The phase-variable pilus-associated protein PilC is commonly expressed in clinical isolates of *Neisseria gonorrhoeae*, and shows sequence variability among strains

Marianne Bäckman,[†] Helena Källström and Ann-Beth Jonsson

Author for correspondence: Ann-Beth Jonsson. Tel: +46 8 728 71 74. Fax: +46 8 34 26 51.
e-mail: Ann-Beth.Jonsson@mtc.ki.se

Microbiology and
Tumorbiology Center,
Karolinska Institutet, S-171
77 Stockholm, Sweden

PilC is a phase-variable protein associated with pilus-mediated adherence of pathogenic *Neisseria* to target cells. In this study, 24 strains of *Neisseria gonorrhoeae* with known epidemiological data were examined for expression of PilC. All strains produced PilC independently of serovar and site of isolation. To investigate whether the PilC protein is conserved or variable among gonococcal strains, the complete nucleotide sequence of *pilC* in four strains, isolated from either rectum, throat or blood, was determined. The deduced amino acid sequence in these strains differed from each other and from the two PilC proteins of *N. gonorrhoeae* MS11. These data demonstrate that PilC is commonly expressed, but the PilC sequence may vary among gonococcal strains.

Keywords: *Neisseria gonorrhoeae*, PilC, pili, adherence

INTRODUCTION

Surface-exposed antigens of *Neisseria gonorrhoeae* can have highly variable structural expression, including differences in their pili, opacity proteins and lipooligosaccharides (Lambden *et al.*, 1981; Hagblom *et al.*, 1985; Diaz & Heckels, 1982; Barritt *et al.*, 1987; Mandrell *et al.*, 1986). Pili of *N. gonorrhoeae* are a major virulence factor and promote attachment of the bacteria to eukaryotic cells (Swanson, 1973; Buchanan & Pearce, 1976). Adherence of piliated (P⁺) bacteria can be modulated by sequence changes in the major pilus subunit protein, PilE (Virji & Everson, 1981; Virji *et al.*, 1982, 1992, 1993; Nassif *et al.*, 1993; Rudel *et al.*, 1992; Jonsson *et al.*, 1994).

PilC is a 110 kDa pilus-associated minor outer-membrane protein involved in pilus biogenesis and pilus-mediated attachment (Jonsson *et al.*, 1991, 1992; Rudel *et al.*, 1992, 1995a, b). *N. gonorrhoeae* strain MS11 contains two *pilC* genes, *pilC1* and *pilC2*, which are highly homologous but not identical (Jonsson *et al.*,

1991; Rudel *et al.*, 1995a). The expression of PilC is frequently turned on and off by frameshift mutations in a poly(G) tract located within the signal peptide coding region. PilC is involved in pilus biogenesis since *pilC* double knock-out mutants are non-piliated (Jonsson *et al.*, 1991; Nassif *et al.*, 1994; Rudel *et al.*, 1995a). Mutants of *N. gonorrhoeae* MS11 expressing either PilC1 or PilC2 can both mediate attachment to target cells at equal levels (Jonsson *et al.*, 1994; Rudel *et al.*, 1995a), whereas in *Neisseria meningitidis* 8013, PilC1, but not PilC2, expression is correlated with adherence to Hec-I-B and HUVEC cell lines (Nassif *et al.*, 1994; Rahman *et al.*, 1997). High PilC expression in the meningococcal strain MC58 is correlated with hyperadherence and superior anchorage of the bacteria to the host cells (Virji *et al.*, 1995). Recently, PilC was suggested to act as an adhesin at the tip of the pilus fibre (Rudel *et al.*, 1995b). The PilC protein is also associated with the bacterial cell surface (Rahman *et al.*, 1997).

In this study, we examined 24 clinical isolates with known serovar and epidemiological data for production of the PilC protein. We also determined the complete *pilC* nucleotide sequence of four gonococcal strains, isolated from the blood, throat or rectum. Comparison of these sequences with known gonococcal *pilC* sequences of strain MS11 and the *pilC* gene of strain FA1090 suggest that there are sequence variations among PilC in gonococcal strains of different origin.

[†] **Present address:** Swedish Institute of Infectious Disease Control, S-105 21 Stockholm, Sweden.

Abbreviation: TEM, transmission electron microscopy.

The EMBL accession numbers for the sequences reported in this paper are Y13022, AJ001120 and AJ001121.

