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Bioinformatic analysis of outer membrane proteome of *Neisseria meningitidis* and *Neisseria lactamica*

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Summary. Two-dimensional electrophoresis (isoelectric focusing/SDS-PAGE) and Western-blotting techniques were used to analyze and compare common and/or specific outer-membrane proteins and antigens from *Neisseria meningitidis* and *Neisseria lactamica*. Bioinformatic image analyses of proteome and immunoproteome maps indicated the presence of numerous proteins and several antigens shared by *N. meningitidis* and *N. lactamica*, although the inter-strain variation in the maps was of similar magnitude to the inter-species variation, and digital comparison of the maps did not reveal proteins found to be identical by MALDI-TOF fingerprinting analysis. PorA and RmpM, two relevant outer-membrane antigens, manifested as various spots at several different positions. While some of these were common to all the strains analyzed, others were exclusive to *N. meningitidis* and their electrophoretic mobilities were different than expected. One such spot, with a molecular mass of 19 kDa, may be the C-terminal fragment of RmpM (RmpM-Cter). The results demonstrate that computer-driven analysis based exclusively on spot positions in the proteome or immunoproteome maps is not a reliable approach to predict the identity of proteins or antigens; rather, other identification techniques are necessary to obtain accurate comparisons. [**Int Microbiol** 2007; 10(1):5-11]

Key words: *Neisseria meningitides* · *Neisseria lactamica* · immunoproteome · common antigens · protein identification

Introduction

Considerable research effort has been dedicated to the development of vaccines able to confer protection against meningococcal meningitis and showing long-term immunological memory as well as wide cross-reactivity. Conjugated vaccines against serogroup C have been some of the most important achievements to date [8], but efforts to develop vaccines against serogroup B have been less successful, despite recent advances [10,14]. Vaccines based on outer-membrane vesicles were developed in Cuba and Norway in the 1980s in response to disease outbreaks in those countries [2,15]; however, they have only been useful against homologous strains. For this reason, there is great interest in the identification of conserved antigens able to induce functional bactericidal antibodies, such as those involved in the development of naturally acquired immunity [4]. Epidemiological evidence suggests that the non-pathogenic *Neisseria lactamica* may be involved in natural immunity, and it is likely that the use of antigens from this species in vaccines would be effective against *N. meningitidis* [9,18].

One-dimensional SDS-PAGE combined with mass spectrometry has proven to be very efficient for the resolution and identification of the proteins present in meningococcal outer-membrane vesicles [11,19–21]. Nevertheless, identification and analysis of single antigenic proteins in these vesicles requires two-dimensional separation techniques to

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obtain immunoproteome maps that can be used to assess the efficacy of vaccine formulations containing specific antigens. The aim of this study was to determine the usefulness of two-dimensional electrophoresis (isoelectric focusing /SDS-PAGE) combined with Western-blotting in the identification and analysis of the cross-reactivity of outer-membrane antigenic proteins from *N. meningitidis* and *N. lactamica.*

Materials and methods

Bacterial strains and outer-membrane vesicles. Three *N. meningitidis* and three *N. lactamica* strains were used in this work. *N. meningitidis* strains B16B6 (B:2a:P1.5,2; ST-11/ET-37), M982 (B:9:P1.9; ST-5), and Nm30 (C:2b:P1.2,5; ST-8/Cluster A4) were isolated from the cerebrospinal fluid of patients suffering from meningococcal meningitis. The first two are reference strains, whereas the third is from our laboratory collection. *N. lactamica* strains NlP0, NlP2, and NlP5 were isolated from the oropharynx of volunteers in our community. The strains were maintained and cultured as described previously [13]. All bacteria were cultured under iron restriction in Mueller-Hinton (MH) broth containing 100 μM Desferal. Outer-membrane vesicles (OMVs) were obtained as described previously [13].

