Chelating HP column (GE Healthcare, Mississauga, Ontario Canada) pre-charged with 100 nM NiCl2. The column was washed in a stepwise manner with 15, 30, and 45 mM imidazole followed by elution of bound proteins with 300 mM imidazole. The elution fraction was dialyzed into 20 mM Tris, pH 8, 100 mM NaCl, treated with tobacco etch virus protease at a final concentration of 0.04 mg/ml for 3 h at room temperature, and applied to a second
nicked affinity chromatography column as above. Unlabeled PilEΔ1–28 was collected in the flow-through fraction, buffer-exchanged into 20 mM Tris, pH 8, 50 mM NaCl, and concentrated to 4 mg/ml.

**Crystallization and Structure Determination**—SeMet PilEΔ1–28 crystals were grown using the hanging drop/vapor diffusion method in a 1:1 ratio of protein (4 mg/ml SeMet PilEΔ1–28 in 20 mM Tris, pH 8, 50 mM NaCl and precipitant (0.2 M ammonium tartrate dibasic, 20% (w/v) PEG 3350) over 1.5 M ammonium sulfate at 20 °C. Crystals were flash-frozen in a nitrogen cold stream with no further cryo-protection. Diffraction data were collected at the X25 beamline of National Synchrotron Light Source (NSLS) in Brookhaven, NY with a wavelength of 0.979 Å.

Single anomalous diffraction data were processed using the HKL2000 program suite (29). The HySS submodule was used to locate the single SeMet site followed by phasing, density modification, automated model building, and refinement in the Phenix suite of programs (30, 31). Iterative rounds of manual model building and refinement were performed in Coot (32) until R_work and R_free values converged and could no longer be improved. Further details of data collection and model refinement statistics are listed in Table 2.

**Construction of Fluorescently Tagged PilE**—PAO1 pilE with the gene encoding mCherry fused on the 3' end was synthesized by GenScript (Piscataway, NJ) with flanking EcoRI and HindIII restriction sites. The insert was subcloned into the EcoRI and HindIII sites of the P. aeruginosa-compatible and arabinose-inducible vector pBADGr (33) to generate pBADGr-pilE-mCherry.

**Twitching Motility Assay**—Twitching motility stab assays were performed as described previously (7). Briefly, strains of interest were stab-inoculated in duplicate to the plastic-agar interface of an LB 1% agar plate, which was incubated at 37 °C for 24 h. The agar was carefully removed, and adherent bacteria were stained with 1% crystal violet. The experiment was performed in triplicate.

**Sheared Surface Protein Preparation**—Proteins were sheared from the surface of P. aeruginosa cells as described previously (10). Briefly, bacterial strains were streaked in a cross-hatched manner on a 150-mm diameter LB agar (1.5%) plate containing gentamicin (30 μg/ml) and grown overnight at 37 °C. Cells were scraped using a glass coverslip, resuspended in 1× PBS, and vortexed for 30 s to shear off surface proteins. Bacterial cells were pelleted by centrifugation at 16,100 × g for 5 min followed by a second spin of the supernatant for 20 min. Sheared surface proteins in the clarified supernatant were precipitated on ice for 1 h using 0.4 M NaCl and 2.4% (w/v) PEG 8000 followed by centrifugation at 16,100 × g for 30 min. The pellets containing surface proteins (pilin and flagellin) were resuspended in 150 μl of 1× SDS loading buffer and boiled for 10 min. Samples were separated on 15% SDS-PAGE gels and stained with Coomassie Brilliant Blue for visualization. Densitometry was performed using ImageJ (34), where pilin levels were standardized against the flagellin band.

For Western blot analysis of PilE in surface fractions, the sheared surface protein samples were separated by SDS-PAGE and transferred to nitrocellulose as described (10), followed by detection with 1:1000 dilution of rabbit polyclonal PilE antibody (7) and 1:3000 dilution of goat anti-rabbit IgG-alkaline phosphatase-conjugated secondary antibody, developed using nitro-blue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP).

**Intracellular PilE Protein Levels**—Bacterial cell pellets, recovered after removal of surface proteins by mechanical shearing as described above, were resuspended in 1× PBS to an A600 of 0.6. Two ml of bacterial suspension were centrifuged at 16,100 × g for 2 min, and the pellets were resuspended in 200 μl of 1× SDS loading buffer and boiled for 10 min. The lysates were separated by SDS-PAGE and transferred to nitrocellulose for immunoblot analysis with anti-PilE polyclonal antibody as described above.

