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Molecular Analysis of the Serotyping Antigens of *Neisseria meningitidis*

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Molecular approaches to the rapid analysis of the serotyping antigens of *Neisseria meningitidis*, the class 2 and 3 outer membrane proteins (OMPs), were developed, evaluated, and used to study 12 antigenic variants of these proteins. A primer set for the polymerase chain reaction (PCR) amplification of the genes encoding these antigens was devised. Low-stringency amplification of meningococcal chromosomal DNA with this primer set resulted in the amplification of two products from each strain, whereas at higher stringencies only one product was amplified in most strains. Southern hybridization techniques and restriction analyses were used to differentiate the PCR products amplified at high stringencies from strains expressing class 2 or class 3 OMPs; these PCR products were further characterized by the determination of their nucleotide sequences, confirming that they represented the amplified class 2 and class 3 OMP genes. Analyses of these and other nucleotide sequences enabled the construction of a phenogram illustrating the interrelationships between *Neisseria* OMP genes. The comparative analysis of deduced amino acid sequences revealed conserved and variable regions of the proteins; the latter probably correspond to surface loops on the protein and hence are potentially exposed to the immune system. Further analyses of the primary structures of these related porins from *Neisseria* species enabled construction of models of the secondary structure of these antigens and comparison of these models with those previously published. The methods reported in the present work are rapid reproducible procedures for the analysis of antigenic variants of these proteins.

Neisseria meningitidis is an important cause of meningitis and septicemia worldwide (27, 33), occurring either as a sporadic disease, mainly of infants and adolescents, or in pandemic waves (2). Capsular polysaccharide vaccines are available for serogroup A, C, W-135, and Y organisms but not for serogroup B organisms, which account for up to 60% of cases of meningococcal meningitis in western Europe and the United States and for large-scale epidemic disease in Brazil and Cuba. The currently available polysaccharide vaccines are relatively ineffective in young children and do not produce booster effects, which makes the scheduling of vaccinations difficult (31). Recent approaches to the development of new vaccines against this organism have included the use of meningococcal outer membrane proteins (OMPs) in vaccine preparations (12). Although attractive in several respects as potential vaccine components, the meningococcal OMPs, in common with other major antigens of this organism, are antigenically variable. This antigenic variation is used in serotyping and subtyping schemes used for this organism (1, 14, 23).

Of the five main classes (classes 1 through 5) of OMP in *N. meningitidis* (36), the class 2 and class 3 OMPs are serotyping antigens (14, 23) and the class 1 OMPs are targets for serosubtyping antibodies (1). In addition, these proteins are major components of candidate vaccines. Most meningococcal strains possess a class 1 and either a class 2 or class 3 OMP. Serotype-specific, protective, bactericidal antibody responses against these proteins have been demonstrated (29, 32). The class 4 and 5 OMPs are not considered suitable for use as components of vaccines or as targets for typing antibodies (17, 28, 35).

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Immunological experiments have demonstrated that the meningococcal class 1, class 2, and class 3 OMPs and the gonococcal PIA and PIB OMPs are related proteins. These relationships have been confirmed by nucleotide sequencing of genes encoding these OMPs (3–6, 15, 18, 22, 24, 39). A structural model for all of these related proteins has been proposed (22, 37). The designations *porA* and *porB* have been proposed for the meningococcal class 1 and class 2/3 genes, respectively (16).

In the present work, the polymerase chain reaction (PCR) technique (10) has been exploited to amplify the genes encoding meningococcal class 1 and class 2 or 3 OMPs either separately or together, establishing the presence of genes encoding class 2 or class 3 OMPs without sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or serological analysis. The amplified genes were further defined by nucleotide sequence analysis and comparative analyses of the nucleotide and derived amino acid sequences. These data may be applied to the development of techniques for the rapid identification and typing of meningococcal isolates directly from clinical specimens.

MATERIALS AND METHODS

Growth and maintenance of bacterial strains. Strains of *N. meningitidis* (Table 1) were maintained at −70°C in 50% Mueller-Hinton broth plus 50% freezing mix (10% glycerol, 3 mM sodium citrate, 1 mM ammonium sulfate, 100 mM potassium phosphate [pH 6.6]) and were propagated on heated blood (chocolate) agar at 37°C in a 5% CO_2 atmosphere for 8 to 16 h.

Membrane preparation and analysis. *N. meningitidis* membranes were prepared by a spheroplast lysis method modified from that of Witholt et al. (38). A single colony from

TABLE 1. Bacterial strains

NIBSC no.	Strain no.	Serogroup	Serotype	Subtype	Epidemiology (reference)	Source[a]
2001	M1080	B	1	P1.1,7	(36)	PHLS
2002	M990	B	6	P1.6	(36)	PHLS
2003	B16B6	B	2a	P1.2,5	(13)	PHLS
2004	M982	B	9	P1.9	(36)	PHLS
2005	2996	B	2b	P1.2,5	(12)	PHLS
2006	35E	C		P1.1	(12)	PHLS
2007	S3032	B	12	P1.12,16	(36)	PHLS
2008	H355	B	15	P1.15	(36)	PHLS
2052	G1960	B	15	P1.7,16	U.K.,[b] 1986 (7)	PHLS
2055	J129	B	4	P1.15	U.K., 1988	PHLS
2183	B54	A	4,21	P1.9,X	Finland, 1975 (26)	MPIG
2184	B227	A	4,21	P1.5,9	Denmark, 1974 (26)	MPIG

