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Phase variation of gonococcal pili by frameshift mutation in *pilC*, a novel gene for pilus assembly

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Pili prepared from *Neisseria gonorrhoeae* contain minor amounts of a 110 kd outer membrane protein denoted PilC. The corresponding gene exists in two copies, *pilC1* and *pilC2*, in most strains of *N.gonorrhoeae*. In the piliated strain MS11(P^+), only one of the genes, *pilC2*, was expressed. Inactivation of *pilC2* by a mTnCm insertion resulted in a nonpiliated phenotype, while a mTnCm insertion in *pilC1* had no effect on piliation. Expression of *pilC* was found to be controlled at the translational level by frameshift mutations in a run of G residues positioned in the region encoding the signal peptide. Nonpilated (P^-), pilin expressing colony variants that did not express detectable levels of PilC were selected; all P^+ backswitchers from these P^-, $PilC^-$ clones were found to be $PilC^+$. The structural gene for pilin, *pilE*, was sequenced and found to be identical in one P^-, $PilC^-$ and P^+, $PilC^+$ pair. Most $PilC^-$ cells were completely bald whereas the $PilC^+$ backswitcher had 10-40 pili per cell. Thus, a turn ON and turn OFF in the expression of PilC results in gonococcal pili phase variation. These results suggest that PilC is required for pilus assembly and/or translocation across the gonococcal outer membrane.

Key words: N.gonorrhoeae/phase switch/*pilC*/pili expression/translational frameshifting

Introduction

Neisseria gonorrhoeae is an organism completely adapted to the human host, having no other ecological niche. It has acquired a large arsenal of strategies to overcome the human host defence system. The first step in infection with *N.gonorrhoeae* is its adherence to target cells. It is thought that pili of *N.gonorrhoeae* are a major virulence factor. Piliated (P^+) variants attach much better to susceptible tissues than non-piliated (P^-) derivatives (Swanson, 1973; Pearce and Buchanan, 1978). Moreover, P^+, unlike P^- variants, are able to establish an infection in human volunteers (Kellogg *et al.*, 1968). It is believed that the pili of *N.gonorrhoeae* bind to specific eukaryotic receptors. However, the receptor structure(s) on epithelial cells has not been characterized, nor has it been clearly established whether the receptor binding domain is part of the pilin subunit or resides in a minor pilus-associated adhesin. The difficulty in analyzing the exact role of pili in attachment is partly due to the lack of understanding of the biogenesis pathway of gonococcal pili.

Pilin is the major subunit of the pilus. Expression of pilin is controlled at the *pilE* locus. Most strains of *N.gonorrhoeae* contain one *pilE* expression site. However, strain $MS11_{ms}$ contains two loci for pilin expression, *pilE1* and *pilE2*. Two closely linked genes, *pilA* and *pilB*, have been shown to regulate transcription from *pilE* (Taha *et al.*, 1988).

In *Escherichia coli*, various pili have been examined in detail and in at least three classes of pili, Pap, Type 1 and S, the receptor binding adhesin is a minor component of the pilus located primarily at the tip (Lindberg *et al.*, 1987; Moch *et al.*, 1987; Hanson and Brinton, 1988). These *E.coli* pili are each encoded by a set of closely linked genes which have greatly facilitated the genetic dissection of pilus biogenesis. The biogenesis of *E.coli* pili requires the expression of a periplasmic chaperone protein that forms periplasmic complexes with each of the pilus subunit proteins (Hultgren *et al.*, 1989; Lindberg *et al.*, 1989). Pilus assembly in *E.coli* also requires an outer membrane protein encoded by each pili gene cluster (Mooi *et al.*, 1983; Orndorff *et al.*, 1984; Norgren *et al.*, 1987). In the absence of this outer membrane protein, preassembled chaperone-pilus subunit complexes are trapped in the periplasm (Hultgren *et al.*, 1989). It is therefore believed that this class of proteins, ranging in molecular weight from 80 to 90 kd, form a channel or an assembly center in the outer membrane necessary for pilus biogenesis (Norgren *et al.*, 1987).

Type 4 pili expressed by *N.gonorrhoeae, Bacteroides nodosus, Pseudomonas aeruginosa, Moraxella bovis* and *Vibrio cholerae* share extensive sequence homologies especially in the amino terminal part of the pilin subunit (McKern *et al.*, 1983; Elleman and Hoyne, 1984; Meyer *et al.*, 1984; Marrs *et al.*, 1985; Pasloske *et al.*, 1985; Faast *et al.*, 1989; Shaw and Taylor, 1990). Expression of the cloned pilin subunits from *B.nodosus* and *M.bovis* in *P.aeruginosa*, but not in *E.coli*, leads to the formation of pili (Elleman *et al.*, 1986; Mattick *et al.*, 1987; Beard *et al.*, 1990), suggesting that there is a specific assembly machinery for Type 4 pili present only in those species that produce this type of pili. Nevertheless, it has not been possible to assemble gonococcal pili in an heterologous background.

In this paper we identify a 110 kd outer membrane protein that is enriched in highly purified pili preparations from *N.gonorrhoeae* strain MS11. We demonstrate that most strains of *N.gonorrhoeae* carry two copies of the corresponding gene, denoted *pilC*, and that expression from these two loci is regulated by frequent frameshift mutations within a run of G residues in the region encoding the signal peptide. The two *pilC* genes are not identical. Hence, alternate expression from either *pilC1* or *pilC2* gives rise to two different forms of PilC. Among nonpiliated (P^-) descendants from P^+ clones we find clones that express pilin but not PilC. All P^+ revertants from such $PilC^-$ non-

piliated clones have regained expression of PilC. Hence, phase variation of gonococcal pili can be caused by frameshift mutations in *pilC*. Transposon inactivation of the expressed *pilC2* copy resulted in a nonpiliated, pilin producing revertible phenotype. We therefore propose that PilC is required for the assembly of pilin subunits into a polymerized pilus fiber in *N.gonorrhoeae*.

Results

Neisseria gonorrhoeae MS11 pili preparations contain a 110 kd minor protein

An outer membrane preparation from *N.gonorrhoeae* strain $MS11_{ms}(P^+)$ contains small amounts of a 110 kd protein (Figure 1). This protein was enriched during alternate cycles of crystallization and solubilization of pili, unlike other outer membrane proteins that decreased in abundance by this procedure.

The 110 kd protein present in purified $MS11_{ms}$ pili preparations was eluted from SDS polyacrylamide gels and rabbit antibodies were generated against the gel purified protein. The antiserum cross reacted extensively with the pilin protein in immunoblots and was therefore absorbed with extracts of *Pseudomonas putida* expressing the pilin subunit of *N.gonorrhoeae* on plasmid pGC02. The absorbed antiserum was used in immunoblots with whole cell extracts of a number of *N.gonorrhoeae* strains as well as commensal strains of *Neisseria* (Figure 2A). All strains of *N.gonorrhoeae*, except strain 605103, contained one or two high molecular weight protein species reacting with the antiserum. Strain 605103, unlike the other strains tested, was nonpiliated and no piliated variants could be obtained suggesting that it is a P^-n variant (Swanson *et al.*, 1985). This was confirmed by Southern blot hybridization using an oligonucleotide probe corresponding to the 5′ end of the *pilE* gene. No hybridization was obtained with this probe. The commensal *N.lactamica* Nctc10618, but not *N.subflava* GN01, contained a high molecular weight protein reacting with the 110 kd antiserum. Immunoblots against outer membrane preparations of P^+ and P^-n $MS11_{mk}$ (Figure 2B), showed the 110 kd protein to be present in the outer membrane in both of these MS11 variants.

Molecular cloning of the pilC1 gene encoding a 110 kd protein

Chromosomal DNA from *N.gonorrhoeae* $MS11_{mk}(P^+)$ was used to construct a λgt11 library. The library was screened with the absorbed 110 kd antiserum and one positive clone out of 10 000 plaques was found, containing an 800 bp insert. A lysogen of this positive λgt11 clone was examined in immunoblots and a fusion protein with an estimated size of 150 kd reacted with the antiserum (data not shown). The 800 bp insert was purified, labeled with ^{32}P, and used as a probe to screen a plasmid library from *N.gonorrhoeae* $MS11_{ms}$. Six clones out of 10 000 hybridized with the probe. Restriction maps for these partially overlapping six clones are shown in Figure 3.

The six plasmid clones were transformed into the minicell producing strain AA10 to monitor expression of plasmid encoded [^{35}S]methionine-labeled proteins. Plasmid pABJ04 expressed minute amounts of three high molecular weight proteins, 113, 111 and 108 kd in size, as well as a number of lower molecular weight protein species not produced from the vector control (Figure 4). The three high molecular

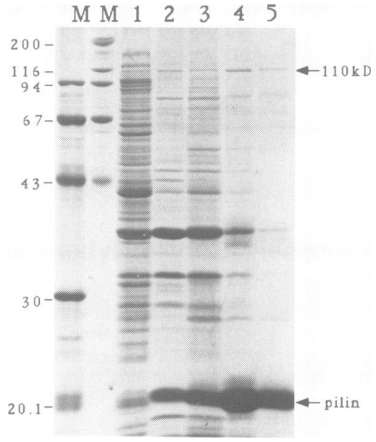

Fig. 1. Pili preparations of *N.gonorrhoeae* $MS11_{ms}(P^+)$ contain a minor protein 110 kd in size. Polyacrylamide gel electrophoresis of purified pili from $MS11_{ms}(P^+)$ stained with Coomassie brilliant blue. Lanes: 1, whole cell lysates; 2, outer membranes; 3, crude pili preparation crystallized once; 4, pili preparation crystallized three times (preparation in lane 3 continued); 5, pili preparation crystallized five times (preparation in lane 4 continued). M, molecular mass standard (size in kd at left). The positions of the 110 kd protein and the pilin are indicated on the right.

