ARTICULOS ORIGINALES COMPLETOS

COMPARISON OF THE DNA SEQUENCE OF NINE DIFFERENT GENES FOR THE CLASS 1 OUTER MEMBRANE PROTEIN FROM *Neisseria meningitidis*

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Recibido en enero de 1993. Aprobado en mayo de 1993.

Key words: P1 protein, DNA sequence homology, *Neiesseria meningitidis*

SUMMARY

The genes coding for nine different class 1 outer membrane proteins of *Neisseria meningitidis* were isolated by polymerase chain reaction (PCR) using primers designed from conserved regions in the signal sequence and the C-terminus. The isolated genes were cloned and sequenced in M13mp18 vector. Here we show a complete comparison of the DNA sequences and the differences found among these and other available published sequences.

RESUMEN

Mediante el empleo de la reacción en cadena de la polimerasa se aislaron los genes codificantes para 9 proteínas de clase 1, localizadas en la membrana externa de la *Neisseria meningitidis.* Los oligonucleotidos utilizados en la amplificación se diseñaron en base a secuencias conservadas del péptido señal y del extremo C-terminal. Los genes se clonaron y secuenciaron en el vector M13mp18. Aquí se muestra la comparación de las secuencias de ADN y las diferencias con respecto a otras secuencias publicadas.

INTRODUCTION

Neisseria meningitidis is a major ethiological agent of bacterial meningitis and septicemia, causing one third of the epidemic and endemic bacterial meningitis cases throughout the world (Peltola, 1983). The disease produces a fulminant effect with a high mortality rate.

There are at least 12 different known serogroups of *N. meningitidis* and more than 20 different serotypes (Frasch *et al.*, 1985) and subtypes. The capsular polysaccharide is the criterium by which meningococci are classified into serogroups and the differences in outer membrane protein (OMP) composition is the criterium for serotype (class 2 and class 3 proteins) and subtype (class 1 protein) classification. The same serotypes are seen in group B, C, Y and W135. The class 3 proteins of serogroup A were found to be antigenically homogeneous and were designated serotype 21 (Frasch *et al.*, 1985). The reported epidemics have been due to serogroups A, B and C. Commercially available polysaccharide vaccines provide limited protection against infection caused by serogroups A and C. The group B polysaccharide is poorly immunogenic in humans (Wyle *et al.*, 1972; Frasch, 1985; Frasch, 1989), probably due to antigenic similarities with human brain components (Finne *et al.*, 1983; Finne, 1985).

Several approaches to provide protection against serogroup B using OMP have failed (Poolman *et al.*, 1987; Boslego *et al.*, 1990; Bjune *et al.*, 1991a, 1991b). Only the cuban vaccine (VA-MENGOC-BCR) has proved its efficacy against serogroups B and C (Sierra *et al.*, 1991).

So far, attempts to obtain an antimeningococcal vaccine based on the use of a cloned OMP have been hampered by the antigenic variability of these proteins (Meyer, 1990) and by the fact that most of these antigens fail to induce bactericidal antibodies. To obtain a wider rank of protection, more than one antigen will probably have to be included in a vaccine preparation.

The serotype classification of *N. meningitidis* is based on the antibody recognition of the variable regions of the Class 1 OMP.

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It has been shown that these proteins , coded by the porA locus (Hitchcock, 1989) are capable of eliciting bactericidal antibodies (Frasch *et al.*, 1986; Van Der Ley *et al.*, 1991) and moreover, monoclonal antibodies directed against their variable regions provide complete protection against challenge to meningococci in the infant rat infection model (Saukkonen *et al.*, 1987). Accordingly, these antigens have been proposed as potential components of any antimeningococcal vaccine. Here we demonstrate that some of the variable regions responsible for the induction of bactericidal antibodies may be identical between Class 1 OMP belonging to different subtypes and that, differences between specific variable regions may be found when comparing Class 1 OMP of the same subtype (Barlow *et al.*, 1989; McGuinness *et al.*, 1990; Seid *et al.*, 1990; Butcher *et al.*, 1991; Maiden *et al.*, 1991). Thus, there are actually possible mutations and selections that might occur in critical epitopes that are missed by the sero- and subtyping methods (Heckels, 1991). For vaccine design, the information derived from the sequence data is important in order to select those proteins more suitable for any immunological schedule.

