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Characterization of a Class II Pilin Expression Locus from *Neisseria meningitidis*: Evidence for Increased Diversity among Pilin Genes in Pathogenic *Neisseria* Species

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Strains of *Neisseria meningitidis* elaborate one of two classes of pili. Meningococcal class I pili have many features in common with pili produced by *N. gonorrhoeae*, including the ability to bind monoclonal antibody SM1 and a common gene and protein structure consisting of conserved, semivariable, and hypervariable regions. Class II pili are SM1 nonreactive and display smaller subunit molecular weights than do gonococcal or meningococcal class I pili. In this study, we have determined the N-terminal amino acid sequence for class II pilin and isolated the expression locus encoding class II pilin from *N. meningitidis* FAM18. Meningococcal class II pilin displays features typical of type IV pili and shares extensive amino acid identity with the N-terminal conserved regions of other neisserial pilin proteins. However, the deduced class II pilin sequence displays several unique features compared with previously reported meningococcal class I and gonococcal pilin sequences. Class II pilin lacks several conserved peptide regions found within the semivariable and hypervariable regions of other neisserial pilins and displays a large deletion in a hypervariable region of the protein believed to be exposed on the pilus face in gonococcal pili. DNA sequence comparisons within all three regions of the coding sequence also suggest that the meningococcal class II pilin gene is the most dissimilar of the three types of neisserial *pilE* loci. Additionally, the class II locus fails to display flanking-sequence homology to class I and gonococcal genes and lacks a downstream Sma/Cla repeat sequence, a feature present in all other neisserial pilin genes examined to date. These data indicate meningococcal class II pili represent a structurally distinct class of pili and suggest that relationships among pilin genes in pathogenic *Neisseria* do not necessarily follow species boundaries.

Pili are filamentous surface structures which often play a role in bacterial attachment. Within the genus *Neisseria*, pili have been observed on the pathogenic species *Neisseria gonorrhoeae* and *N. meningitidis*, as well as on several commensal *Neisseria* species (1, 16, 42). The structure of neisserial pili has been most extensively studied in *N. gonorrhoeae*. Gonococcal pili are composed primarily of multimers of the protein pilin. The subunit molecular weight of gonococcal pilin ranges from 17,000 to 22,000, and all gonococcal pilins that have been examined react with the monoclonal antibody SM1 (38). Gonococcal pilin exhibits features characteristic of the type IV family of bacterial pili. These include a short leader sequence, the presence of *N*-methyl phenylalanine as the first amino acid of the mature protein, a highly conserved N-terminal domain, and the presence of two conserved cysteine residues in the C-terminal region of the protein (34). Crystallographic analysis of gonococcal pilin from strain MS11 reveals a ladle-shaped molecule divided into several distinct structural regions: an N-terminal hydrophobic α-helix, a sugar loop carrying an O-linked disaccharide modification, a four-stranded antiparallel β-sheet containing a loop connection, and a disulfide region demarcated by the conserved cysteine residues and followed by a C-terminal tail (19).

Although numerous features are shared among all gonococcal pili, these structures are also known for their variability. Gonococcal pilins display antigenic differences between strains as well as antigenic variation within a strain. DNA sequence analysis of pilin expression loci (*pilE*) isolated from several gonococcal strains, as well as from different variants within a strain, reveals a gene structure composed of a highly conserved 5′ region encoding amino acids 1 to 53, which comprise the N-terminal α-helix of the protein (11, 19, 28). This region also encodes the SM1 epitope, which has been mapped to amino acids 49 to 53 (38). The conserved region is followed by a semivariable region encoding amino acids 54 to 114. Although this central region of the gene displays an increased degree of sequence divergence, it nonetheless contains several strongly conserved sequence elements designated SV1 to SV5. The remaining 3′ portion of *pilE*, encoding the disulfide region and C-terminal tail of pilin, is often collectively referred to as the hypervariable region. However, the greatest degree of variability among *pilE* loci is localized to sequences encoding part of the disulfide region centrally located between two additional short conserved sequence elements designated cys1 and cys2 (11, 19, 28).

The genetic events responsible for generating the sequence variation observed among different pilin variants are well understood in *N. gonorrhoeae*. In addition to the presence of one or in some cases two expression loci, gonococci possess numerous truncated silent pilin genes (*pilS*) that lack promoters and 5′ coding sequences found in *pilE* loci. The *pilS* loci donate information to *pilE* loci through nonreciprocal recombination events. These events result in the generation of altered *pilE* loci encoding pilin variants that differ primarily in the composition of amino acids found within the hypervariable portion of their disulfide regions (see reference 28 for a review). The cys2 sequence element and the Sma/Cla repeat, a second conserved sequence located downstream of all gonococcal pilin genes,

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play a role in facilitating at least some of these recombination events (12, 41).

Strains of *N. meningitidis* express one of two structurally distinct types of pili termed class I and class II pili. Both class I and class II pilin-producing strains have been isolated from patients with meningococcal disease. Both classes of pili can also facilitate the attachment of meningococci to various types of human cells in in vitro cell culture systems, although differences in binding ability have been observed both between classes and among variants within a class (6, 22, 39, 40). Meningococcal class I pili are similar to gonococcal pili in that they have subunit molecular weights of 17,000 to 23,000 and uniformly react with monoclonal antibody SM1 (6, 20, 38). Pilin expression loci from two strains of class I pilin-producing *N. meningitidis* have been analyzed. Class I meningococcal *pilE* loci exhibit the same overall structure as gonococcal *pilE* loci and a high degree of overall DNA homology to gonococcal *pilE* loci, including near-perfect sequence identity in the conserved 5′ region (18, 23, 40). Glycosylation of meningococcal class I pilin has been observed in the same region of the protein that is glycosylated in gonococcal pilin; however, the O-linked sugar observed in *N. meningitidis* is a trisaccharide (32). Class I pilin also carries an α-glycerophosphate modification (33). Antigenic variation has been observed in class I pilin-producing strains of *N. meningitidis*, and *pilE* loci isolated from antigenic variants differ in the DNA sequences found within the hypervariable portion of their disulfide regions (18, 40).

