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Structure and function of repetitive sequence elements associated with a highly polymorphic domain of the *Neisseria meningitidis* PilQ protein

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Summary

Secretins are a large family of proteins associated with membrane translocation of macromolecular complexes, and a subset of this family, termed PilQ proteins, is required for type IV pilus biogenesis. We analysed the status of PilQ expression in *Neisseria meningitidis* (Mc) and found that PilQ$^-$ mutants were non-piliated and deficient in the expression of pilus-associated phenotypes. Sequence analysis of the 5′ portion of the *pilQ* ORF of the serogroup B Mc strain 44/76 showed the presence of seven copies of a repetitive sequence element, in contrast to the situation in *N. gonorrhoeae* (Gc) strains, which carry either two or three copies of the repeat. The derived amino acid sequence of the consensus nucleotide repeat was an octapeptide PAKQQAAA, designated as the small basic repeat (SBR). This gene segment was studied in more detail in a collection of 52 Mc strains of diverse origin by screening for variability in the size of the PCR-generated DNA fragments spanning the SBRs. These strains were found to harbour from four to seven copies of the repetitive element. No association between the number of copies and the serogroup, geographic origin or multilocus genotype of the strains was evident. The presence of polymorphic repeat elements in Mc PilQ is unprecedented within the secretin family. To address the potential function of the repeat containing domain, Mc strains were constructed so as to express chimeric PilQ molecules in which the number of SBR repeats was increased or in which the repeat containing domain was replaced *in toto* by the corresponding region of the *Pseudomonas aeruginosa* (Pa) PilQ protein. Although the strain expressing PilQ with an increased number of SBRs was identical to the parent strain in pilus phenotypes, a strain expressing PilQ with the equivalent Pa domain had an eightfold reduction in pilus expression level. The findings suggest that the repeat containing domain of PilQ influences Mc pilus expression quantitatively but not qualitatively.

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Introduction

Neisseria meningitidis or the meningococcus (Mc) is a major cause of bacterial meningitis and of septicaemia worldwide. Although normally colonizing the upper respiratory tract without causing symptoms, Mc may disseminate to the bloodstream and, in the absence of bactericidal serum activity, cause sudden onset of disease. Systemic meningococcal disease affects primarily small children and adolescents, often leading to neurological sequelae or fatal outcome. The very closely related bacterium *Neisseria gonorrhoeae* (Gc) is the cause of the localized sexually transmitted disease gonorrhoea. Despite the unique disease manifestations associated with Mc and Gc, many of their basic strategies for successful colonization of their exclusive human hosts are highly conserved.

The influence of pili, filamentous-like structures emanating from the bacterial surface, on neisserial attachment to mucosal surfaces is well established (Swanson, 1973; Heckels, 1989). Neisserial pili are ordered arrays of polymerized protein subunits termed pilin that show a high degree of homology with pilins of other Gram-negative human pathogens, including *Vibrio cholerae*, enteropathogenic *Escherichia coli* and other important mucosal pathogens of man, as well as opportunistic pathogens within the genera *Eikenella*, *Moraxella* and *Pseudomonas* (Tønjum and Koomey, 1997). Collectively, this family of surface appendages containing a highly conserved N-terminal domain in the structural subunit have been termed type IV pili (Ottow, 1975). Further evidence for the relatedness of type IV pili in different species can also be found in the conservation of genes and gene products required for their biogenesis. Homologues corresponding to one or more of these molecules have been implicated in the main terminal branch of the general secretory pathway (GSP)

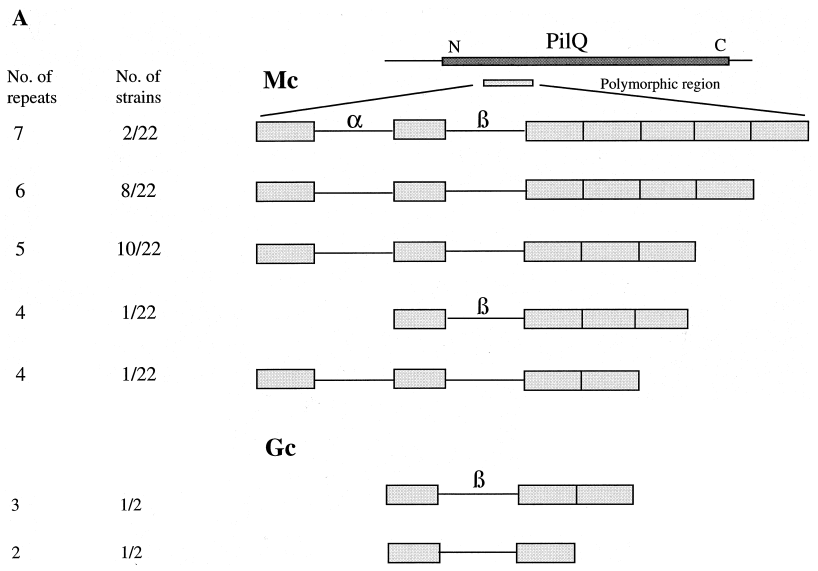


Fig. 1. A. Schematic diagram of the patterns of SBR and α/β domain polymorphisms detected in Mc and Gc PilQ (see also Table 2).
B. DNA sequences and corresponding primary structure of the polymorphic PilQ region from Mc strains. The data from a number of strains are compared with that derived from Mc strain 44/76 shown as the top line with the SBR segments in bold, the α and β domains underlined and residues altered by nucleotide changes shown above. Gene segments from various strains are shown below with dots denoting DNA sequence identity, dashes denoting gaps and base substitutions indicated. The bottom two segments of data are for gonococcal strains MS11 and 2686. Numbering of residues are for $PilQ_{44/76}$.
C. Alignment of the Mc PilQ α and β domains.

and expression of competence for transformation in *Bacillus subtilis* (Pugsley, 1993). The Mc components so far described in this respect are pilin, PilG, an inner membrane protein engaged in pilus assembly (Tønjum *et al.*, 1995), and PilC, functioning as a putative adhesin and assembly factor (Nassif *et al.*, 1994).

Related outer membrane proteins, termed secretins, participate in a broad array of processes involving translocation of macromolecules across the outer membrane of Gram-negative species. These include pIV (engaged in the morphogenesis/export of filamentous phage) (Brissette and Russel, 1990), PulD (required for pullulanase secretion in *Klebsiella oxytoca*) (d'Enfert *et al.*, 1989), as well as molecules shown to be active in type III, contact-dependent secretion systems (Salmond and Reeves, 1993), competence for transformation in *Haemophilus influenzae* (Tomb *et al.*, 1991) and type IV pilus biogenesis (Drake and Koomey, 1995; Martin *et al.*, 1993). Many of the secretins have been demonstrated to form stable homomultimers, composed of 10–12 subunits, that have been proposed to constitute channels through which substrates are translocated (Russel, 1994). The capacity of these molecules to oligomerize and associate with the outer membrane requires the structural integrity of the C-terminal domain that is conserved in the secretins and encompasses a 200-amino-acid span rich in polar residues (Genin and Boucher, 1994). The N-terminal portions of the homologues are more heterogeneous and domain-swapping experiments (Daefler *et al.*, 1997a,b) as well as direct biophysical data (Shevchik *et al.*, 1997) support the notion that these parts of the molecules are responsible for substrate specificity.

