Sequencing of the *porB* Gene: a Step toward a True Characterization of *Neisseria meningitidis*

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Variations in class 2/3 (PorB) proteins form the basis for meningococcal serotyping. Antibodies against these proteins are bactericidal, making serotyping results useful not only for epidemiological surveillance of meningococcal disease but also for identifying potential vaccine components. A total of 20 to 60% of meningococcal B and C isolates from any given population are nontypeable (NT) using a panel of monoclonal antibodies. To analyze the mechanisms responsible for the nonsero typing characteristic in *Neisseria meningitidis*, we (i) established the nucleotide sequences of *porB* gene in 146 meningococcal strains (95 not recognized by the serotyping panel), (ii) identified 18 new allelic variants of the *porB* gene, (iii) correlated allelic variants with serotypes, (iv) suggest the nontypeability characteristic in those 95 NT strains, and (v) reject the possibility of variation in the levels of PorB expression.

*Neisseria meningitidis* remains an important cause of meningitis and septicemia worldwide (9, 14).

For routine epidemiological surveillance, meningococci are classified by immunological reagents into serogroups (by type of capsular polysaccharide), serotypes (PorB, class 2 or 3 outer membrane proteins [OMPs]), and serosubtypes (PorA, class 1 of capsular polysaccharide), serotypes (PorB, class 2 or 3 outer membrane proteins [OMPs]), and serosubtypes (PorA, class 1 OMP) (7).

*N. meningitidis* can be subdivided into serotypes based on the detection of serologically distinct epitopes present on their class 2 or 3 (PorB) OMPs. These PorB proteins are encoded by either one of their respective *porB* gene alleles (PorB2 or class 2 protein encoded by *porB2* allele, and PorB3 or class 3 protein encoded by *porB3* allele), which are mutually exclusive.

PorB OMPs are transmembrane proteins with eight predicted surface-exposed loops (I to VIII) that are variable in terms of their lengths and amino acid sequences. These surface-exposed loops are interspaced and anchored by nine membrane-spanning regions that are relatively conserved (20). Sequence analysis of PorB proteins from different serotypes of meningococci identifies four regions with a high level of amino acid exposed (loops I, V, VI, and VII) (6, 23).

The use of monoclonal antibodies (MAbs) is the standard method for identifying the PorB type, and a widely used panel of MAbs has been developed (1). These MAbs are directed against some of the four variable regions (VRs) of PorB proteins, with the exception of MAAb22, which fails to react with any of the four VRs of PorB (11). Nevertheless, a large number of meningococci cannot be typed using the serotyping antibodies currently available, which is particularly underlined in strains isolated from carriers. From 20 to 60% of meningococcal B and C isolates from any given population can be nontypeable (NT) using that panel of MAbs (11). This problem is already distorting the serotype prevalence data in defined areas (12, 13, 16).

The aim of the present study was to analyze the mechanisms responsible for the nontypeability characteristic in *N. meningitidis* by sequencing the *porB* gene.

**MATERIALS AND METHODS**

**Bacterial strains and serotyping.** All *N. meningitidis* strains received in the Reference Laboratory for *Neisseria* are routinely serotyped by whole-cell enzyme-linked immunosorben t assay (ELISA) (21) using a set of MAbs provided by the National Institute for Biological Standards and Control, including the serotypes 1 (MN3C6B), 2a (5D4-5), 2b (MN2C3B), 4 (5DC4C8G8), 14 (MN5C8C), 15 (855-509), and 21 (6B11F2B5). A total of 146 *N. meningitidis* strains (51 with defined serotypes and 95 NT isolates) recovered from blood or cerebrospinal fluid of patients with meningococcal disease isolated in Spain from 1992 to 2004 were included. The strains were randomly selected among more than 7,000 strains received over the stated period.

