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PilT mutations lead to simultaneous defects in competence for natural transformation and twitching motility in piliated *Neisseria gonorrhoeae*

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Summary

***Neisseria gonorrhoeae*, the Gram-negative aetiological agent of gonorrhoea, is one of many mucosal pathogens of man that expresses competence for natural transformation. Expression of this phenotype by gonococci appears to rely on the expression of type IV pili (Tfp), but the mechanistic basis for this relationship remains unknown. During studies of gonococcal pilus biogenesis, a homologue of the PilT family of proteins, required for Tfp-dependent twitching motility in *Pseudomonas aeruginosa* and social gliding motility in *Myxococcus xanthus*, was discovered. Like the findings in these other species, we show here that gonococcal *pilT* mutants constructed *in vitro* no longer display twitching motility. In addition, we demonstrate that they have concurrently lost the ability to undergo natural transformation, despite the expression of structurally and morphologically normal Tfp. These results were confirmed by the findings that two classes of spontaneous mutants that failed to express twitching motility and transformability carried mutations in *pilT*. Piliated *pilT* mutants and a panel of pilus assembly mutants were found to be deficient in sequence-specific DNA uptake into the cell, the earliest demonstrable step in neisserial competence. The PilT-deficient strains represent the first genetically defined mutants that are defective in DNA uptake but retain Tfp expression.**

Abbreviations: Tfp, type IV pili; ORF, open reading frame; cfu, colony-forming units.

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Introduction

Bacterial genetic competence has been defined as a physiological state that permits the uptake of exogenous DNA. *Neisseria gonorrhoeae*, the Gram-negative aetiological agent of gonorrhoea, is one of many mucosal pathogens of man that express natural competence. Transformation in gonococci is particularly important to genetic exchange and diversity as it accounts for all cell to cell transmission of chromosomal loci (Catlin, 1982). As such, it is solely responsible for the extensive polymorphism and mosaicism that has been shown to occur in single-copy genes in the gonococcal genome (Halter *et al.*, 1989; Bowler *et al.*, 1994; Feil *et al.*, 1996). There is however, no direct evidence that transformation contributes as a mechanism to the impressive antigenic variability of pilin (Zhang *et al.*, 1992). Gonococci express competence in a constitutive fashion (Biswas *et al.*, 1977), unlike the situations in *Bacillus subtilis*, *Streptococcus pneumoniae* and *Haemophilus influenzae* in which competence development is a highly regulated and environmentally responsive event (Herriott *et al.*, 1970; Magnuson *et al.*, 1994; Havarstein *et al.*, 1995). However, gonococci and *H. influenzae* are similar in that they show specificity for DNA uptake based on unrelated sequences of 10 and 11 bp, respectively, that are distributed throughout their genomes (Danner *et al.*, 1980; Elkins *et al.*, 1991).

The process of genetic transformation can be dissected into a number of discrete, sequential steps in which DNA is bound, taken up, translocated across the outer (in the case of Gram-negative bacteria) and inner membranes and subsequently undergoes recombination with its homologous counterpart in the genome. Early studies by Sparling and colleagues (Biswas *et al.*, 1989) established a link between gonococcal transformation and the expression of Tfp by finding that spontaneous non-piliated variants had lost the capacity to take up DNA into a DNase-resistant state. More recently, a 10 000-fold reduction in transformation frequencies has been demonstrated for a large array of defined Tfp biogenesis mutants (Zhang *et al.*, 1992; Drake and Koomey, 1995; Freitag *et al.*, 1995; Tønjum *et al.*, 1995; Drake *et al.*, 1997). Furthermore, a *pilE* deletion

mutant lacking the pilin subunit and a PilC$^-$ mutant were found to be defective at the level of DNA uptake (Facius *et al*., 1996). Other gonococcal genes whose products function in the terminal steps of transformation and recombination have been identified and characterized. These genes include *comA*, *comL* and *tpc* (Facius and Meyer, 1993; Fussenegger *et al*., 1996a,b), which are proposed to be involved in inner membrane translocation, and *recA*, which is required for recombination (Koomey and Falkow, 1987). Mutations that affect the terminal steps of transformation do not alter Tfp expression or DNA uptake.

Given the uniformity of the findings, a strong correlation appears to exist between Tfp expression and the initial phase of the transformation process. This interpretation is complicated by reports in the literature that some non-piliated variants are competent for DNA uptake (Gibbs *et al*., 1989; Meyer *et al*., 1990), and the fact that it has not yet been possible to demonstrate DNA-binding activity with pilin or pili (Dorward and Garon, 1989; Mathis and Scocca, 1984). In addition, a mutant has been derived by chemical mutagenesis that expresses abundant piliation but is defective in DNA uptake (Biswas *et al*., 1989). The nature of the mutation for what has been termed the *dud1* (DNA uptake deficient) allele is unknown. A direct connection has therefore yet to be established between Tfp and DNA uptake.

