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Identification and characterization of *pilG*, a highly conserved pilus-assembly gene in pathogenic *Neisseria*

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Summary

Expression of type IV pili appears to be a requisite determinant of infectivity for the strict human pathogens *Neisseria gonorrhoeae* and *Neisseria meningitidis*. The assembly of these colonization factors is a complex process. This report describes a new pilus-assembly gene, *pilG*, that immediately precedes the gonococcal (Gc) *pilD* gene encoding the pre-pilin leader peptidase. The nucleotide sequence of this region revealed a single complete open reading frame whose derived polypeptide displayed significant identities to the pilus-assembly protein PilC of *Pseudomonas aeruginosa* and other polytopic integral cytoplasmic membrane constituents involved in protein export and competence. A unique polypeptide of M_r 38 kDa corresponding to the gene product was identified. A highly related gene and flanking sequences were cloned from a group B polysaccharide-producing strain of *N. meningitidis* (Mc). The results indicate that the *pilG* genes and genetic organization at these loci in Gc and Mc are extremely conserved. Hybridization studies strongly suggest that *pilG*-related genes exist in commensal *Neisseria* species and other species known to express type IV pili. Defined genetic lesions were created by using insertional and transposon mutagenesis and moved into the Gc and Mc chromosomes by allelic replacement. Chromosomal *pilG* insertion mutants were devoid of pili and displayed dramatically reduced competence for transformation. These findings could not be ascribed to pilin-gene alterations or to polarity exerted on *pilD* expression. The results indicated that PilG exerts its own independent role in neisserial pilus biogenesis.

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Introduction

Infections caused by *Neisseria gonorrhoeae* (Gc) and *Neisseria meningitidis* (Mc), pathogenic members of the genus *Neisseria*, are associated with significant morbidity and mortality for their exclusively human hosts. Despite the unique disease manifestations associated with these species, it appears that their basic strategies for successful colonization of man are highly conserved. Many studies have focused on understanding the structures and functions of surface-localized and extracellular neisserial components. The expression of pili, which are filamentous-like structures emanating from the bacterial surface, appears to be of paramount importance to the pathogenic process (Heckels, 1989). Gc and Mc pilus filaments are ordered arrays of polymerized protein subunits termed pilin. The short leader sequences and proximal 30 amino acids of their pre-pilins (Meyer *et al.*, 1984; Potts *et al.*, 1988) show a high degree of homology with pre-pilins of other Gram-negative human pathogens including *Pseudomonas aeruginosa* (Pasloske *et al.*, 1985), *Vibrio cholerae* (Shaw and Taylor, 1990) and certain strains of enteropathogenic *Escherichia coli* (Giron *et al.*, 1991). Collectively, members of this family of pilus colonization factors have been termed type IV pili. Evidence for critical roles of Gc and Mc pili can be found in the invariable recoveries of piliated organisms from primary cultures (Jyssum and Lie, 1965; Kellogg *et al.*, 1968; Swanson *et al.*, 1987) and the capacities of these structures to undergo antigenic variation (Tinsley and Heckels, 1986; Swanson *et al.*, 1987). Gc pilus variation results from homologous recombination between a single complete pilin gene or expression locus and multiple partial pilin gene copies or silent alleles (Haas and Meyer, 1986; Swanson *et al.*, 1986; Koomey *et al.*, 1987) and an analogous mechanism is thought to operate in Mc pilus variation (Aho and Cannon, 1988; Perry *et al.*, 1988; Blake *et al.*, 1989).

Neisserial pili appear to promote infectivity by mediating attachment to mucosal epithelia (Swanson, 1973; Stephens

and McGee, 1981) although their presence is also associated with other phenotypes which may be relevant to the host–parasite interaction. Pilus expression is correlated with high-level (sequence-specific) competence for DNA transformation with frequencies of recovered transformants being reduced 1000-fold in non-piliated mutants (Frøholm *et al.*, 1973; Seifert *et al.*, 1991; Zhang *et al.*, 1992). Bacterial autoagglutination (independent of that associated with Opa proteins) is a pilus-dependent phenomenon (Swanson *et al.*, 1971) and a form of bacterial surface translocation termed 'twitching motility' is only displayed by piliated organisms (Swanson, 1978). The basis for the associations between these phenotypes and piliation is not well understood.

Studies directed at understanding neisserial pilus biogenesis appear to be particularly well suited to addressing basic questions about organelle structure, function and related phenotypes. Until recently, relatively little was known about this aspect of Gc and Mc pili. Studies of spontaneously arising Gc pilin mutants, as well as those created by site-specific mutagenesis and allelic replacement, have led to the identification of specific amino acid residues and domains of pilin which are required for pilus biogenesis (Koomey *et al.*, 1991; Zhang *et al.*, 1992). A 110 kDa protein, PilC, that co-purifies with the Gc pilin subunit and its corresponding gene has been identified (Jonsson *et al.*, 1991). Gc mutants failing to express PilC were reported to have a significant reduction in piliation (Jonsson *et al.*, 1991) and analogous observations have been made in Mc (Nassif *et al.*, 1994).

The assembly of pili invariably involves the interaction of pilin and other accessory proteins which act to modify, transport and polymerize pilin into the extracellular structures (Hultgren and Normark, 1991). In the type IV pilus systems of *P. aeruginosa* and *V. cholerae*, the genes encoding these ancillary components are linked to the gene encoding the pilus subunit (Nunn *et al.*, 1990; Kaufmann *et al.*, 1993) but this gene clustering does not exist in Gc and Mc. Evidence derived from many prokaryotic systems has revealed that components required for the translocation of proteins to the cell surface are structurally and functionally related to proteins necessary for type IV pilus biogenesis (Whitchurch *et al.*, 1991; Pugsley 1993; Hobbs and Mattick, 1993). At least three distinct families of genes and corresponding proteins which share significant sequence identity across diverse species boundaries have been proposed to be part of the conserved apparatus of extracellular protein localization. Proteins corresponding to one or more of these families have been implicated in the extracellular localization of toxins and hydrolases by many pathogenic Gram-negative bacteria including *V. cholerae* (Sandkvist *et al.*, 1993), *Bordetella pertussis* (Weiss *et al.*, 1993), *Aeromonas hydrophila* (Jiang and Howard, 1992), and *P. aeruginosa* (Bally *et al.*, 1991b; Strom *et al.*, 1991). Related proteins are also involved in competence for DNA transformation in *Bacillus subtilis* (Albano *et al.*, 1989).

We have previously identified the Gc *pilF* and *pilD* genes which encode products displaying significant identities with the NTP-binding domain containing homologues and the family of pre-pilin leader peptidases (Lauer *et al.*, 1993). The Gc gene products are most closely related to the type IV pilus-assembly proteins PilB and PilD of *P. aeruginosa*, respectively (Nunn *et al.*, 1990; Bally *et al.*, 1991a). In this report, we describe the identification of the Gc and Mc *pilG* genes which map immediately upstream of their pre-pilin peptidase-encoding *pilD* genes. Their products are highly related to the pilus-assembly protein PilC of *P. aeruginosa* and other polytopic integral cytoplasmic membrane constituents involved in the formation of surface-localized protein complexes. Gc and Mc *pilG* mutants were constructed by allelic replacement and found to be absolutely deficient in the assembly of pili. In addition, DNA hybridization studies strongly suggest that *pilG*-like genes are present in related bacterial species.

