Journal of General Microbiology (1989), **135**, 3239–3251. *Printed in Great Britain*

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.

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(*Received 24 April 1989; revised 10 August 1989; accepted 15 August 1989*)

The pili expressed by all isolates of *Neisseria gonorrhoeae* react with two monoclonal antibodies, SM1 and SM2. In contrast, although many isolates of *Neisseria meningitidis* also express pili (class I) which react with antibodies SM1 and SM2, a proportion express pili (class II) which fail to react. In order to define the epitopes recognized by these antibodies, a series of overlapping peptides corresponding to the amino acid sequence of conserved regions of gonococcal pili have been synthesized. The minimum epitope recognized by antibody SM1 was found to comprise a linear peptide EYYLN, corresponding to residues 49–53 of mature pilin. In contrast, antibody SM2 reacted with a number of peptides from around the cysteine residue (Cys 1) at position 120, suggesting that an extended region may contribute to a conformational epitope recognized by this antibody in the native protein. The identification of the two epitopes defines structural differences between the classes of pili expessed by meningococci. In order to determine the distribution of pilin gene sequences in *Neisseria* we used as hybridization probes an oligonucleotide (PS1) with the sequence 5′-GAGTATTACCTGAATCA-3′ which spans the coding region for the SM1 epitope, and a fragment of the 3′ end of the gonococcal *pilE* gene which contains conserved sequences flanking the two Cys codons and encodes the SM2 epitope. All strains of *N. gonorrhoeae* and *N. meningitidis* tested, regardless of piliation phenotype, harboured DNA sequences homologous to those encoding the carboxy-terminus of meningococcal class I pilin. Furthermore, all gonococci and all meningococcoci producing class I pili hybridized with oligonucleotide probe PS1. Non-reverting non-piliated derivatives of previously class I pilus-producing strains showed reduced hybridization signals with this probe, but nevertheless retained sequences homologous to the coding sequence for the SM1 epitope. However, meningococci producing class II pili could be divided into two groups on the basis of their reaction with the PS1 probe: half the strains tested failed to react, which is consistent with our previous analysis of silent class I pilin sequences; the remainder reacted (relatively weakly) with the probe, suggesting that the silent *pil* sequences in these strains extend further towards the 5′ end of the pilin gene than in strains studied previously. Some strains of *Neisseria lactamica* reacted weakly with both types of probe but failed to produce SM1-reactive pili. In contrast,

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Abbreviation: mAb, monoclonal antibody.

isolates of *Neisseria flava*, *Neisseria pharyngis*, *Nesseria sicca* and a series of unrelated bacteria failed to react with both SM1 antibody and the DNA probes. This confirms that possession of 'gonococcal' pilin sequences is limited to the pathogenic neisseriae.

INTRODUCTION

Pili (fimbriae) are filamentous appendages present on the surface of pathogenic neisseriae which are thought to play an essential role in virulence (Swanson, 1973; Ward *et al.*, 1974). Fresh gonococcal isolates are invariably piliated, and loss of pilus expression on laboratory subculture has been correlated with loss of the ability to cause infection in human volunteers (Kellog *et al.*, 1963; Swanson *et al.*, 1971). Many studies have subsequently reported that the association between pilus expression and virulence results from the ability of pili to facilitate adhesion of gonococci to the mucosal surfaces of the genital tract, the essential first stage in pathogenesis of gonococcal infection (reviewed by Heckels, 1989). Although the association of piliation with meningococcal disease is less well established, fresh meningococcal isolates from both blood and cerebrospinal fluid are invariably piliated, and pili facilitate adhesion of meningococci to nasopharyngeal cells *in vitro*, suggesting that they play an important role in colonization of the mucosal surfaces of the nasopharynx (Stephens & McGee, 1981; Trust *et al.*, 1983).

Gonococcal pili exhibit considerable structural and antigenic diversity not only between strains but also within a strain so that antigenic shift in pilus expression during the course of an infection allows gonococci to evade the consequences of the host immune response (Zak *et al.*, 1984). Analysis of the predicted amino acid sequence of cloned genes has revealed that pili are assembled from a repeating array of a pilin polypeptide subunit which varies in apparent M_r in the range 17000–22000 between antigenic variants of a strain (Hagblom *et al.*, 1985; Nicolson *et al.*, 1987*b*). Such pilins contain distinct conserved (C) and variable (SV and HV) regions but the variable determinants are immunodominant so that the variant pili show little or no antigenic cross-reactivity (Virji *et al.*, 1982). Despite this antigenic heterogeneity, two monoclonal antibodies (mAbs), SM1 and SM2, have been produced that react with pili on all gonococcal isolates so far tested (Virji & Heckels, 1983).

Use of mAbs SM1 and SM2 has revealed that meningococcal pili show even greater antigenic diversity than those of gonococci. Many strains produce pilins which closely resemble gonococcal pilins in apparent M_r and reactivity with mAbs SM1 and SM2 (Diaz *et al.*, 1984), in possessing strong homology at the amino acid sequence level (Potts & Saunders, 1988) and in undergoing antigenic variation during infection (Tinsley & Heckels, 1986). Other strains produce pilins that fail to react with either mAb but react with polyclonal antisera raised against gonococcal pili to reveal pilins with apparent M_r in the range 13000–16000 (Diaz *et al.*, 1984; Perry *et al.*, 1987*a*). These two types of meningococcal pili have been given a variety of designations by different authors, namely group 1 and group 2 (Diaz *et al.*, 1984), SM1-reactive and SM2-nonreactive (Achtman *et al.*, 1988) and class I and class II (Perry *et al.*, 1988); the last has subsequently been adopted as the recognized nomenclature (Hitchcock, 1989).