The structure of meningococcal class II pili has not been well characterized. Class II pili display slightly smaller subunit molecular weights of 15,000 to 17,000 and are distinguished from meningococcal class I pili and gonococcal pili by their failure to bind monoclonal antibody SM1 (6, 20). Additional antigenic differences have also been observed among meningococcal pili in regions other than the SM1 epitope (30, 31). Silent loci similar to those found in *N. gonorrhoeae* have been identified in class II pilin-producing strains of *N. meningitidis*. However, studies with DNA probes targeted to conserved 5′ sequences shared by meningococcal class I and gonococcal *pilE* genes have failed to detect expression loci in class II pilin-producing strains of *N. meningitidis* (2, 21). This unexpected finding, taken together with the differences in size, antigenicity, and cell-binding properties between class I and class II pili, has led to the suggestion that there may be fundamental structural differences between the two classes of meningococcal pili. To better understand the relationships among different classes of neisserial pilin, we have isolated and determined the DNA sequence of a meningococcal class II *pilE* locus. This report presents the characterization of pilin expression in the class II pilin-producing strain FAM18, together with a comparative analysis of gonococcal, meningococcal class I, and meningococcal class II pilin genes.

MATERIALS AND METHODS

Bacterial strains. *N. meningitidis* FAM18 (serogroup C, serotype 2a) was isolated from a patient with meningococcal septicemia. *Neisseria* cells were grown at 37°C under 5% CO_2 on GC medium base agar or broth (Difco Laboratories) containing the supplements described by Kellogg et al. (14). *Escherichia coli* cells were grown on Luria-Bertani agar or broth with or without 50 μg of ampicillin per ml (25).

Pilin purification and protein sequencing. The piliation state of *N. meningitidis* FAM18 was determined by electron microscopy as previously described (1). Meningococcal pili were purified by the method of Brinton et al. (5). Purified pilin was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis on 15% gels as previously described (3) with the addition of 0.1 mM thioglycolic acid to the top running buffer. The protein was subsequently electrotransferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories) with CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) transfer buffer (10 mM CAPS, 0.5 mM dithiothreitol, 10% methanol [pH 11.0]). The membrane was stained with Coomassie brilliant blue, and the M_r 16,000 protein band was excised. Amino acid sequencing was performed on a Porton model 2090 sequencer by using gas-phase Edman chemistry (North Dakota State University Biopolymers Service Center).

Oligonucleotides. Oligonucleotides were obtained from Genosys. Degenerate oligonucleotide aa2/8 (5′-GGCGCATGCCARYTIATHGARYTIATGAT3′, where I is inosine, H is A+T+C, R is A+G, and Y is C+T) represents all possible sequences encoding amino acids 2 to 8 of mature FAM18 pilin. Degenerate oligonucleotide aa23/29 (5′GGCGGATCCGCIGTRTARTCYTGRTAIGC3′) represents the reverse complement of all possible sequences encoding amino acids 23 to 29 of mature pilin. Oligonucleotide CII5prime (5′CGCCATCGTCGGTATCTTGGCAGCCG3′) represents the region of FAM18 *pilE* at nucleotides 294 to 319 as numbered in Fig. 2. The vectorette-specific oligonucleotide is a 25-mer complementary to a conserved region in both the blunt and *Eco*RI vectorette sequences (Genosys). The primers −25 (5′GTCACAACTGACAAGAAACGTCAGC3′) and 802RC (5′GCACCTAAGCCTCACCCTACAG3′) represent sequences immediately flanking the sequence displayed in Fig. 2. The nomenclature for the remaining oligonucleotides used as internal DNA sequencing primers is as follows. Oligonucleotides with numerical designations consist of sequences contained in FAM18 *pilE* as displayed in Fig. 2 beginning at the designated position. Oligonucleotides with numerical designations followed by RC are composed of nucleotides representing the reverse complement of sequences displayed in Fig. 2, with the first base of the oligonucleotide consisting of the complement of the nucleotide at the designated position. These primers include 237 (5′CATCCAAATGAAAGCAATCC3′), 288RC (5′GATCATCAGCTCGATCAGGG3′), 331RC (5′CAGGGCGACGGCTGCCAAG3′), 356 (5′CCCAAATGTCCGAAGCCCTG3′), 435 (5′CCCGAACAGCAATACTTCC3′), 453RC (5′GGAAGTATTCGTGTTCGGG3′), 675 (5′CTGCCGCACCAAATAAGG3′), and 692RC (5′CCTTATTTGGTGCGGCAG3′).

PCR, cloning, and DNA sequencing techniques. Oligonucleotides aa2/8 and aa23/29 (see above) were used to amplify a 102-bp product from undigested chromosomal DNA from *N. meningitidis* FAM18 in 100-μl reaction mixtures consisting of 100 ng of template DNA, 100 pmol of primers, 200 μM deoxynucleoside triphosphates, 4 mM $MgCl_2$, 1 U of *Taq* polymerase, and 1× reaction buffer as supplied by the enzyme manufacturer (Perkin-Elmer). Forty reaction cycles of 1 min at 94°C, 1 min at 50°C, and 2.5 min at 72°C were run. The PCR product was purified from 2% agarose gels with the QIAquick gel extraction kit (Qiagen Inc.). The purified product was cloned into plasmid pCRII and transformed into *Escherichia coli* INVαF′ as directed in the Invitrogen TA cloning kit. Recombinant plasmids were purified using the QIAfilter Plasmid Midi Kit (Qiagen Inc.). Double-stranded DNA sequence analysis of the cloned PCR product was determined by the dideoxy chain termination method (26) with the Sequenase 2.0 system and M13 forward and reverse primers (United States Biochemical).

Vectorette libraries were constructed by digesting FAM18 chromosomal DNA with *Rsa*I and *Eco*RI (Promega) and ligating blunt and *Eco*RI vectorettes, respectively, to the digested chromosomal DNA by using the protocol provided by the vectorette supplier (Genosys). PCR was performed on 1-μl aliquots of both vectorette libraries with the vectorette-specific primer and CII5prime under the conditions described above with the annealing temperature modified to 68°C. Additional PCRs were performed under these conditions on the *Rsa*I vectorette library with the vectorette-specific primer and oligonucleotide aa23/29. The resulting amplimers were purified and cloned as indicated. The DNA sequences of both strands of relevant portions of the cloned products were obtained as indicated by using M13 forward and reverse primers and the internal DNA sequencing primers listed above.

