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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_-phenylnaphthylamine was not achieved at concentrations 100- to 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 Mg^{2+}. 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 polymxyin.

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* PAO1 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 Laboratoies, Detroit, Mich.) medium. For the experiments desribed 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 dodecyl 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 650-10S 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 $-1/I_{50}$, where the I_{50} 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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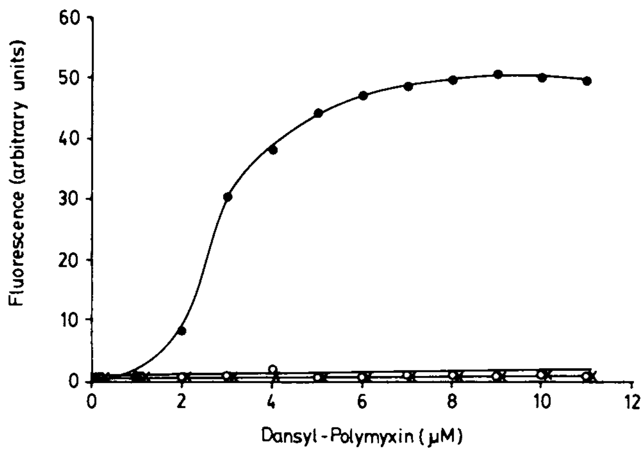

FIG. 1. Binding of dansyl-polymyxin to intact cells. Dansyl-polymyxin was titrated into a cuvette containing 1 ml of 5 mM HEPES buffer (pH 7.35), 10 mM sodium azide, and 20 µl of cells which had been centrifuged and suspended in HEPES-azide to an optical density of approximately 1.0 at 600 nm. The fluorescence was recorded as described in Materials and Methods. Symbols: ●, *P. aeruginosa* H103; ○, *P. cepacia* ATCC 25609; ×, *P. cepacia* K61-3.

cence 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

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

Antibiotic	Concn (µM)	Stimulation of NPN uptake in [a]: *P. aeruginosa* H103	*P. cepacia* ATCC 25609	*P. cepacia* K61-3	*P. cepacia* PC715J
Tobramycin	8.55	73	<1	<1	<1
	8,550	>9,000	22	23	24
Polymyxin	0.83	120	<1	<1	<1
	83	3,700	<1	<1	<1
Gentamicin	2	30	<1	<1	<1
	2,000	245	<1	<1	<1
Poly-L-lysine	4.0	2,457	<1	9.5	10
EDTA	5,000	>9,000	<1	ND[b]	<1

[a] 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.
[b] ND, Not determined.

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.

Binding of dansyl-polymyxin to LPS from *P. cepacia*. Since dansyl-polymyxin binds with high affinity to *P. aeruginosa* LPS, we were interested to determine whether the lack of binding of dansyl-polymyxin by intact cells of *P. cepacia* was due to a lack of binding sites on the LPS. To determine this we examined the ability of dansyl-polymyxin to bind to purified LPS isolated from *P. cepacia* and *P. aeruginosa*. Binding of dansyl-polymyxin to LPS from both organisms was quite similar and displayed saturation binding kinetics (Fig. 2). The binding curves, when transformed into Hill plots (Table 2), revealed that purified LPS from *P. cepacia* bound dansyl-polymyxin cooperatively with approximately the same affinity, as assessed by the $S_{0.5}$ value, as did LPS isolated from *P. aeruginosa*. The *n* value (Hill number) calculated from the Hill plots (Table 2) was also similar for both species (1.9 to 2.3), suggesting that the degree of

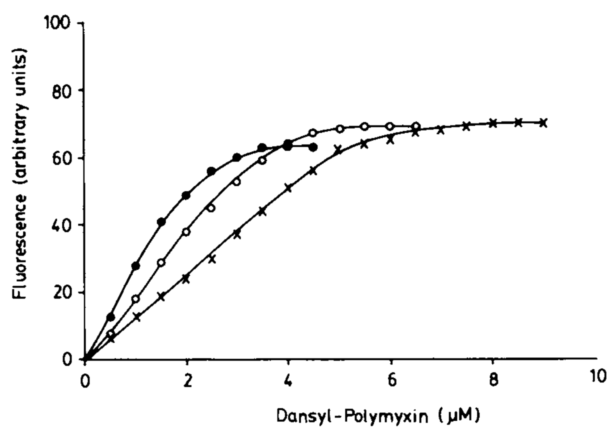

FIG. 2. Binding of dansyl-polymyxin to LPS. Dansyl-polymyxin was titrated into a cuvette containing 1 ml of 5 mM HEPES buffer (pH 7.35) and 3 µg of the specified LPS. Symbols: ●, *P. aeruginosa* H103; ○, *P. cepacia* ATCC 25609; ×, *P. cepacia* K61-3.

