Neisseria meningitidis antigen NMB0088: sequence variability, protein topology and vaccine potential

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The significance of Neisseria meningitidis serogroup B membrane proteins as vaccine candidates is continually growing. Here, we studied different aspects of antigen NMB0088, a protein that is abundant in outer-membrane vesicle preparations and is thought to be a surface protein. The gene encoding protein NMB0088 was sequenced in a panel of 34 different meningococcal strains with clinical and epidemiological relevance. After this analysis, four variants of NMB0088 were identified; the variability was confined to three specific segments, designated VR1, VR2 and VR3. Secondary structure predictions, refined with alignment analysis and homology modelling using FadL of Escherichia coli, revealed that almost all the variable regions were located in extracellular loop domains. In addition, the NMB0088 antigen was expressed in E. coli and a procedure for obtaining purified recombinant NMB0088 is described. The humoral immune response elicited in BALB/c mice was measured by ELISA and Western blotting, while the functional activity of these antibodies was determined in a serum bactericidal assay and an animal protection model. After immunization in mice, the recombinant protein was capable of inducing a protective response when it was administered inserted into liposomes. According to our results, the recombinant NMB0088 protein may represent a novel antigen for a vaccine against meningococcal disease. However, results from the variability study should be considered for designing a cross-protective formulation in future studies.

INTRODUCTION

Neisseria meningitidis is a Gram-negative encapsulated diplococcus that is a major cause of meningitis and sepsis. Among the 13 distinct N. meningitidis serogroups that have been defined on the basis of the immunochemistry of their capsular polysaccharide, groups A, B and C are responsible for approximately 90% of all cases (Rosenstein et al., 2001). The disease mainly affects infants and young children, with case fatality rates of 10–15%, despite the availability of effective antibiotics. Early diagnosis and antibiotic treatment greatly enhance survival, but prevention through vaccination would appear the best way to limit meningococcal disease (Jodar et al., 2002). While relative success has been achieved against serogroups A, C, Y and W135 with polysaccharide-based vaccines (Mitka, 2005), the development of a protective vaccine against serogroup B disease has been challenging. The capsular polysaccharide of serogroup B is a polymer of polysialic acid, which is also present in human tissues (Finne et al., 1983).

Vaccine research against serogroup B meningococcus has mostly focused on cell-surface protein antigens contained in outer-membrane vesicles (OMVs) (Fredriksen et al., 1991; Sierra et al., 1991) or recombinant proteins (Giuliani et al., 2006). The most important vaccines so far evaluated in clinical trials are OMV-based vaccines (Bjune et al., 1991; Sierra et al., 1991). The drawback with the OMV vaccines is that the most immunogenic components are highly variable and, consequently, these vaccines are not able to confer protection against a wide range of heterologous strains (Martin et al., 2000; Tappero et al., 2002).

Abbreviations: CREE, Correia repeat-enclosed element; DRVs, dried-reconstituted vesicles; i.n., intranasal; i.p., intraperitoneal; OMVs, outer-membrane vesicles; TM, transmembrane; VCN, vancomycin/colistin/nystatin; VRs, variable regions.

The GenBank/EMBL/DDJB accession numbers for the new nmb0088 gene sequences are EU645484–EU645512.
A previous study described the analysis by two-dimensional gel electrophoresis and mass spectrometry of N. meningitidis proteins present as the active ingredient of the Cuban vaccine VA-MENGOC-BC\(^9\), and the consequent identification of major and minor proteins in the preparation not previously reported as components of this antimeningococcal vaccine (Uli et al., 2006). A set of these antigens that show promise as vaccine candidates is currently under preclinical evaluation (Delgado et al., 2007; Hsu et al., 2008).

One of these antigens is the protein NMB0088 with an apparent molecular mass of approximately 50 kDa. After several proteomic studies, NMB0088 appears to be abundant in N. meningitidis serogroup B OMV preparations obtained from different strains (Vipond et al., 2006; Williams et al., 2007; Uli et al., 2006; Vaughan et al., 2006). In the present study, the nmb0088 gene and the deduced protein sequence were analyzed for a broader survey of its sequence conservation across a collection of isolates and in order to predict the topographic localization of regions exposed to the immune system. We also report the purification of a recombinant variant of NMB0088 protein, as well as the characterization of the antibodies elicited in mice against this antigen.

