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Polycation Binding to Isolated Lipopolysaccharide from Antibiotic-Hypersusceptible Mutant Strains of *Escherichia coli*

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Lipopolysaccharide (LPS) samples isolated from a parent and two antibiotic-hypersusceptible mutant strains of *Escherichia coli* were analyzed for polycation affinity and level of binding. Purified salts of the LPSs from the parent strain, UB1005, and from one of the mutant strains, DC1, bound similar amounts of sodium and magnesium, but the samples from the second mutant strain, DC2, had significantly greater amounts of counterions bound per phosphate than did the other two isolates. The ^{31}P nuclear magnetic resonance spectra indicated that, compared with LPS from the parental strain, the sample from strain DC1 was similar but the DC2 sample contained fewer diphosphodiester and more diphosphomonoester groups. Motion within the lipid A head group regions of the magnesium salts of the three isolates was dramatically different, as revealed by an electron spin resonance probe. The binding of the cations to the LPS aggregates was measured by the displacement of this cationic spin probe from the LPS samples. The polycations polymyxin, gentamicin, and spermine displaced more probe from samples of the two mutant strains than from that of the parental strain. The sample from the most antibiotic-susceptible strain, DC2, had the highest affinity for all the polyvalent cations tested. The results indicate that antibiotic hypersusceptibility can result from at least two distinct alterations in LPS structure. The decrease in diphosphodiesters and increase in diphosphomonoesters in the LPS of the DC2 sample resulted in more acidic phosphate moieties and a more antibiotic-susceptible cell. In contrast, the alterations in the LPS of DC1 that resulted in antibiotic hypersusceptibility of the cell were not in the phosphate substituents. In both mutants, however, hypersusceptibility resulted in an alteration in LPS structure that increased the affinity of the molecules for polycations.

Gram-negative bacteria are resistant to many antibiotics and amphipathic and hydrophobic compounds because these materials apparently are unable to penetrate the outer membrane (13, 16). The interactions of lipopolysaccharide (LPS) with itself or with outer membrane proteins seem critical for maintaining this barrier function. Mutations which result in a loss of most of the LPS core sugars (deep rough mutants) bring about an increased permeability of the outer membrane to hydrophobic agents (9, 23, 24). These mutants are thought to be hypersusceptible either because there are fewer LPS-LPS interactions or because they have increased levels of phospholipid in the outer leaflet of the outer membrane (16). Ionic interactions within the core and lipid A region are known to be critical in forming the permeability barrier, since removal of divalent cations with chelators makes the cells more sensitive to hydrophobic compounds (13), presumably by causing a rearrangement in the outer membrane organization (16). Mutant strains of *Salmonella typhimurium* which are resistant to chelators and to polycation-induced permeability changes have been shown to have altered phosphate substitutions in lipid A (26, 27). In *S. typhimurium* (25) and *Escherichia coli* (10, 15, 17) several genetic loci have been identified that, upon mutation, result in increased outer membrane permeability and hydrophobic-antibiotic susceptibility. Mutations in these genes, however, have not been shown to result in a change in LPS structure. In contrast, a set of mutants of *E. coli* K-12 which are hypersusceptible to a wide range of dyes and antibiotics, such as β-lactams and chloramphenicol, have been described which have an altered LPS structure (22). The LPSs from these mutant strains (DC1, DC2, and DC3) are reported to have an altered amino-group-to-phosphate ratio (3).

By spectroscopic methods, we studied the LPSs from two of these antibiotic-hypersusceptible mutant strains, DC1 and DC2, and compared the LPS structures and physical properties to those of the parent strain, UB1005. We showed that the LPSs from both of the mutant strains have an increased affinity for polycations compared with that of the parent; the structural alterations in the DC2 LPS appear to be in the phosphate substituents, whereas the LPS from strain DC1 is similar to that of the parent in the level and type of phosphate substitutions.

