Pilus biogenesis gene, *pilC*, of *Neisseria gonorrhoeae*: *pilC1* and *pilC2* are each part of a larger duplication of the gonococcal genome and share upstream and downstream homologous sequences with *opa* and *pil* loci

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Pili of *Neisseria gonorrhoeae* mediate attachment of the bacteria to target cells and undergo both phase and antigenic variation. PilC is a 110 kDa minor pilus-associated protein involved in pilus biogenesis and attachment. The expression of PilC is turned on and off at high frequency and is controlled by frameshift mutations in a run of G residues positioned in the region encoding the signal peptide. Most strains of *N. gonorrhoeae* carry two copies of *pilC*. The DNA sequence of *pilC1* of strain MS11 is presented and compared to the sequence of the 3' end of *pilC2*. These two genes are highly homologous, but not identical. The putative transcriptional terminator of *pilC1* contains a pair of inverted uptake sequences for gonococcal DNA (5'-GCCGTCTGAA-3'). An 88 bp sequence located upstream of the *pilC1* gene has also been reported to precede several *opa* genes of *N. gonorrhoeae*. Shorter regions positioned both downstream and upstream of *pilC1* can also be found in silent *pil* loci as well as close to *opa* genes. The *pilC* genes are part of a duplication of a larger DNA region extending more than 2 kb downstream of the coding region.

Keywords: *Neisseria gonorrhoeae*, pilus biogenesis, repetitive DNA, *pilC*

INTRODUCTION

Surface components of *Neisseria gonorrhoeae* have been the subject of intense examination in order to lead to a better understanding of the biology of this organism and to identify vaccine candidates for gonorrhoea. Many of the surface-exposed antigens, among which are pili, opacity protein (Opa) and lipopolysaccharide (Lambden *et al.*, 1981; Hagblom *et al.*, 1985; Diaz & Heckels, 1982; Barritt *et al.*, 1987; Mandrell *et al.*, 1986), exhibit extensive structural and antigenic variations. Pili are filamentous protein appendages that facilitate the attachment of the gonococcus to human tissues (Swanson, 1973; Pearce & Buchanan, 1978), and are one of the components that can be unambiguously correlated with virulence. All primary isolates are pilated and non-piliated mutants were shown

to be avirulent in male volunteers (Kellogg *et al.*, 1968; Swanson *et al.*, 1987).

The major pilus subunit (pilin) is expressed from the *pilE* locus (Meyer *et al.*, 1982, 1984). Most *N. gonorrhoeae* strains carry only one copy of the expressed pilin gene (*pilE*) and several silent partial pilin gene copies (*pilS*) that lack the 5' end and promoter region (Haas & Meyer, 1986). Pilin antigenic variation occurs at a high rate, such that a single strain can give rise to a broad spectrum of pilus variants, and is mediated by recombination events that result in the transfer of variable sequences from any of the silent loci to the expressed locus (Haas & Meyer, 1986; Swanson *et al.*, 1986).

PilC is a 110 kDa pilus-associated protein involved in pilus biogenesis (Jonsson *et al.*, 1991, 1992). Expression of PilC is controlled by frequent frameshift mutations in a stretch of G residues within the signal peptide coding region (Jonsson *et al.*, 1991). Strain MS11_{mk}(P⁺)-u contains two *pilC* loci, of which only one, *pilC2*, is expressed. Insertional mutants expressing either PilC1 or PilC2 formed equal amounts of pili and both mutants

Abbreviation: OMP, outer-membrane protein.

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bound at equal levels to target cell lines and tissues (Jonsson *et al.*, 1994).

It has been demonstrated that different Pile sequences in *N. gonorrhoeae* and *Neisseria meningitidis* are associated with different abilities to mediate binding to epithelial cells (Rudel *et al.*, 1992; Virji *et al.*, 1992, 1993; Nassif *et al.*, 1993; Jonsson *et al.*, 1994). The PilC protein has been shown to be important in pilus-mediated attachment (Rudel *et al.*, 1992) and PilC has been suggested to be localized at the tip of the pilus fibre (Rudel *et al.*, 1995). In *N. meningitidis*, PilC1, but not PilC2, could mediate binding of the bacteria to target cell lines (Nassif *et al.*, 1994), and inactivation of both *pilC1* and *pilC2* resulted in non-piliated clones.

