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Construction and Characterization of *Pseudomonas aeruginosa* Protein F-Deficient Mutants after In Vitro and In Vivo Insertion Mutagenesis of the Cloned Gene

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Mutants with insertion mutations in the *Pseudomonas aeruginosa* protein F (*oprF*) gene were created in vivo by Tn*1* mutagenesis of the cloned gene in *Escherichia coli* and in vitro by insertion of the streptomycin resistance-encoding Ω fragment into the cloned gene, followed by transfer of the mutated protein F gene back to *P. aeruginosa*. Homologous recombination into the *P. aeruginosa* chromosome was driven by a bacteriophage F116L transduction method in the *oprF*::Tn*1* mutants or Tn*5*-instability in the *oprF*::Ω mutants. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western immunoblotting demonstrated that the resultant *oprF* insertion mutants had lost protein F, whereas restriction digestion and Southern blotting experiments proved that the mutants contained a single chromosomal *oprF* gene with either Tn*1* or Ω inserted into it. It has been proposed that protein F has a role in antibiotic uptake in *P. aeruginosa*. Measurement of antibiotic resistance levels showed small to marginal increases in resistance, compared with that of the parent *P. aeruginosa* strain, to a variety of β-lactam antibiotics. Protein F-deficient mutants had altered barrier properties as revealed by a three- to fivefold increase in the uptake of the hydrophobic fluorescent probe 1-*N*-phenylnaphthylamine.

Porins from a variety of bacteria have been shown to be responsible for the permeability of small hydrophilic solutes both in vivo and in vitro (14, 15). A role for some porins in antibiotic uptake has been proposed. This proposal is based largely on observations, in strains of the family *Enterobacteriaceae*, showing that cells lacking porin proteins in their outer membranes were more resistant than wild-type strains to hydrophilic antibiotics, particularly β-lactams (7, 17, 18, 20, 23). However, for some of the more recently introduced β-lactams, there is still some dispute, based on similar studies, as to the role of *Escherichia coli* porins in their uptake across the outer membrane (7, 17). In many instances, however, model membrane studies have supported a role for porins in antibiotic uptake (14, 29, 38).

Pseudomonas aeruginosa is inherently extremely resistant to antibiotics when compared with many other gram-negative organisms; this phenomenon is attributed in part to low outer membrane permeability (1, 12, 28, 37). Indeed, the outer membrane permeability of wild-type *P. aeruginosa* has been determined to be 12-fold (28) to 100-fold (37) lower than that of wild-type *E. coli*. Two possible explanations for this low permeability have been proposed that are based on experiments designed to measure the molecular sieving ability of the *P. aeruginosa* outer membrane. One possibility is that the *P. aeruginosa* outer membrane largely excludes β-lactams, an explanation consistent with the experiments of Caulcott et al. (5) and Yonegama et al. (36), who suggested that the largest sugars capable of passing through the outer membrane were disaccharides (molecular weight 342) and monosaccharides (molecular weight 180), respectively. Alternatively, it has been suggested that the exclusion limit is large (molecular weight 3,000), but that there are very few porin molecules containing channels that allow the passage of large molecules (1, 3). This explanation is consistent with the relatively large size of some β-lactams (molecular weight >500) that are known to enter and kill *P. aeruginosa* and with model membrane studies, in which protein F preparations were shown to contain large channels at a low frequency (3) and small channels at a high frequency (34).

In an attempt to resolve this controversy and determine whether protein F is involved in β-lactam uptake in *P. aeruginosa*, we constructed stable protein F-deficient mutants by insertion mutagenesis of the cloned gene in *E. coli* followed by gene replacement in *P. aeruginosa*. The mutant bacteria were characterized with respect to antibiotic uptake and permeability to a hydrophobic fluorophor.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The bacterial strains and plasmids used in this study are listed in Table 1. For rich media, LB medium (34), TY medium (34), or Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) was used. Media were solidified when necessary with 2% (wt/vol) Bacto-Agar (Difco). Minimal medium was Vogel-Bonner medium (22) supplemented with required amino acids at 10 μg/ml and with 0.5% (wt/vol) glucose as the carbon source. When required, the following antibiotics were used: for *E. coli*, tetracycline at 25 μg/ml, ampicillin at 50 μg/ml, kanamycin at 25 μg/ml, and streptomycin at 200 μg/ml; for *P. aeruginosa*, streptomycin at 500 μg/ml, tetracycline at 100 μg/ml, and kanamycin at 500 μg/ml.