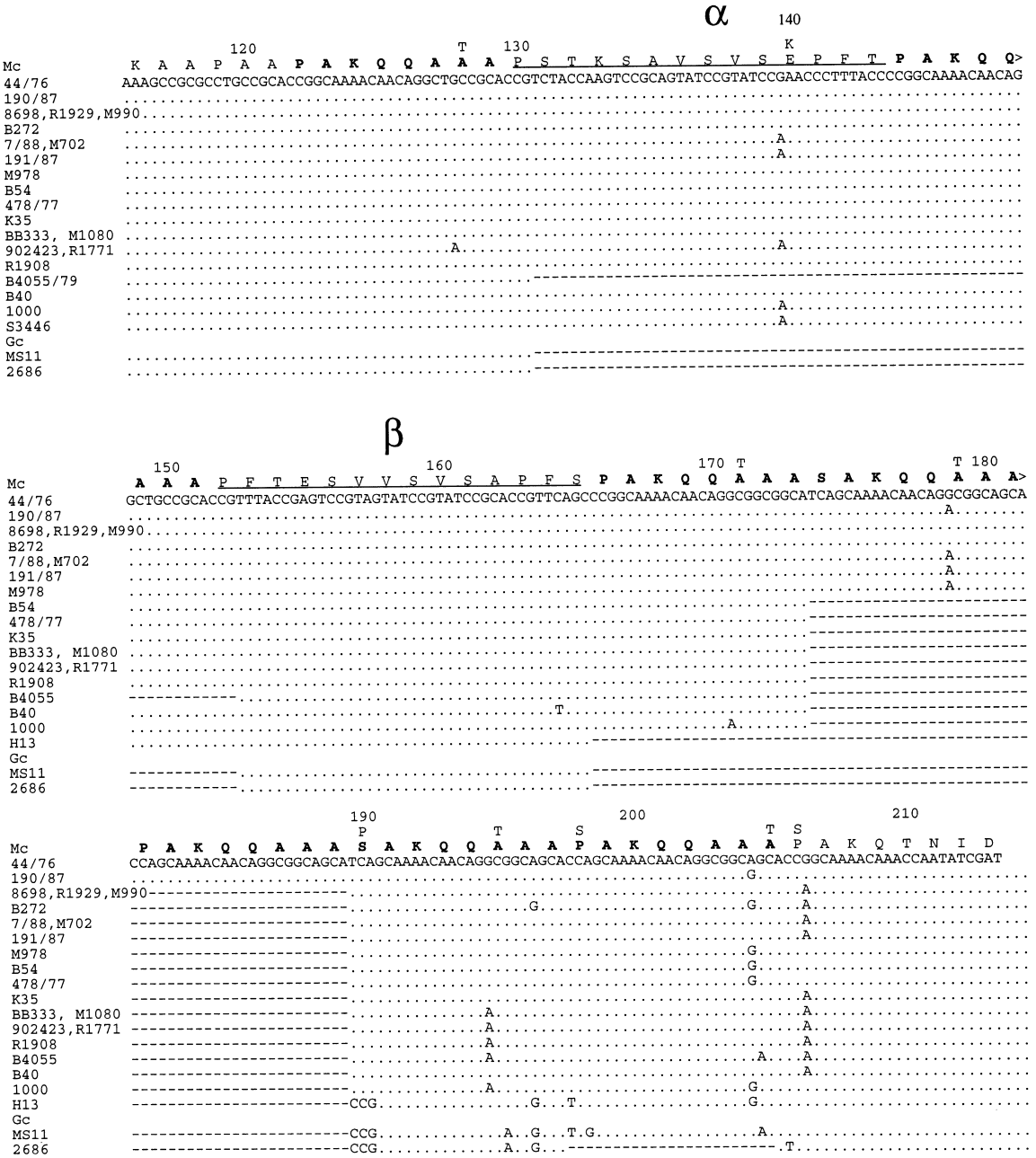
The PilQ proteins are secretins required for type IV pilus biogenesis in Gc and *P. aeruginosa* (Pa). Although early studies suggested that PilQ was a Gc-restricted antigen, subsequent studies indicated that proteins of related antigenicity and molecular mass were found in other neisserial species including Mc (Hansen and Wilde, 1984). To investigate the status of PilQ expression in Mc, we cloned and sequenced the *pilQ* gene from the serogroup B strain H44/76 and examined the effects of *pilQ* mutations in this background. The results were similar to what was previously documented in Gc, indicating a high degree of structural and functional conservation. However, the predicted product of the Mc *pilQ* was unique in that it contained seven copies of a repetitive sequence element within its amino terminus, which is present in only two to three copies in Gc PilQ. An extensive study of 52 Mc strains revealed that the repeat encoding gene segment was highly polymorphic. These findings led us to examine the function of this region of PilQ by constructing strains expressing a protein carrying an increased number of repeats and one in which the repeat domain was swapped with the equivalent but unrelated domain of Pa PilQ.

Results

Cloning and characterization of the Mc 44/76 pilQ *gene*

Recombinant clones containing the *pilQ* gene from Mc serogroup B strain 44/76 were identified within a genomic DNA library propagated in a bacteriophage vector and characterized. Restriction fragments from the phage clones that hybridized with probes corresponding to the Gc *pilQ* 5′ and 3′ ends were subcloned into the high-copy-number vector pBSKII and their nucleotide sequences were determined. Analyses of a 2.7 kb region encompassing the hybridizing segments of this region showed an open reading frame (ORF) of 2298 bp (the DNA sequence of this

B



C

locus appears in the EMBL/GenBank/DDBJ Nucleotide Sequence Data Libraries under the accession no. AF066056). The derived amino acid sequence of 44/76 PilQ is a polypeptide of 765 residues with a predicted molecular mass of 83 202 Da and a pI of 9.90. Like its Gc counterpart, Mc PilQ is predicted to contain a signal sequence at the N-terminus (Tsai *et al.*, 1989) and nine putative β-strands intersected by loops near the C-terminus, as analysed by PREDICT-PROTEIN (EMBL Heidelberg, Germany) (Linderoth *et al.*, 1996; Rost and Sander, 1993). Sequences immediately downstream of the predicted translational termination site demonstrated a strong potential to form a stable stem–loop structure, whose presence in RNA might act as a signal for transcription termination/attenuation or nucleolytic processing (Jaeger *et al.*, 1989).

Although having strong sequence homologies, the deduced protein encoded by Mc *pilQ* was considerably larger than that predicted for Gc PilQ. Alignment of the protein sequences showed a region of discontinuity beginning at residue 131 of the mature PilQs and ending at residue 191 of Mc $PilQ_{44/76}$ and residue 145 of Gc $PilQ_{MS11}$ (Fig. 1A and B). The extra residues within the corresponding segment of Mc PilQ consisted almost entirely of the repeat sequence PAKQQAAA or slight variants thereof, designated here as the small basic repeat (SBR). $PilQ_{44/76}$ also showed a unique 14-amino-acid segment (termed α) that bridged the first two SBR copies, corresponding to a similar element (termed β) located between the first two SBR copies of Gc $PilQ_{MS11}$ with 9 out of 14 residues being identical (Fig. 1C). Further comparisons of the two complete Mc *pilQ* and one Gc *pilQ* genes and their deduced amino acid sequences revealed absolute identities within the first 130 residues and 21 single amino acid differences distributed throughout the region C-terminal to the SBR-containing domain. The vast majority of substitutions in the latter region were conservative and none was predicted to have significant consequences with regard to the structure of this part of the molecule. As such, the major differences in primary structure between $PilQ_{44/76}$ and $PilQ_{MS11}$ could be accounted for by the presence in the former of four additional copies of the SBR and the unique α domain.

Characterization of Mc pilQ *transposon mutants*

To assess directly the potential role of *pilQ* in pilus biogenesis, the plasmid pTT21 was subjected to transposon mutagenesis (Seifert *et al.*, 1991). The insertion mutations were then moved into the chromosome of Mc strain 44/76-A, a background in which RecA expression and recombination functions are under the control of an inducible promoter (Seifert, 1997). All mutants bearing transposon insertions within the *pilQ* ORF lacked the property of autoagglutination, which is reliant on pilus expression, and failed to express pili detectable by electron microscopy (Table 1). The status of PilQ expression in these isogenic strains was examined by immunoblotting analysis of whole-cell extracts using rabbit antibodies raised against the recombinant protein expressed in *E. coli*. The parent strain 44/76-A displayed a predominant reactive species with M_r of 80 kDa, which was absent in the transposon mutant with the insertion in the *pilQ* start codon, whereas faster migrating forms appeared in the mutants with transposon insertions within the carboxy terminus of the ORF

Table 1. Phenotypic characteristics of *N. meningitidis* 44/76 and *pilQ* mutants.

