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Analysis of the *Pseudomonas aeruginosa* Major Outer Membrane Protein OprF by Use of Truncated OprF Derivatives and Monoclonal Antibodies

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Tn*phoA* mutagenesis of the cloned *oprF* gene was utilized to generate 16 classes of fusions encoding differing lengths of the amino terminus of OprF fused to either alkaline phosphatase or to peptide tags of 1 to 20 amino acids, depending on the orientation and reading frame into which Tn*phoA* was inserted. Representatives of each of the 16 classes were sequenced to determine the precise fusion joint. Four of these 16 representatives which produced in-frame fusions to alkaline phosphatase and another 8 with fusion joints in the amino-terminal half of OprF failed to react with a panel of 10 specific monoclonal antibodies. In contrast, OprF derivatives with predicted fusion joints at amino acids 180, 204, 289, and 299 reacted with one to five of the monoclonal antibodies. Four other immunoreactive OprF derivatives were created by subcloning and encoded amino acids 1 to 187, 188 to 326, 1 to 273 and 1 to 170 plus 301 to 326. On the basis of reactivity with the Tn*phoA*-truncated derivatives and subclones of *oprF*, the epitopes for all 10 monoclonal antibodies were localized, in part, to specific regions of OprF. Nine of the 10 monoclonal antibodies, 8 of which recognize surface-exposed epitopes, mapped within the carboxy-terminal region of OprF that is homologous to the *Escherichia coli* outer membrane protein OmpA. Thus, we concluded that parts of the carboxy terminus of OprF are exposed on the external face of the outer membrane. In addition, a clone containing only the first two cysteine residues of OprF demonstrated reactivity with monoclonal antibodies MA4-4 and MA7-8 that was destroyed by 2-mercaptoethanol treatment, as was reactivity with intact OprF. Thus, we conclude that this first pair of cysteines at residues 176 and 185 of mature OprF form a disulfide bond.

The major outer membrane protein OprF of *Pseudomonas aeruginosa* plays a dual role, acting both as a porin, forming water-filled channels through the outer membrane (26, 40), and as a structural protein important for the maintenance of cell shape and growth on low-osmolarity media (39). There has been some controversy, based on in vitro model membrane data, regarding the size exclusion limit of pores formed by OprF (27, 31, 41). However, recent in vivo data from our laboratory, utilizing *P. aeruginosa* strains containing a cloned raffinose utilization operon in growth and plasmolysis studies, have indicated that OprF represents the major porin permitting uptake of di- to tetrasaccharides (1). The structural role of OprF is analogous to that of OmpA, a major outer membrane protein of *Escherichia coli* (32, 39). OprF and OmpA are immunologically cross-reactive, the two proteins demonstrate 33% identity and 55% similarity of amino acid sequences in their carboxy-terminal halves, and cloned OprF appeared to be able to substitute for the structural role of OmpA in an *lpp* mutant background (39). Recently, Nikaido et al. (27, 33) demonstrated that OmpA can reconstitute a porin function in liposomes.

Models of the arrangement of OmpA within the outer membrane have placed the amino-terminal half in the membrane and the carboxy-terminal half of the protein in the periplasm (2, 9, 21, 36). Although there is reasonable data favoring the disposition of the amino-terminal half of OmpA as β-strands crossing the membrane with intervening loop regions (21), evidence that the entire carboxy-terminal half of OmpA is periplasmic is largely indirect. This evidence includes cleavage of OmpA by pronase to a protease-resistant core structure comprising the amino-terminal 177 amino acids (4), demonstration by molecular genetic manipulations that less than the amino-terminal 193 residues must be present to result in an outer membrane-associated product (2), and exclusive localization of amino acids involved in binding of OmpA-specific phage to the amino-terminal half (21). Interestingly, as demonstrated in part both previously (24) and in this study, OprF shares the first two of these properties.

We previously published (31) a model for OprF in which the entire protein was presented as transmembrane β-strands connected by loops of amino acids. This model was based on estimates of the total amount of β-sheet structure by using circular dichroism (19, 31) and the protein structure prediction methods of Paul and Rosenbusch (28). However, while the model presented for OprF was compatible with models for other porin proteins (3, 35, 36), the proposed membrane disposition of the carboxy-terminal half differed markedly from the proposed periplasmic localization of the equivalent region from OmpA (2, 9, 21). In this study, we have utilized a series of monoclonal antibodies, as well as the products of molecular genetic manipulations of the cloned *oprF* gene, in part to assess which of these proposed arrangements of the carboxy terminus applied to OprF.

Many investigators have applied the Tn*phoA* mutagenesis system developed by Manoil and Beckwith (17) to study the topology and export of cytoplasmic membrane proteins (16, 37), although it has shown limited value for studying topol-

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ogy of outer membrane proteins (5, 11, 22, 23). We attempted Tn*phoA* mutagenesis on OprF to create a series of fusion proteins containing various amino-terminal lengths of OprF. We report here the reactivity of OprF fusion proteins and truncated forms of OprF produced by various subclones of *oprF* with a bank of anti-OprF monoclonal antibodies. The implications of the monoclonal antibody reactivity patterns of these OprF derivatives on our proposed model are discussed. A preliminary report of these data was presented at the International *Pseudomonas* 1991 meeting (1).

