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Analysis of *Neisseria meningitidis* Class 3 Outer Membrane Protein Gene Variable Regions and Type Identification Using Genetic Techniques

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The class 3 porin proteins of *Neisseria meningitidis* stimulate bactericidal antibodies and express serotype-specific antigenic epitopes. Sequence analysis of *porB* genes for the class 3 proteins revealed regions of variability that map to surface-exposed loops. To evaluate the relationship between serotype and variable-region (VR) genotype, sequences from the 11 class 3-expressing serotype strains and 3 additional serotype 4 strains were analyzed by molecular techniques. Multiple-sequence alignment revealed a limited number of unique sequences at each of four VRs (VR1 to VR4), ranging from four unique sequences at VR1 to seven sequence patterns at VR2 and VR4. Serotype-specific VR sequences were found in each of the four VRs, suggesting that each VR has immunologic importance. Five serotypes had at least one VR sequence that was unique. Three serotypes which had sequences in common with other serotypes at each VR were distinguished by examining multiple VRs. Serotype 3 was identical to serotype 19 at each VR, and serotype 8 was identical to serotype 18 at each VR. Serotypes 4 and 21 were identical at VR1 and significantly different at VR3 and VR4. A subpopulation of serotype 4 strains with a unique VR2 sequence was identified. The serotypes which were grouped with closely related or identical sequences at one VR were grouped with different serotypes at other VRs consistent with the pattern of genetic mosaicism described for the *porA* (class 1 protein) gene. Hybridization assays demonstrated the ability to identify VR genotypes and distinguish serotypes using biotin-labelled oligonucleotide probes. This information may be useful in strain selection for vaccine development, in epidemiologic studies to determine the prevalence of the individual VR genotype (especially among nonserotypeable strains) and, combined with PCR, in the identification of culture-negative suspected meningococcal cases.

Neisseria meningitidis causes both endemic and epidemic meningitis and septicemia worldwide. Epidemic group A meningococcal disease occurs at rates of 100 to 500 cases per 100,000 people per year. Rates of endemic disease, primarily caused by groups B and C, range from 1 to 3 cases per 100,000 people and increase to 10 to 50 cases per 100,000 people per year during outbreaks. A serotype 15 group B clone has been responsible for outbreaks in northern Europe, and a serotype 4 clone has been responsible for epidemic group B disease in Brazil and Cuba (18). Currently, capsular polysaccharide vaccines are available only for serogroups A, C, Y, and W-135. The group B capsular polysaccharide is poorly immunogenic and is chemically identical to the carbohydrate moiety of the fetal neural cell adhesion molecule (5), so the feasibility of a group B-specific conjugate vaccine has been questioned (5, 27). Also, the polysaccharide vaccines currently available are not useful in very young children, who constitute the population with the highest attack rates. The meningococcal outer membrane proteins (OMPs) are currently under investigation as alternative vaccine candidates (6).

All meningococci express either a class 2 or a class 3 OMP. The class 2 proteins are approximately 39,000 to 41,000 Da, and the class 3 proteins are between 35,000 and 38,000 Da (20). These are the predominant proteins in the outer membrane and function as porins (1, 20). These two proteins are mutually exclusive (1, 20) and are presumed alleles of the single-copy *porB* gene locus (8, 26). The class 2 and class 3 proteins show approximately 70% homology with each other based on DNA-derived amino acid sequences and show approximately 78% homology with the gonococcal proteins P1B and P1A, respectively (2, 15, 25).

Antibodies to the class 2 and 3 OMPs are bactericidal (17), and antigenic diversity between these proteins forms the basis of the current serotyping classification (7). There are approximately 20 different known serotypes within serogroups A, B, and C. Eleven of these serotypes express class 3 OMP antigens, and the remainder express class 2 OMP. Monoclonal antibodies are available for only a portion of these serotypes, and approximately one half of clinical isolates are nontypeable by these methods.

The class 3 protein gene has been sequenced, and several areas of variability have been described (2, 4, 23, 25, 26). Zapata et al. (26) examined two variable regions (VRs), VR1 and VR2, in the class 3 proteins of five serotypes, and these VRs were mapped by using the structural model of van der Ley et al. (21) to the surface-exposed loops I and V of the mature porin protein. Feavers et al. (4) described variability in these loops and additionally in regions corresponding to loops VI and VII in four class 3-containing serotypes. It is expected that these loops or associations of these loops form the antigenic epitopes for serotyping antibodies. These epitopes appear to be conformational rather than linear (11). A detailed analysis of VR sequence as it relates to serotype has not previously been reported.