Pili of pathogenic *Escherichia coli* have been extensively studied. The fimbrial structures of P-, S- and type 1 pili are composed of a major subunit and several minor components including an adhesin (Hanson & Brinton, 1988; Lindberg *et al.*, 1987; Moch *et al.*, 1987). The components involved in the biogenesis of these surface organelles are encoded by genes closely linked to the pilin structural gene (Mooi *et al.*, 1983; Orndorff & Falkow, 1984; Hacker *et al.*, 1985; Norgren *et al.*, 1984). Two accessory proteins required for pilus formation have been identified for P-pili (Norgren *et al.*, 1987; Lindberg *et al.*, 1989; Hultgren *et al.*, 1989); a large (88 kDa) outer-membrane protein (OMP) is thought to play a role in the assembly of pilin subunits and in their translocation across the outer membrane, and a smaller protein (28 kDa) acts as a chaperone by complexing with the pilus subunit proteins in the periplasm prior to polymerization, thereby preventing premature aggregation of the subunits.

The pili of *N. gonorrhoeae* belong to the N-methyl-Phe (or type 4) class of fimbriae also found in *N. meningitidis* (Hermondson *et al.*, 1978; Potts & Saunders, 1988), *Moraxella bovis* (Marrs *et al.*, 1985), *Vibrio cholerae* (Faast *et al.*, 1989), *Pseudomonas aeruginosa* (Pasloske *et al.*, 1985) and *Dichelobacter (Bacteroides) nodosus* (McKern *et al.*, 1983). The major pilus subunit (pilin) of these species share extensive sequence homologies in the N-terminal hydrophobic portion of the protein. Recently, the region upstream of the pilin structural gene of *P. aeruginosa* was shown to encode three proteins necessary for proper processing of the pilin subunits and assembly of pili (Nunn *et al.*, 1990). One of the products (PilD) is necessary for the cleavage of the leader sequence from the precursor pilin subunit (PilA). Several *N. gonorrhoeae* gene products have been identified that bear homology to proteins involved in pilus assembly and protein export of other bacterial systems (Hobbs & Mattick, 1993).

Opa proteins are a family of OMPs associated with opacity, adhesion, serum resistance and invasion (Virji & Everson, 1981; Swanson, 1982; James *et al.*, 1982; Fischer & Rest, 1988; Makino *et al.*, 1991). There are several *opa* genes in the gonococcal genome, most of which have sequence differences (Stern *et al.*, 1986; Connell *et al.*, 1988; Bhat *et al.*, 1991). Opa proteins show both phase and antigenic variation; each gene is independently regulated by frameshift mutations in a CTCTT repeat

located within the region encoding the signal peptide (Stern *et al.*, 1986).

In this paper we present the complete nucleotide sequence of *pilC1*, the first gene identified to be involved in the biogenesis of *N. gonorrhoeae* pili. The *pilC1* gene is preceded by an 88 bp sequence, also found upstream of the promoter region of several *opa* genes, as well as in other known gonococcal sequences. Two shorter regions, homologous to non-coding sequences in *opa* and *pil* loci, are present on either side of the *pilC1* gene. These repetitive elements are possible sites for recombination, but might also be involved in regulation.

METHODS

Bacterial strains and growth conditions. *N. gonorrhoeae* MS11_{mk}(P⁺) (Swanson *et al.*, 1985) was kindly provided by Dr M. Koomey. The specific MS11_{mk}(P⁺) used in our studies is designated MS11_{mk}(P⁺)-u, in order to distinguish it from other MS11_{mk}(P⁺) strain samples. The bacteria were grown at 37 °C in a 5% CO₂ atmosphere on Difco GCB agar containing 10 ml supplement l⁻¹. The supplement contains 400 mg glucose, 5 mg glutamine, 0.5 mg Fe(NO₃)₃·9H₂O and 0.02 mg thiaminpyrophosphate ml⁻¹. Piliated (P⁺) and non-piliated (P⁻) variants were passaged every 18–20 h. *E. coli* strain DH5 (Hanahan, 1985) was used for molecular cloning and TG1 (Gill *et al.*, 1986) for propagation of M13 clones.

