

Enhanced Binding of Polycationic Antibiotics to Lipopolysaccharide from an Aminoglycoside-Supersusceptible, *tolA* Mutant Strain of *Pseudomonas aeruginosa*

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The lipopolysaccharide (LPS) of the aminoglycoside-supersusceptible *Pseudomonas aeruginosa tolA* mutant PAO1715 was compared with its parent strain PAO1670 and *tol*⁺ revertant PAO1716. Electrophoretic separation of purified LPSs from the three isolates showed similar LPS banding patterns. Analysis of the Western blots of these LPSs from the three isolates with O-antigen-specific monoclonal antibody indicated that the ladder pattern consisted of doublet bands, which presumably reflected a modification of core or lipid A; the level of one of the bands in the doublet was in much lower amounts in the isolate from the *tolA* mutant than in that from the parent or revertant. Results of competitive displacement experiments, in which the cationic spin probe 4-dodecyldimethylammonium-1-oxyl-2,2,6,6-tetramethylpiperidine bromide was displaced from its LPS-binding site by polycations, revealed that the *tolA* mutant had a much higher affinity for gentamicin, polymyxin, Ca²⁺, and Mg²⁺ than did the parent or revertant. The order of affinity for all samples was polymyxin B >> gentamicin C >> Ca²⁺ > Mg²⁺. Both gentamicin and polymyxin induced rigidification of all of the LPS samples, but for the sample from the *tolA* mutant, rigidification occurred at substantially lower concentrations. Dansyl polymyxin titration experiments with intact cells demonstrated that the increased affinity of the LPS from the *tolA* mutant for polycations was reflected in an increase in the affinity of binding to the cell. Together these data suggest that the *tolA* mutant is supersusceptible to aminoglycosides by virtue of an LPS change which increases the binding affinity of the LPS for polycations, including gentamicin.

Lipopolysaccharide (LPS) is one of the major components found in the outer monolayer of the outer membrane of gram-negative bacteria. It is involved in one of the major functions of the outer membrane, that is, protection of cells from amphipathic and hydrophobic antibacterial compounds (18, 25). LPS contains a variety of ionic groups, with acidic phosphate and carboxyl moieties concentrated within the core and lipid A head group region (17, 18, 25). Enterobacterial LPS contains about four phosphate residues in the inner core region and an additional two or three phosphate residues in lipid A (25); in contrast, LPS from *Pseudomonas aeruginosa* may contain 10 or more phosphate residues per molecule (42). As a consequence, the LPS carries a net negative charge at physiological pHs, resulting in a strong negative charge on the surface of gram-negative cells (8). The O-antigen chain of LPS from *P. aeruginosa* is often high in uronic acid residues, but the acidic groups are usually modified (13). LPS is apparently associated with a variety of cations, including Mg²⁺ and Ca²⁺ (2, 25), which may form ionic bridges between phosphate groups on neighboring LPS molecules, stabilizing the outer membrane structure (15, 25).

It has been proposed that in some bacteria the ability of cationic antibiotics to penetrate the outer membrane is related to their ability to bind LPS near the lipid A head group, destroying the LPS-LPS cross-bridging and destabilizing the outer membrane bilayer structure (8, 25). Cationic antibiotics, such as polymyxins and aminoglycosides, increase the permeability of the outer membrane to lysozyme and hydrophobic compounds (9, 10, 16, 36, 39). In addition, both classes of antibiotics induce blebbing and other struc-

tural perturbations of bacterial outer membranes, which may serve to make the outer membrane more permeable to the antibiotic (11, 19, 33, 36). The initial action of these antibiotics may be to disrupt the outer membrane structure, allowing themselves and other compounds to enter the cell and inhibit specific metabolic processes (8, 25). This process has been termed self-promoted uptake (8, 9). There has been little direct evidence that the disruption of the outer membrane permeability barrier by polycationic antibiotics initiates an uptake pathway that results in the killing of cells. Most evidence has been derived from the study of two classes of resistant mutants. *Salmonella typhimurium pmrA* mutants demonstrate low-level resistance to polymyxin, are impermeable to lysozyme and deoxycholate upon treatment with polymyxin (38), and are more resistant to Tris-EDTA and polycations such as polylysine and protamine (37). Compared with the parental isolate, the LPSs from the *pmrA* mutants were shown to contain elevated amounts of 4-aminoarabinose and ethanolamine, making the LPS less negatively charged (41), and thus less able to interact with polycations (28, 41). Similarly, *P. aeruginosa* mutant H181 was cross resistant to the polycationic antibiotics polymyxin and aminoglycosides and to the divalent cation chelator EDTA due to an altered outer membrane (24). In wild-type *P. aeruginosa* cells, a statistically significant relationship was observed between a pseudoaffinity constant for the outer membrane-permeabilizing ability and the MICs of eight different aminoglycosides (16).

