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Research Paper

Proteomic study via a non-gel based approach of meningococcal outer membrane vesicle vaccine obtained from strain CU385

A road map for discovery new antigens

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Abbreviations: OMV, outer membrane vesicles; 2-DE, two-dimensional gel electrophoresis; SCAPE, selective capture of peptides; SCX, strong cation exchanger; nHnR, neither histidine nor arginine; LC-MS/MS, liquid chromatography with tandem mass spectrometry; m/z, mass-to-charge; LEP, lysyl-endopeptidase; NHS-Msc, 2-(Methylsulfonyl)ethyl succinimidyl carbonate

Key words: OMV-vaccine, *Neisseria meningitidis*, SCAPE, proteomics

This work presents the results from a study of the protein composition of outer membrane vesicles from VA-MENGOC-BC® (Finlay Institute, Cuba), an available vaccine against serogroup B *Neisseria meningitidis*. Proteins were identified by means of SCAPE, a 2DE-free method for proteome studies. More than one hundred proteins were detected by tandem liquid chromatography-mass spectrometry analysis of fractions enriched in peptides devoid of histidine or arginine residues, providing a detailed description of the vaccine. A bioinformatic analysis of the identified components resulted in the identification of 31 outer membrane proteins and three conserved hypothetical proteins, allowing the cloning, expression, purification and immunological study of two of them (NMB0088 and NMB1796) as new antigens.

Introduction

The fast clinical progression of meningococcal disease and its tendency to cause outbreaks and epidemics have kept it for years as a high-priority target of public health authorities worldwide. However, the development of an effective vaccine against the causal agent of meningococcal disease (the gram-negative diplococcus *Neisseria meningitidis*) has remained an unfinished affair.[1] Polysaccharide-based vaccines for serogroup B have failed to induce protective immunity,[2] leading to the current focus of serogroup B meningococcal vaccine research efforts on membrane proteins. Outer membrane protein-based candidates have been developed either as outer membrane vesicles (OMV)[3-5] or, more recently, as purified recombinant proteins.[6] The vaccine manufactured by the Finlay Institute in Cuba (commercially marketed as VA-MENGOC-BC®) is based on OMV from serogroup B strain CU385 plus serogroup C polysaccharide.[4] This vaccine contributed to the rapid decline of the epidemic in Cuba[7] and has been incorporated into the National Immunization Program since 1992. In addition, it has successfully been used for epidemic control in Brazil, Colombia and Uruguay.[7-9]

Although the use of OMV-based vaccines in single-strain epidemic scenarios is generally accepted, it is also understood that this approach is likely to be of limited benefit for endemic serogroup B disease, due to the rapid evolution of antigenic variants among meningococcal populations. Still, the presence of a modest cross-protective response during an efficacy study of VA-MENGOC-BC® in elder Brazilian children[10] suggested the existence of conserved antigens that can induce functionally cross-reactive antibodies, and has stimulated further works towards a more comprehensive characterization of the OMV components of the vaccine.

The first published result in this direction was the development of a proteomic map of this OMV-derived product, based on two-dimensional gel electrophoresis (2-DE) and mass spectrometry,[11] which identified new potentially protective antigens not previously reported as a component of an antimeningococcal vaccine[12] and confirmed the remarkable low batch-to-batch variability of the main active pharmaceutical ingredient of VA-MENGOC-BC®. It also described the presence of 31 different non-redundant proteins, including five major membrane proteins. The limited number of proteins identified by 2-DE together with the known shortcomings of this technique in the analysis of hydrophobic proteins, and proteins with extreme isoelectric points or molecular weights, have driven our efforts to investigate the vaccine product using a novel strategy.

Recently, a method called SCAPE (Selective CApture of PEptides) for gel-free proteomics was published.[13] SCAPE simplifies the

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complexity introduced as a result of the enzymatic digestion of protein mixtures by selecting a subset of peptides representative of the complete population. This method has been further improved by optimizing the chromatographic conditions and using N-Methylsulfonylethoxycarbonyl as the amino group reversible modifier, with a positive effect in the recovery and specificity of the method (unpublished results). Furthermore, a previous in silico analysis has shown that about 94% of the proteins from *N. meningitidis* serogroup B could be identified via SCAPE with an average of 5 peptides per protein.[13]

In the present work SCAPE was applied to the characterization of the protein composition of the active ingredient of VA-MENGOC-BC®, resulting in the identification of more than one-hundred proteins by LC/MS/MS. Additionally, the potential of two of these proteins (NMB0088 and NMB1796) as candidates for a protective antimeningococcal vaccine was assessed.

Results

Proteomic study of OMV from *Neisseria meningitidis* CU385. The OMV proteins were reduced and carbamidomethylated, digested with lysyl-endopeptidase (LEP) and trypsin, and the generated peptides were derivatized with NHS-Msc. The peptides were purified by RP-HPLC and 13 fractions were collected (Fig. 2). The RP-fractions were individually loaded onto the SCX deposited in an ultrafiltration tube. The nHnR peptides collected in the flow-through (filtrate), were submitted to the procedure for the regeneration of amino groups, and isolated peptides were then analyzed in four mass-to-charge (m/z) ranges i.e., four LC-MS/MS runs in a so called Gas-Phase Fractionation experiment.[14] This enabled the selection of a larger numbers of peptide ion signals for MS/MS and hence an increase in the number of detected species. Finally, protein identification was achieved by the combination of automatic analysis of MS/MS spectra by the MASCOT software application with the use of stringent selection criteria (see Discussion). The results were matched with peptides predicted from a protein sequence database derived from genome data of meningococcal strain MC58.

