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# Insertion Mutagenesis of the *Pseudomonas aeruginosa* Phosphate-Specific Porin OprP

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**The gene encoding the *Pseudomonas aeruginosa* phosphate-specific porin OprP was subjected to both linker and epitope insertion mutageneses. Nine of the 13 linker mutant genes expressed protein at levels comparable to those obtained with the wild-type gene. These mutant proteins were shown, by indirect immunofluorescence with an OprP-specific antiserum, to be properly exposed at the cell surface. Four of the linker mutant genes expressed protein at reduced levels which were not detectable at the cell surface. A foreign epitope from the circumsporozoite form of the malarial parasite *Plasmodium falciparum* was cloned into the linker sites of 12 of the 13 mutant genes. Seven of the resultant epitope insertion mutant genes expressed surface-exposed protein. Two of these mutant genes presented the foreign epitope at surface-accessible regions as assessed by indirect immunofluorescence with a malarial epitope-specific monoclonal antibody. The data from these experiments were used to create a topological model of the OprP monomer.**

The transport of hydrophilic molecules across the outer membranes of gram-negative bacteria is facilitated by a class of proteins known as porins. These proteins form water-filled, membrane-spanning channels which permit the passage of molecules through the membrane and into the periplasmic space (13). Porins have been divided into two categories, nonspecific or general diffusion porins and specific porins (29, 30). General diffusion porins allow the passage of any hydrophilic molecule through their channels provided it is smaller than the channels' exclusion limit. Specific porins possess a substrate-binding site which makes it possible, at rate-limiting concentrations, for them to transport molecules that bind to that site at a much higher rate than other molecules of comparable size. The structures of a number of general diffusion porins have been determined by both crystallographic (12, 40) and mutagenic (5, 10) techniques. They are composed of three identical subunits, each of which is a 16-stranded β-barrel. The β-strands are tilted at an angle with respect to the plane of the membrane and are connected by loops exposed to either the outer surface or the periplasmic space. The periplasmically exposed loops tend to be short and regular, while the surface-exposed loops are longer and more variable. The third surface loop actually folds back into the channel so as to constrict its internal diameter (12).

While there exists a great deal of data regarding the structure of general porins, there is considerably less information pertaining to the structure of specific porins. The list of specific porins is rather short in comparison with that of general diffusion porins. They include LamB (26) and Tsx (20) in *Escherichia coli* and OprB (15), OprD (39), OprO (16), and OprP (17) in *Pseudomonas aeruginosa*. The maltoporin LamB is a well-studied example of a specific porin. This protein, like the general diffusion porins, is composed of three identical subunits, each of which has been proposed to be in the form of a β-barrel (10, 21, 32). However, since it is a specific porin, LamB possesses a substrate binding site located within the channel. This allows LamB to transport maltodextrins at much higher rates than other compounds of comparable size (26). More recently, a 16-stranded β-barrel model has been proposed for the *P. aeruginosa* imipenem-basic amino acid-specific porin OprD (23).

The *P. aeruginosa* phosphate-inducible porin OprP is a specific porin. Reconstitution of purified OprP trimers into lipid bilayer membranes has demonstrated that at low concentrations, these channels transport phosphate ions at least 100-fold more efficiently than chloride ions (14). Detailed model membrane studies have defined the mechanism of phosphate passage through the channel. Chemical modification experiments have shown that this specificity is due, in part, to the presence of lysine residues within the channel that create a phosphate-binding site (14, 18). Circular dichroism studies revealed that the secondary structure of OprP is composed of over 60% β-sheet structure with the remainder existing as random coil or α-helix (44). The *oprP* gene was cloned and sequenced and was found to have little homology with any of the other previously characterized porins (24, 34). Hence, it has not proven possible to create a model of OprP based on sequence alignment or comparisons with the crystal structures of other porins.

The purpose of this study was to create a testable model of the structure of the OprP monomer based on linker and epitope insertion mutagenesis experiments to provide the basis for understanding structure-function relationships in this porin.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** *E. coli* DH5α was used for all procedures involved in creating *oprP* mutant plasmids. Strain CE1248 was utilized in all expression experiments. This strain was deficient in the expression of OmpC, OmpF, and PhoE, allowing overexpression of cloned outer membrane proteins.

Plasmid pRSP-3 (35) was used as the source of the *oprP* gene, which was subcloned into phagemid vector pTZ19U (Pharmacia). Plasmid pUC4KAPA (obtained from J. Smit, Department of Microbiology and Immunology, University of British Columbia) was used as the source of the *Hin*cII fragment which contained the kanamycin resistance cassette.

