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

Gretel Sardiñas,^{1†} Daniel Yero,^{1,2†} Yanet Climent,^{1,2} Evelin Caballero,¹ Karem Cobas¹ and Olivia Niebla¹

Correspondence

Gretel Sardiñas
gretel.sardinias@cigb.edu.cu

Daniel Yero
dyero@finlay.edu.cu

¹Meningococcal Research Department, Division of Vaccines, Center for Genetic Engineering and Biotechnology, Avenue 31, Cubanacan, Habana 10600, Cuba

²Department of Molecular Biology, Division of Biotechnology, Finlay Institute, Avenue 27, La Lisa, Habana 11600, Cuba

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.

Received 7 July 2008

Accepted 4 October 2008

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.*,

†These authors contributed equally to this work.

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/DDBJ accession numbers for the new *nmb0088* gene sequences are EU645484–EU645512.

1999). Alternatively, highly conserved *N. meningitidis* serogroup B membrane antigens have been sought and evaluated as vaccine candidates (Comanducci *et al.*, 2002; Martin *et al.*, 1997; Giuliani *et al.*, 2006). The successful development of a broad-specificity serogroup B vaccine may be attained as a consequence of the genome sequencing of the serogroup B *N. meningitidis* strain MC58 (Tettelin *et al.*, 2000), which has opened up new possibilities for identifying antigens to be included in candidate vaccines (Pizza *et al.*, 2000). In parallel, techniques for proteomic and immunoproteomic analysis have been applied to characterize the antigen composition of commercial complex vaccines, and particularly some applications have arisen for the characterization of OMVs of pathogenic bacteria (Nally *et al.*, 2005; Wheeler *et al.*, 2007). For the meningococcus, such analytical approaches have recently identified new vaccine candidates (Delgado *et al.*, 2007; Hsu *et al.*, 2008).

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[®], 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). 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 analysed 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% CO₂ 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 NI-ST640) 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.psorb.org/psorb/) 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'-CGC AGA TCT CTA CCA CTT CGG CAC AC-3'; and 0088R1, 5'-TTA CTC GAG TTT GTA GGT GTA TTG 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 (dihydrolipoamide 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 (rNMB0088) 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

