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Penetration of β-Lactams through *Pseudomonas aeruginosa* Porin Channels

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Diminished permeation of β-lactam antibiotics in a mutant (PCC23) of *Pseudomonas aeruginosa*, PAO503, was investigated. Resistance to β-lactam antibiotics could not be correlated to a change in the β-lactamase or target proteins in strain PCC23 but was correlated with decreased permeability. In liposome swelling assays, the permeability defect was associated with strain PCC23 porin. Amino acid analysis did not show significant difference of the porin of the mutant (PCC23) from that of the parent (PAO503). Changes in the behavior of isolated porin from PCC23 in migration in sodium dodecyl sulfate-polyacrylamide gels and in response to trypsin digestion as well as preferential labeling of PCC23 by a monoclonal antibody with a preference for the modified form of porin F (F*) indicate that a structural alteration had occurred in this strain and correlated with the change in permeability.

The penetration of hydrophilic molecules through the outer membrane of gram-negative bacteria is governed by water-filled pores consisting of a specific class of proteins, called porins (15, 19). In *Escherichia coli* and *Salmonella typhimurium*, these pores form molecular sieves for neutral molecules up to about 600 daltons (14, 15, 17). In *Pseudomonas aeruginosa*, the molecular sieving function attributed to porin channels is increased to about 9,000 daltons (2, 8).

Purified porin molecules have been incorporated into reconstituted liposomes to provide a "swelling assay," in which purified porin is incorporated into lipid vesicles and, in the presence of a permeable solute, the influx of solute and water causes swelling of the liposomes and reduction in the optical density at 400 nm (20).

The uptake of β-lactam antibiotics has been shown to be dependent upon their penetration through porin channels (9, 21) and to be influenced by the type of lipopolysaccharide (LPS) incorporated into the outer membrane (1, 5).

We describe here a mutant of *P. aeruginosa* which is defective in permeability because of an alteration in the major porin protein (protein F).

MATERIALS AND METHODS

Bacterial strains. *P. aeruginosa* PAO503 *met-9011* and its ticarcillin-resistant derivative PCC23 (5) were used. The organisms were cultured in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) or on brain heart infusion agar (Difco).

Bacterial susceptibility to antibiotics was determined by disk zone sites and broth dilution as described previously (4).

Whole-cell permeability assays were done as described previously (5, 25).

Preparation of porin protein. Two methods were used to obtain purified porin. The first, described by Nakae and Ishii (16), used sodium dodecyl sulfate (SDS) as the extraction detergent. The second method used Triton X-100 for solubilization and was modified slightly from that originally described by Hancock et al. (7). After extraction with 2% Triton X-100 in 0.05 M Tris hydrochloride buffer (pH 7.4), the insoluble fraction was suspended in 1% Triton X-100–10 mM EDTA–20 mM Tris hydrochloride (pH 7.4) at 8 mg/ml. The sample was sonicated (Sonifier; Branson Sonic Power Co., Danbury, Conn.) for 30 s on ice, before centrifugation at 177,000 × *g* for 60 min. The supernatant was loaded onto a Sepharose (6B) column (2.7 by 50 cm) previously equilibrated with 0.1% Triton X-100–10 mM EDTA–50 mM Tris hydrochloride (pH 7.4). Forty 6.7-ml fractions were collected by using column buffer, followed by elution with 50 ml of column buffer containing 0.1 M NaCl, and 6.7-ml fractions were again collected. Column buffer (300 ml) containing 0.3 M NaCl was added, and 6.7-ml fractions were again collected. Each fraction was assayed for protein and plotted, and fractions corresponding to a protein peak were pooled. Pooled fractions were concentrated by dialysis against 30% (wt/vol) polyethylene glycol 4000, before reduction of Triton X-100 by dialysis against 5% (vol/vol) ethanol in water for 22 days at 4°C.

Liposomes. Liposomes were constructed by the procedure of Nakae and Nakae (13) with minor modification. Phosphatidylethanolamine from *E. coli* (Sigma Chemical Co., St. Louis, Mo.) was used as the sole phospholipid. Porin protein, when included, was added at 10 μg/μmol of phospholipid. LPS, extracted from the test strains (5), was added at 60 μg/μmol of phospholipid. Liposomes were processed and handled as described previously (13).

Swelling assays in the presence or absence of test β-lactams, or glucose, were done as described previously (13).

