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RESEARCH ARTICLE

Outer membrane vesicles of the VA-MENGOC-BC® vaccine against serogroup B *of Neisseria meningitidis*: Analysis of protein components by two-dimensional gel electrophoresis and mass spectrometry

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Neisseria meningitidis is a Gram-negative bacterium responsible for significant mortality worldwide. While effective polysaccharides-based vaccines exist against serogroups A, C, W135, and Y, no similar vaccine is suitable for children under 4 years against disease caused by serogroup B strains. Therefore, major vaccine efforts against this serogroup are based on outer membrane vesicles (OMVs), containing major outer membrane proteins. The OMV-based vaccine produced by the Finlay Institute in Cuba (VA-MENGOC-BC®) contributed to the rapid decline of the epidemic in this Caribbean island. While the content of major proteins in this vaccine has been discussed, no detailed work of an outer membrane proteomic map of this, or any other, commercially available OMV-derived product has been published so far. Since OMVs exhibit a large bias toward a few major proteins and usually contain a high content of lipids, establishing the adequate conditions for high resolution, 2-DE of this kind of preparation was definitely a technical challenge. In this work, 2-DE and MS have been used to generate a proteomic map of this product, detailing the presence of 31 different proteins, and it allows the identification of new putative protective protein components it contains.

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1 Introduction

Meningococcal disease remains as one of the most feared infections to be faced by man due to its rapid progression and its tendency to cause outbreaks and epidemics. Every country suffers from meningococcal disease, but its pattern and frequency varies widely between different regions. In temperate countries, it is usually an endemic disease, with annual incidence between 1 and 20 *per* 100 000 inhabitants [1, 2], and true epidemics are rare [2–6], which contrasts sharply with the pattern seen in the "meningitis belt" of sub-Saharan Africa [7], where annual incidence may exceed 200 *per* 100 000 population. Mortality from meningococcal septicemia may be as high as 20–50% and, in some countries, meningococcal disease is now the most frequent cause of death due to infection in childhood [1, 8, 9]. Exposure to the causal agent, *Neisseria meningitidis*, commonly results in asymptomatic nasopharyngeal carriage of the bacteria. Around 10% of adults and up to 30% of teenagers have positive nasopharyngeal cultures; while carriage is believed to

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Abbreviations: DC, dendritic cell; **LPS**, lipopolysaccharide; **OMPs**, outer membrane proteins; **OMVs**, outer membrane vesicles

be transient in most adults, the cause of progression from carriage to invasive disease is unclear. The highest incidence of the disease is among children between 6 and 24 months old with a steady decline in incidence with age [1, 10] until the teenage years when there is a small secondary peak [4].

N. meningitidis is a Gram-negative diplococcus and has both an outer and an inner (cytoplasmic) cell membrane that are separated by a peptidoglycan cell wall. The outer membrane is surrounded by a polysaccharide capsule that is essential for pathogenicity [11, 12]. The chemically distinct polysaccharide capsules are at the heart of the present serogroup-based typing, depending on which one of the 12 capsule antigens are expressed (A, B, C, H, I, K, L, W135, X, Y, Z, and 29E). Additionally, the outer membrane contains several proteins which enable the organism to interact with host cells. It also contains the lipopolysaccharide (endotoxin, LPS) that is involved in the pathogenesis of meningococcal disease [11]. The outer membrane proteins (OMPs), LPS, and the capsular polysaccharide are the principal surface antigens of the organism and are highly variable, with variation driven by environmental and host factors [13–18].

The global impact of infections due to *N. meningitidis* has prompted the scientific community to place a substantial emphasis on the development of preventive universal vaccines against this deadly pathogen. Immunization against the causative organism is likely to be the only measure that will further reduce the morbidity and mortality of the disease. Unfortunately, the quest for the universal meningococcal vaccine has been far from being an easy task.

Several strategies have been developed, the first approach being the use of capsular polysaccharides. Tetra- and trivalent glycoconjugate vaccines, based on capsular polysaccharides, are able to elicit protective antibodies that are serogroup-specific [19] and those conferring protection against serogroups A, C, Y, and W-135 are on schedule to be licensed in several countries. However, there are specific problems related to the production of a polysaccharide-based vaccine against serogroup B meningococcal disease [20]. In this case, the development of vaccines against this serogroup has concentrated on the use of subcapsular antigens and the development of vaccines containing outer membrane vesicles (OMVs).

A number of efficacy trials have been carried out using soluble OMV vaccines of different formulations. The two vaccines most extensively studied were developed in the 1980s in response to outbreaks of disease in Cuba [21] and Norway [22], respectively. The vaccine developed at the Finlay Institute in Cuba (commercially marketed as VA-MENGOC-BC®) is produced by employing OMVs from strain CU-385 plus serogroup C polysaccharide, and is adsorbed to aluminum hydroxide [21]. This vaccine contributed to the rapid decline of the epidemic in Cuba [23]; it has been incorporated into the National Immunization Program since 1992, and is commercially available with more than 25 million vaccinees worldwide.

OMV-based vaccines appear to effectively present OMPs in a sufficiently natural conformation to allow the generation of functional bactericidal antibodies, at least in teenagers and adults. The antibody response generated has also been shown to increase opsonophagocytosis of meningococci [24–26]. Obviously, the precise formulation of the vaccines (*i.e.*, content of OMPs and LPS, and the presence and nature of adjuvant) has a significant impact on immunogenicity [24–26] and the control of every component and batch-to-batch consistency are top priorities.

Only recently, *N. meningitidis* partial and more detailed proteome maps have been published for the serogroup B MC58 strain [27], serogroup C (http:, www.abdn.ac.uk/~mmb023/neismen/neisf5_f.htm, *Web page* 1997), and serogroup A [28]. Techniques for proteome analysis have also been applied to characterize the antigen composition of commercial vaccines [29], and some applications have arisen for the characterization of OMVs of pathogenic bacteria [30].

Bacterial outer membrane preparations are characterized by the presence of major protein components. In the case of *N. meningitidis* vaccine against serotype B, the OMVs are composed mainly of five major outer membrane antigens and exhibit a high content of lipids [31]; in consequence, establishing the adequate conditions for high resolution, 2-DE of this preparation is a particularly difficult task. In this paper we describe the successful analysis by 2-DE of three different batches of *N. meningitidis* OMVs present as the active ingredient of VA-MENGOC-BC, and the consequent identification of minor protein components in the preparation. This work is, to our knowledge, the first systematic proteome mapping of a commercially available OMV-based neisserial vaccine, and it allows the identification of putative protective protein components it contains.

2 Materials and methods

2.1 *N. meningitidis* OMVs

Three batches of the active pharmaceutical ingredient of Cuban OMV vaccine VA-MENGOC-BC (Finlay Institute, Cuba) were received and studied. OMVs were prepared by the manufacturer from the outer membranes of *N. meningitidis* serogroup B strain CU385 (B:4:P1.19.15; L3,7,9) by a detergent extraction method [32]. Briefly, OMVs were obtained from live bacteria by gentle extraction with 10% deoxycholate (Merck, Darmstadt, Germany). Bacterial debris was removed by centrifugation, and nucleic acids were eliminated by enzymatic treatment with nucleases (5 μg/mL) (Merck). OMVs were purified by gel filtration chromatography on Sephacryl S-300 (Pharmacia Fine Chemicals, Uppsala, Sweden) followed by precipitation with 96% ethanol.

