PilN Binding Modulates the Structure and Binding Partners of the *Pseudomonas aeruginosa* Type IVa Pilus Protein PilM

Matthew McCallum†,‡§1, Stephanie Tamam†,‡§1, Dustin J. Little†,‡§2, Howard Robinson‡, Jason Koo‡, Megha Shah‡, Charles Calmettes‡, Trevor F. Moraes‡, Lori L. Burrows‡,§3, and P. Lynne Howell‡,§4

From the †Department of Biochemistry, University of Toronto, Toronto, Ontario MSS 1A8, Canada, the ‡Program in Molecular Structure and Function, Peter Gilgan Centre for Research and Learning, Hospital for Sick Children, Toronto, Ontario MSG 0A4, Canada, the §Photon Sciences Division, Brookhaven National Laboratory, Upton, New York 11973-5000, and the ¶Department of Biochemistry and Biomedical Sciences and the Michael G. DeGroote Institute for Infectious Disease Research, McMaster University, Hamilton, Ontario L8N 3Z5, Canada

*Pseudomonas aeruginosa* is an opportunistic bacterial pathogen that expresses type IVa pili. The pilus assembly system, which promotes surface-associated twitching motility and virulence, is composed of inner and outer membrane subcomplexes, connected by an alignment subcomplex composed of PilMNOP. PilM binds to the N terminus of PilN, and we hypothesize that this interaction causes functionally significant structural changes in PilM. To characterize this interaction, we determined the crystal structures of PilM and a PilM chimera where PilM was fused to the first 12 residues of PilN (PilM-PilN(1–12)). Structural analysis, multilayer light scattering coupled with size exclusion chromatography, and bacterial two-hybrid data revealed that PilM forms dimers mediated by the binding of a novel conserved motif in the N terminus of PilM, and binding PilN abrogates this binding interface, resulting in PilM monomerization. Structural comparison of PilM with PilM-PilN(1–12) revealed that upon PilN binding, there is a large domain closure in PilM that alters its ATP binding site. Using bio-layer interferometry, we found that the association rate of PilN with PilM is higher in the presence of ATP compared with ADP. Bacterial two-hybrid data suggested the connectivity of the cytoplasmic and inner membrane components of the type IVa pilus machinery in *P. aeruginosa*, with PilM binding to PilB, PilT, and PilC in addition to PilN. Pull-down experiments demonstrated direct interactions of PilM with PilB and PilT. We propose a working model in which dynamic binding of PilN facilitates functionally relevant structural changes in PilM.

Type IVa pili (T4aP) are hair-like protein filaments critical for initial and irreversible bacterial attachment, pro-biofilm cyclic diguanylate signaling, spatial differentiation in biofilms, the dispersal of mature biofilms, swarming motility, twitching motility, competence for DNA uptake, and pathogenesis in *Pseudomonas aeruginosa* (1–6). After decades of research, many of the essential *P. aeruginosa* T4aP proteins have been characterized (7–9) (Fig. 1). The pilus fiber is composed predominantly of the major subunit PilA plus small amounts of minor pilins and is proposed to be assembled and disassembled by the cytoplasmic motor subcomplex, composed of the inner membrane protein PilC and orthologous AAA+ ATPases PilB, PilT, and PilU (9). PilB and PilT power assembly and disassembly, respectively (10–12). The exact function of PilU remains unclear. The pilus extends through a multimeric outer membrane PilQ secretin (6, 13). PilQ interacts with PilP, which in turn interacts with PilNO heterodimers (14). In the cytoplasm, PilM associates with this complex by binding the conserved cytoplasmic N terminus of PilN (14, 15), although PilM is not dependent on PilN, PilO, or PilP for stability (16). Little is known of the exact molecular function of PilM, but ∆pilM mutants lack T4aP (16–23).

The structure of PilM bound to a peptide corresponding to the first 15 N-terminal residues of PilN from *Thermus thermophilus* was solved previously, revealing that PilM is an actin homolog with structural similarities to BfpC, GspL, and FtsA (15, 24). GspL and BfpC are components of the type II secretion (T2S) and type IVb pilus (T4bP) systems, respectively. These systems share evolutionary origins with the T4aP because they share similar pilin types, pilus fiber organization, and ATPase architecture.

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‡§1 To whom correspondence may be addressed: Dept. of Biochemistry and Biomedical Sciences and the Michael G. DeGroote Institute for Infectious Disease Research, McMaster University, Hamilton, Ontario L8N 3Z5, Canada. E-mail: howell@sickkids.ca.

‡§2 To whom correspondence may be addressed: Program in Molecular Structure and Function, Peter Gilgan Centre for Research and Learning, Hospital for Sick Children, 686 Bay St., Toronto, Ontario MSG 0A4, Canada. E-mail: burrowl@mcmaster.ca.

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The atomic coordinates and structure factors (codes SEOX, SEOY, SEOU, and SEQ6) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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Clonal, Expression, and Purification of PilM, PilB, and PilT—Cloning of PilM, PilB, and PilT into pET28a with a thrombin-cleavable hexahistadine tag was described previously (10). pET28a:PilM was further modified by round-the-horn PCR mutagenesis using primers P1 and P2 to generate pET28a:PilM-PilN(1–12) (PilM C-terminally fused to the first 12 residues of PilN with a 3-residue linker) and primers P11 and P51 to generate pET28a:PilMΔ2–8 (Table 1). pET28a:PilM was also mutated by site-directed mutagenesis to generate pET28a:PilMΔ2–8 using primers P13 and P14 and pET28a:PilMΔ19A using primers P17 and P18. The fidelity of the sequences was verified by TCAG sequencing facilities (Hospital for Sick Children). Escherichia coli BL21-CodonPlus® cells (Stratagene) transformed with pET28a:PilM, pET28a:PilM-PilN(1–12), pET28a:PilMΔ2–8, and pET28a:PilT and E. coli C41(DE3) transformed with pET28a:PilB were grown in 4 liters of lysogeny broth (LB) with 100 μg/ml kanamycin and 1% (w/v) glucose at 37 °C to an A600 of 0.5–0.6, and then protein expression was induced by the addition of isopropyl-d-1-thiogalactopyranoside to a final concentration of 1 mM, and the cells were grown for 16 h at 18 °C. For production of selenomethionine (SeMet)-incorporated PilM, pET28a:PilM was transformed into E. coli B834 Met− (Novagen) and expressed as described elsewhere (35). Cells were pelleted by centrifugation at 9000 × g for 20 min.