General DNA techniques. DNA manipulations followed the methods outlined by Maniatis et al. (22), with the exceptions described previously (34).

Insertion mutagenesis and gene replacement. Transposon mutagenesis was done by the methods of Ohman et al. (31). Plasmid pWW13 (34), containing an 11-kilobase (kb) insert including the *oprF* gene, was chosen for mutagenesis because it was too small to be packaged by bacteriophage λ, but upon insertion of a transposon of 5 to 14 kb it became large enough to package. Briefly, plasmid RSF1010::Tn*1* was transferred into *E. coli* HB101(pWW13). Cells harboring

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TABLE 1. Bacterial strains, plasmids, and bacteriophages

Strain or plasmid	Characteristics	Source or reference
E. coli K-12		
HB101	*hsdR hsdM recA13 ara-14 thi-1 proA2 lacY1 galK2 mtl-1 xyl-5 recA13 supE44* λ^s λ^-	22
MM294	*pro thi endA hsdW*	22
DH5α	F^- *endA1 hsdR17* (r_x^- m_k^+) *supE44 thi-1 recA1 gyrA96 relA1* Δ(*argF-lacZYA*) U169 φ80d*lacZ* ΔM15 λ^-	Bethesda Research Laboratories
P. aeruginosa PA0		
H103	Cm^r auxotroph	16
H608	H103 *oprF*::Tn*1–3*	This study
H636	H103 *oprF*::Ω	This study
H103(RP1)	H103 (RP1 Tc^r Km^r Ap^r [Tn*1*])	28
Plasmids		
pLAFr1	IncP1 Tc^r *rlx* λ*cos*	11
pWW13	pLAFr1 carrying an 11-kb *Eco*RI fragment insert including the *oprF* gene	34
pRK404	IncPI (RK2 replicon) Tc^r carrying p*lac* and the multicloning site of pUC18	10
pWW2200	pRK404 with an insertion of a 2.5-kb *Pst*I fragment from pWW13 including the *oprF* gene	This study
pUC18	Ap^r	35
pWW2300	pUC18 carrying *Pst*I-*Sal*I fragment from pWW2200	This study
pWW2300Ω	pWW2300 with Ω inserted at the *Sma*I site in the *oprF* gene	This study
pWW2400	pWW2300Ω × pRK404	This study
pRZ102	ColE1::Tn*4 mob* Km^r	19
pWW2500	pRZ102 with *Sal*I fragment from pWW2400 including *oprF*::Ω	This study
pWW13.Tn*1–3*	pWW13 *oprF*::Tn*1–3*	This study
pRK2013	ColE1-Tra^+ Km^r	8
RSF1010::Tn*1*	IncQ Sm^r carrying Tn*1*	13
RSF1010::Tn*501*	IncQ Sm^r carrying Tn*501*	13
Phages		
F116L	*P. aeruginosa*-transducing phage	1
λ *c*I857	*c*I857	22

