PilP, a pilus biogenesis lipoprotein in *Neisseria gonorrhoeae*, affects expression of PilQ as a high-molecular-mass multimer

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Summary

Studies of gonococcal pilus biogenesis are fundamental to understanding organelle structure/function relationships and identifying new approaches to controlling disease. This area of research is also relevant to elucidating the basic mechanisms of outer membrane translocation of macromolecules, which requires components highly related to those involved in type IV pilus expression. Previous studies have shown that products of several ancillary *pil* genes are required for organelle biogenesis but of these only PilQ, a member of the GspD protein family, is a component of the outer membrane. DNA sequencing of the region upstream of *pilQ* revealed the presence of two open reading frames (ORFs) whose deduced polypeptides shared significant identities with proteins required for pilus expression in *Pseudomonas aeruginosa* and *Pseudomonas syringae*, the genes for which are arrayed upstream of a gene encoding a PilQ homologue. Gonococcal mutants bearing transposon insertions in these ORFs were non-piliated and failed to express pilus-associated phenotypes, and the corresponding genes were designated *pilO* and *pilP*. The piliation defects in the mutants could not be ascribed to polarity on distal *pilQ* expression as shown by direct measurement of PilQ antigen in those backgrounds and the use of a novel technique to create tandem duplications in the gonococcus (Gc) genome. As predicted by the presence of a consensus lipoprotein signal sequence, PilP expressed in both *Escherichia coli* and Gc could be labelled with [³H]-palmitic acid. PilP⁻ as well as PilQ⁻ mutants shed PilC, a protein which facilitates pilus assembly and is implicated in epithelial cell adherence, in a soluble form. Combined with the finding that levels of multimerized PilQ were greatly reduced in PilP⁻ mutants, the results suggest that PilP is required for PilQ function and that PilQ and PilC may interact during the terminal stages of pilus biogenesis. The findings also support the hypothesis that the Gc PilQ multimer corresponds to a physiologically relevant form of the protein required for pilus biogenesis.

Introduction

Expression of type IV pili or fimbriae appears to play a critical role in both the colonization of the human host by *Neisseria gonorrhoeae* (Gc) and the elaboration of a number of distinct phenotypes, including competence for natural transformation (Seifert *et al*., 1990; Zhang *et al*., 1992), twitching motility (Swanson, 1978), autoagglutination (Swanson *et al*., 1971), and adherence to epithelial tissue (Swanson, 1973; Heckels, 1989; Rudel *et al*., 1992). Type IV pili, originally defined by their related morphologies and polarized localizations on the cell (Ottow, 1975), have been demonstrated in many other important mucosal pathogens of humans (Hermodson *et al*., 1978; Strom and Lory, 1986; Giron *et al*., 1991; Shaw and Taylor, 1990). Studies have shown that this broad classification scheme corresponds well with the presence of a highly conserved N-terminal domain in the structural subunit which accounts for the shared antigenicity and immunogenicity demonstrated by these molecules (Patel *et al*., 1991). Evidence for the relatedness of these organelles can also be found in the conservation of genes and gene products required for their expression. These components include related prepilin peptidases, soluble proteins with consensus nucleotide-binding motifs and polytopic cytoplasmic membrane proteins but, with the exception of the prepilin peptidases, the functions served by these molecules remains unclear (Lory, 1992; Pugsley, 1993). Homologues corresponding to one or more of these molecules have been implicated in the main terminal branch of the general secretory pathway (GSP (Pugsley, 1993), which is responsible for extracellular localization of a wide variety of toxins and hydrolases by Gram-negative bacteria) and expression of competence for transformation in *Bacillus subtilis* (Albano *et al*., 1989). The fundamental

question then arises as to how these processes might be related to the expression and biogenesis of type IV pili and vice versa.

In the context of Gram-negative species, one obvious connection involves the translocation of large, macromolecular complexes across the outer membrane. This process presumably requires an integral outer membrane component and, based on the conservation of the components detailed above, one might anticipate that structurally related molecules might also be found at this site for each system. Strong candidates for such molecules are homologues of the GspD protein family as exemplified by 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)) and PilQ (involved in type IV pilus biogenesis in *N. gonorrhoeae* (Drake and Koomey, 1995)) and *Pseudomonas aeruginosa* (Pa) (Martin *et al.*, 1993). Furthermore, outer membrane proteins with a high degree of identity to the GspD protein family have also been shown to be active in the type III, contact-depedenent secretion systems (Michiels *et al.*, 1991; Allaoui *et al.*, 1993; Kaniga *et al.*, 1994), competence for transformation in *Haemophilus influenzae* (Tomb *et al.*, 1991), and secretion of S-layer surface-array subunits in *Aeromonas hydrophila* (Thomas and Trust, 1995). The GspD homologues all possess N-terminal signal sequences and show high levels of sequence conservation in a C-terminal domain encompassing a 200-amino-acid span rich in polar residues (Genin and Boucher, 1994).

Insights into the structure/function relationships of these proteins have come most readily from studies of pIV and its role in filamentous phage morphogenesis (Russel, 1991). Using a variety of biochemical techniques, it has been demonstrated that pIV forms a homomultimer composed of 10–12 subunits (Russel and Kazmierczak, 1993; Russel, 1994a; Kazmierczak *et al.*, 1994). In addition, the conserved C-terminal domain of pIV has been shown to mediate its insertion into the outer membrane and nonfunctional pIV missense mutants altered at highly conserved residues in the C-terminus (P375) fail to multimerize despite their membrane localization (Russel, 1994a). Analogous findings relating to XpsD, a GspD homologue involved in hydrolase secretion in *Xanthomonas campestris* pv. *campestris* have been reported (Hu *et al.*, 1995; Chen *et al.*, 1996). More recent findings in the phage system indicated that the abundance and relative SDS and heat stability of high-molecular-mass (HMM) forms of mutant pIVs correlated with their levels of function (Linderoth *et al.*, 1996). Based on these observations, it was proposed that the pIV multimer forms a channel through which phage particles are assembled and extruded and that the other GspD homologues might form analogous export channels (Russel, 1994b; 1995). Gonococcal PilQ

forms a homomultimer composed of 10–12 subunits which is resistant to SDS and heating (Newhall *et al.*, 1980) and we have shown that the integrity of the conserved C-terminal residues is necessary for function and detection of the HMM form (Drake and Koomey, 1995). Evidence for SDS- and heat-stable HMM forms of other GspD homologues has also been accumulating (Hardie *et al.*, 1996; Plano and Straley, 1995).

