Chapter 5

Structural characterization of outer membrane components of the Type IV pili system in pathogenic *Neisseria* by single particle electron microscopy

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* contributed equally.

ABSTRACT

The structure of the PilQ secretins from *Neisseria gonorrhoeae* and *Neisseria meningitidis*, embedded in isolated outer membranes, was investigated by transmission electron microscopy. Two-dimensional projection maps of the PilQ oligomers were obtained by single particle averaging. Remarkably, by analyzing oligomers within the native membrane, additional domains could be revealed which were not observed in studies using purified proteins. PilQ complexes of *N. gonorrhoeae* showed a double ring structure with a clear 14-fold symmetry of the outer ring, and a 14-15-fold symmetry in the inner ring. From the 14 protein densities of the outer ring 7 external spikes are protruding. PilQ complexes of *N. meningitidis* also consisted of two rings, but also complexes partly or completely lacking the outer ring were observed. The outer ring of the *N. meningitidis* PilQ had a 19-fold symmetry. In the *N. meningitidis* structures, no external spikes, were observed. The inner ring structures observed for both *N. gonorrhoeae* and *N. meningitidis* were of similar size as the structures previously observed from the isolated PilP/PilQ complexes, demonstrating that the second ring and the external spikes do not contain domains of the PilQ or PilP proteins. To investigate the composition of the outer ring and the spikes, the structure of PilQ complexes...
within membranes of several N. gonorrhoeae knock-out mutants was investigated. A knock-out of PilP, a lipoprotein shown to interact with PilQ did not show spikes, indicating that this protein might be directly or indirectly involved in the spike composition. Remarkably the PilP knock-out also showed a change in the symmetry of the outer ring from 14 to 19 fold. A PilF mutant also did not show spikes, but maintained the 14-fold symmetry of the outer membrane. PilF is an ATPase located in the inner membrane, involved in pilus assembly, suggesting that localization of the spike-protein depends on functional assembly of the pilus. The PilC2 and PilW mutants yielded similar structures as observed for the wild-type, indicating that they are not involved in these structures. Single particle analysis also showed that the structure composed of two rings and spikes has a high rotational flexibility, especially the connection of the two rings is flexible. This suggests that the second ring and spikes may have a role in stabilizing the anchoring of the PilP/PilQ complexes with the membranes.

INTRODUCTION

Neisseria species are gram-negative bacteria, whose pathogenic members are associated with significant morbidity and mortality for their exclusively human hosts. Neisseria meningitidis, which normally inhabits the human nasopharynx, and Neisseria gonorrhoeae, which normally colonizes mucosal surfaces, are responsible for bacterial meningitis and septicemia, and the sexually transmitted disease gonorrhea, respectively (Tønjum and Koomey 1997). During the infection process, a remarkable variety of adhesins is involved in the modulation of the interaction with the host cell. Among these adhesins are type IV pili which mediate binding of the bacteria to host cells. Type IV pili are long fibrous structures emanating from the bacterial surface involved in a variety of processes. Type IV pili not only mediate cellular attachment to tissue receptors (Jönsson et al. 1994), but are also involved in several other processes, including bacterial auto-agglutination (Frøholm et al. 1973; Swanson et al. 1971), twitching motility (Henrichsen et al. 1972), biofilm formation and natural competence for DNA uptake (Frøholm et al. 1973; Mathis and Scocca 1984).

Type IV pili are long (>1–5 μm), thin (60–70 Å), mechanically strong polymeric fibers which can be extended and retracted. The pilus consists of 500–2000 subunits of the major pilin protein, PilE (Parge et al, 1995) and is assembled by a complex machinery consisting of up to 30 proteins (Wolfgang et al. 2000, Morand et al, 2004, Carbonnelle et al. 2005; Tønjum
and Koomey 1997). This machinery shows similarity to the apparatus for secretion of proteins of the general (type II) secretory pathway (Nunn, 1999; Peabody et al. 2003; Pugsley 1993). The first step of assembly of the pilus is the insertion of PilE into the cytoplasmic membrane. In the cytoplasmic membrane the hydrophobic leader peptide is removed and the freed N-terminal amino acid is methylated by the pre-pilin peptidase PilD (Strom et al. 1993). It was proposed that the PilE subunits are assembled and extruded from the inner membrane by the PilF ATPase (a homologue of GspE, and a member of the AAA chaperone/mechanico-enzyme family) with the aid of a polytopic inner membrane protein PilG. Remarkably a similar ATPase as PilF, PilT is involved in the disassembly of the PilE subunits at the cytoplasmic membrane, resulting in retraction of the pilus. Several other neisserial proteins, called pseudopilins or minor pilins, which show homology in their N-terminal hydrophobic leader peptide with the PilE signal peptide, are processed by PilD and can also be integrated into the growing pilus. The pilus passes the outer membrane through PilQ (Drake and Koomey 1995; Tønjum et al. 1995). PilQ is an antigenically conserved, abundant outer membrane protein, and a member of the GspD secretin superfamily of integral outer membrane proteins involved in different processes like type II and type III secretion (Pugsley 1993).