DNA isolation and manipulation. Total DNA from *N. gonorrhoeae* was isolated from bacteria grown on plates. Gonococci were removed from the GCB-plates with a cotton swab, washed once in PBS and resuspended in 0.2 ml of a solution containing 0.1 M NaCl, 10 mM Tris (pH 8.0) and 10 mM EDTA. The cells were lysed by addition of 25 µl 10% (w/v) SDS followed by incubation at 65 °C for 10 min. After lysis of the bacteria, 7 µl Proteinase K (20 mg ml⁻¹) was added and the incubation was continued at 37 °C for 4 h. The mixture was then extracted with phenol:chloroform (1:1) three times and precipitated with ethanol.

Plasmid DNA extraction and preparation of lambda DNA were performed essentially as described by Sambrook *et al.* (1989). The preparation of lambda DNA was modified so that at least six phenol:chloroform extractions, instead of two, were performed. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs.

Southern blotting and hybridization. Digested genomic DNA was separated on a 0.7% agarose gel and transferred to a Hybond N filter (Southern, 1975). After transfer and baking, the filter was pre-hybridized with a mixture of 5 × SSC, 0.1% SDS, 5 mM EDTA, 10 × Denhardt's solution and 100 µg sonicated calf thymus DNA ml⁻¹ at 65 °C for 2–6 h. ³²P-labelled probe (multiprimer DNA labelling system, Amersham International) was added and hybridization was performed for 12–15 h at the same temperature. The filter was washed twice in 2 × SSC with 0.1% SDS for 15 min, in 0.2 × SSC with 0.1% SDS for 15 min, dried and exposed to Kodak X-Omat AR film at -80 °C. All probes were labelled and hybridized to one single filter that contained *Clai*I- and *Sma*I-digested gonococcal DNA of MS11_{mk}(P⁺)-u. After exposure to X-ray film, the radioactive probe was removed from the filter by incubation in 0.4 M NaOH at 45 °C for 30 min, after which the filter was soaked in 0.2 M Tris/Cl, pH 7.4, 0.1% SDS, 0.1 × SSC at 45 °C for 60 min. The washed Hybond-N filter was exposed to X-ray film for 2 d at -80 °C and hybridized to a second probe if no residual hybridization signal was observed. If weak hybridization signals were still detected, the filter was left for 1–2

Fig. 1. Physical map of the *pilC* loci in *N. gonorrhoeae* MS11. Restriction maps of loci 1 and 2 were obtained by hybridization using the three probes indicated by thicker black bars, i.e. probe F (the *EcoRV*₁–*EcoRV*₂ fragment spanning the central region of *pilC1*), probe G (the *EcoRV*₃–*HindIII*₄ fragment containing the 5' end of *pilC1*) and probe H (an 800 bp fragment from the 3' end of *pilC2*). Differences in the strength of hybridization signals were used to distinguish between fragments from locus 1 or 2. DNA fragments outside the *pilC1* gene used as probes in Southern hybridizations are marked with solid black bars and designated A–E and I. Plasmid pABJ04 contains a full-length copy of *pilC1*; the direction of transcription is indicated by an arrow. The 800 bp insert from λ gt11 contains the 3' end of *pilC2*, and has an additional *SalI* site not found in the plasmid clone pABJ04.

months and then exposed again for 2 d, at which time the filter was always totally blank.

DNA sequencing. Purified DNA fragments from pABJ04 [*MluI*₁–*MluI*₂; *MluI*₂–*MluI*₃; *MluI*₃–*EcoRV*₁; *EcoRV*₁–*SalI*; *SalI*–*EcoRV*₂; *EcoRV*₂–*EcoRV*₃; *HindIII*₃–*HindIII*₄; *HindIII*₄–*BamHI* (in the pBA vector upstream of *pilC1*, 50 bp from the start of the gonococcal insert in pABJ04); *HindIII*₂–*HindIII*₃; and *SalI*–*BamHI*] were subcloned into M13mp18 and M13mp19 vectors (Sanger *et al.*, 1980) and sequenced using the chain-termination method of Sanger *et al.* (1977). Primers used were the M13 17-mer universal primer and oligonucleotides synthesized at Symbicom, Umeå, Sweden, or at the Dept. of Biochemistry, Washington University, St Louis, MO, USA. Each fragment was initially sequenced from both ends, after which appropriate primers were chosen from the obtained sequence to get the complete nucleotide sequence of both strands of each fragment.