**Sequencing of porB gene.** For DNA extraction, samples were heated at 100°C (20 min), subjected to one freeze (2 min)-thaw cycle, and then centrifuged for 5 min at 10,000 × g. Amplification of the *porB* gene from extracted DNA samples was as described previously (17), with some minor modifications. The reactions were carried out with 1 × reaction buffer (PE Applied Biosystems); 200 μM (each) dATP, dCTP, dGTP, and dTTP; 1 μM concentrations of PCR primers PBA1 (5'-TAAATGCAAAAGCTAAGCGGCTTG-3'), PBA2 (5'-TTGTTT GATACCAATCTTTTCAG-3'), 2.5 U of AmpliTaq Gold (PE Applied Biosystems); and 10 μl of template DNA (in 100-μl reaction mixtures). Reaction conditions were 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, followed by incubation at 72°C for a further 2 min. PCR products were purified by using a QIAquick PCR purification kit. For full-length *porB* gene sequencing, a subset of seven oligonucleotides was used (PBS1, PBS2, 8U, 8L, 244U, 244L, and PB260) (23).

The alleles obtained were appointed through the website http://neisseria.org/nmotyping/porB. Those identified as new alleles were submitted to this database, and new allele numbers were assigned.

**Sequencing of porB promoter region.** The *porB* promoter region was amplified and sequenced using the primers PromBF (5'-TTTGTGCGCTTGTCTGATT TTTG-3') and PromBR (5'-GTGTTCTACGGCCGTTTGTGGT-3'). The reaction mixture and conditions were as described for the *porB* gene with only a minor modification (the concentration of the primers was 0.5 μM).

**Western blotting.** OMPs were extracted as described previously (4) and were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in vertical gradient gels by using the Xcell SureLock Mini-Cell kit (Invitrogen) according to the manufacturer’s instruction s. The separated proteins (200-V constant...
voltage) were transferred from the gel to nitrocellulose membranes using the Xcell II blot module (Invitrogen) according to the manufacturer’s instructions. Membranes were blocked with 10% milk in 1/100 phosphate-buffered saline (PBS) with shaking overnight at 4°C. Membranes were then washed three times with 1/100 PBS containing 0.05% Tween 20 (PBS-Tween) and incubated with the corresponding MAb for 1 hour at room temperature. After a new washing cycle, the membranes were incubated with -mouse–peroxidase conjugate in blocking solution (1:500) for 1 hour at room temperature and then washed again three times. Finally, the membranes were developed with 4-chloro-1-naphthol (22).

**Nucleotide sequence accession numbers.** The sequences of the porB gene obtained during the study and identified as new alleles were submitted to the GenBank database under accession numbers listed in Table 1. The accession numbers for the upstream regions are DQ485291 to DQ485304.

**RESULTS**

**Serotyping.** Ninety-five strains were considered NT strains because they were not recognized by the serotyping panel. In the remaining 51 isolates, the serotypes 1, 2a, 2b, 4, 14, 15, and 21 were detected (Table 1).

**porB gene analysis.** The porB gene was sequenced from all 146 strains, with 37 different porB alleles among them, 18 of which were defined as new variants (Table 1). After the analysis of the porB alleles, the correlation between allelic variants and serotypes was established according to the scheme developed by Sacchi et al. (11), which defines the serotypes based on a combination of the sequences of the VRs (Table 1).

The relationships among all porB gene sequences obtained was represented by the split decomposition method (3). With this method, networks of interconnected nodes appear when recombination events are occurring, while a start indicates that the variation is being caused by point mutations. The split graph obtained (Fig. 1) illustrates a small network, showing recombination events only among the porB2 allele sequences.