2.2 Sample preparation and gel electrophoresis

To remove lipids, the aqueous suspension of *N. meningitidis* OMVs was vortexed for 10 min at room temperature with four volumes of ethyl ether. After a brief centrifugation at $10\,000 \times g$, the upper (organic) phase was removed and the procedure was repeated twice. This step was evaluated by

mini-SDS gel electrophoresis. The ether extracts derived from delipidating 300 μg of sample were dried, and the pellet was redissolved in 10 μL of sample buffer containing SDS, and loaded to the 12.5% polyacrylamide gel. Samples of 10 μg taken before and after delipidation of protein containing aqueous phase were also evaluated in 1-D mini-SDS gels. All gels were silver stained.

The aqueous phase was dried by centrifugal evaporation and the pellet dissolved in the rehydration solution (7.5 M urea, 1.5 M thiourea, 2% CHAPS, 0.5% ASB-14, 15% glycerol, 1% DTT, 1% carrier ampholytes p*I* range 3.5–10). 2-DE was performed by combing IEF in IPG strips (Amersham Biosciences, Amersham, UK) with vertical (20 cm) SDS-PAGE. Proteins were reduced and alkylated with iodoacetamide during the equilibration step between the first and the second separation as recommended by Amersham: 10 min in 10 mL equilibrating solution containing 100 mg DTT followed by 10 min in 10 mL equilibrating solution containing 250 mg iodoacetamide. For analytical gels, the sample (30 μg for separation range 3–10, 50 μg for separation range 4–7) was incorporated in 11 cm strips during overnight gel rehydration. For preparative gels, up to 500 μg of protein was loaded on previously rehydrated strips (18 cm) on a paper bridge positioned between the anodic electrode and the acidic border of the strip [33]. Focusing was done using a Multiphore II equipment from Amersham (Amersham) For analytical gels (11 cm strips) voltage was programmed as follows: 200 V (15 min), to 1750 V in 50 min, to 2600 V in 38 min, to 3500 V in 45 min, then at 3500 V until total 15.5 kVh for range 4–7, and 13.5 kVh for range 3–10, respectively. For preparative gels (18 cm strips) focusing was prolonged to 40 kVh. Second dimension was done in 16.5% polyacrylamide gels, 20 cm × 20 cm using the Protean II system from BioRad. Silver staining for analytical gels was done according to Heukeshoven and Dernick [34], while preparative gels were silver stained according to Jensen *et al.* [35].

2.3 Image analysis

Gel images were recorded using the ScanJet 6300C (Hewlett Packard, Palo Alto, CA, USA) at a resolution of 1200 dpi. Gels were simultaneously stained, by using a single batch of staining solution. Images were acquired at fixed time.

Raw images of 2-D gels were processed and analyzed using the Melanie III software (GeneBio, Geneva, Switzerland). Spots were automatically detected without previous contrast enhancement, followed by manual editing when necessary. For each gel, automatic detection, spot edition, and area calculation was done twice and values were compared for evaluating data consistency. Batch-to-batch gel reproducibility was evaluated by using the available tools (pair reports and scatter plot analysis).

2.4 Protein digestion and peptide extraction protocol

Protein digestion with trypsin followed standard procedures while peptide extraction followed a recently published improved protocol [36]. The proteolytic peptides were recovered through a tandem extraction procedure: first at a basic and finally at an acid pH by using the ZipTip C18 from Millipore (MA, USA). The peptides absorbed onto the ZipTip were extensively washed with formic acid (5% v/v) and eluted successively in two separate solutions of 1.5 μL each: first in 60% ACN, 1% formic acid, and then in 50ï sopropanol, 5% formic acid. Finally, both solutions were mixed and loaded into the gold-coated borosilicate nanotip (Micromass, Manchester, UK) for the mass spectrometric analysis.

2.5 MS

The low-energy ESI-MS and MS/MS spectra were acquired using a hybrid quadrupole orthogonal acceleration tandem mass spectrometer QTOF-2™ from Micromass fitted with a Z-spray nanoflow electrospray ion source. Other measuring conditions and data processing were the same as reported previously [36].

2.6 Protein identification

The ESI-MS spectra of the proteolytic peptides were deconvoluted by using the MaxEnt 3.0 (Micromass) and a list containing the molecular masses of the most intense signals was loaded into the ProFound program [37], for the identification by PMF. The criteria for considering a correct identification by PMF were similar to those previously described [36]. The ESI-MS/MS spectra of the peptides were deconvoluted by MaxEnt 3.0, exported as a DTA file and loaded into MASCOT search engine [38]. MS/MS ion search was performed with a peptide mass tolerance of ±2 Da and a fragment mass tolerance of ±0.8 Da. Partial enzyme cleavages, oxidation of methionine, and deamidation of asparagine were considered in these searches. Results from MASCOT searches were carefully scrutinized. Matches with a significant score, indicating identity with a protein in the database, were accepted. Some ESI-MS/MS spectra were manually interpreted in order to obtain partial or complete sequence information and were further analyzed with the computer program Pepsea (http://www.unb.br/cbsp/paginiciais/pepseaseqtag.htm).

3 Results and discussion

The OMV-based active pharmaceutical ingredient of VA-MENGOC-BC vaccine is composed essentially of two kinds of components: proteins, with a predominant contribution of OMPs, and lipids from the outer membrane of this Gram-negative bacterium. Major protein components of the vaccine, clearly identified by using specific antibodies raised against pure OMPs of the organism, are PorA, PorB, RmpM, Opa, and OpcA proteins [32]. A first attempt to get a detailed panorama of minor components by 2-DE analysis of the intact vaccine preparation was unsuccessful. Apparently the high lipid composition promoted severe streaking of the major components, while minor components were not

detected. The presence of major protein components and a large amount of lipids certainly complicated the already complex task of resolving membrane proteins in 2-D gels. In consequence, considerable effort was devoted to the search for optimal sample preparation procedures. Our first objective was the efficient removal of lipids, while keeping the protein composition unaltered.

Membrane proteins tend to be hydrophobic; in consequence, some procedures for removal of lipids may also remove some hydrophobic proteins from the aqueous preparation. Therefore, we evaluated five procedures for lipid extraction while observing possible modifications of protein profiles in SDS gels: protein precipitation in TCA–acetone, or extraction of lipids from the aqueous vaccine preparation with chloroform, ether, ether–hexane, or ethyl acetate–hexane. Some protein bands were diminished or absent when the acid–acetone pellet and the pellet obtained after extraction with chloroform were redissolved in SDS sample buffer and analyzed by 1-D PAGE (not shown). SDS-gel electrophoresis of the organic extracts (previously concentrated to dryness) did not show the presence of proteins while the band profile of the aqueous fraction after extraction with ether, ether–hexane, and ethyl acetate–hexane was highly similar to the intact vaccine preparation. In consequence, for lipid removal, we introduced an ether extraction as the first step in sample preparation (Fig. 1).

