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# *Pseudomonas aeruginosa* Outer Membrane Permeability: Isolation of a Porin Protein F-Deficient Mutant

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A mutant of *Pseudomonas aeruginosa* severely deficient in outer membrane protein F levels was isolated by screening heavily mutagenized strains for membrane protein alterations on sodium dodecyl sulphate-polyacrylamide gel electrophoresis. To provide a basis for phenotypic comparison, three independent spontaneous revertants with normal protein F levels were isolated. Neither the protein F-deficient mutant nor its revertants had gross surface alterations as judged by their sensitivities to 31 phages with diverse receptors and their low degrees of leakage of periplasmic β-lactamase into the supernatant. Outer membrane permeability was measured in whole cells by examining the rates of hydrolysis of a chromogenic β-lactam, nitrocefin, by periplasmic RP1-encoded β-lactamase. It was found that the outer membrane permeabilities of wild-type and protein F revertant strains were similar, but low when compared with those of *Escherichia coli* and an antibiotic-supersusceptible mutant Z61 of *P. aeruginosa*. The loss of protein F caused a further significant decrease in outer membrane permeability. The results suggest that protein F is a pore-forming protein in vivo and that only a small proportion, as few as 1 in 400, of the protein F molecules form active functional channels in vivo.

The cell envelope of gram-negative bacteria consists of a cytoplasmic (inner) membrane, a layer of peptidoglycan, and an outer membrane. The outer membrane has been shown to act as a molecular sieve which determines, mainly on the basis of molecular size, whether a given substrate will enter the cell (14). This property results from a species of transmembrane proteins called porins, which form large water-filled pores through the hydrophobic core of the outer membrane (14, 15). Our laboratory has previously demonstrated the presence in the outer membrane of *Pseudomonas aeruginosa* of one constitutive porin, protein F (4, 9), and two inducible porins, proteins D1 (8) and P (10). These properties were demonstrated in vitro with purified proteins in model membrane systems. The results with protein F suggest that this protein forms substantially larger channels than enteric porins (with an exclusion limit for saccharides of approximately 6,000 daltons instead of 600 daltons [4, 9, 14]), but that protein F has relatively low in vitro activity (i.e., only a small proportion of the porin polypeptides present form functional channels [2, 4]). In agreement with this, we demonstrated by the technique of Zimmermann and Rosselet (20) the low outer membrane permeability in vivo of wild-type *P. aeruginosa* cells (2) and provided evidence that this results in the observed high intrinsic resistance of *P. aeruginosa* to antibiotics (2). However, these data did raise an important problem: given the circumstantial evidence of low in vitro and in vivo activity of protein F, we were unable to conclude with certainty that protein F, rather than a minor protein which copurified with it, was responsible for the observed in vitro permeability data. Therefore, we undertook to isolate a mutant deficient in protein F. The results below confirm the pore-forming function of protein F and indicate that less than 1% of protein F molecules form functional channels across the outer membrane.

## MATERIALS AND METHODS

**Bacterial strains.** *P. aeruginosa* PAO1 strain H103 and its gentamicin-resistant mutant strain H181 (13), as well as the antibiotic-supersusceptible *P. aeruginosa* mutant Z61 and its full revertant H251 (2), have been described previously. *Escherichia coli* strain UB1636 *trp his lys rpsL lac lamB*(RP1) was provided by P. M. Bennett (Department of Bacteriology, University of Bristol, Bristol, England). *P. aeruginosa* strain H283 was isolated by random heavy mutagenesis with the general technique outlined by Suzuki et al. (19). *P. aeruginosa* strain H103 was mutagenized with 1 mg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanadine per ml as suggested by Adelberg et al. (1) to between 0.003 and 0.04% survival. A total of 500 survivors were selected for further study. The cell envelopes of each of the 500 clones were isolated and screened for cell envelope alterations by sodium dodecyl sulfate-poly-

acrylamide gel electrophoresis (SDS-PAGE). Only one survivor, strain H283, was found to lack protein F. Isolation of revertants of strain H283 was facilitated by the relatively low growth rate of the protein F-deficient mutant in both minimal and complex media in the absence of added NaCl (see below). Strains H284, H321, and H324 were isolated as independent spontaneous revertants of strain H283 on minimal glucose medium. The plasmid RP1, encoding the TEM-2 β-lactamase, was introduced into each of the above *P. aeruginosa* strains by conjugation with *E. coli* strain UB1636 and selection on 200 μg of tetracycline per ml (for strain H103), 500 μg of carbenicillin per ml (for strains H181, H251, H283, and H284), or 200 μg of neomycin per ml (for strain Z61).