Examination of the purified secretins of members of this superfamily, such as XcpQ and PilQ from *Pseudomonas aeruginosa* (Bitter et al. 1998), PulD from *Klebsiella oxytoca* (Hardie et al. 1996), the pIV protein, through which filamentous phages are secreted (Daefler et al. 1997; Linderoth et al. 1996) and PilQ of *N. meningitidis* (Collins et al. 2001, Collins et al. 2003) by transmission electron microscopy (TEM) indicated that these proteins form a multimeric ring-like structure. A 3D structure of the PilQ complex was determined by using single particle averaging methods applied to transmission electron microscopy (EM) images of the purified multimer visualized by cryo-negative EM staining. This structure showed a large central cavity, which was closed at the poles by ‘plug’ and ‘cap’ features, and four ‘arm’ features at the sides with an apparent cavity around 60 Å in diameter). A higher resolution 3D structure of a secretin was obtained for PulD of *K. oxytoca*. This complex consists of a dodecameric structure composed of two rings that sandwich a closed disc. The two rings form chambers on either side of a central plug that is part of the middle disc.

Members of the secretin superfamily often strongly interact with small lipoproteins, also known as pilotins, or pilot proteins. These lipoproteins are involved in oligomerization, stabilization, and/or outer membrane localization of the secretin. For example, the *K. oxytoca*
PulD requires PulS for proper outer membrane association; in its absence, PulD remains associated to the inner membrane. MxiM of *Shigella flexneri* and YscW of *Yersinia enterocolitica* are required for outer membrane localization of the Type III secretion secretins MxiD and YscC respectively (Burghout et al. 2004; Schuch and Maurelli 2001). The interaction between MxiM and MxiD has been studied using NMR spectroscopy (Okon et al. 2008). It has been demonstrated that PilP and PilW interact with the Neisserial PilQ. PilP was shown to co-purify with PilQ and to localize to the cap region of the PilQ complex. Although PilQ does not need PilP for its stabilization or membrane localization pilP mutants showed a loss of piliation and natural competence and greatly reduced amounts of PilQ in the outer membrane. In a pilW mutant, the total amount or outer membrane localization of PilQ was not changed, but the stability of the PilQ multimer was strongly affected. Another protein that has been proposed to interact with PilQ is PilC. Within the pathogenic *Neisseria* species, two copies of PilC (PilC1 and PilC2) are found. In *N. gonorrhoeae*, both copies can be exchanged and function as pilus tip adhesins, while in *N. meningitidis*, only PilC1 promotes adhesion. The PilC proteins are associated with the outer membrane but can also be recovered from purified pili, were they seem to be located at the top of the pilus (Rahman et al. 1997).

Although the observations made by different authors have been useful in establishing that secretins adopt ring-like structures with 12-14 fold symmetry, there are still many questions remaining. Structural information about the interaction of the secretins with other components is lacking. Since all structural information about the secretins obtained to date has been obtained from purified proteins, and multi-subunit membrane complexes can loose subunits during the purification procedure (Folea et al. 2008), we set out to study the structure of the PilQ secretin within the membrane using Transmission Electron Microscopy and single particle averaging. Using isolated outer membranes we were able to determine the 2-dimensional structure of the PilQ oligomers of *N. gonorrhoeae* and *N. meningitidis*. Remarkably, using this approach, additional domains could be observed which were not observed in studies using purified proteins. PilQ complexes of *N. gonorrhoeae* showed a double ring structure with a 14-fold symmetry of the outer ring, and external spikes on the outer ring with a 7-fold symmetry. PilQ complexes of *N. meningitidis* also consisted of complexes consisting of two rings, but also structures lacking or having a partial outer ring were observed. The outer ring of the *N. meningitidis* PilQ had a 19-fold symmetry. In the *N. meningitidis* structures, no external spikes were observed. The inner ring structures observed for both *N. gonorrhoeae* and *N. meningitidis* were of similar size and shape as the structures...
previously observed from the isolated PilP/PilQ complexes demonstrating that the second ring and the external spikes consist of proteins most likely do not consist of PilQ or PilP proteins.

To obtain further information on the PilQ complex, we studied the structure of the complex in membrane derived from pilP, pilW, pilF and pilC2 knock outs. While no differences were observed in the pilW and pilC2 knock-outs, remarkable difference were observed for the pilF and pilP knock-outs. The pilF knock-out lost the outer spikes, while the pilP not only lost the outer spikes, but also changed the symmetry of the outer ring from 14 to 19. Implications for the assembly and structure of the observed PilQ mega-complex are discussed.

