A cross-reactive neisserial antigen encoded by the NMB0035 locus shows high sequence conservation but variable surface accessibility

Jesús Arenas,¹ Ana Abel,¹ Sandra Sánchez,¹ Juan Marzoa,¹ Sonsoles Berrón,² Peter van der Ley,³ María-Teresa Criado¹ and Carlos M. Ferreirós¹

Correspondence
Carlos M. Ferreirós
mpcfytc@usc.es

¹Departamento de Microbiología y Parasitología, Facultad de Farmacia, Campus Sur, Universidad de Santiago de Compostela, 15782 Santiago de Compostela, Spain

²Centro Nacional de Microbiología, Instituto de Salud Carlos III, Ctra. Majadahonda-Pozuelo Km2, 28220 Majadahonda, Madrid, Spain

³Laboratory for Vaccine Research, Netherlands Vaccine Institute, PO Box 457, 3720 AL Bilthoven, The Netherlands

The meningococcal NMB0035 locus encodes a 47 kDa outer-membrane protein that is highly conserved antigenically, and is able to induce antibodies during infection and bactericidal responses *in vitro*. This study analysed the surface exposure of this protein using specific antibodies in flow cytometry assays and determined its nucleotide sequence in 33 *Neisseria* strains. Genomic analyses revealed no significant differences in the nucleotide or amino acid sequences, but flow cytometry showed that surface accessibility was highly variable among the strains. These results suggest that masking by and/or association with lipo-oligosaccharides or other membrane molecules can be crucial for antigen accessibility, which must be thoroughly analysed in new vaccine candidates.

Received 19 January 2007
Accepted 13 September 2007

INTRODUCTION

Current research into vaccine candidates suitable for the prevention of meningitis caused by serogroup B meningococci has focused on outer-membrane proteins (OMPs) and lipo-oligosaccharides (LOSs), either purified or naturally incorporated into outer-membrane vesicles (OMVs) (Morley & Pollard, 2001). The high antigenic variability of many OMPs limits the efficacy of single-protein vaccines, and vaccines based on OMVs that contain all of the outer-membrane antigens in their native conformations have failed to induce cross-protection due to the immune dominance of PorA, the serosubtyping antigen, which presents a high degree of variability (Rosenqvist *et al.*, 1995; Tappero *et al.*, 1999; Morley *et al.*, 2001). The prevalence of commensal species as colonizing neisseriae during childhood and the existence of common antigens, particularly between *Neisseria meningitidis* and *Neisseria lactamica*, has raised interest in using commensals for vaccination (Gorringe *et al.*, 2005) or as

delivery vehicles for meningococcal vaccine antigens (O'Dwyer *et al.*, 2004). Thus, the investigation of new vaccine candidates is using all of the technologies derived from advances in molecular biology (Leclerc, 2003; Khan *et al.*, 2006) and the information obtained from the DNA sequences published for several meningococcal strains. Bioinformatic analyses applied to genetic, molecular and antigenic characteristics of vaccine candidates (Zagursky & Russell, 2001; Petrovsky & Brusic, 2002) can help to determine their genetic divergence, and to predict their structural conformation and antigenic domains (Doytchinova & Flower, 2002) on the basis of comparison with previously well-characterized molecules registered in molecular databases.

The most important challenge for obtaining a good meningococcal vaccine based on OMPs is to find an antigen or a group of antigens common to all strains that is easily accessible and is able to induce long-term protective immune responses in both adults and children. Knowledge of the variation of many microbial characteristics, particularly surface-antigen expression, has been conditioned by the general assumption that the behaviour of individual bacteria in a population is homogeneous and can be analysed by measuring the population as a whole. Flow cytometry is having a major impact on antigenic analysis,

Abbreviations: LOS, lipo-oligosaccharide; OMP, outer-membrane protein; OMV, outer-membrane vesicle.