MATERIALS AND METHODS

Bacterial strains, bacteriophage, plasmids, and growth conditions. *E. coli* strains TB1 [*rec ara* Δ(*lac proAB*) *thi rpsL*/ϕ80*dlac*ΔM15 *hsdR* (20)] and CC118 [F⁻ *araD139* Δ(*ara leu*)*7697* Δ*lacX74 phoA*Δ*20 galE galK thi rpsL rpoB argE*(Am) *recA1* (20)] were utilized as the host strains for most plasmids. Plasmids pWW2200, expressing the intact *oprF* gene (38), and pWW5, expressing a truncated product (40), were described previously. Plasmid pWW12 was created during subcloning of *oprF* into the plasmid pLAFr1 (38). It contained an 11.2-kbp *Eco*RI fragment encoding a truncated *P. aeruginosa* OprF. Plasmids pWW1901 and pWW1602 were produced by subcloning 3.0- and 1.2-kbp *Sal*I fragments from plasmid pWW13 (40) into plasmid vector pUC8 (20). These fragments represented the amino and carboxy terminus-encoding parts of the *oprF* gene, respectively. The culture medium used for all *E. coli* strains in this study was Luria broth (LB) (1% tryptone, 0.5% yeast extract, 1% NaCl). Solid LB medium contained 2% agar. All medium components were from Difco Laboratories, Detroit, Mich. Strains were grown at 37°C with agitation overnight. Cells destined to be made competent were grown at 37°C with agitation to an optical density at 600 nm of 0.2 to 0.4. Antibiotic concentrations used were 12.5 μg of tetracycline (Tc) per ml and 50 μg of kanamycin (Km) per ml. To test for clones producing alkaline phosphatase, 5-bromo-4-chloro-3-indolyl phosphate (X-P; *p*-toluidine salt; BaChem, Philadelphia, Pa.) was dissolved in dimethyl sulfoxide and used at a concentration of 20 mg/ml in LB agar. Strains were stored for short terms on plates at 4°C and for long terms in 8% dimethyl sulfoxide at −70°C. Bacteriophage λTn*phoA* (*b*221 *c*I857 *P*am3 with Tn*phoA* in or near *rex* [12]) was propagated in a 0.6% LB agar overlay on the permissive host *E. coli* LE392[F⁻ *hsdr514*(r_K^- m_K^-) *supE44 supF58 lacY1* or Δ(*lacIZY*)*6 galK2 galT22 metB1 trpR55* λ⁻ (29)].

Tn*phoA* mutagenesis. Tn*phoA* mutagenesis of the OprF-encoding plasmid pWW2200 (38) was carried out as follows. *E. coli* TB1/pWW2200 grown in LB supplemented with 10 mM $MgSO_4$ to an optical density at 600 nm of 0.5 were infected with λTn*phoA* at a multiplicity of infection of 1. Successful transpositions were selected on LB plates containing tetracycline to select for maintenance of the plasmid and kanamycin to select for the presence of the transposon. To separate successful transpositions onto plasmid DNA from chromosomal transposon mutants, plasmid DNA was isolated from the pool of doubly resistant colonies and the pooled plasmid preparation was used to transform strain CC118. Transformants were again selected for tetracycline and kanamycin resistance on LB plates and screened for production of alkaline phosphatase (PhoA) by inclusion of X-P in the medium. A total of 96 blue colonies and 500 white colonies from 10 separate transfections of *E. coli* TB1/pWW2200 were collected from LB agar plates containing kanamycin, tetracycline, and the alkaline phosphatase indicator X-P. The overall observed frequency of blue colonies (alkaline phosphatase positive) was approximately 1%. All 596 colonies were screened by colony immunoblotting against 10 anti-OprF monoclonal antibodies to determine which colonies were producing immunodetectable OprF derivatives. Surprisingly, no colonies reacted with all 10 monoclonal antibodies, suggesting that no fusion plasmids were obtained with transposition events that had occurred outside the *oprF* gene. On the basis of patterns of immunoreactivity determined by colony immunoblotting with all 10 monoclonal antibodies and then by *Sal*I restriction mapping of 361 of the plasmids, 16 different classes of insertion sites representing 1 to 75 mutants each were located within the *oprF* gene (Table 1).

TABLE 1. Fusion joints with Tn*phoA* of sequenced OprF fusion plasmids

Fusion plasmid (observed no. of similar plasmids[a])	Fusion site		Predicted size of peptide fused to OprF[b]
	Last *oprF* bp	Last OprF amino acid	
pRF1 (17)	73	Ala-1	PhoA[c]
pRF2 (48)	91	Val-6	PhoA
pRF3 (7)	119	Tyr-15	1
pRF4 (2)	205	Ala-44	PhoA
pRF5 (13)	206	Ala-44	20
pRF6 (28)	215	Tyr-47	1
pRF7 (11)	351	Ala-93	1
pRF8 (4)	360	Asn-96	6
pRF9 (43)	382	Asp-103	18
pRF10 (1)	422	Gly-116	1
pRF11 (11)	505	Asn-144	18
pRF12 (22)	532	Gly-153	PhoA
pRF13 (2)	614	Asp-180	1
pRF14 (39)	685	Gly-204	18
pRF15 (75)	941	Gly-289	20
pRF16 (38)	970	Ser-299	PhoA

[a] Indicates number of plasmids from a total of 361 colonies with identical monoclonal antibody reactivity patterns on colony immunoblots with all 10 monoclonal antibodies and apparently identical restriction patterns.
[b] Length of peptide was determined by the number of amino acids encoded before the first stop codon; the orientation of Tn*phoA* is given in Fig. 5.
[c] Indicates predicted in-frame fusion to PhoA.

Monoclonal antibodies and polyclonal sera. The OprF-specific monoclonal antibodies MA4-4 and MA5-8 were described previously (24). Monoclonal antibody 7-3, proposed for use in identifying *P. aeruginosa* in in vivo samples, was previously designated IG1 (6). Monoclonal antibodies MA7-1, MA7-2, and MA7-4 through MA7-8 were isolated by a similar protocol at Oncogen Co., Seattle, Wash. (6). Antiserum to *E. coli* bacterial alkaline phosphatase, α-BAP, was purchased from 5 Prime → 3 Prime (Boulder, Colo.).

DNA techniques. For large-scale isolation of plasmid DNA, alkaline lysis and then centrifugation in ethidium bromide-cesium chloride density gradients were used (32). For rapid small-scale isolation of plasmid DNA, the same alkaline lysis method was used without subsequent density gradient centrifugation. Plasmid DNA was quantitated by a fluorescence spectrophotometric assay (15) with the DNA-binding fluorochrome H33258 (Hoefer, San Francisco, Calif.).