TABLE 2. Hill coefficient (n) and $S_{0.5}$ for dansyl-polymyxin binding to LPS from *P. cepacia* and *P. aeruginosa*[a]

Strain	$S_{0.5}$ (μM)	Hill coefficient (n)	Maximum binding sites/molecule of LPS[b]
P. aeruginosa H103	0.96	2.3	6.6
P. cepacia K61-3	0.93	2.6	2.4
P. cepacia PC715J	1.78	1.9	2.4

[a] 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.

[b] 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-polyacrylammide gel electrophoretograms as *P. aeruginosa* LPS (unpublished data), we assumed these LPS species had similar average molecular weights.

cooperativity in the binding of dansyl-polymyxin to the respective LPS molecules was similar.

The other major difference observed was in the binding capacity per unit weight of LPS. Assuming similar molecular weights, *P. cepacia* LPS bound less than half as much dansyl-polymyxin per mole of LPS as *P. aeruginosa* LPS.

Inhibition of dansyl-polymyxin binding to LPS by $MgCl_2$. Dansyl-polymyxin binds to at least two types of binding sites on LPS from *P. aeruginosa* (9). Binding of dansyl-polymyxin at one of these sites can be inhibited by the presence of Mg^{2+} and a variety of other polycations (9). However, inhibition by Mg^{2+} is partial (approximately 60% [Fig. 3]), suggesting the existence of a second class of sites with low or no affinity for polymyxin. In contrast, approximately 90% of the dansyl-polymyxin bound to LPS from *P. cepacia* was displaced by Mg^{2+} (Fig. 3). It is possible that the remaining 10% is due to nonspecific hydrophobic binding of dansyl-polymyxin to the lipid A region of the LPS molecule. These data suggest that only one class of binding sites existed on the LPS molecule from this organism.

Streptomycin, gentamicin, and underivitized polymyxin B were also effective at displaying dansyl-polymyxin bound to LPS from *P. cepacia* (Table 3) at levels quite similar to those obtained when the experiments were performed with *P. aeruginosa* LPS. These results indicate that these polycationic antibiotics are capable of binding to purified LPS from *P. cepacia* but not to intact whole cells.

DISCUSSION

P. cepacia is characteristically resistant to a wide range of commonly used antibiotics including β-lactams, polymyxin B, and aminoglycosides (3). The data presented here are consistent with the proposal that the resistance of *P. cepacia* to aminoglycosides and polymyxin B results in part from the inability of these compounds to permeabilize the *P. cepacia* outer membrane. This inability to permeabilize the outer membrane of *P. cepacia* was not due to a reduced affinity for LPS, since dansyl-polymyxin was able to bind to purified LPS from *P. cepacia* and *P. aeruginosa* (Fig. 2) with approximately equal affinities (Table 2). As well, the ability of a variety of aminoglycoside antibiotics to compete with dansyl-polymyxin for binding to LPS from *P. aeruginosa* and *P. cepacia* was similar (Fig. 3). One notable difference in the binding of polycations to LPS from the two organisms was the fact that Mg^{2+} was able to competitively displace approximately 90% of dansyl-polymyxin bound to *P. cepacia* LPS (Fig. 3), but only 60% of dansyl-polymyxin bound to *P. aeruginosa* LPS (9). These results suggest that, unlike the case of *P. aeruginosa* (9), all the dansyl-polymyxin-binding sites on *P. cepacia* LPS bound Mg^{2+} with equal affinity.

