Topology of the outer-membrane secretin PilQ from *Neisseria meningitidis*

Stephan A. Frye,^{1,2} Reza Assalkhou,¹ Richard F. Collins,³ Robert C. Ford,³ Christoffer Petersson,⁴ Jeremy P. Derrick³ and Tone Tønjum^{1,2}

Correspondence

Tone Tønjum

tone.tonjum@medisin.uio.no

¹Centre for Molecular Biology and Neuroscience and Institute of Microbiology, University of Oslo, Norway

²Centre for Molecular Biology and Neuroscience and Institute of Microbiology, Rikshospitalet-Radiumhospitalet Medical Centre, Norway

³Faculty of Life Sciences, The University of Manchester, Sackville Street, PO Box 88, Manchester M60 1QD, UK

⁴Division of Medical Microbiology, Department of Molecular and Clinical Medicine, Faculty of Health Sciences, Linköping University, Sweden

Neisseria meningitidis is the causative agent of epidemic meningococcal meningitis and septicaemia. Type IV pili are surface organelles that mediate a variety of functions, including adhesion, twitching motility, and competence for DNA binding and uptake in transformation. The secretin PilQ is required for type IV pilus expression at the cell surface, and forms a dodecameric cage-like macromolecular complex in the meningococcal outer membrane. PilQ-null mutants are devoid of surface pili, and prevailing evidence suggests that the PilQ complex facilitates extrusion and retraction of type IV pili across the outer membrane. Defining the orientation of the meningococcal PilQ complex in the membrane is a prerequisite for understanding the structure–function relationships of this important protein in pilus biology. In order to begin to define the topology of the PilQ complex in the outer membrane, polyhistidine insertions in N- and C-terminal regions of PilQ were constructed, and their subcellular locations examined. Notably, the insertion epitopes at residues 205 and 678 were located within the periplasm, whereas residue 656 was exposed at the outer surface of the outer membrane. Using electron microscopy with Ni-NTA gold labelling, it was demonstrated that the insertion at residue 205 within the N-terminus mapped to a site on the arm-like features of the 3D structure of the PilQ multimer. Interestingly, mutation of the same region gave rise to an increase in vancomycin permeability through the PilQ complex. The results yield novel information on the PilQ N-terminal location and function in the periplasm, and reveal a complex organization of the membrane-spanning secretin *in vivo*.

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INTRODUCTION

Neisseria meningitidis, or the meningococcus, is both a member of the normal flora in the nasopharynx and the cause of epidemic meningitis. A prerequisite for meningococcal adherence to nasopharyngeal mucosal surfaces, and colonization of its exclusive human host, is the presence of type IV pili. Type IV pili are long ordered arrays of polymerized protein subunits, termed pilin. Type IV pili mediate cellular attachment to epithelial tissue receptors

(Jönsson *et al.*, 1994). Pili are also involved in several other cell biological processes, including bacterial auto-agglutination (Frøholm *et al.*, 1973; Swanson *et al.*, 1971), twitching motility (Henrichsen *et al.*, 1972), and natural competence for DNA uptake (Frøholm *et al.*, 1973; Mathis & Scocca, 1984).

Neisserial type IV pilus biogenesis represents one of the terminal branches of the type II secretion pathway (Pugsley, 1993), and it requires the complex interactions of 15 proteins (Carbonnelle *et al.*, 2005; Tønjum & Koomey, 1997), which are similar, if not orthologous, to those involved in type II secretion in general (Nunn, 1999; Peabody *et al.*, 2003; Pugsley, 1993). To initiate pilus assembly, the pilin subunit PilE, in its membrane bound state, is processed by the pre-pilin peptidase PilD, removing

Abbreviations: EM, electron microscopy; FSC, forward scatter; SBR, small basic repeat; SOEing, splicing by overlapping extension; SPA, single-particle averaging; SSC, side scatter.

A supplementary table and supplementary figures are available with the online version of this paper.

a short hydrophilic N-terminal peptide, and methylating the N-terminal amino acid (Nunn & Lory, 1991). Type IV pili are retractable, and this retraction process is responsible for the twitching motility of neisserial cells observed on solid media and mucosal epithelium (Merz *et al.*, 2000). The cytoplasmic protein PilT is essential for retraction, but dispensable for pilus assembly (Wolfgang *et al.*, 1998). The putative type IV pilus-associated adhesin PilC (Jönsson *et al.*, 1994) is thought to act by binding to epithelial receptors suggested to be CD46- (Rudel *et al.*, 1995) and C4B-binding protein (Winther-Larsen *et al.*, 2001). PilC also plays a role in type IV pilus dynamics by acting as a mediator of pilus homeostasis, and promotes the processivity of organelle elongation in the presence of PilT. PilV, a pilin-like protein, has been implicated in the functional presentation of PilC. In addition to PilD, other components required for the biogenesis of type IV pili include a cytoplasmic protein, PilF, and a polytopic inner-membrane protein, PilG (Tønnum *et al.*, 1995).

Despite the identification and individual characterization of pilus biogenesis components, their coordinated functions and interactions in pilus assembly, and extrusion and retraction across the outer membrane, are poorly understood. An integral element in these processes is the PilQ multimer. PilQ is an antigenically conserved outer-membrane protein (Corbett *et al.*, 1988; Hansen & Wilde, 1984), which is essential for meningococcal pilus expression at the cell surface (Tønnum *et al.*, 1998). Meningococcal PilQ is unique among secretins because of its abundance in the outer membrane, and the presence in its N-terminal domain of four to seven copies of an octapeptide, PAKQAAAA, termed small basic repeats (SBRs) (Tønnum *et al.*, 1998). We previously purified the PilQ oligomer from meningococcal outer membranes, and showed that it forms a symmetric structure of approximately 900 kDa, consisting of 12 identical subunits (Collins *et al.*, 2001). The 3D structure of the PilQ complex has been determined by using single-particle averaging (SPA) methods applied to transmission electron microscopy (EM) images of the purified multimer visualized by cryo-negative EM staining (Collins *et al.*, 2004). The structure seen showed a large central cavity, which was closed at the poles by 'plug' and 'cap' features, and four 'arm' features at the sides. The results suggested that the PilQ complex is the channel through which the moving pilus fibre (polymerized PilE) is extruded and retracted from the bacterial surface (Collins *et al.*, 2005). PilQ subunits form dodecameric complex structures that are extremely stable to heat and SDS. PilQ oligomer formation and stability are compromised in lipoprotein PilP-null mutants (Drake *et al.*, 1997), although the exact role of PilP in this context is unclear. The C terminus, which is responsible for secretin oligomer formation, and is conserved between secretins in diverse Gram-negative bacterial species (Genin & Boucher, 1994), has been predicted to contain 13 β -strands, which could be embedded in the outer membrane (Bitter *et al.*, 1998). PilQ is a member of the GspD secretin superfamily, members of which