Immune sera. Mouse immune sera against OMVs from *N. meningitidis* strains Nm30 and B16B6 and *N. lactamica* strains NlP2 and NlP5 were obtained by intraperitoneal injection of CBA mice [12]. Monoclonal serum (mAb 185,H-8) specific for the RmpM protein was kindly donated by Dr. Einar Rosenqvist (National Institute of Public Health, Oslo, Norway). Monoclonal serum against a conserved epitope in the PorA protein (mAb 9-1-P-1.c) was kindly provided by Elizabeth Moran (Walter Reed Army Institute, USA). Two rabbit sera, one against OMVs from *N. meningitidis* strain Nm30 and the other against a purified 47-kDa outer-membrane protein (P47; locus NMB0035), were obtained in our laboratory [1].

Two-dimensional electrophoresis. OMVs (1 μg/μl) for two-dimensional electrophoresis were solubilized in a solution of 5 M urea, 2 M thiourea, 2 mM tributyl phosphine (TBP), 2% (w/v) 3[(3-cholamidopropyl)dimethylammonio]-propanesulfonic acid (CHAPS), 2% (w/v) *N*-decyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate (SB 3-10), 0.2% (v/v) Bio-lyte 3/10 (Bio-Rad, Richmond, VA, USA), 40 mM Tris, and 0.0002% (v/v) bromophenol blue. For the first dimension, sample (125 μl) was loaded in a 7-cm immobilized pH gradient (IPG) strip (pH 3–10), and actively rehydrated by applying 50 V at 20ºC for 16 h in a Protean IEF cell (Bio-Rad).