Due to the known difficulties for the analysis of membrane proteins by 2-DE, variations in the detergent composition of 7.5 M urea, 1.5 M thiourea sample solutions were evaluated for their ability to keep the largest number of species correctly focused in the 2-D gel as well as the quality of the separation. We did a careful evaluation of several detergents and their combinations, including CHAPS, ASB 14, and SB 3-10. We found improvement in gel quality when 4% CHAPS was replaced by a mixure of CHAPS and ASB 14 (a fact previously described by several authors). Curiously, there was no difference between adding 2% ASB 14 or 0.5% ASB 14 to 2% CHAPS, in consequence, we selected 2% CHAPS, 0.5% ASB 14 as the detergent composition.

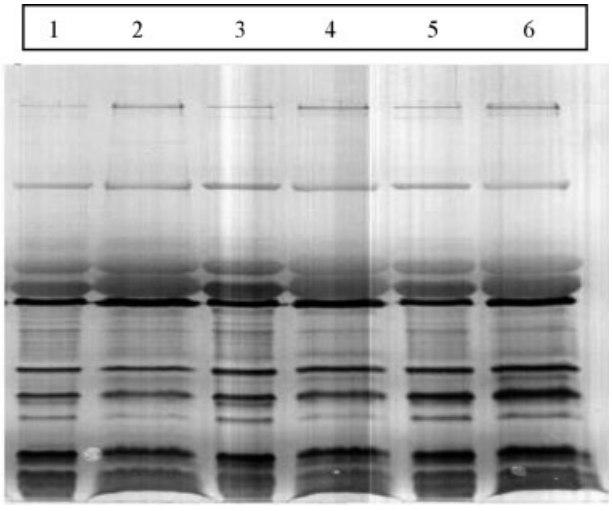

Figure 1. Analysis of delipidated active ingredients of VA-MENGOC-BC. Lipids in the active ingredients were extracted with ethyl ether as described in Section 2.2. SDS-polyacrylamide gel (12.5% PA) shows the protein profile corresponding to 10 μg of the aqueous suspension before and after lipid removal. (1) Batch A; (2) Batch A, delipidated; (3) Batch B; (4) Batch B, delipidated; (5) Batch C; (6) Batch C, delipidated.

Once the conditions for delipidation and sample solubilization were established, the maximum protein load allowing an adequate focusing in analytical gels was investigated. This was a critical parameter, due to the presence of five major species dominating the 2-DE pattern. For gels in the separation range from p*I* 3 to 10, the maximum protein loading during strip rehydration overnight for the analytical gels was 30 μg, while it was 50 μg for gels in the separating range from 4 to 7. Higher loadings generated horizontal streaking at the MW region corresponding to the predominant components. Figure 2 shows the 2-DE maps for three production batches of the main active component of VA-MENGOC-BC vaccine. A complex protein panorama is revealed after 2-DE, showing more than 260 spots in the 3–10 p*I* range gels. Due to the higher protein load and higher resolution, the number of spots significantly increased (more than two-fold) in gels covering the separation range from p*I* 4 to 7.

Batch-to-batch consistency for three production batches of *N. meningitidis* OMVs was evaluated by estimating the spot volume corresponding to the five major components (Table 1) and by using the scatter plot option of the Melanie III software (Fig. 3). Batch A was selected as the reference batch for comparisons, as it showed the highest number of resolved spots in both systems.

For the separation range p*I* 3–10, there were 245 common spots in the three batches under comparison, and 16 spots were only present in one or two of the compared maps. After considering only intense spots, defined as those whose contribution to total spot volume was equal or higher than 0.1% of the total volume, 78 spots were identified as common to all gels and only 7 spots were absent in one or in two of them.

Table 1. Evaluating batch-to-batch reproducibility: Spot number in three batches and contribution of major proteins of the main active component of VA-MENGOC-BC

Spot number and major proteins	Batch		
	A	B	C
Number of spots. Gel p*I* 3–10	297	282	264
Number of spots. Gel p*I* 4–7	630	623	628
FrpB	9.0%	9.1%	9.2%
PorA	18.8%	19.8%	21.8%
PorB	13.9%	15.8%	18.7%
RmpM	9.0%	10.7%	8.3%
Opa + OpcA	7.5%	8.5%	7.5%
Contribution of major proteins to the total protein content	58.2%	69.3%	65.6%

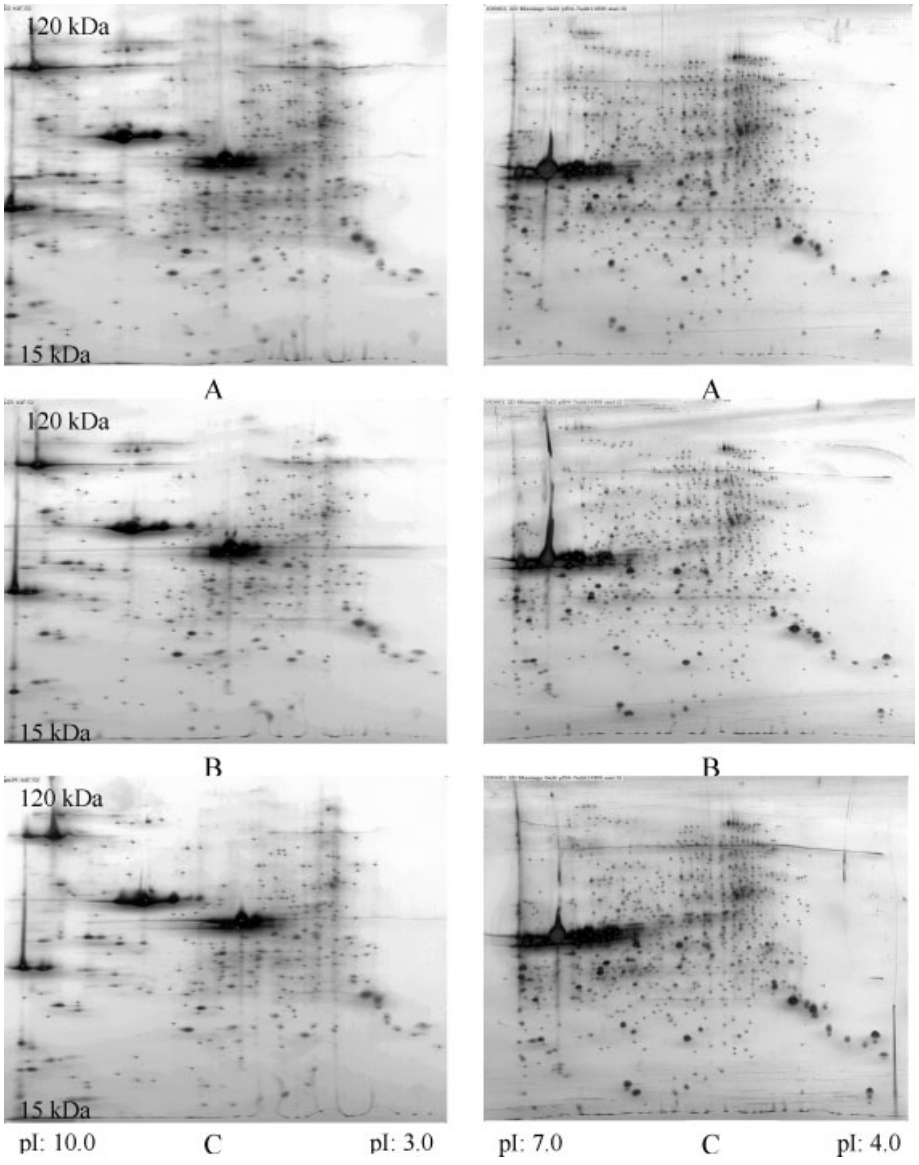

Figure 2. 2-DE of three production batches of the OMV active ingredient. Left: separation range p*I* 3–10; right: separation range p*I* 4–7.