both plasmids were infected with phage λ. A lysate was prepared, treated with $CHCl_3$ to kill residual cells, and then used to transduce a culture of *E. coli* HB101. Transductants were selected for further manipulation which were tetracycline resistant (to demonstrate the presence of plasmid pWW13), carbenicillin resistant (from the transposition of Tn*1* onto pWW13), and protein F deficient as assayed by a colony immunoblot procedure with a protein F-specific monoclonal antibody, MA5-8 (22). The site of transposon insertion in the *oprF* gene was demonstrated by restriction mapping and Southern hybridization with a 2.5-kb *Pst*I fragment containing the entire *oprF* gene (Fig. 1). The resultant plasmids were transferred to *P. aeruginosa* by triparental mating and packaged in vivo into phage F116L, and the resulting phage lysate was used to infect *P. aeruginosa* PA01. Carbenicillin-resistant, tetracycline-sensitive transductants were screened for protein F deficiency as above. Two of the colonies with the desired phenotype, strains H607 and H608, were selected for further characterization. Insertion mutagenesis with other transposons was attempted exactly as described above, except that compatible Tn*5*-, Tn*7*-, or Tn*501*-loaded plasmids were used in place of plasmid RSF1010::Tn*1* at the first step.

Ω mutagenesis and gene replacement. The strategy for mutagenesis with the streptomycin resistance Ω DNA cartridge (Amersham Corp.) is described in Fig. 1. The resultant plasmid, pWW2500, contained an Ω insertion flanked by sequences from the *oprF* gene which was inserted into the *Sal*I site within transposon Tn*5* of plasmid pRZ102. As described by Goldberg and Ohman (13), a combination of the lack of a suitable replication origin for *P. aeruginosa*, the presence of a *mob* site to allow triparental mating into *P. aeruginosa*, and the instability of Tn*5* in this host promotes homologous recombination of sequences cloned between the IS*50* elements of Tn*5* after transfer from *E. coli* to *P. aeruginosa*. After triparental mating of plasmid pWW2500 to *P. aeruginosa*, streptomycin-resistant recombinants containing Ω were obtained at a frequency of about 10^{-7} per recipient. Of these recombinants, 60 to 100% (depending on the recipient) were protein F deficient as assessed by colony immunoblot procedures with specific monoclonal antibodies (26).

Outer membrane characterizations. Isolation of outer membrane and sodium dodecyl sulfate-polyacrylamide gel electrophoresis were performed as described previously (13). Western immunoblot analysis was done by the method of Mutharia and Hancock (25). Colony immunoblots were performed as described previously (34) with MA4-4 and MA5-8 (29). MA4-4 and MA5-8 are monoclonal antibodies specific for two different epitopes on protein F.

Antibiotic MIC determinations. MICs were determined by the agar dilution method of the National Committee for Clinical Laboratory Standards (27a). Approximately 10^4 cells, grown to logarithmic phase on Mueller-Hinton broth, were spotted onto Mueller-Hinton agar plates containing doubling twofold dilutions of a variety of antibiotics. MIC endpoints were determined after 24 h of growth at 37°C. Antibiotics were gifts from the manufacturers.