Strain	Pili	Aggregation	Transformation frequencies
44/76	+	+	3×10^{-3}
44/76 (no DNA)	NA	NA	$<10^{-8}$
MQ0	−	−	10^{-6}–10^{-7}[a]
MQ22	−	−	10^{-6}–10^{-7}[a]
MQ23	−	−	10^{-6}–10^{-7}[a]

+, present; −, absent; NA, not applicable.
a. The transformation frequencies for these strains were 10^{-6}–10^{-7} and there were no statistically significant differences between them.

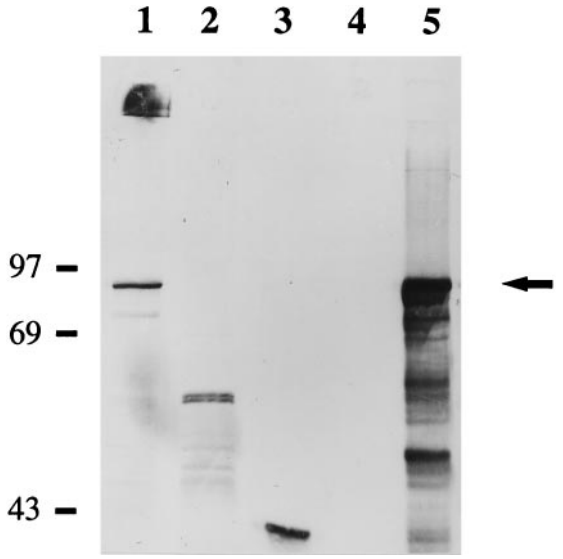


Fig. 2. Detection of Mc PilQ antigen in whole-cell lysates from wild type and mutants by immunoblotting. The filter was reacted with anti-Mc PilQ rabbit antiserum. The arrow denotes migration of the PilQ monomer (80 kDa). Lanes: 1; *N. meningitidis* 44/76-A wild type; 2, mutant containing the *pilQ::mTnerm*#23 allele; 3, mutant containing the *pilQ::mTnerm*#22 allele; 4, mutant containing the *pilQ::mTncm*#21 allele; 5, *N. meningitidis* 44/76-A wild type treated with 1 M iodoacetamide after reduction. Reactive material immediately below the numbering of lane 1 corresponds to HMM form of PilQ that is retained in the well of the stacking gel. Note in lane 5 the disappearance of the HMM form and increase in the amount of the 80 kDa PilQ species after reduction/alkylation. The position of the 69 and 97 kDa protein size standards are marked.

(Fig. 2). A large amount of the Mc PilQ antigen in the wild-type strain was retained at the buffer/stacking gel interface after SDS–PAGE but was not seen in the mutants. The unique species of PilQ with retarded mobility appears to correspond to a high-molecular-mass (HMM) species. This HMM form was shown to be an SDS and heat-resistant multimer that could be dissociated into monomers by reduction and alkylation, resulting in loss of the material at the stacking gel and increased levels of the M_r 80 kDa species as well as what appeared to be degradation products (Fig. 2, lanes 1 and 5).

Immunoblotting of whole-cell lysates of the isogenic Mc PilQ strains was performed to assess the levels of pilin expression in these backgrounds. Although the amounts of pilin antigen detected were equivalent in all cases, the patterns of pilin species were specifically and identically altered in all Mc *pilQ* mutants with some pilin antigen migrating at a position characteristic of S pilin (soluble, truncated pilin) (data not shown).

Previous studies have shown that non-piliated Mc mutants are dramatically reduced in competence for natural transformation (Nassif *et al*., 1994; Tønjum *et al*., 1995). Considering that natural competence for transformation is a highly sensitive marker for pilus expression, we examined the relative competence of Mc strain 44/76-A and its isogenic PilQ mutant derivatives. All mutants had over a 1000-fold reduction in their transformation competence (Table 1).

Identification of a polymorphic region in the N-terminus of PilQ

Given the differences found between $pilQ_{44/76}$ and $pilQ_{MS11}$, we examined the degree of heterogeneity within *pilQ* from other Mc strains. A PCR-based assay was developed using two oligonucleotides that flanked the SBR-encoding region such that gross gene differences could be detected as differences in the relative mobilities of the PCR products. A collection of 52 *N. meningitidis* strains from various geographic origins, including different serogroups and the dominant clone complexes associated with meningococcal disease was investigated. Five distinct size classes of PCR products were seen (data not shown) and DNA sequencing of the products from 22 strains representative of the whole range of size variation (Table 2) revealed differences in the number of repetitive sequence elements encoding the SBRs. Mc strains were found to harbour from four to seven copies of the nucleotide sequence CCGGCAAAACAACAGGCTGCCGCA (or slight variants thereof) (Fig. 1A and B). The derived amino acids of this consensus DNA sequence repeat was PAKQQAAA with single nucleotide changes leading to slightly deviant forms with the structures SAKQQAAA and PAKQQTAA (Fig. 1B).

Also, between the three first repeat cassettes were found additional homologous elements, designated α and β (Fig. 1B and C), of which the α region was specific for Mc strains. In one single Mc strain (B4055/79) the

Table 2. Characteristics of the *N. meningitidis* isolates used in this study.

Strain	Number of PilQ repeats	Clonal group	Serogroup	Serotype	Subtype	Country	Year of isolation
44/76	7	ET-5 complex	B	15	P1.7,16	Norway	1976
190/87	7	ET-5 complex	B	15	P1.16	Norway	1987
8698	6	ET-5 complex	B	15	P1.3	Chile	1985
R1929	6	ET-5 complex	B	4	P1.15	Spain	1985
7/88	6	ET-5 complex	B	NT	P1.16	Norway	1988
191/87	6	ET-37 complex	C	2a	P1.2	Norway	1987
M702/91	6	ET-37 complex	C	2a	NST	Iceland	1991
M990	6		B	6	P1.6		
M978	6	Cluster A1	B	8	P1.7,1		
B272	6	Subgroup IV-2	A	4,21	P1.10	USA	1936
477/78	5	Not analysed					
B40	5	Subgroup I	A	4,21	P1.10	Morocco	1967
B54	5	Subgroup III	A	4,21	P1.9	Finland	1975
K35	5	ET-5 complex	B	4	P1.15	Cuba	1980s
BB333	5	Cluster A4	B	2b	P1.2	USA	1984
M1080	5		B	1	P1.1,7	USA	1984
902423	5	Cluster A3	B	4,14	P1.7,1	Netherlands	1990
R1771/92	5	ET-19	C	21	P1.16	Iceland	1992
R1908	5	ET-5 complex	B	14	P1.1	Spain	1985
1000	5	ET 61	B	ND	ND	USSR	1988
B4055/79	4	Cluster A4	B	2b	P1.2	Iceland	1979
S3446	4	Cluster A1	B	14	NST		

nucleotide sequences encoding the α repeat domain was absent (Fig. 1B), similar to what is seen in Gc strains. Single nucleotide substitutions also contribute to the genetic diversity seen within this segment of the Mc *pilQ* gene. For example, 7 of 22 strains have a single base change within the codon for the single glutamate within the α repeat yielding a lysine at this position (Fig. 1).

No consistent associations between the structure or number of SBR copies and serogroup, serotype, subtype, geographic origin or multilocus genotype of the Mc strains were evident (Table 2). Strains belonging to the ET-5 clone complex (Caugant *et al*., 1986), for example, carried either five (R1908), six (7/88) or seven (44/76) copies of the element and strains belonging to cluster A4 carried either four (B4045) or five (BB333) copies.

PilQ polymorphisms are reflected in altered mobility of the molecule

Given the differences in PilQ predicted from the nucleotide sequences, we sought to determine if we could detect corresponding alterations in its primary structure. This was addressed by examining the relative mobilities of Mc PilQ derived from whole-cell lysates by SDS–PAGE and immunoblotting. In the strains examined, there was a strict correspondence between the SBR number and relative mobility with variability in repeat structure being reflected in altered migration of the major immunoreactive species that had M_r ranging from 70 kDa to 80 kDa (Fig. 3). In addition to these forms of the molecules, faster migrating reacting species were seen with the most prominent of these migrating in the M_r 45–50 kDa range. In each case, the relative pattern seen for the faster migrating forms coincided with what was seen for the monomeric species. Given the related shifts in these two major forms and the location of the repetitive elements within PilQ, it appears that the faster migrating species retains the SBR domains but lacked a significant portion of their C-terminal domains. The biological significance and physiological basis for Mc PilQ degradation in wild-type cells remains unknown.

