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Characterization of the *pilF–pilD* pilus-assembly locus of *Neisseria gonorrhoeae*

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Summary

Expression of Type IV pili by the bacterial pathogen *Neisseria gonorrhoeae* appears to be essential for colonization of the human host. Several *N. gonorrhoeae* gene products have been recently identified which bear homology to proteins involved in pilus assembly and protein export in other bacterial systems. We report here the isolation and characterization of transposon insertion mutants in *N. gonorrhoeae* whose phenotypes indicate that the *N. gonorrhoeae pilF* and *pilD* gene products are required for gonococcal pilus biogenesis. Mutants lacking the *pilD* gene product, a pre-pilin peptidase, were unable to process the pre-pilin subunit into pilin and thus were non-piliated. *pilF* mutants processed pilin but did not assemble the mature subunit. Both classes of mutants released S-pilin, a soluble, truncated form of the pilin subunit previously correlated with defects in pilus assembly. In addition, mutants containing transposon insertions in *pilD* or in a downstream gene, *orfX*, exhibited a severely restricted growth phenotype. Deletion analysis of *pilD* indicated that the poor growth phenotype observed for the *pilD* transposon mutants was a result of polar effects of the insertions on *orfX* expression. *orfX* encodes a predicted polypeptide of 23 kDa which contains a consensus nucleotide-binding domain and has apparent homologues in *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Thermus thermophilus*, and the eukaryote *Caenorhabditis elegans*. Although expression of *orfX* and *pilD* appears to be transcriptionally coupled, mutants containing transposon insertions in *orfX* expressed pili. Unlike either *pilF* or *pilD* mutants, *orfX* mutants were also competent for DNA transformation.

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Introduction

The expression of pili by *Neisseria gonorrhoeae* plays an essential role in gonoccocal colonization of the human host. Pili are filamentous appendages which consist of a polymerized protein subunit of 18–24 kDa known as pilin; this polypeptide is to date the only recognized integral component of the pilus organelle. The short leader sequence and proximal 30 amino acids of gonococcal pre-pilin have a high degree of homology with Type IV pre-pilins of other Gram-negative organisms including *Neisseria meningitidis* (Potts and Saunders, 1988), *Moraxella* species (Tønjum *et al.*, 1991; Marrs *et al.*, 1985), *Dichelobacter nodosus* (Elleman and Hoyne, 1984), *Pseudomonas aeruginosa* (Strom and Lory, 1986), *Vibrio cholerae* (Shaw and Taylor, 1990), and the pilin of certain strains of enteropathogenic *Escherichia coli* (Giron *et al.*, 1991).

A strong correlation exists between pilus expression and the ability of *N. gonorrhoeae* to colonize humans, its sole host. Non-piliated mutants fail to colonize the urethras of male volunteers (Kellog *et al.*, 1968; Swanson *et al.*, 1987) and results from several studies support the notion that pili facilitate adherence of the bacterium to the mucosal surface (Swanson, 1973; McGee *et al.*, 1981; Heckels, 1989). Pilus expression therefore appears to be a requisite determinant of infectivity and the organelle may be thought of as a colonization factor.

Previous attempts to isolate and characterize pilus assembly mutants in gonococci have met with limited success. Mutations within the pilin subunit occur frequently as a consequence of the recombination process responsible for antigenic variation (Koomey *et al.*, 1987), and even in recombination-deficient strains, pilin mutations account for a majority of non-piliated mutants. Despite these difficulties, PilC, a 110 kDa protein which co-purifies with the gonococcal pilin subunit has been identified, and gonococcal mutants which fail to express PilC were reported to express few, if any, pili (Jonsson *et al.*, 1991). Recent reports indicate, however, that it is possible to isolate gonococcal suppressor mutants which regain pilus expression in the absence of PilC; thus the precise role of PilC in pilus assembly remains unclear (Rudel *et al.*, 1992).

An examination of proteins involved in Type IV pilus biogenesis in other systems has revealed that they are structurally and functionally related to components required for

the two-step (signal-sequence dependent) translocation of proteins to the exterior of the cell in many prokaryotic systems. Type IV pilus biogenesis proteins share identity with proteins involved in protein secretion, morphogenesis of filamentous bacteriophages, and DNA transfer by conjugation and transformation (Hobbs and Mattick, 1993; Pugsley, 1993; Strom and Lory, 1993). Based on these homologies, several genes were recently identified in *N. gonorrhoeae* whose products appear related to proteins required for Type IV pilus assembly and for transport of large molecules across the bacterial cell surface (Lauer *et al.*, 1993). Two of these genes, *pilD* and *pilF*, were found to map to a single locus remote from the pilin expression locus, *pilE*, where the pilus subunit-encoding gene is found (Lauer *et al.*, 1993). Both *pilD* and *pilF* share significant structural relatedness to proteins found in virtually all of the two-step protein export pathways that have been genetically dissected. The *pilD* gene product has a high degree of similarity with the *P. aeruginosa* cytoplasmic membrane-localized pre-pilin peptidase encoded by *pilD/xcpA* (Nunn *et al.*, 1990; Bally *et al.*, 1991) as well as the *pulO* gene of *Klebsiella oxytoca* (Pugsley and Reyss, 1990), the *comC* gene of *Bacillus subtilis* (Mohan *et al.*, 1989), and the *tcpJ* gene of *V. cholerae* (Kaufman *et al.*, 1991). When expressed in *E. coli*, the *N. gonorrhoeae pilD* gene product was shown to process gonoccocal pre-pilin (Lauer *et al.*, 1993; Dupuy and Pugsley, 1994) as well as the pilin-like proteins PulG of *K. oxytoca* (Dupuy and Pugsley, 1994) and ComGC of *B. subtilis* (Chung and Dubnau, 1995), indicating that PilD is indeed a pre-pilin leader peptidase. *pilF*, which shares homology with *P. aeruginosa pilB* (Nunn *et al.*, 1990), *K. oxytoca pulE* (Pugsley *et al.*, 1990), *Xanthomonas campestris xpsE* (Dums *et al.*, 1991), *V. cholerae epsE* (Sandkvist *et al.*, 1993), and *comG1* of *B. subtilis* (Albano *et al.*, 1989), encodes a gene product which has an overall hydrophilic character with a lack of obvious membrane-spanning regions, and possesses a consensus nucleotide-binding site (Walker *et al.*, 1982). The structural integrity of the nucleotide-binding domain has been shown to be essential for PilB-dependent pilus assembly and XcpR-dependent protein secretion in *P. aeruginosa* (Turner *et al.*, 1993) and PulE-dependent secretion of pullulanase in *K. oxytoca* (Possot and Pugsley, 1994). In summary, *N. gonorrhoeae* PilD and PilF share significant homology with proteins involved in Type IV pilus assembly and protein-export pathways; however, it remained to be demonstrated whether these two gene products participated in gonococcal pilus expression.