MATERIALS AND METHODS

LPS preparation. *E. coli* K-12 UB1005, DC1, and DC2 were grown at 37°C in nutrient broth, and LPS was extracted by using hot aqueous phenol as described previously (6). Cultures were routinely tested for sensitivity to chloramphenicol before LPS isolation. The sodium salts of the LPS samples were formed by extensive dialysis of the samples against 10 mM sodium EDTA (pH 7.2) and then against distilled water. The magnesium salts of the LPS samples were formed by dialyzing the sodium salts against 10 mM $MgCl_2$ and then against distilled water. LPS recovery was determined by weighing lyophilized samples and was 1 to 2% of the dry weight of the cells for all three isolates.

Spin probe analysis. Electron spin resonance spectroscopy was carried out with a Varian X-band spectrometer (model E-112). Sample temperature, measured with a thermocouple placed within the cuvette, was held at 37°C. Cation titrations of LPS were performed by using the spin probe 4-dodecyldimethylammonium-1-oxyl-2,2,6,6-tetramethylpiperidine bromide (CAT_{12}) as described previously (18).

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TABLE 1. Ionic composition of purified LPS salts[a]

LPS salt	Strain	Ca/P	Mg/P	Na/P	+/P[b]	P/LPS[c]
Sodium	UB1005	0.02 ± 0.01	0 ± 0	2.24 ± 0.05	2.30 ± 0.07	5.3 ± 0.3
	DC1	0.02 ± 0.02	0 ± 0	2.17 ± 0.03	2.22 ± 0.07	5.3 ± 0.2
	DC2	0.01 ± 0.02	0 ± 0	3.12 ± 0.07	3.26 ± 0.11	4.2 ± 0.3
Magnesium	UB1005	0.01 ± 0.01	0.51 ± 0.01	0.26 ± 0.04	1.30 ± 0.08	ND[d]
	DC1	0.01 ± 0.02	0.53 ± 0.02	0.09 ± 0.05	1.17 ± 0.13	ND
	DC2	0.02 ± 0.03	0.66 ± 0.03	0.03 ± 0.07	1.37 ± 0.19	ND

[a] The protein content of the samples was less than 5%. Cation and phosphate levels are expressed as moles per mole. Results are expressed as means ± standard deviations.
[b] Total positive charges per phosphate.
[c] LPS molar levels were determined by weight, assuming a molecular weight of 4,500.
[d] ND, Not determined.

Upon the successive addition of cations to samples of LPS, the spectra were analyzed for $2T_{\parallel}$, 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, calculated as follows: $\psi_i = -\log\{([B]/[F])/C_i\}$, where $C_i = [B]/[F]$ with no added ions (4).

^{31}P NMR measurements. Samples were prepared for nuclear magnetic resonance (NMR) analysis by dissolving 25 to 29 mg of the sodium salt of the LPS in 2.0 ml of D_2O containing 40 mg of sodium dodecyl sulfate (SDS) and 10 mM EDTA. The pH of the samples was adjusted to 9.0 with NaOD and DCl and is reported as the uncorrected pH meter reading. To create a uniform suspension, NMR samples were sonicated for 10 min by using a Branson model 220 bath sonicator (Branson Sonic Power Co., Danbury, Conn.).

^{31}P NMR spectra were recorded on a General Electric GN-300 NMR spectrometer (General Electric Co., Schenectady, N.Y.) as described previously (18). ^{31}P NMR chemical shifts are referenced relative to external phosphoric acid (85%) (20).

SDS-polyacrylamide gel electrophoresis. Gels for SDS-polyacrylamide gel electrophoresis were prepared and run as described previously (21) by using the buffer system of Laemmli (12) and the silver staining procedure of Dubray and Bezard (8). The separating gel was formed with 15% acrylamide and 0.1% SDS, with a 7.5% acrylamide stacking gel.

