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Sequence Variation in the *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 *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 *porB* gene, multilocus restriction typing validated against multilocus sequence typing was used. This determined that B:P1.7–2,4 meningococci with a *porB* gene that was other than type 4 had the same clonal origin. It was concluded that strains with alternative *porB* genes had diverged from the original type 4 *porB*. Variation in *porB* was also shown to be associated with the uptake of DNA encoding one or two of the PorB variable regions leading to mosaic *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 *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 intrastrain 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 *porB* locus. PorA and PorB are important epidemiological markers that are the targets of serosubtyping and serotyping 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 *porB*, or the protein it encodes, do not provide information on the clonal origins of isolates. This is because clonally diverse meningococci share common *porB* genes (6). Meningococci exchange DNA with high frequencies such that organisms with the same *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 *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.

MATERIALS AND METHODS

Meningococcal isolates. Meningococci used in this study originated from cases of meningococcal disease in New Zealand and were referred to the Meningococcal Reference Laboratory, Institute of Environmental Science and Research (ESR), under New Zealand's national surveillance program. All meningococci were assigned an isolate number consisting of the last two numbers of the year in which they were isolated, followed by a unique identifying number.

Meningococci referred to the reference laboratory were routinely serogrouped, serotyped, and serosubtyped (1) and were maintained at −70°C in glycerol broth suspensions (Trypticase soy broth, 15% [vol/vol] glycerol). Cul-

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tures were grown on 5% sheep blood agar plates (Fort Richard Laboratories, Auckland, New Zealand) at 36°C, in an atmosphere of 5% CO_2, 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 cellular components with cetyltrimethyl ammonium bromide (Sigma, St. Louis, Mo.) followed by phenol and chloroform extractions (16). DNA was quantitated using PicoGreen fluorescent 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 undef (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) isolates 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 defined as ST-2672, which differs at all seven loci compared to the multilocus sequence types causing disease in New Zealand (ST-42 and ST-154). Strain NZ98/66 was also unusual in that it contained a *porB* type other than type 4, 14, or 19,D,7,14a (VR1,VR2,VR3, and VR4).

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.

TABLE 1. *porB* types of meningococci isolated during the epidemic identified as having a mosaic *porB*

Strain	Phenotype	*porB* type[a] (VR1, VR2, VR3, VR4)	GenBank accession no.
NZ96/201[b]	B:NT:P1.4	19,D,7,14a	AY586259
NZ97/358	B:NT:P1.4	19,Db,7c,1	AY586261
NZ99/185	B:NT:P1.4	19,Db,7c,14a	AY586265
NZ99/186	B:NT:P1.4	19,D,7,1	AY586266
NZ99/243	B:14:P1.4	4,Db,7c,14	AY586267
NZ01/207	B:4,15:P1.4	4,A,A,Ba	AY745812

[a] *porB* VR sequences as defined by Sacchi *et al.* (17).
[b] Strain NZ96/201 was 1 of 90 meningococci predicted to contain this *porB* type by *porB* PCR-AREA.

Comparison of the four *porB* VR sequences in strain NZ97/358 (B:NT:P1.7–2,4) indicated that this meningococcus might have acquired the *porB* VR4-1 sequence, as all its 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:P1.7-2,4) and strain NZ99/243 (B:14:P1.7-2,4) 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:P1.7-2,4) 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*

TABLE 2. Single-nucleotide differences between *porB* VR sequences from meningococci with various *porB* types

Strain	Phenotype	Nucleotide at indicated position in *porB* sequence[a]												GenBank accession no.
		−35	VR1[b]	207	258	297	568	VR2	624	673	651	VR3	VR4	944	
NZ92/18	B:4:P1.4	T	**4**	C	C	C	G	**D**	A	C	T	**7**	**14a**	A	AY586255
NZ99/38	B:4:P1.4	T	4	C	C	C	G	D	A	C	T	7	14a	A	AY342211
NZ99/243[c]	B:14:P1.4	T	4	C	C	C	A	Db	G	T	C	7c	14	G	AY586267
NZ99/18	B:14:P1.4	T	19	T	T	T	A	Db	G	T	C	7c	14	G	AY586263
NZ99/95	B:14:P1.4	G	19	T	T	T	A	Db	G	T	C	7c	14	G	AY586270
NZ99/300	B:14:P1.4	G	19	T	C	T	A	Db	G	T	C	7c	14	G	AY586268
NZ97/358[c]	B:NT:P1.4	G	19	T	T	T	A	Db	G	T	C	7c	1	G	AY586261
NZ99/185[c]	B:NT:P1.4	G	19	T	T	T	A	Db	G	T	C	7c	14a	A	AY586265
NZ96/201[c]	B:NT:P1.4	G	19	T	T	T	G	D	A	C	T	7	14a	A	AY586259
NZ97/111[c]	B:NT:P1.4	G	19	T	T	T	G	D	A	C	T	7	14a	A	AY586260
NZ99/186[c]	B:NT:P1.4	G	19	T	T	T	G	D	A	C	T	7	1	G	AY586266
NZ99/86	B:1:P1.4	T	19	T	T	C	G	Ac	G	T	T	7a	1	G	AY586269
NZ99/117	B:1:P1.4	G	19	T	T	C	G	Ac	G	T	T	7a	1	G	AY586262
NZ99/190	B:1:P1.4	T	19	T	T	C	G	Ac	G	T	T	7a	1	G	AY586264
NZ96/120	B:NT:P1.4	T	undef	C	T	C	G	Aa	G	T	T	10	14a	A	AY586258
NZ03/011	B:NT:P1.4	T	undef	C	T	C	G	D	G	C	T	7	14a	A	AY586271
NZ95/170	B:NT:P1.4	T	undef	C	T	C	G	D	G	T	C	7	14a	A	AY586257
NZ94/178	B:NT:P1.4	T	undef	C	T	C	G	undef	A	T	C	7b	14a	A	AY586256

[a] 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]).
[b] *porB* VR types as defined by Sacchi et al. (17).
[c] Meningococci with a mosaic *porB* gene.

