Variation in the *Neisseria lactamica* porin, and its relationship to meningococcal PorB

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One potential vaccine strategy in the fight against meningococcal disease involves the exploitation of outer-membrane components of *Neisseria lactamica*, a commensal bacterium closely related to the meningococcus, *Neisseria meningitidis*. Although *N. lactamica* shares many surface structures with the meningococcus, little is known about the antigenic diversity of this commensal bacterium or the antigenic relationships between *N. lactamica* and *N. meningitidis*. Here, the *N. lactamica* porin protein (Por) was examined and compared to the related PorB antigens of *N. meningitidis*, to investigate potential involvement in anti-meningococcal immunity. Relationships among porin sequences were determined using distance-based methods and *F*<sub>ST</sub>, and maximum-likelihood analyses were used to compare the selection pressures acting on the encoded proteins. These analyses demonstrated that the *N. lactamica* porin was less diverse than meningococcal PorB and although it was subject to positive selection, this was not as strong as the positive selection pressures acting on the meningococcal porin. In addition, the *N. lactamica* porin gene sequences and the protein sequences of the loop regions predicted to be exposed to the human immune system were dissimilar to the corresponding sequences in the meningococcus. This suggests that *N. lactamica* Por, contrary to previous suggestions, may have limited involvement in the development of natural immunity to meningococcal disease and might not be effective as a meningococcal vaccine component.

INTRODUCTION

Meningococcal disease, caused by the Gram-negative bacterium *Neisseria meningitidis*, is a global health problem which cannot be comprehensively controlled by vaccination. Effective vaccines directed against the serogrouping antigen, the polysaccharide capsule, are available for disease caused by serogroups A, C, Y and W135 meningococci. However, a vaccine based on serogroup B polysaccharide has not been developed due to its poor immunological reactivity and similarity to host antigens (Finne et al., 1987). As serogroup B strains cause over 85% of meningococcal disease in the UK (Gray et al., 2006), alternative vaccine candidates are under investigation, including outer-membrane proteins (OMPs), either purified (Boslego et al., 1995) or as part of outer-membrane vesicle (OMV) formulations (Bjune et al., 1991; van der Ley et al., 1995).

The commensal bacterium *Neisseria lactamica*, which is closely related to both *N. meningitidis* and *Neisseria gonorrhoeae* (Guibourdenche et al., 1986), is carried in the upper respiratory tracts of young children, in whom carriage of the meningococcus is rare. As natural immunity to meningococcal disease develops during childhood, carriage of *N. lactamica* may be involved in the acquisition of this immunity (Gold et al., 1978). *N. lactamica* is only associated with disease in exceptional circumstances (Schifman & Ryan, 1983; Wilson & Overman, 1976) and as it does not possess a polysaccharide capsule (Griffiss et al., 1987; Kim et al., 1989) or the outer-membrane protein PorA (Ward et al., 1992), this commensal or its antigens can be used in anti-meningococcal vaccines that are independent of both serogroup and serosubtype. Vaccines based on *N. lactamica* whole cells, OMPs, or OMVs have been proposed (Griffiss et al., 1991; Oliver et al., 2002) and research and development of a vaccine
based on *N. lactamica* is ongoing (Finney et al., 2007; Li et al., 2006). However, the antigenic profiles of *N. lactamica* isolates are not well understood, and the cross-reactive epitopes that induce protection against meningococcal infection have not been defined (Tang et al., 1999). This may constitute a major obstacle to the development of vaccines based on this species.

One protein that could be involved in the induction of cross-protective immune responses is the *N. lactamica* porin (Troncoso et al., 2002). Porins, which are essential for growth, are pore-forming membrane proteins that create channels, allowing transport of hydrophilic molecules across lipid bilayers (Achouak et al., 2001) and are generally highly expressed. These proteins exist as trimers and consist of 16 anti-parallel β-strands connected by short periplasmic turns and eight surface-exposed loops (Maiden et al., 1991; van der Ley et al., 1991). The regions corresponding to the surface-exposed loops are less well conserved than the domains corresponding to the β-sheets, and show variation in both length and sequence, presumably as a consequence of immune selection (Maiden et al., 1991; van der Ley et al., 1991).