In *P. aeruginosa*, twitching motility requires Tfp expression (Henrichsen, 1983). To further examine this relationship Bradley identified motility-impaired piliated mutants of *P. aeruginosa*, and based on electron microscopic studies it was concluded that twitching was a consequence of pilus retraction (Bradley, 1980). The mutation responsible for this defect was subsequently mapped to a gene designated *pilT* (Whitchurch *et al*., 1991). In a strikingly similar scenario, Tfp and a homologue of PilT have been found to be essential for social gliding motility exhibited by *Myxococcus xanthus* (Wu and Kaiser, 1995; Wu *et al*., 1997). PilT proteins are structurally related to a large family of proteins bearing consensus nucleotide-binding motifs that are engaged in membrane translocation of macromolecular complexes (Whitchurch *et al*., 1991; Pugsley, 1992; Lessl and Lanka, 1994). It has been speculated that these molecules fuel the translocation process by virtue of their intrinsic ATPase/kinase activities.

Piliated gonococci are known to display patterns of cell movement that are analogous to that described in other species as being twitching motility (Henrichsen, 1983; Swanson, 1978) and a gonococcal *pilT* homologue has been identified and characterized (Brossay *et al*., 1994). In this study, we show that piliated gonococcal mutants expressing null alleles of *pilT* show absolute defects in twitching motility and competence for natural transformation but retain other Tfp-associated phenotypes. Moreover, the defect in transformation in these backgrounds is at the level of DNA uptake, the same step at which transformation is blocked in non-piliated pilus biogenesis mutants.

Results

Construction and characterization of gonococcal PilT mutants

As a first approach to constructing PilT mutants, the plasmid clone p11/2/7, containing the complete *pilT* gene from strain VD300, was subjected to transposon mutagenesis and the precise sites of insertion determined by DNA sequencing. An additional unmarked frameshift mutation was created by filling in a unique *Eco*RI site within *pilT*, yielding the $pilT_{fs164}$ allele (Brossay *et al*., 1994). These mutations were then introduced into the genome of strain N400 by transformation. Their locations are indicated in Fig. 1.

The mutants were then examined for expression of phenotypes associated with gonococcal pilus expression. All mutants in which the integrity of the *pilT* open reading frames (ORFs) were disrupted as well as transposon mutants whose sites of insertion mapped just 5′ of the ORF (Fig. 1) exhibited abnormal colony morphologies that reflected increased intercellular adhesion (data not shown). This increased autoagglutination was most evident when the mutants were grown in liquid media where large clumps of cells were seen.

Gonococcal PilT migrates with a M_r of 40 kDa in SDS–PAGE and can be detected by immunoblotting with mAb 13C5 (Fig. 2, lane 1). Reactivity with this mAb was abolished in all the mutants with altered patterns of growth (data not shown). Electron microscopy of the PilT$^-$ mutants showed that they were piliated and no differences in either levels of piliation or fibre morphology (length and diameter) were discernible from what was seen for wild-type strains (Fig. 3). In addition, there were no detectable differences between the wild-type and mutant strains in levels of pilin (measured by immunoblotting), nor were there dramatic changes in piliation as measured by yield of purified pili (data not shown).

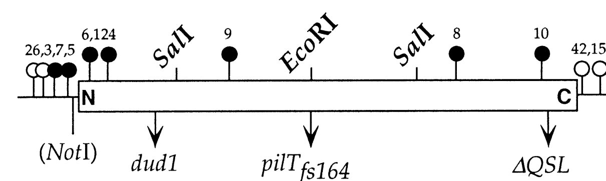

Fig. 1. Physical map of the gonococcal *pilT* locus and mutations. Transposon insertions are indicated by circles (precise positions of insertions are available from authors). Filled circles indicating insertions that result in a PilT$^-$ phenotype. Names and sites of other mutations referred to in the text are shown. Also shown are restriction sites relevant to this study including the *Not*I site inserted in the construction of the $pilT_{ind}$ allele.

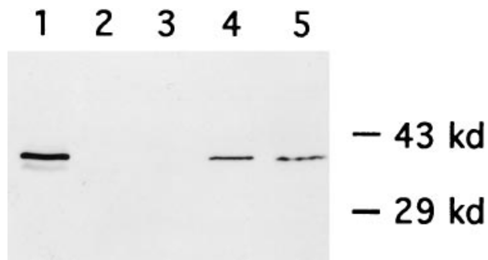

Fig. 2. PilT expression in wild-type and mutant strains assessed by immunoblotting with mAb 13C5. Lane 1, N400 (wild-type); lane 2, GT105; lane 3, GT101; lanes 4–5, two independently derived N400 transformants carrying the $pilT_{\Delta QSL}$ allele.

Based on the high degree of identity of gonococcal PilT with its counterpart in *P. aeruginosa* (67% over their complete length), the gonococcal mutants were examined microscopically for the expression of twitching motility. Zones of spreading cells at the periphery of colonies, although readily detectable in the wild-type parent, were totally absent in the *pilT* mutants. Even when examined at higher resolution with the more sensitive slide culture technique, cell movement was absent in the mutants. As negative controls, a diverse panel of non-piliated biogenesis mutants were assayed in parallel with no evidence of cell movement (Table 1). Finally, the capacity of the mutants to undergo genetic transformation was examined under conditions of saturating concentrations of DNA. Although between 2% and 4% of the wild-type cells acquired resistance to the antibiotic marker used, the frequencies of recoverable recombinants in the mutants were reduced over 10 000-fold (Table 1). These levels of transformation were in fact essentially the same as those found for all the classes of pilus biogenesis mutants tested concurrently.

