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PilC of *Neisseria meningitidis* is involved in class II pilus formation and restores pilus assembly, natural transformation competence and adherence to epithelial cells in PilC-deficient gonococci

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Summary

Type 4 pili produced by the pathogenic *Neisseria* species constitute primary determinants for the adherence to host tissues. In addition to the major pilin subunit (PilE), neisserial pili contain the variable PilC proteins represented by two variant gene copies in most pathogenic *Neisseria* isolates. Based upon structural differences in the conserved regions of PilE, two pilus classes can be distinguished in *Neisseria meningitidis*. For class I pili found in both *Neisseria gonorrhoeae* and *N. meningitidis*, PilC proteins have been implicated in pilus assembly, natural transformation competence and adherence to epithelial cells. In this study, we used primers specific for the *pilC2* gene of *N. gonorrhoeae* strain MS11 to amplify, by the polymerase chain reaction, and clone a homologous *pilC* gene from *N. meningitidis* strain A1493 which produces class II pili. This gene was sequenced and the deduced amino acid sequence showed 75.4% and 73.8% identity with the gonococcal PilC1 and PilC2, respectively. These values match the identity value of 74.1% calculated for the two *N. gonorrhoeae* MS11 PilC proteins, indicating a horizontal relationship between the *N. gonorrhoeae* and *N. meningitidis pilC* genes. We provide evidence that PilC functions in meningococcal class II pilus assembly and adherence. Furthermore, expression of the cloned *N. meningitidis pilC* gene in a gonococcal *pilC1,2* mutant restores pilus assembly, adherence to ME-180 epithelial cells, and transformation competence to the wild-type level. Thus, PilC proteins exhibit indistinguishable functions in the context of class I and class II pili.

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Introduction

Both *Neisseria gonorrhoeae* and *Neisseria meningitidis* colonize mucosal surfaces of their human host (De Voe, 1982; Kellogg *et al*., 1963; Swanson *et al*., 1987). Although the pathogenesis and clinical pictures of gonorrhoea and the meningococcal carrier state differ, many pathogenic factors expressed by *N. gonorrhoeae* and *N. meningitidis* share extensive homologies. One of these factors is the type 4 pilus; filamentous, surface-associated protein structures which have been implicated in mediating adherence of both *N. gonorrhoeae* and *N. meningitidis* to epithelial cells (Ward and Watt, 1975; McGee and Stephens, 1984; Craven and Frasch, 1978), to erythrocytes (Lambden *et al*., 1980; Trust *et al*., 1983; Rudel *et al*., 1992), and to endothelial cells (Virji *et al*., 1991; 1992). Most of the *N. meningitidis* strains express so-called class I pili consisting of a major subunit structurally related to *N. gonorrhoeae* PilE (Virji and Heckels, 1983). Class I pilin is distinguished from class II pilin produced by some *N. meningitidis* strains (Virji *et al*., 1989) by reactivity with the monoclonal antibody SM1 (Virji and Heckels, 1983) which recognizes a conserved epitope in the structure of class I pili (Parge *et al*., 1995).

The pili of *N. meningitidis* undergo both on/off transitions (phase variation) and structural changes (antigenic variation), probably via similar mechanisms that have been extensively investigated in *N. gonorrhoeae* (for a review, see Robertson and Meyer, 1992; Meyer *et al*., 1994; Seifert, 1996). The *N. gonorrhoeae* chromosome contains one or two pilin expression loci (*pilE*) and various silent variant *pil* genes. Recombination between *pilS* and *pilE* leads to changes in the pilin-coding sequence and the expression of antigenically different pili (Haas and Meyer, 1986; Swanson *et al*., 1986; Hagblom *et al*., 1985; Seifert *et al*., 1988; Gibbs *et al*., 1989). Intragenic recombination in the *pilE* genes may also lead to phase transitions resulting in the production of PilE molecules which are not assembled into pilus fibres (Haas and Meyer,

1986; Swanson *et al*., 1986; Haas *et al*., 1987; Manning *et al*., 1991; Hill *et al*., 1990).

Jonsson *et al*. (1991) reported an additional mechanism of pilus phase variation which is based on the variation of the accessory assembly factor PilC. The PilC proteins are 110 kDa in size and encoded by two slightly variant genes termed *pilC1* and *pilC2* in *N. gonorrhoeae* strain MS11 (Jonsson *et al*., 1991; Rudel *et al*., 1995a). The expression of *pilC* genes is subjected to phase variation via frequent frameshift mutations in a poly(G) tract of the signal-peptide-encoding sequence creating in-frame or out-of-frame configurations of the *pilC* genes (Jonsson *et al*., 1991). However, besides their role in pilus biosynthesis, both gonococcal PilC proteins function as an adhesin for epithelial cells and represent an integral part of the *N. gonorrhoeae* pilus (Rudel *et al*., 1995a,b). Moreover, the natural competence for the uptake of DNA (Sparling, 1966), which is connected with the expression of type 4 pilin in *N. gonorrhoeae* (Biswas *et al*., 1977; Gibbs *et al*., 1989; Zhang *et al*., 1992), depends on the presence of PilC protein (Rudel *et al*., 1995c). Evidence exists also for a role of PilC proteins in *N. meningitidis* class I pilus biosynthesis and in pilus-mediated adherence to epithelial and endothelial cells (Nassif *et al*., 1994). However, in contrast to *N. gonorrhoeae* (Rudel *et al*., 1995a), only one of the PilC proteins in *N. meningitidis* strain 8013, tentatively termed PilC1, operates as an adhesin whereas PilC2 does not (Nassif *et al*., 1994). Purified PilC protein efficiently competes with the binding of type 4 pili of both *N. gonorrhoeae* and *N. meningitidis* suggesting that the receptors recognized on the target-cell surface are at least homologous if not identical (Rudel *et al*., 1995b).

In this study we report the isolation and characterization of a *pilC* gene from *N. meningitidis* strain A1493. The PilC protein analysed in this study functions in assembly and binding of the class II pili of the parental strain. Expression of the cloned *pilC* gene from A1493 in a genetically defined *pilC1,2* mutant of *N. gonorrhoeae* strain MS11 restores the wild-type phenotype with respect to pilus assembly and pilus-mediated adherence. Also, transformation competence can be restored in *N. gonorrhoeae*. The cloned *N. meningitidis pilC* gene therefore exhibits homologous functions with respect to pilus assembly and adherence in the two neisserial type 4 pilus classes, i.e. the gonococcal class I and the meningococcal class II pili.