Analysis of porin peptides. Partially purified porin (50 μg) was suspended in gel-loading buffer, minus 2-mercaptoethanol (25 μl), incubated at room temperature for 10 min before being loaded onto 10% SDS-polyacrylamide gels. Porin monomers were recovered by slicing Coomassie brilliant blue-stained proteins from polyacrylamide slab gels of partially purified porin and electroelution through 1-ml polyacrylamide tube gels into dialysis tubing (3,500 M_w cutoff; Spectrum Medical Industries Inc., Los Angeles, Calif.).

Material collected in the dialysis tubing was dialyzed against a 1,000-fold excess of 10 mM Tris hydrochloride (pH 8.1)–10% methanol for 48 h at 4°C. The dialysate was collected and lyophilized to dryness. The protein concentra-

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TABLE 1. MICs for strains used

Antibiotic	MIC (μg/ml) for strain:	
	PAO503	PCC23
Azlocillin	6.25	250.0
Carbenicillin	25.0	125.0
Ticarcillin	12.5	125.0
Piperacillin	1.5	125.0
Cefotaxime	1.5	500.0
Cefsulodin	1.5	12.5
Ceftazidime	0.7	15.0
Moxalactam	6.25	125.0
Imipenem	0.375	1.5
Tetracycline	6.25	3.1
Streptomycin	25.0	12.5
Gentamicin	3.1	1.5
Polymyxin	3.1	0.75
Rifampin	25.0	25.0
Norfloxacin	0.6	0.15
Enoxacin	1.5	0.75

tion was determined by the method of Lowry et al. (10). Trypsin digests were done by treatment of 1 mg of the test protein at 37°C with 10 μg of trypsin (Sigma) for 3 h, followed by lyophilization of the end products. Lyophilized material was suspended in a minimal amount of 35 mM pyridine-acetate (pH 3.0) and applied to a cation exchange column (Aminex A5; Bio-Rad Laboratories, Rockville Centre, N.Y.) previously equilibrated in 35 mM pyridine-acetate (pH 3.0). After a brief wash with 35 mM pyridine acetate (pH 3.0; 10 ml), peptides were eluted with two pyridine-acetate gradients. The first gradient was 25 ml of 35 mM pyridine-acetate (pH 3) plus 25 ml of 200 mM pyridine-acetate (pH 3.2). This was followed by a 50-ml gradient of 25 ml of 200 mM pyridine-acetate (pH 3.2) plus 25 ml of 2 M pyridine-acetate (pH 5.0). The columns were finally washed with 2 M pyridine-acetate (pH 5.0). Fractions (1.5 ml) collected from these columns were lyophilized and suspended in 10 μl of *n*-butanol-acetic acid-water (20:80:20) before being spotted onto silica gel thin-layer plates (Bio-Rad). The dried thin-layer chromatographic plates were developed in *n*-butanol-acetic acid-water by ascending chromatography until the solvent front had traveled approximately 15 cm. Plates were removed from the chromatographic tank and dried. Peptide spots were detected by interference fluorescence under short-wave UV illumination.

Peptide cleavage products of [^{125}I]porin. Porin monomers were excised and eluted from SDS-polyacrylamide gels and concentrated either by dialysis against polyethylene glycol (PEG 20,000) or in an Amicon filtration apparatus (Amicon Canada Corp., Oakville, Ontario, Canada). The protein concentration of the concentrated material was determined by the method of Lowry et al. (10), and purity was checked by running 10 μg of protein on SDS-polyacrylamide gels. A 180-μg portion of each porin was iodinated by adding protein to 500 μl of phosphate-buffered saline (0.01 M sodium phosphate, pH 7.2, 0.89% NaCl) in a capped polypropylene tube. A 0.1-mCi solution of Na^{125}I (Amersham Corp., Arlington Heights, Ill.) (10 μl) was added, and the reaction was initiated by the addition of two washed iodobeads (Pierce Chemical Co., Rockford, Ill.). Preparations were incubated, with gentle agitation, at room temperature for 15 min, followed by transfer of the fluid to another tube containing sufficient NaI to give a final concentration of 0.5 M.

