Molecular and immunological characterization of OprL, the 18 kDa outer-membrane peptidoglycan-associated lipoprotein (PAL) of *Pseudomonas aeruginosa*

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Immunological screening of a *Pseudomonas aeruginosa* cosmid library led to the identification of clones producing an 18 kDa outer-membrane protein. This protein reacted in Western blots with a polyclonal antiserum against outer-membrane proteins of *P. aeruginosa* and with a monoclonal antibody (MA1-6) specific for OprL, the peptidoglycan-associated outer-membrane lipoprotein (PAL). Sequencing of pOML7, a subclone expressing *oprL*, revealed an ORF of 504 bp encoding a polypeptide with a typical lipoprotein signal recognition sequence. Another ORF was found upstream of *oprL*, with homology to the TolB protein of *Escherichia coli* and *Haemophilus influenzae*. Downstream of *oprL*, a second ORF, of 321 bp, was found (*orf2*), encoding a protein with a signal peptide and with no homology with proteins of known biological function. After the stop codon of *orf2*, a rho-independent terminator sequence was detected which is part of the *P. aeruginosa* PAO1 insertion element IS222. OprL showed homologies with all known PALs from Gram-negative bacteria, especially in the C-terminal part. mAb MA1-6 reacted with *P. aeruginosa* cells in immunofluorescence, and with *E. coli* cells expressing *oprL*, which had an abnormal, elongated morphology, an indication that production of the protein perturbed the division process.

Keywords: peptidoglycan-associated lipoprotein, *Pseudomonas aeruginosa*, OprL, TolB, IS222

INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous micro-organism and an important nosocomial bacterial pathogen, especially among immunocompromised and cystic fibrosis patients (Bodey *et al.*, 1985). Infections are difficult to treat, due to the bacterium's intrinsic resistance to several antibiotics (Hancock, 1986; Masuda *et al.*, 1995). The outer membrane of this bacterium contains a limited number of major proteins (Hancock *et al.*, 1990) which are recognized by antibodies present in the sera of patients previously exposed to the pathogen (Anwar *et al.*, 1985; Aronoff & Stern, 1988; Hancock *et al.*, 1984;

Lam *et al.*, 1983). Antibodies have indeed been detected against OprF, the 38 kDa porin, OprL (the peptidoglycan-associated lipoprotein: PAL, formerly protein H2), and the small lipoprotein OprI, in sera from cystic fibrosis patients (Hancock *et al.*, 1984). The gene for OprF, a major structural protein (Woodruff & Hancock, 1989), has been cloned and sequenced (Duchene *et al.*, 1988). The membrane topology and the main epitopes recognized by mAbs have been determined for OprF (Rawling *et al.*, 1995; Wong *et al.*, 1993, 1995). Mice immunized with recombinant OprF were partially protected when challenged with *P. aeruginosa* (Gilleland *et al.*, 1992, 1993), and peptides corresponding to linear B-cell epitopes of OprF elicited antibodies recognizing whole *P. aeruginosa* cells (Gilleland & Gilleland, 1995). The gene for OprI, the small outer-membrane lipoprotein, has also been cloned and sequenced (Cornelis *et al.*, 1989). The product of *oprI* is very immunogenic and

Abbreviations: FITC, fluorescein isothiocyanate; PAL, peptidoglycan-associated lipoprotein.

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can be used as a carrier for fused peptides to elicit antibodies without adjuvant (Cornelis *et al.*, 1996). The same protein seems to confer some degree of protection against *P. aeruginosa* experimental infections in a mouse model (Finke *et al.*, 1990, 1991). Mice fed with recombinant *Salmonella dublin* expressing the *P. aeruginosa* *oprI* gene developed both mucosal and humoral immunity (Toth *et al.*, 1994). Hybrid glutathione *S*-transferase proteins containing the C-terminal domain of OprF fused with OprI also protect mice against experimental *P. aeruginosa* infections (von Specht *et al.*, 1995). OprL is a PAL of about 20 kDa (Mizuno, 1979; Hancock *et al.*, 1981). It is the last major outer-membrane protein from *P. aeruginosa* whose gene has not yet been cloned, although recently the *oprL* gene for the 18 kDa PAL from *P. putida* has been cloned and sequenced, together with the characterization of an *oprL*-negative mutant (Rodriguez-Herva *et al.*, 1996; Rodriguez-Herva & Ramos, 1996). We describe here, for the first time, the cloning and molecular characterization of *oprL*, the gene coding for the PAL of *P. aeruginosa*, together with an immunological characterization of its product, OprL.