**Fluorescence Microscopy**—Overnight cultures of the strains of interest were stab-inoculated into individual chambers of 1.0 borosilicate chambered cover glass slides (Lab-Tek) containing 1% LB agar. Slides were incubated in the dark for 1 h at 37 °C. Cells were then visualized using an EVOS FL Auto microscope (Life Technologies) with a Plan Apochromat 60× oil immersion objective, using either transmitted (white) light or a Texas Red LED light cube (emission 585/29, excitation 624/40). Images were acquired using the EVOS FL Auto Cell Imaging System software (Life Technologies) and exported as TIFF files. TIFF images were processed in ImageJ (34) by cropping representative regions of interest and then adjusting their brightness to improve visualization of mCherry.

**Results**

**PilE Interacts with Major and Minor Pilins**—We showed previously (10) that incorporation of the P. aeruginosa minor pilin PilE into T4aP depended on the presence of the putative PilVVXY1 priming subcomplex and that pilus assembly required (at a minimum) PilVWXY1 plus either FimU or PilE as putative connectors of the priming subcomplex to the major subunit, PilA. We tested potential interactions of full-length, mature PilE with PilA and the other minor pilins using a bacterial adenylate cyclase two-hybrid assay (28). PilE was N-terminally tagged with the T18 fragment of the Bordetella pertussis adenylate cyclase, whereas PilA, FimU, PilV, PilW, and PilX were N-terminally tagged with T25. Interactions were identified on LB + X-gal and MacConkey + maltose indicator plates, which result in blue or red colonies if there is an interaction. A leucine zipper was used as a positive control.

**FIGURE 2. Interactions of PilE with minor pilins and PilA.** Protein-protein interactions were tested using a bacterial adenylate cyclase two-hybrid system (BACTH). Mature PilE was N-terminally tagged with T18, whereas PilA, FimU, PilV, PilW, and PilX were N-terminally tagged with T25. Interactions were tested in E. coli cyA mutant strain BTH101 on LB agar + X-gal and MacConkey + maltose indicator plates, which result in blue or red colonies if there is an interaction. A leucine zipper was used as a positive control.
other putative connector, FimU, PilE interacted with the major pilin PilA and the minor pilins PilV and PilX, but not with PilW, supporting its proposed role in stably linking the minor pilin PilVWX1 priming subcomplex to the major subunit PilA.

The High-resolution Crystal Structure of PilE Reveals Characteristic Pilin Architecture—To gain further insight into the function of PilE, we solved a 1.25 Å high-resolution x-ray crystal structure of SeMet-labeled PilE_{1–28}. The structure lacks the first 28 N-terminal hydrophobic residues of the mature minor pilin, which were removed to improve its solubility. We also obtained native PilE_{1–28} crystals and collected data; the protein crystallized in the same space group, C2, as the SeMet form, but the data were of lower resolution. Crystallographic data collection and refinement statistics are detailed in Table 2.

PilE_{1–28} has a typical type IV pilin fold, characterized by an N-terminal α-helix connected to a four-stranded antiparallel β-sheet, terminating with a disulfide-bonded loop (Fig. 3A). The N-terminal residues from Asn-32 to Ser-52 form the α1-C helix, which likely extends in the full-length protein from the N-terminal hydrophobic α1-N-helix of a full-length pilin. The α1-C helix is packed against a four-stranded antiparallel β-sheet, to which it is connected by a 26-residue loop containing a 3_{10} helix. Between the β2 and β3 strands of the β-sheet, residues Ile-97 to Lys-108 form a long loop with a 3_{10} helix. Cys-106 and Cys-132 form a disulfide bond encompassing 25 residues that make up the D-region of PilE. In type IV pilins, the D-region is hypervariable in sequence, and in major pilins, the Cys residues typically staple the C terminus to the last β-strand (20). In contrast, the C terminus of PilE is linked by a disulfide bond to the β2-β3 loop, and the D-region encompasses β3, β4, and a short two-turn helix. Mutation of Cys-132 to Ala resulted in protein instability and loss of pilus assembly and twitching motility (data not shown), suggesting that the disulfide bond is a critical stabilizing feature of PilE. Similar results have been reported for Cys point mutants of the major pilin, PilA (33).