NMB0088 variant	Strain	Sequence of VR*			Serological phenotype†	Country (source)‡
		VR1	VR2	VR3		
1	MC58	KSKAEILTAKPPKPNGVAEAAKIQ	GAAAKAMWST-MLAANGYTAN	VVKGK-SD	B:15:P1.7,16b	UK (d)
	FAM18	KSKAEILTAKPPKPNGVAEAAKIQ	GAAAKAMWST-MLAANGYTAN	VVKGK-SD	C:2a:P1.5,2	USA (d)
	CU385	KSKAEILTAKPPKPNGVAEAAKIQ	GAAAKAMWST-MLAANGYTAN	VVKGK-SD	B:4:P1.19,15	Cuba (d)
	54/91	KSKAEILTAKPPKPNGVAEAAKIQ	GAAAKAMWST-MLAANGYTAN	VVKGK-SD	B:4:P1.19,15	Cuba (c)
	7/92	KSKAEILTAKPPKPNGVAEAAKIQ	GAAAKAMWST-MLAANGYTAN	VVKGK-SD	B:4:P1.19,15	Cuba (d)
	16/86	KSKAEILTAKPPKPNGVAEAAKIQ	GAAAKAMWST-MLAANGYTAN	VVKGK-SD	B:4:P1.19,15	Cuba (d)
	419/85	KSKAEILTAKPPKPNGVAEAAKIQ	GAAAKAMWST-MLAANGYTAN	VVKGK-SD	B:4:P1.2,5	Cuba (d)
	67/91	KSKAEILTAKPPKPNGVAEAAKIQ	GAAAKAMWST-MLAANGYTAN	VVKGK-SD	ND	Cuba (c)
	32/89	KSKAEILTAKPPKPNGVAEAAKIQ	GAAAKAMWST-MLAANGYTAN	VVKGK-SD	ND	Cuba (d)
	156/85	KSKAEILTAKPPKPNGVAEAAKIQ	GAAAKAMWST-MLAANGYTAN	VVKGK-SD	NG:NT:P1.19,15	Cuba (d)
	411/86	KSKAEILTAKPPKPNGVAEAAKIQ	GAAAKAMWST-MLAANGYTAN	VVKGK-SD	B:4:P1.19,15	Cuba (d)
	12/99	KSKAEILTAKPPKPNGVAEAAKIQ	GAAAKAMWST-MLAANGYTAN	VVKGK-SG	B:4:P1.19,15	Cuba (d)
	57/91	KSKAEILTAKPPKPNGVAEAAKIQ	GAAAKAMWST-MLAANGYTAN	VVKGK-SD	B:4:P1.19,15	Cuba (c)
	87/91	KSKAEILTAKPPKPNGVAEAAKIQ	GAAAKAMWST-MLAANGYTAN	VVKGK-SG	B:4:P1.19,15	Cuba (c)
	811/86	KSKAEILTAKPPKPNGVAEAAKIQ	GAAAKAMWST-MLAANGYTAN	VVKGK-SG	B:4:P1.19,15	Cuba (d)
	322/90	KSKAEILTAKPPKPNGVAEAAKIQ	GAAAKAMWST-MLAANGYTAN	VVKGK-SG	B:4:P1.19,15	Cuba (d)
	B16B6	KSKAEILTAKPPKPNGVAEAAKIQ	GAAAKAMWST-MLAANGYTAN	VVKGK-SG	B:2a:P1.5,2	USA (d)