Results

Amplification and sequence analysis of a meningococcal pilC *gene*

In order to identify *pilC* genes *in N. meningitidis*, *Cla*I-digested chromosomal DNA of different isolated strains was hybridized with the cloned *pilC1* gene of *N. gonorrhoeae* strain MS11 on plasmid pTR25 (Rudel *et al*., 1995a). In *N. meningitidis* strain A1493 one band reacted, indicating the presence of only one *pilC* gene (data not shown). By using specific primers, including the ribosome-binding site and the downstream region of *N. gonorrhoeae* MS11 *pilC2* (Rudel *et al*., 1995a), a fragment of ≈3 kb was amplified by the polymerase chain reaction (PCR) and subsequently cloned in the pBluescript-II SK+ vector resulting in plasmid pRR3 (Fig. 1) which was used for sequencing. The 5′ terminal sequence exhibited the expected ATG start codon downstream of the ribosome-binding site; however, this translational start site gave rise to a very short open reading frame (ORF). At position 79 the short ORF contained a succession of G residues, typical of the variable homopolymeric repeat previously identified in the gonococcal *pilC* genes, comprising a location for frameshift mutations responsible for in-frame and out-of-frame transitions in *pilC* genes (Jonsson *et al*., 1991; Rudel *et al*., 1992; Nassif *et al*., 1994). If one G residue was added to the nine G residues present, a long ORF of 3113 nucleotides encoding a protein of 114 kDa could be deduced from the inserted fragment in pRR3. The deduced amino acid sequence showed 75.4% and 73.8% identity to PilC1 and PilC2 of *N. gonorrhoeae* strain MS11, respectively (Jonsson *et al*., 1995; Rudel *et al*., 1995a), while both gonococcal proteins are 74.1% identical. A multiple alignment of all three proteins yielded 62.9% identity (Fig. 2). It was possible to discern regions of complete homology from others tolerating the accumulation of mostly conservative amino acid exchanges. Regions of pronounced heterology, however, distinguishing the meningococcal PilC from its gonococcal counterparts were not detected. The sequence data indicate that *N. gonorrhoeae* PilC1 is slightly more closely related to *N. meningitidis* PilC than to *N. gonorrhoeae* PilC2, although the differences are marginal. In order to confirm that A1493 possesses only one pilC gene, blots were performed with genomic DNA digested with enzymes that do not cleave within its entire sequence, including *Bst*EII, *Hin*dIII, *Pst*I, *Stu*I and *Xba*I. Hybridization with a labelled *pilC* gene as probe revealed, in all cases, single genomic bands (see the *Experimental procedures*). This almost completely excludes the existence of additional highly homologous genes in strain A1493.

Role of PilC in meningococcal class II pilus assembly and adherence

The parental variant N860 of strain *N. meningitidis* A1493 did not produce any PilC protein that reacted in immunoblots with sera raised against a gonococcal PilC fusion protein (Rudel *et al*., 1992; Fig. 3A). Further characterization revealed that N860 expressed class II pilin at a low level (Fig. 4) but did not form pili (Fig. 5b). In order to investigate whether strain A1493 is capable of forming

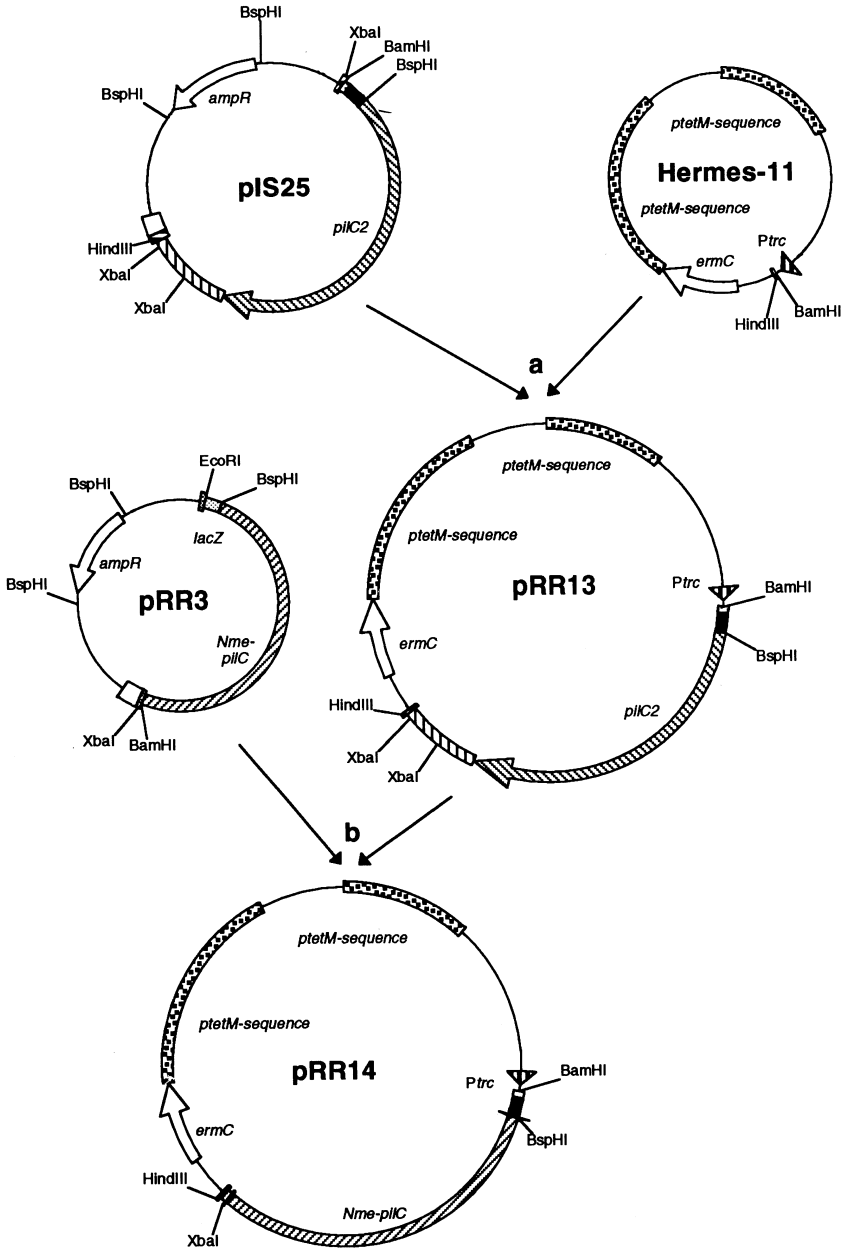


Fig. 1. Construction of plasmids encoding the *N. meningitidis pilC* gene.
a. The *Bam*HI–*Hin*dIII fragment of pIS25 containing the MS11 *pilC2* gene with invariant G residues and histidine tag ($pilC2_{MS11\text{-}His\text{-}6}$) was inserted into the same sites of the shuttle plasmid Hermes-11 resulting in pRR13.
b. pRR3 contains the cloned PCR product of the meningococcal *pilC* gene in pBluescript-II SK+. Replacement of the *Bsp*HI–*Xba*I fragment in pRR13 by the corresponding fragment of pRR3 exchanged the majority of $pilC2_{MS11\text{-}His\text{-}6}$ with the *pilC* gene of strain A1493, resulting in pRR14. The common 5′ end of both *N. gonorrhoeae* and *N. meningitidis pilC* genes containing the invariant G residues and the histidine tag extending to the *Bsp*HI site is drawn as a black bar. The 5′ end of both $pilC2_{His\text{-}6}$ genes has the following sequence: 5′-ATGAATAAA-ACTTTAAAAAGGCGGGTTTTCCGCCATA-CCGCGCTTTATGCCGCCATCTTGATGTT-TTCCCATACCGGCGGGGGGGGGGGGGC-GCAGGCGCAAACC**CATCACCATCATCAC**-CGTAAATACGCTATTATCATGA-3′ (nucleotides shown in bold encode the His tag; the final six nucleotides represent the *Bsp*HI site).

pili we grew pellicles of N860, a method which has been used to enrich piliated gonococcal variants (Koomey *et al.*, 1987). Bacteria from pellicles were subsequently selected for adherence to ME-180 epithelial cells (Rudel *et al.*, 1992). By this procedure, a variant was obtained (N862) which clearly produced pili (Fig. 5c) and strongly adhered to ME-180 cells (Fig. 6c). N862 produced PilC protein and pilin of the same apparent molecular weight as the parental strain (Figs 3 and 4). We therefore asked whether the *pilC* gene which we were able to amplify by PCR and clone had undergone a DNA sequence change upon transition from the non-piliated phase to the piliated phase from strain N860 to N862. Direct DNA sequence determination of the corresponding phase-variable G residues indeed revealed an insertion of one guanine nucleotide rendering this *pilC* gene of N862 in the 'on' configuration (Fig. 7). In parallel experiments, the pilin of strains N860 and N862 was prepared and subjected to V8 protease digestion. This revealed identical peptide patterns (data not shown), ruling out antigenic variation of the pilin as the reason for the observed phase transition. Taken together, these data indicate that the PilC protein encoded by the cloned *N. meningitidis pilC* gene functions in assembly and adherence of the class II pili of variant N862.