Restriction enzyme digests, agarose gel electrophoresis, and transformations were carried out as described by Sambrook et al. (29). The restriction endonuclease patterns of all plasmids were checked regularly to ensure that the patterns were stable and that no recombination events occurred. *E.*

coli strains were made competent by using 0.1 M $CaCl_2$ (29). Agarose gel electrophoresis was performed in Bio-Rad (Richmond, Calif.) DNA Sub cells or Mini Sub DNA cells with 1× TBE (Tris-borate-EDTA) as the running buffer.

DNA sequencing. DNA sequencing was carried out to determine the precise locations of the fusions between *oprF* and Tn*phoA*. Double-stranded, cesium chloride gradient-purified, whole plasmid DNA was used as the template for sequencing these fusion joints. The reaction primer used was a generous gift from Jeff Greenwood (U.B.C., Vancouver, Canada). This primer hybridized to a region 47 to 66 bases upstream of the left end of Tn*phoA*, around the junction between IS*50*L and *'phoA*. All sequencing was carried out with the Applied Biosystems (Foster City, Calif.) model 373A DNA sequencing system. Preparation of sequencing gels, buffers, and other reagents was done according to Applied Biosystems protocols. The *Taq* DyeDeoxy Terminator Cycle Sequencing Kit from Applied Biosystems was used for all sequencing reactions. Polymerase chain reactions were carried out in an Ericomp (San Diego, Calif.) model TCX15 thermal cycler. Excess DyeDeoxy Terminators from completed sequencing reactions were removed by passing the reaction mixture over a 1-ml Sephadex G-50 column (5 Prime → 3 Prime). Column eluants were concentrated, loaded on to sequencing gels, and run according to Applied Biosystems protocols. To locate fusion joints, the DNA sequences generated were comparatively aligned with the published DNA sequence of *oprF* from *P. aeruginosa* (7). This analysis was assisted by the SeqEd 675 DNA Sequence Editor program from Applied Biosystems. The *oprF* gene of pWW12 was sequenced by the method described previously (34), whereas that of pWW5 used a similar approach except that the automated sequencer was employed as described above.

Cell fractionation and SDS-PAGE. Total cellular protein was solubilized as described previously (25). Outer membranes were isolated by the method of Schnaitman (30). Proteins in the fractions were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with 11% polyacrylamide gels run in Bio-Rad Mini-Protean II cells. The running buffer used was 25 mM Tris-HCl, 0.1% SDS, and 192 mM glycine (pH 8.3). Protein gels were stained with Coomassie blue.

Immunodetection techniques. Colony immunoblotting was performed as described by Mutharia and Hancock (24). Western immunoblotting after separation by SDS-PAGE was performed as described previously (39, 40). Immunodetection, with a horseradish peroxidase-conjugated second antibody, was by the method of Harlow and Lane (14). Indirect immunofluorescence was performed by coating acetone-washed slides with poly-L-lysine for 15 min. Fifteen milliliters of logarithmic-growth-phase cells was washed once in phosphate-buffered saline (PBS) (24), and 50 μl was spotted onto the slides and allowed to air dry. The slides were then washed with PBS and then incubated for 30 min at 23°C with OprF-specific monoclonal antibody diluted 100-fold in PBS containing 1% fetal calf serum (GIBCO). Subsequently, cells were washed twice with PBS and then incubated for 30 min at 23°C in fluorescein isothiocyanate-labelled goat anti-mouse immunoglobulin G resuspended in PBS-fetal calf serum. Cells were then washed again, one drop of Sigma mounting medium was added, and the cells were examined under a coverslip by fluorescence microscopy. During these manipulations, cells maintained their shapes, i.e., rods in the case of cells containing no plasmid or pWW200 or pRF16 and long filaments in the case of cells containing plasmids pRF13 or pRF14.

RESULTS

Representative mutants from each of the 16 *oprF*::Tn*phoA* mutant classes identified were characterized in detail. Plasmid DNA from each representative mutant was sequenced to determine the precise location of fusions between *oprF* and Tn*phoA* and also to determine the orientation of transposition. The fusion proteins consisted of a truncated OprF region fused either to 'PhoA or to peptides of 1 to 20 amino acids in length derived from the IS*50*R or IS*50*L elements of Tn*phoA* (Table 1), depending on the orientation and reading frame of the Tn*phoA* insertion. Insertions in the correct orientation and reading frame placed *'phoA* under the control of the *oprF* promoter and were predicted to consist of a truncated OprF' fused to 'PhoA. The product of out-of-frame fusions would consist of a truncated OprF fused to peptides of 1 or 6 amino acids in length. Insertions in the opposite orientation could occur in three different reading frames, resulting in truncated OprF fused to predicted peptide tags of 1, 18, or 20 amino acids in length (on the basis of the known sequence of IS*50*L).

In-frame fusions. Only four of the sequenced transposition sites located in the *P. aeruginosa oprF* gene (representing 89 fusions) gave rise to functional expression of 'PhoA as defined by the production of blue colonies on X-P medium, sequencing to confirm that the insertions were in frame with respect to the *'phoA* coding region and production of bands corresponding to the predicted molecular mass of the full fusion proteins as demonstrated on Western blots of whole cell lysates developed with antiserum to bacterial alkaline phosphatase (anti-BAP) (Table 1). All functional fusions to 'PhoA occurred in the amino terminus, and none were observed in the carboxy terminus of *P. aeruginosa* OprF. The fusion at amino acid 299 in plasmid pRF16 was interesting in that it appeared from sequencing to be in frame with respect to *'phoA* and yet did not give rise to blue colonies on X-P. Nor was a band corresponding to the full fusion protein detectable with anti-BAP. One possibility was that the alkaline phosphatase portion of the predicted fusion was degraded. Overall, we concluded, in agreement with the results of Murphy et al. (22) for FepA, that Tn*phoA* mutagenesis gave limited information per se about the location of fusion sites relative to the internal and external faces of the outer membrane.