The purpose of this study was to further examine the VRs of class 3 OMPs as they relate to serotype to improve our understanding of the antigenic epitopes of these proteins and to

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develop a system of identification and classification using genetic techniques. The present work describes patterns of VR sequence variation between serotypes, in which both unique sequences and sequences that are identical among several serotypes are found. This pattern is consistent with that of horizontal genetic exchange as described for the class 1 OMP (3). A method of distinguishing types by using biotin-labelled oligonucleotide probes is described.

MATERIALS AND METHODS

Bacterial strains and plasmids. All strains were maintained at −70°C in Greave's solution (5% bovine serum albumin [BSA], 0.5% monosodium glutamate, and 10% glycerol) and were cultured on brain heart infusion (Difco) agar plates with 5% horse serum (BHI-HS) at 37°C in 5% CO_2. Cells for DNA preparation were obtained directly from BHI-HS plates or from 200-ml overnight cultures with tryptic soy broth (soybean-casein digest medium; Difco) at 37°C with shaking. The T-7 expression vector pT7-5 has been previously described (19, 26).

DNA preparation. Genomic DNA was obtained in the manner of Moxon et al. (14) with the following changes: DNase-free RNase (Boehringer Mannheim) was added and incubated at 37°C for 30 min prior to the phenol extractions, and the butanol extraction was omitted. Concentration and purity were measured spectrophotometrically at 280 and 260 nm. Plasmid DNA was isolated using plasmid purification kits with maxipreps (Qiagen, Chatsworth, Calif.).

Recombinant DNA techniques. PCR amplification of the class 3 gene from group B meningococcal strains S3446, M978, 6557, 126E, 190I, 6940, and BB1350 was performed with primers C3CW (5′-CGTCGTCTAGAAAGGAGATATA CATATGAAAAAATCCCTGATTGCCCTG-3′) and C3CCW (5′-CGTGCAA GCTTTTATTAGAATTTGTGACG-3′) as previously described (26) with the exception that an annealing temperature of 37°C and an extension temperature of 72°C were used. PCR products were purified with gel permeation columns, digested with *Xba*I and *Hin*dIII restriction endonucleases, isolated from low-melting-point agarose, ligated into pT7-5 vectors, and transformed into *Escherichia coli* DH5α.

Sequence analysis. The class 3 genes were sequenced by Lofstrand Inc. (strains 6557, 190I, and 6940) and Procons Laboratories Inc. (strains S3446, M978, 126E, and BB1350). VR sequences showing one or more single-base-pair changes from closely related sequences were confirmed by sequencing multiple PCR clones with Bst 7-deaza-dGTP (Bio-Rad, Melville, N.Y.) by the manufacturer's double-stranded alkaline denaturation procedure. Nucleotide sequences of these genes and of *N. meningitidis* class 3 genes obtained from GenBank (see Table 2) were analyzed using PC/Gene software (release 6.6; IntelliGenetics, Inc.). Multiple-sequence alignment and phenogram analysis for the full-length protein were performed with translated amino acid sequences shortened so as to begin with the GVETSRS sequence and end with the VSTAGG sequence as the published sequences do. Multiple-sequence alignment analysis for the VRs was performed with nucleic acid sequences which included approximately 5 to 10 bp of constant region on either end to ensure proper alignment.

Oligonucleotide probes. The 5′-end-biotinylated oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer according to a standard phosphoramidite protocol with Biotin-ON phosphoramidite (Clontech Laboratories, Palo Alto, Calif.) as a 5′-biotinylation reagent. Before synthesis, the reagent was dried overnight under vacuum in the presence of phosphorus pentoxide. As recommended by the manufacturer, the coupling time for the Biotin-ON incorporation was increased to 10 min and the time for deprotection was shortened to 6 h. The syntheses were carried on in trityl-ON mode, and the oligonucleotides were purified by high-performance liquid chromatography on a Poros II R/M (10 by 100 mm) column (PerSeptive Biosystems, Cambridge, Mass.) with a linear gradient of acetonitrile in 0.1 M triethylammonium bicarbonate, pH 7.2, or alternatively with Nensorb Prep nucleic acid purification columns (NEN Research Products, Boston, Mass.).