MATERIALS AND METHODS

Strains, plasmids, primers and media

Neisseria gonorrhoeae strain MS11, Neisseria meningitidis strains HB1 and M986 and further strains created in this study are described in Table 1. N. gonorrhoeae strains were grown at 37 ºC in 5% CO2 on GCB (Difco) plates containing Kellog’s supplement (Kellog et al. 1963) or GCB liquid medium (GCBL) containing 0.042% NaHCO3 and Kellog’s supplements. N. meningitidis was also grown at 37 ºC in 5% CO2 on GC-agar plates or in tryptic soy broth (TSB). When necessary, erythromycin was used at 5 µg/ml.

Construction of knock-out strains

Knock-outs in PilC, PilW and PilF were made using the insertion-duplication mutagenesis method (Hamilton et al. 2005). Using this method, the gene is disrupted and expression of genes downstream of the disrupted gene is driven from the erythromycin promotor. PCR products encoding 541 bp (primers PilC-for and PilC-rev), 452 bp (primers PilW-for and PilW-rev) and 524 bp (primers PilF-for and PilF-rev) fragments of PilC, PilW and PilF were generated by performing a PCR on isolated chromosomal DNA of N. gonorrhoeae strain MS11 (for a list of used primers, see Table 2). PilC and PilW PCR fragments were digested with BamHI and KpnI and ligated into the BamHI/KpnI sites of pIND3 (Hamilton et al. 2001) resulting in plasmids pSJ030 and pSJ032 respectively. The PilF PCR fragment was digested with XhoI and KpnI and ligated into XhoI/KpnI site of pIND3 resulting in plasmid pEP057. Plasmids pSJ030 and pSJ032 were transformed to MS11 and plasmid pEP057 was transformed to EP019 (derivative of MS11A strain) and colonies were selected on GCB plates containing erythromycin. Correct clones were identified by performing a PCR on isolated
chromosomal DNA of these colonies resulting in strains SJ030-MS, SJ032-MS and EP060 respectively (Table 1B). To create marker-less non-polar knock-outs of PilP and PilQ, PCR fragments of the flanking regions of the respective genes were annealed using the splicing by overlapping extension PCR (SOE-PCR) (Horton et al. 1990) method. To create the PCR products for PilP and PilQ, the primer combinations of PilP-for1/PilP-rev1 and PilP-for2/PilP-rev2 and PilQ-for1/PilQ-rev1 and PLiqQ-for2/PilQ-rev2) were used. The obtained PCR products were diluted and amplified with the external primers which also contained the gonococcal DNA uptake sequence (DUS). The PCR product was transformed to strain MS11 and the mutant colonies were checked using colony PCR. Correct clones were identified by performing a PCR on isolated chromosomal DNA of several colonies resulting in strains SJ031-MS and SJ001-MS (Table 1B). To further confirm the correct deletion of the gene, the deletion site and the flanking regions were determined by sequencing.

**Membrane Preparation**

To isolate membranes of *N. gonorrhoeae*, the strain was plated on GCB plates with the appropriate antibiotic, and (when possible) piliated cells were scraped from the plate and transferred to 3 ml GCBL medium. Cells were grown to an OD660 of 0.6 and consecutively diluted into increasing volumes until a final volume of 1 liter with an OD660 of 1.0 was obtained. Cells were centrifuged at 8000 rpm and resuspended in 50 mM Tris-HCl pH 7.5. Cells were broken by three passes through a French press at 15psi. Cell debris was removed by centrifugation at 6000 rpm for 10 min. The membranes were spun down at 40,000 rpm for 1 hr, resuspended in 1 ml of 50 mM Tris-HCl pH 7.5 and overlaid on a 4 step (1, 1.8, 0.8 and 0.8 ml) sucrose gradient of 54, 51, 45 and 36 (w/v) sucrose) and centrifuged at 80,000 rpm for 30 mins. The lower two fractions were collected, diluted in 50 mM Tris-HCl pH 7.5 and membranes were collected by centrifugation at 40,000 rpm for 1 hr and resuspended in 1 ml 50 mM Tris-HCl pH 7.5. To isolate membranes of *N. meningitidis*, the strain was inoculated from an overnight GC-agar plate in 40 ml tryptic soy broth (TSB) at an OD550 of 0.1 and grown for 7 hours to OD550 of 4. Cells were collected by centrifugation, resuspended in 50 mM Tris/HCl, 5 mM EDTA pH 8 and frozen at -80°C. After thawing, aliquots were plated to verify killing of the bacteria. The suspensions were sonicated for 4 minutes (Branson 450, setting 6, output 40%) and spun at 10,000 rpm (12,000xg) in an SS34 rotor in a Sorvall RC 5B centrifuge. The resulting supernatant was spun for 8 minutes at 40000 rpm (100,000xg) in a Ti-70 rotor in a Beckmann LE-80K ultracentrifuge. Cell envelope pellets were dissolved in 2 mM Tris/HCl pH 7.6 and overlaid on a 4 step (1, 1.8, 0.8 and 0.8 ml) sucrose gradient of 54,
51, 45 and 36 (w/v) sucrose) and centrifuged at 80,000 rpm for 30 mins. The lower two fractions were collected, diluted in 50 mM Tris-HCl pH 7.5 and membranes were collected by centrifugation at 40,000 rpm for 1 hr. The final membrane preparation was resuspended in 50mM Tris-HCl, pH 7.5, for EM analysis.