Gonococcal and meningococcal pili are characteristically subject, both *in vivo* and *in vitro*, to phase and antigenic variation caused by recombinational events that involve the insertion of alternative silent partial pilin (*pilS*) sequences into a pilin expression (*pilE*) locus (Haas & Meyer, 1986; Hagblom *et al.*, 1985; Potts & Saunders, 1988; Segal *et al.*, 1986; Seiffert *et al.*, 1988). The presence of DNA sequences homologous to the entire structural gene for gonococcal pilin (*pilE*) appears to be limited to the pathogenic members of the genus *Neisseria* (Aho *et al.*, 1987). Despite failing to encode class I pili, the chromosomes of two independently isolated class II pilus-producing meningococci have been shown to contain silent *pil* sequences that are homologous to regions of the central semivariable (SV) and carboxy-terminal hypervariable (HV) coding portions of the gonococcal *pilE* pilin structural gene (Aho & Cannon, 1988; Perry *et al.*, 1988).

mAbs SM1 and SM2 have been used in several studies of meningococcal piliation to detect expression, to differentiate between the two classes of pili and to examine pilus antigenic variation (Diaz *et al.*, 1984; Olafson *et al.*, 1985; Achtman *et al.*, 1988; Potts & Saunders, 1988).

Here we describe the synthesis of peptides corresponding to conserved regions of the gonococcal pilin molecule to locate the epitopes recognized by these mAbs which define the two classes of meningococcal pili. This paper also reports a study of the distribution of the coding sequences for the SM1 epitope and the carboxy-terminus of class I pilin among pathogenic and commensal *Neisseria* species.

METHODS

Bacterial strains and plasmids. N. gonorrhoeae P9 (Lambden *et al.*, 1981) and *N. meningitidis* strains C311 (a class I pilus producer) and C114 (a class II pilus producer) (Perry *et al.*, 1987*a*) have been described previously. *Escherichia coli* strain DH1 (Hanahan, 1983) was used for the propagation of plasmid recombinants containing *pilE* gene probes. The remaining bacteria were all clinical isolates from Alder Hey Children's Hospital, Liverpool, that had been subcultured not more than twice prior to screening. All *Neisseria* strains were cultured on heated blood or clear typing agar (CTA) as described previously (Perry *et al.*, 1987*a*). The remaining bacteria were grown aerobically [with the exception of *Bacteroides* spp., which were grown anaerobically in Gaspak jars (Becton Dickinson)] at 37 °C on fresh horse blood agar plates (Oxoid blood agar base, 12 g; horse blood, 70 ml; distilled water to 1000 ml).

Reagents and enzymes. Reagents, with the exception of Tris (Boehringer Mannheim) and bovine serum albumin (Sigma), were supplied by BDH. Restriction endonucleases, polymerases, ribonuclease A and proteinase K were supplied by Boehringer Mannheim.

mAbs and immunological blotting. The production and properties of mAbs SM1 and SM2 have been described in detail previously (Virji *et al.*, 1983; Virji & Heckels, 1983). Procedures for immunological dot-blotting and Western blotting were as described previously (Nicolson *et al.*, 1987*a*).

Synthesis of peptides and detection of immunological reactivity. Solid-phase peptide synthesis was carried out using a commercially available kit (Cambridge Research Biochemicals) in which peptides are synthesized on to polyethylene rods (Geyson *et al.*, 1987). Peptides were synthesized as described previously (Virji & Heckels, 1989) with pentafluorophenyl active esters of fluorenylmethoxycarbonyl-L-amino acids with t-butyl side-chain-protecting groups (Milligen), except in the case of serine and threonine, for which the oxybenzotriazine active ester was used. After synthesis, the terminal amino group was acetylated by reaction with acetic anhydride. Each synthesis was done in duplicate, and control peptides reacting with a mAb supplied by the manufacturer were included to check the coupling during each synthesis.

The immunological reactivities of the synthesized peptides on the rods were tested by ELISA as described previously (Virji & Heckels, 1989). The rods were incubated with mAbs diluted 1:1000 in PBS containing 1% (w/v) BSA, 1% (w/v) ovalbumin and 0·1% (v/v) Tween 20. After washing and reaction with goat anti-mouse IgG conjugated to horseradish peroxidase (Zymed), colour was developed using 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonate) as substrate (Sigma). The solid-phase peptides were re-used after dissociation of bound antibody by sonication of the rods in 1% SDS, 0·1% 2-mercaptoethanol in 0·1 M-phosphate buffer at 60 °C for 30 min. Immunological reactivity was always observed with duplicate peptides and in assays repeated on at least two occasions.

Preparation of DNA. Meningococcal genomic DNA was extracted from the bacteria obtained from three CTA plates spread to produce confluent growth, using a modification of the method of Kristiansen *et al.* (1984). Cells were resuspended in 2 ml 50 μm-Tris/HCl (pH 8·0), 25% (w/v) sucrose and then pelleted by centrifugation at 12000 *g* for 2 min. The supernatant was discarded and 150 μl of a solution containing 50 μm-Tris/HCl, 25% (w/v) sucrose, 5 mg lysozyme ml^{-1} was added to the pellet. The mixture was vortexed briefly and incubated at 4 °C for 5 min, followed by addition of 60 μl ice-cold 0·25 M-EDTA, vortexing, and incubation for a further 5 min at 4 °C. Then 300 μl lysis solution (50 mM-Tris/HCl, 10 mM-EDTA, 2%, v/v, Triton X-100) and 100 μl of a solution of proteinase K (5 mg ml^{-1}) was added and, following vortexing, the mixture was incubated at 4 °C for 60 min. The resulting cell lysates were extracted three times with an equal volume of phenol/chloroform (100 g phenol, 0·2 g hydroxyquinoline, 100 ml chloroform, equilibrated with 0·1 M-Tris/HCl pH 8·0) and once with chloroform. Ethidium bromide was added to 0·2 mg ml^{-1} and CsCl added to give a refractive index of 1·392. The DNA was then banded by equilibrium density-gradient centrifugation at 180000 *g* for 18 h in an LKB Ultrospin 55 ultracentrifuge. DNA bands were extracted from the centrifuge tubes with 19G needles and syringes, and ethidium bromide removed by extraction with NaCl/water-saturated 2-propanol. The DNA solution was then dialysed against two changes of 1 litre of TE buffer (0·01 M-Tris/HCl, 0·001 M-EDTA, pH 8·0) over 6 h at 4 °C. Finally the solution was phenol-extracted, ethanol-precipitated and resuspended in sterile double-distilled water.

