

EXPRESSION IN *Escherichia coli* AND IMMUNOLOGICAL CHARACTERIZATION OF A HYBRID CLASS 1-P64k PROTEIN FROM *Neisseria meningitidis*

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ABSTRACT

Class 1 outer membrane protein of *Neisseria meningitidis* induce bactericidal antibodies, have been used for the classification of meningococci in subtypes, and combinations of these epitopes have been considered for a new vaccine. Attempts to express Class 1 proteins in *Escherichia coli* have until now been unsuccessful; possibly due to the lethality of these proteins. We have previously described a new high molecular weight protein (P64k), present in all serogroups and serotypes of *N. meningitidis* tested. Here, we report the expression of a 219 aminoacid fragment of the P1.15 proteins as a fusion with the meningococcal P64k in two *E. coli* strains. It was easily purified and it was immunogenic in mice, capable of eliciting bactericidal antibodies.

Key words: *porA*, fusion protein

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RESUMEN

Las proteínas de membrana externa de Clase 1 de *Neisseria meningitidis* son buenos candidatos como antígenos vacunales, capaces de inducir anticuerpos bactericidas. Estas proteínas han sido usadas para la clasificación del meningococo en subtipos. La combinación de diferentes epitopos, reconocidos por anticuerpos bactericidas se considera actualmente como una estrategia promisoriosa para la nueva generación de vacunas. El intento de expresar las proteínas de Clase 1 en *Escherichia coli* ha sido fallido probablemente debido a la letalidad de éstas. Previamente nosotros hemos reportado una nueva proteína de alto peso molecular (P64k) que está presente en todos los serogrupos y serotipos de *N. meningitidis*. En este trabajo reportamos la expresión en *E. coli* de una proteína fusionada que contiene la P64k y la proteína de Clase 1, así como el estudio de inmunogenicidad del polipéptido recombinante.

Palabras claves: *porA*, proteína fusionada

Introduction

Neisseria meningitidis is the major etiologic agent of bacterial meningitis and septicemia, causing one third of the epidemic and endemic bacterial meningitis cases throughout the world (1). Three main serogroups (A, B, C) have been identified based on the composition of their capsules, and vaccines consisting of the capsular polysaccharide have been shown to protect against the disease caused by serogroups A and C. The capsular polysaccharide of serogroup B is, however, poorly immunogenic and unsuitable for vaccine purposes; possibly because of its cross-reactivity with the saccharide part of a glycoprotein in certain human tissues (2, 3).

Meningococcal outer membrane proteins (OMPs) have been studied as alternative components of a vaccine against *N. meningitidis* serogroup B. The meningococcal outer membrane contains five major classes of proteins of which, Class 1 and Class 2/3 have attracted most interest as possible vaccine can-

didates. All are porins which work as nutrient carriers through the membrane (4), and monoclonal antibodies (Mabs) directed against these proteins; particularly the Class 1 protein, are the most effective in bacterial killing and in animal protection experiments (5).

The Class 1 protein has been used for the classification of *N. meningitidis* strains into subtypes, and combinations of different epitopes recognized by bactericidal antibodies are being considered as new vaccine candidates (6, 7).

A new high molecular weight protein (P64k), has previously been demonstrated by our group to be present in all serogroups and serotypes of *N. meningitidis* tested (8). The *lpd A* gene, which encodes this protein, was isolated from a genomic library of *N. meningitidis*, cloned and expressed in *Escherichia coli* and accounted for 25 % of the total cellular protein (9).

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Attempts to express the Class 1 protein in *E. coli* have failed; probably due to the destabilization of the outer membrane (10). To overcome this difficulty, the Class 1 protein was expressed as a fusion with the OmpA in *Bacillus subtilis* (11). Here, the cloning in *E. coli*, the expression and the immunological characterization of a fusion protein (PM14) containing the P64k and a fragment of the Class 1 (P1.15) protein of the *N. meningitidis* strain B:4:P1.15 is reported.