translocate a variety of macromolecules across the outer membrane (Pugsley, 1993). Among these secretins are XcpQ and PilQ from *Pseudomonas aeruginosa* (Bitter *et al.*, 1998), PulD from *Klebsiella oxytoca* (Hardie *et al.*, 1996), and the pIV protein, through which filamentous phages are secreted (Daefler *et al.*, 1997; Linderoth *et al.*, 1996). Pioneering work using cysteine residue insertions in pIV revealed the valency and structure of the multimer in the outer membrane, and identified some surface-exposed regions (Linderoth *et al.*, 1996). However, it remains unclear if the situation in this relatively small phage secretin is generally applicable to secretins associated with the type IV pilus biogenesis systems.

Mutations in the C-terminal domain of secretins can result in increased sensitivity to vancomycin, a phenotype thought to reflect leakage of this antibiotic through an aberrant channel gate (Russel, 1994, 1997; Schmidt *et al.*, 2001; Wall *et al.*, 1999). Normally the secretin gate is closed, but it must open when the pilus elongates or retracts. A *pilQ*⁺*pilA*⁻ strain has the same level of vancomycin resistance as the wild-type (WT), but a *pilQ1* mutant with a missense mutation in the C-terminal domain is more sensitive, because the gate has been compromised (Wall *et al.*, 1999). However, the effect on sensitivity to vancomycin of changes in the secretin N terminus has until now not been monitored.

The topology and organization of the PilQ oligomer within the outer membrane has yet to be defined. In order to begin to address this problem, we constructed polyhistidine insertions in the PilQ N-terminal SBR region, and in predicted loop regions in the C terminus. We show, for the first time, direct evidence that significant portions of the PilQ multimer are exposed within the periplasm, and map one of these regions to the 3D structure of the secretin. In addition, we assessed the potential of meningococcal PilQ to influence the integrity of the outer membrane, as viewed by its ability to allow passage of vancomycin. This new information is important for interpretation of 3D structural data, and further modelling of the PilQ complex (Collins *et al.*, 2003, 2004), as well as for studies of PilQ antigenicity and immunogenicity (Corbett *et al.*, 1988; Hansen & Wilde, 1984). The findings may also be relevant for homologous secretins from the Gram-negative type II and III secretion systems.

METHODS

Bacterial strains, media and plasmids. The *N. meningitidis* strains used in this study are listed in Table 1. *Escherichia coli* strain ER2566 (New England Biolabs) was used for plasmid propagation, and was grown in Luria Bertani (LB) medium or on LB plates containing kanamycin (50 $\mu\text{g ml}^{-1}$) or ampicillin (100 $\mu\text{g ml}^{-1}$), at 37 °C. Meningococcal strains were grown as previously described (Tønnum *et al.*, 1995). The M1080- Δ *pilQ* mutant has a transposon insertion in the *pilQ* start codon (Tønnum *et al.*, 1998). The Mc-2 mutant is a chimeric strain in which the SBR region of the PilQ monomer is replaced by the corresponding but non-homologous region from *P. aeruginosa* PilQ (Tønnum *et al.*, 1998). The plasmid

Table 1. Bacterial strains, and associated pilus-related phenotypes

Strain	Pili	Agglutination (solid medium/liquid)	PilQ complex	PilQ monomer	Transformation ratio	Source
M1080	+	+ / +	+	+	1	Frasch & Chapman (1972)
M1080- Δ <i>pilQ</i>	–	– / –	–	+	$< 10^{-6}$	Tønnum <i>et al.</i> (1998)
Mc-205	+	+ / +	+	+	10^{-2} – 10^{-3}	This study
Mc-656	–	–	–	++	$< 10^{-5}$	This study
Mc-678	– / (+)	–	(+) / –	++	$< 10^{-5}$	This study

pUP6 (Wolfgang *et al.*, 1999) is a derivative of pHSS6 (Seifert *et al.*, 1990), and contains two neisserial DNA-uptake sequences as an inverted repeat. The plasmid pMF121 contains the *Neisseria cps* cluster, which is disrupted by an *ermC* cassette (Frosch *et al.*, 1990). All standard methods of DNA manipulation were performed as described by Sambrook *et al.* (1989).

Prediction of PilQ secondary structure. M1080 PilQ secondary structure prediction was based on the deduced amino acid sequences and computational β -strand prediction (Gromiha *et al.*, 1997), complemented with manual inspection and alignments with other secretins by using the JPred program (Cuff *et al.*, 1998). In addition, information was implemented from sequence alignments with the secretin XcpQ from *P. aeruginosa*; this secretin has been analysed for possible β -strands in the C-terminal domain (Bitter *et al.*, 1998).

Cloning of the full-length and partial *pilQ*. The full-length and partial *pilQ* gene was amplified by PCR from genomic M1080 DNA. For the generation of recombinant PilQ with an N-terminal His tag, the vector pQE30 was used (Qiagen, Germany), and for the C-terminal His tag, the pET28b(+) vector (Novagen, USA) was used. The *pilQ* full-length gene, corresponding to the mature PilQ, was PCR amplified with the primers QBamHI and PilQ4B, generating the plasmid pSAF12-31. The 5' part of *pilQ*, encoding nearly 50% of the monomer, was amplified by using primers QBamHI and QEcoRI, generating plasmid pPilQ-1. The very 5' part of *pilQ*, not encoding the SBR region, was amplified with the primers QBamHI and Rez1, resulting in plasmid pPilQ-2. The central portion of *pilQ* was amplified with the primers SF21 and SF22, resulting in plasmid pPilQ-3. The 3' part of *pilQ* was amplified with the primers SF20 and SF19, and subcloned into pET28b(+) (Novagen), resulting in the plasmid pPilQ-4.

