Involvement of Outer Membrane of Pseudomonas cepacia in
Aminoglycoside and Polymyxin Resistance

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Pseudomonas cepacia was found to be resistant to the outer membrane-permeabilizing effects of aminoglycoside antibiotics, polymyxin B, and EDTA. Permeabilization of P. cepacia to the fluorescent probe 1-N-phenylalanthylamine was not achieved at concentrations 100-1,000-fold above those required to permeabilize Pseudomonas aeruginosa. Furthermore, in contrast to P. aeruginosa cells, intact cells of P. cepacia did not bind the fluorescent probe dansyl-polymyxin. However, purified lipopolysaccharide (LPS) from P. cepacia bound dansyl-polymyxin with approximately the same affinity as did LPS from P. aeruginosa. Also, binding of dansyl-polymyxin to P. cepacia (and P. aeruginosa) LPS was inhibited by polymyxin B, streptomycin, gentamicin, and Mg2++. These data suggest that P. cepacia does not utilize the self-promoted pathway for aminoglycoside uptake and that the outer membrane is arranged in a way that conceals or protects cation-binding sites on LPS which are capable of binding polycations such as aminoglycosides or polymyxin.

Pseudomonas cepacia has become increasingly important as an opportunistic pathogen. Clinical isolates are usually highly resistant to polymyxin as well as many β-lactam and aminoglycoside antibiotics (3). Consequently, P. cepacia infections are difficult to treat and often life threatening.

Penetration of polymyxin B and, at least in some cases, aminoglycosides into the gram-negative bacterium involves an initial interaction with the outer membrane (5, 10, 12, 14). For Pseudomonas aeruginosa, a self-promoted uptake pathway has been proposed to explain the mechanism of aminoglycoside uptake across the outer membrane (4, 5, 10). In this model, an aminoglycoside or other polycation must interact with a divalent-cation-binding site which is involved in the stabilization of outer membranes by cross bridging adjacent lipopolysaccharide (LPS) molecules. The result of this interaction is the displacement of the divalent cation (9) and subsequent disruption and permeabilization of the outer membrane (5,7,10).

The reported inherent resistance of P. cepacia to aminoglycosides (3) and polymyxin (12) led us to examine the interaction of these compounds with this organism. Our findings suggest that a major factor explaining aminoglycoside resistance in P. cepacia is the inability of the antibiotic to disrupt and permeabilize the outer membrane.

MATERIALS AND METHODS

Bacterial strains and growth conditions. P. aeruginosa PA01 strain H103 was used in this study and has been previously described (11). P. cepacia ATCC 25609, the type strain, was obtained from the American Type Culture Collection (Rockville, Md.). P. cepacia K61-3 and PC715J were clinical isolates from cystic fibrosis patients and were obtained from D. Woods, University of Calgary, Calgary, Alberta, Canada. Cells were grown in 1% Proteose Peptone no. 2 (Difco Laboratoires, Detroit, Mich.) medium. For the experiments described below, fresh medium (20 ml) was inoculated with an overnight culture to a final dilution of 1:20 and grown with vigorous aeration at 37°C to an optical density at 600 nm of approximately 0.8.

LPS isolation. LPS was isolated as described by Darveau and Hancock (2). Isolated LPS was extracted twice with an equal volume of chloroform-methanol to remove trace amounts of sodium dodecy1 sulfate and phospholipids resulting from the isolation procedure (2). The LPS from the P. cepacia strains was quantitated by dry weight since it was only weakly reactive in standard assays used to detect the LPS-specific saccharide 2-keto-3-deoxyoctonate.

Dansyl-polymyxin binding experiments. Dansyl-polymyxin was prepared as described by Schindler and Teuber (13) and quantitated by dinitrophenylation (1). Dansyl-polymyxin binding to LPS or to whole cells was monitored by measuring the fluorescence intensity with a Perkin-Elmer 650-105 fluorescence spectrophotometer set with an excitation wavelength of 340 nm and an emission wavelength of 485 nm as previously described (10). Inhibition of dansyl-polymyxin binding to LPS was performed as previously described (9). Briefly, inhibitors of dansyl-polymyxin binding were titrated into a cuvette containing 1 to 3 μg of LPS and approximately 1 to 2 μM dansyl-polymyxin (resulting in 85 to 90% saturation of the LPS by dansyl-polymyxin) in 1 ml of 5 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (pH 7.35), and the decrease in the observed fluorescence (percent inhibition) was recorded. Maximum inhibition of a given inhibitor was calculated as the extrapolated y intercept of a plot of 1 percent inhibition versus 1/inhibitor concentration. The x intercept gave −1/I_{so}, where the I_{so} was the concentration of inhibitor giving 50% maximal inhibition at the given concentration of dansyl-polymyxin and LPS used.