METHODS

Bacterial strains and growth conditions. The neisserial strains used in the present study as a source of chromosomal DNA for gene amplification are listed in Table 1. Bacteria employed as target strains for the immunological experiments were grown in a humidified atmosphere of 5% CO\(_2\) in air on brain heart infusion (BHI) agar (Oxoid) supplemented with vancomycin/colistin/nystatin (VCN) (Oxoid). For the infant rat protection assay, strain CU385 was grown on BHI agar supplemented with 7% (v/v) defibrinated goat blood (BHI-blood agar) and containing VCN. The Neisseria lactamica strain 102-CIGB (Sardinas et al., 2006) used for the immunoblotting was obtained from the culture collection of the Center for Genetic Engineering and Biotechnology (Havana, Cuba).

Escherichia coli XL1-Blue (Invitrogen) was used for cloning purposes and strain E. coli K-12 W3110 (New England BioLabs) was employed for the expression of the recombinant protein. E. coli cells with plasmids were cultured aerobically at 37 °C in Luria–Bertani medium or expression medium supplemented with 12.5 μg tetracycline ml\(^{-1}\) and 100 μg ampicillin ml\(^{-1}\) for strain XL1-blue, and with 100 μg ampicillin ml\(^{-1}\) for strain W3110. Expression medium consists of M9 synthetic medium (Yero et al., 2006) supplemented with 10 mg tryptone ml\(^{-1}\) and 1% (v/v) glycerol.

Sequence analysis of the nmb0088 gene and the deduced protein. Genomic DNA sequences flanking the nmb0088 gene were obtained from the annotated MC58 genome sequence (accession no. NC_003112). The homologue of nmb0088 and flanking sequences of the N. meningitidis strains Z2491 and FAM18 and a N. lactamica strain (an ST-640 strain identified here as ST-640) were obtained from the Sanger Institute (http://www.sanger.ac.uk/Projects/). The incompletely assembled genome sequence of the N. lactamica vaccine strain Y92-1009 (Vaughan et al., 2006) was used. The Neisseria gonorrhoeae strain FA1090 genome was accessed from the University of Oklahoma website (http://www.genome.ou.edu/gono.html). The recently published sequence from the serogroup C isolate 053442 (ST-4821) (Peng et al., 2008) was also employed. CLUSTAL W version 2.0 (Larkin et al., 2007) was used to perform sequence alignments.

Deduced polypeptide sequences of the open reading frames were analysed with the PSORTb program (www.psort.org/psortb/) to predict their cellular localization and the presence of a signal peptide. BLAST (http://ncbi.nlm.nih.gov/) was used to search the National Center for Biotechnology Information (NCBI) databases to identify previously reported sequences with homology to those that were sequenced. For secondary structure analysis, the prediction server JPred3 (www.compbio.dundee.ac.uk/~www-jpred/) was applied. An amino acid hydrophilicity plot of NMB0088 was made with the Kyte–Doolittle algorithm available at the ExPASy website (www.expasy.ch/tools/protscale.html).

PCR and nucleotide sequencing. Genomic DNA from the N. meningitidis strains listed in Table 1 was used as a template for the amplification of an nmb0088 fragment (from amino acid 26 to 464) with the following primers: 0088F1, 5′-CGA AGC TTA CCA CTA CCT CGG CAC AC-3′; and 0088R1, 5′-TTA CTC GAG TTA GTG GTA TGG CAG ACC-3′. Primers were designed for amplification of the segment encoding the mature form of antigen NMB0088, based on the nucleotide sequence reported for this gene in strain MC58. The PCR products were purified and nucleotide sequencing was performed by Macrogen (Seoul, Korea). Nucleotide sequences were edited, aligned and analysed using the program AlignX (vector NTI Suite 7.1; InforMax).

Cloning and expression of the nmb0088 gene. The PCR product corresponding to the NMB0088 sequence from strain CU385 was cloned into the expression vector pM238 using primers 0088F1 and 0088R1 as previously described (Yero et al., 2006). This vector provides six His residues at the C-terminus of the expressed protein, and at the mature N-terminus a stabilizer sequence that consists of the first 47 amino acids of the LpDA (dihydrodiploamidine dehydrogenase A) protein from N. meningitidis. The recombinant expression plasmid was transferred to the expression host strain E. coli W3110 and protein expression was induced by growing the bacteria in expression medium for 16 h at 37 °C.

