Interactions between the Lipoprotein PilP and the Secretin PilQ in *Neisseria meningitidis*^{▽†}

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Neisseria meningitidis can be the causative agent of meningitis or septicemia. This bacterium expresses type IV pili, which mediate a variety of functions, including autoagglutination, twitching motility, biofilm formation, adherence, and DNA uptake during transformation. The secretin PilQ supports type IV pilus extrusion and retraction, but it also requires auxiliary proteins for its assembly and localization in the outer membrane. Here we have studied the physical properties of the lipoprotein PilP and examined its interaction with PilQ. We found that PilP was an inner membrane protein required for pilus expression and transformation, since *pilP* mutants were nonpiliated and noncompetent. These mutant phenotypes were restored by the expression of PilP *in trans*. The *pilP* gene is located upstream of *pilQ*, and analysis of their transcripts indicated that *pilP* and *pilQ* were cotranscribed. Furthermore, analysis of the level of PilQ expression in *pilP* mutants revealed greatly reduced amounts of PilQ only in the deletion mutant, exhibiting a polar effect on *pilQ* transcription. *In vitro* experiments using recombinant fragments of PilP and PilQ showed that the N-terminal region of PilP interacted with the middle part of the PilQ polypeptide. A three-dimensional reconstruction of the PilQ-PilP interacting complex was obtained at low resolution by transmission electron microscopy, and PilP was shown to localize around the cap region of the PilQ oligomer. These findings suggest a role for PilP in pilus biogenesis. Although PilQ does not need PilP for its stabilization or membrane localization, the specific interaction between these two proteins suggests that they might have another coordinated activity in pilus extrusion/retraction or related functions.

Neisseria meningitidis, or the meningococcus, is the cause of significant morbidity and mortality worldwide. Meningococci express type IV pili, which mediate the colonization of its exclusive human host by adherence (50, 51). These surface organelles are therefore important in the pathogenesis of meningococcal disease and also for infections caused by the closely related *N. gonorrhoeae*, the gonococcus, as well as for many other gram-negative bacteria. In the pathogenic *Neisseria*, type IV pili are involved in autoagglutination, twitching motility (26, 35), and natural transformation (6, 47, 62). *Neisseria* twitching motility has been shown to be due to pilus retraction (35). At least 18 proteins have so far been found to be involved in the pilus biogenesis machinery in *Pseudomonas aeruginosa* (1) and, at minimum, 15 proteins in *Neisseria* sp. (8). Among these 15 Pil proteins, in addition to pilin, which is the principal structural protein in the pilus fiber, only 6 proteins, including PilP, were suggested to be required for the pilus assembly (7). A complete description of the structure-function relationships of pilus biogenesis components and how they

participate in pilus assembly, extrusion, and retraction is still to be produced.

Neisseria type IV pili are thought to be extruded and retracted through the secretin PilQ (10). This secretin is a member of a large family of integral outer membrane proteins with conserved C-terminal domains engaged in type II secretion, including type IV pilus biogenesis, and type III secretion (18, 23, 54). The C-terminal domain of PilQ is likely to be important for multimer assembly of this secretin (5, 18, 54). The N-terminal domains of secretins are generally less conserved and confer substrate specificity (37, 43). Examination of purified secretins, such as PilQ and XcpQ, by transmission electron microscopy (TEM) indicates a ring-like structure in projection (5, 10, 12, 13, 18, 32, 44, 57, 61). The meningococcal secretin PilQ oligomer consists of 12 identical monomers (10, 12–14, 54). A three-dimensional (3D) structure of meningococcal PilQ, determined by single-particle averaging from TEM images after cryonegative staining, showed a lantern-like assembly with a large central chamber measuring up to 6.5 nm across (14). Interestingly, there are some structural similarities between the PilQ oligomer and Wza, an outer membrane protein involved in the transport of capsular polysaccharide across the outer membrane (11). PilQ spontaneously associates with type IV pili when they are incubated together *in vitro*: the PilQ oligomer binds at one end of the pilus fiber, which potentially fills the central chamber (13). This observation suggests that the PilQ secretin interacts specifically with its secreted substrate, and could therefore play a role in pilus assembly or disassembly. In *N. meningitidis*, PilQ occurs as the sole secretin

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TABLE 1. Plasmids and bacterial strains included in the study

Plasmid or strain	Relevant characteristic(s)	Source or reference
Plasmids		
pET28b(+)	C-terminal His tag expression vector	Novagen, United States
pCR7 TOPO	N-terminal expression vector	Invitrogen, United States
pUC4K	Drug-resistance marker encoding aminoglycoside 3'-phosphotransferase	Amersham PB 47
pUP6	pHSS6 derivative carrying two neisserial DNA uptake sequences	This study
pET28b(+)- <i>pilP</i>	pET28b(+) contains <i>pilP</i> from MC58	This study
pUP6- <i>pilP</i> _{Δ1-27::kan}	pUP6 contains <i>pilP</i> from MC58 with inserted kanamycin cassette	This study
pUP6 <i>pilP</i> _{74-6xHis}	pUP6 carries <i>pilP</i> from MC58 with six-His tag inserted after amino acid 74	This study
pUP6:: <i>pilP</i> fs	pUP6 contains <i>pilP</i> from MC58 with frame shift in first 14 bases	This study
p2/16/1	Derivative of pUP6 that allows insertion of DNA fragments into <i>iga</i> locus	59
pSY6	Plasmid containing nalidixic acid-resistance-conferring gene	49
Strains		
ER2566	<i>E. coli</i> strain; F ⁻ λ ⁻ <i>fhuA2</i> [<i>lon</i>] <i>ompT</i> <i>lacZ</i> ::T7 <i>gene1</i> <i>gal</i> <i>sulA11</i> Δ(<i>mcrC-mrr</i>)114::IS10 <i>R</i> (<i>mcr-73</i> ::mini-Tn10-Tet ^s) 2 <i>R</i> (<i>zgb-210</i> ::Tn10)(Tet ^s) <i>endA1</i> [<i>dcm</i>]	New England Biolabs
BL21(DE3)*	<i>E. coli</i> strain; F ⁻ <i>ompT</i> <i>hsdS_B</i> (r _B ⁻ m _B ⁻) <i>gal</i> <i>dcm</i> <i>rne131</i> (DE3)	Invitrogen, United States
M1080	<i>N. meningitidis</i> serogroup B; B:1:P1.1,7; United States	20
MC58	<i>N. meningitidis</i> serogroup B; ET-5 complex; England	34
M1080Δ <i>pilP</i>	<i>N. meningitidis</i> M1080 strain with <i>pilP</i> ::Kan	This study
M1080Δ <i>pilP</i> , <i>iga</i> :: <i>pilP</i>	<i>N. meningitidis</i> M1080Δ <i>pilP</i> strain containing ectopic <i>pilP</i> gene in <i>iga</i> locus	This study
M1080 <i>pilP</i> fs	<i>N. meningitidis</i> M1080 with frame shift in <i>pilP</i> gene	This study
M1080 <i>pilP</i> fs, <i>iga</i> :: <i>pilP</i>	<i>N. meningitidis</i> M1080 <i>pilP</i> fs strain containing ectopic <i>pilP</i> gene in <i>iga</i> locus	This study
M1080-PilP _{74-6xHis}	<i>N. meningitidis</i> M1080 strain with six-His tag in <i>pilP</i> gene after amino acid 74	This study
M1080Δ <i>pilQ</i>	<i>N. meningitidis</i> M1080 strain with mini-Tn _{cm21} in <i>pilQ</i>	54

