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Sequence Variation in the \textit{porB} Gene from B:P1.4 Meningococci Causing New Zealand’s Epidemic

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Since mid-1991, New Zealand has experienced an epidemic of meningococcal disease. The epidemic has been caused by serogroup B meningococci expressing PorA type P1.7–2,4, belonging to the ST-41/ST-44 complex, lineage III. Most B:P1.7–2,4 meningococci express type 4 PorB (87.0%), although case isolates with \textit{porB} other than type 4 have been identified throughout the duration of the epidemic. To assess the genetic relatedness of case isolates with an alternative \textit{porB} gene, multilocus restriction typing validated against multilocus sequence typing was used. This determined that B:P1.7–2,4 meningococci with a \textit{porB} gene that was other than type 4 had the same clonal origin. It was concluded that strains with alternative \textit{porB} genes had diverged from the original type 4 \textit{porB}. Variation in \textit{porB} was also shown to be associated with the uptake of DNA encoding one or two of the \textit{porB} variable regions leading to mosaic \textit{porB}. Point mutation rather than horizontal transfer and recombination was implicated as the mechanism of sequence variation in some strains. This work will serve as a reference point to determine if the administration of a strain-specific vaccine increases the level of \textit{porB} divergence and variation already observed in New Zealand case isolates. It also complements the study undertaken of PorA stability which showed that variation in P1.7–2,4 PorA was almost exclusively due to deletions in the P1.4 epitope of the epidemic strain.

The inability to use a polysaccharide vaccine to protect against group B meningococci has limited the ability to control group B meningococcal disease by vaccination. The weak immunogenicity of the group B polysaccharide means that subcapsular antigens, and in particular the PorA and PorB outer membrane proteins, have become a focus as vaccine components.

The five most abundant outer membrane proteins on the meningococcal cell surface do not exhibit intrastain variability, although antigenic differences exist between different strains (11). The class 1 protein is PorA. Expression of the class 2 and class 3 proteins (PorB) is mutually exclusive, and they are products of the \textit{porB} locus. PorA and PorB are important epidemiological markers that are the targets of serotyping and serosubtyping antibodies, respectively (11), and are the targets of bactericidal antibodies (22).

The PorB protein consists of eight surface-exposed loops that vary by strain, labeled loops I to VIII (20). The longest loops (loops I, V, VI, and VII) show the greatest variation and are labeled variable region 1 (VR1), VR2, VR3, and VR4, respectively (20, 23). Monoclonal antibodies used for serotyping recognize conformational epitopes, although the significance of each VR in forming an epitope is not clear (3, 10, 23).

Methodologies that assess variation in \textit{porB}, or the protein it encodes, do not provide information on the clonal origins of isolates. This is because clonally diverse meningococci share common \textit{porB} genes (6). Meningococci exchange DNA with high frequencies such that organisms with the same \textit{porB} may not share other common genetic loci (13). Analysis of the genetic relatedness of meningococci has been achieved by the use of multilocus sequence typing (MLST) (14) and, more recently, multilocus restriction typing (MLRT) (5, 8).

New Zealand has experienced an epidemic of serogroup B meningococcal disease since mid-1991 (15). Two subclones (ST-42 and ST-154) of the hypervirulent ST-41/ST-44 complex, lineage III, have been the dominant cause of meningococcal disease throughout the epidemic (7). Meningococci of both subclones have the PorA serosubtype P1.4 with the genetic description P1.7–2,4. The P1.7–2 epitope is not identified by serosubtyping due to a 3-amino-acid deletion in the VR1 loop of the mature protein. Most group B meningococci with the P1.4 PorA serosubtype express type 4 PorB, although a number of different PorB types have been identified in association with B:P1.7–2,4 meningococci (8). This study was undertaken to determine the nature of the genetic events that, during the course of the epidemic, had resulted in either alternative PorB types being associated with B:P1.7–2,4 meningococci or the failure of the expressed PorB protein to type with the monoclonal antibodies available. This work will serve as a reference point to determine if the administration of a strain-specific vaccine increases \textit{porB} variation in New Zealand case isolates. It also complements a study analyzing the stability of PorA in the context of New Zealand’s epidemic.