MATERIALS AND METHODS

E. coli strains

E. coli strain JM109: F'traD36 LacIq (LacZ) M15proAB/recA1 endA1 gyrA96 (Nalr) thi hsdR17 (rk-mk+) supE44 relA1 (Lac-proAB) (Yanisch *et al., 1985).*

Meningococcal strains

Meningococcal strain B385 (B:4:P1.15) was isolated from a patient with meningococcal disease and obtained from the National Center for Antimeningococcal Vaccine (Havana, Cuba). Strains IHN 36109 (B:4:P1.2), IHN 36117 (B:14:P1.7), IHN 36152 (B:NT:P1.6), IHN 5363 (B:4:P1.9), 866 (B:15:P1.16) were kindly given by Dr. L.Saarinen from the National Public Health Institute (Helsinki, Finland). Strain H 44/76 (B:15:P1.7,16) was obtained from the Provincial Center for Hygiene and Epidemiology (Havana, Cuba). Strain 882066 (B:NT:P1.4) originally from Dr. Jan T. Poolman from the National Institute of Public Health and Environmental Protection (The Netherlands), and strain 8529 (B:15:P1.3) originally from Dr. W.D. Zollinger from the Walter Reed Army Institute (Washington, USA), were obtained from Dr. Mark Achtman from the Max Planck Institut für Molekulare Genetik (Berlin, FRG).

Genomic DNA preparation

Neisseria meningitidis strains were grown in Muller-Hinton Broth (OXOID, UK.) or BHI (OXOID, UK.). The cells were harvested from the culture by low speed centrifugation, resuspended in 8 ml of TE (10 mM Tris-hydroxymethylaminomethane, 1 mM Ethylene diamine tetraacetic acid (EDTA), pH 8.0) and incubated for 1 h at 37^{0}C in a solution of 10 mg/ml lysozyme (Sigma, UK.), 0.2 ml of 20 mg/ml pronase E (Merck, FRG.) and 1.1 ml of 10% sodium dodecyl sulphate (SDS) (BDH, UK.); followed by extraction with phenol:chloroform:isoamyl alcohol (25:24:1), addition of an equal volume of 2-butanol and precipitation with 2.5 volumes of absolute ethanol. The tRNA was removed by incubation with RNAse A (Sigma, UK.).

Polymerase chain reaction (PCR) and gel electrophoresis

The PCRs were performed with Taq DNA polymerase (Enzibiot, Cuba) using 2 units per reaction. The reaction mixture (25 mM Tris-HCL, pH 9.0, 50 mM KCl, 10 mM $MgCl_2$, 0.1% gelatin, 1 mM Dithiothreitol (DTT), 200 μM each of dNTP, primers at 1 M and 1 μg of genomic DNA) in a 1.5 ml microcentrifugue tube, was incubated in a programmable heat block HYBAID (Cera Labo SA, France) during 30 cycles for 1 min. at 95^{0}C (denaturation), 30 sec. at 55$C (annealing), and 40 sec. at 72^{0}C (extension), and the last cycle of extension was carried out for 3 min. at 72^{0}C. Then, the reaction mixture was extracted with 100 μl of phenol-chloroform-isoamyl alcohol (25:24:1) and the aqueous phase washed with diethyl ether saturated with TE. After centrifuging for 1 min., the ether was discarded and the remainder was removed by heating for 5 min. at 50^{0}C. The gene amplification product was checked on 0.8% agarose gel electrophoresis in TA (40 mM Tris-acetate ph 8.0, 1 mM EDTA) at 120 volts using 5μl of each sample. The rest of the samples were separated on 0.8% Low Melting Temperature (LMT) agarose gels and the amplified genes were isolated as described by Sambrook *et al.* (1989).

DNA cloning and sequencing

DNA fragments isolated by phenol extraction from the LMT agarose gels were ligated without further modification into the SmaI cut M13mp18 phage vector and used to transfect the *E. coli* strain JM-109, the positive phage plaques were selected by non blue color development in 100 g/ml X-gal and 20 g/ml IPTG containing medium followed by DNA hybridization, NcoI restriction analysis and single-stranded hybridization test to select clones with opposite orientation. Single-stranded DNA from each clone was sequenced using Multiwell microtitre plate DNA sequencing system-T7 DNA polymerase (Amersham, UK.). All the procedures were carried out essentially as described by Sambrook *et al.* (1989) and following the instructions given by the manufacturers.

Sequence alignment was done using the CLUSTAL V software (Higgins *et al.*, 1991).

DNA hybridization

One microliter (50-500 ng) of purified replicative DNA form from each phage was spotted onto Hybond N+ membrane filters (Amersham, UK.). The P1.15 gene cloned into M13mp18 and the M13mp18 vector alone were used as positive and negative controls respectively. Hybridization was carried out using the ECL gene detection system (Amersham, UK.) and the P1.15 gene as hybridization probe.