To provide further evidence for a relationship between an antibiotic interaction with the outer membrane and eventual cell killing, we decided to examine mutants that were supersusceptible to killing by polycations for outer membrane

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alterations. The *P. aeruginosa* PAO *tolA* mutant 1715 was isolated by Mills and Holloway (20) as an aeruginocin 41-tolerant mutant of strain 1670 and was shown to be supersusceptible to all 10 aminoglycosides tested; but it was normally susceptible to carbenicillin, tetracycline, mercury, and erythromycin. The rate of uptake of [³H]streptomycin was much greater for the mutant, and it was suggested that this strain had a cell envelope mutation. In this study we measured the relative affinities of two polycationic antibiotics for purified LPS from these strains using a cationic spin probe. Our results suggest that the aminoglycoside supersusceptibility is due to an alteration in cell envelope LPS such that it binds aminoglycosides with a higher affinity.

MATERIALS AND METHODS

Bacterial strains and growth media. *P. aeruginosa* 1670 *ade-136 leu-8 rif-1*; its aminoglycoside-supersusceptible, aeruginocin 41-tolerant derivative strain 1715 *ade-136 leu-8 tolA12*; and a *tol*⁺ revertant, strain 1716 *ade-136 leu-8 rif-1* (20), were obtained from B. Holloway (Department of Genetics, Monash University, Clayton, Australia). All three strains were grown to the mid-logarithmic phase in a 100-liter fermentor containing 80 liters of protease peptone no. 2 medium after inoculation with 1 liter of an overnight culture grown in the same medium. After growth, strains were tested for their susceptibilities to aminoglycosides to ensure retention of the appropriate phenotype.

Isolation of LPS. LPS was isolated by the method of Darveau and Hancock (3), followed by two extractions in chloroform-methanol (1:1 [vol/vol]) to remove residual sodium dodecyl sulfate (SDS) and phospholipids, resulting in recovery of approximately 80% of the total LPS. The LPSs from the isolates were dialyzed extensively against a buffer containing 0.2 M NaCl, 10 mM Tris, 1 mM EDTA, and 0.01% NaN₃ (pH 8.0) at 37°C, followed by dialysis against distilled water. The magnesium salts of the LPSs from the isolates (Mg-LPS) were formed by dialysis against 10 mM MgCl₂ followed by distilled water. All the LPSs from the isolates were dialyzed simultaneously to decrease the variation of the ion content between preparations. Samples were lyophilized and stored at -20°C.

Chemical analysis. Inductively coupled plasma emission spectroscopy of wet-ashed LPS samples was used to quantify phosphorus and metal ion content, as described previously (2). Levels of 2-keto-3-deoxyoctulosonic acid (KDO) were quantitated by the thiobarbituric acid assay (4). The protein content in LPS was determined by the BCA protein assay (Pierce Chemical Co., Rockford, Ill.), using bovine serum albumin as a standard.

SDS-PAGE and Western blots. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out as described previously (28), using the buffer system of Laemmli (14) and the silver staining procedure of Dubray and Bezar (5). The separating gel was formed with 15% acrylamide and 0.1% SDS, with a 7.5% acrylamide stacking gel. Cell envelope and outer membrane isolation and SDS-PAGE were carried out as described previously (24).

Western blots of SDS-polyacrylamide gels (11% acrylamide) were prepared as described previously (26). The gels were electrotransferred with an electrophoresis apparatus (model TE Transphor; Hoefer Scientific Instruments) at a constant current of 150 mA for 18 h by using the electrode buffer described by Otten and co-workers (26). The nitrocellulose blots were visualized as described previously (26) with monoclonal anti-strain 503 LPS antibody (6) (titer, ~1:100,000) diluted 1:10,000 in a blocking solution.

Partitioning of spin probe. Electron spin resonance spectroscopy was carried out with an X-band spectrometer (model E-112; Varian Instruments, Inc.). The sample temperature was measured with a thermocouple placed within the cuvette. Titrations of Mg-LPS suspended at 10 mg/ml were performed by measuring the spectral parameters of the spin probe 4-dodecyldimethylammonium-1-oxyl-2,2,6,6-tetramethylpiperidine bromide (CAT₁₂; LPS-CAT₁₂, 18:1 molar ratio) after successive additions of cations to samples at 37°C. All the cations and antibiotics, as well as the LPS, were dissolved in 50 mM KOH-HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.0).