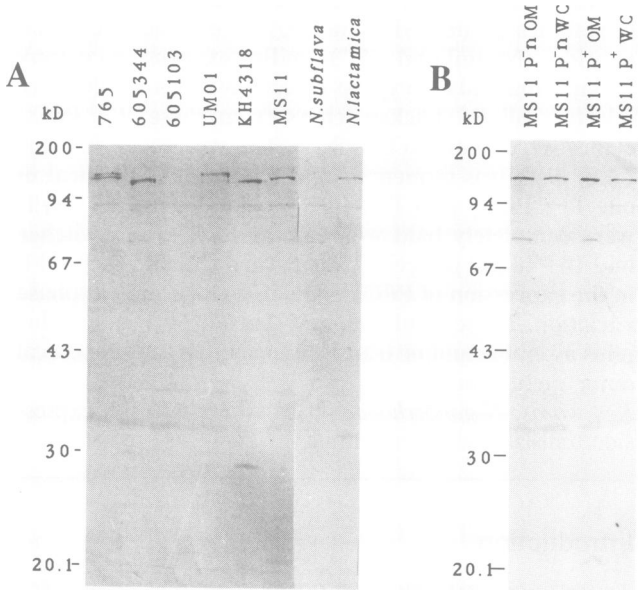

Fig. 2. Immunoblotting of *Neisseria* strains with the antiserum against the 110 kd protein. (**A**) Whole cell lysates separated on SDS–PAGE were immunoblotted with the absorbed antiserum against the 110 kd protein from $MS11_{ms}(P^+)$. *N.gonorrhoeae* strains: 765(P^+), 605344 (P^+), 605103(P^-n), UM01(P^+), KH4318(P^+) and $MS11_{ms}(P^+)$. Commensal *Neisseria* strains: *N.subflava* GN01 and *N.lactamica* Nctc 10618. (**B**) Outer membranes (OM) and whole cell lysates (WC) of $MS11_{mk}P^+$ and $MS11_{mk}P^-n$ were immunoblotted with the 110 kd antiserum. Size of molecular mass standards in kd is shown on the left.

weight bands were missing in pABJ05 and pABJ06 but three novel lower molecular weight protein species had appeared, suggesting that pABJ05 and pABJ06 are deleted for the 3′ end of a gene, denoted *pilC1*, and that this gene is responsible for all three high molecular weight species. This suggested that the distal end of the gene must be located between the $MluI_1$ and $MluI_2$ sites (Figure 3). The observation that

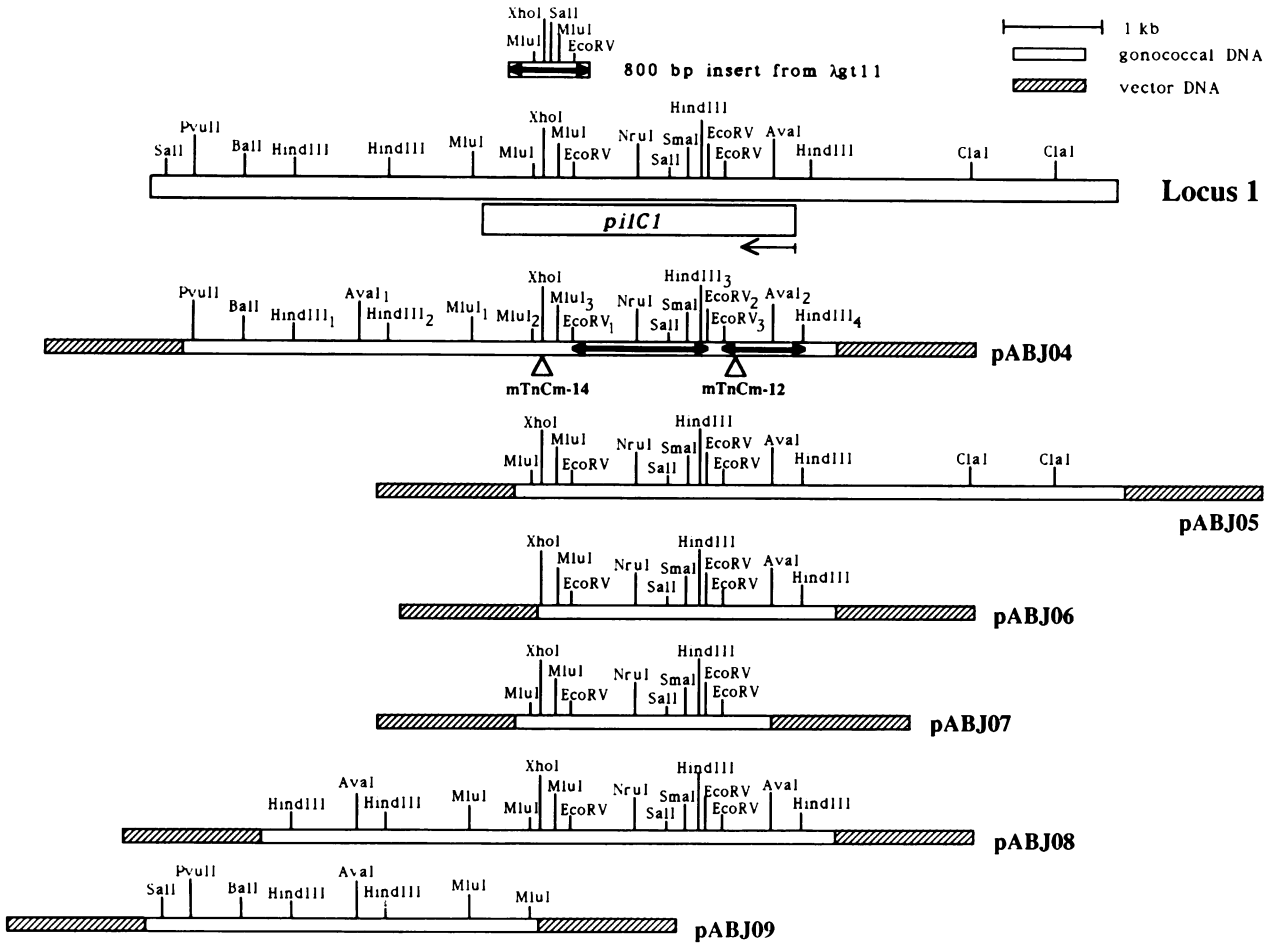

Fig. 3. Genetic and physical map of *pilC* locus 1. Plasmids pABJ04-09, all found to belong to locus 1, were isolated from a plasmid library using the 800 bp insert from λgt11 as a probe. The λgt11 insert (from locus 2) has an additional *Sal*I site not found in the plasmid clones. The position of the *pilC1* gene and direction of its transcription (indicated by an arrow) were determined in *E.coli* minicells (Figure 4). Three thickened lines, with an arrow-head at each end, indicate fragments used as probes in Southern hybridizations, i.e., the 800 bp insert from λgt11, the $EcoRV_1-EcoRV_2$ (1.3 kb) and the $EcoRV_3-HindIII_4$ (0.8 kb) fragments of pABJ04. Triangles mark the location of two mTnCm insertions in pABJ04. The resulting plasmids, pABJ04::mTnCm-12 and pABJ04::mTnCm-14, were used to inactivate *pilC1* and *pilC2*.

plasmid pABJ07 did not express any high molecular proteins tentatively located the 5′ end of the gene to a region 0.5−1.2 kb to the right of the $EcoRV_3$ site. The size for a gene encoding a 110 kd protein is ~3 kb which is in agreement with these mapping data.

The Neisseria gonorrhoeae MS11 genome contains two complete copies of pilC

The 800 bp insert in λgt11 contains a single *Sal*I site not present in the region on pABJ04 which hybridized to this fragment, suggesting that there is more than one *pilC* locus in the genome of *N.gonorrhoeae* MS11. This was confirmed in Southern blot hybridizations in which three different *pilC* fragments were used to probe *Sma*I and *Cla*I digested genomic DNA. The 800 bp fragment from λgt11 hybridized in a Southern blot to two *Cla*I (18 and 8 kb) and *Sma*I (13 and 4.5 kb) fragments of DNA prepared from *N.gonorrhoeae* $MS11_{mk}$ (Figure 5). Since the probe does not contain any internal *Cla*I or *Sma*I sites, there are, presumably, two copies of the 3′ end of *pilC* in the MS11 genome. The 1.3 kb $EcoRV_1-EcoRV_2$ fragment of pABJ04 carries the central region of *pilC1*. This probe hybridized to the same two *Cla*I fragments and to four *Sma*I fragments, two of which are the same size as the two *Sma*I fragments identified with the 800 bp probe (13 kb and 4.5 kb). Hybridization with the 800 bp probe was more extensive to the 8 kb *Cla*I and the 4 kb *Sma*I fragment whereas the reverse was found with the 1.3 kb $EcoRV_1-EcoRV_2$ fragment from pABJ04 strongly suggesting that the two genomic copies of *pilC* show a significant sequence variation in the 3′ as well as in the central region. A probe corresponding to the 5′ region of *pilC1* was also used in Southern hybridization experiments. This 0.8 kb $HindIII_4-EcoRV_3$ fragment hybridized to two *Cla*I (18 kb and 4 kb) and *Sma*I (25 kb and 7 kb) fragments with seemingly equal efficiency. The hybridization pattern was identical using DNA from $MS11_{ms}$ (data not shown). Taken together these hybridization data indicate that *N.gonorrhoeae* MS11 contains two complete copies of *pilC*. Furthermore the two genes appear to be more homologous in their 5′ as compared to their central and 3′ regions.

The 800 bp insert from λgt11 must carry information from *pilC2* whereas the clones pABJ04−09 must carry information from *pilC1*. Finally *pilC2* must be located >2 kb from either end of *pilC1*.