In order to define the adherence properties of the piliated variant N862, competition experiments were performed

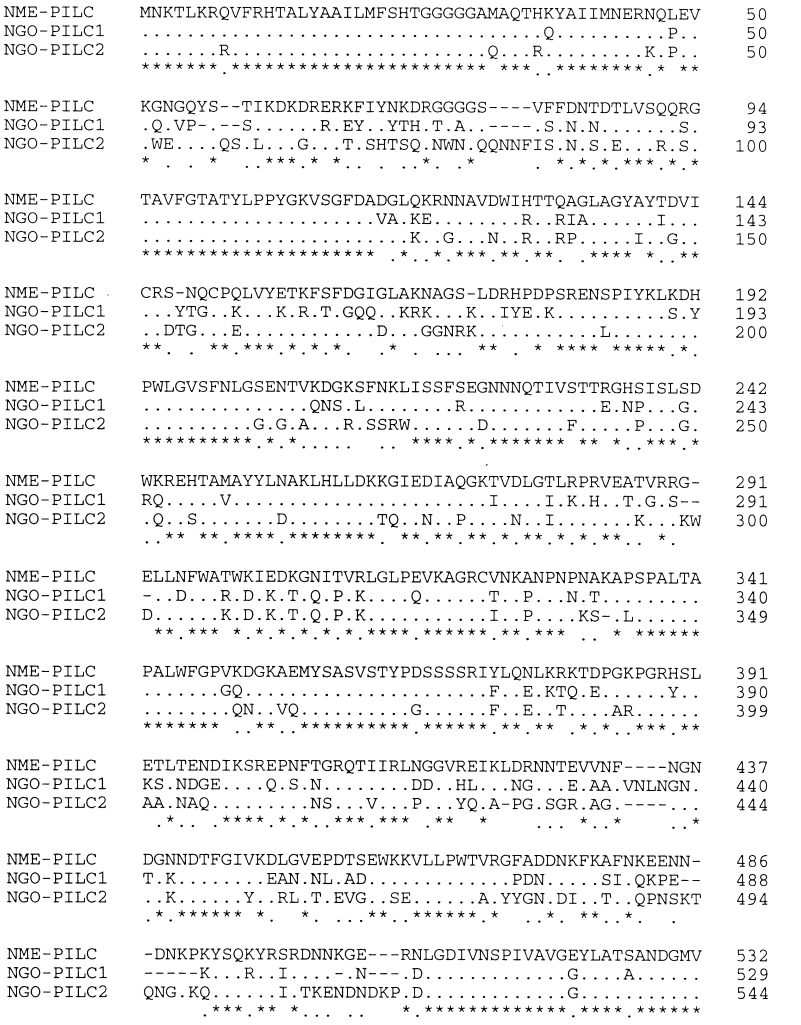


Fig. 2. Comparison of the deduced amino acid sequences of *N. meningitidis* A1493 PilC protein and the *N. gonorrhoeae* MS11 PilC1 and PilC2 proteins (Jonsson *et al.*, 1995; Rudel *et al.*, 1995a). Positions of identical or similar amino acids are marked by asterisks and dots, respectively. The *N. meningitidis* PilC protein sequence is based on a nucleotide sequence with a run of G residues arbitrarily set to 13 G residues. The EMBL/GenBank accession number of NME-PILC is Z54202.

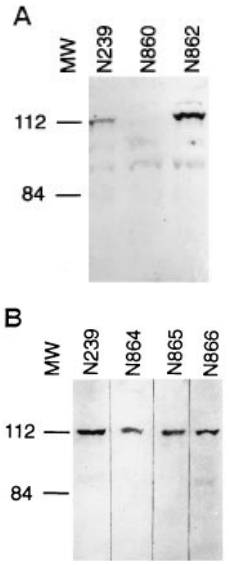


Fig. 3. Western blot analysis of PilC protein expression using antiserum (AK203) raised against a gonococcal PilC fusion protein.
A. PilC expression of *N. gonorrhoeae* MS11 N239 and *N. meningitidis* A1493 N860 (P^-) and N862 (P^+) selected for adherence to ME-180 epithelial cells.
B. PilC expression of *N. gonorrhoeae* N239 (chromosome encoded) and N864, N865 and N866 (plasmid encoded).

using purified PilC adhesin (Rudel *et al.*, 1995b). $PilC2_{His\text{-}6}$ protein prepared from *N. gonorrhoeae* MS11 N560 was affinity purified by virtue of its histidine tag over a nitriloacetic acid–Ni^{2+} column and added at a concentration of $\approx 2\,\mu g\,ml^{-1}$ to ME-180 cells to which the protein bound efficiently (Rudel *et al.*, 1995b; I. Scheuerpflug *et al.*, submitted). Such pretreatment almost completely prevented N862 from binding to ME-180 human epithelial cells (Fig. 6h), indicating that purified $PilC2_{His\text{-}6}$ protein competes for the binding of *N. meningitidis* N862 to ME-180 cells.

Cloning of N. meningitidis pilC *for functional analysis*

As the isolated *N. meningitidis pilC* gene was out-of-frame, it had to be modified in order to stably express PilC for functional analysis. For this purpose we constructed a generalized cloning vector for the stable expression of cloned *pilC* genes, based on plasmid pIS25 (Fig. 1; Rudel *et al.*, 1995a,b; I. Scheuerpflug *et al.*, submitted). In this plasmid the homopolymeric G residues of the *N. gonorrhoeae* MS11 *pilC2* gene was replaced by an invariable sequence and an additional DNA segment was inserted encoding six histidine residues in the N-terminal part of the processed PilC protein in order to facilitate purification. Immediately downstream of the G residues at position 122 a unique *Bsp*HI site present in all known *pilC* genes is located in pIS25. The gonococcal *pilC2* gene of pIS25 was inserted into Hermes-11 to yield pRR13 (Fig. 1). Subsequently, the *N. gonorrhoeae pilC2* gene was replaced by fusion of the *N. meningitidis pilC* gene at this *Bsp*HI site. This led to plasmid pRR14 in which the *N. meningitidis pilC* can be stably expressed under the control of the promoter P_{trc} (Fig. 1). As well as the added histidine tag, the newly created *N. meningitidis* PilC protein exhibits only one conservative amino acid exchange at position 3 of the mature protein, with a histidine being replaced by an arginine (corresponding to amino acid 36 in Fig. 2). Considering the insertion of the histidine tag, the mature PilC sequence begins with QTHHHHHHR.