Labeled porin was separated from free ^{125}I by passage through a PD-10 column (Pharmacia, Uppsala, Sweden), preequilibrated with 50 ml of double-distilled H_2O. Twenty 0.5-ml fractions were collected from the column, and 2-μl samples from each fraction were assayed on a gamma counter (Beckman Instruments, Inc., Fullerton, Calif.). Labeled porin eluted in the first peak, fractions of which were pooled and concentrated by dialysis against solid polyethylene glycol. Protein concentrations were determined. Labeled porin (40 μg for PAO503, 200 μg for PCC23) was digested with 30 μg of trypsin per ml for 3 h at 37°C and loaded onto 15% SDS-polyacrylamide gels. Porin peptides and digests were detected by autoradiography of the dried gels on X-Omat film (Eastman Kodak Co., Rochester, N.Y.). Exposure times varied from 2 h to 7 days, depending on the protein concentration and the strain used.

Detection of porin and LPS antigens on whole cells. Cellular surface antigens, detectable by porin-specific monoclonal antibody (MCA 48H3), and an anti-O-specific LPS monoclonal antibody (6) with conjugated protein A-gold (S.P.I. Supplies, Toronto, Ontario, Canada) were observed as described previously (6).

RESULTS

P. aeruginosa PCC23 was derived from strain PAO503 *met-9011* by ethane methane sulfonate mutagenesis and selection for resistance to ticarcillin (5). The mutant acquired resistance to β-lactams other than ticarcillin (Table 1), and the MIC was reduced for all of the non-β-lactam antibiotics tested, with the exception of rifampin (Table 1).

Strain PCC23 had a normal, inducible β-lactamase and bound ^{3}H-labeled penicillin G (New England Nuclear Corp., Boston, Mass.) with affinities and a pattern similar to those of parent PAO503 (data not shown).

The protein profile of the outer membrane in strain PCC23 showed a minor variation, as discussed previously (5). Partial purification of the porin proteins from both strains PAO503 and PCC23 indicated that this difference may be a modification of some of the porin molecules in PCC23 (Fig. 1). In this figure, silver-stained partially purified porin from both strains is shown in lanes a through c, and corresponding immunoblots of the same porin preparations are also shown (lanes d to f). The mutant (PCC23) (Fig. 1, lanes c and d) displayed two prominent fractions recognized by monoclonal antibody MCA-48H3, whereas the parent (PAO503) had only one band recognized by this antibody. The band from strain PAO503 corresponds to the unmodified porin protein in silver-stained preparations.

Liposome swelling assays. The porin preparations, when associated with LPS, were capable of diffusion pore formation in phosphatidylethanolamine-containing liposomes. Swelling could be detected by microscopic examination. Greater than 90% of vesicles containing porin increased significantly in size after 1 min of incubation in moxalactam, compared with vesicles lacking added porin (data not shown). Liposome swelling was detected also by changes in optical density in a spectrophotometer. Swelling rates are shown in Table 2. Various combinations of porin and LPS were attempted, but significant rates were obtained only when both LPS and porin were inserted into the liposomes. This observation is different from that reported by Yoshimura et al. (24), who reported no significant differences in swelling rates with the addition of LPS, and probably reflects the different liposome systems used rather than a real influence of LPS on penetration. Strain PCC23 porin prepa-

TABLE 2. Liposome swelling rates[a]

Liposome containing:		Liposome swelling rate with:				
Porin	LPS	Moxalactam	Carbenicillin	Ticarcillin	Piperacillin	Glucose
		0.001	<0.001			
PCC23		0.004 ± 0.001	0.003 ± 0.001	0.004 ± 0.002	0.004 ± 0.001	0.007 ± 0.003
PAO503		0.005 ± 0.002	0.004 ± 0.002	0.006 ± 0.001	0.003 ± 0.002	0.005 ± 0.003
	PCC23	<0.001	<0.001			
	PAO503	<0.001	<0.001			
PCC23	PCC23	0.003 ± 0.001	0.005 ± 0.001	0.005 ± 0.002	0.006 ± 0.001	0.014 ± 0.003
PCC23	PAO503	0.003 ± 0.002	0.002 ± 0.001	0.006 ± 0.001	0.007	0.019 ± 0.005
PAO503	PAO503	0.075 ± 0.014	0.024 ± 0.008	0.027 ± 0.004	0.020 ± 0.004	0.022 ± 0.005
PAO503	PCC23	0.072 ± 0.017	0.027 ± 0.01	0.067 ± 0.01	0.024 ± 0.007	0.024 ± 0.003