Exposure of Mc PilQ at the cell surface

Prior studies using protease accessibility (Tsai *et al*., 1989) and surface iodination (Hansen and Wilde, 1984) have supported the contention that part of Gc PilQ is exposed to the extracellular milieu. To assess whether Mc PilQ might be surface exposed, intact Mc cells were labelled with sulphosuccinimido-biotin, a reagent that is unable to penetrate the outer membrane. The results showed that an 80 kDa protein, which was confirmed to be PilQ by parallel immunoblotting of duplicate samples, was present in Mc 44/76 wild-type whole-cell lysates but was absent in the mutant that carries a transposon insertion within the initiation codon of the *pilQ* ORF (Fig. 4). To confirm that conditions used for biotinylation labelled specifically detected outer membrane proteins, parallel immunoblotting was performed using antibodies to the class 1 and class 3 proteins, which are the major constituents of the Mc bacterial outer membrane. These two proteins were the most abundantly labelled molecules in whole-cell lysates (Fig. 4). Moreover, the patterns found using biotinylation were identical to that found for Coomassie-stained gels of outer membrane preparations (data not shown).

Functional analysis of PilQ chimeric proteins

Chimeric proteins in which the N-terminal domains of related pIV molecules, the secretins dedicated to filamentous phage morphogenesis, are swapped, have been very useful in identifying segments critical to imparting functional specificity (Daefler *et al*., 1997b). To use a similar approach to assess what influence the Mc PilQ polymorphic repeat region might have on pilus biogenesis and pilus-associated phenotypes, it was first necessary to devise a protocol to construct strains expressing hybrid alleles. A genetic strategy was chosen using duplication of the *pilQ* gene by plasmid integration into the genome (outlined in Fig. 5). This approach made it possible to manipulate the gene copy 5′ of the integrated plasmid

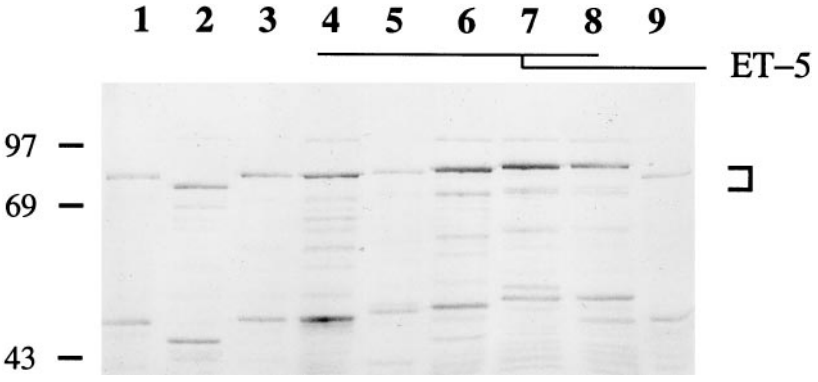


Fig. 3. Repeat polymorphisms alter Mc PilQ migration during SDS–PAGE. Immunoblotting of whole-cell lysates using Mc PilQ rabbit antibodies was used to detect PilQ. Shown are Lanes 1 and 9, strain S3446 (4× SBR); 2, B4055/79 (4× SBR minus the α domain); 3, B54 (5× SBR); 4, K35 (5× SBR); 5, 7/88 (6× SBR); 6, 8698 (6× SBR); 7,190/87 (7× SBR); 8, 44/76-A (7× SBR). Location of monomeric PilQ forms are indicated on right by the bracket. Note the correspondence between the relative migration of the reactive species in the M_r 45–50 kDa range with that seen for the monomeric forms.

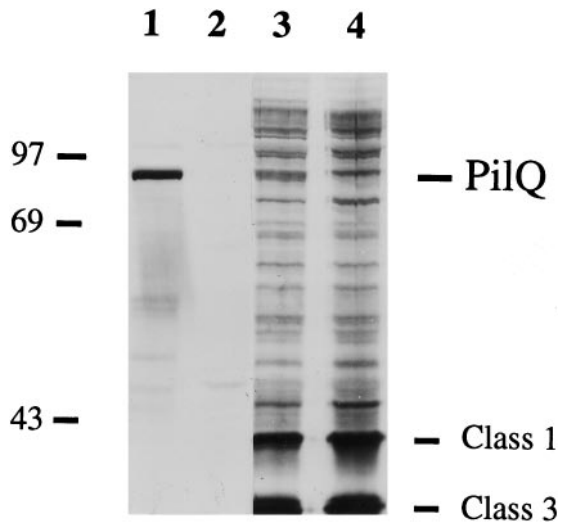


Fig. 4. Exposure of Mc PilQ on intact cells detected by surface biotinylation. Whole cell lysates of Mc strain 44/76-A wild type (lanes 1 and 3) and its isogenic derivative carrying the *pilQ::Tncm#21* allele (lanes 2 and 4) treated with sulphosuccinimido-biotin were fractionated by SDS–PAGE and then transferred to an Immobilon filter. Lanes 1 and 2 were developed as an immunoblot using Mc PilQ rabbit antiserum while lanes 3 and 4 were developed using streptavidin conjugated to alkaline phosphatase. The profile of the biotinylation pattern was also compared and correlated with parallel immunblots developed with antibodies to the class 1 and class 3 proteins (marked for lanes 3 and 4) and Coomassie-stained gels of enriched outer membranes OM (data not shown). The position of the 43, 69 and 97 kDa protein size standards are also shown.

vector, whereas the intact gene copy 3′ of the vector provides wild-type PilQ, maintaining pilus expression and competence for transformation. After the reconstitution of the hybrid gene copy, expression from the unaltered 3′ gene was inactivated by introduction of a mTn*Cm* transposon insertion. Attempts at using this protocol with strain 44/76-A were unsuccessful but the constructs could be introduced into strain M1080-A, a background that expresses a PilQ with five copies of the SBR.

Two strains were constructed in which the PilQ polymorphic region was replaced by either the same segment from strain 44/76-A (containing seven copies of the SBR, Fig. 1) or the equivalent domain from *P. aeruginosa* (Pa) PilQ. The latter construct was made because the corresponding region of Pa PilQ shows no sequence identity to the polymorphic region of neisserial PilQ nor does it contain any repetitive motifs while adjacent residues are conserved between the proteins. It is in fact the conservation of these flanking residues and DNA sequence homology that made it possible to create the chimeras by ligating restriction fragments as the *Sty*I–*Nco*I and *Cla*I restriction sites reside within the nucleotide sequences that encode the residues PARIALD and NIDF, respectively, which are conserved in PilQ of the two species (Martin *et al*., 1993). As such, the Mc/Pa PilQ hybrid contained residues 67–164 of Pa PilQ, which are flanked by the conserved PARIALD/NIDF residues. The first 76 residues (67–143) of this segment of Pa PilQ shares greater than 26% identity with the equivalent portion of neisserial PilQs, whereas the remainder (corresponding to the SBR region) shows no identity.

When these strains were tested for PilQ expression by immunoblotting, two monomeric protein species were detected, one encoded by the genes in which the N-terminal modules had been swapped, the other encoded by the wild-type gene bearing five copies of the SBR (Fig. 6, lanes 4 and 6). The chimeric gene products were expressed at high levels while the wild-type gene product was reduced in quantity as it is expressed from the gene mapping 3′ of the integrated plasmid and this distal gene copy lacks the major promoter driving *pilQ* expression (S. A. Dunham and M. Koomey, unpublished). Disruption of the distal wild-type gene copy by crossing in a transposon insertion mutation yielded strains in which only the hybrid PilQ was expressed (Fig. 6, lanes 5 and 7).