Of the P. aeruginosa minor pilins, PilE is the most similar to PilA, with 38% sequence similarity between full-length proteins. Despite the sequence differences and the differences in the connectivity of the disulfide bond in the D-region, 96 of 108 residues of PilE_{1–28} could be aligned with the structure of PilAPAK (Protein Data Bank (PDB) 1OQW), with a root mean square deviation (RMSD) of 3.8 Å (Fig. 3B). The α1-helix of major pilins has a characteristic shallow S-shaped curve created by residues Pro-22 and Pro- or Gly-42 (35–37). Mature PilE has an Asp residue at position 42, and its truncated α1-C helix was not curved in our structure (Fig. 3, A and B). However, there is a Pro-22 residue in the PilE sequence that likely creates a kink in the α1-N helix region of PilE. The αβ-loops of major pilins are involved in inter-subunit interactions between pilin subunits in the pilus (38). In P. aeruginosa PilAPAK, this region forms a minor β-sheet, whereas in PilE_{1–28}, this region has a 3_{10} helix and is less extended, possibly to accommodate interactions with the other minor pilins. Although β3 and β4 of PilE_{1–28} are part of the D-region, the length and orientation of the four β-strands of central β-sheet are generally conserved between PilAPAK and PilE_{1–28}, as is the packing of the β-sheet against α1-C.

![Figure 3](image_url)

**FIGURE 3.** X-ray crystal structure of PilE_{1–28} A, the x-ray crystal structure of selenomethionine-labeled PilE_{1–28} was solved to 1.25 Å (PDB code: 4NOA). The N-terminal α-helix is colored cyan, αβ-loop is in magenta, β-sheet is in gray, and D-region is in blue. Cys residues are represented as sticks and colored yellow. B, structural alignment between PilE_{1–28} (purple) and PilAPAK (gray; PDB code: 1OQW). 96 residues aligned with an RMSD of 3.8 Å. The arrow indicates the hook-like protrusion in both structures. C, mapping of residues differing between PilE_{PAO1} and PilE_{PA14}. Non-conservative residues are colored green. Structural illustrations and alignments were generated with PyMOL (version 1.3, Schrödinger, LLC).

| TABLE 2 |
| ———— | ———— |
| **PilE data collection and refinement statistics** | |
| **SeMet-PilE** | **NSLS X25** |
| **Beamline** | 0.979 |
| **Wavelength** | C2 |
| **Space group** | 76.16, 35.56, 43.54 |
| **Unit cell parameters a, b, c (Å)** | 90.0, 97.32, 90.0 |
| **Unit cell parameters α, β, γ (°)** | 6.4 (4.5) |
| **No. of molecules in ASU** | 95.4 (89.6) |
| **Resolution range (Å)** | 20.7 (7.6) |
| **Rmerge (%)** | 8.4 (21.0) |
| **Rwork (%)** | 5.2 (4.7) |
| **Rfree (%)** | 50.0–1.25 (1.27–1.25) |
| **Unique reflections** | 30,749 |
| **Data redundancy** | 50.0–1.25 (1.27–1.25) |
| **Completeness (%)** | 831 |
| **No. of amino acid residues/atoms** | 248 |
| **No. of molecules in ASU** | 831 |
| **No. of waters** | 76.16, 35.56, 43.54 |
| **No. of reflections** | 76.16, 35.56, 43.54 |
| **RMSD bond lengths (Å)** | 76.16, 35.56, 43.54 |
| **RMSD bond angles (°)** | 50.0–1.25 (1.27–1.25) |
| **Average B (Å²)** | 50.0–1.25 (1.27–1.25) |
| **Ramachandran statistics (%)** | 50.0–1.25 (1.27–1.25) |
| **Favored** | 50.0–1.25 (1.27–1.25) |
| **Allowed** | 50.0–1.25 (1.27–1.25) |
| **PDB code** | 4NOA |