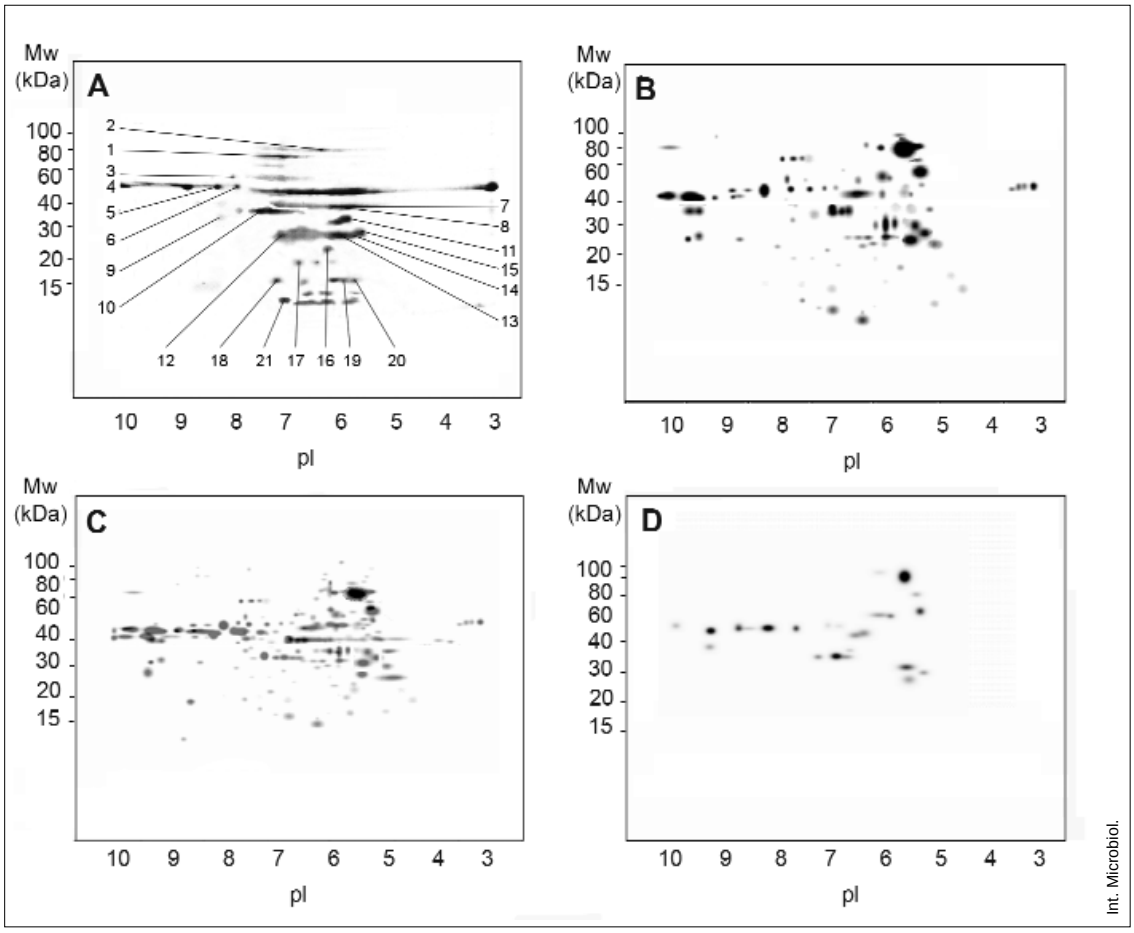

Fig. 1. Coomassie-blue-stained 2-D protein maps of *Neisseria meningitidis* strain B16B6 (**A**), and *N. lactamica* NlP0 (**B**), and the predicted common spots maps obtained for all *N. meningitidis* (**C**) and *N. lactamica* (**D**) strains analyzed. Dashed line indicates a PorA spot train. Numbers indicate relevant spots identified by MALDI-TOF (see Tables 1 and 2).

Isoelectric focusing was conducted using a three-step program: (i) linear voltage increase from 0 to 500 V over 20 min, (ii) linear voltage increase from 500 to 4000 V over 4 h, and (iii) focusing until 10,000 V-h were reached (at a maximum of 4000 V). Before the samples were separated in the second dimension, the IPG strip was equilibrated first in a solution containing 6 M urea, 2% (w/v) SDS, 0.05 M Tris/HCl (pH 8.8), 20% (v/v) glycerol, and 2% (w/v) dithiothreitol (DTT) and then in a solution containing 6 M urea, 2% (w/v) SDS, 0.05 M Tris/HCl (pH 8.8), 20% (v/v) glycerol, and 2.5% (w/v) iodoacetamide. Separation in the second dimension was carried out by SDS-PAGE in 9–18% acrylamide gradient gels using a Mini-PROTEAN 3 Cell (Bio-Rad). The separated proteins were visualized by Coomasie staining.

Detection and identification of proteins and antigens. Some of the proteins were identified by MALDI-TOF analysis of the most relevant spots (those more clearly visible and common to all strains analyzed), which were excised from the 2-D gels. The antigenic patterns and immunological identification of some of the antigens were determined by transferring two-dimensional electrophoresis gels to polyvinylidene fluoride (PVDF) membranes using a Mini-Trans-Blot Electrophoretic transfer cell (Bio-Rad) according to the manufacturer's instructions. Membranes were then sequentially probed with the above-listed sera, and bound immunoglobulins were visualized with peroxidase-conjugated antibodies in combination with standard colorimetric techniques.

Image analysis. Stained gels and PVDF membranes were scanned with a GS-800 densitometer, and image analysis was done using PDQuest 7.2 software (Bio-Rad). Images were improved by the application of a weighted-mean (3´3 pixel matrix) noise-reduction filtering algorithm. Protein spots were then detected and gaussian images created for each spot. Proteomic and immunoproteomic maps from OMV preparations obtained on at least three different days were used to construct reference maps for each strain. Data analysis for comparisons was done using the most restrictive matching method provided in the software.

Results

Stringent solubilization conditions and conventional 2-D analysis (IEF/SDS-PAGE) were used to obtain reference proteome maps. Figure 1 shows the maps for strains *N. meningitidis* B16B6 (Fig. 1A) and *N. lactamica* NlP2 (Fig. 1C). Two main "trains" of spots (at various points in the pI range, with the same molecular masses), corresponding to PorA (42 kDa) and RmpM (36 kDa), were detected in the *N. meningitidis* strains. As shown in Fig. 1B, the software identified a total of

Table 1. MASCOT search results obtained by MALDI-TOF analysis of relevant spots of the *Neisseria meningitidis* master strain (B16B6)