The entire FAM18 class II *pilE* locus was amplified from undigested chromosomal DNA with primers −25 and 462RC at an annealing temperature of 55°C. The resulting 825-bp fragment was purified as described above. The purified PCR product was sequenced at the University of North Carolina—Chapel Hill Automated DNA Sequencing Facility on a model 373A DNA sequencer (Applied Biosystems) with the Taq DyeDeoxy Terminator cycle-sequencing kit (Applied Biosystems). The DNA sequences of both strands of the 825-bp fragment were determined by using −25, 462RC, and the internal DNA sequencing primers indicated above. DNA sequence alignments were initially constructed with the program Seqaid II, version 3.5, and then modified based on known structural features of the pilin proteins.

Nucleotide sequence accession number. The GenBank accession number of the *N. meningitidis* FAM18 class II *pilE* sequence reported in this study is U81551.

RESULTS

We have analyzed pilin expression in the class II pilin-producing *N. meningitidis* FAM18 (serogroup C). In a previous study, we used Southern blot analysis to examine chromosomal DNA from this strain for homology to a cloned gonococcal *pilE* gene. Under high-stringency conditions, the gonococcal pilin gene hybridized strongly to a single fragment in the

FAM18 chromosome. Characterization of this hybridizing fragment revealed the presence of a *pilS* locus encoding two tandem, in-frame *pilS* genes (2). We now describe a meningococcal class II *pilE* locus from strain FAM18.

Pilin purification and amino acid sequencing. We purified pilin from a piliated variant of strain FAM18. This pilin displayed a molecular weight of 16,000 on SDS-polyacrylamide gel electrophoresis and failed to bind monoclonal antibody SM1 on either dot blots or Western blots (data not shown). Purified class II pilin was transferred from polyacrylamide gels to polyvinylidene difluoride membranes, and the first 32 amino acids were analyzed by gas phase sequencing (see Fig. 3). The sequence of amino acids 2 to 32 is typical of the consensus type IV pilin N-terminal sequence, and these residues display perfect identity with the corresponding amino acids found in gonococcal and class I meningococcal pilins. The identity of the first amino acid of mature FAM18 pilin could not be determined by this method.

Isolation and characterization of meningococcal class II *pilE*. We generated an oligonucleotide probe specific for the 5′ region of the FAM18 class II pilin expression locus by first using amino acid sequence data to design pools of degenerate PCR primers representing all possible nucleotide combinations encoding amino acids 2 to 8 (aa2/8) and 23 to 29 (aa23/29). The degenerate primers were used to amplify a single 102-bp fragment from purified FAM18 chromosomal DNA (data not shown). We determined the DNA sequence of this fragment and used the data to design oligonucleotide CII5prime, which represents a perfect DNA sequence match with the nucleotides encoding amino acids 11 to 18 of mature FAM18 pilin. Chromosomal DNA from strain FAM18 digested with *Cla*I, *Eco*RI, *Hae*III, or *Hin*cII was examined by Southern blot analysis with CII5prime as a probe under moderate-stringency conditions. A single hybridizing fragment was identified in each digest (data not shown).

We also used CII5prime as a tool to clone a class II pilin expression locus from *N. meningitidis* FAM18. Initial attempts to clone the *pilE* gene by screening DNA libraries generated in plasmid vectors with CII5prime were uniformly unsuccessful. Although initial screens would yield hybridizing clones, these clones were unstable and hybridizing inserts could not be maintained on subculture. Consequently, we adopted a vectorette PCR strategy to isolate class II *pilE*. We digested FAM18 chromosomal DNA with either *Rsa*I or *Eco*RI and ligated blunt or *Eco*RI vectorettes, respectively, to the chromosomal restriction fragments. Vectorettes, which are small, partially double-stranded DNA fragments, facilitate PCR amplification of the region between a specific internal primer and the end of the restriction fragment to which the vectorette is ligated (24). We used the specific primer CII5prime and a vectorette annealing primer in reactions that amplified 340-bp (from the *Rsa*I digest) and 1,150-bp (from the *Eco*RI digest) products from FAM18 DNA. Additionally, we used degenerate primer aa23/29 and the vectorette primer to amplify a 975-bp product representing upstream portions of *pilE* from the *Rsa*I vectorette population (Fig. 1). These PCR products, which represent partial gene fragments, were cloned into plasmid pCRII, and their DNA sequence was determined. Using sequence data from the cloned partial gene fragments we designed PCR primers complementary to flanking sequences on either side of *pilE* and amplified an 825-bp product containing the entire class II *pilE* gene from undigested FAM18 chromosomal DNA (Fig. 1). The DNA sequence of the 825-bp fragment was determined directly from the PCR product. The sequences of each of the initial cloned fragments and the 825-bp PCR product containing the intact gene yielded identical results.

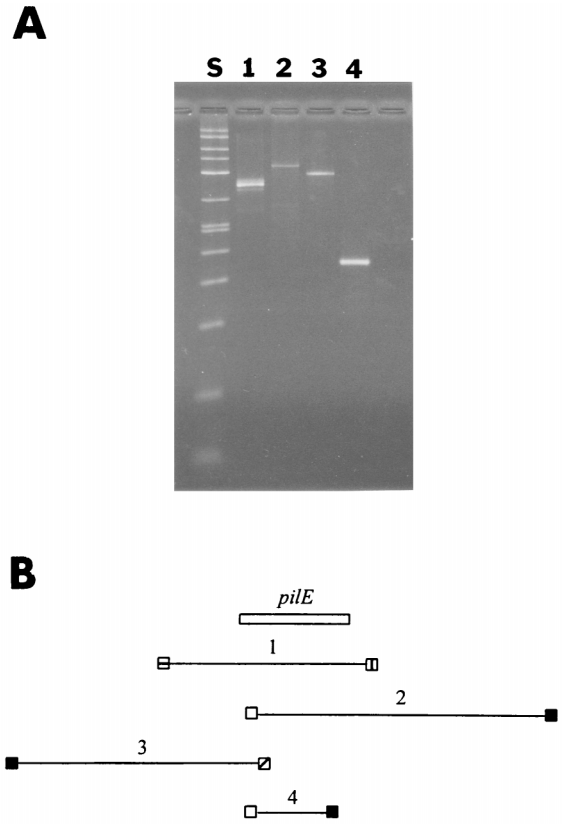

FIG. 1. PCR products containing class II *pilE* sequences. (A) Purified PCR products were electrophoresed in 2% agarose and stained with ethidium bromide. Lanes: S, size standards (2,500, 2,000, 1,500, 1,250, 1,000, 750, 525, 500, 400, 300, 200, 100, and 50 bp); 1, 825-bp product containing the entire *pilE* gene amplified from undigested *N. meningitidis* FAM18 chromosomal DNA; 2, 1,150-bp product amplified from the FAM18 *Eco*RI vectorette library; 3, 975-bp product amplified from the FAM18 *Rsa*I vectorette library; 4, 340-bp product amplified from the FAM18 *Rsa*I vectorette library. (B) Diagrammatic representation of the PCR products relative to *N. meningitidis* FAM18 class II *pilE*. 1, 2, 3, and 4 are the 825-, 1,150-, 975-, and 340-bp products indicated above, respectively. Boxes indicate primers: box with horizontal line, −25; box with vertical line, 462RC; open box, CII5prime; solid box, vectorette-specific primer; box with diagonal line, aa23/29.