**Media, enzymes, and reagents.** Cells were grown at 37°C with shaking in L.B. broth (Accumedia) supplemented with 50 μg of ampicillin per ml. When strain CE1248 was used, 0.4% glucose was included to inhibit expression of LamB. In addition, 5 μM isopropyl-β-D-thiogalactopyranoside (IPTG) was included in cultures used in indirect immunofluorescence experiments. Restriction endo-

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nucleases and T4 DNA ligase purchased from Gibco/BRL and Boehringer Mannheim were used in accordance with the accompanying literature.

**Antibodies.** Monomer-specific anti-OprP rabbit serum was made during the course of a previous study (31). Trimer-specific antiserum was made as previously described (31), with fast protein liquid chromatography-purified OprP. An anti-malarial epitope monoclonal antibody was obtained from R. Wirtz (Department of Entomology, Walter Reed Army Institute of Research, Washington, D.C.).

**Development of plasmid pAS27.** The *oprP* gene was subcloned into pTZ19U in two separate steps. First, a vector lacking *Eco*RI and *Pst*I recognition sites was created by ligating a 900-bp *Sca*I-*Hin*dIII fragment from pUC18 to a 1.7-kb *Sca*I-*Hin*dIII fragment from pUC19 to form vector pUC18/19. A 700-bp *Hin*dIII-*Hin*dIII fragment from pRSP-3 encoding the first 95 amino acids of OprP was gel purified and cloned into the *Hin*dIII site of pUC18/19. This plasmid was then digested with *Eco*RI and *Pst*I, releasing a 27-bp fragment. The vector was then ligated to a 27-bp synthetic oligonucleotide which replaced the excised fragment but contained a single-base-pair change which destroyed the *Pst*I recognition site while maintaining the encoded amino acid sequence. After verification of the proper orientation of the oligonucleotide by DNA sequencing, the *Hin*dIII-*Hin*dIII fragment was excised and gel purified.

A 1.2-kb *Pst*I-*Pst*I fragment from pRSP-3 encoding amino acids 35 to 406 of OprP was cloned into the *Pst*I site of pTZ19U. This construct was then digested with *Hin*dIII and ligated to the gel-purified 700-bp *Hin*dIII-*Hin*dIII fragment. Finally, the plasmid was digested with *Pst*I and ligated to an 18-bp synthetic oligonucleotide which encoded the last four amino acid residues of OprP and contained a single-base-pair change which destroyed the *Pst*I recognition site. Proper incorporation of the oligonucleotide was verified by DNA sequencing.

**Linker insertion mutagenesis.** Insertion of 12-bp linkers into the *oprP* gene was accomplished as described previously (41). Briefly, plasmid pAS27 was partially digested with frequently cutting blunt-end restriction endonucleases *Alu*I, *Rsa*I, and *Tha*I in the presence of ethidium bromide (10 to 50 ng/μl). After digestion, the singly cut plasmid was purified and ligated in equimolar ratios to a 1.3-kb *Hin*cII fragment which was isolated from kanamycin resistance plasmid pUC4KAPA. The ligation products were transformed into *E. coli* DH5α, and clones were selected for both ampicillin resistance and kanamycin resistance. The resultant colonies were picked onto grids and screened for OprP expression by immunoblotting (43) with anti-OprP (trimer-specific) polyclonal rabbit serum. Plasmids were isolated from OprP-negative clones and digested with *Pst*I, releasing the kanamycin resistance gene and leaving a 12-bp residual linker sequence. The religated linker mutant plasmids were transformed back into DH5α.

**Epitope insertion.** The linker mutant plasmids were linearized by digestion with *Pst*I and ligated to 47-bp synthetic oligonucleotides encoding the malarial epitope at a molar ratio of 1:100. The oligonucleotides, which were synthesized on an Applied Biosystems 392 DNA synthesizer, were engineered so as to introduce a unique restriction enzyme recognition site (*Sph*I or *Xba*I as underlined below) when inserted into the plasmid. Separate oligonucleotides were utilized for each reading frame to ensure correct translation. This resulted in inserted sequence CCG AAC GCC AAC CCG AAC GCC AAC CCG AAC GCC GGG CAT GCA, AC CCG AAC GCC AAC CCG AAC GCC AAC CCG AAC GCA TGC A, or G AAC GCC AAC CCA AAC GCG AAT CCG AAT GCT CTA GAC TTG CA.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting.** Overnight cultures were pelleted, resuspended in 20% sucrose, and broken by passage at 14,000 lb/in$^2$ through a French press. Outer membranes were isolated with sucrose density gradients as described previously (46). Isolated outer membranes were heated at 100°C for 10 min, and 10 μg was loaded per well. Electrophoresis was carried out by SDS-PAGE on discontinuous 12% polyacrylamide gels (27). Proteins were stained with Coomassie brilliant blue.