			Molecular mass		Isoelectric point	
Spot	GenBank accession number	Protein name	Observed	Predicted	Observed	Predicted
1	CAA61894	OMP P64k61.0	61.0	62.2	6.4	5.4
2	P42385	60-kDa chaperonin[a]	66.4	57.5	7.0	5.0
3	CAB83479	Putative periplasmatic protein	47.7	45.6	5.5	7.8
4	CAA40469	Class 1 OMP (PorA)	42.8	42.8	4.8	4.8
5	AAD52970	Class 1 OMP (PorA)	43.7	43.7	5.3	5.3
6	CAA40469	Class 1 OMP (PorA)	41.2	41.2	5.6	5.6
7	CAA46064	Major ferric-iron-binding protein[a]	35.7	35.8	7.1	9.6
8	1904330A	Ferric iron-binding protein[a]	31.6	33.5	7.3	9.5
9	A37004	Class 4 OMP (RmpM)[a]	31.2	26.2	5.3	6.5
10	A37004	Class 4 OMP (RmpM)[a]	33.7	26.2	6.0	6.5
11	CAB85320	Class 4 OMP (RmpM)[a]	33.5	26.3	6.1	7.0
12	CAB85353	Hypothetical protein	27.5	27.3	6.3	5.6
13	CAB85420	Putative thiol:disulfide interchange protein[a]	27.3	25.4	7.1	5.8
14	CAB85036	Histidine-binding periplasmatic protein[a]	27.6	29.1	7.5	5.2
15	CAB85036	Histidine-binding periplasmatic protein[a]	29.0	29.1	7.6	5.2
16	AAF40732	Thiol:disulfide interchange protein DsbA[a]	24.6	25.4	7.1	5.8
17	D81800	Probable peptidil-propyl isomerase	22.8	28.9	7.5	5.7
18	AAF41888	Putative peptidil-propyl isomerase	19.7	28.9	6.0	5.7
19	A81072	Azurin precursor	19.8	18.7	7.4	4.8
20	A81072	Azurin precursor	19.8	18.7	7.5	4.8
21	D81234	Translation-elongation factor Tu	16.9	43.1	6.3	5.1

[a]Coincident with proteins identified also in *Neisseria lactamica.*

Table 2. MASCOT search results obtained by MALDI-TOF analysis of relevant spots of the *N. lactamica master* strain (NlP0)

			Molecular mass		Isoelectric point	
Spot	GenBank accession no.	Protein name	Observed	Predicted	Observed	Predicted
1	CAA61902	Fe-regulated protein B (*N. meningitidis*)	72.7	79.6	6.3	9.4
2	CAB83768	Chaperonin 60-kDa subunit[a]	63.0	57.5	7.1	5.0
3	C81021	Chaperonin 60-kDa (*N. meningitidis*)[a]	66.1	45.6	9.0	5.0
4	CAA70491	Porin	36.2	31.6	4.8	7.2
5	CAA70491	Porin	35.6	31.6	5.3	7.2
6	CAA70491	Porin	36.2	31.6	5.9	7.2
7	CAA70491	Porin	37.7	31.6	9.6	7.2
8	CAA46071	Periplasmic iron-binding protein[a]	29.9	34.2	8.9	9.5
9	A37004	Class 4 OMP (precursor)[a]	28.8	26.2	5.6	7.0
10	A37004	Class 4 OMP (precursor)[a]	28.8	26.2	5.8	7.0
11	A37004	Class 4 OMP (precursor)[a]	28.7	26.2	7.3	7.0
12	AAC44892	Hemoglobin-haptoglobin utilization protein A (*N. meningitidis*)	29.7	36.5	7.3	8.7
13	CAB85036	Histidine-binding periplasmic protein (*N. meningitidis*)[a]	30.1	29.0	8.7	6.0
14	AAK08056	Ex13L	29.7	47.0	5.9	4.5
15	AAW90622	Putative ABC transporter, periplasmic binding protein (*N. gonorrhoeae*)	26.6	30.2	6.8	6.2
16	YP_209148	Genome-derived neisserial antigen 1946 (*N. gonorrhoeae*)	25.7	31.4	7.6	5.3
17	AAK08056	Ex13L	23.7	47.0	6.5	4.5
18	AAF40732	Thiol:disulfide interchange protein DsbA (*N. meningitidis*)[a]	24.1	25.4	6.8	5.8
19	CAA29561	Unnamed protein product (*N. gonorrhoeae*)	20.3	18.7	7.0	4.7
20	AAF41888	H.8 outer-membrane protein (*N. meningitidis*)	20.3	18.7	7.2	4.7

[a]Coincident with proteins identified also in *Neisseria meningitidis*.