### Table 1. Allelic variants of porB gene obtained from 146 meningococcal strainsa

| Allele (GenBank accession no.)b | No. of strainsc | PorB VR type (genotype): | No. of strains that were: |
|-------------------------------|-----------------|--------------------------|--------------------------|
|                               | VR1  | VR2  | VR3  | VR4  | NTd  | Typeable (serotype)e |
| porB3-1                       | 37   | 4    | D    | 7    | 14a  | 26   | 11 (4) |
| porB3-14                      | 4    | 19   | Ac   | 7a   | 1    | 3    | 1 (1)  |
| porB3-16                      | 5    | 19   | Aa   | 10   | 14a  | 5    | 0      |
| porB3-25                      | 10   | B    | C    | 7    | 14b  | 10   | 0      |
| porB3-35                      | 2    | 19   | A    | 10   | Aa   | 2    | 0      |
| porB3-36                      | 2    | 19   | Db   | 7c   | 14a  | 3    | 0      |
| porB3-38                      | 3    | E    | D    | 7b   | 14a  | 2    | 4 (4)  |
| porB3-39                      | 6    | 4    | B    | 7d   | 14a  | 2    | 0      |
| porB3-41                      | 2    | 19   | Aa   | 7c   | 1    | 2    | 0      |
| porB3-45                      | 4    | 4    | D    | 7    | 14a  | 4    | 0      |
| porB3-60                      | 2    | E    | D    | 7b   | 21   | 1    | 1 (21) |
| porB3-64                      | 1    | C    | Dc   | 7b   | Bc   | 1    | 0      |
| porB3-71                      | 4    | 19   | Db   | 7c   | 14   | 2    | 2 (14) |
| porB3-80 (AY699311)           | 1    | 4c   | B    | 7d   | 14g  | 1    | 0      |
| porB3-81 (AY699312)           | 3    | 19   | De   | 7b   | 14a  | 3    | 0      |
| porB3-82 (AY699313)           | 3    | 19   | Ac   | 7a   | 1    | 3    | 0      |
| porB3-83 (AY699314)           | 1    | 19b  | Db   | 7c   | 14   | 0    | 1 (14) |
| porB3-84 (AY699315)           | 4    | A    | [A]  | A    | Ba   | 3    | 1 (15) |
| porB3-85 (AY699316)           | 1    | 19   | B    | 7d   | 14a  | 1    | 0      |
| porB3-86 (AY699317)           | 1    | 4    | D    | 7    | 14b  | 1    | 0      |
| porB3-87 (AY699318)           | 1    | A    | A    | 14a  | 14a  | 3    | 0      |
| porB3-88 (AY699319)           | 1    | B    | 7    | 14b  | 1    | 0      |
| porB3-89 (AY699320)           | 1    | 4    | D    | 7    | 14a  | 0    | 1 (4)  |
| porB3-90 (AY699321)           | 1    | 4c   | D    | 7    | 14c  | 1    | 0      |
| porB3-91 (AY699322)           | 1    | 19   | D    | 7    | 14a  | 1    | 0      |
| porB3-92 (AY699323)           | 1    | 19   | Aa   | 10   | 14a  | 1    | 0      |
| porB3-93 (AY699324)           | 1    | 4e   | D    | 7    | 14a  | 0    | 1 (4)  |
| porB3-116 (AY966903)          | 1    | 4    | Db   | 7c   | 14   | 0    | 1 (14) |
| porB2-2                       | 12   | C    | Eb   | 2a   | C    | 0    | 12 (2a) |
| porB2-3                       | 15   | C    | Ea   | 2b   | C    | 0    | 15 (2b) |
| porB2-11                      | 1    | C    | Eh   | 5a   | Cb   | 1    | 0      |
| porB2-22                      | 2    | C    | Ef   | 2aa  | Db   | 2    | 0      |
| porB2-23                      | 6    | D    | Ed   | 5b   | Db   | 6    | 0      |
| porB2-27                      | 2    | Cf   | Eb   | 2ba  | Cc   | 2    | 0      |
| porB2-48 (AY699308)           | 2    | C    | Eb   | 2ad  | C    | 2    | 0      |
| porB2-49 (AY699309)           | 1    | C    | Ed   | 5b   | Db   | 1    | 0      |
| porB2-54 (AY699310)           | 1    | D    | Ed   | 5b   | Cc   | 1    | 0      |

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a Correlation with serotypes defined by MAbs.
b Nomenclature for the alleles already appointed in the Web site http://neisseria.org/nm/typing/porB/. GenBank accession numbers only for the new allelic variants found in the present study are indicated in parentheses.
c That is, the number of strains in each VR combination.
d That is, the number of strains showing a specific allelic variant which were not recognized by MAbs (NT strains with MAbs).
e That is, the number of strains showing that specific allelic variant which were recognized by MAbs (typeable strains with MAbs). The serotype obtained by MAbs is given in parentheses.

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obtained (including both known and new allelic variants), detecting also the punctual mutations at this level. However, all porB3 allele sequences obtained in the present study appeared only as the result of point mutations (Fig. 1).