[a] Swelling rates are calculated from the initial decrease (60 s) in optical density at 400 nm after dilution and mixing of the liposomes and a 3-s delay. Averages ± standard deviations are shown.

rations were inefficient at permitting permeation of the tested β-lactams, whether associated with homologous LPS or with strain PAO503 LPS. Porin preparations of strain PAO503, whether associated with strain PAO503 or strain PCC23 LPS, allowed penetration of the β-lactams at rates comparable to (or better than) that for glucose, the control sugar used for each liposome preparation. LPS on its own in liposomes showed no swelling either on microscopy or in the spectrophotometer assay (Table 2). Porin incorporated by itself showed a slight rate for all of the tested solutes. There were no apparent differences between the rates for strains PAO503 or PCC23 LPS and strain PAO503 porin.

Data from the test assays (both porin and LPS incorporated) indicate that the defect was associated with the porin of the mutant (PCC23), since decreased permeation was observed with both species of LPS and no decrease in permeation was observed when parental (PAO503) porin was incorporated with either LPS type.

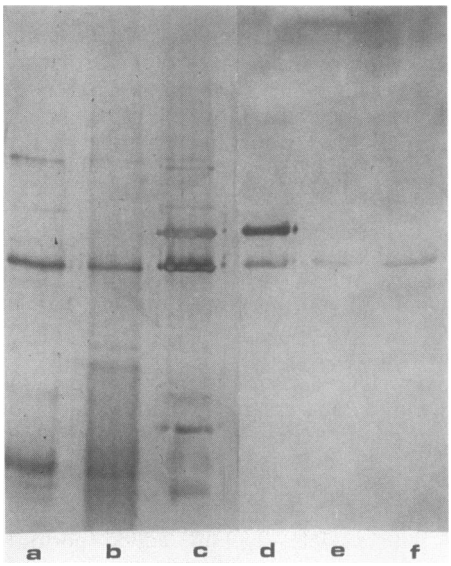

FIG. 1. Silver stains and immunoblots of partially purified preparations (10 μg per well) of strain PAO503 (lanes a, b, e, and f) and strain PCC23 (lanes c and d) porins. Protein preparations were solubilized at room temperature for 10 min in the absence of 2-mercaptoethanol. Silver stains (lanes a to c) indicate some contamination in the preparations prepared with both Triton X-100 (lanes a and c) and sodium lauryl sulfonate (lane b) solubilization. Duplicate lanes, run on the same gel, were used for blotting to nitrocellulose, and porin was detected with a specific antibody (MCA-48H3) (lanes d to f).

Amino acid composition of porin. Porin monomers isolated from SDS-polyacrylamide gel electrophoresis were subjected to amino acid analysis. No significant differences were detected between porin monomers from the two strains or from the composition of *P. aeruginosa* PAO porin published previously (11) (data not shown). The method used was not precise enough to detect single amino acid differences. Attempts at N-terminal sequencing failed for both porin preparations.

Porin modifications. The behavior of porin monomers electroeluted from SDS-polyacrylamide gels with or without heat during the second immersion in loading buffer was evaluated (Fig. 2). Heating at 100°C for 3 min (in the absence of β-mercaptoethanol) did not produce significant conversion of strain PAO503 porin to the modified form (Fig. 2, lane A2). Although the unmodified form of strain PCC23 porin was excised from the original gel, this protein migrated as the modified porin in the second gel (Fig. 2 A, B, and C), suggesting that modification had occurred during electroelution. This observation may explain the apparent aberrant behavior of the mutant (PCC23) porin in response to enzymatic digest (see below).