A total of 208 peptide assignments were made, all of them belonging to the nHnR group. This resulted in the identification of 106 non-redundant proteins (Tables 1 and 2; and Suppl. materials). Approximately 43% of the proteins (46) were identified on the basis of two or more peptides; the remainder (60) was identified based on a single peptide match. Additional information for each protein identified, such as peptide amino acid sequence, flanking residues, detected modifications, MASCOT score values, experimental and calculated m/z values and charge of precursors ions can be found in the Supplementary electronic material.

In silico analysis of potential antigens. The identified proteins were classified on the basis of their predicted locations within the bacterial cell using the PSORTb algorithm, which predicts the subcellular locations of proteins in gram-negative bacteria according to the presence or absence of signal peptides, homologies to known proteins, transmembrane domains, and outer membrane anchoring motifs.[15] A total of 31 proteins were predicted by the PSORTb analysis to be located in the outer membrane (Table 1). Most of them have been already identified as major components of the outer membrane, namely, the PorA and PorB porins, OpcA, PilQ, RmpM and the iron-regulated outer membrane protein FetA.[11,16] Two other proteins appear to be abundant in this formulation, OmpP1 (NMB0088) and the hemagglutinin/hemolysin-related protein HrpA (NMB0497); the latter was recently found to be involved in a functional two-partner secretion system in *N. meningitidis* as a secreted effector protein.[17] As NMB0497 represent an almost identical copy of NMB1779 in the MC58 genome,[18] this fact could explain in part the abundance of such peptides after proteomic studies.

To identify novel potential antigens, in addition to the outer membrane proteins, we analyzed those proteins classified by PSORTb as periplasmatic or with unknown localization and function. From the identified proteins three were periplasmic and a set of nine were classified as hypothetical. When the 43 supposed surface antigens was filtered for proteins from which experimental results existed in the literature, there were 13 remaining proteins identified in the OMV without evidences of their use as vaccine candidates.

Immunological analysis of novel antigens identified. Having identified 43 putative surface-exposed proteins in the OMV preparation, we tested the immunogenicity and protective capacity after immunization with two proteins classified here as novel putative antigens: NMB0088 and NMB1796. Protein NMB0088 was selected for two reasons. Firstly, this protein is a homologue ($E = 1e^{-23}$) of the long-chain fatty acid transporter FadL from *E. coli*,[19] indicating that NMB0088 forms probably a β-barrel inside the meningococcal outer membrane. Secondly, it has been detected in meningococcal OMV by other proteomic approaches as a relatively abundant protein.[16,20,21] NMB1796 was chosen as an example of a protein annotated as a conserved hypothetical protein with unknown function.[18] NMB0928 was also included in the analysis as an already known immunoprotective antigens since this is a membrane-associated lipoprotein previously characterized by our group as a potential vaccine candidate.[12]

The immunogenic capacity of these recombinant meningococcal antigens was tested in a murine immunization model. Antisera produced against the three purified recombinant variants demonstrated reactivity with surface antigens of the CU385 strain (Table 3). In addition, western blot analysis of OMV obtained from the homologous strains using the antisera against these recombinant proteins corroborated that the selected antigens are present in the preparation (Fig. 3). Nevertheless, the level of recognition of NMB0928 was significantly lower than that observed for NMB0088 and NMB1796, suggesting that this membrane lipoprotein could be less abundant in the OMV preparation. This finding agrees with the IgG ELISA results obtained against the OMV (Table 3). More important was the fact that the anti-NMB0088 and anti-NMB1796 sera showed bactericidal activity against the homologous strain CU385 with titers of 1:32 and 1:64 respectively. Serum obtained after immunization with recombinant protein NMB1796, administered with aluminium hydroxide, did not induce complement-mediated killing of the strain tested. A negative control group, immunized with a non-related recombinant protein, did not show positive results in any of the assays (Table 3). Functional activity against recombinant NMB0928 has been previously reported.[12]

Discussion

Bacterial outer membrane preparations are characterized by the presence of major protein components. In the case of VA-MENGOC-BC®, the OMV are composed mainly by five outer

Table 1 **Predicted outer membrane proteins[a] identified in this study**

No.	NCBI locus tag (NMB no.)	Protein name	No. peptides	Predicted function (COGS)[b]
1	NMB1988	Iron-regulated outer membrane protein (FetA)	13	Inorganic ion transport and metabolism
2	NMB2039	outer membrane protein class III (PorB)	12	Transport and binding proteins; Porins
3	NMB1429	outer membrane protein class 1 (PorA)	8	Transport and binding proteins; Porins
4	NMB1053	outer membrane protein class 5c (OpcA)	9	Cell envelope
5	NMB1812	Secretin (PilQ secretin)	7	Intracellular trafficking, secretion and vesicular transport
6	NMB0382	outer membrane protein class 4 (RmpM)	3	Cell wall/membrane/envelope biogenesis
7	Opa[c]	Opacity protein	3	Colony opacity-associated adhesin
8	Opa[c]	Opacity protein	3	Colony opacity-associated adhesin
9	NMB0280	organic solvent tolerance protein, probable	3	Cell wall/membrane/envelope biogenesis
10	NMB1994	Putative adhesin/invasion (NadA)	3	Cellular processes; Pathogenesis
11	NMB0663	outer membrane protein (NspA)	1	Cell wall/membrane/envelope biogenesis
12	NMB1497	TonB-dependent receptor	3	Inorganic ion transport and metabolism
13	NMB1829	TonB-dependent receptor	2	Inorganic ion transport and metabolism
14	NMB0088	outer membrane protein P1, FadL probable	5	Lipid transport and metabolism
15	NMB1985	adhesion and penetration protein	3	Cell wall/membrane/envelope biogenesis
16	NMB1540 or NMB1541	lactoferrin-binding protein A or B	2	Inorganic ion transport and metabolism
17	NMB0497 and/or NMB1779	hemagglutinin/hemolysin-related protein	5	Cellular processes; Pathogenesis
18	NMB1214	hemagglutinin/hemolysin-related protein	3	Cellular processes; Pathogenesis
19	NMB0493	hemagglutinin/hemolysin-related protein	3	Cellular processes; Pathogenesis
20	NMB1762	hemolysin activation protein HecB, probable	1	Intracellular trafficking, secretion and vesicular transport
21	NMB0461	transferrin-binding protein A	2	Inorganic ion transport and metabolism
22	NMB0182	outer membrane protein Omp85	3	Cell wall/membrane/envelope biogenesis
23	NMB0700	IgA1 protease	2	Cell wall/membrane/envelope biogenesis
24	NMB1668	hemoglobin receptor	2	Inorganic ion transport and metabolism
25	NMB0464	phospholipase A1, putative	2	Cell wall/membrane/envelope biogenesis
26	NMB1333	conserved hypothetical protein	1	Cell cycle control, cell division, chromosome partitioning
27	NMB1971	conserved hypothetical protein	1	Function unknown
28	NMB1737	secretion protein, probable	1	Cell wall/membrane/envelope biogenesis
29	NMA0474	hemoglobin-haptoglobin-utilization protein (HpuB)	2	Inorganic ion transport and metabolism
30	NMA0475	hemoglobin-haptoglobin utilization protein (HpuA)	1	Inorganic ion transport and metabolism
31	NMB0703	competence lipoprotein ComL	1	Function unknown

