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Role of Protein F in Maintaining Structural Integrity of the *Pseudomonas aeruginosa* Outer Membrane

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To investigate the functional role of protein F of the outer membrane of *Pseudomonas aeruginosa*, we isolated mutants devoid of protein F, and the defective gene was transferred to a wild-type strain by plasmid FP5-mediated conjugation. Chemical analyses of the protein F-deficient outer membrane revealed that the amount of outer membrane protein was reduced to 72 to 74% of that of the protein F-sufficient strain and that lipopolysaccharides and phospholipids increased to 117 to 123% and 135 to 136%, respectively. The mutants and the transconjugant showed the following characteristics: (i) growth rates of protein F-deficient strains in low-osmolarity medium (e.g., L broth containing 0.1% NaCl) were less than 1/10 the rate of the protein F-sufficient strain; (ii) protein F-deficient cells were rounded, and the outer membrane formed large protruded blebs; and (iii) the outer membrane became physically fragile, since a significant amount of periplasmic proteins leaked out and the cells became highly sensitive to osmotic shock. The results suggested that protein F plays an important role in morphogenesis and in maintaining the integrity of the outer membrane. Determination of the diffusion rates of saccharides and β-lactam antibiotics showed that the protein F-deficient outer membrane had no detectable transport defect compared with the protein F-sufficient outer membrane. The MICs of antibiotics for the protein F-deficient strains were nearly identical to those for the protein F-sufficient strain.

The outer membrane proteins of gram-negative bacteria play roles in forming diffusion pores stabilizing the outer membrane; that is, they form porins (22) for small hydrophilic solutes. These outer membrane proteins, which include OmpF, OmpC, and OmpA (20), are involved in the receptor function for bacteriophages, bacteriocins, and sex pili (20). *Pseudomonas aeruginosa*, a pathogen in immunocompromised patients, is highly resistant to a wide range of antibiotics (44). This intrinsic drug resistance is known to be due mainly to the diffusion barrier at the outer membrane (1, 42). In permeability studies of the outer membrane of *P. aeruginosa*, protein F was reported to be the porin forming the large diffusion pore. This pore allows the passage of uncharged polysaccharides with M_rs of several thousands as determined by the equilibrium of the labeled polysaccharides in vitro (11) and in vivo (10) and rate measurement by the liposome-swelling method (43). Determination of ion conductivity in a black lipid bilayer supported these results, and the pore diameter was calculated to be about 2.2 nm (2). Nicas and Hancock had isolated a mutant devoid of protein F and showed that the diffusion rate of a chromogenic β-lactam, nitrocefin, across such an outer membrane was lowered to one-sixth that of the protein F-sufficient outer membrane, but the antibiotic susceptibility was essentially unchanged (8, 27). To explain this apparent discrepancy, they proposed that the majority of the protein F pores were nonfunctional, being in a closed state (2, 30). It was also found that more than 99% of protein F formed the small pore and that less than 1% formed the large pore (39).

On the other hand, Caulcott et al. (4) and Yoneyama et al. (40, 41) recently showed that the outer membrane pore(s) in *P. aeruginosa* excluded di- or trisaccharides as determined by measuring the equilibrium of the test solutes across the intact outer membrane. If these recently reported results are correct, one can explain the intrinsic drug resistance of *P. aeruginosa* to structurally unrelated antibiotics without difficulty. To clarify the underlying problems associated with the intrinsic drug resistance of *P. aeruginosa*, we examined the functional role of protein F and found that this protein plays a role in stabilizing the outer membrane.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *P. aeruginosa* PAO1 (a wild-type strain) (13), PAO2354 (*oru-325 puuC10 tyu-9018 ben-9010*) (37), and PAO4089 (*met-9020 pro-9024 blaJ9111 blaP9202*) (18) were kindly provided by H. Matsumoto, Shinshu University. Cells were grown aerobically with vigorous shaking at 37°C in L broth consisting of 1% tryptone (Difco Laboratories, Detroit, Mich.) and 0.5% yeast extract (Difco) supplemented with an appropriate amount of NaCl.

Isolation of protein F-deficient mutants. Strain PAO4089 was treated with 1 mg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml in 100 mM sodium acetate buffer, pH 5.8. The cultures were inoculated on L agar and incubated at 37°C for 48 h. Small colonies were scored. Protein F was extracted by the rapid extraction method (7) and subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE).