The *N. lactamica* porin is related to gonococcal PorB1a and PorB1b and to meningococcal PorB (Derrick et al., 1999; Ward et al., 2002) and is essentially identical to the *Neisseria polysaccharea* porin (Derrick et al., 1999). Only these members of the genus contain three conserved lysine residues, found in close proximity within the pore, which form a potential GTP-binding site implicated in pathogenesis, consistent with a role in regulating pore function when inserted into host cells (Derrick et al., 1999; Rudel et al., 1996). Meningococcal PorB proteins are divided into two distinct, mutually exclusive classes designated PorB2 and PorB3. These proteins exhibit high levels of genetic diversity, especially in the surface-exposed regions, and these regions, with the exception of putative loops II and III, are subject to the diversifying influence of immunological selection (Urwin et al., 2002). Putative loops II and III have structural roles; loop II is important in monomer–monomer interactions within the porin trimer, while loop III is sequestered in the pore of each monomer, and may be involved in regulating pore function (Derrick et al., 1999).

Here, *N. lactamica* por sequences, in particular the regions encoding the putative surface-exposed loops, were examined and compared to *porB* of *N. meningitidis*, to investigate the diversity of the encoded proteins and to assess whether these *N. lactamica* proteins could be involved in the production of cross-protective immune responses to the meningococcus. This was achieved by comparing 69 unique *N. lactamica* por gene sequences, obtained from isolates previously characterized by multilocus sequence typing (MLST) (Bennett et al., 2005) with a diverse collection of meningococcal *porB* sequences (Urwin et al., 2002). Distance-based methods and FST were used to determine relationships among the porins and maximum-likelihood analyses were used to investigate the selection pressures acting on these proteins in *N. lactamica*. The results indicated that the *N. lactamica* porin was only subject to weak positive selection pressures, had limited sequence similarity to meningococcal PorB and was less diverse than meningococcal PorB. This suggests that it might not be suitable for inclusion in anti-menningococcal vaccine formulations.

**METHODS**

**Isolates.** A total of 587 individuals, including infants and a small number of siblings and parents, were sampled in Oxfordshire, UK, during two longitudinal studies of nasopharyngeal bacterial carriage in infants, resulting in 275 *N. lactamica* isolates. The genetic diversity of these *N. lactamica* isolates was similar to that seen in isolates from other locations and likely to be representative of the global diversity of this organism. There is no evidence that the Oxfordshire isolates were a separate population and the collection contained representatives from five of the six clonal complexes currently defined for *N. lactamica* (Bennett, 2006). Details of these isolates are available from the Neisseria MLST database: http://pubmlst.org/neisseria/ (Jolley et al., 2004). Nucleotide sequences were determined for the entire *por* gene from all isolates. The majority had been collected from the same infants on successive occasions, enabling an analysis of the effect of carriage on the *N. lactamica* porin. The nucleotide and amino acid sequences from the *N. lactamica* porins were compared to the porin sequences from a diverse collection of 324 meningococcal isolates: http://neisseria.org/nm/typing/porb/ (Urwin et al., 2002) which included sequences from both carried and disease-associated isolates.

**PCR amplification and sequence determination of por genes.** Amplification of the *por* gene was carried out by PCR with oligonucleotide primers B21 and B22 (Table 1). Amplification reaction mixtures contained reaction buffer [10 mM Tris/HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001 % (w/v) gelatin]; 200 μM each of dATP, dCTP, dGTP, dTTP; 1 μM of each primer; 1.25 units *Taq* polymerase (AmpliTaq; Applied Biosystems) per 50 μl; and 0.5 μl template DNA per 50 μl (approx. 50 ng μl⁻¹). The PCR conditions consisted of an initial denaturation step of 94 °C for 2 min, followed by 35–40 cycles of denaturation (94 °C for 1 min), annealing (50–5 °C for 1 min), extension (72 °C for 2 min), and then a final extension step of 72 °C for 2 min. The PCR products were precipitated by incubation at room temperature for 30 min with 20 % (w/v) polyethylene glycol 8000, 2.5 M NaCl. After centrifugation for 60 min at 2750 g, the precipitates were washed twice in 70 % (v/v) ethanol, dried and resuspended in 5–10 μl sterile distilled water.