**Electron Microscopy and single particle analysis**

For image processing, whole membranes from *Neisseria gonorrhoeae* and *Neisseria meningitidis* were negatively stained with 2% uranyl acetate on glow-discharged carbon-coated copper grids. Electron microscopy was performed on a Philips CM120 equipped with a LaB₆ tip operating at 120 kV. The “GRACE” system for semi-automated specimen selection and data acquisition (Oostergetel et al. 1998) was used to record 2048 x 2048 pixel images at 60000x calibrated magnification with a Gatan 4000 SP 4K slow-scan CCD camera. About 8000 images were recorded, and about 20000 single particle projections of the PilQ complex from *Neisseria gonorrhoea*, 8000 projections of the PilQ complex from *Neisseria meningitidis*, 7000 projections of the PilC knock-out, 6000 of PilW knock-out and around 5000 of the PilP knock-out projections from *N. gonorrhoea* were selected, respectively. Single particle analysis was performed using the Groningen Image Processing (“GRIP”) software packages on a PC cluster. Single particles of PilQ were repeatedly aligned and classified and finally the best 100% of the data set was taken for the final sums.
Table 1: strains used in the study

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<th>Strains</th>
<th>Description</th>
<th>Reference</th>
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<tr>
<td>HB1</td>
<td><em>Neisseria meningitidis</em> strain</td>
<td>Bos et al, 2005</td>
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<td>M986</td>
<td><em>Neisseria meningitidis</em> strain</td>
<td>Tsai et al, 1980</td>
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<td>MS11</td>
<td><em>Neisseria gonorrhoeae</em> strain</td>
<td>Swanson et al, 1971</td>
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<td>SJ001-MS</td>
<td>MS11 strain with PilQ truncation</td>
<td>This work</td>
</tr>
<tr>
<td>SJ030-MS</td>
<td>MS11 strain transformed with pSJ030; non polar insertion in PilC, ErmC</td>
<td>This work</td>
</tr>
<tr>
<td>SJ031-MS</td>
<td>MS11 strain transformed with PCR product to introduce truncation in PilP</td>
<td>This work</td>
</tr>
<tr>
<td>SJ032-MS</td>
<td>MS11 strain transformed with pSJ032; non polar insertion in PilW, ErmC</td>
<td>This work</td>
</tr>
<tr>
<td>EP060</td>
<td>EP019 strain transformed with pEP057; non polar Insertion in PilF, ErmC</td>
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**Table 2: Primers used in this study.**

<table>
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<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>PilC-for</td>
<td>5’-TGGCGGTACCCTCGCTGCCCAAATTGAAAG-3’</td>
</tr>
<tr>
<td>PilC-rev</td>
<td>5’-GCGCGGTACCTTGTCGAGATCAGCTATTATCATGGGACG-3’</td>
</tr>
<tr>
<td>PilW-for</td>
<td>5’-GCGCGGTACCTTGTCCGCGATGCAAGAATG-3’</td>
</tr>
<tr>
<td>PilW-rev</td>
<td>5’-GCGCGGATCCCGACCGCATAGGCGATTGACCAC-3’</td>
</tr>
<tr>
<td>PilF-for</td>
<td>5’-ATGCTCGAGACGCGCGACACCCATATCC-3’</td>
</tr>
<tr>
<td>PilF-rev</td>
<td>5’-TACGCTACCCGGAAGCTGTCGATTTC-3’</td>
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<tr>
<td>PilP-for1</td>
<td>5’-GGTTCCTCCAACGTAAGTTATTTTTCGCGCATTTTGTG-3’</td>
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<td>PilP-rev1</td>
<td>5’-ATGCCGTCTCTGACTTCACCTGCTCAACCTTC-3’</td>
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<tr>
<td>PilP-for2</td>
<td>5’-TTCAGACGGCATGCCAATTCTGATAATGCC-3’</td>
</tr>
<tr>
<td>PilP-rev2</td>
<td>5’-ACTTACGTTAGGGAAACCATGAAATACCAAAACTGACAAAATC-3’</td>
</tr>
<tr>
<td>PilQ-for1</td>
<td>5’-GTGACTATGACGCTGCACATCAAAGTTTCCTCCCTGAC-3’</td>
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<tr>
<td>PilQ-rev1</td>
<td>5’-TTTCAGACGGCATGCCAATTCTGATAATGCC-3’</td>
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<tr>
<td>PilQ-for2</td>
<td>5’-ATGCCGTCTGAAACCTTTCCAACCTACCC-3’</td>
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<tr>
<td>PilQ-rev2</td>
<td>5’-GACGTCATAGTCAAGGGAGAATCTCTCTTCTTAT-3’</td>
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**RESULTS**