Materials and Methods

Bacterial strains and growth conditions

E. coli strains W3110 *thyA* 36 *deoc2* *IN(rrnD-rrnE)1 rph pyrE* lambda⁻ F⁻ (12) and MM294 F⁻ *endA1 hsdR17(rk⁻mk⁺)supE44 thi-1 relA1? rfbD1? spoT1?* (13) were grown overnight at 37 °C in Luria Broth (LB).

The meningococcal strain B385 (B:4:P1.15) isolated from a patient with the meningococcal disease was kindly given by the Finlay Institute for Sera and Vaccines (Havana, Cuba). Meningococcal strains B:4:P1.15, B:4:P1.1, B:4:P1.9, B:4:P1.2, B:4:P1.7, B:4:P1.16, NT:P1.6 and a different isolate from the strain B:4:P1.15 were obtained from the Center for Epidemiology and Hygiene of Havana. The strains were grown overnight at 37 °C in Brain Heart Infusion (OXOID, UK), in a 5 % CO₂ atmosphere.

DNA cloning and sequencing

The *lpd* A gene and the *por* A gene were previously cloned into the plasmids pM-6 (9) and pM-7 (14), respectively.

Restriction and modification enzymes *Mlu* I, *Kpn* I, *Pvu* II, Klenow fragment of *E. coli* polymerase I, and T4 DNA ligase were obtained from Heber-Biotec (Cuba). DNA sequencing was done using the multiwell microtitre plate DNA sequencing system (Amersham, UK). All procedures were carried out according to the instructions given by the manufacturers.

SDS-PAGE and western blotting

Twenty µg of *E. coli* crude extracts or whole cells of *N. meningitidis* B385 (B:4:P1.15) were separated in 10 % SDS-PAGE as previously described by Laemmli UK (15) and the proteins were transferred to nitrocellulose filters BA 85 (Schleicher and Shuell) (16). The filters were blocked with 5 % Skim milk powder in phosphate buffered saline (PBS) 1 h at room temperature (RT) and incubated with the Mab anti Class 1 protein subtype 15 (CB Nm-1) and the Mab anti-P64k (Mab-114), respectively, 1 h at RT. As a second antibody, anti-mouse IgG peroxidase-linked whole antibody was used and the reaction was developed with Enhanced Chemiluminescence (ECL-Kit) from Amersham. Mabs against P64k and P1.15 were supplied by the Division of Immunotechnology (CIGB, Cuba).

Recognition of *N. meningitidis* outer membrane proteins by murine polyclonal antibodies was assessed on western-blotting. To do this, whole cells from strains classified in different subtypes were separated by electrophoresis, transferred onto nitrocellulose sheets, and incubated with polyclonal antibodies to the proper dilution. The conjugation and detection steps were performed as in the immunoblotting with Mabs.

Electron microscopy

To confirm the localization of the fusion protein in *E. coli*, electron microscopy was done as described (17). Briefly, the samples were fixed with 3.2 % glutaraldehyde and post-fixed 1 h in 1 % OsO₄, rinsed with 0.1 M PBS pH 7.2, and dehydrated on increasing ethanol concentrations. The inclusions were performed in Spurr and the blocks were sectioned with an ultramicrotome (LKB 2188). The ultrathin sections were placed on 400 mesh supports without membrane. The sections were examined in a transmission electronic microscope JEOL-JEM 2000 EX.

Protein purification

E. coli expressing the hybrid protein PM14 was grown in enriched M9 medium during 12 h at 37 °C. After cell collection, bacterial disruption was achieved using an ultrasonic disrupter (Braun, Germany). Partial purification was performed by a washing of the bacterial pellet procedure (11). The inclusion bodies formed were solubilized using 6 M guanidium hydrochloride in PBS pH 7.2. To remove the chaotropic salt, the protein solution was extensively dialyzed against PBS. The protein content was determined as described by Lowry OH (18) and the preparation was kept at -20 °C until required.