The GenBank/EMBL/DDBJ accession numbers for the *p47* gene sequences reported in this paper are DQ788636–DQ788662 (*N. meningitidis* strains) and DQ788663–DQ788666 (*N. lactamica* strains).

and is a valuable technique that allows the simultaneous determination of the accessibility and population variability of surface antigens to specific antibodies (Valdivia & Falkow, 1998). Previous studies in our laboratory have demonstrated the cross-reactivity of a 47 kDa antigenic neisserial OMP by Western blotting and dot blotting. This antigen is inducible by iron restriction and has been identified by matrix-assisted laser desorption/ionization time-of-flight MS as the product encoded by the NMB0035 (*N. meningitidis* strain MC58) and NMA0281 (*N. meningitidis* strain Z2491) loci (Arenas *et al.*, 2006). Experiments with sera from patients convalescing from meningococcal disease (Troncoso *et al.*, 2000) and with a polyvalent specific rabbit serum against this protein (Arenas *et al.*, 2006) have shown that this antigen is able to induce antibodies during infection and bactericidal responses *in vitro*. To investigate further the vaccine potential of this antigen, which we called P47, this study has extended the previous analysis by using a specific antiserum and flow cytometry techniques to evaluate the accessibility of P47 epitopes on the surface of 27 *N. meningitidis* and 6 commensal *Neisseria* strains and, by sequencing the gene in all of them, to establish its conservation.

METHODS

Bacterial strains and culture conditions. The characteristics of all strains used in this work have been described previously (Arenas *et al.*, 2006). *N. meningitidis* strains GLD and B16B6 were kindly provided by Dr Borriello (Queens Medical Centre, University Hospital, Nottingham, UK), strain NMB by Dr Granoff (Children's Hospital, Oakland Research Institute, Oakland, CA, USA) and strain M7 (a capsule-deficient mutant of NMB) by Dr Pizza (Immunobiological Research Institute, Siena, Italy). The three strains *galE*, *rfaC* and *lpxA* are different LOS mutants obtained by Dr van der Ley from strain H44/76 (Gorter *et al.*, 2003). All other strains were from our laboratory collection. Multilocus sequence typing (MLST) of each strain was carried out by determination of the allelic profiles obtained from a 450 bp internal region in the *abcZ*, *adk*, *aroE*, *fum*, *gdh*, *pdhC* and *pgm* genes. Assignment to clonal complexes was done by comparison of the profiles with those in the meningococcal MLST database (<http://pubmlst.org/neisseria/>).

Maintenance of the strains and culture under iron-restricted conditions were as described previously (Pintor *et al.*, 1996). For flow cytometry, cells were fixed by adding formaldehyde (1%, v/v) to the culture just after growth and incubating for 30 min at room temperature with constant shaking at 100 r.p.m. Cells were then washed twice with PBS, suspended at a concentration of 10^9 cells ml^{-1} in PBS containing 0.05 mM PMSF and maintained at 4 °C until use. For some experiments, the cells were permeabilized with 70% ethanol (30 min at room temperature with constant shaking at 100 r.p.m.) prior to treatment with formalin.

Flow cytometry assays. Flow cytometry assays were carried out using a modification of the method described by Welsch *et al.* (2003) using rabbit sera against both the purified 47 kDa protein and OMVs obtained as described previously (Arenas *et al.*, 2006). Formaldehyde-fixed bacteria were suspended at a concentration of 10^7 cells ml^{-1} in 1% BSA (w/v) in Tris-buffered saline with 0.05% Tween 20 (TBSA) and blocked for 1 h at 37 °C with constant gentle shaking. Aliquots (1 ml) were then stained with BacLight (Molecular Probes) according