Low-abundance components that were not detected in gels stained with MS compatible-silver stain were not selected here as they were also not amenable to identification.

A similar comparison was performed for gels in the separation range 4–7. There were 512 spots common to all gels and 47 spots absent in one or in two gels. After selection of the spots contributing equally or higher that 0.1% to the total spot volume, 275 spots were identified as common to the three production batches while only 9 spots were absent in one or two batches. The analysis of batch reproducibility after selecting spots according to their contribution to the total spot volume was a valuable tool for identifying those components that substantially contribute to total protein mass in the vaccine and discriminating batch-to-batch variability introduced by very low-abundance components. Inter-batch variation in the number of spots was very low when very minor components (representing below 0.1% of the total spot volume) were not considered, confirming the high reproducibility in the protein pattern observed by visual inspection (Fig. 3).

Once a thorough evaluation of batch-to-batch consistency had been assessed with the analytical gels, a key technical challenge was to increase protein load as high as possible to allow efficient identification of most of the protein species resolved by the gels, while keeping horizontal streaking under limits compatible with image analysis and adequate spot definition. Three sample loading procedures were evaluated: sample loading during overnight strip rehydration, sample loading in cups, and sample loading on paper strips [33]. In addition, the two last methods were evaluated at both the anodic and the cathodic region of the strip. Best results were obtained after sample loading (500 μg) on paper strips

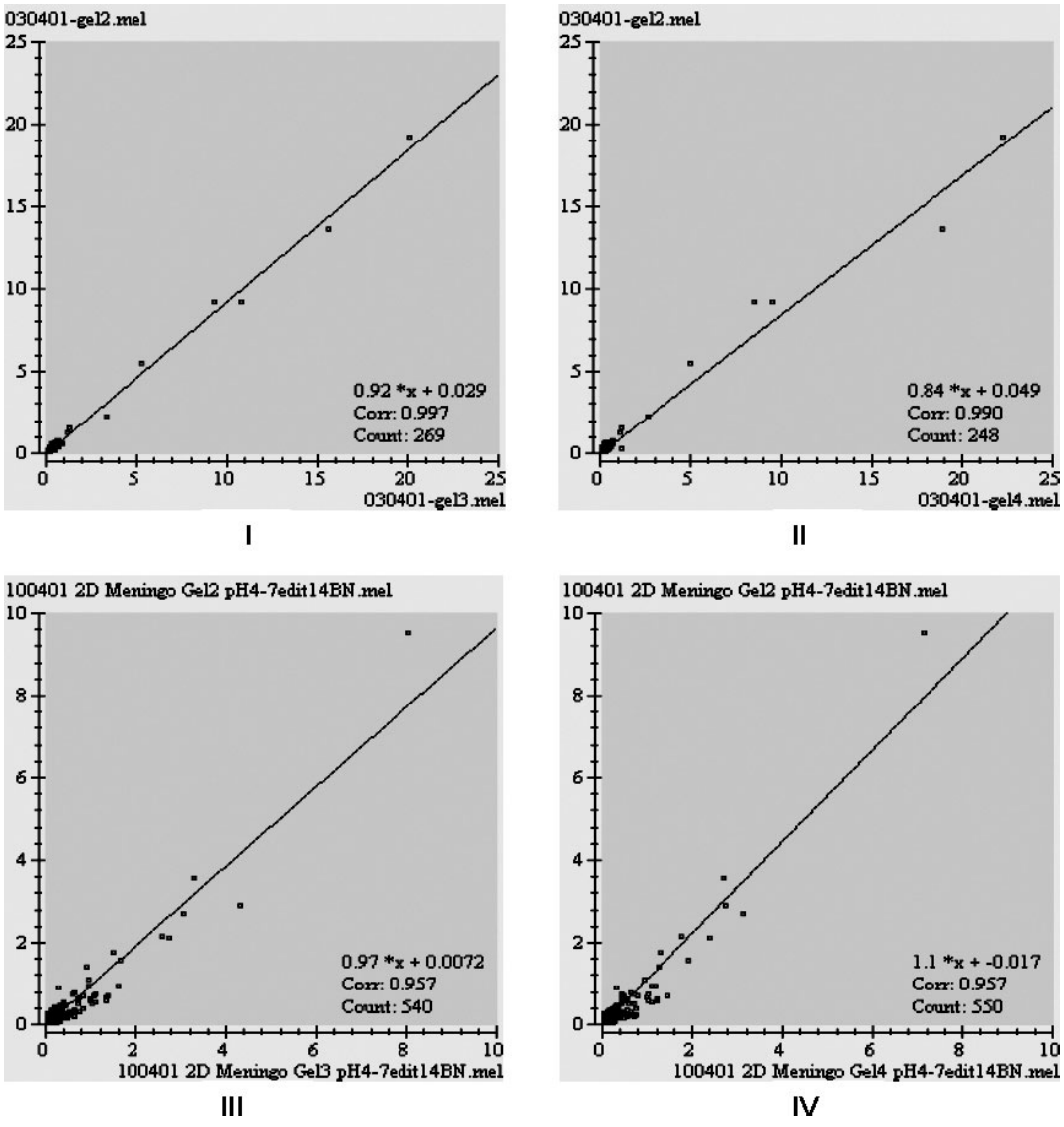

Figure 3. Scatter plot for spot volume (%). Upper panel: separation range: p*I* 3–10. (I) Comparison between Batch B and Batch A; (II) comparison between Batch C and Batch A. Lower panel: separation range 4–7. (III) Comparison between Batch B and Batch A. (IV) Comparison between Batch C and Batch A.

placed at the anodic end of strips (Fig. 4); these gels were used for spot identification. Preparative gels for protein identification by MS were obtained in the ranges p*I* 3–10, p*I* 4–7, and p*I* 6–11.

2-DE spots were digested with trypsin and analyzed by ESI-MS and ESI-MS/MS. Proteins were identified by PMF using the consensus of several criteria: (a) PMF identification using two search programs (ProFound and MASCOT), two databases (NCBI and Swiss-Prot), and bacteria as the taxonomy category placed the same *N. meningitidis* protein as the top candidate; (b) sequence coverage (20% or higher); (c) the most intense signals of the spectrum are explained from the sequence; and (d) a minimum of five matching peptides with a mass accuracy better than 0.05 Da.

When the above criteria were not fulfilled, the identification was further confirmed or accomplished through the analysis of MS/MS spectra by using the MASCOT program and also by manually sequencing aided with the Pepsea program.