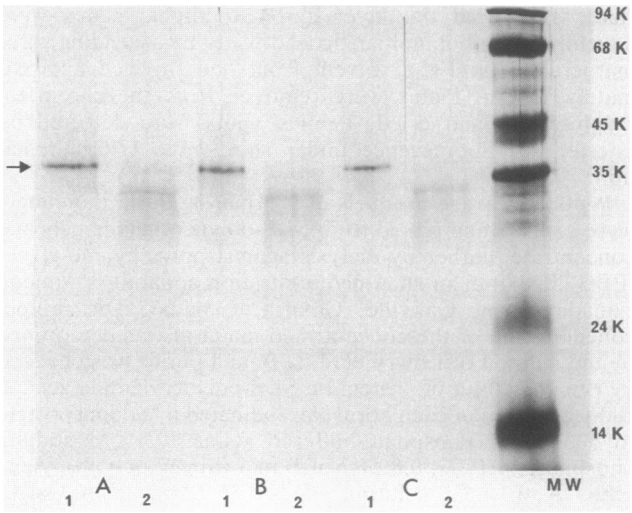

FIG. 2. Silver-stained electroeluted porins from strains PAO503 and PCC23 resolubilized and run on 15% polyacrylamide gels. Resolubilization was done at 100°C for 3 min (A); 37°C for 3 min (B); and 20°C for 3 min (C). The porin from PCC23 is shown in lanes 1, and that from PAO503 is shown in lanes 2. MW, Molecular weight; K, 1,000. The position of the modified porin (F*) is indicated by the arrow.

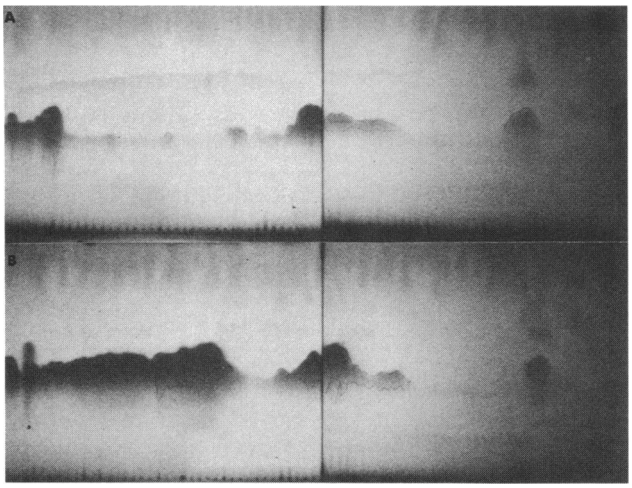

FIG. 3. Trypsin digest products of the porins from PCC23 (A) and PAO503 (B) detected by UV interference fluorescence on silica gel thin-layer plates. Degradation products were separated on an Aminex A-5 column, concentrated, and spotted into the thin-layer chromatographic plates. Plates were developed in (20:80:20) *n*-butanol-acetic acid-water, dried, and photographed under short-wave UV light.

Porin peptides. Trypsin digests of both porins were collected from Aminex A-5 columns, concentrated, and visualized by interference fluorescence on thin-layer chromatographic plates (Fig. 3). The resultant peptide patterns indicated that the protein from strain PAO503 (Fig. 3B) had cleavage products present which were missing in the protein from strain PCC23 (Fig. 3A).

Trypsin digests of electroeluted ^{125}I-labeled proteins were separated on polyacrylamide gels, and the digest products were detected by autoradiography (Fig. 4). Several interesting observations were made during the preparation of iodinated proteins to produce this figure. Although identical protein bands and concentrations were used, the iodination of the mutant (PCC23) porin was consistently reduced 10-fold or more compared with that observed with the parent (PAO503) porin. The iodination was repeated five times with similar results. Consequently, the exposure times and protein concentrations applied to the two gels in Fig. 4 are different. It was evident, however, that trypsin digest was more efficient in the mutant (PCC23), resulting in almost complete degradation of the protein, than in the parent (PAO503), in which two prominent trypsin by-products were observed (Fig. 4). One of these bands (the upper), with a molecular weight of approximately 31,000, corresponds to that observed in the trypsin digest of Mutharia and Hancock (12). The lower band probably resulted from the more extensive enzymatic degradation used here.

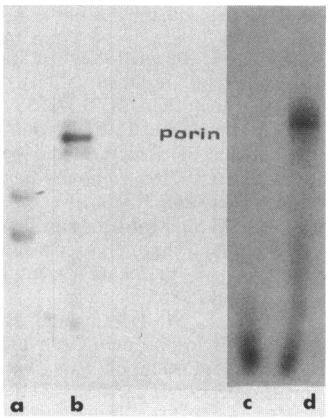

FIG. 4. ^{125}I-labeled porin and trypsin digests of porin from strains PAO503 and PCC25. Trypsin-digested (a) and labeled (b) porin from PAO503 (10 μg of protein) are compared with the digested (c) and undigested (d) porin from PCC23 (40 μg of protein). Exposure times for autoradiography were 24 h (a and b) and 7 days (c and d).