[a] The reference strains of *N. meningitidis* used in the present work were obtained from the Public Health Laboratory Service collection (PHLS), courtesy of D. M. Jones, and from the Max-Planck-Institut für Molekulare Genetik, Berlin, Germany (MPIG), courtesy of M. Achtman.
[b] U.K., United Kingdom.

cultures of *Neisseria meningitidis* was inoculated into Mueller-Hinton broth (10 ml) and grown for 6 h at 37°C. A portion of this culture (1 ml) was used to inoculate Mueller-Hinton broth (100 ml), and this was incubated overnight at 37°C. The cells were harvested and resuspended in 5 ml of 200 mM Tris-Cl buffer (pH 8.0). To this suspension was added, with stirring, 5 ml of 200 mM Tris-Cl–1 M sucrose (pH 8.0). After 90 s, 1 mg of egg white lysozyme (Sigma) was added, and after a further 45 s, 10 ml of deionized water was added. The suspension was incubated with gentle shaking at room temperature for 30 min. The spheroplasts thus formed were collected by centrifugation (40,000 × *g* for 20 min at 20°C) and lysed by the addition of ice-cold water (20 ml). The membranes were collected by centrifugation (40,000 × *g* for 20 min at 20°C) and resuspended in 50 mM potassium phosphate buffer (pH 7.5). Protein concentrations were estimated by the method of Markwell et al. (20), and the membrane proteins were separated on a 12.5% polyacrylamide gel. Proteins were visualized by staining with Coomassie brilliant blue.

DNA preparation. Confluent (overnight) growth from five chocolate agar plates was scraped into 5 ml of TE buffer (10 mM Tris-Cl, 1 mM EDTA [pH 8.0]), and then 5 ml of TNE buffer (100 mM Tris-Cl, 100 mM NaCl, 10 mM EDTA, 1% Sarkosyl [pH 7.5]) plus proteinase K (100 μg/ml) was added and the sample was incubated at 55°C for 2 h. This was followed by sequential extractions with equal volumes of phenol (saturated with TE buffer), phenol-chloroform (1:1, saturated with TE buffer), and chloroform. Boiled RNase A (Sigma Chemical Co.) was added, and the extract was incubated for 2 h at room temperature and then subjected to sequential phenol-chloroform and chloroform extractions and dialysis against two overnight changes of 0.1× TE buffer. The DNA concentration and purity were estimated spectrophotometrically.

Oligodeoxyribonucleotide primer sequences. Oligodeoxyribonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer. Primers 21 (5′-CTG TAC GGC GAA ATC AAA GCC GGC GT-3′) and 22 (5′-TTA GAA TTT GTG GCG CAA ACC GAC-3′) were complementary to the nucleotide sequence of the class 1 OMP gene and have been described previously (18). Primers 27 (5′-TTG TAC GGT ACA ATT AAA GCA GGC GT-3′) and 28 (5′-TTA GAA TTT GTG ACG CAG ACC AAC-3′) were complementary to the equivalent regions of the class 2 OMP gene (24), corresponding to the N- and C-terminal ends, respectively, of the mature protein. Primers 33 (5′-GGG GTA GAT CTG CAG GTT ACC TTG TAC GGT ACA ATT AAA GCA GGC GT-3′) and 34 (5′-GGG GGG GTG ACC CTC GAG TTA GAA TTT GTG ACG CAG ACC AAC-3′) were versions of primers 27 and 28, respectively, modified to include restriction endonuclease recognition sites (*Bgl*II and *Pst*I in primer 33 and *Bst*EII and *Xho*I in primer 34). The locations of the primers relative to the coding sequence of the class 2 gene are shown in Fig. 1.

PCR. PCR components were as follows: 50 ng of template (meningococcal chromosomal DNA, prepared as described above) per μl; 10 mM Tris-Cl (pH 8.0); 50 mM KCl; 1.5 mM $MgCl_2$; 0.001% gelatin; 200 μM each dATP, dCTP, dGTP, and dTTP; the required primers at 2 μM; and 0.5 U of *Taq* polymerase (Cetus Corp.). The reactions were incubated for 30 cycles in a PHC-1 programmable heat block (Techne Instruments Ltd.) for 2 min at 94°C, 2 min at the required annealing temperature, and 3 min at 72°C. At the end of the 30 cycles, the reactions were incubated for a further 3 min at 72°C.

Restriction digestion, agarose gel electrophoresis, and DNA-DNA (Southern) hybridization. The products of PCRs were digested with restriction endonucleases essentially as described by Maniatis et al. (19); restriction endonucleases and buffers were supplied by New England BioLabs. DNA samples were separated on agarose gels (between 0.7 and 2%, depending on the expected fragment size) in 40 mM Tris-acetate–1 mM EDTA (pH 8.0) at 50 to 100 mA.

Agarose gels were blotted with a Vacugene vacuum-blotting unit (Pharmacia-LKB) onto nylon membrane filters (GeneScreen; DuPont). The filters were fixed by irradiation with 254-nm UV light for 4 min. Prior to hybridization, the filters were soaked in 4× SET buffer (19) for 1 h at 42°C. Prehybridization was carried out for 1 h in 4× SET containing 0.1% sodium PP_i, 50% formamide, 0.2 mg of heparin per

FIG. 1. Locations of primers relative to the class 2 OMP gene. The class 2 gene is represented by the rectangle. The arrows indicate the locations of primers 27 and 28, and the region of the gene corresponding to the leader peptide is shaded. The portion of the gene sequenced in this study is indicated in black.

ml, and 2% SDS. For hybridization, radiolabeled probe, produced by end-filling of PCR products with DNA polymerase (Klenow fragment; Boehringer Corp.), with [α-^{32}P]dATP as the label, was heated to 100°C for 2 min, added to the prehybridization mixture, and incubated with shaking overnight at 42°C. The filters were washed twice in 4× SET–0.1% SDS at 42°C and twice in 4× SET–0.1% SDS at 65°C and autoradiographed at −70°C with an intensifying screen on Hyperfilm (Amersham International). The washes were repeated at higher temperatures and lower SET concentrations as required, and the filters were autoradiographed again.