METHODS

Bacterial strains and isolation. *N. gonorrhoeae* MS11_{mk}(P⁺) and MS11_{mk}(P⁻n) (Swanson *et al.*, 1986) were obtained from

Dr M. Koomey (University of Michigan, Ann Arbor, USA). The specific MS11_{mk} used in our studies is designated MS11_{mk}-u to distinguish it from other MS11_{mk} strain samples. The remaining clinical isolates described in this study were

Table 1. Characteristics of *N. gonorrhoeae* strains

Isolate	Comments	Date of isolation (year-month-day)	Sex/ year of birth	Site of isolation	Serovar*	Pili†	PilC‡
GC-456 } GC-457 }	Partners	90-10-09 90-10-12	m/1970 f/1969	Urethra Urethra	Bpyvut Bpyvut	+	+
GC-469 } GC-470 }	Isolated from the same individual	90-11-12 } 90-11-12 }	m/1962	Urethra Throat	Bpyvut Bpyvut	+	+
GC-614 } GC-626 } GC-624 } GC-625 }	Isolated from the same individual	93-02-04 } 93-02-26 } 93-02-26 } 93-02-26 }	m	Urethra Rectum Rectum Throat	Brput Brpyvut Bpyust Brpyut	+	+
GC-620 } GC-621 }	Isolated from the same individual	93-02-17 } 93-02-17 }	m	Urethra Rectum	Bpyust Brpyut	+	+
GC-645 } GC-646 } GC-647 } GC-653 } GC-655 }	Isolated from the same individual who was infected for a long period of time	93-07-20 } 93-07-29 } 93-07-29 } 93-08-22 } 93-09-15 }	f/1950	Urethra Cervix Throat Throat Throat	Bpyvu Bpyvu Bpyvu Bpyvu Bpyvu	+	+
GC-689 } GC-690 }	Isolated from the same individual, mother to newborn infected with GC-692, and partner to male infected with GC- 693	94-05-19 94-05-19	f/1977	Urethra Rectum	Bpyvut Bpyvut	+	+
GC-692	Infant, infected during birth, see GC-689/690 and GC-693	94-05-17	m/1994	Eye	Bpyvut	+	+
GC-693	Father to newborn infected with GC-692, and partner to female infected with GC- 689/690	94-06-01	m/1966	Urethra	Bpyvut	+	+
GC-504	Partner to female infected with GC- 508/509	91-04-18	f/1956	Cervix	Arst	+	+
GC-508 } GC-509 }	Isolated from the same individual, partner to female infected with GC-504	91-04-30 91-04-30	m/1956	Urethra Throat	Arst Arst	+	+
GC-640		93-06-07	m/1941	Blood	Arost	-	+
GC-673		94-01-30	m/1966	Urethra	Brpyust	+	+
MS11 _{mk} (P ⁺)-u		71	m	Urethra	Bopvt	+	+
MS11 _{mk} (P ⁻ n)-u						-	+
MS11-8 (P ⁻ rev)						-	-

* The strains were classified into serovars with mAbs directed to PI (Ph-panel, Boule Diagnostics) (Knapp *et al.*, 1984; Sandström *et al.*, 1985).

† Determined by TEM.

‡ Determined by immunoblotting.

Table 2. Primers used in this study

Primer	DNA sequence	Position in GC-640 <i>pilC</i>
C103	5' TCATTTCGACAATACCGAT 3'	264–281
C105	5' CAGGCTACTCCTACACCG 3'	430–447
C106	5' GCAGATCATCCAACAAAT 3'	658–675
C107	5' TCGTCCTGTCGTCTCGAC 3'	896–879
C108	5' AATATATCGTCATTACCG 3'	1457–1474
C104	5' TTGACGATGTCGCCCAA 3'	1597–1580
C208	5' TTCCTCGCCTCCGGTTAT 3'	2100–2117
C112	5' TGGCAGACAAACGCGTCG 3'	2413–2430
C800	5' CGGATACGGACAAATGTG 3'	2734–2751
C206	5' GTTTACGACAAGCCGTT 3'	2943–2960
C1	5' CCGCGAAGGCAAACTTAAGGAAT 3'	–21*–2
C14	5' ATTCTGCTTTACCTCGGGCTGGTT 3'	164–141
C30	5' CAAACCCATCAATACGCT 3'	105–122
C100	5' GTCGCCCACATAGCCTTT 3'	1811–1794
C31	5' GGGTATTTGGCAACCGCCG 3'	1620–1638
C4	5' TTGCCGCCCCCTCCCGAAAAAGC 3'	3259–3236*

* Approximate location and size. The primers designed from the MS11 *pilC1* sequence are located outside the obtained GC-640 *pilC* sequence.