Meningococcal PilC restores the pilus-assembly function in PilC-deficient gonococci

In order to functionally compare the meningococcal and gonococcal PilC proteins we intended to introduce and express the cloned meningococcal $pilC_{A1493\text{-}His\text{-}6}$ gene in a gonococcal *pilC1,2* mutant using the Hermes shuttle-vector system (Kupsch *et al.*, 1996). The Hermes vectors can be propagated in *Escherichia coli* and can recombine via allelic exchange with the large conjugative plasmid ptetM25.2 upon transformation of an appropriate recipient *Neisseria* strain. The system has been used previously to complement non-transformable *Neisseria* derivatives including *N. gonorrhoeae pilC1,2* mutants via conjugation (Rudel *et al.*, 1995c). Accordingly, strain N219 was transformed with plasmid pRR14 to recombine with ptetM25.2 and to generate pHRR14 in strain N863. Conjugative transfer of pHRR14 from N863 into the *pilC1,2* mutant N477 ($PilC^-$) generated strain N864 which was used for further analysis. Strains N865 expressing *N. gonorrhoeae*

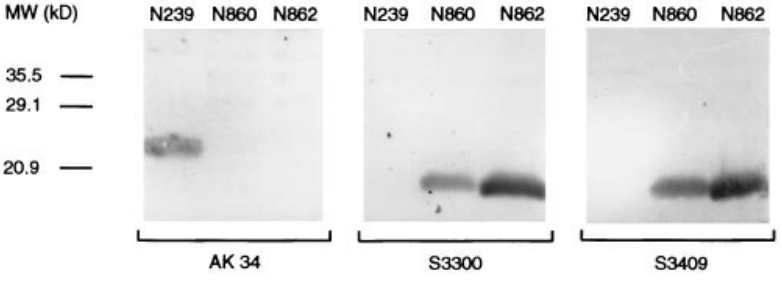
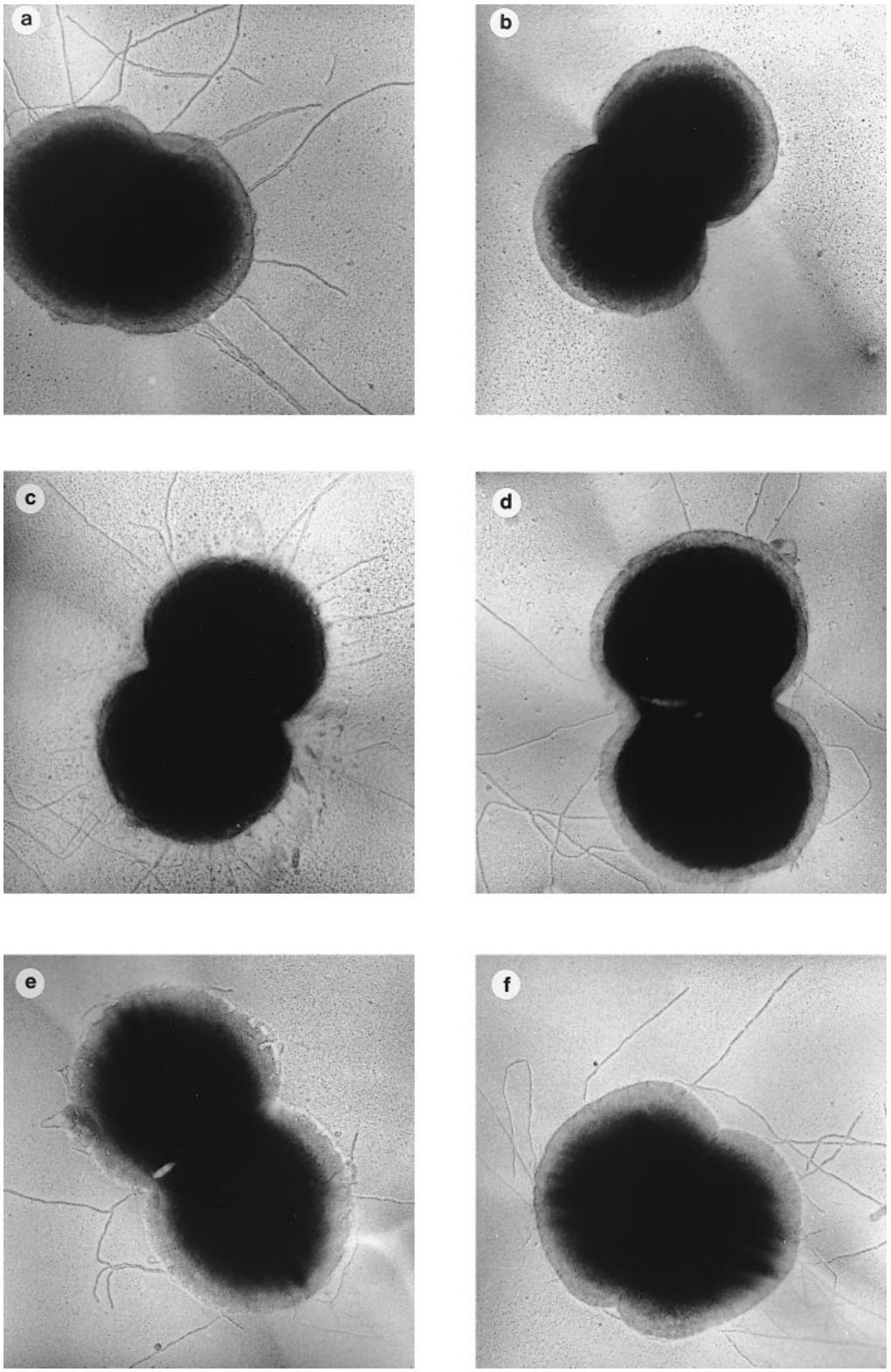


Fig. 4. Western blot analysis of whole-cell lysates of *N. gonorrhoeae* N239 and *N. meningitidis* N860 and N862. Rabbit antiserum raised against a gonococcal PilE fusion protein (AK34) was used for detection of class I pilin, and monoclonal antibodies U101 (S3300) and AG123 (S3409) recognizing pilin classes IIa and IIb, respectively, were used for determining class II pilin expression.

Fig. 5.