Fatty acid analysis. Samples (10 mg) of the LPS sodium salts from strains UB1005 and DC1 were added to 5 ml of 1% methanolic sulfuric acid (vol/vol). The samples were maintained at 70°C for 16 h. After the addition of 5 ml of water, the fatty acid methyl esters were extracted with three portions of light petroleum ether (boiling point, 35 to 60°C). Samples (2 to 3 μl) of the extracts were injected into a Hewlett-Packard Model 402 gas chromatograph (Hewlett-Packard Co., Palo Alto, Calif.) equipped with a flame ionization detector. The column (6 ft [ca. 183 cm] by 2 mm [inside diameter]) was packed with 20% DEGS Gas Chrom Q 80/100 (Anspec, Ann Arbor, Mich.). The oven was set at 140°C initially and temperature programmed to 205°C (at 4°C/min). The injection port temperature was 220°C, and the detector temperature was 230°C. The fatty acid methyl ester standards methyl laurate, methyl myristate, methyl palmitate, and methyl 3-hydroxymyristate were purchased from the Foxboro Co., North Haven, Conn. Peak areas were determined by the cut-and-weigh method.

Chemical analyses. Inductively coupled plasma emission spectroscopy of wet-ashed LPS samples was used to quantitate phosphorus and metal ion content as described previously (6). Levels of 2-keto-3-deoxyoctulosonic acid (KDO) were quantitated by the thiobarbituric acid assay (7). Protein levels were determined by the method of Lowry et al. (14). Nucleic acid contamination was monitored by A_{260}, and heptose was quantitated by the cysteine-sulfuric acid method (29).

RESULTS

The magnesium and sodium salts of the LPSs from strains UB1005, DC1, and DC2 were analyzed for metal ion and phosphate levels by inductively coupled plasma emission spectroscopy (Table 1). The DC2 isolate had more bound counterions per phosphate, whereas the DC1 isolate had slightly less than the parental sample. Analysis of the phosphate content indicated that the UB1005 and DC1 isolates had similar phosphate levels but the DC2 isolate contained approximately one less phosphate group.

The levels of KDO were also measured for samples of known weight. Assuming a molecular weight of 4,500 for the LPS samples, it was found that the parent and DC1 strains had 1.3 reactive KDO groups per LPS and the DC2 LPS had 1.8 reactive groups.

To ascertain that the LPSs from the three strains had approximately the same molecular weights, the samples were run on SDS-polyacrylamide gels and their mobilities were compared with those of LPSs from R_a and R_e chemotypes. The results showed that the mobilities of the LPSs from strains UB1005, DC1, and DC2 were rather similar to each other and to that of the R_a LPS (data not shown). Thus, an approximate molecular weight of 4,500 appears to be a reasonable assumption for the three isolates. In addition, the three isolates were assayed for heptose; the levels detected in the samples from UB1005, DC1, and DC2 were 1.0 ± 0.2, 0.9 ± 0.1, and 0.9 ± 0.1 μmol of heptose per mg of LPS. Since the heptose assay measures the relative molar amounts of LPS, these results lend further support to the contention that the molecular weights of the three samples were not significantly different.

The results indicate that the parental and DC1 isolates had similar phosphate levels but the DC2 sample was unique, containing fewer phosphate groups but more reactive KDO residues. To characterize the differences in the phosphate moieties, ^{31}P NMR experiments were run with the three isolates (Fig. 1). The NMR spectra were very similar for the samples from strains UB1005 and DC1, but again the DC2 isolate was distinctly different. Peak assignments were made as previously described (18). The DC2 isolate had decreased levels of diphosphodiester groups and an increase in diphosphomonoester groups. The dramatic increase in diphosphomonoesters concomitant with a decrease in diphosphodiesters would account for the increased acidity of the