**Medium conditions.** Cells were generally grown in either 1% proteose peptone no. 2 medium or basal medium no. 2 of Gilleland et al. (6), containing 0.5 mM $MgCl_2$ and either 20 mM potassium succinate, pH 7.2, or 0.4% (wt/vol) glucose as a carbon source. Protein F deficiency was tested after each experiment by examining the cell envelope protein profile with SDS-PAGE.

**Membrane isolation and SDS-PAGE.** Cell envelopes and outer membranes were isolated as previously described (7). SDS-PAGE was carried out as described by Hancock and Carey (7) with both 14 and 11% acrylamide running gels and a solubilization temperature of 88°C for 10 min.

**Phage susceptibility testing.** Bacteriophage susceptibility was tested with a multiple-syringe phage applicator as described by Hancock and Reeves (12). The bacteriophage sets which we described previously (13) were used with the addition of phage V4, which was isolated from Vancouver sewage as a phage able to plate on strain H103 and on lipopolysaccharide-altered or pilus-altered variants of H103 (13) but unable to form plaques on the protein F-deficient strain H283.

**Outer membrane permeability.** A method based on the technique of Zimmermann and Rosselet (20) as modified by Angus et al. (2) was used since neither the original technique nor the modification enabled a measurement of outer membrane permeability for strain H283. In particular, we found relatively substantial release of periplasmic β-lactamase during the suspension of cells after centrifugation. Therefore, we further modified the technique as follows. *P. aeruginosa* or *E. coli* strains containing the RP1 plasmid were grown overnight in proteose peptone no. 2 broth at 37°C in the presence of 200 μg of tetracycline per ml or, in the case of strain Z61(RP1), with 20 μg of tetracycline per ml to ensure retention of the plasmid. The overnight cultures were diluted at 1/20 into fresh proteose peptone no. 2 broth and were grown to an optical density at 600 nm of 0.6 to 0.8. A 0.1-ml sample of cells was placed in the sample cuvette of a Lambda 3 dual beam spectrophotometer (Perkin-Elmer Corp., Oak Brook, Ill.). Another 1.5-ml sample was taken at the same time and centrifuged for 1 min at 9,000 × *g* in an Eppendorf microcentrifuge model 5412 (Brinkmann Instruments, Inc., Westbury, New York). The cell-free supernatant was decanted, and 0.1 ml was added to the reference cuvette of the Perkin-Elmer Lambda 3 spectrophotometer. To both reference and sample cuvettes, 0.8 ml of a 0.1 mg/ml solution of the chromogenic β-lactam nitrocefin (16) was added. The differential rate of conversion of nitrocefin to nitrocefoic acid was followed over time at an absorbance of 540 nm with a coupled Perkin-Elmer model 58 strip chart recorder. Since both sample and reference cuvettes contained cell supernatants in common, the differential rate of hydrolysis was a measure of the whole cell hydrolysis of nitrocefin.

## RESULTS

**Characterization of the protein F-deficient strain.** The protein F-deficient strain H283 was isolated after heavy mutagenesis. Thus, one might expect more than one mutation in this strain. We were unable to transfer the specific mutant gene to another strain by genetic means since the only reliable method of screening for the mutant phenotype was by the SDS-PAGE of purified cell envelopes and since frequencies of conjugation and transduction in *P. aeruginosa* tend to be quite low. Therefore, three independent spontaneous revertants which arose during growth on liquid medium were isolated to provide an isogenic wild type for comparison purposes. The revertants H284, H321, and H324 contained wild-type levels of protein F. The isogenicity of these strains with the protein F-deficient mutant H283 was indicated by the following experiment. In an attempt to isolate a protein F-specific phage, we selected phage V4, which could plate on the parent strain H103 but not on the mutant H283. However, the revertants H284, H321, and H324 were also unable to plate on this phage, suggesting that the mutant strain H283 and the revertants H284, H321, and H324 contain at least one other mutation not present in the parent strain H103. Thus, although the phage was not protein F specific, these data do favor the conclusion that the mutant and revertants only differ in the mutation affecting protein F.