[a]Proteins localization on the outer membrane predicted by the PSORTb algorithm. [b]Predicted functional categories were derived from the Clusters of Orthologous Groups of proteins (COGS) database (located at http://www.ncbi.nlm.nih.gov/). [c]These proteins were not annotated in the MC58 genome database. In CU385 the two Opa proteins detected appeared to be different.

membrane antigens; a fact that limits the detection of minor components of the preparation, as evidenced during initial attempts to characterize the active protein ingredient of VA-MENGOC-BC® by a combination of 2-DE and mass spectrometry.[11] In the present work, a gel-free method called SCAPE[13] was applied to the identification of proteins present in such OMV in order to achieve a more comprehensive characterization of the vaccine. This resulted in the identification of 106 proteins, based on the specific isolation and analysis of nHnR peptides. The specificity of our approach is highlighted by the fact that among all the meningococcal peptides reported by MASCOT (212 in total); only 4 non-nHnR species were detected. These were discarded based in the selected acceptance criteria.

Several features of our proteomic strategy deserve further comments. In general, the ability to select a specific peptide subset greatly decreases the false-positive rate during proteomics data validation.[22] On this regard, it has been suggested that confident peptide and protein identifications should be reported based on the use of two or more algorithms, validated manually or by statistical models. More recently, other criteria have been explored to accept or refute identifications. These include the retention time of peptide during reversed-phase chromatography and the isolectric point of peptide after isolectrofocusing. In the present study, we have addressed the issue above by following a stringent set of criteria which combines a wide database search of MS/MS spectra (i.e., tryptic peptides from all bacteria), and partial manual validation. These criteria yield a positive identification only when the Mascot probability analysis chooses a *N. meningitidis* peptide or protein as the top candidate, the identified peptide belongs to the nHnR subset, the peptide score is

Table 2 **Proteins identified that are not predicted to have an outer membrane location**

No.	NCBI locus tag	Protein name	No. peptides	Cellular location[a]	Predicted function (COGS)[b]
32	NMB0550	thiol-disulfide interchange protein dsbC homolog	1	P	Posttranslational modification, protein turnover, chaperones
33	NMB0634	iron(III) ABC transporter, periplasmic binding protein	1	P	Inorganic ion transport and metabolism
34	NMB0928	hypothetical protein (NlpB probable)	1	P	Cell wall/membrane/envelope biogenesis
35	NMB0387	ABC transporter, ATP-binding protein	1	CM	Function unknown
36	NMB0401	proline dehydrogenase	4	C	Energy metabolism; Amino acids and amines
37	NMB0139 and/or NMB0124	translation elongation factor Tu	2	C	Translation, ribosomal structure and biogenesis
38	NMB1604	phosphoglycerate mutase	2	C	Carbohydrate transport and metabolism
39	NMB0138	translation elongation factor EF-G	2	C	Translation, ribosomal structure and biogenesis
40	NMB1445	recA protein	2	C	Replication, recombination and repair
41	NMB0815	Adenylosuccinate synthetase	1	C	Nucleotide transport and metabolism
42	NMB0944	homocysteine methyltransferase	2	C	Amino acid transport and metabolism
43	NMB0431	citrate (si)-synthase	1	C	Energy production and conversion
44	NMB1339	prolyl-tRNA synthetase	1	C	Translation, ribosomal structure and biogenesis
45	NMB1390	glucokinase	2	C	Carbohydrate transport and metabolism
46	NMB1127 or NMB1165	short chain dehydrogenase	1	C	Secondary metabolites biosynthesis, transport and catabolism
47	NMB1379	Cysteine desulfurase	1	C	Amino acid transport and metabolism
48	NMB1506	Arginyl-tRNA synthetase	1	C	Translation, ribosomal structure and biogenesis
49	NMB0693	dihydrofolate synthase	2	C	Coenzyme transport and metabolism
50	NMB0214	oligopeptidase A	1	C	Amino acid transport and metabolism
51	NMB0045	Cell division protein ftsY homolog	1	C	Intracellular trafficking, secretion and vesicular transport
52	NMB1861	acetyl-CoA carboxylase, biotin carboxylase	1	C	Lipid transport and metabolism
53	NMB1932	glycyl-tRNA synthetase, alpha chain	1	C	Translation, ribosomal structure and biogenesis
54	NMB1934	ATP synthase F1 sector subunit beta	1	C	Energy production and conversion
55	NMB0010	phosphoglycerate kinase	1	C	Carbohydrate transport and metabolism
56	NMB1833	isoleucyl-tRNA synthetase	1	C	Translation, ribosomal structure and biogenesis
57	NMB0351	transaldolase	1	C	Carbohydrate transport and metabolism
58	NMB1526	SsrA-binding protein	1	C	Posttranslational modification, protein turnover, chaperones
59	NMB1897	Leucyl-tRNA synthetase	1	ML	Translation, ribosomal structure and biogenesis
60	NMB0295	signal recognition particle protein	2	ML	Intracellular trafficking, secretion and vesicular transport
61	NMB2101	30S ribosomal protein S2	2	U	Translation, ribosomal structure and biogenesis
62	NMB0137	30S ribosomal protein S7	1	U	Translation, ribosomal structure and biogenesis
63	NMB0156	30S ribosomal protein S8	1	U	Translation, ribosomal structure and biogenesis
64	NMB0166	30S ribosomal protein S11	1	U	Translation, ribosomal structure and biogenesis
65	NMB0136	30S ribosomal protein S12	1	U	Translation, ribosomal structure and biogenesis
66	NMB0165	30S ribosomal protein S13	1	U	Translation, ribosomal structure and biogenesis
67	NMB1321	30S ribosomal protein S18	1	U	Translation, ribosomal structure and biogenesis
68	NMB0463	30S ribosomal protein S20	1	U	Translation, ribosomal structure and biogenesis
69	NMB0157	50S ribosomal protein L6	1	U	Translation, ribosomal structure and biogenesis
70	NMB1320	50S ribosomal protein L9	1	U	Translation, ribosomal structure and biogenesis
71	NMB0127	50S ribosomal protein L11	2	U	Translation, ribosomal structure and biogenesis
72	NMB2057	50S ribosomal protein L13	1	U	Translation, ribosomal structure and biogenesis
73	NMB0161	50S ribosomal protein L15	1	U	Translation, ribosomal structure and biogenesis
74	NMB0149	50S ribosomal protein L16	1	U	Translation, ribosomal structure and biogenesis
75	NMB0158	50S ribosomal protein L18	1	U	Translation, ribosomal structure and biogenesis