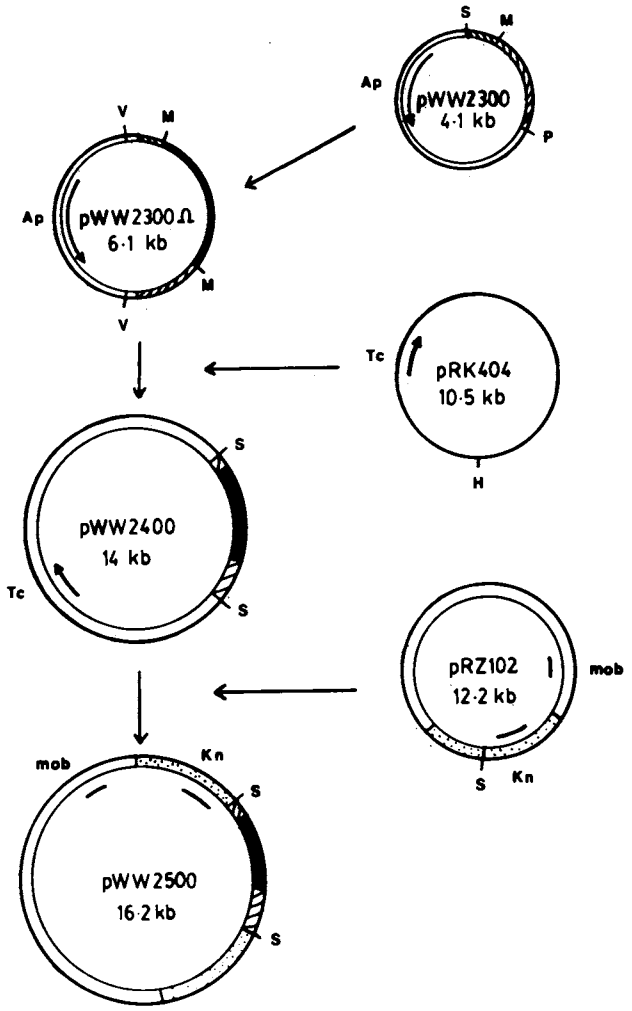

FIG. 1. Strategy for isolation of plasmid pWW2500 for Ω mutagenesis of the *oprF* gene. A 1.4-kb *PstI-SalI* fragment containing the amino-terminal half of the protein F gene (pWW2300) was insertionally mutagenized in vitro by the insertion of the streptomycin resistance cartridge Ω into the *SmaI* site. The 3.5-kb *PvuII* fragment of this plasmid, pWW2300Ω, was purified and ligated into pRK404 which had been digested with *Hin*dIII and blunt ended with the Klenow fragment. The resultant plasmid, pWW2400, was digested with *SalI*, and the 3.4-kb *SalI* fragment containing Ω was ligated into the *SalI* site of pRZ102 to create pWW2500. Symbols: ▨, *oprF* DNA; ▬, Ω DNA; ▩, Tn*5* DNA. The restriction sites are indicated by one-letter codes as follows: H, *Hin*dIII, M, *Sma*I; P, *Pst*I; S, *Sal*I; V, *Pvu*II. Other abbreviations: Kn, kanamycin resistance gene; Tc, tetracycline resistance gene; Ap, ampicillin resistance gene; mob, mobilization site for triparental mating. The plasmids are not drawn to scale, but within each plasmid representation the relative insert sizes are proportional.

NPN uptake assays. Uptake of 1-*N*-phenylnaphthylamine (NPN) was assessed exactly as described by Loh et al. (21).

RESULTS

Transposon mutagenesis of the *oprF* gene in *E. coli* (pWW13) and gene replacement in *P. aeruginosa*. Attempts were made to insert transposons Tn*1*, Tn*5*, Tn*7*, and Tn*501* into the cloned *oprF* gene of plasmid pWW13. We were able to construct plasmids with transposon inserts of Tn*1*, Tn*5*, and Tn*7* which mapped within the *oprF* gene. However, no

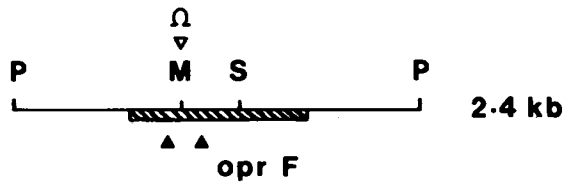

FIG. 2. Restriction map of the *oprF* gene showing the location of the Tn*1* insertions in two *P. aeruginosa* strains (marked on the map with arrowheads). The site of the Ω insertion in strain H636 is marked Ω.

Tn*501* inserts within the *oprF* gene were isolated, although over 3,000 Tc^r Hg^r transductants (i.e., transductants containing plasmid pWW13::Tn*501*) were screened for the loss of ability to express protein F. The transposon-mutagenized plasmids with insertions within the *oprF* gene were conjugated into *P. aeruginosa*. Lysates of phage F116L were made on *P. aeruginosa* strains containing such plasmids and used to infect *P. aeruginosa* PA01. We selected transductants which had lost the tetracycline marker of the plasmid, retained the transposon marker, and did not interact with the protein F-specific antibody, indicating that gene replacement by homologous recombination had occurred. We achieved gene replacement only with our Tn*1*-mutagenized plasmids. Protein F-lacking mutants were constructed by using plasmids with Tn*1* inserted at one of two sites within the *oprF* gene (Fig. 2). A resultant *P. aeruginosa* strain containing the upstream Tn*1* insertion was designated H608.