The DNA sequence of the FAM18 class II *pilE* locus is shown in Fig. 2. This gene consists of a single 444-bp open reading frame. A consensus ribosome binding site is located 9 bp upstream of the initiation codon, and an inverted repeat representing a potential transcription termination signal is located 22 bp downstream of the termination codon. Nucleotides 729 to 738 beginning within the first inverted repeat match 8 of 10 bases in the neisserial DNA uptake sequence (GCCGTCT GAA) (10). A second region which also matches 8 of 10 bases in the uptake sequence is located slightly further downstream at nucleotide positions 769 to 777. Several potential transcription initiation sequences are located upstream of the start codon, including possible −10 and extended −10 sequences at positions −155 and −200 and two possible −24/−12 promoters at positions −58 and −121, with the latter demonstrating the strongest similarity to the consensus −24/−12 sequence. The predicted translation product of class II *pilE* displays an N-terminal signal peptide of 7 amino acids followed by a phenylalanine and 32 amino acids that are identical to the sequence determined from the purified protein. The predicted molecular weight of the mature protein is 14,800.

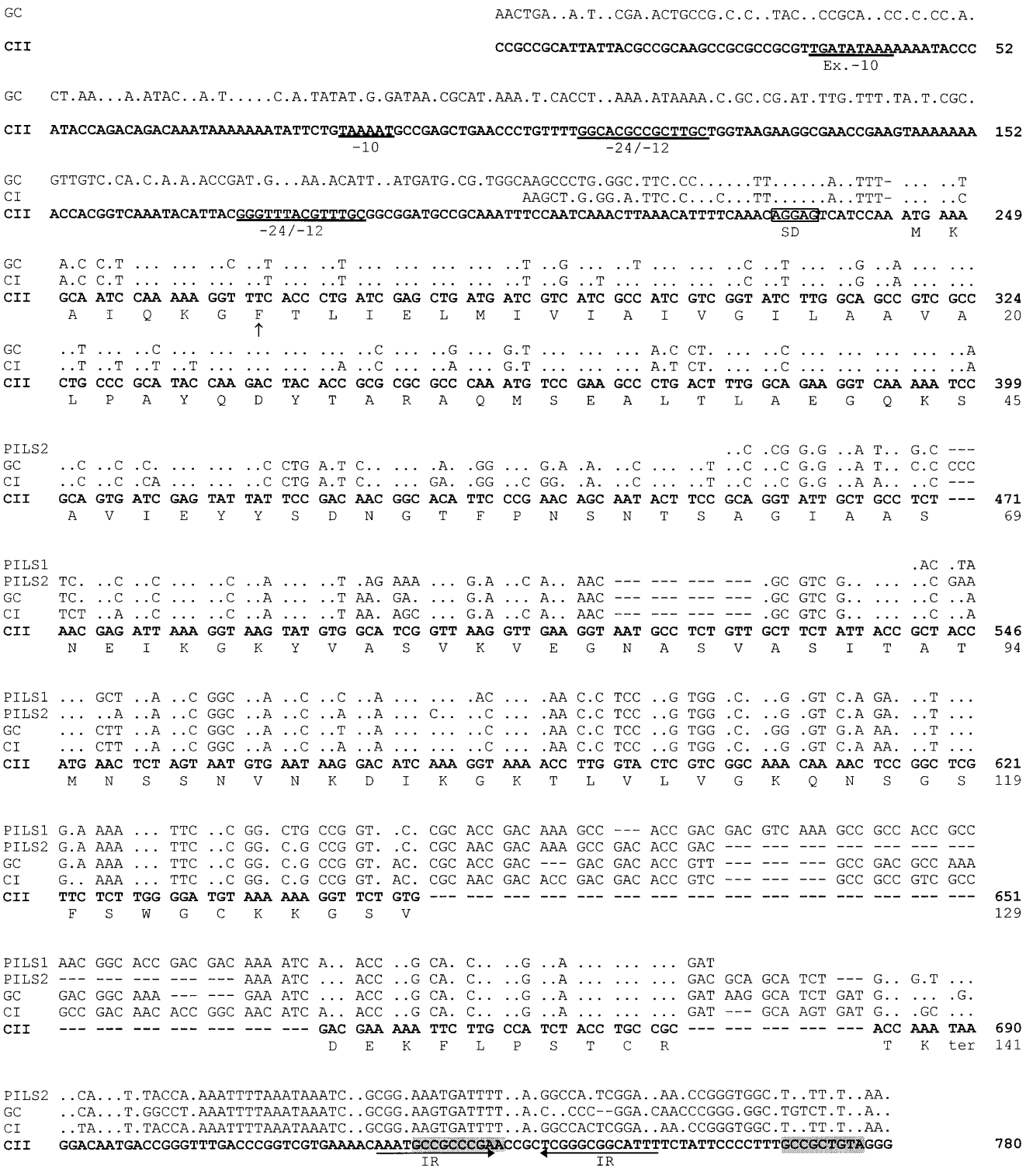

FIG. 2. Nucleotide and deduced amino acid sequences of the *N. meningitidis* FAM18 class II *pilE* locus and comparison to gonococcal *pilE*, meningococcal class I *pilE*, and FAM18 *pilS* loci. The sequence of the 825-bp PCR product generated from FAM18 chromosomal DNA with primers −25 and 462RC is designated CII. Other loci displayed are as follows: GC, gonococcal *pilE* locus from strain MS11 (17); CI, meningococcal class I *pilE* locus from strain C311 (23); PILS1 and PILS2, silent loci from strain FAM18 (2). Dots in the GC, CI, and PILS lines indicate bases that are identical to the CII sequence. A dash represents deletion of a base, and nucleotide exchanges are as indicated. Potential promoter elements are underlined, and inverted repeats representing a possible transcription terminator are underlined with arrows. A likely Shine-Dalgarno sequence is indicated by an open box. Shaded boxes indicate regions with partial homology to the neisserial DNA uptake sequence.