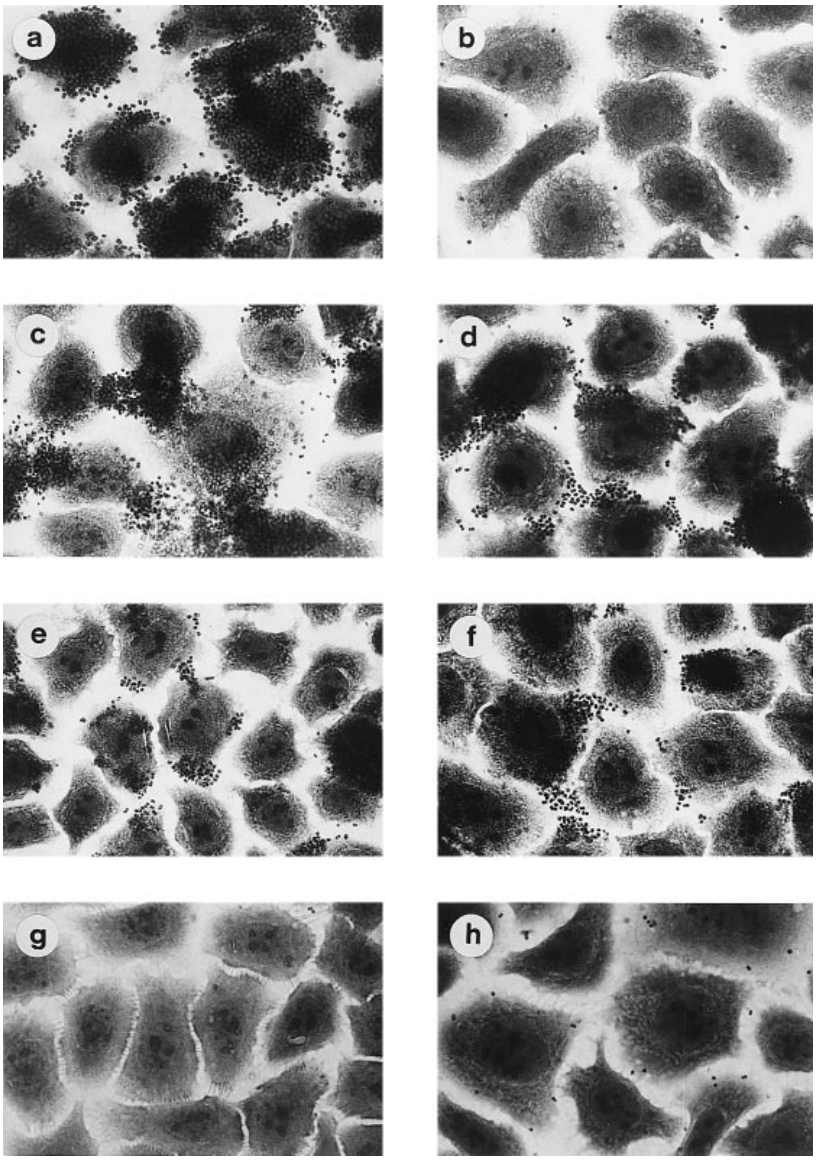


Fig. 6. Adherence of various *N. gonorrhoeae* and *N. meningitidis* strains to ME-180 epithelial cells.
a. N239 (+++).
b. N860 (+).
c. N862 (+++).
d. N864 (++).
e. N865 (++).
f. N866 (++).
g. N867 (−).
h. N862 after pre-incubation of epithelial cells with purified PilC (+).
−, < 1 bacterium per epithelial cell; +, 1–5 bacteria per epithelial cell; ++, 6–20 bacteria per epithelial cell; +++, > 20 bacteria per epithelial cell. The number of adherent bacteria was calculated on the basis of three independent experiments, in each of which at least 100 epithelial cells were counted.

pilC2^{iv} from pHTR102 (Rudel *et al*., 1995a) and N866 expressing *N. gonorrhoeae pilC2*$_{MS11\text{-}His\text{-}6}$ from pHIS26 (I. Scheuerpflug *et al*., submitted) in N477 were used as controls. All strains harbouring a *pilC* gene on ptetM25.2 expressed normal quantities of PilC protein under the control of the LacI-repressed P_{trc} promoter (Fig. 3B; Rudel *et al*., 1995a). Whereas the recipient strain N477 showed a P^- morphology when viewed under a binocular microscope, the complemented strains expressing either of the *pilC* genes exhibited a P^+ colony morphology indicating that the ability to form pili was restored. This finding was confirmed by electron microscopy (Fig. 5). On average, N864 produces 10–20 pili per diplococcus, which often appear as bundles. Differences in size or structure when compared to the wild-type strain, N336, N865 or N866 were not observed. Thus, the *N. meningitidis*

Fig. 5. Piliation of various *N. meningitidis* and *N. gonorrhoeae* strains analysed by transmission electron microscopy.
a. N239 (wild-type P^+).
b. Parental *N. meningitidis* A1493 variant N860 (P^-).
c. *N. meningitidis* A1493 variant N862 (P^+) selected for binding to ME-180 epithelial cells.
d. N864 (N477 producing $PilC_{A1493\text{-}His\text{-}6}$).
e. N865 (N477 producing $PilC2_{MS11}{}^{iv}$).
f. N866 (N477 producing $PilC2_{MS11\text{-}His\text{-}6}$).

N860 ATG ... ... GGC GGG GGG GGG *CGA TGG CGC AAA CCC ATA AAT ACG CTA TTA TCA stop
N862 ATG ... ... GGC GGG GGG GGG GCG ATG GCG CAA ACC CAT AAA TAC GCT ATT ATC ...

Fig. 7. Direct sequencing of the homopolymeric G region in the *pilC* genes of N860 and N862 chromosomal DNA. The insertion of one G residue in N862 renders the *pilC* gene translationally in frame (see Fig. 2).

$pilC_{A1493\text{-}His\text{-}6}$ gene fully restores the gonococcal *pilC* defect of pilus formation.

Meningococcal PilC expressed in PilC-deficient gonococci confers adherence to ME-180 epithelial cells

Piliated derivatives of *N. gonorrhoeae* and *N. meningitidis* strains (see Table 1) were tested for binding to ME-180 epithelial cells. The control strain *N. gonorrhoeae* N239 ($PilE_{F3}$) strongly adhered to the majority of cells (Fig. 6a), whereas the non-piliated variant N860 containing the out-of-frame *pilC* hardly adhered at all (Fig. 6b). In contrast, *N. meningitidis* N862, the piliated variant of A1493 expressing *pilC*, bound efficiently to ME-180 cells (Fig. 6c). Similarly, N864 (Fig. 6d), N865 (Fig. 6e) and N866 (Fig. 6f) adhered to ME-180 cells with approximately the same efficiency as the wild-type strain N239 (Fig. 6a). It can therefore be concluded that the *N. meningitidis* PilC complements the adherence function of *N. gonorrhoeae* PilC. As shown previously with strain N862, adherence of all *pilC* recombinant gonococci could be prevented by adding purified $PilC2_{MS11\text{-}His\text{-}6}$ protein prior to infection.

Meningococcal PilC restores transformation competence in PilC-deficient gonococci

Besides their roles in pilus assembly and adherence, PilC proteins were shown to be crucial determinants for the natural transformation competence in *N. gonorrhoeae* (Rudel *et al*., 1995c). Because PilE protein is additionally required for DNA uptake (Gibbs *et al*., 1989; Zhang *et al*., 1992), typical P^+ colonies of N864, N865 and N866 were assessed for their transformation competence with plasmid pRB2 conferring kanamycin resistance. All gonococcal *pilC* mutants expressing either a *N. gonorrhoeae pilC* gene or the *N. meningitidis pilC* gene were competent for transformation to kanamycin resistance to the same degree (Table 2). The influence of the histidine tag in PilC on transformation competence was not observed. Hence, the cloned *N. meningitidis* PilC protein originally associated with class II pili functions equally well in gonococcal DNA uptake as the native gonococcal PilC proteins.

Discussion

PilC proteins constitute important determinants for *Neisseria* type 4 pilus assembly, adherence and natural transformation competence. Both variant *pilC* genes of *N. gonorrhoeae* MS11 were cloned and characterized (Jonsson *et al*., 1991; 1995; Rudel *et al*., 1995b), thus allowing the investigation of the diverse PilC functions with the help of genetically defined strains (Rudel *et al*., 1995a,b,c). In *N. meningitidis*, which in contrast to *N. gonorrhoeae* produces two different pilus classes, PilC function has been investigated for class I pilus biosynthesis and adherence (Nassif *et al*., 1994). In the current study, we describe the isolation and functional characterization of a *pilC* gene of the *N. meningitidis* class II pilus-forming strain A1493. Our DNA sequence data indicate that there is a high degree of homology between the *N. meningitidis* PilC and the PilC proteins of *N. gonorrhoeae* MS11 (Jonsson *et al*., 1995; Rudel *et al*., 1995b).

The two variant PilC proteins detected in strain 8013NA were suggested to exhibit different functions (Nassif *et al*., 1994): one of the PilC proteins, tentatively termed PilC1, supported both class I pilus assembly and binding, whereas expression of the other PilC resulted in pilus formation but

Table 1. *N. gonorrhoeae* and *N. meningitidis* strains used in this work.