For PilM and PilM-PilN(1–12), cell pellets were subsequently resuspended in 30 ml of buffer A1 (50 mM Tris, pH 7.0, 150 mM NaCl, 30 mM citrate, 10% (v/v) glycerol, 2 mM tris(2-carboxyethyl)phosphine, 5 mM MgCl2, 5 mM ATP, and 50 mM imidazole). ATP was omitted when PilM was used in the ATPase assays. Resuspended cells were lysed by passage through an EmulsiFlex-c3 high pressure homogenizer, and the cell debris was removed by centrifugation for 30 min at 40,000 × g. The resulting supernatant was passed over a column containing 5 ml of pre-equilibrated Ni-NTA-agarose resin (Life Technologies, Inc.). The resin was first washed with 10 column volumes (CV) of buffer A1 and then 10 CV of buffer A2 (buffer A1 adjusted to pH 6.0) before elution with 5 ml of buffer A2 plus 300 mM imidazole. The protein was then further purified by size exclusion chromatography on a HiLoad™ 16/600 Superdex™ 200 pg column pre-equilibrated with buffer A2 without ATP or imidazole. Purified PilM constructs, with the exception of PilMΔ2–8, which precipitated at room temperature, were treated with thrombin (EMD Millipore) for 16 h at room temperature and separated from the tagged protein using a second round of nickel affinity purification.
For the interaction studies between PilM, PilB, and PilT, cell pellets were resuspended in 30 ml of buffer B1 (100 mM Tris, pH 7.0, 150 mM NaCl, 30 mM citrate, 150 mM (NH₄)₂SO₄, 15% (v/v) glycerol, 5 mM MgSO₄, 50 mM imidazole) before adjusting the pH with 10 CV of buffer B3 (buffer B1 adjusted to pH 7.0). The protein was eluted in 5 ml of buffer B3 plus 300 mM imidazole and then further purified by size exclusion chromatography on a HiLoad™/16/600 Superdex™/200pg column pre-equilibrated with buffer B3 without imidazole.

For PilB, after the cell pellets were homogenized and centrifuged, insoluble inclusion bodies were solubilized by gentle stirring in 40 ml of buffer B3 plus 8 M urea for 45 min at room temperature. The resulting supernatant was passed over a HisTrap HP 5-ml (GE Healthcare) nickel affinity column. The resin was washed with 4 CV of buffer B3 before elution with a gradient of buffer B3 and 300 mM imidazole. Eluted fractions containing PilB were then concentrated to 12 mg/ml in an ultrafiltration device, and 300 µl were added (in 50-µl increments every minute for 6 min) to 50 ml of gently stirring renaturation buffer (buffer B3 without imidazole plus 5% (v/v) glycerol, 0.5 M arginine, 2 mM tris(2-carboxyethyl)phosphine, and 1 mM ATP) and incubated at 4 °C for 16 h. Then the 50-ml solution was exchanged for buffer B3 without imidazole and concentrated to 500 µl over an ultrafiltration device. The protein was then further purified by size exclusion chromatography on a HiLoad™/16/600 Superdex™/200pg column pre-equilibrated with buffer B3 without imidazole. Purified proteins were stored at 4 °C for less than 2 days before use.

Crystalization, Data Collection, and Structure Solution—For crystallization, purified PilM and PilM-PilN(1–12) were concentrated to 15 mg/ml at 3000 g in an ultrafiltration device, and ATP or AMP-PNP (Sigma-Aldrich) was added to a final concentration of 5 mM as detailed below. Crystallography conditions were screened using the complete MCSG suite (MCSG 1–4) (Microlytic), using a Gryphon LCP robot (Art Robbins Instruments). Crystal conditions were screened and optimized using vapor diffusion at 20 °C using Art Robbins Instruments Intelli-Plates (96-3 Shallow Well Hampton Research) with equal amounts of protein and reservoir solution. For PilM, the reservoir solution was 27% (w/v) PEG3350, 0.2 M MgCl₂, 0.1 M BisTris, pH 7.0. For PilM-PilN(1–12), the reservoir solution was 2 M (NH₄)₂SO₄ and 0.1 M Tris, pH 8.0. The crystals were cryoprotected for 5 s in reservoir solution supplemented with 20% (v/v) ethylene glycol before vitrification in liquid nitrogen. A wavelength anomalous diffraction or native data, as appropriate, were collected on Beamline X29 at the National Synchrotron Light Source (see Table 2). The data were indexed and scaled using HKL2000 (36). Native data were also collected on PilM with AMP-PNP at the x-ray diffraction facilities at the Hospital for Sick Children on a Bruker D8 Venture machine. These data were indexed and scaled with SAINT (Bruker AXS Inc., Madison, WI). PHENIX Autosol (38) was used to identify the 10 selenium atom positions in the asymmetric unit of SeMet PilM. The resulting electron density map was of high quality and enabled PHENIX AutoBuild (39) to build 90% of the protein. The remaining residues were built manually in COOT (40). The structures of native PilM and PilM-PilN(1–12) complexed with ATP or AMP-PNP were solved by molecular replacement using the SeMet-PilM struc-
Structure and Binding Partners of PilM