Nucleotide sequence analysis of OMP genes. The nucleotide sequences of the PCR products were determined by a combination of cloning (class 2 genes) and direct sequencing (class 2 and class 3 genes). For cloning, amplification was carried out with primers 33 and 34 and the PCR products were digested with the restriction endonucleases *Pst*I (recognition site present in primer 33), *Xho*I (recognition site present in primer 34), and *Kpn*I (recognition site within the gene). The two parts of each gene were cloned into bacteriophage M13 vectors mp18 and mp19, and the resultant clones were sequenced with modified bacteriophage T7 DNA polymerase (34) by using Sequenase kits (United States Biochemical Corp.) as specified by the manufacturer and substituting primers internal to the gene where necessary. Amplified PCR products were sequenced directly by the method of Embley (9).

The protein sequences were aligned (see Fig. 5) on the basis of the amino acid alignment of Maiden et al. (18) and Feavers et al. (11). The nucleotide sequences of meningococcal class 1, 2, and 3 and gonococcal PIA and PIB genes were aligned to conform with the sequences presented in reference 11. The similarity between sequences was illustrated by the construction of a phenogram, or tree, using an agglomerative distance matrix approach (25). The distance (number of mismatched nucleotides as a percentage of sequence length) between each pair of sequences was calculated by using the University of Wisconsin Genetics Computer Group software package (8). The sequences were grouped by using the average-linkage or UPGMA clustering algorithm with the SAS statistical and graphical software package (SAS Institute Inc., Cary, N.C.).

RESULTS

PCR amplification with primers derived from the meningococcal class 2 OMP gene sequence. Chromosomal DNA from serological reference strains (NIBSC 2001 through NIBSC 2008 [Table 1]) was used to optimize PCR amplifications with primers 27 and 28. At low annealing stringencies (40°C; 50 mM KCl), PCRs with this primer set amplified two products in each of the eight strains tested (Fig. 2A). These products were of about 1,100 bp (the same size as the amplified class 1 OMP gene) and between 900 and 1,000 bp. The amplified genes separated by agarose gel electrophoresis had similar size relationships to each other as did the major OMPs themselves, separated by SDS-PAGE of membrane preparations (Fig. 3; Table 2); i.e., the strains with smaller OMPs had smaller PCR products. At higher stringencies (60°C; 50 mM KCl), only the smaller products were amplified for all strains except strain NIBSC 2002: in this strain both products were amplified with primers 27 and 28 under all annealing conditions used (Fig. 2B).

The relationships between the PCR products were established by restriction endonuclease digestion and Southern hybridization. Restriction endonuclease analyses showed

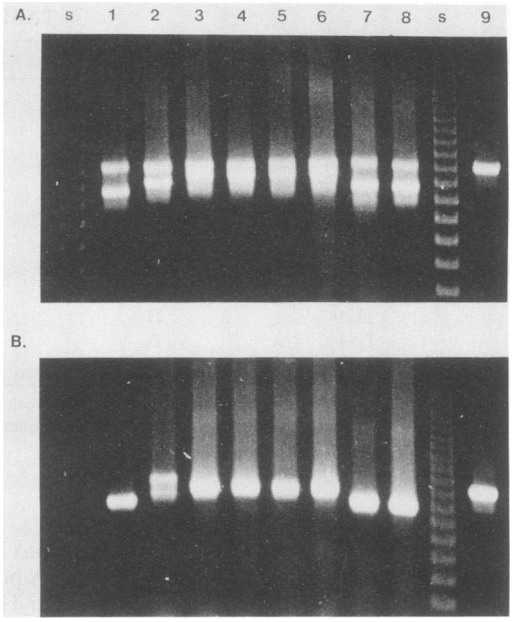

FIG. 2. PCR amplification products. Primers 27 and 28 were used at an annealing stringency of 40°C (A) and 60°C (B), separated by agarose gel electrophoresis, and stained with ethidium bromide. Lanes 1 to 8 contain PCR amplifications of chromosomal DNA from each of the meningococcal reference strains NIBSC 2001 through NIBSC 2008 (Table 1), with various primers at different annealing stringencies. Lane 9 shows a control amplification of the class 1 outer membrane protein from strain NIBSC 2001 amplified with primers 21 and 22 (18). Lanes s contain standards (123-bp ladder).

that the smaller products from strains NIBSC 2003 through NIBSC 2006 had restriction maps similar to those deduced from the class 2 nucleotide sequence of Murakami et al. (24), whereas the restriction endonuclease maps of the products from strains NIBSC 2001, NIBSC 2007, and NIBSC 2008 were different (data not shown). Southern hybridization experiments were carried out with agarose gels similar to that shown in Fig. 2A, with PCR-derived probes for the class 1 OMP gene (18; data not shown), the class 2 gene from strain NIBSC 2002, or the smaller PCR product, presumed to be the amplified class 3 gene, from strain NIBSC 2001 (Table 2). These analyses demonstrated that the larger product, amplified from strains NIBSC 2001 through NIBSC 2008 at low annealing stringency and from NIBSC 2002 at low and high stringencies, corresponded to the class 1 OMP gene. The amplified class 1 OMP genes did not cross-hybridize with the smaller products. The smaller products from all eight strains cross-hybridized, but the products from strains NIBSC 2002 through NIBSC 2006 were distinguished from the products of strains NIBSC 2001, NIBSC 2007, and NIBSC 2008 after the blots were washed at higher stringencies.