Table 2 Proteins identified that are not predicted to have an outer membrane location (continued)

76	NMB0589	50S ribosomal protein L19	1	U	Translation, ribosomal structure and biogenesis
77	NMB0147	50S ribosomal protein L22	3	U	Translation, ribosomal structure and biogenesis
78	NMB0144	50S ribosomal protein L23	1	U	Translation, ribosomal structure and biogenesis
79	NMB0876	50S ribosomal protein L25	1	U	Translation, ribosomal structure and biogenesis
80	NMB0652	adhesin mafA	2	U	Function unknown
81	NMB0039	hypothetical protein	1	U	Function unknown
82	NMB0763	cysteine synthase	2	U	Amino acid transport and metabolism
83	NMB1212	hypothetical protein	2	U	Function unknown
84	NMB1916	3-oxoacyl-(acyl-carrier-protein) synthase III	2	U	Lipid transport and metabolism
85	NMB2095	adhesin complex protein, probable	3	U	Function unknown
86	NMB0476	hypothetical protein	3	U	Function unknown
87	NMB1291	ribonucleoside-diphosphate reductase, alpha chain	2	U	Nucleotide transport and metabolism
88	NMB1125 or NMB1163	probable periplasmic protein	1	U	Function unknown
89	NMB0323	UbiH family protein	2	U	Coenzyme transport and metabolism
90	NMB1457	transketolase	1	U	Carbohydrate transport and metabolism
91	NMB1389	RpiR/YebK/YfhH family protein	1	U	Transcription
92	NMB1874	probable orotate phosphoribosyltransferase	2	U	Nucleotide transport and metabolism
93	NMB1887	triosephosphate isomerase	1	U	Carbohydrate transport and metabolism
94	NMB1870	Factor H binding protein (Lipoprotein GNA1870)	1	U	Function unknown
95	NMB1572	aconitate hydratase 2	1	U	Energy production and conversion
96	NMB1690	phosphoglucomutase	1	U	Carbohydrate transport and metabolism
97	NMB1468	hypothetical protein	1	U	Function unknown
98	NMB0337	branched-chain amino acid aminotransferase	1	U	Amino acid transport and metabolism
99	NMB1230	DNA-binding protein HU-beta	1	U	Replication, recombination and repair
100	NMB1581	histidinol dehydrogenase	1	U	Amino acid transport and metabolism
101	NMB2135	conserved hypothetical protein	1	U	Function unknown
102	NMB1796	conserved hypothetical protein	1	U	Function unknown (Predicted FMN-oxidoreductase)
103	NMB2069	thiamin-phosphate pyrophosphorylase	1	U	Coenzyme transport and metabolism
104	NMB0415[c]	competence factor in gonococcus (Dca)	1	U	Function unknown
105	NMB0895	conserved hypothetical protein	1	U	Function unknown
106	NMB0313	Putative outer membrane protein OmpU	1	U	Function unknown

[a]Proteins localization predicted by the PSORTb algorithm: C, cytoplasmic; P, periplasmic; CM, cytoplasmic membrane; U, unknown; ML multiple locations. [b]Predicted functional categories were derived from the Clusters of Orthologous Groups of proteins (COGS) database (located at http://www.ncbi.nlm.nih.gov/). [c]NMB0415 is a pseudogene in MC58 due to a frameshift caused by the presence of nine guanines.