isolated from patient samples at the Department of Microbiology Stockholm Söder Hospital, Stockholm, Sweden, with the exception of GC-692 obtained from Dr S. Ringertz, Karolinska Hospital, Stockholm, Sweden. The strains were verified as previously described (Bäckman *et al.*, 1995). The strains were classified into serovars with mAbs directed against PI (Ph-panel, Boule Diagnostics) (Knapp *et al.*, 1984; Sandström *et al.*, 1985) and the antigens were prepared as previously described (Bäckman *et al.*, 1985). The strains were passaged two to four times on haematin agar plates [36 g GCII agar base (BBL), 83 ml horse blood (National Veterinary Institute), 2 ampoules yeast autolysate (Oxoid product no. SR105B) per litre] before being stored at -70°C . The characteristics of the strains are shown in Table 1. The control strain MS11_{mk}(P⁺)-u produces pili and PilC (P⁺, PilC⁺), the MS11_{mk}(P⁺)-u variant 8 (P⁺, PilC⁺) is a spontaneous revertible derivative of MS11_{mk}(P⁺)-u (Jonsson *et al.*, 1992). These strains are referred to as MS11 and MS11-8 in the text. The strains were grown on GC agar [GC medium base with Kellogg's supplement (Jonsson *et al.*, 1991)].

Immunoblotting. Gonococcal cells from a GC agar plate were harvested in PBS (0.145 M sodium chloride, 0.15 M sodium phosphate) and the concentration was adjusted to OD₆₀₀ 5.0. Ten microlitres whole cell lysates of each strain were electrophoresed on SDS-PAGE gels (Laemmli, 1970). The proteins were transferred onto a nitrocellulose filter and tested for reaction with polyclonal anti-PilC serum (Towbin *et al.*, 1979). The polyclonal anti-PilC serum was directed against PilC2 of MS11 and was prepared as previously described (Jonsson *et al.*, 1991).

Transmission electron microscopy (TEM). For TEM, strains of gonococci were grown overnight on GC agar plates, suspended in 10 mM Tris/HCl (pH 7.5), 10 mM MgCl₂ buffer and overlaid on 200-mesh carbon-coated copper grids for 10 min. The grids were washed with water, negatively stained with 1% sodium silicotungstate and examined in a Philips 100xx microscope.

Assays for adherence to cultured ME180 cells. ME180 (ATCC HTB33), an epithelial-like cell line derived from a human cervical carcinoma was grown in McCoy's 5A medium supplemented with 10% foetal calf serum and 1% L-glutamine at 37°C in 5% CO₂. For adherence assays, the cells were seeded in four-well tissue-culture chamber slides (Nunc) and incubated at 37°C for 2 d. The bacterial isolates were taken from the -70°C storage supply, and several colonies of each strain were streaked on a GC agar plate for use in the binding assay. Non-confluent layers of ME180 cells were infected with 10^7 bacteria ml⁻¹ for 1 h at 37°C in 5% CO₂. The cells were washed four times with minimal essential medium (MEM) and fixed with 0.3% glutaraldehyde. After blocking with 2% BSA for 1 h, attached bacteria were detected with gonococcal antiserum (diluted 1:200 in 0.2% BSA in PBS, pH 7.4) for 30 min, washed three times in PBS, incubated with FITC-conjugated anti-rabbit IgG (Sigma) diluted 1:1000 in PBS for 60 min and finally washed three times in PBS. The mean number of attached bacteria per cell was determined by counting the bacteria on 100 randomly chosen cells. The binding assay was performed on three independent occasions, with bacteria taken from the -70°C storage vial each time.

PCR amplification. Genomic DNA was prepared as previously described (Jonsson *et al.*, 1991). The PCR reactions were carried out according to the protocol of Perkin Elmer Cetus. Chromosomal DNA from GC-640 was used as the template and the primers used were synthesized according to the DNA sequence of MS11 *pilC1* (Jonsson *et al.*, 1995) by Pharmacia Biotech. DNA polymerase from Dynazyme was used in the PCR assay. The samples were subjected to 30 cycles of 1 min at 94°C , 1 min at 44°C and 3 min at 72°C . The samples were separated by agarose gel electrophoresis and the sizes of the fragments obtained were calculated by comparing with DNA molecular mass markers (Boehringer Mannheim). DNA fragments of the expected size were cut out, electroeluted and purified by Elutip-d (Schleicher & Schuell).

DNA sequencing. The *pilC* gene of GC-640 was PCR-amplified in three overlapping fragments. Fragment 1 (185 bp) and fragment 2 (1707 bp) were PCR-amplified using the primer pairs C1, C14 and C30, C100, respectively. These two fragments were ligated into M13mp18 and M13mp19 vectors and transformed into *Escherichia coli* XL1-Blue supercompetent cells (Epicurian Coli, Stratagene) according to the manufacturer's instructions. Fragment 3 (1571 bp), containing the 3' end of *pilC*, was amplified using the primers C31 and C4, and sequenced directly. Sequencing was performed using the T7 Sequencing kit (Pharmacia) for manual sequencing, or the Taq DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) for automated sequencing. The fragments were sequenced by primer walking using oligonucleotides synthesized according to the MS11 *pilC1* sequence or oligonucleotides synthesized according to the sequence of GC-640 obtained in the previous step (Table 2). More than one M13 clone was sequenced, and no sequence differences between clones were found. The *pilC* gene of GC-647, GC-653 and GC-626 was PCR-amplified using primers C1 and C4 (Table 2), and then gel-purified. Automated sequencing of the PCR products was performed using internal *pilC* primers (Table 2) and the Taq DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems). The sequences obtained were analysed with the help of the Wisconsin Sequence Analysis package, version 8.0 (CGC 8) (Genetics Computer Group, Madison, WI, USA).