We report here the characterization of the region 1.3 kb upstream of the *N. gonorrhoeae pilQ* gene and identification of two new genes, *pilO* and *pilP*. The product of *pilP* is a lipoprotein which is essential for pilus biogenesis and HMM PilQ stability. Based on the unique phenotypes found for both PilP⁻ and PilQ⁻ mutants, PilP appears to be required for PilQ function. The product of the *pilO* gene is necessary for competence for transformation but formal evidence for its role in organelle biogenesis is lacking.

Results

Nucleotide sequence analysis of the pilQ *upstream region*

Gc PilQ⁻ mutants are defective in pilus biogenesis (Drake and Koomey, 1995) and it was therefore of interest to examine if other assembly-related genes might be linked to *pilQ*. As sequence analyses and mutagenesis of the region immediately 3′ to *pilQ* failed to reveal such genes (Drake and Koomey, 1995), restriction fragments from the cosmid clone pAE8 which hybridized with probes corresponding to the *pilQ* 5′ end were subcloned into a high-copy-number vector. The nucleotide sequence of the 1.6 kb *Eco*RV–*Cla*I fragment in pKSD showed that the clone began at the most 5′ *Cla*I site in *pilQ* and extended 1.3 kb upstream of the gene. Two seemingly significant open reading frames (ORFs) arrayed in the same orientation as *pilQ* were detected (designated ORFs 5 and 6) and high scoring matches to each were found using the BLASTX program. ORF5, which began at the *Eco*RV site, was ≈33% identical to the central 80 residues of both the Pa and *Pseudomonas syringae* (Ps) PilO proteins (Martin *et al.*, 1995; Roine *et al.*, 1996), while ORF6 was 30% identical (over 110 residues) to the Pa PilP protein (Martin *et al.*, 1995) and sightly less related to Ps PilP (Roine *et al.*, 1996). Based on the alignment of the most 5′ ORF with Pa/Ps PilO, it appeared that part of the ORF corresponding to the PilO N-terminal domain was missing from pKSD. DNA sequencing of a plasmid carrying a 9 kb *Eco*RI fragment derived from pAE8 showed that a contiguous ORF with N-terminal identity to Pa/Ps PilO did in fact span the *Eco*RV site. Further characterization of this clone showed that sequences starting 0.5 kb 5′ of the *Eco*RV site were different from that present in the Gc genome based on Southern hybridization studies and,
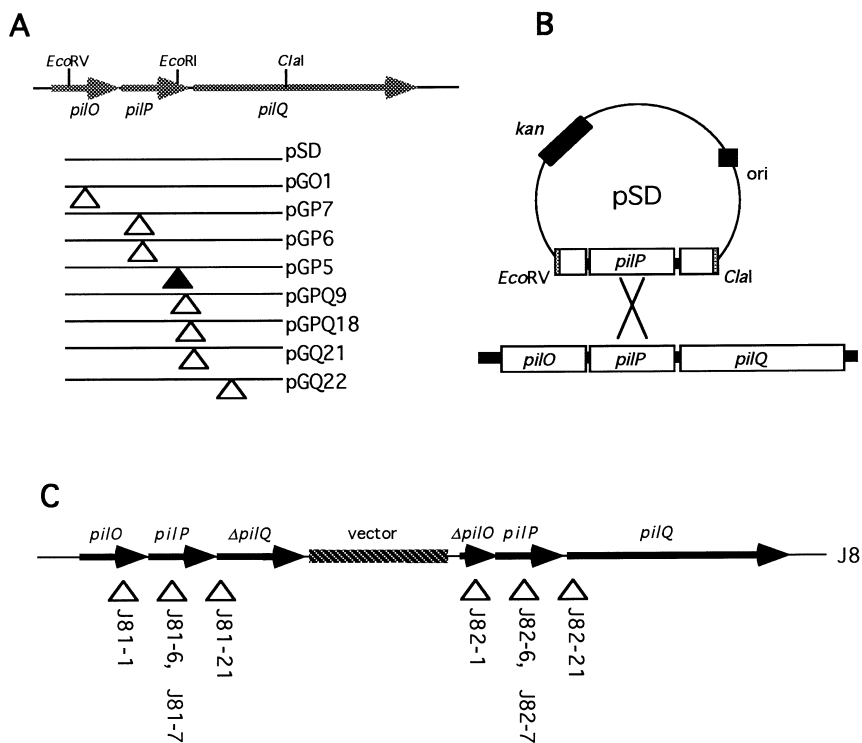
Fig. 1. Genomic organizations and physical maps of the constructs and strains used in this study.
A. Plasmid derivatives of pSD containing transposon insertions (denoted by triangles) and a *cat* gene cassette insertion (filled triangle at *Eco*RI site).
B. Depiction of the recombination event leading to integration of pSD into the genome of N400 to create strain J8. Note that although it is shown as a single cross-over between plasmid and genome, the actual mechanism does not involve a circular form of pSD (see the *Discussion*).
C. Positions of transposon insertions in strain J8 derivatives which carry a duplication of the *pilP* gene and flanking region (strain designations are shown below the triangles).

as such, this clone was not used for further characterization of the upstream locus.

ORF5, 645-bp long, potentially encodes a polypeptide of 215 residues with a molecular mass of 23 327 Da which, while lacking an apparent signal sequence, would contain one putative membrane-spanning segment near the N-terminus. Other than Pa/Ps PilO, no other significant matches were found in the databases. Starting with a initiation codon 18 bp downstream of the termination codon for ORF5 and ending 18 bp 5′ of the start codon for PilQ, ORF6 consists of 543 bp and encodes a product of 181 residues with a molecular mass of 20 110 Da. The derived polypeptide of ORF6 contains an N-terminal signal sequence characteristic of lipoproteins with a consensus tetrapeptide cleavage site of LSAC. In addition to the Pa/Ps PilP proteins, the only other potential match to ORF6 was the OspA lipoprotein of *Borrelia burgdorferi* with which it shares 22% identity. Overall, the content and organization of the Gc ORF5/ORF6/*pilQ* locus is strikingly similar to that of the Pa and Ps *pilO*, *pilP* and *pilQ* loci (Martin *et al.*, 1995; Roine *et al.*, 1996).