Recombinant PilQ expression and purification. For recombinant PilQ expression, the *pilQ* plasmids were transformed into *E. coli* ER2566. The full-length and partial PilQ proteins were overexpressed, and purified to homogeneity. The cells were grown at 37 °C in LB broth until the OD₆₀₀ reached 0.5, and then protein overexpression was induced with 1 mM IPTG. Cells were grown for another 4 h, harvested by centrifugation, and lysed by means of a French Press (Thermo Electron Corporation) at 20 000 p.s.i. (138 000 kPa). The His-tagged proteins were purified by immobilized metal affinity chromatography, with Ni-coupled NTA agarose (Qiagen, Germany), under native or denaturing conditions. Protein separation was performed by SDS-PAGE (Schägger & von Jagow, 1987). Protein purity and concentrations were determined by Coomassie blue staining and Bradford analysis (Bradford, 1976). Protein specificity was detected by immunoblotting using polyclonal rabbit antisera in dilution 1:1000.

Construction of meningococcal in-frame PilQ–His fusions

Genetic constructs. His-tag insertions were designed to insert into the predicted random coils (supplementary Table S1). For the generation of internally His-tagged PilQ splicing by overlapping

extension (SOEing) PCR (Horton *et al.*, 1990) was employed. The 3' and 5' fragments were produced with the primer combinations SF9/SF16 and SF17/SF12b, PilQ1/PilQ2 and PilQ3/PilQ4c, and PilQ1/SF4 and SF5/PilQ4c, for constructs Mc-205, Mc-656 and Mc-678, respectively. The SOEing PCR final products were amplified with the external primers SF9/SF12b, PilQ1/PilQ4c and PilQ1/PilQ4c for constructs Mc-205, Mc-656 and Mc-678, respectively, and cloned into the vector pUP6. The final constructs were used for transformation of WT Mc-M1080.

Non-selective transformation of *N. meningitidis*. For the transformation assays, meningococcal cells were grown overnight, and the colony morphology was monitored by microscopy. Agglutinating colonies were restreaked on blood agar plates, and grown for 4–6 h at 34 °C in 5% CO₂. Cells were harvested, and suspended in pre-warmed transformation medium (GC broth supplemented with IsoVitalax; Becton Dickinson) containing 7 mM MgCl₂. DNA was added to this cell suspension. The sample was briefly mixed, and incubated without shaking for 30 min at 34 °C. Pre-warmed transformation medium was added to dilute the sample, and this dilution was inoculated on blood agar. After overnight incubation, single clones were streaked onto fresh blood agar plates for growth.

Screening for positive meningococcal His-tag insertions. PCR size determination was used to monitor successful transformation and recombination of the mutated *pilQ* genes into the meningococcal genome. Confirmation of correct His-encoding insertions was performed by DNA sequencing employing an ABI 3730 Genetic Analyser (Applied Biosystems). Expression of the PilQ–His fusions was monitored by immunoblotting using the monoclonal anti-His-tag antibody (Qiagen).

Purification of the native PilQ complex. Purification of the PilQ complex from meningococcal outer membranes was performed as previously described (Collins *et al.*, 2001, 2003, 2004).

Purification of meningococcal type IV pilus fibres. Type IV pili were purified from the meningococcal cell surface using ammonium sulfate precipitation of a shearing fraction (Brinton *et al.*, 1978). Meningococci were vortexed for 1 min in 0.15 M ethanolamine buffer (pH 10.5), and cellular debris was removed by centrifugation. Pilus fibres were precipitated at room temperature for 30 min by addition of 0.1 vols ammonium sulfate-saturated 0.15 M ethanolamine buffer and collected by centrifugation. Pili were subsequently washed twice with 50 mM Tris-buffered saline (TBS), pH 7.5.

Rabbit immunization, and antibody purification. Rabbit polyclonal antibodies were raised against the native PilQ complex and purified recombinant His-tagged PilQ fragments (Table 2). Rabbit antisera were purified by ammonium sulfate precipitation. Shortly afterwards, the sera were diluted with 2 vols TBS, and the immunoglobulins were precipitated with a final concentration of 45% saturated ammonium sulfate, and collected by centrifugation. The pellet

Table 2. Ratio of the increase of antibody binding to permeabilized *N. meningitidis* cells in relation to non-permeabilized cells, indicated by fluorescent secondary-antibody binding, and analysed by plate fluorometer (VICTOR²D) analysis

Mean results $\pm$ SD (in parentheses) are given.

Cells	Antibody				
	K913- α -PilQ _{25–132}	K010- α -PilQ _{25–354}	K914- α -PilQ _{217–478}	K850- α -PilQ _{350–761}	K824- α -PilP
Mc-WT	7.31 (5.08)	3.41 (0.19)	6.16 (5.36)	2.06 (0.28)	2.14 (0.23)
Mc- Δ PilQ	1.58 (0.38)	2.31 (0.12)	1.65 (0.39)	1.60 (0.27)	1.79 (0.41)
Mc-WT/Mc- Δ PilQ	4.6	1.5	3.7	1.3	1.2

was washed once with 40 % saturated ammonium sulfate, and resuspended in 1 vol. TBS with 50 % (w/v) glycerol. Antibodies were dialysed overnight against TBS with 50 % glycerol, and stored at -20°C .