Results

Cloning and characterization of the DNA region upstream of pilD *in* N. gonorrhoeae

The Gc *pilD* gene encodes a pre-pilin leader peptidase which has been demonstrated to be essential for pilus biogenesis (Freitag *et al.*, 1995). In order to detect other genes potentially involved in pilus biogenesis, the region of plasmid clones (Fig. 1) containing sequences upstream of *pilD* was further analysed by subcloning and DNA sequencing. An open reading frame (ORF) was identified upstream of, and in the same orientation as, *pilD* (Fig. 2). The DNA sequence of this locus appears in the EMBL/Genbank/DDBJ Nucleotide Sequence Data Libraries under the accession number U19579 (data not shown). This ORF had an in-frame ATG start codon at nucleotide position 219. Its derived polypeptide beginning at that site would consist of 393 amino acids, having a molecular mass of 45.3 kDa and a pI of 9.4. The deduced polypeptide did not appear to possess a typical secretion signal sequence but was mainly hydrophobic in character with four potential membrane-spanning helices (Rao and Argos, 1986). Sequences immediately downstream of the predicted translation termination site demonstrated a strong potential to form a stable stem and loop RNA structure (ΔG (25°C) = −21.3 kcal mol^{-1} using the FOLD program of the GCG software package). Its presence in RNA might act as a signal for transcription termination/attenuation or nucleolytic processing (Jaeger *et al.*, 1989). The intergenic region between this ORF and the *pilD* ORF encompassed

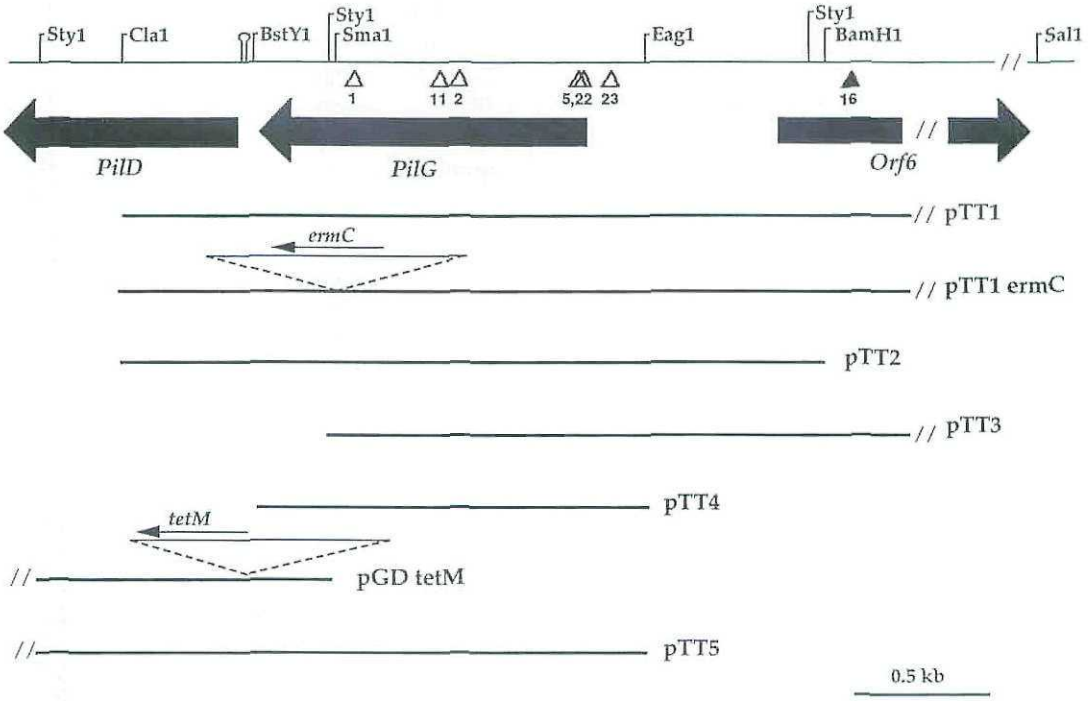

Fig. 1. Physical map of the *pilG* locus in pathogenic *Neisseria* and plasmid constructs derived from these regions. The nucleotide sequences of the *pilG* genes of *N. gonorrhoeae* N400 and *N. meningitidis* 44/76-A appear in the EMBL/GenBank/DDBJ Nucleotide Sequence Data Libraries under the accession numbers U19579 and U19580, respectively . Seven mTnErm insertions generated in the plasmid pTT3 in *E. coli* and recombined into the Gc and Mc genomes are shown. Precise sites of *pilG*::mTnErm insertions within this nucleotide sequence are GG23, position 88; GG5, position 215; GG22, position 219; GG2, position 697; GG11, position 787; and GG1, position 1099. Symbols: open triangles, locations of insertions resulting in loss of piliation; filled triangle, site of insertion which does not alter pilus phenotype. Details of plasmid constructs are described in Table 4 and in the text. Note that the inserts containing the *ermC* and *tetM* genes are not drawn to scale. The region of dyad symmetry downstream of *pilG* is depicted by a stem and loop structure.

73 nucleotides. Based on its linkage with *pilD*, the findings cited below and the nomenclature established in our other studies, we have designated the corresponding gene *pilG*.

The derived polypeptide of the pilG *ORF is a homologue of components required for type IV pilus assembly and protein export*

In a computer-assisted database search, the deduced PilG polypeptide exhibited significant sequence similarity to these proteins in the following order of decreasing identity: *P. aeruginosa* PilC (Nunn *et al.*, 1990), *P. aeruginosa* XcpS (Bally *et al.*, 1991b), *Xanthomonas campestris* XpsF (Dums *et al.*, 1991), *Erwinia chrysanthemi* OutF (He *et al.*, 1991), *A. hydrophila* ExeF (Jiang and Howard, 1992), and *Klebsiella oxytoca* PulF (Pugsley and Reyss, 1990) (Table 1). It also displayed regions of similarity to *B. subtilis* Com-G2 (Albano *et al.*, 1989) and *V. cholerae* TcpE (Kaufmann *et al.*, 1993), but to a lesser extent than the others cited above. The regions of similarity shared by these proteins were equally distributed over their entire lengths (Fig. 3). These proteins are components involved in pilus assembly or export of other proteins (Hobbs and Mattick, 1993; Pugsley, 1993). All members of the protein family display an overall hydrophobic character and are predicted to be located in the cytoplasmic membrane (Nunn *et al.*, 1990; Bally *et al.*, 1991b).

Identification of an expressed polypeptide corresponding to Gc PilG

We next sought to identify a polypeptide expressed by

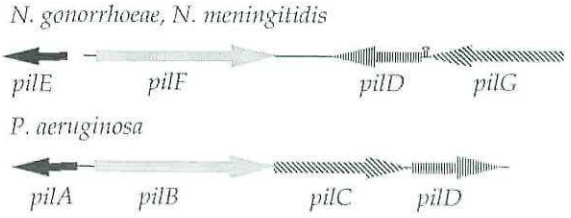

Fig. 2. Relative genetic organization of the *pilG* assembly gene clusters of *N. gonorrhoeae* N400 and *N. meningitidis* 44/76-A, in comparison with the pilus-assembly gene cluster of *P. aeruginosa*. Note that *pilE*, the pilin subunit-encoding gene of Gc and Mc, is not linked to this region as it is in *P. aeruginosa*.

Table 1. Overall sequence similarity between Gc PilG and other members of the PilC/PulF family of proteins.