Upon the successive addition of cations to samples of LPS, the spectra were analyzed for $2T_{||}$, the hyperfine splitting parameter, whose value is an indicator of probe mobility, and for Ψ_i , the partitioning of probe between aqueous (*F*) and LPS-bound (*B*) environments, which was calculated as previously described (1).

Scatchard plots of the binding of CAT₁₂ to LPS isolates from *P. aeruginosa* 1715, 1716, and 1670 were determined by using the Mg-LPS complexes suspended at 5 μM in 50 mM KOH-HEPES (pH 7.0). The LPS samples were mixed with different concentrations of CAT₁₂, and the concentrations of bound (*B*) and free (*F*) probe were determined from the resultant electron spin resonance spectra, which were measured at 25°C.

Dansyl polymyxin titrations. Whole-cell dansyl polymyxin-binding experiments were performed as described previously (21). Cells were grown to an optical density at 600 nm of 0.5 and then centrifuged, washed twice, and suspended in 5 mM sodium HEPES buffer-10 mM sodium azide (pH 7.2) to a final optical density of 0.5. Cells (10 μl) were then added to 1 ml of 5 mM sodium HEPES buffer (pH 7.2)-10 mM sodium azide in a cuvette, and portions of dansyl polymyxin were titrated into this cuvette. Fluorescence emission intensity was measured at 485 nm after excitation at 340 nm.

Chemicals. Polymyxin B sulfate, gentamicin C sulfate, and spermine were purchased from Sigma Chemical Co. (St. Louis, Mo.), the monoclonal anti-strain 503 LPS antibody (6) was a gift from L. E. Bryan, and CAT₁₂ was synthesized as described previously (1). All other chemicals were of reagent grade or better.

RESULTS

Characterization of the cell envelopes. We confirmed the conclusions of Mills and Holloway (20) that the *tolA-12* strain 1715 was more susceptible to the aminoglycosides gentamicin (15-fold), streptomycin (10-fold), and tobramycin (20-fold) compared with the susceptibilities of its *tol*⁺ parent 1670 and revertant 1716. In addition, strain 1715 was twofold more susceptible to polymyxin than was its parent or revertant. However, it was unaltered in susceptibility to the β-lactams, chloramphenicol, or tetracycline. Phage typing with 24 phages with different cell envelope receptors (24) demonstrated no differences in susceptibility between the parent strain 1670 and its *tolA-12* mutant 1715, suggesting that no gross surface alterations occurred due to the *tolA-12* mutation and that many surface structures serving as receptors were unaltered. In addition, the amounts of LPS per cell (8.3%, based on hydroxy fatty acid analysis [3]) and the SDS-PAGE patterns of cell envelope and outer membrane proteins were unaltered in the *tolA-12* mutant and its parent and revertant.

LPS characterizations. Silver staining of SDS-PAGE-separated LPS from the *tolA* mutant 1715 and its parent strain

1670 and revertant 1716 revealed three major sets of bands (Fig. 1A, lanes 1 to 3), indicating as many as three populations of molecules differing in O-antigen length (3, 7, 27). At low concentrations, there was no difference in the migration pattern of the short-chain LPSs from the three strains (Fig. 1A, lanes 4 to 6). Analysis of Western blots of LPSs from the isolates with anti-O-antigen-specific monoclonal antibodies were performed on gels containing 11% acrylamide to resolve the closely spaced bands (Fig. 1B). This blot indicated that the ladder consisted of doublet bands, that the level of one of the doublet bands was lower in the isolate from the *tolA* mutant 1715 than it was from the parent 1670, and that the levels in 1716 were intermediate between those in the parent and mutant isolates. Presumably, this reflects a difference in the levels of molecules containing a specific modification within the core or lipid A regions (27, 41).

The levels of metal ions bound and the phosphate content of the magnesium salts from the LPSs from the three isolates were determined by inductively coupled plasma emission spectroscopy. There were similar amounts of phosphate bound per LPS molecule for all three isolates (Table 1). There was no detectable protein associated with the LPS salts. The thiobarbituric acid assay showed two reactive KDO residues per molecule of LPS for the three strains. No significant differences in the fatty acid compositions or heptose or hexose contents of the three LPS samples were observed. In addition, both reactivities to the LPSs with an O-antigen-specific monoclonal antibody (Fig. 1B) and the susceptibilities of the strains to several smooth LPS-specific phages (24) suggested that the LPS O-antigen composition was unaltered.