Smaller amounts of DNA for dot blotting were isolated by a modification of the method of O'Reilly *et al.* (1986) from the growth of bacteria on the surface of one CTA or blood agar plate, depending on the organism. Cells were harvested in 2 ml TES buffer (100 mM-NaCl, 1 mM-EDTA, 20 mM-Tris/HCl, pH 8·0) and pelleted in a microcentrifuge at 12000 *g* for 2 min. Then 1 ml lysis mixture (10 mM-Tris/HCl, 10 mM-EDTA, 100 mM-NaCl,

2%, w.v SDS, 39 mM-dithiothreitol, 50 μg proteinase K ml^{-1}, pH 8·0; Gill *et al.*, 1985) was added and, after vortexing, the mixture was incubated at 37 °C for 30 min. The lysate was extracted twice with phenol/$CHCl_3$ and used immediately for dot-blot hybridization. Alternatively, if the DNA was to be digested with restriction endonuclease, it was extracted once more with phenol/$CHCl_3$, ethanol-precipitated and resuspended in 150 μl TE buffer. Ribonuclease A (10 μl of a 10 mg ml^{-1} solution) was added and the mixture incubated at 37 °C for 1 h. The DNA was then ethanol-precipitated and finally resuspended in double-distilled water.

Plasmid DNA was extracted as described previously (Nicolson *et al.*, 1987*a*).

DNA/DNA hybridization. The synthetic oligonucleotide PS1 (alias SM1) (5′-GAGTATTACCTGAATCA-3′) (Potts & Saunders, 1988) was produced using a Biosearch 3810 automatic DNA synthesizer employing phosphoramidite chemistry. Following purification by reverse-phase FPLC the probe was 5′-end labelled using phage T4 polynucleotide kinase (Boehringer) and [γ-^{32}P]ATP (Amersham) by the procedure recommended by the manufacturers. The 0·42 kb *BglI–ClaI* fragment of recombinant pLV830, which harbours sequences encoding the carboxy-terminal portion of the gene corresponding to CNBr3 fragment (Schoolnik *et al.*, 1984) of pilin from *N. meningitidis* C311 (Potts & Saunders, 1988), was used as a general probe for gonococcal and class I meningococcal pilin sequences. This restriction fragment was purified from agarose gels and labelled with [α-^{32}P]CTP (Amersham) using the random hexanucleotide priming reaction of Feinberg & Vogelstein (1983) as described previously (Nicolson *et al.*, 1987*a*). Hybridizations were carried out essentially as described previously (Sullivan & Saunders, 1989) at 68 °C with subsequent washing at room temperature for low-stringency and at 68 °C for high-stringency conditions. Dot-blotting was performed by applying approximately 10 μg genomic DNA to nitrocellulose filters (BA85, Schleicher and Schuell) that had been presoaked in 6 × SSC (1 × SSC is 0·15 M-NaCl, 0·015 M-trisodium citrate, pH 7·0) and fixed in a Schleicher and Schuell Minifold I apparatus. Filters were removed and placed sequentially for two separate 5 min periods each time on three sheets of Whatman 3MM paper soaked in (*a*) 0·5 M-NaOH, then (*b*) 1 M-Tris/HCl (pH 8·0) and finally (*c*) neutralizing solution (1·0 M-Tris/HCl pH 8·0, 1·5 M-NaCl). After drying in air, filters were baked *in vacuo* on a single sheet of 3MM paper at 80 °C for 2 h. Filters were then incubated at 68 °C for 16 h in prehybridization buffer containing 6 × SSC, 10 × Denhardt's solution (0·2% Ficoll; 0·2% polyvinylpyrrolidone; 0·2% bovine serum albumin) and denatured, sonicated calf thymus DNA (50 μg ml^{-1}). Hybridizations with oligonucleotide probes were done in prehybridization buffer containing 0·2 ng of the oligonucleotide ml^{-1}, for 2 h. All hybridizations with DNA fragments as probes were carried out as described previously (Nicolson *et al.*, 1987*a*). Hybridizations were carried out three times on each sample, with three separate DNA preparations in each case.

Electron microscopy. Bacteria were scraped from agar plates, resuspended to a concentration of approximately 10^8 c.f.u. ml^{-1} in nutrient broth containing 2% (v/v) horse serum and incubated at 37 °C for 1 h. The bacteria were then placed on carbon-Formvar-coated 200 mesh copper grids, stained with phosphotungstic acid (2%, v/v) and examined using a Philips 301 electron microscope.