Several proteins – generally the major components of the preparation – were repeatedly identified in multiple locations (Fig. 4 and Suppl. Table 1), indicating the presence of fragments differentially migrating at unpredictable p*I* and mass values. The most ubiquitous entity was the OMP Class 3, identified in 12 spots. Additionally not only major components but also minor components were identified in multiple locations; this was the case of the elongation factor G that was identified in four spots (Suppl. Table 1). The presence of translation elongation factors was in good agreement with

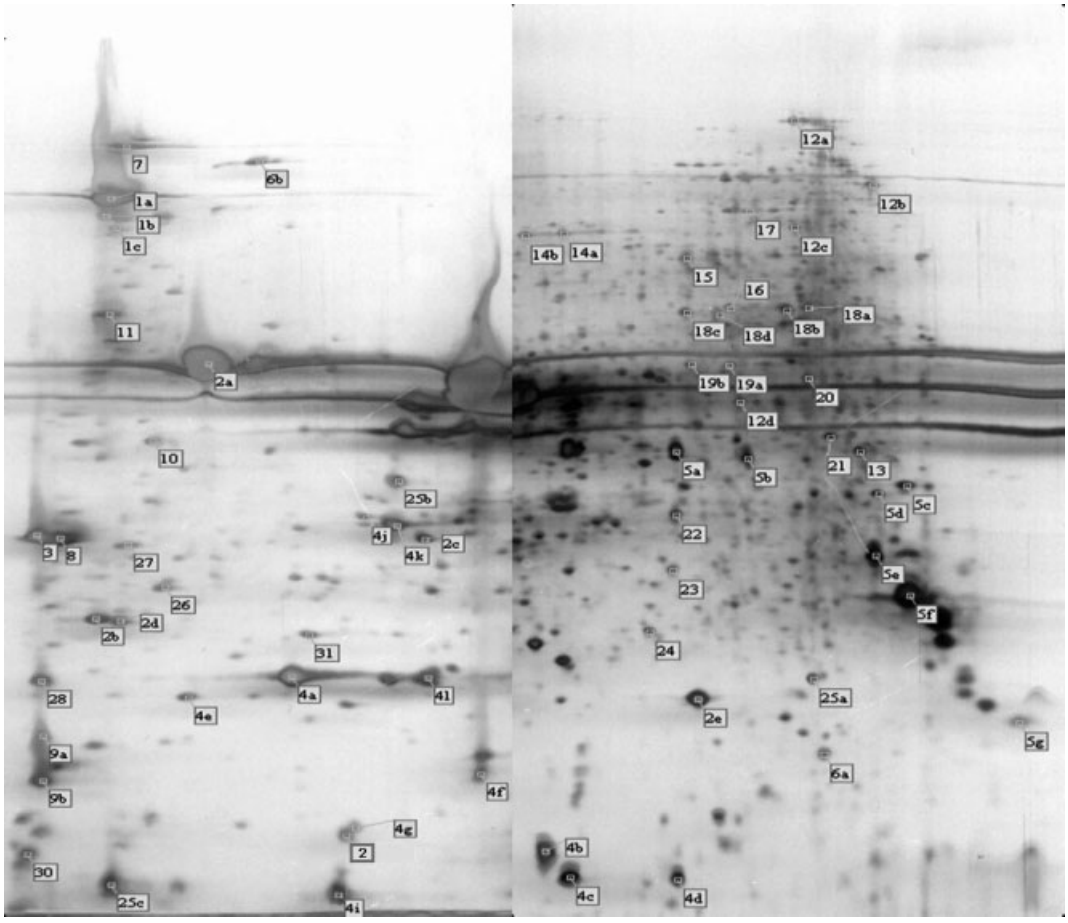

Figure 4. Preparative gels showing the identified spots. Image was composed by combining gel images from separation ranges p*I* 4–7 and p*I* 6–11. Spot numbering corresponds to proteins shown in Suppl. Table 1.

previous reports for *N. meningitidis* [28] and other pathogens [39] and it is correlated not only with their role in translation elongation but also with the chaperone properties reported for some of them [40, 41]. In general, heterogeneity was not only due to variability in p*I* values (horizontal patterns) but also due to some variability in M_r and combination of both types were observed.

The occurrence of unspecific cleavage by trypsin at Tyr residue or putative ion source fragments from larger peptides was detected in the spots 5b, 5d, and 23. For proteins OMP Class 3, OMP Class 4, and suface protein A in spots 4a, 4b, 4f, 4k, 5c, 5f, 5g, and 9b, signals corresponding to their predicted tryptic N-terminus, after removal of the signal peptide, were clearly identified.

The presence of a protein mixture was detected in the spot 29. This assignment was possible by PMF identification of the more abundant protein in the spot (50S ribosomal protein L9) and the ESI-MS/MS analysis of a single peptide from the OMP Class 3.

In five OMPs we identified peptides shifted to a mass value higher than the expected by 80 Da (Table 2), which suggested the presence of protein phosphorylation or sulfation. Upon collision dissociation experiments the modification group was readily lost and, by further increasing the collision energy, it was possible to fragment the nonmodified pseudo molecular ion and to obtain detailed sequence information. A typical case is shown in the spectrum from the tryptic digest of spot 3, corresponding to OMP Class 5c, and in the MS/MS spectrum from the doubly charged peptide with *m/z* 1226.08 (Fig. 5A). However, it was not possible to define the site of modification by MS/MS as none of the original precursor ions were present at the time of peptide backbone fragmentation. Furthermore, these peptides contain several amino acids such as serine, threonine, tyrosine, histidine, aspartic, or glutamic acids which may be considered as potential sites for modification. This array of possibilities made the determination of the actual nature and site of the modified residue quite a challenge that will require further experiments.

In total, from 78 selected spots (Fig. 4), 65 were successfully characterized corresponding to 31 unique proteins, while the remaining 13 corresponded mainly to extremely minor components from which no valuable information was obtained. Summaries of the identifications are shown in Suppl. Table 1.

Table 2. Identified peptides with a modification of 80 Da

Protein	In-gel ID	*m/z* Experimentally modified peptide	*m/z* Theoretical peptide	Charge	Peptide sequence
	4b	1225.09	1185.10	2	SVEHNGGQVVSVETGTGIVDLGSK
OMP class 3 (PorB)	4l	1225.09	1185.10	2	SVEHNGGQVVSVETGTGIVDLGSK
	4c	1231.08	1191.07	2	LVEDNYSHNSQTEVAATLAYR
OMP class 4 RmpM	5f	788.70	762.02	3	VEGHTDFMGSDKYNQALSER
		1031.02	990.99	2	GEASVQGYTVSGQSNEIVR
		2614.24	1268.12	2	AQELQTANEFTVHTDLSSISSTR
OMP class 5c (OpcA)	3	1303.65	1263.28	2	VHADLLSQLGNGWYINPWSEVK
		1226.08	1186.08	2	IDLYTGYTYTQTLSDSLNFR
		778.84	738.86	2	TYKESGEFSVTTK
Opacity protein (Opa)	8	935.41	895.42	2	ETTTTFSPPAQGATVPGK
		999.46	959.41/	2	LENTRFKTHEVSLGMR
Surface protein A NsgA	9b	851.39	811.38	2	EGASGFYVQADAAHAK