and is unique among the secretin family members because of its abundance in the outer membrane and a polymorphic N-terminal region containing repetitive octapeptides, or small basic repeats (54). Recently, it has been reported that PilQ has the ability to bind DNA, with a binding site in the central part of the structure (3).

The oligomerization, stabilization, and/or outer membrane localization of secretins is often influenced by small lipoproteins. Until now, *Klebsiella oxytoca* PulS (16, 25), *N. gonorrhoeae* PilP (19), *Erwinia chrysanthemi* OutS (48), *Salmonella* InvH (15, 17), *Yersinia enterocolitica* YscW (29), and *Shigella flexneri* MxiM (45), as well as the orthologs *N. meningitidis* PilW (8) and *Myxococcus xanthus* Tgl (38), have been reported to affect secretin assembly. *S. flexneri* MxiM has been shown to interact with the C-terminal part of its cognate secretin (30, 46). These lipoproteins are all relatively small and are predicted to have the lipid moiety covalently attached to their N termini. They have low sequence identity, ranging from 17 to 19%.

Involvement of PilP in gonococcal pilus biogenesis was first reported by Drake et al. (19). The neisserial *pilP* gene is located upstream of *pilQ* in a cluster with other pilus biogenesis genes: *pilM*, *pilN*, and *pilO* (19, 40, 52). Gonococcal *pilP* mutants carrying transposon insertions in the central part of the open reading frame were nonpilated and failed to exhibit pilus-associated phenotypes. The levels of oligomerized PilQ were also reduced in those *pilP* mutants. Based on these findings, it was suggested that PilP functions by stabilizing the PilQ complex (19). In *N. meningitidis* and *M. xanthus*, no role for PilP in PilQ multimer stabilization has been defined (8, 38). Recently, it has been reported that another meningococcal

pilus biogenesis protein, the 28-kDa lipoprotein PilW, influences PilQ oligomer levels (8). In *M. xanthus*, however, the lipoprotein Tgl was reported to be required for PilQ complex assembly (38).

Recently, we have determined the structure of a recombinant fragment of PilP by nuclear magnetic resonance (NMR) and shown that it adopts a novel β-sandwich-type fold (24). The protein appears to form a hydrophobic binding site for a small ligand, in a fashion similar to MxiM (30), although the two proteins share no sequence or structural homology. The results of this structural work therefore indicate that PilP might interact with another protein or component.

In this work, we studied the physical properties of meningococcal PilP and found that it was copurified with the inner membrane. We provide evidence that *pilP* and *pilQ* are co-transcribed and show that there is a specific interaction between the two proteins, suggesting that they act coordinately in pilus biogenesis and transformation or related functions.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Meningococcal strains were grown overnight in 5% CO₂ at 37°C on GC agar plates supplemented with IsoVitalEx (Becton Dickinson, United States). When required, kanamycin at a final concentration of 100 μg/ml was added. *Escherichia coli* strain ER2566 (New England Biolabs, United Kingdom) was used for plasmid propagation and was grown in LB medium or on LB plates containing kanamycin (50 μg/ml) or ampicillin (100 μg/ml) at 37°C. The bacterial strains and plasmids employed in this study are listed in Table 1.

Search for signature motifs. A search for functional domains and signatures in the meningococcal MC58 *pilP* deduced amino acid sequence (NMB1811) was carried out using DOLOP (31) and PROSITE (28). The MC58 PilP sequence was assessed for disorder prediction on DISPROD2 (58) and VSL1 (56).

Cloning and overexpression of PilP. The *pilP* gene was PCR amplified from MC58 genomic DNA using primers as listed in Tables S1 and S2 in the supplemental material. The gene fragments encoding full-length, N-terminal, C-terminal, and internal deletions of the *pilP* gene were cloned into the vector pET28-b(+)(Novagen, United States), with the C-terminal six-His tag in frame, or into the vector pCR7 TOPO (Invitrogen, United States), with an N-terminal His tag. For the generation of the internal deletion mutants, splicing by overlap extension (SOEing) PCR was employed (27). All recombinant proteins were overexpressed in *E. coli* ER2566 (New England Biolabs, MA) or *E. coli* BL21(DE3)* (Invitrogen, United States).

Construction of *pilP* mutants. The *pilP* knockout mutant was made by deleting 81 bp beginning from the start codon and inserting a kanamycin resistance cassette (Kan^r) into a SalI site created with the primers SVB25 and SVB26. The plasmid obtained, pUP6-*pilP*_{Δ1-27}:kan, was transformed into meningococcus strain M1080 by natural transformation using kanamycin resistance selection (55). A frameshift mutation was constructed by SOEing PCR using the primers SVB24, SVB77, SVB27, and SVB78 (see Tables S1 and S2 in the supplemental material). A single base at the start codon was deleted and a restriction site (EcoRI) was created to verify the frameshift mutation. The PCR product was digested with BamHI and ligated into the BamHI site of pUP6 to create the plasmid pUP6::*pilP*_{fs}. Plasmid pUP6::*pilP*_{fs} was transformed into *N. meningitidis* M1080, and transformants were detected by PCR after nonselective transformation. For the complementation studies, cloning of *pilP* into vector p2/16/1 (59) was performed using the primers SVB71-SVB74 (see Tables S1 and S2 in the supplemental material) and the resulting product was digested with SacI and cloned into a unique SacI site in the vector.