to the manufacturer's instructions, incubated with working dilutions (1:1000) of the different sera for 90 min at 37 °C, washed with Tris-buffered saline and finally incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes) in TBSA for 1 h at 37 °C with constant gentle shaking. Fluorescence was measured in a Coulter-XL flow cytometer. Bacteria incubated with non-immune rabbit or mouse sera were used to adjust the instrument for non-specific cell-associated fluorescence. Controls using unstained bacteria were included to check for overlapping of fluorescence. For each strain, 10^4 events were recorded and the fluorescence associated with bacterial cells was determined by gating with the BacLight channel values. Fluorescence produced by cells incubated with non-immune serum was used to adjust the counter so that 99% of cells lay under the 10^1 fluorescence intensity region. Thus, fluorescence intensities reported for immune sera in each experiment were always relative to those obtained using non-immune serum. All experiments were repeated on 2 to 4 different days, and fluorescence intensities reported for each strain were the means of all replicates in all experiments. Data were plotted using WinMDI software (version 2.9; <http://www.bio-soft.net/other.html>).

Isolation of DNA and PCR amplification. For DNA extraction, cells were suspended in protease- and DNase-free deionized water (approx. 3×10^8 cells ml^{-1}) and treated for 20 min at 100 °C and then at -20 °C for 2 min. The suspension was then centrifuged at 10 000 g for 5 min and the supernatant recovered and stored in aliquots at -20 °C until use.

Detection of the *p47* gene was carried out using a primer pair (D-L/D-R; Table 1) designed following analysis of the NMA0281 (*N. meningitidis* Z2491) and NMB0035 (*N. meningitidis* MC58) loci from public databases (The Institute for Genomic Research, www.tigr.org), which corresponded to an inner fragment of approximately 600 bp. PCRs were performed in a MyCycler 1.06 system (Bio-Rad) after optimization of the optimal detection conditions. Each reaction mixture (20 µl) contained: 0.5 µl DNA solution, 1.5 mM MgCl_2 , 10 µM dNTPs, 0.8 µM oligonucleotides, 2 U BioTaq, 16 mM $(\text{NH}_4)_2\text{SO}_4$, 67 mM Tris/HCl (pH 8.8) and 0.01% (v/v) Tween 20. The PCR program comprised pre-incubation at 94 °C for 5 min, followed by 27 cycles of 94 °C for 30 s, 67 °C for 30 s and 72 °C for 1 min, with a final extension at 72 °C for 4 min. Positive and negative controls were included in each experiment.

DNA sequencing and phylogenetic analysis. Sequencing of the *p47* gene in the different strains was carried out after PCR amplification of five overlapping fragments (Table 1). Primers for amplification (F1 to F5) were designed on the basis of sequence data from the homologous locus NMA0281. Different primer pairs were necessary to amplify fragment 5. PCR products were purified using a QIAquick gel extraction kit (Qiagen) and the DNA sequenced using a BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems). Each reaction (10 µl) contained 4 µl purified PCR product, 3 µl BigDye terminator RR-100, 1 µl primer (5 µM) and 2 µl H_2O . Amplification and labelling reactions were carried out independently and repeated twice for each chain. Sequencing of fragments was carried out at the Instituto de Salud Carlos III (www.isciii.es) and Sistemas Genomicos S.A. (www.sistemasgenomicos.com), Spain.

Assembly of the complete *p47* gene sequences was carried out using CAP3 software (Huang & Madan, 1999). Alignments of the *p47* gene sequences and the deduced amino acid sequences were done using CLUSTAL_X (version 1.81). Phylogenetic analyses were carried out using TREECON (version 3.1b) using the Kimura model and the neighbour-joining method (Van de Peer & De Wachter, 1994). Substitutions (synonymous and non-synonymous), types of site and *p* distances (*pS* and *pN*) were calculated by the Nei-Gojobori method, whereas overall mean distances and group distances were computed using the Kimura two-parameter model, using MEGA3 (version 3.1) (Kumar *et al.*, 2004). Fingerprints of multiple amino acid sequence