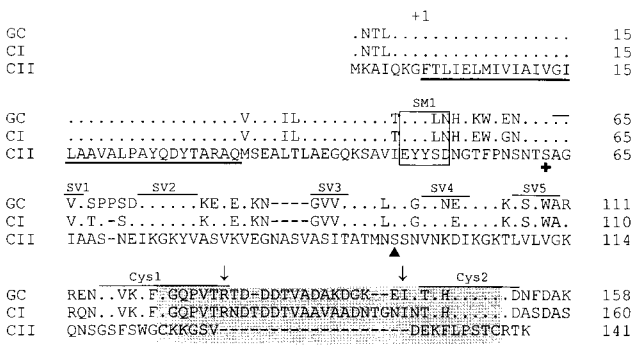

FIG. 3. Predicted amino acid sequence of *N. meningitidis* FAM18 class II pilin and comparisons to gonococcal and meningococcal class I pilin sequences. CII indicates the amino acid sequence deduced by translation of the FAM18 class II *pilE* locus reported in this study. CI indicates the amino acid sequence deduced from the *N. meningitidis* C311 (23) class I *pilE* locus. GC indicates the amino acid sequence determined by crystallographic analysis of *N. gonorrhoeae* MS11 (19). Dots in the GC and CI lines indicate residues identical to the CII sequence. Dashes represent deletions of amino acids, and amino acid differences are as indicated. Numbering begins with the first amino acid of the mature proteins, indicated by +1. The SV1, SV2, SV3, SV4, SV5, cys1, and cys2 regions are overlined. The region encoding the SM1 epitope in GC and CI is boxed. Serine-63, which represents a potential glycosylation site in the CII sequence, is indicated by a plus sign, and serine-97, which represents a potential site of α-glycerophosphate modification, is indicated by a solid triangle. The disulfide region is shaded, and conserved arginine and isoleucine residues in this region of the GC and CI sequences are indicated by arrows. The underlined CII residues represent amino acids analyzed by gas-phase sequencing of purified FAM18 pilin.

Comparison of neisserial *pilE* genes. The predicted protein sequence of meningococcal class II pilin has many features in common with gonococcal and meningococcal class I pilins. Figure 3 displays a comparison of the deduced amino acid sequences for FAM18 class II pilin and representative examples of class I pilin from *N. meningitidis* C311 (23) and gonococcal pilin from *N. gonorrhoeae* MS11 (17, 19). The N-terminal region of the protein is highly conserved in all three strains. As expected from the protein-sequencing data, the first 32 amino acids following the leader peptide display perfect identity. The gonococcal and class I sequences are also identical to one another from amino acids 33 to 55, a region in which the class II sequence differs by 7 amino acids. Interestingly, the SM1 epitope, which has been mapped to residues 49 to 53, lies just at the beginning of a region in which the class II sequence begins to diverge from the other sequences. The first three residues of this epitope are conserved in the class II sequence, but the last two lie in a region of divergence. Although the class II sequence displays an increased level of divergence from the gonococcal and class I sequences beyond amino acid 55, several small stretches of perfect identity among all three sequences can be observed, including two 6-amino-acid stretches at positions 72 to 77 and 134 to 139. Two potential modification sites at serine-63 and serine-97 are also conserved in the meningococcal class II sequence. Serine-63 has been identified as a site of O-linked glycosylation in both *N. gonorrhoeae* MS11 and *N. meningitidis* C311 (19, 32). Both of these strains also carry an α-glycerophosphate modification at the position equivalent to serine-97 in strain FAM18 (33). Two C-terminal cysteine residues located at positions 124 and 138 in the class II sequence also represent a conserved feature, not only of gonococcal and class I meningococcal pilins but also of type IV pilin proteins in general.

Despite the presence of certain conserved residues, overall the central and C-terminal regions of the predicted meningococcal class II pilin protein sequence differ from the gonococcal and meningococcal class I sequences. The region from amino acids 54 to 117 encompasses the portion of neisserial pilin generally described as semivariable. The class II sequence exhibits a more pronounced overall degree of divergence in this region than has been previously observed among different strains of *Neisseria* within a species or between the meningococcal class I and gonococcal sequences (Fig. 3; Table 1). Additionally, striking differences between the meningococcal class II sequence and the gonococcal and meningococcal class I sequences are evident in the C-terminal region of the protein following amino acid 117. Comparative sequence analyses of multiple gonococcal strains, together with analysis of the crystallographic structure of *N. gonorrhoeae* pilin, have demonstrated that the hypervariable portion of pilin is located between the highly conserved amino acids arginine-127 and isoleucine-142 (the numbering is for *N. gonorrhoeae* MS11 [Fig. 3]) within the disulfide region of the protein (19). Comparable arginine and isoleucine residues are also conserved in meningococcal class I pilin. Surprisingly, arginine-127, isoleucine-142, and the entire intervening hypervariable region are absent from the meningococcal class II sequence. A further deletion is also found in the extreme C terminus of class II pilin. In gonococcal and class I meningococcal pilins, the second cysteine of the disulfide region is followed by a region of at least 7 amino acids that forms a C-terminal tail. This tail region is truncated in the class II sequence, which displays only 3 amino acids following cysteine-138.

DNA sequence comparisons also suggest that class II pilin is a divergent member of the neisserial pilin family. Within the region of *pilE* encoding the conserved N-terminal 53 amino acids of the mature protein, the class II sequence differs at several nucleotide positions which are identical in the class I and gonococcal sequences (Fig. 2; Table 1). As expected, the DNA sequence divergence becomes more pronounced among all three *pilE* loci in the semivariable region encoding amino acids 54 to 117. However, the gonococcal and class I meningococcal sequences are clearly more closely related to one another in this region than to the class II sequence (Fig. 2; Table 1). The large deletions found in the 3′ region of the class II sequence also set it apart from the class I and gonococcal sequences, which continue to display stretches of near-perfect homology with one another even within their hypervariable regions (Fig. 2). Additionally, flanking sequences both upstream and downstream of the class II *pilE* gene differ from the class I and gonococcal sequences, which are quite similar to one another. The class II expression locus is also unique in that it lacks the Sma/Cla repeat commonly found downstream of neisserial pilin genes. We have examined an additional 139 bp downstream of the nucleotides shown in Fig. 2 on the cloned 1,150-bp *Eco*RI vectorette PCR product and found no evi-

TABLE 1. Sequence comparisons among representative neisserial *pilE* loci

Loci[a]	% Identity (protein/DNA) in:	
	Conserved region[b]	Semivariable region[c]
Class II, class I	88.7/78.6	55.4/53.8
Class II, gonococcal	88.7/81.1	46.2/53.8
Class I, gonococcal	100.0/94.3	84.6/90.8

[a] Sequences are from the following strains: class II, *N. meningitidis* FAM18; class I, *N. meningitidis* C311; gonococcal, *N. gonorrhoeae* MS11.
[b] The conserved region is amino acids 1 to 53 of mature pilin.
[c] The semivariable region is amino acids 54 to 117 of mature pilin.

dence of homology to the Sma/Cla repeat element (data not shown).