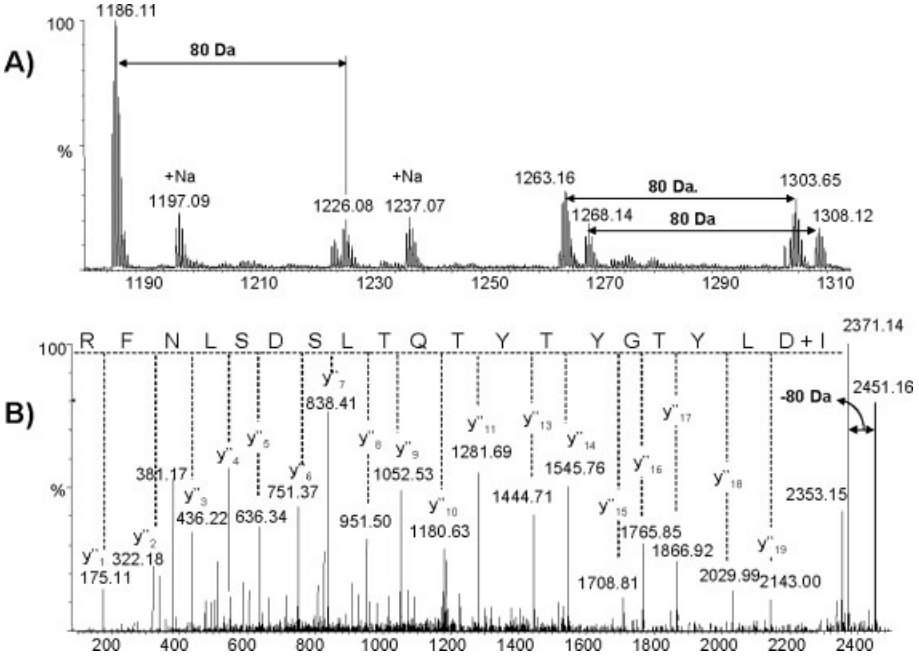

Figure 5. Detection of modified tryptic peptides from the spot 3 corresponding to protein OMP Class 5c. (A) Section of the ESI-MS showing four peptides presenting a modification of 80 Da. (B) MS/MS from modified peptide of *m/z* 1226.08, assigned to the sequence IDLYTGY-TYTQTLSDSLNFR.

It is noteworthy to point out that the presence of different spots representing each protein also followed a conserved pattern among gels, being clearly superimposable, and confirming the high consistency appreciated through the comparative analysis of the three batches (Table 1).

The functional classification of the proteins present in the OMV was largely facilitated by the availability of several Neisserial genome sequences [42–44]. As expected, the cell envelope was the most abundant functional class represented in the map in terms of total mass contribution. So far, the published genome analysis of meningococcus predicts several OMPs (26 for serogroup A, and 22 for serogroup B). In this work 11 of them were positively identified; and five corresponded to major OMPs representing between 58 and 65% of the total protein composition of the OMVs. While both, the known solubilization problems for membrane proteins that are expected to act as limiting factors for protein resolution by 2-DE mapping [45] and the large bias in total protein content in favor of major proteins, were partially addressed, a higher number of OMPs were expected. Since several OMPs were not detected, probably because they may not be expressed at significant levels during *in vitro* culture, it is obvious that this must be the central point of further improvement of the techniques shown in this work, and certainly the motivation for the development of new methodologies.

Several cytosolic proteins were also found to be present in the analyzed OMVs. In the published proteome for serogroup A [28], energy metabolism was the functional class with the highest representation, covering about 17% of the total number of identified species, being the proteins belonging to the class of synthesis and modification of macromolecules being the second most represented functional class. In this work, of the 20 minor cytosolic proteins identified, 11 belonged to the small molecule metabolism including energy and amino-acid biosynthesis functional classes, and 9 to macromolecule metabolism including protein synthesis and modification. An important finding in this last class was a putative amino-peptidase, since its impact and participation in the generation of minor degradation of major proteins deserves further study.

The presence of a large number of nontypically predicted membrane proteins raised very interesting points to be considered in the characterization of present OMV-based vaccines, and OMV-based products in general, and most important in the development of future meningococcal vaccines. There are reasons to believe that some of these proteins, not previously estimated to have any hydrophobic/trans-membrane regions, could have become membrane-associated due to functional rather than structural constraints. Well-documented examples of this, some of them relevant to proteins identified in our study, are ATP synthases [46], bacterioferritin (Pessolani *et al.*, 1994) [47] and heat shock proteins [48]. Interestingly, some heat shock proteins (HSP70 and others) have been found to be present even in the detergent-resistant membrane fractions [49]. Ribosomal proteins are also among the documented cases of membrane association [50]. For example, Gu *et al*. (2003) [51] reported the membrane association of a large number of ribosomal proteins in *Mycobacterium tuberculosis*. These results are consistent with the observation that membrane-bound or exported proteins are cotranslationally inserted into the membrane by ribosomes [50].

In the neisserial OMV-based vaccines field, most attention has being traditionally devoted to major OMPs; however, the impact of these minor components in the induction of a significant immune response, and in the immuno-stimulating and carrier properties of OMV-based products [52], must not be overlooked. For different pathogens, albeit mostly intracellular, some of the most promising antigens are cytosolic proteins. In *Neisseria* these proteins are less variable than the typically exposed outer membrane antigens, and they may contribute to the modulation of the protective immune response induced by this kind of vesicles, through the presence of pathogen-specific T-cell epitopes as has being reported in other microorganisms [53].

In *M. tuberculosis* the study on membrane-associated protein containing fractions [54] revealed that the immunogenicity of most potent linfoproliferation-inducing fractions was most probably imparted by ribosomal proteins present in particular membrane protein fractions. Additionally they found that all mycobacterial ribosomal proteins did not appear to be equally potent, RplE being the most immunogenic.

While alternative explanations like the presence of some other, yet unidentified, major T-cell antigens in trace amounts cannot be ruled out [54], there are several reports suggesting strong immunogenicity of ribosomal proteins of pathogens [55, 56]. Antigenic specificity and high abundance could be considered as two main reasons behind the strong immunogenicity of ribosomal proteins. Ribosomes constitute nearly 25% of the dry weight of a bacterial cell, and while there is a high (>85%) sequence identity between these ribosomal proteins S2, L6, L9, and L25 detected here from various bacteria, there is hardly any (<30%) between the bacterial and human ribosomal proteins [57]. Accordingly with that, of the detected proteins at least two of them (S2 and L6) are predicted to contain strong human DRB1 CD4(+) T-Cell epitopes (data not shown).

Valuable work has been done by several groups that give clues about the contribution of individual OMV components in the induction a potent immune response. OMVs from serogroup B *N. meningitidis* are capable of activating mouse and human dendritic cells (DC) [58–61], and OMV-derivates are also being reported as strong adjuvants [62–64]. As a whole, OMVs are capable of inducing up-regulation of MHC-II, MHC-I, CD40, CD80, and CD86 expression on the surface of murine bone marrow-derived DC and macrophages [61]; and it has been demonstrated that individual components of the OM, other than LPS, are also likely to be involved in determining the levels of DC activation and the nature of the T-helper immune response [65].

Although the nature of all OM components that activate DC responses is not well characterized, valuable evidence of it has been presented for meningococcal porins. For example, meningococcal PorB protein was able to mediate signals through TLR2 on B cells [66, 67], while recombinant PorA was capable of inducing DC maturation and most importantly was also capable of influencing the nature of the T-helper immune response. These two are important well-recognized properties for generating antibody responses required for protective immunity against meningococci and for determining the immuno-adjuvant effects of this protein [68].

Thus, it is accepted that the appropriate response mounted against meningococcal OMVs *in vivo* is likely to involve a combination of all of these factors, and the modulation of these biological activities suggests the possibility of inducing or manipulating the desired innate and/or adaptive immune responses with appropriately designed vaccines [65]. Therefore, it is important to note that, while at this moment, little is known about the impact on CD4(+) T-cell response induced by the minor individual components of meningoccocal OMV-based vaccines, these observations are expanding a field of research that certainly deserves further exploration.