Dot blots and hybridization assays. Genomic DNA was applied to nitrocellulose filters (Schleicher & Schuell, Keene, N.H.) in 20-μg dots by a standard method (28) and fixed by UV cross-linking. DNA concentrations ranging from 10 to 50 μg per dot were tested, and a slight decrease in signal intensity was noted at 10 μg. Filters were stored in plastic bags at −20°C or used immediately. Filters were prehybridized at the hybridization temperature for 1 h in 0.2 ml of prehybridization solution (6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0], 5× Denhardt's solution, 0.5% [wt/vol] sodium dodecyl sulfate, 100 μg of sheared denatured salmon sperm DNA [Gibco BRL] per ml) per cm^2 of filter. Filters were then hybridized at the hybridization temperature overnight in 0.05 ml of hybridization solution (prehybridization solution with 0.01 M EDTA) per cm^2 with 30 ng of biotin-labelled oligonucleotide probe per ml. Except where otherwise noted, results are shown for a hybridization temperature of 54°C. Similar results were seen with 4-h hybridizations. Filters were then processed as described by Podbielski et al. (16) with the exception that 3% BSA (in 100 mM Tris-HCl [pH 7.4]–150 mM NaCl) was used as a blocking agent and avidin-alkaline phosphatase (Boehringer Mannheim) at 20 μl/10-ml block was used as a conjugate.

TABLE 1. Sequence accession data for class 3 protein genes sequenced for VR analysis

Strain	Serotype	Calculated protein mol wt[a]	Sequence length (nucleotides)	ORF (amino acids)[b]	GenBank accession no.
126E	3	33,779	996	332	U07191
BB1350	4	33,721	990	330	U07193
M978	8	33,843	996	332	U07189
S3446	14	33,577	987	329	U07188
6557	17	33,948	990	330	U07190
190I	18	33,814	996	332	U07192
6940	19	33,800	996	332	U11030

[a] Calculated from the leader sequence cleavage point (26).
[b] ORF, open reading frame.

RESULTS

Sequencing of six class 3 protein genes. To further examine the relationship between VR sequence and serotype, the complete nucleotide sequences of the class 3 gene were obtained from five prototypic strains of *N. meningitidis* representing serotypes not previously reported in the literature. Strains 126E (serotype 3), S3446 (serotype 14), 6557 (serotype 17), 190I (serotype 18), and 6940 (serotype 19) were used. A Brazilian outbreak strain, BB1350 (serotype 4), and M978 (serotype 8), for which VR1 and VR2 were previously described (26), were also sequenced in full and analyzed. Nucleotide sequence length, open reading frame length, and predicted molecular weight are shown in Table 1. The first 44 nucleotides were encoded by using an upstream primer for PCR and include the restriction enzyme site, the ribosomal binding site, and the beginning of the leader sequence. The last 24 nucleotides of the sequences were encoded by a downstream primer. Predicted molecular weights for the mature proteins calculated from the leader sequence cleavage point (26) were approximately 33,600 to 33,900, though their estimated molecular weights on polyacrylamide gels were much greater. All seven sequences showed regions of identical sequence corresponding to conserved regions of previously reported class 3 gene sequences (4, 23, 26).

Protein sequence analysis. Multiple-sequence-alignment analysis of the seven new (Table 1) and seven published (Table 2) translated protein sequences revealed large areas of highly conserved sequence and several well-defined areas of sequence variability. Amino acid differences between the 14 strains were noted in 46 different positions, and 43 of these positions were located in areas corresponding to outer exposed loops when the protein is folded by using the model for neisserial porin proteins described by van der Ley et al. (21). As shown in Fig. 1, all nonconserved amino acid differences between the 14 strains analyzed are localized to the four regions of major variability. These VRs (VR1, VR2, VR3,

TABLE 2. Sources of class 3 gene sequences from the GenBank or EMBL database used in the VR analysis

Strain	Serotype	Reference(s)
M1080	1	4, 23
M981	4a	23
Cu385	4a	26
J129	4b	4
S3032	12	4, 23
H355	15	2, 4
B54	21	4

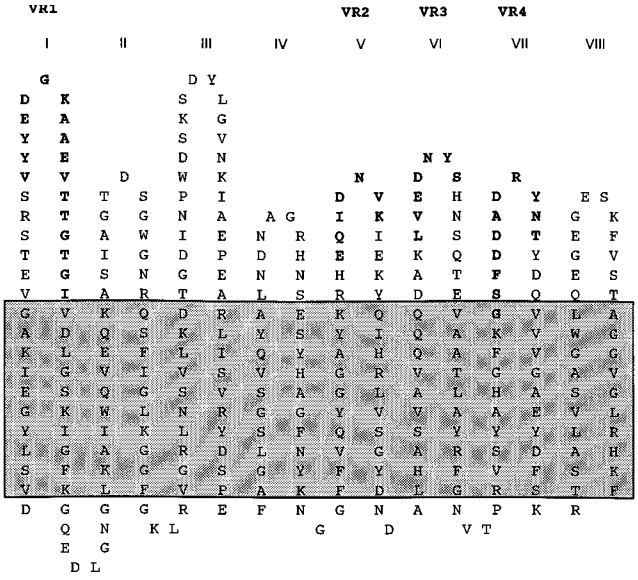

FIG. 1. Class 3 OMP from serotype 17 (strain 6557). The protein is folded by using the model for porin proteins by van der Ley et al. (21). The shaded region represents the area spanning the membrane. The VRs are shown in bold type, and the outer loops are labelled I to VIII.