RESULTS

Localization of epitope recognized by mAb SM1

Previous studies with synthetic peptides corresponding to the conserved region of the gonococcal pilin molecule revealed that mAb SM1 reacted with a peptide corresponding to residues 48–60 (Heckels & Virji, 1986). In addition, comparison of the predicted amino acid sequence of cloned gonococcal pilin genes showed significant variation between residues 54–60, suggesting that residues 48–53 must be sufficient for antibody binding (Nicolson *et al.*, 1987*b*). With one exception, the amino acid sequences of all class I meningococcal pilins so far examined are identical in this region (Hermodsen *et al.*, 1978; Potts & Saunders, 1988). Pilin from *N. meningitidis* strain SP3428 reacts with mAb SM1 but contains valine at residue 48 (Olafson *et al.*, 1985) rather than the more typical threonine (Potts & Saunders, 1988). This indicates that the SM1 epitope should be no larger than the pentapeptide EYYLN. Synthetic decapeptides spanning the region 43–62 were therefore synthesized, with adjacent peptides overlapping by five residues. mAb SM1 reacted strongly with a single peptide corresponding to residues 48–57 (Fig. 1*a*). A series of hexapeptides spanning this region were synthesized, with adjacent peptides differing by a single amino acid. Two peptides, TEYYLN (48–53) and EYYLNH (49–54), reacted (Fig. 1*b*), as did the additional hexapeptide VEYYLN (Fig. 1*c*), corresponding to the equivalent residues 48–53 in pilin of *N. meningitidis* SP3428 (Olafson *et al.*, 1985). Since the data suggested that the minimum epitope recognized by mAb SM1 is the sequence EYYLN (49–53), this peptide was synthesized and also found to react (Fig. 1*c*).

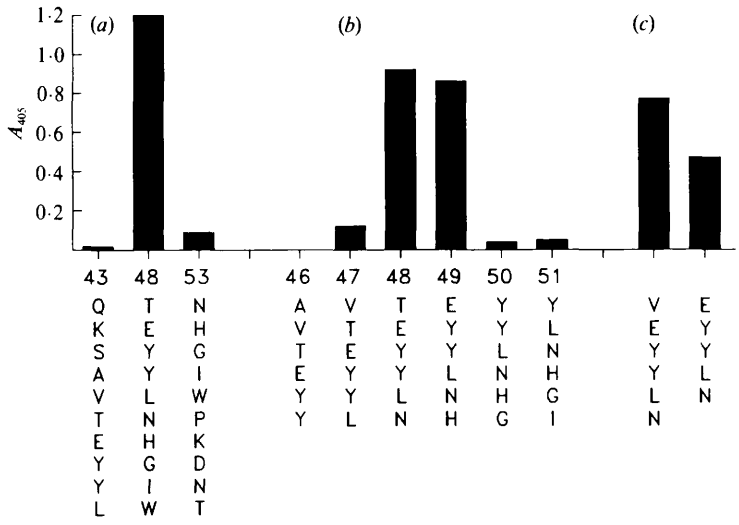

Fig. 1. Reaction of mAb SM1 with solid-phase synthetic peptides spanning the region of 43–62 of the gonococcal pilin molecule: (*a*) decapeptides synthesized with a five amino acid overlap; (*b*) adjacent hexapeptides synthesized with a single amino acid difference; (*c*) variant peptide 48–53 from meningococcal strain SP3428 (Olafson *et al.*, 1985) and minimum reacting pentapeptide 49–53. For (*a*) and (*b*) the residue number denotes the position within the pilin molecule of the first amino acid in each peptide.

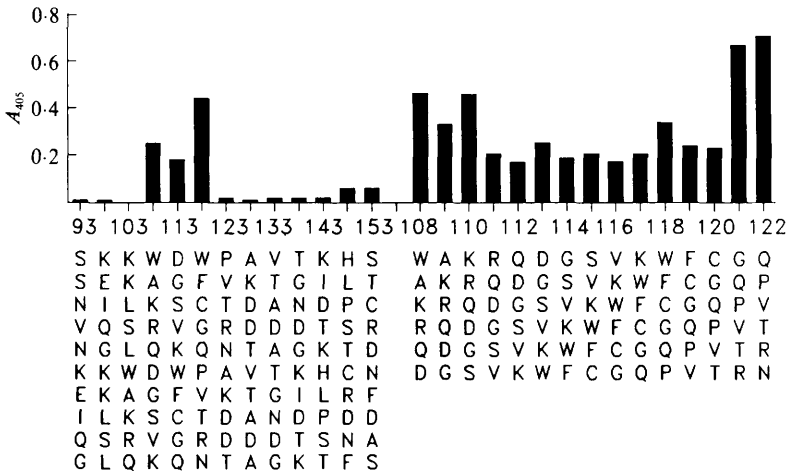

Fig. 2. Reaction of mAb SM2 with solid-phase synthetic peptides spanning the CNBr3 fragment of the gonococcal pilin molecule deduced from the nucleotide sequence of plasmid recombinant pLV260. (*a*) Decapeptides synthesized with a five amino acid overlap; (*b*) adjacent hexapeptides synthesized with a single amino acid difference. The residue number denotes the position within the pilin molecule of the first amino acid in each peptide.

Reaction of mAb SM2 with synthetic peptides

Previous studies with peptides derived from cyanogen bromide cleavage of pilin revealed that mAb SM2 reacted with fragment CNBr3, comprising the carboxy-terminal portion of the molecule (Virji & Heckels, 1985). Using the deduced amino acid sequence of the cloned pilin gene on plasmid pLV260, derived from *N. gonorrhoeae* P9-2 (Nicolson *et al.*, 1987*a*, *b*), a series of decapeptides were synthesized corresponding to fragment CNBr3 with adjacent peptides overlapping by five residues. Antibody SM2 reacted with the three adjacent peptides covering the sequence 108–127 (Fig. 2). Strongest reactivity was with the peptide WFCGQPVTRN,