It has been shown that preparations of neisserial OMPs only elicit immunity in humans when presented in vesicle form [69, 70]. In this scenario, vesicle stability, consistency, and reproducibility are key issues to control during vaccine production and, at the same time, they constitute formidable challenges that are difficult to guarantee.

As we understand, the immunogenicity and reactogenicity of OMVs employed largely depend on the amount and nature of proteins and LPS present in the final preparation. These elements vary from producer to producer and for some manufacturers, even from batch-to-batch, affecting the induction of a crossreactive, crossprotective immune response, and the generalization and standardization of OMV-based products.

At present, a substantial body of experience in vesicle production has been accrued in vaccine manufacture and the currently produced vaccines are subjected to thorough quality control [71–73]. Nevertheless, we are facing a regulatory environment where better defined vaccines must be generated and, in accordance, more sophisticated control methodologies, while costly, must be already in development [74], and will be available in the near future. As a result of applying proteomic-based techniques, we found that there is remarkable low batch-to-batch variability in VA-MENGOC-BC.

It is now accepted that the use of these OMV-based vaccines is likely to be effective in a single-strain epidemic [75], but questions were raised pointing that this approach is likely to be of limited benefit for endemic serogroup B disease where there is rapid evolution of antigenic types amongst meningococcal populations [76, 77]. In this direction, however, the Cuban experience gave essential clues. In the efficacy study in Brazil [78] the Finlay Institute vaccine showed the induction of partial crossprotection in older children, and obviously offered some promise in the sense that a further refinement of this type of vaccine can be attained, as new crossreactive components are identified, leading to a new crossprotective product. This modest crossreactive immunity induced by a OMV-based vaccine has fuelled our search for the characterization of specific OMV components and new outer-membrane antigens (or group of antigens), that are consistently present in different batches of this vaccine, capable of inducing functional crossreactive antibodies. Such antigens, if they were also conserved on all strains irrespective of serogroup, might form the basis of a truly universal meningococcal vaccine.

4 Concluding remarks

In this study we report the first proteomic analysis of a commercially available outer membrane-based vaccine against *N. meningitidis* serogroup B (VA-MENGOC-BC) confirming the low batch-to-batch variability of the main active pharmaceutical ingredient. Eleven OMPs were clearly identified, in conjunction with minor cytosolic components.

This work, in combination with genomic data, has demonstrated the underlying complexity of present OMV-based vaccines and therefore the importance of their comprehensive characterization in order to truly standardize these vaccines and the products derived from them, and it is conceived as an essential step in the direction of creating a new generation of antimeningococcal vaccines.

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5 References

[1] Rosenstein, N. E., Perkins, B. A., Stephens, D. S., Lefkowitz, L. *et al.*, *J. Infect. Dis.* 1999, *180*, 1894–1901.

[2] Connolly, M., Noah, N., *Epidemiol. Infect.* 1999, *122*, 41–49.

[3] Jefferies, C., Lennon, D., Stewart, J., Martin, D., *N. Z. Med. J.* 1999, *112*, 115–117.

[4] Kaczmarski, E. B., *Commun. Dis. Rep. CDR Rev.* 1997, *7*, R55–R59.

[5] Hussey, G., Schaaf, H., Hanslo, D., Hitchcock, J. *et al.*, *S. Afr. Med. J.* 1997, *87*, 51–56.

[6] Annual report of the Australian Meningococcal Surveillance Programme 1999, *Commun. Dis. Intell.* 2000, *24*, 181–189.

[7] Lapeyssonnie, L., *Bull. World Health Organ.* 1963, *28*, SUPPL: 1–114.

[8] Loughlin, A. M., Marchant, C. D., Lett, S. M., *Am. J. Public Health* 1995, *85*, 392–394.

[9] Phillips, E. J., Simor, A. E., *Postgrad. Med.* 1998, *103*, 102–117.

[10] Campagne, G., Schuchat, A., Djibo, S., Ousseini, A. *et al.*, *Bull. World Health Organ.* 1999, *77*, 499–508.

[11] Vogel, U., Frosch, M., *Mol. Microbiol.* 1999, *32*, 1133–1139.

[12] McNeil, G., Virji, M., Moxon, E. R., *Microb. Pathog.* 1994, *16*, 153–163.

[13] Achtman, M., *Gene* 1997, *192*, 135–140.

[14] Bart, A., Dankert, J., van der, E. A., *Infect. Immun.* 1999, *67*, 3842–3846.

[15] Froholm, L. O., Kolsto, A. B., Berner, J. M., Caugant, D. A., *Curr. Microbiol.* 2000, *40*, 372–379.

[16] Jelfs, J., Munro, R., Wedege, E., Caugant, D. A., *Clin. Diagn. Lab. Immunol.* 2000, *7*, 390–395.

[17] Linz, B., Schenker, M., Zhu, P., Achtman, M., *Mol. Microbiol.* 2000, *36*, 1049–1058.

[18] Rokbi, B., Renauld-Mongenie, G., Mignon, M., Danve, B. *et al.*, *Infect. Immun.* 2000, *68*, 4938–4947.

[19] Rosenstein, N. E., Perkins, B. A., Stephens, D. S., Popovic, T., Hughes, J. M., *New Engl. J. Med.* 2001, *344*, 1378–1388.

[20] Finne, J., Bitter-Suermann, D., Goridis, C., Finne, U., *J. Immunol.* 1987, *138*, 4402–4407.

[21] Sierra, G. V., Campa, H. C., Varcacel, N. M., Garcia, I. L. *et al.*, *NIPH Ann.* 1991, *14*, 195–207.

[22] Bjune, G., Hoiby, E. A., Gronnesby, J. K., Arnesen, O. *et al.*, *Lancet* 1991, *338*, 1093–1096.

[23] Rodriguez, A. P., Dickinson, F., Baly, A., Martinez, R., *Mem. Inst. Oswaldo Cruz* 1999, *94*, 433–440.

[24] Lehmann, A. K., Halstensen, A., Naess, A., Vollset, S. E. *et al.*, *APMIS* 1991, *99*, 769–772.

[25] Gomez, J. A., Hernandez, E., Criado, M. T., Ferreiros, C. M., *Vaccine* 1998, *16*, 1633–1639.

[26] Steeghs, L., Kuipers, B., Hamstra, H. J., Kersten, G. *et al.*, *Infect. Immun.* 1999, *67*, 4988–4993.

[27] Norais, N., Nogarotto, R., Lacobini, E. T., Garaguso, I. *et al.*, *Proteomics* 2001, *1*, 1378–1389.

[28] Bernardini, G., Renzone, G., Comanducci, M., Mini, R. *et al.*, *Proteomics* 2004, *4*, 2893–2926.

[29] Whiting, G. C., Rijpkema, S., Adams, T., Corbel, M. J., *Vaccine* 2004, *22*, 4245–4251.

[30] Nally, J. E., Whitelegge, J. P., Aguilera, R., Pereira, M. M. *et al.*, *Proteomics* 2005, *5*, 144–152.

[31] Tsai, C. M., Frasch, C. E., Rivera, E., Hochstein, H. D., *J. Biol. Stand.* 1989, *17*, 249–258.