The nucleotide sequences of the amplified gene fragments were determined at least once on each DNA strand by cycle sequencing with BigDye Ready Reaction Mix (Applied Biosystems), used in accordance with the manufacturer’s instructions. Sequencing reactions were performed with the oligonucleotide primers listed in Table 1. Unincorporated dye terminators were removed by precipitation of the termination products with 95 % (v/v) ethanol and the labelled extension products were separated and detected with either a Prism 3700 DNA analyser, or a Prism 377 XL DNA analyser (Applied Biosystems).

**Analysis of sequence data.** Nucleotide sequence data from forward- and reverse-strand chromatograms were assembled into single contiguous sequences using the Staden suite of computer programs (Staden, 1996). Sequences were manually aligned using the SeqLab program, part of the GCG Wisconsin package, version 10.3 (Womble, 2000). The alignment (available on request) was based on amino acid sequence similarity, with codon integrity maintained.
Table 1. Oligonucleotide primers used in N. lactamica por sequencing

| Primer       | Sequence (5′–3′)                     | Function               |
|--------------|--------------------------------------|------------------------|
| B21 (forward)* | CCAAAAAAGGAATACAGC                  | Amplification and sequencing |
| B22 (reverse)* | GCAGATTAGAATTGTGG                    | Amplification and sequencing |
| 8U (forward)† | TCCGTACGCTAGATTCTCC                  | Sequencing              |
| 8L (reverse)† | GGGAATCCTAGGATACGGA                  | Sequencing              |
| 94U (forward)† | CTCAAAACGAAATGTGGCG                  | Sequencing              |
| 94L (reverse)† | CGGGCAGTTTGGTTGAG                    | Sequencing              |

*Ward et al. (1992).
†Suker (1997).

Each unique por allele was given a number in order of discovery, so that alleles were named por-1 to por-69. To allow comparisons of N. lactamica por with the published meningococcal porB sequences (http://neisseria.org/nm/typing/porb/) (Urwin et al., 2002), all sequences were truncated to the same length, so that the amino acid sequences began at the 32nd amino acid of the N. lactamica Por sequence (starting motif EITYRT) and ended with the 336th amino acid (motif STAST). MEGA version 2.1 (Kumar et al., 2001) was used to calculate genetic distances between sequences and to produce neighbour-joining trees. To construct the tree from nucleotide sequences, all three coding positions were examined and the Kimura two-parameter distance correction (Kimura, 1980) was applied. A gamma shape parameter was not calculated as the inclusion of this parameter in the analysis had a minimal effect on the phylogeny produced. To produce the trees from amino acid sequences, p-distances were used. The reliability of the inferred trees was assessed using the bootstrap test (2000 replications). FST values were calculated using Arlequin version 2.000 (Schneider et al., 2000). Alignment gaps were excluded from all analyses.

**Analysis of selection pressures.** A maximum-likelihood approach was used to examine selection pressures acting on individual codon sites of the N. lactamica por gene. A phylogenetic tree of the aligned sequences was constructed using PAUP, version 4 (Swofford, 1998), using the HKY85 model of nucleotide substitution. Selection pressures on the aligned por sequences were examined using CODEML, implemented in the PAML program (Yang, 1997), in which codon substitution models were compared using the maximum-likelihood tree and the nucleotide sequence data. The $d_{0}/d_{2}$ ratio (parameter $\omega$) was calculated codon by codon using different models of codon substitution that differed in how $\omega$ varied along the sequences. Model M0 estimated a single $\omega$ parameter for all sites, whereas the M1 model divided codons into two site categories: conserved sites ($p_0$), with $\omega_0$ set at 0, and neutral sites ($p_1$), with $\omega_1$ set at 1. To account for positive selection the M2 model was used, with the same two classes as M1 plus a third category of sites ($p_2$), with $\omega_2$ estimated from the data. The M3 model was a more sensitive test of selection as it estimated $\omega$ values for three classes of sites, all of which could be >1. Models M7 and M8 both used a discrete beta distribution (with ten categories described by parameters $p$ and $q$) to model $\omega$ ratios among sites, although M8 also included an additional class of sites for which $\omega$ could be >1.