Surface-exposure analyses

Surface-exposure immunofluorescence. For surface-exposure analysis, the cells were grown for 18 h on blood agar plates, and then harvested, washed and resuspended in PBS, and fixed with 2 % (w/v) formaldehyde for 1 h. The cells were collected by centrifugation for 3 min at 400 g, and blocked with 1 % BSA in PBS containing 0.25 mM CaCl_2 and 0.5 mM MgCl_2 , at room temperature for 30 min. The optical density was measured at 660 nm, and cells equivalent to 250 μl with an OD_{660} of 2.5 were used for each sample. Subsequent steps were carried out at room temperature. When required, permeabilization was done in 0.5 ml PBS containing 0.5 % Triton X-100 and 10 mM EDTA. Incubation with the first antibody was done in PBS containing 1 % BSA, for 1 h. The cells were washed with PBS containing 0.01 % Tween-20. The secondary antibody Alexa-488 (Molecular Probes) was diluted 1:3000 in PBS with 1 % BSA, and incubated with the samples for 1 h in darkness. After washing with PBS containing 0.01 % Tween-20, the cells were resuspended in 300 μl PBS, and 100 μl of each sample was transferred to flat-bottom microtitre plates. Fluorescence and OD_{620} were measured with a microplate fluorometer (Wallac 1420 VICTOR² multilabel counter; Perkin-Elmer Life Sciences), using the standard settings for FITC (E, 485 nm; A, 535 nm).

Flow cytometry of immunostained cells. Cells prepared as described above were used for flow cytometry analysis using a BD LSR flow cytometer (BD Biosciences). During data collection, events were triggered on side scatter (SSC) with a threshold of 0. For all analyses, forward scatter (FSC) and SSC were set in log mode; the FSC gain was EO1, and the SSC gain and voltage were typically 1 and 580 V, respectively. FL1 was also in log mode, with gains of 1–2, and voltages set to 600–750 V. The suspension was sampled on a low setting (12 $\mu\text{l min}^{-1}$), giving a data acquisition rate of 100–400 events s^{-1} . Data analysis was performed with the program WinMDI.

Immunofluorescence microscopy. For immunofluorescence microscopy, meningococcal cells were resuspended in PBS, applied onto polylysine-precoated slides, and fixed with 4 % formaldehyde for 30 min at room temperature. Permeabilization was performed with 0.2 % Triton X-100 in PBS for 10 min at room temperature. The samples were blocked with 1 % BSA in PBS, followed by incubation with the primary antibody in the same solution for 2 h. After washing with PBS, the slides were incubated for 1 h with Alexa-488-coupled secondary antibody (Molecular Probes) in PBS containing

1 % BSA and 0.05 % Tween 20. The samples were washed with PBS, embedded with Fluorescence Mounting Medium (Dako-Cytomation), and sealed for microscopy.

Ni-NTA nanogold labelling of PilQ, and image analysis. A 10 μl volume of His₆-PilQ (100 $\mu\text{g ml}^{-1}$) from Mc-205 was incubated with 5 μl Ni-NTA nanogold (Nanoprobes) for 24 h at 4°C , with agitation. Samples were then centrifuged at 16 000 g in a bench-top centrifuge for 5 min, before preparing for cryo-negative staining, as previously described (Collins *et al.*, 2006). Grids were placed in an Oxford system cryo-stage, and data were recorded at ≈ 100 K in a FEG CM200, used in conjunction with a Gatan 4K CCD camera. Individual gold-labelled particles were interactively selected in 64 pixel boxes ($\text{\AA}$ per pixel, 3.1) using BOXER. Following contrast transfer function (CTF) correction, data were contrast normalized, centred, and low-pass filtered to 25 $\text{\AA}$ resolution. After applying a 190 $\text{\AA}$ circular mask to the data, six rounds of iterative refinement were performed in C4 symmetry, using our previously calculated 3D structure of PilQ (Collins *et al.*, 2004) filtered to 25 $\text{\AA}$ resolution, as a start model.

Freeze-fracture immunogold labelling of meningococci. Cells were inoculated from the agar plate to double-replica-device discs. The samples were quick frozen in liquid propane, and rapidly transferred to the freeze-fracture plant (BAF 400D; Balzers), and fractured at -115°C . Replication was carried out by evaporation of Pt and C (Petersson *et al.*, 2000). After washing in PBS, the replicas were transferred to 20 mM Zwittergent 3-12 (Roche Diagnostics) in 10 mM Tris/HCl, 30 mM sucrose, pH 8.3, and incubated overnight at room temperature. Replicas were rinsed in PBS, blocked with 1 % BSA in PBS, pH 7.3, for 30 min at room temperature, and placed in drops with polyclonal rabbit antiserum directed against N-terminal PilQ (K010), and diluted 1:100 in PBS with 1 % BSA, and incubated for 1 h at room temperature in a moisture chamber. After labelling, the replicas were washed in PBS containing 1 % BSA and 0.05 % Tween 20, pH 7.3. To detect the primary antibody, the replicas were placed in drops of biotinylated polyspecies antibody (E0453; DakoCytomation Norden) diluted 1:100 in PBS containing 1 % BSA, and incubated for 1 h at room temperature in a moisture chamber. Control immunolabelling was performed by omission of the primary antibody. As a negative control, the PilQ-negative strain M1080- Δ PilQ was employed. The replicas were washed in PBS with 1 % BSA and 0.05 % Tween 20, incubated for 1 h at room temperature with 10 nm streptavidin-conjugated colloidal gold (Aurion), and diluted 1:50 in PBS. Following immunogold labelling, replicas were rinsed in PBS, fixed with 0.5 % glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, for 10 min at room temperature, washed in distilled water, and mounted on gold-coated grids. Replicas were examined with a JEM-1200 EX/II electron microscope (JEOL). Images were handled by the Adobe Photoshop software. All micrographs were taken at a magnification of $\times 40\,000$, and scale bars displayed in the figures represent 0.2 μm .

PilQ pore function assays

Vancomycin susceptibility testing by MIC determination.

Meningococcal suspensions of between 1×10^6 and 5×10^6 bacteria ml^{-1} were applied to agar plates, and surplus fluid was removed. The plates were allowed to dry for at least 15 min before the vancomycin-containing E-test strips (AB Biodisk) or diffusion discs (Becton-Dickinson) were applied to the surface. E-test strips contained vancomycin concentrations ranging from 0.016 to 256 μg , while the disks contained 30 μg vancomycin. The plates were pre-incubated at room temperature for 30 min, and then incubated overnight at 37 °C, with readings taken after 24 h. The inhibitory zones for the vancomycin E-test and diffusion disks were measured at the transitional point where growth abruptly decreased. The experiment was repeated three times.