A comparison between the neisserial *pilE* loci and the *pilS* (2) locus previously characterized from strain FAM18 is also shown in Fig. 2. Neither of the silent gene copies in the *pilS* locus contains the information found in the FAM18 class II *pilE* locus, nor do they share flanking homology with class II *pilE*. However, the *pilS* locus from this class II pilin-producing strain shares a great deal of similarity with gonococcal and meningococcal class I *pilE* loci, including an intact hypervariable region demarcated by conserved arginine and isoleucine residues and the presence of downstream flanking sequence similarity, including a Sma/Cla repeat.

DISCUSSION

Class II pili form a structurally distinct group of pili expressed by some strains of *N. meningitidis*. We have used a vectorette PCR strategy to isolate a class II *pilE* locus from *N. meningitidis* FAM18. This expression locus consists of a single open reading frame preceded by a consensus ribosome binding site. A potential transcription termination signal is located downstream of the termination codon, and several potential transcription initiation signals are located upstream of the start codon. These include a possible −10 sequence, a potential extended −10 sequence (13), and two regions with homology to the consensus −24/−12 promoter sequence (37). Both −10 and −24/−12 promoters, as well as a more typical −35/−10 promoter, initiate gonococcal pilin expression under appropriate in vitro conditions (4, 9, 17, 35). Taha et al. have examined the *pilE* promoter region of the class I pilin-producing *N. meningitidis* 8013 and found a −10 consensus sequence but no −24/−12 promoter elements. These authors postulate that DNA sequence diversity among neisserial *pilE* promoters may result in differing levels of responsiveness to the regulatory protein PilA and therefore in potentially different regulatory pathways for controlling pilin expression in response to environmental stimuli (36). The presence of potential −24/−12 promoter sequences upstream of the FAM18 class II pilin gene provides further evidence of diversity among neisserial *pilE* promoters.

DNA and protein sequence data indicate that meningococcal class II pili are typical type IV pili. Other organisms that display type IV pili include class I pilin-producing *N. meningitidis* strains, *N. gonorrhoeae*, *Pseudomonas aeruginosa*, *Dichelobacter nodosus*, *Moraxella bovis*, *Vibrio cholerae*, and enterotoxigenic *E. coli* (see reference 34 for a review). *N. meningitidis* FAM18 displays the following features that are common to type IV pilin: a short (7-amino-acid) leader peptide, strong conservation of the N-terminal 32 amino acids, and conserved cysteine residues located in the C-terminal portion of the protein. Another feature typical of type IV pilins is the presence of *N*-methyl phenylalanine as the first residue of the mature protein. While we have not experimentally determined the identity of this amino acid in FAM18 pilin, DNA sequence analysis indicates the codon for phenylalanine, and the failure of standard protein-sequencing techniques to identify this residue is consistent with the presence of a modified amino acid.

In addition to the features typical of type IV pili in general, the predicted meningococcal class II pilin protein sequence has many features in common with meningococcal class I and gonococcal pilins. Strong protein sequence conservation is displayed throughout the N-terminal conserved region of the proteins, although the class II sequence does differ from the other two sequences at 6 of 53 residues in this region. Other, smaller areas of protein sequence conservation among all three pilin types are worthy of note. Residues 72 to 77 represent a conserved region previously designated SV2, and residues 134 to 139 represent the majority of the cys2 region (Fig. 3). A specific function has not been postulated for SV2; however, conservation of this peptide region in class II pilin further supports the hypothesis that it may represent a region of the protein under selective pressures to remain invariant. DNA sequences encoding cys2 are believed to play a role in facilitating recombination among *pil* loci (12). The degree of DNA sequence conservation in this region (nucleotides 664 to 681 [Fig. 2]) is more limited in the class II sequence; nonetheless, it represents one of the most notable regions of identity among the 3′ regions of neisserial pilin genes. Residues that represent sites of potential posttranslational modification are also conserved in the meningococcal class II predicted protein sequence. Consequently, glycosylation and/or the addition of α-glycerophosphate may explain the size discrepancy between the predicted molecular weight of FAM18 pilin based on DNA sequence analysis (14,800) and the observed molecular weight (16,000) of the purified protein on SDS-polyacrylamide gel electrophoresis.

The meningococcal class II pilin deduced amino acid sequence also displays interesting differences from the meningococcal class I and gonococcal pilin sequences, particularly in the semivariable and hypervariable regions of the protein (Fig. 3; Table 1). Several previously identified peptide regions commonly conserved among gonococcal and class I meningococcal pilin sequences, including SV1, SV3, SV4, SV5, and cys1, display extensive divergence in the class II sequence. The most striking difference in class II pilin is the deletion of the entire hypervariable portion of the disulfide region of the protein. Pilus fiber assembly models and antigenicity data suggest that this portion of pilin may protrude from the face of the assembled gonococcal pilus, masking more highly conserved regions of the protein located in the center of the pilus fiber (8, 19). Thus, sequence differences that we have observed within the disulfide region of meningococcal class II pilin may influence pilus structure and immunogenicity by allowing surface exposure of alternate regions of the pilin molecule.