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tasures were grown on 5% sheep blood agar plates (Fort Richard Laboratories, Auckland, New Zealand) at 36°C, in an atmosphere of 5% CO₂, for 18 h. All nonserotypeable meningococci were typed by use of porB-PCR-amplicon restriction endonuclease analysis (AREA) (8). Variation in porB sequence data in New Zealand case isolates was assessed using the same 30 isolates used to develop porB-PCR-AREA (8). They were selected on the basis of their diverse serological typing results.

DNA extraction. Genomic DNA was purified from other meningococcal cecal components with cetyltrimethyl ammonium bromide (Sigma, St. Louis, Mo.) followed by phenol and chloroform extractions (16). DNA was quantitated using Picogreen fluorescein dye (Molecular Probes, Eugene, Oreg.) in a fluorometer (BMG LabTechnologies, Offenburg, Germany). Stock DNA was stored at −70°C, and 5-ng/μl dilutions made in TE buffer (10 mM Tris, 1 mM EDTA) were stored at 4°C.

PCR-based typing. A PCR master mix (QIAGEN, Hilden, Germany) was used to amplify the PCR products. The porB PCR products were amplified and sequenced using the PorB forward (F) and PorB reverse (R) primers as described previously (8). Fragments of porB were amplified to determine if an undefined sequence was present in porB VR1 by using the PorB under (5′-GAT TAC CAG GAC GGT CAA GTT-3′) and the PorB R primers at 1 μM. Amplification reaction mixtures were incubated at 94°C for 2 min, followed by 30 cycles at 94°C for 40 s, 56°C for 40 s, and 70°C for 60 s. Extension was completed at 72°C for 3 min. MLRT (9) and MLST (14) were carried out as described previously, with the addition of primers to amplify fumC (fumC-A1, 5′-CAC CGA ACA CGA CAC GAT GG-3′, and fumC-A2, 5′-ACG ACC AGT TCG TCA AAC TC-3′).

Sequencing. Sequencing was carried out using a model 3100 genetic sequencer (Applied Biosystems, Foster City, Calif.). Sequence data analysis was carried out using sequence analysis programs (DNASTAR, Inc., Madison, Wis.). To assign porB VR sequences to families, nucleotide sequences were compared with previously described VR sequences (17).

Nucleotide sequence accession numbers. The porB sequence data from New Zealand case isolates were submitted to GenBank under accession numbers AY342211, AY586255 to AY586271, and AY745812.

RESULTS

Acquisition of a new porB. Most of the 2,026 B:P1.4 meningococci isolated from clinical cases of disease in New Zealand from 1990 through 2003 had type 4 porB (87.0%, 1,762 of 2,026). This total included the five isolates obtained in 1990, the year prior to the recognized start of the epidemic. Of the remaining B:P1.7-2,4 meningococci, 4.6% (94 of 2,026) were type 14 porB and 4.4% (90 of 2,026) contained the porB VR1-19, VR2-D, VR3-7, and VR4-14a sequences. When the clonal origins of 131 of the B:x:P1.7–2,4 (where x is other than 4) meningococci were assessed using MLRT, all except one had restriction profiles indicating that they belonged to the ST-41/ST-44 complex, lineage III. The one exception (strain NZ98/66) was sequenced using the PorB forward (F) and PorB reverse (R) primers as described previously (8). Fragments of porB were amplified to determine if an undefined sequence was present in porB VR1 by using the PorB under (5′-GAT TAC CAG GAC GGT CAA GTT-3′) and the PorB R primers at 1 μM. Amplification reaction mixtures were incubated at 94°C for 2 min, followed by 30 cycles at 94°C for 40 s, 56°C for 40 s, and 70°C for 60 s. Extension was completed at 72°C for 3 min. MLRT (9) and MLST (14) were carried out as described previously, with the addition of primers to amplify fumC (fumC-A1, 5′-CAC CGA ACA CGA CAC GAT GG-3′, and fumC-A2, 5′-ACG ACC AGT TCG TCA AAC TC-3′).