Purification of the recombinant fusion protein. After growth in expression medium, E. coli cells expressing the recombinant NMB0088 (nMB0088) protein were collected by centrifugation for 15 min at 4000 g. The recombinant polypeptide was then purified by the washed pellet procedure. Briefly, the pellet was suspended by gentle stirring in chilled rupture buffer (10 mM Tris-Cl, 1 mM EDTA, pH 7.5), sonicated (five cycles, 30 s pulses at 60 s intervals on...
Table 1. Alignment of three VRs of NMB0088 from the strains used in this study

<table>
<thead>
<tr>
<th>NMB0088 variant</th>
<th>Strain</th>
<th>Sequence of VR*</th>
<th>Serological phenotype†</th>
<th>Country (source)‡</th>
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<td>Cuba (d)</td>
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*Sequencing data for Neisseria strains MC58, Z2491, FAM18 and 053442 were obtained from GenBank (NP_273150, NP_283028, YP_974211 and YP_001598273, respectively).
†NG, Non-groupable; NT, non-typable; NST, non-subtypable; ND, not determined; NA, not applicable.
‡Source in parentheses: d, disease; c, carrier.
ice) and the insoluble material was collected by centrifugation for 10 min at 7000 \( \text{g} \) at 4 °C. The inclusion bodies contained in the isolated pellet were washed successively with rupture buffer containing 10 mM MgCl\(_2\), 0.8 M NaCl and 0.1% Nonidet P-40 and recentrifuged. After solubilization in loading buffer [6 M urea in carbonate–bicarbonate buffer solution (0.1 M sodium carbonate and 0.1 M sodium hydroxide, pH 10.0)], the recombinant protein was purified by affinity chromatography with a Chelexing Sepharose Fast Flow column (Amersham Pharmacia Biotech) previously loaded with Cu\(^{2+}\) according to the manufacturer’s instructions. The column was washed with loading buffer containing 10 mM imidazole and contaminants bound to the column were eluted. Finally, the recombinant protein was eluted with 50 mM imidazole in loading buffer. The eluates were analysed by SDS-PAGE, the fractions containing a single protein band were pooled and dialysed against phosphate buffer (pH 8.0), and the protein concentrations were determined. The expression of recombinant protein, after each purification step, was analysed by gel electrophoresis and the proportions were determined by densitometry analysis using software Image J 1.37h (National Institutes of Health, USA).

**Detection of immune response by ELISA.** Antibodies against the protein on the surface of the bacteria were detected by whole-cell ELISA as described previously (Rosenqvist et al., 1990). Strains were grown overnight at 37 °C on BHI agar and then subcultured onto a fresh plate under the same conditions for a further 4 h. The bacteria were harvested and suspended in PBS with 0.02% sodium azide. The \( \text{OD}_{620} \) of the suspension was adjusted to 0.1. One hundred microlitres of this suspension was added to individual wells in polystyrene plates and left to evaporate overnight at 37 °C. The rest of the experiment followed a conventional ELISA protocol. Titres were expressed as the reciprocal of the dilution showing a twofold increase in absorbance over that obtained with a negative control.

**Bactericidal antibody assay.** The bactericidal assay was carried out using homologous strain CU385 in 96-well plates, as described previously (Maslanka et al., 1997), with few modifications. Baby rabbit serum was used as a source of exogenous complement, and this serum was adsorbed on ice with inactivated meningococcal cells immediately before use. Briefly, bacteria (target strain) were adjusted to \( \text{OD}_{620} \) 1.0 in PBS and the suspension was incubated for 30 min at 56 °C. A bacterial pellet, corresponding to 2.5 ml heat-inactivated bacterial suspension, was added to 1.0 ml rabbit serum and the mixture was incubated for 50 min on ice with agitation. Finally, bacteria were removed by centrifugation in a refrigerated centrifuge. The pre-adsorbed baby rabbit serum was used in the bactericidal assays. The bactericidal titre of the serum was defined as the reciprocal of the highest serum dilution giving \( \geq 50 \% \) killing compared to the mean from complement-independent control wells for each assay plate.