Reconstitution of proteoliposome and the permeability assay. Proteoliposome reconstitution and the permeability assay were carried out as described previously (22, 23, 28). Briefly, 1 μmol of phospholipids (97 parts phosphatidylcholine and 3 parts dicetylphosphate) in chloroform was dried by a stream of dried N_2 gas. The tube was kept for more than 2 h in an evacuated desiccator. The phospholipid film was suspended in 200 μl of distilled water and mixed with the purified outer membrane (40 or 50 μg of protein), and the

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mixture was subjected to sonic oscillation for 7 min in a water bath at 20°C. The mixture was dried again and kept in a desiccator as described above. The contents were suspended in 167 μl of an aqueous solution containing 12 mM stachyose, 4 mM sodium NAD, and 1 mM imidazole-NAD buffer, pH 6.0 (29), for antibiotic diffusion assays or 40 mM stachyose and 1 mM 3-(*N*-morpholino)propanesulfonate (MOPS) buffer, pH 7.2, by blending in a Vortex mixer (The Vortex Manufacturing Co., Cleveland, Ohio) for 30 s.

Proteoliposomes (35 μl) were diluted with 665 μl of the isotonic test solution in a 1-ml cuvette. Immediately after mixing, the optical density at 450 nm was recorded at a range of 0.1 with a chart speed of 60 mm/min for 60 s. The stock solution of β-lactams was made to 25 mM in 1 mM sodium NAD–1 mM imidazole-NAD buffer, pH 6.0. The pH of the test solution was carefully adjusted to 5.8 to 6.2 with NaOH solution. The stock solution was then diluted with 1 mM sodium NAD–1 mM imidazole-NAD, pH 6.0 to 18, 9, or 6 mM for zwitterionic, monoanionic, or dianionic β-lactams, respectively. The saccharide solutes were prepared to 40 mM in 1 mM MOPS buffer, pH 7.2

Electron microscopy. Cells were grown in L broth for 3.5 h at 37°C after dilution of a fully grown preculture with 9 volumes of fresh medium. Cells were prefixed with 2.5% glutaraldehyde for 30 min at 4°C. After two washes by centrifugation at 15,000 × *g* for 5 min each, the cells were postfixed with 1% osmium tetroxide for 16 h at 24°C. The sample was dehydrated with absolute ethanol, stained with 2% uranyl acetate, and embedded in Epon resin. Thin sections were stained with lead citrate and uranyl acetate and observed with a JEOL (Tokyo) 1200EX electron microscope. For scanning electron microscopy, bacterial cells were dehydrated with alcohol, suspended in isoamyl acetate, dried with a Hitachi (Tokyo) HCP-2 critical point drier and then further evaporated with Pt-Pd. The surface structure of the bacterial cells was observed with a JEOL JSM-35 scanning electron microscope.

Leakage of β-lactamase. A culture of *P. aeruginosa* harboring plasmid R68.45 grown overnight in L broth was diluted with 20-fold fresh L broth and grown to an optical density of 0.7 at 540 nm. Part of the culture (1.5 ml) was harvested by centrifugation for 5 min at 15,000 × *g*. β-Lactamase activity in the supernatant was determined. Another part of the cell suspension (1.5 ml) was subjected to sonic oscillation in a Heat Systems ultrasonicator for 7 min. β-Lactamase activity was determined at 540 nm in a solution containing 100 μl of the crude extracts and 80 μg of the chromogenic cephalosporin nitrocefin (32) in a 1-ml cuvette. The extent of β-lactamase leakage was calculated by dividing the V_{max} of the 15,000 × *g* supernatant by the V_{max} of the sonic extracts.

Isolation of outer membrane and fatty acid analysis. The outer membrane (3 mg, dry weight) was suspended in 500 μl of freshly prepared 2 N HCl in methanol. Tubes were sealed under N_2 and heated at 100°C for 16 h. The internal reference, 5 μl of tetradecanoic acid methyl ester in methyloctonate (42.93 mg/ml, wt/vol), was added, and the fatty acid methyl esters were quantified at a split ratio of 1:50 with a Shimadzu (Kyoto) 14A gas-liquid chromatography system equipped with a glass capillary column (ca. 0.2 mm by 25 m) containing OV-101. The temperature gradient varied from 80 to 250°C at a rate of 6°C/min. Helium was used as the carrier gas at a flow rate of 0.67 ml/min. Peaks were integrated with a Shimadzu C-R6A Chromatopac.