METHODS

Bacterial strains, plasmids and growth conditions. Bacteria were grown in Luria-Bertani (LB) broth or plates solidified with 1.5% (w/v) agar at 37 °C. *P. aeruginosa* PAO1 (ATCC 15692) was used throughout these experiments.

The following *Escherichia coli* strains were used: HB101 (*supE44 hsdS20* ($r_B^- m_B^-$) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*) (Boyer & Roulland-Dussoix, 1969), and DH5 α (*supE44 Δ lacU169* (ϕ 80 *lacZ* Δ M15 *recA hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) (Hanahan, 1983). As plasmid cloning vectors, cosmid pRG930 (Sm^r Sp^r) (van den Eede *et al.*, 1992) was used for the construction of the PAO1 genomic library, and plasmid pBluescript (pBS) for the subcloning experiments (Stratagene). Antibiotics were added in the following concentrations: ampicillin, 100 μ g ml⁻¹, streptomycin, 25 μ g ml⁻¹; spectinomycin, 50 μ g ml⁻¹.

Total *P. aeruginosa* extracts and outer membranes. Bacterial cell pellets, obtained after centrifugation from a 1.5 ml overnight culture, were lysed in 100 μ l GTE (50 mM glucose, 25 mM Tris/HCl, pH 8, 10 mM EDTA) mixed with the same volume of twofold concentrated Laemmli sample buffer (Laemmli, 1970) by boiling the tube for 10 min, followed by sonication at 35 A (Braun Labsonic) for 1 min. The tube was further centrifuged for 30 s in a mini-centrifuge at 14000 *g* and the supernatant kept at -20 °C until further use. Total outer membranes were obtained from bacterial pellet of 250 ml culture by the Sarkosyl differential membrane solubilization method (Filip *et al.*, 1973) as previously described (Cornelis *et al.*, 1989).

Polyclonal and monoclonal antibodies. A New Zealand White rabbit was immunized subcutaneously with 1 mg total outer membranes from *P. aeruginosa* PAO1, prepared as previously described (Cornelis *et al.*, 1989), and emulsified in complete Freund's adjuvant. A second immunization was done 4 weeks later in incomplete Freund's adjuvant. Blood samples were taken from the ear vein before the first immunization and 10 d after the second immunization. Immune serum was absorbed twice overnight at 4 °C with an equal volume of *E. coli* cell lysate (from a 50 ml culture, vol. of lysate: 1 ml). To generate

monoclonal antibodies, five female F1 (BALB/c $\times$ C57B1/6) mice between 6 and 10 weeks old were immunized intra-footpad with 5 μ g outer membranes (Holmdahl *et al.*, 1985). Nine days later, the popliteal lymph nodes were aseptically collected and lymphocytes were extracted and fused with the NSO B myeloma cell line using standard techniques. Hybridomas were tested for their reactivity with total outer membranes from *P. aeruginosa* in ELISA. Positive clones were further tested by Western blotting of outer-membrane proteins (Towbin *et al.*, 1979). Monoclonal antibody MA1-6, specific for OprL lipoprotein was a gift of Professor R. E. W. Hancock (Mutharia & Hancock, 1985).

Quantification of proteins and SDS-PAGE. Proteins were quantified using the Bradford (1976) method with a commercially available kit (Bio-Rad), using proteinase K to establish a standard curve. Around 15–20 μ g outer-membrane proteins or 10 μ l total cell lysate were resolved in 15% polyacrylamide running gels in a mini-gel apparatus (Bio-Rad) at 100 V. The following molecular mass markers (Pharmacia) were used: phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and α -lactalbumin (14 kDa).