Ω mutagenesis of the protein F gene. The above approach resulted in the isolation of strains with only Tn*1* insertion mutations in the *oprF* gene. This was a disadvantage for further studies of the function of protein F in β-lactam uptake across the outer membrane, since Tn*1* encodes a β-lactamase enzyme. Therefore, we attempted to insert aminoglycoside resistance genes. Since the use of aminoglycoside resistance-encoding transposons Tn*5* and Tn*7* in the above gene replacement strategy was unsuccessful, we used in vitro mutagenesis with the commercially available DNA fragment Ω (encoding streptomycin and spectinomycin resistance and containing multiple transcriptional stop signals) and the gene replacement vector pRZ102 (19) to make *oprF*::Ω mutants of *P. aeruginosa*. After construction of plasmid pWW2500 (Fig. 1) and triparental mating into *P. aeruginosa*, mutants derived from homologous recombination and replacement of the $oprF^+$ gene were easily obtained in a number of different genetic backgrounds (data not shown). One of these, a derivative of strain H103, was strain H636.

Characterization of the protein F-deficient mutants. Outer membranes were isolated from both the *P. aeruginosa* protein F-deficient Tn*1* mutants and the Ω mutant. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles of membranes of these mutants (Fig. 3A, lanes 2 and 3) were almost identical to that of the parent strain H103 (Fig. 3A, lane 1), except that protein F was conspicuously absent in the mutants. There was no substantial increase in the size or relative content of any other protein, indicating that the cells did not compensate for the loss of protein F by substituting another outer membrane protein.

The loss of protein F from the outer membrane was confirmed by Western immunoblots of the outer membrane proteins. In the mutants, no interaction was observed with a protein F-specific monoclonal antibody (Fig. 3B). Chromosomal DNA was isolated from the mutant strains, digested

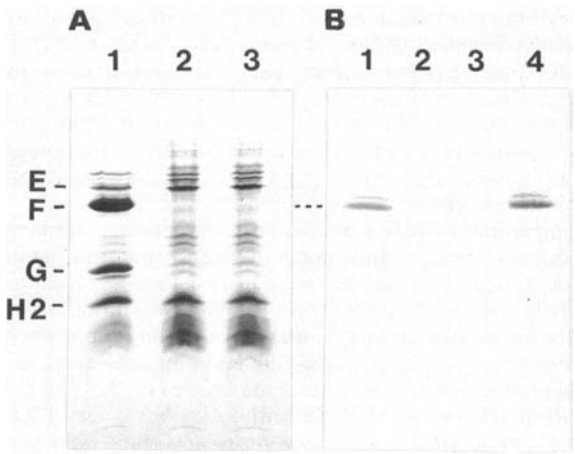

FIG. 3. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoretogram of outer membranes of *P. aeruginosa* H103 (lane 1) and its protein F-deficient mutants H608 *oprF*::Tn*1* (lane 2) and H636 *oprF*::Ω (lane 3). The running position of protein F is indicated. (B) Corresponding Western immunoblot of the outer membranes shown in panel A after transfer to nitrocellulose and interaction with a protein F-specific monoclonal antibody as described previously (29). Lane numbering corresponds to panel A, except for lane 4, which represents purified protein F. The appearance and relative amount of protein G is variable depending on the growth conditions, the stage of growth, and the specific strain. Less protein G has been observed in other outer membrane preparations of strain H103 (16). The upper band which reacts with MA5-8 in panel B is heat-modified protein F and is commonly observed in outer membrane preparations of H103 (16, 34).

with restriction enzymes, electrophoresed in agarose, and analyzed by Southern blot hybridization with a radioactively labeled probe of the *oprF* gene. The hybridization patterns for the mutants compared with that of the parent strain H103 indicated that the Tn*1* or Ω insertions were in the *oprF* structural gene and were present at only one site in the mutant chromosomes (data not shown).