Immunization

Ten Balb/c mice were immunized subcutaneously with three doses of 50 µg of hybrid protein or P64k using aluminum hydroxide as an adjuvant, the same schedule with aluminum hydroxide alone was used as a negative control. The doses were given on days 0, 7 and 21. Blood samples were taken on days 0, 7, 14, and 28. The sera were separated, pooled and stored at -20 °C until required.

ELISA

Five µg of the recombinant hybrid protein PM14 or 10 µg of the meningococcal outer membrane proteins (9) from B385 strain were used to coat polystyrene microtiter plates. The sera were diluted 1:1000 in PBS-Tween 0.05 % and incubated in the coated plates. As a second antibody anti-mouse IgG, peroxidase-linked whole antibody was used. The reaction was developed by using a substrate solution containing hydrogen peroxide and *o*-phenylenediamine. The absorbance was measured in a Titertek Multiscan MC340 spectrophotometer at 492 nm.

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Bactericidal test

The bactericidal test against *N. meningitidis* B:4:P1:15 was made as described by Larrick JW (19) but with modifications. The bacteria were grown 12 h in Brain Heart Infusion Agar (BHI, Oxoid) at 37 °C in a 5 % CO₂ atmosphere. Then, a 25 mL culture was inoculated at a starting O.D. (620 nm) of 0.05 and allowed to grow for 6 h at 37 °C with gentle shaking. The bacteria for the assay were prepared by diluting this culture in PBS containing Ca²⁺ and Mg²⁺. A total reaction mixture of 125 µL that contained 25 µL of bacterial suspension (100 bacteria), 50 µL of PBS, 25 µL of heat-inactivated (30 min at 56 °C) target serum, and 25 µL of baby rabbit serum (source of complement), was incubated 30 min at 37 °C with gentle shaking. After this time, 125 µL of Mueller Hinton Agar, containing 5 % of calf serum, were added to each well and the plate was incubated 16 h at 37 °C as described before. Controls included heat-inactivated baby rabbit serum, target serum and the bacterium alone. The end point of the titration was the highest dilution of serum that killed 50 % or more of the bacteria inoculum.

Results and Discussion**Construction and expression of pM-14**

The 657 bp fragment of the *por A* gene coding for 219 amino acids of the Class 1 protein and including the immunodominant variable region (VR) from subtype P1.15, was obtained from clone pM-7 by PCR, using the primers PPVU (5'-end) and PKPN (3'-end); and cloned into the *Mlu* I site of the *lpd A* gene within the pM-6 construct, which was treated with Klenow enzyme (Figure 1).

The DNA sequence coding for the P1.15 protein used for the construction of pM-14 is shown in Figure 2. The fusion sites between both genes are shown and as a result, an asparagine was created.

```

      G   D   A   L   Q   L
5'- GGC GAC GCG CTG CAG TTG A - 3'
      lpdA   P1.15

      E   A   N   A   Y   E
5'- GAA GCC AAC GCG TAC GAA - 3'
      P1.15           lpdA
  
```

A fusion protein accounting for more than 10 % of the total cellular protein was expressed in *E. coli*, under the control of the tryptophan promoter, in two strains of this microorganism. Figure 3 shows the electrophoretic pattern of W3110 and MM294 strains expressing the hybrid protein PM14. In western blotting, this protein was recognized by Mabs against natural P1.15 and P64k proteins (Figure 4).

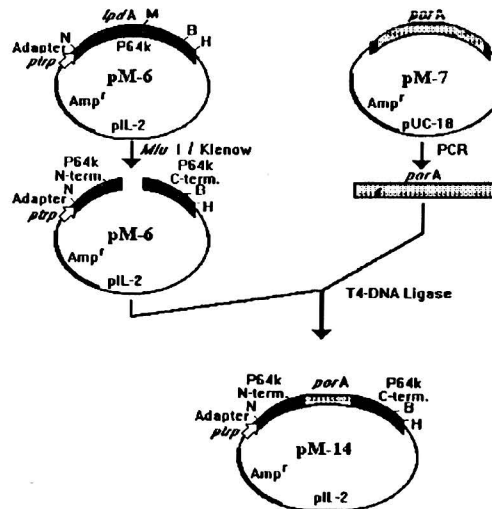


Figure 1. Cloning strategy for the expression in *E. coli* of the *por A* gene from the *N. meningitidis* strain B385 (B:4:P1.15) fused to the *lpd A* gene. The final construction (pM-14) contains the *por A* gene with the amino-terminal-end sequence of the *lpd A* gene and the *lpd A* terminator. The tryptophan promoter controls the expression of the fusion protein.