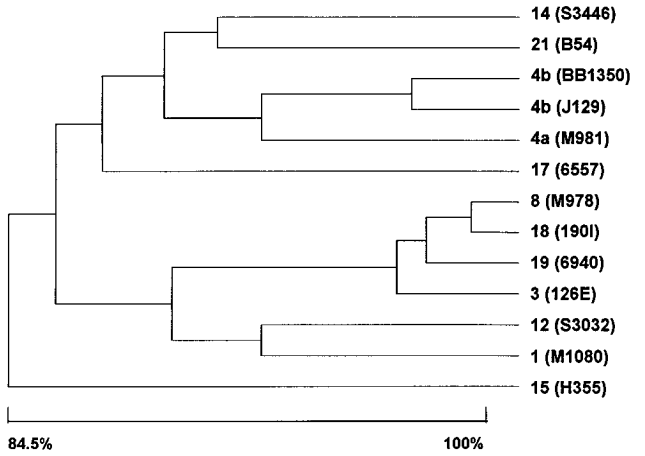

FIG. 2. Multiple-sequence alignment of the translated amino acid sequences from 13 strains of *N. meningitidis*. The phenogram represents the hypothetical phylogenetic tree calculated from pairwise alignment scores. The consensus length was 296 residues with an identity of 250 (84.5%) and a similarity score of 34 (11.5%). The scale at the bottom represents percent identity.

and VR4) correspond to loops I, V, VI, and VII of the folded protein.

A phenogram of the multiple-sequence alignment of the class 3 protein is shown in Fig. 2, illustrating the overall degree of variability among class 3-containing serotypes. The amino acid sequences for M981 and Cu385 are identical, and only M981 is shown. Serotypes 17 and 15 are the most unique, while serotypes 3, 8, 18, and 19 are the most closely related and show less overall variability than the four examples of serotype 4 examined. Serotype 4 is quite distinct from serotype 21, and among the serotype 4 strains there appear to be two subtypes. We have referred to these as serotype 4a, corresponding to prototype strains M981 and Cu385, and serotype 4b, corresponding to strains J129 and BB1350.

Variable region analysis. Analyses of individual VRs using nucleic acid sequences as shown in Fig. 3 were performed with the PC/Gene Clustal program, and the resultant phenograms are shown next to the VR sequences. The VR sequences of J129 (4) were excluded from this portion of the analyses because they were identical to BB1350 except for a single base change from T to G in the region of VR1. Analysis of VR1 shows four distinct subgroups. Serotype 15 and serotype 17 each have unique sequences, serotype 4 and 21 share a sequence which differs significantly from the other serotypes. The fourth group consists of serotypes 1, 3, 8, 14, 18, and 19, all of which are identical in this region.

In VR2, unique sequences for serotype 4a (M981) and serotype 17 were found. The remaining serotypes fall into two major groups. In the first group, serotypes 8 and 18 are identical and serotypes 1, 3, and 19 are identical. These two sequences differ from each other by three bases (two amino acids), and each sequence differs from that of serotype 15 by one base (one amino acid). Serotype 15 and serotype 12 differ by three bases (three amino acids). In the second group, strain BB1350 (4b) differs from serotype 21 by one base, which does not translate into an amino acid change. Serotype 21 and serotype 14 differ by two bases (two amino acids).

For VR3, serotypes 1 and 12 are identical, all strains of serotype 4 and serotype 17 are identical, serotypes 21 and 14 differ by one base (one amino acid), and serotypes 3, 8, 18, and 19 are identical. Serotype 15 and the subgroup containing serotypes 3, 8, 18, and 19 have the two most unique sequences in this VR.

In VR4, serotypes 1, 12, 15, 17, and 21 each have distinctly different sequences. Serotypes 3, 4, 8, 18, and 19 are identical and differ from serotype 14 by three bases (two amino acids).