Further experiments were carried out on strains NIBSC 2052 and NIBSC 2055 isolated in Stroud, United Kingdom in 1986 and 1988, respectively (7), and NIBSC 2183 and NIBSC 2184, isolated in Finland in 1974 and Denmark in 1975, respectively (26). These strains gave similar results (Table 2; data not shown).

Nucleotide sequences of PCR products. Nucleotide sequences of the PCR products of the 12 strains were determined and are shown in Fig. 4, together with a consensus

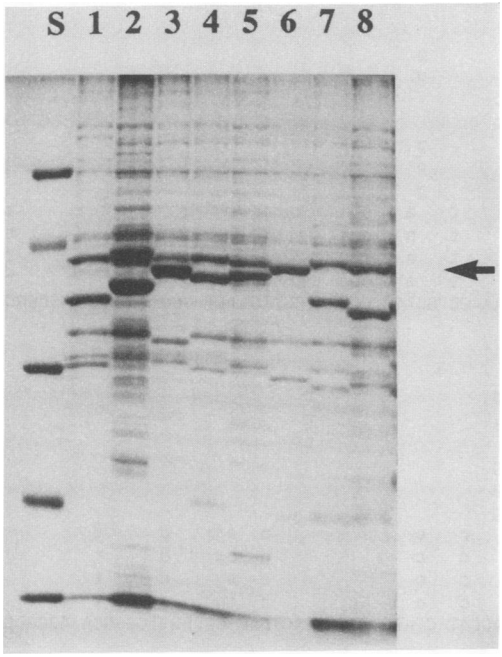

FIG. 3. SDS-PAGE profiles of type strains. Lanes 1 to 8 contain membrane preparations (25 μg of protein for each track) of the eight reference strains of *N. meningitidis* NIBSC 2001 through NIBSC 2008 (Table 1), which were separated by SDS-PAGE on a 12.5% gel and stained with Coomassie brillant blue to visualize the proteins: the class 1 OMPs are indicated with an arrow, and the class 2 and class 3 OMPs are the major OMPs varying in size. Strains NIBSC 2001, NIBSC 2007, and NIBSC 2008 express class 3 OMPs, and strains NIBSC 2002 through NIBSC 2006 express class 2 OMPs. Standards (lane S) were lysozyme, carbonic anhydrase, ovalbumin, and bovine serum albumin.

sequence. The design of the PCR primers was such that the first 35 and last 21 bases of the gene encoding the mature proteins were not determined (Fig. 1). These regions corresponded to regions that are highly conserved between *Neisseria* OMPs. The nucleotide sequences of the PCR products from strains NIBSC 2002 through NIBSC 2006 were very similar to those obtained from the class 2 OMP gene by Murakami et al. (24), whereas those from the remaining strains were similar to the published nucleotide sequences of class 3 OMP genes (4, 39). The nucleotide sequence of strain NIBSC 2008 (H355, serotype 15) was identical to the sequence reported previously for this strain (4). The nucleotide sequence of strain NIBSC 2052 (serotype 15) was identical to that of strain H44/76 (serotype 15) (4). The genes from strains H44/76 and NIBSC 2052 have a single-base substitution relative to the otherwise identical sequence from strain NIBSC 2008 (H355). This established that the base substitution between these two genes encoding serotype 15 proteins, noted previously (position 829, Fig. 4) (4), was a genuine sequence change between these two variants. The amino acid sequences deduced from these data are compared with the protein sequence deduced from the PCR product of strain NIBSC 2001 in Fig. 5. Most of the amino acid sequence changes within classes were located in six regions of the sequence (I, IV, V, VI, VII, and VIII [Fig. 5]); a further region (region III [Fig. 5]) showed variation between strains NIBSC 2002 through NIBSC 2006 and the sequences from the remaining strains.

Relationships between nucleotide sequences. The nucleotide sequences of the PCR products were aligned with equivalent sequences from *Neisseria* OMP genes by the method of Maiden et al. (18), and the aligned sequences were used to produce a dendrogram (Fig. 6). The meningococcal class 1 OMP genes form a distinct cluster, and the PCR products from strains NIBSC 2002 through NIBSC 2006 cluster with the class 2 sequence from strain M986. The remaining strains form a cluster. The gonococcal PIB proteins are closely linked, but the PIA protein does not cluster closely with any other *Neisseria* protein.

DISCUSSION

Primers 27 and 28 can be used in the PCR to amplify selectively the class 2 or class 3 gene from meningococcal strains. The PCR products are consistent with the sizes of

TABLE 2. Properties of serotyping antigens from each strain

NIBSC no.	Peptide map protein class (if known)[a]	Protein class from DNA sequence homology	Protein mol wt from SDS-PAGE	Protein mol wt calculated from PCR product sequence[b]	Protein class from cross-hybridization of PCR products[c]
2001	Class 3	Class 3	35,400	34,037	Class 3
2002	Class 2	Class 2	36,500	35,565	Class 2
2003	Class 2[d]	Class 2	39,200	36,674	Class 2
2004	Class 2	Class 2	38,600	37,050	Class 2
2005		Class 2	40,200	36,829	Class 2
2006		Class 2	41,400	36,891	Class 2
2007		Class 3	36,000	34,060	Class 3
2008	Class 3	Class 3	34,800	33,934	Class 3
2052		Class 3	35,200	34,934	ND[e]
2055		Class 3	35,900	33,911	ND
2183		Class 3	35,200	33,762	ND
2184		Class 3	35,200	33,782	ND

[a] Defined by the peptide map (36).
[b] Calculated by adding the calculated M_r from the PCR-derived sequence to the amino acids not present in the amplified gene, derived from previously published sequences (4, 24, 35).
[c] Determined by cross-hybridizing separately with the PCR-derived probe from strains NIBSC 2001(M1080, serotype 1) or NIBSC 2002(M990, serotype 6).
[d] This strain was not peptide mapped by Tsai et al. (36), but M986, which has the same serotype (serotype 2a), is a class 2 protein (24).
[e] ND, not determined.