	M982	KSKAEILTAKPPKPNGVAEAAKIQ	GAAAKAMWST-MLAANGYTAN	IVNGKKS	B:9:P1.22,9	USA (d)
	52/91	KSKAEILTAKPPKPNGVAEAAKIQ	GAAAKAMWST-MLAANGYTAN	VVKGK-SG	B:4:P1.19,15	Cuba (c)
	375/91	KSKAEILTAKPPKPNGVAEAAKIQ	GAAAKAMWST-MLAANGYTAN	IVNGK-SG	C	Cuba (d)
2	135/99	MEKAKALKETPPNP---TKAAQIK	GAAAKAMWST-MLAANGYTAN	VVKGK-SD	Z:NT:P1.NST	Cuba (c)
	159/99	MEKAKALKETPPNP---TKAAQIK	GAAAKAMWST-MLAANGYTAN	VVKGK-SD	NG:NT:P1.NST	Cuba (c)
	67/89	MEKAKALKETPPNP---TKAAQIK	GAAAKAMWST-MLAANGYTAN	IVNGK-SG	NG	Cuba (c)
	111/02	MEKAKALKETPPNP---TKAAQIK	GAAAKAMWST-MLAANGYTAN	IVNGK-SG	NG:15:P1.6	Cuba (c)
	57/89	IEKAKALKETPPNS---TKAAQIK	GAAAKAMWST-MLAANGYTAN	IVNGK-SG	B:15:P1.15	Cuba (c)
	Z4181	MEKAKALKETPPNP---TKAAQIK	GAAAKAMWST-MLAANGYTAN	IVNGK-SG	C:2a:P1.5	Mali (d)
	053442	MEKAKALKETPPNP---TKAAQIK	DAAAKAMWST-MLAANGYTAN	IGMGKKS	C:ND:P1.7-2,14	China (d)
3	Z2491	MEKAKALKETPPNP---TKAAQIK	DAMAKQLWDANKLALLGYTPS	IVNGK-SD	A:4,21:P1.7-2,13a	Gambia (d)
	Z1127	MEKAKALKETPPNP---TKAAQIK	DAMAKQLWDANKLALLGYTPS	IVNGK-SD	A:4:P1.9	B. Faso (d)
4	1/03	PKKAQMLQATPSNP---TAAQIK	DAMAKQLWNSNMLAPLGYTAN	IVNGK-SD	B	Cuba (d)
	NZ124	PKKAQMLQATPSNP---TAAQIK	DAMAKQLWNSNMLAPLGYTAN	IVNGK-SD	B:4:P1.7-2,4	New Zealand (d)
	131/89	PKKAQMLQATPSNP---TAAQIK	DAMAKQLWNSNMLAPLGYTAN	IVNGK-SD	C	Cuba (d)
	133/83	PKKAQMLQATPSNP---TAAQIK	DAMAKQLWNSNMLAPLGYTAN	IVNGK-SD	C	Cuba (d)
	102/98	PKKAQMLQATPSNP---TAAQIK	DAMAKQLWNSNMLAPLGYTAN	IVNGK-SG	W135:NT:P1.NST	Cuba (c)
5	Y92-1009	MEKAKALQETPPNP---TKAAQIK	GAAAKALWST-TLATNGYTAN	IVNGQ-SD	NA	UK (NA)
6	NI-ST640	RQKAAMLNN-----LAGAAAIQ	GAAAKAMWNT-MLAKIGYTAN	VVKGK-SD	NA	UK (NA)