[32] Campa, C., Sierra, G. V., Gutierrez, M. M., Biset, G. *et al.*, US Patent US005597572, 1997.

[33] Sabounchi-Schutt, F., Astrom, J., Olsson, I., Eklund, A. *et al.*, *Electrophoresis* 2000, *21*, 3649–3656.

[34] Heukeshoven, J., Dernick, R., *J. Chromatogr.* 1985, *326*, 91–101.

[35] Jensen, O. N., Wilm, M., Shevchenko, A., Mann, M., *Methods Mol. Biol.* 1999, *112*, 513–530.

[36] Gonzalez, L. J., Castellanos-Serra, L., Badock, V., Diaz, M. *et al.*, *Electrophoresis* 2003, *24*, 237–252.

[37] Zhang, W., Chait, B. T., *Anal. Chem.* 2000, *72*, 2482–2489.

[38] Perkins, D. N., Pappin, D. J., Creasy, D. M., Cottrell, J. S., *Electrophoresis* 1999, *20*, 3551–3567.

[39] Vandahl, B. B., Birkelund, S., Demol, H., Hoorelbeke, B. *et al.*, *Electrophoresis* 2001, *22*, 1204–1223.

[40] Caldas, T., Laalami, S., Richarme, G., *J. Biol. Chem.* 2000, *275*, 855–860.

[41] Caldas, T. D., El Yaagoubi, A., Richarme, G., *J. Biol. Chem.* 1998, *273*, 11478–11482.

[42] Tettelin, H., Saunders, N. J., Heidelberg, J., Jeffries, A. C. *et al.*, *Science* 2000, *287*, 1809–1815.

[43] Tettelin, H., Parkhill, J., *Nat. Genet.* 2004, *36*, 1028–1029.

[44] Parkhill, J., Achtman, M., James, K. D., Bentley, S. D. *et al.*, *Nature* 2000, *404*, 502–506.

[45] Churchward, M. A., Butt, R. H., Lang, J. C., Hsu, K. K., Coorssen, J. R., *Proteome Sci.* 2005, *3*, 5.

[46] Futai, M., Noumi, T., Maeda, M., *Annu. Rev. Biochem.* 1989, *58*, 111–136.

[47] Pessolani, M. C., Smith, D. R., Rivoire, B., McCormick, J. *et al.*, *J. Exp. Med.* 1994, *180*, 319–327.

[48] Shin, B. K., Wang, H., Yim, A. M., Le Naour, F. *et al.*, *J. Biol. Chem.* 2003, *278*, 7607–7616.

[49] Broquet, A. H., Thomas, G., Masliah, J., Trugnan, G., Bachelet, M., *J. Biol. Chem.* 2003, *278*, 21601–21606.

[50] Driessen, A. J., Manting, E. H., van der, D. C., *Nat. Struct. Biol.* 2001, *8*, 492–498.

[51] Gu, S., Chen, J., Dobos, K. M. *et al.*, *Mol. Cell Proteomics* 2003, *2*, 1284–1296.

[52] Bitton, R. J., Guthmann, M. D., Gabri, M. R., Carnero, A. J. *et al.*, *Oncol. Rep.* 2002, *9*, 267–276.

[53] Charo, J., Geluk, A., Sundback, M., Mirzai, B. *et al.*, *Eur. J. Immunol.* 2001, *31*, 3602–3611.

[54] Sinha, S., Kosalai, K., Arora, S., Namane, A. *et al.*, *Microbiology* 2005, *151*, 2411–2419.

[55] Iborra, S., Soto, M., Carrion, J., Nieto, A. *et al.*, *Infect. Immun.* 2003, *71*, 6562–6572.

[56] Matsuoka, M., Nomaguchi, H., Yukitake, H., Ohara, N. *et al.*, *Vaccine* 1997, *15*, 1214–1217.

[57] Lecompte, O., Ripp, R., Thierry, J. C., Moras, D., Poch, O., *Nucleic Acids Res.* 2002, *30*, 5382–5390.

[58] Dixon, G. L., Newton, P. J., Chain, B. M., Katz, D. *et al.*, *Infect. Immun.* 2001, *69*, 4351–4357.

[59] Kolb-Maurer, A., Unkmeir, A., Kammerer, U., Hubner, C. *et al.*, *Infect. Immun.* 2001, *69*, 6912–6922.

[60] Unkmeir, A., Kammerer, U., Stade, A., Hubner, C. *et al.*, *Infect. Immun.* 2002, *70*, 2454–2462.

[61] Rodriguez, T., Perez, O., Menager, N., Ugrinovic, S. *et al.*, *Vaccine* 2005, *23*, 1312–1321.

[62] Wetzler, L. M., *Ann. N. Y. Acad. Sci.* 1994, *730*, 367–370.

[63] Perez, O., Bracho, G., Lastre, M., Mora, N. *et al.*, *Immunol. Cell Biol.* 2004, *82*, 603–610.

[64] Mesa, C., De Leon, J., Rigley, K., Fernandez, L. E., *Vaccine* 2004, *22*, 3045–3052.

[65] Al Bader, T., Christodoulides, M., Heckels, J. E., Holloway, J. *et al.*, *Infect. Immun.* 2003, *71*, 5590–5597.

[66] Massari, P., Henneke, P., Ho, Y., Latz, E. *et al.*, *J. Immunol.* 2002, *168*, 1533–1537.

[67] Singleton, T. E., Massari, P., Wetzler, L. M., *J. Immunol.* 2005, *174*, 3545–3550.

[68] Al Bader, T., Jolley, K. A., Humphries, H. E., Holloway, J. *et al.*, *Cell Microbiol.* 2004, *6*, 651–662.

[69] Zollinger, W. D., Mandrell, R. E., Griffiss, J. M., Altieri, P., Berman, S., *J. Clin. Invest.* 1979, *63*, 836–848.

[70] Zollinger, W. D., Mandrell, R. E., Altieri, P., Berman, S. *et al.*, *J. Infect. Dis.* 1978, *137*, 728–739.

[71] Claassen, I., Meylis, J., van der, L. P., Peeters, C. *et al.*, *Vaccine* 1996, *14*, 1001–1008.

[72] Fredriksen, J. H., Rosenqvist, E., Wedege, E., Bryn, K. *et al.*, *NIPH Ann.* 1991, *14*, 67–79.

[73] Norheim., G., Aase, A., Caugant, D. A., Hoiby, E. A. *et al.*, *Vaccine* 2005, *23*, 3762–3774.

[74] Mukhopadhyay, T. K., Halliwell, D., O'Dwyer, C., Shamlou, P. A. *et al.*, *Biotechnol. Appl. Biochem.* 2005, *41*, 175–182.

[75] Holst, J., Feiring, B., Naess, L. M., Norheim, G. *et al.*, *Vaccine* 2005, *23*, 2202–2205.

[76] Morley, S. L., Cole, M. J., Ison, C. A., Camaraza, M. A. *et al.*, *Pediatr. Infect. Dis. J.* 2001, *20*, 1054–1061.

[77] Morley, S. L., Pollard, A. J., *Vaccine* 2001, *20*, 666–687.

[78] de Moraes, J. C., Perkins, B. A., Camargo, M. C., Hidalgo, N. T. *et al.*, *Lancet* 1992, *340*, 1074–1078.