In this work, we show that both the *pilD* and the *pilF* gene products are essential for pilus assembly in *N. gonorrhoeae* since strains containing mutations in either gene were absolutely defective in pilus expression. The mutants were also found to release S-pilin, a truncated form of the pilin subunit protein which has been associated with reduced levels of piliation or absence of pilus assembly (Koomey *et al.*, 1991; Haas *et al.*, 1987; Koomey *et al.*, 1987; Swanson *et al.*, 1986). In addition, the *pilD* and *pilF* gene products were found to be required for competence, a property tightly associated with pilus expression in *N. gonorrhoeae* (Sparling, 1966; Biswas *et al.*, 1977; Biswas *et al.*, 1989) although the relationships between the two processes are unclear. Lastly, we have identified an additional gene, *orfX*, located downstream of *pilD*. Although *orfX* and *pilD* are co-transcribed, mutants containing transposon insertions in *orfX* assembled pili and were competent for DNA transformation but exhibited a severely restricted growth phenotype.

Results

Transposon mutagenesis of the N. gonorrhoeae pilF–pilD *region*

To determine if the *pilF* and *pilD* gene products are

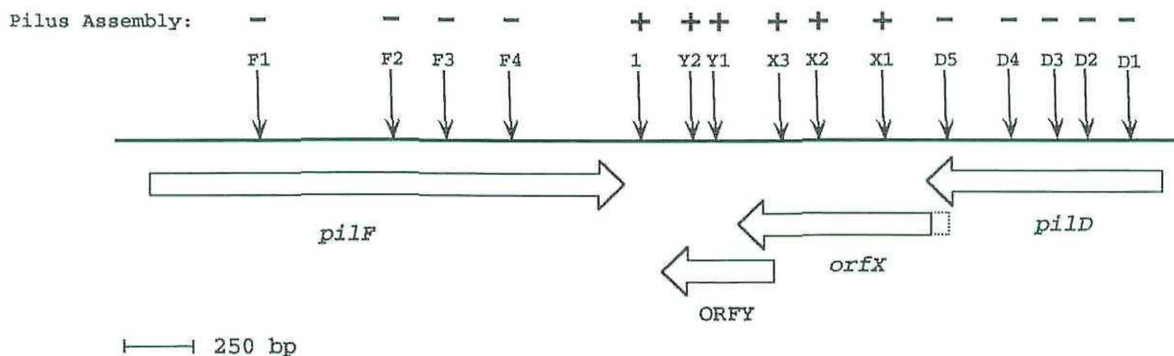

Fig. 1. Map of the *pilF–pilD* locus and location of transposon insertion mutations. Large open arrows indicate gene location and orientation. Vertical arrows designate sites of m–Tn*3erm* transposon insertions. Gonococcal strains containing transposon insertion mutations are designated with a G followed by the location of the m–Tn*3erm* as depicted above (for example, GF1 represents the gonococcal mutant carrying the F1 *pilF*::m–Tn*3erm*).

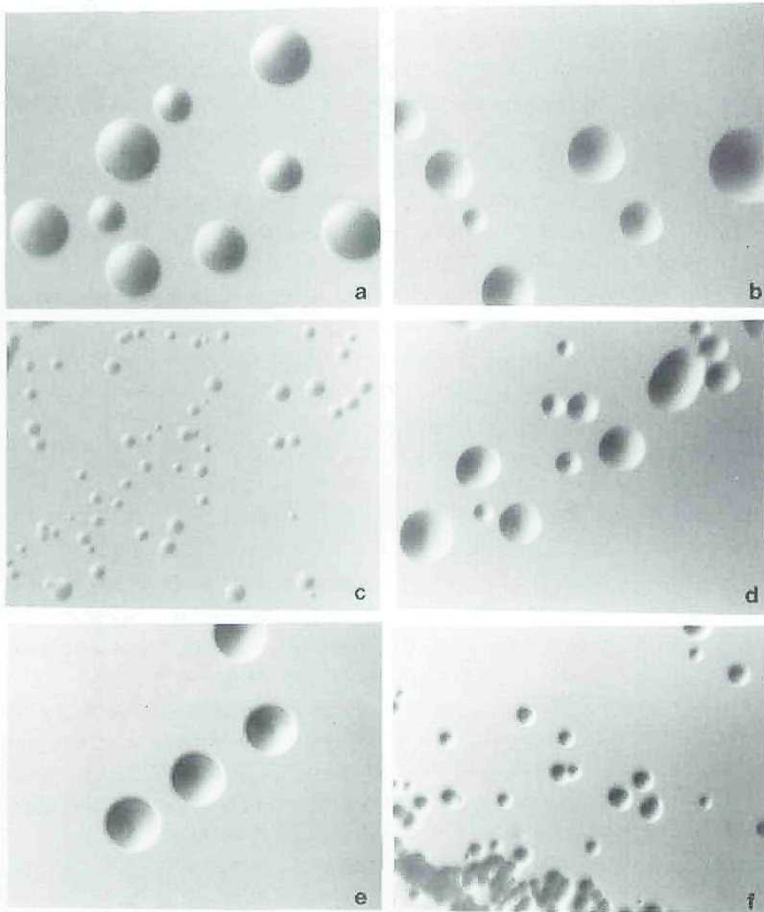

Fig. 2. Colony morphologies of gonococcal transposon insertion mutants. (a) N400 (wild type); (b) GF2 (*pilF*::m–Tn*3erm*); (c) GD3 (*pilD*::m–Tn*3erm*); (d) GD (*Cla*I → *Xho*I), (*pilD* frameshift mutant); (e) GY1 (ORFY::m–Tn*3erm*); (f) GX2 (*orfX*::m–Tn*3erm*).