Table 3 Reactivity of antisera obtained following immunization with selected protein candidates

Formulation (Antigen-Adjuvant)	ELISA titer[a] measured against: Homologous protein	OMV (CU385)	Whole cells (CU385)
NMB0088-Aluminum	409600	12800	1600
NMB1796-Freund's	25600	100	3200
NMB0928-Aluminum	204800	<100	12800
OMV-Aluminum	ND	>12800	>12800
LpdA-P6-Aluminum[b]	ND	<100	<100

[a]Data are IgG titers of pooled sera from groups of ten mice. ND, Not determined. [b]A non-related recombinant protein used as negative control (P6 protein from *H. influenzae* fused to meningococcal LpdA).

higher than 25, at least 4 consecutive *y"* ion fragments in the MS/MS spectrum are assigned, and the most intense signals of the spectrum are explained from the sequence.

Furthermore, for a number of proteins detected based on single peptide matches, auxiliary information assisted in the validation of their identification. The proteins NMB0663, NMB0550, NMB0351, NMB0157, NMB1320 and NMB0876 were previously identified in the vaccine with more than 25% of sequence coverage.[11] For the proteins NMB1796 and NMB0928, with single peptide hits of relative median-to-low MASCOT score values, the antibody-based assay carried out here to demonstrate immunogenicity was also useful as an alternative validation tool, to confirm their presence in the OMV.

In particular, for NMB0928, the comparison of the recognition of OMV and whole bacterial cells by sera from animals immunized with the recombinant protein showed that despite it being a relatively

abundant protein on the surface of intact bacteria, it appears to be poorly represented in the OMV preparation. The extraction with deoxycholate, one of the steps for the manufacture of OMV,[23] may have reduced the amount of this lipoprotein below the detection threshold for the immunochemical assays used in this work. Ferrari et al.,[24] have recently demonstrated that the treatment to obtain detergent-extracted OMV may selectively remove membrane proteins, particularly lipoproteins.

The proteomic study identified 31 (58%) out of the 53 potential outer membrane proteins predicted by PSORTdb from the genomic sequence of *N. meningitidis* MC58. The number of peptide hits was biased towards the identification of outer membrane proteins (2 or more nHnR peptides) compared to proteins not predicted to localize to this compartment. This result is a logical consequence of the enrichment in outer membrane antigens achieved by the preparation of OMV, given that the number of peptide hits usually correlates with abundance in the sample.[25] However, the overall data indicate that OMV are not exclusively constituted by outer membrane proteins, since 70% of the identified components were classified by PSORTb as cytoplasmic, periplasmic or with unknown location. Only one cytoplasmic membrane protein was detected (NMB0387), and the cytosolic components corresponded to proteins involved in housekeeping functions, which tend to be most abundant in this compartment. The presence of "non-outer membrane" antigens has been systematically reported for *N. meningitidis* OMV analyzed by proteomic techniques.[16,21,24,26] Whether this fact (particularly for predicted periplasmic proteins) highlights the shortcomings of the method for OMV preparation or is an indication of actual membrane association for these molecules is still a matter of research and debate. However, it has been demonstrated that certain periplasmic and cytosolic proteins in *N. meningitidis* are exposed[24] and they elicit serum bactericidal activity after immunization in mice.[27,28] Grifantini et al.,[29] have recently proposed a model in which proteins not located in the outer membrane could migrate to the bacterial surface due to membrane remodeling during infection.

As part of the meningococcal serogroup B genome sequence project,[18] a large number of proteins has been proposed as novel antigens;[6,30] two of which (NMB1870,[31] and NadA[32]) were also identified in the present study. In addition, the outer membrane proteins NspA[33] and Omp85,[34] that have been likewise proposed as vaccine candidates, were also detected. On the other hand, several proteins that have been postulated as putative vaccine candidates were not detected in the OMV, including: AspA, NMB2132, AutA, membrane-bound lytic murein transglycosylase A (NMB0033) and TspA (NMB0341). However, the failure to detect a protein cannot be taken as absolute evidence of absence, since the nature of the LC-MS/MS approach, as well as the huge difference in the dynamic detection range for different proteins, suggests undetected antigens are often simply present at very low levels.

Here we have shown that meningococcal proteins OmpP1 (NMB0088) and NMB1796, not previously characterized as vaccine candidates, are immunogenic and able to induce bactericidal antibodies in mice. Nevertheless, further studies should be conducted to fully demonstrate the potential of these antigens in *N. meningitidis* as vaccine candidates and also to understand the precise function of both proteins. The homologous to OmpP1 in *Haemophilus influenzae* is a major surface antigen bearing B-cell and T-cell epitopes,[35] and is bound by complement component C3 during opsonisation.[36] A homologue protein to NMB0088 is also present in the related commensal organism *Neisseria lactamica*. This protein has been identified as an abundant component of OMV obtained from the *N. lactamica* Y92-1009 strain.[21,37] In MC58, NMB1796 is annotated as a conserved hypothetical protein with unknown function, and we have found that it is likely to be surface exposed. This protein shares homology ($E = 5e^{-16}$) with a conserved domain characteristic of a group of NADPH-dependent FMN reductases. Recently, it has been demonstrated that expression of gene *nmb1796* is regulated by iron[38] via Fur,[39] indicating that the produced protein could be a pathogenic attribute for the meningococcus probably involved during the infection.

It is important to note that other conserved uncharacterized minor proteins (Tables 1 and 2) were identified in the OMV of CU385 strain, two of which (NMB1333 and NMB1971) are probably located in the outer membrane. The NCBI Conserved Domain Search reveals that NMB1333 contains a sequence with homology to the Peptidase family M23/M37, which in addition to the eukaryotic proteins of the family, includes bacterial lipoproteins that have no peptidase activity. NMB1971 was identified through signature-tagged mutagenesis as a gene essential for bacteremic disease.[40] In addition we also identified protein NMB1468 that it has been recently proposed as a novel lipoprotein with vaccine potential.[41] All these conserved proteins could also be involved in the protection provided by VA-MENGOC-BC® in some individuals against serogroup B meningococcal strains other than the vaccine type-strain.[9,10,42,43] We suggest that these proteins should be considered in future studies of new subunit vaccines against the meningococcus.