Table 1. Primers used for detection (D) and sequencing (F) of the *p47* gene

Primer	Sequence (5'→3')	Location (nt)*	Annealing temperature (°C)	Product (bp)	Strain
D-L	GTTGCCGGGCGAATACGAAATGACTT	322–346	67	514	All
D-R	GACGCGCCGCCGACCACCTT	833–814			
F1-L	GTGTGCTGCTCGCGCGATTTTTC	<146–<124	60	380	All
F1-R	GCTTGCGGCCGCTGTTGTTTTTA	232–210			
F2-L	AGTATCGCCGTCAACGACA	138–156	61	228	All
F2-R	ATTGGTCAAAAGACCGCAAG	365–346			
F3-L	GCCGCAAGCTCGAATGGGAAATC	225–247	68†	228	All
F3-R	CGGCGAGCGGTTGGGACAGTT	453–433			
F4-L	TTGCCGGGCGAATACGAAATGACT	322–345	68	512	All
F4-R	GACGCGCCGCCGACCAC	833–817			
F5A-L	AAAGATGCCGGRTTTACYG	664–682	56	993	GLD, M7, NMB, Nm16, Nm22, Nm68, NmP17
F5A-R	AGGCGCACATAATCGAAAA	>489–>471			
F5B-L	GAAGGAAATTGCAGCGAAAC	737–756	58	513	Nm62, Nm63, NmP0, NmP2, NmP3, NmP5, NmP7, NmP18, NmP19
F5B-R	GCAATGCCGTACTGGTTTTT	>82–>63			
F5B-L	GAAGGAAATTGCAGCGAAAC	737–756	58	638	Nm59, Nm60, Nm70
F5C-R	ATTAACCGATAGCGGCAGGT	>207–>188			
F5D-L	AGCTTTTGARCGAACTGCAC	602–621	63	1304	B16b6, Nm9, Nm17, Nm20, Nm21, Nm30, Nm39, Nm40, NIP3, NIP5, NIP6, NIP9
F5D-R	GCGGTGTGTTTGATGATGTY	>738–>719			

*<, Position upstream of the *p47* gene; >, position downstream of the *p47* gene.

†64 °C for NmP7.

alignments were produced using the alignment shading software TeXshade (Beitz, 2000).

RESULTS

Surface expression and accessibility of P47

Fig. 1 shows the results of the flow cytometry analysis of the reactivity of anti-P47 and anti-OMV rabbit sera with cells from some representative *N. meningitidis* and commensal *Neisseria* strains. Binding of anti-P47 antibodies was highly variable among *N. meningitidis* strains, but this variability did not occur with anti-OMV antibodies (Fig. 1, top and middle rows). The same variability was observed for the commensal *Neisseria* strains, with much higher binding values for *N. lactamica* strains, in which anti-P47 antibodies reacted with intensities similar to those seen in many *N. meningitidis* strains (Fig. 1, bottom row). Quantitative expression of P47 on the cell surface of all strains tested is shown in Fig. 2. Results shown are the means of at least two assays carried out on different days. Fluorescence intensity was widely variable among strains, although the mean for all strains was higher in *N. meningitidis*. As described above, the reactivity of some single commensal *Neisseria* was comparable to that of some *N. meningitidis* strains.

Flow cytometry was also used to evaluate the surface accessibility in some selected strains after permeabilization

with ethanol (Fig. 3). Permeabilization increased the accessibility of P47 to specific antibodies ($P<0.05$) in four of the seven *N. meningitidis* strains, whereas it decreased accessibility in the other three *N. meningitidis* ($P<0.05$) and in both *N. lactamica* strains (not statistically significant). Reactivity with anti-OMV serum after permeabilization was also variable, although the differences between permeabilized and non-permeabilized cells were much less significant (results not shown).

Differences in surface accessibility of P47 in the strain H44/76 and three homologous LOS-deficient mutants (*lpxA*, *rfaC* and *galE* strains) are shown in Fig. 4. Binding of anti-P47 antibodies to the wild-type strain H44/76 was low, but was significantly increased in the three mutants (see also Fig. 2).