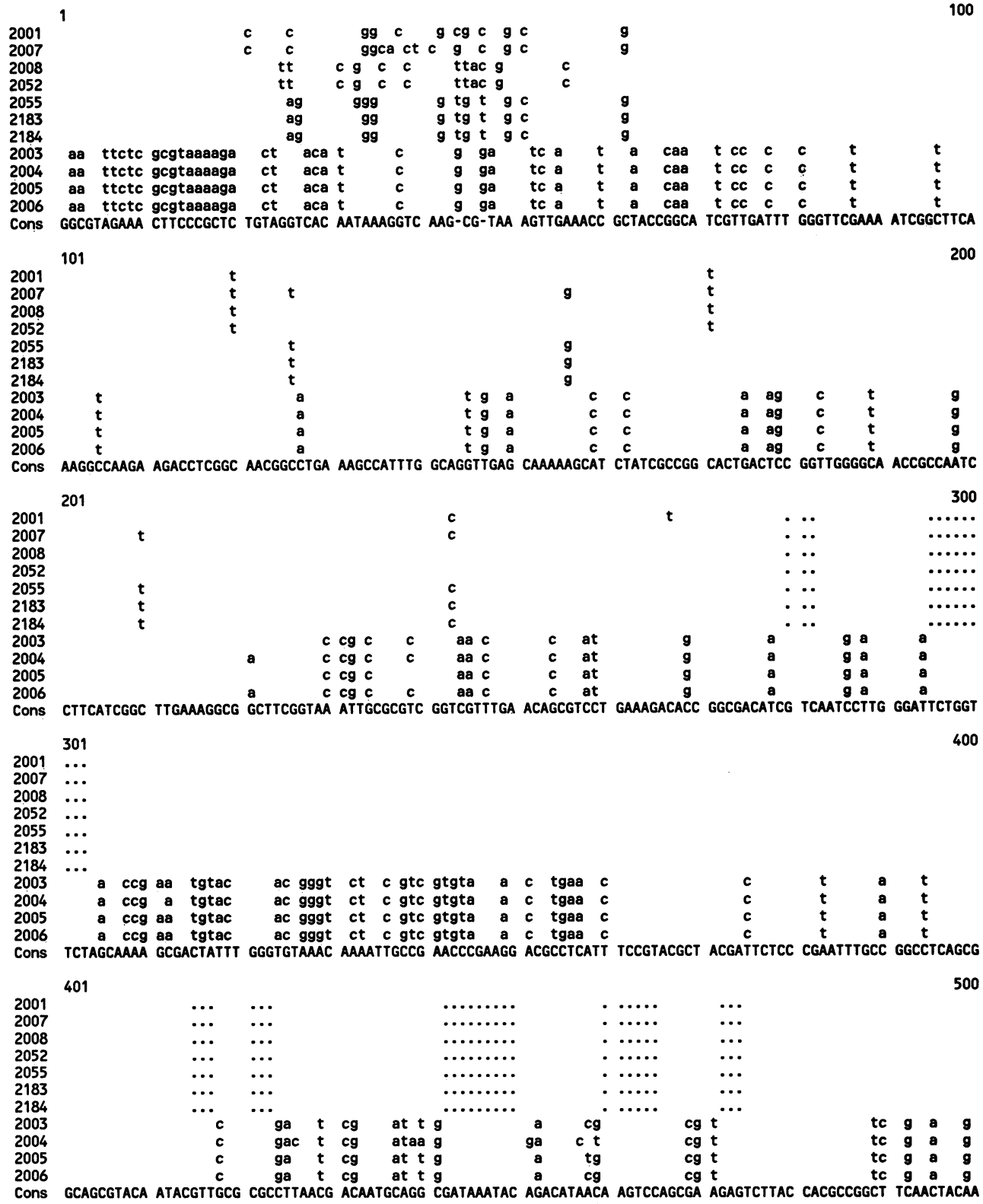

FIG. 4. Nucleotide sequences of PCR-amplified class 2 and class 3 OMP genes. The aligned nucleotide sequences from strains NIBSC 2001 through NIBSC 2008, NIBSC 2052, NIBSC 2055, NIBSC 2183, and NIBSC 2184 (Table 1) are shown, together with a consensus sequence. Differences from the consensus are illustrated with lowercase letters; periods indicate deletions. Base positions for which it is not possible to calculate a consensus are shown by dashes. Strain NIBSC 2008 is equivalent to strain H355 (Table 1). The serotypes of the meningococcal strains are given in Table 1.