Table 2

Data collection and refinement statistics of PilM and PilM:PilN(1–12) structures

Values in parentheses correspond to the highest resolution shell.

| Data Collection | PilM-lof-ADP | PilM-ADP | PilM-AMP-PNP | PilM-PiI(1–12)-ATP |
|-----------------|-------------|----------|--------------|-------------------|
| Beamsource      | NSLS X29    | NSLS X29 | Bruker D8 Venture | NSLS X29         |
| Wavelength (Å)  | 0.97920     | 1.0750   | 1.5406       | 1.0750            |
| Space group     | P2₁ 2 2,    | P2₁ 2 2, | P2₁ 2 2,     | P2₁ 2 2,         |
| a, b, c (Å)     | 110.4, 152.0, 41.6 | 111.4, 150.4, 41.6 | 112.6, 151.8, 45.9 | 131.8, 131.8, 79.0 |
| Resolution (Å)  | 46–2.50 (2.55–2.50) | 46–2.40 (2.45–2.40) | 20–3.51 (3.63–3.51) | 50–2.43 (2.49–2.43) |
| Total reflections | 402,920     | 406,860  | 24,279       | 742,571           |
| Unique reflections | 26,503 (1531)| 30,127 (1742) | 9479 (895) | 29,964 (3574) |
| Redundancy (%)  | 13.0 (12.9) | 11.7 (11.3) | 2.6 (1.7) | 19.9 (19.8) |
| Completeness (%)| 100 (98)    | 100 (96) | 92 (80) | 100 (97) |
| Mean I/σ | 18.3 (1.5) | 30.6 (1.6) | 7.0 (2.9) | 21.2 (1.3) |
| Rsym (%) | 11.5 (100) | 6.9 (100) | 12.9 (26.7) | 13.5 (100) |
| Rfree (%) | 4.1 (44.0) | 2.4 (14.1) | 9.4 (23.2) | 4.1 (44.0) |

| Refinement | PilM-lof/| PilM-lof/ | PilM-lof/ | PilM-lof/ |
|------------|---------|----------|----------|----------|
| Rwork/Rfree (%) | 20.7/26.2 | 22.4/25.7 | 24.1/27.4 | 21.1/23.8 |
| RMSD | 0.006 | 0.005 | 0.004 | 0.003 |
| Bond lengths (Å) | 0.91 | 0.86 | 0.96 | 0.62 |
| Ramachandran plot (%) | 98 | 98 | 98 | 99 |
| Total favored (%) | 100 | 100 | 100 | 100 |
| Coordinate error (Å) | 0.28 | 0.37 | 0.30 | 0.37 |
| Atoms | 5244 | 5122 | 5198 | 5081 |
| Protein | 5100 | 4978 | 5088 | 4901 |
| Water | 87 | 88 | 46 | 101 |
| Magnesium | 3 | 2 | 2 | 2 |
| ATP | 0 | 0 | 0 | 31 |
| AMP-PNP | 0 | 0 | 0 | 0 |
| ADP | 54 | 54 | 0 | 0 |
| Sulfate | 0 | 0 | 0 | 40 |
| Sodium | 0 | 0 | 0 | 2 |
| Chlorine | 0 | 0 | 0 | 4 |
| Average B-factors (Å²) | 66.40 | 79.00 | 28.09 | 70.40 |
| Protein | 66.50 | 79.30 | 28.30 | 70.00 |
| Water | 58.50 | 68.50 | 20.10 | 61.20 |
| Ligands | 65.70 | 72.30 | 20.70 | 107.60 |
| PDB code | 5EOY | 5EOX | 5EQ6 | 5EOU |

* Rsym = ΣΣI(1)/(ΣΣI(1)–ΣΣI(2))/(ΣΣI(1)–ΣΣI(2)), Rfree = ΣΣI(obs)–ΣΣI(calc)/ΣΣI(obs)–ΣΣI(calc), and CC² = (ΣΣI(obs)–ΣΣI(calc))/(ΣΣI(obs)–ΣΣI(calc) + ΣΣI(calc)–ΣΣI(obs)), where ΣΣI(obs) is the Pearson correlation coefficient of two half-data sets, as calculated elsewhere (69).

** Rwork = ΣΣI(model)–ΣΣI(calc)/ΣΣI(model)–ΣΣI(calc), where ΣΣI(model) and ΣΣI(calc) are the observed and calculated structure factors, respectively. Rfree is the sum extended over a subset of reflections (5%) excluded from all stages of the refinement.

*** Maximum likelihood-based coordinate error, as determined by PHENIX (42).

**Bioinformatics Analysis of the N Terminus of PilM**—Using BLAST (44), protein sequences with significant homology to PilM were identified. Sequences that were not annotated as a pilus or fimbrial protein, duplicate sequences, and sequences with duplicate species identifiers were removed, leaving 2231 sequences that were aligned in MEGA (45). We compared the alignment with our structure of PilM to identify sequences with N termini of greater than 7 residues, which was the size of the...
motif bound by PilM. We searched these N termini for the $hXXhhX^+$ motif (where $h$ is a hydrophilic residue, $X$ is any residue, and + is a positive residue) and the $hX(X)hh(X) +$ motif (where $(X)$ represents an optional single residue deletion) using the SMS program (46). The motif sequences were extracted by the SMS program, and the consensus motif was visualized using WebLogo (47).