The 800 bp *EcoRI*–*EcoRI* insert of the λ gt11 clone containing the 3' end of *pilC2* (Jonsson *et al.*, 1991) was transferred to

plasmid pACYC184, generating pABJ01. The gel-purified 800 bp insert of this clone was digested with *SalI*, which has a site positioned in the central region of the 800 bp *EcoRI*–*EcoRI* fragment, to give two smaller fragments of DNA. The complete 800 bp fragment, as well as the two *SalI*–*EcoRI* fragments, were cloned into M13mp18 and M13mp19 and sequenced from both ends. The exact size of the '800 bp' fragment turned out to be 739 bp.

RESULTS

The complete nucleotide sequence of *pilC1* of *N. gonorrhoeae* MS11

PilC is involved in pilus biogenesis of *N. gonorrhoeae* and *N. meningitidis*. In strain *N. gonorrhoeae* MS11, PilC can be expressed from either of two loci, *pilC1* or *pilC2*. Linear chromosomal maps of the *pilC* loci were constructed by Southern hybridizations using three *pilC*-specific probes, i.e. probe F, G and H in Fig. 1. Differences in the strength

Fig. 2. For legend see facing page.

Fig. 3. Homologies between *pilC1* and *pilC2*. Nucleotide sequence and deduced amino acid sequence of the 800 bp insert from λ gt11 (bp 1–739) that contains the 3' end of *pilC2*. This sequence is compared to that of the 3' end of *pilC1* (bp 2446–3178 of the *pilC1* gene shown in Fig. 2). Identical nucleotides are indicated by small dots; large dots indicate identical amino acids in *PilC1* and *PilC2*. Asterisks mark base pairs not existing/deleted in either *pilC1* or *pilC2*. Selected restriction enzyme sites are underlined.

of the hybridization signals were used to determine which loci the hybridizing restriction fragments belonged to (data not shown).

The DNA sequence of the entire coding region of *pilC1* was determined. Plasmid pABJ04, which carries a complete copy of the *pilC1* gene, was sequenced from the *Bam*HI site to the *Mlu*I site (Fig. 1). One ORF that starts at position 475 and ends at 3468 was identified (Fig. 2). This cloned *pilC1* gene of MS11 has previously been shown to be out-of-frame in a run of G residues within the region encoding the signal peptide (Jonsson *et al.*, 1991). Addition of one G residue to the 12-residue-long G stretch (position 460–471) within the signal peptide coding region would result in an in-frame mature protein of 996 (1029 with signal peptide) amino acids with a

predicted molecular mass of 108.3 kDa (112.4 kDa with signal peptide), which is close to the estimated molecular mass of 110 kDa for the protein subjected to SDS-PAGE (Jonsson *et al.*, 1991).

The identified ORF is preceded by an RBS (-AGGAA-) located at position 371–375. No sequences in the –10 and –35 regions were found that conformed to the consensus sequence for a Pribnow box, which binds to RpoD (σ^{70}) containing RNA polymerase. Promoters recognized by the alternative sigma factor RpoN (σ^{54} in *E. coli*) have the consensus sequence TGGCAC-N5-TTGC situated between –26 and –12. Such a promoter sequence has been found to precede the pilin structural gene, *pilE*, of *N. gonorrhoeae* MS11 (Meyer *et al.*, 1984). The G residues in the consensus are totally conserved in all identified