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,

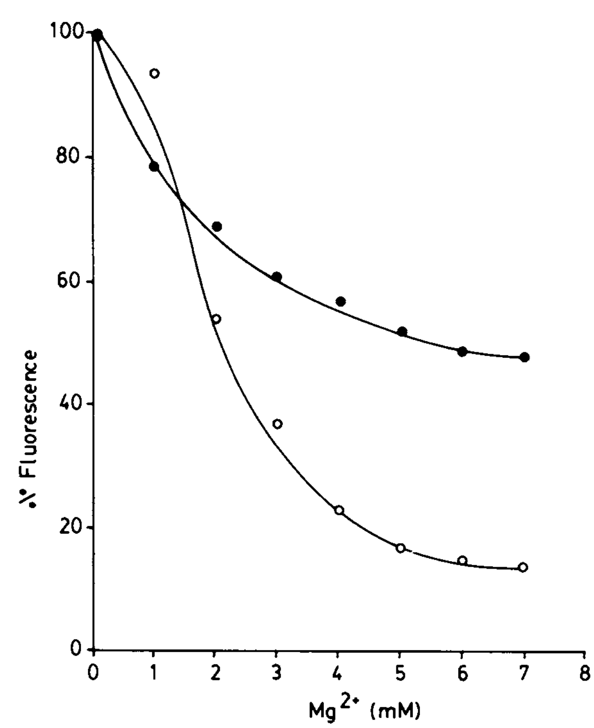

FIG. 3. Inhibition of dansyl-polymyxin binding to LPS by Mg^{2+}. $MgCl_2$ was titrated into a cuvette containing 3 μg of the specified LPS and 2 μM dansyl-polymyxin, and the decrease in fluorescence was recorded. Maximum inhibition was calculated as described in Materials and Methods. Symbols: ●, *P. aeruginosa* H103; ○, *P. cepacia* ATCC 25609. Similar data were obtained with other *P. cepacia* strains.

TABLE 3. Inhibition of dansyl-polymyxin binding to LPS

Strain	I_{50}[a]			
	Gentamicin	Streptomycin	Polymyxin B	Mg^{2+}
P. aeruginosa H103	149	284	4.0	1,600
P. cepacia K61-3	75	180	3.7	2,600
P. cepacia PC715J	160	204	6.0	2,500

[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.

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LITERATURE CITED

1. **Bader, J., and M. Teuber.** 1973. Binding of the O-antigenic lipopolysaccharide of *Salmonella typhimurium*. Z. Naturforsch. Sect. C **28**:422–430.
2. **Darveau, R. P., and R. E. W. Hancock.** 1983. Procedure for isolation of bacterial lipopolysaccharides from both smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains. J. Bacteriol. **155**:831–838.
3. **Fass, R. J., and J. Barnishan.** 1980. *In vitro* susceptibilities of nonfermentative Gram-negative bacilli other than *Pseudomonas aeruginosa* to 32 antimicrobial agents. Rev. Infect. Dis. **2**:841–853.
4. **Hancock, R. E. W.** 1984. Alterations in outer membrane permeability. Annu. Rev. Microbiol. **38**:237–264.
5. **Hancock, R. E. W., V. J. Raffle, and T. I. Nicas.** 1981. Involvement of the outer membrane in gentamicin and streptomycin uptake and killing in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **19**:777–785.
6. **Hancock, R. E. W., and P. G. W. Wong.** 1984. Compounds which increase the permeability of the *Pseudomonas aeruginosa* outer membrane. Antimicrob. Agents Chemother. **26**:48–52.
7. **Loh, B., C. Grant, and R. E. W. Hancock.** 1984. Use of the fluorescent probe 1-*N*-phenylnaphthylamine to study the interactions of aminoglycoside antibiotics with the outer membrane of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **26**:546–551.
8. **Manniello, J. M., H. Heymann, and F. W. Adair.** 1979. Isolation of atypical lipopolysaccharides from purified cell walls of *Pseudomonas cepacia*. J. Gen. Microbiol. **112**:397–400.
9. **Moore, R. A., N. C. Bates, and R. E. W. Hancock.** 1986. Interaction of polycationic antibiotics with *Pseudomonas aeruginosa* lipopolysaccharide and lipid A studied using dansyl-polymyxin. Antimicrob. Agents Chemother. **29**:496–500.
10. **Moore, R. A., L. Chan, and R. E. W. Hancock.** 1984. Evidence for two distinct mechanisms of resistance to polymyxin B in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **26**:539–545.
11. **Nicas, T. I., and R. E. W. Hancock.** 1980. Outer membrane protein H1 of *Pseudomonas aeruginosa*: involvement in adaptive and mutational resistance to ethylenediaminetetraacetate, polymyxin B, and gentamicin. J. Bacteriol. **143**:872–878.
12. **Richards, R. M. E., and R. H. Cavill.** 1980. Electron microscope study of the effect of benzalkonium, chlorhexidine and polymyxin on *Pseudomonas cepacia*. Microbios **29**:29–31.
13. **Schindler, P. R. G., and M. Teuber.** 1975. Action of polymyxin B on bacterial membranes; morphological changes in the cytoplasm and in the outer membrane of *Salmonella typhimurium* and *Escherichia coli* B. Antimicrob. Agents Chemother. **8**:95–104.
14. **Vaara, M., and T. Vaara.** 1983. Polycations sensitize enteric bacteria to antibiotics. Antimicrob. Agents Chemother. **24**:107–113.
15. **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.