Immunoreactivity of truncated Opr derivatives. Only 154 of the 361 *oprF*::Tn*phoA* fusion clones, for which the fusion site was restriction mapped, gave rise to colonies which reacted with any of the 10 OprF-specific monoclonal antibodies on colony immunoblots. These 154 clones represented 4 of the 16 classes of fusions based on the size of *Sal*I fragments produced from their fusion plasmids and their pattern of reactivity with OprF-specific monoclonal antibodies (Table 1). The fusion joints from plasmids pRF13-16 (Table 1), which represented these four immunoreactive classes, were determined by DNA sequencing, and *E. coli* CC118 clones containing each of these representative plasmids were examined by Western immunoblot analysis with all 10 OprF-specific monoclonal antibodies. Both whole cell lysates and outer membrane preparations were examined, and patterns of reactivity were the same as those observed by colony immunoblotting for isolates of the class (Table 1) for which the given fusion clone was representative. As indicated in Table 2, the apparent molecular masses of the

TABLE 2. Molecular masses of anti-OprF immunoreactive OprF fusion proteins

Encoding plasmid	Fusion site in OprF (amino acid)	Predicted molecular mass[a] (kDa)		Apparent molecular mass[b] (kDa)	Immunoreactivity
		Full fusion protein	OprF portion		
pRF12	Gly-153	62.5	15.5	53.2	—[c]
pRF13	Asp-180	18.2	18.1	18.5[d]	MA7-1
pRF14	Gly-204	22.8	20.7	24.5	MA7-1, MA4-4, MA7-8
pRF15	Gly-289	31.6	29.3	23.5 (28.5)	MA7-1, MA4-4, MA7-8 (MA7-2, MA7-6)[e]
pRF16	Ser-299	77.3	30.3	23.5 (29.3)	MA7-1, MA4-4, MA7-8 (MA7-2, MA7-6)[e]

[a] Calculated from sequencing data.
[b] Estimated by migration distance relative to prestained markers of known molecular size on Western blots developed with either anti-bacterial alkaline phosphatase or anti-OprF monoclonal antibodies. First numbers were the main products detected, numbers in brackets were other weakly detected products.
[c] Identified by reactivity with antisera to bacterial alkaline phosphatase but failing to react with OprF-specific monoclonal antibodies.
[d] This was the product detected in 2-mercaptoethanol-treated samples; in non-2-mercaptoethanol-treated samples the main product detected was 40 kDa in size, while the 18.5-kDa product was more weakly detectable (Fig. 3).
[e] See Fig. 1; only the minor high-molecular-weight product reacted with MA7-2 and MA7-6.

immunoreactive truncated OprF fusion proteins derived from pRF13 and pRF14 corresponded well with the molecular masses predicted from sequencing data. However, for the fusions at amino acids 289 (in plasmid pRF15) and 299 (in pRF16), the main immunodetectable products (Fig. 1, lanes E and D, respectively) did not correspond to the molecular masses predicted for the full fusion protein or the OprF portion of the fusion protein (Table 2). Other weakly immunodetectable products, detectable only by monoclonal antibodies MA7-2 (Fig. 1, lanes B and C) and MA7-6, corresponded better to the predicted molecular mass of the OprF portion of the fusion protein (Table 2). These fusion proteins, which apparently contained all but the last 27 or 37 amino acids of OprF, appeared to be unstable and subject to degradation. The degradation products observed in the case of both fusions ran at the same molecular weight as an OprF degradation product frequently seen when this protein is expressed in *E. coli* (Fig. 1, cf. lanes E and F). Attempts to overcome degradation by expressing the fusion proteins in *E. coli* backgrounds deficient in protease activity (8, 10) were unsuccessful. The main immunodetectable product in both backgrounds was still the degradation product.

In the case of the fusion at amino acid 180 in plasmid pRF13, two immunodetectable products corresponding to the predicted molecular mass (Fig. 2, M) and to more than twice the predicted molecular mass (Fig. 2, lane A) were observed after solubilization in the absence of 2-mercapto-

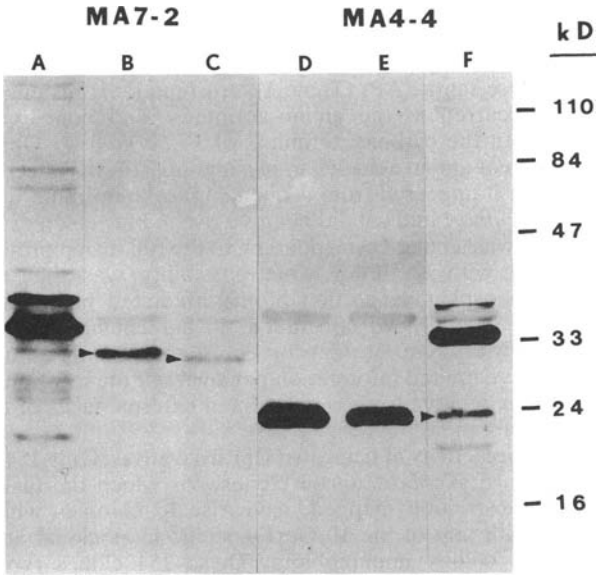

FIG. 1. Immunoreactivity of fusions of OprF at amino acids 289 (in pRF15) and 299 (in pRF16) to PhoA. Whole cell lysates were heated at 100°C for 10 min, run on SDS–11% PAGE, and transferred to nitrocellulose. The heat-modified (F*) and non-heat-modified (F) forms of OprF are indicated on the left, and molecular size markers in kilodaltons are indicated on the right. Lanes A and F, CC118(pWW2200); lanes B and D, CC118(pRF16); lanes C and E, CC118(pRF15). Lanes A to C were reacted with MA7-2; lanes D to F were reacted with MA4-4. The faint bands observed in lanes B to E at the running positions of 34 and 40 kDa were present in the CC118 negative controls and represent OmpA and an unknown outer membrane protein, respectively. In addition, the minor reactivities of monoclonal antibodies with oligomeric forms and protease degradation products of OprF, as previously described (24), occurred in the positive control (lanes A and F).