*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 g at 4 °C. The inclusion bodies contained in the isolated pellet were washed successively with rupture buffer containing 10 mM MgCl₂, 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 hydrogen carbonate, pH 10.0)], the recombinant protein was purified by affinity chromatography with a Chelating Sepharose Fast Flow column (Amersham Pharmacia Biotech) previously loaded with Cu²⁺ 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).

Insertion of the recombinant protein into liposomes. Liposomes were obtained using the dried-reconstituted vesicles (DRVs) method (Kirby & Gregoriadis, 1984). Briefly, a mixture of 18 mg dipalmitoylphosphatidylcholine and 9 mg cholesterol in chloroform was dried by rotatory evaporation at 30 °C for 1 h. Dried phospholipids were suspended in 5 ml PBS (pH 8.0) by vigorous agitation. The suspension was homogenized in an ultrasonic water bath (five bursts of 2 min), resulting in a suspension of multilamellar vesicles. The suspension of empty liposomes was mixed with 6 mg rNMB0088 and freeze-dried three times in order to achieve the insertion of rNMB0088 into the vesicles. DRVs were centrifuged at 100 000 g for 2 h at 4 °C, and the pellet was suspended in 1 ml PBS.

Immunization of animals. To evaluate the immunogenicity of the recombinant protein, female BALB/c mice (CENPALAB; Havana, Cuba) 8–10 weeks of age were immunized by the intraperitoneal (i.p.) or the intranasal (i.n.) routes. The animals used in the study were housed and used strictly in accordance with the Institutional Guidelines for Care and Use of Laboratory Animals. Three groups of 10 mice each received three i.p. doses at 2-week intervals (100 µl per dose) containing 10 µg rNMB0088 in Freund's or aluminium hydroxide adjuvants or inserted in liposomes (rNMB0088-DRVs). An additional group received 50 µl (25 µl per nostril) rNMB0088-DRVs, containing 10 µg of the recombinant protein, by the i.n. route. As positive control, a mouse group was immunized i.p. following the same schedule with 10 µg per dose of meningococcal OMVs (with aluminium adjuvant). The sera were collected by centrifugation from blood extracted on days 0 (preimmune) and 45 and samples were stored at –20 °C.

SDS-PAGE and Western blotting. Cell lysates of *N. meningitidis* strains were separated by SDS-PAGE, transferred onto a nitrocellulose membrane (Hybond ECL; Amersham Pharmacia Biotech) and analyses were performed as described previously (Wedge & Froholm, 1986), with the following modifications. Membrane strips containing transferred proteins were blocked with 5% (w/v) skim milk powder (Oxoid) in PBS for 3 h at room temperature. Pooled sera from immunized and control mice were diluted 1:100 in PBS containing 0.05% (v/v) Tween 20 (PBS-T) and 1% (w/v) skim milk, and incubated with the membranes overnight at 4 °C with continuous shaking. The membranes were washed three times with PBS-T and incubated for 1 h at room temperature with goat anti-mouse IgG peroxidase conjugate (Sigma-Aldrich) diluted 1:10 000 in PBS-T. The membranes were washed again and the antigen-antibody reactions were visualized using a substrate solution containing 3-amino-9-ethylcarbazole (Sigma-Aldrich) as the chromogen.