The 800 bp fragment from *pilC2* was also used to probe digested genomic DNA from *N.gonorrhoeae* strains UM01, 765 and 605103. The latter isolate does not express detectable

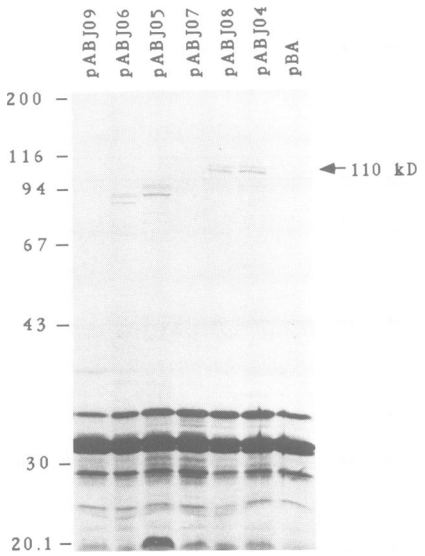

Fig. 4. Synthesis of PilC1 in minicells. Autoradiograph of [^{35}S]methionine-labeled proteins expressed in *E.coli* minicells containing the indicated plasmids. Plasmids pABJ04-09 are shown in Figure 3 and pBA is the vector control coding for β-lactamase. The location of the 110 kd protein and molecular mass standards (in kd) are shown on either side.

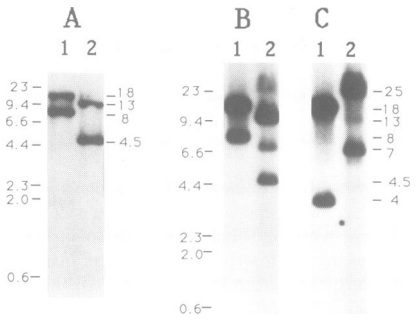

Fig. 5. Southern hybridization of *pilC* specific probes to *N.gonorrhoeae* MS11$_{ms}$(P$^+$) chromosomal DNA. Digests in blots are *Cla*I (lanes 1) and *Sma*I (lanes 2). (**A**) digested chromosomal DNA probed with the 800 bp insert from λgt11, containing the 3′ end of *pilC2*. (**B**) the same DNA, probed with a 1.3 kb *Eco*RV$_1$−*Eco*RV$_2$ fragment of pABJ04, i.e., the central region of *pilC1*. (**C**) blot probed with the 0.8 kb *Eco*RV$_3$−*Hin*dIII$_4$ fragment of pABJ04, containing the 5′ region of *pilC1*. Sizes in kb of λ/*Hin*dIII standard fragments (on the left) and estimated size of the hybridizing fragments (on the right) are indicated.

levels of the 110 kd protein. Strain UM01, unlike MS11, contained only one *Cla*I fragment of 15 kb that hybridized to the probe (data not shown). Hence, this strain may contain only one copy of *pilC*. Strain 605103 and 765, on the other hand, each seem to contain two copies of *pilC* since two *Cla*I and two *Sma*I fragments hybridized to the 800 bp probe (data not shown).

The commensal *N.lactamica* Nctc10618 DNA digested with *Cla*I and *Sma*I also hybridized with the 800 bp probe. Since only one band hybridized in each case this strain may contain only one copy of *pilC*. In contrast, *N.subflava* GN01 did not hybridize to the 800 bp *pilC2* probe using the same stringency (data not shown).

The pilC1 gene on pABJ04 is translationally out of frame

The amino terminal sequence of gel purified 110 kd protein from strain MS11$_{ms}$ (P$^+$) was determined by sequential Edman degradation. Considerable difficulties were encountered in the sequencing probably due to blocking of the N-terminus. As a result, only the residues from position 4 to 10 were obtained (Figure 6). The 3.3 kb *Hin*dIII$_4$−*Mlu*I$_1$ fragment encompassing the entire *pilC1* gene was sequenced on both strands using the dideoxy sequencing method adapted for single stranded DNA (to be published). One single open reading frame of 997 codons was found (from left to right in Figure 6) and starting at an AUG codon 195 bp from the *Hin*dIII$_4$ site. Codons 7−12 in this open reading frame corresponded to amino acids 5−10 in the sequence of the gel purified protein. The AUG codon in the beginning of the long open reading frame was not preceded by a typical Shine−Dalgarno sequence. Moreover, since the 110 kd protein is located in the outer membrane of *N.gonorrhoeae*, we expected the protein to be translated with a signal sequence. When examining the nucleotide sequence, an AUG codon was found in frame 1 that was preceded by a typical Shine−Dalgarno sequence (−AGGAA−). The sequence following this AUG codon would encode a typical signal peptide with basic amino acids in the amino terminal region and a hydrophobic central region. However, no signal peptidase cleavage site could be predicted following the rules of von Heijne (1983). A tract of 12 G residues was found in the region encoding the putative signal peptide for PilC. Addition of one G residue or the loss of two would align the long open reading frame with the AUG codon in frame 1. The translated region in frame 2 contains a putative signal peptidase cleavage site between Ala and Gln. A cleavage at this site would align the determined amino acid sequence at positions 5−10 for the 110 kd protein with the deduced amino acid sequence. The data therefore suggested that the cloned *pilC1* gene is out of frame due to frameshifting in the region encoding the signal peptide.

Genetic inactivation of pilC2 but not pilC1 abolishes expression of the 110 kd protein in MS11

Plasmid pABJ04 was mutagenized in *E.coli* by a transposon mini-Tn3 derivative, mTnCm (Seifert *et al.*, 1990), and the map positions for 30 insertions were determined. Only two mTnCm insertions had occurred in *pilC1* (Figure 3). Truncated protein species were seen in minicells with the mTnCm-14 insertion located 0.5 kb from the 3′ end of *pilC1* but not with the mTnCm-12 insertion located 0.5 kb from the 5′ end of the gene (data not shown). Both insertion mutants were used in a gene replacement experiment. Plasmids pABJ04::mTnCm-12 and pABJ04::mTnCm-14 were linearized with *Bam*HI and transformed into *N.gonorrhoeae* MS11$_{mk}$(P$^+$) and transformants resistant to 10 μg/ml of chloramphenicol were selected. Forty-eight P$^+$ transformants (24 from each experiment) were assayed for the presence of PilC in immunoblots. All these transformants remained capable of expressing the PilC protein (data not shown). Genomic DNA was prepared from seven of the chloramphenicol resistant transformants (five from pABJ04::mTnCm-12 and two from pABJ04::mTnCm-14), cleaved with *Cla*I and *Pvu*II and used in Southern blot experiments using the *Eco*RV$_1$−*Eco*RV$_2$ fragment of pABJ04 as a probe (Figure 7A). The 8 kb *Cla*I fragment

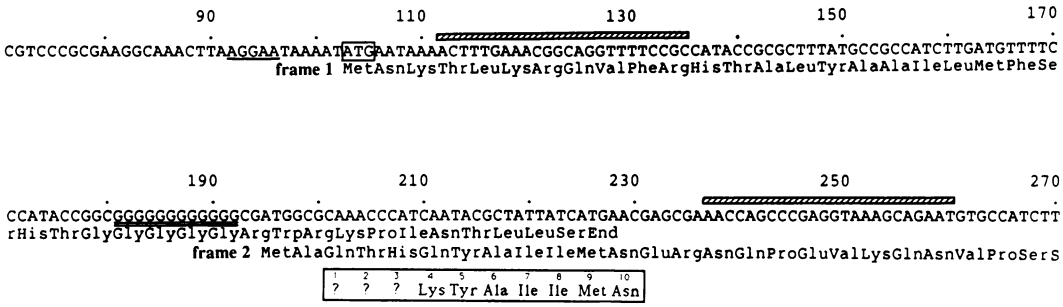

Fig. 6. Nucleotide sequence and the deduced amino acid sequence of the 5′ end of *pilC1*. The amino-terminal sequence of gel purified PilC from $MS11_{ms}(P^+)$ is shown in a box below frame 2, a 997 amino acid long open reading frame that would code for a protein 110 kd in size (to be published). Frame 1 contains 41 amino acids and is preceded by a putative Shine−Dalgarno sequence (underlined). Two horizontal lines mark a stretch of 12 G residues. An addition of one G in this region would align the ATG (boxed) in frame 1 with frame 2. Numbers above the sequence show base positions relative to the *Hin*dIII$_4$ site (= 0) located on pABJ04. The position of two 24 bp oligonucleotide primers (opposite strands), used for PCR amplification, are indicated above the sequence by hatched bars.

was unaffected in the mutants whereas the 18 kb *Cla*I fragment had been replaced by a 20 kb fragment. *Pvu*II cleaves within the 1.6 kb mTnCm element. The probe detected an 8 kb *Pvu*II fragment in both parent and mutant DNA. In the mutants, a novel *Pvu*II fragment appeared that was 6.2 kb in size in five transformants obtained with pABJ04::mTnCm-12 and 4.8 kb in size in two transformants with pABJ04::mTnCm-14. To confirm the insertion of mTnCm, a 250 bp *Eco*RI−*Hin*dIII fragment of the CAT GenBlock (Pharmacia, Sweden), containing the *Pvu*II site, was used as a probe. It detected the larger of the two *Cla*I fragments as well as the 6.2 kb *Pvu*II fragment (Figure 7B). In addition, a 2 kb *Pvu*II fragment not covered with the *pilC* probe was detected. These data demonstrate that we have obtained gene replacements in *pilC1*, whereas *pilC2* was unaffected in all seven P^+, $PilC^+$ transformants. A rapid hybridization was done to screen the remaining 41 P^+ transformants. All but one had mTnCm inserted in *pilC1*. The remaining transformant had an intact locus 1 and 2 and must therefore contain mTnCm elsewhere in the gonococcal chromosome (data not shown).

In the same transformation experiments, the frequency of P^- colony variants was about five-fold higher as compared with that occurring normally in strain $MS11_{mk}(P^+)$. Two P^-mTnCm-12 transformants isolated at 10 μg/ml of chloramphenicol were also analyzed by Southern blot hybridization using the *Eco*RV_1−*Eco*RV_2 fragment of pABJ04 and the *Eco*RI−*Hin*dIII fragment of the CAT GenBlock. Each of these mutants carried mTnCm in *pilC2* as evidenced by a replacement of the 8 kb *Cla*I fragment by a fragment 9.5 kb in size that hybridizes to both probes (Figures 7C and D). These *pilC2*::mTnCm insertion mutants did not express PilC as determined by immunoblot analysis (Figure 8, lane 3).