Porin and LPS antigens on whole cells. Comparison of the distribution of O side chain of LPS and porin on PCC23 and PAO503 are shown in Fig. 5. Strain PCC23 (Fig. 5A) was observed to bind both antibodies with an elevated frequency when compared with the parent (Fig. 5B). The increased binding of the O-specific antibody correlated well with the predicted phenotype on the basis of chemical analysis of the isolated LPS from strain PCC23 (5).

DISCUSSION

The resistance of strain PCC23 to β-lactam antibiotics (Table 1) could not be attributed to β-lactamase or changes in target proteins. The resistance did correlate with a decrease in the permeability of this strain (5).

Changes, within the LPS of strain PCC23, should result in an increase in the number of side chain-capped LPS polymers. This situation should result in an increase in the hydrophilic domain on the cell surface and should not hinder penetration of hydrophilic solutes (for a review, see reference 22). But PCC23 was resistant to a range of β-lactam antibiotics which are relatively hydrophilic molecules (3). Permeation of β-lactams was reduced in liposome swelling assays (13) and correlated with the presence of strain PCC23 porin.

Liposome swelling assays and their potential usefulness in the determination of permeability have been reviewed recently (22). The potential interference in the assay caused by charged solutes (22) may induce complex movement of buffer and ions, but qualitative differences between the porins from the two strains should be observable. Given these limitations, it was evident that the porin of strain PCC23 was the limiting factor (Table 2) in the penetration of β-lactams.

The porin of the mutant strain (PCC23) was observed to have several characteristics, which indicated that it was altered when compared with the parent strain (PAO503) porin. *P. aeruginosa* PAO porin, when banded by SDS-polyacrylamide gel electrophoresis, has been shown to have an anomalous migration dependent upon the solubilizing temperature in SDS solutions and the presence of β-mercaptoethanol (11, 12). This behavior was observed with both strain PCC23 and strain PAO503. However, strain PCC23, when solubilized and electrophoresed under conditions which did not modify the parental (PAO503) porin, gave two bands, identifiable by monoclonal antibody, on SDS-polyacrylamide gel electrophoresis. One of the bands corresponded to the strain PAO503 porin, and the other corresponded to the modified F* (11) position (Fig. 1). Further, the lower, unmodified form from strain PCC23 was readily converted to the F* form (Fig. 2). The increased reactivity, on Western blots (immunoblots), of the higher-molecular-weight form in PCC23 reflects the preference of this antibody for the modified form of the porin molecule (K. E. Williams, D. E. Woods, A. J. Godfrey, L. E. Bryan,