Antibiotic MICs. Two factors, potentially relevant to our protein F-deficient insertion mutants, can markedly affect levels of resistance to β-lactam antibiotics: growth rates and β-lactamase levels. With respect to growth rates, we found that both mutants H608 and H636 grew poorly on Difco Proteose Peptone no. 2 medium compared with the parent strain H103. This low growth rate could be partially restored to that of H103 by addition of NaCl to Proteose Peptone no. 2 medium, as previously described for chemically induced mutants deficient in protein F (28). For our antibiotic MIC measurements we used Mueller-Hinton medium, since similar doubling times were observed for strain H103 (51 min) and its derivatives H608 *oprF*::Tn*1* (54 min) and H636 *oprF*::Ω (58 min) on this medium.

TABLE 3. Mean MICs for strain H103 and its protein F-deficient Ω insertion derivative H636

Antibiotic	Geometric mean MIC (μg/ml) for[a]:		MIC ratios for porin-deficient and porin-sufficient strains	
	H103 *oprF*$^+$	H636 *oprF*::Ω	*P. aeruginosa*[b]	*E. coli*[c]
Cefpirome	1.0	3.0[d]	3.0	–
Cefotaxime	4.0	12.7	3.2	2
Cefepime	0.8	2.0	2.5	–
Aztreonam	1.4	2.4	1.7	4
Carbenicillin	16	28[d]	1.8	8
Ceftazidime	1.0	1.6[e]	1.6	2
Piperacillin	1.7	2.3	1.4	2
Cefpiramide	2.0	2.5	1.3	–
Cefsulodin	1.6	2.0	1.3	–
Imipenem	3.2	2.5	0.8	–
Norfloxacin	3.0	2.6	0.9	–

[a] Geometric means of three to five determinations.
[b] Ratio of MICs for strains H636 and H103.
[c] Data from references 7, 17, 18, and 20. –, No information available.
[d] $P = 0.05$ by Fisher's exact test, compared with the MIC for strain H103.
[e] $P = 0.1$; all other results, $P = 0.2$.

Strain H608 contained transposon Tn*1*, which expressed a β-lactamase. Therefore, we were unable to assess MICs, for this strain, of β-lactam antibiotics which were hydrolyzable by this β-lactamase. However, as a control for β-lactamase we used strain H103(RP1), which carried Tn*1* on plasmid RP1 and expressed levels of β-lactamase similar to those for strain H608 (Table 2), and were thus able to demonstrate small increases in the resistance of strain H608 to the β-lactamase-stable β-lactams aztreonam and ceftazidime. In contrast, marginal differences in resistance were observed for the quinolone antibiotic norfloxacin or the aminoglycosides tobramycin and gentamicin (Table 2).

Comparison of strain H103 and its Ω insertion-mutated, protein F-deficient derivative H636 demonstrated small increases in the levels of resistance of strain H636 to three β-lactam antibiotics, but less-than-twofold changes in resistance to seven other β-lactams (Table 3). For some β-lactam antibiotics in Tables 2 and 3, the increases in MIC were statistically significant ($P < 0.05$ by Fisher's exact test), but it must be noted that the changes were always small. As observed for the Tn*1* protein F-deficient mutant, resistance to norfloxacin was unaffected by the *oprF*::Ω mutation.

Altered outer membrane barrier properties of protein F-deficient mutants. The levels of increases in MICs for the protein F-deficient mutants, compared with their parent strains, were less extreme than expected. We considered the possibility that the loss of protein F caused a change in the overall structure of the outer membrane, thus enhancing antibiotic uptake via nonporin pathways and counteracting the effects of the loss of protein F in our insertion mutants.