A P^+, *pilC1*::mTnCm-12 mutant was retransformed with DNA prepared from a P^-, *pilC2*::mTnCm-12 mutant and colonies growing at 30 μg/ml of chloramphenicol were selected to obtain double mutants in *pilC*. All resistant transformants were P^-, and when analyzed by Southern blot hybridization all contained mTnCm in both *pilC1* and *pilC2* (Figure 7C and D). Electron microscopy revealed that the P^+, *pilC1*::mTnCm-12 mutant still expressed pili albeit at a slightly lower level than the $MS11_{mk}(P^+)$ parental clone, whereas the P^-, *pilC2*::mTnCm-12 was completely bald as was the *pilC1, pilC2* double mutant (data not shown).

Immunoblot analyses were performed on the P^+, *pilC1*::mTnCm-12 mutant, the P^-, *pilC2*::mTnCm-12 mutant and the P^-, *pilC1*::mTnCm-12, *pilC2*::mTnCm-12 double mutant, using PilC and pili antisera (Figure 8).

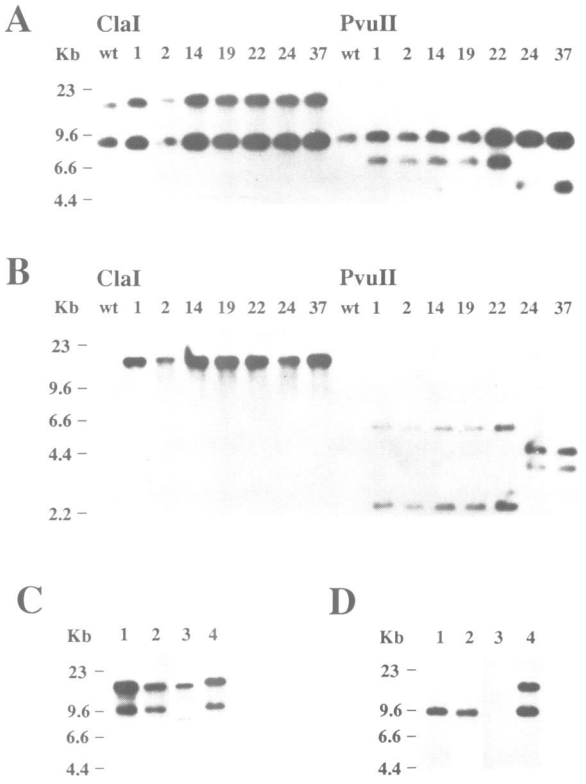

Fig. 7. Southern blot hybridizations demonstrating insertional inactivation of *pilC1* and *pilC2*. (**A**) Piliated transformants isolated at 10 μg/ml of chloramphenicol were digested with *Cla*I or *Pvu*II and probed with the 1.3 kb *Eco*RV_1−*Eco*RV_2 fragment of pABJ04 (central region of *pilC1*). Chloramphenicol resistant clones 1, 2, 14, 19 and 22 were transformed with pABJ04::mTn3-12 and clones 24 and 37 with pABJ04::mTn3-14 (see Figure 3). $MS11_{mk}(P^+)$ is marked as wt. (**B**) The same piliated transformants as in A, probed with the 250 bp *Eco*RV−*Hin*dIII fragment of the CAT GenBlock, containing the *Pvu*II site. (**C**) Two nonpiliated transformants isolated at 10 μg/ml chloramphenicol (lane 1 and 2), $MS11_{mk}(P^+)$ in lane 3 and a double mutant isolated at 30 μg/ml of chloramphenicol (lane 4) digested with *Cla*I and probed with the 1.3 kb *Eco*RV_1−*Eco*RV_2 fragment of pABJ04. (**D**) The same transformants as in C, probed with the 250 bp large *Eco*RV−*Hin*dIII fragment of the CAT GenBlock. Location and sizes of λ/*Hin*dIII fragments are shown on the left.

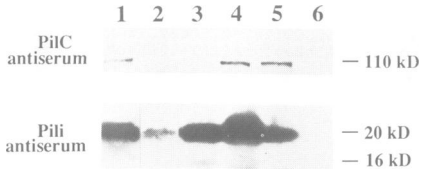

Fig. 8. Immunoblotting of *pilC1*::mTnCm-12 and *pilC2*::mTnCm-12 single and double mutants with both PilC and pili antiserum. The wild type $MS11_{mk}(P^+)$ and *pilC* mutants were subjected to SDS−PAGE as whole cell lysates. Lanes: 1, piliated revertant of the *pilC2*::mTnCm-12 mutant in lane 3; 2, double mutant (P^-); 3, *pilC2*::mTnCm-12 mutant (P^-); 4, *pilC1*::mTnCm-12 mutant (P^+); 5, $MS11_{mk}(P^+)$; 6, 605103 (P^-n, $PilC^-$).

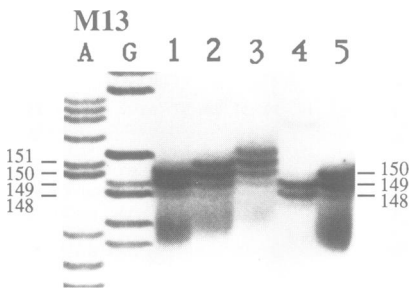

Fig. 9. Variation in the length of the signal peptide coding region of *pilC* visualized as a ladder of PCR amplified products. A region containing the G stretch at the 5′ end of *pilC* was amplified from four *N.gonorrhoeae* strains and from the *E.coli* plasmid pABJ04 using the two 24 base long primers shown in Figure 6. The amplified products were separated on a standard sequencing gel and autoradiographed. A dideoxy sequence of M13mp18 (A and G) was used to mark the size of amplified fragments. The size in bases is given in the margins. Lanes: 1, $MS11_{ms}(P^+, PilC^+)$; 2, UM01(P^+, $PilC^+$); 3, 765(P^+, $PilC^+$); 4, 605103(P^-n, $PilC^-$); 5, pABJ04 purified from *E.coli* strain AA10 (*recA*).

Inactivation of *pilC1* did not abolish expression of PilC or the pilin. Inactivation of *pilC2* totally abolished expression of PilC but did not affect expression of pilin. The *pilC1, pilC2* double mutant was $PilC^-$ but produced only low levels of pilin. Taken together these data imply that *pilC2* but not *pilC1* is expressing PilC in the MS11 variant under study. Moreover, inactivation of *pilC2* but not *pilC1* was associated with a loss of piliation.

P^+ revertants occurred spontaneously at a low frequency in the *pilC2*::mTnCm-12 mutants. These revertants expressed pili as determined by electron microscopy and also expressed PilC (Figure 8, lane 1). It is likely that PilC expression is due to in-frame switching in *pilC1*.

The pilC genes of N.gonorrhoeae vary in the length of the G tract

Polymerase chain reaction (PCR) with *Taq* polymerase was used to analyze the 5′ region of *pilC* using two 24 base long synthetic oligonucleotides based on the sequence of *pilC1* (Figure 6). These oligonucleotides would generate an amplified fragment of 149 bases as judged from the sequence obtained from pABJ04.

The amplified products from $MS11_{mk}(P^+)$ DNA were 149 and 150 bases long respectively (Figure 9). In addition, two less abundant products of 151 and 148 bases were seen. The amplified products were electroeluted and cloned into M13mp18, and twenty phage clones were sequenced using a universal primer. Four different sequences were obtained (Figure 10). Variant patterns 1a and 1b were identical to each other and to the cloned sequence on pABJ04 except for the presence of 11 instead of 12 G residues in the G tract of 1b. The G tract of sequence 2a was 13 residues long indicating that the sequence is in frame. In addition, this sequence differed from *pilC1* by four 1 bp substitutions outside the G tract, including an AAA lysine codon four triplets downstream of the putative signal peptide processing site which is in agreement with the lysine residue found in the fourth position of the gel purified 110 kd protein. Sequences 1a and 1b contained CAA, the codon for Gln, at the same position. Sequence 2b was identical to 2a except for the presence of 12 G residues in the G tract. These data are compatible with sequence 1 being from *pilC1* and sequence 2 from *pilC2* and give further support for the hypothesis that *pilC2* must be the expressed locus in the $MS11(P^+)$ variant we are studying.

Strain UM01 apparently only contains one copy of *pilC*. DNA from this strain generated five amplified fragments ranging in size from 148 to 152 bp in the PCR reaction (Figure 9). The most abundant fragments were 149−151 bp long. Among ten M13 clones, three variant 1 sequences were found (a,b,c) that differed only in the number of G residues (11−13) in the G tract (Figure 10) supporting the hybridization data that this strain contains only one *pilC* gene. Since a PilC protein is expressed from UM01 we suggest that the majority of cells has 13 Gs in the G tract.

Strain 765 contains two *pilC* loci, both of which seem to be translationally ON based on the presence of two high molecular weight proteins reacting with the absorbed PilC antiserum (Figure 2). A number of amplified fragments were seen after the PCR reaction ranging in size from 149 to 153 bases. Three variant sequences were found among nine clones (Figure 10). The G tract was 13 residues long in variant 3a (in frame) and 14 (out of frame) in variant 3b, whereas sequence variant 4 contained 11 G residues in the G tract. Variant sequence 4 contained four additional nucleotides (−CAGG−) distal to the G tract relative to variant sequences 1,2 and 3, indicating that the amplified product with 11 Gs from this variant sequence is 152 bases long and out of frame. Two PCR amplified products 152 and 153 bases in length were obtained from strain 765 (Figure 9) suggesting that in frame variants of sequence 4 might be present in the DNA prepared from this strain.