Mutagenesis of the pilQ *upstream region*

As a first approach, mutagenesis was used to assess if the Gc ORFs did, in fact, correspond to genes and gene products involved in pilus biogenesis. Defined mutations were constructed in *Escherichia coli* using a transposon shuttle mutagenesis system (Seifert *et al.*, 1986) and

these mutations were returned to the genome of Gc strain N400 by transformation. Seven transposon insertion mutants, predicted to disrupt ORF5, ORF6 and the 5′ portion of *pilQ*, were selected for further study (Fig. 1A). An eighth mutation was constructed by insertion of a promoterless *cat* gene cassette into the *Eco*RI site within ORF6. After ensuring the correct introduction of the mutations by Southern hybridization and that no changes in *pilE* (the pilin subunit gene) had occurred, pilus expression and associated phenotypes were assessed. With one exception, all of the Gc transposon mutants (i) lacked pili detectable by transmission electron microscopy (TEM); (ii) had greatly reduced rates of competence for natural transformation; and (iii) displayed non-autoagglutinating phenotypes. The one exception, strain GPQ18 which carried a transposon insertion 6 bp 5′ of the start codon for PilQ, was piliated (albeit at reduced levels), had wild-type frequencies for transformation and displayed a diminished but readily detectable capacity to autoagglutinate. All of the above mutants released S-pilin (a soluble form of proteolytically degraded PilE lacking the 40 N-terminal residues) into culture supernatants. The strain containing the *cat* gene-cassette insertion was indistinguishable from the wild-type parent.

PilQ expression in mutant backgrounds

PilQ has been demonstrated to be essential for pilus expression. To determine whether the non-piliated state
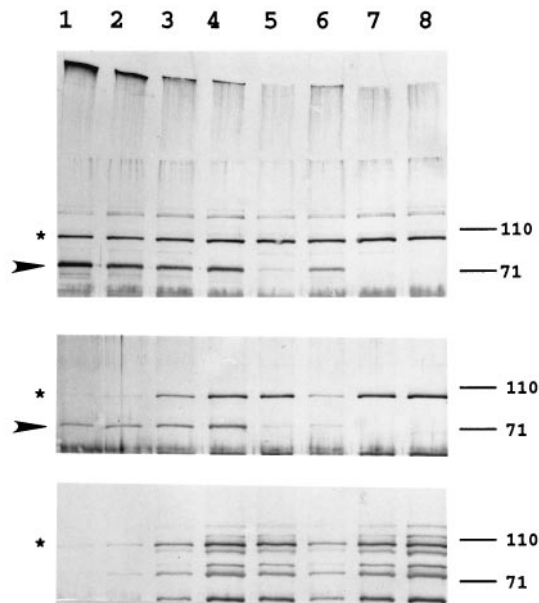
Fig. 2. Detection of PilQ and PilC antigen forms in N400 and derivatives. Lanes: 1, N400; 2, GO1; 3, GP6; 4, GP7; 5, GPQ9; 6, GPQ18; 7, GQ21, 8, GQ22. Top panel, immunoblot of whole-cell lysates using PilQ rabbit antibodies. Reactive material at the top of the panel corresponds to HMM PilQ retained in the well of the stacking gel. Middle panel, immunoblot of soluble proteins in culture supernatants using PilQ rabbit antibodies. Arrows for top and middle panels denote migration of the PilQ monomer (75 kDa). Bottom panel, immunoblot of soluble proteins in culture supernatants using PilC rabbit antibodies. PilC and apparently identically reactive species seen using PilQ antibodies are denoted by asterisks. The relative mobilities of molecular mass standards are shown in kDa.

of the mutants was a consequence of polarity on *pilQ* expression imposed by the transposon insertions, the levels of PilQ in these backgrounds were examined by immunoblotting (Fig. 2, top panel). GQ21 and GQ22 carry insertions in the *pilQ* ORF and make no detectable antigen (lanes 7 and 8). GPQ9 and GPQ18, containing insertion mutations 5 bp apart in the 18 bp region between ORF6 and the *pilQ* ORF, had greatly reduced levels of the 75 kDa PilQ with the level in GPQ18 being greater than in GPQ9 (lanes 5 and 6). Unless transcription can proceed across the transposons, PilQ expression in these mutants appears to be dependent on promoters within the element and it is worth noting, in this regard, that the transposons in GPQ9 and GPQ18 are in opposite orientations. The mutants with insertions in ORF5 and ORF6 expressed slightly less of the 75 kDa species (lanes 2, 3 and 4) but, given the piliated state of GPQ18 (which expresses far less PilQ; lane 6), their assembly defects cannot be solely due to polarity exerted on *pilQ*. Thus, a polypeptide corresponding to ORF6 appeared to be required for pilus assembly and, in accord with this and other data, ORF6 was designated *pilP* and its product PilP. A direct role for a product corresponding to ORF5 cannot be surmised

from this data because its transposon insertion mutation might exhibit polarity on *pilP*. However, based on its relationship to Pa/Ps *pilO* and data detailed below, ORF5 was designated *pilO*.

Altered forms of PilC and PilQ in mutants

A significant amount of the Gc PilQ antigen is retained at the buffer/stacking-gel interface following SDS–PAGE (Drake and Koomey, 1995). This unique HMM species of PilQ appears to reflect its propensity to form multimers consisting of 10–12 monomers which can be completely dissociated only after reduction and alkylation (Newhall *et al.*, 1980). In the immunoblot studies examining PilQ expression, we found that the levels of HMM PilQ were reduced in all of the Gc transposon mutants (Fig. 2, top panel). In GPQ9, no antigen in this form could be detected (lane 5), while in GPQ18, HMM PilQ was detected (lane 6) and the relative ratio of the HMM form to the 75 kDa species was close to that seen in the wild-type parent. However, the ratios of HMM PilQ to monomer were dramatically reduced in both PilP⁻ mutants (lanes 3 and 4). A slight reduction in the level of HMM PilQ relative to monomer was noted in whole-cell lysates of the PilO⁻ insertion mutant (lane 2). Reduced levels of HMM PilQ were not seen in any other class of pilus biogenesis mutants (e.g. PilD⁻, PilE⁻, PilF⁻ mutants; data not shown).