25 spots common to the three strains, corresponding to 34% of the total number of spots detected in strain B16B6, 25% of those in strain Nm30, and 22% of those in strain M982. All common spots had molecular masses between 20 and 60 kDa, and pI values between 5 and 8. By contrast, the software detected only 17 common spots in the *N. lactamica* strains (Fig. 1D), corresponding to 40% of the total number of spots in strain NlP0, 19% of those in strain NlP2, and 25% of those in strain NlP5. As in the *N. meningitidis* strains, many of these spots formed a "train" at 31–34 kDa, with pI values ranging from 5 to 10.

The reference maps (Figs. 1B, D) obtained for the common spots detected in both species were compared using the above-described software. Only a small number of proteins common to the two species were detected (not shown). Identification of the main antigenic spots (see below) found in *N. meningitidis* and *N. lactamica* by MALDI-TOF peptide map fingerprinting revealed only five coincident proteins (indicated in Tables 1, 2). These corresponded with ten spots in the *N. meningitidis* maps and eight in the *N. lactamica* maps.

Western-blotting of the proteome gels using sequential detection with specific mAbs and the anti-P47 and anti-OMVs sera allowed the generation of immunoproteome maps (summarized in Fig. 2). The anti-PorA mAb reacted with several spots in the *N. meningitidis* strains. The molecular masses of the spots were between 31 and 41 kDa and their pI values were variable (Fig. 2A, B). As expected, no reaction with the anti-PorA mAb was observed in the *N. lactamica* maps (Fig. 2C, D). The anti-RmpM mAb reacted with spots in the *N. lactamica* strains that were of lower molecular mass (32–34 kDa) than those in the *N. meningitidis* strains (37 kDa). RmpM spots either common to both species or exclusive to *N. meningitidis* were seen. The molecular masses of the RmpM spots differed from those reported on the basis of one-dimensional SDS-PAGE: three of these spots were located around 53 kDa, and another three around 19 kDa, with pI values between 6 and 6.5 in all cases. The anti-P47 serum detected three spots in all *N. meningitidis* strains, with molecular masses of 27, 43, and 56 kDa and each with a pI of 7.2, but this serum failed to react with any spot in the *N. lactamica* strains under the assay conditions used. When