Construction of a meningococcal in-frame PilP-His fusion. (i) Genetic construct. A meningococcal in-frame *pilP*-His fusion was designed to encode a six-histidine tag in the predicted loop region at amino acid 74. For the generation of the construct encoding the internal PilP-His fusion, PCR SOEing was employed. The 5' and 3' fragments were produced with the primer combinations SVB31/SVB19 and SVB20/SVB32 (see Table S1 in the supplemental material), respectively. The final PCR product was amplified with the external primers SVB31 and SVB32, digested with BamHI, and ligated into the BamHI-digested vector pUP6 (47). The final vector, pUP6-*pilP*_{74-6xHis}, was used for the transformation of wild-type meningococcus M1080.

(ii) Nonselective transformation. Agglutinating colonies were densely streaked on blood agar plates and grown for 4 to 6 h at 37°C in 5% CO₂. Cells were harvested and resuspended in prewarmed GC broth supplemented with IsoVitalEx (Becton Dickinson, United States) and 7 mM MgCl₂. Plasmid DNA was added to the cell suspension. The sample was briefly mixed and incubated without agitation for 30 min at 34°C. Prewarmed supplemented medium was added, and the suspension was spread on blood agar plates and incubated overnight.

(iii) PilP-His fusion screening. Crude DNA samples for PCR testing from clones were made by freezing and thawing in distilled water and removal of debris by centrifugation. Specific primers SVB22 and SVB23 were designed to produce small PCR products covering the points of mutation (see Table S1 in the supplemental material).

Purification of recombinant PilP and PilQ proteins and pilus fibers. The full-length and partial PilP and PilQ recombinant proteins were overexpressed in *E. coli* and purified to homogeneity. All recombinant PilP proteins were affinity purified using Ni-nitrilotriacetic acid (NTA) agarose (QIAGEN, Germany) or, for PilP_{Δ1-19} only, Talon cobalt metal affinity resin (Clontech, United States) under native conditions according to the manufacturer's protocol. The eluted proteins, except PilP_{Δ1-19}, were immediately dialyzed against 50 mM sodium phosphate buffer (pH 7.0) containing 300 mM NaCl and 10% glycerol. PilP_{Δ1-19} was subjected to a second purification step by anion exchange using a MonoQ HR 10/10 column (GE Healthcare, United Kingdom). Purification of recombinant PilQ proteins and meningococcus type IV pilus fibers was performed as previously described (13).

Native purification of PilQ complex and His-tagged PilP. The PilQ complex was purified from meningococcus outer membranes by detergent solubilization and size exclusion as previously described (14). For native PilP purification, meningococcus M1080-PilP_{74-6xHis} cells were grown on blood agar plates in 5% CO₂ at 34°C overnight, harvested in cold phosphate-buffered saline (PBS) buffer and collected by centrifugation at 4,000 × *g* for 15 min. The cell pellet was resuspended in cold Tris-buffered saline (pH 7.5) containing 1× EDTA-free complete protease inhibitor cocktail (Roche Applied Science, Germany) and passed twice through a French press at 25,000 lb/in². Cell debris was removed by two succeeding centrifugations at 4,000 × *g* for 10 min. The supernatant was collected, and the membrane fraction pelleted by centrifugation at 35,000 rpm for 35 min in an SW40Ti rotor (Beckman, Ireland). The membrane pellet was

dissolved in lysis buffer (10 mM imidazole [Sigma, Germany], 300 mM NaCl, 50 mM NaH₂PO₄, 0.5% Triton X-100, 10 mM β-mercaptoethanol [Sigma, Germany], 3 mM MgCl₂, pH 8.0) containing 1× EDTA-free complete protease inhibitor cocktail and incubated with rotation at 4°C overnight. The dissolved membrane fraction was centrifuged at 4,000 × *g* for 15 min, and the clear supernatant was loaded onto a Ni-NTA agarose column (QIAGEN, Germany). Bound proteins were eluted with elution buffer (200 mM imidazole, 300 mM NaCl, 50 mM NaH₂PO₄, pH 8.0) and immediately dialyzed against 50 mM sodium phosphate buffer (pH 7.0) containing 300 mM NaCl and 10% glycerol.

Antibody production. Rabbit polyclonal antibodies were raised against the purified full-length recombinant PilP proteins to generate antiserum K824 as previously described (54, 55). Antisera against PilQ were produced as previously described (22, 54).

Reverse transcription-PCR (RT-PCR). Total RNA was isolated from MC58 cells using TRIzol (GIBCO Life Technologies, United States). After treatment with DNase I, the RNA concentration was spectrophotometrically quantitated, and the quality was assessed by agarose gel electrophoresis. RT was performed in a final volume of 30 μl containing 1× RT buffer, 7 units AMV reverse transcriptase (Promega, United States), 28 units RNasin RNase inhibitor (Promega, United States), 15 mM deoxynucleoside triphosphates (GE Healthcare, Sweden), 10 μM reverse primer *pilQ*-6485, and 25 ng RNA template. The primer and RNA were denatured at 94°C for 1 min and immediately cooled on ice for 5 min before being added to the reaction mixture. The reaction was incubated at 42°C for 1 h. One microliter of 10 μg/ml RNase A was added to the controls prior to incubation, and samples were incubated at room temperature for 5 to 20 min. RT products were incubated at 98°C for 3 min to separate the strands. Five microliters of the cDNA-containing product was amplified in a 50-μl PCR mixture containing 1× PCR buffer and 1 unit *Taq* polymerase (BioLine, CA), as well as 10 μM of forward primer *pilQ*-5675 or *pilP*-5075 and reverse primer *pilQ*-6485. After denaturation for 5 min at 95°C, PCR was performed for 30 cycles (30 s at 95°C, 30 s at 59°C, and 60 s at 72°C) with a 7-min final extension of the products at 72°C. Controls were included to verify the absence of DNA in the RNA preparation, as well as nonspecific RT in the absence of the reverse primer. The PCR products were analyzed by agarose gel electrophoresis.