Analysis of cation binding of LPS. A cationic electron spin resonance probe, CAT₁₂, was used to measure cation binding to the Mg²⁺ salt form of LPS from the parent (1670),

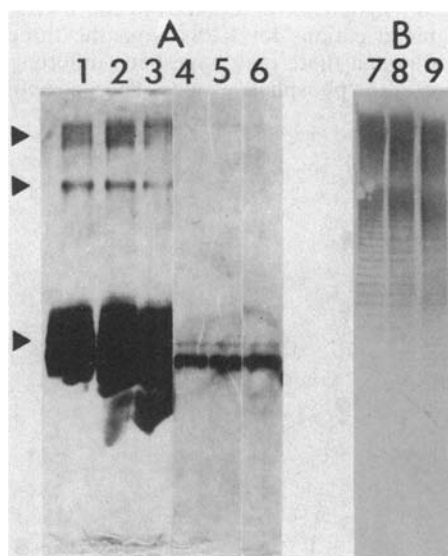


FIG. 1. (A) Silver-stained SDS-polyacrylamide gel of LPS from *P. aeruginosa* PAO1670 (lanes 1 and 4), PAO1716 (lanes 2 and 5), and PAO1715 (lanes 3 and 6). Samples of either 5 µg (lanes 1 to 3) or 0.10 µg (lanes 4 to 6) were applied to a 15% polyacrylamide gel which had polymerized overnight with a butanol overlay. Arrowheads indicate the three regions of intensive staining. (B) Western blots of an 11% acrylamide gel with 2.5 µg of LPS from *P. aeruginosa* PAO1715 (lane 7), PAO1716 (lane 8), and PAO1670 (lane 9) reacted with monoclonal anti-strain 503 LPS O-antigen-specific antibody.

TABLE 1. Elemental composition of purified LPSs from *P. aeruginosa* isolates

Strain	mol/mol (mean ± SD) of ^a :			
	Mg/P	+P ^b	Mg/LPS ^c	P/LPS ^c
1670	0.76 ± 0.01	1.55 ± 0.04	10.20 ± 0.13	13.4 ± 0.1
1716	0.75 ± 0.01	1.52 ± 0.02	9.30 ± 0.56	12.4 ± 0.6
1715	0.72 ± 0.01	1.48 ± 0.01	8.39 ± 2.14	11.6 ± 2.7

^a The metal and phosphate contents of the magnesium salts of the three LPS isolates were determined by inductively coupled plasma emission spectroscopy. There was less than 0.01 metal per phosphate (mole/mole) of Na, K, Ca, Fe, Al, and Zn ions associated with the samples. Comparisons of the values were made by using the randomized block analysis of variance statistical analysis at $P < 0.05$ (35).

^b Total cation charges on metal ions bound to the LPS per LPS phosphate.

^c Assumes an average LPS molecular mass of 9,000 (30).

revertant (1716), and mutant (1715) strains. Scatchard analysis of CAT₁₂ binding was used to obtain K_D , the apparent dissociation constant, and N , the number of binding sites for the probe. Also, the Hill constant α_H was calculated to give an index of the cooperativity of probe binding. Results from the Scatchard analysis indicated that there were approximately 3.5 probe-binding sites per LPS molecule, 1 high-affinity site and approximately 2.5 low-affinity sites (Table 2). The apparent K_D of the probe for the high-affinity site in the LPS from the *tolA* mutant 1715 was higher than for that from the parent strain 1670, which indicates that the mutant has a lower affinity for the binding of probe to this site, and thus, the probe could be more readily displaced by other cations. The apparent K_D of the high-affinity site for the revertant 1716 sample also appeared to be higher than that for the parental strain, although the difference was not significant ($P = 0.05$). However, the Hill coefficients were significantly different for binding in these two samples. The apparent K_D s of the probe for the low-affinity sites and the total number of sites were similar for LPSs from all three strains. At low ligand concentrations, a concave-down shape of the Scatchard plot was observed (Fig. 2). This, along with the Hill coefficient (Table 2), indicated positive cooperativity binding of CAT₁₂ to Mg-LPS aggregates. Positive cooperativity has also been observed for CAT₁₂ binding to the Mg-LPS complexes of *Escherichia coli* (28) and to the sodium salt of lipid A (E. J. McGroarty and G. E. Chessen, unpublished data).

Competitive displacement of the cationic spin probe CAT₁₂ from LPS. Titrations of LPS samples containing CAT₁₂ with various cations resulted in displacement of different amounts of the probe, which was reflected in the partitioning parameter Ψ_i (Fig. 3). The addition of the highly charged cations polymyxin B and gentamicin resulted in displacement of CAT₁₂ at lower concentrations than did the addition of cations with a lower charge, e.g., Mg²⁺ and Ca²⁺. Displacement of the spin probe from the Mg-LPS complex of the *tolA* mutant strain 1715 by any cation occurred at much lower levels of added cation than did those of the parent strain 1670 or revertant 1716 isolates (Fig. 3). LPSs from the *tol*⁺ strains 1716 and 1670 exhibited similar cation-binding affinities which were lower than those observed for the *tolA* mutant. The binding affinities of the compounds for Mg-LPSs from all three strains were as follows: polymyxin B \gg gentamicin = spermine \gg Ca²⁺ > Mg²⁺ (Fig. 3A to C; data not shown).