Interestingly, we detected two proteins in the CU385 OMV, not previously annotated in the MC58 genome,[18] with homologies to the serogroup A hemoglobin-haptoglobin utilization proteins NMA0474 (HpuB) and NMA0475 (HpuA). HpuAB is a two-component receptor system that mediates binding to haemoglobin, haemoglobin-haptoglobin and apo-haptoglobin, and both proteins are also known to be phase variable in Neisseria species.[44]

In conclusion, our work expanded the protein catalogue of the active ingredient of VA-MENGOC-BC®, since it was possible to annotate less abundant proteins not initially detected.[11] Still, seven proteins (ATP synthase F1 alpha subunit, putative aminopeptidase, homoserine dehydrogenase, electron transfer flavoprotein, alpha subunit, DNA-binding response regulator, 2,3,4,5-tetrahydropyridine-2-carboxylate N-succinyltransferase and glyceraldehyde 3-phosphate dehydrogenase) that were identified by the 2-DE approach,[11] escaped detection in the present study in spite of having nHnR peptides. This confirms the fact that different proteomic techniques provide complementary results. Thus, having a comprehensive picture of the protein composition of the vaccine will undoubtedly contribute to understand the mechanisms by which the OMV stimulate the immune system.

Furthermore, our study contributes to confirm that some proteins predicted from the genome sequence as hypothetical, are indeed expressed. It also demonstrates that utilization of genome sequence by application of proteomics and bioinformatics can expedite the vaccine discovery process, providing a set of potential candidates for further testing, and paving the way for a further refinement of OMV-based vaccines through their enrichment with minor conserved

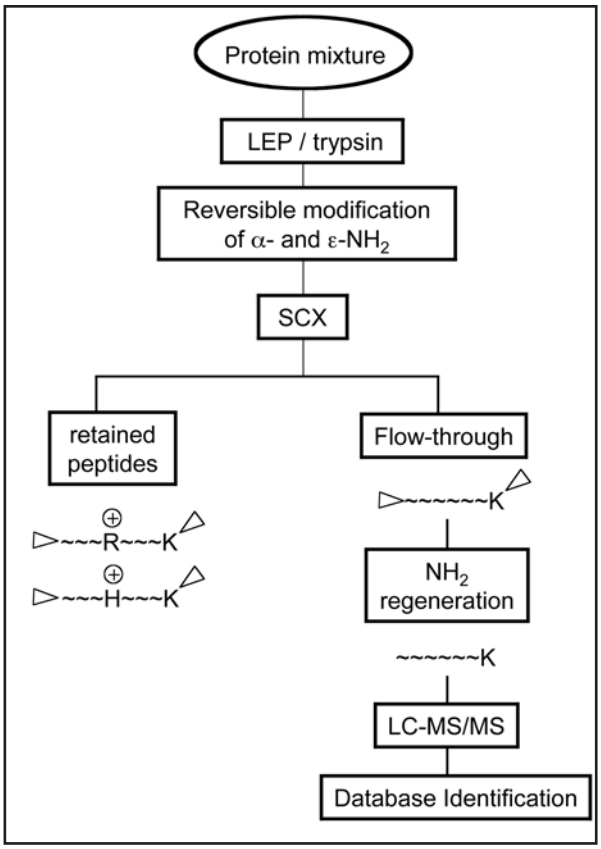

Figure 1. Schematic representation of the SCAPE method. The triangle represents the reversible blocking group attached to α- and ε-amino groups, in this case N-Methylsulfonylethoxycarbonyl.

antigens. Such a strategy should result on a wide-range protective response that could lead to a truly cross-protective product.

Materials and Methods

Proteomics-related materials. The enzymes Lysyl-endopeptidase (LEP) and trypsin were from Wako (Japan) and Promega (USA), respectively. Iodoacetamide and 2-(Methylsulfonyl)ethyl succinimidyl carbonate (NHS-Msc) were obtained from SIGMA (USA). The OMV, the main active ingredient of VA-MENGOC-BC®, were obtained from meningococcal strain CU385 (B:4,7:P1.19,15; ST = 33), and were manufactured by the Finlay Institute (Cuba).[4,23] Other reagents and solvents were HPLC-grade or the highest quality available.

Workflow of the SCAPE method. Figure 1 depicts a schematic representation of SCAPE. Briefly, the protein mixture is first enzymatically digested and the α- and ε-amino groups are chemically modified through a reversible reaction. The modification abolishes the positive charge of α- and ε-amino groups under acidic conditions. The mixture of derivatized peptides is loaded onto a strong cation exchanger (SCX), which traps positively charged species while non-charged peptides, containing neither histidine nor arginine (nHnR) peptides are captured in the flow-through. The amino modifier group is then released from nHnR peptides to regenerate free amino groups, and finally the nHnR fraction is analyzed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) detection in order to identify the proteins present in the mixture.

Reduction and carbamidomethylation. The OMV extract (400 μg) was dissolved in 100 μL of 150 mM HEPES, pH 8.1 containing 3 M guanidine chloride and incubated for 1 hour with 10 mM DTT in a nitrogen atmosphere at 37°C. Iodoacetamide was then added to a final concentration of 20 mM, and the reaction was allowed to proceed at room temperature in the dark for 30 minutes.

LEP and trypsin digests. The mixture of alkylated OMV proteins was two-fold diluted with water and the proteins were digested with LEP at an enzyme-to-substrate mass ratio of 1/50, for 12 hours at 37°C. After this time, the digest was again two-fold diluted with water, and trypsin was added at an enzyme-to-substrate mass ratio of 1/100, for 8 hours at 37°C.