**Negative Stain Electron Microscopy**—EM grids (400-mesh Cu/Rh, Electron Microscopy Sciences) were overlaid with continuous carbon and subjected to glow discharge before PilM, Cu/Rh, Electron Microscopy Sciences) were overlaid with carbon. The grids were then floated on a drop of 50 mM Na$_2$HPO$_4$, pH 7.4, 20 mM KCl, 2 mM MgSO$_4$, 0.8 mg/ml cetyltrimethylammonium bromide detergent, 0.4 mg/ml sodium deoxycholate, and 0.54% (v/v) $\beta$-mercaptoethanol. 150 $\mu$L of a second solution was then added: 60 mM Na$_2$HPO$_4$, pH 7.4, 40 mM KCl, 1 mM ortho-nitrophenyl-$\beta$-galactoside, 20 $\mu$g/ml cetyltrimethylammonium bromide, 10 $\mu$g/ml sodium deoxycholate, and 0.27% (v/v) $\beta$-mercaptoethanol. The $A_{420}$ and $A_{550}$ were measured every 5 min for 1 h at 30 °C in a plate reader. $\beta$-Galactosidase activity was calculated by finding the slope of $(A_{420} - 1.75 \times A_{550})/(0.5 \times$ absorbance units $\times 0.5$ ml) over time (min) relative to a positive control (pKT25:zip with pUT18C:zip). PilN, PilM, and PilO pKT25 and pUT18C constructs were described previously (50).

**Immunoblotting**—Equal amounts of total cell extract were separated on SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked with 5% (w/v) nonfat dry milk in TBS containing 0.1% (v/v) Tween 20 and incubated with either anti-PilM (16), anti-PilB (10), and anti-PilT (17) antibodies. Goat anti-rabbit HRP conjugate (Bio-Rad) was used to detect PilM, PilT, or PilB by chemiluminescence (Thermo Fisher Scientific).

**Isothermal Titration Calorimetry**—PilM was incubated with ATP and ADP—The dissociation constant ($K_d$) for nucleotide binding was determined using isothermal titration calorimetry (ITC) using a MicroCal Auto-iTC200 (Malvern Instruments Ltd.) at 10 °C. PilM was used directly from the SEC column without further concentration to avoid buffer mismatch. 200 $\mu$L of 96 $\mu$M PilM, 73 $\mu$M PilM-PilN(1–12), 77 $\mu$M PilM K52A, or 55 $\mu$M PilM S19A were injected into the cell. 2 $\mu$L of SEC buffer or 1.5 mM ATP or 1.5 mM ADP was then injected into the cell containing PilM. A total of 25 injections were performed with an equilibration time of 150 s between injections. The data were fitted with Origin 7 ITC analysis software using a single-site binding model.

**Results**

**Improving the Solubility of P. aeruginosa PilM**—Initial E. coli expression and purification of P. aeruginosa PilM revealed that the protein was poorly expressed, found predominantly in high molecular weight aggregates, and that it precipitated rapidly following purification. The crystallizability of T. thermophilus PilM (PilM$_{79}$) was improved by incubating it with a peptide corresponding to the cytoplasmic N-terminal portion (residues 1–15) of PilM$_{79}$, although only residues 1–8 were identified in the resulting electron density (15). Similarly, in the crystal structure of cytoplasmic GspL, residues 1–12 of its PilN-like portion could be modeled (51). In an attempt to improve the solubility of P. aeruginosa PilM, we fused the cytoplasmic portion of PilN (residues 1–12) via a 3-residue linker to the C terminus of PilM (PilM-PilN(1–12)). Although PilM-PilN(1–12) also precipitated after purification, we exploited this property in a solubility screen to find a more compatible purification buffer. The solubility of PilM-PilN(1–12) was improved in 50 mM Tris and 30 mM citrate, pH 6.0, 150 mM NaCl, 10% (v/v) glycerol, 2 mM tris(2-carboxyethyl)phosphine, 5 mM MgCl$_2$, and 5 mM ATP. Conveniently, this buffer also improved solubility and reduced aggregation of native PilM.

**Structure Determination of PilM and PilM-PilN(1–12)**—To study potential structural changes upon PilM-PilN interaction, we determined the crystal structures of PilM and PilM-PilN(1–12) with varying concentrations (1–100 $\mu$M) of PilM. The signal from reference wells without PilN(1–12) conjugated to the biosensors or from which PilM had been omitted were subtracted from all binding curves before curve-fitting analysis was performed using ForteBio Data Analysis version 8.1.

**In Vitro Pull-down Experiments**—For pull-downs, 100 $\mu$M PilM or PilM-PilN(1–12) was incubated with 100 $\mu$M PilB or PilT in 50 $\mu$L of buffer B3 for 1 h at 20 °C. As described above, the PilM constructs were treated with thrombin, and the His tag was removed while both PilB and PilT were His-tagged. The proteins were then applied to 50 $\mu$L of pre-equilibrated Ni-NTA-agarose resin, washed with 35 ml of buffer B3 plus 10 mM arginine, and eluted in 50 $\mu$L of buffer B3 plus 300 mM imidazole. Eight microliters were then separated on SDS-PAGE, and Western blot analysis was performed using rabbit anti-PilM (16), anti-PilB (10), and anti-PilT (17) antibodies. Goat anti-rabbit HRP conjugate (Bio-Rad) was used to detect PilM, PilT, or PilB by chemiluminescence (Thermo Fisher Scientific).

**Biological Interferometry**—PilM and PilN(1–12) binding kinetics were determined by biolayer interferometry using an Octet RED96 (ForteBio). All binding reactions were performed at 25 °C in buffer B3 without imidazole with 0.2% (v/v) Triton X-100, 0.01% (w/v) bovine serum albumin, and 2 mM ATP or ADP. Streptavidin biosensors were loaded with 1 $\mu$m synthetic PilN(1–12) C-terminally linked to lysine-biotin by PEG6 (GenScript), quenched with 10 $\mu$g/ml biocytin, and then exposed to multiple washes before being incubated with 0.25 $\mu$L of a library of test solutions, and the time course of the response was monitored.