**Infant rat protection assay.** The ability of the sera from immunized mice to confer protection against \( \text{N. meningitidis} \) bacteraemia in infant rats was performed as previously described (Yero et al., 2005). In brief, 5–6-day-old infant rats (outbred Wistar; CENPALAB) were randomly distributed in groups of six animals per pool of sera. Rat pups were injected i.p. with 100 \( \mu \text{l} \) serum diluted 1:10, 1 h before the i.p. bacterial challenge with approximately 10\(^7\) c.f.u. per pup in a final volume of 150 \( \mu \text{l} \). For the challenge, bacteria were suspended in sterile PBS containing iron dextran. A pool of preimmune sera was used as negative control and a pool of hyperimmune sera from OMV-immunized mice was used as positive control for protection. Development of bacteraemia was assessed by culturing diluted blood samples taken 4 h after challenge on BHI-blood agar containing VCN. The results of blood cultures were transformed to logarithmic values to calculate the geometric mean value of the c.f.u. in each group of six animals. Statistical significance was evaluated using a one-way analysis of variance (ANOVA) followed by a Dunnett’s post test. GraphPad Prism version 4.0 (GraphPad Software) was used and a \( P \) value less than 0.05 was considered statistically significant.

**RESULTS**

**nmb0088 and deduced protein features.**

The gene encoding protein NMB0088 is an isolated open reading frame located downstream of the gene encoding pyruvate kinase II (nmb0089) in the meningococcal genome. A characteristic feature of this region is the presence of Correia repeat-enclosed elements (CREEs) (Liu et al., 2002) flanking gene nmb0088 (Fig. 1). CREEs have been divided into four families (\( \alpha, \alpha', \beta \) and \( \beta' \)), distinguished by a 50 bp internal deletion and five point mutations (Buisine et al., 2002). In \( \text{N. meningitidis} \) strain MC58, the downstream region of nmb0088 contains a...
155 bp \(\alpha\)-family CREE with 26 bp terminal inverted repeats. In strains MC58 and FAM18, the upstream regions of this gene contain a member of the \(\alpha\)-family CREE that is 106 bp long and has terminal inverted repeats of 26 and 27 bp. These upstream CREEs carry putative \(-35\) and \(-10\) promoter elements that could act as transcription regulators for the \(nmb0088\) gene. In strains Z2491 and 053442, and in \(N.\) lactamica Nl-ST640, the upstream CREEs are 101 bp long due to a five nucleotide internal deletion (Fig. 1). In \(N.\) lactamica Y92-1009, this upstream repetitive element is absent, and different promoter elements could act on the expression of the \(nmb0088\) gene.

NMB0088 is a protein of 466 amino acids in strain MC58 with a possible signal peptide of 24 amino acids. The mature protein has a predicted molecular mass of 48 133 Da and an isoelectric point of 9.28. Analysis of the predicted secondary structure revealed several features that are summarized in Fig. 2. We detected stretches with secondary structure typical of transmembrane (TM) domains, three \(\alpha\)-helical motifs at the N-terminus of the protein and several regions with \(\beta\)-sheet prediction (reliability of accuracy >7) along the rest of the amino acidic sequence.

The function of NMB0088 is still unclear but this protein is a putative outer-membrane protein which shares homology (\(E=3e^{-39}\)) with a family of transporters (FadL) in a number of organisms. This family includes TodX from \textit{Pseudomonas putida} F1 and TbuX from \textit{Ralstonia pickettii} PKO1. These are membrane proteins of uncertain function that are involved in aromatic hydrocarbon biodegradation (Hearn \textit{et al.}, 2008). The family also includes FadL of \(E.\) coli involved in translocation of long-chain fatty acids across the outer membrane (Black \textit{et al.}, 1987). When NMB0088 was aligned onto the primary sequence of the FadL protein, weak conservation could be observed. However, the majority of the secondary structure elements, which could contribute to creating a properly native conformation, were maintained along the sequence (Fig. 2). The crystal structure of FadL from \(E.\) coli has been reported indicating this protein forms a 14-stranded \(\beta\)-barrel that is occluded by a central hatch domain (van den Berg \textit{et al.}, 2004). The N-terminal region of the protein contains three short helices, which plug the barrel. One of the helices is capped by the conserved sequence NPA, a signature sequence in aquaporins (Fu \textit{et al.}, 2000) that is also present in NMB0088 (amino acids 56–58).