Quantitative transformation assay. Natural competence for DNA transformation was quantified by counting the number of CFU obtained after the transformation with plasmid pSY6 harboring a nalidixic acid-resistance-confering gene (49). Transformation was performed as described above for nonselective transformation, except that 1 μg/ml nalidixic acid was used for selection. The ratio of nalidixic acid-resistant CFU to the total number of CFU obtained without antibiotic selection yielded the transformation rate.

SDS-PAGE, immunoblotting, and solid-phase overlay assays. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting, and solid-phase overlay assays in the form of far-Western analysis were carried out as previously described (13). For phenol treatment, equal volumes of the sample and phenol were mixed by vortexing for 2 min, and samples were further vortexed after adding 2 volumes of ice-cold acetone. The proteins were pelleted by centrifugation at 12,000 × *g* for 15 min after overnight incubation at -20°C. The pellet was washed twice with 70% ethanol before it was resuspended in SDS-PAGE sample buffer.

Isolation of outer membrane vesicles (OMV). Bacteria were grown on blood agar plates at 37°C for 15 h, harvested in serotype antigen buffer (0.2 M LiCl, 0.1 M NaAc, pH 5.8) (21) and inactivated at 60°C for 30 min. A bacterial suspension with an optical density at 600 nm of 20 was prepared and shaken with 2-mm glass beads at 230 rpm for 15 min at room temperature. Cell debris was removed by two rounds of centrifugation at 4,000 × *g* for 20 min and 18,000 × *g* for 15 min, respectively. The supernatant was ultracentrifuged at 140,000 × *g* for 90 min and the pellet resuspended in H₂O and ultracentrifuged again at 140,000 × *g* for 90 min. The resulting pellet was resuspended in H₂O and analyzed by immunoblotting.

Separation of outer and inner membranes by sucrose gradient centrifugation. Meningococcal cells were grown on blood agar plates at 37°C in 5% CO₂, harvested in PBS buffer, and washed three times with PBS. The cells were resuspended in 50 mM Tris buffer, pH 8.0, containing 50 μg RNase and DNase (Sigma Aldrich, United Kingdom) and processed twice in a French press at 15,000 lb/in². The cell debris was removed by two centrifugations at 10,000 × *g* for 10 min. Sucrose gradient analysis was carried out as described by Masson and Holbein (33), with slight modifications. The supernatant was transferred onto two layers of sucrose consisting of 3 ml of 55% (wt/wt) sucrose and 4 ml 15% sucrose in H₂O with 3 mM EDTA, pH 8.0, and centrifuged at 217,000 × *g* for 5 h at 4°C in an SW40Ti rotor (Beckman, Ireland). The membrane fraction positioned at the interface of the two sucrose layers was collected and diluted in H₂O with 3 mM EDTA, pH 8.0, to a sucrose concentration of 30%. The membrane

FIG. 1. Gene organization and *pilP* constructs. (A) Chromosomal organization of the *pilP* locus of *N. meningitidis*. (B) Structural features of PilP. The lipobox and proline-rich regions are shaded in gray. Diagonal and dotted boxes indicate disordered and folded regions, respectively. (C) Schematic representation of the PilP clones used in this study. Internal deletions of the PilP protein are shaded in gray. Positions of the six-His tag are marked with H's, and the kanamycin cassette insertion in *N. meningitidis* M1080 *pilP* is also indicated. Relative molecular masses in kDa are given. na, not applicable; *, the PilP Δ ₁₋₁₉ product contains an N-terminal His tag with 35 amino acids derived from the vector.

fraction was then applied to a discontinuous sucrose gradient, consisting of 3 ml of 50, 45, 40, and 35% sucrose in 3 mM EDTA, pH 8.0. This gradient was centrifuged in an SW40Ti rotor for 35 h at $180,000 \times g$ at 4°C and fractionated from the top into 1-ml fractions. The density of each fraction was measured by using a refractometer from Precision Instruments (Bellingham and Stanley, United Kingdom). An aliquot of each fraction was diluted in 3 mM EDTA and ultracentrifuged at 35,000 rpm in an SW40Ti rotor for 3 h. The resulting pellet was resuspended in 50 μ l 3 mM EDTA, and the level of D-lactate dehydrogenase (LDH) activity was measured as described by Osborn et al. (39).

PilP-PilQ interaction image analysis. Sample preparation, cryonegative staining, TEM, and image analysis were carried out as described previously (13). Natively purified recombinant PilP Δ ₁₋₁₉ (100 μ g/ml) was incubated with PilQ complex purified from outer membranes, with or without Ni-NTA-nanogold (Nanoprobes, United States), with agitation for 24 h at 4°C. Samples were then centrifuged at 13,000 rpm in a benchtop centrifuge for 5 min before being prepared for cryonegative staining as previously described (9). Grids were placed in an Oxford system cryostage, and data were recorded at ~ 100 K in a FEG CM200 used in conjunction with a Gatan 4K charge-coupled-device (CCD) camera. Individual gold-labeled particles were interactively selected in 64-pixel boxes ($\text{\AA}/\text{pixel} = 3.1$) by using BOXER. Following contrast transfer function correction, data were contrast normalized, centered, and low-pass filtered to a 25- $\text{\AA}$ resolution. After a 190- $\text{\AA}$ circular mask was applied to the data, six rounds of iterative refinement were performed in C4 symmetry, using our previously calculated 3D structure of PilQ (14) filtered to a 25- $\text{\AA}$ resolution as a start model.