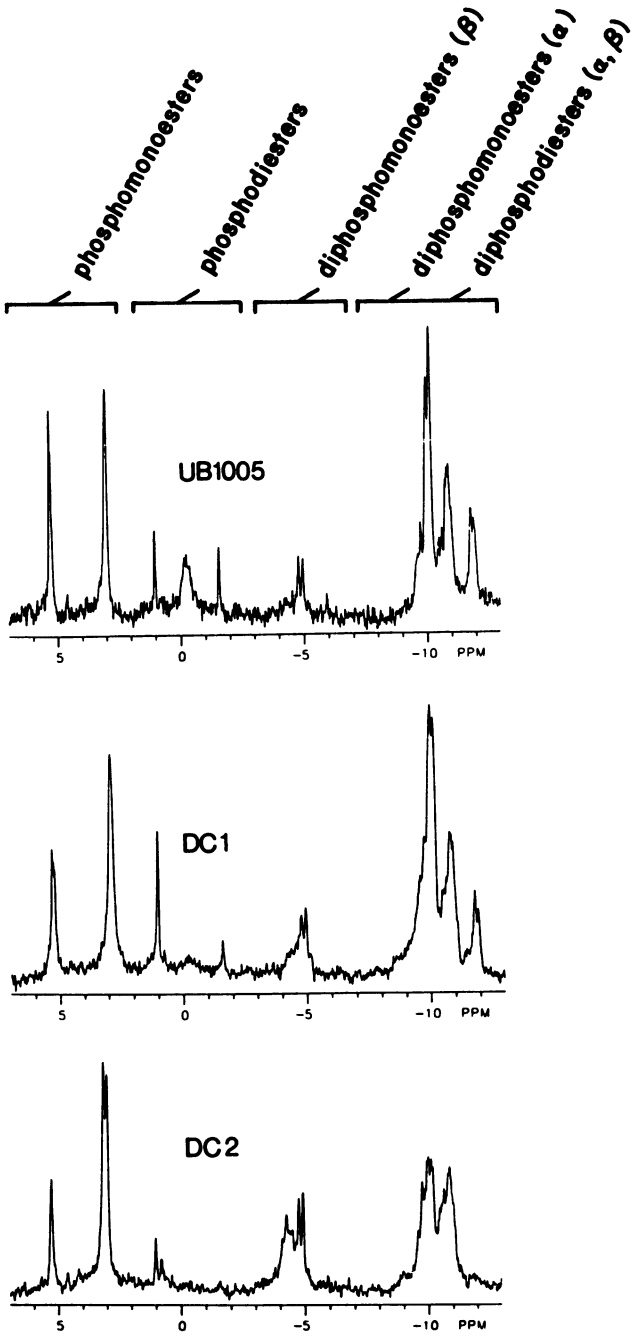

FIG. 1. ^{31}P NMR spectra of LPSs isolated from *E. coli* UB1005, DC1, and DC2. The ^{31}P NMR spectra were obtained at 25°C at pH 9.0.

phosphate groups of the DC2 samples compared with that of the UB1005 isolate, as reflected in the levels of bound ions (Table 1).

To assess whether there were acylation differences in the UB1005 and DC1 samples, the fatty acid compositions of these two isolates were characterized. Only four fatty acids were present at levels greater than 1%, and the relative proportions of these four fatty acids by weight were the same for both isolates ($C_{12:0}$, 21%; $C_{14:0}$, 27 to 28%; $C_{16:0}$, 2 to 3%; and 3OH-$C_{14:0}$, 47 to 50%).

The relative affinities of cations for the magnesium salts of the LPSs from the three strains and the ability of polycations to alter the structures of the magnesium salts were monitored with the electron spin resonance probe CAT_{12}. The magnesium salt was used for these titrations because it best mimics the structure of the native sample (5, 18). Upon addition of the polycation spermine, gentamicin C, or polymyxin B, CAT_{12} was displaced from the LPS, as revealed by the relative increases in the CAT_{12} displacement parameter, ψ_i. All three cations displaced CAT_{12} at lower concentrations when the LPSs from the two mutant strains were used rather than that from the parental sample (Fig. 2). This reflects a higher affinity of the cations for the mutant isolates. The standard deviation of ψ_i for titrations of independent isolates was ±0.04 and was approximately constant across the concentration range of added cations. Thus, the standard deviation for the data shown in Fig. 2 is equal to or less than the size of the symbols used, indicating that the differences in cation affinities between the mutant and parental isolates were significant. Also, the DC2 isolate had a slightly higher affinity for spermine and gentamicin than did the DC1 isolate, but both samples bound polymyxin in a similar manner. The differences in the affinities of the three isolates for gentamicin were small but were significant and reproducible. The apparently higher affinity of the DC2 isolate for polycations compared with that of the parental sample may