Permeabilization of whole cells to NPN. 1-N-Phenylnaphthylamine (NPN) assays were performed as previously described (6, 7). Cells were centrifuged at room temperature and suspended to an optical density of 0.5 at 600 nm in 5 mM HEPES buffer (pH 7.35) containing 10 mM sodium azide. Cells (1 ml) were placed in a cuvette, and NPN was added to a final concentration of 10 μM. Compounds tested for the ability to permeabilize cells to NPN were added at the specified concentrations, and the increase in NPN fluorescence intensity was monitored with a Perkin-Elmer fluores-

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ence spectrophotometer attached to a Perkin-Elmer Coleman 165 strip-chart recorder. The excitation and emission wavelengths were set at 350 and 420 nm, respectively.

Chemicals. Chemicals were of the highest quality commercially available and were obtained from Sigma Chemical Co., St. Louis, Mo., with the exception of HEPES buffer (Calbiochem-Behring, La Jolla, Calif.). Polymyxin B sulfate and gentamicin sulfate were obtained from Sigma. Tobramycin sulfate was obtained from Eli Lilly & Co., Toronto, Ontario, Canada.

RESULTS

Binding of dansyl-polymyxin to whole cells of P. aeruginosa and P. cepacia. We had shown previously (10) that dansyl-polymyxin will bind to whole cells of P. aeruginosa resulting in an enhancement of the fluorescence intensity of the dansyl-polymyxin molecule and a characteristic blue shift in the emission maximum. The kinetics of dansyl-polymyxin binding to whole cells of P. aeruginosa was found in this study to be similar to the previously described (9) kinetics of binding to LPS (data not shown). In contrast to our findings with P. aeruginosa, we observed that whole cells of P. cepacia did not bind dansyl-polymyxin as indicated by the lack of increase in fluorescence of dansyl-polymyxin upon addition to cells (Fig. 1). The lack of enhanced fluorescence was not due to an inability of dansyl-polymyxin to interact with cell components since enhanced fluorescence was observed when dansyl-polymyxin was titrated into a cuvette containing French-passed P. cepacia cells (data not shown).

Polycation-mediated permeabilization of P. aeruginosa and P. cepacia. The outer membranes of many gram-negative bacteria constitute a barrier to the uptake of hydrophobic substances. Dansyl-polymyxin and other polycationic antibiotics can interact with the outer membrane at divalent-cation-binding sites on LPS, resulting in permeabilization of the outer membrane to hydrophobic compounds such as the fluorescent probe NPN (6, 7). The results displayed in Fig. 1 suggested that dansyl-polymyxin was unable to interact with intact cells of P. cepacia. To determine whether the outer membrane of P. cepacia could be permeabilized by using higher concentrations of other polycationic antibiotics, we examined the ability of these compounds to permeabilize the outer membrane of P. cepacia to NPN. The results (Table 1) illustrated that the outer membrane of P. cepacia was resistant to the permeabilizing action of polymyxin B, gentamicin, tobramycin, poly-L-lysine, and the Mg\(^{2+}\) chelator EDTA.

**Table 1. Polycation-mediated permeabilization of P. cepacia and P. aeruginosa to the hydrophobic fluorescent probe NPN**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Concentration (μM)</th>
<th>P. aeruginosa H103</th>
<th>P. cepacia ATCC 25609</th>
<th>P. cepacia K61-3</th>
<th>P. cepacia PC715J</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobramycin</td>
<td>8.55</td>
<td>&gt;9,000</td>
<td>22</td>
<td>23</td>
<td>24</td>
</tr>
<tr>
<td>Polymyxin</td>
<td>0.83</td>
<td>3,700</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>2</td>
<td>30</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Polys-L-lysine</td>
<td>4.0</td>
<td>2,457</td>
<td>&lt;1</td>
<td>9.5</td>
<td>10</td>
</tr>
<tr>
<td>EDTA</td>
<td>5,000</td>
<td>&gt;9,000</td>
<td>ND*</td>
<td>ND*</td>
<td>ND*</td>
</tr>
</tbody>
</table>

* Stimulation of NPN uptake refers to the fluorescence increase after addition of permeabilizer and is expressed as the increase in fluorescence intensity (arbitrary units) per milliliter.