Previous data have indicated that polycationic antibiotics rigidify *P. aeruginosa* LPS (29). To study the influence of the *tolA* mutation on this property, the relative fluidity of the bound CAT₁₂ probe was measured by calculating $2T_{||}$ as a

TABLE 2. Scatchard analysis of CAT₁₂ binding to Mg-LPS isolated from *P. aeruginosa*^a

Strain	High-affinity site			Low-affinity site		
	K_D (μM)	N^b	α_H^c	K_D (μM)	N	α_H^c
1670 (<i>tol</i> ⁺)	0.11 \pm 0.03	1.0 \pm 0.1	2.6	2.2 \pm 0.50	2.5 \pm 0.3	1.0
1716 (<i>tol</i> ⁺)	0.32 \pm 0.10	1.3 \pm 0.2 ^d	1.5 ^d	2.2 \pm 0.2	2.2 \pm 0.1	0.9
1715 (<i>tolA-12</i>)	0.44 \pm 0.09 ^d	1.4 \pm 0.1 ^d	1.3 ^d	2.4 \pm 0.4	1.9 \pm 0.3	1.1

^a Samples of 5 μM of the magnesium salts were suspended in 50 mM KOH-HEPES (pH 7.2) at 25°C and mixed with different concentrations of CAT₁₂. The levels of bound and free probe were determined from the resultant electron spin resonance spectra. Results are averages of three samples.

^b N represents the number of probe-binding sites per LPS molecule.

^c α_H is the Hill coefficient (34).

^d Values differed significantly at $P = 0.05$ from the value for the sample from strain 1670. Analysis was determined by using the randomized analysis of variance statistical analysis (35).

function of added cation concentration (Fig. 4). The hyperfine splitting parameter $2T_{||}$ is related to the rotational mobility of the spin label, and therefore reports the local motion within the LPS head groups. High values of $2T_{||}$ reflect low motion (rigidification). It can be determined from the data that on titration of Mg-LPS with polycations, a structural change in the *tolA* mutant 1715 sample occurred at lower cation concentrations than it did for LPS from strain 1670 (Fig. 4). The antibiotics polymyxin B and gentamicin rigidified the Mg-LPS of strain 1715 to a greater extent than they did that of the parent strain (Fig. 4B and C). Of these two antibiotics, polymyxin B rigidified the LPS aggregate to a greater extent. The addition of Ca²⁺ (Fig. 4A) or Mg²⁺ (data not shown) had only a slight effect on the motion of the Mg-LPS aggregates, slightly rigidifying the 1715 sample at intermediate concentrations. Similar changes in head group motion on cation addition have been observed previously for *P. aeruginosa* PAO1 (29).

Dansyl polymyxin binding to intact cells. To demonstrate that the alterations in cation binding associated with the *tolA* mutation were also observable in intact cells, a fluorescent analog of polymyxin B, dansyl polymyxin (23), was used. This compound has been shown to bind to LPS sites in both isolated LPS and intact cells (21, 22). Dansyl polymyxin was demonstrated to bind more readily to its LPS-binding sites in intact cells of the *tolA* mutant strain 1715 than to those of the *tolA*⁺ parent 1670 (Fig. 5). Furthermore, competitive displacement experiments demonstrated an apparently higher affinity of polymyxin, gentamicin, and Mg²⁺ for LPS sites in the sample from strain 1715, with concentrations resulting in 50% maximal displacement of dansyl polymyxin being approximately half of those observed for strain 1670 (data not shown).

DISCUSSION

The data presented here suggest that the *tolA* mutation causes an alteration in the LPS such that it binds polycations with a higher affinity. As a consequence of this, polycationic antibiotics interact more readily with the cell surface of the *tolA* mutant 1715 than they do with its *tolA*⁺ parent 1670 (as judged by the interaction of dansyl polymyxin with intact cells), are transported more readily into cells (based on results of [³H]streptomycin uptake experiments [19]), and kill the *tolA* mutant at substantially lower concentrations (19). These data support the concept (8, 9, 39) that the interaction of polycations with the cell surface, to disrupt the outer membrane permeability barrier, is an obligate component of cell killing by these polycations. The observation that the *tolA* mutant strain 1715 was relatively more affected in susceptibility to aminoglycosides (5- to 15-fold) than to polymyxin B (2-fold) is probably related to the relative

numbers and affinities of LPS-binding sites for these different types of antibiotics, since it is known that these parameters differ for the two antibiotics in wild-type cells (21).