Homologue	% identity	% similarity
PilC	45.6	69.2
XcpS	31.2	56.9
XpsF	30.6	56.7
OutF	30.3	57.3
ExeF	28.7	58.7
PulF	28.6	56.4

Scores were obtained using the BESTFIT program of the GCG sequence analysis package from the University of Wisconsin.

cloned DNAs that corresponded to PilG. This was done using *in vitro* transcription–translation analysis of plasmid DNAs. By comparison with the pattern found using the cloning vector pACYC184, pTT1 (Fig. 1) primed reactions yielded major unique polypeptides with molecular masses of 55 kDa and 38 kDa (Fig. 4, lanes 1 and 2). Further comparison with the pattern found using $pTT1_{fs\,XmaI}$ which carries a specific frameshift mutation in *pilG* confirmed that the 38 kDa species corresponded to PilG (Fig. 4, lane 3). In addition, the unique polypeptide of molecular mass 24 kDa detected for $pTT2_{fs\,XmaI}$ correlated well with the size predicted for truncated PilG resulting from premature translational termination. Analogous results were found using pTT4 DNA and other plasmids as templates (data not shown). It should be noted that the *P. aeruginosa* PilC protein, which is most closely related to PilG, has a predicted molecular mass of 44.5 kDa but migrates with an apparent molecular mass of 37 kDa (Nunn *et al.*, 1990).

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           1                                                  50                                                 100
PilG       MAKNGGFSLFAKKEKRFIFEGRHSASDKLVNGEVSAFTEEEARKKLAKRGIRPLQIT          RVKTS   SKRKITQEDITVFTRQLSTMIKAGLPL
PilC       MADK       ALKTSVFIWEGTDKKGAK VKGELTGQNPMLVKAHLRKQGINPLKVR          KKGISLLGAGKKVKPMDIALFTRQMATMMGAGVPL
XcpS                     MAAFEY LALDPSGRQQKGVLEADSARQVRQLLRERQLAPLDVKPTRTREQSGQGGRLTFAR GLSARDLALVTRQLATLVQAALPI
ExeF                                                VTEGDSARQVRQQLREQGLTPLEVNETTEKAK REANRFVLFRRGASTSELALITRQLATLVGAGLTI
PulF                     MALFRY QALDEQGKPRRGVQQADSARHARQLLREKGWLALDIDPAAGGGRP       SRFMRRTSARDLALVTRQLATLVAAAIPL
XpsF                                          MLDGQMEAANDAEVALRLQEQGHLPVETRLATG ENGSPSLRMLLRKKPFDNAALVQFTQQLATLIGAGQPL
OutF                     MALFQY QALNAQGKKSQGMQEADSARHARQLLREKGLVPVKIEEQRGEAAPRSGFSLSFGRSHRIASDLALLTRQLATLV AALPL
Consensus                   F Y        K   G  EADSAR VRQ LRE G  PL                L          DLALFTRQLATLV AGLPL

           101                                                150                                                200
PilG       MQAFEIVARGHGNPSMTEMLMEIRGQVEQGSSLSRAFSNHPKYFDRFYCNLVAAGETGGVLESLLDKLAIYKEKTQAIRKKVKTALTYPVSVIAVAIGLV
PilC       LQSFDIIGEGFDNPNMRKLVDEIKQEVSSGNSLANSLRKKPQYFDELYCNLVDAGEQSGALENLLDRVATYKEKTESLKAKIKKAMTYPIAVIIVALIVS
XcpS       EEALRAAAAQSTSQRIQSMLLAVRAKVLEGHSLAGSLREFPTAFPELYRATVAAGEHAGHLGPVLEQLADYTEQRQQSRQKIQLALLYPVILMVASLAIV
ExeF       EEALRAVAEQCEKAHLRSLVATVRSKVVEGYSLADSLGAFPHVFDQLFRSMVAAGEKSGHLEKVLNRLADYTEQRQHMRTKLLQAMIYPIVLTLVAVGVI
PulF       EKALDAVAQQSEKPQLKTLIAGVRGKVLEGHSLAEAMRGHPGCFDALYCAMVAAGEASGH                      RLLQAMIYPIVLTLVAVSVI
XpsF       DRALSILMDLPEDDKSRRVIADIRDTV.RAAPLSVALERQHGLFSKLYINMVRAGEAGGSMQDTLQRLADYLERSRALKGKVINALIYPAILLAVVGCAL
OutF       EEALDAVAKQSEKPKLSALMAAVRAKVVEGHSLAEAMGNFPGSFERLYCAMVAAGEASGHLDAVLNRLADYTEQRHEMRSRIQQAMIYPCVLTLVAISVV
Consensus  E ALDAVA Q E P L  L A VR KV EG SLA AL   P  FD LYC MVAAGE SGHLE  L RLADY E     R KI  AMIYPIVL  VA  VV

           201                                                250                                                300
PilG       FVMMIFVLPAFKEVYANMGAELPPLTQTVMDMSDFFVSYGWMVLIALGFAIYGFLKLKARSIKIQRRMDAILLRMPIFGDIVRKGTIARWGRTTATLFAA
PilC       AILLIKVVPQFQSVFEGFGAELPAFTQMIVNLSEFMQEWWFFIILAIAIFGFAFKELHKRSQKFRDTLDRTILKLPIFGGIVYKSAVARYARTLSTTFAA
XcpS       GFLLGYVVPDVVRVFIDSGQTLPLLTRVLIGVSDWVKAWGALAFVAAIGGVIGF RYALRKDAFRERWHGFLLRVPLVGRLVRSTDTARFASTLAILTRS
ExeF       SILLTAVVPKVVAQFEHMGQQLPATTRFLIGTSELMQHYGLWFLLLLFIGGFVW RWWLTDEKRRRHWHQVVLRLPVIGRVSRGLNTARFARTLSILNAS
PulF       VILLSTVVPKVVEQFIHLKQALPFSTRLLMAMSDMLRAAGPWLLLAILLLILLL RYLLRQPAKRLAWHRLLLRLPLIGRVARSVNSARYARTLSILNAS
XpsF       LFLLGYVVPQFAQMYESLDVALPWFTQAVLSVGLLVRDWWLVLVVIPGVLGL WLDRKRRNAAFRAALDAWLLRQKVIGSLIARLETARLTRTLGTLLRN
OutF       SILLSAVVPKVVEQFIHMKQALPLSTRLLMSASDAVRTYGPWVVLLLVLAIMGF RVLLRQEKHRLVFHRRLLFLPVVGRVARGLNTARYARTLSILNSS
Consensus   ILL  VVP VV  F  MGQ LPL TRLLM  SD M   GLW LLA    I  F RY LR  K R  WH  LLRLPVIGRVVR   TARYARTLSIL AS

           301                                                350                                                400
PilG       GVPLVDVLDSTAGAAGNLIYEEATREIRTRVIQGLSMTSGMRATELFPNMMLQMSSIGEESGSLDDMLNKAAEFYEDEVDNAVGRLSAMMEPIIIVILGL
PilC       GVPLVDALDSVSGATGNIVFKNAVSKIKQDVSTGMQLNFSMRTTSVFPNMAIQMTAIGEESGSLDEMLSKVASYYEEEVDNAVDNLTTLMEPMIMAVLGV
XcpS       GVPLVEALAIAAEVIANRIIRNEVVKAAQKVREGASLTRSLEATGQFPPMMLHMIASGERSGELDQMLARTARNQENDLAAQIGLMVGLFEPFMLIFMGA
ExeF       AVPLLEGMKIAGEVLSNDPARTRIGEATERVREGTSLRKALDETKIFPPMMLHMIASGEQSGELDSMLERAADNQDREFETQVNIALGVFEPLLVVSMAG
PulF       AVPLLLAMRISADVLSNAWAKRQLEAASDAVREGVSLHRALEMTQLFPPMMRYMVASGERSGELNSMLERAADNQDRDLSAQIQLALSLFEPLLVVAMAG
XpsF       GVPLLAAIGIARNVMSNTALVEDVAAAADDVKNGHGLSMSLARGKRFPRLALQMIQVGEESAALDTMLLKTADTFELETAQAIDRALAALVPLITLVLAS
OutF       AVPLLQAMRISGDVLTNDYARFRLGQATDAVREGVTLHKALEQTALFPPMMRHMIASERRRARRH  VNPRGDNQDREFSAQMTLVLGLFEPLLVVSMAG
Consensus  GVPLL A  I   VL N      V  A D VREG SL   LE T  FPPMML MIASGE SG LD ML   ADNQE E   Q   L LFEPLLVV MA