After confirming the DNA sequences and integrities of both the *pilQ* and the *pilE* genes in these backgrounds, the strains were examined by electron microscopy. Although no differences in pilus fibre morphology were found (data not shown), the abundance of pili in the strain expressing only PilQ with the Pa-derived N-terminal module was reduced (Fig. 7). The diminution of piliation in this background does not appear to be solely due to a reduction in the levels of the HMM form as strain TT31, which expresses both the 5× and 0× SBR PilQs, also shows reduced levels of the HMM form (Fig. 6, lanes 4 and 5) but is wild type with respect to piliation levels (Tables 3 and 4). It may be possible that in strain TT31, which expresses both the 5× and 0× SBR PilQs (Fig. 6, lane 4),

Table 3. Phenotypic characteristics of *N. meningitidis pilQ* chimeric strains.

Strain	PilQ expressed (no. of SBRs)	Purified pili[a]	Transformation frequency	Adherence for ME180 cells[b]
M1080-A	5×	++	10^{-3}	++
TT1	5×	++	10^{-3}	++
TT31	0×, 5×	++	10^{-3}	++
TT31-121	5×	++	10^{-3}	++
TT31-221	0×	+[c]	10^{-3}	++
TT32	7×, 5×	++	10^{-3}	++
TT32-121	5×	++	10^{-3}	++
TT32-221	7×	++	10^{-3}	++
TT0	–	–	$<10^{-7}$	–

+, Present; –, absent.
[a] See Fig. 7.
[b] ++, greater than 50 colony-forming units per epithelial cell; –, less than 0.1 colony-forming units per epithelial cell.
[c] The amount of pili purified from this chimeric strain was one-eighth the amount of pili purified from the other strains.

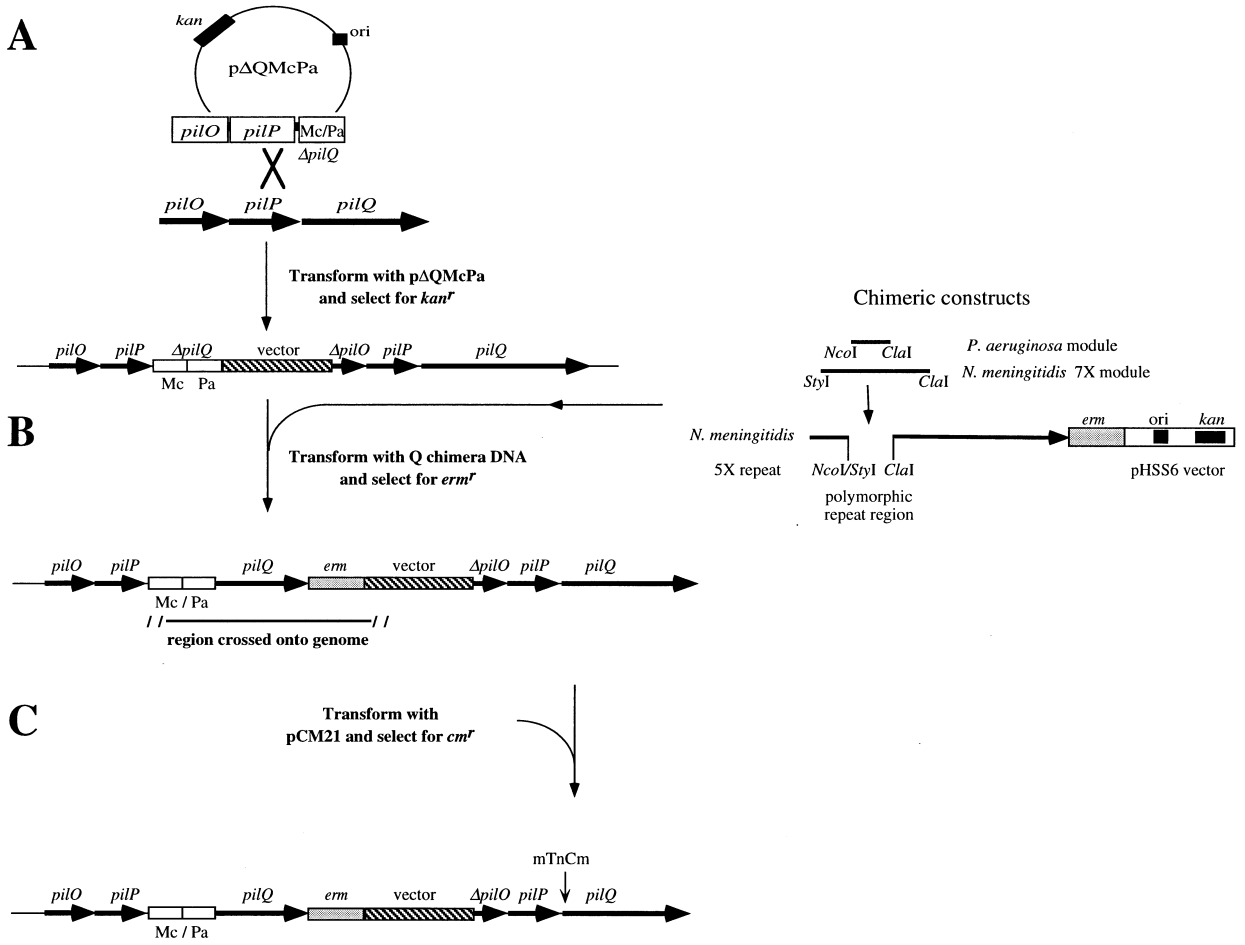


Fig. 5. Construction of Mc strains expressing PilQ polypeptides altered in the polymorphic repeat region (PilQ chimeras).
A. The plasmid pΔQMcPa contains a truncated Mc *pilQ* gene with a translational fusion to residues 77–179 derived from the *P. aeruginosa* (Pa) PilQ protein. This construct was made using the *Sty*I–*Nco*I and *Cla*I restriction sites, which are conserved in the *pilQ* genes of both species and which flank the neisserial polymorphic repeat encoding region and the equivalent coding region from the Pa *pilQ* gene. Transformation using this plasmid DNA and selecting for kanamycin resistance gives rise to strain containing one truncated, chimeric Mc/Pa hybrid *pilQ* gene and one complete Mc *pilQ* gene. The non-homologous Pa segment is necessary to force crossing-over 5′ of the repeat encoding segments in the next step.
B. Strain TT1 was transformed with linearized DNA from *pilQ* chimeras of Mc and Pa (listed as Q chimeras) and erythromycin resistance selection. A complete but chimeric *pilQ* gene is formed that contains the repeat encoding region from either the Mc (7× repeats) or Pa (no repeats) *pilQ* gene.
C. Inactivation of the original complete Mc *pilQ* gene by recombination of mTn*cm*#21 insertion mutation in the start codon of the gene, leading to expression of only the upstream chimeric gene.

functional mixed multimers may be formed that contain only a small level of the 5× SBR molecule. Moreover, pathogenic *Neisseria* appear to express PilQ (both as HMM and monomeric forms) at levels well in excess of that required for normal piliation (unpublished data, Drake *et al.*, 1997). Thus, it is difficult to establish a strict correlation between the reduction in levels of PilQ HMM forms and the defect seen with the PilQ molecule containing the Pa domain.

When examined by SDS–PAGE and staining, purified pili from these strains showed no qualitative differences in pilin but the yield of pili from the strain expressing PilQ with the Pa-derived N-terminal module was reduced approximately eightfold (Fig. 7). To assess the functionality of the pili expressed in the strains, the pilus-associated phenotypes of adherence for human epithelial cells and competence for natural transformation were measured (Table 3). Surprisingly, all three strains were identical with respect to their levels of epithelial cell adherence and transformability. In summary, no discernible alterations could be detected in association with changing the number of SBRs from five to seven while replacing the repeat containing domain with the equivalent module from Pa PilQ decreased piliation quantitatively but had no effect on the two pilus-associated phenotypes examined.