For Western immunoblotting, unstained gels were transferred to nitrocellulose membranes (Schleicher & Schuell) as previously described (38). After blocking with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) (pH 7.4), the nitrocellulose membranes were incubated with either a 1/1,000 dilution of anti-OprP rabbit serum or a 1/5,000 dilution of an anti-malarial epitope monoclonal antibody in 1% BSA-PBS for 2 h at room temperature on a shaker. After being washed with PBS, the membranes were incubated with a 1/2,000 dilution of an alkaline phosphatase-conjugated secondary antibody (Bio-Rad). The bound antibodies were detected with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium.

**Indirect immunofluorescence.** Detection of surface-exposed proteins and epitopes was accomplished by the method of Hofstra et al. (22). Aliquots of cells (100 or 200 μl) grown for 14 to 17 h were pelleted, washed, and incubated with a 1/100 dilution of the primary antibody for 1.5 h, washed twice with PBS, and incubated with a fluorescein isothiocyanate-conjugated secondary antibody (Gibco/BRL). Cells were then washed, resuspended in 1% BSA-PBS, and dried on poly-L-lysine-coated slides (Sigma). Fluorescence was monitored with a Zeiss microscope fitted with a halogen lamp and filters set for emission at 525 nm.

**DNA sequencing.** Plasmid DNA was sequenced with the Applied Biosystems automated fluorescent sequencing system with a model 373 sequencer and PCR protocols provided by Applied Biosystems. Template DNA was prepared with Quiawell-8 plasmid kits (Quiagen). Primers were synthesized on an Applied Biosystems DNA synthesizer.

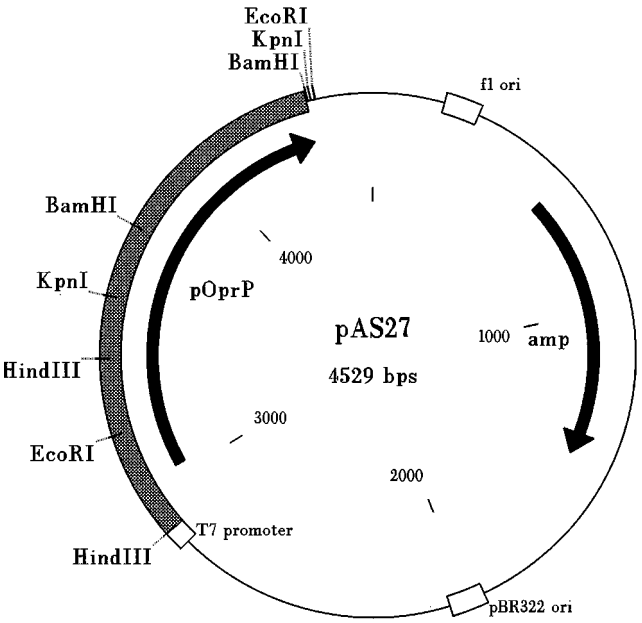


FIG. 1. Restriction digest map of plasmid pAS27. The fragment containing the *oprP* gene (shaded) was cloned into the multiple cloning site of phagemid pTZ19U downstream of the T7 promoter. ori, origin of replication.

**Planar lipid bilayer experiments.** Analysis of the pore-forming ability of mutant proteins was accomplished with the planar lipid bilayer technique as previously described (6, 7). Membranes were composed of oxidized cholesterol. At least 100 channels were observed for each mutant protein examined.

## RESULTS

**Construction of plasmid pAS27.** The *oprP* gene had been previously cloned into broad-host-range plasmid pMMB66HE (35). The strategy employed for linker insertion mutagenesis required the removal of two *Pst*I recognition sites in the *oprP* gene. This was accomplished by subcloning the gene into pTZ19U as described in Materials and Methods. The resulting plasmid, pAS27 (Fig. 1), was transformed into *E. coli* DH5α. Western immunoblotting of outer membranes with anti-OprP polyclonal serum demonstrated that the protein was efficiently expressed.

**Linker insertion mutagenesis.** Twelve unique linker insertion *oprP* mutants were generated by ligating a kanamycin resistance cassette to plasmid pAS27 partially digested with frequently cutting restriction enzymes *Alu*I, *Rsa*I, and *Tha*I. One mutant was created by ligating the cassette to the *Eco*RV-linearized plasmid. After transformation and selection for kanamycin resistance, colonies were picked and screened for loss of OprP expression by immunoblotting with anti-OprP antibodies (data not shown). Clones which did not express OprP would possess plasmids with the cassette inserted within the gene. The plasmids from these clones were isolated and digested with *Pst*I, releasing the kanamycin resistance cassette. The religated plasmids contained a 12-bp linker (including a unique *Pst*I recognition sequence) at the original insertion site of the cassette. The mutants were grouped according to restriction digestion patterns, and representative plasmids were sequenced by using the dideoxy termination method. The mutant plasmids were named pPL1 to pPL13 for OprP linker insertion (Table 1) and the corresponding sites at which these insertions occurred, PL1 to PL13.