**Alignment of NMB0088 sequences and variable region definition**

Proteins homologous to NMB0088 have been found in other meningococcal genomes with identities ranging from 93% to 99%, and in \(N.\) lactamica strains NI-ST640 and Y92-1009 with 93% and 89% identity, respectively. However, in the gonococcus, the homologue to \(nmb0088\) appears to be a pseudogene. A first alignment analysis using these reported sequences showed discrete regions of high variability in the deduced amino acid sequence of NMB0088. To confirm the existence of variable regions (VRs) within the NMB0088 sequence and in order to define the topology model of this protein, we refined the alignment analysing orthologous sequences in a panel of meningococcal strains belonging to different serogroups, serotypes and sero-subtypes.

Comparison of the deduced amino acid sequences from 34 meningococcal strains and those from the two \(N.\) lactamica
strains revealed large areas of highly conserved sequence and several well-defined regions of variation (Table 1 and Fig. 2). The alignment showed that NMB0088 variability is confined to three specific segments, designated VR1 (193–216), VR2 (268–287) and VR3 (339–345). The two first regions were the most variable among strains at both the nucleotide and amino acid level. The number of amino acids in the three regions is different between strains tested, a fact that contributes to the variability of the VRs. Comparison of the per cent similarities in NMB0088 sequences among N. meningitidis isolates identified four variants with amino acid sequences of $\leq 96\%$ similarity (Table 1). The two N. lactamica sequences added to the alignment represented two new variants. In the N. meningitidis strains tested, the DNA regions coding for the VR1 in NMB0088 variants 2, 3 and 4 are distinguished by a 9 bp internal deletion. No correlation was found between the serological classification of strains and their NMB0088 variant, except for the two serogroup A strains that were grouped into variant 3. However, it was interesting that 70% of the strains belonging to variant 2 were isolated from healthy carriers (Table 1).

**Membrane topology model for protein NMB0088**

In an attempt to construct a topology model for this membrane protein, we compared sequences of different NMB0088 proteins, and hypervariable regions (putative exposed regions) were predicted from the variability profile of the alignment (Fig. 2). The pattern of alternating $\beta$-sheets and VRs is similar to that of other neisserial porins and TonB-dependent proteins, where it has been demonstrated that variable stretches are located at the surface, while conserved regions form membrane-spanning $\beta$-sheets (van der Ley et al., 1991; Derrick et al., 1999). We assumed that VRs are present on the surface, and used the location of hydrophilic maxima in the hydropathy profile (Kyte-Doolittle) as an additional criterion for surface exposure.

The membrane-spanning segments in the proposed model for NMB0088 were predicted based on a number of features concerning TM $\beta$-strands and their organization in well-known $\beta$-barrel proteins (Schulz, 2000). The number of TM $\beta$-strands in barrels has been shown to range from 6 to 22 (most frequently 12) residues. TM $\beta$-strands show an inside–outside dyad repeat motif of alternating residues facing the lipid bilayer and the inside of the barrel. Outside (lipid bilayer facing) residues are typically hydrophobic whilst inside (facing inside of barrel) residues are of intermediate polarity. TM $\beta$-strands are also often flanked by a layer of aromatic residues, believed to be involved in maintaining the protein’s stability within the membrane (Yau et al., 1998). Subsequently, we examined the NMB0088 alignment for stretches of 12 amino acid residues, which should be amphipathic when present in a $\beta$-configuration, flanked by aromatic amino acids and located in regions predicted as $\beta$-sheets by Jpred and by homology with E. coli FadL.

Finally, the topographic map was built starting at the C-terminal region with the last TM segment directed towards the periplasm. In our model, protein NMB0088 is thought to span the membrane 14 times, thereby exposing seven hydrophilic loops beyond the outer membrane. Based on the topology model proposed here, the VRs were located in the third, fourth and fifth extracellular loops, respectively (Fig. 2). Regardless of whether or not the first 11 residues in the mature protein were included in the alignment, the N-terminal portion of protein NMB0088 appears to form an $\alpha$-helical plug similarly to the homologous region in E. coli FadL.