The protein F-deficient strain H283 grew more slowly than both its parent and revertant strains in both minimal and rich media. Generation times for the mutant in basal medium no. 2 (6) with succinate as a carbon source were 85 to 95 min, compared with 42 to 45 min for the parent strain H103 and the revertant strain H284. This lowered growth rate was probably not directly related to the porin deficiency discussed below since there was no significant enhancement of growth rate ($P > 0.5$ by the Student *t* test) of the mutant when succinate was replaced by glucose as a carbon source, despite the presence of a glucose-induced porin protein D1. In addition, a 10-fold reduction in the medium concentration of the carbon source did not alter the growth rate of either the mutant strain H283 or its revertant H284. Also, doubling the concentrations of each of the components of the minimal salts medium did not enhance the growth rate. However, the growth rate of the protein F-deficient mutants

could be substantially enhanced by adding 50 mM NaCl to the growth medium, although this addition caused no apparent induction of outer membrane proteins, including protein F. Similar enhancement of the growth rates of three porin-deficient (*ompB*) mutants of *E. coli* by NaCl addition were observed (E. Buenaventura and R. E. W. Hancock, unpublished data) and may well be related to the loss of a major structural outer membrane protein, i.e., porin (18), in these strains.

Protein F has been shown previously to be present in about $10^5$ to $3 \times 10^5$ copies per cell of strain H103. The revertants H284 (Fig. 1C), H321, and H324 contained normal levels of protein F. In contrast, the outer membranes of mutant strain H283 did not contain observable levels of protein F (Fig. 1B). When the electrophoretic mobility of protein F from strain H103 and the revertants was increased by omitting 2-mercaptoethanol from the solubilization buffer (7), no outer membrane polypeptide from strain H283 was similarly modified by 2-mercaptoethanol (Fig. 1D and E).

**Phage sensitivity and surface properties.** To obtain more information about the surface properties of the protein F mutant H283, we screened this strain and the revertants with a set of 31 phages which were able to form plaques on our parent strain H103 and which apparently had a variety of different receptors (13). Strain H283 and its revertants were sensitive to all phages tested, with the above-noted exception of phage V4. This allowed us to conclude that strain H283 and its revertants had smooth lipopolysaccharide, were piliated, and had no gross surface alterations. This last feature was further indicated by the low release of periplasmic β-lactamase (<5% of the enzyme in the periplasm) during growth of the strains containing plasmid RP1 and by the lack of large changes in the levels of

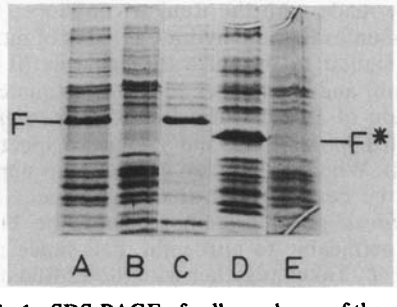


FIG. 1. SDS-PAGE of cell envelopes of the protein F-deficient mutant H283 (lanes B and E), its parent, H103 (lanes A and D), and a revertant, H284 (lane C). For lanes D and E, 2-mercaptoethanol was omitted from the solubilization buffer so that protein F ran in the F* position (7).

any outer membrane proteins other than protein F (Fig. 1). Strain H283 and its revertants were normally inducible for the glucose-inducible porin protein D1 (8) and for major outer membrane protein H1 (13).