Permeability of fluorescence-labelled vancomycin. The permissibility of the cell membrane for macromolecules was monitored by the uptake of fluorescence-labelled vancomycin (Molecular Probes). Meningococcal WT and Mc-Pa-2 mutant cells, as well as Mc-205, Mc-656 and Mc-678, were resuspended in PBS, and incubated with fluorescence-labelled vancomycin for 10 min at room temperature. The cells were then washed briefly in PBS, fixed in 1.5% formaldehyde, and flow cytometry analysis was performed as described above. Three independent experiments were performed.

RESULTS

Prediction of the secondary structure of PilQ

Secondary structure predictions for the C-terminal region of PilQ were derived using a variety of bioinformatics methods in order to build a consensus based on alignments with related secretins. A synthesis of these results displays how the PilQ C terminus is conserved between homologous proteins, with homology in the region where 13 β -strands have been predicted for other secretins (supplementary Fig. S1,

Table S1). Together with the sequence alignments (supplementary Fig. S2), these data corresponded well with previous observations on other secretins, such as *P. aeruginosa* XcpQ (Bitter *et al.*, 1998) and *K. oxytoca* PulD (Guilvout *et al.*, 1999).

Expression of PilQ-His insertion mutants

Based on the predicted PilQ monomer secondary structure, a 6-His in-frame insertion mutant was constructed between N-terminal SBRs 4 and 5, at residue 205 (Fig. 1). Two additional His insertions were made into two predicted loop regions at residue numbers 656 and 678. The PilQ-His insertion mutants were designated Mc-205, Mc-656 and Mc-678, respectively (Fig. 1), and each of these PilQ-His fusions was stably expressed in meningococci (Fig. 2a). The phenotypes of the His-tagged PilQ mutants were assessed by colony morphology microscopy inspection, agglutination and transformation assays (Table 1). The isolation of pili yielded WT levels for the N-terminal Mc-205 mutant, but only trace levels of pili were found in the shearing fractions of C-terminal mutants Mc-656 and Mc-678, indicating that the pilus assembly and/or extrusion function, as expected, were severely affected in these backgrounds. These findings were paralleled by the fact that while liquid-grown Mc-205 agglutinated strongly (forming aggregates similar to those of the WT parent), Mc-656 and Mc-678 showed no and reduced agglutination under these conditions, respectively. Mc-205 exhibited transformation ratios similar to the WT strain, while Mc-656 was as non-competent as the PilQ-null mutant, and Mc-678 was barely competent.

Mc-205, Mc-656 and Mc-678 were tested for the presence of PilQ multimers and monomers by immunoblotting using anti-PilQ and anti-His antibodies (Fig. 2). Mc-205 exhibited

Fig. 1. Recombinant PilQ-His constructs. Positions of the three His-Tag inserts in the *pilQ* gene (GenBank accession no. AJ564200). The tag termed Mc-4H5 is situated between small basic repeats, SBR4 and SBR5. The tags Mc-565 and Mc-678 are positioned between consecutive predicted β -strands. The *Pst*I site was used to separate PilQ into an N-terminal and a C-terminal part. The PilQ rabbit polyclonal antisera K913, K010, K914 and K850 raised were directed against the amino acid residues PilQ₂₅₋₁₃₂, PilQ₂₅₋₃₅₄, PilQ₂₁₇₋₄₇₈ and PilQ₃₅₀₋₇₆₁ of the predicted polypeptide, respectively.

Fig. 2. Detection of the PilQ in meningococcal PilQ mutants. (a) Detection of PilQ in whole-cell lysates of *N. meningitidis* WT and the *pilQ*-null mutant, as well as in whole-cell lysates of Mc-205, Mc-656 and Mc-678, by immunoblotting using the antibody K010. (b) Detection of His-tagged PilQ by immunoblotting of whole-cell lysates using the anti-5x-His mAb. Samples were native or treated with phenol, as indicated. (c) Purification of the His-tagged PilQ protein from *N. meningitidis* Mc-205: affinity purification of PilQ under native conditions. Lanes 1–5 show the peak eluate fractions. PilQ was detected with the K010 antibody.

WT levels of PilQ complex (Fig. 2a). In contrast, the C-terminal Mc-678 mutant showed a reduction in the amount of oligomerized PilQ, while Mc-656 did not exhibit PilQ complex detectable with the α -PilQ polyclonal antibody (Fig. 2a). Given the established importance of the C-terminal regions of PilQ in oligomer formation (Bitter *et al.*, 1998), this result is understandable. A reduction in the amount of PilQ multimer detected by the full-length PilQ antibody was accompanied by an increase in the monomer, indicating either that the ability of PilQ to oligomerize or that the stability of the PilQ multimer was detrimentally affected by the C-terminal His insertions. The amount of PilQ monomer in each mutant was further verified by immunoblotting of phenol-treated cellular lysates (Fig. 2a). PilQ-complex formation could be detected by the His antibody in Mc-205 and 678 only, and even the Mc-656 monomer exhibited low reactivity with His antibody, as if the His epitope was present at very low levels, maybe due to degradation (data not shown). PilQ multimer and monomer could be isolated by affinity chromatography from Mc-205 under both native and denaturing conditions (Fig. 2a), whereas only PilQ monomer and minute amounts of multimer could be isolated from Mc-678 under native conditions.

Meningococcal PilQ is exposed at the cellular surface

Immunofluorescence microscopy was used to monitor the expression and exposure of PilQ at the cell surface (data not

shown). WT meningococcal cells showed staining around the surface of the cells when using antibody K010 recognizing full-length PilQ, indicating a surface- or membrane-associated fluorescence staining of the cells, while the M1080- Δ *pilQ* mutant showed no such staining. Mc-205 cells exhibited the same ring-like staining as the WT meningococci with K010, indicating the presence of PilQ in the same cellular compartment as in the WT strain. The Mc-656 and Mc-678 mutants, however, exposed no staining, and were similar to the PilQ-null mutant, indicating that PilQ in these strains was not surface exposed. The distribution of PilQ in the outer membrane was therefore identical to that observed by Tommassen and co-workers (Voulhoux *et al.*, 2003). The entire surface of the meningococcal cells was uniformly labelled with the PilQ-specific antibody, indicating no preferred localization or polarity of PilQ.