Purification of the PM14 and analysis of the immune response

The hybrid protein PM14 was located in the insoluble fraction after cell disruption. This localization was confirmed by electron microscopy Figure (5),

```

Leu Glu Leu T G P P S K S Q P Q V K V
ctg cag ttg acc gaa cgg ccc tca aag agt caa cct cag gta aaa gtt
T K A K S R I R T K I S D F G F
act aag gcc aaa agc cgc atc agg acg aaa atc agt gat ttc ggc tcg
I G F K G S E D L G E G L K A V
ttt atc gcc ttt aag ggg agt gag gat ttg ggc gaa ggg ctg aag gct
W Q L E Q D V S V A G G G A T Q
ggt tgg cag ctt gag caa gac gta tcc gtt gcc ggc ggc ggc ggc acc
W G N R E S F V G L A G E F G T
cag tgg ggc aac agg gaa tcc ttt gtc ggc ttg gca ggc gaa ttc ggt
L R A G R V A N Q F D D A S Q A
acg ctg cgc cgg tgg ctt ggc aat cag ttt gac gat gcc agc caa gcc
I D P W D S N N D V A S Q L G I
att gat cct tgg gac agc aac aat gat gtg get tcg caa ttg ggt att
F K R H D D M P V S V R Y D S P
ttc aaa cgc cac gac gat atg cgg gtt tcc gta cgc tac gac tct ccg
D F S G F S G S V Q F V P I Q N
gac ttt tcc ggt ttc agc ggc agt gtc caa ttc gtt ccg atc caa aac
S K S A Y T P A Y H Y T R Q N N
agc aag tcc gcc tat acg cgg gct tac cat tat act agg cag aac aat
A D V F V P A V V G K P G S D V
get gat gtt ttc gtt ccg get gtt gtc ggc aag ccc gga tcg gat gtg
Y V A G L N Y K N G G F A G S Y
tat tat gcc ggt ctg aat tac aaa aat ggc ggt ttt gcc ggc agc tat
A F K Y A R H A N V G R N A F E
gcc ttt aaa tat gcg aga cac gcc aat gtc gga cgt aat gct ttt gag
L F L L G S T S Asp Glu Ala
ttg ttc ttg ctc ggc agc acg agt gat gaa gcc aa
  
```

Figure 2. The primers used for PCR amplification of the *por A* gene coding for P1.15, as well as the VR within the protein are shown in bold face letters. The derived amino acid sequence is shown in the one letter code and the amino acids corresponding to the ends are shown in the three letter code.

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where the inclusion bodies (CI) were seen. This finding was not surprising, considering the high percentage of expression of the recombinant protein in the host. Similar results have been obtained by others that have expressed the P1.7,16 protein as a fusion with the *E. coli* OmpA protein (11).

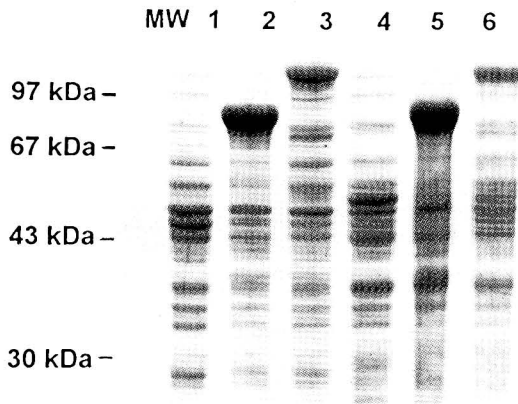


Figure 3. SDS-PAGE analysis of the expression of PM14 fusion protein in two *E. coli* strains. Lane 1: *E. coli* strain W3110, lane 2 and 3: *E. coli* strain W3110 expressing P64k and PM14 proteins, respectively, lane 4: *E. coli* strain MM294, lane 5 and 6: *E. coli* strain MM294 expressing P64k and PM14 protein, respectively. Low molecular weight markers: 97 kDa: phosphorylase B, 67 kDa: bovine serum albumin, 43 kDa: ovalbumin, 30 kDa: carbon anhydrase.