TABLE 2. Geometric mean MIC determinations for the *oprF*::Tn*1* mutant H608 and the wild-type strain H103(RP1::Tn*1*)

Strain	β-Lactamase level (nmol of nitrocefin hydrolyzed/min per mg of cells)	MIC (μg/ml) of[a]:				
		Aztreonam	Ceftazidime	Norfloxacin	Tobramycin	Gentamicin
H103(RP1::Tn*1*)	2.2	1.7	1.0	0.5	2.0	2.0
H608 *oprF*::Tn*1*	1.5	4.0[b]	3.4[b]	0.8	0.7	2.8

[a] Geometric means of four or more independent results.
[b] Significantly different from H103(RP1::Tn*1*) result ($P = 0.05$) by Fisher's exact test. All other strain H608 results were not significantly different from these for this control strain.

TABLE 4. Uptake of NPN by strain H103 and its protein F-deficient derivatives H608 *oprF*::Tn*1* and H636 *oprF*::Ω

Strain	Relative fluorescence (% of maximum)	
	Untreated cells	EDTA-treated cells
H103 *oprF*$^+$	0.95 ± 0.00 (10.5%)	9.05 ± 0.05 (100%)
H608 *oprF*::Tn*1*	5.43 ± 0.46 (50.9%)	10.67 ± 0.17 (100%)
H636 *oprF*::Ω	2.94 ± 0.06 (28.8%)	10.20 ± 0.69 (100%)

Recently, Angus et al. (2) demonstrated that a mutation at the *P. aeruginosa absA* locus caused increased permeability to the hydrophobic fluorescent probe NPN and increased susceptibility to not only hydrophobic antibiotics, but also a wide range of β-lactams. Therefore, we tested our *oprF* mutants for increased permeability to NPN. The protein F-deficient mutants H608 and H636 took up three- to fivefold more NPN than did their protein F-sufficient parent strain, H103, (Table 4). This was about 30 to 50% of the maximum level of NPN that could be taken up by cells after EDTA treatment (Table 4).

DISCUSSION

In this paper we have demonstrated the applicability of recent genetic methodologies, pioneered by Ohman and co-workers (13, 31), for making mutants with insertion mutations in the structural gene, *oprF*, of porin protein F. Transposon mutagenesis in *P. aeruginosa* is still somewhat difficult owing to the tendency of existing transposon delivery suicide plasmids to form whole-plasmid insertion cointegrates with the *P. aeruginosa* chromosome and the low rate of resolution of these cointegrates (32). This becomes an especially difficult problem when coupled to the negative selection (i.e., loss of antigenicity) that one must use to select porin-deficient mutants in the absence of a defined phenotype. Despite the success of researchers in our laboratory in isolating Tn*501* insertion mutants deficient in porin protein P, by using specific antibody screening of transposon insertion mutants following mutagenesis with the suicide plasmid pMT1000, a similar approach was not successful for the *oprF* gene (W. Woodruff, unpublished results). This can be explained in part by the fact that of 3,000 plasmid pWW13 derivatives with a Tn*501* insertion that were screened in this study, none had a Tn*501* inserted in the *oprF* gene, suggesting that there is probably no target site for transposition of Tn*501* into this gene. Nevertheless, the difficulty with which protein F-deficient, nitrosoguanidine-induced mutants were obtained, the large number of background mutations, and the high reversion frequency of the resultant mutants (28), made the isolation of stable insertion mutants highly desirable. Therefore, we used the cloned gene and either in vivo mutagenesis with transposons in *E. coli* or in vitro insertion of a commercially available fragment Ω, together with different gene replacement technologies, to construct mutants with *oprF* insertion mutations in *P. aeruginosa*. Although the creation of the final plasmid, pWW2500, for Ω mutagenesis was quite involved because of problems with convenient restriction sites, we favor this method for the following reasons: first, once such a plasmid is constructed, the gene replacement technology is relatively simple and occurs at a frequency of 60 to 100% per streptomycin-resistant recombinant in our hands; second, the position of insertion of the fragment is controlled by the investigator, whereas with transposons the insertion site or even the possibility of insertion is not known until the plasmid is mapped; third, in general, the plasmid size used in the Ω mutagenesis strategy is smaller because of the relatively small size of Ω (2 kb); fourth, since Ω is not a transposon, excision of Ω is not a substantial cause of concern; fifth, relatively small regions of homology (i.e., 1.2 kb on one side of Ω and 0.35 kb on the other side) were clearly sufficient to drive homologous recombination; and, last, streptomycin, unlike many other antibiotics, is convenient to use in *P. aeruginosa*.