Nested models were compared using a likelihood ratio test (LRT). Twice the difference in log-likelihood between models was compared with the value obtained under a $\chi^2$ distribution, with degrees of freedom equal to the difference in the number of parameters between models. Finally, empirical Bayesian methods were used to calculate the probability that a particular codon site belonged to a specific class.

**RESULTS**

**Comparative diversity and relationships between N. lactamica por and meningococcal porB allele sequences**

A total of 69 unique N. lactamica por alleles, obtained from 275 isolates, were aligned with 46 porB2 and 77 porB3 sequences, obtained from 324 diverse meningococcal isolates (Urwin et al., 2002). A neighbour-joining tree was constructed from these sequences (Fig. 1), grouping the porin classes into three distinct clusters, supported by bootstrap values of 99%. The N. lactamica por sequences were more closely related to porB3 than porB2, with less diversity than both meningococcal porB classes. $F_{ST}$ analysis of por, porB2 and porB3 was used to assess levels of gene flow among the populations and gave values >0.85 ($P \leq 0.05$) for all three comparisons, indicating separate populations with little genetic exchange among them. A neighbour-joining tree constructed from the nucleotide sequences from the $\beta$-barrel-encoding regions alone, using the same alignment, produced a tree topology almost identical to the $F_{ST}$ analysis of the full sequence.

![Fig. 1. Neighbour-joining tree constructed from aligned gene sequences showing the relationship of N. lactamica por to meningococcal porB2 and porB3. Bootstrap values are shown.](http://mic.sgmjournals.org)
identical to that shown in Fig. 1 (data not shown). The diversity within meningococcal porB and N. lactamica por alleles was assessed (Table 2). The number of variable sites was comparable, with 147 for porB2, 136 for porB3 and 132 for por. Diversity, however, was greater within porB2 and porB3 (4.34% and 4.03% respectively) than in por (2.31%). There were 44 unique amino acid sequences for porB2, 65 for porB3 and 62 for por, with more variable sites within porB2 (70) and porB3 (61) than in por (49).

**Comparative diversity and relationships between N. lactamica Por and meningococcal PorB loop regions**

The amino acid sequence variants of the putative Por loop regions were named in accordance with the meningococcal PorB nomenclature used in the PorB database (http://neisseria.org/nm/typing/porb/), although the location and length of the loops were as defined previously (Derrick et al., 1999). Six variants were found in loop I, eight in loop II, 17 in loop III, four in loop IV, 10 in loop V, five in loop VI, and three in loops VII and VIII. The loops were of variable length (11 amino acids in loops II, IV and VI, 13 in loop VII, 16 in loop VIII, 18 in loop V, 23 in loop I and 41 in loop III). Some of the amino acid sequences were encoded by more than one nucleotide sequence, and one of the variants found in loop III was longer than the other variants by two dipeptides: ST and GI. The eight loop regions in N. lactamica Por were compared to the corresponding loops in meningococcal PorB (Table 2). Mean percentage p-distances within the loops of Por, PorB2 and PorB3 were determined, and for each loop, with the exception of loops II and III, divergence was greater within PorB2 and PorB3 than within Por.