Other methods. The outer membrane was prepared according to the method of Hancock and Carey (9). SDS-PAGE was performed according to the method of Neville (26). Protein was quantified by the procedure of Lowry et al. (16). MICs of antibiotics were determined by the agar dilution method.

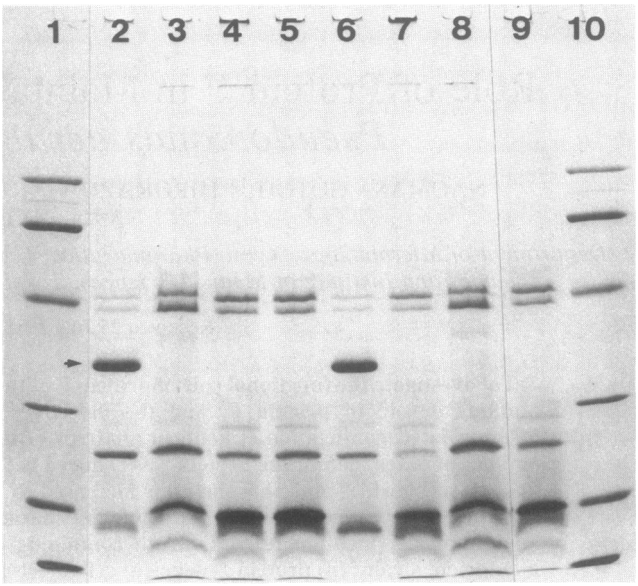

FIG. 1. SDS-PAGE of outer membrane proteins. Cells were grown in L broth containing 0.5% NaCl for 3.5 h after 10-fold dilution of a fully grown preculture, and the outer membrane was isolated according to the method of Hancock and Carey (9). The buffer system used for electrophoresis was that described by Neville (26). A sample containing 40 μg of protein was applied. Lanes: 2, PAO4089; 3, KG1077; 4, KG1080; 5, KG1086; 6, PAO2354; 7, KG1078; 8, KG1079; 9, KG1081. Lanes 1 and 10 are M_r references that contained rabbit muscle phosphorylase *b* (M_r, 974,000), bovine serum albumin (M_r, 662,000), hen ovalbumin (M_r 427,000), soy bean trypsin inhibitor (M_r, 215,000), and lysozyme (M_r, 144,000). Arrow indicates protein F.

Chemicals. NAD and imidazole were purchased from Wako Pure Chemicals, Osuka, Japan. Egg yolk phosphatidylcholine (type V-E) and dicetylphosphate were purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Isolation and general properties of protein F-deficient mutants. Three protein F-deficient mutants, KG1077, KG1080, and KG1086, were selected as reported earlier (7). To avoid the effects of unwanted mutations, the mutation was transferred from KG1077 via plasmid FP5 to a protein F-sufficient recipient, PAO2354. Three protein F-deficient recombinants, KG1078, KG1079, and KG1081, were obtained at a frequency of 5% upon selection for Tyu^+. The SDS-PAGE profile of the outer membrane proteins of the mutants and recombinants showed no visible band corresponding to protein F (Fig. 1). Most data presented here were obtained from experiments using these transconjugants. The lack of protein F in KG1077 was also confirmed by the Western blotting (immunoblotting) method, using monoclonal antibody against protein F (R.E.W. Hancock, personal communication).

Growth rates of the protein F-deficient strains were very low. In fact, the generation times of KG1077 in L broth containing 2.0, 1.0, 0.5, 0.25, and 0.1% NaCl were 139, 134,

TABLE 1. General properties of protein F-deficient strains

Strain	Generation time[a] (min) at NaCl content (%) of:					Viability upon osmotic shock[b] (%)	Extracellular β-lactamase[c] at NaCl content (%) of:		Bleb formation[d] (%)
	2.0	1.0	0.5	0.25	0.1		0.5	2.0	
PAO4089	ND[e]	46	45	46	43	91	7.5	0	0
KG1077	139	134	151	301	1,204	27	ND	7.0	11.5
KG1080	98	79	86	125	452	13	17.3	6.6	8.7
PAO2354	ND	53	57	54	56	86	15.1	0	0
KG1078	ND	42	56	144	1,806	36	31.0	8.5	12.0