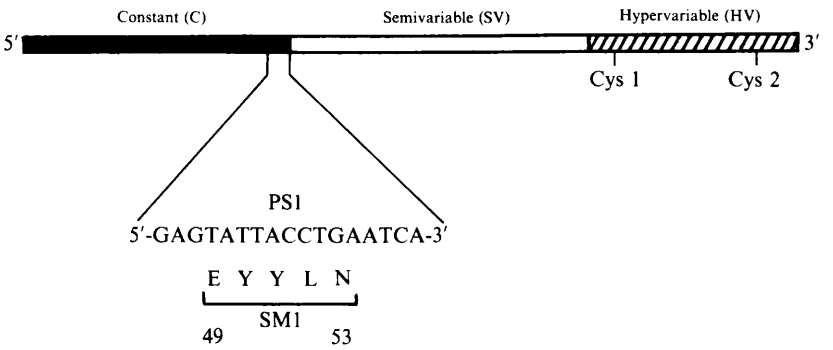

Fig. 3. Arrangement of the coding sequence for the mature gonococcal and meningococcal class I pilin molecule. The 5′ end represents the codon for phenylalanine at the amino-terminus of mature pilin. Promoter sequences and the coding region for the prepilin leader sequence are not shown. SM1 and PS1 represent the extent of the epitope and oligonucleotide probe, respectively.

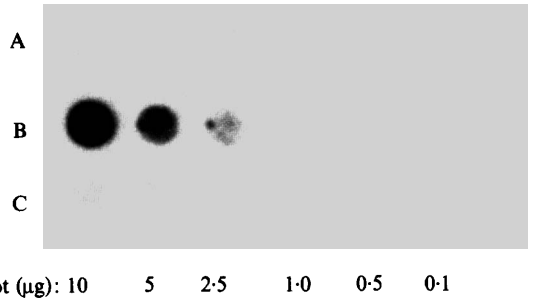

Fig. 4. DNA dot-blot hybridization of meningococcal DNA with oligonucleotide probe PS1. Columns represent hybridization signals from: A, C114 (P^+) DNA; B, C311 (P^+) DNA; C, C311 (P^-) DNA.

corresponding to residues 118–127. A series of hexapeptides spanning the region 108–127, with adjacent peptides differing by one amino acid, were synthesized. Antibody SM2 reacted to a variable extent with each of the hexapeptides, greatest reactivity being seen with the peptide 122–127.

Analysis of genomic sequences that encode the SM1-reactive epitope

The 17-mer synthetic oligonucleotide PS1, which consists of the codon for pilin residue 49 through to the first two (conserved) nucleotides of the codon for residue 54 (Potts & Saunders, 1988), was used as the hybridization probe for the coding region of the SM1 epitope (Fig. 3). This 17-mer sequence is conserved in all expressed pilin (*pilE*) genes in gonococci and class I pilus-producing meningococci for which DNA sequence data are available. The specificity of the PS1 oligonucleotide probe was tested by hybridization to genomic DNA extracted from SM1-reactive and nonreactive meningococcal strains. No background hybridization was observed with DNA from strain C114 (Fig. 4, A), which expresses class II pili and possesses truncated silent class I pilin sequences that do not include the coding sequence for the SM1-reactive epitope (Perry *et al.*, 1987*a*, 1988). Meningococcal strain C311, which expressed class I pili, showed strong hybridization with the oligonucleotide PS1, while a non-piliated variant derived from it gave a much weaker signal (Fig. 4, B, C). Since strain C311 exhibits distinct chromosomal rearrangements associated with pilus phase variation (Perry *et al.*, 1987*a*), this presumably reflects the loss of one or more tracts of the genome that harbour PS1-homologous sequences.

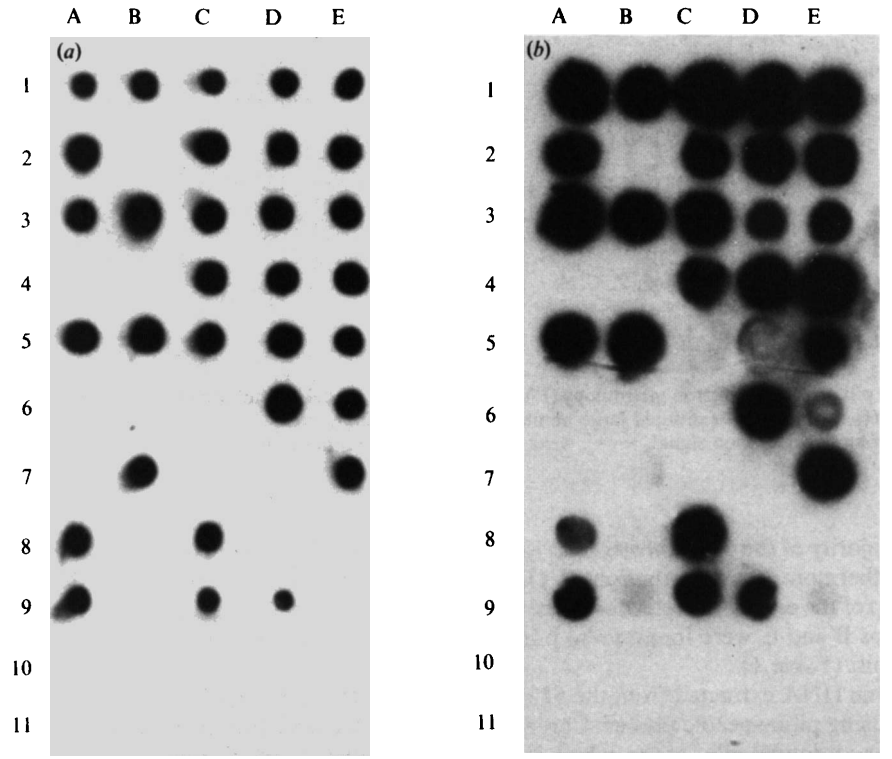

Fig. 5. Analysis of *Neisseria* isolates by immunological and DNA dot-blotting. Panels (*a*) and (*b*) represent autoradiographs of immunological dot-blotting with antibody SM1 and DNA dot-blot hybridization with oligonucleotide PS1 respectively. Each individual organism is arranged identically in each blot. Horizontal rows: 1–5, *N. meningitidis* strains of serogroup B; 6–7, *N. meningitidis* strains of serogroup C; 8, *N. meningitidis* strains of serogroups W135, X, Z, A; 9, *N. gonorrhoeae* strains; 10, *N. lactamica* strains; 11, commensal *Neisseria* species (*N. pharyngis*, *N. flava*, *N. sicca*). Position 11E represents *E. coli* DH1. Vertical rows represent different isolates, sometimes from different sites in the same patient, of each serogroup.