RESULTS

Analysis of PilP structure. In the pathogenic members of the *Neisseriae*, the *pilP* gene is located downstream of *pilO* and upstream of *pilQ* (Fig. 1A), and these genes all encode proteins

required for pilus biogenesis (19, 32). In order to identify functional domains or signatures of the PilP protein, computational predictions were made based on the MC58 *pilP* deduced amino acid sequence. According to the DOLOP database (31), the PilP signal sequence was predicted to be 16 amino acids long, with a C-terminal lipobox (signal peptide cleavage site), LSAC, at positions 13 to 16 (Fig. 1B). In addition, a prokaryotic membrane attachment site (PROSITE entry PS00013) was identified, suggesting that PilP might be anchored in the membrane. A proline-rich region was identified from amino acid 40 to 60 (Fig. 1B). Proline-rich regions tend to be very flexible and are often implicated in protein-protein interaction domains (2). Previous NMR studies of a PilP fragment spanning residues 20 to 181 and a 3D structure determination of the C-terminal region covering residues 69 to 181 (24) showed a single folded domain from residue 85 to 158; the remaining N-terminal (amino acids 25 to 84) and C-terminal (amino acids 159 to 181) regions are disordered in solution. The folded domain is a novel β -sandwich structure, made up of a single 3_{10} -helix and seven β -strands. Based on these results, a scheme for the generation of full-length and truncated forms of recombinant PilP (Fig. 1C).

PilP is expressed in all meningococcal strains examined. Using rabbit antibodies raised against full-length recombinant

FIG. 2. PilP immunodetection. (A) Purified recombinant PilP proteins were immunodetected. Lanes: 1, full-length PilP; 2, PilP Δ 1-12; 3, PilP Δ 1-15; 4, PilP Δ 1-16; 5, PilP Δ 1-77; 6, PilP Δ 61-80; 7, PilP Δ 114-181. (B) Detection of M1080-PilP $_{74-6xHis}$ with anti-PilP antiserum K824 (lanes 1 and 2) and anti-His (lanes 3 and 4). Lanes 1 and 3, wild-type M1080; lanes 2 and 4, M1080-PilP $_{74-6xHis}$. PilP protein is denoted with arrowheads I and II. Positions of the molecular mass standard proteins are indicated on the left in kDa.

PilP, PilP expression was examined in 16 clinical meningococcal isolates (data not shown). PilP was present in all of these strains, indicating that it is an important cellular component. When recombinant PilP proteins were reacted with rabbit antiserum raised against the full-length recombinant PilP, all partial proteins displayed strong reactivity, except the C-terminal PilP Δ 1-77 (Fig. 2A). An in-frame PilP polyhistidine insertion construct was made and introduced into the meningococcal genome in order to purify native PilP from meningococcal membranes. When the cell lysate from this meningococcus M1080-PilP $_{74-6xHis}$ was tested by immunoblotting, the migration of PilP $_{74-6xHis}$ (Fig. 2B, lane 2) was retarded relative to the migration of the wild type (Fig. 2B, lane 1), consistent with its increased molecular mass. The presence of the polyhistidine tag was demonstrated with anti-His antibody (Fig. 2B, lane 4). Based on the results of the immunoblot analysis of truncated PilP proteins, the N-terminal region seemed to be immunodominant with respect to denatured epitopes compared to the C-terminal region. The C terminus was recognized only after semipreparative gel electrophoresis (data not shown), suggesting that this region contains immunogenic epitopes in the tertiary structure. This observation agrees with the results of our NMR studies, which established that the C terminus of the PilP protein consisted of a folded domain but that the N terminus is disordered (24).

PilP copurifies with the meningococcal inner membrane. We demonstrated that PilP was not present in OMV (Fig. 3). In order to determine the subcellular localization of PilP, we isolated the inner and outer membranes by using sucrose density gradient centrifugation. The cellular fractions were assessed for LDH activity as a marker for the inner membrane fraction and PilQ and OpcA as markers for the outer membrane fraction (Fig. 4). The LDH activity was detected at a lower density, corresponding to fractions 4 to 8, whereas PilQ and OpcA were mainly localized at a higher density, corresponding to fractions 10 to 13 (Fig. 4). The analysis of the fractions by immunoblotting, using the PilP antiserum, demonstrated that PilP was located in the inner membrane fractions. The presence of PilP in the inner membrane gradient fractions, in combination with the absence of PilP in OMV, strongly suggests that PilP is associated with the inner membrane. Immunoblot analysis of membrane fractions from the *pilP* frameshift mutant M1080*pilPfs* demonstrated that PilQ was still localized in the outer membrane (data not shown),

FIG. 3. PilP is not present in OMV. Immunoblotting of the whole-cell lysates (lane 1) and purified OMV (lane 2) from *N. meningitidis* MC58 detected with anti-PilP antiserum (K824), anti-PilQ antiserum (K010), and anti-OpcA antibody. α , anti.

indicating that PilP does not have an effect on PilQ membrane localization.

The *pilP* and *pilQ* genes are cotranscribed. Since the genes encoding PilP and PilQ are located in the same locus, RT-PCR was employed to analyze the transcription patterns of the *pilP* and *pilQ* genes (Fig. 5). As expected, 1,410-bp and 810-bp products were obtained when primer pairs *pilP*-5075/*pilQ*-6485 and *pilQ*-5675/*pilQ*-6485 (see Table S1 in the supplemental material), respectively, were used, indicating that *pilP* and *pilQ* are cotranscribed.

PilP is not required for PilQ multimer stability. Previous reports have yielded contradictory results on PilQ stability in *pilP*-knockout mutants (8, 19). Drake et al. (19) found that an *N. gonorrhoeae pilP* null mutant expressed reduced amounts of PilQ complex. In contrast, Carbonnelle and coworkers (8) reported that in an *N. meningitidis pilP* null mutant, the amounts of PilQ monomer and complex formation were not affected. We therefore tested the PilQ multimer stability in our *pilP* mutants. Immunoblotting of whole cell lysates revealed that, in the M1080PilP $_{74-6xHis}$ and the M1080*pilPfs* mutants, PilQ multimers were detected in the stacking gel as for the wild type, but PilQ complex was found in greatly reduced amounts in the M1080*pilP* mutant (Fig. 6). The reduction of PilQ multimers in M1080*pilP* was due to reduced PilQ monomer production, since the overall amount of this protein after phenol treatment of the extract also was reduced (Fig. 6B). Complementation of the *pilPfs* and Δ *pilP* mutants with PilP in *trans* did not exert any effect on PilQ expression or complex levels (Fig. 6A). The analysis of PilP expression in the *pilQ* mutant exhibited partial degradation of PilP (Fig. 6C). These experiments clearly show that PilP is not required for PilQ stability and therefore cannot be classified as a pilotin.