Antibody accessibility of PilQ in cells

By using polyclonal PilQ-fragment-specific antibodies, in combination with fluorescence-labelled secondary antibodies, with and without membrane permeabilization, antibody accessibility of membrane-bound PilQ complex was assessed (Table 2). The WT and Mc-205 cells showed a 7.31- and 6.4-fold increase, respectively, in binding of K913 antibody-targeting residues PilQ_{25–132} to permeabilized cells, compared to non-permeabilized cells. The corresponding factors for the WT cells and the sera K010, K914

and K850 were values of 3.4, 6.1 and 2.0, respectively. On the other hand, the negative control M1080- Δ *pilQ* showed only a 1.2- to 2.4-fold increase in antibody binding for permeabilized cells compared with cells without permeabilization (Table 2). Thus, there was an increase in antibody binding to Mc-WT and Mc-205 after permeabilization, and this increase was stronger with antibodies recognizing N-terminal PilQ compared with those recognizing C-terminal PilQ. In summary, the difference in polyclonal-antibody-binding intensity showed that epitopes of the N-terminal part of PilQ were more exposed after detergent/chelator treatment, and therefore must be buried inside or under the outer cell membrane. The fluorimetric assay described above allowed an overall analysis of the antibody accessibility of PilQ epitopes, but exhibited relatively high variability for the positive signal. Therefore, flow cytometry was also used to assess antibody accessibility in meningococcal cells (Fig. 3). The polyclonal rabbit antisera K913, K010, K914 and K850 were employed in this analysis (Table 2, Figs 1 and 3). The antisera directed against the N-terminal parts of PilQ (K913, K010 and K914) showed double-peaked broad-intensity spectra for non-permeabilized cells, and a single high-intensity peak for permeabilized meningococcal cells, whereas serum directed against the C-terminal part of PilQ (K850) showed only one low-intensity peak under both conditions, and no significant intensity difference (Fig. 3a). These results indicate that the N-terminal part of the PilQ complex is inaccessible in intact cells, but becomes available to antibodies after membrane permeabilization. An increase in anti-His antibody binding after permeabilization was detected for Mc-205 and Mc-678, but not for Mc-656 (Fig. 3b). Flow cytometry of fluorescence-labelled meningococcal cells demonstrated that, in addition to the N-terminal PilQ domain, the loop surrounding aa 678 is not normally accessible on the outside surface. The findings in the non-piliated Mc-656, which produced minute amounts of PilQ multimer, most probably because it is not properly folded (Fig. 2b), might also be complicated by the fact that the conformation of the PilQ complex changes with its reaction state with its pilus substrate (Collins *et al.*, 2005). Our findings that the mutations introduced into the Mc-656 and Mc-678 mutants compromised PilQ multimer formation and/or stability complicated the interpretation of the data obtained from these variants. The N-terminal mutant Mc-205 apparently assembles normally, and is therefore of more value to the investigation of the topology of the PilQ multimer.

Freeze-fracture EM analysis of PilQ

A morphological study of the PilQ protein localization was performed using the Zwittergent-digested freeze-fracture technique, allowing exposure of the membrane surfaces facing the periplasm (Petersson *et al.*, 2000). Previously, we have reported the adoption of the SDS-digested freeze-fracture replica labelling method termed SDS-FRL (Fujimoto, 1995) for studies of membranes of Gram-negative bacteria (Petersson *et al.*, 2000). The gold freeze-fracture

cytochemistry using the antibody K010, which recognizes N-terminal PilQ, showed that this domain of PilQ was localized in the inner leaf of the outer membrane, facing the periplasm (Fig. 4A). Our results also showed that the PilQ protein could be visualized associated with the extracellular face of the inner membrane (Fig. 4B). Thus, the EM data presented here strongly imply that N-terminal PilQ is situated in the periplasm of the meningococcal cell. The colloid gold particles clustered to some extent around ring-like structures seen in the membrane, but the majority of gold particles showed a dispersed distribution. The original protocol using 2.5 % SDS digestion (Fujimoto, 1995) was found to give high levels of non-digested debris from the bacteria (results not shown), probably mainly as a consequence of the viscous surface polysaccharide capsule. These remains of biological material resulted in non-specific labelling with colloidal gold, and blurring of the images. Through adopting the detergent Zwittergent 3-12 (Petersson *et al.*, 2000), we managed to overcome these obstacles. Due to non-specific adsorption of gold-antibody complexes to the Pt/C cast, labelling can be seen adjacent to the exo- and protoplasmic faces in the micrographs (Fig. 4A–D, white arrows). Considering that PilQ is a pilus secretin component located in connection with the periplasmic space, some displacement and shedding of the protein during the freeze-fracture process cannot be excluded. It is noteworthy that there is a complete absence of immunogold labelling in the micrographs of M1080- Δ *pilQ* (Fig. 4C, D), supporting the specificity of the immunogold-label binding. In addition, the control immunolabelling with another non-reactive antibody correspondingly displayed absence of gold labelling (data not shown).

Mutation of the SBR region of PilQ increases channel permeability

Due to its size, vancomycin will not act on Gram-negative bacteria since it cannot pass the outer membrane; thus, meningococci are normally resistant to this antibiotic (Valu, 1976). The MICs to vancomycin of Mc-205, Mc-656 and Mc-678 exhibited the same vancomycin-resistant phenotype as WT meningococci ($32 \mu\text{g ml}^{-1}$). However, a meningococcal PilQ chimera, Mc-Pa-2, in which the SBR region is replaced by the corresponding but non-homologous region of *P. aeruginosa* PilQ (Tønnum *et al.*, 1998), exhibited an MIC to vancomycin of $>256 \mu\text{g ml}^{-1}$. The permeability of fluorescence-labelled vancomycin was also monitored by flow cytometry in the WT and the PilQ chimera Mc-Pa-2. The PilQ chimera was clearly permeable to fluorescent vancomycin, as compared with the non-permeable WT cells and M1080- Δ *pilQ* (Fig. 5). Mc-205, Mc-656 and Mc-678 were impermeable to vancomycin, indistinguishable from meningococci with WT PilQ (data not shown). These findings demonstrate that changes in the N-terminal portion of PilQ can affect the secretin channel function, such that PilQ becomes more permeable to larger molecules (perturbed channel size and conformation) and/or that the selective property of the channel becomes reduced. Alternatively, these effects may be indirect with the