**Immunogenicity of the recombinant protein and functional activities of the elicited antibodies**

As described in detail in Methods, the complete nmb0088 DNA sequence of CU385, minus its signal sequence, was amplified and inserted into an E. coli expression vector. The recombinant protein formed insoluble cytoplasmic inclusion bodies that were harvested, purified and concentrated. To study the immunogenicity of rNMB0088 using different formulations, BALB/c mice were immunized (i.p.) with different adjuvants. Taking into account that NMB0088 is probably an integral $\beta$-barrel protein and to attempt to present the protein in its native conformation for immunization experiments, rNMB0088 was also incorporated into liposomes and administered by the i.p. and i.n. routes. Sera collected after immunization with the recombinant protein were analysed by whole-cell ELISA and Western blotting experiments.

The specificity of antisera produced against purified rNMB0088 was tested by Western blotting and results showed that this protein was expressed by a panel of meningococcal strains belonging to different serogroups and different NMB0088 variants, and also by a N. lactamica strain (Fig. 3). An easily identifiable band was recognized in each strip corresponding to a meningococcal protein with an apparent molecular mass of approximately 50 kDa. These antisera produced against purified rNMB0088 in all the immunization variants recognized epitopes contained in the sample of homologous strain CU385 in a whole-cell ELISA (Fig. 4a). Similar results were obtained in the immunoassay when we evaluated pooled sera from mice immunized with rNMB0088 in aluminium hydroxide using as target cells strains B16B6 (titre 3690), Z4181 (titre 4200), M982 (titre 5890), NZ124 (titre 1750) and Z1127 (titre 2100). These results indicate that NMB0088 was expressed by the N. meningitidis strains tested and levels of expression appear to be nearly the same.

Antisera raised against the rNMB0088 were also tested for the ability to promote in vitro complement-mediated killing of the homologous meningococcal strain (Table 2). Only sera from mice immunized with protein emulsified in Freund’s adjuvant showed no detectable bactericidal titres. The other antisera promoted bactericidal activity against meningococci, with titres ranging from 1:16 to 1:64.
addition, sera from mice immunized with the recombinant protein were bactericidal against two heterologous strains (B16B6 and M982) with the same NMB0088 variant but with different serological classification and sequence type (Table 2). These sera did not kill two other heterologous strains with different sequence variants (Table 2).

To determine whether the sera from mice immunized with rNMB0088 with different adjuvants were also able to confer protection in vivo, we tested them for their ability to induce passive protection in the infant rat challenge model using serogroup B strain CU385 (Fig. 4b). Animals passively immunized with pooled sera from mice injected with formulation containing rNMB0088-DRVs by the i.n. or i.p. route had a significant reduction in the level of bacteraemia compared to the control group. No significant differences were detected between the other formulations.

**DISCUSSION**

In the neisserial OMV vaccine field, most attention has traditionally been devoted to major outer-membrane proteins. However, the impact of minor components in
Fig. 2. Amino acid sequence of 33 unique meningococcal NMB0088 proteins deduced from the nucleotide sequence and aligned with CLUSTAL W. Shaded residues are those that differ from the majority consensus sequence (Consen.). The secondary-structure prediction of the NMB0088 antigen is shown based on the Jpred service (α-helices and β-strands predicted with higher scores are represented by H and E, respectively). Alignment of the NMB0088 consensus sequence with the sequence and secondary structure of the E. coli FadL protein is exposed. β-Sheets and α-helices are indicated by arrows and helices, respectively. ‘|’ Residues are identical; ‘:’, conserved substitutions; ‘.’, semi-conserved substitutions. The figure also shows a proposed topology model for protein NMB0088. The regions predicted by homology to form β-strands of the β-barrel are indicated by arrows and are marked from β1 to β14. Membrane-spanning segments in the final proposed model are boxed. The variable regions (VRs) are indicated.
the induction of a significant immune response should also be considered. One of these minor proteins, NMB0088, was previously identified using proteomic approaches as a component of the OMVs produced from meningococcal strain CU385 (Uli et al., 2006). Taking into account the impact of antigen variability on the immune response exerted by OMV-based vaccines, characterization of sequence conservation of each protein contained in such formulations is also an important consideration for serogroup B vaccine design and development. In the present study, the gene encoding protein NMB0088 was sequenced in a panel of 25 N. meningitidis strains isolated in Cuba between 1983 and 2003, and in several standard strains with a worldwide distribution. After this analysis, four variants of NMB0088 were identified; the variation was confined to three specific segments, designated VR1, VR2 and VR3.