*Values in parentheses represent highest resolution shell.*
Each *P. aeruginosa* strain carries one of two different sets of T4aP minor pilin genes (exemplified by those of common laboratory strains PAO1 and PA14), which are encoded with specific major pilin genes in a “pilin island” that bears signatures of horizontal gene transfer (39). The sequence similarities between the minor pilin orthologues encoded by the two sets of genes range from ~60 to 75%, with higher similarity in the N termini and lower in the C termini of each pair (40). With the exception of pilX<sub>PA14</sub>, cross-complementation of PAO1 minor pilin mutants with PA14 minor pilin genes restored surface pilation and twitching motility to various degrees, suggesting that most subunits can make functional interactions with heterologous partners (39). PilE<sub>PAO1</sub> and PilE<sub>PA14</sub> share 61% amino acid sequence similarity (39), and we found that most of the divergent residues map to loops or solvent-exposed surfaces (Fig. 3C). These results suggest that overall conservation of PilE architecture is important for its function.

**Comparison of PilE and PilX<sub>Nm</sub> Structures**—The top hit from a structural comparison of PilE<sub>31–28</sub> with others in the PDB using DALI (41) was the PilX<sub>Nm</sub> minor pilin from *N. meningitidis* (15). PilX<sub>Nm</sub> is encoded with the *Neisseria* PilHIJK equivalents of the *P. aeruginosa* core minor pilins FimU–PilVWX and has been implicated in controlling efficient pilus biogenesis, as well as in attachment and aggregation of surface-exposed pili (8, 15, 27). PilE and PilX<sub>Nm</sub> share 25% overall sequence identity, concentrated in the α1-N region (18 of 28 residues, 64%) that was deleted for structural studies (Fig. 1). The structure of N-terminally truncated PilX<sub>Nm</sub> is composed of the typical N-terminal α1-C helix connected to a four-stranded antiparallel β-sheet (15) (Fig. 4A). Although there is only 14% sequence identity between the C-terminal domains of PilE and PilX<sub>Nm</sub>, the critical pilin structural elements are maintained, and the Ca<sub>α</sub> molecules align over 104 residues with an RMSD of 4.3 Å (Fig. 4B).

Like PilE, the N-terminal α1-C helix in the PilX<sub>Nm</sub> structure lacks a kink at position 42, although it has a Gly at this position (15), which in other pilin structures allows for a second bend in the S-shaped α1 helix (36–38). Of note, the D-regions of both PilE and PilX<sub>Nm</sub> have hook-like protrusions (Fig. 4B). This feature was previously suggested to be important for protein-protein interactions between PilX<sub>Nm</sub> subunits on neighboring but antiparallel pilus fibers, opposing pilus retraction and thereby promoting aggregation (15). Complementation of a pilE mutant with a construct encoding PilE with an in-frame deletion of the corresponding region (residues 120–127) restored wild-type pilus assembly and twitching motility (data not shown), suggesting that this region of PilE is not crucial for function in *P. aeruginosa*.

**PilV<sub>Nm</sub> Is Predicted to Be Structurally Similar to PilE**—Although there is no structure yet available for PilV<sub>Nm</sub>, it has higher sequence identity to PilE (35% overall identity) than the obvious structural homologue PilX<sub>Nm</sub> (27% overall identity) (Figs. 1 and 4A). We used the Phyre<sup>2</sup> structural prediction algorithm (42) to search for the best homology model of PilV<sub>Nm</sub> using only its C-terminal region (residues 29–122, mature PilV<sub>Nm</sub> numbering). The top hit for PilV<sub>Nm</sub> was our PilE structure, with a confidence level of 99.9% over an alignment of 90 residues (Fig. 4C). The next three hits were *N. meningitidis* minor pilin ComP (PDB 2MK3), *P. aeruginosa* major pilin PilA (PDB 1OQW), and *N. gonorrhoeae* major pilin PilE (PDB 2PIL), with decreasing levels of confidence. Of note, the PilV<sub>Nm</sub> structure was not among the hits for the PilV<sub>Nm</sub> C-terminal region, suggesting that the level of sequence identity between them was too low to generate even a low-confidence model. Repeating the search with the full-length mature PilV<sub>Nm</sub> sequence returned similar results (data not shown).

Although PilV<sub>Nm</sub> has Cys residues in the same position as PilX<sub>Nm</sub>, according to the alignment, no disulfide bond was present in the Phyre<sup>2</sup>-generated models of PilV<sub>Nm</sub>. These structures do not model the last four residues of PilV<sub>Nm</sub> after the last Cys, suggesting sufficient differences within this region to preclude high-confidence predictions. Nevertheless, PilV<sub>Nm</sub> likely has a fold similar to PilE including a disulfide bond in its D-region, consistent with reports that it is incorporated into pili (26, 43).