Construction and screening of the genomic bank. Standard protocols for DNA manipulation were followed (Sambrook *et al.*, 1989). Genomic DNA obtained from *P. aeruginosa* PAO1 (20 μ g) was partially restricted with *Hind*III, using increasing dilutions of the restriction enzyme in 100 μ l volume. After 30 min, the reaction was stopped by addition of EDTA and 5 μ l aliquots from each tube analysed by agarose gel electrophoresis (7%, w/v). Tubes containing fragments averaging 30 kb were pooled and their DNA ligated to *Hind*III-restricted cosmid pRG930 (van den Eede *et al.*, 1992). Packaging of ligated DNA and transformation of *E. coli* HB101 was done using a commercially available kit, following the recommendations of the manufacturer (Promega). Colonies were selected on LB agar plates with streptomycin and spectinomycin and transferred with sterile toothpicks to individual wells of 96-well microtitre plates containing 100 μ l of the same medium. After incubation at 37 °C overnight, the clones were replicated using an alcohol-and-flame-sterilized replicator on agar plates. The plates were incubated overnight and an autoclave-sterilized nitrocellulose filter (Hybond C, Amersham) was overlaid on top and allowed to absorb for 1 h at room temperature. The filter was then laid on 10 ml 1% SDS (w/v) to lyse the cells and washed with running tap water to remove the debris. After blocking for 1 h with 3% (w/v) BSA in PBS (pH 7.4) on a shaker, the filter was incubated with the *E. coli*-absorbed anti-PAO1 outer-membrane proteins polyclonal rabbit serum (1:500 dilution) for 90 min on a shaker, washed with PBS/Tween 20 (0.2%, v/v) and further incubated for 1 h with a 1:1000 dilution of protein A-horseradish peroxidase conjugate (Amersham) in PBS. After washing, the blot was revealed with 4-chloro-1-naphthol (Janssen) as previously described (Cornelis *et al.*, 1989). Positive clones were further reacted with mAbs SH2.2 (anti OprL) and SD6.3 (anti OprF) to discard clones expressing *oprI* or *oprF*.

DNA manipulations and sequencing. DNA was manipulated according to Sambrook *et al.* (1989). DNA fragments were purified from the agarose electrophoresis gel using a commercial kit (Jet-Sorb, Genomed) according to the manufacturer's recommendations. Nucleotide sequencing was done on both strands from subclones in the plasmid pBluescript (pBS SK+, Stratagene) using the dideoxy chain-termination method (Sanger *et al.*, 1977) with a PCR-cycle sequencing kit and the universal forward and reverse primers (Sequitheerm, Biozyme). To eliminate false stops, the reaction was further incubated

with terminal transferase. Briefly, at the end of the PCR cycles, 1 µl of a mixture containing 4 mM of each dNTP, 8 mM CoCl₂, 1 × terminal transferase buffer (Sambrook *et al.*, 1989) and 0.75 units terminal transferase ml⁻¹ was added to each tube. After incubation at 37 °C for 1 h, the reaction was terminated by adding the stop solution from the sequencing kit. The fragments were separated using a Bio-Rad electrophoresis system. Three internal primers were also used to complete the sequence: L1, a 23-mer (5'-GGCCGTTCA-GCGCTACCTGGTGC-3') corresponding to the coding strand, and representing nucleotides 366 to 388 of the ORF of *oprL*, L2, a 22-mer (5'-AGAGCGGCTTCGTCGCTCAGGC-3') of the complementary strand, representing nucleotides 161 to 182 of the coding strand, and L3, a 17-mer (5'-AGGT-CGGAGCTGTCGTA-3'), also of the complementary strand, representing nucleotides 211 to 227 of the coding strand. DNA sequences were analysed using PC Gene (Intelligenetics) and GCG (Genetic Computing Group, programs FASTA and PILEUP) software.

Immunofluorescence assays of bacterial cells. Cell pellets were obtained from 1.5 ml overnight cultures. After washing with PBS, cells were incubated for 1 h with 0.1 % BSA (w/v), in PBS, to block unspecific binding sites. Then, 500 µl *E. coli*-absorbed anti-PAO1 serum (1:500 in PBS) or mAb MA1-6 (1:1000 in PBS) was allowed to react for 2 h, followed by three washing steps with PBS/BSA. Cells reacted with anti-PAO1 serum were further added with 50 µl sheep anti-rabbit Ig-FITC conjugate (1:1000 in PBS) and allowed to react for 1 h while cells treated with mAb MA1-6 were further incubated with FITC-labelled goat anti-mouse serum (1:1000 in PBS). After washing, each pellet was resuspended in 10 µl PBS/BSA and examined under normal and UV light using a Leitz Wetzlar microscope.

RESULTS

Construction and immunological screening of a *P. aeruginosa* cosmid bank

PAO1 DNA was partially cut with *Hind*III and ligated to the cosmid pRG930 (van den Eede *et al.*, 1992).