The presence of PilQ in concentrated culture supernatants was examined because mutants expressing truncated (and non-functional) PilQ have been shown to release detectable levels of the antigen (Drake and Koomey, 1995). Culture supernatants of both of the PilP⁻ mutants appeared to contain elevated levels of PilQ monomer (Fig. 2, middle panel, lanes 3 and 4). Interestingly, a PilQ antibody cross-reactive protein with a molecular mass of approximately 105 kDa present in whole-cell lysates was seen in culture supernatants of PilP⁻ and PilQ⁻ mutants (denoted by the asterisks in Fig. 2). Additionally, while lower levels of this antigen were detected in the GPQ18 supernatant (lane 6), it was absent from those of the wild type and the PilO⁻ mutant (lanes 1 and 2). Given its molecular mass and altered localization in the mutants, we asked if this molecule might be PilC, an assembly-facilitating protein implicated in pilus-dependent adherence to epithelial cells. A pattern virtually identical to that seen with PilQ antibodies was seen for PilC antibodies with a 105 kDa species being found in culture supernatants of the PilP⁻ and PilQ⁻ mutants and reduced amounts of it present in the GPQ18 sample (Fig. 2, bottom panel). This 105 kDa species in supernatants was not seen in any other class of pilus biogenesis mutants (data not shown). As such, PilC appeared to be shed in a soluble form only in PilQ⁻ mutants, mutants expressing reduced amounts of PilQ, and in PilP⁻ mutants. A second
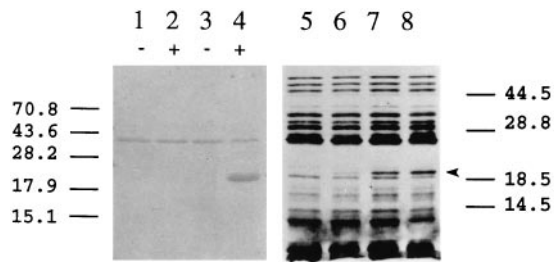
Fig. 3. Autoradiographic detection of PilP by labelling with [^{3}H]-palmitic acid. Detection using the T7-based expression system in *E. coli* BL21(DE3) containing pT7-5 (the vector; lanes 1 and 2) or pT7-P (lanes 3 and 4) in the absence (−) and presence (+) of IPTG-induced T7 RNA polymerase. Detection of PilP in Gc strains. Lanes: 5, GP7; 6, GO1; 7, GQ22; 8, N400. The [^{3}H]-palmitic acid-labelled PilP (≈20 kDa) is denoted by the arrowhead. The relative mobilities of molecular mass standards are shown in kDa.

protein species with a molecular mass of 58 kDa was also seen with both sera and its presence coincided with that of the 105 kDa antigen. To determine if the two sera were indeed reacting with the same proteins, supernatants from GQ21 were immunoblotted, the filter strip cut to divide the lane in half, and each half reacted with one of the sera. After developing the strips, it was found by re-aligning them that the 105 kDa and 58 kDa proteins observed with each sera were identical within the limitations of the technique (data not shown).

Identification of PilP in E. coli *and* N. gonorrhoeae

The T7 expression system (Studier and Moffatt, 1986; Tabor and Richardson, 1985) was used to determine if the product of the Gc *pilP* gene could be detected in *E. coli*, and if, as predicted by the presence of a consensus signal peptidase II cleavage site, it was a lipoprotein. Cultures of *E. coli* BL21(DE3) with pT7P plasmid (constructed in such a way as to orientate the T7 promoter upstream of the *pilP* gene) and pT7-5 (the vector) were labelled with [^{3}H]-palmitic acid. A 20 kDa protein species was detected only in the pT7P sample and its detection was strictly dependent on T7 RNA polymerase induction (Fig. 3, lane 4), while a non-specific radiolabelled band of ≈38 kDa was observed in all samples. For reasons not yet understood, attempts to visualize a product for *pilO* using the T7 system and intrinsic amino acid incorporation were unsuccessful (data not shown).

In light of these results, we asked if PilP could be detected using whole-cell labelling of Gc with [^{3}H]-palmitic acid. Although many radiolabelled proteins were seen, a species of 20 kDa was found in proteins from the wild-type strain and the PilQ$^-$ strain GQ2 but absent from the PilP$^-$ transposon insertion mutant (Fig. 3, lanes 5–8). A significant reduction in the level of the same 20 kDa protein was seen in the *pilO* transposon insertion mutant (lane 6).

The latter finding may be an indication that *pilO* and *pilP* are co-transcribed and that the reduction in PilP level in that strain is a consequence of polarity exerted by the upstream transposon insertion.

Analyses of strains carrying tandem duplications of the pilP *gene*

When constructing the Gc transposon mutants, we occasionally noted the presence of both piliated and non-piliated colony types among the primary chloramphenicol-resistant Gc transformants. Examination of the circumstances under which this heterogeneity arose showed that it occurred with pGO1 and pGQ22 plasmid DNAs (Fig. 1A) and only when undigested (i.e. circular) DNA was used. Given that these two plasmids carry transposon insertions close to the ends of the Gc DNA insert in pSD, it seemed that the piliated transformants derived from these constructs might arise by integration of the whole plasmid DNA into the Gc genome (as depicted in Fig. 1B). This was in fact the case because all the chloramphenicol-resistant, piliated transformants were resistant to kanamycin (the gene for kanamycin resistance is carried on the vector part of pSD) and shown by hybridization analyses to carry a whole copy of the plasmid integrated at the *pilP* locus (data not shown). By transforming N400 with intact pSD DNA and selecting for kanamycin resistance, we constructed strain J8 which carries a duplication of the *pilP* gene separated by a vector junction (Fig. 1C) and is indistinguishable from N400 in piliation and pilus-associated phenotypes.