Acylation of amino groups with NHS-Msc. The mixture of proteolytic peptides was diluted with 200 μL of 500 mM HEPES, pH 7.1, to give a final pH around 7.5. NHS-Msc, dissolved in DMSO, was added to the peptide solutions in a 25 molar excess over the total concentration of amino groups. The reaction was allowed to proceed for 15 minutes at 4°C.

RP-HPLC. The acylated peptides were purified by HPLC (LKB-Pharmacia) in a RP-C18 column (250 x 4.6 mm, Vydac). The peptides were eluted using a linear gradient of solvent B (0.05% TFA in acetonitrile) from 1 to 60% in 150 minutes at a flow rate of 0.8 ml/min. The eluate was monitored at 226 nm.

Capture of nHnR peptides. About 70 mg of the strong cation exchange resin (SCX, PolySULFOETHYL A, PolyLC, USA) was placed in an ultrafiltration tube of 0.1 μm (UFC30VV00, Millipore, USA). The resin was extensively washed and equilibrated with 5 mM potassium phosphate buffer, pH 2.7 containing 5% acetonitrile. The reverse-phase fractions containing the N-acylated peptides were dissolved in 200 μl of the equilibrium solution, independently loaded onto the resin and the ultrafiltration tube was spun in a centrifuge at 6000 rpm. The filtrated eluate containing nHnR peptides was submitted to a procedure for the regeneration of the amino groups, while the peptides retained by the SCX column were eluted with a solution of 1 M NaCl.

Regeneration of amino groups. The Msc groups were released by incubation of modified peptides with 100 mM NaOH at room temperature for 15 minutes. Afterward, the peptide fractions were acidified with formic acid 5%, concentrated in speed-vac and analyzed on the LC-MS/MS system.

LC-MS/MS experiments. The LC-MS/MS analysis were performed on an integrated nano LC-MS/MS system (Agilent Series 1100, USA) equipped with a microautosampler. The injected samples (40 μL) were trapped and desalted on an LC-Packings PepMap C18 Precolumn Cartridge (5 mm x 300 μm I.D, USA) for 40 min with 0.1% formic acid delivered by an auxiliary pump at 25 μl/min. By valve switching the trapping column was back-flushed and the sample was loaded onto a C18 capillary column (15 cm x 75 μm i.D., packed with 5 μm Zorbax 300 SB, USA), at 200 nl/min using a 90 min gradient of 5% to 45% acetonitrile in 0.1% formic acid.

The outlet of the column was connected to a quadrupole orthogonal acceleration tandem mass spectrometer QTof-2™ (Micromass, UK). Online nanoESI-MS survey scan and data dependent acquisition of CID MS/MS were fully automated and synchronized with the nanoLC runs under the software control of MassLynx 4.0. Argon was used as the collision gas for CID MS/MS. For routine

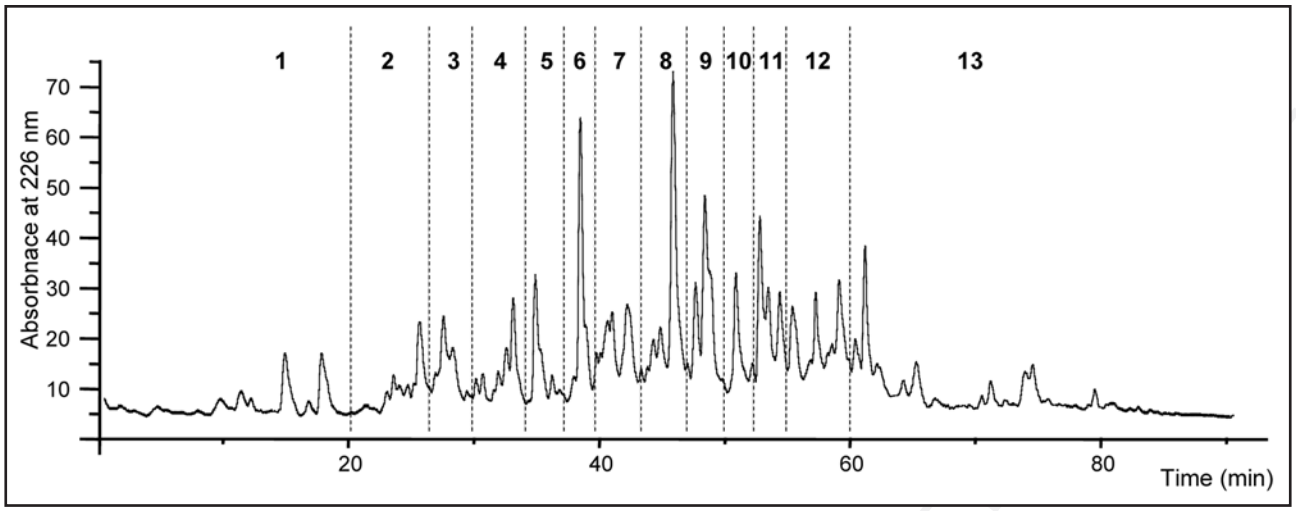

Figure 2. RP-HPLC profile of the enzymatic digest with LEP and trypsin of the OMV-based vaccine. The generated peptides were previously derivatized with NHS-Msc and 13 fractions were collected.

protein identification analysis, 1 s survey scans were acquired over the predefined mass range and a maximum of 4 concurrent MS/MS acquisitions were triggered for 2+, 3+ charged precursors detected at an intensity above a 15 counts threshold. Each MS/MS acquisition was completed and switched back to survey scan when the precursor intensity fell below a 2 counts threshold or after a maximum of 5 s acquisition. For each nHnR peptide fraction four different m/z ranges (4 LC-MS/MS runs) were employed for ion selection. They were: a single broad m/z range of 400–1600 and 3 narrow m/z ranges covering 400–600, 590–800 and 790–1300.