**Fig. 2.** Binding of dansyl-polymyxin to LPS. Dansyl-polymyxin was titrated into a cuvette containing 1 ml of 5 mM HEPES buffer (pH 7.35) and 3 μg of the specified LPS. Symbols: ●, P. aeruginosa H103; ○, P. cepacia ATCC 25609; ×, P. cepacia K61-3.
TABLE 2. Hill coefficient (n) and $S_{0.5}$ for dansyl-polymyxin binding to LPS from *P. cepacia* and *P. aeruginosa*

<table>
<thead>
<tr>
<th>Strain</th>
<th>$S_{0.5}$ (μM)</th>
<th>Hill coefficient (n)</th>
<th>Maximum binding sites/molecule of LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em> H103</td>
<td>0.96</td>
<td>2.3</td>
<td>6.6</td>
</tr>
<tr>
<td><em>P. cepacia</em> K61-3</td>
<td>0.93</td>
<td>2.6</td>
<td>2.4</td>
</tr>
<tr>
<td><em>P. cepacia</em> PC715J</td>
<td>1.78</td>
<td>1.9</td>
<td>2.4</td>
</tr>
</tbody>
</table>

* The results were derived from Hill plots. The $S_{0.5}$ is the concentration of dansyl-polymyxin at which half of the binding sites on the LPS molecule are saturated. The Hill coefficient indicates the degree of binding cooperativity. An n value greater than 1 indicates that binding is cooperative.
* Assuming LPS had a molecular weight of 9,000. For *P. aeruginosa* this was obtained by assuming that a measured weight of LPS contained two reactive 2-keto-3-deoxyoctonate molecules per LPS molecule (2.8). Since *P. cepacia* LPS had a similar pattern of distribution of LPS species on sodium dodecyl sulfate-polyacrylamide gel electrophoretograms as *P. aeruginosa* LPS (unpublished data), we assumed these LPS species had similar average molecular weights.

The apparent difference in $I_{50}$ values obtained in this study versus previously published values (9) was due to lower initial concentrations of dansyl-polymyxin used in the inhibition experiments reported here (Table 2). However, the relative ability of the compounds to compete with dansyl-polymyxin for binding to LPS remained the same (Table 3).

Whole cells of *P. cepacia* were not permeabilized to the hydrophobic fluorescent probe NPN with antibiotic levels 100 to 1,000-fold in excess of those required to permeabilize *P. aeruginosa* (Table 1). The same antibiotics were also unable to permeabilize *P. cepacia* to the chromogenic β-lactam nitrocefin (15) (data not shown). In addition, dansyl-polymyxin did not bind to whole cells of *P. cepacia* (Fig. 1). It is possible that the LPS of *P. cepacia* is arranged in the outer membrane in a way that masks the negative charges found on the LPS molecule, thus making them unavailable to bind polycationic antibiotics. Alternatively, by analogy to *P. aeruginosa* outer membrane protein H1 (11), *P. cepacia* may have outer membrane proteins associated with the LPS which serve to replace cations which would otherwise be required to stabilize the outer membrane by cross bridging adjacent LPS molecules (4). This idea is supported by the observation that *P. cepacia* was not permeabilized by EDTA (Table 1). Finally, it is possible that LPS from *P. cepacia* lacks a critical polycation-binding site required for polycation-mediated permeabilization of the outer membrane, e.g., a polycation-binding site on lipid A (9). Consistent with this,
TABLE 3. Inhibition of dansyl-polymyxin binding to LPS

<table>
<thead>
<tr>
<th>Strain</th>
<th>$I_{50}^a$</th>
<th>Gentamicin</th>
<th>Streptomycin</th>
<th>Polymyxin B</th>
<th>Mg$^{2+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. aeruginosa H103</td>
<td>149</td>
<td>284</td>
<td>4.0</td>
<td>1,600</td>
<td></td>
</tr>
<tr>
<td>P. cepacia K61-3</td>
<td>75</td>
<td>180</td>
<td>3.7</td>
<td>2,600</td>
<td></td>
</tr>
<tr>
<td>P. cepacia PC715J</td>
<td>160</td>
<td>204</td>
<td>6.0</td>
<td>2,500</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Concentration (micromolar) resulting in 50% inhibition of dansyl-polymyxin binding.

the phosphate content of P. cepacia LPS was determined in one study (8) to be only one-third of that of P. aeruginosa LPS.

The results presented here suggest that P. cepacia does not utilize the self-promoted uptake pathway for aminoglycoside antibiotic uptake. We propose that, as a consequence, the cell is resistant to high levels of polycationic antibiotics and to the permeabilizing effects of EDTA.

ACKNOWLEDGMENTS

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LITERATURE CITED