One consequence of the duplication in J8 is that both truncated PilQ (from the 5′ *pilQ* copy) and intact PilQ (from the 3′ complete gene) should be expressed. As seen by immunoblotting, J8 expressed slightly reduced levels of PilQ monomer as well as species of 23 and 20 kDa detectable with PilQ antibodies (Fig. 4, lane 2, asterisks). By transforming J8 with pGQ21 DNA (carrying a m-Tn*Cm* insertion in the PilQ initiation codon), piliated derivatives, in which the transposon had recombined into the incomplete 5′ *pilQ* gene copy (J81-21), and non-pliated derivatives, in which the transposon had recombined into the 3′ *pilQ* gene copy (J82-21), were recovered (Fig. 1C). Immunoblotting confirmed that the 23 kDa and 20 kDa antigen forms were products of the defective *pilQ* allele (Fig. 4, lanes 3 and 4).

Based on the structure of the duplication in strain J8, it became possible to test directly whether the piliation defects associated with the *pilO* and *pilP* transposon insertions were a consequence of altered or reduced *pilQ* expression. J8 derivatives carrying the transposons in the 3′ copies of *pilO* and *pilP* (J82-1, J82-6 and J82-7) were indistinguishable from the J8 parent, formally proving that sufficient levels of PilQ were expressed in the context
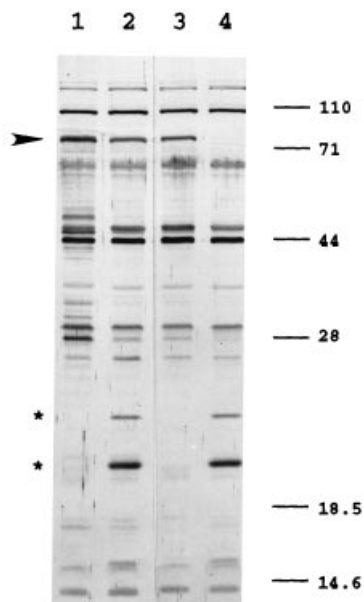
Fig. 4. Immunoblot detection of PilQ antigen forms in whole-cell lysates from strains carrying a duplication of the *pilP* gene and flanking sequence. Lanes: 1, N400; 2, J8; 3, J81-21; 4, J82-21. The relative mobility of wild-type PilQ (75 kDa) is denoted by the arrowhead while those of the truncated forms expressed from the defective *pilQ* allele are indicated by the asterisks. The relative mobilities of molecular mass standards are shown in kDa.

of the insertions (Table 1). Strains J81-6 and J81-7 with the *pilP* transposon insertions in the upstream gene copy were non-piliated, implying that functional levels of PilP were not expressed from the downstream copy. Strain J81-1, with an insertion in the upstream intact *pilO* gene copy, was also non-piliated. When competence for transformation was examined, the frequencies for strains J81-7 and J81-6 were 1% of that found for piliated derivatives, while the other non-piliated mutants showed approximately 10-fold lower levels than this (0.1% of wild type; Table 1). The intermediate levels of transformability found for J81-7 and J81-6 argue that some functional PilP is expressed from the 3′ gene copy in the J8 backgrounds, while the more absolute defect found for J81-1 proves that PilO plays an essential role in competence and strengthens the concept that it functions in pilus biogenesis.

Radiolabelling of Gc with [3H]-palmitic acid was used to assess if PilP was expressed from the 3′ gene copy in the J8 derivatives (Fig. 5). When compared with strain GP7 (which carries an insertion in its single *pilP* gene copy and makes no product), it is clear that strain J82-7 expresses detectable but significantly reduced levels of the tritiated form of PilP. In addition, the fact that J82-7 expresses less PilP than J82-1 (the PilO⁻ mutant) but has a 10-fold higher level of transformability corroborates the other data, indicating that PilO functions in competence for transformation and, presumably, pilus biogenesis.

Discussion

This study has led to the identification of two genes which map immediately upstream of the gene that encodes PilQ, an essential pilus biogenesis component and member of the GspD protein family. The deduced polypeptides of the gene ORFs share significant identities with proteins required for pilus expression in Pa and Ps, the genes for which are arrayed upstream of a gene encoding a GspD homologue (Martin *et al.*, 1995; Roine *et al.*, 1996). The findings extend the previously documented conservation found for pilus-biogenesis components in Gc and Pa/Ps. Overall, the gross organization of these particular gene clusters are strikingly similar and recent studies have shown that Gc ORFs corresponding to Pa/Ps *pilM* and *pilN* can be found immediately 5′ of the *pilO* gene (S. Dunham and M. Koomey, unpublished). However, subtle differences can be found. For example, in *Pseudomonas* species, the termination codons of the *pilO* ORFs overlap the initiation codon for *pilP*, while in Gc there is a 17 bp intergenic region which posesses a potential ribosome-binding site. Mutant analyses in each system appear to indicate that transcriptional organization of *pilO*, *pilP* and *pilQ* may also be conserved. In Pa/Ps, *pilO* and *pilP* appear to be co-transcribed while *pilQ* seems to have a functional promoter within the 300 bp region upstream of its initiation codon within *pilP* (Martin *et al.*, 1995; Roine *et al.*, 1996). Based on previous studies which indicate that the transposons used in this study do

Table 1. Phenotypic characteristics of *N. gonorrhoeae* strains.