Single-stranded hybridization test

To identify phages carrying inserts on opposite orientation, 20 μl were withdrawn from lysates of each pair of putative recombinants to be tested (prepared according to Sambrook *et al.*,1989) and mixed together along with 1 μl of 20% SDS before heating at 55^{0}C for 1 h. Then 2 μl of sample buffer (2%

Bromophenol blue, 25 mM EDTA, 50% Glycerol) were added and the whole mixture run on a 0.8% agarose gel in TA buffer. Clones from samples giving bands of retarded electrophoretic mobility were selected.

All the chemicals used were from Merck (FRG) and BDH (UK). SmaI and NcoI endonucleases, T4 DNA ligase modification enzyme and primers used for PCR amplification and DNA sequencing were from Enzibiot (Cuba).

RESULTS AND DISCUSSION

For PCR amplification genomic DNA of nine different class 1 OMPs from *N. meningitidis* (Table. 1) was isolated as described in Experimental Procedures.

The porA genes that codify for the class 1 OMP were amplified by PCR using primers from the signal sequence and the C-Terminal constant regions, selected on the basis of the P1.16 gene sequence previously published (Barlow *et al.*, 1989). The primers designed for PCR amplification were:

N-Terminal

P674 5' TTCCATGGATGCGAAAAAAACTTACCGCC 3'

NcoI

C-Terminal

P675 5' TTGGATCCGAATTTGTGCAAACCGAC 3'

BamHI

Primers 674 and 675 were designed including the NcoI and BamHI restriction sites with the aim of cloning the isolated genes in an expression vector (data pending publication). The NcoI site was also used for restriction analysis.

Amplified DNA fragments were ligated into the SmaI cut M13mp18 phage vector used to transfect the *E. coli* strain JM-109 and screened for positive clones as described in Experimental Procedures.

The following set of primers, located in the constant regions within the gene were constructed for DNA sequencing.

P1053 5' GGCATAGCTCCCGGCAAAACC 3'

P1054 5' GTGTATTATGCCGGTCTG 3'

P1055 5' GCTGGCATCGTCAAACTG 3'

P1056 5' TTGGCAGGCGAATTCGGTACG 3'

The location of these primers is shown schematically in figure 1. Differences between the porA sequences of each subtype and a consensus sequence were determined with the CLUSTAL V software (Fig. 2).

The derived aminoacid sequences of variable domains from nine class 1 proteins are shown in Table. 1.

The sequence for three of these proteins (P1.3, P1.4 and P1.7) have not been reported before, and except for strain H 44/76, the rest of the strains sequenced were different from those previously reported and presented several aminoacid changes. There are three regions (VR1, VR2 and VR3) which show high frequency of variation among strains (Maiden *et al.*, 1991; Van Der Ley *et al.*, 1991; Butcher *et al.*, 1991). The high degree of variation of VR1 and VR2 and the implication of these regions in binding and

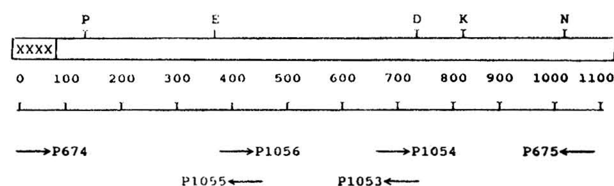

Fig. 1. Schematic diagram showing the strategy followed for DNA sequencing. The signal peptide and the open reading frame are shown as the filled and open boxes respectively. The restriction endonucleases indicated are: E:EcoRI, P:PvuII, K:KpnI, D:DraI, N:NcoI. Arrows indicate the location and direction of primers used to sequence the genes.

Table 1

Translation into aminoacids of variable regions 1, 2, and 3 of nine porA genes sequences

CV number	Strain number	Subtype	VR1	VR2	VR3
113	B385	P1.15	PPSKSQP	HYTRQNNADVF	STSDE
15	IHN36109	P1.2	PLQNIQP	HFVQQTSKSQPTL	SGSDE
--	H44/76	P1.7,16	AQAANGGASG	YYTKDTNNNLTL	SGSDE
16	IHN36117	P1.7	AQAANGGASG	YWTTVNTGSATTT	SGSDE
19	IHN36152	P1.6	PPSKGQTGNK	TYTVDSSGVVTP	RIGEDDE
14	IHN5363	P1.9	QPSKGQVGNK	YVDEQSKYHA	SGSGSDQ
182	882066	P1.4	AQAANGGASG	HVVVNNKVATH	SATSDQ
214	8529	P1.3	AQAANGGASG	TLANGANNTIIR	RIGDDDE
73	866	P1.16	QPQVTNGVQGN	YYTKDTNNNLTL	SATSDE

CV number is the identification number in the Center for Genetic Engineering & Biotechnology collection.