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Evidence for mosaic porB. Four porB VR combinations that differed from the combinations described by Sacchi et al. (17) were identified on one occasion each (in strains NZ97/358, NZ99/185, NZ99/186, and NZ99/243) (Table 1). Comparison of the porB VR sequences from these strains with the porB VR sequences previously described indicated that strains NZ97/358, NZ99/185, NZ99/186, and NZ99/243 might have acquired foreign DNA encoding one porB VR. To determine if evidence for horizontal transfer could be found, porB sequence data from strains NZ97/358, NZ99/185, NZ99/186, and NZ99/243 were compared to porB sequence data from 30 New Zealand meningococci with the combinations of porB VR sequences previously described. Outside the regions encoding the four porB VRs, there were 26 nucleotide positions that differed between strains. Of the 26 positions, 9 are illustrated in Table 2 and these highlight the differences between the porB sequences. All 12 serotype 4 meningococci with the porB VR1-4, VR2-D, VR3-7, and VR4-14a sequences had identical sequence data over the 1.1-kb region sequenced. They are represented by strains NZ92/18 and NZ99/38 (Table 2). Three type 14 meningococci contained identical porB genes, and this sequence is represented by strain NZ99/95 (Table 2). Sequences from the other two serotype 14 meningococci each differed by a single-nucleotide substitution compared to porB from strain NZ99/95. The porB sequence data from isolates shown in Table 2 were submitted to GenBank under accession numbers AY342211 and AY586255 to AY586271.

Comparison of the four porB VR sequences in strain NZ97/358 (B:NT:1,7–2,4) indicated that this meningococcus might have acquired the porB VR4-1 sequence, as all other VRs were identical to those found in serotype 14 meningococci (Table 1). The single-nucleotide substitutions in porB from strain NZ97/358 were identical to those found in the serotype 14 meningococcus NZ99/95 (Table 2). The only nucleotide substitution after porB VR3 was identical to that found in both serotype 1 and serotype 14 meningococci (Table 2). Similarly, comparison of the four porB VR sequences and single-nucleotide substitutions in strain NZ99/185 (B:NT:1,7–2,4) and strain NZ99/243 (B:14:1,7–2,4) indicated that these meningococci might have acquired the porB VR4-14a and porB VR1-4 sequences, respectively.

Comparison of the four porB VR sequences in strain NZ99/186 (B:1,7–2,4) indicated that this meningococcus might have acquired the porB VR2-D and VR3-7 sequences from a serotype 4 meningococcus. However, the VR2 and VR3 sequences in strain NZ99/186 differed from other serotype 1 meningococci either found in this study or reported on by Sacchi and coworkers (17) but were identical to those found in serotype 4 meningococci. The polymorphisms in strain NZ99/186 (including those not illustrated in Table 2) appeared unique to this meningococcus.

Ninety nonserotypeable meningococci isolated between 1990 and 2003 inclusive had the porB VR1-19, VR2-D, VR3-7, and VR4-14a sequences. This included strain NZ96/201 (Tables 1 and 2). Comparison of the VR sequences in this porB
indicated that it might have originated following the horizontal transfer and recombination of DNA encoding the porB VR1-19 sequence by a meningococcus with the porB VR1-4, VR2-D, VR3-7, and VR4-14a sequences. Comparison of single-nucleotide polymorphisms supported this hypothesis (Table 2).