Transformation defect of pilT *mutants is at the level of DNA uptake*

Natural transformation involves sequential events in which DNA is taken up into a DNase-resistant state, translocated across the membranes, processed and integrated into the genome. As defects at any one of these steps could account for diminished transformation capabilities, a DNA uptake assay was designed to assess the immediate fate of transforming DNA (see *Experimental procedures*). Using this technique, we examined a group of defined pilus biogenesis mutants created in the N400 background and found that in every case DNA uptake proficiency was abolished. In contrast to the wild-type parent, however (which protected more than 4% of the DNA added), piliated *pilT* mutants were indistinguishable from non-piliated mutants with no significant DNA uptake or DNase protection being observed (Table 1).

dud1 *is a defective allele of* pilT

In addition to its defect in DNA uptake, the *dud1* mutant characterized originally by Biswas and colleagues shared another property with *pilT* mutants, that being a propensity to hyperautoagglutinate, which was reflected as an abnormal colony morphology. We therefore tested a derivative of MS11 (the progenitor to strains VD300 and N400) carrying the *dud1* allele (Seifert *et al*., 1988) for expression of PilT and found using immunoblotting that it failed to react with the PilT-specific mAb 13C5, which recognizes a linear epitope within the carboxy-terminal 25 residues of the protein (Brossay *et al*., 1994) (Fig. 2, lane 3). The lack of PilT reactivity seen could have resulted from a mutation in the *pilT* gene itself or an extragenic mutation in gene(s)

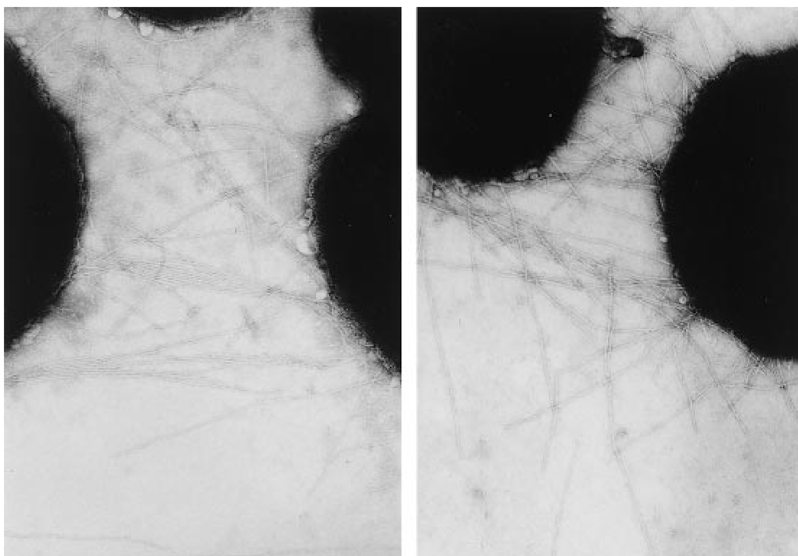

Fig. 3. Transmission electron micrographs of wild-type and *pilT* mutant strains of *N. gonorrhoeae*. Left, N400 (wild type). Right, GT105 ($pilT_{fs164}$ allele). Magnification 39 000×.

Table 1. *N. gonorrhoeae* strains used in this study with genotypes/phenotypes.

Strain	Relevant genotype	Piliation status[a]	Twitching motility[b]	DNase-resistant DNA uptake[c] (%)	Transformation efficiency[d] (%)	Epithelial cell adherence[e]	Reference
N400[f]	–	+++	+	100	100	+++	Tønjum *et al.* (1995)
GF3	*pilF*	–	–	<5	<0.01	–	Freitag *et al.* (1995)
$GD_{(ClaI-XhoI)}$	*pilD*	–	–	<5	<0.01	–	Freitag *et al.* (1995)
GQ21	*pilQ*	–	–	<5	<0.01	–	Drake and Koomey (1995)
GG2	*pilG*	–	–	<5	<0.01	–	Tønjum *et al.* (1995)
GE68[g]	*pilE*	–	–	<5	<0.01	–	This work
GT101	$pilT_{dud1}$	+++	–	<5	<0.01	+++	This work
GT102	$pilT_{\Delta QSL}$	+++	–	<5	<0.01	+++	This work
GT103	*pilT*::Tn*#7*	+++	–	<5	<0.01	+++	This work
GT104[h]	$pilT_{ind}$	+++	–	<5	<0.01	+++	This work
GT105	$pilT_{fs164}$	+++	–	<5	<0.01	+++	This work

a. Piliation status was determined by transmission electron microscopy.
b. Twitching motility assessed by slide culture method.
c. Values are relative to the wild-type parent strain and represent cell-associated DNA that becomes protected from DNase I treatment.
d. Values represent the frequency of transformation to nalidixic acid resistance relative to wild type.
e. Values represent the number of bacteria/epithelial cell: +++, greater than 50; –, less than 1.
f. N400 is a derivative of VD300 that carries the inducible *recA6* allele.
g. GE68 carries a *pilE* frameshift mutation in the codon for pilin residue 68 (unpublished).
h. GT104 grown in the absence of inducer.