Strain	Relevant features	Source/Reference
N. gonorrhoeae N219	P^S, $PilE_{B1}$, p*tetM*25.2	Rudel *et al.* (1995a)
N. gonorrhoeae N239	P^+, $PilE_{F3}$ (variant F3)	Haas *et al.* (1987)
N. gonorrhoeae N419	P^+, $PilE_A$ (variant A)	Meyer *et al.* (1984)
N. gonorrhoeae N477	P^-, $PilE_{F1}$, $PilC^-$	Rudel *et al.* (1995a)
N. gonorrhoeae N560	P^-, $PilC_{MS11\text{-}His\text{-}6}$	Rudel *et al.* (1995b)
N. gonorrhoeae N556	P^+, $PilE_{F1}$, $PilC^-$	Rudel *et al.* (1995a)
N. meningitidis N860	P^-, wild-type isolate A1493	U. Berger, Heidelberg
N. meningitidis N862	N860; P^+	This work
N. gonorrhoeae N863	N219; P^S, $PilC_{A1493\text{-}His\text{-}6}$	This work
N. gonorrhoeae N864	N477; P^+, $PilC_{A1493\text{-}His\text{-}6}$	This work
N. gonorrhoeae N865	N477; P^+, $PilC_{MS11}{}^{iv}$	This work
N. gonorrhoeae N866	N477; P^+, $PilC_{MS11\text{-}His\text{-}6}$	This work
N. gonorrhoeae N867	N864; P^-, $PilC_{A1493\text{-}His\text{-}6}$	This work

Table 2. Transformation frequencies of *pilC*-complemented gonococcal strains.

Strain/variant designation	Transformants per colony-forming unit
N864 ($PilC_{A1493\text{-}His\text{-}6}$)	1.5×10^{-5}
N865 ($PilC_{MS11}^{iv}$)	1.3×10^{-5}
N866 ($PilC_{MS11\text{-}His\text{-}6}$)	2.0×10^{-5}
N419 (P^+, wild type)	4.0×10^{-5}
N477 (P^-, *pilC1,2*)	$< 6.0 \times 10^{-9}$
N556 (P^+, *pilC1,2*)	$< 5.0 \times 10^{-9}$

did not confer adhesive properties to the pili composed of the same PilE protein in this *N. meningitidis* strain. A difference with regard to the adhesive properties has not been observed for the PilC1 and PilC2 proteins of *N. gonorrhoeae* MS11 (Rudel *et al*., 1995b,c). Our correlation of the numbers of variable G residues in a distinct *pilC* gene with the expression of PilC protein and the formation of adhesive pili indicates that the investigated PilC protein is functionally similar to the already described PilC1 of strain 8013NA (Nassif *et al*., 1994). In agreement with our data, Virji *et al*. (1995) reported a strongly adherent variant of *N. meningitidis* MC58, termed 58#18.18, characterized by increased PilC expression compared to the less adherent parent strain. Inactivation of the *pilC* gene led to a loss of piliation and abolished adherence to human umbilical vein epithelial cells (HUVEC) cells, although class I pilin could still be detected.

The pili of *N. meningitidis* have been classified based on the reactivity of pilin with the monoclonal antibody SM1 (Diaz *et al*., 1984; Virji *et al*., 1989); however, additional antigenic differences must exist in the conserved regions of the class I and class II pilins. However, little is known about the relationship of both pilus classes. The gene encoding the major subunit of class I pili but not class II pili has been cloned (Potts and Saunders, 1988). In this study we show that the same PilC protein can act as an accessory assembly factor of both class II and class I pili. The PilC proteins of *N. gonorrhoeae* MS11 and of *N. meningitidis* A1493 forming class II pili share extensive sequence homologies. Therefore, class I and class II pili probably share similarities regarding their biogenesis and overall structural features, as already suggested by Virji *et al*. (1989). Furthermore, the function of PilC in transformation competence as determined in *N. gonorrhoeae* (Rudel *et al*., 1995c) is presumably interchangeable between *N. meningitidis* and *N. gonorrhoeae* as well as between the two pilus classes in *N. meningitidis.*

Little is known about the distinct functions of class I and class II pili. Data from Virji *et al*. (1992) suggest a different binding behaviour of class I- and class II-forming *N. meningitidis* strains to epithelial cells. However, purified *N. gonorrhoeae* $PilC2_{MS11\text{-}His\text{-}6}$ protein was shown to inhibit binding of *N. meningitidis* strains forming either class I or class II pili to ME-180 epithelial cells (Rudel *et al*., 1995b). Thus, irrespective of their association with a distinct pilus class, neisserial type 4 pili recognize the same or closely related receptors via their PilC adhesins. This is confirmed by the results presented here where the same PilC protein conferring class II pilus binding in A1493 is able to restore pilus binding in gonococci which produce class I pili. Nonetheless, a large amount of evidence suggests that the variation of pilin as well as the expression of different pilus classes modulates the binding to epithelial cells (Rudel *et al*., 1992; Virji *et al*., 1992; 1993; Nassif *et al*., 1993; 1994; Dunn *et al*., 1995). This may also be reflected by a slightly different binding pattern expressed depending on whether the *N. meningitidis* PilC protein is produced in the context of the *N. meningitidis* strain or the *N. gonorrhoeae pilC1,2* mutant. This modulating function of the major subunit on the adherence to epithelial cells indicates additional adherence functions expressed by the neisserial pili which might reside in either or both of the major pilin subunits (Rudel *et al*., 1992;1995a) and perhaps additional minor subunits present in the neisserial pili (Muir *et al*., 1988; Parge *et al*., 1990). In conclusion, it is now evident that *N. meningitidis* and *N. gonorrhoeae* use the same principle of pilus adherence with PilC as the major adhesin despite the difference in their naturally preferred (but not exclusive) colonization sites, the nasopharynx and the genital tract in humans. Which factors ultimately determine, and how they influence, the tissue tropisms in pathogenic *Neisseria* species remains to be investigated.

Experimental procedures

Bacterial strains and plasmids

Neisserial strains and derivatives used in this study are listed in Table 1. All *Neisseria* strains were grown on GC agar base (Difco) enriched with vitamin mix at 37°C in a humified atmosphere at 5% CO_2. For the selection of resistance markers, $7\,\mu g\,ml^{-1}$ chloramphenicol, $10\,\mu g\,ml^{-1}$ erythromycin and/or $50\,\mu g\,ml^{-1}$ kanamycin were added. The cloning procedures were performed in *E. coli* DH5α (BRL) grown on Luria (L) broth agar supplemented with $100\,\mu g\,ml^{-1}$ ampicillin or $250\,\mu g\,ml^{-1}$ erythromycin for selection of plasmids. pBluescript-II SK+ was purchased from Stratagene. The shuttle vector Hermes-11 has been described previously (Kupsch *et al*., 1996).