Fig. 2. DNA sequence and deduced amino acid sequence of *pilC1*. Both the in-frame and the out-of-frame variant of the *pilC1* gene on plasmid pABJ04 are shown. The ATG start codon (position 382–384) and cleavage sites for a number of restriction enzymes are underlined. Double underlining indicates the variable G stretch in the signal peptide coding region and the arrow marks the proposed signal peptidase cleavage site (Jonsson *et al.*, 1991). The complete *pilC1* gene in plasmid pABJ04 is out-of-frame. Two possible reading frames for the out-of-frame copy (12 Gs) are shown, one starts at position 382 and ends at 504 and the other starts at 475 and ends at 3478. The in-frame variant of *pilC1* starts at 382 and ends at 3478. A stem-loop terminator structure at position 3498–3530 is also underlined and carries inverted copies of the consensus uptake sequence (5'-GCCGTCTGAA-3') for gonococcal DNA. Two repetitive elements, an 88 bp stretch (position 1–88) and a 38 bp stretch (position 176–213), in the region preceding the start codon are found in many other gonococcal DNA sequences such as *opa* and *pil* loci (see Fig. 4). Black bars mark three putative σ^{54} -dependent promoter regions (consensus 26-TGGCAC-N5-TTGC-12). The box indicates the part of *pilC1* that is compared to *pilC2* in Fig. 3. Numbering starts at the *Sau*3A1 site at the junction between the pBA vector and gonococcal DNA upstream of *pilC1* and ends at the *Mlu*I site in plasmid pABJ04 (Fig. 1).

Fig. 4. Repetitive DNA elements are positioned upstream of the *pilC1* gene. The nucleotide sequence that precedes the translational start of *pilC1* is compared to other gonococcal DNA sequences. An 88 and 38 bp region highly homologous to stretches in *opa* and *pil* loci are boxed. The 38 bp repeat is also present downstream of *pilC1* (see Fig. 5). Internal 26 bp repeats within the 88 bp repeat and within the *PilC* sequence are marked. The -35 regions of the *opa* genes are underlined, the distance from the last bp shown in the -35 region to the ATG is 82–85 bp for the *opa* genes shown here. Dots denote nucleotides identical to those in *pilC1* or to bp 1–74 in the *PilC* gene sequence. Nucleotides are numbered according to the published sequences. The position of each sequence in relation to the start or stop codon (if one exists) is indicated by the number of base pairs between the last base given and the start/stop codon (underlined, with an arrow indicating the direction of transcription). The *opaD*, *opaE*, *opaF*, *opaI*, *opaJ* and *opaK* loci were from *N. gonorrhoeae* strain MS11 (Stern *et al.*, 1986; Bhat *et al.*, 1991), the *opa* locus encoding *PilC* was from strain JS3 (van der Ley *et al.*, 1988) and pFLOB1100, pFLOB790 and pFLOB210 were from strain FA1090 (Connell *et al.*, 1988). The small repeat elements, pNG273, pNG268 and pNG294, were from *N. gonorrhoeae* strain 5019 (Correia *et al.*, 1986). *opaE1*, *pilE*, *pilA* and *pilS1* of strain MS11 are described by Meyer *et al.* (1984), Taha *et al.* (1988) and Haas & Meyer (1986). The *rmp* sequences are from strain R10 (Gotschlich *et al.*, 1987).

promoters of this kind (Morett & Buck, 1989). Three putative binding sites that contain the conserved three G residues and at least two or three additional bases of the consensus could be identified upstream of the *pilC1* gene.

A potential terminator was identified with the stems at positions 3498–3511 and 3517–3530. This dyad symmetry arrangement contains the signal for recognition of transforming DNA (5'-GCCGTCTGAA-3'); DNA containing these uptake targets has been shown to interact efficiently with the cellular DNA receptor (Goodman & Scooca, 1988; Elkins *et al.*, 1991).

The deduced amino acid sequence of *pilC1* was compared to other known DNA and amino acid sequences in the EMBL database. No significant homologies to other reported sequences were found.

The two *pilC* genes are not identical

In *N. gonorrhoeae* strain MS11_{mk}(P⁺)-u, *pilC2* is expressed and *pilC1* is silent. Inactivation of *pilC2* but not *pilC1* abolishes expression of pili. Pilated revertants of a *pilC2::mTnCm* mutant arise at high frequency and accordingly should express *PilC1*. Thus, both *pilC1* and *pilC2* encode functional *PilC* proteins (Jonsson *et al.*, 1991).