controlling *pilT* expression. To determine if a mutation resided within the *pilT* ORF in this background, translational fusions between MalE and the ORFs from the wild-type and *dud1* strain were constructed in *E. coli* (Fig. 4). This was carried out using PCR such that the amino-terminal segment of the fusion was MalE, whereas the carboxy-terminal part comprised the complete PilT polypeptide beginning at residue 28. Immunoblotting of lysates containing the chimeric molecules with mAb 13C5 showed that the wild-type construct expressed a mAb-binding species of the expected molecular weight, but no reactive species was seen with the *dud1*-derived fusion product. This indicated that a missense or chain terminating mutation was contained within the *pilT* ORF of the *dud1* strain. The mAb reactivities of additional fusions generated by swapping restriction fragments between the wild-type and *dud1 pilT* alleles revealed that the mutation mapped 5′ of the unique *Eco*RI site in *pilT*. Further mapping of the mutation was carried out by making an internal deletion in both clones using the two *Sal*I sites that are in the same reading frame. Deletion of the *Sal*I fragment in the wild-type clone produced a smaller but reactive polypeptide. Deletion of this fragment from the *dud1* clone failed to restore mAb reactivity, mapping the lesion to a 120 bp region 5′ of the *Sal*I site. DNA sequencing of this region showed the insertion of an extra thymidine within the codon for Met-50. Therefore, although other mutations may have arisen in the creation of the *dud1* mutant, the frameshift mutation in *pilT* is sufficient to account for all the altered phenotypes.

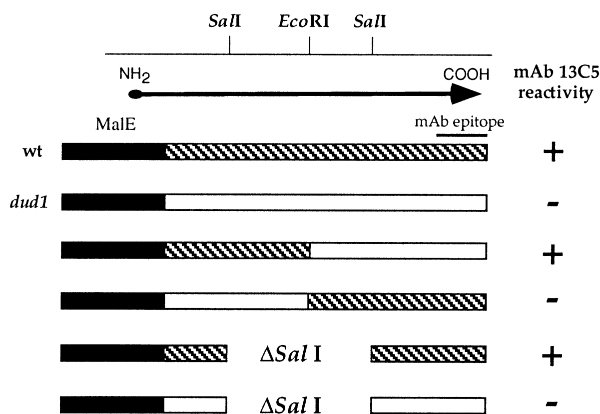

Fig. 4. Identification of *dud1* as a defective allele of *pilT*. Construction of MalE–PilT fusions is described in *Experimental procedures*. Solid shading, MalE protein; stripped shading, wild-type derived segment; no shading, *dud1*-derived segment. Restriction sites used in the construction of chimeric fusions are shown. The position of the mAb 13C5 epitope is indicated at the carboxy-terminus of PilT. Reactivity of fusion proteins with mAb 13C5 is indicated as + or –.

A microdeletion accounts for spontaneous pilT *missense mutants*

In earlier attempts to obtain unique gonococcal pilus mutants, colony morphology piliation variants were selected from strain MSO1, which carries two functional *pilE* genes. This genetic background leads to extremely stable pilus expression and colony morphology. After growth for 72 h on Gc agar, all colonies contained sectors of hyperagglutinating variants that were found to be defective for twitching motility. At that time, it was shown that these variants had no alterations in *pilE* nor did they express Opa proteins (known to effect autoagglutination), but the basis for their

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1020                                    1050
   *                                       *
CAGACGATGGACCAATCGCTGCAATCGCTGGTGCGCCAAGGG
 Q  T  M  D | Q  S  L | Q  S  L | V  R  Q  G
            319             324