Hybridization assays. Hybridization assays using biotin end-labelled oligonucleotide probes (Table 3) were performed. For Fig. 4, a class 2 probe and probes for VR1 serotype 4 (VR1-4), VR4-21, VR4-12, VR1-15, and VR1-17 were hybridized with blots containing 20 μg of genomic DNA from each prototype serotype strain. The class 2 probe hybridized with every class 2-expressing serotype and none of the class 3-expressing serotypes. The VR1-4 probe hybridized only to serotype 4 and serotype 21 strains. The VR4-21 probe hybridized only to type 21 strains. By using the information from these two probes, serotypes 4 and 21 were correctly identified. The VR4-12 probe hybridized to the prototypic strain S3032 without cross-reaction with other serotypes. The VR1-15 probe hybridized to all four serotype 15 strains. The VR1-17 probe hybridized to the prototype strain 6557 and to a nontypeable strain, 337/90. These data suggest that the probes described above are specific for prototypic serotype strains.

To further investigate the subgroups of serotype 4 and to evaluate the consistency of VR sequences among a number of strains of the same serotype, a second series of hybridizations was performed (Fig. 5). Nitrocellulose membranes dotted with DNA from 14 different serotype 4 strains and 8 different serotype 21 strains were hybridized with probes VR1-4, VR2-4a, VR4-4, and VR4-21. The VR1-4 probe hybridized to all 14 serotype 4 strains and 7 of 8 serotype 21 strains. The VR2-4a probe corresponding to the VR2 region of serotype M981 hybridized to strains M981, Cu385, BB1432, and BB1438. The VR4-4 probe reacted with all serotype 4 strains as well as serotypes 3, 8, 14, 18, and 19. At higher hybridization temperatures (58°C) serotype 14 does not react with this probe. The VR4-21 probe hybridized to all eight serotype 21 strains.