[a] Determined on the basis of the rate of increase in optical density in medium containing 1% tryptone, 0.5% yeast extract, and the indicated concentrations of NaCl.
[b] Cells were grown in L broth supplemented with 0.5% NaCl and 20% sucrose to $A_{600}^{1\,cm} = 1.0$, and a portion of the culture was diluted into a 1,000-fold volume of fresh medium without sucrose at 37°C. The number of CFU in the shock fluid was determined.
[c] Leakage of β-lactamase was determined in L broth containing 0.5% NaCl for 3.5 h at 37°C after dilution of a fully grown preculture.
[d] Cells with blebs were scored under a phase-contrast microscope. Culture medium contained 0.5% NaCl.
[e] ND, Not determined.

151, 301, and 1,204 min, respectively (Table 1). The generation times of the F-sufficient strains PAO4089 and PAO2354 were nearly constant in these media (Table 1). Viability of the protein F-deficient strains rapidly dropped in L broth containing less than 0.5% NaCl. Stability against osmotic shock was examined by diluting cells grown in L broth supplemented with 20% sucrose to a 1,000-fold volume of L broth without sucrose. Viabilities of KG1077 and KG1080 were 27 and 13%, respectively, whereas that of PAO4089 was 91% (Table 1). Similar results were obtained for the protein F-deficient transconjugant KG1078 (Table 1).

Leakage of periplasmic proteins was also measured. Extracellular β-lactamase from the protein F-deficient strains appeared to be about twofold higher than that of the respective parent strains (Table 1). Leakage of β-lactamase could be suppressed to about 70% of that in the protein F-sufficient strains as NaCl in the medium was raised to 2%. These results suggest that the outer membrane of the protein F-deficient strains is physically fragile.

Permeability properties of the protein F-deficient outer membrane. Protein F was reported to be the porin forming the large diffusion pore and small pore (2, 10, 11, 39, 43). Therefore, one can assume that the low growth rates of the mutants (Table 1) were the cause of poor nutrient transport at the outer membrane. To test this assumption, the diffusion rates of saccharides and the β-lactam antibiotics were determined by the liposome-swelling technique. The diffusion rates of ribose appeared to be 50, 49, 64, and 40 arbitrary units in liposomes containing the outer membranes of PAO1, KG1078, KG1079, and KG1081, respectively (Table 2). The relative diffusion rates of ribose, glucose, galactose, α-methylgalactoside, and α-methylmannoside in liposomes containing the outer membrane of PAO1 were 100, 58, 40, 10, and 3, respectively (Table 2). Similar results were obtained for liposomes containing the outer membranes of KG1078, KG1079, and KG1081. The relative diffusion rates of imipenem in liposomes containing the outer membranes of PAO1, KG1087, KG1079, and KG1081 were 100, 130, 125, and 141 arbitrary units, respectively (Table 3).

Diffusion rates of two new β-lactams were also examined. The relative diffusion rates of CGP31608 (M_r, 262) (25) and CS-533 (M_r, 339) (24) among these strains were similar to those of imipinem, although the former was more and the latter was less permeable than imipenem (Table 3). Attempts to determine the diffusion rates of several other β-lactams were unsuccessful because the rates were too low to detect by the method used. These results suggest that protein F plays little role in formation of the hydrophilic diffusion pores. The higher diffusion rates of saccharides and β-lactam antibiotics in liposomes containing the protein F-deficient outer membrane than in liposomes containing the protein F-sufficient outer membrane were probably due to incorporation of a relatively large amount of porin(s) into the liposomes, since comparison was made on the basis of total outer membrane proteins.

MICs (in micrograms per milliliter) of antibiotics for PAO4089, a protein F-sufficient strain, were as follows: imipenem, 0.2; CGP31608, 0.78; CS-533, 3.13; piperacillin, 6.25; carbenicillin, 6.25; cefsulodin, 0.78; cefoperazone, 3.13; ceftazidime, 0.39; kanamycin, 50; gentamicin, 1.56; tobramycin, 0.39; amikacin, 3.13; tetracycline, 12.5; and polymyxin B, 0.78. MICs for all mutants and recombinants tested were indistinguishable from those for the parent strain (data not shown). These MIC determinations fully support the results of the permeability experiments.