The *pilE* gene was PCR-amplified and sequenced directly as previously described (Jonsson *et al.*, 1994).

RESULTS

Expression of PilC in clinical isolates of *N. gonorrhoeae*

We examined 24 strains of *N. gonorrhoeae* (Table 1) for the production of PilC. Whole bacterial cell lysates were examined by immunoblotting using a PilC-specific antiserum, and all isolates demonstrated a 105–110 kDa band, indicating that PilC was expressed. The negative control used was MS11-8, which is a spontaneous non-piliated, PilC⁻ variant of MS11_{mk}(P⁺)-u. This variant reverts to P⁺, PilC⁺ at a frequency of 10⁻³–10⁻⁴ per cell per generation, so it presumably had a minor population of PilC-expressing bacteria. However, this was not detected by immunoblotting. We thus concluded that the PilC-positive signal of the isolates came from true PilC⁺ bacteria and not from P⁻, PilC⁻ clones containing a small population of revertants. Isolate GC-626 displayed a weaker band in immunoblots, indicating that PilC production was down-regulated in this clone or that the PilC sequence of this isolate was very different compared to that of PilC2 of *N. gonorrhoeae* MS11, against which the antiserum was raised.

Variation in adherence to ME180 cells

Twenty-three of the strains in Table 1 expressed pili as determined by TEM, and one strain, GC-640, isolated from blood was not able to produce pili. One set of related strains, GC-645, GC-646, GC-647, GC-653 and

Fig. 1. Attachment of *N. gonorrhoeae* clinical isolates to the epithelial cell line ME180. The bacteria (10⁷ per well) were added to a non-confluent layer of ME180 cells and incubated at 37 °C in 5% CO₂ for 1 h. Mean values and SD of three experiments are shown.

GC-655, was studied for adherence to the epithelial cervical cell line ME180. These strains were isolated from a patient not responding to antibiotic treatment, and differed from other sets of related strains in that they did not produce Opa, as detected by SDS-PAGE and Coomassie-brilliant-blue-staining of outer-membrane preparations. Non-confluent layers of cells were infected with 10⁷ bacteria for 1 h and bound bacteria were detected by immunofluorescence microscopy. All five strains were of identical serovar, but differed in attachment levels from 5 to 60 bacteria per cell (Fig. 1). Southern hybridization and PCR amplification revealed that the strains carried only one copy of *pilC* (data not shown). According to careful examination by TEM, this set of strains produced equal amounts of pili. However, the strains contained different *pilE* gene sequences relative to each other (Fig. 2), as determined by direct sequencing of PCR-amplified fragments. Therefore, it is likely that the variation in adherence was due to *PilE* variation. These data are in agreement with previous studies *in vitro*, which showed that sequence changes in *PilE* lead to variation in tropism to target cells (Virji *et al.*, 1993; Rudel *et al.*, 1992; Jonsson *et al.*, 1994; Nassif *et al.*, 1993). The non-piliated DGI (disseminated gonococcal infection) strain GC-640 expressed Opa, and bound to ME180 cells at levels of 10 bacteria per cell, which is higher than the non-piliated MS11 control strain with five bacteria per cell. Opa proteins of *N. gonorrhoeae* mediate adherence and invasion into target cells (Virji & Everson, 1981; Kupsch *et al.*, 1993). It is therefore likely that the increased adherence of GC-640 was due to expression of Opa.

Fig. 2. Deduced amino acid sequence of *pilE* in a set of related strains. The *pilE* coding region was PCR-amplified and sequenced directly. The six variable mini-cassettes are marked MC1–MC6. Amino acids that differ from *PilE* of GC-645 are shown. Identical stretches of amino acids are indicated by dashes. Numbering above the sequences starts at position 1 of the mature *PilE* protein.