The LPSs from *P. aeruginosa* 1670, 1716, and 1715 isolates were shown to contain as many as three major size populations (Fig. 1A). The Western blots of the LPS (Fig. 1B) revealed a ladderlike banding pattern with regular spacing. In addition, the bands were resolved as doublets, which presumably represents modifications in the core or lipid A on only a fraction of the total molecules, similar to the doublets seen in the LPSs from *Salmonella* spp. and *E. coli* (25, 27, 28, 41); such variability in substitutions has been observed in ³¹P-nuclear magnetic resonance analysis of *P. aeruginosa* lipid A phosphates (M. Bateley and R. E. W. Hancock, unpublished data). The levels of the two bands in the doublet were different for the three isolates (Fig. 1B). The parental isolate had about equal amounts of the two bands, while the isolate from 1715 had much greater amounts of one of the two bands. LPS from 1716 appeared to have intermediate levels of the two bands. Elemental analysis indicated that there were no significant differences in the levels of phosphate and metal cations for LPSs from the three isolates (Table 1), although there may have been differences in the esterification of the phosphate groups. The susceptibilities of

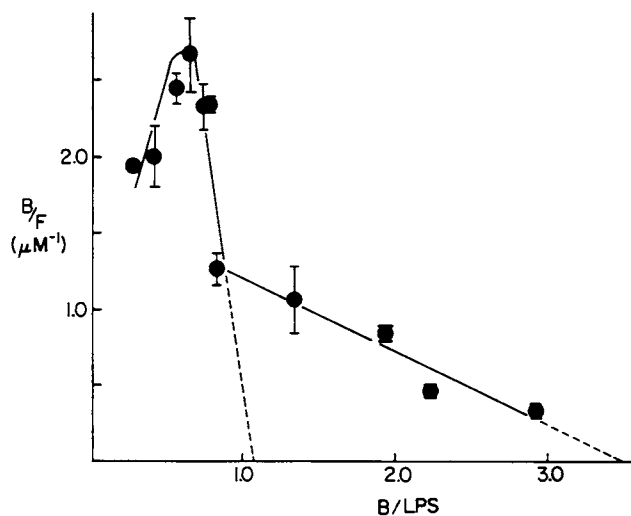


FIG. 2. Scatchard plots of CAT₁₂ binding to the magnesium salt of LPS from *P. aeruginosa* PAO1670. Samples were prepared as described in the text. Bound probe (B) is presented as moles of bound CAT₁₂ relative to moles of LPS. Error bars indicate the standard deviation for the triplicate measurements made on the samples. F is the free CAT₁₂ concentration.

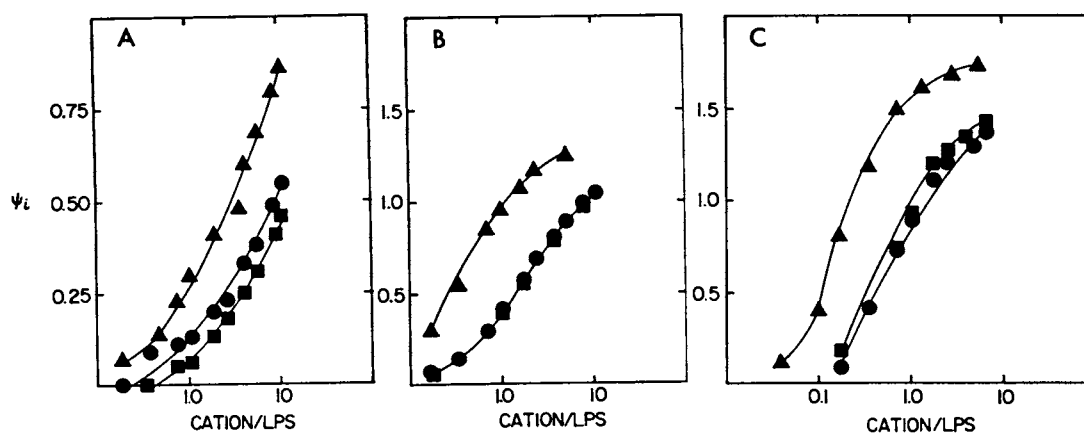


FIG. 3. Competitive displacement of CAT₁₂ detected by using the partitioning parameter Ψ_i measured as a function of added CaCl₂ (A), gentamicin (B), and polymyxin B (C). Partitioning of the spin probe CAT₁₂ was determined on the addition of cation to Mg-LPSs from *P. aeruginosa* PAO1670 (parent; ●), PAO1715 (antibiotic-supersusceptible mutant; ▲), and PAO1716 (revertant; ■). The amount of added cation is plotted as the number of cations per LPS (mole/mole).

the cells to polycationic compounds may have resulted from differences in the levels of specific substituents in the core or lipid A regions of the LPS, such as groups esterified to the phosphate moieties which could affect the cation interactions in this region.