To compare *pilC2* to *pilC1* at the nucleotide level, an 800 bp clone containing the 3' end of *pilC2* (Fig. 1) was subcloned and sequenced by the dideoxy chain-termination method. One ORF of 729 bp was identified that covered the complete fragment. The nucleotide sequence and the deduced amino acid sequence of this region were aligned to the corresponding region of *pilC1* (Fig. 3). The

Fig. 5. Repetitive region 0.7 kb downstream of *pilC1*. The nucleotide sequence of a region that starts 0.7 kb from the end of *pilC1* and ends at the *HindIII*₂ site in pABJ04 is given. The *HindIII*₂ site is underlined, as is the stop codon of *pilC1* and the start codon of *pilE1*. The 152 bp repetitive region is boxed. A 38 bp stretch within the larger repetitive boxed region, which also shows homology to a region upstream of *pilC1* (Fig. 4), is indicated. Sequences are numbered corresponding to the published sequences. The position of each fragment in relation to the start or stop codon (if one exists) is indicated by the number of base pairs between the last base given and the start/stop codon (underlined, with an arrow indicating the direction of transcription). The *pilE* upstream sequence was from strain P9 (Perry *et al.*, 1987), the *opaE1* and the *pilS1* loci were from MS11 (Stern *et al.*, 1986; Haas & Meyer, 1986), pFLOB210 and pFLOB709 contain *opa* loci from strain FA1090 (Connell *et al.*, 1988) and *pilA* was from strain MS11 (Taha *et al.*, 1988).

sequence of these two loci are highly homologous but not identical. Triplets of base pairs were often found to be deleted or inserted in this DNA sequence. The deduced amino acid sequence of *pilC1* shows an 85% identity and a 90% similarity to the deduced amino acid sequence of *pilC2*. Thus, two structurally different forms of PilC protein can be produced by *N. gonorrhoeae* MS11.

Short stretches of DNA flanking *pilC1* show high homology to repetitive regions in *opa* and *pil* loci

Upstream of the transcriptional start of *pilC1*, an 88 bp stretch of DNA was identified that is highly homologous to the region that precedes the *opa* gene encoding PilC of strain JS3 (van der Ley *et al.*, 1988), the *opaD*, *opaE*, *opaF*, *opaI*, *opaJ* and *opaK* genes of strain MS11 (Stern *et al.*, 1986; Bhat *et al.*, 1991), and the *opa* gene of clone pFLOB1100 from strain FA1090 (Connell *et al.*, 1988) of *N. gonorrhoeae* (Fig. 4). The homologies between the 88 bp *pilC1* upstream region and the *opa* sequences are 93–98%. The -35 region of the *opa* σ^{70} -dependent promoter and one of the putative σ^{54} -dependent promoters of the *pilC1* gene are located immediately after the 88 bp repeats. The *pilC1* upstream region is also highly homologous to the small-repeat-element of *N. gonorrhoeae* DNA originally identified as DNA fragments that contain a 26 bp

repetitive sequence (Correia *et al.*, 1986). The 26 bp repetitive sequence, present in at least 20 copies in the gonococcal chromosome, is specific for *N. gonorrhoeae* and *N. meningitidis* and has been suggested to be part of a transposon-like element (Correia *et al.*, 1986); the last 26 bp of the 88 bp upstream region of *pilC1* correspond to this 26 bp repeat. Homologies to *pilC1* were found in small-repeat-element clones pNG273, pNG268 and pNG294 of strain 5019 (Correia *et al.*, 1986) (Fig. 4). The 26 bp sequence is also present downstream of *rmp* (PIII) (Gotschlich *et al.*, 1987) and upstream of *pilE* (Meyer *et al.*, 1984; see Fig. 4). The homology between *pilC1* and the *opa* clone pFLOB709 of strain FA1090 (Connell *et al.*, 1988) is limited to the 26 bp repeat element.

In addition to the 88 bp region, a 38 bp repetitive sequence was identified in the region preceding *pilC1*. The 38 bp sequence is homologous to repeats in silent and expressed *pil* loci as well as in the region preceding the *opa* gene of clone pFLOB210 (Connell *et al.*, 1988), the region downstream the *opa* gene of clone pFLOB790 (Connell *et al.*, 1988) and the region in between the stop codons of *opaE1* and *pilA* (Stern *et al.*, 1986; Taha *et al.*, 1988) (Fig. 4).