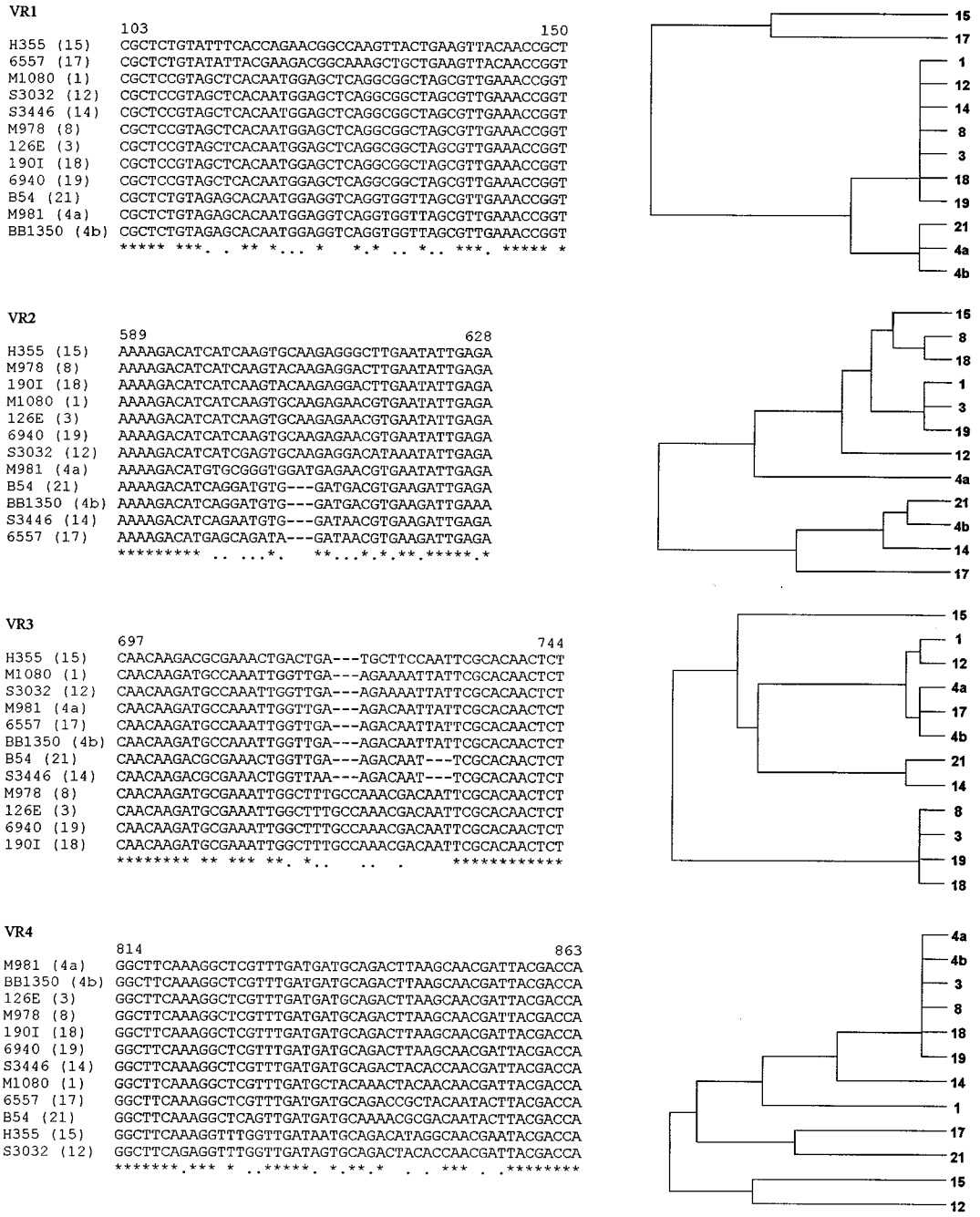

FIG. 3. Individual VR nucleic acid sequence alignments made with the Clustal program of PC/Gene. The positions of the first and last nucleic acid based on the consensus sequence are shown above the alignment. Symbols: *, perfectly conserved positions; ., well-conserved positions. Phenograms for each VR are shown on the right. In VR1, the phenogram represents 50% identity at the far left and 100% identity at the last branch point to the right. The VR2 phenogram spans from 51.2 to 100% identity, the VR3 phenogram spans from 43.8 to 100% identity, and the VR4 phenogram spans from 62 to 100% identity.

These results are consistent with the sequence data discussed earlier.

Similar hybridization assays were performed with the VR1-15 probe and the VR4-12 probe. The VR1-15 probe hybridized to nine of nine strains which had been identified as serotype 15 by whole-cell dot blot monoclonal antibody assays. One additional strain which reacted weakly with the serotype 15 monoclonal antibody did not hybridize to this probe. The VR4-12 probe hybridized only to the prototypic S3032 strain. Because a monoclonal antibody for serotype 12 is not currently available, comparison with a "gold standard" cannot be made. Two strains identified as serotype 12 by polyclonal rabbit antisera did not react with the VR4-12 probe.

The results of hybridization reactions for each of the probes evaluated are summarized in Table 3. In this study, the probes for class 2, VR1-15, VR1-17, VR4-12, and VR4-21 appear to be specific for DNA from strains of the corresponding serotypes. VR1-15 positively identified a series of serotype 15 strains, and the probes VR1-4 and VR4-21, when used in

TABLE 3. Oligonucleotide probes used in the hybridization assays

Probe	Sequence (5′→3′)	Target strains
VR1-4	GAGCACAATGGAGGTCAGGTGGTTAGCGTT	Types 4 and 21
VR2-4	GTGCGGGTGGATGAGAACGTG	Type 4a
VR4-4	AAAGGCTCGTTTGATGATGCAGACTTAAGC	Types 3, 4, 8, 18, and 19
VR1-15	TTTCACCAGAACGGCCAAGTTACTACTGAAGTT	Type 15
VR1-17	TATTACGAAGACGGCAAAGCTGCTGAA	Type 17
VR4-12	GAGGTTTGGTTGATAGTGCAGACTACAC	Type 12
VR4-21	CAGTTGATGATGCAAAACGCGACAATACTT	Type 21
Class 2	CTGTACGTTTCTGTTGCCGGTCAGTATGAAGCT	All class 2 strains

tandem, correctly identified a series of serotype 4 and serotype 21 strains, suggesting a useful degree of sensitivity.

DISCUSSION

Regions of variability. The nucleic acid sequences of *porB* for six additional serotypes of *N. meningitidis* expressing the class 3 protein were obtained from cloned *porB* genes with prototypic serotype strains. All six sequences were consistent with the sequences of previously published class 3 protein genes with the majority of variations limited to well-defined VRs. In addition, the *porB* gene sequence for the Brazilian strain, BB1350, was obtained and found to be identical to the clinical serotype 4 isolate J129 from England described by Feavers et al. (4) except for a single T-to-G substitution in VR1 resulting in an amino acid change from Asn to Lys. Significant variability between class 3-containing serotypes appears to be limited to loops I, V, VI, and VII, and we have extended the previous VR nomenclature to label the regions coding for these loops as VR1, VR2, VR3, and VR4, respectively.

Linear epitope analysis as performed for class 1 proteins (13) has not been successful for the class 2 and class 3 proteins, suggesting that the antigenic determinants are conformational. Therefore, it is not known which VRs are of greatest immunologic importance. The presence of unique VR sequences among some serotypes and identical VR sequences among other serotypes at each of the four regions of major variability suggests that each of these VRs, alone or in combination, has immunologic significance. The relative antigenicity and immunogenicity of individual loops and/or the presence of discontinuous epitopes involving more than one loop can now be investigated by using a series of strains which differ only at specific known regions.