Strain	Pili[a]	Autoagglutination[b]	% Natural competence[c]
N400	+	+++	100
GO1	−	−	< 0.1
GP6	−	−	< 0.1
GP7	−	−	< 0.1
GP5	+	+++	100
GPQ9	−	−	< 0.1
GPQ18	+[d]	+	100
GQ21	−	−	< 0.1
GQ22	−	−	< 0.1
J8	+	+++	100
J81-1	−	−	< 0.1
J81-6	−	−	1
J81-7	−	−	1
J81-21	+	+++	100
J81-22	+	+++	100
J82-1	+	+++	100
J82-6	+	+++	100
J82-7	+	+++	100
J82-21	−	−	< 0.1
J82-22	−	−	< 0.1
GF-2[e]	−	−	< 0.1

a. Determined by transmission electron microscopy.
b. Determined by colony morphology and growth in liquid media.
c. Determined using a DNA transformation assay under RecA-induced conditions.
d. Reduced number of pili noted by TEM.
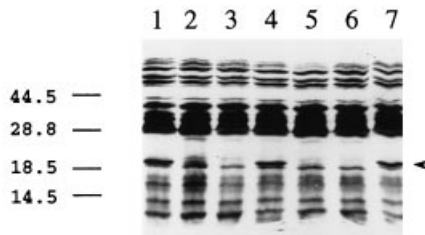**e.** Non-piliated PilF⁻ control strain (Freitag *et al.*, 1995).

Fig. 5. Autoradiographic detection of [³H]-palmitic acid-labelled PilP in whole-cell lysates from Gc strains carrying a duplication of the *pilP* gene and flanking sequence. Lanes: 1, N400; 2, GO1; 3, GP7; 4, J8; 5, J81-1; 6, J81-7; 7, J82- 7. The [³H]-palmitic acid-labelled PilP (≈20 kDa) is denoted by the arrowhead. The relative mobilities of molecular mass standards are shown in kDa.

exhibit polarity (Freitag *et al.*, 1995) and the reduced levels of palmitate-labelled PilP in the *pilO* insertion mutant, the Gc counterparts are probably transcriptionally coupled. This probability is also supported by the low levels of expression of the downstream *pilP* gene copy in the J8 derivatives. Similarly, the levels of PilQ expressed in the *pilP* transposon mutants and also from the downstream *pilQ* gene copy in the J8 derivatives suggest the presence of a distinct *pilQ* promoter within the 300 bp upstream of its initiation codon. Nonetheless, slightly reduced levels of monomeric PilQ are found in the *pilO* and *pilP* transposon mutants and the J8 derivatives. This may mean that PilP stabilizes PilQ in a post-translational fashion and/or that correct stoichiometries of each component are required for stabilization. However, evidence for decreased PilQ stability is lacking because an increase in proteolytic degradation products, such as those found in other PilQ mutants (Drake and Koomey, 1995), is not seen in the absence of PilP (data not shown). Alternatively, the reduced PilQ levels in the mutants may reflect a role for *pilO/pilP* readthrough transcription in activating a *pilQ* promoter. More studies using gene fusions and RNA analysis are required to further elucidate this situation.

The genomic integration of whole-plasmid DNA was an unanticipated result stemming from this study. This recombination event appeared to result from a single cross-over between the chromosome and a circular plasmid molecule in a Campbell-type integration fashion (as depicted in Fig. 1B). Intact plasmid DNA is normally rapidly linearized during the uptake process, making it difficult to envision how this process might occur. A similar finding of plasmid integration, made in *Neisseria meningitidis*, was attributed to a minority of plasmid DNA molecules which remain circular following uptake (van der Ley *et al.*, 1995). However, results using genetically marked constructs indicate that this process does not involve a single cross-over event but utilizes linearized substrates and seems to involve a third DNA molecule in addition to the plasmid and genomic DNA (S. Sandstedt and M. Koomey, unpublished). In

addition to using this methodology to create tandem duplications and assess operon structure as described in this study, this technique has been exploited for *in situ* cloning, creating gene-fusion reporters while retaining an intact copy of the gene of interest, and stable introduction into the genome of foreign (heterologous) genes (M. Koomey, unpublished). Just as these types of manipulations have become part of the standard repertoire for gene manipulation and strain construction in most prokaryotic systems, their introduction to pathogenic *Neisseriae* species represents a significant advancement, employing a simple technique. This is especially true because the methodology employs a standard cloning vector already in use by many workers.

While these findings and the fact that functional Gc homologues for the Pa/Ps *pilO* and *pilP* genes exist are interesting, the more pertinent contributions of this work involve the observations of altered localization of PilC in PilQ⁻ and PilP⁻ mutants, aberrant PilQ behaviour in PilP⁻ mutants, together with other evidence that PilP is required for PilQ function. PilC, originally identified in enriched pilus preparations (Jonsson *et al.*, 1991), acts as a facilitator of pilus biogenesis and appears to be responsible for the epithelial cell-binding activity associated with Gc pili (Rudel *et al.*, 1995b). PilC has also been reported to fractionate with the outer membrane and, using antibody-immunogold labelling, an as yet undetermined proportion of PilC can be found associated with the ends of pilus filaments (Rudel *et al.*, 1995b). The aberrant PilC localization found specifically in PilP and PilQ mutants implies that these components interact with one another at the terminal stage of pilus biogenesis. Given that the PilQ serum which detects PilC was raised against the 800 kDa multimer, it is possible that the cross-reaction reflects the presence of PilC in the complex. However, antibodies raised against recombinant meningococcal PilQ expressed in *E. coli* also display PilC cross-reactivity (T. Tønjum, unpublished). Rudel and colleagues (1995a) have described the presence of a 75 kDa polypeptide in pili purified from Gc PilC⁻ pseudorevertants expressing high levels of piliation, a peptide which may be PilQ. Although the mutation responsible for alleviating the biogenesis requirement for PilC is undefined, the probability that the 75 kDa polypeptide is PilQ strengthens the notion that these two molecules interact. If PilC and PilQ interactions are essential for pilus biogenesis, it is curious that a PilC-like molecule has yet to be detected in Pa. In fact, although Gc pilin can be expressed as pili in *P. aeruginosa* (Hoyne *et al.*, 1993), it was reported that this species does not possess a PilC homologue (Rudel *et al.*, 1995a). Nonetheless, given the similarities between Gc/Pa/Ps PilP and PilQ and other assembly components, the functional equivalents of PilC *vis à vis* assembly will probably be found in these *Pseudomonas* species.