required for *N. gonorrhoeae* pilus assembly, a plasmid containing *pilF–pilD* sequences was subjected to saturated transposon mutagenesis as described in the *Experimental procedures*. Selected insertions (shown in Fig. 1) were then moved by transformations onto the chromosome of strain N400 which carries the *recA* gene under the control of an IPTG-inducible promoter. In the absence of induction, N400 bacteria are essentially $RecA^-$, thereby preventing *pilE* gene rearrangements (Koomey *et al.*, 1987). Transformations were carried out in the presence of IPTG to enable homologous recombination of the mutations into the chromosome. Distinct differences in colony morphologies of transposon insertion mutants were readily apparent in the transformants (Fig. 2). All *pilF* and *pilD* mutants lost the aggregative phenotype (a well-defined colony edge) observed for the piliated (P^+) parent N400 strain and similarly failed to autoagglutinate when grown in liquid culture. *N. gonorrhoeae* mutants containing transposon insertions within *pilD* and in a 600 bp region located immediately downstream of *pilD* formed petite, slow-growing colonies, although transposon mutants carrying insertions downstream of *pilD* retained a piliated colony

Table 1. Phenotypes of *pilF–pilD* region transposon insertion mutants.

Strain	Description	Pili [a]	% Competence for DNA transformation [b]	Growth [c]	Pre-pilin processing
N400	Wild type	+	100%	+++	+
GF2	*pilF*::m–Tn*3erm*	–	< 0.1%	+++	+
GD3	*pilD*::m–Tn*3erm*	–	< 0.1%	+	–
GX2	*orfX*::m–Tn*3erm*	+	100%	+	+
GY1	ORFY::m–Tn*3erm*	+	100%	+++	+
G-1	Intergenic m–Tn*3erm*	+	100%	+++	+

a. As assayed by transmission electron microscopy.
b. Measured under *recA*-induced conditions.
c. In rich peptone-based media.

morphology (Fig. 2). In contrast, transposon insertion mutants G-1 and GY1 were indistinguishable in their patterns of growth from the parent strain.

N. gonorrhoeae pilF *and* pilD *are required for pilus assembly and competence*

The transposon insertion mutants were examined for the expression of assembled pili. Southern analysis of the *pilF* and *pilD* mutants indicated that these genes are present in single copy in the gonococcal chromosome (data not shown). Transposon insertions within *pilF* or *pilD* eliminated pilus assembly as detected by transmission electron microscopy (Table 1, data not shown). The remaining transposon mutants expressed pili on their cell surfaces, therefore gene products encoded by potential genes located between *pilF* and *pilD* are not required for pilus assembly. The nucleotide sequences of *pilE* (the pilin subunit gene) were determined for the non-piliated mutants and found to be unaltered. The results indicated that defective pilus expression in these mutants could not be ascribed to changes in pilin gene status. The phenotypes of *pilF* and *pilD* mutants thus demonstrate that both gene products are required for pilus assembly.

Competence for DNA transformation is a pilus-associated phenotype (Sparling, 1966; Biswas *et al.*, 1977; Biswas *et al.*, 1989). The transposon insertion mutants were examined for relative competence by selection for a drug-resistance marker following DNA transformation using genomic DNA from either rifampicin- (Rif^R) or nalidixic acid-resistant (Nal^R) gonococcal strains. The *pilF* and *pilD* transposon insertion mutants had greatly reduced transformation frequencies in comparison to the parent strain, whereas the remaining transposon insertion mutants appeared fully competent (Table 1). The transformation frequencies observed for both *pilF* and *pilD* mutants are similar to those observed for non-piliated *N. gonorrhoeae* strains containing mutations located within *pilE*, the gene encoding the pilin subunit (Seifert *et al.*, 1990; Zhang *et al.*, 1992) and *pilG*, encoding an essential pilus assembly factor (Tønjum *et al.*, 1995).

Identification of two open reading frames located between pilF *and* pilD

Analysis of sequence data from the intergenic region between *pilF* and *pilD* indicated the presence of two large open reading frames (ORFs), designated here as ORFX and ORFY (Fig. 1). Based on sequence analysis, the ATG initiation codon of ORFX may overlap the stop codon of *pilD*; alternatively, the protein may begin from an ATG located within *pilD* coding sequences. Neither start codon appears to have a consensus ribosome-binding site preceding it (Gold, 1988). ORFX was predicted to