           401              423
PilG       VIGTLLVAMYLPLFNLGNVVA*
PilC       LVGGLIVAMYLPIFQLGNVVG*
XcpS       VVLVIVLAILLPILSLNQLVG*
ExeF       VVLFIVMSILQPILELNNMVNL*
PulF       MVLFIVLAILQPILQLNTLMSM*
XpsF       VVGLVIISVLVPLYDLTNAIG*
OutF       IVLPIVLAILQPILQLNTLMSM*
Consensus  VVLFIVLAIL PIL LNNLV
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Fig. 3 . Comparative alignment of the deduced amino acid sequences of PilG and homologues was performed by using the GAP Program of the UWGCG package. A consensus sequence based on sites in which a majority of sequences display identical residues is shown beneath. Underlined residues represent those regions with a strong potential to form membrane-spanning helices according to a specified algorithm (Rao and Argos, 1986). The precise sequence identities and similarities are shown in Table 2.

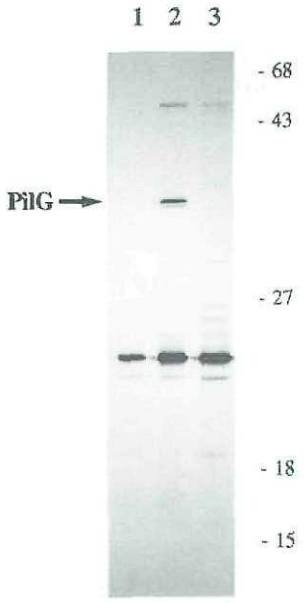

Fig. 4. *In vitro* coupled transcription/translation analysis of pACYC 184 (lane 1), pTT1 (lane 2) and pTT1$_{fsXmaI}$ (lane 3) as analysed by SDS–PAGE. The relative mobilities of protein molecular mass standards are shown in kDa on the left. Proteins were labelled by the incorporation of [^{35}S]-methionine using an S30 extract. The *pilG* gene product is indicated by an arrow.

Characterization of the meningococcal pilG *gene and linked gene organization*

Southern hybridization analysis using Gc *pilG* as a probe against *N. meningitidis* genomic DNA gave a strong signal under stringent conditions, suggesting a high homology between Gc *pilG* and its counterpart in *N. meningitidis* (data not shown). To further assess this possibility, the homologous locus from Mc strain 44/76-A was cloned from a genomic phage library and subcloned into plasmid vectors. The DNA sequence of this locus appears in the EMBL/Genbank/DDBJ Nucleotide Sequence Data Libraries under the accession number U19580. Thirteen nucleotide differences were found between Gc and Mc sequences over this whole region and of these, only six result in PilG amino acid substitutions. Further characterization of the Mc locus in pTT5 (Fig. 1) by restriction mapping and partial DNA sequencing (data not shown) has revealed that the relative organization of the *pilD*, *pilF* and *pilG* genes is also highly conserved between Gc and Mc (Fig. 2).

Characterization of gonococcal and meningococcal pilG *transposon and insertion mutants*

To assess directly the potential role of *pilG* in pilus biogenesis, the plasmid pTT3 was subjected to transposon mutagenesis. These insertions were then moved into the chromosome of Gc strain N400, a background in which RecA expression and recombination functions are under the control of an inducible promoter. After ensuring the correct introduction of the mutations by Southern hybridization analysis (data not shown) and that no changes in *pilE* sequence (the pilin subunit gene) had been incurred during the process, pilus expression and associated phenotypes were assessed. Mutants carrying insertions in and immediately upstream of *pilG* displayed non-piliated colony morphotypes and were devoid of pili when examined by transmission electron microscopy (TEM) (Fig. 5). Identical results were found using a derivative which carried an insertion of an *ermC* gene cassette at the unique *Sma*I site in *pilG*. These results were in direct contrast to mutants carrying insertions further upstream from *pilG* which were unperturbed in pilus expression and its associated pattern of growth. This class of mutants was exemplified by strain GG16, whose site of insertion maps within a large ORF (designated here as ORF6) that is oriented divergently from *pilG*. Expression of this ORF accounts for the 55 kDa polypeptide seen in the *in vitro* transcription–translation analysis and its derived polypeptide shows significant identity with the phosphoglucoseisomerase of *E. coli* (T. Tønjum, unpublished).

Immunoblotting of whole-cell lysates of the isogenic Gc 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 the *pilG* mutants (Fig. 6). In N400, pilin was detected as a doublet of approximately 18 kDa, and the faster migrating species seen here co-migrated with pilin present in pili purified by repeated cycles of solubilization and isoelectric precipitation (data not shown; Koomey *et al.*, 1991). When compared with the pattern found using a mutant that expresses pilin incapable of being processed by Gc PilD (the pre-pilin leader peptidase), it is clear that both bands of the doublet seen in N400 appear to have been N-terminally processed. In all the *pilG* mutants, however, only the slower migrating species of the doublet seen in N400 was found. Both doublet species present in N400 and the slower migrating species seen in *pilG* mutants reacted with *P. aeruginosa* PAK pilus-specific antiserum, which has been demonstrated to recognize only pilins processed by pre-pilin leader peptidase (Koomey *et al.*, 1991; data not shown). In addition, pilin antigen migrating at a position characteristic of S-pilin (soluble, truncated pilin) was seen for each mutant (Fig. 6). Immunoblotting of concentrated culture supernatants confirmed that all the *pilG* mutants elaborated S-pilin. The presence of S-pilin has previously been correlated with defects in pilus assembly arising from pilin gene alterations (Haas *et al.*, 1987; Koomey *et al.*, 1991) and *pilC* mutations (Jonsson *et al.*, 1992).