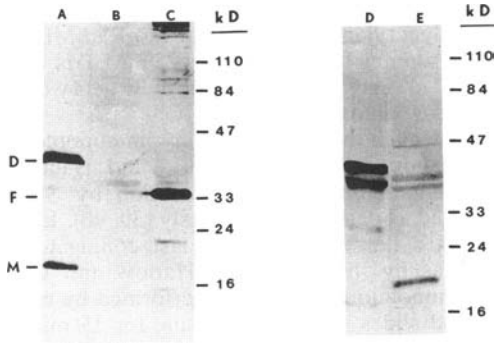

FIG. 2. Immunoreactivity with MA7-1 of OprF fusions to PhoA at amino acids 144 (in pRF11) and 180 (in pRF13). Outer membrane preparations were heated at 100°C for 10 min, run on SDS–11% PAGE, and transferred to nitrocellulose. The heat-modified, mercaptoethanol-modified (F'*); heat-unmodified, mercaptoethanol-modified (F'); and heat-unmodified, non-mercaptoethanol-modified (F) forms of OprF and the monomeric (M) and proposed oligomeric (D) form of the fusion at amino acid 180 are indicated. Molecular size markers in kilodaltons are indicated on the right. Lanes A and E, CC118(pRF13); lane B, CC118(pRF11); lane C and D, CC118(pWW2200). Lanes A to C, untreated; lanes D and E, treated with 2-mercaptoethanol (nota bene: OprF changes mobility upon reduction of its disulfides with 2-mercaptoethanol, running at a higher apparent molecular weight and with a greater tendency to heat modify [13]).

TABLE 3. Reactivities of intact cells with monoclonal antibodies as assessed by indirect immunofluorescence assays

Plasmid content of cells	Reactivity[a] with following monoclonal antibody:		
	MA7-1	MA4-4	MA5-8
None	−	−	−
pWW2200 (*oprF*)	+	+	+
pRF13 (*oprF*::Tn*phoA*)	+	+	−
pRF14 (*oprF*::Tn*phoA*)	+	+	ND
pRF16 (*oprF*::Tn*phoA*)	+	+	+

[a] −, no detectable fluorescence; +, positive fluorescence along the entire length of the cell; ND, not done.

ethanol. The latter major product, presumably a dimer (labelled D in Fig. 2, lane A), was lost upon treatment with 2-mercaptoethanol (Fig. 2, lane E), and an immunoreactive peptide of the predicted apparent molecular weight, presumably a monomer, appeared. A fusion at amino acid 180 would interrupt the predicted disulfide bonds of OprF by eliminating three of the four cysteines (retaining only Cys-176). The monomeric form, therefore, would possess one unpaired cysteine residue which could bond to the cysteine of another monomer, resulting in stable dimer formation. Only MA7-1 was able to react with this fusion at amino acid 180, and fusions containing even fewer N-terminal amino acids, including the *oprF*::Tn*phoA* in-frame fusion at amino acid 153 (Table 2), failed to react with any of the 10 monoclonal antibodies tested.

Outer membranes were prepared from strains producing fusion proteins detectable by anti-OprF monoclonal antibodies (i.e., those from plasmids pRF13 to pRF16). The fusion proteins were detectable in the outer membrane fractions of the cells but not in the cytoplasmic and periplasmic fractions. To confirm this, we examined, by indirect immunofluorescence, the reactivities of three specific monoclonal antibodies with cells producing no OprF, intact OprF, or one of the OprF fusion peptides. The data confirmed surface localization of the epitopes of monoclonal antibodies against the N terminus (MA7-1), middle (MA4-4), and C terminus (MA5-8) in the *E. coli* clones (Table 3). In addition, the pattern of reactivity was in direct agreement with the results from colony and Western immunoblots. This indicated that these products were transported to the *E. coli* outer membrane. The carboxy-terminal half of OprF, therefore, appeared to be nonessential for export to the outer membrane.

Epitope localization with OprF::Tn*phoA* derivatives. The MA4-4 epitope (24) and MA7-8 epitope (Fig. 3) were both lost upon treatment of OprF with 2-mercaptoethanol (see also Table 4). This implicated one or both of the predicted (31) disulfide bonds as being essential for the formation of these epitopes. The fusion at Asp-180 (in plasmid pRF13) which eliminated three of the four cysteines, and hence the ability to form disulfides, was not reactive with MA4-4 or MA7-8. In contrast, the fusion at Gly-204 (in plasmid pRF14), which retained the first pair of cysteine residues but lacked the second pair, was still reactive with these monoclonal antibodies (Fig. 3). Consequently, the MA4-4 and MA7-8 epitopes could be localized in part to between Cys-176 and Gly-204. MA4-4 and MA7-8 were raised against OprF in separate procedures in separate laboratories, but there is nevertheless a distinct possibility that they recognize the same epitope. Reactivity, with MA4-4 of the pRF14-encoded fusion (Fig. 3, lane E), like that with intact OprF