Fig. 4. Freeze-fracture replica labelling. (A, B) Zwittergent-digested freeze-fracture replica labelling of *N. meningitidis* strain M1080. Immunolabelling was performed with polyclonal antibodies raised against the N-terminal domain of PilQ, followed by addition of a biotinylated polyspecies antibody and 10 nm streptavidin-conjugated colloidal gold particles. The PilQ protein is gold-labelled (black arrows). (C, D) Control immunolabelling incubations were done using the non-PilQ-expressing strain *N. meningitidis* M1080- $\Delta pilQ$. Labelling was performed with antibodies against the N-terminal part of the PilQ protein, followed by addition of a biotinylated polyspecies antibody and 10 nm streptavidin-conjugated gold. Binding to unspecific components is visualized (white arrowheads). Remains of undissolved cell components are marked with an asterisk. See text for details. EF, extracellular face; OM, outer membrane; bars, 0.2 μ m.

PilQ structure influencing the activities of other components influencing vancomycin sensitivity.

Identification of the location of the SBR region of PilQ by *in situ* nanogold labelling

The demonstration that vancomycin sensitivity and permeability could be influenced by a change in the SBR region of PilQ raised the question of the location of this region within the 3D structure of the complex. We have previously shown that the structure of the PilQ multimer can be determined by SPA applied to transmission EM images of PilQ particles in cryonegative stain (Collins *et al.*, 2004). The results of the application of Ni-NTA labelling to the Mc-205 His-insertion mutant, whose insertion maps within the SBR region, are shown in Fig. 6. Fig. 6(a) shows a representative montage of PilQ complexes labelled with Ni-NTA nanogold; each individual particle is presented in a random orientation relative to the support surface, and so the number and position of gold particles vary from particle to particle. SPA applied to images of several hundred gold-labelled PilQ particles allowed us to localize the SBR region of the protein within the oligomeric 3D structure (Fig. 6b, c). PilQ is a dodecameric complex, and under EM conditions it displays

a preference for a C4 subarrangement. After processing in C4 symmetry, viewing the complex from the top shows that radial gold densities can be clearly seen bound around the periphery of the oligomer. Viewing the complex from the side, the characteristic three-banded appearance, common to secretins, can be readily observed, and reveals gold densities bound above the portion of the complex that we previously termed the 'plug' (Collins *et al.*, 2004). The results suggest that this segment of the N-terminal region of PilQ is located at the outside of the multimer, and in a central location.

DISCUSSION

The secretin PilQ is a key component of the type IV pilus secretion machinery in a number of Gram-negative pathogens; it is therefore important to establish its orientation in the outer membrane, and to define surface-exposed parts. Currently, little is known about the relative location of secretin substructures in cellular compartments. Previous surface-biotinylation experiments have demonstrated that meningococcal PilQ is surface exposed (Tønnum *et al.*, 1998), but until now it has not been known which parts of the complex are situated at the surface. Likewise,

Fig. 3. Meningococcal surface exposure analysis by flow cytometry using PilQ- and His-specific antibodies. Flow cytometry analysis of non-permeabilized (filled) and permeabilized (open) meningococcal cells labelled with antibodies. (a) Flow cytometry analysis using polyclonal rabbit antibodies. (b) Flow cytometry analysis using monoclonal anti-His antibodies. The polyhistidine tag in the SBR region, and at aa 678, became more accessible after permeabilization, indicating that SBR and residue 678 are surface exposed at the periplasmic side of the outer membrane.

Fig. 5. Permeability of PilQ SBR insertion mutant to vancomycin. The permeability of fluorescence-labelled vancomycin was monitored by flow cytometry in WT and PilQ chimeric cells. In the meningococcal PilQ chimeras, the SBR region is replaced by the corresponding, but non-homologous, region of *P. aeruginosa* PilQ (Tønjum *et al.*, 1998).

Wilde and colleagues demonstrated that antibodies raised to the PilQ multimer had bactericidal activities (Corbett *et al.*, 1988). Some information on secretin orientation can be derived from work on the filamentous phage secretin pIV (Linderoth *et al.*, 1996; Opalka *et al.*, 2003), although that secretin is only distantly related to the PilQ proteins. In order to begin to define which parts of the meningococcal PilQ complex are exposed from the outer leaf of the outer membrane, and which are directed towards the periplasm, we constructed in-frame His insertions within the N- and C-terminal portions of PilQ. Since changes in the PilQ C terminus inherently will affect complex stability, the N-terminal mutant was the most important target in this study. We probed the mutants with His-specific and PilQ-fragment-specific antibodies, in combination with membrane fixation and permeabilization, followed by flow cytometry and EM analysis. We demonstrated that the regions around residue 205 and 678 are likely to be located within the periplasm: the demonstration that segments of both the N- and C-terminal sections of the PilQ polypeptide are located within the periplasm suggests that substantial parts of the secretin lie within the periplasmic space.

Impaired stability of secretins has previously been observed in XcpQ (Bitter *et al.*, 1998), PulD (Guilvout *et al.*, 1999) and neisserial PilQ mutants (Drake & Koomey, 1995; Tønjum *et al.*, 1998; Zhao *et al.*, 2005). The meningococcal PilQ complex forms a homo-dodecamer (Collins *et al.*, 2001) with high stability. The secretin C terminus is likely to play a central part in PilQ monomer interactions, and,

accordingly, the Mc-656 and Mc-678 modifications had a commensurate effect on PilQ multimer stability. Thus, since the PilQ multimer formation and/or stability are severely affected in the C-terminal Mc-656 and Mc-678 mutants, the results obtained from these constructs are of considerably less value than the important and reliable findings on the N-terminal Mc-205 mutant.