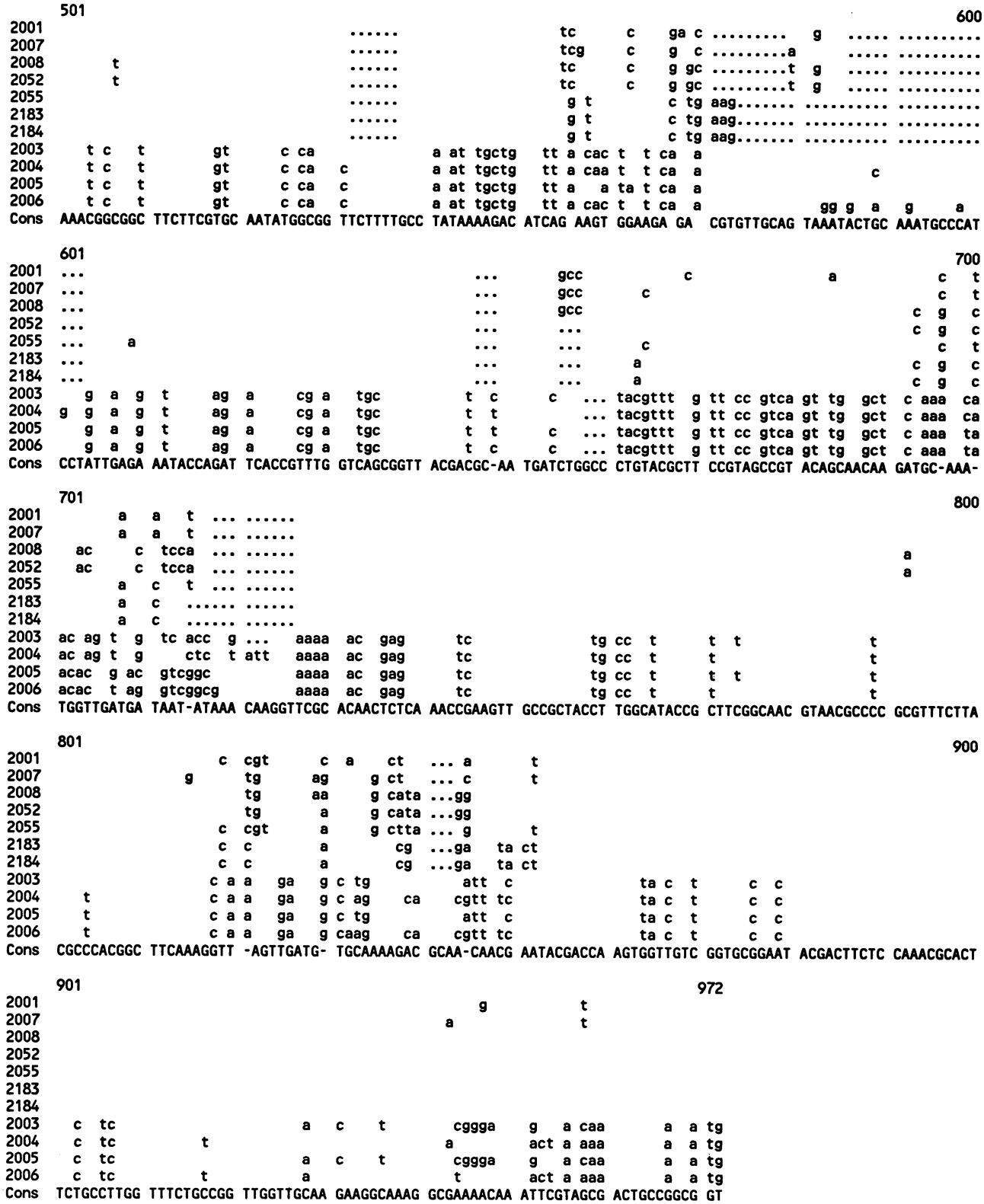

FIG. 4—*Continued.*

the proteins present on SDS-PAGE gels, and their nucleotide sequences are consistent with previously published class 2 and 3 genes. The strains for which the protein classes have been defined by peptide mapping give PCR products with the appropriate nucleotide sequence characteristics (Table 2). Further, the amplified class 3 OMPs all encode the peptide sequence -G-V-E-T-S-R-S-V- determined for the class 3 protein from strain H44/76 (30).

