# Role of Porins in Intrinsic Antibiotic Resistance of *Pseudomonas cepacia*

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The measured outer membrane permeability of *Pseudomonas cepacia* to the β-lactam nitrocefin was low: approximately 10 times less than that of *Escherichia coli* and comparable to that of *Pseudomonas aeruginosa*. The purified *P. cepacia* porin demonstrated an average single channel conductance in 1 M KCl of 0.23 nS.

The free diffusion of hydrophilic antibiotics, such as β-lactams, to their targets in gram-negative bacteria is retarded by the outer membranes of these organisms (7, 13). Water-filled transmembrane channels (porins) allow hydrophilic solutes to penetrate the membrane, subject to molecular size limits. The permeation properties of a solute are dependent on molecular size and other physiochemical properties of the diffusing molecule (7, 13). The hydrophilic permeability barrier of some species of gram-negative bacteria is considerable, for example, that in *Pseudomonas aeruginosa* (1, 12). In such species reduced diffusion of certain antibiotics across the outer membrane may contribute significantly to the high antibiotic resistance that is observed.

*Pseudomonas cepacia* is being increasingly recognized as a pathogen, particularly in patients suffering from cystic fibrosis (10). Infections with *P. cepacia* may be life-threatening, in part because of the high level of antibiotic resistance that is seen in isolates of this species. Many isolates of *P. cepacia* are highly resistant to most, if not all, β-lactams, as well as to aminoglycosides (6, 9, 13). The mechanism(s) of this intrinsic resistance include the possession by *P. cepacia* of a β-lactamase (15). In this report we demonstrate that the outer membrane makes a major contribution to resistance to hydrophilic antibiotics.

The outer membrane permeability of *P. cepacia* was assessed by the method of Zimmermann and Rosselet (17), as modified by Nicas and Hancock (12). Results of these assays are shown in Table 1. The outer membrane permeability coefficient for *P. cepacia* PC715J were found to be approximately 10-fold less than that found for the *Escherichia coli* control strain. This high outer membrane permeability barrier was equal to that found in the *P. aeruginosa* control strain. These data suggest that *P. cepacia* PC715J has a substantial outer membrane hydrophilic permeability barrier.

To assess further the role of the hydrophilic diffusion pathway in antibiotic diffusion across the outer membrane of *P. cepacia*, the major outer membrane porin was isolated from three strains, PC715J, K61-3, and ATCC 25609. Outer membranes that were isolated as described previously (8) were treated with 2% sodium dodecyl sulfate (SDS) in 10 mM Tris hydrochloride buffer (pH 8.0). The mixture was sonicated and centrifuged at 100,000 × g for 1 h at 20°C. The pellet was extracted with 2% SDS–0.4 M NaCl–10 mM Tris hydrochloride (pH 8.0). The supernatant was acetone precipitated (2 volumes of acetone per volume of solution) at

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4°C for 1 h to concentrate the protein, and the precipitate was suspended at a concentration of 17 mg of protein per ml in 1% SDS–0.4 M NaCl–10 mM Tris hydrochloride (pH 8.0) and then added to a Sephacryl S200 superfine (Pharmacia, Dorval, Quebec, Canada) column (1 m by 25 mm) and eluted with a column buffer consisting of 1% SDS–0.4 M NaCl–10 mM Tris hydrochloride (pH 8.0). The peak fraction that eluted after the void volume was used for studies of porin function.

The preparation of *P. cepacia* porin obtained from strain ATCC 25609 was greater than 95% pure in the oligomer form, as determined by densitometry (Fig. 1, lane 1). After solubilization at room temperature the protein ran as an oligomer with an apparent molecular weight of 81,000 (Fig. 1, lane 1). Solubilization at temperatures to 100°C for periods of less than 30 min resulted in the incomplete breakdown of the oligomer (data not shown). After heating at 100°C for 30 min, however, the oligomer band dissociated into one major and one minor polypeptide species (Fig. 1, lane 2) with apparent molecular weights of 36,000 and 27,000, respectively. Two-dimensional (unheated and heated) SDS-polyacrylamide gel electrophoresis (14) confirmed that both bands were present in the unheated multimer (data not shown). We were unable to find conditions that allowed the separation of these two bands. It is our working hypothesis that the 36,000-molecular-weight band is the true pore-forming protein, because it had a molecular weight that is typical of bacterial porins (7, 13). If this is true, then the lower molecular weight band may be noncovalently but strongly associated with the oligomer band. Alternatively, we cannot exclude with certainty the possibility that the oligomer contained heterologous subunits or that the smaller subunit was a proteolytic digestion product. The electrophoretic mobilities of the bands in Fig. 1 were unaffected by 2-mercaptoethanol.