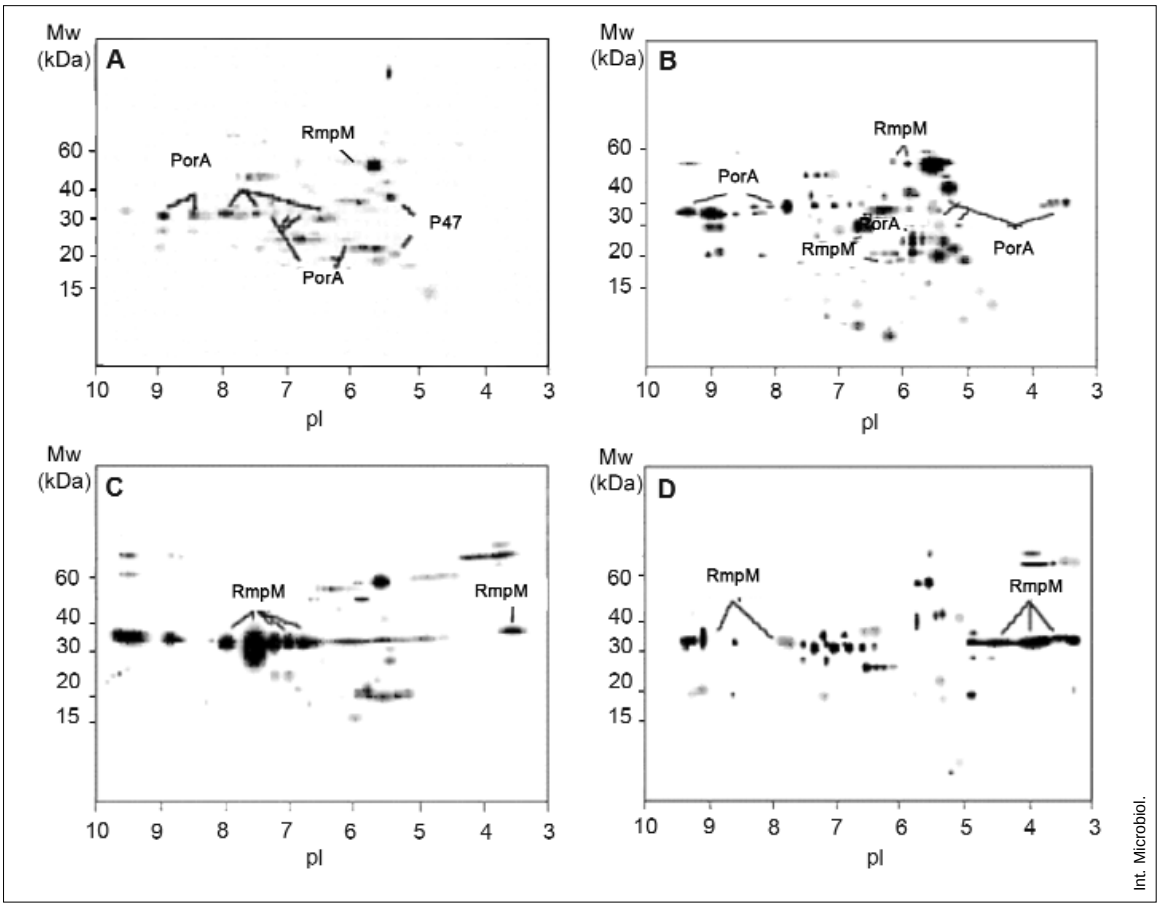

Fig. 2. Immunoproteome maps of the outer-membrane antigens from *N. meningitidis* strains B16B6 (**A**) and Nm30 (**B**), and from *N. lactamica* strains NlP2 (**C**) and NlP5 (**D**). Spots marked in the figure were identified by sequential probing with monoclonal antibodies against PorA, PorB, and RmpM, and two polyclonal sera against P47 and whole OMVs.

individual immunoproteome maps from *N. meningitidis* and *N. lactamica* were digitally compared, only nine spots common to the two species were detected (not shown).

Discussion

Proteomic approaches facilitate the identification, quantification, and structural, biochemical and functional characterization of proteins. They also allow the evaluation of how these characteristics vary in space and time and with physiological state. In the case of pathogenic microorganisms, proteomic techniques have been extensively used to identify new biomarkers and determinants of virulence, as well as candidate antigens for use in vaccine development [6]. In the present study, we constructed proteome and immunome maps of outer-membrane proteins from different strains of *N. meningitidis* and *N. lactamica*. Our immediate aim was to identify antigens common to the two species, or exclusive to each one, and then evaluate their potential in the development of effective vaccines against *N. meningitidis* serogroup B.

The construction of such maps requires good separation of the proteins present in the samples. Two-dimensional electrophoresis, based on IEF and subsequent SDS-PAGE, yields good protein resolution [3,19,21]. Moreover, when used in combination with Western-blotting with specific antisera, it is the only technique available for the construction of immunoproteome maps in which individual antigens can be detected and compared. However, it should be noted that some antigenic membrane proteins cannot be analyzed due to their extreme pI or to solubilization problems during IEF separation in the first dimension. In the present study, the best resolution and a high reproducibility were obtained using 5 M urea and 2 M thiourea for solubilization. Other important factors were appropriate incubation times and the use of zwitterionic detergents to avoid increases in the ionic charge of the solution, which allowed the application of high voltages without a major increase in current. Although the detection of

proteins present at low concentrations is difficult, when the eventual aim is to detect antigenic proteins this problem is often corrected because proteins can be distinguished with specific antibodies. We also observed that some of the spots detected after immunoblotting did not correspond to any of the spots visualized in the gels by Coomassie staining.