Pilus expression and transformation are abolished in *pilP* mutants. Phenotypic analyses of the *pilP* mutants were carried out by testing piliation, agglutination, and competence for transformation with the M1080*pilQ* mutant as a negative control (Table 2). The results showed wild-type levels of pili in

FIG. 4. PilP copurifies with the inner membrane. The subcellular localization of *N. meningitidis* PilP was assessed by sucrose gradient centrifuge analysis of membrane fractions. Fractions enriched in the inner membranes were identified by monitoring LDH activity (graph, fractions 4 to 8). The presence of PilP protein was detected by immunoblotting using anti-PilP antiserum K824 (upper gel) and coincided with the LDH activity (fractions 4 to 8). Outer membrane fractions (10 to 13) enriched in the outer membrane proteins PilQ and OpcA (middle and lower gels) were detected by immunoblotting with anti-PilQ and anti-OpcA antibodies, respectively. OD/min, optical density per minute; ●, LDH activity; ■, sucrose density; α, anti.

M1080-PilP_{74-6xHis}, whereas the expression of pili was abolished in the M1080Δ*pilP*s and M1080Δ*pilP* mutants (Table 2). Complementation of *pilP* deletion and frameshift mutants restored the pilus expression to the wild-type level (Table 2). Compared to the competence for transformation in wild-type M1080 and M1080-PilP_{74-6xHis}, competence for transformation was abolished in the M1080Δ*pilP* and M1080Δ*pilP*s mutants (Table 2). These observations clearly showed that PilP is important for pilus biogenesis and competence for transformation.

The N-terminal and C-terminal segments of PilP interact with the central portion of PilQ. Solid-phase overlay assay was employed to assess the interaction between the PilP and PilQ proteins, including the full-length, N-terminal, central, and C-terminal portions of the proteins (Fig. 7A) (13, 22). The interactions of another neisserial outer membrane protein, hemoglobin binding receptor (HmbR), as well as bovine serum albumin, with PilP were also included as controls. Full-length PilP exhibited an interaction with full-length PilQ and the central portion of PilQ and a weak interaction with N-terminal

PilQ, but no interaction with the C-terminal PilQ section (Fig. 7B, panel I). PilP proteins with the deletion of amino acids 1 to 12, 1 to 15, 40 to 60, 61 to 80, and polyhistidine-tagged protein (M1080-PilP_{74-6xHis}) exhibited a binding pattern similar to that of full-length PilP protein (Table 3). Deletion of residues 1 to 77 and only residues 1 to 16 abolished binding completely (Table 3). C-terminal PilP deletions which spanned residues 81 to 102, 107 to 116, and 114 to 181 of the protein abolished binding to all three truncated recombinant PilQ fragments, but not to the full-length PilQ. The results suggest that the N-terminal region of PilP, around the lipidated residue Cys₁₆, mediates substantial interaction with PilQ and that the region around the folded domain at the C terminus also contributes to PilQ recognition. For PilQ, the middle section of the polypeptide chain, between residues 213 and 349, seems to be the major site for PilP binding.

3D reconstruction of the PilP-PilQ oligomer complex. Having established the location of the PilP binding site within the primary structure of the PilQ oligomer, we extended the investigation to examine the interaction within the context of the

FIG. 5. The genes encoding PilP and PilQ are cotranscribed. RT-PCR analysis of *pilP* and *pilQ* from *N. meningitidis* M1080. Ten microliters of each reaction mixture was subjected to electrophoresis on a 2% agarose gel. Lanes: 1, DNA size marker; 2 and 3, RT-PCRs on RNA treated with RNase for 5 and 20 min, respectively; 4, RT-PCR on RNA template using *pilP*- and *pilQ*-specific primers *pilP*-5075 and *pilQ*-6485; 5, PCR on RNA with *pilQ*-specific primers *pilQ*-5675 and *pilQ*-6485 using *Taq* polymerase; 6, RT-PCR on RNA without *pilQ* reverse primer; 7, RT-PCR on RNA using *pilQ*-specific primers *pilQ*-5675 and *pilQ*-6485. The products in lane 6, obscuring the interpretation of results, indicate a fold-back artifact of RNA, which can provide a 3' terminus appropriate for reverse transcriptase to act on even in the absence of reverse primer. Molecular size markers are indicated on the left in kilobases.

3D structure of the PilQ oligomer. PilP is a much smaller protein than PilQ, and it is therefore difficult to identify the density attributable to PilP from a low-resolution PilQ-PilP complex reconstruction determined by TEM. Consequently, two separate structures were determined. The first structural

analysis involved the incubation of a recombinant version of PilP, with a polyhistidine tag at the N terminus (Fig. 1C), with the PilQ oligomer. The second involved the incubation of PilP Δ_{1-19} with PilQ and a Ni-NTA-nanogold probe, which would specifically adhere to the polyhistidine tag within

FIG. 6. PilP is not required for PilQ multimer stability. Meningococcal PilQ and PilP expression in whole-cell lysates monitored by immunoblotting. (A) Samples detected with anti-PilQ antiserum (K010); (B) samples detected with anti-PilQ antiserum after phenol extraction; (C) samples detected with anti-PilP antiserum K824. Lanes: 1, wild-type M1080; 2, M1080*pilP*_{74-6xHis}; 3, M1080 Δ *pilP*; 4, M1080 Δ *pilP* *iga::pilP*; 5, M1080*pilPfs*; 6, M1080*pilPfs* *iga::pilP*; 7, M1080 Δ *pilQ*. Arrowheads indicate the positions of proteins. Equal amounts of starting materials were used to make cell lysates. Positions of the molecular mass standard proteins are indicated on the left in kDa.

TABLE 2. Phenotypes of the *N. meningitidis* strains

Strain	PilP ^a	Agglutination in liquid	Transformability
M1080	+	+	+
M1080-PilP _{74-6xHis}	+	+	+
M1080 <i>pilPfs</i>	—	—	—
M1080 <i>pilPfs iga::pilP</i>	+	+	+
M1080Δ <i>pilP</i>	—	—	—
M1080Δ <i>pilP iga::pilP</i>	+	+	+
M1080Δ <i>pilQ</i>	—	—	—

^a Assessed by isolating pilus-shearing fractions.

PilPΔ₁₋₁₉. The recombinant fragment PilPΔ₁₋₁₉ was selected for these studies because we have reported its structural characterization by NMR elsewhere (24).