Morphological appearance of protein F-deficient cells. Scanning electron microscopy of the protein F-deficient cells revealed a rounded cell shape, compared with the rod shape of the protein F-sufficient cells (Fig. 2B and C). The ratio of latitudinal/longtitudinal length of the mutants appeared to be 0.59 (n = 115), significantly higher than the ratio for the parent strain, 0.20 (n = 105). Electron microscopy of thin sections showed large extruded blebs in the outer membrane (Fig. 2E and F). The blebs were not limited to a specific

TABLE 2. Relative diffusion rates of saccharides through liposome membranes containing the outer membrane of protein F-sufficient and -deficient strains

Saccharide	Relative diffusion rate[a]			
	PAO1	KG1078	KG1079	KG1081
Ribose	100	100	100	100
Glucose	58	90	74	64
Galactose	40	49	49	45
α-Methylgalactoside	10	23	16	13
α-Methylmannoside	3	5	6	1
Sucrose	0	0	0	0
Raffinose	0	0	0	0

[a] Determined by the liposome-swelling assay, using proteoliposomes reconstituted from 50 μg of protein of the purified outer membrane per μmol of phospholipid as described in Materials and Methods. The buffer system was 1 mM MOPS, pH 7.2. Diffusion rates were calculated from dA^{-1}/dt as described earlier (29, 43; Yoshihara et al., in press) and expressed as values relative to those for ribose.

TABLE 3. Relative diffusion rates of β-lactam antibiotics through liposome membranes containing the outer membrane of protein F-sufficient and -deficient strains

β-Lactam	M_r	Relative diffusion rate[a]			
		PAO1	KG1078	KG1079	KG1081
Zwitterionic compound					
CGP31608	262	120	147	150	159
Imipenem	299	100	130	125	141
CS-533	339	73	93	90	98
Cefaloridine	415	24	23	26	25
Aspoxicillin	475	25	24	25	23
Monoanionic compound					
Cefroxime	410	19	20	18	17
Cefoxitin	426	17	15	17	18
Cefazolin	453	19	20	22	21
Cefotaxime	454	29	25	23	22
Cefmetazole	470	22	22	20	25
Dianionic compound					
Sulbenicillin	412	27	25	28	28
Moxalactam	518	22	21	23	25
Cefriaxone	552	22	23	25	21
Compound with one positive and two negative charges					
Nocardicin A	499	20	19	19	22
Cefsulodin	531	25	26	24	22
Ceftazidime	545	18	19	16	20

[a] Determined by the liposome-swelling assay, using proteoliposomes reconstituted from 40 μg of protein of the purified outer membrane per μmol of phospholipid as described in Materials and Methods. The buffer system was 1 mM MOPS, pH 6.0. See the footnote to Table 2 for additional details. Values of less than 30 were interpreted as indicating that the solutes were not permeable under the assay conditions used.

surface area but were scattered over the cell. The blebs were not of uniform size. It might be thought that the blebs seen in Fig. 2 are an artifact of fixation. This is unlikely, however, since the frequency of blebs in live cells under phase-contrast microscopy was higher (about 10% of the cell population) than was seen under electron microscopy (Fig. 3 and Table 1). This difference might be due to shearing of the blebs during preparation of the specimen for electron microscopy. Bleb formation became more significant in low-salt medium. The morphology of the mutant cells strongly supports the notion that protein F may be involved in maintaining outer membrane integrity.

Chemical analysis of the outer membrane. The amount of phospholipids was calculated on the basis of the sum of hexadecenoic acid, hexadecanoic acid, and octadecenoic acid as reported previously (21). By this calculation, phospholipids in the mutant outer membrane increased 36% compared with phospholipids in the protein F-sufficient outer membrane (Table 4). The amount of lipopolysaccharide was quantified from the amount of 3-hydroxydecanoic acid, 2-hydroxydodecanoic acid, 3-hydroxydodecanoic acid, and dodecanoic acid according to the method of Kropinski et al. (14). Results showed that the lipopolysaccharide in the protein F-deficient outer membrane was 117% of that in the protein F-sufficient outer membrane (Table 4). Although the precise surface area of the mutant cells could not be calculated, these quantitative analyses suggested that the gap in the surface area caused by the protein F deficiency was replenished with phospholipids and lipopolysaccharide. This surface alteration in the mutant outer membrane reflected the diffusion rate of a hydrophobic probe, as shown by the increased rate of fluorescence of 1-*N*-phenylnaphthylamine (data not shown).