pilC in gonococcal strains differs from *pilC* in strain MS11

To determine the variability of *pilC* among gonococcal strains from different sites of isolation, the entire *pilC* gene was sequenced in four strains: GC-640, GC-626, GC-647 and GC-653. Strain GC-640 was isolated from blood and carried only one *pilC* gene, as determined by Southern blotting of *Cla*I-digested genomic DNA, and by *Cla*I digestion of PCR-amplified *pilC* fragments of GC-640 (data not shown). Strain GC-626, isolated from the rectum, expressed low levels of *PilC* in immunoblots. The two related strains, GC-647 and GC-653, isolated from the throat, bound at low (5 bacteria per cell) or high (60 bacteria per cell) numbers to ME180 cells. For DNA sequencing of GC-640, PCR was used to amplify the gene in three overlapping pieces. Two fragments of 1.7 kb and 1.5 kb covered the region encoding the mature protein, and overlapped by 250 bp. The third fragment of 185 bp covered the signal peptide coding region. Each fragment was sequenced by primer walking using the oligonucleotides shown in Table 2. The sequenced *pilC* gene of GC-640 contained 12 G residues within the poly(G) tract in the signal peptide coding region (EMBL accession number Y13022). The gene showed 84% homology with *pilC1* of MS11 (Jonsson *et al.*, 1995) and 88% homology with *pilC2* (Rudel *et al.*, 1995a) of MS11, according to analysis with the GCG 8 software. In the case of GC-626, GC-647 and GC-653, the complete *pilC* gene was PCR-amplified and sequenced directly by using internal primers. The *pilC* gene of GC-647 was identical to *pilC* of GC-653 (EMBL accession number Y13021), which proved that *pilC* sequence variation did not mediate the different levels of adhesion seen. *pilC* of GC-647/653 is 86% homologous to MS11 *pilC1*, and 84% homologous to MS11 *pilC2*. This *pilC* gene sequence carries 10 G residues within the poly(G) tract. GC-626 *pilC* (EMBL accession number Y13020) contains 13 G residues in the poly(G) tract, and showed 89% homology with *pilC1* and 91% homology with *pilC2* of MS11.

The *PilC* sequence varies among strains

The deduced amino acid sequences of *pilC* in GC-640, GC-626 and GC-647/653 are shown in Fig. 3. The

mature *PilC* proteins had a predicted molecular mass of around 110 kDa and an isoelectric point close to 10. Several variable and conserved regions were identified when comparing the obtained amino acid sequences with MS11 *PilC1* and *PilC2*, and with the preliminary *PilC* sequence of FA1090 (obtained during the Gonococcal Genome Sequencing project) (B. A. Roe, S. Clifton & D. W. Dyer, <http://dna1.chem.uoknor.edu>). The first 50 amino acids and the last 350 amino acids were more conserved than the rest of the protein. *PilC* of GC-640 was 73% identical (83% similar) to MS11 *PilC1*, and 77% identical (84% similar) to MS11 *PilC2*. GC-626 *PilC* was 81% identical (88% similar) to MS11 *PilC1*, and 76% identical (84% similar) to MS11 *PilC2*. The *PilC* protein of GC-647/653 was 84% identical (90% similar) to MS11 *PilC1*, and 74% identical (83% similar) to MS11 *PilC2*. *PilC* of GC-626 reacted weakly in immunoblots using an antiserum raised against *PilC1* of MS11. Since this sequence did not differ more from MS11 *PilC1* compared to the other strains, it is likely that the weaker *PilC* band seen in immunoblots was due to down-regulation in *PilC* production. Hydrophobic and hydrophilic domains were analysed by the GCG 8 software and showed no apparent variation between the different sequences (data not shown).

DISCUSSION

The aim of this study was to determine if *PilC* is expressed in gonococcal strains of different origin, and to determine whether the *PilC* sequence is conserved or variable. We found that all the 24 clinical isolates expressed *PilC*, independently of serovar and site of isolation. The DGI strain GC-640 expressed *PilC*, although *pili* were not demonstrated by TEM. However, the isolate GC-626 showed a weaker *PilC* band in immunoblots. Since the *pilC* sequence of this strain did not show increased variability compared to the other strains examined, it is likely that *PilC* production may be down-regulated in GC-626. In a previous study of *N. meningitidis*, *PilC* was detected in 89% of the mucosal isolates and in 54–60% of the disseminated isolates studied, as determined by immunoblotting using a *PilC* antiserum (Virji *et al.*, 1995). It could be that meningococci and gonococci differ in *PilC* expression. How-