**Outer membrane permeability.** We have previously hypothesized that protein F is a porin, although only indirect in vitro evidence has been provided. To test this hypothesis further, we studied the outer membrane permeability of our protein F-deficient strain H283 and a variety of other strains by the technique of Zimmermann and Rosselet (20). This technique is based on the concept that if enough β-lactamase is present in the periplasm, then the β-lactamase activity of intact cells will be limited by the rate of diffusion of β-lactam across the outer membrane to the periplasmic β-lactamase. Thus, the equilibrium rate of hydrolysis of β-lactam by intact cells ($V_{intact}$) is equal to the rate of diffusion ($V_{diff}$). This allows calculation of an outer membrane permeability parameter $C$ by the equation of Zimmermann and Rosselet $V_{intact} = V_{diff} = C(S_{out} - S_{in})$, where $S_{out}$ is the β-lactam concentration added and $S_{in}$ is the periplasmic concentration of β-lactam, which can be calculated from the Michaelis-Menten equation. The β-lactam used in these measurements was the chromogenic cephalosporin nitrocefin (16), whereas the β-lactamase was introduced into the periplasm of each of the different strains by conjugating in the RP1 plasmid. It was shown that in each case, periplasmic β-lactamase activity was in excess with crypticity values (rate of hydrolysis by broken cells divided by the rate of hydrolysis by intact cells) in the range of 5 to 200 for the various cell types. Control experiments were previously performed (2) to demonstrate that the temperature coefficient of nitrocefin hydrolysis by intact cells ($=V_{diff}$) was consistent with nitrocefin entering the cell via a hydrophilic pathway. In addition, we were able to demonstrate that nitrocefin hydrolysis in intact cells was directly proportional to the concentration of added nitrocefin ($S_{out}$) for strain H103(RP1) and *E. coli* UB1636(RP1) over an eightfold range of substrate concentrations, as predicted by the above diffusion equation (given that under these conditions $S_{in} \ll S_{out}$). It should be noted that although early work suggested that plasmid RP1 caused membrane permeability alterations this has recently been disproved (5).

The results shown in Table 1 reveal that strain H283 is significantly less permeable than its parent strain H103 or the revertant H284. Although the standard deviations of these results were rather high, the ranges of rates of hydrolysis of nitrocefin in intact cells of strains H283 and H103 or H284 did not overlap, and the means were clearly different as judged by the

TABLE 1. Nitrocefin hydrolysis and outer membrane permeability of *P. aeruginosa* H103(RP1) and other organisms

| Organism[a] | | No. of determinations | Rate of nitrocefin hydrolysis in intact cells[b] | Outer membrane permeability coefficient $C$ ($10^4$)[c] | $P$[d] |
|---|---|---|---|---|---|
| Strain | Description | | | | |
| H103(RP1) | Parent | 12 | 60 ± 17 | 4.1 | |
| H181(RP1) | Resistant to gentamicin, polymyxin, and EDTA; overproduces protein H1 | 9 | 62 ± 36 | 4.2 | >0.5 |
| H251(RP1) | Revertant of Z61 | 7 | 58 ± 13 | 3.9 | >0.5 |
| H284(RP1) | Revertant of H283 | 7 | 59 ± 30 | 4.0 | >0.5 |
| H283(RP1) | Protein F deficient | 5 | 9.8 ± 7.6 | 0.7 | <0.01 |
| Z61(RP1) | Antibiotic supersusceptible | 12 | 360 ± 170 | 24.9 | <0.001 |
| UB1636(RP1) | *E. coli* | 13 | 740 ± 390 | 50.6 | <0.001 |

[a] *P. aeruginosa* except where noted.
[b] Picomoles of nitrocefin per minute per milligram of cells (dry weight); means ± standard deviations are shown.
[c] Per second per milligram of cells (dry weight).
[d] Compared with strain H103(RP1) by the Student $t$ test.

Student $t$ test. As a control, we confirmed by this modified permeability assay our previous results (2), which demonstrated that the antibiotic-supersusceptible mutant Z61 was significantly more permeable than either strain H103 or the full revertant strain H251. In contrast, there was no significant alteration in permeability of the aminoglycoside- and polymyxin-resistant mutant H181. An *E. coli* K-12 strain UB1636 had much greater permeability than any of the *P. aeruginosa* strains studied here.

## DISCUSSION

The results presented here provide the first in vivo evidence that protein F is indeed a porin in that a protein F-deficient mutant has a significantly lower mean outer membrane permeability parameter $C$ than its parent or revertant strains. From Fick's diffusion law, we know that this parameter $C = DA_0/d$, where $D$ is the diffusion coefficient for nitrocefin, $A_0$ is the total area of pores available for diffusion, and $d$ is the width of the membrane. Thus, since the diffusion coefficient should remain fairly similar in all strains (with the below-noted exceptions) and the membrane width is generally accepted as a constant, $C$ remains a fairly sensitive measure of the total area of pores available for diffusion. Since strain H283 does have a measurable $C$ value, it may well not be totally porin deficient but rather porin protein F-deficient, with other porin proteins present at lower levels in the outer membrane. Black lipid bilayer studies of fractions from porin-deficient mutants of *E. coli* (3) have provided evidence that a more cation-selective channel is responsible for residual porin activity in these strains. In the case of *P. aeruginosa*, two other inducible porin proteins, P and D1 (8, 10), are possible candidates for providing the residual porin activity of protein F-deficient strain H283.