DNA amplification, cloning of meningococcal pilC *and Southern blotting*

Meningococcal *pilC* was amplified by PCR from chromosomal DNA using two primers designated BS10 and TR33 and directed against the ribosome-binding site of the *N. gonorrhoeae* MS11 *pilC2* gene and a conserved downstream sequence

of both gonococcal *pilC* genes, respectively. The primers were synthesized with several restriction endonuclease sites, including *Eco*RI in BS10 and *Bam*HI in TR33, to facilitate directional cloning of the PCR products. Chromosomal DNA was purified using the protocol of Boom *et al.* (1990). PCR was performed in a volume of 100 μl containing 10 μl of 10× Vent Polymerase buffer, 200 μM of each nucleotide triphosphate, 50 pmol of each primer and 4 U of Vent Polymerase. The samples were heated to 94°C for 3 min. Subsequently, the following PCR cycle was repeated 25 times: 1 min at 94°C, 2 min at 48°C, and 5 min at 72°C with an extension of 5 s at each step. After completion of all cycles the samples were cooled to 4°C until analysis in 0.8% agarose gels was performed. Before digestion with *Eco*RI and *Bam*HI, a proteinase K digestion was performed, according to the method of Crowe *et al.* (1991), followed by desalting the samples using sephacryl S-300 columns (Maniatis *et al.*, 1989). For Southern blotting, genomic DNA of strain N862 was digested with *Bst*EII, *Hin*dIII, *Pst*I, *Stu*I and *Xba*I, separated by agarose gel electrophoresis and transferred to Nylon membranes (Hybond N+; Amersham) using a Vacuum Blotter (Appligene). An almost complete fragment of *pilC* was amplified, using PCR, from genomic DNA of strain N862 using primers TR47 and TR64 and ECL-labelled according to the manufacturer's protocol (ECL nucleic acid labelling kit; Amersham). After hybridization using the conditions described in the kit (primary washing buffer: 6 M urea, 0.5× SSC) and addition of the chemoluminescent reagents, exposure of the membranes to X-ray film yielded one band of different sizes in each of the digests.

Construction of strains and plasmids

After PCR amplification of meningococcal *pilC* the gene was inserted into the multiple cloning site of vector pBluescript-II SK+ making use of the *Eco*RI and *Bam*HI sites introduced by the primers BS10 and TR33, resulting in plasmid pRR3. In order to construct a generalized vector for the cloning of *pilC* genes, the *Bam*HI–*Hin*dIII backbone of plasmid pIS25 harbouring *N. gonorrhoeae pilC2* with invariant G residues and His-6 tag was replaced by the Hermes-11 *Bam*HI–*Hin*dIII backbone devoid of any *Bsp*HI sites, thus giving rise to pRR13. Subsequently, the *Bsp*HI–*Xba*I fragment was replaced by the corresponding fragment of pRR3 resulting in pRR14. pRR14 was used to transform N219 which harbours the conjugative ptetM25.2 plasmid (Kupsch *et al.*, 1996). This resulted in N863 where the cloned *pilC* gene in pRR14 was integrated in ptetM25.2 by homologous recombination to yield pHRR14. Conjugation of N863 with strain N477 (*pilC1,2*) resulted in N864 where the cloned *pilC* was expressed in a gonococcal context.

S1 heteroduplex analysis

The exchange of the fragments was verified by restriction analysis of pRR14. Additional confirmation was obtained by S1 nuclease digestion. *PilC* was amplified by PCR from genomic DNA of strain N862 using primers TR47 and TR58, yielding a 1.9 kb fragment. Similarly, fragments of 2.3 kb were amplified from plasmid DNA of strains N864 (pHRR14) and N866 (pHIS26) using primers TR49 and TR62. Approximately 100 ng of the *pilC* fragment amplified from genomic DNA, and those fragments amplified from the recombinant *N. gonorrhoeae* plasmids pHRR14 and pHIS26, respectively, were mixed, denatured on ice for 5 min in the presence of 1 M NaOH and neutralized by adding 1 M Tris-acetate (pH 4.0) to give a final volume of 16 μl. After adding PCR oil, the samples were heated to 80°C and allowed to cool to room temperature within a period of 2 h. A volume of 15 μl was transferred to a fresh Eppendorf tube, 1.8 μl of 10× S1 nuclease buffer (33 mM Na-acetate, 200 mM NaCl, 0.03 mM $ZnSO_4$, pH 4.5) and 1.2 μl of a 1:100 dilution of S1 nuclease (Boehringer Mannheim) were added and incubated at 37°C for 30 min. The samples were then examined by agarose gel electrophoresis. The hybridization of N862- and pHRR14-derived *pilC* fragments yielded a band of the expected size of 1.6 kb resistant to S1 nuclease digestion whereas the hybridization product of N862- and pHIS26-derived *pilC* fragments was degraded during S1-nuclease digestion, therefore providing additional evidence that pHRR14 contained in *N. gonorrhoeae* N864 harbours an identical copy of the *pilC* gene of *N. meningitidis* N862.

Selection of piliated N. meningitidis *A1493 derivatives*

Adhesive derivatives of *N. meningitidis* strain A1493 variant N860 were obtained by growing pellicles (Koomey *et al.*, 1987) and their subsequent selection for adherence to the human epithelial cell line ME-180 (Rudel *et al.*, 1992). Specifically, 7×10^7 cells ml^{-1} medium were allowed to adhere for 60 min to ME-180 epithelial cells grown on cover slips. The cell layer of the cover slips with the adherent bacteria was removed and plated on GC agar, thus enabling bacterial growth. The procedure was repeated twice (with 5×10^7 bacteria ml^{-1} for 60 min and with 3×10^7 bacteria ml^{-1} for 30 min) to yield highly adherent *N. meningitidis* variants. One variant clone (N862) was studied in detail.

DNA sequence determination

N. meningitidis pilC fragments were amplified by PCR using primers directed against maximally conserved regions of *N. gonorrhoeae pilC1,2* spaced at average intervals of 300 bp. Primers with odd numbers (TR47–TR65) were derived from the plus-strand sequence; accordingly, primers with even numbers (TR48–TR66) were derived from the minus-strand sequence. Combination of two adjacent primers (e.g. TR47 and TR48) in a PCR reaction yielded a fragment of ≈300 bp ideally overlapping with the fragment amplified by the following set of primers. Additionally, the primers carried the M13 forward and reverse sequences which allowed direct sequencing as previously described (Kupsch *et al.*, 1993). Oligonucleotides used in this study are listed in Table 3. The distal regions of the gene were determined by using the M13 forward and reverse primers adjacent to the multiple cloning site of the vector thus allowing sequencing into the insert. After the PCR reaction the fragments were run on a preparative 1.2% agarose gel, stained with 0.1% methylene blue and isolated using a GeneClean kit (Bio 101). Subsequently, the fragments were sequenced using the Taq Primer

Table 3. Oligonucleotides used for PCR amplification of the *N. meningitidis pilC* gene.

Strand (+ or −)	Sequence
+ Strand	
BS10	5′-GGAATTCTGCAGCCGAAAAGGAAATACGATG-3′
TR31	5′-TGTAAAACGACGGCCAGTCCGAAAAGGAAATACGATG-3′
TR47	5′-TGTAAAACGACGGCCAGTTATTATCATCAACGAGCG-3′
TR49	5′-TGTAAAACGACGGCCAGTAAGGTTTCCGGTTTTGATG-3′
TR51	5′-TGTAAAACGACGGCCAGTGCGTATCTTTCAATTTGG-3′
TR53	5′-TGTAAAACGACGGCCAGTAGTCAAAGCAGGCCGCTG-3′
TR55	5′-TGTAAAACGACGGCCAGTAAAAACGACACTTTCGGC-3′
TR57	5′-TGTAAAACGACGGCCTACAATCTGAAGCTCAGC-3′
TR59	5′-TGTAAAACGACGGCCAGTACACCGTCGGTACGCCGC-3′
TR61	5′-TGTAAAACGACGGCCAGTAGTAAATGGTCTGCAAAG-3′
TR63	5′-TGTAAAACGACGGCCAGTTGAGGGAAGGAGAACGCG-3′
TR65	5′-TGTAAAACGACGGCCAGTAACCGTCTGCCCGAACGG-3′
− Strand	
TR33	5′-AATCTAGAGGATCCCCCTCCCGAAAAACGC-3′
TR48	5′-CAGGAAACAGCTATGACCCGGGTGGTACGAATCCAA-3′
TR50	5′-CAGGAAACAGCTATGACCACGATGGTTTGATTATTA-3′
TR52	5′-CAGGAAACAGCTATGACCACAGCGCGGGGGCGGTCAG-3′
TR54	5′-CAGGAAACAGCTATGACCCGTCCAAGGCAGCAGCAC-3′
TR56	5′-CAGGAAACAGCTATGACCTCCACGCCGTAGCGGTCG-3′
TR58	5′-CAGGAAACAGCTATGACCAGGAAGGCGGCGTATTTG-3′
TR60	5′-CAGGAAACAGCTATGACCTGCCAGTCGGGAAACGGC-3′
TR62	5′-CAGGAAACAGCTATGACCTACGCAATACCACGGTCG-3′
TR64	5′-CAGGAAACAGCTATGACCAG(A/G)TAACGCACATTAACC-3′
TR66	5′-CAGGAAACAGCTATGACCTTC-3′