Strain 605103 carries two *pilC* copies, both of which seem to be translationally OFF (Figure 2). The amplified fragments were 148 and 149 bases in size (Figure 9). Out of eight M13 clones only variant 1a and 1b sequences were found, with 11 and 12 Gs in the G tract respectively. Consequently, we were unable to find an in frame sequence variant from this strain. We do not know if the 5′ ends of the two *pilC* genes are identical in this strain or if one *pilC* gene differed from *pilC1* in the region corresponding to the oligonucleotides used for amplification. In the latter case we would not expect to obtain any amplified products from the second copy.

The only in frame variant found in DNA amplified from *N.gonorrhoeae* carried 13 Gs in the G tract. To see if variants with 10 Gs arise in *E.coli*, PCR amplified products were generated from pABJ04 purified from *E.coli* strain AA10, using the same two oligonucleotide primers as before. Out of 12 sequenced clones, two carried 10 Gs in the G tract

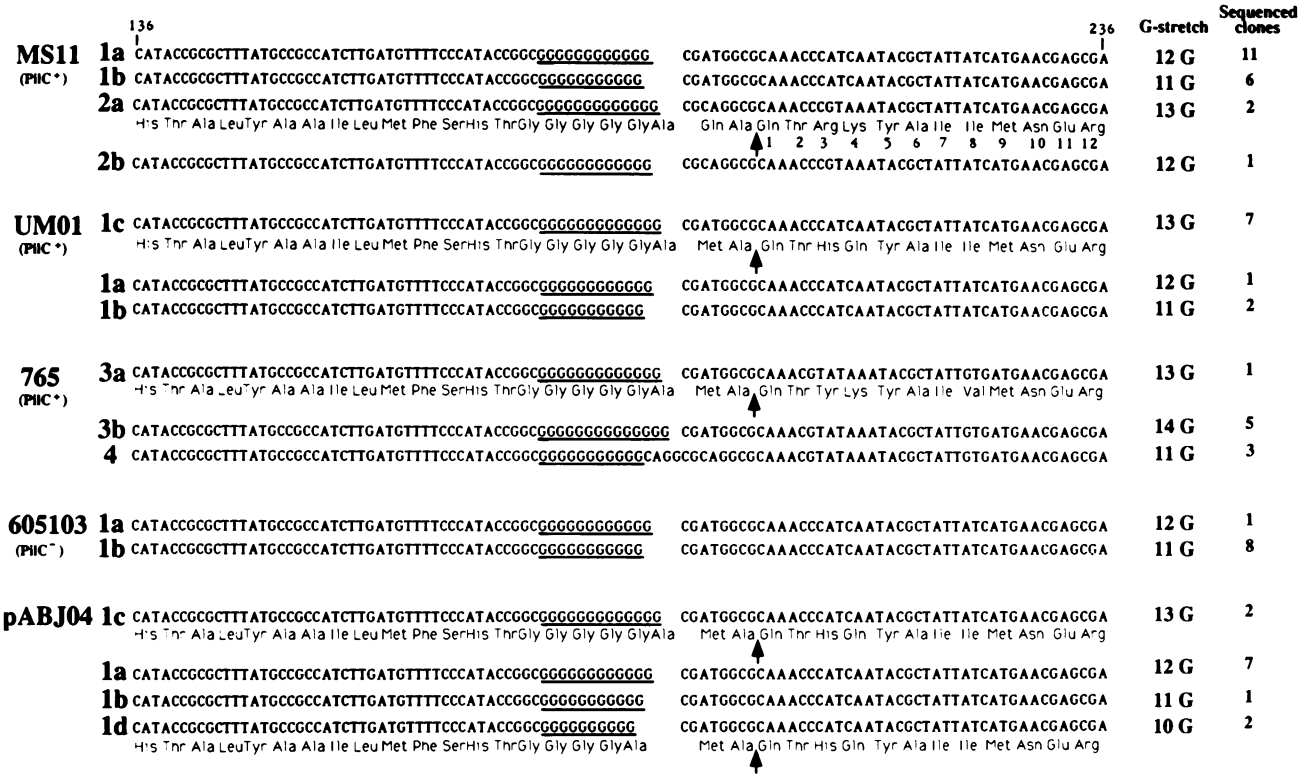

Fig. 10. Nucleotide sequence of PCR amplified fragments demonstrating a variation in the length of the G tract and sequence differences in the 5′ region of the *pilC* genes. The two oligonucleotide primers used for the PCR are shown in Figure 6. Amplified DNA was cloned into M13mp8 and sequenced. Shown are the complete nucleotide sequence in between the two primers. In-frame sequences are translated and the G stretches are underlined. The putative cleavage sites are marked with arrows. Genomic DNA from *N.gonorrhoeae* strains MS11(P^+, $PilC^+$), UM01(P^+, $PilC^+$), 765 (P^+, $PilC^+$) and 605103 (P^-n, $PilC^-$), and purified plasmid DNA from pABJ04/AA10(*recA*) was used in the PCR.

(Figure 10). The majority of clones (seven) carried 12 Gs as expected. It is likely that the PCR amplification products are not representative of the original DNA population. However, the distribution of variation in the G tract is consistent with a model in which only one G residue is gained or lost at one given event. Since AA10 is *recA*, frameshift mutations in the G tract in *E.coli* occur independently of the RecA protein.

Phase variation in gonococcal pili expression can be caused by frameshift mutations in pilC

If PilC is required for pilus formation, we would expect some P^- progeny arising from a P^+ clone to accumulate unassembled pilin in the absence of PilC. Nonpiliated (P^-) colonies were derived from $MS11_{mk}(P^+)$, restreaked, and tested for the presence of PilC and pilin in immunoblots with the PilC and pili antisera. Five out of eight P^- clones did not produce detectable levels of PilC, but expressed the pilin subunit. The remaining three P^- clones expressed PilC but not pilin (data not shown). The molecular mass of the pilin subunit was the same in the P^-, $PilC^-$ variants as in $MS11_{mk}(P^+, PilC^+)$. However, the former in addition produced a protein reacting with the pili antiserum that was 16 kd in size (Figure 11, lane 11). Since $MS11_{mk}$ only contains one expression site for pilin we believe that this protein species represents a proteolytic degradation product of the pilin and may be identical to the S-pilin previously described (Haas *et al.*, 1987). Several independent P^- clones were isolated from one P^-, $PilC^-$ clone. They all remained $PilC^-$ and retained expression of pilin (Figure

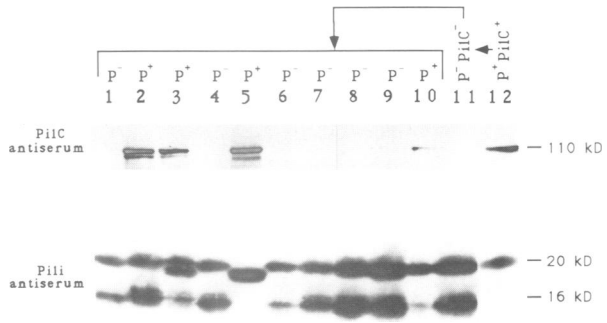

Fig. 11. Immunoblotting of a P^-, $PilC^-$ descendant of $MS11_{mk}(P^+, PilC^+)$ and its P^+ revertants with both PilC and pili antiserum. Nonpiliated (P^-) descendants of $MS11_{mk}(P^+, PilC^+)$ often lack PilC. Piliated as well as nonpiliated colonies from such a P^-, $PilC^-$ variant were isolated over a period of several days. The original $MS11_{mk}(P^+, PilC^+)$, its P^-, $PilC^-$ derivative and P^+ revertants as well as P^- colonies of this derivative were subjected to SDS–PAGE as whole cell lysates and immunoblotted with both PilC antiserum and pili antiserum. Lanes: 1–10, piliated, P^+, and nonpiliated, P^-, clones isolated from variant 8 (P^-, $PilC^-$); 11, variant 8 (P^-, $PilC^-$); 12, $MS11_{mk}(P^+, PilC^+)$.

11). Piliated (P^+) revertants were also obtained from the same P^-, $PilC^-$ clone. These P^+ revertants occurred at about a tenfold lower frequency (10^{-4}) than P^- derivatives from a P^+ clone. All P^+ revertants from a P^-, $PilC^-$ clone had regained expression of PilC (Figure 11). All but one expressed a pilin with the same molecular weight as the

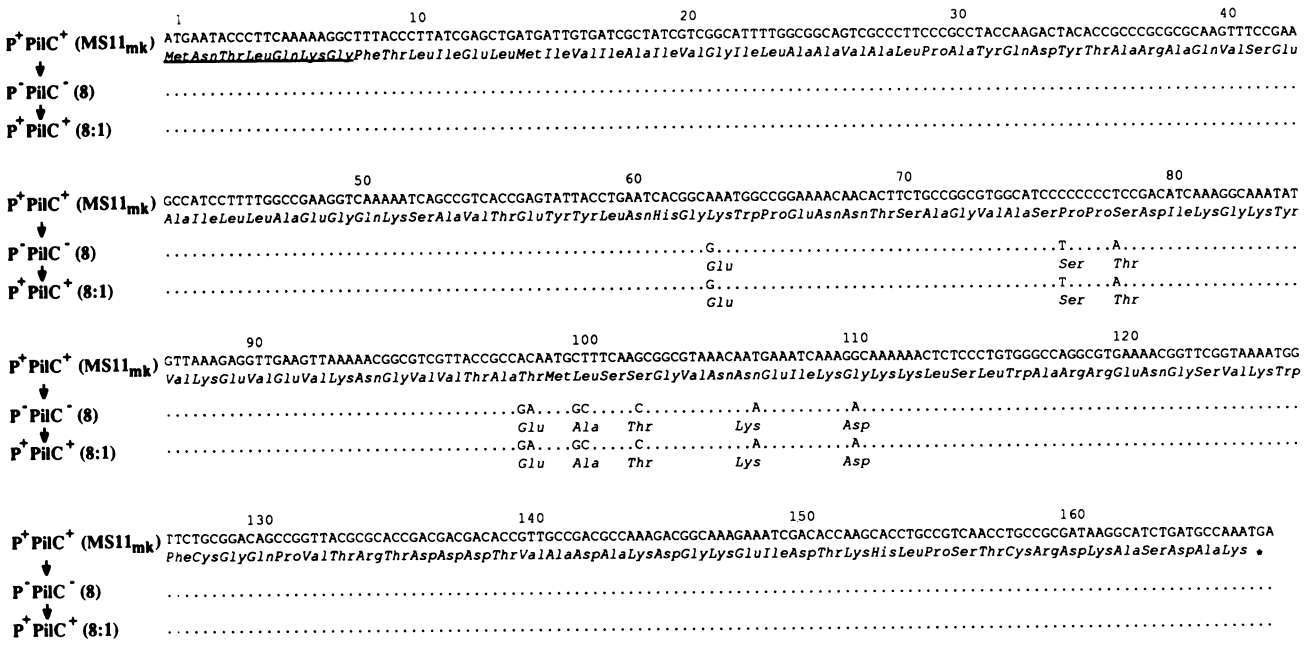

Fig. 12. DNA sequence and deduced amino acid sequence of the pilin structural gene (*pilE*) from MS11$_{mk}$(P$^+$, PilC$^+$), its P$^-$, PilC$^-$ derivative (8) and a P$^+$, PilC$^+$ revertant (8:1). The sequences were obtained by direct sequencing of PCR amplified *pilE*. Amino acids 1−7 represent the putative pilin leader polypeptide. Dots denote nucleotides identical to those in MS11$_{mk}$(P$^+$, PilC$^+$).