| Table 2. Diversity within meningococcal porB and N. lactamica por genes |
|-----------------|-----------------|-----------------|
|                 | porB2           | porB3           | por             |
| No. of alleles  | 46              | 77              | 69              |
| Length of aligned sequences (bp) | 1053           | 894            | 1029           |
| No. of variable sites | 147            | 136            | 132            |
| Mean percentage genetic distance (Kimura’s two-parameter model) | 4.34           | 4.03           | 2.31           |
| No. of amino acid sequences | 44             | 65             | 62             |
| No. of variable sites | 70             | 61             | 49             |
| Mean percentage p-distance | 10.60         | 24.80          | 8.39           |
| Loop I          | 6.06            | 6.06           | 12.00          |
| Loop II         | 4.89            | 2.27           | 6.87           |
| Loop III        | 15.23           | 13.89          | 12.12          |
| Loop IV         | 24.51           | 30.67          | 13.77          |
| Loop V          | 32.78           | 28.86          | 14.72          |
| Loop VII        | 15.71           | 34.84          | 7.69           |
| Loop VIII       | 24.11           | 11.93          | 7.29           |

Neighbour-joining trees were constructed from the amino acid sequences of the loop regions from Por, PorB2 and PorB3 (Fig. 2). For the majority of the loops, distinct clusters, corresponding to the three different classes, were formed. In loop II the groups were indistinct and not well supported by bootstrap values (not included), with two PorB2 and two PorB3 amino acid sequences identical to Por sequences from N. lactamica. One of these PorB2 sequences was encoded by a nucleotide sequence identical to that of N. lactamica. Three distinct clusters were evident for loop III, with this loop more uniform in the meningococcal porins than in the N. lactamica porin. In loop VI the PorB2 variants formed a distinct group but the PorB3 variants were more closely related to N. lactamica Por. A loop VI PorB3 amino acid sequence was identical to the amino acid sequences from three N. lactamica alleles, and clustered with the N. lactamica sequences. This PorB3 variant was encoded by a nucleotide sequence identical to one of the N. lactamica variants.

**Maximum-likelihood analysis**

Maximum-likelihood analysis provided evidence for positive selection pressures acting on the por gene of N. lactamica (Table 3), as models with site classes where parameter \( \omega \) was permitted to be >1 (models M2, M3 and M8) were statistically significantly better than those in which it was not (models M0, M1, M7). However, the M3 model (a sensitive test of positive selection) was not significantly better supported than the more conservative M2 model (\( P=0.251 \)). Under the M2 model, 7.0% of sites fell into a positively selected class, where \( \omega=4.195 \). Under model M3, 7.1% of sites fell into a positively selected class, where \( \omega=3.936 \), 8.5% of sites fell into a neutrally evolving class where \( \omega=1.002 \) and the remaining 84.4% of sites were highly conserved (\( \omega=0.013 \)). Under model M8 7.3% of sites fell into a positively selected class, where \( \omega=3.886 \).

Bayesian methods were used to identify the sites with the highest probability of falling into the positively selected class under models M2, M3 and M8. A \( d_S/d_K \) ratio (parameter \( \omega \)) >1.5 was considered to be indicative of positive selection, and for model M2, there were nine selected sites with \( d_S/d_K \) ratios of 4.068–4.195. The same nine sites were identified using model M8, although the \( d_S/d_K \) ratios were 3.825–3.886. Model M3, which had previously been used to demonstrate selection in meningococcal PorB (Urwin et al., 2002) and a more sensitive test of selection than model M2, identified 16 additional selected sites with \( d_S/d_K \) ratios of 1.900–3.936. The majority of selected sites fell within predicted loop regions, with positive selection identified in loops I, II, IV, VI and VIII using all three models. Only model M3 identified selected sites within loops III, V and VII. All selected sites were mapped onto the translated sequence of por-2 (Fig. 3).

**A N. lactamica porin structural model**

A homology model for the N. lactamica porin was constructed in the same way as corresponding models for
meningococcal PorB2 and PorB3 (Urwin et al., 2002). The translated sequence of *N. lactamica* por-2 was aligned against that of the porin Omp32 from *Comamonas acidovorans* (Zeth et al., 2000), which was used as a template for structural alignment and refinement, as implemented by the software package MODELLER (Sali et al., 1995). The three-dimensional structural model of the *N. lactamica* porin is shown along with the published models of meningococcal PorB2 and PorB3 (Urwin et al., 2002) in Fig. 4. Positively selected sites, as inferred from maximum-likelihood analysis using model M3, were mapped onto this model, with model M2 and model M8 positively selected sites highlighted. Whereas a number of strongly selected sites were located within the surface-exposed loops for both meningococcal PorB2 and PorB3, only weakly positively selected sites were present in the *N. lactamica* porin structure. In the *N. lactamica* porin, using model M3, weakly positively selected sites were evident