Distribution of the SM1 epitope and DNA sequences homologous to class I meningococcal pilin genes among pathogenic and commensal Neisseria spp.

A panel of 52 clinical isolates, including pathogenic and commensal *Neisseria* species, was analysed for the presence of pili, reaction with monoclonal antibody SM1, and presence of pilin-specific gene sequences (Fig. 5). Table 1 summarizes the responses of isolates of *N. meningitidis* to these tests. Several groups of meningococci were obtained from different locations in the same patient but did not necessarily show identical characteristics with respect to pilus expression, pilin production and hybridization to pilin probes. This suggests that these strains may be isogenic variants derived from a single parental isolate or, less probably, that patients may harbour a number of different meningococcal strains. This finding is consistent with previous observations showing that antigenic variation of surface structures takes place during the course of meningococcal infection (Tinsley & Heckels, 1986). Meningococcal isolates either elaborated numerous individual pili or expressed pili that were aggregated in large bundles (data not shown), as has been observed by Greenblatt *et al.* (1988). Some meningococcal strains exhibited pili that were detached from the cell surface. Those isolates reacting with SM1 were considered to express class I pili. Piliated menincococci that failed to react with SM1 were presumed to produce class II pili. However, it was not possible in this limited study to determine any correlation between morphological differences and immunological reaction of meningococcal pili.

Table 1. *Piliation phenotypes of N. meningitidis isolates*

No. of isolates	Serogroup/serotype	Pili visible under EM*	Reaction with SM1	Hybridization with PS1†
16	B.15, B.NT, B.16 NT.12	P^+	+	+
8	B.15, B.NT, B.16 C.NT, X	P^-	+	+
4	B.15/16, NT.15/16 C.NT, C.2B	P^{+d}	+	+/−
5	C.2A, W145, Z, C.2B	P^+	−	+/−
1	B.15	P^-	−	+/−
5	B.NT, C.2A, C.2B	P^+	−	−
1	A	P^-	−	−

* P^-, no pili visible by electron microscopy (EM); P^+, up to 15% of cells in culture piliated; P^{+d} indicates that some isolates in this group exhibited large numbers of detached pili.

† +, Strong hybridization signal, +/−, weak signal; −, no detectable signal.

The majority of the 40 *N. meningitidis* isolates analysed in this study elaborated SM1-reactive pili. Furthermore, the distribution of class I pili was apparently unrelated to the capsular serogroup of the organism tested, or to the source of the isolate. Meningococci belonging to both serogroups B and C were found to be piliated, some isolates possessing class I pili and others class II pili (Table 1).

Genomic DNA extracted from the 52 isolates was subjected to DNA dot-blot hybridization analysis using pilin-specific probes. Chromosomal DNA from all gonococci tested reacted with the carboxy-terminal pilin probe, which includes invariant domains around the two Cys codons of the gonococcal *pilE* gene (Hagblom *et al.*, 1985; Perry *et al.*, 1987*b*), and also the codons for the SM2 epitope (residues 118–127).

All meningococcal isolates that reacted with SM1, and all gonococcal strains, harboured genomic sequences that hybridized to the synthetic oligonucleotide probe PS1 (Fig. 5, Table 1) even though examination of these strains by electron microscopy failed to reveal intact pili in some isolates. However, such non-piliated isolates showed hybridization signals with the same intensity as those of piliated isolates (Fig. 5) and contained SM1-reactive pilin when examined by Western blotting (data not shown). Four SM1-reactive meningococcal strains harboured DNA sequences that showed weak hybridization to the probe PS1 and all produced complete pilus structures. The most likely explanation of the reduced hybridization signal is that such strains retain only a single copy of the PS1 homologous sequence at the *pilE* locus and none at *pilS* loci. Presumably in most strains several of the *pilS* partial pilin copies retain the PS1-homologous sequence and produce a more intense signal in dot-blots. A single meningococcal isolate, C786, that was non-piliated and failed to express SM1-reactive pilin, gave reproducibly low levels of hybridization to the probe PS1 (Fig. 5, position 2B). Similarly, non-piliated isogenic variants that do not produce SM1-reactive pilin that have been derived from *N. meningitidis* C311 and *N. gonorrhoeae* P9-2 show a reduced hybridization signal with the probe PS1 compared to their piliated or pilin-producing isogenic counterparts [Fig. 4; Fig. 5, positions 9A (P9-2 P^+) and 9B (P9-2 P^-)].

A single non-piliated isolate that did not react with SM1 and apparently did not possess genomic sequences encoding the SM1-reactive epitope was observed. It is possible that this isolate is a non-reverting P^- variant derived from a class II pilus-producing strain. Of the ten class II pilus-producing isolates, five showed no detectable hybridization to the oligonucleotide PS1, and five showed weak hybridization with this probe (Table 1; Fig. 5).