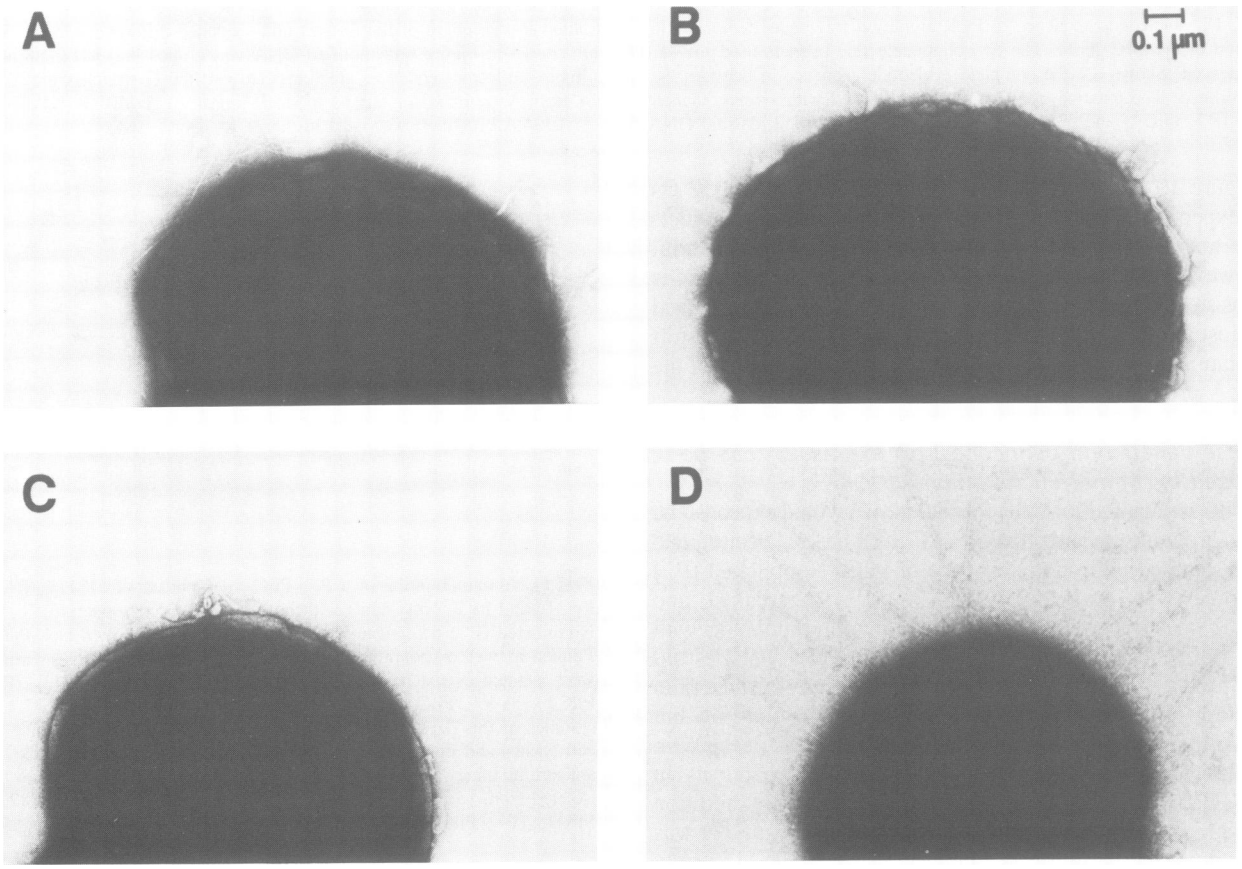

Fig. 13. Piliation of PilC$^+$ and PilC$^-$ gonococcal variants observed by transmission electron microscopy (70 000×). Representative samples are shown. (**A**) MS11$_{mk}$(P$^+$, PilC$^+$), (**B**) Variant 8 (P$^-$, PilC$^-$) with no pili, (**C**) variant 8 with one pilus and (**D**) variant 8:1 (P$^+$, PilC$^+$).

nonpiliated parent. However, the low molecular weight pilin degradation product was much less abundant in the P$^+$, PilC$^+$ revertants. It was possible to obtain P$^+$ revertants from other P$^-$, PilC$^-$ clones as well, all of which expressed PilC (data not shown).

The *pilE* gene from one set of PilC switches was PCR

amplified and sequenced directly. The P^-, $PilC^-$, $pilin^+$ variant 8 carried eight amino acid changes in the pilin relative to the parental clone $MS11_{mk}$ (Figure 12). The pilin sequence of the P^+, $PilC^+$ backswitcher 8:1 was identical to variant 8. Thus, the backswitching from P^- to P^+ colony morphology was not associated with any alteration in the pilus subunit protein implying that the change in colonial morphology was due to the switch in PilC expression.

Strain $MS11_{mk}(P^+)$, variants $8(P^-)$ and 8:1 (P^+) were also examined by transmission electron microscopy (Figure 13). The $MS11(P^+)$ parental cells were heavily piliated and pili were often seen to aggregate. In contrast most cells of variant $8(P^-)$ were nonpiliated. One or two pili were found on ~10% of these cells. All cells of variant 8:1 (P^+) were piliated, carrying ~10–40 fibers/cells. No aggregation of individual fibers was seen. These data confirm that the observed changes in colonial morphology reflect alterations in expression of pili. Therefore, phase variation of gonococcal pili may not only be caused by recombination events occurring in the *pilE* locus (Bergstrom *et al.*, 1986; Swanson *et al.*, 1986) but also by frameshift mutations in *pilC*.

Discussion

Even though Type 4 pili have been extensively studied in several laboratories, little is known about their assembly. The presence of a specific assembly machinery for this class of pili is evident from the fact that the pilin gene of *B.nodosus* and *M.bovis* can be properly processed and assembled into a pilus in *P.aeruginosa* but not in *E.coli* (Elleman *et al.*, 1986; Mattick *et al.*, 1987; Beard *et al.*, 1990). Furthermore, the recent genetic characterization of TCP pili of *Vibrio cholerae* has revealed that a number of closely linked genes are required for pilin processing and assembly into a structure (Taylor *et al.*, 1988). The TCP pilin does not carry an *N*-methylphenylalanine but its primary sequence is highly homologous to the Type 4 class of pilins.

The *N.gonorrhoeae* pilus facilitates adherence of the bacterium to a number of eukaryotic cell types (Watt *et al.*, 1980) and is thought to play a role in bacterial interaction with neutrophils (Fischer and Rest, 1988). The pilin is encoded from one or two *pilE* loci (Meyer *et al.*, 1984; Swanson *et al.*, 1986) which most likely each form a monocistronic operon. Hence, there have been no suggestions that genes closely linked to *pilE* are involved in pilus assembly. A dispersed location of genes involved in gonococcal pilus assembly as well as the rapid occurence of nonpiliated variants generated via recombination with pilin sequences from silent loci, *pilS*, have made it extremely difficult to identify putative assembly genes for gonococcal pili.

The PilC protein described in this paper is an outer membrane protein expressed in small amounts by *N.gonorrhoeae* MS11 and most other gonococcal strains. It is the only protein that is enriched in highly purified preparations of MS11 pili. PilC was not released from a nonpiliated MS11 (P^-n) variant using the same procedure (data not shown) suggesting that this protein interacts with the polymerized pilus fiber.

DNA sequence analysis of the cloned *pilC1* gene revealed one long open reading frame that was out of frame with its putative AUG initiation codon and 5′ end encoding the signal peptide. Minute amounts of PilC were expressed in *E.coli* from pABJ04. It is not known if this represents expression from in frame variants present in the population or frameshift suppression at the translational level. Gel purified PilC from MS11 contained a lysine residue in position four, whereas *pilC1* had a glutamine codon at this position. A lysine codon was, however, found at position four in a number of PCR amplified 5′ *pilC* fragments suggesting that these fragments represent the 5′ end of *pilC2*, which then must be ON in MS11. The finding that a miniTnCm insertion in *pilC2* abolished PilC expression, whereas insertional inactivation of *pilC1* did not abolish PilC expression further argues that *pilC1* is translationally out of frame and *pilC2* translationally in frame in the MS11 variant we are studying.