Many studies have shown that non-piliated Gc mutants

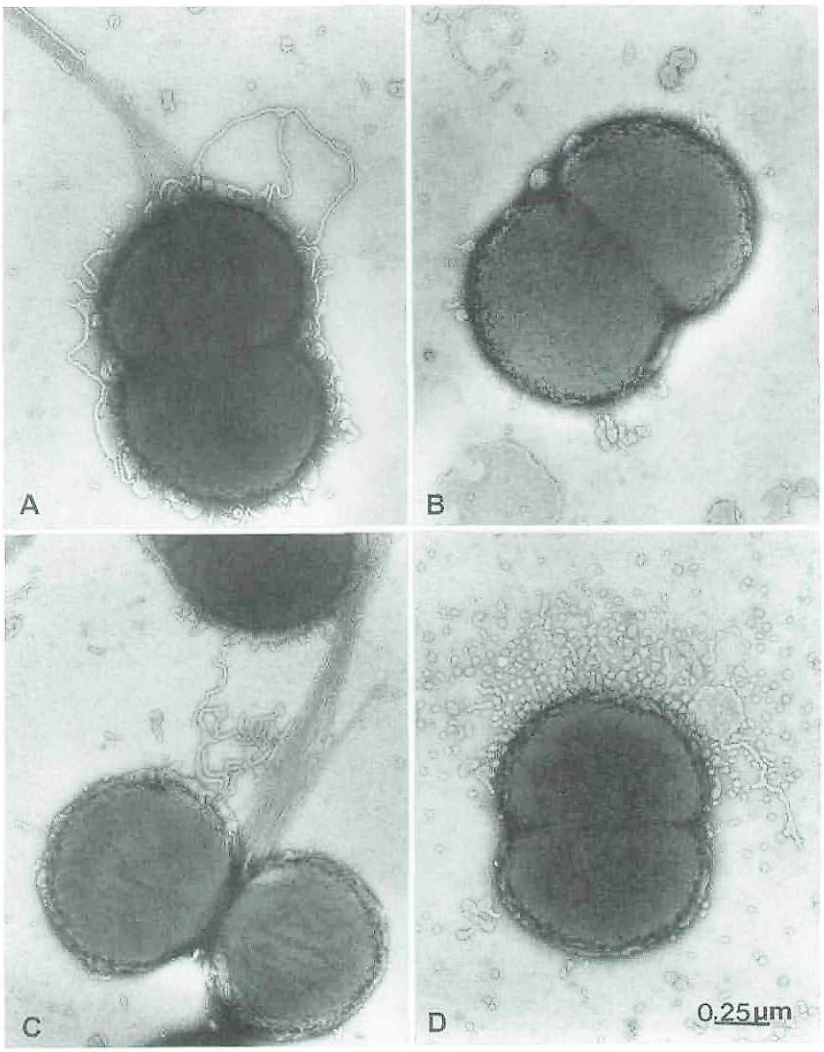

Fig. 5. Transmission electron micrographs of *N. gonorrhoeae* strains N400 (A) and GG2 (B) and *N. meningitidis* strains 44/76-A (C) and MG1 (D). Other mutant *pilG* derivatives gave results identical to that seen with GG2. The specimens were negatively stained with 0.25% (Gc) and 0.5% (Mc) phosphotungstic acid. Magnification 29 250×.

are dramatically reduced in competence for natural transformation and, thus, pilus expression appears to be necessary for this property. However, antigenic variants that express extremely low levels of pili are transformable at frequencies equivalent to that seen for extensively piliated strains (Gibbs *et al.*, 1989). Given that competence for transformation is an extremely sensitive marker for pilus expression, we examined the relative competence of N400 and its isogenic derivatives. All mutants shown to lack pili by TEM had over a 1000-fold reduction in their transformation competence (Table 2). These levels of competence are equivalent to that found for non-piliated mutants resulting from *pilE* lesions (Seifert *et al.*, 1991; Zhang *et al.*, 1992).

The same transposon and insertion mutants were moved onto the chromosome of Mc strain 44/76-A. As anticipated from the results in Gc, the Mc *pilG* mutants were non-piliated (Fig. 6) and expressed dramatically reduced competence for transformation (data not shown). Although the pilin gene status was not specifically examined in this background (because this strain is wild type with regard to homologous recombination functions), it is important to note that all primary transformants were

Table 2. Phenotypic characteristics of *Neisseria gonorrhoeae* N400 and *pilG*::mTnErm mutants.

Strain	Pili	Aggregation	S-pilin expression	Transformation frequencies
N400	+	+	−	3.4×10^{-3}
N400, no DNA	NA	NA	NA	$<10^{-8}$
GG1	−	−	+	10^{-6}–10^{-7}*
GG2	−	−	+	10^{-6}–10^{-7}*
GG5	−	−	+	10^{-6}–10^{-7}*
GG11	−	−	+	10^{-6}–10^{-7}*
GG22	−	−	+	10^{-6}–10^{-7}*
GGermC	−	−	+	10^{-6}–10^{-7}*
GG16	+	+	−	4.7×10^{-3}

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

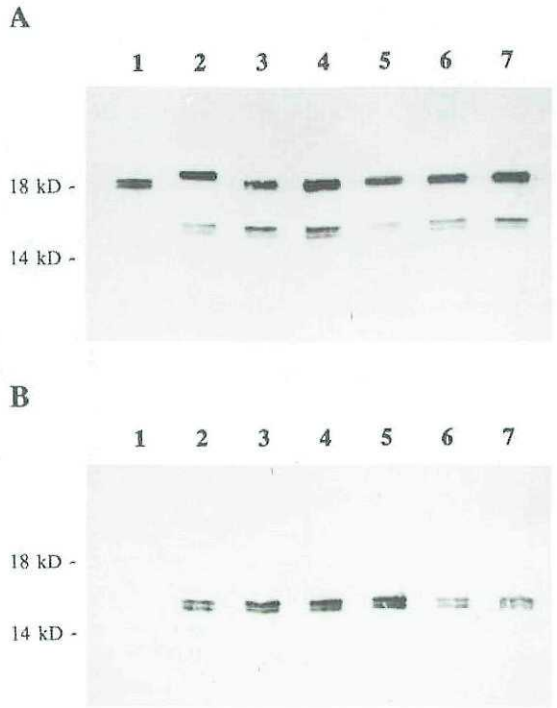

Fig. 6. Immunoblotting of whole-cell lysates (A) and concentrated culture supernatants (B) of wild-type and mutant gonococcal strains. The filter was reacted with rabbit antiserum raised against gonococcal pili at a dilution of 1:2000. The positions of 14 and 18 kDa protein molecular mass standards are shown on the left. Lanes 1, N400; 2, VD302 $P_{gly\text{-}1 \rightarrow ser\text{-}1}$; 3, GG5; 4, GG22; 5, GG2; 6, GG11; 7, GGermC.

non-piliated and that these mutants failed to give rise to piliated revertants at detectable levels. Reversion might be expected to occur readily if their non-piliated status resulted from pilin variation generated by recombination at *pilE*.

Direct assessment of pilG *function in non-polar mutant strains of gonococci*

The organization of the *pilG–pilD* genes suggested that they may be part of an operon or that a promoter for *pilD* may map within *pilG*. This situation raised the possibility that insertions in *pilG* might exhibit polarity on *pilD*. This did not appear to be the case as pilin in the mutants appeared to be completely processed. None the less, it seemed remotely possible that a reduced level of *pilD* expression might account for non-piliation if the kinetics of the polymerization were disrupted. In such a scenario, the rate of cleavage might be reduced in a way that precluded assembly, although sufficient PilD was present to cleave all pilin detected as a steady-state population. Therefore, we constructed a strain in which *pilD* expression was placed under the control of an exogenous promoter such that potential polarity from the *pilG* mutations was alleviated. This was done by placing an 8 kb restriction fragment that contained the *tetM* gene into a unique intergenic *Bst*YI site (pGDtetM, Fig. 1) and then moving this construct into the genome of strain VD300 by transformation to yield strain GDtetM. This strain was piliated and expressed a colony morphology phenotype identical to its progenitor, VD300. Moreover, pilin detected in this background by immunoblotting yielded a pattern indistinguishable from that seen for N400 (Fig. 7, lanes 1 and 3) with no antigen detected corresponding to unprocessed pilin (Fig. 7, lane 6). This indicated that sufficient PilD was expressed by this strain to promote pilus assembly in an otherwise wild-type background.