	1					50				100
GC-640	MNKLTKPVQF	RHTALYAAIL	MFSHTGGGGG	AMAQT..HQY	AIVMNERNP	EVKWHGQYQK	SQLREKDGGR	KFTYTNQKTG	GTNNKNFISF	DNTDTLVSQR
pilC2	-----RR--	-----	-----	-Q--..RK-	--I--K--	-----SQ	-T-KD-GR--	T-SH-S-RNW	NGQQ-----	N-S-E-----
pilC1	-----R--	-----	-----	-----	-----	-----	-----NVP	-SIKD--RR-	EY--YTHR--	-GS...V--N-N-----Q-
GC-626	-----R--	-----	-----	E--K-..LK-	P-I--GQ--	K--...NVP	-SI-D-R--	TYI-NT-SNY	-IS...V--	N-S-E--Q-
GC-647/653	-----R--	-----	-----	-----	-K--IIN-Q-	-----QNV-F..	PPIKD-N-D-	V-N-PT--NP	LGRT-----	N-NN-----Q-
FA1090	-----RR--	-----	-Q-----	-Q-----	-QTR--	--I-D--	-----E-S-ST	-T-----	-I-----RNK	LNQQ-----
	101					150				200
GC-640	SGTAVFGTAT	YLPYGVKVS	FOIAELNKR	NAVNIHTTR	AALSGYSYTG	VTCRSGKCRP	KLVIYKTRFSF	DNTGLAKNAG	R.LNRHPDPS	RENSPIYKLE
pilC2	-----ADG-K-	-----	-----	-----	PG-A-I--	I--DTGQC-	E--E-K--	-GID--GGN	-K-D--	-----L-K--
pilC1	-----AVA-KE-N	-----	-D-R--	I--A--ID	I--YTGC-	-----	-----T	GQQ--KRK--	SK-DIYE-K-	-----S
GC-626	-----	-----	-----	-----	-T--NITGC-	-----	-----Q-T	-QQ--K-K--	SK-DIYE-K-	-----S
GC-647/653	-----	-----	-TDS-KG-A	-----	PG-A-NA-D	-I--N--	Q--Q-T	GQQ--QRKDS	K.-DIYE-K-	-----K
FA1090	-----	-----	-T-----	-----	-G-A-V--	-I--DTGQC-	Q-----	-----T	.-D--TE--	.S
	201					250				300
GC-640	DHPWLGVSN	LGAEGTDKDG	RSSNKLVSF	SDNNSN..QI	IVCTTEGHRI	SLGDWQREHT	AMAYYLNAKL	HLDDKKQIEN	IAPGKTVQLG	TLRPRVETTG
pilC2	-----G--A--	-----	-SRWI--	-ED-N--T	-F--R--P-	-----S	-----D-	-----T	-----N	I-----AKV
pilC1	-Y-----	-S-N-VQNS	KLF--I--	REG-N--T	-S--NP-	-----R--	-V-----	-----G-D	-Q--I-D-	I-K-H--
GC-626	-Y-----	-S-N-V--	K-F--I--	GED-N--T	-S--DYP-	-----H	-F-L--	-----G-Q	-----R	V-K-SIDVRT
GC-647/653	-Y-----	-S--A--	-VT--Y--	DEK--..NN	L-Y--RN-	-M-N--S-	-----	-----K--	-----D	V-K--S
FA1090	-Y-----	-S--A--	-----	DE--SN-N	L-Y--	-----S	-----	-----QR--	-----D	-----KT
	301					350				400
GC-640	R...SWLDF	WATWKIEDK	NITVRLDPE	VKAGRCINKP	NPNKNTKAPS	PALTAPALWF	GPVQNGKVQM	YSASVSTYPG	SSSSRIFLQE	LKTQTDPARP
pilC2	-RKW,DL-N-	-K-D-K-T-	Q-P-K-G--	-----	-PKS.-L-	-----	-----	-----	-----	-----K
pilC1	-----L--	-R-D-K-T-	Q-P-K-G-Q	-----	-N-----	-----	-G-D--AE-	-----	-----D	-----E-GK-
GC-626	-----	-NTGFGF-N-	-K-D-K-N-	Q-P-K-G--	-----	-----	-----	-----	-----D	-----SRND-KNK-
GC-647/653	-----	-----	-----	-----	-P-S.-L-	-----	-----	-----	-----D	-----E-E-GK-
FA1090	NNWGN L-S-	-----	-----	-T-TA	H--P-A--	-----	-----	-----	-----	-----R
	401					450				500
GC-640	GRHSLAALNA	QDIKSREPNF	NSRQTVIRLP	GGVYKISSGK	NGGRVAGI..	..NGNDGKND	TFGIYKDKLV	TPEADEWSNI	LLPWATYRYG	NDIDIFKTFNQ
pilC2	-----	-----	-----	-Q-AP--	-S--F--	-----	-----R--	-----VG--EV	-----	-----
pilC1	--Y--KS--D	GE--Q-S-	-G--I--D	D--HL-KLNG	SKDE--AFVN	LNGN-T--	-----V-EAN-	NLD--KKV	-----V-GPD	--NK--SI--
GC-626	--Y--QP--E	NE--Q-S-	TI--I--D	D--HL-RLSR	ARDE-IDFVN	LNGN-T--	-----V-EAN-	NLN--KKV	-----V-GPD	--NK--SI--
GC-647/653	--Y--KP-SE	NE--VK--R-	-R-----	D--QL-NLNW	-NDE-VTF..	..G-A-N-G	-----V-EAD-	NL--KKV	-----V-AP-	--NK--SI--
FA1090	-----	-DT-----	-----	-N-----	-----	-----	-----R--	-----	-----	-----
	501					550				600
GC-640	PNNKKQSDKK	QYSQKYRIRT	KEDDNDN.RD	LGDIVNSPIT	AVGGYLTATSA	NDGMVHIFKK	NGT.DQRGYE	LKLSYIPGTM	PRKDIESN..	...DSTLAKE
pilC2	--S-T-NG--	-----	-N--KP--	-----	-V-----	-----	T--E-S-N	-----	E--G--	...D--