The results shown in Table 1 are also consistent with our previous hypothesis that the well-known intrinsic antibiotic resistance of *P. aeruginosa* can be explained on the basis of the low permeability of the *P. aeruginosa* outer membrane owing to the properties of porin protein F. Although in vitro experiments have indicated that the area of individual protein F channels is up to threefold larger than the area of *E. coli* porin channels (4) and that *P. aeruginosa* and *E. coli* cells have around the same numbers of molecules of their respective porins per cell (2, 18), *P. aeruginosa* has a significantly lower outer membrane permeability parameter $C$ than *E. coli* does (Table 1). What makes these data more impressive is that the size of nitrocefin (520 daltons) approaches the exclusion limit of the *E. coli* porins. Thus, in this case, the diffusion coefficient for nitrocefin, which would be affected by the frictional and steric interaction of the antibiotic with the walls of the channel (15), would be substantially decreased when compared with, for example, the larger protein F channels which should allow relatively freer nitrocefin diffusion. Correction for these factors can be made with the Renkin equations (17) if one assumes that the hydrated radius of nitrocefin is similar to that of a disaccharide (0.44 to 0.53 nm) and if one uses previous estimates for the radii of *E. coli* porin Ib and *P. aeruginosa* protein F pores (0.65 and 1.1 nm, respectively [4, 15]). When this Renkin correction is applied, it can be demonstrated that single pores of *P. aeruginosa* porin protein F should be 11-fold more permeable to nitrocefin than single pores of *E. coli*. Taken together with the 12-fold difference in $C$ values demonstrated in Table 1, this makes a total difference of 132-fold in the outer membrane pore area available for nitrocefin diffusion. Since the actual area of a single *P. aeruginosa* channel is three times that of a single *E. coli* channel, the total number of active and

functional protein F channels per milligram of cells (dry weight) can be estimated by the formula {[total area of *P. aeruginosa* channels per milligram of cells (dry wt)]/[total area of *E. coli* channels per milligram of cells (dry wt)]} × [(single channel area of *E. coli*)/(single channel area of *P. aeruginosa*)] = (1/132) × (1/3), or 1/400 that of *E. coli*. Chemical cross-linking data have suggested that protein F, like *E. coli* porins, is arranged as a trimer in the membrane (B. L. Angus and R. E. W. Hancock, Abstr. Int. Cong. Microbiol., P516, p. 135, 1982). The number of trimers per cell of both *E. coli* (14, 18) and *P. aeruginosa* (2) have been calculated as approximately $4 \times 10^4$. Thus, the number of active functional channels in *P. aeruginosa* is about 100, assuming one channel per trimer (3, 4), or about 300 if one assumes three channels per trimer (14). Although we have previously demonstrated that the number of active, functional porin channels can be increased by a lipopolysaccharide mutation in mutant Z61 (2), we have as yet no definitive explanation why >99% of protein F molecules apparently do not form functional pores in the outer membranes of wild-type cells.

We have previously suggested that aminoglycosides and polymyxins cross the outer membrane of *P. aeruginosa* via a pathway that does not involve protein F pores (11, 13). It was suggested that this pathway involves self-promoted uptake in that cationic antibiotics like aminoglycosides and polymyxins interact with a $Mg^{2+}$ binding site on the outer membrane lipopolysaccharide and permeabilize the outer membrane to other antibiotic molecules. The properties of the polymyxin-, aminoglycoside-, and EDTA-resistant mutant H181, in which this site is apparently masked by an outer membrane protein H1, lent strong support to this model. In agreement with this, it was demonstrated (Table 1) that there was no significant difference in outer membrane permeability between the mutant H181(RP1) and its parent strain, H103. Should future data confirm our hypothesis regarding this alternative outer membrane antibiotic uptake route, it would provide an interesting demonstration of adaptation to the decreased porin function of wild-type *P. aeruginosa*.

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