Genetic mosaicism typical of horizontal genetic exchange has been well described for *porA*, the class 1 protein gene (3). The data presented here support a similar mechanism of genetic exchange for the *porB* gene since serotypes which are closely related or identical at one VR are distinct from each other and in some cases identical to a different group of serotypes at another VR. This is illustrated by the different groupings found in the phenograms for individual VRs and for the full-length protein.

Serotype 4 versus serotype 21 and subgroups of serotype 4. Serotypes 4 and 21 have previously been distinguished on the basis of monoclonal antibody testing and multilocus enzyme electrophoresis (24). It was noted by Wedege et al. that a subgroup of serotype 21 strains also reacted with several serotype 4 monoclonal antibodies. Our data are consistent with these observations in that serotype 21 strains had a unique VR4 region sequence which was found in all 8 strains tested in the hybridization assays. At VR3 the serotype 21 and serotype 4 sequences are also distinct. VR1 is the only VR where the serotype 4 sequence is shared only by serotype 21 and therefore may represent the epitope for monoclonal antibodies which react with both serotypes. At VR2, two different sequences have been described for serotype 4 strains. One, which we have referred to as VR2-4a, appears to be unique and in hybridization assays was found in 4 of 14 serotype 4 strains but was not detected in any other serotype strains. The other sequence, VR2-4b, has one nucleic acid difference between serotypes 4b and 21, but this does not translate into an amino acid difference. This is an unlikely epitope for either serotype 4 or serotype 21 monoclonal antibodies because this subgroup distinction has not been previously observed. The epitope for serologic distinction of serotype 4 remains unclear. At every VR the serotype 4 sequence is shared by one or more other serotypes. A monoclonal antibody would therefore require a discontinuous epitope to be unique for serotype 4.

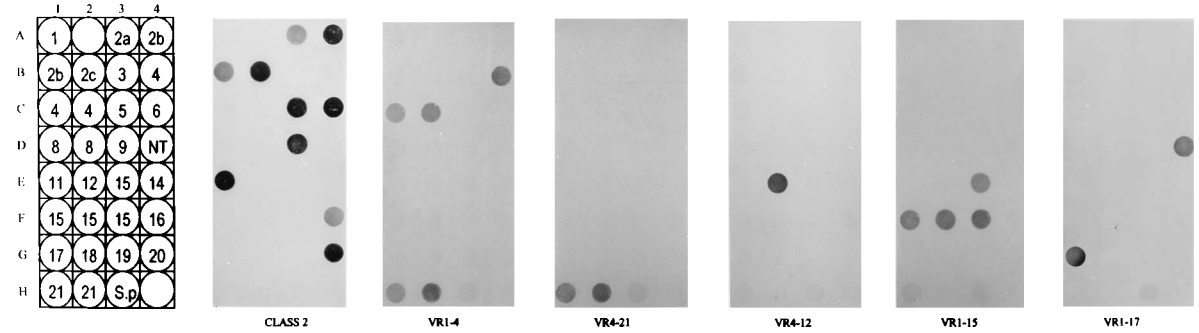

FIG. 4. Hybridization assays using dot blots of genomic DNA from all prototype serotype strains hybridized with biotin-labelled oligonucleotide probes. The serotypes of blotted strains are shown in the key on the left, and the probe used is listed below each blot. The strains used (and their positions on the blot; see the key) were M1080 (A1), M986 (A2), B16B6 (A3), 3006 (A4), 1002/90 (B1), 3459 (B2), 126E (B3), M981 (B4), Cu385 (C1), BB1350 (C2), M992 (C3), M990 (C4), M978 (D1), 735/90 (D2), M982 (D3), 337/90 (D4), M136 (E1), S3032 (E2), BB364 (E3), S3446 (E4), 44/77 (F1), H355 (F2), BB512 (F3), C-11 (F4), 6557 (G1), 190I (G2), 6940 (G3), 35E (G4), GA G-11 (H1), ATCC 13077 (H2), *Streptococcus pneumoniae* R61 (H3), and no strain (H4).