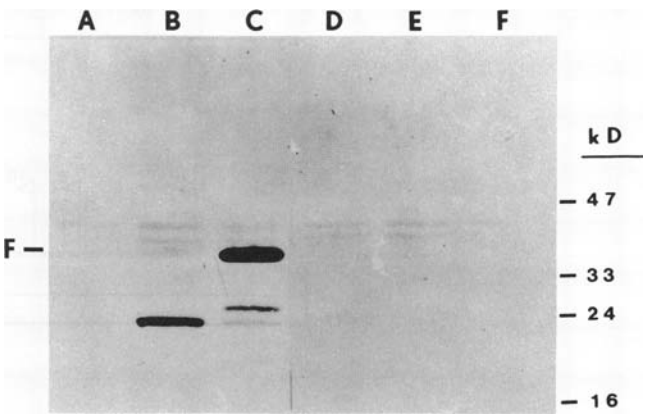

FIG. 3. Immunoreactivity with MA7-8 of OprF::PhoA fusions at amino acids 180 (in pRF13) and 204 (in pRF14), after solubilization in the presence (lanes D, E, and F) and absence (lanes A, B, and C) of 2-mercaptoethanol. Outer membrane preparations were heated at 100°C for 10 min, run on SDS–11% PAGE, and transferred to nitrocellulose. The unheated (F) form of OprF is indicated on the left, and molecular size markers in kilodaltons are indicated on the right. Lanes A and D, CC118(pRF13); lanes B and E, CC118 (pRF14); lanes C and F, CC118(pWW2200).

(Fig. 3, lane F), was destroyed by 2-mercaptoethanol treatment.

The epitopes for MA7-2 and MA7-6 could be localized in part to the region between Gly-204 and Gly-289 on the basis of the observation that OprF fusions at Gly-204 (in plasmid pRF14) were nonreactive, while OprF fusions at Gly-289 (in plasmid pRF15) and Ser-299 (in plasmid pRF16) both retained reactivity with these monoclonal antibodies (Table 2). The immunoreactive product in the case of both of these fusions was not the predominant degradation product but the more weakly detectable band corresponding to the predicted molecular weights of the OprF portions (Fig. 1, lanes B and C; Table 2). The degradation products were not detectable by either MA7-2 or MA7-6. The MA7-2 and MA7-6 epitopes appeared to be distinct, since reactivity of intact OprF with MA7-2 was unaffected by 2-mercaptoethanol treatment

TABLE 4. Immunoreactivities of OprF derivatives expressed by *oprF subclones*

Anti-OprF monoclonal antibody	Reactivity[a] with following *oprF* subclone:					
	pWW2200[b]		pWW5	pWW12[c]	pWW1901	pWW1602
	−2ME	+2ME				
MA4-4	+	−	−	+	+	−
MA5-8	+	+	+	−	−	+
MA7-1	+	+	+	+	+	−
MA7-2	+	+	−	W	−	+
MA7-3	+	W	−	−	−	+
MA7-4	+	W	−	−	−	+
MA7-5	+	+	−	−	−	+
MA7-6	+	W	−	W	−	+
MA7-7	+	W	−	−	−	+
MA7-8	+	−	−	+	+	−

[a] +, positive reaction; −, negative reaction; W, weak reaction.
[b] 2-mercaptoethanol was either added (+2ME) or not added (−2ME) with the solubilization reduction mix prior to electrophoresis.
[c] The product of subclone pWW12 was subject to degradation; degradation products were not immunoreactive with MA7-2 or MA7-6.

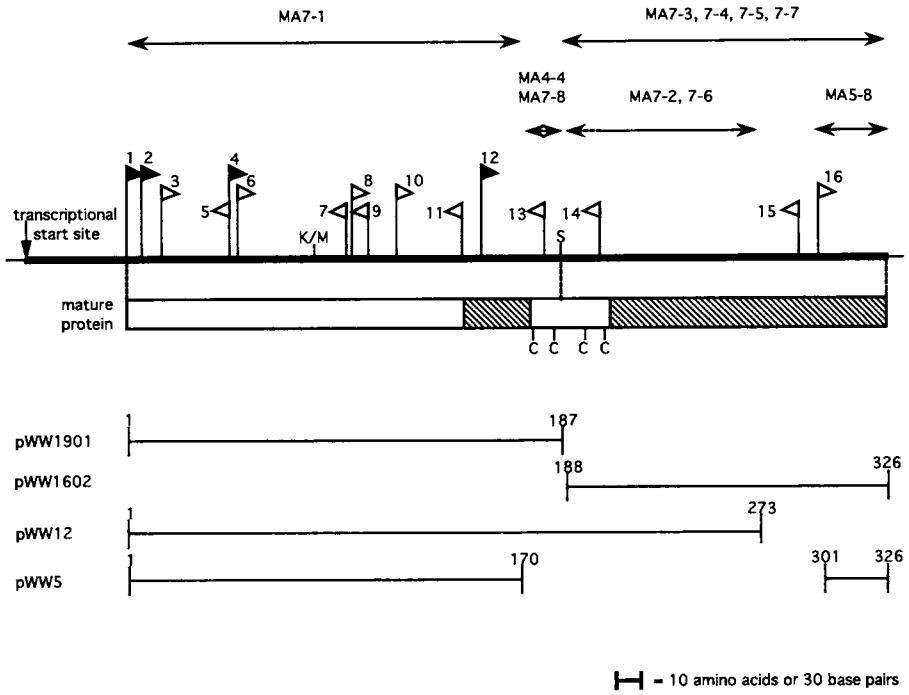

FIG. 4. Summary of the location of fusions, subclones, and epitopes of monoclonal antibodies on a linear map of *P. aeruginosa* OprF. For the fusions indicated above the line, dark arrows indicate functional expression of PhoA, open arrows indicate white colonies on X-P medium, and the direction of the arrow indicates the orientation of transposition. The *ompA* homologous regions of OprF (hatched region) and cysteine(s) (C) are indicated below the line. Subclones and the portions of OprF that they encode are indicated. Monoclonal antibody epitopes are indicated by solid lines (representing regions defined here as belonging to the epitope).

whereas reactivity with MA7-6 was weakened by 2-mercaptoethanol treatment (Table 4).

None of the OprF fusion proteins generated in this study were reactive with MA5-8, MA7-3, MA7-4, MA7-5, and MA7-7 (Table 2).