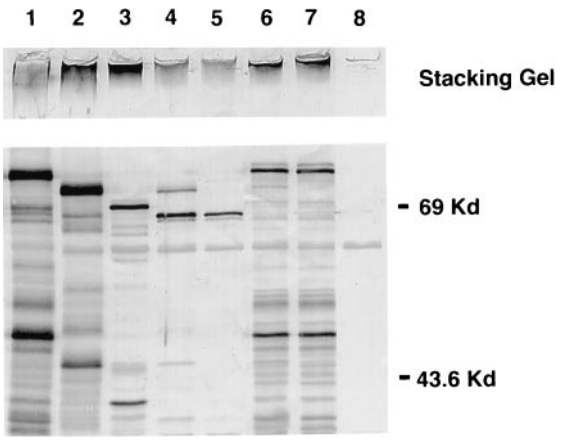


Fig. 6. Detection of PilQ antigen in Mc wild type and chimeric strains by immunoblotting of whole-cell lysates. The filter was reacted with Mc PilQ rabbit antiserum. Lanes: 1, Mc 44/76-A (7×); 2, Mc 1080-A (5×); 3, Gc N400 (3×); 4, TT31, *pilQ* duplication (0× SBR and 5× SBR); 5, TT31–221, *pilQ* chimera (0× SBR); 6, TT32, *pilQ* duplication (7× SBR and 5× SBR); 7, TT32-221, *pilQ* chimera (7× SBR); 8, TT0 (negative control).

Discussion

Mc PilQ$^-$ mutants exhibited gross defects in the expression of pili and pilus-associated phenotypes, indicating that Mc PilQ is engaged in pilus biogenesis. These findings are consistent with other studies that demonstrate that pilus biogenesis components of Mc and Gc are highly conserved. However, genetic and biochemical evidence derived from this study reveal some unique facets of Mc PilQ. Most striking perhaps is the variability in the number of tandem repeats of the sequence PAKQQAAA (and minor variants thereof), which varies from four to seven copies. Although the sequences for only two Gc *pilQ* genes are currently available, PCR-based studies analogous to those detailed here using a large number of Gc isolates indicate that these strains carry either two or three copies of the SBR (S. Drake and M. Koomey, unpublished), as found previously for strains 2686 and MS11 respectively (Drake and Koomey, 1995). It appears that the presence of four or more SBRs is unique to Mc, with the majority of strains (17 out of 22) possessing five or six SBRs.

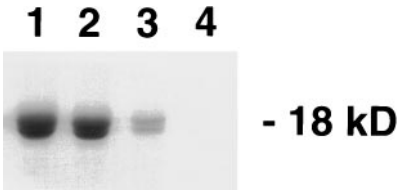


Fig. 7. Pilus expression monitored by detection of purified PilE antigen from Mc wild-type and chimeric strains. Lanes: 1, Mc 1080-A (5× SBR); 2, TT32–221 (7× SBR); 3, TT31-221 (0× SBR); 4, Mc 1080-A with mTn*cm*#21 insertion (in *pilQ* start codon – negative control).

Another result of this work entails the identification of the unique α domain that bridges the first two SBRs in Mc PilQs. This domain is highly related to the β domain that spans the second and third SBR in Mc isolates and the first two SBRs in the Gc strains, sharing 9 out of 14 residues and a high degree of nucleotide homology. We speculate that α (or β) arose by tandem duplication by recombination and unequal crossing-over or via transformation. Given that the majority (51 out of 52) Mc strains carry the unique α domain, it appears that its presence along with increased numbers of SBRs are Mc specific. In light of the high degree of identities between other Mc and Gc pilus biogenesis factors (Nassif *et al*., 1994; Tønjum *et al*., 1995;), it is curious that Mc PilQ would possess these somewhat subtle but consistent allele differences. There are obvious differences in the pathogenesis of Mc and Gc infection *vis-à-vis* sites of, and potential for, colonization, and there may be Mc-specific features that influence this situation. For example, subtractive hybridization studies have identified relatively large genome segments that carry Mc-specific DNA (Tinsley and Nassif, 1996). However, it is worth noting that subtractive hybridization methodologies detect only relatively large genomic differences but miss the more subtle yet distinct genetic nuances, such as those found in this study.

No clear associations were evident between *pilQ* gene polymorphisms and Mc genotypes or phenotypes. In fact, within this somewhat limited survey, one can find evidence for both genetic stability and instability. The high stability of intrastrain repeat number is reflected by the fact that there was no change in repeat number during more than 25 passages of strain 44/76 and no differences were observed between cultures derived from the original stock of 44/76 (stored in 1976) and the strain independently maintained in another laboratory over the course of 15+ years (T. Tønjum and D. A. Caugant, unpublished). However, *in vitro* stability is not representative of the selective pressures and conditions that exist *in vivo*. Furthermore, the variability in number of repeats within clones of the ET-5 complex and cluster A4 (Table 2) suggest that such variations occur in the human habitat (although these are formally not isogenic strains). Pathogenic *Neisseria* are particularly adept at using DNA rearrangements to modify gene expression. In some instances these rearrangements entail combinatorial diversification and gene recombination (such as in pilin variation) (Haas and Meyer, 1986; Zhang *et al*., 1992), whereas in others they involve slipped strand mispairing within repetitive or monotonous stretches of nucleotides such as documented in *opc*, the serogroup B capsule and the LOS biosynthetic gene systems (Sarkari *et al*., 1994; Hammerschmidt *et al*., 1996; Yang and Gotschlich, 1996). Given the organization of the gene segments encoding the SBRs, either forms of DNA metabolism may alter DNA repeat copy number.

Structurally, the PAKQQAAA SBR and adjacent sequences share similarities with other repetitive regions in proteins (Williamson, 1994). This segment of Mc PilQ is similar to a 30-residue linker in the bacterial dihydrolipoyl acetyltransferase component of 2-oxoacid dehydrogenase complexes that is proposed to act as a torsionally restricted but flexible hinge (Hannavy *et al.*, 1990). Like this domain and other proline-containing repeats, the SBR also contains an abundance of alanine residues. Mixed proline–alanine sequences/moieties appear to impart more mobility than either residue alone (Williamson, 1994). In this regard, it is important to note that none of the other secretins (including Pa PilQ) has repeat structures analogous or homologous to the neisserial SBR, although the XpsD and OutD proteins do carry a glycine/serine-rich domain in their N-termini (Chen *et al.*, 1996). As originally suggested by Genin and Boucher (1994), it may indeed be that these repetitive regions act as linkers that define or delineate two functional domains in these forms of secretins. Whatever potential functions are served by this domain of PilQ, alignment of all the neisserial sequences currently available suggest that the minimal structure consists of the β domain flanked on each side by at least one SBR.

In the simplest of interpretations, the variation in the Mc PilQ repeat region may be entirely random. Given its role in neisserial pilus biogenesis, changes in PilQ may represent a means of fine tuning assembly to accommodate variability in pilin and PilC expression. The surface structures of many microbial pathogens have repetitive peptide motifs but only in select instances have these domains been shown to undergo significant alterations in copy number. It is difficult to envision that significant changes in antigenicity ensue from the simple gain or loss of SBRs. However, recent findings on variation in tandem repeat number within the Alpha C protein of group B streptococci indicate that even although all polymorphic variants were highly immunogenic, the number of repeats was inversely proportionate to antigenicity (Gravekamp *et al.*, 1996). Analogous changes in tandem repeat structures in M proteins of group A streptococci have been demonstrated and in some instances, reduced reactivity with certain opsonic antibodies was noted (Jones *et al.*, 1988). The potential role of repeat polymorphism in alternating the antigenicity of PilQ clearly requires more knowledge of which parts of the molecule are surface exposed.