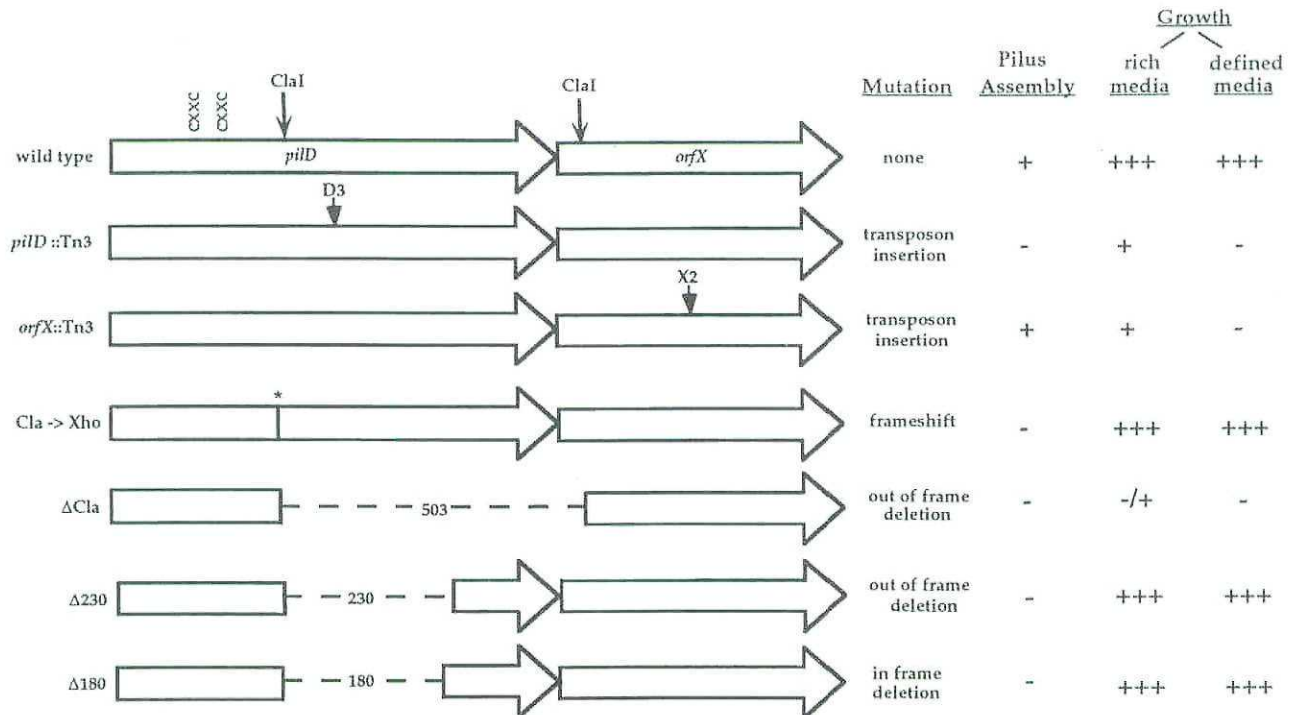

Fig. 3. Genotypes and phenotypes of *pilD* and *orfX* mutants. Unmarked mutations were introduced into N400 by selection for a downstream transposon-encoded antibiotic-resistance marker as described in the *Experimental procedures*.

encode either a 206- or 223-amino-acid polypeptide of 23 kDa or 24 kDa, depending on the location of its translation initiation codon. The putative initiation codon for ORFY appears to be located within ORFX coding sequences, and ORFY was predicted to encode a 106-amino-acid polypeptide of approximately 12 kDa. The isoelectric points (pIs) of both predicted polypeptides were very basic, with values of 9.5 (or 9.1 for the larger polypeptide) for the ORFX polypeptide and 9.8 for the ORFY polypeptide.

The orfX *gene product is required for normal growth*

All *pilD*- and ORFX-disrupting transposon insertion mutants exhibited a severe growth-restricted phenotype in either rich peptone-based liquid or solid media (Koomey *et al.*, 1987), and no growth was detected for them in a chemically defined media (Catlin, 1973) (Fig. 3). The *pilE* genes in the ORFX transposon insertion mutants were unchanged and so pilus variation could not account for their unique colony morphologies. Based on the orientation of *pilD* and ORFX coding sequences, it was possible that the transposon insertions isolated within *pilD* were exerting polarity on expression of ORFX. If this were true, then mutants carrying non-polar *pilD* mutations would not exhibit the growth-restricted phenotype. To determine directly if the restricted growth phenotype observed for the *pilD* insertion mutants reflected a loss or reduction of *orfX* expression, in-frame and out-of-frame deletion mutations and a frameshift mutation generated by *Xho*I–linker insertion were constructed in *pilD*. These alleles were introduced into N400 by selection for a downstream transposon-encoded antibiotic-resistance gene (see the *Experimental procedures*) (Fig. 3). Both in-frame and out-of-frame *pilD* deletion mutants as well as the frameshift mutant failed to express pili; however, all mutants exhibited growth phenotypes similar to the wild-type parent strain and to the *pilF* mutants (Fig. 3). The petite, restricted-growth colony phenotype observed

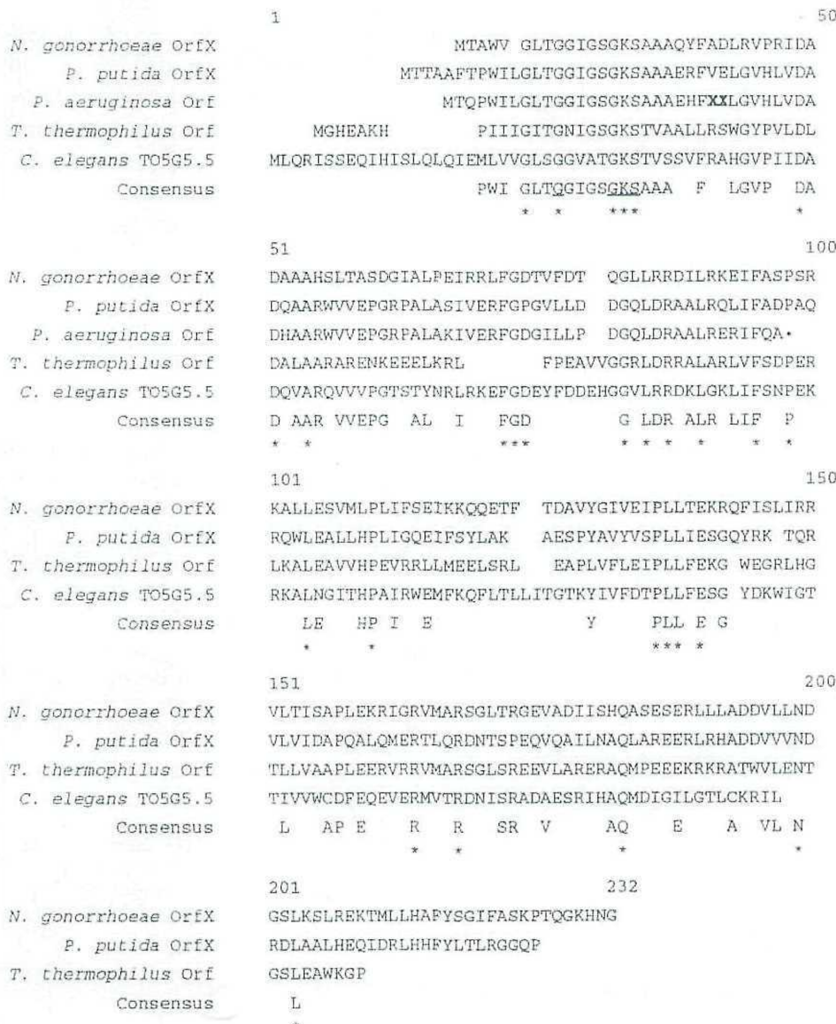

Fig. 4. Comparison of *N. gonorrhoeae* OrfX to open reading frames previously identified in *P. aeruginosa, P. putida, T. thermophilus,* and *C. elegans.* Comparative alignment of the deduced amino acid sequences was performed using the GAP program of the UWGCG package. Amino acid homology observed for the partial *P. aeruginosa* ORF was extended by switching to an alternative reading frame at the position denoted by the two bold X's. The • symbol represents the end of the reported *P. aeruginosa* sequence. A consensus sequence based on residues shared by three or more members is shown below. Amino acid residues conserved in all ORFs are indicated by an asterisk. The consensus nucleotide-binding site has been underlined.

for the *pilD* transposon insertion mutants therefore appears to be the result of polar effects of the transposon on ORFX expression and suggests that *pilD* and ORFX are transcriptionally linked. We infer from this genetic evidence that OrfX is indeed a functional product expressed in *N. gonorrhoeae*, and have designated the gene *orfX*.