*Transmission electron microscopy on isolated membranes of Neisseria gonorrhoeae*

To study the structure of the PilQ secretin of *N. gonorrhoeae* in its native environment, total membranes were isolated and separated on a sucrose gradient. Fractions containing the highest amount of PilQ (from 45 to 51 % (w/v) sucrose) were collected and concentrated. This fraction contained both inner and outer membranes as determined by antibodies against SecY and DsbA (as inner membrane markers) and Omp85 and Imp (as outer membrane markers) (data not shown). These mixed membranes were analyzed with transmission electron microscopy. Indeed both inner membranes which appear as vesicles and outer membranes which appear as flattened sheets could be identified (data not shown). The membranes form rather intact closed vesicles, because upon air-dried negative staining, the
membranes collapse and became superimposed. This can be seen at the edges where a white rim marks the curvature (Fig. 1A). The outer membranes contained prominent stain-filled indentations (Fig. 1A) which were absent in the inner membranes, with an average density of 350 indentations per $\mu\text{m}^2$. Since these stain-filled indentations could be formed by the PilQ secretin, a $\textit{pilQ}$ knock-out strain was constructed. The absence of PilQ within membranes isolated from this strain was confirmed by Western blotting using an anti-PilQ antibody (data not shown), and isolated membranes of this strain were checked by electron microscopy (Fig. 1B). The stain filled indentations were absent in the membranes of the $\textit{pilQ}$ knock-out demonstrating that the stain filled indentations are indeed related to PilQ.

![Figure 1](image)

**Figure 1.** Overview of negatively stained Neisseria gonorrhoeae membranes. **(A)** Membranes isolated from strain MS11. **(B)** Membranes isolated from a $\textit{pilQ}$ knock-out strain. Space bar for frames in A and B is 1000 Å.

**Projection structure of N. gonorrhoeae PilQ**

To further analyze the PilQ structure, a large data set of about 20,000 single projections of the stain-filled indentations was obtained from EM images and this data set was analyzed by single particle analysis. After several cycles of multi-reference alignment, multivariate statistical analysis and classification, final class sums from all analyzed particles were obtained (Fig. 2A). The 2D map shows a circular particle composed of a double ring with extending spike-like densities. The inner ring has a diameter of 150 Å and has a large central cavity, whereas the second ring has a diameter of 210 Å. The spikes further extend the
diameter to 310 Å. The second ring clearly has a 14-fold symmetry, while the spike-like densities show a 7-fold symmetry. After applying 7-fold symmetry, the features of both the second ring and the spikes improve (Fig. 2B). If this figure is used for further improvement as a reference in a next alignment procedure, it appears that the spike features become stronger, but at the cost of the resolution of the outer ring, which now becomes less well defined (Fig. 2C). This suggests that the structure has some flexibility between the second ring and the spikes. The symmetry of the inner ring could not be resolved from either the class averages without symmetry applied or from the class averages with 7-fold symmetry applied. In an attempt to determine the symmetry of the inner ring, the second ring was masked out during analysis. After repeated alignment and classification the final projection map showed two striking features. First, densities in the inner ring come into focus (Fig. 2D). At least 11 densities are well separated, with a average center-to-center distance of about 25 Å (red bars, Fig. 2E). However, in two areas the features are not well resolved (blurred red bars), despite the fact that we increased the number of analyzed projections from 20,000 to 36,000. This indicates that at the current resolution of this map, which is in the range of the 25 Å of the center-to-center distance of the inner ring densities, we can not prove the symmetry. It appears most likely to be 14 or 15. By imposing 14-fold symmetry, as performed in Fig. 2F, the features become stronger enhanced than with any other imposed symmetry between 12 and 16. A second striking result from this analysis is the total disappearance of the features of the second ring. This shows that also the association of the inner and outer ring is remarkably flexible, much more flexible than between the second ring and the outer spikes.
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Figure 2. Class averages of single particle electron microscopy images of the PilQ complex from *N. gonorrhoeae*. (A) wild-type projection map (B) with 7-fold symmetry imposed on the outer spikes (C) with 7-fold symmetry imposed using the class average of (C) as template (D) class average of the inner ring, after masking out the second ring and spikes (E) inner ring 2D map with positions of densities indicated (F) inner ring 2D map with imposed 14-fold symmetry. The projection map of the PilQ complex in frame A has a resolution of 20 Å. The scale bar is 100 Å.