The degree of antigenic variation exhibited by class II pilin-producing strains of *N. meningitidis* has not been well documented. Phase variation between piliated and nonpiliated phenotypes has been observed in strain FAM18, and both functionally and antigenically distinct pilin variants have been observed in this strain (22; our unpublished data). Typical neisserial *pilS* loci have been identified in class II pilin-producing strains, including FAM18 (2, 21). Unfortunately, the relationship between the FAM18 silent locus and class II expression locus examined in this study remains unclear. The two silent gene copies comprising the FAM18 *pilS* locus display a greater degree of overall similarity to gonococcal and class I meningococcal *pilE* loci than they do to the class II expression locus (Fig. 2). Furthermore, the FAM18 class II *pilE* locus does not contain the hypervariable sequence information encoded by either silent gene copy. While our data do not preclude the possibility of recombination between the identified *pilS* and *pilE* loci in strain FAM18, they fail to provide evidence documenting the recent existence of such recombination events. Nonetheless, a functional relationship between these loci remains a possibility. Alternatively, the *pilS* loci in class II pilin-producing strains of *N. meningitidis* may have been acquired via transformation from a class I pilin-producing strain of *N. meningitidis* or from *N. gonorrhoeae.*

DNA sequence comparisons among representative examples of meningococcal class II, class I, and gonococcal *pilE* loci suggest that similarities among neisserial pilin genes may not

necessarily follow species boundaries. The meningococcal class I and gonococcal loci share a higher level of sequence identity with one another than either shares with the meningococcal class II locus. Pilin genes from other species of *Neisseria* have not been examined. However, cloned gonococcal pilin genes failed to hybridize to DNA from piliated strains of *N. flava* and *N. cinerea* on dot blot analysis (1), suggesting that some commensal *Neisseria* species may also possess divergent members of the neisserial pilin gene family. Saunders et al. have suggested that pilin expressed by *N. lactamica* may share genetic ancestry with meningococcal class II pilin based on the development of a pilus-specific monoclonal antibody which recognizes both of these organisms (27). Gene transfer between commensal *Neisseria* species and *N. meningitidis* has been documented for other loci, including *penA*, *dhps*, *recA*, *argF*, and *adk* (7; for reviews, see references 15 and 29), and may have occurred for pilin genes as well. Analyses of *pilE* loci from multiple meningococcal strains of defined lineages and identification of the genes encoding pilin in commensal *Neisseria* species are needed to further understand the degree of diversity and evolutionary history of the neisserial pilin gene family.