N. gonorrhoeae *OrfX has potential homologues in* P. aeruginosa, Pseudomonas putida, Thermus thermophilus, *and* Caenorhabditis elegans

A database search revealed no obvious homologies for the ORFY open reading frame. OrfX, however, shared significant identity with three other ORFs, all of which contain a consensus nucleotide-binding sequence or Walker box A (Walker *et al.*, 1982) (Fig. 4). An ORF located downstream from a putative insertion sequence element in *T. thermophilus* shares 37% identity and 54% similarity (Ashby and Berquist, 1990), and an ORF identified in the nematode *C. elegans* shares 32% identity and 52% similarity (Wilson *et al.*, 1994). However, neither of the protein products encoded by these putative genes has been assigned any function. An additional ORF homology was found encoded by a potential gene, *orfX*, located immediately downstream of the *pilD* peptidase gene of *P. putida* (deGroot *et al.*, 1994). The orientation of the *P. putida orfX* with respect to the *pilD* encoded peptidase is identical to the orientation of *pilD* and *orfX* observed in *N. gonorrhoeae*, and includes the overlap of the potential *orfX* translation intitiation codon with the stop codon of *pilD*. *N. gonorrhoeae* OrfX and *P. putida* OrfX share 40% identity and 56% similarity. It is worth noting that a similar partial ORF exists downstream of *P. aeruginosa pilD* (Bally *et al.*, 1991) but the entire sequence of this region has not been reported. OrfX homology, including the nucleotide-binding consensus domain, can be recognized in the translation of the partial sequence immediately downstream of *P. aeruginosa pilD*, and additional homology extends through an alternative reading frame within the reported sequence (Fig. 4).

N. gonorrhoeae pilD *mutants fail to process pre-pilin*

Expression and processing of the pre-pilin subunit protein was examined in the transposon and *pilD* deletion mutants (Fig. 5). Polyclonal antibodies generated against purified pili isolated from *N. gonorrhoeae* were used in Western analysis of whole-cell lysates. Strains containing transposon insertions or deletions in *pilD* did not process pre-pilin as evidenced by the presence of a slower migrating form of the pilin subunit in these strains, which co-migrates with a pre-pilin resistant to pre-pilin peptidase cleavage (Koomey *et al.*, 1991; Fig. 5, top panel, lanes 7–9). It has been previously demonstrated that antibodies generated against *P. aeruginosa* PAK pili recognize only processed pilin in *N. gonorrhoeae* (Koomey *et al.*, 1991). The failure of these antibodies to recognize the pilin subunit in *pilD* mutants confirms the lack of processing (Fig. 5, lower panel, lanes 7–9). Mutants with transposon insertions in *pilF*, *orfX*, ORFY, and the intergenic region between *pilF* and ORFY all processed pre-pilin to the faster migrating form (Fig. 5, lanes 3–6). The production of S-pilin was also observed in the *pilF* and *pilD* mutants indicating that neither PilF nor PilD function is required for this altered form of processing.

In the parent strain, the processed pilin subunit has been observed to migrate as a doublet and pilin purified from assembled pili migrates as the lower band of the doublet (Koomey *et al.*, 1991). Interestingly, *pilF* mutants and, to a lesser extent, *orfX* mutants, display the upper band of the doublet as the predominant subunit species, whereas in the parent strain the lower band predominated. 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 and significance remains unknown. Studies in *P. aeruginosa* have indicated that methylation does not alter the

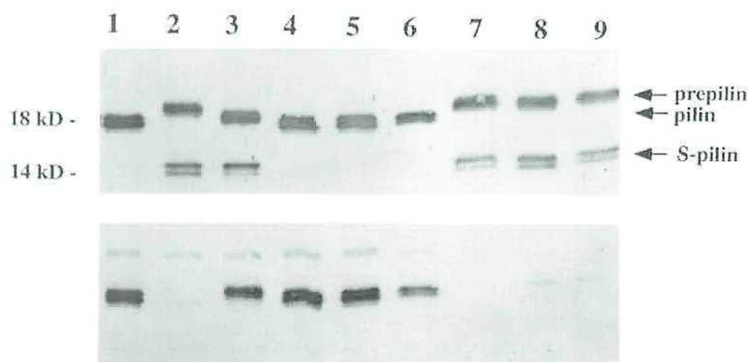

Fig. 5. Immunoblotting of whole cell lysates to detect pilin expression. Polyclonal GC2-66 antibody generated against purified gonococcal pili (upper panel) or polyclonal PAK 2-567 antibody generated against purified pili from *P. aeruginosa* PAK (lower panel) was used to identify pilin antigen. Samples were standardized for total protein. Lanes: (1) N400 (wild type); (2) VD302 P$^-$ (Gly-1 → Ser-1); (3) GF2 (*pilF*::m–Tn*3erm*); (4) G-1 (intergenic m–Tn*3erm*); (5) GY1 (ORFY::m–Tn*3erm*); (6) GX2 (*orfX*::m–Tn*3erm*); (7) GD3 (*pilD*::m–Tn*3erm*); (8) GD (*Cla*I → *Xho*I); (9) GDΔ180 (in-frame *pilD* deletion).

relative mobility of pilin in that system (Strom *et al.*, 1993); however, we have not yet determined the methylation status of the pilin doublet.