Transmission electron microscopy on isolated membranes of *Neisseria meningitidis*

The PilQ and PilP proteins from *N. gonorrhoeae* and *N. meningitidis* are highly homologous (89 % identity and 91 % similarity for PilQ and 98 % identity and 98 % similarity for PilP). The structure of the purified PilQ/PilP complex was previously analyzed by transmission electron microscopy (Collins et al. 2001, Collins et al. 2003, Collins et al. 2004). To enable us to compare the previously published structure of the solubilized *N. meningitidis* PilQ/PilP complex to the structures of *N. gonorrhoeae* and *N. meningitidis* PilP/PilQ in the membrane, membranes of *N. meningitidis* were isolated and analyzed by transmission electron microscopy. The membranes of *N. meningitidis* also showed the presence of pores, but in much smaller numbers compared to *N. gonorrhoeae*. Moreover, the pores were less homogeneously distributed in the membrane, and some of them are found in
small clusters (Fig 3A). Single particle analysis showed a structure composed of only one ring (Fig. 3B) or with an additional second ring in about 20% of the data set (Fig. 3C). Some particles (10%) showed an incomplete second ring (Fig. 3D). The inner and outer rings have the same diameter as observed in the *N. gonorrhoeae* structure (Fig. 3E). Remarkably, there is no indication for spikes attached to the second ring. The motif in the second ring, is also substantially smaller than in *N. gonorrhoeae*, indicating that it is possibly composed of a larger number of copies of a smaller protein. Symmetry analysis was performed to evaluate the number of copies. It appears that imposing 2-, 3-, or 7-fold does not enhance the features (not shown). Since the motif is smaller than in *N. gonorrhoeae*, symmetries above 14 were evaluated (Fig. 3F-J). This approach strongly points to a 19-fold symmetry in the second ring. Based on the high similarities of the PilP and PilQ proteins of *N. meningitidis* and *N. gonorrhoeae*, the observed differences were unexpected. To ensure that the lack of spikes is a general feature of *N. meningitidis*, and not caused by the strain used, two different *N. meningitidis* strains, M986 and HB-1, which are different clonal lineages were used. Both strains gave very similar results. To further exclude that the *N. meningitidis* strains used in our study contained any mutations affecting pilP or pilQ, the entire PilP/PilQ operon and flanking regions were sequenced, but no differences were observed with the published sequence of strain (Bentley et al. 2007).

Previously, a purified *N. meningitidis* PilQ complex was studied by electron microscopy. The PilQ complex appeared as single rings with a diameter of approximately 15.5 to 16.5 nm and 6.0- to 6.5-nm-diameter cavities in the central region (Collins et al. 2001). These rings are comparable to the inner ring of both the *N. meningitidis* and *N. gonorrhoeae* structures. Data reported for oligomeric rings of PilQ and XcpQ of *Pseudomonas aeruginosa* show rings of 198 Å and 183 Å (Bitter et al 1998), but it is not clear if these structures are actually composed of two rings, since no image processing was performed.
Figure 3. Electron microscopical analysis of the PilQ complexes in isolated membranes. (A) Neisseria meningitidis membranes negatively stained with 2% uranyl acetate (B-D) Average of 8,000 projections of the PilQ complexes from N. meningitidis showing classes with single, double and incomplete rings, respectively. (E) Average of 10,000 projections of PilQ complex from N. gonorrhoeae showing inner and the outer ring having 14-fold symmetry. (F-J) To investigate the number of copies of the outer ring, 17- to 21-fold rotational symmetry (white numbering) was imposed on the classes after completion of the analysis. Strongest rotational symmetry within the second ring is in frame H. Scale bar in equals 1000 in (A) and 100 Å in (B-J).

Structure and assembly of the PilQ complex of N. gonorrhoeae

Our analysis of the structure of the PilQ complex in isolated outer membranes unequivocally shows extra subunits not observed in purified PilQ/PilP complexes. To try to identify the different subunits, we set-out to knock-out possible candidates for the extra observed densities. PilC is a protein normally present in two copies, involved in adhesion to
epithelial cells, and is located in the outer membrane and at the tip of the pilus. Since the MS11 strain used for our study, contains a non-functional copy of pilC1 (Rudel et al. 1995), we first generated a pilC2 knock-out. PilC2 is a protein with a molecular weight of 110 kDa. Single particle analysis was performed as on WT membrane pore complexes, and 6,000 projections were analyzed. The pilC knock-out yielded projection maps showing a similar structure as observed for WT with the presence of double rings plus 7 spikes (Fig. 4C). Again the features become more visible after imposing symmetry (Fig. 4D). This demonstrates that PilC2 is not a subunit of the observed PilQ complex. In a next step, a knock-out of pilW, a lipoprotein with a molecular mass of 28 kDa of the mature protein which was shown to be involved in stabilization of the PilQ oligomer was generated. As observed for the pilC knock-out no differences from WT were observed when 6,000 projections were analyzed. The membranes also contained a similar density of particles. This demonstrates that stable PilQ complexes can indeed still be formed within the membrane in the absence of PilW, and that PilW is not a subunit of the PilQ complex we observed.