DISCUSSION

Among the opportunistic pathogens to which immunocompromised patients are susceptible, *P. aeruginosa* persists longest during extensive antibiotic treatments (41). This antibiotic resistance has been though to be due mostly to low antibiotic permeability across the outer membrane (1). The cause of this low outer membrane permeability has been assumed to be the presence of small diffusion pores that allow the diffusion of uncharged saccharides of M_r less than about 350 to 400 (4, 40, 41). However, it was reported earlier that protein F of the outer membrane of *P. aeruginosa* forms the large pore that allows the diffusion of uncharged saccharides with M_rs of several thousands (2, 10, 11, 30). To clarify this apparent discrepancy, we isolated mutants devoid of protein F (similar to the mutant isolated by Nicas and Hancock [27]), and the transconjugants were tested for permeability of saccharide and antibiotics. The results of these experiments suggested that the diffusion rates of saccharides and the low-M_r antibiotics in protein F-deficient outer membranes were comparable to or slightly higher than those of the protein F-sufficient outer membrane (Tables 2 and 3). The fact that the diffusion rates of saccharides and antibiotics in the protein F-deficient strain were unchanged was consistent with the observation that MICs of various antibiotics for the protein F-deficient strains were comparable with those for the protein F-sufficient strains (see Results). These results indicated that protein F does not form the general diffusion pore.

The following lines of evidence support our conclusion. (i) Analyses of the outer membrane proteins of imipenem-resistant strains isolated from imipenem-treated patients and from imipenem-impregnated medium in vitro showed that