### Table 3. Parameter estimates for the maximum-likelihood analysis of selection pressures acting on the *N. lactamica* porin

| Model | Site categories (p) and d_\text{dN/dS} (\omega) | Likelihood test | \chi^2 | P |
|-------|---------------------------------------------|----------------|-------|---|
| M0    | \omega=0.306                               |                |       |   |
| M1    | \rho_0=0.816, \rho_1=0.184                  | M0 vs M2       | 387.144 | <0.000 |
| M2    | \rho_0=0.818, \rho_1=0.116, \rho_2=0.070, \omega=4.195 | M1 vs M2       | 70.442 | <0.000 |
| M3    | \rho_0=0.844, \rho_1=0.085, \rho_2=0.071, \omega_0=0.013, \omega_1=1.002, \omega_2=3.936 | M0 vs M3       | 389.907 | <0.000 |
|       |                                             | M1 vs M3       | 73.205 | <0.000 |
|       |                                             | M2 vs M3       | 2.763  | 0.251 |
| M7    | \rho_1=0.026, \rho_0=0.118                  | M7 vs M8       | 72.475 | <0.000 |
| M8    | \rho_0=0.035, \rho_1=0.330, \rho_2=0.073, \omega_0=3.886 | M7 vs M8       | 72.475 | <0.000 |

Fig. 2. Neighbour-joining trees constructed from the aligned amino acid sequences from the loop regions of *N. lactamica* Por and meningococcal PorB2 and PorB3. Amino acid sequences identical in *N. lactamica* and *N. meningitidis* are indicated by dashed circles. Bootstrap values are shown.
within the pore. Weakly positively selected sites were also present within the pore of meningococcal PorB2, but not PorB3. The general structure of all three porins was similar, although the *N. lactamica* porin was closer to meningococcal PorB3, as some of the surface-exposed loops were shorter in both Por and PorB3.

### Selection pressures acting on *N. lactamica* porins during carriage

As the majority of *N. lactamica* isolates were collected from the same infants sampled on successive occasions, it was possible to examine the effects of carriage on the porins of these sequential isolates. There was long-term carriage of particular variants, with little evidence for replacement of variants. One child carried isolates with the MLST genotype ST-638 on seven successive samplings, between the ages of 12 weeks and 48 weeks, but at age 48 weeks the porin allele associated with these isolates altered by one nonsynonymous nucleotide substitution in loop VI. This allele was not present among the isolates obtained from any other subjects and is likely to have arisen by point mutation as a result of immune selection during carriage by this particular child. This site was also variable in other *N. lactamica* porin alleles (position 253, Fig. 3), with a \( d_N/d_S \) value of ~3.648, except for position 95 (\( d_N/d_S \) 1.487).

### DISCUSSION

Establishing the diversity of antigen genes from non-pathogenic *neisseriae* is important, as antigens common to *N. meningitidis*, *N. lactamica* and other non-pathogenic commensals may be involved in conferring natural immunity to meningococcal disease (Troncoso *et al.*, 2000, 2002). *N. lactamica* antigens are also components of anti-meningococcal vaccines currently being developed (Gorringe, 2005) and the identity and characterization of the key cross-reactive antigenic components is of great importance (Finney *et al.*, 2007). Although the porins of *N. meningitidis* have been thoroughly examined (Russell *et al.*, 2004; Urwin *et al.*, 2002), little information was available on the diversity of the *N. lactamica* porin and its relationship to meningococcal porins (Derrick *et al.*, 1999; Ward *et al.*, 1992). Here, the diversity of 69 *N. lactamica* por alleles was determined and compared to the related meningococcal *porB* alleles to assess the potential for antigenic cross-reactivity.