PilP is an 18 kDa lipoprotein shown to co-purify stoichiometrically with PilQ. Both in N. meningitidis and N. gonorrhoeae PilP and PilQ are located within one operon and are cotranscribed (Balasingham et al. 2007). PilP mutants show a loss of piliation and natural competence and strongly reduced amounts of PilQ in the outer membrane (Drake et al. 1997). A previous study in which a comparison was made of the 3D reconstructions of N. meningitidis PilQ complexes derived from WT strains and of a strain lacking the PilP protein showed that PilP localized to the cap region of the PilQ complex (Balasingham et al. 2007). Comparison of these results with our structures suggests that the PilP protein is a component of the outer ring or involved in its assembly. To study the localization of PilP in PilQ complexes within the membrane, a pilP knock-out was created in N. gonorrhoeae. As expected, the density of PilQ complexes observed in the membranes derived from the pilP knock-out was reduced to only ~30% compared to the WT membranes, showing that our pilP mutant either affects the expression or the stability of the pilQ mutant. More remarkable differences were observed when the class averages of 6,000 particles without (Fig. 4G) and with applied symmetry (Fig. 4H) were compared to the class averages obtained from WT membranes. The pilP knock-out has not only lost the extending spike-like densities, but also the symmetry of the outer ring has changed from 14 to 19. Based on a possible localization of PilP between the inner and outer rings of the PilQ complex (see above), it could be proposed that deletion of PilP affects the interface between the inner and outer ring, resulting in a re-
assembly of the second ring. The reassembled second ring does not appear to be able to bind the spike-like extensions. Even more surprisingly, the structure of the PilQ complex in membranes derived of the *N. gonorrhoeae pilP* knock-out shows a remarkable similarity to the structure of the PilQ complex isolated from WT *N.meningitidis* membranes. Why the absence of PilP in the structure of the *N. gonorrhoeae* PilQ complex results in a structural change to a complex resembling the PilQ complex in *N. meningitidis* remains an open question (See discussion).

It has previously been shown that incubation of purified PilQ complexes with isolated pili can induce structural changes in PilQ (Collins et al. 2005). It was proposed that these structural changes could be explained by an outward movement of the arms and dissociation of the cap feature of the PilQ oligomer on binding to the pilus. To compare the structures of PilQ complexes that have interacted with the pilus and PilQ complexes that have not interacted with the pilus, membranes derived from a *pilF* knock-out were studied. PilF is an ATPase, localized in the inner membrane and essential for the assembly and extrusion of pilin sub-units. Remarkably, when the class averages of 6,000 particles obtained from membranes of the *pilF* knock-out without (Fig. 4I) and with applied symmetry (Fig. 4J) were compared to the class averages obtained from WT membranes, it appeared that the PilQ structure has lost the extending spike-like densities. This suggest that either the PilQ complex changes its conformation upon interaction with the pilus resulting in assembly of the extending spike-like structures, or that the spike-like structures are either formed by the pilus protein PilE or by a protein transported across the outer membrane along with the extension of the pilus.
**Figure 4.** Single particle analysis of particles derived of isolated membranes of different *N. gonorrhoeae* knock-out strains. Projection maps derived from membranes isolated from (A) *N. gonorrhoeae* strain MS11, (B) with 7-fold symmetry imposed, (C) the PilC mutant, (D) the PilC mutant with 7-fold symmetry imposed, (E) the PilW mutant, (F) the PilW mutant with 7-fold symmetry imposed, (G) the PilP mutant, (H) the PilP mutant with 19-fold symmetry imposed (I) the pilF mutant, and (J) the PilF mutant with 14-fold symmetry imposed on I. Scale bar equals 100 Å.