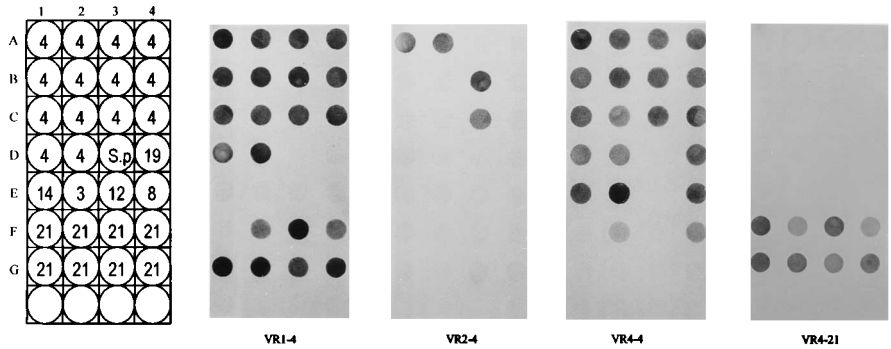

FIG. 5. Hybridization assays of serotype 4 and serotype 21 strains using three type 4 probes and the type 21 probe. The serotypes of blotted strains are shown in the key on the left, and the probe used is listed below each blot. The strains used (and their positions on the blot; see the key) were M981 (A1), Cu385 (A2), BB1350 (A3), BB1352 (A4), 44/89 (B1), BB1361 (B2), BB1438 (B3), BB475 (B4), BB482 (C1), BB1314 (C2), BB1432 (C3), BB490 (C4), BB470 (D1), BB487 (D2), *S. pneumoniae* R61 (D3), 6940 (D4), S3446 (E1), 126E (E2), S3032 (E3), M978 (E4), BB168 (F1), BB1396 (F2), BB1397 (F3), BB1398 (F4), BB1399 (G1), BB1400 (G2), GAG11 (G3), ATCC 13077 (G4).

Serotypes 3, 8, 18, and 19. The sequences for serotypes 8 and 18 are identical at each VR, as are those for serotypes 3 and 19. Serotypes 3 and 19 differ from serotypes 8 and 18 by an Ala-to-Val amino acid change in loop II and a two-amino-acid change (AsnVal to AspLeu) in VR2. The serotype 3, 8, 18, and 19 prototype strains react with the current serotype 8 monoclonal antibody (data not shown), and monoclonal antibodies to serotypes 3, 18, and 19 have not been developed. These data suggest that there may not be significant antigenic differences between serotypes 3 and 19 or between serotypes 8 and 18.

Use of defined oligonucleotides as a typing system. DNA probes for use as typing tools have been well described for a number of organisms, e.g., M typing of group A streptococcus (16) and serosubtyping of *N. meningitidis* (10). Analysis of the individual VRs of the serotyping protein shows between four and seven unique sequences at each VR. The present work demonstrates that it is possible to classify strains on the basis of their ability to hybridize with probes to these regions. Expansion of this technique would avoid many of the problems of monoclonal antibody availability and supply and would allow for more consistent classification of strains among laboratories. The system described here is nonradioactive and non-PCR based and therefore has broad application and availability. This methodology is easily applied in conjunction with PCR techniques which would potentially permit classification of suspected cases of meningococcal disease for which cultures are negative (9). This is especially important given that clinical trials utilizing OMP vaccines may be conducted in countries where antibiotic use prior to clinical evaluation is common and where as many as 50% of clinically suspected cases may have negative cultures.

Implications for vaccine development. Development of meningococcal OMP vaccines must address the problem of wide antigenic variation among strains. This has been addressed primarily by inclusion of the most epidemiologically prevalent strains in vaccine preparations. Genetic engineering of the class 1 protein utilizing antigenic hybrids (22) or expression of multiple recombinant epitopes (12) has also been proposed. The patterns of identical VR sequences found among serotypes described here may predict patterns of immunologic cross-reactivity and may have important implications regarding strain selection for vaccine use. If VR1 codes for an immunologically important epitope, then all 11 serotypes examined would be covered by including four strains, one from each group of VR1 sequences. If all VRs code for immunologic sites that stimulate bactericidal antibodies, then inclusion of serotype 4 in a vaccine would stimulate antibodies to serotypes 4 and 21 (VR1 and VR2), serotype 17 (VR3), and serotypes 3, 8, 18, and 19 (VR4). Additionally, it may be desirable epidemiologically to determine the prevalence of individual VR types in addition to serotype to determine cases which would be expected to be prevented by a given vaccine. We are presently examining nonserotypeable strains to determine whether they may possess one or more VRs in common with the serotypes described.

Conclusions. We have expanded the sequence data of *N. meningitidis* class 3 proteins to include six additional serotypes and have described both significant similarities and differences between VR sequences from a variety of distinct serotypes. A subgroup of serotype 4 strains differing in one VR was demonstrated. Neither the unique sequences nor the identical sequences are concentrated at a specific VR, suggesting that at least several VRs have antigenic and immunogenic significance. We have shown the feasibility of a nonradioactive DNA probe technique for identifying specific types of *N. meningitidis* which may be useful in both epidemiological investigations and vaccine trials.

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