CAGACGATGGACCAATCGCTGGTGCGCCAAGGG
 Q  T  M  D | Q  S  L | V  R  Q  G
```

Fig. 5. A spontaneous microdeletion accounts for spontaneous *pilT* missense mutations. The top figure represents the wild-type *pilT* allele. Bottom figure depicts the $pilT_{\Delta QSL}$ allele. Nucleotide numbering is indicated above the wild-type DNA sequence and refers to GenBank accession number S72391. Positions of relevant amino acids are identified below boxed residues. Note the highlighted 9 bp tandem repeat and the deletion of one repeat copy in the $pilT_{\Delta QSL}$ allele resulting in the in-frame deletion.

altered phenotypes was unknown. Realizing subsequently that those variants had similar properties to *pilT* mutants, they were examined for PilT expression. Using immunoblotting with mAb 13C5, independently derived variants of this type were shown to produce PilT, albeit at what appeared to be reduced levels, and that migrated at a slightly faster mobility (Fig. 2, lanes 4 and 5). DNA sequencing of PCR products derived from *pilT* in two such independently derived variants showed that in each case, one copy of a 9 bp segment that was part of a direct, tandem duplication was deleted. The consequence of this in-frame deletion is the loss of the three residues QSL from the QSLQSL direct repeats that span residues 319–324 (Fig. 5). The altered *pilT* allele was designated $pilT_{\Delta QSL}$. To confirm that this mutation was responsible for loss of function, DNAs from plasmid clones containing a 400 bp fragment encompassing the altered region from either the wild-type or deletion alleles were used to transform strain N400. Transformants expressing altered colony morphology were only found in cells exposed to the cloned mutant DNA and those transformants (such as GT102) were piliated, defective in DNA uptake, non-twitching (Table 1) and possessed the in-frame deletion but no other changes as seen by direct sequencing of PCR products. Thus, the defects in twitching motility and DNA uptake proficiency as well as the other altered phenotypes can be attributed to the *pilT* deletion mutation itself, rather than to a polar effect on distal gene expression.

Influence of regulated transcription of pilT *on pilus-associated properties*

To confirm the role of PilT in pilus-associated properties, a strain was constructed in which the gene was placed under the transcriptional control of an inducible promoter. A selectable gene cassette containing the *tac*-UV5 promoter and operator elements (and the $lacI^{Q}$ gene, such that repression can be alleviated by the addition of gratuitous inducer) (Seifert, 1997) was placed immediately upstream of the *pilT* ribosome binding site at a created *Not*I site (Fig. 1), and the construct was placed into the genome of strain VD300. In the absence of induction, the resulting gonococcal strain (GT104) had all the phenotypes found in *pilT* null mutants, although when propagated in the presence of IPTG, the strain displayed twitching motility, normal colony morphology and wild-type frequencies of transformation (Table 2). Using the DNase protection assay, induction led to an increase in DNA uptake proficiency from a level indistinguishable from that seen for non-piliated mutants to 30% of the value seen in the wild-type parent (Table 2). The failure to achieve maximal levels of uptake suggests that PilT expression may still be limiting after conditions of maximal induction. This explanation is supported by the findings that using the highest concentrations of IPTG attainable, the level of mAb 13C5 reactive material is 30–40% of that seen in the wild type (data not shown). The discrepancy between transformation efficiency and uptake proficiency after induction suggests that DNA uptake is not a limiting step in transformation under the conditions used.

Effects of pilT *mutations on pilus proteins and pilus-associated adherence for human epithelial cells*

As previously documented and found in this work, mutation of any one of a large number of genes whose products are required for pilus biogenesis led to a profound reduction in levels of transformability and the defect in these mutants is at the level of DNA uptake. Given that pilus expression appears to be necessary for DNA uptake and that piliated *pilT* mutants lack this property, it was a formal possibility that pili expressed in the mutant background were structurally or functionally altered.

To address if compositional differences might account for the DNA uptake defect, we set out to compare profiles of pili and putative pilus-associated polypeptides from the isogenic strains. For this purpose, pili were prepared from

Table 2. DNA uptake and transformation restored by ectopic *pilT* expression.

Strain	Relevant genotype	IPTG[a]	DNase-resistant DNA uptake[b] (%)	Transformation efficiency[c] (%)
VD300[d]	–	NA	100	100
GF3	*pilF*	NA	<5	<0.01
GT104	$pilT_{ind}$	–	<5	<0.01
GT104	$pilT_{ind}$	+	<5	10
GT104	$pilT_{ind}$	++	30	100

a. Strains are induced with IPTG immediately before DNA uptake and transformation assays (+) or for 16 h before the assays (++).
b. Values are relative to those of the wild-type parent strains (VD300 or N400 in the case of GF3) and represent cell-associated DNA that becomes protected from DNase I treatment.
c. Values represent the frequency of transformation to nalidixic acid resistance relative to wild type.
d. VD300 is a wild-type derivative of MS11