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 OD₆₂₀ 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 OD₆₂₀ 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 ≥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 *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 µl serum diluted 1:10, 1 h before the i.p. bacterial challenge with approximately 10⁷ c.f.u. per pup in a final volume of 150 µl. 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 (α , α' , β and β'), distinguished by a 50 bp internal deletion and five point mutations (Buisine *et al.*, 2002). In *N. meningitidis* strain MC58, the downstream region of *nmb0088* contains a

Fig. 1. Physical map of the MC58 genome region (from positions 99 527 to 101 427) comprising the *nmb0088* gene and nearby Correia repeat-enclosed elements (CREE), indicating the 26 or 27 bp repeat sequences. Neisserial DNA uptake sequences (DUS) are shown by arrows. The DNA sequence alignment of the region upstream of the *nmb0088* gene in four *N. meningitidis* strains and two *N. lactamica* strains is shown. The CREE upstream to the *nmb0088* gene in the MC58 DNA sequence is in boldface and the inverted repeats are underlined. Putative promoter sequences are boxed. RBS marks the position of a putative ribosome-binding site.

155 bp α -family CREE with 26 bp terminal inverted repeats. In strains MC58 and FAM18, the upstream regions of this gene contain a member of the α' -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* N1-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 α -helical motifs at the N-terminus of the protein and several regions with β -sheet prediction (reliability of accuracy >7) along the rest of the amino acid sequence.

The function of NMB0088 is still unclear but this protein is a putative outer-membrane protein which shares homology ($E=3e^{-58}$) with a family of transporters (FadL) in a number of organisms. This family includes TodX from *Pseudomonas putida* F1 and TbuX from *Ralstonia pickettii* PKO1. These are membrane proteins of uncertain function that are involved in aromatic hydrocarbon biodegradation (Hearn *et al.*, 2008). The family also includes FadL of *E. coli* involved in translocation of long-chain fatty acids across the outer membrane (Black *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 β -barrel that is occluded by a central hatch domain (van den Berg *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 *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 N1-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 β -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 β -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 β -strands and their organization in well-known β -barrel proteins (Schulz, 2000). The number of TM β -strands in barrels has been shown to range from 6 to 22 (most frequently 12) residues. TM β -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 β -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 β -configuration, flanked by aromatic amino acids and located in regions predicted as β -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 α -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 β -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. In

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 $\beta 1$ to $\beta 14$. Membrane-spanning segments in the final proposed model are boxed. The variable regions (VRs) are indicated.

Fig. 3. Expression of NMB0088 in several *Neisseria* strains. Western blotting of an SDS-PAGE gel loaded with total cell lysates of different strains incubated with pooled sera from mice immunized (i.p.) with rNMB0088 in liposomes. Lanes: 1, strain CU385 (serogroup B); 2, strain 52/91 (serogroup B); 3, strain Z4181 (serogroup C); 4, strain M982 (serogroup B); 5, strain NZ124 (serogroup B); 6, *N. lactamica* strain 102-CIGB; 7, strain Z1127 (serogroup A); 8, strain 135/99 (serogroup Z); 9, *E. coli* W3110 as negative control.

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

Fig. 4. Analysis of antisera elicited against rNMB0088 administered in different formulations and routes. (a) Recognition of antigens present in whole meningococci as detected by whole-cell ELISA, using pooled sera. (b) Passive protection in infant rats challenged with strain CU385. The results shown in (b) are the mean (plus standard deviation) of the level of bacteraemia of individual rat pups within each group. Asterisks indicate a significant statistical difference (* $P < 0.05$; ** $P < 0.01$: ANOVA followed by a Dunnett's post test) between bacterial counts in the blood of immunized mice and the preimmune treated group. In both cases, a pool of preimmune sera (PI) or pooled sera from mice immunized with meningococcal OMVs were included as controls.

long CREEs has been considered to be a transcriptional terminator in the *dcw* cluster of *N. gonorrhoeae* CH811 (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

Table 2. Bactericidal activity of antiserum from mice immunized with recombinant NMB0088 against different *N. meningitidis* strains