DNA sequencing and phylogenetic analysis

One possible explanation for apparent strain differences in reactivity with the anti-P47 antisera in flow cytometry is polymorphisms in the P47 protein. To test this possibility, we sequenced the *p47* gene. PCR amplifications using the D-L/D-R primer pair demonstrated that the *p47* gene was present in all strains analysed. Sequencing of the entire gene was carried out by amplification of five overlapping fragments using the F1 to F5 primer pairs shown in Table 1. Amplification of the fifth fragment required four different primer pairs, depending on the strain, due to the existence

Fig. 1. Flow cytometry analysis of the reactivity of anti-P47 (filled peaks) and anti-OMV (peaks with thick lines) rabbit sera with formalin-treated cells from representative *N. meningitidis* and commensal *Neisseria* strains. Reactivity with a non-immune rabbit serum (peaks with thin lines) was used as a negative control.

of a hypervariable non-coding region between the annotated NMB0035 (*p47*) and NMB0036 loci. The assembled gene had the same length in all strains (1167 bp), showing only single nucleotide substitutions with no insertions, deletions or intragenic codon stops, and coding for 13 different amino acid sequences (Fig. 5). The overall mean distance, type and number of substitutions, and potential site and *p* distances, as well as ratios for synonymous and non-synonymous substitutions, are shown in Table 2. Mean distances between groups are shown in the inset of Fig. 5.

To perform phylogenetic analyses, the registered sequences of the homologous genes from strains *N. meningitidis* Z2491, *N. meningitidis* MC58, *Neisseria gonorrhoeae* FA1090 and *N. lactamica* NISANGER (currently being sequenced at the Sanger Institute) were also included.

The degree of similarity between the amino acid sequences derived from alignment analysis ranged from 97.4 to 99.7% for *N. meningitidis* and from 97.7 to 99.0% for *N. lactamica*. The sequence of the protein from the *N. gonorrhoeae* strain analysed showed a 95.4% similarity to those of the most divergent *N. meningitidis* strains. Fig. 6 shows an alignment fingerprint of the 13 amino acid sequence types.

DISCUSSION

Development of vaccines effective against serogroup B meningococci is one of the major challenges for researchers in this field. Antigens suitable for effective vaccines should be highly conserved, easily accessible at the bacterial surface

Fig. 2. Interstrain variability of the reactivity of anti-P47 rabbit serum with formalin-treated cells, measured by quantitative flow cytometry. Bars represent the mean fluorescence intensity ($\pm$ SEM) of all measured cells in a single strain (dark grey bars, *N. meningitidis*; light grey bars, commensal *Neisseria*). Lines indicate the geometric mean for each group (*N. meningitidis* or commensal *Neisseria*) and shaded boxes show the 95% confidence interval of the means.

Fig. 3. Reactivity of anti-P47 rabbit serum with formalin-treated cells non-permeabilized (light grey bars) or permeabilized with ethanol (dark grey bars), measured by quantitative flow cytometry. Bars represent the mean fluorescence intensity ($\pm$ SEM) of all measured cells in a single strain.

Fig. 4. Reactivity of anti-P47 rabbit serum with formalin-treated cells obtained from strain H44/76 (LOS, lipid A-KDO₂-Hep₂-Glc-GlcNAc₂-Gal₂; shaded profile) and the LOS-deficient mutants *lpxA* (complete lack of LOS), *rfaC* (LOS, lipid A-KDO₂) and *galE* (LOS, lipid A-KDO₂-Hep₂-Glc-GlcNAc), measured by quantitative flow cytometry.

and able to elicit long-lasting bactericidal responses. Currently, the most promising approaches are based on OMPs, which are being investigated using several strategies, including reverse vaccinology (Adu-Bobie *et al.*, 2003), heterologous commensal strains (Gorringe *et al.*,

2005) and multiple PorA expression mutants (Martin *et al.*, 2000). In previous studies, we found a 47 kDa meningococcal OMP inducible by iron restriction, which showed high antigenic conservation among strains and the ability to induce bactericidal responses *in vitro* (Arenas *et al.*, 2006).