**Expression of linker mutant proteins.** The effect that the

TABLE 1. Expression and characterization of OprP linker insertion mutant proteins

| Plasmid | Insertion site (amino acid) | Protein expression (by SDS-PAGE)[a] | Surface reactivity[a,b] | Avg conductance (nS)[c] ± SD | Amino acids inserted |
|---|---|---|---|---|---|
| pAS27 | | ++ | ++ | 0.23 ± 0.04 | |
| pPL1 | 9 | ++ | ++ | 0.23 ± 0.04 | DLQV |
| pPL2 | 60 | ++ | ++ | 0.22 ± 0.04 | TCRS |
| pPL3 | 75 | + | − | ND[d] | PAGP |
| pPL4 | 82 | ++ | ++ | 0.25 ± 0.04 | DLQV |
| pPL5 | 124 | ++ | ++ | 0.24 ± 0.04 | TCRS |
| pPL6 | 148 | ++ | ++ | ND | DLQV |
| pPL7 | 154 | ++ | ++ | ND | TCRS |
| pPL8 | 190 | + | − | ND | PAGP |
| pPL9 | 208 | ++ | ++ | 0.23 ± 0.04 | TCRS |
| pPL10 | 226 | ++ | ++ | 0.23 ± 0.04 | TCRS |
| pPL11 | 287 | ++ | ++ | 0.24 ± 0.05 | TCRS |
| pPL12 | 309 | + | − | ND | DLQV |
| pPL13 | 333 | + | − | ND | DLQV |

[a] ++, level comparable to that of the wild type; +, reduced level compared with the wild type; −, undetectable level.
[b] As assessed by indirect immunofluorescence with anti-OprP (trimer-specific) serum.
[c] Average single-channel conductance of 100 individual events.
[d] ND, not done.

linker insertion mutations had on the expression of OprP was determined by SDS-PAGE and Western immunoblotting of outer membranes prepared from clones containing the mutant plasmids. The 13 linker mutant plasmids, along with pAS27 and pTZ19U, were transformed into the *E. coli* CE1248.

All 13 linker insertion mutant plasmids were found to be capable of directing the expression of OprP as determined by Western immunoblotting with anti-OprP antibodies (Fig. 2). However, four of the mutant plasmids (pPL3, pPL8, pPL12, and pPL13) led to expression of the protein at greatly reduced levels in comparison with the parent plasmid. All of the mutant proteins exhibited electrophoretic mobilities similar to that of wild-type OprP.

Insertion of the linker into the PL2 site appeared to induce the production of a degradation product with an apparent molecular mass of approximately 30 kDa and two smaller products, while the insert in PL13 appeared to induce the production of a small amount of a degradation product with a molecular mass slightly less than that of the native protein.

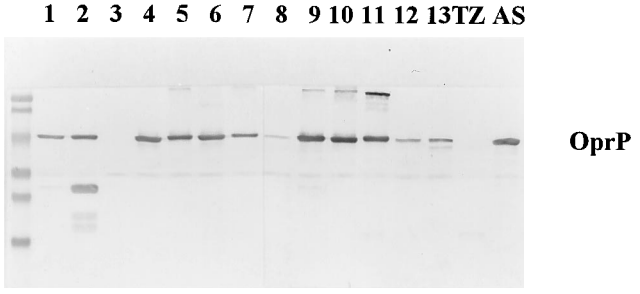


FIG. 2. Western immunoblot of outer membranes of *E. coli* CE1248 containing OprP linker insertion plasmids pPL1 to pPL13 (lanes 1 to 13, respectively) and positive and negative control plasmids pAS27 (lane AS) and pTZ19U (lane TZ). The blot was reacted with anti-OprP (monomer-specific) antiserum. The prestained molecular mass standards shown on the left were (from the top) 106, 80, 49.5, 32.5, 27.5, and 18.5 kDa. Although not observed here, a band corresponding to OprP appeared for CE1248/pPL3 when higher concentrations of protein were used.

A number of the mutant proteins appeared to possess increased trimer stability. Lanes 5, 7, 9, 10, and 11 of Fig. 2 all contained a lower-mobility band which migrated at the position of nondenatured OprP. Normally, nondenatured trimers do not react with monomer-specific antisera (31). The fact that these mutant trimers were able to bind the antibody indicated that the heat treatment affected the secondary structure of the protein without causing dissociation of the individual monomers.