Strain	Serological classification	Sequence type (ST) (electrophoretic type [ET] cluster)	NMB0088 variant	Bactericidal titre*		
				Alum (i.p.)	DRV (i.p.)	DRV (i.n.)
CU385	B:4:P1.19,15	ST-33 (ET5 complex)	1	16	32	64
B16B6	B:2a:P1.5,2	ST-11 (ET37 complex)	1	32	<8	128
M982	B:9:P1.22,9	ST-3790 (other)	1	<8	<8	8
Z4181	C:2a:P1.5	ST-11 (ET37 complex)	2	<8	<8	<8
NZ124	B:4:P1.7-2,4	ST-44 complex (ET lineage 3)	4	<8	<8	<8

*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.

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 proteomic 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. meningi-*

tidis (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

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*.

ACKNOWLEDGEMENTS

The authors would like to thank Thomas E. Vaughan (HPA, Porton Down, UK) for providing the unassembled *N. lactamica* Y92-1009 genome. We also would like to thank to Dr Andrew Gorrington from the UK for critical review of this manuscript.

REFERENCES

- Bentley, S. D., Vernikos, G. S., Snyder, L. A., Churcher, C., Arrowsmith, C., Chillingworth, T., Cronin, A., Davis, P. H., Holroyd, N. E. & other authors (2007). Meningococcal genetic variation mechanisms viewed through comparative analysis of serogroup C strain FAM18. *PLoS Genet* **3**, e23.
- Bjune, G., Hoiby, E. A., Gronnesby, J. K., Arnesen, O., Fredriksen, J. H., Halstensen, A., Holten, E., Lindbak, A. K., Nøkleby, H. & other authors (1991). Effect of outer membrane vesicle vaccine against group B meningococcal disease in Norway. *Lancet* **338**, 1093–1096.
- Black, P. N., Said, B., Ghosn, C. R., Beach, J. V. & Nunn, W. D. (1987). Purification and characterization of an outer membrane-bound protein involved in long-chain fatty acid transport in *Escherichia coli*. *J Biol Chem* **262**, 1412–1419.
- Bolduc, G. R., Bouchet, V., Jiang, R. Z., Geisselsoder, J., Truong-Bolduc, Q. C., Rice, P. A., Pelton, S. I. & Goldstein, R. (2000). Variability of outer membrane protein P1 and its evaluation as a vaccine candidate against experimental otitis media due to nontypeable *Haemophilus influenzae*: an unambiguous, multifaceted approach. *Infect Immun* **68**, 4505–4517.
- Buchanan, S. K. (1999). Beta-barrel proteins from bacterial outer membranes: structure, function and refolding. *Curr Opin Struct Biol* **9**, 455–461.
- Buisine, N., Tang, C. M. & Chalmers, R. (2002). Transposon-like Correia elements: structure, distribution and genetic exchange between pathogenic *Neisseria* sp. *FEBS Lett* **522**, 52–58.
- Christodoulides, M., Brooks, J. L., Rattue, E. & Heckels, J. E. (1998). Immunization with recombinant class 1 outer-membrane protein from *Neisseria meningitidis*: influence of liposomes and adjuvants on antibody avidity, recognition of native protein and the induction of a bactericidal immune response against meningococci. *Microbiology* **144**, 3027–3037.
- Comanducci, M., Bambini, S., Brunelli, B., Adu-Bobie, J., Aricò, B., Capecci, B., Giuliani, M. M., Massignani, V., Santini, L. & other authors (2002). NadA, a novel vaccine candidate of *Neisseria meningitidis*. *J Exp Med* **195**, 1445–1454.
- De Gregorio, E., Abrescia, C., Carlomagno, M. S. & Di Nocera, P. P. (2003a). Asymmetrical distribution of *Neisseria* miniature insertion sequence DNA repeats among pathogenic and nonpathogenic *Neisseria* strains. *Infect Immun* **71**, 4217–4221.
- De Gregorio, E., Abrescia, C., Carlomagno, M. S. & Di Nocera, P. P. (2003b). Ribonuclease III-mediated processing of specific *Neisseria meningitidis* mRNAs. *Biochem J* **374**, 799–805.
- Delgado, M., Yero, D., Niebla, O., González, S., Climent, Y., Pérez, Y., Cobas, K., Caballero, E., García, D. & Pajón, R. (2007). Lipoprotein NMB0928 from *Neisseria meningitidis* serogroup B as a novel vaccine candidate. *Vaccine* **25**, 8420–8431.
- Derrick, J. P., Urwin, R., Suker, J., Feavers, I. M. & Maiden, M. C. (1999). Structural and evolutionary inference from molecular variation in *Neisseria* porins. *Infect Immun* **67**, 2406–2413.
- Finne, J., Leinonen, M. & Makela, P. H. (1983). Antigenic similarities between brain components and bacteria causing meningitis. Implications for vaccine development and pathogenesis. *Lancet* **2**, 355–357.
- Francis, F., Ramirez-Arcos, S., Salimnia, H., Victor, C. & Dillon, J. R. (2000). Organization and transcription of the division cell wall (*dcw*) cluster in *Neisseria gonorrhoeae*. *Gene* **251**, 141–151.
- Fredriksen, J. H., Rosenqvist, E., Wedege, E., Bryn, K., Bjune, G., Frøholm, L. O., Lindbak, A. K., Møgster, B., Namork, E. & other authors (1991). Production, characterization and control of MenB-vaccine “Folkehelsa”: an outer membrane vesicle vaccine against group B meningococcal disease. *NIPH Ann* **14**, 67–79.
- Fu, D., Libson, A., Miercke, L. J., Weitzman, C., Nollert, P., Krucinski, J. & Stroud, R. M. (2000). Structure of a glycerol-conducting channel and the basis for its selectivity. *Science* **290**, 481–486.
- Giuliani, M. M., Adu-Bobie, J., Comanducci, M., Aricò, B., Savino, S., Santini, L., Brunelli, B., Bambini, S., Biolchi, A. & other authors (2006). A universal vaccine for serogroup B meningococcus. *Proc Natl Acad Sci U S A* **103**, 10834–10839.
- Gorrington, A., Halliwell, D., Matheson, M., Reddin, K., Finney, M. & Hudson, M. (2005). The development of a meningococcal disease vaccine based on *Neisseria lactamica* outer membrane vesicles. *Vaccine* **23**, 2210–2213.