## Table 2. Single-nucleotide differences between porB VR sequences from meningococci with various porB types

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype</th>
<th>VR1 at indicated position in porB sequence</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NZ92/18</td>
<td>B:4:P1.4</td>
<td>T  4  C  C  C  G  D  A  C  T  7  14a</td>
<td>AY586255</td>
</tr>
<tr>
<td>NZ99/38</td>
<td>B:4:P1.4</td>
<td>T  4  C  C  C  G  D  A  C  T  7  14a  A</td>
<td>AY342211</td>
</tr>
<tr>
<td>NZ99/243</td>
<td>B:14:P1.4</td>
<td>T  4  C  C  C  A  Db  G  T  C  7c  14  G</td>
<td>AY586267</td>
</tr>
<tr>
<td>NZ99/18</td>
<td>B:14:P1.4</td>
<td>T  19  T  T  T  A  Db  G  T  C  7c  14  G</td>
<td>AY586263</td>
</tr>
<tr>
<td>NZ99/95</td>
<td>B:14:P1.4</td>
<td>G  19  T  T  T  A  Db  G  T  C  14  G</td>
<td>AY586270</td>
</tr>
<tr>
<td>NZ99/300</td>
<td>B:14:P1.4</td>
<td>G  19  T  T  T  A  Db  G  T  C  14  G</td>
<td>AY586268</td>
</tr>
<tr>
<td>NZ97/358</td>
<td>B:NT:P1.4</td>
<td>G  19  T  T  T  A  Db  G  T  C  7c  1  G</td>
<td>AY586261</td>
</tr>
<tr>
<td>NZ99/185</td>
<td>B:NT:P1.4</td>
<td>G  19  T  T  T  A  Db  G  T  C  14a  A</td>
<td>AY586265</td>
</tr>
<tr>
<td>NZ96/201</td>
<td>B:NT:P1.4</td>
<td>G  19  T  T  T  A  Db  G  T  C  14a  A</td>
<td>AY586259</td>
</tr>
<tr>
<td>NZ79/111</td>
<td>B:NT:P1.4</td>
<td>G  19  T  T  T  G  D  A  C  T  7  14a  A</td>
<td>AY586260</td>
</tr>
<tr>
<td>NZ99/186</td>
<td>B:NT:P1.4</td>
<td>G  19  T  T  T  G  D  A  C  T  7  14a  A</td>
<td>AY586266</td>
</tr>
<tr>
<td>NZ99/86</td>
<td>B:1:P1.4</td>
<td>T  19  T  T  C  G  Ac  G  T  T  7a  1  G</td>
<td>AY586262</td>
</tr>
<tr>
<td>NZ99/117</td>
<td>B:1:P1.4</td>
<td>G  19  T  T  C  G  Ac  G  T  T  7a  1  G</td>
<td>AY586264</td>
</tr>
<tr>
<td>NZ99/243</td>
<td>B:1:P1.4</td>
<td>G  19  T  T  C  G  Ac  G  T  T  7a  1  G</td>
<td>AY586258</td>
</tr>
<tr>
<td>NZ99/190</td>
<td>B:1:P1.4</td>
<td>T  19  T  T  C  G  Ac  G  T  T  7a  1  G</td>
<td>AY586264</td>
</tr>
<tr>
<td>NZ96/120</td>
<td>B:NT:P1.4</td>
<td>T  undef  C  T  C  G  Ac  G  T  T  10  14a</td>
<td>AY586258</td>
</tr>
<tr>
<td>NZ97/358</td>
<td>B:NT:P1.4</td>
<td>T  undef  C  T  C  G  Ac  G  T  T  10  14a</td>
<td>AY586258</td>
</tr>
<tr>
<td>NZ99/185</td>
<td>B:NT:P1.4</td>
<td>G  19  T  T  T  A  Db  G  T  C  7c  14a  A</td>
<td>AY586265</td>
</tr>
<tr>
<td>NZ96/201</td>
<td>B:NT:P1.4</td>
<td>G  19  T  T  T  A  Db  G  T  C  7c  14a  A</td>
<td>AY586259</td>
</tr>
<tr>
<td>NZ295/170</td>
<td>B:NT:P1.4</td>
<td>T  undef  C  T  C  G  D  G  T  C  7  14a  A</td>
<td>AY586257</td>
</tr>
<tr>
<td>NZ94/178</td>
<td>B:NT:P1.4</td>
<td>T  undef  C  T  C  G  undef  A  T  C  7b  14a  A</td>
<td>AY586256</td>
</tr>
</tbody>
</table>

* Numbers indicating the position of the mutation refer to the nucleotide position in NZ99/38, with positions one to three coding for the first amino acid (van der Ley et al. [20]).
* porB VR types as defined by Sacchi et al. (17).
* Meningococci with a mosaic porB gene.