A 3D reconstruction of the PilPΔ₁₋₁₉-PilQ complex was determined from approximately 8,000 particles, using a method similar to that described for the determination of the 3D structure of the PilQ oligomer alone (14) (Fig. 8A and B). The resulting structure was determined at a resolution of 16 Å: comparison with the original structure of the unliganded PilQ oligomer revealed changes within the “arm” and “cap” regions, as defined previously (11). As was the case for the structure of the PilQ oligomer alone, the PilPΔ₁₋₁₉-PilQ complex exhibited strong C4 symmetry (compare the unsymmetrized class averages in Fig. 8B with the back projections determined assuming C4 symmetry). For the second independent experiment, PilQ and PilPΔ₁₋₁₉ were incubated with Ni-NTA-nanogold: around one thousand particles were analyzed and processed to produce a 3D volume from which the gold particles could be readily identified based on their differential scattering characteristics. The gold particles, shown as yellow density peaks in Fig. 8C, were located around the cap feature of the complex, confirming that this is the location of the binding site for PilPΔ₁₋₁₉. The gold particles identify the location of the polyhistidine tag in PilPΔ₁₋₁₉; we have shown that the N terminus of this recombinant fragment of PilP is highly flexible in solution and does not adopt any regular secondary structure (24). Such an observation would explain why the locations of the gold labels in Fig. 8C appear to be adjacent to regions of density, rather than within them. Nevertheless, it is clear that the labeling is concentrated around the cap end of the PilQ complex.

DISCUSSION

Insights into the structure-function relationship of components involved in type IV pilus biogenesis/type II secretion and their interactions are of importance to completely understand this pilus-generating machinery. Here we addressed the sub-cellular location of the meningococcal lipoprotein PilP and its role in pilus biogenesis and visualized its direct interaction with the secretin PilQ. We showed that PilP copurifies with the inner membrane and established that the N- and C-terminal regions of PilP recognize the central part of the PilQ monomer.

PilP is predicted to be a lipoprotein with its lipid attachment site at Cys₁₆, which most likely anchors it to the inner membrane. Results from computational predictions, which sug-

gested a disordered predicted N-terminal domain, and our solution NMR studies (24) have both shown that N-terminal residues 20 to 85 in PilP are flexible and probably unstructured in solution. Disordered regions can be partially or completely extended in solution (41); one of the functions of disorder may be to promote the molecular recognition of proteins and nucleic acids. It has been speculated that multiple metastable conformations adopted by disordered binding sites allow the recognition of multiple targets with high specificity and low affinity (41). Disorder-to-order transitions may therefore play a critical role in macromolecular recognition. In this case, it could mean that the N-terminal region of PilP be-

FIG. 7. Meningococcal PilP and PilQ monomers directly interact. Interaction of recombinant meningococcal PilP with recombinant PilQ protein detected by a solid-phase overlay assay (far-Western analysis). (A) Schematic diagram showing the *pilQ* gene constructs encoding the four recombinant proteins used in this study. H represents the position of the polyhistidine tag. (B) Far-Western analysis; the three panels, I to III, show gels with the same samples in each lane, but overlaid with different recombinant PilP proteins before immunodetection with anti-PilP antiserum K824. Panels: I, full-length recombinant PilP; II, recombinant PilP_{Δ1-12}; III, recombinant PilP_{Δ61-80}. Lanes: 1, full-length PilQ; 2, PilQ₂₅₋₃₅₄; 3, PilQ₂₁₇₋₄₇₈; 4, PilQ₃₅₀₋₇₆₁; 5, Hemoglobin-binding outer membrane receptor protein (HmbR); 6, bovine serum albumin. Positions of the molecular mass standard proteins are indicated on the left in kDa.

TABLE 3. Interaction of recombinant PilP proteins with recombinant PilQ proteins as monitored by solid-phase overlay assays (far-Western analysis)^a

PilP protein	Full-length PilQ	PilQ _{25–354}	PilQ _{217–478}	PilQ _{350–761}
PilP _{FL}	+	(+)	+	–
PilP _{Δ1–12}	+	(+)	+	–
PilP _{Δ1–15}	+	(+)	+	–
PilP _{Δ1–16}	–	–	–	–
PilP _{Δ1–77}	–	–	–	–
PilP _{Δ40–60}	+	(+)	+	–
PilP _{Δ61–80}	+	(+)	+	–
PilP _{Δ81–102}	+	–	–	–
PilP _{Δ107–116}	+	–	–	–
PilP _{Δ114–181}	+	–	–	–
M1080-PilP _{74–6xHis}	+	(+)	+	–

^a +, strong interaction; (+), weak interaction; –, no interaction.

comes more structured upon binding to the PilQ oligomer. It is certainly striking that there is relatively little sequence conservation within the N-terminal region of the PilP family of lipoproteins.

The insertion of a polyhistidine tag into the meningococcal PilP disordered region to produce M1080-PilP_{74–6xHis} did not affect the PilQ and type IV pilus expression levels or the efficiency of natural transformation. The *pilP* frameshift and deletion mutants, however, lacked the ability to form type IV pili and could not be naturally transformed (Table 2). This is due to a direct effect of PilP on pilus assembly. The levels of

PilQ complex and monomer expression were not affected in the *pilP* frameshift mutant but were reduced in the *pilP* deletion mutant. The reduced amounts of PilQ monomer and complex in the *pilP* deletion mutant were merely due to the genes encoding PilP and PilQ being cotranscribed. In two previous reports, *pilP*-knockout mutants had different effects on PilQ expression (8, 19). Drake et al. (19) showed that a *pilP* null mutant had a reduced amount of PilQ monomer and complex in *N. gonorrhoeae*, whereas Carbone et al. (8) reported no effect on the expression of PilQ in an *N. meningitidis pilP* null mutant. The inactivation of the *pilP* gene in its 3' region can therefore lead to a reduced quantity of *pilQ* transcript, due to a polar effect. Here we have clearly shown that PilQ does not need PilP for its membrane localization and/or stabilization.