**Surface exposure of linker mutant proteins.** To determine if the mutant forms of OprP were efficiently folded and incorporated in the outer membrane of host cells, clones were analyzed by indirect immunofluorescence for surface exposure of the mutant proteins. Cells containing the pPL plasmids, pAS27, or pTZ19U were incubated with anti-OprP (trimer-specific) rabbit serum followed by a goat anti-rabbit fluorescein isothiocyanate-conjugated antibody and viewed under a fluorescence microscope. Nine of the 13 mutant clones fluoresced at levels comparable to those exhibited by clones expressing the wild-type protein (Fig. 3A and Table 1). The other four clones did not fluoresce perceptibly more than the negative control (Fig. 3B). These four mutant clones were those previously shown to produce reduced levels of the protein in the outer membrane. The absence of detectable surface exposure displayed by these mutant clones could be due to either an inability of the proteins to be properly incorporated into the outer membrane or an inability to discriminate between such low levels of antibody binding and background levels.

**Pore-forming activity of linker mutant proteins.** To further determine the effect of insertion mutagenesis on the folding of the mutant proteins, a number of the linker mutant proteins which were expressed at wild-type levels were purified and subjected to analysis by the planar lipid bilayer method. All of the mutant proteins tested formed channels with sizes that varied by less than 10% from those formed by wild-type OprP (Table 1), suggesting that the linkers did not drastically affect folding in these mutant proteins or alter the architecture of the anion-binding site within the channel. Outer membranes from cells containing pTZ19U had no detectable channel activity (data not shown).

**Epitope insertion mutagenesis.** Twelve of the 13 linker mutant proteins were subjected to further mutagenesis by inserting a foreign epitope into the linker site. This was done to determine the acceptance of the insertion sites of a larger stretch of amino acids. In addition, the epitope provided a tag for determination of the surface exposure of the sites. The malarial epitope from the circumsporozoite form of *Plasmodium falciparum* was chosen for this purpose. Previous studies done in this laboratory have shown that this epitope is both tolerated and antigenic when inserted into outer membrane proteins (42). The mutant plasmids were linearized by digestion with *Pst*I and ligated to a 47-bp oligonucleotide encoding the repeating malarial epitope $(PNAN)_3$. Clones were screened both by restriction enzyme digestion analysis and by colony immunoblotting with an antimalarial epitope-specific monoclonal antibody. Correct insertion was verified by DNA sequencing.

Outer membranes from clones containing the epitope insertion mutant plasmids were isolated and analyzed by Western immunoblotting with both anti-OprP and antimalarial epitope antibodies. All of the mutant clones produced OprP, but at reduced levels in comparison with the parent linker mutant clones (Fig. 4A). The three mutants whose linker mutant parents displayed greatly reduced levels of expression (inserts at sites PL8, PL12, and PL13) also exhibited low expression. In addition, two other mutant clones, whose expression was un-