Reactivity of anti-OprF monoclonal antibodies with other OprF derivatives. Data regarding the locations of the epitopes recognized by the anti-OprF monoclonal antibodies, based on analyses of the truncated OprF derivatives created by Tn*phoA* mutagenesis, were complemented by the monoclonal antibody reactivity patterns of OprF derivatives created by subcloning. The amino-terminal (start to the *Sal*I site) and carboxy-terminal (*Sal*I to end) coding regions of *oprF* were subcloned to give pWW1901 and pWW1602, respectively (Fig. 4). The other two OprF derivatives, pWW5 (40) and pWW12, arose during the original subcloning of the *oprF* gene. Sequencing of pWW5 and pWW12 in the present study revealed that pWW5 encoded an OprF with an internal deletion from amino acid 171 through 300, whereas pWW12 encoded an OprF derivative which was truncated at amino acid 273. The sequence data for these subclones are summarized in Fig. 4, and the immunoreactivities of the proteins produced by these subclones are presented in Table 4 and Fig. 5. Consistent with the above data, monoclonal antibodies MA7-1, MA4-4, and MA7-8 reacted with the N-terminal first 187 amino acids, the product of pWW1901. The other seven monoclonals all interacted with the carboxy-terminal product of pWW1602 expressing amino acids 188 to 326. The apparent molecular weight of this immunoreactive product on Western immunoblots of 2-mercaptoethanol-reduced protein was 16,000 (18). The product of pWW12, expressing amino acids 1 to 273, behaved similarly to the products of pRF15 and pRF16, expressing amino acids 1 to 289 and 1 to 299, respectively, in that pWW12 produced a protein, with the predicted molecular weight of the OprF-encoding portion, which was reactive with MA7-2 and MA7-6. This protein tended to break down to a product of the same apparent molecular weight as a normal proteolysis product of OprF (Fig. 5, lanes E and F). Both of these products lost reactivity to MA7-2 and MA7-6 but retained reactivity with MA7-1, MA4-4, and MA7-8

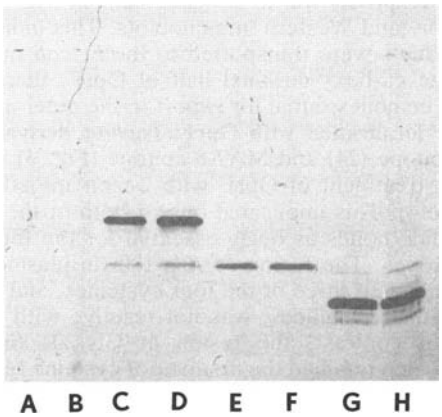

FIG. 5. Immunoreactivity with MA7-1 of various subclones of *oprF* demonstrated by Western immunoblotting with whole cell proteins separated by SDS-PAGE and transferred to nitrocellulose. Lanes A and B, CC118 negative control; lanes C and D, CC118 (pWW2200) positive control (nota bene: the minor band is the protease digestion product referred to in the legend to Fig. 1); lanes E and F, CC118(pWW12); lanes G and H, CC118(pWW5).

(Tables 2 and 4). Plasmid pWW5 arose as a result of an internal deletion due to an apparent recombination between the identical DNA sequences, CCGGTTGCCGAC, that begin at nucleotide positions 583 and 973 of the *oprF* coding sequence (1,053 bp total). The resultant deletion protein had lost the region encoding the four cysteines of OprF and most of the carboxy-terminal region of OprF except amino acids 301 to 326. The resultant protein, which had a predicted molecular weight of 22,000 and an apparent molecular weight of 24,000 (40) (Fig. 5), retained reactivity only with MA7-1 and MA5-8.

DISCUSSION

In this manuscript, we have utilized a series of truncated OprF derivatives, created by Tn*phoA* mutagenesis, in addition to others created by subcloning, to map the epitopes of a series of monoclonal antibodies within the OprF gene. We have previously presented data suggesting that OprF, when expressed in *E. coli*, is exported to the outer membrane and capable of reconstituting channels with characteristics the same as those of OprF from *P. aeruginosa* (40). Furthermore, on the bases of 2-mercaptoethanol reduction experiments and the ability of OprF to react with two OprF-specific monoclonal antibodies (Table 4), it would seem that the intrachain cysteine disulfide(s) formed by this protein in *P. aeruginosa* is also formed in *E. coli*. As judged by colony immunoblotting (for antibodies MA4-4 and MA7-8) and indirect immunofluorescence studies (for MA4-4), the epitopes for both antibodies are recognized in *E. coli* cells.

These results strongly suggest that OprF is exported to the outer membrane of *E. coli* and folds in a manner similar to that of native OprF in *P. aeruginosa*. Consistent with this we have shown that a further eight OprF-specific monoclonal antibodies react with *E. coli*-derived OprF (Table 4), which is consistent with the above results. Although we cannot conclude that the immunoreactive, truncated OprF derivatives studied here were also folded correctly, the observations that they were apparently exported to the outer membrane (Table 3), were capable of reacting on colony and Western immunoblots with between 1 and 7 of the 10 OprF-specific monoclonal antibodies, and for those truncated OprF derivatives with more than one pair of cysteines, produced at least one cysteine disulfide (Fig. 3) suggested that these derivatives might have retained some of the folding patterns of native OprF. Nevertheless, it must be stressed that the conclusions made below regarding the localization of epitopes are based on Western immunoblot studies after transfer of the truncated OprF derivatives from SDS-PAGE to nitrocellulose. Therefore, the conclusions regarding epitope localization are based on the immunoreactivity of partially denatured truncated OprF derivatives and not on a presumption of retention of secondary or tertiary structure in these derivatives.