**Variation in porB as a result of single-nucleotide substitutions.** Five meningococci that encoded porB with a single-nucleotide substitution in one of the porB VR sequences were identified. The porB VR4 sequence in strain NZ98/17 (B:NT: P1.4, 5'-TCG TT TAT GAT GTA GAC TTA AGC AAC GAT 3') differed from the porB VR4-14a sequence (5'-TCG TT TAT GAT GTA GAC TTA AGC AAC GAT 3') by the substitution of an adenine residue for a guanine residue. This substitution would have resulted in the expression of glycine in strain NZ98/17 instead of the asparagine residue expressed by meningococci encoding the VR4-14a sequence. Four meningococci were identified with the undef, undef, 7b, 14a porB type. The porB VR2 sequence found in these meningococci (5'-CAG GAT GTG GAT AAC GTG AAG 3') differed from the porB VR2-D sequence (5'-CAG GAT GTG GAT AAC GTG AAG 3') by the substitution of a guanine residue for an adenine residue and the porB VR2-Db sequence (5'-CAG AAT GTG GAT AAC GTG AAG 3') by the substitution of an adenine residue for a guanine residue. This substitution (underlined below) would have resulted in the expression of the QDVDDVK VR amino acids in meningococci with the porB VR2-D sequence, the QNDVNDVK VR amino acids in meningococci with the porB VR2-Db sequence, and the QDVDDVK VR amino acids in meningococci with the undefined porB VR2 sequence.
DNA sequencing confirmed the value of the genetic characterization of \textit{porB} to demonstrate \textit{porB} sequence variability in isolates with the same group and PorA subtype. \textit{PorB} variation in New Zealand \textit{B:P1.7–2,4} case isolates was detected by serotyping, although serotyping failed to show the full extent of \textit{porB} variation.

Identification of phenotype \textit{B:4:P1.4} has tended to be a good marker of meningococci belonging to the ST-41/ST-44 complex, lineage III (7). However, meningococci that belong to this complex yet have a different serotype and/or serosubtype have been identified (2, 18, 21). Since all except one B:x:P1.4 meningococcus belonged to the ST-41/ST-44 complex of lineage III, the B:x:P1.4 phenotypes most likely occurred as the result of horizontal transfer and recombination of DNA encoding part of the \textit{porB} genes. B:P1.7–2,4 meningococci with \textit{porB} types 1 and 14 were found in both subclones (RT-42 and RT-154), indicating that the DNA encoding these \textit{porB} was acquired independently by meningococci belonging to both subclones.

As serotyping cannot differentiate between all \textit{porB} sequence variants, it was logical to conclude that there could be \textit{porB} variants within the New Zealand population that serological typing could not detect. Analysis of some nonserotypeable meningococci showed that they had mosaic \textit{porB}, probably resulting from the horizontal transfer and recombination of DNA encoding part of the \textit{porB}. There was no evidence to suggest that partial deletion of the epitope-encoding region led to \textit{PorB} variation, as was found to be the case for PorA variation in lineage III (ST-41/ST-44) meningococci from The Netherlands (2) and New Zealand (6a).