Comparison of MICs of a variety of β-lactam antibiotics for porin-sufficient and porin-deficient mutants demonstrated 1.3- to 3.4-fold increases in resistance in the porin-deficient mutants (Tables 2 and 3). This was in contrast to results for *E. coli* and other members of the family *Enterobacteriaceae* in which porin-deficient mutants were 8- to 32-fold more resistant to certain antibiotics (4, 7, 17, 20, 23). Unfortunately, most of these antibiotics have very high or immeasurable MICs for *P. aeruginosa* and could not be used in these studies. For many of the modern β-lactam antibiotics which have been tested against *E. coli* porin-deficient mutants, only a two- to fourfold increase in MIC was observed over the MIC for *E. coli* porin-sufficient strains (Table 3). This dichotomy in levels of increase in resistance to β-lactam antibiotics of porin-deficient *E. coli* strains (i.e., large versus small increases) has been previously noted by other researchers (7, 17).

It could be argued that the antibiotic MICs for our protein F-deficient mutants were influenced by the perturbation of outer membrane barrier properties caused by the loss of protein F (Table 4). Nevertheless, the data presented in Tables 2 and 3 place some doubt on the proposed (28, 39) role of protein F in the uptake of β-lactam antibiotics across the outer membrane of *P. aeruginosa*. This role was proposed on the basis of the observed in vitro channel-forming properties of protein F preparations (3), the decreased rate of permeation of β-lactam antibiotics through the outer membrane of a heavily mutagenized, protein F-deficient *P. aeruginosa* strain (26), and the substantial increases in resistance to β-lactams of a putative protein F-altered mutant (12). However, one possibility that must be considered is that the small (and presumably antibiotic-impermeable) channels observed by Woodruff et al. (34) in protein F preparations represent the actual protein channel size. Thus, the larger channels observed at a low frequency in protein F preparations (3) could represent a contaminating porin protein present in small copy number, as previously discussed (14). We are currently attempting to generate mutants with defined protein F alterations in order to address these issues.

The characterization of the *oprF* mutants with respect to NPN uptake showed that the loss of protein F significantly changed the outer membrane. Protein F constitutes about 15% of the outer membrane proteins and is present at an estimated 200,000 copies per cell (1, 3, 25). The mutants did not appear to compensate for the loss of protein F by an increase in existing outer membrane proteins or by the synthesis of new proteins. In addition, the outer membranes of the *oprF* mutants were considerably more permeable to NPN, a hydrophobic probe which is largely excluded by the wild-type cells (19). A possible explanation for this observation could be that the gaps left by the loss of protein F are simply filled in by lipidic material. Thus, these NPN data are consistent with a structural role for protein F in the outer membrane.

Recently, the gene for protein F was sequenced (7). Sequence homology between protein F and other outer membrane proteins was not extensive. Since the highest sequence homology observed was between protein F and the

OmpA proteins of members of the family *Enterobacteriaceae*, the question of further analogy between these proteins could be raised. In addition to sequence homology, protein F shares the following characteristics with the OmpA protein: they have similar molecular weights (6, 9), strong peptidoglycan and lipopolysaccharide LPS association (24, 39), and substantial stable β-sheet structure (24, 39), and they are similarly heat modifiable on SDS-polyacrylamide gels (16, 27). The OmpA proteins have not been characterized with respect to channel-forming ability, but are reported to play a structural role in the outer membrane (33). Although some of the above characteristics are also found in the *E. coli* porin proteins OmpC and OmpF, the possibility does exist that protein F and OmpA are structurally analogous and evolutionarily related.

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