The *pilG* transposon mutations were then moved into the genome of GDtetM by transformation. After ensuring the correct introduction of the mutations and retention of the *tetM* construct by Southern hybridization analysis, pilus expression was assessed by TEM. With the single exception of transformants carrying mTnErm-16 (Fig. 1), all GDtetM transformants were non-piliated (data not shown). As stated previously for the Mc mutants, it is important to note that all primary mTnErm transformants (excluding those derived from mTnErm-16) were non-piliated and that these mutants failed to give rise to piliated revertants at detectable levels. When pilin expression was examined by immunoblotting in the non-piliated transformants, patterns identical to those previously seen in N400-derived mutants were found (Fig. 7, lanes 2, 4, and 5); i.e. only the slower migrating species of the doublet seen in GDtetM was found along with a form corresponding to S-pilin. Given the concordance between the data obtained here and that found for N400 derivatives, it is reasonable to surmise that the loss of pilus expression in *pilG* mutants cannot be ascribed to polarity on *pilD* expression.

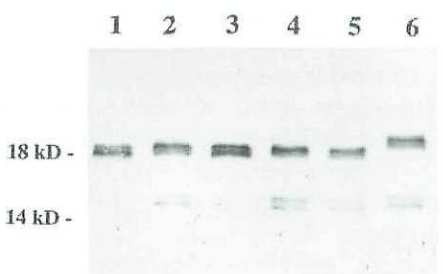

Fig. 7. Immunoblotting of whole-cell lysates of wild-type and mutant gonococcal strains. The filter was reacted with polyclonal rabbit antiserum raised against purified gonococcal pili at a dilution of 1:2000. The position of 14 and 18 kDa protein size standards are shown on the left. Lanes 1, N400; 2, GG2; 3, GDtetM; 4, GDtetM with mTnErm-2 mutation; 5, GDtetM with mTnErm-1 mutation; 6, GDΔ180 (N400 carrying a deletion mutation in *pilD*; Freitag *et al.*, 1995).

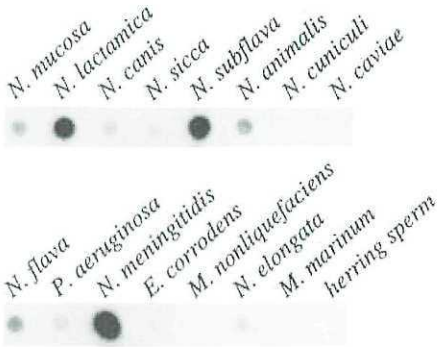

Fig. 8. Dot-blot hybridization of genomic DNAs from a variety of commensal *Neisseria* species using a radioactively labelled *pilG*-specific PCR probe. Lanes 1, *Neisseria mucosa* ATCC 25996; 2, *Neisseria lactamica* ATCC 23970; 3, *Neisseria canis* ATCC 14687; 4, *Neisseria sicca* ATCC 29193; 5, *Neisseria subflava* ATCC 10555; 6, *Neisseria animalis* CPH8; 7, *Neisseria cuniculi* ATCC 14688; 8, *Neisseria caviae* ATCC 14659; 9, *Neisseria flava* ATCC 14221; 10, *P. aeruginosa* PAK; 11, *N. meningitidis* 44/76-A; 12, *E. corrodens* 31745/80; 13, *M. nonliquefaciens* NCTC 7784; 14, *Neisseria elongata* 6171; 15, *M. marinum* TMC 1218; 14, herring sperm.

Presence of pilG *gene homologues in commensal neisseriae and other species*

DNAs from a variety of commensal *Neisseria* and other species known to express type IV pili or type IV pilus-associated phenotypes were screened by dot-blot hybridization using a Mc *pilG*-specific polymerase chain reaction (PCR) probe (Fig. 8). The probe hybridized strongly to DNAs of most of the commensal *Neisseria* analysed, while a weaker hybridization signal was detected with DNAs of *P. aeruginosa* and *Eikenella corrodens* (a member of the family Neisseriaceae). The *P. aeruginosa* DNA signal serves as an internal standard as the precise nucleic acid homologies between the probe and the *pilC* and *xcpS* genes are known to be 54% and 45%, respectively (Nunn *et al.*, 1990; Bally *et al.*, 1991a, b). Less homology was detected with DNAs of *Neisseria cuniculi*, *Neisseria caviae* and *Moraxella nonliquefaciens* (members of the family Moraxellaceae). Southern blotting indicated that the hybridization signals seen are restricted to distinct DNA fragments and therefore do not represent spurious or random reactivities (data not shown). No hybridization signals were detected with control DNAs from *Mycobacterium marinum* and herring sperm.

Discussion

It has been demonstrated previously that *N. gonorrhoeae* possesses genes which encode proteins with substantial similarities to those in other species responsible for type IV pili assembly and a specialised pathway of protein export (Lauer *et al.*, 1993). This work represents an extension of that study and describes the identification and characterization of the *pilG* genes of both *N. gonorrhoeae* and *N. meningitidis*. The Gc gene was identified by virtue of its presence upstream of *pilD* and the Mc gene was identified and cloned by virtue of homology to the Gc gene. We have shown that Gc and Mc mutants bearing defined lesions in *pilG* are completely devoid of pili. Efforts have been made to ensure that these results cannot be explained by pilin gene alterations or polarity exerted on downstream gene (i.e. *pilD*) expression. As such, these studies demonstrate an essential role for PilG in Gc pilus biogenesis. Although the experiments in Mc were not subject to the same degree of rigour, analogous results in this background can be most easily explained by a similar interpretation.

Prior to this work, only two genes have been directly implicated in Gc and Mc pilus assembly: *pilE*, encoding the pilin subunit and *pilC*, encoding large (>110 kDa) proteins which appear to be associated with the outer membrane. While an essential role for pilin in assembly is obvious, the situation for PilC is less clear. The original description of spontaneous *pilC* mutants in Gc reported that they were not bald but, in fact, expressed low levels of pili (Jonsson *et al.*, 1991). Earlier studies using the very same recombination-deficient strains noted that the most frequent class of variants expressed reduced levels of pili (Koomey *et al.*, 1991) and these have been subsequently demonstrated to be *pilC* mutants (H. Seifert, personal communication).

A consistent theme of these studies is the striking similarities between the Gc and Mc pilus-assembly components and those identified in *P. aeruginosa* (Nunn *et al.*, 1990) and *Pseudomonas putida* (deGroot *et al.*, 1994). The results with *pilG* are complementary to and in agreement with those found for *pilC* in *P. aeruginosa* (Koga *et al.*, 1993). In each case, the lack of pilus expression cannot be accounted for by polarity of upstream mutations on *pilD* expression and thus members of this protein family play their own distinctive and essential role in pilus assembly. Although their precise role(s) remains enigmatic, their predicted or demonstrated localization in the cytoplasmic membrane and linkage of their genes to those encoding pre-pilin leader peptidases suggest that they function in the initial stages of pilus assembly.

One distinctive feature of the organization of the Gc and Mc *pilG* and *pilD* genes is the presence of 73 bp intergenic regions, whereas in *P. aeruginosa* and *P. putida*, the corresponding intergenic spacings are 3 and 2 bp in size, respectively (Nunn *et al.*, 1990; deGroot *et al.*, 1994). Dupuy and Pugsley (1994) noted the presence of four potential promoters for Gc *pilD* which, based on the data presented here, would all map within the *pilG* open reading frame. None of these putative promoters would be disrupted in the mutants examined here. Koga and colleagues (1993) raised the formal possibility that *pilC* (the homologue

of Gc *pilG*) and *pilD* are co-transcribed from the *pilC* promoter in *P. aeruginosa*. Given the extremely low levels of PilD needed for pilin processing in Gc (N. Freitag and M. Koomey, unpublished), the absence of any readily identifiable promoters 3′ of the *pilG* termination codon and the presence of potential transcription attenuating/RNA processing signals 3′ of *pilG*, it is conceivable that a similar operon structure exists for the Gc and Mc *pilG–pilD* genes. If this is true, then *pilD* expression in the mTnErm mutants may be dependent on transcription initiated in the transposon. Clarification of this aspect of Gc *pilG* and *pilD* expression warrants further study. Additional mutant analysis has shown that the genes upstream of *pilG* are non-essential for pilus assembly and, taken together with other results (Freitag *et al.*, 1995), it appears that the locus spanning *pilF* to *pilG* appears to define the immediate gene cluster engaged in Gc and Mc pilus biogenesis.