**Structure and Binding Partners of PilM**
Structure and Binding Partners of PilM

![Diagram of PilM binding partners and structures](Image)

**FIGURE 2. Structural comparison of PilM with homologs.** A, PilM homologs were aligned to PilM-PilN(1–12) using SPalign-NS (68). From left to right, BfpC from *E. coli* (PDB code 3VHJ, RMSD_{cα} = 2.2 Å over 106 residues); GspL from *Vibrio cholerae* (PDB code 2BH1, RMSD_{cα} = 2.0 Å over 159 residues); FtsA from *Thermotoga maritima* (PDB code 4A2A, RMSD_{cα} = 1.9 Å over 257 residues); actin α1 from *Oryctolagus cuniculus* (PDB code 2A3Z, RMSD_{cα} = 2.0 Å over 192 residues); DnaK from *E. coli* (PDB code 4B9Q, RMSD_{cα} = 2.1 Å over 216 residues); and hexokinase-1 from *Schistosoma mansoni* (PDB code 1BDG, RMSD_{cα} = 2.4 Å over 196 residues). The individual domains are color-coded according to actin and FtsA domain definitions (29, 30): yellow, 1C domain; blue, 1B domain; red, 2A domain; green, 2B domain; orange, 1A domain; gray, not annotated here; faded gray, not in the crystal structure. Protein segments that lie across the 1B/2B interface are represented as black spheres. B, the domain architecture is also represented in schematic blocks and color-coded as in A. C, the linear domain architecture for each protein is represented to scale.

12). PilM and PilM-PilN(1–12) crystallized in a number of different conditions. Those that favored the growth of large single crystals of PilM and PilM-PilN(1–12) were identified after optimization (Table 2). The crystals of PilM and PilM-PilN(1–12) indexed to space groups P2_12_1 and P2_12_1, respectively, and diffracted to 2.4 Å. Because we were unable to solve the structure of PilM or PilM-PilN(1–12) by molecular replacement using the available structure of PilM_PilN(1–15), a SeMet derivative of PilM was crystallized, and its structure was determined using the single wavelength anomalous diffraction technique to 2.5 Å resolution. The structures of native PilM and PilM-PilN(1–12) were subsequently solved by molecular replacement using our SeMet-PilM structure as a template, and both were found to have two PilM molecules in the asymmetric unit. Model building and refinement produced final models of native PilM and both were found to have two PilM molecules in the asymmetric unit. Model building and refinement produced final models of native PilM and PilM-PilN(1–12) with R_{work}/R_{free} values of 20.7/26.2, 22.4/25.7, and 21.1/23.8%, respectively. Some residues could not be modeled due to poor electron density. The entire PilM sequence could be modeled into both molecules in the asymmetric unit of the SeMet-PilM structure, except for residues 9 of one molecule. In both molecules of the asymmetric unit in the native PilM structure, the entire PilM sequence could be modeled, except for residues 8 and 9. In one molecule of the PilM-PilN(1–12) structure, residues 9–99, 116–128, 132–241, 244–354, and 359–367 were modeled, and in the other molecule, residues 10–132, 134–186, 189–355, and 358–366 were modeled.

The PilN Binding Pocket of PilM Is Highly Conserved—As previously reported for the structure of PilM(17–15) (15, 24, 28), our PilM structures were similar to those of FtsA, GspL, and BfpC (Fig. 2) as well as to Hsp70 family chaperones such as DnaK, eukaryotic and bacterial actins such as actin α1, and sugar kinases like hexokinase 1. Aligning the structures of PilM with FtsA, GspL, BfpC, DnaK, actin α1, and hexokinase 1 not only illustrates the diversity of domains found in the actin/Hsp70/hexokinase superfamily but also reveals that domains 1A and 1B are present in each actin/Hsp70/hexokinase superfamily member identified herein, with domain 2A inserted within domain 1B.

The 1B and 2A domains also create an interface that binds inter- or intramolecular peptides in all superfamily members identified herein. For PilM, this peptide is the cytoplasmic N terminus of PilN. For GspL and BfpC, this peptide corresponds to the cytoplasmic PilN-like portion of GspL or BfpC, respectively. For FtsA, this peptide corresponds to the N terminus of FtsN (32, 34), as mentioned above, although as yet there are no corroborating structural data. This peptide in DnaK corresponds to the linker region between the actin-like portion of DnaK and its substrate-binding domain. For hexokinase, this peptide corresponds to the linker region between an N-terminal domain and the actin-like portion of hexokinase. For actin, this peptide corresponds to the WH2 motif of DnaE I, and several other peptides bind actin at this same interface (53). Peptide binding at this interface is thus a conserved feature of PilM paralogs.

Comparison of our *P. aeruginosa* PilM-PilN(1–12) structure with that of PilM_PilN(1–15) (15) revealed that the mode of binding of PilM to PilN was highly conserved and amphipathic (Fig. 3A). In both species, PilN Ile-4 (Leu-4 in *T. thermophilus*), Leu-6, and Leu-7 are tucked into hydrophobic pockets; Asn-5 forms hydrogen bonds with the backbone of residues Lys-166 (Val-168) and Val-168 (Leu-170); and Arg-3 forms a salt bridge
with a residue near the C terminus. In T. thermophilus, Arg-3 forms a salt bridge with Glu-374 in PilM, whereas in P. aeruginosa, Arg-3 forms a salt bridge with the backbone of Phe-353 in PilM. The PilM-PilN interface buries 610 Å² with a binding free energy of $-4.9$ kcal/mol, as calculated by PDBePISA (52).