Twelve clones were selected at random and their plasmid DNA extracted for analysis of the size of their inserts after restriction with *Hind*III; the insert size was found to be between 28 and 35 kb. A total of 2200 clones were screened with a rabbit polyclonal antiserum raised against outer membranes from PAO1, after it had been absorbed against *E. coli* extracts. This antiserum recognized, in Western blots, four outer-membrane proteins, OprL (8 kDa), OprL (18 kDa), OprF (38 kDa) and OprD (45 kDa) (see Fig. 2a, lane 1). A total of 119 clones gave a positive reaction with the antiserum and 11 reacted with mAb SH2.2 against OprL. From the remaining clones, 16 were randomly selected and probed in Western blots with the anti-PAO1 antiserum; 13 clones showed the production of an immunoreactive outer-membrane protein of about 18 kDa (OprL) while the three other clones produced a 38 kDa immunoreactive outer-membrane, presumably OprF (results not shown). Restriction analysis of the 13 clones mentioned above with *Hind*III, *Pst*I and *Sal*I indicated that they contained inserts ranging in size between 25 and 35 kb. Common bands were detected, indicating that overlapping fragments had been cloned (results not shown).

Subcloning and localization of *oprL*

One clone containing a 30 kb *Hind*III insert was restricted with *Hind*III and re-ligated, resulting in a positive clone, pOML1, containing a 8.5 kb *Hind*III fragment (Fig. 1). This clone was shown by Western blotting to produce an 18 kDa outer-membrane protein in *E. coli* HB101 (Fig. 2a, lane 3 shows the reaction with the polyclonal antiserum; Fig. 2b, lane 3 shows the reaction with the mAb MA1-6). Further subcloning was done in pBS (Fig. 1) and each clone was analysed for its immunoreactivity in Western blot using both the polyclonal antiserum and the mAb MA1-6 (results not shown).

Fig. 1. Restriction map of clone pOML1 (8.5 kb *Hind*III fragment in pRG930) and subclones. All subcloning was done in pBS. The reaction in Western blots with the polyclonal antiserum against *P. aeruginosa* PAO1 proteins is also shown. The locations and direction of transcription of the different ORFs as deduced from the sequence, together with the position of IS222, are shown at the bottom of the figure.

Fig. 2. Western blot of outer-membrane proteins from *P. aeruginosa* PAO1 (lane 1), *E. coli* HB101(pRG930) (lane 2), *E. coli* HB101(pOML1) (lane 3), *E. coli* DH5 α (pBS) (lane 4), and *E. coli* DH5 α (pOML7) (lane 5). (a) Incubation with the polyclonal rabbit antiserum raised against outer membranes of *P. aeruginosa* PAO1; (b) incubation with mAb MA1-6 against OprL.

A 2.2 kb *KpnI*–*XhoI* fragment was sub-cloned in pBS, resulting in clone pOML7, which produced the immunoreactive 18 kDa protein in *E. coli* DH5 α (Fig. 2a, b, lane 5). Since the pOML7 fragment probably contained the complete gene, including the promoter sequences, it was sequenced, using subclones and internal primers as described in Methods.

Sequence analysis

The sequence of a 1200 bp fragment is shown in Fig. 3. The *oprL* coding region was found to be flanked by two other reading frames, one upstream, incomplete, and one downstream of *oprL*, complete (*orf2*). The ORF upstream of *oprL* was found to encode the 52 C-terminal amino acids of a putative protein which showed a high degree of homology with the TolB protein of *E. coli* (Sun & Webster, 1987) and the TolB protein of *H. influenzae* (Sen *et al.*, 1996) (Fig. 4). Three possible ATG start codons were found for OprL, at positions 209, 212 and 218, however only the second ATG was preceded by a potential ribosome-binding site, eight nucleotides upstream (AGGAG). The first 21 residues of OprL form a potential signal sequence, typical of lipoproteins, with the sequence AVG↓CSS and a cleavage site between the G and the C, leaving the cysteine as the first amino acid of the mature lipoprotein (Hayashi & Wu, 1990). The

Fig. 3. Sequence of 1200 bp of DNA and the different translation products (TolB, OprL and ORF2). The nucleotides in italics represent the sequence corresponding to the PAO1 insertion element IS222. Underlined sequences represent the restriction sites for *SalI*, *PstI* and *XhoI*, while the double-underlined sequences represent potential ribosome-binding sites before *oprL* and *orf2*. Amino acids in italics represent potential secretory signal peptides in OprL and ORF2 proteins.