Altered pilin profiles in Gc *pilG* mutants were similar to those seen previously in non-piliated pilin gene mutants and mutants expressing reduced levels of pili. As originally proposed, the inverse correlation between these alterations and levels of piliation can most easily be ascribed to S-pilin processing being a consequence of non-assembly (Koomey *et al.*, 1991). The findings of S-pilin in *pilG* mutants further supports this model. Another consistent perturbation in pilin expression has been seen in *pilG* and *pilC* Gc mutants (Koomey *et al.*, 1991; Jonsson *et al.*, 1992). In wild-type Gc expressing the particular *pilE* allele found in VD300 and its derivatives, pilin was detected as a doublet with the faster migrating species co-migrating with pilin present in purified pili. However, only the slower migrating species was detected in *pilG* and *pilC* mutants (Koomey *et al.*, 1991; Jonsson *et al.*, 1992). That both species appear to have been processed by pre-pilin leader peptidase suggests that an additional form of post-translational pilin processing exists but its nature remains obscure. Studies in *P. aeruginosa* have shown that undermethylation of pilins occurs in mutants failing to express PilC (the homologue to Gc PilG) but methylation did not alter the relative mobility of pilin in that system (Strom *et al.*, 1993). Recent evidence suggests that the pilin subunits in meningococci may be differentially glycosylated and that glycosylation appears to influence pilin mobility in SDS–PAGE (Virji *et al.*, 1993). Whatever the basis for the two species, the failure to find the faster migrating form in Gc *pilG* and *pilC* mutants may indicate that this as yet uncharacterized processing does not occur in these backgrounds. Alternatively, we speculate that specific degradation of the mature (faster migrating) pilin into S-pilin may account for its absence in the mutants. Efforts directed at deciphering the basis for these observations are currently underway.

Much of the interest in Gc and Mc pili stems from their apparent significance to the pathogenesis of human infection. This correlation is most easily rationalized by the increased adherence of piliated cells for epithelial tissue as seen using *in vitro* models; however, not all piliated variants display this property (Rudel *et al.*, 1992; Virji *et al.*, 1993; Jonsson *et al.*, 1994; Nassif *et al.*, 1994). Autoagglutination, twitching motility, and competence for transformation are other pilus-associated phenotypes which may influence the biology of host–parasite interactions. While the strong correlation between pilus expression and competence has been demonstrated in many studies (Frøholm *et al.*,1973; Seifert *et al.*, 1991; Zhang *et al.*, 1992), its molecular basis has remained elusive. Some have the opinion that pilin production alone, rather than the actual formation of pili, is the essential prerequisite for transformation competence (Gibbs *et al.*, 1989; Meyer *et al.*, 1990), while data from our prior studies are most consistent with competence requiring the expression of the assembled organelle (Zhang *et al.*, 1992). The data obtained here with *pilG* mutants lend strong support to the latter position. Along with virtually identical results found for Gc *pilF* and *pilD* mutants (Freitag *et al.*, 1995), these findings represent the first reports of defined, non-pilin gene mutations that directly link pilus expression and competence. These results have particular significance for studies of competence in *Bacillus subtilis* where an essential surface-localized structure analogous to type IV pili has been predicted based on genetic data (Dubnau, 1991; Hobbs and Mattick, 1993), but not yet demonstrated. In addition to the defects in bacterial aggregation and transformation ability manifested in *pilG* mutants, twitching motility and adherence to human epithelial cells are abolished or greatly diminished, respectively, in this background (S. Drake and M. Koomey, unpublished data).

A major finding stemming from these studies is that much of the basic machinery for Gc and Mc pilus assembly is highly conserved. Further evidence of this conservation can be found at the level of gene organization. Macrorestriction mapping of the Mc strain 44/76 genome using DNA probes derived from the *pilF–pilG* locus and other genes (*pilQ* (S. Drake and M. Koomey, submitted), *pilT* (Lauer *et al.*, 1993) and *pilE* (Meyer *et al.*, 1984; Potts and Saunders, 1988)) has revealed that the genes modifying pilus expression are distributed throughout the genome (A. B. Kolstø and L. O. Frøholm, personal communication). These data closely mirror that reported for the localization of these same genes in Gc strain FA 1090 (Dempsey and Cannon, 1994). In addition, *pilG* homologous sequences were detected in related commensal bacterial species expressing type IV pili or exhibiting phenotypes associated with type IV pilus expression. These findings were reminiscent of analogous results found using the Gc *pilD* gene as a probe (Dupuy and Pugsley,

1994). Taken together, the observations imply that type IV pilus-assembly genes are conserved and widely distributed in these species as well. Given the existence of highly related genes involved solely in protein export and nucleic acid transport, the specific dedication of the putative genes to pilus assembly in these species remains to be demonstrated. These studies represent one part of our general approach to understanding the mechanisms of neisserial pilus biogenesis and the role(s) which pili play in the pathogenesis of gonococcal and meningococcal disease.

Experimental procedures

Bacterial strains, media and plasmids

All bacterial strains used are listed in Table 3. *N. gonorrhoeae* (Gc) and *N. meningitidis* (Mc) were propagated on 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. *N. meningitidis* strain 44/76 was isolated from a fatal case of meningitis and septicaemia (Holten *et al.*, 1979) and is the source for current meningococcal vaccine development in Norway. Gc and Mc transformants were selected on solid GC media containing 8 μg ml^{-1} erythromycin or 5 μg ml^{-1} rifampin (Sigma). *E. coli* strains HB101 and DH5αF′IQ were used in plasmid cloning experiments while strain KW251 was used in the propagation of recombinant phage clones. *E. coli* strains were grown at 37°C in Luria–Bertani (LB) medium supplemented with appropriate antibiotics: chloramphenicol, 10 μg ml^{-1} (Sigma), erythromycin, 300 μg ml^{-1} (Sigma) or carbenicillin, 100 μg ml^{-1} (Sigma) and X-gal (40 μg ml^{-1}) when appropriate. The plasmids used in this study are listed in Table 4.

DNA preparation and manipulation

Gonococcal and meningococcal genomic DNAs were prepared by standard methods (Sambrook *et al.*, 1989). Isolation and purification of plasmid and phage DNAs were performed using Qiagen columns (Diagen, Germany) according to the manufacturer's specifications. For the phage library, meningococcal 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). Other standard methods of DNA manipulation were performed as described (Sambrook *et al.*, 1989). DNA sequencing was performed by the dideoxy chain-termination method (Sanger *et al.*, 1977) using a modified form of T7 DNA polymerase (Sequenase version 2.0, United States Biochemicals). The complete sequence of both strands was determined by subcloning, as well as by the use of appropriate deletion constructs and unique oligonucleotide primers. High stringency Southern and dot-blot hybridations were performed according to published methodologies (Tønjum *et al.*, 1992). The *pilG*-specific hybridization probe covered nucleotides 272–1123 in the sequence (accession number U19580) and was generated by a PCR protocol using standard procedures (Perkin–Elmer Cetus) and the primers TT36 (5′-CTTTGAAGGCAGGCATTC-3′) and TT37 (5′-GCGCCGGCAGTGGAGTCC-3′).