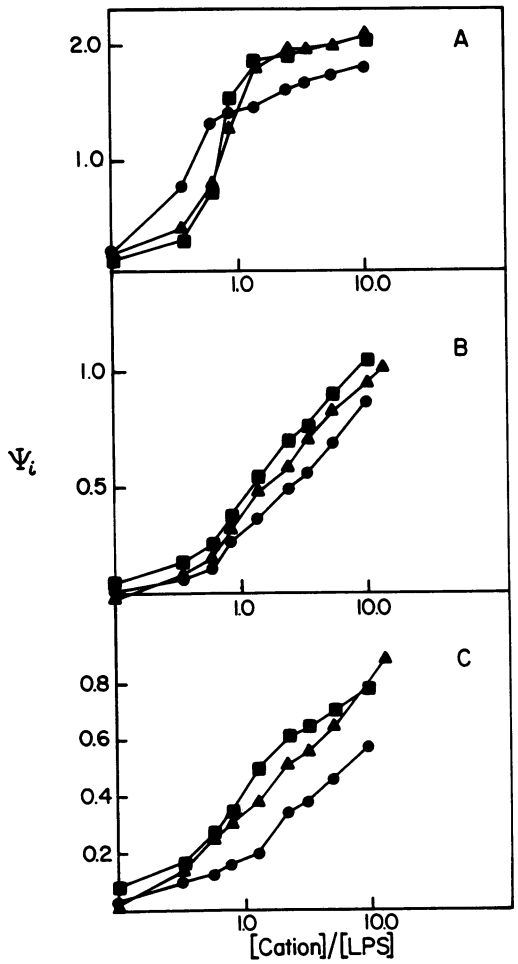

FIG. 2. Partitioning of the spin probe CAT_{12} upon addition of polymyxin (A), gentamicin (B), or spermine (C) to the magnesium salts of LPSs isolated from strains UB1005 (●), DC1 (▲), and DC2 (■).

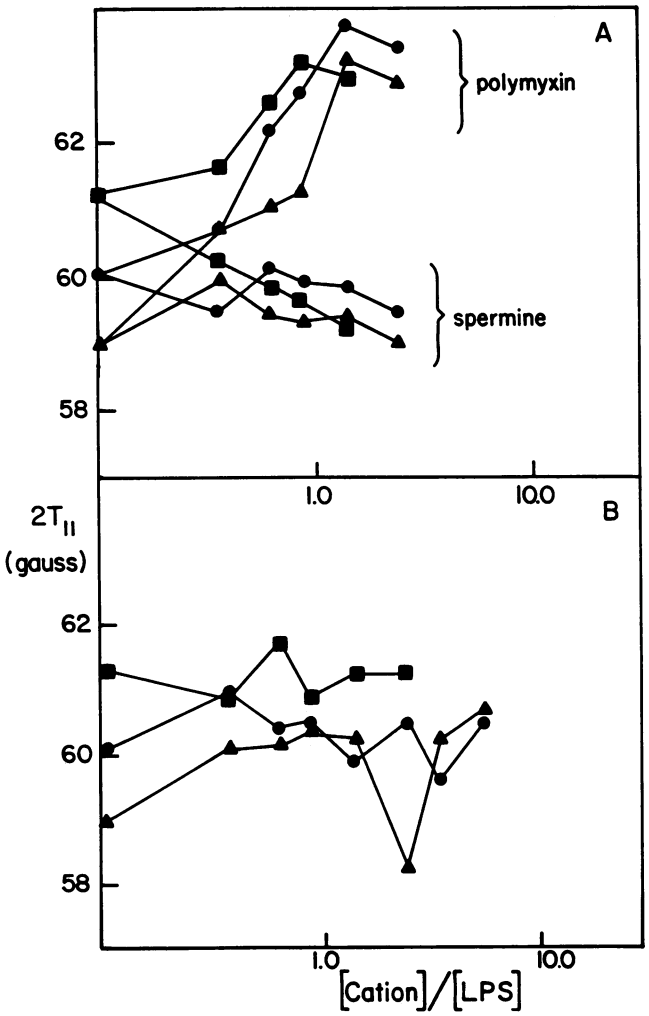

FIG. 3. Head group mobility of the magnesium salts of LPSs from *E. coli* UB1005 (●), DC1 (▲), and DC2 (■), as measured by the hyperfine splitting parameter ($2T_{||}$) of bound CAT_{12}. This parameter was measured as a function of increasing concentrations of polymyxin or spermine (A) or gentamicin (B).