**VR sequence analysis.** The analysis of VR sequences allowed us to identify two different groups. (i) Of the 95 NT strains, 42 showed the allelic variants porB2-11, porB2-22, porB2-23, porB2-27, porB2-48, porB2-49, porB2-54, porB3-16, porB3-25, porB3-35, porB3-38, porB3-64, porB3-80, porB3-85, porB3-88, porB3-90, porB3-91, and porB3-92, which cannot be recognize by the MAbs routinely used (Table 1). (ii) In the remaining 53 NT strains, any change found in the VR sequences could explain the nonreactivity with any of the antibodies included in the panel currently available (Table 1).

**Sequence of the promoter region of the porB gene.** To determine whether these 53 strains were NT because of no expression or only a low level of expression of PorB protein, the upstream region of the start codon of porB gene, containing the promoter of porB, was analyzed. The porB upstream region of the start codon of these 53 NT strains and also 10 additional isolates expressing several types was amplified and sequenced, yielding 14 different sequences at this level.

Comparison and alignment of the sequences showed that the porB promoter sequences were very similar in all analyzed strains. The few changes found do not seem to be associated with the level of expression of PorB protein because they were present in both NT and typeable strains.

Close examination of the sequences revealed that the putative transcriptional start point is located at position 292, 95 bp upstream of the ATG initiation codon (position 387). A putative −10 sequence (TATAGT) is found 7 bp upstream of the transcriptional start, with only one different residue with respect to the consensus −10 sequence of Escherichia coli (TATAAT). The putative −35 sequence of the porB promoter is more difficult to recognize, and the best candidate is TTGTTR, with three residues of homology to the consensus −35 sequence of E. coli (TTGACA) and located 17 bp upstream of the −10 sequence.

**MAb reactivity.** To determine whether the nonreactivity of MAbs with the 53 NT strains was because of a masking of the epitopes, Western blot assays using MAb types 1, 4, 14, 15, and 21 were performed. In this case, the MAbs should recognize the epitopes because the proteins are linear. Strains expressing serotypes 1, 4, 14, 15, and 21 and confirmed by sequencing of the porB gene were used as a control. A good recognition epitope MAb was observed in all cases, and one example is shown in Fig. 2.
DISCUSSION

Serological characterization of \textit{N. meningitidis} antigens is valuable for epidemiological studies, as well as for identifying immunologically important antigens in vaccine development (13, 10). This serological characterization allows us to classify the meningococcal strains into serogroups, serotypes, and serosubtypes. However, a large number of meningococcus strains cannot be serotyped. The aim of the present study was to explain the mechanisms responsible for the nonserotypeability in \textit{N. meningitidis} strains.

In the present study, 42 of 95 NT strains showed the non-serotypeability characteristic, either because MAbs that might be able to react with some of the four VRs (the VRs designated with a letter [see Table 1]) have not been developed or because the set of MAbs routinely used do not include these MAbs (types 5, 19, 10, 7, and its variants). A third possibility is that these VRs, which are variants as a result of changes in the original VR DNA sequence, are not being recognized by the panel of MAbs (2aa, 2ad, 2ba, 4c, 14a, 14b, 14c, and 14g). Because the serotype is defined by the combination of their VRs, different combinations of the three possibilities already mentioned can be present in each strain (Table 1).

In the remaining 53 NT strains, any change found in the VR sequences could explain the lack of binding with any of the antibodies included in the panel currently available. The analysis of the \textit{porB} promoter sequence disproved the hypothesis of alterations in the expression of these proteins. To check whether the lack of reactivity of the MAbs was due to accessibility to the epitopes, Western blot assays using MAbs types 1, 4, 14, 15, and 21 were carried out. A good recognition epitope MAb was observed in all cases. Therefore, a possible reason for the failure of the MAbs to identify these strains might be the very limited accessibility of the epitopes. Either a large amount of capsular polysaccharide or lipooligosaccharide masking the PorB VR epitopes or a less exposed epitope might explain this finding. Less exposition could be explained either because of alterations in the class 2/3 proteins affecting their conformation or because of the development of membrane-protein complexes.