Pili were seen on three strains of *N. lactamica* examined by electron microscopy, although none of these isolates expressed SM1-reactive pilin. DNA from these strains of *N. lactamica* exhibited only faint hybridization signals to the oligonucleotide probe PS1 (Fig. 5) or to the 0·42 kb *BglI–ClaI* fragment of recombinant pLV830 (data not shown). This may reflect the

presence of sequences with only limited homology to the pilin gene. Three non-pathogenic isolates of the genus *Neisseria* (*N. pharyngis*, *N. flava* and *N. sicca*) did not contain sequences homologous to the probe PS1, nor did they produce SM1-reactive pilin despite being demonstrably piliated.

Occurrence of the SM1 epitope and coding sequences in other bacterial genera

Comparisons of the N-terminal amino acid sequences of a number of the so called methylphenylalanine-type pilins have revealed homologies between pilins produced by *Neisseria* and those expressed by *Bacteroides nodosus*, *Moraxella bovis*, *M. nonliquefaciens* and *Pseudomonas aeruginosa* (Marrs *et al.*, 1985; Olafson *et al.*, 1985; Elleman *et al.*, 1986; Pasloske *et al.*, 1988). A number of clinical isolates of Gram-negative bacteria, including *Aeromonas hydrophila*, *Bordetella pertussis*, *Pasteurella haemolytica*, *Moraxella liquefaciens*, *Pseudomonas stutzeri* and *Bacteroides fragilis* were therefore tested for the expression of SM1-reactive pilin and the possession of the DNA sequences encoding this epitope. All isolates, with the exception of some strains of *B. fragilis* which exhibited very weak reactions, showed no homology to the oligonucleotide probe PS1 and failed to produce SM1-reactive pilin (data not shown). The amino acid sequence corresponding to the SM1-reactive epitope of pilin from *Neisseria* species is not evident in the analogous region of *B. nodosus* pilin. It is possible that there are partial homologies between DNA and amino acid sequences for pilins from *B. fragilis* (which have not yet been sequenced) and *Neisseria* species.

DISCUSSION

Physical confirmation of the minimum epitope recognized by monoclonal antibody SM1 to the sequence EYYLN, corresponding to amino acids 49–53 of mature neisserial pilin, is in accord with structural analysis of pilins from a number of different species. Mature gonococcal pilins contain three distinct domains, a highly conserved (C) region encompassing the first 53 amino acids, a semivariable (SV) region (approx. residues 54–114) in which amino acid substitutions occur, and a hypervariable (HV) region at the carboxy-terminus in which insertions and deletions of up to four amino acids occur (Hagblom *et al.*, 1985; Nicolson *et al.*, 1987*b*; Swanson *et al.*, 1987). Sequence analysis of cloned class I meningococcal *pilE* (Potts & Saunders, 1988) and *pilS* (Perry *et al.*, 1988) loci suggest that pilins produced by most strains of *N. meningitidis* also conform to this model, with complete homology between amino acids 1–55. In each case the sequence between residues 49 and 53 is preserved and the pili react with antibody SM1. In contrast, N-terminal homology between *Neisseria* pilin and the pilins produced by *Moraxella*, *Pseudomonas* and *Bacteroides* species does not extend beyond approximately residue 30 (Marrs *et al.*, 1985; Saastry *et al.*, 1983; Elleman *et al.*, 1986; Pasloske *et al.*, 1988), the EYYLN sequence is missing and the pili do not react with antibody SM1.

Previous studies (Virji & Heckels, 1985) which suggested that mAb SM2 recognizes a conserved determinant in the carboxy-terminal portion of the pilin molecule have now been confirmed. Although this region contains the hypervariable determinants, conserved sequences occur around the two cysteine residues which form the disulphide loop (Hagblom *et al.*, 1985). In contrast to the SM1 epitope, the studies described here do not define a simple linear peptide for the SM2 epitope. mAb SM2 reacts with a number of peptides from around the Cys 1 at position 120, suggesting that this extended region may contribute to a conformational epitope recognized by the antibody in native pilin. This would be in accord with previous observations that mAb SM2 reacts much less strongly on Western blots of SDS-denatured pilin than does mAb SM1 (Virji *et al.*, 1983). As would be expected from the reactivity of SM2, this region is also highly conserved not only in gonococci but also in the class I pilin gene of meningococcal strain C311 (Potts & Saunders, 1988) but is absent from the *Moraxella*, *Bacteroides* and *Pseudomonas* pilins.

Pilin genes encoding class II meningococcal pilin have not yet been cloned and no sequence information is available. It is likely from hybridization and sequence data that the structural gene for class II pilin will possess only limited nucleotide sequence homology to the class I *pilE* gene (Perry *et al.*, 1988). The current studies reveal that differences between the two classes of

meningococcal pilin are likely to be extensive and not simply the result of the deletion of a limited number of amino acids in a single domain. Despite the quite different locations of the determinants recognized by the two mAbs, class II pili do not react with either antibody. These pili also fail to react on Western blots with polyclonal sera raised against gonococcal pili, although they do react with the same sera in immune precipitation under less denaturing conditions (Diaz *et al.*, 1984). This suggests that the class II pili differ at the level of primary amino acid sequence but nevertheless contain conformational determinants similar to those found on class I and gonococcal pili. If this is the case it would imply conservation at the functional level and indicate a common role for two pilus classes.

This study confirms that the majority of strains of *N. meningitidis*, regardless of serotype, elaborate class I pili which are characterized by the possession of an amino-terminal C region containing the relatively weakly immunogenic linear EYYLN epitope recognized by mAb SM1. All SM1-reactive isolates of *N. meningitidis*, and all gonococcal isolates tested, not surprisingly harboured genomic sequences that hybridized to the oligonucleotide probe PS1. Some meningococcal strains were non-piliated, but produced SM1-reactive pilin. Pilus phase variation in *N. gonorrhoeae* can result from recombination between *pilS* loci and the *pilE* locus, producing defective pilin gene products. Such defective pilins are immunologically reactive, but are not assembled into pili, giving rise to a non-piliated phenotype (Bergstrom *et al.*, 1986; Haas *et al.*, 1987; Swanson *et al.*, 1986). Such non-piliated gonococcal variants can readily revert to the piliated phenotype. It is evident that pilin-producing P^- variants are also generated in *N. meningitidis* (Stephens *et al.*, 1985; this study).