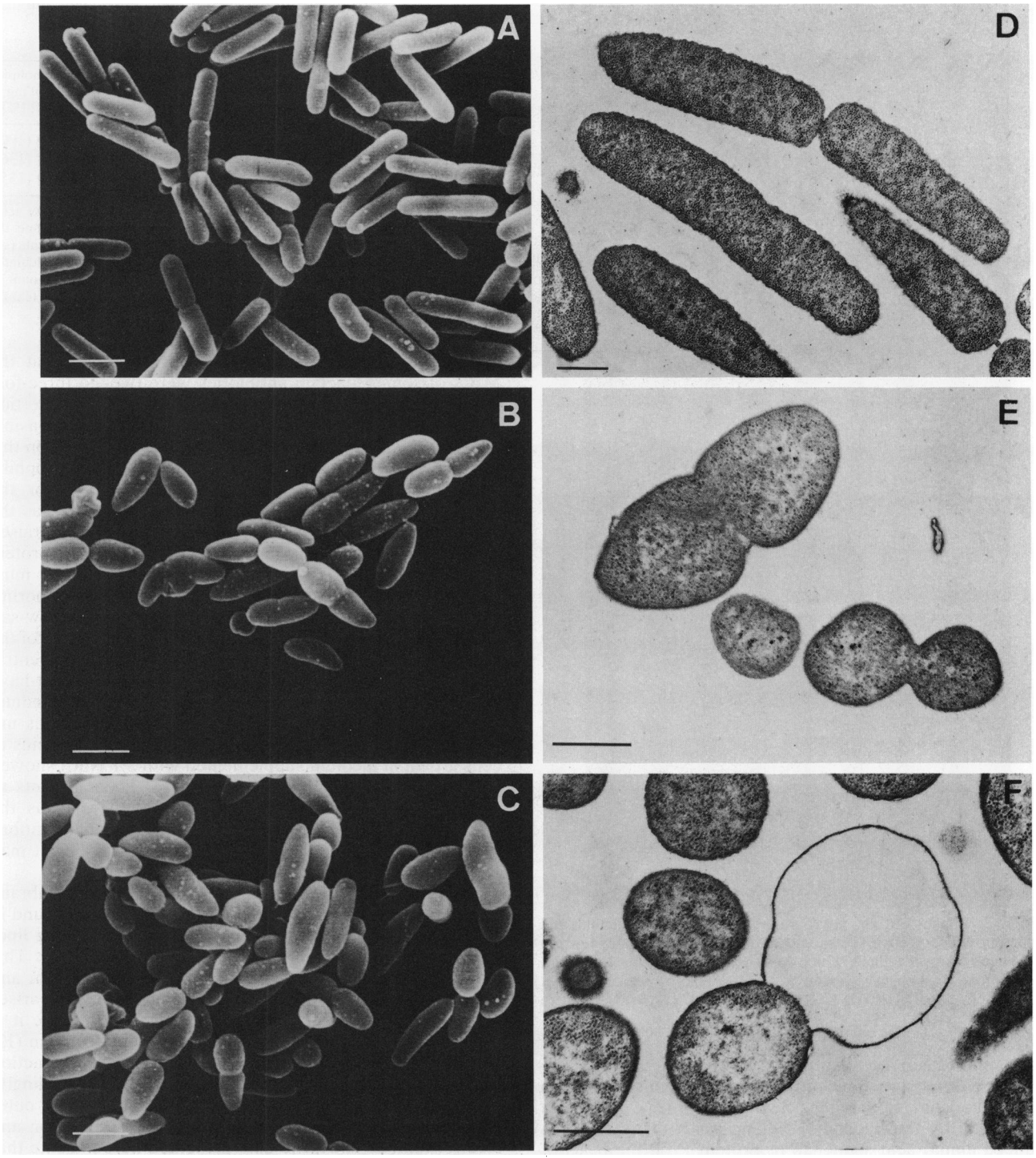

FIG. 2. Electron micrographic comparison of protein F-deficient and -sufficient cells. Experimental details are given in Materials and Methods. (A, B, and C) Scanning electron micrographs of PAO4089, KG1077, and KG1080, respectively (bar, 1.0 μm). (D, E, and F) Transmission electron micrographs of PAO4089, KG1077, and KG1080, respectively (bar, 0.5 μm). Similar studies using transconjugants produced the same results (data not shown).

protein D was missing but that protein F was fully retained (3, 17, 34). Although this observation suggests that protein D forms the imipenem-specific channel, we have firm evidence that protein D is a porin forming the general pore for the passage of pentoses and hexoses but not of saccharides larger than at least trisaccharides (Yoshihara and Nakae, submitted for publication). Recently, Trias et al. identified protein D1 as the missing outer membrane protein in imipenem-resistant strains (36). They showed that the protein D1 pores were selective for glucose and xylose but not for imipenem. (ii) The relative diffusion rates of saccharides in the liposomes (reconstituted from the protein F-sufficient

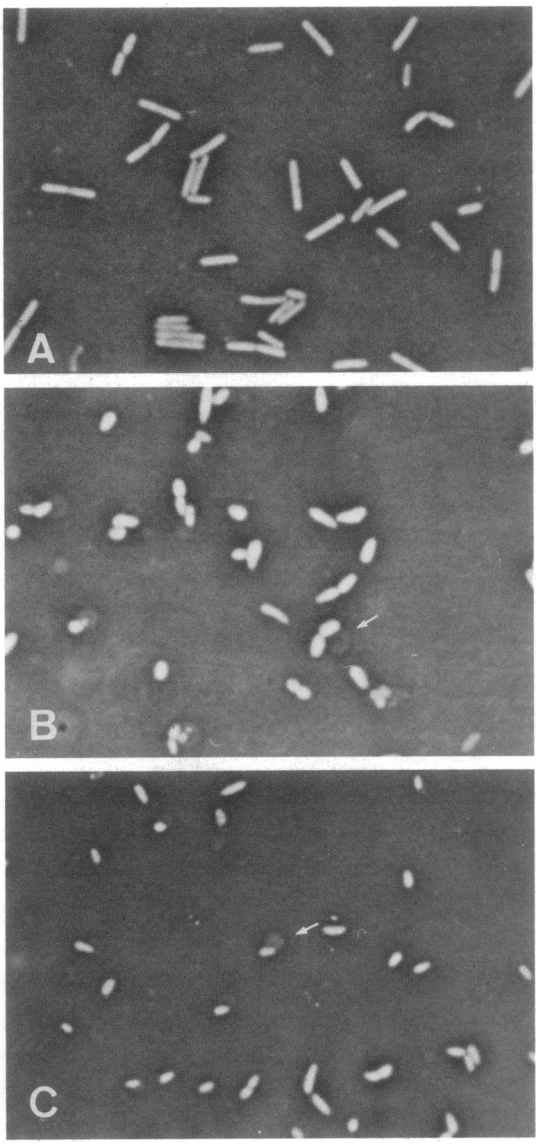

FIG. 3. Phase-contrast micrographs of protein F-deficient and -sufficient cells. A cover glass with a drop of bacterial suspension was placed on a slide glass, which was overlaid with a thin agar film. The sample was observed with a Nikon (Tokyo) phase-contrast microscope. (A) PAO1; (B) KG1077; (C) KG1078. Arrows point to blebs.