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REFERENCES

1. **Aho, E. L., G. L. Murphy, and J. G. Cannon.** 1987. Distribution of specific DNA sequences among pathogenic and commensal *Neisseria* species. Infect. Immun. **55:**1009–1013.
2. **Aho, E. L., and J. G. Cannon.** 1988. Characterization of a silent pilin gene locus from *Neisseria meningitidis* strain FAM18. Microb. Pathog. **5:**391–398.
3. **Black, W. J., R. S. Schwalbe, I. Nachamkin, and J. G. Cannon.** 1984. Characterization of *Neisseria gonorrhoeae* phase variation by use of monoclonal antibodies. Infect. Immun. **45:**453–457.
4. **Boyle-Vavra, S., M. So, and H. S. Seifert.** 1993. Transcriptional control of gonococcal *pilE* expression: involvement of an alternate sigma factor. Gene **137:**233–236.
5. **Brinton, C. C., J. Bryan, J.-A. Dillon, N. Guerina, L. J. Jackobson, A. Labik, S. Lee, A. Levine, S. Lim, J. McMichael, S. A. Polen, K. Rogers, A. C.-C. To, and S. C.-M. To.** 1978. Uses of pili in gonorrhea control: role of bacterial pili in disease, purification and properties of gonococcal pili, and progress in the development of a gonococcal pilus vaccine for gonorrhea, p. 155–178. *In* G. F. Brooks, E. C. Gotschlich, K. K. Holmes, W. D. Sawyer, and F. E. Young (ed.), Immunobiology of *Neisseria gonorrhoeae*. American Society for Microbiology, Washington, D.C.
6. **Diaz, J.-L., M. Virji, and J. E. Heckels.** 1984. Structural and antigenic differences between two types of meningococcal pili. FEMS Microbiol. Lett. **21:**181–184.
7. **Feil, E., G. Carpenter, and B. G. Spratt.** 1995. Electrophoretic variation in adenylate kinase of *Neisseria meningitidis* is due to inter- and intraspecies recombination. Proc. Natl. Acad. Sci. USA **92:**10535–10539.
8. **Forest, K. T., S. L. Bernstein, E. D. Getzoff, M. So, G. Tribbick, H. M. Geysen, C. D. Deal, and J. A. Tainer.** 1996. Assembly and antigenicity of the *Neisseria gonorrhoeae* pilus mapped with antibodies. Infect. Immun. **64:**644–652.
9. **Fyfe, J. A. M., C. S. Carrick, and J. K. Davies.** 1995. The *pilE* gene of *Neisseria gonorrhoeae* MS11 is transcribed from a σ^{70} promoter during growth in vitro. J. Bacteriol. **177:**3781–3787.
10. **Goodman, S. D., and J. Scocca.** 1988. Identification and arrangement of the DNA sequence recognized in specific transformation of *Neisseria gonorrhoeae*. Proc. Natl. Acad. Sci. USA **85:**6982–6986.
11. **Hagblom, P., E. Segal, E. Billyard, and M. So.** 1985. Intragenic recombination leads to pilus antigenic variation in *Neisseria gonorrhoeae*. Nature **315:**156–158.
12. **Howell-Adams, B., L. A. Wainwright, and H. S. Seifert.** 1996. The size and position of heterologous insertions in a silent locus differentially affect pilin recombination in *Neisseria gonorrhoeae*. Mol. Microbiol. **22:**509–522.
13. **Keilty, S., and M. Rosenberg.** 1987. Constitutive function of a positively regulated promoter reveals new sequences essential for activity. J. Biol. Chem. **262:**6389–6395.
14. **Kellogg, D. S., W. L. Peacock, W. E. Deacon, L. Brown, and C. I. Pirkle.** 1963. *Neisseria gonorrhoeae*. I. Virulence genetically linked to clonal variation. J. Bacteriol. **85:**1274–1279.
15. **Maiden, M. C. J.** 1993. Population genetics of a transformable bacterium: the influence of horizontal genetic exchange on the biology of *Neisseria meningitidis*. FEMS Microbiol. Lett. **112:**243–250.
16. **McGee, Z. A., R. R. Dourmashkin, J. G. Gross, J. B. Clark, and D. Taylor-Robinson.** 1977. Relationship of pili to colonial morphology among pathogenic and nonpathogenic species of *Neisseria*. Infect. Immun. **15:**594–600.
17. **Meyer, T. F., E. Billyard, R. Haas, S. Storzbach, and M. So.** 1984. Pilus genes of *Neisseria gonorrhoeae*: chromosomal organization and DNA sequence. Proc. Natl. Acad. Sci. USA **81:**6110–6114.
18. **Nassif, X., J. Lowy, P. Stenberg, P. O'Gaora, A. Ganji, and M. So.** 1993. Antigenic variation of pilin regulates adhesion of *Neisseria meningitidis* to human epithelial cells. Mol. Microbiol. **8:**719–725.
19. **Parge, H. E., K. T. Forest, M. J. Hickey, D. A. Christensen, E. D. Getzoff, and J. A. Tainer.** 1995. Structure of the fibre-forming protein pilin at 2.6 Å resolution. Nature **378:**32–38.
20. **Perry, A. C. F., C. A. Hart, I. J. Nicolson, J. E. Heckels, and J. R. Saunders.** 1987. Inter-strain homology of pilin gene sequences in *Neisseria meningitidis* isolates that express markedly different antigenic pilus types. J. Gen. Microbiol. **133:**1409–1418.
21. **Perry, A. C. F., I. J. Nicolson, and J. R. Saunders.** 1988. *Neisseria meningitidis* C114 contains silent, truncated pilin genes that are homologous to *Neisseria gonorrhoeae pil* sequences. J. Bacteriol. **170:**1691–1697.
22. **Pinner, R. W., P. A. Spellman, and D. S. Stephens.** 1991. Evidence for functionally distinct pili expressed by *Neisseria meningitidis*. Infect. Immun. **59:**3169–3175.
23. **Potts, W. J., and J. R. Saunders.** 1988. Nucleotide sequence of the structural gene for class I pilin from *Neisseria meningitidis*: homologies with the *pilE* locus of *Neisseria gonorrhoeae*. Mol. Microbiol. **2:**647–653.
24. **Riley, J., R. Butler, D. Ogilvie, R. Finniear, D. Jenner, S. Powell, R. Anand, J. C. Smith, and A. F. Markham.** 1990. A novel, rapid method for the isolation of terminal sequences from yeast artificial chromosome (YAC) clones. Nucleic Acids Res. **18:**2887–2980.
25. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
26. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
27. **Saunders, J. R., J. Wakeman, G. Sims, H. O'Sullivan, C. A. Hart, and M. Virji.** 1993. Piliation in *Neisseria meningitidis* and its consequences. J. Med. Microbiol. **39:**7–9.
28. **Seifert, H. S.** 1996. Questions about gonococcal pilus phase- and antigenic variation. Mol. Microbiol. **21:**433–440.
29. **Spratt, B. G., N. H. Smith, J. Zhou, M. O'Rourke, and E. Feil.** 1995. The population genetics of the pathogenic *Neisseria*, p. 143–160. *In* S. Baumberg, J. P. W. Young, E. M. H. Wellington, and J. R. Saunders (ed.), Population genetics of bacteria. Cambridge University Press, Cambridge, United Kingdom.
30. **Stephens, D. S., A. M. Whitney, J. Rothbard, and G. K. Schoolnik.** 1985. Pili of *Neisseria meningitidis*: analysis of structure and investigation of structural and antigenic relationships to gonococcal pili. J. Exp. Med. **161:**1539–1553.
31. **Stephens, D. S., A. M. Whitney, G. K. Schoolnik, and W. D. Zollinger.** 1988. Common epitopes of pilin of *Neisseria meningitidis*. J. Infect. Dis. **158:**332–342.
32. **Stimson, E., M. Virji, K. Makepeace, A. Dell, H. R. Morris, G. Payne, J. R. Saunders, M. P. Jennings, S. Barker, M. Panico, I. Blench, and E. R. Moxon.** 1995. Meningococcal pilin: a glycoprotein substituted with digalactosyl 2,4-diacetamido-2,4,6-trideoxyhexose. Mol. Microbiol. **17:**1201–1214.
33. **Stimson, E., M. Virji, S. Barker, M. Panico, I. Blench, J. Saunders, G. Payne, E. R. Moxon, A. Dell, and H. R. Morris.** 1996. Discovery of a novel protein modification: α-glycerophosphate is a substituent of meningococcal pilin. Biochem. J. **316:**29–33.
34. **Strom, M., and S. Lory.** 1993. Structure-function and biogenesis of the type IV pili. Annu. Rev. Microbiol. **47:**565–596.
35. **Taha, M.-K., and D. Giorgini.** 1995. Phosphorylation and functional analysis of PilA, a protein involved in the transcriptional regulation of the pilin gene in *Neisseria gonorrhoeae*. Mol. Microbiol. **15:**667–677.
36. **Taha, M.-K., D. Giorgini, and X. Nassif.** 1996. The *pilA* regulatory gene modulates the pilus-mediated adhesion of *Neisseria meningitidis* by controlling the transcription of *pilC1*. Mol. Microbiol. **19:**1073–1084.
37. **Thöny, B., and H. Hennecke.** 1989. The −24/−12 promoter comes of age. FEMS Microbiol. Rev. **63:**341–358.
38. **Virji, M., J. E. Heckels, W. J. Potts, C. A. Hart, and J. R. Saunders.** 1989. Identification of epitopes recognized by monoclonal antibodies SM1 and SM2 which react with all pili of *Neisseria gonorrhoeae* but which differentiate between two structural classes of pili expressed by *Neisseria meningitidis* and

the distribution of their encoding sequences in the genomes of *Neisseria* spp. J. Gen. Microbiol. **135:**3239–3251.
39. **Virji, M., C. Alexandrescu, D. J. P. Ferguson, J. R. Saunders, and E. R. Moxon.** 1992. Variations in the expression of pili: the effect on adherence of *Neisseria meningitidis* to human epithelial and endothelial cells. Mol. Microbiol. **6:**1271–1279.
40. **Virji, M., J. R. Saunders, G. Sims, K. Makepeace, D. Maskell, and D. J. P. Ferguson.** 1993. Pilus-facilitated adherence of *Neisseria meningitidis* to human epithelial and endothelial cells: modulation of adherence phenotype occurs concurrently with changes in primary amino acid sequence and the glycosylation status of pilin. Mol. Microbiol. **10:**1013–1028.
41. **Wainwright, L. A., K. H. Pritchard, and H. S. Seifert.** 1994. A conserved DNA sequence is required for efficient gonococcal pilin antigenic variation. Mol. Microbiol. **13:**75–87.
42. **Wistreich, G. A., and R. F. Baker.** 1971. The presence of fimbriae (pili) in three species of *Neisseria*. J. Gen. Microbiol. **65:**167–173.

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