Characterization of a pilD–orfX *deletion mutant*

pilD transposon insertion mutants grew at rates slightly slower than *orfX* mutants. It thus appeared that mutants deficient in both *pilD* and *orfX* expression were more severely growth restricted than mutants carrying only the *orfX* gene lesion. We therefore constructed a deletion mutation, which would disrupt both *pilD* and *orfX*, to assess the phenotypes associated with loss of both gene products. The construction, created by deletion of a 0.5 kb *Cla*I fragment (Fig. 3), was introduced into the chromosome of N400 by transformation and selection for a downstream transposon-encoded antibiotic-resistance gene. This mutation results in the deletion of sequences encoding the C-terminal 141 amino acids of PilD and N-terminal 42 amino acids of the smaller predicted OrfX polypeptide but does not translationally fuse the two reading frames. Two colony types were observed among the erythromycin-resistant transformants. One class of transformants was visible after 24 hours of growth and resembled the P^+ parent strain. These mutants were found to have only crossed in the transposon-linked antibiotic-resistance gene and not the upstream deletion. After four days of incubation, tiny colonies were visible on the transformation plates, which were not visible on plates of bacteria transformed with control DNA lacking the deletion but bearing the linked transposon insertion. The severe growth restriction of these mutants was somewhat alleviated when the cells were grown in the absence of antibiotic (data not shown). Attempts to isolate genomic DNA sufficient for Southern analysis from these ultrapetite colonies were unsuccessful. The extreme restricted growth phenotype exhibited by the *pilD–orfX* double mutant suggests that loss of both gene products has a dramatic effect on cell viability, much greater than that observed with loss of *orfX* alone. However, after several days of growth, non-piliated faster growing variants could be observed within the mutant populations. Although these variants exhibited improved growth in rich media, they remained unable to grow in defined media. Southern analysis of genomic DNA isolated from these latter variants indicated that they indeed carried the 0.5 kb *Cla*I deletion. To assess the status of pilin in the *pilD–orfX* deletion mutants, Western analysis of six independently arising fast-growing variants was performed. The fast-growing pseudorevertants were found to be composed of two distinct classes. In four of the six cases, pilin expression was dramatically decreased (Class I), whereas in the other two cases no pilin antigen was detected (Class II). In the Class I mutants, the pilin subunit migrated at the position of unprocessed pilin (data not shown).

Discussion

The findings detailed here indicate that the *pilD* and *pilF* gene products are required by *N. gonorrhoeae* for pilus assembly. Transposon insertions in either gene or deletions within *pilD* completely eliminated detectable pili on the bacterial cell surface. In addition, *pilF* and *pilD* mutants were found to release S-pilin, a soluble truncated form of pilin which correlates with defects in pilus assembly (Koomey *et al.*, 1991; Haas *et al.*, 1987; Koomey *et al.*, 1987; Swanson *et al.*, 1986). Both classes of mutants expressed the pre-pilin subunit at normal levels, but *pilD* mutants lacked pre-pilin peptidase activity and were unable to process pre-pilin to mature pilin. The results presented here confirm the identities of these two gene products as Type IV pilus-assembly proteins in *N. gonorrhoeae* and as functional members of an extensive family of proteins involved in the transport of macromolecules into and out of the bacterial cell. The *pilF* and *pilD* gene products, in addition to the recently described PilG protein (Tønjum *et al.*, 1995), therefore represent the first unambiguous identifications of proteins absolutely required for pilus assembly in *N. gonorrhoeae*.

In addition to pilus assembly, the data presented here indicate that the *pilF* and *pilD* gene products are required for competence for DNA transformation. Competence has been shown to be a pilus-associated property as non-piliated variants have been shown to be dramatically reduced in competence for DNA transformation (Sparling, 1966; Biswas *et al.*, 1977; Biswas *et al.*, 1989; Seifert *et al.*, 1990; Zhang *et al.*, 1992). However, the relationship between pilus expression and competence is not well understood and it is not known whether pili are structural components of the machinery for DNA binding and uptake or if expression of a competent phenotype is perhaps co-regulated with, or in some other way dependent upon, pilus biogenesis. Efforts to detect DNA-binding activity by purified pilin subunit or by assembled pili have been unsuccessful (Mathis and Scocca, 1984; Sparling *et al.*, 1977). Homologues of PilF and PilD (the ComG-1 and ComC proteins, respectively) are required in *B. subtilis* for natural competence (Albano *et al.*, 1989; Mohan *et al.*, 1989), and it has been proposed that the two *B. subtilis* proteins are necessary for formation of a complex which transports DNA into the bacterium (Dubnau, 1991). It is possible then that PilF and PilD may be necessary for formation of a similar complex in *N. gonorrhoeae* but it remains to be determined whether or not separate complexes exist for pilus assembly and DNA processing.

The *orfX* gene and an additional open reading frame ORFY are located downstream of *pilD*. *orfX* and ORFY

transposon-insertion mutants expressed pili and were transformable, therefore their products were not required for either pilus assembly or competence. Transposon insertion mutations, which would disrupt expression of ORFY, had no discernible phenotypic difference from the parent *N. gonorrhoeae* strain used in these studies, and so far we have no genetic or biochemical data for the existence of an ORFY-derived polypeptide. In contrast, insertions in *orfX* dramatically affected bacterial growth. *orfX* mutants formed petite, dark coloured opaque colonies and displayed extremely low efficiencies of plating. Examination of the mutants by both light and electron microscopy revealed a high level of cell lysis (data not shown). In addition, the mutants failed to grow in defined media. This genetic evidence lends support to the existence and expression of the OrfX polypeptide, encoded by *orfX*, in *N. gonorrhoeae*.

The function of the *orfX* gene product is not known, although it is clearly important for bacterial viability. The extreme degree of conservation within the OrfX homologue family is particularly striking in this regard (Fig. 4). Pairwise aligments of the homologues reveal that they all share at least 30% identity with one another (data not shown) and that the homologies span the full lengths of the polypeptides. The cross-phylum matches found in this family make the similarities even more remarkable and may indicate that OrfX homologues function in an intrinsically fundamental aspect of metabolism. Surprisingly, the *C. elegans orfX* gene contains introns, and the location of these introns may perhaps be useful in defining modular domains of the OrfX protein (Gilbert *et al.*, 1986). Dupuy and Pugsley (1994) used Southern analysis to demonstrate that *Neisseria meningitidis* and other *Neisseria* species all contained a 500–700 bp *Cla*I fragment which hybridized to a *pilD*-derived DNA probe. The conservation of the *Cla*I site downstream of *pilD* coding sequences (defined as a 500 bp fragment) suggests that the organization of *orfX* and *pilD* may be conserved across *Neisseria* species.