Fig. 5. Neighbour-joining phylogenetic tree of the deduced amino acid sequences of the P47 protein. The sequence of the *N. gonorrhoeae* strain (most divergent) was used to root the tree. Thirteen sequence types were identified, eight in *N. meningitidis*, four in *N. lactamica* and one in *N. gonorrhoeae*. Mean distances between groups are shown in the inset.

Table 2. Overall mean distances, synonymous and non-synonymous substitutions and sites, p distances of substitutions and pS/pN ratio in the $p47$ gene

Species	No. of amino acid sequence types	Overall mean distance	Substitution				
			Type	Number	Potential sites	p distance (pS or pN)	pS/pN^*
<i>N. meningitidis</i>	8	0.012	Synonymous	11	270	0.042	14.0
			Non-synonymous	3	894	0.003	
<i>N. lactamica</i>	4	0.037	Synonymous	37	269	0.139	23.2
			Non-synonymous	6	895	0.006	
All	12	0.025	Synonymous	40	270	0.150	13.6
			Non-synonymous	10	895	0.011	

*A pS/pN ratio of <1 indicates a positive selection for amino acid replacement; ratios >1 indicate a negative selection, suggesting that conservation of the protein sequence is critical and only neutral changes at the amino acid sequence level are tolerated.

Flow cytometry results obtained with formalin-treated cells were comparable to those using live intact cells (not shown) and demonstrated the accessibility of P47 at the bacterial surface. As can be seen in Figs 1 and 2, the anti-P47 mean binding intensity was variable, providing evidence for significant differences among the *N. meningitidis* strains, and between these and the commensal *Neisseria* tested. These differences could be due to variability in expression, surface accessibility or P47 epitope arrangements in the different strains. Previous results obtained by Western blotting (in which anti-P47 serum reacted with linear epitopes) showed no differences between meningococcal strains, suggesting that the inter-strain variability observed by flow cytometry was not due to differential protein expression, but was probably caused by differences in the accessibility and/or epitope arrangement of the P47.

Antigen masking by surface structures, such as the capsule and lipopolysaccharides, has already been demonstrated. Ethanol damages bacterial outer structures, partially eliminating the capsule and LOS outer residues, exposing

surface antigens and facilitating their accessibility to antibodies (Jolley *et al.*, 2001; Michaelsen *et al.*, 2001). In *N. meningitidis*, capsular structure and/or the amount and length of LOS can significantly vary among strains, and all commensal *Neisseria* lack a capsule and have particular LOS due to the absence of *lgt* genes (which encode proteins involved in glycosyltransferase synthesis) in most species (Zhu *et al.*, 2002; Kahler *et al.*, 2005).

Thus, we checked the possibility of steric hindrance of the binding of anti-P47 antibodies by capsules or LOS by comparing ethanol-treated and untreated cells for a capsulated strain (NMB) and its non-capsulated mutant (M7), and a wild-type strain (H44/76) and three homologous LOS mutants, one without any LOS and the other two with LOS carrying a truncated oligosaccharide chain (*lpxA* completely lacks LOS, *rfaC* has only the lipid A molecule and two KDO residues, and *galE* has lipid A and two KDO residues linked to two heptoses, glucose and *N*-acetylglucosamine). Our results did not show congruent differences between ethanol-treated and untreated cells, as some strains significantly increased binding of anti-P47

Fig. 6. Alignment fingerprint of the 13 amino acid sequence types produced using the shading software TeXshade. Non-conserved, similar and conserved residues are indicated.

antibodies after treatment, whereas others showed the opposite effect. It is noteworthy that ethanol produced a significant diminution of binding after treatment in most of the strains that showed the higher binding of anti-P47 antibodies by untreated cells. It is possible that, in these cases, ethanol could affect the P47 epitope structure due to a more exposed conformation.