The high variability observed in the NMB0088 VRs suggests that possible insertions, deletions and/or recombination events occurred. The nmb0088 gene is located in a chromosome region that may be genetically unstable, due to the presence of repeat sequences flanking the gene. The N. meningitidis genome contains many hundreds of repetitive sequence elements ranging from simple sequence repeats to duplications of gene clusters. It was suggested that these repetitive arrays may encourage sequence variation in neighbouring genes by increasing the frequency of recombination with exogenous DNA (Bentley et al., 2007). Among the most abundant repeat types are the CREEs, which are often located upstream of genes (Liu et al., 2002; De Gregorio et al., 2003a), have been shown to affect gene expression (De Gregorio et al., 2003b), and may be transposable or mobilizable (Buisine et al., 2002). The downstream CREE reported in the present study could also be involved in the expression of NMB0088 since a family of long CREEs has been considered to be a transcriptional terminator in the dcw cluster of N. gonorrhoeae CH11 (Francis et al., 2000). The CREE upstream of the nmb0088 gene has been previously reported for the MC58 strain (De Gregorio et al., 2003a) and our sequence analysis (Fig. 1) showed that this element is not identical in all reported sequences, and is absent in one N. lactamica strain.

However, the fact that N. lactamica Y92-1009 lacks the 5' CREE does not impair the expression of protein NMB0088 as was previously demonstrated in proteomic studies by Vaughan et al. (2006), indicating that other promoter elements are present on the upstream region of nmb0088. The precise localization of the promoter elements in the nmb0088 gene needs to be demonstrated and further transcription studies should be conducted to clarify the regulation of transcription in this gene.

The function of NMB0088 in the meningococcus is still unclear. This antigen has homology with a family of
bacterial membrane proteins that includes FadL from *E. coli*. The FadL family includes several distantly related proteins, all probably outer-membrane proteins, from the human pathogens *Haemophilus influenzae*, *Pseudomonas* sp. and *Moraxella catarrhalis*. Many researchers have focused on these proteins as vaccine candidates. In *M. catarrhalis*, the protein homologue to FadL is known as the outer-membrane protein OMP-E, and many studies have indicated that OMP-E should be considered a potential vaccine antigen (Murphy *et al*., 2001). Furthermore, NMB0088 is a homologue of OmpP1 from *H. influenzae*, which has been evaluated as a vaccine candidate against experimental otitis media due to nontypable strains (Bolduc *et al*., 2000). Passive immunization with *H. influenzae* OmpP1 induced protection against bacteraemia in the infant rat model (Munson & Hunt, 1989) as was also demonstrated for protein NMB0088 in the present study. OmpP1 has four immunogenic surface-exposed loops which show sequence variability among different strains (Bolduc *et al*., 2000) and contain positively selected codons (Mes & van Putten, 2007). Thus development of a polyvalent vaccine reflecting the variability of OmpP1 would be necessary to construct an efficacious vaccine against *H. influenzae*, and a similar approach could be carried out with protein NMB0088.

A previous comparative proteome study of OMVs produced from *N. meningitidis* and *N. lactamica* strain Y92-1009 revealed substantial differences between the antigen repertoires of the two species (Vaughan *et al*., 2006). However, as most of the *N. lactamica* proteome components have close meningococcal orthologues, like NMB0088, it has been proposed that an OMV vaccine prepared from this bacterium may offer sufficient cross-protection to prevent meningococcal disease (Gorringe *et al*., 2005). In our work, sera from mice immunized with rNMB0088 recognized a protein in Western blotting of a *N. lactamica* strain (Fig. 3), indicating that differences in the sequence do not impair immune recognition. Previous studies have shown that OMVs from *N. lactamica* are immunogenic when given by the i.n. route and they are able to induce bactericidal antibodies against *N. meningitidis* (Sardinas *et al*., 2006). The results of the i.n. vaccination with rNMB0088 in the present work corroborate the utility of this route for developing a protective response and encourage studying the mucosal immune response after immunization with *Neisseria* antigens.