It has been shown previously that the use of an expanded serotype panel can improve the sensitivity of serotyping by resolving a number of formerly NT strains (11). However, the number of NT strains will still be large due to continuous changes in the VRs, and the problem of the accessibility of the epitopes has not been solved.

Split decomposition has been used extensively to analyze the population structures of both bacteria and viruses. Because this method does not make the a priori assumption that the sequences have a tree-like structure, conflicting phylogenetic signals in the data such as evidence of recombination can be visualized, leading to the generation of an interconnected network rather than a tree. In the present study, punctual mutations were the main cause of variation in the \textit{porB} gene, probably because of a very strong selection pressure. In both \textit{porB2} and \textit{porB3} genes codons have been identified that had been subjected to very strong selection pressure (19). Recombination events have been reported as well (19, 5, 18), although our results suggest recombination only among the \textit{porB2} sequences (Fig. 1). Strains showing the PorB2 class protein are usually associated with illness rather than carriers. Because of that these strains might need to produce more-complicated variants to elude the immune system, explaining this rare finding. However, only further studies including a higher number of isolates may confirm it.

Previous studies show that PorA expression could be altered by multiple mechanisms (2). Slipped-strand mispairing during replication in the homopolymeric tract of guanine [poly(G)] and/or thymidine residues between the −12 and −35 domains of the \textit{porA} promoter, as well as the homopolymeric tract of adenine [poly(A)] residues in the \textit{porA} coding region, are the principal mechanism responsible for altered PorA expression. In addition, point mutations or insertion of an IS element in the \textit{porA} coding region or deletion of the complete \textit{porA} gene may result in meningococci lacking PorA expression. However, studies about alterations of PorB expression have not been reported previously. In our study the upstream region of start codon of \textit{porB} was analyzed, and the putative promoter region was located. No mechanisms for altered PorB expression were found, supporting the idea that PorB protein is not subject to phase variation. Mutant meningococcal strains that lack PorB do not grow well (15), suggesting an essential role of this protein as our results have already shown.

In conclusion, our study suggests different reasons for the nontypeability characteristic in \textit{N. meningitidis} and emphasizes that genetic characterization should be preferred over phenotypic characterization for typing meningococcal strains. However, a nomenclature scheme widely accepted and based on the four VR sequences must be designed, as has been done with the PorA protein to define the serosubtype. We propose that each strain might be defined with a VR combination (PorB VR type; Table 1).

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REFERENCES

1. Abdillahi, H., and J. T. Poolman. 1998. Typing of group B \textit{Neisseria meningitidis} with monoclonal antibodies in the whole-cell ELISA. J. Med. Microbiol. 26:177–180.
2. Alcalá, B., C. Salcedo, L. Arreaza, R. Abad, R. Enriquez, L. de la Fuente, M. J. Uria, and J. A. Vázquez. 2004. Antigenic and/or phase variation of PorA protein in non-subtypeable \textit{Neisseria meningitidis} strains isolated in Spain. J. Med. Microbiol. 53:515–518.
3. Bandelt, H. J., and A. W. Dress. 1992. Split decomposition: a new and useful approach to phylogenetic analysis of distance data. Mol. Phylogenet. Evol. 1:242–252.
4. Crowe, B. A., R. A. Wall, B. Kusecek, et al. 1989. Clonal and variable properties of \textit{Neisseria meningitidis} isolated from cases and carriers during and after an epidemic in The Gambia, West Africa. J. Infect. Dis. 159:686–700.
5. Dyet, K. H., and D. R. Martin. 2005. Sequence variation in the \textit{porB} gene from B:P1.4 meningococci causing New Zealand’s epidemic. J. Clin. Microbiol. 43:838–842.
6. Feavers, I. M., J. Saker, A. J. McKenna, A. B. Heath, and M. C. J. Maiden. 1992. Molecular analysis of the serotyping antigens of \textit{Neisseria meningitidis}. Infect. Immun. 60:3620–3629.
7. Frasch, C. E., W. D. Zollinger, and J. T. Poolman. 1985. Serotype antigens of \textit{Neisseria meningitidis} and proposed scheme for designation of serotypes. Rev. Infect. Dis. 7:504–510.
8. Huson, D. H. 1998. SplitsTree: analyzing and visualizing evolutionary data. Bioinformatics 14:68–73.
9. Peláez, H. 1903. Meningococcal disease: still with us. Rev. Infect. Dis. 8:71–91.
10. Poolman, J. T. 1996. Bacterial outer membrane protein vaccines: the meningococcal example. Adv. Exp. Med. Biol. 397:73–77.
11. Sacchi, C. T., A. P. S. Lemos, A. M. Whitney, C. A. Solari, M. E. Brant, C. E. A. Melles, C. E. Frasch, and L. W. Mayer. 1998. Correlation between serological and sequence analysis of the PorB outer membrane protein in the Neisseria meningitidis serotyping scheme. Clin. Diagn. Lab. Immunol. 5:348–354.