The locations of the various epitopes for the 10 monoclonal antibodies studied are described in Fig. 4. The MA7-1 epitope was delineated by the reactivity of MA7-1 with all products containing the amino-terminal 170 amino acids, including pRF13-16 (Table 2), pWW1901, pWW12, and pWW5 (Table 4). The lack of reactivity of the protein produced by pRF12 with MA7-1 (Table 4) was consistent with a conclusion that part of the epitope for MA7-1 lies between amino acids 153 (the fusion joint in pRF12) and 170 (in pWW5). However, we could not conclude that the entire epitope lies in this region, and an 8-kDa variance in the predicted and apparent molecular masses of the *oprF*::Tn*phoA* fusion protein from pRF12 weakened this conclusion. The epitopes for MA7-2 and MA7-6 were localized to amino acids 188 to 326 by reactivity with the product of plasmid pWW1602 (Table 4). The carboxy-terminal extension of these epitopes was further delineated by reactive clones pWW12 (amino acids 1 to 273), pRF15 (1 to 289), and pRF16 (1 to 299) and nonreactive clone pWW5, whereas clones that failed to react with MA7-2 and MA7-6 included pRF14 (1 to 204) and the major (degradation) products of the above reactive clones (estimated to be slightly larger than the pRF14 product on the basis of gel mobility). Therefore, amino acids 204 to 273 appear to contain at least part of these epitopes, whereas the involvement of amino acids 188 to 204 is uncertain. These two monoclonal antibodies have epitopes that are different as determined on the basis of the observations that MA7-6 recognizes a surface epitope whereas MA7-2 apparently does not, and the two monoclonal antibodies have different patterns of reactivity against other fluorescent *Pseudomonas* species (19). Monoclonal antibody MA5-8 recognized an epitope between amino acids 301 and 326 on the basis of its reactivity with the product of pWW1602 (encoding amino acids 188 to 326) and pWW5 (encoding only amino acids 301 to 326 of the pWW1602-encoded region of OprF) but lack of reactivity with the *oprF*::Tn*phoA* fusion products (Table 2) and with the product of pWW12 (Table 4). Monoclonal antibody MA5-8 recognizes a surface epitope as determined on the basis of immunofluorescence of intact cells and phagocytosis opsonization data (24).

The epitopes for monoclonal antibodies MA7-3, MA7-4, MA7-5, and MA7-7 were localized to the carboxy terminus (amino acids 188 to 326) on the basis of reactivity with the product of pWW1602. However, these monoclonal antibodies failed to react with any of the products of the *oprF*::Tn*phoA* fusions or with the products of the pWW12 or pWW5 subclones. The amino-terminal extension (amino acid 204) of the epitope for these monoclonal antibodies was implied from the lack of reactivity of the protein produced by cells containing pRF14 and of the major truncated products produced by cells containing pRF15, pRF16, or pWW12 (we did not consider the lack of reactivity of the minor bands produced from these clones as definitive given their poor production [Fig. 1]). The carboxy-terminal extension of the epitopes was indicated to lie at amino acid 300 on the basis of lack of reactivity with the protein expressed by pWW5.

On the basis of mobility shifts in the presence of 2-mercaptoethanol, Hancock and Carey (15) proposed that OprF contained two disulfide bonds. In a recent model of OprF (31), we placed these bonds for convenience between neighboring disulfide residues. The resultant loop regions between the bonds were arranged at the cell surface on the basis of the observation that reactivity with MA4-4, a monoclonal antibody that recognizes surface epitopes (24), was lost upon treatment of OprF with 2-mercaptoethanol (Fig. 3). The data presented here favor the hypothesis that the secondary structure created by a disulfide bond between Cys-176 and another cysteine residue possibly forms the epitope recognized by MA4-4 and MA7-8. The other cysteine residue involved is probably not Cys-205 or Cys-192, as evidenced by the fact that a clone expressing amino acids 1 to 187 of OprF [in TB1(pWW1901)], which would lack both these cysteines, is still reactive with MA4-4 and MA7-8. Also, the fusion at amino acid 204 in pRF14 (which would lack Cys-205) retained reactivity with both MA4-4 and MA7-8 (Fig. 2). In addition, fusion pRF13 containing amino acids 1 to 180 and subclone pWW5 containing amino acids 1 to 170

failed to react with these two monoclonal antibodies, indicating that both Cys-176 and Cys-185 may be required for this epitope. The OprF proteins of both *P. aeruginosa* and *P. syringae* are identical in sequence between these cysteines but diverge substantially at their amino termini (34). Since both react with MA4-4 (24, 34) and MA7-8 (Fig. 2) (19), we hypothesize that the region of Cys-176 to Cys-185 composes most or all of the epitope for these monoclonal antibodies.

The carboxy-terminal half (amino acids 146 to 326) of OprF appears related (identity, 33%; total similarity, 55%) to that of the *E. coli* outer membrane protein OmpA (amino acids 177 to 335 [39]), and the two proteins have many similarities including a role in cell shape and structure determination and immunological cross-reactivity (39). The major regions of nonidentity in these carboxy termini include the cysteine disulfide region (amino acids 176 to 205) of OprF and a cysteine-containing region of OmpA (amino acids 301 to 317). Henning and colleagues (2, 9, 21) have represented a model for OmpA in which the amino-terminal region traverses the membrane via a series of β-strands connected by loop regions, but the carboxy-terminal half is found in the periplasm. In contrast, we have proposed a model for OprF in which the entire protein comprises β-strands which cross the membrane and are connected by loop regions (31). This model, based on β-sheet structure predictions by the method of Paul and Rosenbusch (28), is consistent with the measured content of β-sheet structure in OprF (19). The results of the present study are consistent with our previous model since several monoclonal antibodies recognizing surface epitopes (19, 24), including MA5-8, MA7-3, MA7-4, MA7-5, MA7-6, and MA7-7, map to the region of OprF related to the carboxy terminus of OmpA, whereas MA4-4 and MA7-8 map to the cysteine region that has been apparently inserted into this region of homology.

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