504 bp coding part of *oprL* ended with a TAA stop codon, immediately followed by a potential Shine–Dalgarno sequence (AGAAG), preceding an ATG start codon for another frame (*orf2*) downstream. This 321 bp frame encodes a polypeptide of 107 amino acids with a calculated molecular mass of 11180 Da. A potential secretory signal peptide was found with a possible cleavage site LAA↓RAE at position 19. This coding sequence ends with a TGA stop codon and is followed, 54 bases downstream, by the insertion element IS222 from *P. aeruginosa* PAO1 (Gertman *et al.*, 1986; Kropinski *et al.*, 1994). Interestingly, the insertion element is inserted from position 1234, according to the published sequence (Kropinski *et al.*, 1994), bringing a potential rho-independent terminator sequence in close

<i>P. a.</i> TolB	SPSVAPNGTMLIRHPPAGPG.VLMLVSTD.AYGYLSPTAQGDVREPSWSPYLN
<i>H. i.</i> TolB	SPSLSPNGIMIIYSSTQGLGKVLQLV.SADGRFKASLPGSDGQVKFPAWSPYLTk
<i>E. c.</i> TolB	TPSLAPNGTMVIYSSSQGMGSVLNLV.STDG.FKARLPATDGQVKFPAWSPYL

Fig. 4. Homology between the translation products of the incomplete ORF upstream of *oprLpal* from *P. aeruginosa* (*P. a.*), and the C-terminal parts of *E. coli* (*E. c.*) (Chen & Henning, 1987) and *Haemophilus influenzae* (*H. i.*) (Sen *et al.*, 1996) TolB proteins.

proximity, after the stop codon of *orf2*. Another hairpin was predicted to be formed, between bases 876 and 911, within the *orf2* coding sequence, with a ΔG of $-33 \text{ kcal mol}^{-1}$ (-138 kJ mol^{-1}); however the significance of this hairpin is not known. The putative product of *orf2* did not show any homology with proteins having a known function but did show some degree of homology with the product of an ORF present downstream of the *oprL* gene of *P. putida* (Rodriguez-Herva *et al.*, 1996) and with the product of *orfD*, downstream of the *Legionella pneumophila pal* gene (Engleberg *et al.*, 1991) (results not shown).

Comparison of *P. aeruginosa* OprL with other Gram-negative PALs

A comparison was made between the *P. aeruginosa* PAL and the known sequences of PALs from *P. putida* (Rodriguez-Herva *et al.*, 1996), *Haemophilus influenzae* (Deich *et al.*, 1988; Nelson *et al.*, 1988), *E. coli* (Chen & Henning, 1987), *Pasteurella multocida* (Kasten *et al.*, 1995), *Brucella abortus* (Tibor *et al.*, 1994), and *Legionella pneumophila* (Engleberg *et al.*, 1991). As could be expected, the highest homology was found with the *P. putida* PAL (96% identity) and was less pronounced for the other PALs (ranging from 57% identity for *H. influenzae* to 64% identity for *L. pneumophila*). As already observed and documented by others, the homology is highest in the C-terminal part of the PALs. Recently, it was proposed that the interaction of proteins with the peptidoglycan layer is dependent on the presence of an α -helical motif which is conserved at the C-terminal region of these proteins; this motif follows the proposed consensus sequence NxxLSxxRAXxVxxxL (Koebnik, 1995). Analysis of the amino acid sequence of OprL showed that indeed amino acids 113 to 128 of the mature lipoprotein contained the sequence NmaLgerRAkaVqryL.

Immunological characterization of OprL

Immunofluorescence experiments were performed using the mAb MA1-6 against OprL of *P. aeruginosa*. Intact, non-fixed, *P. aeruginosa* cells were intensely stained when mAb MA1-6 was used (not shown). mAb MA1-6 also gave a positive reaction with *E. coli* cells expressing *oprL* (Fig. 5d). In both cases the reaction resulted in the staining of elongated cells which were also clearly apparent in visible light (Fig. 5c). No fluorescence was observed when *E. coli* cells containing pBS alone were tested (Fig. 5b), furthermore these cells had a normal

Fig. 5. (a, d) Indirect immunofluorescence of live *E. coli* HB101(pRG930) (b), and *E. coli* HB101(pOML1) (d) cells with mAb MA1-6 against OprL. Panels (a) and (c), *E. coli* HB101(pRG930), and *E. coli* HB101(pOML1) cells, respectively, photographed under visible light.

appearance when observed in visible light (Fig. 5a), confirming that the elongated cell phenotype was not a result of the treatment.