**PilE Incorporation into Pili Is Necessary for Function**—Imhaus and Duménil (27) recently reported that PilX<sub>Nm</sub> and PilV<sub>Nm</sub> are required for efficient pilus biogenesis in *N. meningitidis*. They suggested that the functional pool of PilX<sub>Nm</sub> and PilV<sub>Nm</sub> was located in the periplasm rather than on the cell surface (as might be expected for integral components of...
assumed pili), because mCherry fusions considered too bulky to pass through the PilQ secretin were capable of complementing their cognate mutants. However, the proteins were unable to complement function unless processed by the pre-pilin peptidase (27), an essential prerequisite for pilus incorporation (11). This finding was consistent with other studies showing that minor pilins are present in sheared surface fractions, suggesting incorporation into pili (7, 9).

To reproduce this experiment in *P. aeruginosa*, mCherry was fused to the C-terminal end of PilE and its ability to complement a pilE mutant was tested. Analysis of cells complemented with the fusion protein by fluorescence microscopy revealed circumferential staining, confirming its expected periplasmic localization (Fig. 5A). However, complementation of a pilE mutant with PilE mCherry resulted in no recoverable surface pili or twitching motility, similar to the negative control (Fig. 5, B and C). The levels of PilE mCherry expressed from the pBADGr vector were intermediate between those of unmodified PilE expressed from the same plasmid and those expressed from the chromosomal locus (Fig. 5D), both of which restore similar levels of twitching motility. Therefore, the amount of fusion protein expression is unlikely to underlie the absence of twitching motility or pili in the strain expressing the PilE mCherry fusion. Consistent with our model (in which PilE stabilizes interactions between the major and a minor pilin subcomplex required for efficient pilus biogenesis), these data suggest that *P. aeruginosa* PilE cannot restore pilus biogenesis from a periplasmic location. Alternatively, attachment of a bulky fluorescent protein could impair the ability of PilE to function efficiently in the initiation of pilus assembly, leading to a lack of surface pili under circumstances where retraction is active, due to unbalanced extension/retraction dynamics.

To examine the latter possibility, we tested whether pBADGr-pilE-mCherry could complement pilation in a previously characterized *P. aeruginosa* ΔfimU pilE ΔMPP pilT mutant (10). This strain is retraction-deficient due to inactiva-
ration (and thus efficiency of assembly initiation) to the point where surface piliation and motility are lost when retraction is active.

**Discussion**

Our earlier results (10) suggested that the minor pilins prime T4aP assembly by forming a priming complex, analogous to that formed by minor pseudopilins (19), to initiate fiber polymerization. In our model, PilVWX1Y first form a subcomplex, which is then bound by PilE and coupled to the major subunit PilA through its interactions with both PilE and FinU, which interact with one another (10). Here we show that PilE interacts with the major pilin PilA and minor pilins FinU, PilIV, and PilX (Fig. 2), supporting its role as a connector.

PilE has a typical type IV pilin structure (Fig. 3), likely facilitating its interactions with the major pilin and incorporation into the pilus. However, despite its structural and 38% sequence similarity to PilA, PilE does not form pili on its own, even when overexpressed (data not shown). The assembly process tolerates a wide range of PilE expression levels, while still supporting similar amounts of twitching motility (Fig. 5), likely due to the dependence of PilE on PilVWX1Y for incorporation into pili (10). Simply increasing intracellular levels of PilE would not necessarily affect the amount of PilE in the pilus if stoichiometric interactions with PilVWX1Y are necessary for its inclusion in pili. In addition, the architecture of the minor pilins may preclude their polymerization into a fiber. Major pilins have a Gly or Pro residue at position 42, creating a kink in their α1-C-helices that allows for inter-subunit interactions and flexibility of the fiber (35, 37, 44, 45). Putative connectors PilE (Fig. 3) and FinU (10) lack kinks in their α1-C helices. The absence of such curvature may have implications for the packing of these minor pilins with the priming subcomplex, or possibly their recognition by the assembly machinery, differentiating them from the major pilin proteins.