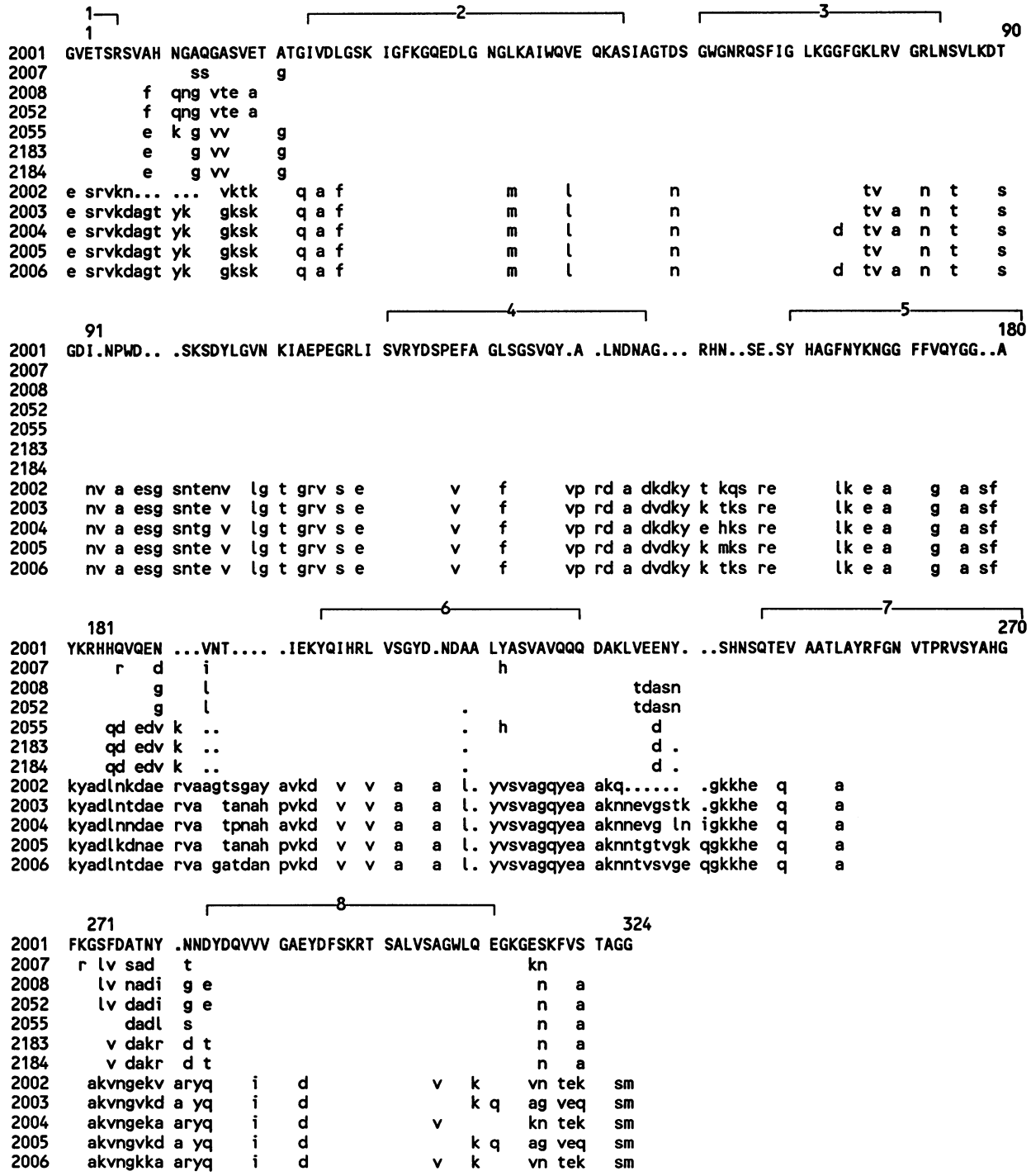

FIG. 5. Amino acid sequences of class 2 and class 3 OMPs deduced from the nucleotide sequences of PCR products. The primary structures of each of the 12 proteins are shown compared with the class 3 OMP sequence of strain NIBSC 2001. Differences from this sequence are shown by lowercase letters; periods indicate deletions. The conserved regions (18, 39) are indicated with brackets, and the putative loops are labeled with roman numerals I to VIII. Strain NIBSC 2008 is equivalent to strain H355 (Table 1). The serotypes of the meningococcal strains are given in Table 1.

Previously, the designation of class 2 or class 3 proteins had been made mainly on the basis of protein size (14, 28). Nucleotide sequence analysis enables a more precise definition of class 2 and class 3 proteins, and PCR technology permits rapid identification and analysis of the genes encoding these antigens. The size difference between class 2 and class 3 OMPs is caused by deletions in loops IV, V, and VI. The apparently anomalous result obtained by Tsai et al. (36) for strain M990 (NIBSC 2002 in this study), whose protein has a class 2 OMP chymotryptic map but is closer to a class

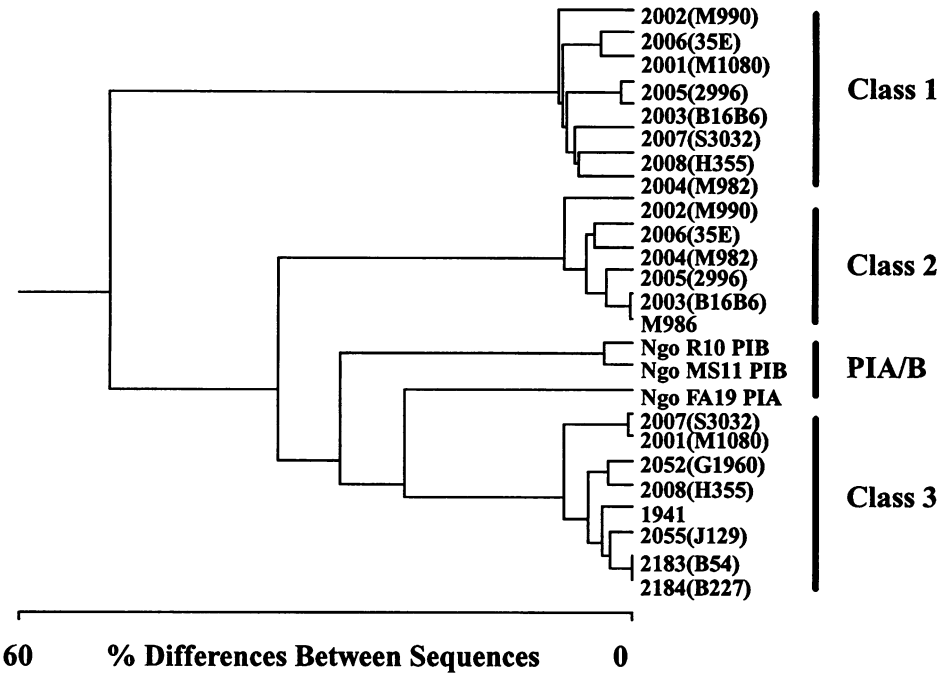

FIG. 6. Phenogram illustrating the relationships between meningococcal and gonococcal genes. The relationships between *Neisseria* OMP genes were calculated on the basis of nucleotide sequence identity between the equivalent primers. The bars indicate the protein classes. The numbers refer to the strain from which the sequences were derived (Table 1; Ngo indicates gonococcal strains); original strain designations are given in parentheses. The nucleotide sequences were derived as follows: class 1 OMP genes sequences, strains NIBSC 2001 to NIBSC 2008 (18); class 2 OMP gene sequences, strains NIBSC 2002 to NIBSC 2006 (this work) and strain M986 (24); class 3 sequences, strains NIBSC 2001, NIBSC 2007, NIBSC 2008, NIBSC 2052, NIBSC 2055, NIBSC 2183, and NIBSC 2184 (this work) and strain 1941 (39); the gonococcal PIA OMP gene sequence, strain FA19 (5); the PIB OMP gene sequence, strain MS11 (6); and the PIB sequence, strain R10 (15). The serotypes of the meningococcal strains are given in Table 1.