Using the advantage of the insolubility of the PM14 protein, it was easy to purify by the washing of the cellular pellet with saline buffer and detergent solutions (11). The partially purified protein was solubilized in 6 M guanidium hydrochloride and dialyzed against PBS. When this preparation was employed to immunize Balb/c mice in three

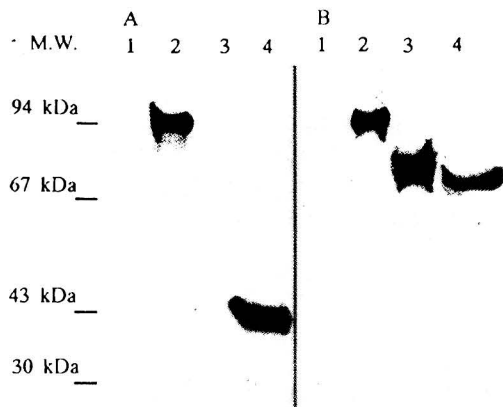


Figure 4. Western blot with Mab CB Nm-1 (A) and Mab 114 (B). Lane 1: *E. coli* strain W3110, lane 2 and 3: *E. coli* strain W3110 expressing pM-14 and P64k, respectively, lane 4: outer membrane proteins from *N. meningitidis* strain B385 as a positive control.

doses, adsorbed to aluminum hydroxide, a strong antibody response was produced. It was measured by ELISA against the P64k protein and outer membrane proteins of *N. meningitidis* strain B.4:P1.15 respectively. As shown in Figure 6, the hybrid protein elicited or produced an antibody response against P64k equivalent to that produced by outer membrane proteins in a control group previously immunized with the referred protein. The response against the outer membrane proteins was better in the case of sera prepared against PM14 (Figure 7), as was expected considering the presence of the highly immunogenic portion of the P1.15 in the fusion protein (20).

The murine polyclonal antibodies anti-PM14 were also assayed by immunoblotting. The results are shown in Figure 8. These antibodies recognized all meningococcal strains tested, which was expected because of the presence of the P64k protein in a high percentage of *N. meningitidis* strains.

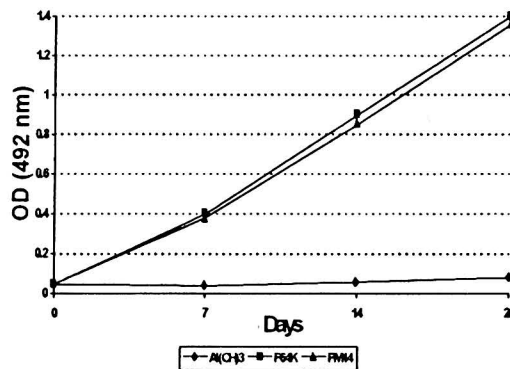


Figure 6. Immune response of mice immunized with the hybrid protein PM14 measured by ELISA with the recombinant P64k protein as coating antigen. Sera were diluted 1:1000.

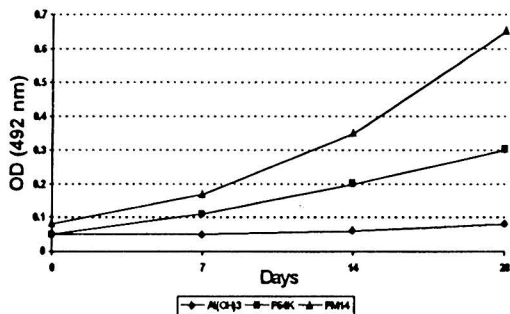


Figure 7. Immune response of mice immunized with the hybrid protein PM14 measured by ELISA with outer membrane proteins of *N. meningitidis* strain B385 as coating antigen. Sera were diluted 1:1000. Al(OH)₃ - Negative control, P64K - Positive control.