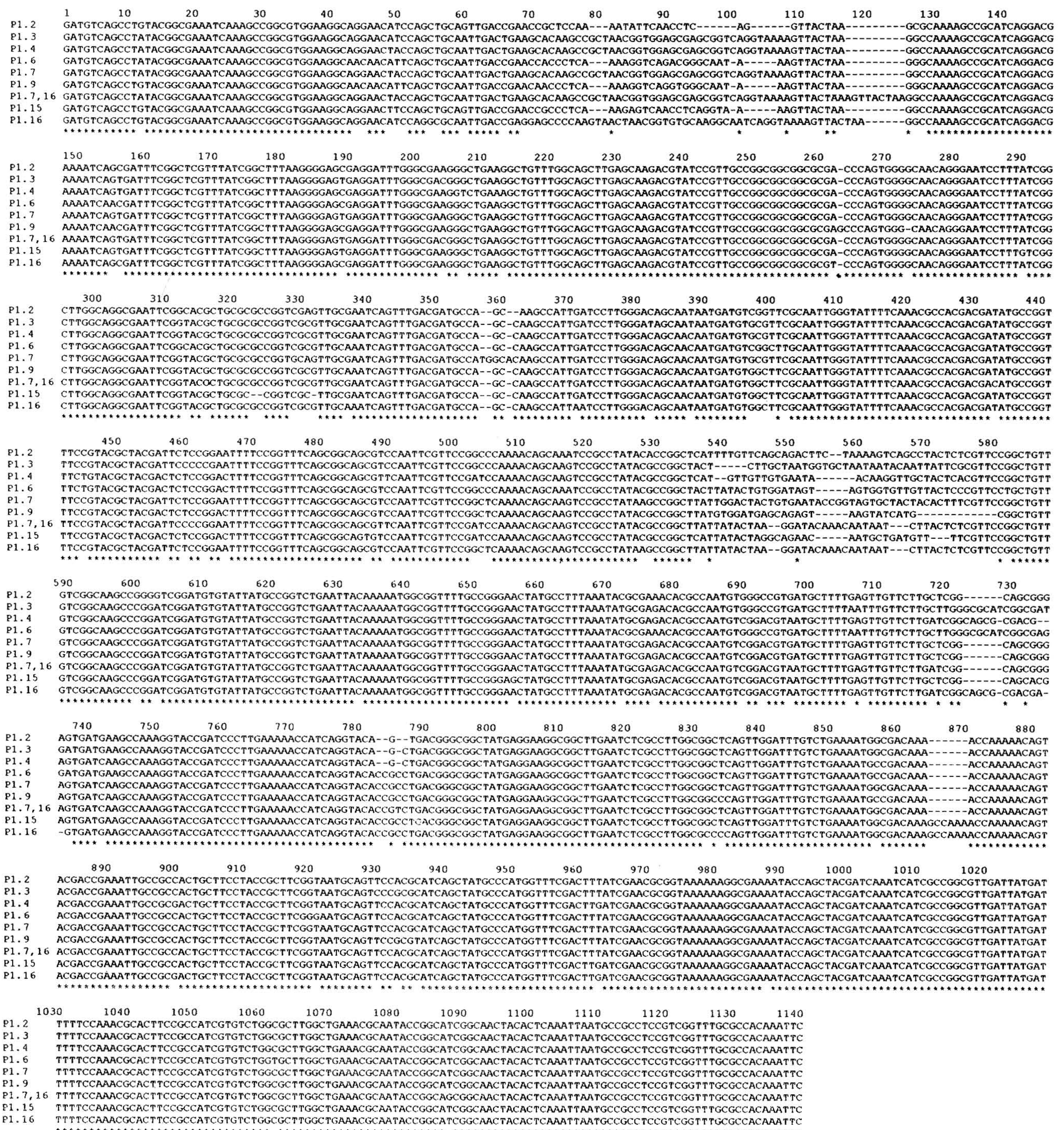

Fig. 2. Comparative alignment of nine meningococcal porA genes nucleotide sequences.(*) indicate identities and (-) indicate variable domains.

bactericidal activity of monoclonal antibodies, were studied using synthetic peptides (McGuiness *et al.*, 1990; Van Der Ley *et al.*, 1991). The antibodies against synthetic peptides of VR3 have been shown to bind the whole cells, and require further study. The variation of some porA genes in this region may be involved in a different grade in the immunological response among strains.