General protocols

Gonococcal and meningococcal mutants failing to express functional PilG were constructed primarily by shuttle mutagenesis using the mini-transposon mTnErm (a derivative of Tn3) as described (Seifert *et al.*, 1991). The precise sites of the transposon insertions were determined by DNA sequencing with primer Tn3L (5′-CTCATGACCAAAATCCC-3′) which is complementary to sequences at one end of the transposon.

Table 3. Bacterial strains used in this study.

Species and strains	Relevant characteristics	Source/Reference
N. gonorrhoeae		
VD300	Piliated, Opa$^-$, derivative of MS11	Koomey *et al.* (1991)
N400	VD300, *recA6* (IPTG-inducible *recA*)	This work
VD302 $P^-_{Gly\text{-}1 \rightarrow Ser\text{-}1}$	Mutant pilin resistant to pre-pilin peptidase activity	Koomey *et al.* (1991)
GGseries	*pilG*::mTnErm transposon insertions in N400	This work
GD*tetM*	VD300 with *pilD* under the control of an exogenous promoter	This work
N. meningitidis		
44/76-A	Piliated variant of 44/76 (serogroup B:15:P1.7, 16:L3,7–selected by pellicle formation)	Holten *et al.* (1979); this work
MG-series	*pilG*::mTnErm transposon insertions in *Neisseria meningitidis* 44/76-A	This work
Commensal and other species		
N. lactamica 23970	Type strain	ATCC
N. subflava 10555	Type strain	ATCC
N. sicca 29193	Type strain	ATCC
N. cinerea 14685	Type strain	ATCC
N. mucosa 25996	Type strain	ATCC
N. animalis CPH 8	Reference strain	K. Bøvre
N. canis 14687	Type strain	ATCC
N. cuniculi 14688	Type strain	ATCC
N. caviae 14659	Type strain	ATCC
N. elongata 6171	Reference strain	Tønjum *et al.* (1992)
P. aeruginosa PAK	Reference strain	D. Bradley
E. corrodens 31745/80	Reference strain	Tønjum *et al.* (1993)
M. nonliquefaciens NCTC 7784	Reference strain	Tønjum *et al.* (1992)
M. marinum 927	Type strain	ATCC
E. coli HB101	F$^-$, *mcrB mrr hsdS20* (r_B^-, m_B^-) *recA13 supE44 ara14 galK2 lacYI proA2 rpsL20 xyl15 leu mtl1*	BRL
E. coli DH5αF′IQ	*hsdR recA laZYA80 lacZ*M15	BRL
E. coli KW251	F$^-$ *supE44 galK2 galT22 metB1 hsdR2 mcrB1 argA81*:Tn*10 recD1014*	Promega

Table 4. Plasmids used in this study.

Plasmids	Relevant characteristics	Source/Reference
pACYC184	Cloning vector, Tet^R	New England Biolabs
pBluescript II SK/KS	Cloning vector, Amp^R	Stratagene
pHSS6	Vector for shuttle mutagenesis, Kan^R	Seifert *et al.* (1991)
pOx::mTnErm	Shuttle mutagenesis, conjugative F factor derivative with mTnErm	Seifert *et al.* (1991)
pTCA	Shuttle mutagenesis, Tet^R, expresses Tn3 transposase constitutively	Seifert *et al.* (1991)
pTT1	*ClaI–SalI* fragment containing Gc *pilG* in pACYC184	This work
$pTT1_{fsXmaI}$	pTT1 with frame-shift mutation in *pilG* at *XmaI* site	This work
pTT2	*BamHI–ClaI* fragment containing Gc *pilG* cloned into pBSIISK	This work
pTT2ermC	pTT2 with *ermC* gene cassette in *SmaI* site	This work
pTT3	*SmaI–SalI* fragment containing part of Gc *pilG* in pHSS6	This work
pTT4	*EagI–BstYI* fragment containing complete Gc *pilG* in pBSIISK	This work
pTT5	*EagI*-fragment containing Mc*pilG*, *pilD*, *pilF* genes in pBSIIKS	This work
pGDtetM	*tetM* gene containing restriction fragment cloned upstream of *pilD*	This work

Tet^R, tetracycline resistant; Amp^R, ampicillin resistant; Kan^R, kanamycin resistant.

One mutation was constructed by site-specific insertion of an erythromycin-resistance gene cassette (Projan *et al.*, 1987). These mutations were introduced into *N. gonorrhoeae* N400 by genetic transformation using linearized plasmid DNAs. The N400 strain harbours the *recA6* allele in which the *recA* gene is under the control of an inducible *taclac* promoter (H. Seifert, personal communication). The *recA6* construct also contains the $lacI^q$ repressor gene and the *tetM* gene as an associated selectable marker. 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. Linearized *pilG*–mTnErm plasmids were also transformed into *N. meningitidis* strain 44/76-A. Introduction of specific transposon insertions into the genome of recipients was verified by Southern hybridization analysis with *ermC*- and *pilG*-specific hybridization probes labelled with ^{32}P. The complete *pilE* nucleotide sequence of each mutant was determined by PCR-based thermocycle sequencing using Vent polymerase (New England Biolabs) and customized oligonucleotide primers.

Polypeptides encoded by plasmids were identified by means of an *in vitro* coupled transcription–translation system analysis of plasmid DNA. Purified plasmid DNAs were used to prime the reactions according to the supplier's recommendations (Promega) with [^{35}S]-methionine (Amersham). Labelled polypeptides were separated by SDS–PAGE, transferred to nitrocellulose filters by electroblotting and visualized by autoradiography.

Competence for natural transformation of Gc and Mc strains was assessed as detailed (Zhang *et al.*, 1992) using chromosomal DNA from a spontaneous rifampin-resistant mutant of VD300.

Detection of pilin antigen in whole-cell lysates and concentrated culture supernatants were performed using rabbit polyclonal pilin-specific antibodies raised against *N. gonorrhoeae* pili (lot 2-66) and *P. aeruginosa* PAK pili (lot 526). Conditions for sample preparation, SDS–PAGE, electroblotting and antigen detection have been described previously (Koomey *et al.*, 1991).

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.

Transmission electron microscopy

Prior to examination in the electron microscope, strains were grown on GC agar for 14–17 h and harvested in 1% ammonium acetate, pH 7.2. Droplets (5 μl) of the solutions were placed on carbon filmed grids and left for 1 min, blotted almost dry and negatively stained for another min with 0.25% (Gc) and 0.50% (Mc) sodium phosphotungstic acid (NaPT), pH 7.0. The grids had previously been glow discharged in air to facilitate spreading of both the bacteria and the stain. The specimens were examined in a Jeol 1010 microscope operated at 100 KeV.

Uncoupling pilD *expression from* pilG *by use of an exogenous promoter*

Plasmid pGD carries the Gc *pilD* gene and flanking sequences on a 2.6 kb *SmaI–HpaI* fragment (N. Freitag, and M. Koomey, submitted). An 8 kb *NotI*-fragment from pHSX–*tetMlacIP* (H. S. Seifert, personal communication) containing the *tetM* gene was isolated and inserted immediately upstream of *pilD* at the *BstYI* site in pGD in the orientation shown in Fig. 1 to create pGDtetM. This construct placed the *pilD* gene under the control of an exogenous promoter present in the inserted restriction fragment, presumably the *tetM* promoter. This construct was introduced into *N. gonorrhoeae* VD300 by transformation and selection for tetracycline resistance to produce strain GDtetM. The mTnErm mutations were then transformed into GDtetM.

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