PCR amplified fragments of *pilC1* and *pilC2* in MS11 differed in the number of G residues found in the G tract. Only 11 or 12 Gs were found in *pilC1* clones (which would both generate an OFF phenotype) while 12 or 13 Gs were found among *pilC2* specific clones. Since *pilC2* is the expressed gene in the MS11 variant under study, we believe that this variant carries 13 Gs in *pilC2* and 12 Gs in *pilC1*. The frequency of frameshift mutations in each locus is not known. However, the lack of 13 Gs among *pilC1* specific fragments and the lack of 14 Gs among *pilC2* specific fragments suggests that a deletion of one G residue occurs at a higher frequency than the insertion of one G residue. We had expected to find amplified fragments from *N.gonorrhoeae* containing 10 G residues in the G tract, but found none in the 48 clones sequenced. If only one G is added or deleted in each mutational event, the frequency of G tracts with 10 residues should be low if G tracts normally are 12 or 13 bp long.

Frameshifting in *pilC1* also occurred in *E.coli*. In this case, however, two variants with 10 residues were found out of 12 clones sequenced. It may therefore be that there is a selection against in frame variants with 10 Gs in *N.gonorrhoeae*. A change from five glycines to four in the signal peptide may for example have an effect on the physical properties of the precursor form of PilC such that the signal peptide is not cleaved off. *E.coli* strain AA10 is *recA*. Therefore, frameshift mutations in the G tract of *pilC* occurs independent of the RecA protein.

Translational frameshifting has been shown to regulate phase and antigenic variation of the gonococcal opacity protein PII that is encoded by a number of *opa* loci showing sequence variations. In this system a number of pentameric CTCTT repeats is present in the region encoding the signal peptide (Stern *et al.*, 1986). Variation in the number of repeats is independent of *recA* in *N.gonorrhoeae* as well as in *E.coli* (Murphy *et al.*, 1989). Variation in the expression of lipopolysaccharide epitopes in *Haemophilus influenzae* was recently explained by translational frameshifting created by alterations in the number of CAAT repeats occurring in the 5′ end of *licA* (Weiser *et al.*, 1989). In *Bordetella pertussis* frameshift mutations in the regulatory *vir* locus occur in a run of C residues positioned internally in the gene (Stibitz *et al.*, 1989). The C tract was in this case varying from 6 (in frame) to 7 residues (out of frame). It is not known if this frameshift mutation is programmed or not. The pilin gene of *Bordetella pertussis* was recently shown to be preceded by a stretch of Cs. Frequent mutations affecting the length of this C tract influenced the transcriptional activity of the pilin gene (Willems *et al.*, 1990).

Variation in the number of the CTCTT repeats in *opa*

genes was recently suggested to be due to recombination-independent slipped strand mispairing (Murphy *et al.*, 1989). Mispairing is thought to occur between strands subjected to local denaturation and should preferentially occur during replication. A number of unusual DNA structures (cruciform, Z form, H form) have been shown to form *in vitro* within a variety of specific DNA sequences. Under normal conditions the B form is the most favorable thermodynamically (Frank-Kamenetskii and Vologodskii, 1984). Transition to alternative conformations requires specific external conditions, supercoiling being the most physiologic. Single stranded $(dG)_n$ and $(dC)_n$ strands renature more slowly than complementary strands with arbitrary sequences, and methylation experiments suggest that a poly dG chain may form a hairpin-like structure stabilized by G−G bp (Panyutin *et al.*, 1990). It is tempting to speculate that the high frequency of frameshift mutations occurring in the G tract of *pilC* is due to the formation of an unusual conformation e.g. G−G pairing in this stretch of DNA.

Four variant sequences differing outside the G tract were obtained by PCR amplification of the 5′ end of *pilC* from four *N.gonorrhoeae* strains (Figure 10). The region 5′ of the G tract was invariant, as was the 3′ end of the amplified region. All variation was confined to a region located 3′ of the G tract. At least some of these sequence variations can be explained by mismatch pairing events. Thus, the addition of four nucleotides distal to the G tract in variant sequence 4 is possible to explain by a two step mispairing event occurring within variant sequence 2. Slip strand mispairing between the two CA residues in −GGCGCAGGCGCA− would yield −GGCGCAGGCGCAGGCGCA−. A second mispairing event occurring between the two C-residues at position 3 and 5 gives rise to the sequence −GGCAGGCGCAGGCGCA− present in variant 4. It may therefore be that a sequence close to a poly(G) tract is prone to slipped strand mispairing.

Gonococcal pilus phase variation is associated with an altered nucleotide sequence of *pilE* via recombinations with silent *pilS* sequences (Haas and Meyer, 1986, Swanson *et al.*, 1986). An irreversible switch OFF in pilus expression results from deletions of the 5′ coding and control regions of the *pilE* locus (Swanson *et al.*, 1985). Reversible gonococcal pilus phase variation is associated with nucleotide changes in *pilE* resulting in an altered pilin product. It has been suggested that the pilins of these variants are assembly defective (Bergstrom *et al.*, 1986; Swanson *et al.*, 1986; Hill *et al.*, 1990). Here we present evidence that switch OFF and ON of PilC expression causes pilus expression to phase vary. Five out of five P^-, pilin producing descendants from $MS11_{mk}$ (P^+, $PilC^+$) that expressed pilin did not express PilC. All tested P^+ revertants from the five P^-, $PilC^-$ variants had regained expression of PilC. The pilin of one nonpiliated PilC OFF-switcher (variant 8) differed by eight amino acids from that of the parent. The fact that one piliated $PilC^+$ backswitcher (8:1) expressed a pilin identical in sequence to the nonpiliated variant (8) strongly suggests that the regained expression of pili is due to an ON-switch in PilC expression. The above results also imply that the nonpiliated phenotype of variant 8 is not due to the alterations in the pilin relative to the parental strain but to an OFF-switch of PilC. The finding that mTnCm insertions resulted in P^+ colonies when inserted into *pilC1* and P^- colonies when inserted into the actively expressing *pilC2* locus offers further evidence that PilC is essential for the biogenesis of gonococcal pili. P^-, *pilC2*::mTnCm-12 insertion mutants reverted to P^+ colony morphology at a low frequency. These revertants most likely represent frameshifting mutants in *pilC1* resulting in expression of PilC from this locus. A double mutant in *pilC1* and *pilC2* was stably nonpiliated, expressed pilin, but did not express any pili when examined by transmission electron microscopy. It is therefore believed that out of frame mutations of both *pilC1* and *pilC2* will abolish pili formation.

At this stage we cannot exclude the possibility that some $PilC^-$ variants from $MS11_{mk}$(P^+, $PilC^+$) are generated by transformation of *pilC1* sequences and homologous recombination with *pilC2* thus generating variants with two *pilC1* 5′ ends at both *pilC* loci. $PilC^+$ revertants from $PilC^-$ clones must, however, all be due to frameshift mutations in either *pilC1* or *pilC2*.

We propose that PilC forms an outer membrane pore or assembly center enabling the pilin subunits to be assembled and translocated across the outer membrane analogous to the proposed function of the high molecular weight proteins required for the assembly of enterobacterial pili (Norgren *et al.*, 1987).

It is possible that the alternate expression of PilC from two structurally different *pilC* loci is yet another example of antigenic variation in *Neisseria gonorrhoeae*. It is however possible that this variation could have functional implications as well. Each class of *E.coli* pili utilize a different outer membrane pore/assembly protein. Hence, pilin subunits and/or periplasmic chaperone complexes may specifically interact with an exposed region of the outer membrane protein allowing polymerization of pilus subunit proteins. The repertoire of antigenic variants of gonococcal pilins is vast (Hagblom *et al.*, 1985). It may be that only certain pilin variants are assembled via PilC1 and PilC2 respectively.

To determine the exact role of PilC in the biogenesis of gonococcal pili it will be necessary to develop a pilus assembly system in an heterologous background. Such studies are under way.

Materials and methods

Bacterial strains and growth conditions

N.gonorrhoeae $MS11_{ms}$ (Meyer *et al.*, 1984) and P^+ and P^-n variants of $MS11_{mk}$ (Swanson *et al.*, 1986) were kindly obtained from Dr M.So and from Dr M.Koomey, respectively. The gonococcal isolates UM01 and KH4318 have previously been described (Norlander *et al.*, 1981). *N.gonorrhoeae* strains 605344 and 605103 were obtained from Dr D.Danielsson, Örebro, Sweden, and strain 765 was isolated at the Department of Bacteriology in Umeå, Sweden. The commensal *Neisseria* species *N.lactamica* Nctc 10618 and *N.subflava* GN01 were obtained from Pharmacia, Uppsala, Sweden. These bacteria were grown at 37°C in a 5% CO_2 atmosphere on Difco GCB agar containing Kellogg's supplement. Piliated (P^+) and nonpiliated (P^-) variants were distinguished by colony morphology and passed as single colonies. *E.coli* strain Y 1090 (obtained from Promega Biotech) was used for plaque screening, DH5 (Hanahan, 1985) for molecular cloning, AA10 *recA* (Stoker *et al.*, 1984) for isolation of minicells and TG1 (Gill *et al.*, 1986) for propagation of M13 clones.

Preparation of pili and outer membranes

Pili were prepared essentially as described by Brinton *et al.* (1978). Gonococci (P^+Tr) from 80 GCB plates, grown for 18 h, were harvested in 0.05 M Tris−HCl pH 8.0 and 0.15 M NaCl, washed twice and resuspended in 40 ml 0.15 M ethanolamine pH 10.5. Pili were sheared off in a Sorvall Omnimixer, setting 3 for 30 s. The cell debris was pelleted at 13 000 *g* for 30 min at 4°C and the supernatant was dialyzed against 0.05 M Tris−HCl pH 8.0 and 0.15 M NaCl. The crystallized pili were

pelleted at 13 000 *g* for 60 min, resuspended in 0.15 M ethanolamine pH 10.5, and centrifuged at 23 000 *g* for 60 min. The supernatant was dialyzed as described above against 0.05 M Tris−HCl pH 8.0 and 0.15 M NaCl. Several cycles of crystallization and solubilization were performed to produce pili preparations with high purity. Outer membranes of *N.gonorrhoeae* were prepared by the sarkosyl method described by Norquist *et al.* (1978).