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).

During routine serological typing, one meningococcus (strain NZ01/207) that was recognized by both the serotype 4 and serotype 15 monoclonal antibodies was identified. Sequencing *porB* from strain NZ01/207 showed that it contained the *porB* VR1-4, VR2-A, VR3-A, and VR4-Ba sequences (Table 1). The *porB* sequence data for a serotype 4 and 15 isolate from Chile (strain Ch501) (4) and strain NZ01/207 were identical around *porB* VR2, VR3, and VR4. Four single-nucleotide substitutions around VR1 differentiated *porB* from strains Ch501 and NZ01/207. Data surrounding *porB* VR1-4 in strain NZ01/207 were identical to those found in type 4 meningococci with the *porB* VR1-4, VR2-D, VR3-7, and VR4-14a sequences. Data surrounding *porB* VR1-4 in strain Ch501 were identical to those found for type 4 meningococci with the *porB* VR1-4, VR2-B, VR3-7, and VR4-14a sequences.

During *porB* sequencing, meningococci that had a sequence in *porB* VR1 (*porB* VR1-undef) and that previously had not been described by Sacchi et al. were identified (17). A forward primer designed to be complementary to the *porB* VR1-undef sequence, together with the PorB R primer, amplified a 900-bp product in 30 of the 251 B:NT:P1.4 meningococci isolated from 1990 through 2003. Sequencing the *porB* PCR product for 17 of these meningococci showed that all contained identical *porB* VR1 sequences, although different sequences were found in the other VRs. The sequences found were seven undef,D,7,14a *porB* genes (where "undef" indicates an undefined nucleotide); five undef,Aa,10,14a *porB* genes; four undef,undef,7b,14a *porB* genes; and one undef,Da,7,14a *porB* gene. All *porB* genes containing the VR1-undef sequence had identical sequences surrounding the *porB* VR1 region. The first meningococcus with the *porB* VR1-undef sequence was isolated in 1994 and had the undef,D,7,14a *porB* type. Comparison of the VR sequences in the undef,D,7,14a *porB* type and type 4 meningococci (4,D,7,14a) suggested that the undef,D,7,14a *porB* type was the result of a type 4 meningococcus acquiring the *porB* VR1-undef sequence. However, there were a large number of differences between the single-nucleotide polymorphisms of the 4,D,7,14a and undef,D,7,14a *porB* types.

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 TTT GAT GGT GCA GAC TTA AGC AAC GAT-3′) differed from the *porB* VR4-14a sequence (5′-TCG TTT GAT GAT GCA 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 GAC 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 QNVDNVK VR amino acids in meningococci with the *porB* VR2-Db sequence, and the QDVDNVK VR amino acids in meningococci with the undefined *porB* VR2 sequence.

DISCUSSION

DNA sequencing confirmed the value of the genetic characterization of *porB* to demonstrate *porB* sequence variability in isolates with the same group and PorA subtype. PorB variation in New Zealand B:P1.7–2,4 case isolates was detected by serotyping, although serotyping failed to show the full extent of *porB* variation.

Identification of phenotype 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 B:4:P1.4 meningococci acquiring alternative *porB* genes. B:P1.7–2,4 meningococci with *porB* types 1 and 14 were found in both subclones (RT-42 and RT-154), indicating that the DNA encoding these *porB* was acquired independently by meningococci belonging to both subclones.

As serotyping cannot differentiate between all *porB* sequence variants, it was logical to conclude that there could be *porB* variants within the New Zealand population that serological typing could not detect. Analysis of some nonserotypeable meningococci showed that they had mosaic *porB*, probably resulting from the horizontal transfer and recombination of DNA encoding part of the *porB*. There was no evidence to suggest that partial deletion of the epitope-encoding region led to 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 *porB* gene is the acquisition of DNA encoding a new VR1 sequence by a meningococcus with a type 4 *porB* gene. This was supported by the single-nucleotide polymorphisms in *porB*. It is more unlikely that DNA encoding the *porB* VR2-D, VR3-7, and VR4-14a sequences was acquired by a meningococcus with the *porB* VR1-19 sequence due to the longer fragment of DNA that would have to have been transferred. The combination of *porB* VR sequences in type undef,D,7,14a *porB* might also have originated following the acquisition of a new *porB* VR1 sequence by a meningococcus with type 4 *porB*. However, the large number of differences in the single-nucleotide polymorphisms between the VRs in these *porB* genes does not support this hypothesis. Rather, it suggests that the undef,D,7,14a *porB* type originated following a number of independent recombinational events.

A number of meningococci that contained a region in *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 undef,D,7b,14a *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 undef,D,7,14a *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 *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 *porB* VR1 sequence serotype 4 meningococci and *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 *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 *porB* VR1-4, VR2-D, VR3-7, and VR4-14a sequences, the common *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 *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 PorB, and these loops would be accessible to antibodies produced during an immune response.

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

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