Previous studies have revealed the complexity of OMV proteomes, in which many periplasmic and cytosolic proteins are present [19–21]; however, a bias toward major proteins such as PorA, PorB, and RmpM is usually evident. We found that the extraction of OMVs by mechanical approaches, although more time-consuming, results in a much lower contamination of OMV preparations with periplasmic and cytosolic proteins (unpublished results) than occurs with the method used by the authors of those other studies, in which OMVs were obtained by detergent solubilization.

Analysis by 2D-PAGE and immunoblotting confirmed that PorA is present in all strains of *N. meningitidis* but absent in all strains of *N. lactamica*, as previously reported [7,20]. In the *N. meningitidis* maps, PorA was detected as a series of spots ("trains"), with molecular masses between 38 and 41 kDa, that were distributed over the entire pI range analyzed. This observation is in agreement with published studies on the composition of OMV-based vaccines [19,21]. The train-like distribution may have been due to many factors, such as post-translational modifications and/or processing [21], or the association of PorA with different membrane complexes of varying isoelectric points that were maintained during IEF separation [13]. The anti-RpmM mAb identified several spots with molecular masses of 35 kDa and pI values between 5.6 and 7.4. In addition, all the *N. meningitidis* strains analyzed showed other spots for RmpM; their pI values were in the range of 6–6.5, and their apparent molecular masses were 53.3 (3 spots) or 19 (3 spots) kDa. These 19-kDa forms could be involved in the process of dimerization of RmpM, as recently described [5]. In fact, the molecular mass of this spot coincides with that of the C-terminal fragment of the protein, named RmpM-Cter, which consists of an 18-kDa OmpA-like domain able to interact with other outer-membrane proteins. The spots of higher molecular mass identified by the antibody used in the present study probably correspond to different conformations of the protein or to associations with other membrane components (PorA and/or PorB), either of which would affect its apparent electrophoretic mobility. The anti-RmpM mAb did not detect any of these higher or lower molecular mass spots in any of the *N. lactamica* strains, which suggests that they represent forms specific to *N. meningitidis*. RmpM in *N. lactamica* appeared as a series of spots with molecular masses of 32–34 kDa, in agreement with previous studies carried out in our laboratory [17].

On the basis of computerized analysis of the results obtained from *N. meningitidis* and *N. lactamica*, our results indicate that different strains may show different proteomes despite having the same protein composition. Indeed, an *N. meningitidis* strain and an *N. lactamica* strain may have a similarity percentage (based on coincident spots) comparable to that between two *N. meningitidis* strains. Furthermore, we have shown that there is no close correspondence between proteomes and immunomes, which hinders the identification of species-specific and shared antigens. When individual spots are identified by MALDI-TOF, it becomes clear that observed mobilities are very unreliable and by no means appropriate for comparisons, even between strains of the same species.

Using image analysis to evaluate and compare the immunome maps obtained, we were able to identify only one antigenic protein, RmpM, common to the two species. However, visual analysis of the results obtained with polyclonal sera revealed the presence of numerous other antigenic proteins expressed in common. It is important to bear in mind that proteins common to the two species show differences in molecular mass, as seen in the case of RmpM. Furthermore, proteins with high molecular masses, complex-forming proteins, and highly hydrophobic proteins (many membrane proteins) may not enter the gels, or may not be focused properly during the first-dimension step. Similarly, proteins with extreme pI values may be excluded from IEF gels, which gives rise to very different protein or antigen patterns in the second dimension than would be obtained by one-dimensional SDS-PAGE. Many antigens may thus seem to disappear or will show anomalous patterns. Similarly, it should be borne in mind that the solubilization conditions used in IEF are very different from those used in one-dimensional SDS-PAGE. We thus consider that adequate analysis of bacterial-membrane immunoproteomes still requires research into separation techniques, more efficient antigen detection, and identification by tryptic digestion, fingerprinting, and comparison with protein databases, already available for *N. meningitidis*.

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