Repetitive DNA is also present downstream of *pilC1*. A short DNA sequence 1 kb downstream of the *pilC1* stop

Fig. 6. Southern blot hybridizations using probes flanking the *pilC1* gene. *ClaI* (lane 1) and *SmaI* (lane 2)-digested gonococcal DNA were hybridized with six different fragments of plasmid pABJ04 (see Fig. 1). (a) *PvuII*–*BalI* fragment (probe A); (b) *BalI*–*HindIII*₁ fragment (probe B); (c) 0.7 kb *HindIII*₁–*AvaI*₁ fragment (probe C); (d) *AvaI*₁–*HindIII*₂ fragment (probe D); (e) *HindIII*₂–*MluI*₁ fragment (probe E); (f) a *pilC*-specific probe, the *EcoRV*₁–*EcoRV*₂ fragment of pABJ04 (probe F), hybridizing to both loci (Jonsson *et al.*, 1991). *HindIII*-digested phage lambda DNA markers are indicated on the left; sizes of hybridizing fragments (in kb) are indicated on the right.

codon was obtained by subcloning the *HindIII*₂–*HindIII*₃ fragment of pABJ04 into M13 vectors and sequencing from the *HindIII*₂ site. Within the 250 bp sequence at the *HindIII*₂ end of this fragment, a central 152 bp region highly homologous to repeat regions in *pilS1* (Haas & Meyer, 1986), to a region upstream of *pilE* (Perry *et al.*, 1987) and to *opa* loci was found (Fig. 5). A region at one end of this 152 bp region is highly homologous to, and in the same orientation as, the 38 bp repeat upstream of *pilC1*. No ORF spanning the 152 bp sequence could be identified. Thus, sequences homologous to intervening silent non-coding sequences of *pil* and *opa* loci are positioned on both sides of the *pilC1* gene.

Each *pilC* gene is a part of a larger duplication of gonococcal DNA

Five probes encompassing different sequences flanking the *pilC1* gene were used in Southern hybridizations of chromosomal DNA of *N. gonorrhoeae* strain MS11_{mk}(P⁺)-u. The 0.6 kb *BalI*–*HindIII*₁ fragment (probe B in Fig. 1), the 0.7 kb *HindIII*₁–*AvaI*₁ fragment (probe C) and the 0.9 kb *HindIII*₂–*MluI*₁ fragment (probe E) from pABJ04 hybridized to the same two *ClaI* fragments (18 kb and 8 kb) and *SmaI* fragments (13.5 kb and 4.5 kb) as did the internal *EcoRV*₁–*EcoRV*₂ fragment (probe F) of *pilC1* (Fig. 6). In addition to these two fragments, weak hybridization was obtained to several other *ClaI* and *SmaI* fragments. The *pilC*-specific probe (probe F) carries, in contrast to the probes B, C and E, an internal *SmaI* site and hybridizes to four fragments of *SmaI*-digested gonococcal DNA. Two probes, the 0.4 kb *PvuII*–*BalI* probe (fragment A) and the 0.2 kb *AvaI*₁–*HindIII*₂ fragment (probe D), hybridized only to the 18 kb *ClaI* fragment and the 13.5 kb *SmaI* fragment of locus 1 (Fig. 6). We conclude that, in addition to the 3 kb long *pilC* genes, more than 2 kb downstream of the *pilC* genes are also duplicated on the gonococcal chromosome. However, this duplication is not identical since probe D only hybridized to locus 1. The repetitive DNA stretch of 152 bp positioned 0.7 kb from the translational stop codon of *pilC1* may be responsible for the multiple weakly hybridizing DNA fragments in the Southern blots. This repeat is present in non-coding regions of *pil* and *opa* loci.

Hybridization with a probe from the upstream region of *pilC1* was also performed. The 0.8 kb *Sau3A1*–*Sau3A1* fragment of pABJ05 (probe I in Fig. 1) hybridized to a 4 kb *ClaI* fragment not detected with the *pilC*-specific probe (probe F) and is likely to be present in only one copy (data not shown). The unexpected hybridization pattern of probe I may be explained by DNA rearrangements in this region in or before the cloning process.