The PilN Binding Pocket of PilM Is Promiscuous—Although the P. aeruginosa PilM and PilN(1–12) structures are remarkably similar (RMSDC = 1.9 Å), our PilM structure, the first to be determined without a PilN peptide bound, exhibits a number of novel features. Examination of the PilM structure revealed that its PilN binding pocket was occupied. We found continuous electron density from the PilN binding groove to the N terminus of the second PilM protomer in the asymmetric unit and were able to build the 7 N-terminal residues of PilM (PilM(1–7)) into this density (Fig. 3B). As detailed above, these residues could not be modeled in the PilM-PilN(1–12) structure. Examination of this interaction revealed that PilM(1–7) was oriented antiparallel to the PilN peptide in the PilM-PilN(1–12) structure, with residues Met-1, Leu-4, and Ile-5 of PilM playing roles similar to Leu-7, Leu-6, and Ile-4 of PilN in PilM-PilN(1–12), respectively. Although the side chain of PilM Lys-7 could not be modeled, the position of the main chain atoms is similar to that of PilN Arg-3. PilM(1–7) adopts a bent configuration compared with the extended conformation of PilN. The bend originates between PilM Leu-4 and Ile-5. PilM Leu-4 and Ile-5 appear to stretch to occupy the sites occupied by PilN Leu-6 and Ile-4 in PilM-PilN(1–12). PilN Leu-6 and Ile-4 do not need to stretch because Asn-5 separates them, and thus PilN remains extended. The bent configuration of PilM(1–7) appears to hold the PilN binding pocket open. Despite these differences, the buried surface area (620 Å²) and binding free energy ($-8.8$ kcal/mol) of the PilM(1–7) interaction are similar to values calculated for the PilM-PilN peptide interaction.

The N Terminus of PilM Contains a Conserved Motif Necessary for PilM Dimerization—Structural comparison of PilM-PilM(1–7) binding with PilM-PilN(1–12) binding indicated that the N-terminal residues Met-1, Leu-4, Ile-5, and Lys-7 play a key role in the binding of the N-terminal peptide to the protein. If the binding of PilM(1–7) had functional implications, we hypothesized that a similar pattern of residues should be found in other PilM orthologues. PilM orthologues from...
unique species were aligned to our structure of PilM to define the flexible N terminus from the globular portion of PilM. This analysis revealed that approximately two-thirds of PilM orthologues have at least an extra 7 flexible N-terminal residues. Due to variations in length and sequence, these PilM N termini align poorly. Despite this, 54% of N termini have an hXXhhX+ motif. Allowing optional single residue deletions, denoted by (X) in hXX(X)hh(X)+, allows this motif to be found in 80% of the sequences examined. Although our motif search was for any hydrophobic or positive residues in the sequences examined. Although our motif search was for any responsible for the dimerization of PilM.

Either of the N-terminal or FtsA-like interfaces might be involved in typical protein-protein interactions between similar domains. Because typical protein-protein interactions have buried surface areas of greater than 600 Å (54), either of the N-terminal or FtsA-like interfaces might be responsible for the dimerization of PilM.

Similar to FtsA, the actin-like interface of PilM suggested that it could potentially form higher order oligomers. However, negative stain EM revealed no PilM oligomers. To further explore the dimer interface, we deleted the first 8 N-terminal residues of our PilM construct (PilM1–8). SEC-MALS analysis showed that PilM1–8 was monomeric in solution (Fig. 3D), indicating that the N terminus of PilM was important for homodimer formation. These data are consistent with PilM (1–7) binding to the PilN binding pocket in the crystallographic dimer. These data are also consistent with the monomeric structure of PilM-PilN(1–12) because the binding of PilN to PilM would prevent the PilM self-interaction.

Lys-52 and Ser-19 Are Important for but Not Essential to ATP Binding—Examination of the electron density map revealed the presence of nucleotide in both the PilM and PilM-PilN(1–12) structures. Although we added ATP to our crystallization buffer, we found that modeling ADP and magnesium best fit the electron density observed in the PilM structure (Fig. 4A). This suggests that either the terminal phosphate was disordered or that ATP was hydrolyzed during the crystallization process. If the latter is the case, hydrolysis was likely to be non-enzymatic, because we and others (15) have been unable to demonstrate in vitro ATPase activity for either PilM or PilM-PilN(1–12). However, aligning the ATP-binding site of PilM with homologous ATPase DnaK (PDB code 4B9Q) revealed a high degree of conservation in the residues necessary for ATP catalysis, including Lys-52 and Glu-171 of PilM, which align with the catalytic residues Lys-70 and Glu-171 of DnaK (55) (Fig. 4B). In the PilM structure, Lys-52 and Glu-171 are shifted away from the γ-phosphate of ATP by 3 Å compared with corresponding Glu-171 and Lys-70 of DnaK. Thus, PilM could potentially have ATPase activity if a conformational change brought Lys-52 and Glu-171 closer to the γ-phosphate.

To model the γ-phosphate, we crystallized PilM in the presence of AMP-PNP (Fig. 4C). These crystals diffracted to lower resolution (3.5 Å), but valuable information could still be derived. Residue Lys-52 was disordered in the PilM-ADP structure but ordered in PilM-AMP-PNP structure. Lys-52 and Ser-19 appear to facilitate coordination of the γ-phosphate of ATP. To confirm that these residues participate in ATP binding, we made S19A and K52A PilM variants and evaluated their ATP-binding affinity using ITC (Fig. 4E). The binding of ATP to PilM was enthalpy-driven, with a $K_d$ of 4.63 ± 0.05 μM, whereas binding of ADP to PilM was too weak to be quantified. Compared with wild type, the PilM(S19A) and PilM(K52A) variants have ~6- and 4-fold reduced affinity for ATP, respectively. These data suggest that Ser-19 and Lys-52 are each important but not essential for ATP binding.

Examination of the active sites of the two protomers in the asymmetric unit of the PilM-PilN(1–12) structure revealed electron density that could be best fit as ATP and magnesium in one protomer (Fig. 4D). In the second protomer, sulfate, which was present in the crystallization buffer, appeared to be bound in the ATP-binding pocket. As in the PilM-AMP-PNP structure, Ser-19 and Lys-52 coordinated the γ-phosphate of ATP. The binding affinity of PilM-PilN(1–12) for ATP was similar to that of PilM (4.37 ± 0.05 μM) (Fig. 4E).