Antisera, immunoblot and aminoterminal and sequence determinations

Pili preparations of *N.gonorrhoeae* $MS11_{ms}(P^+)$ crystallized 5 times were separated on 10% SDS−polyacrylamide gels using the buffer system of Laemmli (1970). These gels were stained in 0.25 M KCl and 1 mM DTT for 5 min, the 110 kd protein band was sliced out, crushed and incubated in a buffer containing 0.05 M Tris−HCl pH 7.9, 0.1 mM EDTA, 5 mM DTT and 0.15 M NaCl at 4°C overnight. Gel pieces were removed by centrifugation prior to immunization of rabbits.

The achieved 110 kd-antiserum was extensively absorbed with *Pseudomonas putida* 2440 (Bagdasarian *et al.*, 1983), carrying a recombinant plasmid, pGC02, constructed as follows. The 1.0 kb *Hpa*I−*Eco*RI fragment of the pilus gene clone pNG1100 (Meyer *et al.*, 1984) obtained from M.So was cloned into the *Hpa*I and *Eco*RI sites of pMMB66 (Fürste *et al.*, 1986). The *pilE* gene is then under control of the *tac* promoter and induction with 1 mM IPTG resulted in high levels of pilin produced in *P.putida* 2440, but no extracellular pili structures were observed. Dense sonicated cultures of *P.putida* 2440/pGC02 were mixed in a 1:1 ratio with the crude antiserum. About 15 cycles of 1 h incubation and 30 min centrifugation at 25 000 *g* in the presence of 1 mM PMSF (phenylmethylsulfonylfluoride) at 4°C were performed.

The pili antiserum used in immunoblots was generated in a rabbit against highly purified pili preparations of *N.gonorrhoeae* $MS11_{ms}$.

In immunoblots 10 μg of boiled bacterial cells or the same amount of outer membranes were electrophoresed on 10% SDS−polyacrylamide gels. The proteins were transferred from the gel onto nitrocellulose sheets where their immunological cross reaction with the 110 kd absorbed antiserum was tested using an immunoblotting protocol as described by Towbin *et al.* (1979).

For aminoterminal sequence determination automated Edman degradations (Edman and Bregg, 1967) were performed in an updated Beckman 890C spinning cup sequencer. The sequencing procedure and the method for analysis of the 3-phenyl-2-thiohydantoin derivatives have been described (Engström *et al.*, 1984).

Genomic libraries

Chromosomal DNA from *N.gonorrhoeae* strains was isolated using the technique of Stern *et al.* (1986). Restriction digestion of the DNA was as recommended by the manufacturers.

An expression library in λgt11 was constructed from *N.gonorrhoeae* $MS11_{mk}$ (P^+) chromosomal DNA. *Eco*RI methylated (New England Biolabs) chromosomal DNA (50 μg) was sheared by sonication, treated with mung bean nuclease (New England Biolabs) to produce blunt ends, and size fractionated over a sucrose gradient. Fractions corresponding to 1−5 kb were ligated to *Eco*RI linkers (New England Biolabs), extensively cut with *Eco*RI and the small oligonucleotides were separated from the genomic DNA pieces over a Sephadex G50 column. Insert and λgt11 vector were ligated and packaged *in vitro* using the Protoclone and Packagene reagents from Promega Biotech. Screening of the λgt11 library in Y1090 with the absorbed 110 kd antiserum was performed by the method described by Gotschlish *et al.* (1986). Plates containing 10^3-10^4 plaques were incubated for 3−4 h at 42°C. A nitrocellulose filter previously impregnated with 10 mM IPTG was placed on top of each plate and the incubation was continued at 37°C for 3 h. The filters were placed in blocking solution (0.15 M NaCl, 50 mM phosphate buffer, pH 7.4 and 0.5% gelatin) for 1 h, incubated with the 110 kd antiserum for 2 h, washed three times, incubated with alkaline phosphatase conjugated anti rabbit IgG for 1 h, washed and finally incubated with alkaline phosphatase substrate.

A purified positive plaque was used to infect Y1089 to produce lysogens. These were induced for phage production by shifting incubation temperature to 44°C and then induced with IPTG for antigen production. The presence of a β-galactosidase fusion protein was examined in Western blots using the absorbed 110 kd antiserum.

The plasmid library used was kindly provided by Dr T.Meyer and had been constructed essentially as previously described (Meyer *et al.*, 1982). However, pBA (Segal *et al.*, 1985), a pBR322 derivative, had been used as cloning vector. Bacterial colonies on LA-plates were transferred directly to nitrocellulose filters (Schleicher & Schuell). The filters were then placed on Whatman 3MM paper soaked in solutions of 0.5 M NaOH, 1.5 M Tris−Cl pH 7.5, and 1.5 M NaCl, 0.5 M Tris−Cl pH 7.4 for 5 min each. After baking for 2 h at 80°C the filters were hybridized with ^{32}P-labeled probe (multiprime DNA labeling system, Amersham International). The filters were washed in 4 × SSPE and 0.1% SDS, 2 × SSPE and 0.1% SDS, 0.2 × SSPE and 0.1% SDS for 2 × 15 min each and thereafter exposed to Kodak X-Omat AR film.

Southern blot

Digested genomic DNA was separated on 0.7% agarose gels and transferred to nitrocellulose filters (Southern, 1975). After transfer and baking the filters were prehybridized in a mixture of 5 × SSC, 0.1% SDS, 5 mM EDTA, 5× Denhardt's solution and 100 μg/ml of sonicated calf thymus DNA at 65°C for 2−6 h. ^{32}P-labeled probe (multiprime DNA labeling system, Amersham International) was added and hybridization was performed for 12−15 h at the same temperature. The filters were washed in 2 × SSC with 0.1% SDS and in 0.2 × SSC with 0.1% SDS for 2 × 15 min each, dried and exposed to Kodak XRP film at −80°C.

A 21 base long oligonucleotide complementary to the signal peptide coding region of *pilE* (5′-GCCTTTTTGAAGGGTATTCAT-3′) was ^{32}P-labeled with T4 polynucleotide kinase and used to probe *Cla*I digested genomic DNA. The blot was prehybridized at 37°C in a mixture containing 2 × Denhardt's, 0.1% SDS, 2.5 mM EDTA, 5 × SSC and 100 μg/ml sonicated calf thymus DNA, hybridized at 37°C and washed in 2 × SSC for 5 min. $MS11_{mk}(P^+)$ gave a 4 kb hybridization fragment, whereas $MS11_{mk}(P^-n)$ and 605103 gave no hybridization signal.

Minicells

The *E.coli* minicell strain AA10 was transformed with plasmid DNA (pABJ04-09) and chromosome deficient minicells from these strains were purified over sucrose gradients (Thompson and Achtman, 1978). The plasmid encoded proteins were labeled in the presence of 80 μCi [^{35}S]methionine in minimal salts medium and 1% methionine assay medium (Difco). After lysis of the minicells in sample buffer (Laemmli, 1970) the proteins were electrophoresed on an SDS−polyacrylamide gel, the gel was dried and exposed to X-ray film (Kodak X-OmatAR).

Mutagenesis with miniTnCm

The shuttle mutagenesis system developed by Seifert *et al.* (1986) using a miniTn3 carrying the chloramphenicol resistance gene was kindly provided by Dr M.So. Mutagenesis of pABJ04 with mTnCm and transformation of *N.gonorrhoeae* were performed as previously described (Seifert *et al.*, 1990). MiniTnCm insertions at 30 different positions in pABJ04 were identified, two of which mapped within the *pilC* gene. Piliated *N.gonorrhoeae* $MS11_{mk}$ were transformed with 2 μg plasmid DNA, transformants were selected for on plates containing 10 μg/ml chloramphenicol for the single mutants and 30 μg/ml chloramphenicol for the double mutants.

DNA sequencing

Purified DNA fragments from pABJ04 and PCR amplified 5′ end of *pilC1* was subcloned into M13 vectors (Sanger *et al.*, 1980; Yanish-Perron *et al.*, 1985) and sequenced using the chain termination method of Sanger *et al.* (1977). Primers used were the M13 17mer universal primer and oligonucleotides synthesized at Symbicom, Umeå, Sweden or at the Department of Biochemistry, Washington University, St Louis, MO, USA.

DNA sequence of the pilin structural gene in different gonococcal variants was determined by direct sequencing of PCR amplified *pilE*. The oligonucleotides used in the PCR reaction were 5′-TTTCCCCTTTCAA-TTAGGAGT-3′ and 5′-AAATTTAAGGCCTAATTTGCC-3′. Amplified fragments were purified from agarose gels and used as templates in asymmetric PCR reactions. The final concentration of the limiting primer was 0.005 μM and of the excess primer was 0.5 μM. DNA from the asymmetric PCR reactions was precipitated with isopropanol and ammoniumacetate and sequenced directly by using the Sequenase sequencing kit together with internal primers of *pilE*.

Electron microscopy

Electron microscopy was performed with a JEOL 100CX microscope with 200-mesh copper grids coated with thin films of 2% Formvar. The bacterial colonies were carefully overlaid with buffer [10 μM Tris−HCl (pH 7.5), 10 μM magnesium chloride] and the cells were allowed to sediment for 15 min on a grid. The grids were washed with water, negatively stained for 10 s with 1% sodium silicotungstate (pH 7.0) and then washed again.

PCR amplification

Polymerase chain reaction was carried out in 100 μl containing 50 ng of genomic DNA or 5 ng of plasmid DNA, 1.0 μM of each oligonucleotide, 200 μM of each nucleotide, 0.001% gelatin, 1.5 mM $MgCl_2$, 10 mM Tris (pH 8.3), 50 mM KCl, 0.25 μl 1 mCi/ml[^{32}P]dATP and 2 U of *Taq*

Polymerase (Perkin Elmer Cetus). The samples were passed through 25 cycles: 2 min at 50°C, 1 min at 94°C and 3 min at 72°C in a Thermal Cycler (Perkin Elmer Cetus). Aliquots of the DNA fragments were denatured at 95°C for 2 min and electrophoresed on standard denaturing sequencing gels.

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