- Hearn, E. M., Patel, D. R. & van den Berg, B. (2008). Outer-membrane transport of aromatic hydrocarbons as a first step in biodegradation. *Proc Natl Acad Sci U S A* **105**, 8601–8606.
- Hsu, C. A., Lin, W. R., Li, J. C., Liu, Y. L., Tseng, Y. T., Chang, C. M., Lee, Y. S. & Yang, C. Y. (2008). Immunoproteomic identification of the hypothetical protein NMB1468 as a novel lipoprotein ubiquitous in *Neisseria meningitidis* with vaccine potential. *Proteomics* **8**, 2115–2125.
- Jodar, L., Feavers, I. M., Salisbury, D. & Granoff, D. M. (2002). Development of vaccines against meningococcal disease. *Lancet* **359**, 1499–1508.
- Kirby, C. & Gregoriadis, G. (1984). Dehydration rehydration vesicles: a simple method for high yield drug entrapment in liposomes. *Biotechnology* **2**, 979–984.
- Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A. & other authors (2007). CLUSTAL W and CLUSTAL_X version 2.0. *Bioinformatics* **23**, 2947–2948.
- Liu, S. V., Saunders, N. J., Jeffries, A. & Rest, R. F. (2002). Genome analysis and strain comparison of Correia repeats and Correia repeat-enclosed elements in pathogenic *Neisseria*. *J Bacteriol* **184**, 6163–6173.
- Martin, D., Cadieux, N., Hamel, J. & Brodeur, B. R. (1997). Highly conserved *Neisseria meningitidis* surface protein confers protection against experimental infection. *J Exp Med* **185**, 1173–1183.
- Martin, S. L., Borrow, R., van der Ley, P., Dawson, M., Fox, A. J. & Cartwright, K. A. (2000). Effect of sequence variation in meningococcal PorA outer membrane protein on the effectiveness of a hexavalent PorA outer membrane vesicle vaccine. *Vaccine* **18**, 2476–2481.
- Maslanka, S. E., Gheesling, L. L., Libutti, D. E., Donaldson, K. B., Harakeh, H. S., Dykes, J. K., Arhin, F. F., Devi, S. J., Frasch, C. E. & other authors (1997). Standardization and a multilaboratory comparison of *Neisseria meningitidis* serogroup A and C serum bactericidal assays. The Multilaboratory Study Group. *Clin Diagn Lab Immunol* **4**, 156–167.
- Mes, T. H. & van Putten, J. P. (2007). Positively selected codons in immune-exposed loops of the vaccine candidate OMP-P1 of *Haemophilus influenzae*. *J Mol Evol* **64**, 411–422.
- Mitka, M. (2005). New vaccine should ease meningitis fears. *JAMA* **293**, 1433–1434.
- Mora, M., Veggi, D., Santini, L., Pizza, M. & Rappuoli, R. (2003). Reverse vaccinology. *Drug Discov Today* **8**, 459–464.
- Munson, R., Jr & Hunt, A. (1989). Isolation and characterization of a mutant of *Haemophilus influenzae* type b deficient in outer membrane protein P1. *Infect Immun* **57**, 1002–1004.
- Murphy, T. F., Brauer, A. L., Yuskiv, N., McNamara, E. R. & Kirkham, C. (2001). Conservation of outer membrane protein E among strains of *Moraxella catarrhalis*. *Infect Immun* **69**, 3576–3580.
- Nally, J. E., Whitelegge, J. P., Aguilera, R., Pereira, M. M., Blanco, D. R. & Lovett, M. A. (2005). Purification and proteomic analysis of outer membrane vesicles from a clinical isolate of *Leptospira interrogans* serovar Copenhageni. *Proteomics* **5**, 144–152.
- Niebla, O., Alvarez, A., Martin, A., Rodriguez, A., Delgado, M., Falcon, V. & Guillen, G. (2001). Immunogenicity of recombinant class I protein from *Neisseria meningitidis* refolded into phospholipid vesicles and detergent. *Vaccine* **19**, 3568–3574.
- Peng, J., Yang, L., Yang, F., Yang, J., Yan, Y., Nie, H., Zhang, X., Xiong, Z., Jiang, Y. & other authors (2008). Characterization of ST-4821 complex, a unique *Neisseria meningitidis* clone. *Genomics* **91**, 78–87.
- Pizza, M., Scarlato, V., Masignani, V., Giuliani, M. M., Aricò, B., Comanducci, M., Jennings, G. T., Baldi, L., Bartolini, E. & other authors (2000). Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing. *Science* **287**, 1816–1820.
- Rosenqvist, E., Wedege, E., Hoiby, E. A. & Froholm, L. O. (1990). Serogroup determination of *Neisseria meningitidis* by whole-cell ELISA, dot-blotting and agglutination. *APMIS* **98**, 501–506.
- Rosenstein, N. E., Perkins, B. A., Stephens, D. S., Popovic, T. & Hughes, J. M. (2001). Meningococcal disease. *N Engl J Med* **344**, 1378–1388.