pilC1	KPE.....	-----R--	..--NGN--	-----	-V-----	-----	-GS-E-S-N	-----	-----Q-Q..	...E--
GC-626	...RE.....	-----R--	..E-G--	-----	-V-----	-----	-GS-E-S-N	-----	G--NQ..	...ESTLAKE
GC-647/653	EPG.....	-----R--	..N-G--	-----	-V-----	-----	-GS-E-S-N	-----	-QYFDNDTS	ALK--Q-
FA1090	--S-T-NG--	-----	-KP--	-----	-V-----	-----	-L-R	-----	E--G--	...D--
	601					650				700
GC-640	LRAFAEKGYV	GDRYGVGGF	VLCPL.QMST	QAEHFFMCGA	MALGGRGYVA	LDSRKINDFF	FPVGRPLFGV	KDGDNNNGKNR	..VKVDLGY	TRRTRNFGTP
pilC2	--T-----	-----	-RRITDDQD	KQK--F--	-G--A--	-LS--DSSN	LT.-VSM-D-	QNDK--NN-K	NDNNR-K--	-VG-PQI-KT
pilC1	-----	-----	-RQV..EL-	GQK-V--F--	-G--A--	-LS--G.N	Y-AAA--D-	-----	-----E--	-VG-PQI-KI
GC-626	-----C--	-----	-RRITDDQD	RQK--F--	-G--A--	-LS--DG.N	Y-AAA--D-	-----KK--	...E-K--	-VG-PQI-KT
GC-647/653	--T-----	-----	-RQV..NLNG	.DRV--	-G--A--	-LS--G.N	Y-AAA--D-	-----	...E-K--	-VG-PQI-KT
FA1090	-----	-----	-RRITDDQD	KQK--F--	-G--A--	-LS--DG.N	Y-AAA--D-	-N-----	...E--	-VG-PQIK--
	701					750				800
GC-640	PKRGKYSVSL	GSYWGKNIG	SGDNKTALYV	YDLGNTLGTP	IAKIDVKGDK	GGLSSPTLVD	KDLGDTADIA	YAGDRGGYMY	RDLNSNDPS	KCSVSTIFEAF
pilC2	QN...AAF-	A--AA--	-----	-T-----	-E-S-SL	-K--EAPG--	-----	-V-----	-----	-N--F--
pilC1	RN...AAF-	A--AA-K-D	DST-----	-----	-K--	-----	-----	-V-----	-----	-N--F--
GC-626	QN...AF-	A--AA-H-A	-PT-----	-----	-----	PG--	-----	-N--V--	-----	-S--F--
GC-647/653	QN...AF-	A--AA-D-D	DPT-----	-----	-----	EAPG--	-----	-D--V--	-----	-N--F--
FA1090	QN...AAF-	A--AA--	-----	-----	-E-GS-SL	-K-E-QG--	-----	-V-----	-----	-N--F--
	801					850				900
GC-640	AKPITSVAAV	SRLADKRVAI	FGTARYLTEE	DVLDKREQYI	YGIFDDDKGT	..VKPTVQNG	TAGGLLEQVL	KEENKTLFL.	..NGSGVSGQS	KGWIVKLKEG
pilC2	D-----AP--	-----	-V--GSD-S-Q	-----	-TDK--	-S--	..N-K-T--	-G-----	-S--S	N--A-G--
pilC1	D-----AP--	-----	-V--GSD--D	-----	-NTG--	-----	-----	-H--	TQ-----	--R-D-G--
GC-626	T-----AP--	-----	-V--GSD-S--	-----	-VGTDQ--	-----	NN--	-----	-G-----	-T--
GC-647/653	T-----AP--	-----	-V--GSD--D	-----	-----	GT-----	-----	-G-----	-N--MQ-----	--R-D-G--
FA1090	D-----AP--	-----	-V--GSD--D	-----	-NTG--	-----	-----	-G-----	..G-D--G--	--V--
	901					950				1000
GC-640	QRLTDKPTVV	LRTALVTIRS	YTADNCGAQ	PAIVVINTAD	GGALTPRSAR	PIVPOHNS.V	AQYSGHKKTA	EGKSPPIGCM	NKCKGTVCPN	GYVDDKADIV
pilC2	E-V-V--	-----	-F--K--	-DT-K--	T--LG--	-----	-----	-Q--M-N--I--	W-NS--	-----
pilC1	E-V-V--	-----	-F--K--	-NDGG--E	T--LG--	-----	-----	-T-S	K--I--	D--
GC-626	E-V-V--	-----	-F--K--	-N-K-D--	T--LG--	-----	-GDQ..	-----	-Q--M-N--V--	W-N--
GC-647/653	E-V-V--	-----	-F--K--	-NDGG--E	T--LG--	-----	-EA-TA	-----	-T-S	K--I--
FA1090	--V-V--	-----	-F--K--	-N-K--	T--LG--	-----	-Q--	-----	-Q--M-N--I--	W-N--
	1001					1050				
GC-640	RYLEKRRKTD	FPVTADGDAG	GSRTFKEGKK	PARNNRCFSG	KGVRTLLMND	LDSLDTGPM	CGIKRLSWREVFF*	-----	-----	-----
pilC2	---DEK--	-----	-----	-----	-----	-----	-----	-----	-----	-----
pilC1	---DETE--G	-ST-----	---IDPA-RR	-GK-----	-----	-----	-----	-----	-----	-----
GC-626	---DEK--	-----	-----	-----	-----	-----	-----	-----	-----	-----
GC-647/653	---DETE--G	-ST-----	---IDPADRR	-GK-----	-----	-----	-----	-----	-----	-----
FA1090	---DFK--	-----	-----	-----	-----	-----	-----	-----	-----	-----