In the present study, results from the sequence alignment also impacted on the topological reconstruction of protein NMB0088. It appears to share common folding with other Gram-negative integral outer-membrane proteins, i.e. they form a TM β-barrel consisting of antiparallel amphipathic β-strands. The identification of β-barrel regions here was based on the X-ray crystallography of the protein FadL of *E. coli* (van den Berg *et al*., 2004). Despite the low level of amino acid sequence congruence, the fit between FadL and NMB0088 was sufficient to pinpoint the approximate localization of TM regions and surface-exposed loops. More divergent structural templates and targets, such as OmpF and PhoE of *E. coli*, have successfully been used for the reconstruction of 3D models of porins in *Neisseria* (Derrick *et al*., 1999). Recently, a homology model for the vaccine candidate OmpP1 of *H. influenzae* has been proposed based on the FadL structure, demonstrating that the VRs are located on surface-exposed loops (Mes & van Putten, 2007).

More results from the present study deserve further comments. Recombinant NMB0088 protein was easily purified by affinity chromatography with Chelating Sepharose Fast Flow, obtaining a preparation with 90% purity. However, this protein was obtained as inclusion bodies and denaturing and renaturing steps were required during its purification. The reconstitution of recombinant bacterial membrane proteins into their native conformations after purification has been a major problem in their use as effective vaccines. Liposomes have been shown to be an attractive approach as they provide a native-like environment for membrane proteins, particularly with a β-barrel structure (Buchanan, 1999). Several studies with recombinant meningococcal antigens including porins PorA (Christodoulides *et al*., 1998; Niebla *et al*., 2001) and PorB (Wright *et al*., 2002) have clearly demonstrated

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serological classification</th>
<th>Sequence type (ST)</th>
<th>NMB0088 variant</th>
<th>Bactericidal titre*</th>
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<tbody>
<tr>
<td></td>
<td>(electrophoretic type [ET] cluster)</td>
<td></td>
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<tr>
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<td>&lt;8</td>
</tr>
</tbody>
</table>

* Bactericidal titres are represented as the reciprocal of the dilution of antiserum that reduced viable cell count by 50%. Preimmune sera showed no bactericidal activity (<8). Serum pools were employed from groups treated with rNMB0088 formulated on aluminium hydroxide (alum) or incorporated into DRV liposomes and administered by the intraperitoneal (i.p.) or intranasal (i.n.) routes.

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that the production of bactericidal antibodies was dependent on refolding of the protein to induce native conformation. In this study, immunization with formulations containing rNMB0088 induced a humoral immune response, characterized by the presence of specific IgG in sera (as detected by whole-cell ELISA and Western blotting) and by the induction of bactericidal antibodies. Nevertheless, the only pooled sera that conferred passive protection in the infant rat model were from the group immunized with the recombinant protein inserted into liposomes, suggesting that some additional protective epitopes of the NMB0088 antigen are better represented in a conformation of the protein resembling that in the outer membrane. However, based on this study and the methods of protein expression and preparation, the antigen would not be a sufficiently good candidate for inclusion in future vaccines. Further studies should be conducted to increase the titre of bactericidal antibodies after immunization with NMB0088, e.g. to change the dose of the antigen, the adjuvant and/or the refolding procedure during purification.

In the fight against human pathogens, a number of the most promising new vaccine candidates have been identified as a result of applying proteomic-based techniques to a large number of antigens, enabling more rapid development of these candidates at a reasonable cost compared with traditional strategies (Mora et al., 2003). The protein presented here is an example of how high-throughput strategies contribute to protein identification, production and evaluation as a vaccine candidate. NMB0088 should be considered in future studies as an attractive antigen for a subunit recombinant vaccine against the meningococcus. However, the level of diversity of the exposed loops in NMB0088 and the result of the bactericidal activity against different strains suggest that vaccines based on this antigen should be carefully designed in order to provide broad coverage in N. meningitidis.

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REFERENCES