the wild-type parent and the *pilT* mutants and examined by SDS–PAGE. As shown in Fig. 6, in which preparations from N400 and its isogenic derivative carrying the $pilT_{dud1}$ allele (GT101) were compared, there are no differences detectable in the migration or level of pilin. The PilC protein has been demonstrated to co-purify with pili, and Rudel and colleagues (1995a) have presented data to suggest that the association of this molecule with pili is essential for DNA uptake. As detected both by immunoblotting and by silver staining, no differences in its level or migration are seen in these or any of the other *pilT* backgrounds. Subtle differences in levels of other minor polypeptides can be seen but were not consistently reproducible with similar fluctuations in profiles detectable in independent preparations taken from identical strains. It has also been reported that preparations of pili may contain the PilQ protein (Rudel *et al*., 1995a), a member of the secretin family that functions in pilus biogenesis (Drake and Koomey, 1997), but no differences in the level or migration of PilQ were found in the *pilT* mutants (Fig. 6).

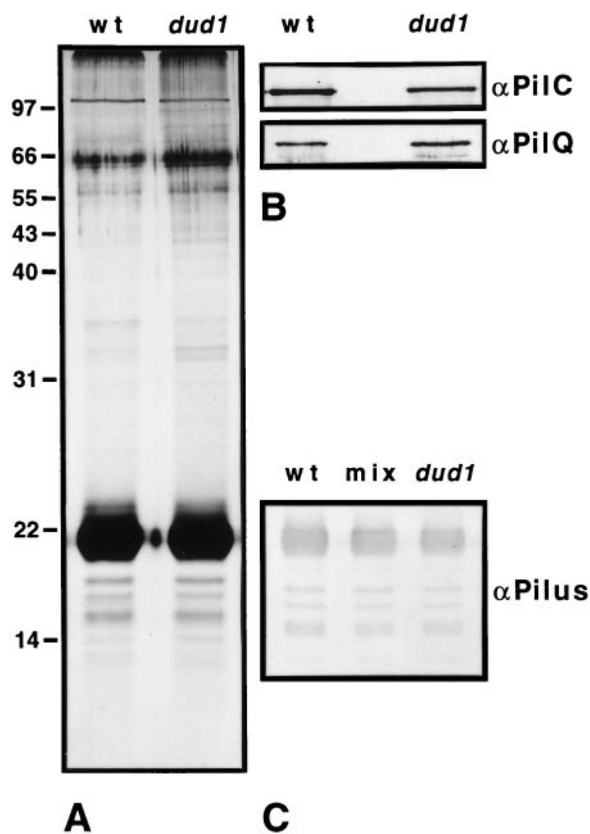

Fig. 6. Pilin and pilus-associated polypeptides are not altered in *pilT* mutants.
A. Silver staining of purified pilus preparations from N400 and GT101 analysed by SDS–PAGE.
B. Immunoblotting of pilus preparations using polyclonal rabbit anti-PilC and anti-PilQ sera.
C. Immunoblotting of pilus preparations with polyclonal rabbit anti-whole pili (anti-pilin) sera.

PilC protein has also been implicated as a pilus-associated adhesin for human epithelial tissue and its ability to promote gonococcal adherence requires pilus expression (Rudel *et al*., 1992;1995b,c). As the capacity of piliated gonococci to adhere to epithelial cells provides an indirect readout of both pili and PilC functionality, we examined the interaction of N400 and the *pilT* mutants with the ME180 human epithelial cell line (Table 1). The wild-type parent and the *pilT* mutants all displayed high levels of cytoadherence for ME180 cells (greater than 50 diplococci per cell), whereas the non-piliated strains were unable to interact productively with this cell line (< 1 diplococcus per cell). Pili and PilC expressed in the absence of PilT therefore appear to be fully functional with regard to epithelial cell adherence.

Discussion

The precise relationships between Tfp expression and its associated phenotypes remain the topic of much investigation and debate. In this study, we demonstrate an essential role of the gonococcal PilT protein in both competence for natural transformation and twitching motility in the absence of discernible alterations in pili and piliation. The defects and alterations observed in the gonococcal mutants are directly related to a loss of PilT function as (i) they can be co-ordinately corrected by induced transcription of *pilT*, and (ii) they are manifest in the $pilT_{\Delta QSL}$ missense mutant. With regard to Tfp associated motility, the findings are remarkably similar to those made previously for the PilT proteins of *P. aeruginosa* and *M. xanthus* that are required for twitching motility and social motility respectively (Whitchurch *et al*., 1991; Wu *et al*., 1997). This conclusion is based on the fact that in all three organisms pilus expression is required for motility and that the PilT proteins are essential for this process but dispensable for organelle biogenesis. The structural and functional similarities of these three proteins strongly suggest a common mechanism of action in promoting Tfp-associated motility. In gonococci, only one other mutant with a $PilT^-$ phenotype has been reported, that being associated with the *dud2* allele (Biswas *et al*., 1989), but it is not known if this lesion maps to *pilT*. In myxococci, some mutations leading to a defect in social gliding motility in piliated organisms have been shown to map outside of *pilT* (Wu *et al*., 1997).

Based on the findings here using a broad array of pilus biogenesis mutants, it appears that pilus expression itself is in some way essential to the initial step in the transformation process, that is sequence-specific uptake of DNA into a DNase-resistant state. Although these results are consistent with most prior studies using uncharacterized pilus mutants, it has been concluded by others that it is the synthesis of pilin rather than pilus filaments that is

essential to competence for natural transformation (Gibbs *et al*., 1989; Meyer *et al*., 1990). It is difficult to reconcile these two discrepant points of view given that all the mutants examined here (save for the *pilE* mutant) express the pilin subunit. The absence of DNA uptake in PilT$^-$ mutants that appear to express normal pilus filaments raises questions for a direct role of pili in the process. One could envision that perhaps the pili in these backgrounds are altered in a manner not detectable by current techniques. For example, they may be altered conformationally or lack a pilus-associated molecule that fails to fractionate with pili during purification. It has been proposed that PilC, a protein engaged in pilus biogenesis and pilus-associated epithelial adherence, functions directly in competence for transformation (Rudel *et al*., 1995a). As shown here, PilC is present in purified pilus preparations from PilT$^-$ mutants at levels indistinguishable from that found for wild-type strains. Moreover, PilC appears to be functional in PilT$^-$ mutants as those cells adhere to human epithelial cells. It seems unlikely then that the phenotypes of PilT mutants relate simply to a PilC defect. Given that PilT might function in pilus retraction, one could envision DNA being physically translocated across the outer membrane in conjunction with organelle retraction.