In an effort to ascribe particular function(s) to the repeat region of Mc PilQ, strains expressing chimeric PilQ molecules in which the number of repeats was increased from five to seven or in which the repeat domain was replaced in its entirety by the equivalent region of Pa PilQ were constructed. Although no clear influence of changing repeat number on pilus biology was evident, the replacement of the repeat region by the equivalent module from Pa PilQ reduced the level of piliation. Although it was not that surprising that the Pa hybrid substitution had no effect on the strain's transformability given that pili were expressed (albeit in reduced amount), it was rather remarkable that no discernible effect on epithelial cell adherence was seen. Although the basis for the quantitative defect in this background remains to be determined, the findings do indicate that the polymorphic repeat region does not play a critical role in the two pilus associated phenotypes examined. That functional pili were expressed at any level in this background is somewhat remarkable given that the N-terminal domains of the pIV and OutD molecules impart those secretins with exquisite substrate specificity. For example, OutD of *Erwinia carotovora* functions in export of its homologous pectinase but not the highly related molecule found in *E. chrysanthemi* (Shevchik *et al.*, 1997) and exchanging the N-terminal domains of pIV from f1 and IKe switches phage morphogenesis specificity (Daefler *et al.*, 1997b). PilQ as type IV pilus secretins may be more relaxed or promiscuous in their substrate specificity.

The methodology used here to construct the chimeric strains has the advantage that the altered alleles are expressed from their native promoter in a single copy on the genome. In addition, it is possible to express altered forms of PilQ while maintaining expression of the wild-type allele, making it feasible to assess if altered forms of PilQ exert negative dominance as might be predicted for molecules that function as a multimer. In light of the findings that the polymorphic repeat region is not essential for pilus biogenesis, we can now use this technique to assess which N-terminal alterations can be tolerated without affecting function and what role this region might play in providing proper spacing to functional domains of PilQ.

Based on its identities with Gc and Pa PilQ, the role of Mc PilQ in pilus expression was not surprising. The demonstration that Mc PilQ can contain within its N-terminal region variable numbers of SBRs none the less provides evidence for a form of gene/protein polymorphism that is unprecedented in neisserial outer membrane proteins. Furthermore, this is a unique finding as it is the first member of the secretins displaying such strain to strain variability. The potential significance of these findings to the biology of type IV pilus biogenesis and PilQ structure and function are the goals of on-going studies.

Experimental procedures

Bacterial strains, media and plasmids

Bacterial strains used are listed in Tables 2 and 4. The strains H44/76 (B:15:P1,7,16) and M1080 (B:1:P1.1,7) were selected for the most detailed analysis as they are most representative of the clone complex that has caused a series of outbreaks of serogroup B meningococcal disease worldwide. *N. meningitidis* strain 44/76 was isolated from a fatal case of

meningitis and septicaemia (Holten, 1979) and is the source for current meningococcal vaccine development in Norway (Bjune *et al*., 1991). *N. meningitidis* strains 44/76-A and M1080-A were constructed by introduction of the *recA6* allele, which places the RecA gene under the control of an IPTG-inducible promoter (Seifert, 1997). In addition, a total of 52 strains recovered from patients ($n = 50$) and healthy carriers ($n = 2$) from 15 countries in four continents were screened for the presence of the *pilQ* gene. The strains were genetically and serologically heterogeneous, including representatives of the five serogroups (A, B, C, W135 and Y) associated with disease. The 22 strains subjected to DNA sequence analysis are listed in Table 2. Mc strains were propagated on 5% blood agar plates or clear solid GC media at 36°C in 5% CO_2 or in liquid GC media that had been preincubated overnight in 5% CO_2. The colony morphology state of Mc strains was monitored using a Leica StereoZoom 7 dissecting microscope. Strain KW251 was used in the propagation of recombinant phage clones, whereas *E. coli* strains HB101 and DH5aF′IQ were used in plasmid cloning experiments. *E. coli* strains were grown at 37°C in Luria–Bertani (LB) medium supplemented with appropriate antibiotics: carbenicillin, 100 μg ml^{-1} (Sigma), chloramphenicol, 10 μg ml^{-1} (Sigma), erythromycin, 300 μg ml^{-1} (Sigma), kanamycin (50 μg ml^{-1}) or streptomycin (50–1000 μg ml^{-1}) and Xgal (40 μg ml^{-1}) when appropriate. The plasmids used in this study are listed in Table 3.

DNA preparation and manipulation

Mc genomic DNAs were prepared by standard methods (Sambrook *et al*., 1989). Isolation and purification of plasmid and phage DNAs were performed using Qiagen columns according to the manufacturer's specifications. For the phage library, Mc DNA partially digested with *Sau*3AI endonuclease was fractionated by electrophoresis and fragments 15–20 kb in size were recovered by electroelution. This DNA was ligated into *Bam*HI-digested EMBL-3 phage arms (Frischauf *et al*., 1983) and encapsidated in phage particles using a Gigapack II Plus packaging extract (Stratagene). The *Eag*I restriction fragments sized 4.1 kb from the cosmid clone that hybridized with probes corresponding to the Gc *pilQ* 5′ end was cloned into the low-copy-number vector pLG339, forming the plasmid pTT17, upon which the *Eag*I–*Bst*XI restriction fragment was subcloned into the high-copy-number vector pBSKII, forming pTT21. Other standard methods of DNA manipulation were performed as described (Sambrook *et al*., 1989). DNA sequencing of the complete *pilQ* genes of strains 44/76-A and M1080 were performed by the dideoxy chain termination method (Sanger *et al*., 1977). Polymerase chain reaction (PCR) products were achieved by using standard procedures (Perkin-Elmer Cetus).

PCR and sequence analysis of the polymorphic region of pilQ

Primers located in regions of the *pilQ* gene conserved in Mc and Gc (5′-CGA ATC GGA CGA TAC CGT GTC CGC CC-3′ and 5′-CGG CAA AGC CCA ATG CCG CCA ATT CG-3′) were used to generate an amplification product of the 5′ region of the gene. DNA sequencing of the PCR

Table 4. Plasmids and strains used in this study.

Plasmid	Relevant characteristic	Source
pHSS6	Cloning vector, kan^r	Seifert *et al*. (1991)
pLG339	Cloning vector, kan^r	Cunningham *et al*. (1993)
pBluescript II SK/KS	Cloning vector, amp^r	Stratagene
pTT5	*EagI* fragment containing $pilQ_{44/76}$ in pACYC184	This study
pTT17	*EagI* fragment containing $pilQ_{44/76}$ in pACYC184	This study
pCM21	mTn*cm*#21 insertion at base 238 of Gc *pilQ* (residue 1) in pHSS6	Drake *et al*. (1997)
pMQ22	mTn*erm*#22 insertion at base 1876 of $pilQ_{44/76}$ (residue 546) in pHSS6	This study
pMQ23	mTn*erm*#23 insertion at base 2119 of $pilQ_{44/76}$ (residue 627) in pHSS6	This study
pAD9	complete $pilQ_{MS11}$ gene (minus promoter) in pHSS6	This study
pDF-R1	*Bgl*II fragment containing $pilQ_{PAO1}$ in pBluescript II SK	Martin *et al*. (1993)
pΔQMcPa	*Eco*RV–*Cla*I fragment containing $pilQ_{44/76}$ with truncated, translational fusion to Pa PilQ (residues 77–179) in pHSS6	This study
pTT31	*Sty*I–*Cla*I fragment from pDF-R1 in pTT30, creates chimeric PilQ with residues 77–179 from *P. aeruginosa*	This study
pTT32	*Nco*I–*Cla*I fragment from Mc 44/76 *pilQ* in pTT30, creates chimeric PilQ with 7X copies of SBR with α and β domains	This study
Strain		
44/76-A	Mc 44/76 with *recA6* allele (IPTG inducible RecA)	Tønjum *et al*. (1995)
M1080-A	Mc M1080 with *recA6* allele (IPTG inducible RecA)	This study
MQ0	44/76-A with mTn*cm*#21 in *pilQ*	This study
TT0	M1080-A with mTn*cm*#21 in *pilQ*	This study
TT1	pΔQMcPa integrated into the genome of M1080-A	This study
TT31	TT1 with McPa *pilQ* chimeric gene (ex pTT31) 5′ of the integrated vector	This study
TT32	TT1 with 7× SBR *pilQ* chimeric gene (ex pTT32) 5′ of the integrated vector	This study
TT31-121	TT31 with mTn*cm*#21 in chimeric *pilQ* gene 5′ of the integrated vector	This study
TT31-221	TT31 with mTn*cm*#21 in wild type *pilQ* gene 3′ of the integrated vector	This study
TT32-121	TT32 with mTn*cm*#21 in chimeric *pilQ* gene 5′ of the integrated vector	This study
TT32-221	TT32 with mTn*cm*#21 in wild type *pilQ* gene 3′ of the integrated vector	This study

products was performed as previously described (Caugant *et al.*, 1995).