Table. 2.
Comparison between variable regions within the same subtype among different reported sequences. The aminoacid changes are indicated in bold letters.

Strain	Classif	Reported seq.	Reg	References.
S3032	B:NT:P1.16	**KLSSTNAKTGNKVE**	VR1	Van Der Ley *et al.*
MC50	C:NT:P1.16	QPQVTNGVQGNQVK	VR1	Barlow *et al.*
866	B:15:P1.16	QPQVTNGVQGNQVK	VR1	In this report
H355	B:15:P1.15	HYTRQNN**A**DVFVP	VR2	Maiden *et al.*
MC51	C:NT:P1.15	HYTRQNN**T**DVFVP	VR2	McGuinness *et al.*
B385	B:4:P1.15	HYTRQNN**A**DVFVP	VR2	In this report
B2106I	B:4:P1.2	PLQNI**Q**QPQVTK**A**	VR1	Seid *et al.*
B16B6	B:2a:P1.2	PLQNIQPQVTK**R**	VR1	Maiden *et al.*
B40	A:4:P1.10	PLPNIQPQV	VR1	Seid *et al.*
B16B6	B:2a:P1.2	HFVQQTP**K**SQPTLV	VR2	Maiden *et al.*
2996	B:2b:P1.2	HFVQQTP**E**SQPTLV	VR2	Maiden *et al.*
2996	B:2b:P1.2	HFVQQTP**Q**SQ**I**TLV	VR2	Van Der Ley *et al.*
B2106I	B:4:P1.2	HFVQQTP**QQ**SPTLV	VR2	Seid *et al.*
IH36109	B:4:P1.2	HFVQQT**SK**SQPTLV	VR2	In this report
M1080	B:1:P1.1,7	AQAANGGA**GA**SGQV	VR1	Maiden *et al.*
H44/76	B:15:P1.716	AQAANGGASGQV	VR1	McGuinness *et al.*
IH36117	B:14:P1.7	AQAANGGASGQV	VR1	In this report
M990	B:6:P1.6	PPSKGQTGNKV**TKG**	VR1	Maiden *et al.*
6940	B:19	PPSKGQTGNKV**KVT**	VR1	Seid *et al.*
M982	B:9:P1.9	QPSK**AQGQTNNQV**	VR1	Maiden *et al.*
IH5363	B:4:P1.9	QPSK**GQVGN**	VR1	In this report

The different antibodies elicited against each variable region imply that each meningococcal strain could exhibit distinct subtype specificities (McGuiness *et al.*, 1990). We observed lack of definition in subtype specificity among strains where only specificity against one variable region has been defined. However, from our sequence comparison data, it can be noted that some P1s grouped in the same subtype on the basis of the antigenic identity of only one variable region can indeed bear variable regions belonging to different serological subtypes. This is shown in the case of the strains 882066, defined as P1.4 subtype and 8529, defined as P1.3; whose VR1 match the P1.7 VR1 sequence. These strains should therefore be correctly redefined as P1.4,7 and P1.3,7 respectively. This is specially relevant; since VR1 from P1.7 subtype is recognized by bactericidal monoclonal antibodies (Van Der Ley *et al.*, 1991) and it have been shown that P1.7 is the main subtype among serogroup A strains (Achtman, 1991).

The comparison between variable regions of published porA gene sequences shows some aminoacid changes in the same P1 subtype (Table. 2). So it would be interesting to know if these variations could influence the cross-reactivity of antibody binding and the bactericidal activity of these antibodies. In this respect, the serotyping may be even more complicated than it looks. In the U.K. for instance, there are two quite distinct populations of B:15:P1.7,16 strains, one of which has a mutation in the P1.16 epitope that still reacts with the typing antibody but it is no longer bactericidal for that strain (Heckels, 1991). Moreover, it has been demonstrated that about 20% of B:15:P1.16 strains of the ET-5 complex, isolated in Norway between 1987 and 1991, carrying the P1.7 epitope, showed no reaction with the P1.7 subtype monoclonal antibody on dot-blots, due to a deletion of tree aminoacids in VR1 (Wedege *et al.*, 1991).

These results indicate that more efforts and extensive epidemiological studies about subtype classification in *N. meningitidis* should be addressed to select those subtypes more representatives, recognized as major causes of the disease, for their inclusion in new generation vaccines in the near future.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. Luis Herrera for fruitful discussions and recommendations concerning technical aspects of this work. They also want to thank Dr. Sonia Dunn for the help given in the translation, and Dr. Maria de Jesús Leal for typing.

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