PilN Binding Causes Tertiary Structure Changes in PilM—Comparing the overall structures of PilM bound to ADP versus AMP-PNP revealed a 5° rotation of domain 1B with respect to domain 2A (Fig. 5A and supplemental Animation 1). Comparison of the AMP-PNP-bound structure of PilM with the ADP-bound structure of PilM-PilN(1–12) revealed a conformational change in PilM upon binding PilN (Fig. 5B and supplemental Animation 2). Using DynDom (56), we determined that Domain 1C of PilM undergoes a hinge-bending motion of ~25° that clamps the PilN peptide between the 1C and 1B domains. In a corresponding lever-like motion, a loop in the 1C domain of PilM (residues 83–85, loop 1B) moves 7 Å, whereas domain 1B rotates ~10° with respect to domain 2A. These motions bring the loop containing Lys-52 closer to ATP by 2 Å. Because binding of AMP-PNP to PilM versus PilN to PilM rotated domain 1B in opposing directions, we hypothesized that ATP binding could modulate PilN binding. We tested this hypothesis using bilayer interferometry with a biotinylated peptide of PilN(1–12) attached to the biosensor (Fig. 5C). The $K_{d}$, $k_{on}$, and $k_{off}$ values of PilM for PilN(1–12) with 2 mM ATP were 3.27 ± 0.03 μM, 20,800 ± 200 M$^{-1}$ s$^{-1}$, and 67,900 ± 200 s$^{-1}$, respectively. The dissociation constant of PilM corresponds to a half-life of ~10 s. The affinity of PilM for PilN(1–12) was 2-fold higher in the presence of ATP than ADP. The
dissociation rate was the same regardless of which nucleotide was present. We also evaluated the binding of the PilMK52A and PilMS19A variants to PilN. Like wild-type PilM, the affinity of PilMS19A for PilN(1–12) was 2-fold higher in the presence of ATP than ADP. Conversely, the affinity of PilMK52A for PilN(1–12) was the same with ADP or ATP. These data suggest that ATP binding increases the association of PilM and PilN(1–12) and that Lys-52 is necessary for ATP to mediate this effect.

PilM Bridges PilN, PilB, PilT, and PilC— Based on data from the homologous T2S and T4bP systems, we hypothesized that in addition to ATP and PilN, PilM will bind to additional components of the T4aP system. BACTH assays (57) have been used previously to analyze the connectivity of cytoplasmic T4aP proteins in Neisseria meningitidis. These data suggested interactions between PilM and PilT2, a PilT paralog not present in P. aeruginosa (58). To determine whether additional binding partners could be identified in P. aeruginosa with our PilM constructs, we performed BACTH analysis with both PilM and PilM/H18528PilN(1–12) using two assay outputs, MacConkey-maltose and the/n-galactosidase assay.

Although the interactions observed for the cytoplasmic proteins in P. aeruginosa are similar to those reported for N. meningitidis, there are several important differences (Fig. 6, A and B). As were seen in N. meningitidis, using the MacConkey-maltose assay, we identified interactions between PilC and PilA, PilC and PilO, PilC and PilT, PilT and PilU, PilT and PilB, PilM and PilN, PilN and PilO, and PilO and PilA as well as PilA/B/C/M/O/T/U self-interactions. We also observed a PilC-PilU

**FIGURE 4.** Structural analysis of ATP binding to PilM. Shown are stick representations of the ATP binding site of the PilM-ATP (A), PilM-AMP-PNP (B), and PilM-PilN(1–12)-ATP (C) structures. Direct polar contacts are shown as dashed lines. ADP, ATP, and AMP-PNP are shown in light gray, waters coordinated by magnesium are shown as small dark gray spheres, and magnesium is shown as a large black sphere. The gray mesh represents the 2Fo−Fc omit electron density calculated without nucleotide and contoured at 1σ. D, DnaK (black) from E. coli (PDB code 2B9Q) bound to ATP aligned with PilM/PilN(1–12) ATP (cyan). Structures were aligned by superimposing the ATP ligand. E, isothermal titration calorimetry analysis of PilM, PilMK52A, PilMS19A, and PilM/PilN(1–12) titrated with ATP or ADP. The raw data of each PilM construct are plotted in μcal/s on the left with the calculated dissociation constant (K) indicated. The integrated data for each ATP titration are plotted on the right; PilM is plotted as a filled circle, PilMS19A as a filled square, PilMK52A as a filled triangle, and PilM/PilN(1–12) as an empty circle.
interaction and PilM-PilB, -PilT, and -PilC interactions. It is not clear whether these additional interactions are absent in the *N. meningitidis* T4aP system or if *P. aeruginosa* proteins are more amenable to BACTH analysis. Interactions between PilM-PilC and PilM-PilB equivalents in the T2S system have been described previously (24, 27). Consistent with our SEC-MALS data, we detected PilM self-interactions but not PilM-PilN(1–12) self-interactions. Our BACTH analysis broadens our understanding of the connectivity of the cytoplasmic T4aP components and highlights the central position of PilM.

Comparing the BACTH signals using the β-galactosidase assay suggests that whereas PilM and PilM-H18528PilN(1–12) bind PilB, PilT, and PilC, their affinities for these proteins are different (Fig. 6C). Specifically, PilM-H18528PilN(1–12) interacts preferen-

![Structure and Binding Partners of PilM](image-url)
tially with PilB, and PilM interacts preferentially with PilC and PilT. The β-galactosidase assay also suggested an interaction between PilA and PilM, which was not identified using the MacConkey-maltose assay, underscoring the necessity for secondary validation of interactions identified by BACTH.