Fig. 3. For legend see facing page.

ever, a different PilC antiserum was used in the meningococcal study.

The strains demonstrated variation in attachment to ME180 cells. GC-647 and GC-655 adhered very poorly although they were piliated and PilC⁺. Thus, adherence to ME180 varied even when the amount of PilC detected by immunoblotting was equal. GC-647 (binds poorly to ME180), and GC-653 (binds well to ME180) carry an identical *pilC* gene, but differ in *pilE* gene sequence. Therefore, the variation in adherence is most likely due to PilE sequence variation. Sequence variation in the major pilus subunit, PilE, has previously been shown to modulate adherence of both *N. gonorrhoeae* and *N. meningitidis* to target cell lines and tissues (Virji *et al.*, 1992, 1993; Jonsson *et al.*, 1994; Rudel *et al.*, 1992; Nassif *et al.*, 1993).

Some of the strains used in this study were passaged one or two times before being frozen. Therefore, we can not exclude the possibility that the phenotypes expressed by these isolates differed from the *in vivo* situation. The number of G residues found indicates that GC-640 *pilC* was out-of-frame. However, expression of PilC in GC-640 was demonstrated by immunoblotting. It may be that the *pilC* gene was in-frame in a subclone of the isolate. It is also possible that during PCR, a frameshift, with loss of a G residue or addition of two G residues, could occur because of the limited proof-reading of the DNA polymerase. Different proportions of in- and out-of-frame copies were found in an earlier study (Jonsson *et al.*, 1991) involving strain MS11, which was found to express PilC. Among the strains studied, we found that only GC-640 and the set of five related strains, i.e. GC-645, GC-646, GC-647, GC-653 and GC-655, carry one *pilC* locus. The remaining isolates contained two *pilC* alleles, according to PCR-amplification with *pilC1*- and *pilC2*-specific primers.

The conserved and variable domains in PilC could represent antigenic variation of the PilC protein. PilC has been suggested as a vaccine candidate, but the antigenicity of the stable domains has to be investigated to determine whether the conserved domains are exposed to the human immune system. If PilC is an important adhesin which interacts with a specific receptor on eukaryotic cells, the receptor-binding domain of the PilC protein should be conserved and be a potential target for development of a vaccine. Further studies are required to determine if the variable and conserved regions of PilC are surface-exposed and to determine the possible clinical importance of the demonstrated variation.

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Fig. 3. Comparison of gonococcal PilC sequences. Shown are the PilC sequence of *N. gonorrhoeae* strain GC-640 (EMBL accession number Y13022), the PilC2 (Rudel *et al.*, 1995a) and PilC1 (Jonsson *et al.*, 1995) sequences of *N. gonorrhoeae* strain MS11, the PilC sequence of GC-626 (EMBL accession number Y13020), and the identical PilC sequences of GC-647 and GC-653 (EMBL accession number Y13021). The PilC sequence of *N. gonorrhoeae* FA1090 is preliminary and originates from the Gonococcal Genome Sequencing project (B. A. Roe, S. Clifton & D. W. Dyer, <http://dna1.chem.uoknor.edu>). The in-frame versions of the proteins are presented. Thick black lines under the sequences represent variable regions of PilC. Dashes represent amino acids identical to those in the GC-640 sequence and dots represent amino acid gaps. Asterisks mark the ends of the proteins.

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