outer membrane) containing stachyose (M_r, 666) in the intravesicular space appeared to be indistinguishable from rates in liposomes containing dextran T-10 (M_r, 10,000) as determined by the liposome-swelling assay (41a). (iii) The deduced amino acid sequence of protein F on the basis of DNA sequencing revealed no homology between protein F and OmpF, OmpC, PhoE, or LamB of *Escherichia coli* or the mitochondrial porin (5). Protein F showed the highest homology to OmpA of *E. coli* (5).

The drawback to this conclusion is the isolation of an enoxacin-resistant mutant from enoxacin-treated patients. This mutant is missing protein F and becomes cross-resistant to certain other antimicrobial agents (33). However, it is not certain whether this mutant lacks only protein F or other outer membrane proteins as well, since no outer membrane protein profiles were available.

TABLE 4. Comparison between protein F-deficient and -sufficient outer membranes in protein, phospholipid, and lipopolysaccharide contents[a]

Strain	Protein (μg/mg, dry wt [%])	Lipopolysaccharide (nmol/mg, dry wt [%])	Phospholipids (nmol/mg, dry wt [%])
PAO1	1,240 (100)	158.1 (100)	336.9 (100)
KG1077	918 (74)	184.5 (117)	468.3 (136)
KG1078	895 (72)	195.2 (123)	455.9 (135)

[a] Protein was determined by the method of Lowry et al. (16). Fatty acids were quantified by gas-liquid chromatography after methanolysis, and the data were used to calculate phospholipid and lipopolysaccharide contents (see Materials and Methods). Total amount of lipopolysacchairde was calculated as the sum of $3OHC_{10:0}$, $2OHC_{12:0}$, $3OHC_{12:0}$, and $C_{12:0}$. Total amount of phospholipids was calculated as the sum of $C_{16:0}$, $C_{16:1}$, and $C_{18:1}$. See text for details.

Woodruff and Hancock (38) recently reported that the MICs of some β-lactam antibiotics were two- to three-fold higher for protein F-deficient mutants made by insertion mutagenesis and concluded that protein F was responsible for the diffusion of hydrophilic antibiotics. We question this interpretation, since MICs of only 3 of 11 hydrophilic antibiotics tested showed a substantial increase for the mutant. Since the mutation seems to be very tight, as the immunoblotting assay using a monoclonal antibody raised against purified protein F showed no detectable protein band, the MICs of most of the antibiotics tested must become high (if one assumes that protein F is a porin). Growth rates of the mutants were very poor in low-salt medium and were increased to close to the growth rate of the protein F-sufficient strain by increasing the NaCl concentration to 2%. One could argue that the new porin might have been induced because the cells were grown in medium containing 2% NaCl. However, this possibility seems unlikely, since SDS-PAGE of the outer membrane proteins of cells grown in medium supplemented with 2% NaCl showed no visible new outer membrane protein. Their mutants as well as ours showed nearly normal growth rates as the medium was supplemented with appropriate concentrations of NaCl. This finding suggests that the growth defect may not be due to poor nutrient transport.

Microscopy revealed that the mutant outer membrane tends to form large extruded blebs similar to those found in *E. coli* and *Salmonella typhimurium* mutants missing lipoprotein, OmpF, OmpC, or OmpA (6, 15, 20, 31, 35). This bleb formation became significant in low-salt medium and was lowered in the presence of 2% NaCl. A question arises as to why protein F-deficient cells form blebs. Since it is known that protein F is associated with peptidoglycan (12, 19), the outer membrane may be stabilized by the interaction with the peptidoglycan sacculi via protein F. Accordingly, deletion of protein F may cause detachment of the outer membrane from peptidoglycan, resulting in protrusion and bleb formation. Thus, the data presented here indicate that protein F of the *P. aeruginosa* outer membrane plays a role in stabilizing the outer membrane but that the protein plays little role in formation of the general diffusion pore. This latter conclusion is supported by the finding that proteins C, D, and E of the outer membrane of *P. aeruginosa* form the small diffusion pores in reconstituted proteoliposomes (Yoshihara et al., to be published).

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