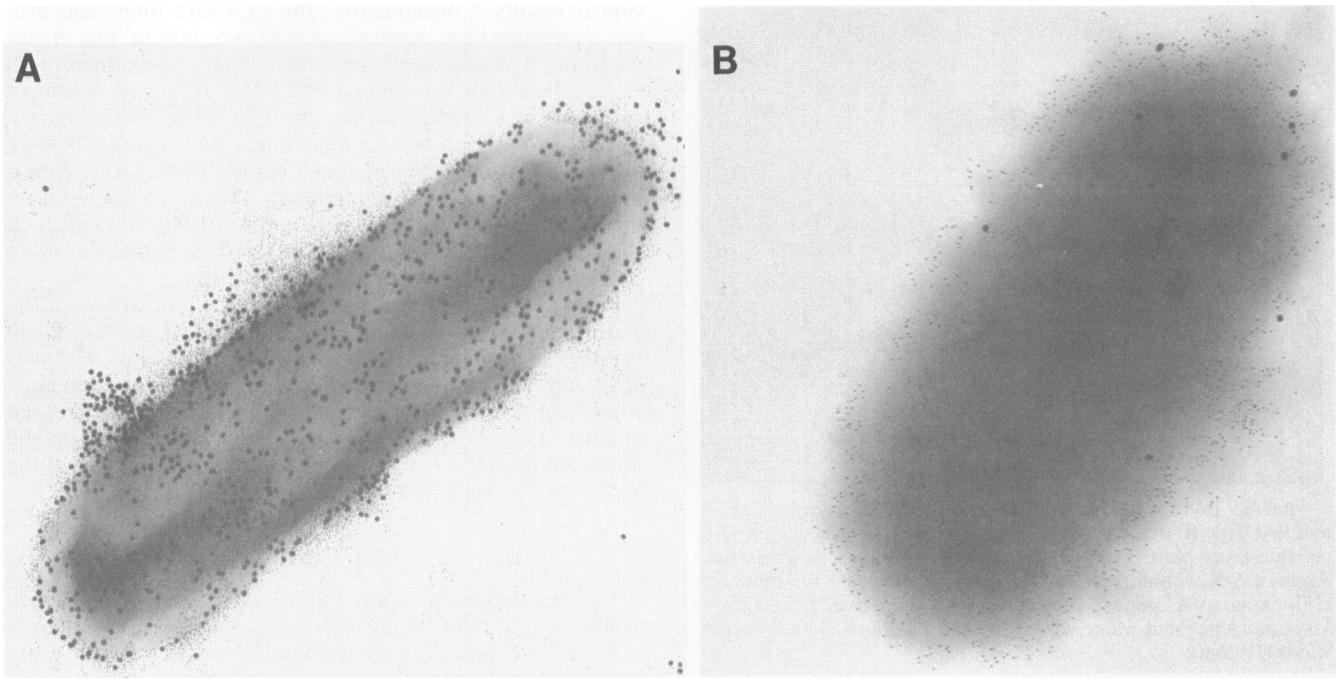

FIG. 5. Distribution of O side chain of LPS (5-nm gold particles) and porin (15-nm gold particles) antigens on strain PCC23 (A) and strain PAO503 (B). Both strains were labeled with monoclonal antibody to LPS and then challenged with the monoclonal antibody against porin.

and H. R. Rabin, submitted for publication). The presence of modified porin occurring naturally (i.e., without heat treatment) on the cell surface is supported by the labeling of the mutant (PCC23) by MCA-48H3 when compared with that for the parent strain (Fig. 5). It is difficult to explain the elevated binding of the anti-porin antibody on a cell displaying a phenotype specifying reduced permeability, unless we speculate that the porin detected was modified (i.e., inactive), or in the small-channel configuration, a situation postulated for the majority of pores in *P. aeruginosa* (1, 18, 22–24). If we accept the latter postulation, then the small-pore configuration would be permeable to glucose but an inefficient pore configuration for β-lactams. In this case, similar rates should be observed for glucose minus the contribution of the larger pores in PAO503, which was observed (Table 2). β-Lactam permeation would then be dependent only on the larger pores, which are presumably absent in PCC23.

The exact nature of the defect in strain PCC23 porin remains to be determined. The amino acid compositions of the porins isolated from both strains were not significantly different from each other or from that previously published for *P. aeruginosa* PAO protein F (11). Trypsin digest and analysis of resulting peptides suggest a difference in the presentation of trypsin-sensitive sites in porins from the two strains. Since total amino acid composition was not significantly altered, the digest data suggest that a single or possibly double amino acid change may have occurred in the mutant porin protein. These changes, in turn, may cause the modified configuration which is associated with a decrease in β-lactam permeation. Such minor changes may be detectable by DNA sequencing of the responsible genes for both the parent and mutant strains.

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LITERATURE CITED

1. **Angus, B. L., A. M. Carey, D. A. Caron, A. M. B. Kropinski, and R. E. W. Hancock.** 1982. Outer membrane permeability in *Pseudomonas aeruginosa*: comparison of a wild-type with an antibiotic-supersusceptible mutant. Antimicrob. Agents Chemother. **21**:299–309.
2. **Benz, R., and R. E. W. Hancock.** 1981. Properties of the large ion-permeable pores formed from protein F of *Pseudomonas aeruginosa* in lipid bilayer membranes. Biochim. Biophys. Acta **646**:298–308.
3. **Biagi, G. L., A. M. Barbaro, M. F. Gamba, and M. C. Guerra.** 1969. Partition data of penicillins determined by means of reversed-phase thin-layer chromatography. J. Chromatogr. **41**: 371–379.
4. **Godfrey, A. J., and L. E. Bryan.** 1982. Mutation of *Pseudomonas aeruginosa* specifying reduced affinity for penicillin G. Antimicrob. Agents Chemother. **21**:216–223.
5. **Godfrey, A. J., L. Hatlelid, and L. E. Bryan.** 1984. Correlation between lipopolysaccharide structure and permeability resistance in β-lactam-resistant *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **26**:181–186.
6. **Godfrey, A. J., M. S. Shahrabadi, and L. E. Bryan.** 1986. Distribution of porin and lipopolysaccharide antigens on a *Pseudomonas aeruginosa* permeability mutant. Antimicrob. Agents Chemother. **30**:802–805.
7. **Hancock, R. E. W., G. M. Decad, and H. Nikaido.** 1979. Identification of the protein producing transmembrane diffusion pores in the outer membrane of *Pseudomonas aeruginosa* PAO1. Biochim. Biophys. Acta **554**:323–331.
8. **Hancock, R. E. W., and H. Nikaido.** 1978. Outer membranes of gram-negative bacteria. XIX. Isolation from *Pseudomonas aeruginosa* PAO1 and use in reconstitution and definition of the permeability barrier. J. Bacteriol. **136**:381–390.
9. **Kobayashi, Y., I. Takahashi, and T. Nakae.** 1982. Diffusion of β-lactam antibiotics through liposome membranes containing purified porins. Antimicrob. Agents Chemother. **22**:775–780.

10. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
11. **Mizumo, T., and M. Kakeyama.** 1979. Isolation and characterization of major outer membrane proteins of *Pseudomonas aeruginosa* strain PAO with special reference to peptidoglycan-association protein. J. Biochem. **86:**979–989.
12. **Mutharia, L. M., and R. E. W. Hancock.** 1985. Characterization of two surface-localized antigenic sites on porin protein F of *Pseudomonas aeruginosa*. Can. J. Microbiol. **31:**381–386.
13. **Nakae, R., and T. Nakae.** 1982. Diffusion of aminoglycoside antibiotics across the outer membrane of *Escherichia coli*. Antimicrob. Agents Chemother. **22:**554–559.
14. **Nakae, T.** 1975. Outer membrane of *Salmonella typhimurium*: reconstitution of sucrose-permeable membrane vesicles. Biochem. Biophys. Res. Commun. **64:**1224–1230.
15. **Nakae, T.** 1976. Outer membrane of *Salmonella*: isolation of protein complex that produces transmembrane channels. J. Biol. Chem. **251:**2176–2178.
16. **Nakae, T., and J. Ishii.** 1980. Permeability properties of *Escherichia coli* outer membrane containing, pore-forming proteins: comparison between lambda receptor protein and porin for saccharide permeation. J. Bacteriol. **142:**735–740.
17. **Nakae, T., and H. Nikaido.** 1975. Outer membrane as a diffusion barrier in *Salmonella typhimurium*: penetration of oligo- and polysaccharides into isolated outer membrane vesicles and cells with degraded peptidoglycan layer. J. Biol. Chem. **250:** 7359–7365.
18. **Nicas, T. I., and R. E. W. Hancock.** 1983. *Pseudomonas aeruginosa* outer membrane permeability: isolation of a porin protein F-deficient mutant. J. Bacteriol. **153:**281–285.
19. **Nikaido, H., and T. Nakae.** 1979. The outer membrane of gram-negative bacteria. Adv. Microb. Physiol. **20:**163–250.
20. **Nikaido, H., and E. Y. Rosenberg.** 1983. Porin channels in *Escherichia coli*: studies with liposomes reconstituted from purified proteins. J. Bacteriol. **153:**241–252.
21. **Nikaido, H., E. Y. Rosenberg, and J. Foulds.** 1983. Porin channels in *Escherichia coli*: studies with β-lactams in intact cells. J. Bacteriol. **153:**232–240.
22. **Nikaido, H., and M. Vaara.** 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. **49:**1–32.
23. **Woodruff, W. A., T. R. Parr, Jr., R. E. W. Hancock, L. F. Hanne, T. I. Nicas, and B. H. Iglewski.** 1986. Expression in *Escherichia coli* and function of *Pseudomonas aeruginosa* outer membrane porin protein F. J. Bacteriol. **167:**473–479.
24. **Yoshimura, F., L. S. Zalman, and H. Nikaido.** 1983. Purification and properties of *Pseudomonas aeruginosa* porin. J. Biol. Chem. **258:**2308–2314.
25. **Zimmerman, W., and A. Rosselet.** 1977. Function of the outer membrane of *Escherichia coli* as a permeability barrier to beta-lactam antibiotics. Antimicrob. Agents Chemother. **12:** 368–372.