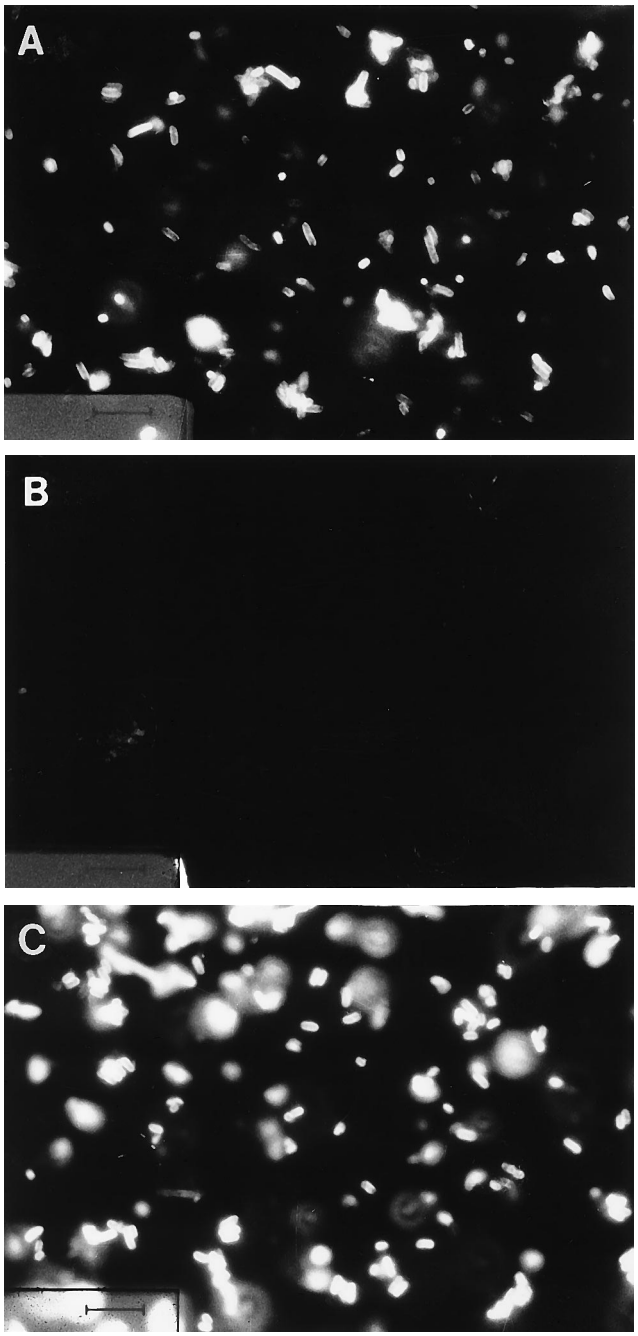


FIG. 3. Indirect immunofluorescence of *E. coli* CE1248 containing plasmid pAS27 reacted with anti-OprP (trimer-specific) serum (A), plasmid pTZ19U reacted with anti-OprP sera (B), and the OprP mutant plasmid with the malarial epitope inserted at site PL11 reacted with anti-malaria epitope monoclonal antibody p.f.2A.10 (C). Bar, 10 μm.

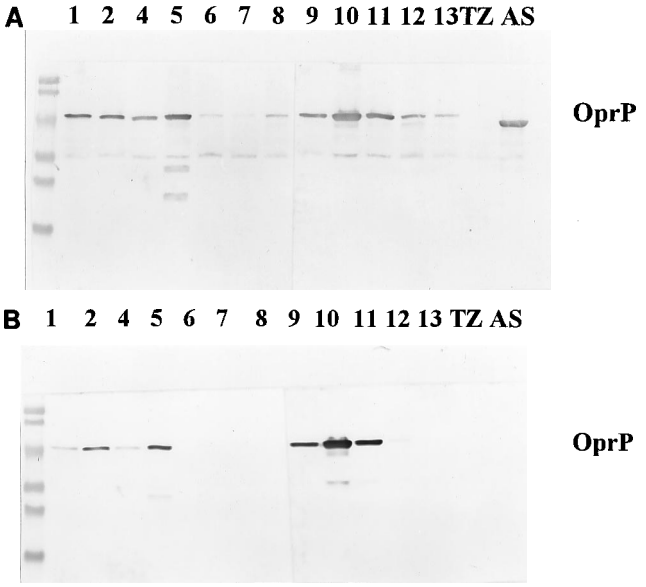


FIG. 4. Western immunoblots of the outer membranes of *E. coli* CE1248 containing OprP-encoding plasmids with malaria epitope insertions at sites PL1, PL2, and PL4 to PL13. Positive and negative controls were plasmids pAS27 (lane AS) and pTZ19U (lane TZ). Blots were reacted with anti-OprP (monomer-specific) serum (A) and anti-malaria epitope monoclonal antibody p.f.2A.10 (B) as the primary antibodies.

affected by insertion of only the 12-bp linker, displayed significantly reduced OprP expression after insertion of the epitope (at sites PL6 and PL7). It may be that these sites are less flexible than those of the remaining seven mutant proteins, whose levels of expression more closely resembled that of the wild-type protein. All of the mutant proteins demonstrated slightly reduced electrophoretic mobilities compared with wild-type OprP.

Insertion of the malarial epitope into linker site PL5 appeared to induce the production of two degradation products with approximate molecular masses of 30 and 25 kDa (Fig. 4A). Surprisingly, inclusion of the epitope at insertion site PL2 did not induce the production of a degradation product even though insertion of the linker alone did (Fig. 2). The increased trimer stability displayed by some of the linker mutant proteins was reversed after insertion of the malarial epitope into the linker sites.

Western immunoblotting of the outer membranes with the anti-malarial epitope antibody was done to verify that the epitope was indeed expressed in the mutants prior to analysis of surface exposure in whole cells (Fig. 4B). Because of the increased specificity of the anti-malarial epitope antibody, not all of the bands are visible. With higher concentrations of protein, all mutants displayed bands of the proper molecular mass.

**Surface exposure of epitope insertion mutant proteins.** The surface exposure of the epitope insertion mutant proteins was assessed by indirect immunofluorescence in the same manner used for the linker mutant proteins. All seven mutant clones whose levels of expression were least affected by incorporation of the epitope exhibited levels of fluorescence, due to reactivity with anti-OprP serum, that were comparable to that of the wild-type clone (Table 2). The other five clones did not fluoresce more than the negative control. This could be due either to the inability of the mutant proteins to be inserted properly into the membrane or to the lack of sensitivity of the method of detection.