Imhaus and Duménil (27) created mCherry fusions to prevent the incorporation of PilXNm or PilVNm into surface-exposed pili, with the assumption that the fusions would be too large to fit through the secretin pore. However, it is difficult to differentiate that phenotype from one in which pilus assembly is impaired due to changes in interactions among major and minor pilin subunits because of the presence of the fusion protein. In *P. aeruginosa*, complementation of a pilE mutant with pilE-mCherry failed to restore surface pilation (Fig. 5C); however, in a retraction-deficient background, we recovered a small amount of pili in which PilE-mCherry could be detected (Fig. 6, A and B). This result is consistent with a model of pilus assembly initiation, in which only one PilE subunit per pilus is required (10). Pilus assembly is likely less efficient with PilEmCherry versus wild type PilE, potentially due to suboptimal interactions with the fusion protein, inefficient priming, and thus no recoverable surface pili if retraction is active.

*Neisseria* non-core minor pilins PilXNm (PilLNg), PilVNm, and Comp all co-purify with pili, suggesting that they are part of the fiber (8, 9, 15, 16, 26, 43). PilXNm and PilVNm variants that cannot be processed by the pre-pilin peptidase were defective for complementation, suggesting that they need to be incorporated into pili for function (27). PilXNm is structurally similar to PilE (Fig. 4). Both are proposed to be involved in efficient initiation of pilus assembly (10, 27). In the absence of PilIXNm, or its *N. gonorrhoeae* homologue, PilIXNg, pilination is reduced (9, 25), whereas without PilE, *P. aeruginosa* cells are non-piliated (7, 46). In trying to understand this difference between model species, we noticed that PilE and the *N. meningitidis* non-core minor pilin PilVNm were potentially orthologous. A Phyre2 analysis of the C-terminal domain of PilVNm yielded a high-confidence structural model on the PilE template, but returned no match with PilIXNm, although DaliLite (41) analysis suggested that PilIXNm is the top structural match for PilE (Fig. 4A). Based on these data, we propose that both PilIVNm and PilIXNm are PilE orthologues, possibly explaining why single *P. aeruginosa* pilE mutants lack surface pili, but both pilVNm and pilIXNm must be deleted in *Neisseria* before pilation is lost (27).

The T2S system lacks both PilE and PilY1 equivalents. We showed previously that PilY1 is required for PilVWX incorporation into pili and that all four members of this putative subcomplex must be present for PilE to be incorporated into pili, suggesting that it recognizes a novel subcomplex interface (10). PilE may act as a quality control point to ensure the incorporation of the important non-pilin protein PilY1 into each pilus fiber. Like *P. aeruginosa*, other species such as *Xylella fastidiosa*, *Ralstonia solanacearum*, and *Chromobacterium violaceum* carry T4aP minor pilin operons that encode both PilY1-like proteins and PilE equivalents (9), suggesting a functional link.

Consistent with having two putative PilE equivalents, *Neisseria* spp. encode two PilY1-like proteins, PilC1 and PilC2 (47, 48). However, they are encoded separately from the minor pilins PilHIJK(X/L) (9), and their expression and regulation are not well understood. In *N. gonorrhoeae*, incorporation of PilINg into pili depended on the core minor pilins PilHIJK as well as PilC1/2 (9). Similarly, PilIVNg was dependent on PilC1/2 for pilus incorporation, although dependence on other minor pilins was not tested (43). Like PilY1, which does not require PilE for pilus incorporation, PilC is still present in surface pilus fractions in the absence of PilIXNm, PilILNg, or PilIVNm, Ng (8, 9, 26, 43). Based on the insights provided by characterization of PilE, we suggest that PilIXNm and PilVNm may interact with one or more of the core minor pilins, plus PilC1 and/or PilC2, to initiate pilus assembly, and that the function of non-core PilE-like minor pilins may be conserved across T4aP-producing species that express PilY1-like proteins.

*Author Contributions*—Y. N. and L. L. B. designed the study, and Y. N., M. S. J., and L. L. B. wrote the paper. Y. N. and S. D. B. purified and crystallized PilE protein, and S. S. M., Y. N., and M. S. J. determined its X-ray structure. Y. N. and H. H. designed and constructed mutants and fusion constructs, and analyzed PilE function. Y. N. and R. N. C. B. performed microscopy experiments. All authors analyzed the results and approved the final version of the manuscript.

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