12. Sacchi, C. T., A. P. S. Lemos, M. C. O. Gorla, and C. E. Frasch. 1995. Monoclonal antibody to serotype 17 of Neisseria meningitidis and its prevalence in Brazilian states. Rev. Inst. Med. Trop. Sao Paulo 37:1–5.

13. Scholten, R. J. P. M., H. A. Bijlmer, J. T., Poolman, B. Kuipers, D. A. Caugant, L. V. Alphen, J. Dankert, and H. A. Valkenburg. 1993. Meningococcal disease in The Netherlands, 1958–1990: a steady increase in the incidence since 1982 partially caused by new serotypes and subtypes of Neisseria meningitidis. Clin. Infect. Dis. 16:237–246.

14. Schwartz, B., P. S. Moore, and C. V. Broome. 1989. Global epidemiology of meningococcal disease. Clin. Microbiol. Rev. 2:S118–S124.

15. Tomesen, J., P. Vermeij, M. Struyve, R. Benz, and J. T. Poolman. 1990. Isolation of Neisseria meningitidis mutants deficient in class 1 (PorA) and class 3 (PorB) outer membrane proteins. Infect. Immun. 58:1355–1359.

16. Tzanakaki, G., C. C. Blackwell, J. Kremastinou, D. M. Weir, A. Mentis, and R. J. Fallon. 1993. Serogroups, serotypes and subtypes of Neisseria meningitidis isolated from patients and carriers in Greece. J. Med. Microbiol. 38:19–22.

17. Urwin, R. 2001. Nucleotide sequencing of antigen genes of Neisseria meningitidis, p. 157–172. In A. J. Pollard and M. C. J. Maiden (ed.), Meningococcal disease: methods and protocols. Humana Press, Inc., Totowa, N.J.

18. Urwin, R., A. J. Fox, M. Musilek, P. Kriz, and M. C. J. Maiden. 1998. Heterogeneity of the PorB protein in serotype 22 Neisseria meningitidis. J. Clin. Microbiol. 36:3680–3682.

19. Urwin, R., E. C. Holmes, A. J. Fox, J. P. Derrick, and M. C. J. Maiden. 2002. Phylogenetic evidence for frequent positive selection and recombination in the meningococcal surface antigen PorB. Mol. Biol. Evol. 19:1686–1694.

20. Van der Ley, P., J. E. Heckels, M. Virji, P. Hoogerheut, and J. T. Poolman. 1991. Topology of outer membrane porins in pathogenic Neisseria spp. Infect. Immun. 59:2963–2971.

21. Wedege, E., E. A. Hulbø, E. Rosenqvist, and L. O. Froholm. 1990. Serotyping and subtyping of Neisseria meningitidis isolates by co-agglutination, dot blotting, and ELISA. J. Med. Microbiol. 31:195–201.

22. Wood, J. N. 1984. Solid-phase screening of monoclonal antibodies, p. 279–826. In J. M. Walker (ed.), Methods in molecular biology. I. Proteins. Humana Press, Clifton, N.J.

23. Zapata, G. A., W. F. Vann, Y. Rubinstein, and C. E. Frasch. 1992. Identification of variable region differences in Neisseria meningitidis class 3 protein sequences among five group B serotypes. Mol. Microbiol. 6:3493–3499.