To determine the surface exposure of the epitope, intact cells expressing the mutant proteins were reacted with an anti-malarial epitope antibody followed by a fluorescein isothiocyanate-conjugated secondary antibody and viewed under a fluorescence microscope. The clones expressing the mutant proteins with epitopes inserted at sites PL10 and PL11 both fluoresced strongly compared with clones expressing native OprP (Table 2). This indicated that the insertion sites were

TABLE 2. Expression and surface exposure of OprP malarial epitope insertion proteins

| Insertion site[a] | Protein expression (by SDS-PAGE)[b] | Surface reactivity | |
|---|---|---|---|
| | | Anti-OprP antibodies | Anti-malarial epitope monoclonal antibody |
| | ++ | ++ | − |
| PL1 | ++ | ++ | − |
| PL2 | ++ | ++ | − |
| PL4 | ++ | ++ | − |
| PL5 | ++ | ++ | − |
| PL6 | + | − | − |
| PL7 | + | − | − |
| PL8 | + | − | − |
| PL9 | ++ | ++ | − |
| PL10 | ++ | ++ | ++ |
| PL11 | ++ | ++ | ++ |
| PL12 | + | − | − |
| PL13 | + | − | − |

[a] Insertion sites correspond to those listed for the equivalent plasmid in Table 1.

[b] ++, level comparable to that of the wild type; +, reduced level compared with the wild type; −, undetectable level.

located in surface-exposed regions. Figure 3C shows the fluorescence of cells expressing OprP with the epitope inserted at site PL11 after reaction with the anti-malarial epitope antibody followed by the secondary antibody. Similar results were obtained with the epitope inserted at site PL10. None of the other mutants displayed any fluorescence, suggesting that the epitopes at these sites were not located at areas accessible to the anti-malarial epitope antibodies or that expression was inadequate for detection.

## DISCUSSION

The predominant conformational feature of the 410-amino-acid residue sequence of OprP is a high β-sheet structure content (44). Since the sequence of OprP was previously shown to have little homology with the sequences of the classical porins (34), a method other than sequence alignment was required to discriminate between sections of the protein which compose the loop regions and those that form the β-strands. We have described the creation and analysis of 13 linker insertion and 12 epitope insertion mutant forms of the *oprP* gene. The results of this study were used to produce a model of the membrane topology of the OprP monomer (Fig. 5).

In keeping with the motif of the previously published porin models, OprP was proposed to contain 16 β-strands, with lengths that varied from 8 to 13 amino acid residues. The proposed β-strands in all of the current porin models are composed of amphipathic stretches of amino acid residues (12, 40). This general property has been maintained in the proposed OprP model. The amphipathic carboxy-terminal β-strand is a feature common to many porins (36), and its specific sequence is conserved in OprP. Deletion of this strand in PhoE completely prevented localization of the protein to the outer membrane (8).

Insertion sites PL3, PL8, PL12, and PL13 were all placed within the β-strands. Incorporation of the four-amino-acid residue insert at these sites resulted in a substantial decrease in the amount of protein expressed in the outer membranes (Fig. 2) and an inability to detect surface-exposed protein in whole cells (Table 1). Insertion and deletion studies with LamB and PhoE have demonstrated that mutagenesis of the proposed

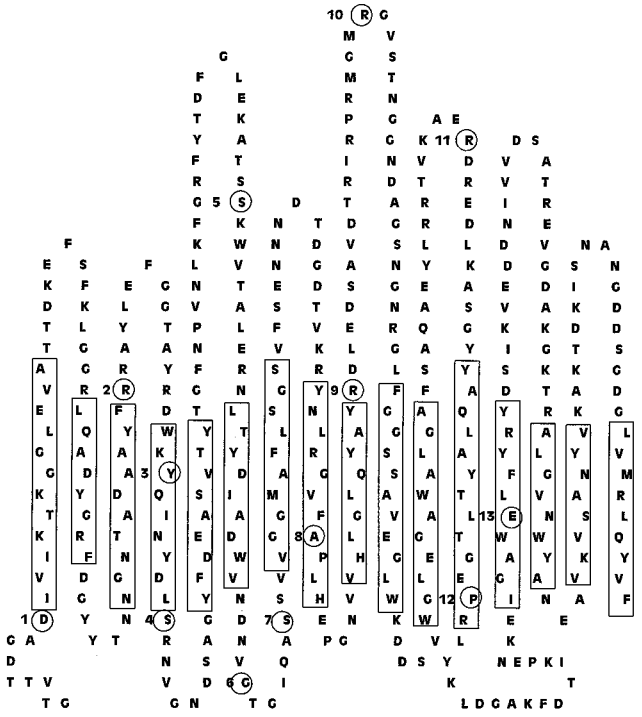

FIG. 5. Membrane topology model of the OprP monomer. Boxes enclose the proposed transmembrane β-strands. Circles indicate insertion sites PL1 to PL13 (numbered 1 to 13, respectively).