DISCUSSION

Pili of *N. gonorrhoeae* are absolutely required for the capability of these organisms to cause disease in male volunteers (Kellogg *et al.*, 1968; Swanson *et al.*, 1987). How these filamentous structures are assembled and polymerized is not yet understood. However, PilC, a 110 kDa protein involved in pilus biogenesis in *N. gonorrhoeae* has recently been identified (Jonsson *et al.*, 1991). Also, pilus biogenesis genes homologous to the biogenesis genes of *P. aeruginosa* have been identified (Lauer *et al.*, 1993). In strain MS11, PilC can be expressed from either of two loci, *pilC1* or *pilC2*; expression is regulated by changes in the number of G residues in a stretch of Gs positioned in the signal peptide coding region. Addition or loss of a G residue puts the initiation codon in- or out-of-frame with respect to the mature protein (Jonsson *et al.*, 1991). PilC has also been implicated in adhesion (Rudel *et al.*, 1992, 1995).

Determination of the primary structure of PilC, as well as characterization of the regions flanking the *pilC* genes, is a prerequisite for a deeper understanding of gonococcal pilus biogenesis and attachment. In this report we present the complete nucleotide sequence of the *pilC1* gene from *N. gonorrhoeae* MS11. This gene was cloned out-of-frame (Jonsson *et al.*, 1991) from a gonococcal genomic library. The inability to clone an in-frame gene is probably due to the lethality of high-level PilC expression in *E. coli*. PilC is associated with the outer membrane of non-piliated gonococci (Jonsson *et al.*, 1991), although it has recently been shown that PilC is localized at the tip of the pilus fibre (Rudel *et al.*, 1995).

Two repetitive sequences, an 88 bp region and a 38 bp region, that both show high homology to non-coding

stretches in *opa*, *pil* and small repeat elements of DNA in *N. gonorrhoeae* are present upstream of the *pilC1* coding region. The small 38 bp repetitive sequence is also present 0.7 kb downstream of the *pilC1* translational stop codon. In *E. coli*, it has been reported that the minimal length necessary for recombination is 20 bp (Watt *et al.*, 1985). Among the sequences that belong to the 88 bp repeat, all except pFLOB210, *rmp* and *pilE*, have a 20 bp or longer exact match to the *pilC1* sequence. Within the 38 bp repeat, four sequences have an exact match 20 bp or longer. The role of the repetitive elements upstream of *pilC1* is unknown, although it is tempting to speculate a role not only in recombination but also in regulation of expression. A characteristic of *N. gonorrhoeae* is the ability to exhibit phase and antigenic variation. Switches and alterations in both the pilus and the Opa OMPs occur at frequencies much higher than the normal mutation rate. It is interesting that both *opa* and *pilC* expression is controlled by changing the number of repeats in the signal peptide coding region. The frequency at which new repeats are added or lost may well be affected by local DNA topology. It is therefore tempting to speculate that the 88 bp repeat may, via protein binding, affect DNA structure and thereby the frequency of mispairing events leading to changes in the number of repeats in both the *opa* and *pilC* genes.

In *E. coli*, the pilus is composed of and assembled by the products of a set of closely linked genes (Mooi *et al.*, 1983; Orndorff & Falkow, 1984; Hacker *et al.*, 1985; Norgren *et al.*, 1984). Each *E. coli* strain can carry two or more homologous gene clusters, each directing the production of a surface organelle with separate adhesive properties. Each gene cluster encodes a large OMP, 80–90 kDa in size, that is proposed to play a role in the assembly or polymerization of pilin molecules into the pilus structure. Immediately adjacent to the gene encoding the large OMP are genes encoding a chaperone and several adhesins. Although *pilE*, encoding the major subunit, pilin, is not physically linked to the *pilC* genes (Dempsey *et al.*, 1991; Bihlmaier *et al.*, 1991), two biogenesis genes, *pilF* and *pilD*, are closely linked on the gonococcal chromosome (Lauer *et al.*, 1993). The region downstream of *pilC1* is currently being characterized in order to identify possible adjacent genes involved in pilus assembly or adhesion.

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