DISCUSSION

The gene coding for the PAL from *P. aeruginosa* has been cloned using an immunological screening approach. The production of an 18 kDa outer-membrane protein in *E. coli* was confirmed by Western blotting with both a polyspecific polyclonal antiserum and a previously characterized monoclonal antibody against OprL (Mutharia & Hancock, 1985). Expression of *pal* genes from other Gram-negative bacteria in *E. coli* has been confirmed in several reports, including *Legionella pneumophila* (Engleberg *et al.*, 1991), *Brucella abortus* (Tibor *et al.*, 1994) and *Haemophilus influenzae* (Deich *et al.*,

1988). The analysis of the *P. aeruginosa oprL* sequence confirmed that it encoded a protein of 168 residues with an N-terminal signal peptide typical of bacterial lipoproteins (Hayashi & Wu, 1990; Pugsley, 1993). The sequence, G↓CSS, was found also in OprI from *P. aeruginosa* (Cornelis *et al.*, 1989). The *oprL* coding sequence was preceded by an incomplete ORF, corresponding to the C-terminal part of a protein showing strong homology with the C-terminal domains of the TolB proteins from *E. coli* (Levengood & Webster, 1989) and *H. influenzae* (Sen *et al.*, 1996). Interestingly, *tolB* is localized upstream of the *pal* gene in *E. coli* and upstream of the gene for the P6 Pal protein in *H. influenzae* (Lazzaroni & Portalier, 1992; Sen *et al.*, 1996). In *E. coli*, among the Tol proteins TolB in particular is in association with Pal at contact sites between the inner and the outer membranes, forming, with the other Tol proteins, a complex facilitating the uptake of colicins (Bouveret *et al.*, 1995; Lazdunski, 1995). Other roles have not yet been assigned for these proteins except that *tol* and *pal* mutants are detergent-sensitive and release periplasmic proteins into the extracellular medium (Fognini-Lefebvre *et al.*, 1987). A Tn5 mutant of *P. putida* with the transposon inserted in the *oprL* gene, and a gene-replacement mutant of *oprL*, were both found to be very sensitive to osmotic pressure and to SDS, deoxycholate and EDTA, suggesting that OprL is needed for the maintenance of cell envelope integrity (Rodriguez-Herva *et al.*, 1996; Rodriguez-Herva & Ramos, 1996).

Downstream from *oprL* is an ORF which encodes a protein with a molecular mass of 11180 Da. Similar ORFs are found downstream from other *pal* genes but the biological role of the proteins they encode is, so far, not known (Vianney *et al.*, 1996). The presence, downstream of this ORF, of the insertion element IS222 from *P. aeruginosa* PAO1 (Kropinski *et al.*, 1994) is of interest. This insertion element brings with it a typical rho-independent terminator sequence, with a hairpin, followed by a stretch of T residues. It would be interesting to see whether this organization is conserved in other *P. aeruginosa* strains, since IS222 is mentioned as being specific for PAO1 (Kropinski *et al.*, 1994).

Not surprisingly, OprL showed a very high identity with the PAL from *P. putida* (Rodriguez-Herva *et al.*, 1996), and a high degree of homology at the C-terminal end with other PALs. It also had some homology with the C-terminal region of other outer-membrane proteins known to interact with peptidoglycan, such as OmpA from *E. coli* and OprF from fluorescent pseudomonads (De Mot *et al.*, 1994; Koebnik, 1995). In all these proteins a motif supposed to interact non-covalently with peptidoglycan is conserved.

The mAb against OprL reacted with intact *P. aeruginosa* cells without prior treatment with EDTA, indicating that part of OprL is surface exposed. Such a surface exposure of PALs (or part of the protein) has been demonstrated in the case of *H. influenzae* (Green *et al.*, 1987), *Brucella* spp. (Clockaert *et al.*, 1990), and *L. pneumophila* (Engleberg *et al.*, 1984). These results are

also in agreement with those of Mutharia & Hancock (1985), who demonstrated the accessibility of OprL at the surface of *P. aeruginosa* cells by a colony blot technique. The same authors also stressed that treatment of cells with TBS containing 0.1 M NaCl was necessary to achieve recognition of OprL by mAb MA1-6. We used PBS with a similar ionic strength as the TBS used in their study.

In conclusion, we think that OprL, the last major constitutive outer-membrane protein from *P. aeruginosa* to be molecularly characterized, could be, along with OprI and OprF, an interesting target molecule for the development of a vaccine against *P. aeruginosa*.

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