In contrast, binding to the LOS mutants was significantly higher than to the homologous wild-type strain. It is noteworthy that binding to the *lpxA* strain, which completely lacks LOS, was lower than that to the *rfaC* and *galE* strains with incomplete LOS molecules (Gorter *et al.*, 2003). It is possible that the presence of LOS molecules, even incomplete, could affect the correct composition of the whole outer membrane or the location of some of their protein components. Previous studies on the proteome of wild-type and LOS-deficient OMVs in fact point to differences in their protein composition (Williams *et al.*, 2007). In addition, the iron-regulated lipoproteins TbpB and LbpB have been found in much lower amounts in outer membranes of the LPS-deficient *lpxA* mutant (Steeghs *et al.*, 2001). As P47 is also an iron-regulated lipoprotein, its expression may be similarly reduced in the *lpxA* mutant.

Other authors have not noted an effect of capsular material on surface-antigen accessibility (Bowden *et al.*, 1995), which, together with our results, strongly point to LOS as being responsible for the differences in accessibility of P47 to antibodies. The observed variability among strains and the opposing effects of ethanol treatment could be explained by the significant differences in LOS structure and variability found in *Neisseria*, even among cells in a single strain population (Zhu *et al.*, 2002; Kahler *et al.*, 2005).

The information obtained by analysis of the nucleotide and/or amino acid sequence data using bioinformatic tools can be useful to predict some characteristics of the molecules and to help in the selection of the most promising antigenic variants. Specialized web-based servers and analysis software allow a statistical prediction of characteristics, such as cellular location, hydrophobicity, secondary and tertiary structure, and potential epitopes (Zhang *et al.*, 1998; Yu *et al.*, 2002).

Sequencing of the *p47* gene was done using five primer pairs due to the poor results obtained with primer pairs covering longer fragments. Genetic analyses demonstrated 23 alleles for the *p47* gene, giving rise to 13 amino acid sequences, with an apparent correlation (although not complete) between some allele types and the clonal complexes assigned to the strains (i.e. alleles 1 and 2 with complex ST-32, and alleles 11–16 with complex ST-8). It is noteworthy that the allele carried by the *N. lactamica* NLSANGER strain is closer to those of the *N. meningitidis* strains (particularly to strain NMB and its capsule-deficient mutant M7) than to those of the other *N. lactamica* strains analysed. The amino acid sequence of the P47 protein

showed a 90% similarity to COG2822 (a predicted periplasmic lipoprotein involved in iron transport in *Escherichia coli*, *Yersinia pestis*, *Staphylococcus aureus*, *Bacillus subtilis* and *Listeria innocua*) and a 65% similarity with DUF451 (predicted putative lipoprotein in *E. coli*, *S. aureus* and *B. subtilis*), which is in agreement with the iron-regulated character of P47. Predictions also showed a high probability that the molecule is located in the outer membrane ($P=0.79$) and a very low probability ($P=0.06$) that it is located in the periplasm.

We believe that differences in reactivity with the anti-P47 antisera in flow cytometry are not due to polymorphisms in the P47 protein because, although 13 amino acid sequence types were demonstrated, the percentage of non-synonymous substitutions found was low, and the very high *pS/pN* ratios indicated that the sequence could be considered extremely conserved (Table 2). The results obtained in this work confirm the antigenic cross-reactivity of P47 in all *N. meningitidis* strains and some commensal *Neisseria*, particularly *N. lactamica*, and demonstrate its surface location. Correlation of the sequence types with antibody binding by flow cytometry was not significant. Thus, the differences observed are likely to be due to masking by LOS or other surface molecules, or to association of P47 forming protein complexes in which conformational and/or shared epitopes could be dominant. This possibility is currently being investigated in depth. Our results suggest that masking by, or association with, LOS or other membrane molecules can be crucial for antigen accessibility, which must be thoroughly analysed in new vaccine candidates.

ACKNOWLEDGEMENTS

We wish to thank Drs Peter Borriello, Dan Granoff and Mariagrazia Pizza for kindly providing some of the strains used in this study. This work was supported by grants PGIDT01BIO20301PR from the Xunta de Galicia and PI050178 from the Fondo de Investigación Sanitaria (Ministerio de Sanidad y Consumo), Spain.

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