Protein identification. After data acquisition, the individual MS/MS spectra acquired for each of the precursors within a single LC run were combined, smoothed and centered using the Micromass ProteinLynx Global Server (PGS) 2.0 data processing software and output as a single Mascot-searchable peak list (.pkl) file. The peak list files were used to query the SwissProt database using the Internet-available search engine Mascot (http://www.matrixscience.com/) with the following parameters: taxonomy, bacteria; enzyme, trypsin; peptide mass tolerance, 0.5 Da; MS/MS ion mass tolerance, 0.08 Da; allow up to one missed cleavage; variable modifications included methionine oxidation and deamidation, while cysteine carbamidomethylation was considered as a fixed modification.

The identification of a protein or peptide was considered positive using the consensus of several criteria: (a) the Mascot probability analysis choose a *N. meningitidis* peptide or protein as the top candidate, (b) the identified peptide is nHnR, (c) the peptide score was greater than 25, (d) the assignment of 4 consecutive *y"* ion fragments in the MS/MS spectrum and (e) the most intense signals of the MS/MS spectrum are explained from the sequence.

Bioinformatic analysis of proteins. The predicted cellular localizations for each protein were determined with the program PSORTb 2.0.4 (http://www.psort.org/psortb/). Prediction of domains or motifs suggestive of putative proteins function was performed by using the CDD (Conserved Domain Database) tool available at the NCBI website (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Functions were assigned in cases where sequence similarity extended over 80% mutual coverage and the expectation value of the alignment was smaller than e^{-10}.

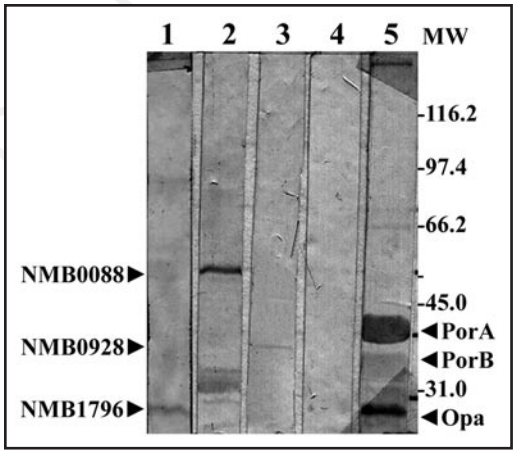

Figure 3. Western blotting analysis of outer membrane vesicles from *N. meningitidis* CU385 (10 μg per lane) with pooled sera from mice immunized with the recombinant antigens NMB1796 (lane 1), NMB0088 (lane 2), NMB0928 (lane 3) and LpdA-P6 as a negative control (lane 4). A pool of sera from OMV-immunized mice (lane 5) was used as a positive control.

Cloning, expression and purification of the selected protein candidates. The pM238 expression system[45] was used for cloning the neisserial gene fragments to express the proteins in *Escherichia coli* as COOH-terminus His-tagged fusion. In addition, with this expression vector the recombinant protein is expressed fused at its N-terminus to the first 47 amino acids of the LpdA (dihydrolipoamide dehydrogenase A) protein from *N. meningitidis*. The sequences coding for proteins NMB0088[45] and NMB0928[12] have been previously cloned and expressed as recombinant fusion proteins using this system. For the amplification of DNA fragment coding for protein NMB1796 the following primers were used: 1796F1, 5'-CCG GTC TAG ATC TAG GAA TGA TTA TGG C-3', and 1796R1, 5'-ATT CCC GGG ATC CTT CAA TCA AAC C-3'. Fusion proteins were purified by affinity chromatography on Cu^{2+}-conjugated chelating fast-flow Sepharose (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Proteins expressed as inclusion bodies were solubilized with Urea and renatured by dialysis after purification.

Immunization of animals. The adjuvants used in the immunization study were Freund's complete adjuvant (FCA), and incomplete adjuvant (both from Sigma) and aluminium hydroxide (Alhydrogel; Superfos, Denmark). The vaccine formulations were prepared according to the manufacturer's instructions. Groups of 10 BALB/c mice (CENPALAB, Havana, Cuba), 4 to 6 weeks old, were immunized subcutaneously with 10 μg of each antigen in one of the previously mentioned adjuvant formulations (see Table 3). Negative control mice received 10 μg of a non-related recombinant protein (outer membrane protein P6 from *Haemophilus influenzae*) expressed in *E. coli* using the same expression vector.[45] The animals were boosted on days 7, 14 and 28 of the immunization schedule. As positive control, a group was immunized following the same schedule with 5 μg per dose of meningococcal OMV (with aluminum adjuvant). The animals were bled on days 0 (preimmune) and 38, and the sera were used for immunoassays. Sera from each immunization group were pooled prior all assays.

Immunological assays. Antibody titers were determined by ELISA on microtiter plates coated with 100 μL/well of the corresponding recombinant protein (5 μg/mL) or OMV (10 μg/mL) diluted in carbonate buffer (0.05 M Na_2CO_3, pH 9.6). Antibodies against the protein exposed on the surface of the bacteria were detected by whole-cell ELISA as described previously.[46] Immunoglobulin titers were expressed as the reciprocal of serum dilution showing more than two-fold increase in absorbance over that obtained with a negative control. Immunoblots were prepared on OMV with 10% polyacrylamide gels and 1:100 sera dilutions.

Bactericidal assay. The bactericidal assay was carried out in 96-well plates as described previously,[12] with adsorbed baby rabbit serum used as complement source. The serum bactericidal titers were reported as the reciprocal of the serum dilution yielding ≥50% bacterial killing.

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