According to the *E. coli* paradigm for lipoprotein membrane sorting, the +2 rule, a lipoprotein with an aspartate residue at position 2 immediately after the fatty acylated cysteine will be retained in the inner membrane (60). Based on the presence of a serine in the +2 position, gonococcal PilP was previously suggested to be located in the outer membrane (19). Meningococcal PilP was thus expected to be an outer membrane lipoprotein, since the amino acid in the +2 position is also a serine. We therefore searched extensively for PilP in the outer membrane and in OMV. Surprisingly, no PilP was found in OMV (Fig. 3), but it copurified with the inner membrane (Fig. 4). The absence of PilP in the outer membrane was also confirmed by surface exposure analysis using flow cytometry and biotinylation experiments (data not shown). An example of

FIG. 8. 3D reconstruction of the complex between PilP_{Δ1–19} and the PilQ oligomer. (A) Sample micrograph of PilQ particles, with PilP_{Δ1–19} bound, in cryonegative stain (14). The positions of individual PilQ-PilP particles are circled. Scale bar = 1,000 Å. (B) PilQ-PilP particle classification. 2D class averages for each of the particle classes following reference-free alignment are shown with the corresponding 2D back projections used for the C4 3D reconstruction. Averages were calculated by using the software package EMAN. Box size = 250 Å². (C) Comparison of 3D reconstructions of PilQ and PilQ-PilP oligomers. Isosurface renderings of volumes are displayed at thresholds appropriate to accommodate 900 to 1,000 kDa of mass. The top structure shows the structure of the PilQ oligomer alone (14). The middle structure shows the PilQ oligomer bound to PilP_{Δ1–19}, determined from 7,700 particles. The structure converged correctly using a symmetrical start model, a class-selected C1 start model, or an elliptical “blob”; model-dependent bias was therefore eliminated. The bottom structure shows the structure of the PilQ oligomer bound to PilP_{Δ1–19} and Ni-NTA-nanogold (*n* = 1,010). The structure was calculated in the same way from a cryonegatively stained sample containing Ni-NTA-nanogold. Scale bar = 100 Å.

another meningococcal inner membrane lipoprotein which contains serine at position +2 is DsbA1 (53).

The PilQ complex is not stabilized by the lipoprotein PilP; however, the results of the solid-phase overlay assays and electron microscopy studies did reveal that PilP and PilQ directly interact. We have shown that the N- and C-terminal regions of PilP interact with the central domain of the PilQ monomer, which is complementary to the sites of interaction between PilQ and the pilus, located in the N- and C-terminal domains of the PilQ (13). We have shown by electron microscopy that the PilP-PilQ interacting site is located in the cap region of the PilQ complex, an observation which was confirmed by NTA-gold affinity labeling. This highly specific binding behavior for a particular part of the PilQ structure rules out any question of nonspecific binding. The recombinant fragment of PilP used for these binding experiments did not contain the Cys residue at position 16, and was therefore not attached to a lipid moiety. Without Cys₁₆, PilP lipidation cannot occur, which may lead to an inappropriate structural conformation not available for optimal promotion of PilP-PilQ interaction. The results summarized in Table 3 established that the folded C-terminal region of PilP also played a role in PilQ recognition. Mutations in this region abolished binding to the PilQ recombinant fragment which covered the central region of the PilQ polypeptide. The binding behavior of PilP for PilQ is therefore likely to involve more than one site on the PilP protein and possibly more than one site on PilQ as well. In addition, we have observed that increased proteolytic degradation of PilP occurred in the *pilQ*-knockout mutant (Fig. 6C, lane 7). This observation implies that PilP needs PilQ for its correct membrane localization.

Although it is clear that a specific structural change has occurred in the cap region of the PilP Δ_{1-19} -PilQ interacting complex, it is difficult to estimate the stoichiometry of binding. Estimates of the amount of mass enclosed within the density envelope are unreliable, partly because the occupancy of PilP Δ_{1-19} binding to PilQ is unknown, and partly because up to half of the PilP fragment may be highly disordered in structure, giving rise to lesser density. The EM results are not inconsistent with the possibility that PilP binds to PilQ in a 1:1 stoichiometry. If this were the case, each mass attributable to PilP Δ_{1-19} (circled in Fig. 8C) would harbor three PilP polypeptides. Other interpretations of binding stoichiometry are also possible from these data.

The type III secretion system in *S. flexneri* requires the outer membrane lipoprotein MxiM (46), which is proposed to facilitate the outer membrane insertion and multimerization of secretin MxiD through an allosteric switch mechanism that involves an acyl chain binding cavity (30). It has been reported that MxiM interacts with the nonconserved part of the secretin C terminus (30). Here, we have shown by in vitro analysis that the central domain of PilQ mainly interacts with N- and C-terminal PilP. This location is therefore slightly different from the C-terminal binding site previously described for MxiM (30, 46). In this context, it should be emphasized that the definition of pilot proteins so far is limited to the *K. oxytoca* PulS and *E. chrysanthemi* OutS homologs, which have been defined as such to describe their chaperone-like and targeting functions (48), although they might remain associated with their cognate secretin once it has assembled in the outer membrane (36). MxiM and other secretin-interacting lipoproteins, such as PilP,

are not necessarily defined as pilot proteins in type II or type III secretion. The proposed role for MxiM involved in type III secretion is rather different from that suggested for either PulS/OutS or PilW/Tgl, and the situation in *Shigella* is complicated by the fact that yet another lipoprotein, MxiJ, also influences MxiD multimerization (46). Furthermore, the lipoprotein gene in the *Thermus thermophilus pilM-pilQ* gene cluster is represented by a gene termed *pilW* (42), whose gene product is nonhomologous to either neisserial PilW or *M. xanthus* Tgl. Therefore, grouping all lipoproteins involved in secretin assembly and stabilization as pilotins (48) or pilot proteins (30) is inappropriate, because targeting to the outer membrane, multimerization, and even complex stabilization might be independent events (4), not ruling out other functions for some of these lipoproteins, including meningococcal PilP. Furthermore, we have noted that the 3D structures of MxiM and the PilP main domain are unrelated (24).

It is clear from this work that PilP could be involved at several different stages of type IV pilus assembly and secretion. In addition to assembly of the pilus fiber, its interaction with PilQ, which has been documented here, suggests other functions which could be important at latter stages of the "pathway" for pilus maturation or for events associated with pilus retraction. Clearly, further work will be required to investigate the details of PilP function and its role in type IV pilus biogenesis.

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