**DISCUSSION**

In this study we set out to study the structure of the PilQ secretin within the outer membrane using transmission electron microscopy and single particle averaging. Our approach revealed features of a large structure not seen before. Compared to the previously published structures derived from purified PilP/PilQ complexes of *N. meningitidis*, the complexes observed in *N. meningitidis* membranes contained an extra proteinous ring with a 19 fold symmetry. In our analysis we also observed structures lacking or having a partial outer ring, indicating that the extra domains are not tightly attached and can possibly also be dissociated during the membrane isolation procedure. Apparently this extra ring structure is also lost during the previously described purification of the PilQ/PilP complexes (Balasingham et al. 2007). Remarkably, the complexes observed in membranes isolated from *N. gonorrhoeae* (which expresses highly similar PilQ and PilP proteins) appear much more stable, and showed a double ring structure with a 14-fold symmetry of the outer ring, from which seven external spikes are protruding. These data demonstrate that the study of these multi-componet membrane inserted complexes within their native lipid environment by electron microscopy can identify extra components and/or structures which are lost after purification.
Compared to the previously published structures derived from purified PilP/PilQ complexes of *N. meningitidis* shows that the inner ring in our structures most likely consists of PilQ and PilP. The symmetry of the inner ring of *N. meningitidis* has previously been determined to be 12, while the symmetries of the *K. oxocya* PulD and the pIV protein of filamentous phages were respectively 12 and 14. We were unfortunately not able to conclusively determine the symmetry of the inner ring of *N. gonorrhoeae*, but our analysis indicates that the symmetry of the inner ring of *N. gonorrhoeae* is most likely 14, and thus could differ from the inner ring of *N. meningitidis*.

Another remarkable feature of the studied secretin complexes is the high flexibility between the different rings and the spikes. In particular, the fact that the number of protein copies in the second ring changes from 14 to 19 in a mutant lacking the spikes, is uncommon but intriguing. There is one other system of flexible interacting membrane proteins, that we like to discuss here in some detail, which has some similar structural aspects. Monomeric photosystem I (PSI) is a membrane protein complex of 330 kDa, which is mainly present as trimers in cyanobacteria. Under stress conditions it forms supercomplexes with a 37 kDa integral membrane protein named IsiA (see for details [http://www.uniprot.org/uniprot/Q55274](http://www.uniprot.org/uniprot/Q55274)). These complexes have been extensively studied by electron microscopy (Yeremenko et al. 2004, Kouřil et al. 2005) and it was shown that IsiA can form complete and incomplete single and double rings around monomeric or trimeric PSI. The number of IsiA copies was variable; in the case of monomers the inner IsiA ring was composed of 12, 13 or 14 copies and these numbers matched with 19, 20 or 21 copies in the outer ring. One conclusion was that within these PSI-IsiA supercomplexes the double rings of IsiA are flexibly attached. For complexes with 14 / 21 IsiA copies, analysis showed that the rotational flexibility between both rings was about 2-3°, on the average. In this work on the secretin complex of the type IV pilus, we noticed a same type of rotational flexibility between the first and second ring and and a smaller fliexibility between the second ring and the spikes.

A second finding on the PSI-IsiA supercomplexes could also be relevant for the type IV secretion complex. On trimers with two IsiA rings, the inner ring is composed of 18 copies and the outer ring is formed by 25. However, the positions of IsiA in incomplete second rings with 12-19 copies were slightly different. If extrapolated to complete rings they appeared to consist of only 24 copies. These data illustrate how IsiA is flexibly attached to PSI.
(Kouřil et al. 2005 and unpublished data). In a similar way, it could be possible that the protein(s) making the second ring around the secretin of the type IV pilus are flexible in their self-association. In one spike-less *N. gonorrhoeae* mutant and in *N. meningitidis* the ring contains 19 copies, but the association of spikes, each binding two second ring proteins, triggers ring formation with only 14 copies. There is, however, no evidence that the second rings of 14 and 19 copies in Neisseria contain the same protein(s).

Within this study we also tried to identify the proteins located within the second ring and in the spike-like extensions, and showed that neither PilC2 or PilW are components of the outer ring or the spike-like extensions. Remarkably, our study showed that the PilQ structure in the *pilP* knock-out has not only lost the extending spike-like densities, but also the symmetry of the outer ring has changed from 14 to 19. A previous study showed, however, that PilP localized to the cap region of the PilQ complex (Balasingham et al. 2007), which would place the PilP protein on the interface between the inner and outer ring, making it unlikely that PilP forms either the second ring or the spike-like extension. Finally, it became apparent that in the PilF knock-out the PilQ structure has lost the extending spike-like densities. Since PilF is an ATPase located in the inner membrane, it is unlikely that this protein is part of the spike-like extensions. Thus inactivation of the assembly and extrusion of the pilus results in loss of the spike-like extensions. This suggests that either the PilQ complex changes its conformation upon interaction with the pilus resulting in assembly of the extending spike-like structures, or that the spike-like structure are either formed by the pilus protein PilE or a protein transported across the outer membrane along with the extension of the pilus. We have therefore still not identified the proteins in the outer ring and spike-like extensions.

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Chapter 5