The major conflict to be reconciled here is whether pili themselves are engaged directly in DNA uptake or if other, as yet unidentified, molecules that parasitize the biogenesis pathway to achieve proper localization at the cell surface are responsible. In either case, a defect in biogenesis would yield the same incompetent phenotype. It is important in this context to note that DNA binding and uptake in *Bacillus subtilis* requires the expression of proteins that are highly related to those engaged in type IV pilus biogenesis, although no pili have been detected (Dubnau, 1997). In fact, the *B. subtilis* ComGB protein, which is related to PilT and its homologues, is needed for DNA binding (Chung and Dubnau, 1998). As no pili are seen in that system however, it is impossible to determine if ComGB functions in a manner similar to PilT or if it functions like the related gonococcal PilF that is essential for pilus biogenesis (Freitag *et al*., 1995). In any event, there must be some commonality between transformation in gonococci and *B. subtilis*. Perhaps the gonococcal organelle provides a means of breaching the outer membrane, a barrier not only to export of the machinery to the cell surface but also to import of DNA.

This work represents the first case in which either twitching motility or DNA uptake, two constitutive properties of piliated gonococci, have been disrupted in genetically defined, pilus-expressing mutants. That these two properties are co-ordinately lost in the mutants demonstrates a potential relationship between uptake of DNA into the cell and surface organelle movement. With regard to gonococcal pathogenesis, it should now be possible to examine the effect of twitching motility on the interaction of the bacterium with human epithelial cells and its influence on colonization in experimental human infections.

The PilT proteins comprise a distinct subgroup of a large family of proteins that have as their common denominator an essential role in membrane translocation of macromolecules. Despite an abundance of work on many members of the family, their mode of action in membrane translocation remains to be elucidated. In attempting to understand the roles of the PilT proteins, it is important to note that these proteins are most similar to PilB in *P. aeruginosa* and *M. xanthus* and PilF in *N. gonorrhoeae*, which are essential for pilus biogenesis in each system. It stands to reason then that PilT proteins interact in a non-essential way with other components of the pilus biogenesis machinery to bring about their associated phenotypes. Experiments designed to address how gonococcal PilT might function in conjunction with the other pilus biogenesis components are actively being pursued.

Experimental procedures

Bacterial strains, plasmids and transposon mutagenesis

The bacterial strains used in this study are described in Table 1. Gonococcal strains were propagated on clear solid medium (Gc agar) at 37°C in 5% CO_2 or in this media lacking agar and preincubated in 5% CO_2 (Gc broth). *E. coli* strain HB101 was used for plasmid cloning and was grown at 37°C in Luria–Bertani medium supplemented with the appropriate antibiotics. The *pilT* gene from strain VD300 was cloned as a 1.7 kb *Pst*I–*Ssp*I fragment from lambda clone 18/4 (Lauer *et al*., 1993) into *Pst*I–*Hinc*II of pBluescript-II KS to produce p11/9/3. A 0.3 kb fragment carrying a gonococcal DNA uptake sequence upstream of the *Pst*I site was added to the clone to produce p1/13/3. This fragment was amplified from 18/4 using primers PT5′3, which overlaps the *Pst*I site (5′-AAATTGGTTCTGCAGGCGGGCAGG-3′), and PT5′4, which created an *Xba*I site for cloning (5′-GCTCTAGACCTCGACACACACCTGCAAGGG-3′). The *Pst*I site and *Xba*I site are underlined. The PCR product was digested and ligated to p11/9/3 digested with *Xba*I and *Pst*I to produce a 2.0 kb fragment containing the *pilT* gene and DNA uptake sequence. To create p11/2/7 the 2.0 kb gonococcal fragments was removed from p1/13/3 by *Xba*I–*Xho*I digestion. The *Xho*I site was filled in and the fragment cloned into *Xba*I and a blunted *Cla*I site of the transposon mutagenesis vector pHSS6 (Seifert *et al*., 1990). Transposon mutagenesis was performed on p11/2/7 as described (Seifert *et al*., 1990). Transposon insertion were isolated, sequencing, and transformed into the gonococcal strain N400 as previously described (Freitag *et al*., 1995). Altered alleles of spontaneously arising *pilT* mutants were cloned by PCR amplification of genomic DNA using primers PT-E (5′-CGGATATCGCAACACCGTCATAGATCATCCC-3′) and PT-D (5′-CCGAAGCTT**GCCGTCTGAA**CGCCTTGTTTTCCCGCCGGCATGG-3′). Primer PT-D adds a *Hin*dIII site and DNA uptake sequence, underlined and highlighted respectively. PCR products were digested with *Sal*I and *Hin*dIII and

cloned into pBluescript-II SK. Cloned wild-type and mutant alleles are designated p2/7/1 and p2/7/5. DNA sequencing of *pilT* and *pilE* alleles was carried out using SEQUENASE version 2.0 (United States Biochemical) as previously described (Freitag *et al.*, 1995). A partial sequence of this region including the entire *pilT* ORF appears in the EMBL/GenBank/DDBJ nucleotide sequence data libraries under accession number S72391, and nucleotide numbering presented in this publication corresponds to that accession. pH11 was constructed by cloning a 200 bp *Hae*III fragment from the intergenic region of *pilF* and OrfY (accession number U32588, nucleotide 1861–2065), into the *Sma*I site of pBluescript-II SK. This fragment carries two copies of the gonococcal DNA uptake sequence spanning bases 1942–78.

Expression of PilT from an inducible promoter