DNA dot-blot analysis of piliated and non-piliated SM1-reactive meningococci revealed that the majority of isolates showed strong hybridization signals with the probe PS1. However, four strains that produced SM1-reactive pilus structures exhibited weak hybridization with this probe, suggesting that there are relatively few copies of PS1-homologous sequences in their genomes. This would be consistent with a variety of Southern hybridization patterns that are observed when different strains of *N. meningitidis* are hybridized with the gonococcal *pilE* gene as probe (Perry *et al.*, 1987*a*). *N. gonorrhoeae* can undergo irreversible P^+ to P^- pilus phase variation as the result of genomic DNA rearrangements that lead to deletion of the 5′ end of the pilin structural gene (Meyer *et al.*, 1982; Nicolson *et al.*, 1987*a*; Segal *et al.*, 1985). Similarly the class I pilus-producing meningococcal strain C311 exhibits phase variation, accompanied in a proportion of cases by deletion of *pilE*-related sequences (Perry *et al.*, 1987*a*). This study indicates that such deletions of pilin gene sequence in non-piliated variants of *N. gonorrhoeae* P9-2 and *N. meningitidis* C311 are correlated with a reduced hybridization signal in DNA dot-blots with the oligonucleotide PS1 when compared to signals obtained with isogenic piliated variants of these strains. Nevertheless, none of the non-reverting P^- derivatives of *N. meningitidis* that we have isolated following growth *in vitro* have exhibited deletion of all PS1-homologous sequences.

Of the 40 meningococcal isolates examined in this study, fewer produced class II pili than produced class I pili. Five of the 10 class II pilus-producing strains showed no detectable homology to the PS1 probe whereas the remainder reacted weakly. All meningococci and gonococci tested harboured sequences homologous to the 3′-proximal SV and HV regions of the class I meningococcal *pilE* gene. This confirms that all class II piliated isolates retain part of the class I pilus sequence which is silent because it lacks 5′ sequences containing the *pilE* promoter. These observations are consistent with our previous studies of the class II pilus-producing strain C114 in which the only sequences homologous to the gonococcal *pilE* gene are two tandem truncated *pilS* copies comprising pilin codons 79–144 (copy 1) and 64–150 (copy 2) (Perry *et al.*, 1988). A similar arrangement has subsequently been observed in the *pilS* locus of FAM18, an unrelated strain of *N. meningitidis* (Aho & Cannon, 1988). The class II pilus-producing meningococci that showed faint hybridization signals with the PS1 probe probably harbour silent pilin gene copies that are not expressed but still retain the coding sequence for the SM1-reactive epitope. Presumably the class I *pilS* sequences present extend further towards the N-terminal coding portion of the pilin gene than those sequences in strains C114 and FAM18. Interestingly, three of the four strains of *N. lactamica* also showed faint hybridization signals

with the probe PS1 but elaborated pili that did not react with mAb SM1. This is reminiscent of the situation observed in this second type of class II pilus-producing strains of *N. meningitidis*. Experience with non-reverting P$^-$ derivatives of gonococci in which pilin and N-terminal coding sequences are deleted (Swanson *et al.*, 1986; Segal *et al.*, 1985) suggests that class II meningococcal strains with either type of residual class I *pil* sequences are unlikely to be able to regain the ability to produce class I pili. However, it is conceivable, by analogy with gonococci (Seifert *et al.*, 1988), that defects in class I pilin production might be repaired following the acquisition of a complete complement of *pilE* sequences by transformation. Nevertheless, these strains have probably elaborated class I pili at some stage in their evolutionary history.

Aho *et al.* (1987) showed that some strains of *N. lactamica* contained sequences homologous to an intact gonococcal *pilE* gene probe. Three of the piliated strains of *N. lactamica* examined in our study exhibited weak hybridization reactions with the PS1 probe. The presence of class I *pil* DNA sequences in *N. lactamica*, which is commonly regarded as a commensal, is consistent with a number of reports that members of this species can occasionally cause infections at different sites in humans (Johnson, 1983; Brown *et al.*, 1987). In addition, *N. lactamica* shares further characteristics with *N. meningitidis* and *N. gonorrhoeae*, including expression of the H8 antigen and possession of DNA sequences related to the gonococcal *opa* (heat-modifiable outer-membrane protein PII) gene (Cannon *et al.*, 1984; Black & Cannon, 1985; Aho *et al.*, 1987; Stern & Meyer, 1987). These observations support the idea that a divergent evolutionary development has separated these three species from other members of the genus. Class I neisserial pilin sequences were not detected in the genomes of a number of non-pathogenic members of the genus *Neisseria* that were tested in this study. This supports the contention that sequences homologous to the gonococcal pilin gene are essentially diagnostic for the pathogenic members of the genus (Aho *et al.*, 1987). However, our study shows that for a proportion of meningococci such sequence conservation is limited to residual *pilS* sequences that lack the 5′ end of the gonococcal *pilE* gene.

This work was supported by project grants from the Medical Research Council and the Meningitis Trust. Part of the work was supported by a Leave Fellowship to J.R.S. from the Wellcome Trust. W.J.P. is grateful for a postgraduate scholarship from the Medical Research Council. We thank T. Knapp, Royal Liverpool School of Tropical Medicine, for synthesis of oligonucleotides.

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