3 OMP in apparent M_r (Table 2), is explained by a deletion in loop I that is not seen in other class 2 or 3 proteins, as well as a deletion in loop VI (Fig. 5). Nucleotide sequence data provide a basis for future development of PCR-based typing and diagnostic techniques. The conditions described in the present work can detect as few as 100 cells (data not shown).

Relationships between meningococcal OMP genes. The meningococcal class 1, 2, and 3 OMPs, together with the gonococcal PIA and PIB OMPs, form a family of porin proteins. The similarity of the class 2 and, particularly, the class 3 OMPs to the gonococcal gene products suggests that these proteins may have been acquired by the meningococcus from the gonococcus or possibly, in the case of the class 2 OMP, from another *Neisseria* species. Horizontal genetic exchange is known to occur between *Neisseria* genes, including the class 1 OMP gene (11). This may have occurred between different class 2 or 3 OMP genes. Given the general diversity of these sequences, the close relationship between the class 3 genes of strain NIBSC 2055 (B:4) and two serogroup A strains (NIBSC 2183 and NIBSC 2184; A:4,21 [Fig. 6]) suggests that genetic exchange may have occurred between these serogroups.

As is found with class 1 OMP genes, base changes between class 2 and class 3 OMP genes occur in clusters; for example, 20 of 21 nucleotide base differences between the class 3 genes of strains NIBSC 2055, NIBSC 2183, and NIBSC 2184 (all serotype 4) occur between bases 609 and 847 (Fig. 4). These indicate regions where horizontal genetic exchange of fragments of genes may have occurred, giving rise to what has been termed localized sex (21).

Structural model for meningococcal OMPs. The determination of the primary structures of the *Neisseria* OMPs has enabled the prediction of possible secondary-structure models for the proteins (18, 37). The derived amino acid sequences presented here are consistent with these models. The conserved regions of the protein (CR1 to CR9 [16], marked 1 through 9 in Fig. 5), which are putatively membrane spanning, are identifiable in each of the strains. The predicted surface loops (I to VIII [Fig. 5]) that occur between the conserved regions are smaller in the class 2 and class 3 OMPs. A notable feature is that loop III is approximately the same size in all the proteins. In contrast to the class 1 OMP, loop III is the largest loop in the class 2, class 3, PIA, and PIB OMP models. The sequence present in this loop does not vary within classes, and antibodies against this loop do not bind to whole cells, although they do bind to outer membrane vesicles (37). These observations suggest that this loop is located within the protein structure, performing a conserved, possibly porin function-related, role unique to each class of protein.

Antigenic variation of meningococcal OMPs. The model of the class 1 OMP has eight surface-exposed loops (I to VIII) (18, 37). The antigenically important variable epitopes of the class 2 and class 3 OMPs are likely to reside in the equivalent surface loops (marked I to VIII in Fig. 5). In contrast to the class 1 OMP, the class 2 and class 3 OMPs possess smaller surface loops and the amino acid substitutions are found in several loops. Loop I, which corresponds to variable region 1 (VR1) in the class 1 OMP, varies between the class 2 and 3 OMPs and within class 3; the class 2 OMP from strain

NIBSC 2002 is an exception in the class 2 proteins in having a deletion causing the loss of six amino acids in this loop. Loop II is essentially invariant between the class 2 and class 3 OMPs, and loop III has been discussed above. Most of the amino acid sequence variation between class 2 and class 3 OMPs occurs in loops V and VI. Loop V corresponds to VR3 in the class 1 OMP (22). A major difference between the class 1 OMP and the class 2 and 3 OMPs is that loop IV (VR2 in class 1) is much shorter and shows less sequence variation in the class 2 and 3 OMPs. Additional sequence variation is located in loop VII and, between class 2 OMPs, loop VIII. In the class 1 OMP, several subtyping monoclonal antibodies have been defined with overlapping synthetic peptides corresponding to the amino acid sequences of either VR1 or VR2 (22). This indicates that there are continuous epitopes recognized in this class of protein, at least by mouse monoclonal antibodies. The presence of smaller surface-exposed loops of the other classes of porin from *Neisseria* strains suggests that discontinuous epitopes may be more important in the different serological variants of these proteins and that the sequence variation in the class 2 and class 3 OMPs is not as great as that seen in the class 1 OMP.

There are seven amino acid substitutions between the class 3 OMP from strain NIBSC 2055 (serotype 4) and strains NIBSC 2183 and NIBSC 2184, both serotype 4,21 (at positions 11, 221, 275, 279, 280, 281, and 283). These presumably account for the 21 serotype. The sequence VDDAKRDNT (loop VII) is a likely candidate for this epitope, although the involvement of other loops in a discontinuous epitope cannot be discounted. A set of serological reagents, based on the protein sequences and directed against specific loops whose sequences have been defined in this study, may prove useful additions to the reagents available for the analysis of these proteins.

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