To characterize the differences in cation affinity and the number of binding sites, we first analyzed the binding of the cationic spin probe CAT₁₂ by Scatchard analysis. For all three strains, the Scatchard plots were nonlinear (Fig. 2). The results indicated that there are two types of binding sites on LPS for which CAT₁₂ and presumably other cations compete. We also analyzed the binding data by calculating the Hill constant (α_H ; Table 2); this constant is an index of the cooperativity of binding. Values of α_H greater than 1 indicate positive cooperativity, whereas α_H values equal to 1 indicate no cooperativity (34). A Hill coefficient with a value of less than 1 or a curvilinear Scatchard plot may indicate either negative cooperative interactions or the heterogeneity of receptor sites (34). Thus, the high-affinity site in LPSs from all three isolates showed positive cooperativity of CAT₁₂ binding, whereas the low-affinity site indicated no cooperativity (Table 2). The LPS from the parent strain 1670 showed a significantly higher level of cooperativity in probe binding, which correlated with the apparent K_D . The coop-

erativity of binding for the revertant strain 1716 was similar to that of strain 1715. The differences in cooperativity may reflect a difference in the ability of CAT₁₂ to intercalate between the head groups of the LPSs from the three isolates. The cooperativity of binding for all the isolates may have been due to a CAT₁₂-induced alteration in LPS aggregate packing at low levels of CAT₁₂ association. The initial barrier to probe penetration at low probe concentrations may result from strong polar and ionic interactions within the core and lipid A, between O polymers, and between core sugars (31). In summary, the high-affinity CAT₁₂-binding sites appeared to be different for LPSs from all three isolates, and the structure of the LPS from the revertant strain did not appear to be identical to that of the parent. This is consistent with the results of the Western blots, which indicated that the doublet banding pattern of the 1716 sample was intermediate between those of the *tolA* mutant and the parental isolates.

The ability to displace the CAT₁₂ probe from the LPSs depended on the cations used. The interaction of polycationic antibiotics with LPS depends on the net charge, size, and structure, as well as the presence of hydrophobic regions on the antibiotic molecule (29, 36, 40). Since the LPS of *P. aeruginosa* has an unusually high phosphate content

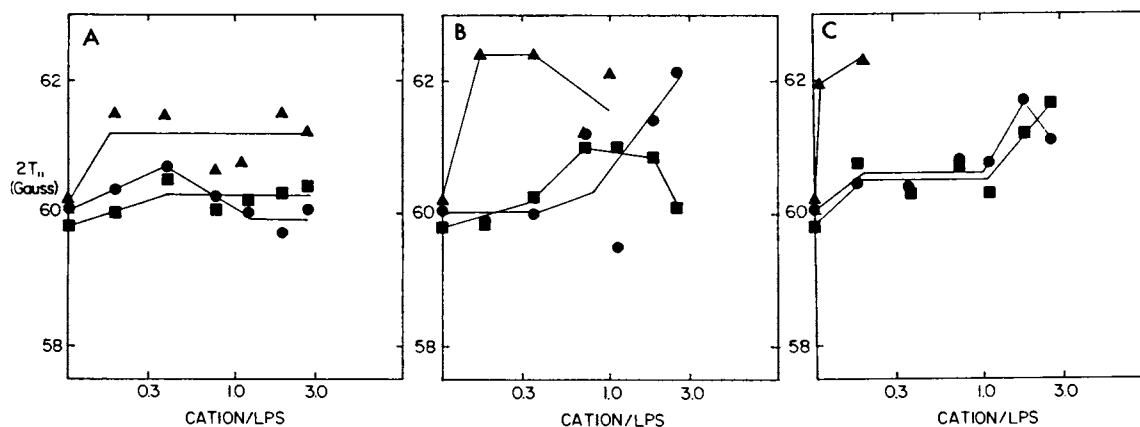


FIG. 4. Head group mobility of LPSs from *P. aeruginosa* PAO1670 (●), PAO1716 (■), and PAO1715 (▲) was measured by the hyperfine splitting parameter ($2T_H$; in gauss) of bound CAT₁₂. This parameter was measured as a function of increasing concentrations of CaCl₂ (A), gentamicin (B), and polymyxin B (C) added to the magnesium salts of LPS.