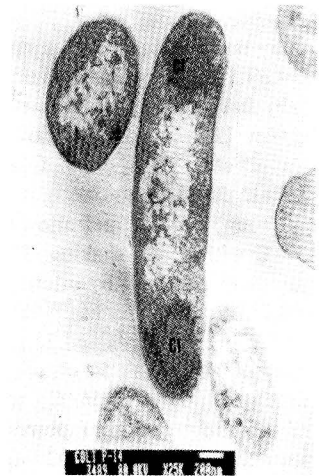


Figure 5. Electron microscopy of *E. coli* cells expressing the hybrid protein PM14. (CI = Inclusion bodies) Al(OH)₃ - Negative control, P64K - Positive control.

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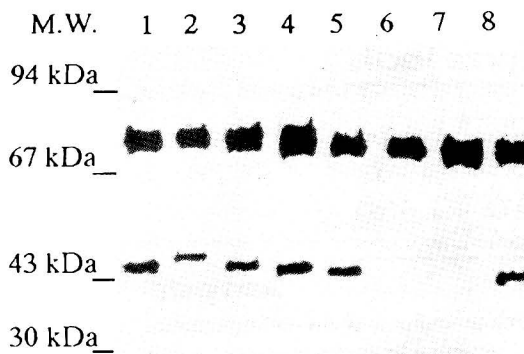


Figure 8. Western blot of several meningococcal strains with murine polyclonal antibodies elicited against the hybrid protein. Lane 1: strain B385 (B:4:P1.15), lane 2: strain B:4:P1.1, lane 3: strain B:4:P1.9, lane 4: strain B:4:P1.2, lane 5: strain B:4:P1.7, lane 6: strain B:4:P1.16, lane 7: strain NT:P1.6, lane 8: a different isolate of strain B:4:P1.15.

In addition, the anti-PM14 antibodies recognized the P1.15 protein in the homologous strain B:4:P1.15 (lanes 1 and 8), but also reacted with P1 from subtypes 1, 2, 7 and 9. This fact could be explained because crossreactive regions, present in several meningococcal subtypes, are mainly included in the constant loops of the protein (21). The Class 1 fragment fused to P64k in this construction lacks three of these conserved loops. Nevertheless, the VR3 (15) is highly conserved among subtypes 2, 7 and 15, and partially conserved in subtype 9, in contrast with subtypes 6 and 16, where the VR3 has no homology with subtype 15, present in the hybrid protein. This fact could explain the lack of recognition of the Class 1 protein from subtypes 6 and 16 on western blotting (Figure 8).

Table I shows the bactericidal titers detected for murine polyclonal antibodies anti-PM14. There was a correlation between the bactericidal activity and the progress of the immunization schedule. A mouse serum against the P64k protein was used as an internal control for the PM14 protein. Also, we used the Mab anti-P1.15 and a serum against Al(OH)₃ as the positive and negative controls, respectively.

In summary, we have expressed a 219 amino acids fragment of the P1.15 protein as a fusion with the meningococcal P64k in two *E. coli* strains. The hybrid protein showed a high percentage of expression in two *E. coli* strains, where it is presumably present as aggregates. It was easily purified, and it was immunogenic in mice; capable of eliciting bactericidal antibodies that reacted with antigens present in the natural source.

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Table 1. Functional activity of murine polyclonal antibodies against the hybrid protein (PM14), measured as a bactericidal effect against the *N. meningitidis* strain B385. Positive control: Murine Mab CB Nm-1.

Antiserum	Dose	Bactericidal titer
Al(OH) ₃	1	0
	2	0
	3	0
P64k	1	0
	2	1:100
	3	1:200
PM14	1	0
	2	1:50
	3	1:100
CB Nm-1	-	1:1600