- Sardinas, G., Reddin, K., Pajon, R. & Gorringer, A. (2006). Outer membrane vesicles of *Neisseria lactamica* as a potential mucosal adjuvant. *Vaccine* **24**, 206–214.
- Schulz, G. E. (2000). beta-Barrel membrane proteins. *Curr Opin Struct Biol* **10**, 443–447.
- Sierra, G. V., Campa, H. C., Varcacel, N. M., Garcia, I. L., Izquierdo, P. L., Sotolongo, P. F., Casanueva, G. V., Rico, C. O., Rodriguez, C. R. & Terry, M. H. (1991). Vaccine against group B *Neisseria meningitidis*: protection trial and mass vaccination results in Cuba. *NIPH Ann* **14**, 195–207.
- Tappero, J. W., Lagos, R., Ballesteros, A. M., Plikaytis, B., Williams, D., Dykes, J., Gheesling, L. L., Carlone, G. M., Hoiby, E. A. & other authors (1999). Immunogenicity of 2 serogroup B outer-membrane protein meningococcal vaccines: a randomized controlled trial in Chile. *JAMA* **281**, 1520–1527.
- Tettelin, H., Saunders, N. J., Heidelberg, J., Jeffries, A. C., Nelson, K. E., Eisen, J. A., Ketchum, K. A., Hood, D. W., Peden, J. F. & other authors (2000). Complete genome sequence of *Neisseria meningitidis* serogroup B strain MC58. *Science* **287**, 1809–1815.
- Uli, L., Castellanos-Serra, L., Betancourt, L., Dominguez, F., Barbera, R., Sotolongo, F., Guillen, G. & Pajon, F. R. (2006). Outer membrane vesicles of the VA-MENGOC-BC(R) vaccine against serogroup B of *Neisseria meningitidis*: analysis of protein components by two-dimensional gel electrophoresis and mass spectrometry. *Proteomics* **6**, 3389–3399.
- van den Berg, B., Black, P. N., Clemons, W. M., Jr & Rapoport, T. A. (2004). Crystal structure of the long-chain fatty acid transporter FadL. *Science* **304**, 1506–1509.
- van der Ley, P., Heckels, J. E., Virji, M., Hoogerhout, P. & Poolman, J. T. (1991). Topology of outer membrane porins in pathogenic *Neisseria* spp. *Infect Immun* **59**, 2963–2971.
- Vaughan, T. E., Skipp, P. J., O'Connor, C. D., Hudson, M. J., Vipond, R., Elmore, M. J. & Gorringer, A. R. (2006). Proteomic analysis of *Neisseria lactamica* and *Neisseria meningitidis* outer membrane vesicle vaccine antigens. *Vaccine* **24**, 5277–5293.
- Vipond, C., Suker, J., Jones, C., Tang, C., Feavers, I. M. & Wheeler, J. X. (2006). Proteomic analysis of a meningococcal outer membrane vesicle vaccine prepared from the group B strain NZ98/254. *Proteomics* **6**, 3400–3413.
- Wedege, E. & Froholm, L. O. (1986). Human antibody response to a group B serotype 2a meningococcal vaccine determined by immunoblotting. *Infect Immun* **51**, 571–578.
- Wheeler, J. X., Vipond, C. & Feavers, I. M. (2007). Exploring the proteome of meningococcal outer membrane vesicle vaccines. *Proteomics Clin Appl* **1**, 1198–1210.
- Williams, J. N., Skipp, P. J., Humphries, H. E., Christodoulides, M., O'Connor, C. D. & Heckels, J. E. (2007). Proteomic analysis of outer membranes and vesicles from wild-type serogroup B *Neisseria meningitidis* and a lipopolysaccharide-deficient mutant. *Infect Immun* **75**, 1364–1372.
- Wright, J. C., Williams, J. N., Christodoulides, M. & Heckels, J. E. (2002). Immunization with the recombinant PorB outer membrane protein induces a bactericidal immune response against *Neisseria meningitidis*. *Infect Immun* **70**, 4028–4034.

Yau, W. M., Wimley, W. C., Gawrisch, K. & White, S. H. (1998). The preference of tryptophan for membrane interfaces. *Biochemistry* **37**, 14713–14718.

Yero, C. D., Pajon, F. R., Caballero, M. E., Cobas, A. K., Lopez, H. Y., Farinas, M. M., Gonzales, B. S. & Acosta, D. A. (2005). Immunization of mice with *Neisseria meningitidis* serogroup B genomic expression

libraries elicits functional antibodies and reduces the level of bacteremia in an infant rat infection model. *Vaccine* **23**, 932–939.

Yero, D., Pajon, R., Niebla, O., Sardiñas, G., Vivar, I., Perera, Y., Garcia, D., Delgado, M. & Cobas, K. (2006). Bistronic expression plasmid for the rapid production of recombinant fused proteins in *Escherichia coli*. *Biotechnol Appl Biochem* **44**, 27–34.