A 2.0 kb fragment *Xba*I–*Kpn*I carrying the *pilT* ORF and DNA uptake sequence from p1/13/3 was cloned into M13mp19 digested with *Xba*I and *Kpn*I and transformed into DH5αF′. Site-directed mutagenesis was performed as described (Sambrook *et al.*, 1989) to insert a *Not*I restriction site 14 nucleotides upstream of the PilT initiation codon between nucleotide positions 57 and 58 (Fig. 1). The site was created using the oligonucleotide PilT–*Not*I (5′-AATGCGGCTCTGTTGCGGCCGCTAGTATAATGTTTC-3′). The mutagenized fragment was then removed from M13mp19 using the polylinker restriction sites *Xho*I and *Hin*dIII and cloned into plasmid pACYC184 (New England Biolabs), digested with *Sal*I and *Hin*dIII. A *Not*I fragment from pVD300recA6 carrying the $lacI^{Q}$–*tac–UV5* control region and *tetM* gene (Seifert, 1997) was cloned into the newly created *Not*I restriction site, resulting in pLacP–pilT. Gonococcal strain VD300 was transformed with pLacP–pilT DNA, and transformants containing the inducible *pilT* allele ($pilT_{ind}$) were selected on Gc agar plates containing 4 μg ml^{-1} tetracyline. The resulting strain was designated GT104.

SDS–PAGE, immunoblotting and silver staining

Procedures for SDS–PAGE and immunoblotting have been described previously (Freitag *et al.*, 1995). PilE, PilQ and PilC were detected by immunoblotting pilus preps using rabbit polyclonal antibodies and alkaline phosphatase coupled goat anti-rabbit antibodies (Tago) PilE- and PilQ-specific sera have been described previously (Drake and Koomey, 1995), PilC-specific serum was a gift from T. F. Meyer. Silver staining was performed using Pierce GelCode Silver Stain as directed by the manufacture. PilT antigen was detected using mAb 13C5 (Brossay *et al.*, 1994) and alkaline phosphatase conjugated goat anti-mouse antibodies (Tago)

Construction of MalE–PilT fusions

MalE–PilT protein fusions were constructed by PCR amplification of *pilT* from lambda clone 18/4 or from MS11 carrying the *dud1* allele using primers PT–*Eco*RV (5′-CCGGATATCATTCGGGTTCACGGCGACATGCGG-3′) and PT–*Hind*III (5′-CCGAAGCTTCGCCTTGTTTTCCCGCCGGCATGG-3′), which create *Eco*RV and *Hind*III sites respectively. PCR products were digested with *Eco*RV–*Hind*III and cloned into *Stu*I–*Hind*III of pMal-c (New England Biolabs) to produce p12/3/1 and p12/7/1 respectively. Expression from p12/3/1 and p12/7/1 results in the production of maltose-binding protein fused to PilT at residue 28. Chimeras of the wild-type and *dud1 pilT* allele ($pilT_{dud1}$) were constructed by exchanging restriction fragments as indicated on Fig. 4.

Electron microscopy

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

Measurement of transformation frequency and DNA uptake

Transformation assays were carried out as previously described using 1 μg ml^{-1} plasmid pSY6 DNA (Stein *et al.*, 1991). Transformants were selected on Gc agar containing 1 μg ml^{-1} nalidixic acid. DNA uptake was accessed using a ^{32}P-labelled PCR fragment that carried two copies of the Gc DNA uptake sequence. Radiolabelled DNA was generated by PCR amplification of the Gc DNA insert carried on pH11 using the commercially available (Promega) pUC/M13 PCR primers, forward (−40) (5′-GTTTTCCCAGTCACGAC-3′) and reverse (5′-CAGGAAACAGCTATGAC-3′) to produce a 450 bp product. PCR was performed in the presence of 200 μM dNTPs supplemented with 1 μM [^{32}P]-dCTP (800 Ci $mmol^{-1}$, Amersham) using *Taq* DNA polymerase (Gibco BRL). Unincorporated nucleotides and primers were removed using G-50 Sephadex Quick Spin Columns (Boehringer Mannheim). The resulting product had a specific activity of 2.5×10^6 c.p.m. μg^{-1}. Uptake assays were performed by adding 300 ng of ^{32}P-labelled PCR product to 1 ml suspensions containing 10^9 cfu of Gc supplemented with 7 mM $MgCl_2$. Suspensions were tumbled for 30 min at 37°C, placed on ice and DNase I was added to a final concentration of 100 μg ml^{-1} and incubated for 5 min. Cells are washed four times in 1 ml of ice-cold Gc broth and counted in a 3 ml scintillation cocktail in a Beckman LS3801 liquid scintillation counter.

Characterization of twitching motility

Twitching motility was assessed by direct visualization of cells at the periphery of colonies observed with a Stereozoom 7 (Bausch and Lomb) stereomicroscope as described (Swanson, 1978) as well as by the slide culture method (Darzins, 1993), in which cells are inoculated onto Gc agar slices on microscope slides, covered with a coverslip and visualized under a Zeiss phase microscope using the 40× objective.

Pilus purification

Pili were purified by the ammonium sulphate procedure previously described (Brinton *et al.*, 1978). Cells from four heavily streaked Petri dishes were suspended in 10 ml of

0.15 M ethanolamine, pH 10.5 and vortexed for 60 s. Cellular debris was removed by centrifugation at $17\,000 \times g$ for 15 min. Pilus crystals were precipitated at room temperature for 30 min with one-tenth volume ammonium sulphate-saturated 0.15 M ethanolamine and collected by centrifugation at $17\,000 \times g$ for 15 min. Pilus filaments were washed twice with 0.05 M Tris-buffered saline.

Epithelial cell adherence

Adherence assays were performed using the human epithelial cell line ME-180 (ATCC) maintained in McCoy's 5A medium (Gibco BRL) supplemented with 10% fetal bovine serum (FBS). Epithelial cells were seeded into a 24-well tissue culture plate at a density of 5×10^5 cells per well the day before infection. For assays, $2–5 \times 10^7$ bacteria in 1 ml of McCoy's 5A medium supplemented with 1% FBS (assay medium) were added to each well of monolayers, and incubated at 37°C in 5% CO_2. After 1 h, non-adherent bacteria were removed by washing five times with assay media, and the monolayers and cell-associated bacteria were recovered by treatment with 0.25% trypsin for 5 min at 37°C. The recovered bacteria were plated on Gc agar after dilution and the number of cell-associated bacteria determined.

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