Sequencing Kit and an Applied Biosystem 373A automatic sequencer. Combining the fragments into the complete sequence of *N. meningitidis pilC* was performed using the program ASSEMBLEGEL of the software package PC/Gene (IntelliGenetics Inc.). The gonococcal *pilC* sequences introduced by the *pilC1,2*-derived primers were neglected in searching overlaps between adjacent fragments. The cleavage site of the signal peptide was determined by the program PSIGNAL. For the sequence determination of the G-residue region of N860 and N862, the 5′ end of meningococcal *pilC* was amplified using a combination of primers TR31 and TR48. The nucleotide sequence of the *N. meningitidis pilC* gene has been submitted to the EMBL Nucleotide Sequence Database under the Accession Number Z54202.

Electrophoresis, immunoblotting and V8 protease digestion

Bacteria grown on GC agar plates were suspended in sample solution, sonicated for between 30 s and 60 s in a Branson sonifier (model 450, 50% duty cycle, power setting 4, microtip) and subsequently boiled for 10 min. Insoluble debris was removed by microcentrifugation (10 min at 10 000 × *g*) and the supernatant was standardized for the amount of total protein. For immunoblotting analysis, samples were analysed by electrophoresis on 10% polyacrylamide gels (Laemmli, 1970) for the detection of PilC and on Tricine–polyacrylamide (Schägger *et al.*, 1987) for the detection of pilin, respectively, and transferred to nitrocellulose (0.1 μm pore size) at 1 mA cm^{-2} using a semidry blot system (Biotec-Fisher) and Tris-glycine buffer with 20% methanol (48 mM Tris, 39 mM glycine, 1.3 mM SDS, pH 9.2). Filters were blocked (1 h, room temperature) with 3% BSA in TBS and then incubated overnight at 4°C with the appropriate antibody dilutions (1:400 of AK203 for detection of PilC, 1:500 of AK34 for the detection of class I pilin and a combination of 1:1000 dilutions of U101, preparation S3300 (Achtman *et al.*, 1992), and AG123 (Wang *et al.*, 1993), preparation S3409, for the detection of meningococcal class II PilE). After three repeated washing steps in TBS with 0.5% Tween 20 for 5 min, the blots were incubated at room temperature for 1 h with the appropriate conjugate antibody (alkaline phosphatase coupled to protein A and anti-rabbit IgG antibody or anti-mouse IgG-antibody, as appropriate and at dilutions of 1:2000 each). The blots were washed three times in TBS with 0.5% Tween 20 for 5 min and developed in 0.1 M Tris-HCl (pH 9.6 at 20°C) containing 7 mM $MgCl_2$, 0.1 mg ml^{-1} nitro blue tetrazolium and 50 μg ml^{-1} 5-bromo-4-chloro-3-indolyl-phosphate *p*-toluidine.

For V8 protease digestion, whole cell lysates of N860 and N862 were subjected to SDS gel electrophoresis and the pilin bands were cut out after Coomassie-brilliant-blue staining of the gel. After restoring neutral pH within the gel slices by incubation in 0.1% SDS, 1 mM EDTA, 2.5 mM dithiothreitol (DTT) and 125 mM Tris-HCl (pH 6.8), the slices were transferred into the wells of a second 15% polyacrylamide gel with a stacking-gel length of ≈3 cm filled with the same buffer, and were subsequently overlaid with 15 μl of 0.1% SDS, 1 mM EDTA, 2.5 mM DTT, 125 mM Tris-HCl (pH 6.8) and 20% glycerol. On top of this, 10 μl of 0.1% SDS, 1 mM EDTA, 2.5 mM DTT, 125 mM Tris-HCl (pH 6.8) and 10% glycerol containing various dilutions of V8 protease (Sigma) were added. Electrophoresis was allowed to proceed at a constant voltage of 125 V until the running front approached the end of the stacking gel. Power was switched off and digestion of pilin was allowed to take place for 45 min. The run was then continued at 125 V until the running front approached the end of the separating gel. The gel was silver stained according to the method of Blum *et al.* (1987).

Electron microscopy

Gonococci grown on GC agar plates were spread on water droplets, collected with Formvar and carbon-coated grids and subjected to oblique-angle carbon shadowing. Grids were examined in a Zeiss EM109 electron microscope at 80 kV.

Transformation of E. coli

Competent cells were obtained by the method described by Hanahan (1983) and stored at −70°C. Before transformation, cells were thawed on ice for 10 min, 10 μl of DNA solution was added to 80 μl of bacterial suspension and left on ice for at least 30 min. Bacteria were then heat shocked for 90 s at 42°C before being placed on ice for 2 min. After adding 2 vols of L medium, bacteria were shaken at 37°C for 60 min. Aliquots of 10, 50 and 100 μl were plated on selective agar and incubated overnight at 37°C.

Transformation of Neisseria

Gonococci were diluted to an OD_{550} of 0.2 in transformation medium (15 g l^{-1} proteose-peptone, 5 g l^{-1} NaCl, 0.5 g l^{-1} starch, 4 g l^{-1} KH_2PO_4, 1 g l^{-1} K_2HPO_4, 10 mM $MgCl_2$, 1% vitamin mix and 5 mM $NaHCO_3$). A total of 500 ng of DNA was added and the suspension was gently shaken for 4 h at 37°C. Aliquots were then plated onto selective agar. Plasmid pRB2 was constructed by inserting the *Bam*HI-excised kan_{GC} cassette from pMF54 (Fussenegger *et al.*, 1996) into the *Bgl*II site of pIP11, a derivative of pIP100 (Halter *et al.*, 1989).

Adherence assays of Neisseria *on ME-180 epithelial cells*

ME-180 epithelial cells were grown in RPMI medium (Biomol) with 5% fetal calf serum (FCS) on cover slips in 24-well culture dishes in a humified atmosphere (37°C, 5% CO_2). Prior to the assay, pre-confluent cells were washed with RPMI. If inhibition of adherence by native PilC was to be measured, purified PilC was added to the well to give a final concentration of 2 μg ml^{-1} and pre-incubated at 37°C for 30 min. To exclude Opa-mediated binding, bacteria were scored for the lack of Opa synthesis, suspended in RPMI, and added to the wells, resulting in a final concentration of 5×10^7 bacteria ml^{-1}. Adherence was allowed to proceed for 2 h at 37°C before non-adherent bacteria were removed by washing five times with 1 ml PBS (pH 7.4, 0.5 mM $CaCl_2$, 0.7 mM $MgCl_2$, 5 mM glucose). The cells were then fixed with 0.5 ml 2% paraformaldehyde in PBS for 10 min, washed once with 1 ml distilled H_2O and stained with 1 ml crystal violet solution (30× stock solution: 2 g of crystal violet dissolved in 100 ml of 96% ethanol and water added to give a final volume of 900 ml).

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