General protocols

Mc mutants failing to express functional PilQ were constructed by shuttle mutagenesis using the minitransposon mTn*erm* (a derivative of Tn*3*) as described (Seifert *et al.*, 1991). Defined mutations of the *Eag*I–*Bst*XI insert were constructed in *E. coli* and these mutations were returned to the genome of Mc strain 44/76 by transformation as described previously (Tønjum *et al.*, 1995). Three transposon mutants predicted to disrupt *pilQ*, one in the ATG start codon (Drake and Koomey, 1995) and two insertions at positions 1876 and 2119 in the gene, were selected for further study. These mutations were introduced into *N. meningitidis* 44/76-A by genetic transformation using linearized plasmid DNAs. For transformation, RecA expression and recombination functions were transiently induced in this background by IPTG addition to a final concentration of 200 $\mu g\ ml^{-1}$ at the same time at which transforming DNA was added. After ensuring the correct introduction of the mutations by Southern hybridization and that no concomitant changes in *pilE* (the pilin subunit gene) had occurred, pilus expression and associated phenotypes were assessed.

DNA and peptide sequence data were compiled and analysed by computer using the MACVECTOR version 3.5 and University of Wisconsin Genetics Computer Group (UWGCG) software packages (Devereux *et al.*, 1984). DNA homologies were found using the FASTA routine and protein homologies were identified using TFASTA. Pairwise alignments of proteins were performed using the GAP and BESTFIT programs with default parameters.

Transformation competence assay

Competence for natural transformation of Mc wild-type and *pilQ* mutant strains was assessed as detailed (Tønjum *et al.*, 1995) using chromosomal DNA from a spontaneous streptomycin-resistant mutant of Mc 44/76-A selected at 1000 $\mu g\ ml^{-1}$ streptomycin. RecA expression and recombination functions were transiently induced by IPTG addition to a final concentration of 200 μM at the same time as transforming DNA was added (see above). Mc transformants were selected on solid GC or 5% blood media containing 50 $\mu g\ ml^{-1}$ streptomycin.

Pilus purification

Pili were purified by the ammonium sulphate procedure previously described (Brinton, 1978). Cells from three to five heavily streaked Petri dishes were suspended in 10 ml of 0.15 M ethanolamine, pH 10.5, and vortexed for 60 s. Cellular debris was removed by centrifugation at 17 000 × *g* for 15 min. Pilus crystals were precipitated at room temperature for 30 min with one-tenth volume ammonium sulphate saturated 0.15 M ethanolamine and collected by centrifugation at 17 000 × *g* for 15 min. Pilus filaments were washed twice with 0.05 M Tris-buffered saline.

Epithelial cell adherence

The Mc adherence assay was performed using the human epithelial cell line ME-180 (ATCC) maintained in McCoy's 5A medium (Gibco-BRL) supplemented with 10% fetal bovine serum (FBS). Epithelial cells were seeded into a 24-well tissue culture plate at a density of 5×10^5 cells per well the day before infection. For assays, $2–5 \times 10^7$ bacteria in 1 ml of McCoy's 5A medium supplemented with 1% FBS (assay medium) were added to each well of monolayers and incubated at 37°C in 5% CO_2.

After 1 h, non-adherent bacteria were removed by washing five times with assay media, and the monolayers and cell-associated bacteria were recovered by treatment with 0.25% trypsin for 5 min at 37°C. The recovered bacteria were plated on agar after dilution and the number of cell-associated bacteria determined by the number of colony-forming units.

Immunoblotting

The presence of PilQ in whole-cell lysates was detected by immunoblotting using rabbit polyclonal antibodies raised against recombinant Mc PilQ. Detection of Mc pilin antigen in whole-cell lysates was performed using rabbit polyclonal pilin-specific antibodies raised against Gc pili (lot 2–66). Conditions for sample preparation, SDS–PAGE, electroblotting and antigen detection have been described previously (Drake and Koomey, 1995).

Isolation of outer membranes (OM)

OM was isolated from Mc cells disrupted in a French pressure cell (Newhall *et al.*, 1980). The lysate from three passes through a French pressure cell at 12 000 $lb\ in^{-2}$ was centrifuged at 10 000 × *g* to remove non-ruptured cells. The supernatant was centrifuged at 40 000 × *g* for 1 h at 4°C onto a 55% (w/w) sucrose cushion. The material banding on top of the cushion was resuspended to $\approx 4\ mg\ ml^{-1}$ in 10 mM HEPES (pH 7.4) containing 1% sarkosyl to remove the inner membranes. After incubation at RT for 30 min, the suspension was centrifuged for 1 h at 40 000 × *g*. The pellet was washed once in 10 mM HEPES (pH 7.4) containing 0.002 M PMSF and 0.02% NaN_3 by recentrifugation. The pellet of enriched OM was suspended in the same buffer.

Biotinylation of cell-surface proteins

The procedures used were essentially the same as those described previously (Kocks *et al.*, 1992). Bacterial cells were washed three times in ice-cold phosphate-buffered saline (PBS, pH 8.0) to remove any contaminating proteins. Cells were suspended at a concentration of 2.5×10^6 cells ml^{-1} in PBS (pH 8.0). Sulfo-NHS-LC-Biotin (Pierce Chemical) (0.5 mg per ml of reaction volume) was added and the suspension was incubated at room temperature for 5 min. The cells were washed three times with ice-cold PBS (pH 8.0) to remove any remaining biotinylating agent. Determination of biotin incorporation in the cells was then performed by SDS–PAGE and electroblotting, after which biotinylated proteins were detected by streptavidin conjugate that was labelled

with alkaline phosphatase (Pierce Chemical). The profile of the biotinylation pattern was then compared and correlated with Coomassie-stained OM and parallel immunoblotting of the biotinylated whole-cell samples.

Construction of hybrid PilQ molecules (PilQ chimeras)

Construction of Mc and Gc strains expressing PilQ polypeptides altered in the polymorphic repeat region (*pilQ* chimeras) was performed as follows (Fig. 5) (note that all strains constructed below carry the *recA6* allele, a background in which RecA function can be repressed until transiently induced via addition of IPTG). The plasmid pΔQMcPa (Fig. 5A) contains a truncated Gc *pilQ* gene with a translational fusion to residues 67–164 derived from the *P. aeruginosa* (Pa) PilQ protein. This construct was made using the *Sty*I–*Nco*I and *Cla*I restriction sites that flank the neisserial polymorphic repeat encoding region and the equivalent coding region from the Pa *pilQ* gene. The *Cla*I restriction site is conserved in the *pilQ* genes of all three species and the *Nco*I was introduced by PCR into the Pa *pilQ* fragment using the primer 5′-CGTGGCTATACCATCGAGCAGCCGG. Transformation using this plasmid DNA and selecting for kanamycin resistance gives rise to a strain containing one truncated, chimeric Mc/Pa hybrid *pilQ* gene and one complete Mc *pilQ* gene. The nonhomologous Pa segment was necessary to force crossing over 5′ of the repeat encoding segments in Fig. 5B. Strain McQPa was transformed (Fig. 5B) with linearized DNA from *pilQ* chimeras of Mc and Pa, and erythromycin resistance selected. A complete but chimeric *pilQ* gene is formed that contains the repeat encoding region from either the Mc (7× repeats) or Pa (no repeats) *pilQ* gene. Inactivation of the original complete Mc *pilQ* gene by recombination of a Tn*cm* insertion mutation in the start codon of the gene, leading to expression of only the upstream chimeric gene (Fig. 5C).

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