Another infant carried ST-608 isolates on eight successive occasions, from age 8 weeks until age 96 weeks, but seven different porin alleles were identified among these isolates. There were six polymorphic sites among these alleles (Fig. 3), with each change nonsynonymous, altering the structure of the porin. Most were found within and around loop III, which potentially influences pore function. One was found within loop V, which may have occurred as a result of immune selection. These sites with \( d_N/d_S \) values of ~3.648, except for position 95 (\( d_N/d_S \) 1.487).

Fig. 3. Positive selection acting on *N. lactamica* porin sequences. The translated sequence of *por*-2, one of the longest sequences, is used as an example. The locations of the putative loop regions (loops I–VIII) are indicated. Amino acid residues subject to positive selection under models M2 and M8 are identified with asterisks. Amino acid residues subject to positive selection under the M3 model are indicated with black boxes. Polymorphic sites in the porin alleles of the genotypically identical (ST-608) isolates carried by one infant are indicated by triangles. Figures at the end of each line of sequence denote residue number.
encode both the β-barrel regions and the loop regions. This suggests that the porins have not diverged recently.

The amino acid sequences of the surface-exposed loop regions were analysed individually and the loops that determine antigenic variability in N. meningitidis (loops I, IV, V, VI, VII and VIII) (Derrick et al., 1999) were dissimilar to the corresponding loops in N. lactamica. Loop II sequences were the most similar between N. lactamica and N. meningitidis, and sequences from two PorB2 and two PorB3 loop II variants were identical to loop II N. lactamica variants. As structural constraints are likely to limit diversity in this region (Derrick et al., 1999), these identical sequences are probably a consequence of shared ancestry rather than recent lateral genetic transfer. This is supported by the detection of minimal gene flow between these three populations. A single example of lateral genetic transfer between species may have occurred in loop VI. This loop was subject to positive immune selection and a meningococcal PorB3 variant was encoded by a nucleotide sequence identical to a N. lactamica sequence. However, two other N. lactamica variants had amino acid sequences identical to the PorB3 variant but were encoded by different nucleotide sequences.

Frequent genetic recombination between species has been described for _tbpB_ (Linz et al., 2000) and may also occur within the variable region of _fetA_ (J. S. Bennett, E. A. L. Thompson, P. Kriz, K. A. Jolley & M. C. J. Maiden, unpublished results). However, genetic exchange frequency decreases rapidly as a function of relatedness of the donor to the recipient (Majewski, 2001). As the _N. lactamica_ porin is distinct from the meningococcal porins, including PorA (Derrick et al., 1999), and does not experience strong selection pressures, frequent lateral genetic transfer of porin sequences between the two species is unlikely, in common with the housekeeping genes of _Neisseria_ (Bennett et al., 2007).

Porins, including those of _Neisseria_, have an internal eyelet region formed by a long loop III folded into the pore, with a negatively charged cluster of side-chains facing the positive charges from R/K residues derived from β-sheets forming the β-barrel wall (Achouak et al., 2001; Schirmer, 1998). This organization produces an electrostatic field in the lumen that regulates the diffusion of molecules through the constricted area (Achouak et al., 2001). Loop III was more variable in _N. lactamica_ Por than in meningococcal PorB. The amino acid sequences encoded by _por-2_ and _por-8_ were the longest _N. lactamica_ sequences due to two dipeptide insertions (S T and G I), occurring at the tip of loop III. As this loop folds back into the centre of the lumen, these insertions could affect pore conductance. The sequence motif found in this region of the translated sequences of _por-2_ and _por-8_ (STKDTGI) was not present in any other _N. lactamica_ or meningococcal sequence examined in this study and may have been transferred from another bacterial species by lateral genetic transfer.

Analysis of the selection pressures acting on the _N. lactamica_ porin revealed codons subject to positive selection, the majority of which were located within the loop regions. However, due to the variability of these regions in both _N. meningitidis_ and _N. lactamica_ and the difficulty in aligning the amino acid sequences unambiguously, it was not possible to determine if the positively selected codons in _N. lactamica_ were the same as those positively selected in _N. meningitidis_. Whereas some residues in meningococcal PorB2 and PorB3 were under strong positive immune selection, with _dS/dK_ ratios (_ω_ > 4) for PorB2 and 13.923 for PorB3 (Urwin et al., 2002), residues in the _N. lactamica_ porin experienced much weaker positive selective pressures, with _dS/dK_ ratios that did not exceed 4.195.