Validating these differential interactions in vitro has not been possible. Purified PilB and PilIT each have ATPase activity, suggesting that they are folded properly, but PilM and PilIT aggregated in our biophysical instruments, impeding analysis. However, we could validate a direct interaction between PilM and PilB or PilIT using in vitro co-purification studies. Whereas PilM showed some nonspecific binding to the Ni-NTA resin, additional PilM was co-purified with His-tagged PilB or PilIT (Fig. 6D). PilM-PilN(1–12) did not nonspecifically bind to the Ni-NTA resin and more clearly co-purified with His-tagged PilB and PilIT, providing evidence that PilM interacts directly with PilB and PilIT in vitro.

**Discussion**

In this study, we characterized two distinct PilM conformations using crystallographic, biophysical, biochemical, and BACTH analyses. We identified conserved peptide motifs that bind to each of these conformations, supporting the relevance of both. Further, we have extended the PilM protein interaction network and demonstrated using two different methods that PilM binds to PilB and PilIT.

An intriguing feature of our PilM crystal structure is the interaction we observed between PilM(1–7) and the adjacent PilM protomer. This peptide occupies the PilN binding groove and facilitates the formation of a PilM homodimer. Although we have not determined whether PilM homodimerizes in vivo, comparison with other species revealed that the residues involved in this interaction are a conserved feature of PilM. PilM(1–7) and PilN(1–8) residues make similar contacts in the binding pocket, and both are followed by positive residues interspersed with polar residues. The N terminus of FtsN bound by the PilM homolog FtsA also has a conserved motif followed by interspersed positive residues (32) extending the number of conserved features between PilN and FtsN. PilN(1–8) and PilM(1–7) have amphipathic characteristics, reflecting the nature of the PilN binding pocket of PilM. These amphipatic qualities and membrane proximity suggest that PilN(1–8) and PilM(1–7) could be membrane-associated when not bound to PilM. The PilM homologues, FtsA and MreB, have membrane-associated C- and N-terminal amphipatic helices, respectively (59, 60). The amphipathic C-terminal helix of FtsA maintains FtsA in a non-polymerized conformation until FtsA is adjacent to a lipid bilayer (61). Thus, PilM(1–7) might keep PilM in a dormant conformation until PilN binding, or it may keep PilM stable when temporally disengaged from PilN.

The structural analysis in this study revealed that peptide binding at an interface created by domains 1B and 2A is highly conserved in actin/Hsp70/hexokinase superfamily proteins. This interface corresponds to the PilN binding pocket of PilM and may be a conserved binding site because it is located at a hinge point that can rotate upon ATP binding (62). Hence, where it has been evaluated, peptide binding to this interface can be modulated by ATP binding and/or catalysis (53, 63).

This is consistent with our finding that ATP binding to PilM increased PilN affinity ~2-fold. In DnaK, ATP binding increases affinity for the interdomain linker peptide, 389VLLL392 (63). When DnaK binds the 389VLLL392 peptide, a substrate peptide bound to a different interface is released (63). Hence, substrate release is dependent on 389VLLL392 binding, which is itself dependent on ATP binding. Mutating Lys-70 in DnaK prevents ATP-dependent release of substrate peptide (64). Thus, it seems likely that Lys-70 in DnaK enables ATP to increase the affinity of DnaK for the 389VLLL392 peptide. PilM Lys-52 aligned structurally with DnaK Lys-70, and mutation of PilM Lys-52 similarly nullified the ATP-mediated increase in PilN binding. Lack of the 2B domain necessary to bind ATP in GspL and BfpC of the T2S and T4bP systems (28) suggests that GspL and BfpC do not need to regulate association to their PilN-like portions, consistent with fusion of these domains.

We found that the half-life of the PilM-PilN(1–12) interaction is ~10 s, suggesting that PilN may disengage from PilM on a time scale of relevance to type IV pilus dynamics. For example, the pilus of *P. aeruginosa* is expected to extend or retract for ~1–14 s, because pili lengths vary from 1 to 7 μm, and the mean extension and retraction rate is 0.5 ± 0.2 μm/s (65, 66). In other words, the PilM-PilN interaction may be just long enough for a cycle of extension or retraction and thus could have functional implications for these or related processes.

At the time of manuscript submission, these analysis provided the first evidence for any T4aP system that PilM directly interacts with PilB and PilIT and provided BACTH data to suggest that PilM might bind PilC. Before manuscript publication, it was demonstrated that PilM directly interacts with PilB in *Mycobacterium xanthus* (67), corroborating our results. Both BfpE, the PilC homologue in the T4bP system, and GspE, the PilB homologue in the T2S, have been shown to bind to the PilM loop 1B equivalents in BfpC and GspL, respectively (24, 51). We predict that PilM interacts with PilB and potentially PilC using a similar interface. Comparing the structures of PilM and PilM-PilN(1–12) revealed that residues in loop 1B move by 7 Å in the presence of the PilN peptide. Likewise, our BACTH data suggested that interaction of PilM with PilN differentially impacts the binding of PilM to PilB, PilT, and PilC. Based on these analyses, we predict that the binding of PilN to PilM dynamically regulates PilM binding to PilB, PilT, and/or PilC.
Structure and Binding Partners of PilM

(Fig. 7). One hypothesis might be that PilN detachment from PilM is part of a molecular switch regulating a T4aP process, such as extension or retraction. The newly identified protein-protein interactions described herein suggest that PilM is not simply a passive scaffold but potentially a key regulator of T4aP dynamics.

Author Contributions—M. M. performed all experiments and wrote the manuscript. S. T. aided in experimental design and edited the manuscript. J. K. performed the EM studies. D. J. L. aided with crystallization, structure solution, and crystallographic data analysis and edited the manuscript. H. R. collected crystallographic data at the X29 beamline. M. S., C. C., and T. F. M. performed SEC-MALS analysis. M. M., L. L. B., and P. L. H. designed the project, analyzed the results, and wrote the manuscript. All authors reviewed and approved the final version of this article.

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