The identification of the location of the SBR region, by determination of the structure of the Mc-205 variant of PilQ in complex with Ni-NTA nanogold, showed a central position within the 'arms' region of the complex (Collins *et al.*, 2004). Labelling of polyhistidine sequence tags with Ni-NTA nanogold particles is a powerful method for the identification of specific sequences within a structure at low resolution for several reasons (Collins *et al.*, 2006). First, the Ni-NTA chelating portion of the label is small, soluble and penetrable, and has a high binding affinity for multiple His sites (Collins *et al.*, 2006). Second, nanogold is strongly electron scattering and provides a high-contrast marker that can be used to provide accurate classification during image analysis. Based on these observations, at least some portions of the N terminus therefore contribute to the large arm-like features that enclose the central cavity of the PilQ multimer. The demonstration that the sequence around position Mc-205 is located in the periplasm provides another indication of the orientation of the PilQ complex in the outer membrane. The exact location of the membrane-spanning portions of PilQ relative to the 3D structure is still unresolved, although these results suggest that they lie towards the top or bottom of the complex.

The detergent-digested freeze-fracture labelling technique is a potent technique for establishing visual information in molecular microbiology (Chirica *et al.*, 2002). To our knowledge, there has been only one report on the use of the freeze-fracture technique for investigation of membrane morphology and potential antigenicity mechanisms of *N. meningitidis* (Devoe & Gilchrist, 1974). Here, we have shown that freeze-fracture immunogold labelling of the periplasmic space successfully can be used to visualize the PilQ molecule in association with this cellular compartment. To confirm the findings we conducted parallel experiments to label the PilQ protein using immunonegative staining (Chirica *et al.*, 2002). This technique allows labelling of surface-localized epitopes only (Beveridge *et al.*, 1994; Harris, 1997). With the K010 antibody, these experiments failed to visualize the PilQ protein on the meningococcal surface (data not shown). Hence, considering these findings, the N-terminal domain of the PilQ complex is found intramembranously and in association with the periplasmic space. However, our data do not establish a complete morphometrical analysis of the actual number of PilQ molecules detected.

It has been suggested that the N-terminal portion of PilQ is exposed on the exterior surface and that the COOH terminal portion is membrane associated (Tsai *et al.*, 1989). In that study, however, the recombinant PilQ protein was

Fig. 6. Nanogold labelling of the N-terminal SBR region of the PilQ multimer. (a) Montage of unaligned raw PilQ oligomers labelled with 18 Å diameter Ni-NTA nanogold. To emphasize the decoration of the nanogold particles (black high density circles) rather than the PilQ density, the data have been low-pass filtered to 25 Å resolution, and then contrast enhanced. Boxes, 200 Å². (b) Back projections from the Ni-NTA gold-labelled 3D structure of PilQ from the top view to the side view (left to right). Boxes, 200 Å². (c) Top and side views of the Ni-NTA gold-labelled 3D structure of PilQ. The PilQ density is shown at 1.09σ (thresholded to accommodate ≈1 MDa of protein and detergent density) in purple mesh. The locations of bound gold densities are shown at -5σ in gold surface render. Scale bar, 100 Å.

expressed from λ gt11, was only 45 kDa in size, and was 95 aa longer than our N-terminal construct (PilQ-2). The likelihood of N-terminal PilQ being exposed to the exterior was initially suggested by the discovery of its N-terminally located polymorphic region containing SBRs (Tønnum *et al.*, 1998), which conceptually could be suggested to contribute to antigenic variation. However, during long-term assessment of meningococcal isolates over time, the number of SBRs did not vary when passed on solid media. As shown here with Mc-205, the SBRs are oriented towards the periplasm, which might indicate a functional role for SBRs in supporting the extruding and retracting pilus. Neisserial type IV pili are modified with post-translational modifications, such as glycosylations and phosphoethanolamines (Hegge *et al.*, 2004; Virji, 1999), and the number of SBRs might vary to accommodate these or other antigenic variations.

The channel function of neisserial secretins has been assessed to some extent (Chen *et al.*, 2004; Zhao *et al.*, 2005). Meningococcal PilQ can behave as a gated channel and regulator of transmembrane flux control. This topic was addressed here by subjecting WT and PilQ-hybrid meningococci to vancomycin. Indeed, the PilQ hybrid strain was

sensitive to vancomycin, as opposed to the WT strain, indicating a role for PilQ in controlling molecule transport across the outer membrane. Likewise, the C-terminal Glu₆₀₆→Lys₆₀₆ PilQ mutant (designated the *penC* allele) has been found to be more permissive to antibiotics than PilQ WT strains (Zhao *et al.*, 2005). Phage transport, as well as pilus locomotion and transmembrane flux, have been associated with the function of PilQ (Bille *et al.*, 2005). Although the secretin C terminus has been implicated in vancomycin permeability (Schmidt *et al.*, 2001; Wall *et al.*, 1999), this is the first description of the role of the secretin N-terminal region in this context.

As in the case of any abundant outer-membrane protein, the secretin PilQ is a potential vaccine candidate. The meningococcus is an ideal bacterium for the study of secretins because, as opposed to what is found in other Gram-negative bacterial species, only a single secretin is present in this organism. In addition, meningococcal PilQ is naturally expressed at very high levels. Furthermore, the meningococcus is naturally transformable and amenable to genetic manipulation throughout its growth cycle, allowing the generation of the constructs described. Based on these constructs, we have defined the orientation of the

meningococcal PilQ complex in the outer membrane, and, in these constructs, we now have new tools available for enhanced yield of meningococcal PilQ complex during native membrane protein purification. These results are a prerequisite for defining the structure–function relationship and modelling of the meningococcal PilQ complex in the outer membrane, as well as suggesting which antigenic epitopes can be relevant for vaccine development.

The results have defined the position of an epitope within the SBR region of the PilQ protein by demonstrating that it is located within the periplasm, and mapping it onto the 3D structure of the secretin. This suggests that a substantial part of the PilQ complex is exposed within the periplasm, possibly traversing part of the periplasmic space. The observation that the same region of the protein also affects vancomycin permeability suggests that the central ‘arms’ regions of the PilQ structure could affect passage of vancomycin through the complex. This is hard to consider based on the 3D structure of PilQ alone; however, *in vivo*, PilQ is part of a larger complex of proteins, possibly including PilP and PilW. Full consideration of the implications of these results will therefore require a better understanding of the assembly, as well as the orientation and membrane embedding, of this interacting macromolecular machinery.

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