Results from the three maximum-likelihood models that took account of positive selection agreed on nine positively
selected codon sites in the \textit{N. lactamica} porin. The M3 model identified an additional 16 positively selected sites. These additional sites were considered in this analysis as five of them were polymorphic in isolates with identical MLST genotypes that were carried by one infant on successive occasions. A sixth site was only polymorphic in the porin of the isolates carried by this particular infant, although the \(d_{S}/d_{V}\) value for this site (1.487) was less than the cut-off value for positive selection (>1.5) used in this analysis. Both the M2 and M8 models failed to detect positive selection at these sites which were biologically relevant as the changes were nonsynonymous, altering the structure of the porin.

Using model M3, three weakly positively selected codon sites were detected in loop II of the \textit{N. lactamica} porin, whereas in the meningococcus, only one weakly positively selected site was present in loop II of PorB3 and none in loop II of PorB2 (Urwin \textit{et al.}, 2002). This suggests that there is less structural constraint in this region in \textit{N. lactamica} than in the meningococcus. Under model M3, four weakly positively selected sites were detected in loop III of the \textit{N. lactamica} porin, three of which were variable among the sequential isolates with identical MLST genotypes carried by one infant. As loop III is sequestered within the lumen, these variations may reflect changes in pore function. Three weakly positively selected sites were present in loop III of meningococcal PorB2 but none in loop III of PorB3 (Urwin \textit{et al.}, 2002), suggesting that \textit{N. lactamica} por is more like PorB2 in this respect. \textit{N. lactamica} has a single porin as opposed to the two in most meningococci, and variations in the permeability of the pore might alter the function of this protein, improving fitness. In the gonococcus, which also has one functional porin (Feavers & Maiden, 1998), mutations in loop III were shown to reduce porin permeability to hydrophilic antibiotics and these changes may therefore have a role in resistance to penicillin and tetracycline (Gill \textit{et al.}, 1998). However, the genotypically identical \textit{N. lactamica} isolates that expressed seven different \textit{por} alleles while carried by a single infant had not been exposed to antibiotic therapy (D.W. Crook, personal communication) and therefore the reasons for the rapid amino acid variation among these isolates remain unexplained.

Although the \textit{N. lactamica} porin undergoes relatively weak positive selection pressures in comparison to the meningococcus, nonsynonymous changes in the porin alleles among isolates carried by two infants showed plasticity of the porin, probably in response to selection, over a very short time period. This supports previous studies that established that meningococcal porins are subject to strong positive selection pressures and evolve rapidly (Jelfs \textit{et al.}, 2000; Urwin \textit{et al.}, 2002), hindering the design of vaccines based on these proteins.

Initially, \textit{N. lactamica} por appeared to be a good candidate for involvement in the production of a cross-protective immune response to meningococci, as the related meningococcal PorB protein is subject to strong immune selection (Urwin \textit{et al.}, 2002). Porins are also essential for bacterial growth and are expressed at high levels, and \textit{por} genes were amplified from all \textit{N. lactamica} isolates examined. However, the \textit{N. lactamica} porin was dissimilar to the porins of \textit{N. meningitidis}, with no alleles shared, and the surface-exposed loop region amino acid sequences were rarely identical in meningococci and \textit{N. lactamica}. The low sequence identity between Por and PorB, the low levels of genetic exchange, as indicated by the \(F_{S}S\) results, together with the relatively weak positive selection pressures acting on the \textit{N. lactamica} porin and the lack of immunological cross-reactivity with meningococcal PorB (Finney \textit{et al.}, 2007; Kim \textit{et al.}, 1989), suggest that this protein may not be important in the induction of any cross-protective immune responses that might protect against meningococcal disease. Therefore, any protection against meningococcal disease provided by \textit{N. lactamica} carriage is likely to be due to antigens other than Por.

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