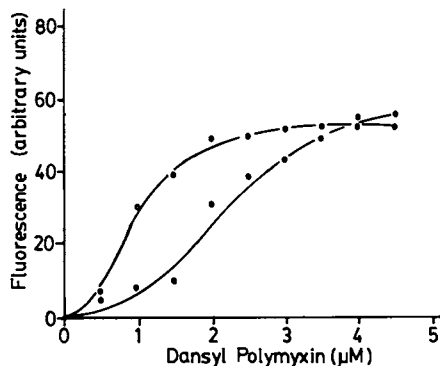


FIG. 5. Interaction of dansyl polymyxin with intact cells assessed by the increase in cell-associated fluorescence on the addition of different concentrations of dansyl polymyxin to intact cells of the *tolA* mutant strain PAO1715 (○) and its *tol*⁺ parent PAO1670 (●). Dansyl polymyxin fluoresces weakly in free solution and strongly when bound to intact cells or LPS (21). Thus, the increased fluorescence as a function of added dansyl polymyxin reflected dansyl polymyxin binding to the LPS molecules of the cells (21).

(42, 43) and two to three KDO residues (12, 43), there are numerous potential cation-binding sites, some of which may have a high affinity for certain cations. Competition between cations could be for the same site (21, 32, 36) or for overlapping sites (21, 29). As a given cation binds to LPS, the LPS aggregate may alter its conformation to optimize ionic interactions, and thus alter other binding sites. This induced change in conformation is suggested by the cooperative binding of polycations to LPSs from *E. coli* and *P. aeruginosa* (16, 21, 29). Thus, partitioning of the spin probe CAT₁₂ on the addition of cation may depend on the affinity of the added cation, the preferential site to which the cation binds, and the ability of the cation to induce a structural alteration on binding.

All of the polycations tested displaced CAT₁₂ more readily from LPS from strain 1715 than it did those from strains 1670 and 1716 (Fig. 3), which is consistent with the affinity of the probe for the three isolates (Table 2). Also, the dansylated polymyxin had a higher affinity for the LPSs from the *tolA* mutant. This difference in antibiotic binding to the LPS might result from differences in LPS structure caused by the differences in the substoichiometric modification observed in the Western blot of the LPS (Fig. 1B). Vaara et al. (41) and Peterson et al. (28) have demonstrated that decreases in polymyxin B binding to enterobacterial LPS is a consequence of increases in the substitution of the core or lipid A phosphates. The nature of the modifications in the LPSs isolated from the *tolA* mutants has yet to be elucidated. Interestingly, cation binding to the LPS from the revertant strain was similar to that from the parental isolate, even though the high-affinity CAT₁₂-binding site was distinctly different for these two samples.

When the Mg-LPS complexes were titrated with divalent cations, there was a higher affinity for Ca²⁺ than for Mg²⁺, suggesting that the Ca²⁺ ions tend to complex more tightly to LPS than do Mg²⁺ ions (31, 44). This is consistent with the ability of calcium to accommodate a variety of stereochemistries and a larger number of coordinating groups (44).

The increased rigidity of LPS aggregates on the addition of cationic antibiotics indicated that there was an antibiotic-induced alteration of the LPS packing (Fig. 4). Peterson et al. (28, 29) have observed similar polycation-induced structural alterations in the Mg-LPS complexes from members of

the family *Enterobacteriaceae* and *Pseudomonas* spp. Electron microscopy studies (11, 19, 33, 39) have shown that polymyxin B and other polycationic compounds alter the cell envelope. It has been proposed that these antibiotics alter the structure of the outer membrane and provide a pathway for their own uptake (8, 25). The alteration in LPS structure seen in the pure LPSs may reflect the changes that occur in the intact membrane, a proposal that is consistent with the observation that the LPS from the aminoglycoside-supersusceptible strain 1715 was rigidified by gentamicin at significantly lower concentrations than was the LPS from *tol*⁺ strains.

In summary, the differences in antibiotic susceptibilities observed among the three strains used in this study probably resulted from modifications of the core or lipid A region, which affected cation affinities and perhaps the conformation of the aggregates. This suggests that changes other than the overall negative charge can affect cation binding. Finally, the Ca²⁺ and Mg²⁺ titration data indicate that the LPS aggregate structure and perhaps the structure of the intact outer membrane may depend in part on the divalent cations present and their physicochemical properties. We are currently investigating the hypothesis that Ca²⁺ can stabilize and protect the cell surface against binding of polycationic antibiotics.

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