Although not essential for pilus biogenesis, several observations suggest that the *orfX* gene product may play a role related to pilus expression or PilD peptidase activity. Expression of *orfX* is transcriptionally coupled to *pilD* and the OrfX$^-$ phenotypes of mutants containing transposon insertions located near the 5′ end of *pilD* (GD1 (Fig. 1), for example) and larger deletions of *pilD* coding sequences (data not shown) support the notion that *orfX* transcription initiates upstream of *pilD*. Western analysis of *orfX* mutants using antibody directed against purified gonoccocal pili revealed the presence of several polypeptide species, distinct from pilin, with altered mobility compared to the parental strain (data not shown). These polypeptides may represent pilus-associated proteins, and the differences in mobility observed may potentially reflect incorrect localization of the proteins or errors in processing or post-translational modification. Finally, construction of a mutant carrying a deletion spanning both *pilD* and *orfX* resulted in ultrapetite colonies which were more severely growth restricted than *orfX* mutants. These ultrapetite colonies gave rise to faster growing sub-populations which had decreased expression of pre-pilin. It appears then that the growth restriction of *pilD–orfX* double mutants in rich media can be compensated for by a reduction in pre-pilin expression. However we have not yet ruled out the possibility that other compensating mutations may have occurred. It is not known at this time if the poor growth phenotype of the *pilD–orfX* double mutants results from the *orfX* mutation in the context of a lack of PilD, pre-pilin processing or pilus assembly in general. If the latter case is true, one would expect a similar phenotype to be observed for *orfX–pilF* double mutants. Although no distinguishable phenotype has yet been observed for ORFY mutants, it is possible that loss of ORFY expression may be contributing to the decreased viability of the *pilD–orfX Cla*I deletion mutants. However, as mentioned above, we have no genetic or biochemical evidence to support the existence and expression of the ORFY polypeptide.

The poor growth of *pilD–orfX* double mutants may explain previous difficulties in generating gonococcal *pilD* mutants (Dupuy and Pugsley, 1994), as the constructs used in those experiments to create *pilD* lesions would in retrospect have eliminated both *pilD* and *orfX* expression. The *pilD–orfX* deletion mutants generated here were more sensitive to erythromycin than the other transposon mutants; it is possible that the double mutants would be hypersensitive to chloramphenicol, the selective marker employed by Dupuy and Pugsley (1994).

The possible relationship between pre-pilin processing/pilus assembly and OrfX, revealed by the dramatic loss in viability of the *pilD–orfX* double mutants, may provide an insight into OrfX function. Indeed, *N. gonorrhoeae* may provide a model system for assigning a role for OrfX and its homologues in other systems based on the phenotypes of *N. gonorrhoeae orfX* mutants. No effect on growth has been reported for transposon insertions isolated in the *pilD* peptidase gene of *P. aeruginosa* (Nunn *et al.*, 1990) although, as previously described, potential *orfX* coding sequences exist downstream of *pilD*. It is possible that the *orfX* gene product is not expressed in *P. aeruginosa*, or that its expression is not linked to that of *pilD* as it is in *N. gonorrhoeae*. To our knowledge, mutants bearing lesions within the potential *orfX* of *P. aeruginosa* have not yet been directly examined; similarly, no relevant mutants have yet been constructed in *C. elegans* or *T. thermophilus orfX*. Pilus assembly has not been observed in *P. putida* despite the presence of Type IV pilus assembly homologues (deGroot *et al.*, 1994), thus a possible link

between pilus assembly and OrfX function may be difficult to assess in this organism.

Preliminary data using antibodies directed against PilD and OrfX suggest the proteins are expressed at very low levels in *N. gonorrhoeae* (data not shown). Koga *et al.* (1993) have reported that low levels of *P. aeruginosa* PilD protein are sufficient to process pre-pilin and precursors of components of the extracellular secretion machinery and that PilD appears to be expressed in *P. aeruginosa* at levels 10-fold higher than needed. A similar situation may exist for PilD (and perhaps OrfX) in *N. gonorrhoeae*. Experiments are currently underway to gain an understanding of OrfX function. We are using reconstitution experiments to determine the basis for the growth defect observed for *orfX* mutants in defined media, and are determining the importance of the consensus nucleotide binding site for OrfX activity. The results obtained from these experiments, and from experiments designed to address the specific functional roles of PilF and PilD, will enable a more detailed understanding of the mechanism of pilus assembly in *N. gonorrhoeae.*

Experimental procedures

Bacterial strains, plasmids, and transposon mutagenesis

The bacterial strains used in this study are described in Table 2.

Table 2. Bacterial strains.

Strain	Relevant characteristics	Source/ Reference
N400	Derived from VD300[a]. Contains the *recA6* allele (IPTG-inducible)	This work
VD302 P$^-$ Gly-1 → Ser-1	Mutant pilin resistant to pre-pilin peptidase activity	Koomey *et al.* (1991)
GF1[b]	*pilF*::m–Tn*3erm* at position 1232[c]	This work
GF2	*pilF*::m–Tn*3erm* at position 1424[c]	This work
GF3	*pilF*::m–Tn*3erm* at position 1620[c]	This work
G-1	m–Tn*3erm* at position 1059[d]	This work
GY1	ORFY::m–Tn*3erm* at position 836[d]	This work
GY2	ORFY::m–Tn*3erm* at position 861[d]	This work
GX1	*orfX*::m–Tn*3erm* at position 281[d]	This work
GX2	*orfX*::m–Tn*3erm* at position 464[d]	This work
GX3	*orfX*::m–Tn*3erm* at position 670[d]	This work
GD1	*pilD*::m–Tn*3erm* at position 317[c]	This work
GD2	*pilD*::m–Tn*3erm* at position 421[c]	This work
GD3	*pilD*::m–Tn*3erm* at position 525[c]	This work
GD4	*pilD*::m–Tn*3erm* at position 655[c]	This work
GD5	*pilD*::m–Tn*3erm* at position 904[c]	This work
GD(*Cla*I → *Xho*I)	*pilD* frameshift mutation–insertion of *Xho*I linker at the *Cla*I site[e]	This work This work
GDΔ180	Deletion of nucleotides 552–731[c] In-frame *pilD* deletion[e]	This work
GDΔ230	Deletion of nucleotides 552–781[c] Out-of-frame *pilD* deletion[e]	This work
GDXΔ*Cla*I	Deletion of 0.5 kb *Cla*I fragment spanning *pilD–orfX*[e]	This work

a. VD300 is an MS11 derivative (Koomey *et al.*, 1987).
b. All remaining listed strains are derived from N400.
c. Nucleotide sequence from Lauer *et al.* (1993).
d. Nucleotide sequence appears in the EMBL/GenBank/DDBJ Nucleotide Sequence Libraries under the accession number U19767.
e. Contains downstream m–Tn*3erm* insertion in ORFY at position 836.