Functional characterization of porin activity in the preparation described above was done by using the black lipid bilayer technology described previously (2, 3, 4). The addition of small amounts (<1 ng/ml) of the purified porin preparation resulted in a stepwise increase in conductance (Fig. 2). The magnitudes of these increments were clustered around a mean, and the average single channel conductance in 1 M KCl of porin preparations from strain ATCC 25609 was found to be 0.23 nS (461 measured events). Similar data (average single channel conductance, 0.21 to 0.25 nS) were obtained for porin preparations from strains PC715J and K61-3. A decrease in the salt concentration to 0.1 M KCl for strain ATCC 25609 resulted in a concomitant decrease in the average single channel conductance, to 0.03 nS (116 mea-

TABLE 1. Outer membrane permeability of *P. cepacia, P. aeruginosa,* and *E. coli* to the chromogenic β-lactam nitrocefin

| Strain[a] | Rate of nitrocefin hydrolysis by intact cells (nmol of nitrocefin hydrolyzed/min per mg of cell [dry wt])[b] | Outer membrane permeability coefficient C (ml/mg of cell [dry wt] per min)[c] |
|---|---|---|
| *E. coli* C127 | 55.76 | 0.38 |
| *P. cepacia* PC715J | 6.47 | 0.044 |
| *P. aeruginosa* H309 | 5.21 | 0.035 |

[a] *P. cepacia* clinical isolates PC715J and K61-3 were obtained from D. W. Woods, University of Calgary, Calgary, Alberta, Canada. *P. cepacia* ATCC 25609 was the type strain obtained from the American Type Culture Collection (Rockville, Md.). *P. aeruginosa* PAO1 H309 (12) and *Escherichia coli* K-12 C127 (12) have been described previously. Assays were done with strains into which the plasmid RP1 had been conjugated (12). All strains were grown aerobically at 37°C in 1% proteose peptone broth (Difco Laboratories, Detroit, Mich.).

[b] Mean of seven independent determinations; standard deviations of less than 10% were observed; according to Zimmermann and Rosselet (17), this provides an estimate of the equilibrium rate of nitrocefin diffusion across the outer membrane to the periplasmic β-lactamase.

[c] Calculated according to the Fick first law of diffusion, as expressed by Zimmermann and Rosselet (17).

sured events), indicating that the channel was water filled. When approximately 100 channels were incorporated into a lipid bilayer membrane, it was found that the measured current was a linear function of the applied voltage and that the current voltage line passed through zero, indicating that the channels were not voltage regulated or gated.

The ion selectivity of the *P. cepacia* porin channel was determined by zero current potential measurements. Such measurements were difficult to make with *P. cepacia* porin because large numbers of porins in a membrane caused membrane instability. Nevertheless, we were able to demonstrate with 7 separate membranes (15 individual determinations) that the channel was slightly anion selective in KCl (ratio of permeabilities of $K^+$ ($P_{K^+}$) to $Cl^-$ [$P_{Cl^-}$] of 0.50 ± 0.27 as obtained by solving the Goldman-Hodgkin-Katz equation [4] for the zero current potential obtained with different lipid bilayer membranes containing 100 or so *P. cepacia* porin channels).

Results of the studies described above indicate that *P.*



FIG. 1. SDS-polyacrylamide gel electrophoretogram of purified porin protein of *P. cepacia* ATCC 25609. Lane 1, purified protein solubilized at 20°C before electrophoresis; lane 2, the same sample as in lane 1 solubilized at 100°C for 30 min before electrophoresis. Molecular weights (in thousands [K]) are indicated to the right of the gels.
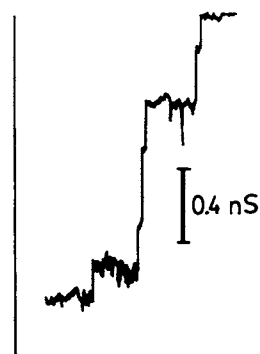


FIG. 2. Conductance steps caused by the addition of a 0.15-ng/ml final concentration of purified porin protein to an aqueous solution (1 M KCl) bathing a small (0.1-mm²) lipid bilayer membrane formed from 1.5% oxidized cholesterol in *n*-decane. The voltage applied was 50 mV, and the temperature was 20°C. The tracing starts at the left. By analogy to other lipid bilayer experiments, these steps represent single channels of *P. cepacia* porin that were incorporated into the membrane. The bar represents 0.4 nS (i.e., 0.4 nA of current measured per volt applied).

*cepacia* has an outer membrane that would considerably retard the diffusion of hydrophilic antibiotics to their targets. Thus, the measured outer membrane permeability of *P. cepacia* for the β-lactam nitrocefin was low; approximately 10-fold lower than that of *E. coli* isolates and comparable to that of *P. aeruginosa* isolates.

The purified porin preparations were highly active, showing pore-forming activity at concentrations in the picogram per milliliter range. The functional characteristics of this porin were analogous to those of the majority of porins that have been investigated to date. The unusual characteristic of this porin channel was its small size, as indicated by its small single channel conductance (0.23 nS), compared with the porins of *E. coli* (1.8 to 2.2 nS) (5). This probably explains the low permeability of the *P. cepacia* outer membrane to nitrocefin. Similarly, *P. aeruginosa*, which also demonstrates high intrinsic resistance to antibiotics, contains a porin, protein F, which predominantly forms small (0.34 nS) channels (16), although in this case it must be pointed out that a small proportion of protein F, unlike *P. cepacia* porin, also forms large (5 nS) channels (16).

Antibiotic resistance is generally high in *P. cepacia* strains (6, 9, 15). The strains used here had resistances to β-lactams, which is typical of most *P. cepacia* strains (6, 9, 15; data not shown). The studies described here indicate that the outer membrane of *P. cepacia* is a major contributing factor in the β-lactam resistance of this species, retarding the diffusion of β-lactams to their penicillin-binding protein targets. Results of other studies in our laboratory (11) have shown that resistance to aminoglycosides and hydrophobic compounds in *P. cepacia* is largely due to low outer membrane permeability as well.

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