β-strands either reduced or completely inhibited expression of the protein in the host cell outer membrane (3, 9). This may be due to a disruption in the folding patterns of the proteins, making them more susceptible to proteolytic degradation and less likely to be inserted into the outer membrane. Studies have shown that the fate of misfolded outer membrane proteins is often to be degraded by periplasmically associated proteases (28, 45). The fact that some level of expression still remained in these mutant clones could be due to the ability of the inserts to maintain the general amphipathic nature of the β-strand, allowing the displaced part of the strand to be forced into the adjacent loop. This could explain why, although all four proteins exhibited reduced expression, the proteins with insertions at sites PL-12 and PL-13, whose four-amino-acid residue insert is the amphipathic sequence DLQV, were expressed at somewhat higher levels than those with inserts at sites PL-3 and PL-8 consisting of the nonpolar sequence PAGP (Fig. 2).

Insertion mutagenesis of LamB and PhoE has shown that permissive insertion sites were located in the loop regions (1, 2, 11). Insertion sites PL1, PL4, PL6, and PL7 have all been placed in periplasmic loops. None of the resulting mutant clones displayed significant decreases in expression after incorporation of the 12-bp linker into the gene. However, mutant clones resulting from insertion of the malarial epitope into linker sites PL6 and PL7 did manifest drastic decreases in expression (Fig. 4). It is possible that inclusion of the longer epitope sequence (14 amino acids) at these sites caused a disruption in an area critical to either correct folding of or maintenance of the stability of the folded structure of the protein, whereas incorporation of only 4 amino acid residues in the linker mutant proteins did not. Mutant proteins with insertions at sites PL1 and PL4 both appeared to tolerate inclusion of the epitope, as demonstrated by the ability of these

mutant proteins to react with anti-OprP antibodies at the cell surface (Table 2). It should be noted that since the anti-OprP antibodies used in the immunofluorescence experiments recognized only the native trimer form of the protein, their binding to the cells reflects proper trimerization of the mutant proteins in positive clones. None of these proposed periplasmic-loop-localized sites displayed the malarial epitope at surface-accessible regions (Table 2).

Linker insertion sites PL2, PL5, PL9, PL10, and PL11 were all placed in surface-exposed loops. All of these sites tolerated insertion of the linker and were able to incorporate the malarial epitope without significant effects on expression or surface exposure (Tables 1 and 2). However, only sites PL10 and PL11 presented the malarial epitope at surface-accessible regions (Table 2). Insertion sites PL2 and PL9 were both situated close to the junction between loop and β-strand regions in our model of OprP. It is possible that the epitopes inserted at those sites were not efficiently exposed at the cell surface. Struyé et al. similarly speculated that the reason that an epitope inserted into the second postulated loop of PhoE was not shown to be surface exposed (1) was because it was at the interface between a loop and an adjacent β-strand region (37).

Insertion site PL5 was localized to the third surface-exposed loop of our model. The analogous loops in the *Rhodobacter capsulatus* porin and PhoE, OmpF, and OmpC have been shown to fold back into the channels and constrict their internal diameters (12, 37, 40). If this loop in OprP also folds back into the channel, an epitope inserted into it might not be accessible to the antibody and therefore might not be recognized as being at a surface-exposed region. The surface exposure of an epitope from the foot-and-mouth disease virus inserted into the third loop of PhoE was not achieved until three copies of the epitope were inserted (37), suggesting that this loop is somewhat shielded from the exterior surface of the cell.

The proposed periplasmic loops of OprP tended to be longer and more irregular than those of the classical porins. Since OprP apparently interacts with the periplasmic phosphate-binding protein in *P. aeruginosa* (19), these longer loops may be necessary to facilitate that interaction. The long periplasmic loops in the proposed topological models of siderophore-iron receptors FhuA (25) and FoxA (4) may similarly be involved in the interactions between these proteins and cytoplasmic membrane-associated proteins such as TonB.

The recently cloned *P. aeruginosa* polyphosphate-selective porin OprO was shown to be highly homologous to OprP, with 76% identity and 16% conserved substitutions (33). Comparison of the amino acid sequence of OprO with our model of OprP revealed that 29 of the 39 nonconservative substitutions and two of the three one-amino-acid gaps are in the loop regions. This seems appropriate, since although the variability between closely related outer membrane proteins is usually concentrated in the loop regions, a certain amount of variation in the amino acid residues which line the interior of the channels formed within these two different proteins is required to account for their differences in substrate specificity.

The proposed model of the OprP monomer represents a starting point from which we may begin to define the structure of this protein. Testing of the model, along with ongoing crystallographic work, should help to better explain the topology of this porin.

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