Plasmid pVD300–*recA6* contains the *N. gonorrhoeae recA* gene under the control of an inducible *lac* promoter as well as repressor *lacI*^{q} and a tetracyline resistance gene (*tetM*), and will be described in detail elsewhere. This plasmid was transformed into *N. gonorrhoeae* VD300 (Koomey *et al.*, 1987) to produce strain N400, which was used as the parent strain for all mutant constructions. N400, with its IPTG-inducible copy of *recA,* allows stable expression of the *pilE* locus in the absence of induction, and in the presence of IPTG permits transformation of selectable markers into the chromosome. N400 was propagated on clear solid medium at 36°C in 5% CO_2 or in this media lacking agar that had been preincubated overnight in 5% CO_2 (Koomey *et al.*, 1987). Chemically defined media containing free amino acids were prepared as previously described (Catlin, 1973). *E. coli* strain HB101 was used in plasmid cloning experiments and was grown at 37°C in Luria–Bertani (LB) medium supplemented with the appropriate antibiotics.

Transposon mutagenesis of the *pilF–pilD* region was carried out as described by Seifert *et al.* (1990). A 3.2 kb *Bam*HI–*Sma*I fragment was isolated from plasmid p12/7/1 (Lauer *et al.*, 1993) and cloned into mutagenesis vector pHSS6 to create plasmid pF. Transposon insertions were isolated in *E. coli* host strains as described (Seifert *et al.*, 1990) and sequenced using primer Tn3L (5′-CTCATGACCAAAATCCC-3′) (Seifert and Wilson, 1992) to determine the location of transposon insertion. Plasmid DNA containing insertions of interest (see Fig. 1) was transformed into N400 and transformants were obtained by selection on agar plates containing 8 $\mu g\ ml^{-1}$ erythromycin. The *pilE* gene was amplified, by polymerase chain reaction (PCR), from selected mutants using appropriate primers and subjected to thermal cycle sequencing as directed by the manufacturer (Circumvent, New England Biolabs).

DNA sequence analysis

DNA sequencing of clones was performed using Sequenase 2.0 (United States Biochemical) as directed by the manufacturer. The complete sequence of both strands was determined by subcloning, as well as by the use of appropriate deletion constructs and unique oligonucleotide primers. This sequence appears in the EMBL/GenBank/DDBJ Nucleotide Sequence Data Libraries under the accession number U19767. The sequence spanning nucleotides 1 to 245 was derived from Lauer *et al.* (1993) and contains corrected sequences for *pilD* corresponding to those described by Dupuy and Pugsley (1994).

DNA and peptide sequence data were 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). Protein homologies were found using the TFASTA command and alignments were done using the PILEUP and PRETTY commands.

Electron microscopy

Sample grids were prepared by touching carbon-coated Formvar 3.05 mm grids (Tousimas Research Corp.) to individual bacterial colonies after approximately 18 h of growth at 37°C on solid media. Grids were air dried for 10 min, then floated on a drop of 0.25% ammmonium molybdate for 30 s and again air dried. Transmission electron microscopy was performed using a Philips CM-10 Transmission Electron Microscope.

Measurements of transformation frequencies

Transformation assays were carried out as previously described (Zhang *et al.*, 1992) using genomic DNA isolated from nalidixic acid- or rifampicin-resistant gonococcal strains. Transformants were selected on media containing 10 $\mu g\, ml^{-1}$ nalidixic acid or 5 $\mu g\, ml^{-1}$ rifampicin.

Construction of non-polar pilD *mutations*

Plasmid pF–Y2, a derivative of plasmid pF which contains a transposon insertion at position 861 (see Fig. 1), was used to construct deletions within *pilD* coding sequences. Following partial digestion of pF–Y2, with *Cla*I and Klenow treatment to produce blunt ends, a *Xho*I linker was inserted into the *pilD Cla*I site. The resulting plasmid was designated pF*pilD–Xho*I. Deletions within *pilD* were generated after digesting to completion with *Xho*I using exonuclease III as described (Ausubel *et al.*, 1991). The *pilD* deletion plasmids were sequenced using primer 5′-GAAACTGTCAGTGCGGGG-3′ to confirm the deletion locations and are designated as follows (nucleotide numbers are from the *pilD* sequence as reported by Lauer *et al.*, 1993): pF*pilD*Δ180 (deletion of nucleotides 552–731), and pF*pilD*Δ230 (deletion of nucleotides 552–781).

Plasmid pFΔCla was constructed by digesting pF–Y2 with *Cla*I followed by re-ligation; this plasmid contains deletions of both *pilD* and *orfX* coding sequences (see Fig. 3).

Gonococcal GD(*Cla*I → *Xho*I), GDΔ180, and GDΔ230 mutants were constructed by transformation of N400 with plasmids pF*pilD–Xho*I, pF*pilD*Δ180, and pF*pilD*Δ230, respectively, followed by selection on solid media containing 8 $\mu g\, ml^{-1}$ erythromycin. Introduction of chromosomal deletion mutations was confirmed by Southern analysis of genomic DNA.

Detection of pre-pilin processing

Assays for pre-pilin processing activity were performed as follows: bacterial cell suspensions were made from overnight plates in 200 μl sterile water and 200 μl 2x sample PAGE buffer containing 0.1 M Tris-HCl (pH 6.8), 0.6 μM β-mercaptoethanol, 400 mM EDTA, 4% SDS, and 20% glycerol (v/v) and heated at 95°C for 5 min. Samples were fractionated by SDS–PAGE on 15% gels and the proteins transferred to nitrocellulose. Filters were incubated with a 1:2000 dilution of polyclonal antibody GC2-66 generated against purified gonococcal pili or a 1:500 dilution of polyclonal antibody PAK 2-567 generated against *P. aeruginosa* PAK purified pili, then exposed to alkaline-phosphatase coupled goat anti-rabbit immunoglobulin antibodies (Tago Inc.). Pilin antigen was detected by the use of a colorimetric alkaline phosphate substrate. Details of the immunoblotting and antigen procedures have been published previously (Koomey *et al.*, 1991).

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