The evidence in favour of a physiologically significant role for the HMM homomultimers seen for Gc PilQ and its homologues continues to mount. The ability of pIV to form SDS- and heat-resistant HMM species has been shown to correlate with function in phage morphogenesis (Linderoth *et al.*, 1996). SDS- and heat-resistant HMM forms have now also been observed for PulD in *Klebsiella oxytoca* (Hardie *et al.*, 1996) and YscC in *Yersinia pestis* (Plano and Straley, 1995), which suggests that this property may be a common feature of this family of molecules. However, the only mutations characterized to date which lead to a reduction or absence of the HMM forms of these homologues map within their respective structural gene. In that sense, Gc PilP appears to be the first molecule identified other than the PulD homologues themselves which significantly influences formation or stability of the HMM species. It is possible that PilP promotes the efficiency or avidity with which PilQ binds to the membrane matrix used for immunodetection. While we cannot rule this out, we would note that we know of no examples in the literature of such a phenomenon. Moreover, recombinant PilQ expressed in the absence of PilP in *E. coli* binds with high efficiency to PVDF membranes (data not shown).

Recently, Hardie and colleagues (1996) reported that the lipoprotein PulS, essential for pullulanase secretion, was required for outer membrane localization of PulD. They observed no reduction in the level of HMM PulD in a PulS⁻ background but noted that membrane-dissociated HMM species consisted solely of stable, proteolytic breakdown products. Based on these findings, PulS was proposed to represent the first member of a new family of periplasmic chaperones required for outer membrane insertion of PilQ-like molecules. It is clear that the absolute pilus defects seen in the Gc PilP⁻ mutants cannot be solely a consequence of the reduced levels of HMM PilQ, given that PilQ mutants expressing lower levels of the HMM species are piliated. The HMM forms seen in PilP⁻ mutants appear, therefore, to be defective, perhaps because they are not localized properly, as suggested by the increased levels of soluble PilQ seen in culture supernatants of PilP⁻ mutants. Alternatively, or in addition, PilP may actually be an integral component of the macromolecular structure. Attempts to begin to address these possible explanations are now in progress. As noted above, similar explanations could account for the findings in the pullulanase system (Hardie *et al.*, 1996) and, thus, although there is no obvious sequence identity between PulS and Gc PilP, the findings and fundamental basis or observations in the two systems may be in concordance. In most Gram-negative species, lipoprotein localization appears to be dependent on the amino acid residue immediately adjacent to the processing site (Yamaguchi *et al.*, 1988). Similar to the case for PulS (d'Enfert and

Pugsley, 1989), the localization of PilP is probably in the outer membrane, as it does not have an acidic residue at position +2. Lipoproteins of unknown function (other than an essential role in each cognate system) have been found in association with other type IV pilus biogenesis (Parsot *et al.*, 1991; Stone *et al.*, 1996) and protein-secretion systems (Michiels *et al.*, 1991; Allaoui *et al.*, 1992; Condemine *et al.*, 1992). Lipoproteins also play essential roles in T-DNA transfer mediated by *Agrobacterium tumefaciens* (Fernandez *et al.*, 1996) and expression of related pilus conjugation functions (Perumal and Minckley, 1984; Doran *et al.*, 1994; Pohlman *et al.*, 1994). The relevance of the relationships defined in this study and in the pullulanase system between lipoproteins and stabilization of macromolecular complexes required for outer membrane trafficking to these other transport processes remains to be determined.

Experimental procedures

Bacterial strains, plasmids, and growth conditions

Bacterial strains and relevant plasmids are detailed in Table 2. Gonococcal strains were propagated on clear solid Gc media at 37°C in 5% CO_2 or in media lacking agar that had been preincubated overnight in 5% CO_2 (Koomey *et al.*, 1987). For examination of secreted/released proteins in culture supernatants, chemically defined media (containing free amino acids) was used as previously described (Catlin, 1973). *E. coli* HB101 (Bethesda Research Laboratories) was used in plasmid-cloning experiments and recombinant strains were grown at 37°C in Luria–Bertani (LB) medium supplemented with the appropriate antibiotics. *E. coli* HB101 was used as the host for subcloning experiments. *E. coli* BL21(DE3) was used for T7 expression experiments. The DNA sequence for the *pilO–pilQ* gene cluster is maintained in the EMBL/GenBank/DDBJ Nucleotide Sequence Data Libraries under Accession Number U40596.

General protocols

Gonococcal transposon mutants were constructed primarily by shuttle mutagenesis using the minitransposon m-Tn*Cm* (a derivative of Tn*3*) as described previously (Seifert *et al.*, 1986). The precise sites of insertions were determined by DNA sequencing with primer Tn*3*L (5′-CTCATGACCAAAATCCC-3′) which is complementary to sequences at the left end of the transposon. These and other mutations were introduced into N400 by transformation using plasmid DNAs as detailed previously (Tønjum *et al.*, 1995) and selection for chloramphenicol resistance (10 μg ml⁻¹). Gc strain J8 was generated by transformation of N400 with pSD DNA and selection for resistance to kanamycin (50 μg ml⁻¹) and the correct introduction of the plasmid into the genome was verified by Southern hybridization. The N400 strain harbours the *recA6* allele in which the *recA* gene is under the control of an inducible *tac–lac* promoter (Freitag *et al.*, 1995). For transformation, RecA expression and recombination functions were

Table 2. Bacterial strains and plasmids used in this study.