The most likely origin of the type 19,D,7,14a \textit{porB} gene is the acquisition of DNA encoding a new VR1 sequence by a meningococcus with a type 4 \textit{porB} gene. This was supported by the single-nucleotide polymorphisms in \textit{porB}. It is more unlikely that DNA encoding the \textit{porB} VR2-D, VR3-7, and VR4-14a sequences was acquired by a meningococcus with the \textit{porB} VR1-19 sequence due to the longer fragment of DNA that would have to have been transferred. The combination of \textit{porB} VR sequences in type undefined,D,7,14a \textit{porB} might also have originated following the acquisition of a new \textit{porB} VR1 sequence by a meningococcus with type 4 \textit{porB}. However, the large number of differences in the single-nucleotide polymorphisms between the VRs in these \textit{porB} genes does not support this hypothesis. Rather, it suggests that the undefined,D,7,14a \textit{porB} type originated following a number of independent recombinational events.

A number of meningococci that contained a region in \textit{porB} VR1 that had not been described by Sacchi et al. (17) were identified. More recently, this VR1 sequence was described by Urwin and coworkers (19) in combination with the VR2-D, VR3-7b, and VR4-14a sequences. Urwin and coworkers reported that meningococci with the undefined,D,7b,14a \textit{porB} type could be serotyped using the MN14G21 serotype 4 monoclonal antibody in a whole-cell enzyme-linked immunosorbent assay. We also used this antibody in a whole-cell enzyme-linked immunosorbent assay, but none of the meningococci identified as containing the undefined,D,7,14a \textit{porB} type were serotypeable. This was most likely due to the presence of the VR3-7 sequence rather than the VR3-7b sequence found in the meningococci typed by Urwin and coworkers (19).

One meningococcus strain, NZ01/207, expressing a mosaic \textit{porB} gene was initially detected because it typed with both serotype 4 and serotype 15 monoclonal antibodies. Serotypes 4 and 15 normally occur on serologically distinct meningococci. Serotype 4 meningococci are associated with a number of hypervirulent meningococcal clones, including serogroup A meningococci belonging to subgroups I, III, and V; the ST-41/ST-44 complex, lineage III; and the ET-5 complex (6). Serotype 15 meningococci are typically associated with meningococci belonging to the ET-5 clonal complex that have phenotype B:15:P1.7,16 (6). Both strain NZ01/207 and the Chilean strain Ch501 (4) had the \textit{porB} VR1 sequence serotype 4 meningococci and \textit{porB} VR2, VR3, and VR4 sequences identical to those found in serotype 15 meningococci. Sequence differences indicated that the first 200 bp of strains NZ01/207 and Ch501 had different origins. The most likely explanation is that the first 200 bp in strain Ch501 came from a meningococcus with \textit{porB} VR1-4, VR2-B, VR3-7, and VR4-14a sequences while the first 200 bp in strain NZ01/207 came from a meningococcus with \textit{porB} VR1-4, VR2-D, VR3-7, and VR4-14a sequences, the common \textit{porB} type found in meningococci causing New Zealand’s epidemic.

Point mutation rather than horizontal transfer and recombination was implicated as the mechanism behind the sequence variation in some of the \textit{porB} variants. As the point mutations identified did not occur in sequences encoding serotyping antibody recognition sites, the implication of these mutations with respect to the binding of antibodies is not known. The mutations encode amino acids in the surface-exposed loops of \textit{PorB}, and these loops would be accessible to antibodies produced during an immune response.

To date, type 4 \textit{PorB} proteins have been dominant on the \textit{B:P1.7–2,4} meningococci causing the epidemic, although a number of other \textit{porB} types have been identified both in this study and in previous work (8). The strain-specific vaccine designed to provide protection against meningococci causing New Zealand’s epidemic contains type 4 \textit{PorB} (12). It is unlikely that antibodies elicited to type 4 \textit{PorB} would recognize a number of the different \textit{porB} VRs identified on the strains described in this study. The description of \textit{porB} variation in New Zealand case isolates identified since 1991 provides a reference for determining the impact on \textit{porB} heterogeneity following a New Zealand-wide vaccination program using a \textit{B:4:P1.7–2,4} strain-specific vaccine.

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