Strain/Plasmid	Relevant characteristics	Source/Reference
Strain		
N. gonorrhoeae		
N400	Derived from VD300[a]. Contains the *recA6* allele (IPTG inducible)	Tønjum *et al.* (1995)
GO1	*pilO*::m-Tn*Cm* at position 365[b]	This work
GP6	*pilP*::m-Tn*Cm* at position 962[b]	This work
GP7	*pilP*::m-Tn*Cm* at position 931[b]	This work
GP5	*pilP*::*cat* (using *Eco*RI site at position 1178[b])	This work
GPQ9	m-Tn*Cm* at position 1235[b]	This work
GPQ18	m-Tn*Cm* at position 1240[b]	This work
GQ21	*pilQ*::m-Tn*Cm* at position 1250[b]	This work
GQ22	*pilQ*::m-Tn*Cm* at position 1420[b]	This work
GQ1	*pilQ*::m-Tn*Erm* at position 2516[b]	Drake and Koomey (1995)
J8	N400 containing integrated pSD	This work
J81-1	J8 with *pilO*::m-Tn*Cm* at position 365[c]	This work
J81-6	J8 with *pilP*::m-Tn*Cm* at position 962[c]	This work
J81-7	J8 with *pilP*::m-Tn*Cm* at position 931[c]	This work
J81-21	J8 with *pilQ*::m-Tn*Cm* at position 1250[c]	This work
J82-1	J8 with *pilO*::m-Tn*Cm* at position 365[c]	This work
J82-6	J8 with *pilP*::m-Tn*Cm* at position 962[c]	This work
J82-7	J8 with *pilP*::m-Tn*Cm* at position 931[c]	This work
J82-21	J8 with *pilQ*::m-Tn*Cm* at position 1250[c]	This work
E. coli		
BL21 (DE3)	F^{−} *ompT*, r_B^- m_B^- with prophage λ carrying the T7 RNA polymerase gene	Studier *et al.* (1990)
HB101	F^{−}, *mcrB mrr hsdS20* (r_B^- m_B^-) *recA13 supE44 ara14 galK2 lacY1 proA2 rpsL20* (Sm[R]) *xyl15* l[−] *leu mtl1*	Gibco BRL
Plasmid		
pAE8	Cosmid clone	Drake and Koomey (1995)
pBluescript-II SK+	Cloning vector	Stratagene
pKSD	*Eco*RV−*Cla*I 1.6 kb fragment in pBluescript-II SK+	This work
pSD	*Eco*RV−*Cla*I 1.6 kb fragment in pHSS6	This work
pGP5	*Eco*RV−*Cla*+I 1.6 kb containing *cat* cassette (derived from pCM7) insertion at the *Eco*RI site	This work
pT7-5	T7 RNA polymerase promoter f10, Ap[R]	Tabor and Richardson (1985)
pT7P	*Xho*I (*Eco*RV)−*Cla*I fragment (derived from pKSD) in pT7-5	This work

a. VD300 is a MS11 derivative (Koomey *et al.*, 1987).
b. Derived by transformation using pSD derivative plasmid DNAs (Figs 1 and 2). Nucleotide positions correspond to those in EMBL/Genbank/DDBJ Nucleotide Sequence Data Libraries under Accession Number U40596.
c. Strains contain insertions 5′ (J81−) or 3′ (J82−) of the integrated vector. Locations of transposon insertions are shown in Fig. 2.

transiently induced in this background by IPTG addition to a final concentration of 200 μM at the same time at which transforming DNA was added.

The complete sequences of cloned DNAs were determined using the dideoxy chain-termination method (Sanger *et al.*, 1977) with Sequenase (USB) in conjunction with standard vector-based and customized oligonucleotide primers. The *pilE* (pilin subunit-encoding) gene nucleotide sequence of each mutant was determined by thermocycle sequencing of polymerase chain reaction (PCR) products using CircumVent polymerase (New England Biolabs) and customized oligonucleotide primers. DNA and protein sequence data was compiled and analysed by computer using both the MacVector 3.5 (International Biotechnologies Inc.) and the University of Wisconsin Genetics Computer Group (UWGCG) software packages (Devereux *et al.*, 1984). Homology searches using the sequenced DNA and translated ORFs were performed by accessing the BLAST network server at the National Centre for Biotechnology Information (Gish and States, 1993).

Gonococcal transformation competence assay

Competence for natural transformation of gonococcal strains was assessed as described using chromosomal DNA from spontaneous rifampicin- and naladixic acid-resistant mutants of VD300 (Zhang *et al.*, 1992). RecA expression and recombination functions were transiently induced by IPTG addition to a final concentration of 200 μM at the same time at which transforming DNA was added (see above).

Transmission electron microscopy

Electron microscopy samples were prepared by placing carbon-coated Formvar 3.05 mm grids (Tousimas Research Corp.) onto individual bacterial colonies propagated for 16 h. Grids were air dried for 10 min, floated on a drop of 0.25% sodium phosphotungstic acid (NaPT), pH 7.0, for 30 s and air dried again. Grids were examined using a Philips CM-10 Transmission Electron Microscope.

SDS−PAGE and immunoblotting

The presence of PilE, PilQ and PilC in whole-cell lysates and concentrated culture supernatants was detected by immunoblotting using rabbit polyclonal antibodies as described previously (Drake and Koomey, 1995) and rabbit polyclonal PilC-specific antibodies (a gift of T. F. Meyer). Antigen detection was performed by use of alkaline phosphatase-coupled goat anti-rabbit immunoglobulin antibodies (Tago Inc.) and a colourimetric alkaline phosphate substrate. Conditions for sample preparation, SDS−PAGE, membrane transfer and antigen detection have been described previously (Blake *et al.*, 1984). Proteins were transferred to Immobilon-P[SQ] PVDF membranes (Millipore Corp.). Pre-stained molecular weight protein standards (Diversified Biotech and Gibco BRL) were used).

[3H]-palmitic acid labelling of lipoproteins

[3H]-palmitic acid (specific activity 50 Ci mM[−1]; Amersham) was concentrated by evaporation of solvent and added to

cultures to achieve a concentration of 20 μCi ml^{-1}. The PilP protein, expressed in *E. coli* by using coupled T7 RNA polymerase–promoter gene fusions in strain BL21 (DE3; Table 2), was labelled under standard conditions used for amino acid incorporation with the exception that LB medium (rather than defined minimal media) was used. Gonococcal liquid cultures were started using suspensions of fresh (16-h old) plate-grown cells diluted to give an initial concentration of 5×10^6 colony-forming units (cfu) ml^{-1} and grown for 5 h at 37°C in the presence of [^{3}H]-palmitic acid. Proteins of whole-cell lysates were separated by SDS–PAGE, transferred to Immobilon-P^{SQ} PVDF membranes (Millipore Corp.) and treated with Enhance (Dupont-NEN) for fluorography, as recommended by the manufacturer. Autoradiography was performed over 2–21 d using Kodak X-ray films.

Acknowledgements

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