have resulted from the higher acidity of the phosphates in the DC2 sample noted above. However, the increased polycation affinity of the DC1 sample cannot be explained on this basis.

The order of cation affinity of the three LPS isolates was generally the same as the order of susceptibility of the strains to β-lactam and hydrophobic antibiotics. For instance, DC2 has been shown to be over 20-fold more susceptible to novobiocin and ampicillin than is UB1005 (3), and its LPS had the highest affinity for polycations; strain DC1 is only twofold more sensitive to the same drugs (3), and its LPS was intermediate in cation affinity. Interestingly, the magnesium salt of the LPS from the parental strain had a higher affinity for polymyxin at low concentrations than did those from the two mutant strains but a lower affinity at higher concentrations (Fig. 2A).

During the cation titrations, the motion of the probe associated with LPS was determined by measuring the hyperfine splitting parameter, $2T_{||}$, at each point (Fig. 3). Differences in $2T_{||}$ of greater than 0.5 G have been shown to be significant (19). In the absence of added cations, the LPSs of the three strains had significantly different head group mobilities. The DC2 sample was more rigid and that of DC1 was less rigid than the parental LPS. The higher LPS rigidity of the DC2 isolate may have been the result of the higher level of bound Mg^{2+} than was found in the UB1005 sample. The additional divalent cation bridges between the LPS molecules may rigidify the head groups. Conversely, the DC1 LPS magnesium salt sample had levels of monovalent cations lower than those of the parental sample (Table 1); the lack of charge neutralization may have resulted in increased head group separation.

Upon addition of polymyxin B, the LPSs of all three strains were rigidified and at high concentrations had approximately the same head group motion (Fig. 3A). Previous studies have shown that polymyxin has a strong tendency to immobilize LPS head groups (18–20). Gentamicin, which also reportedly rigidifies LPS but to a lesser extent than does polymyxin B (19, 20), had little effect on the head group motion of the UB1005 and DC2 samples but rigidified the DC1 sample (Fig. 3B). Finally, spermine addition brought the head group motion of all three samples to approximately the same level (Fig. 3A); the tendency of polyamines to fluidize LPS has been reported previously (19, 20).

DISCUSSION

In recent years, analyses of the chemical alterations of the cell surface that result in increased antibiotic susceptibility have been performed with mutants of several different gram-negative species (1, 3, 11, 25, 27). Alterations in the LPS composition have been detected in antibiotic-hypersusceptible mutants of *Pseudomonas aeruginosa* (11) and *E. coli* (3), but three classes of antibiotic-susceptible mutants of *S. typhimurium* showed no alteration in the LPS structure (27). In the present study, we looked at certain antibiotic-susceptible mutants of *E. coli* to relate the differences in the LPS structure to chemical sensitivity. In addition, we determined how these changes affect cation binding to the LPS.

The two mutants analyzed, viz. strains DC1 and DC2, have significantly enhanced differential susceptibilities to a wide variety of antibiotics, such as β-lactams, chloramphenicol, and dyes, due to increased permeability of the outer membrane of the cell (3, 22). We found that the alterations in the structures of the LPSs from the two mutant strains were significantly different. The DC2 LPS had a lower level of phosphate compared with the parental LPS, missing diphosphodiester groups but containing more phosphate in the diphosphomonoester region of the ^{31}P NMR spectrum. Since both changes would tend to cancel each other, leaving the overall acidity of the molecule unchanged, the difference in distribution of charge in the UB1005 and DC2 isolates apparently caused the more rigid head group in the DC2 sample. This decrease in head group motion may have resulted from a decrease in charge at the diglucosamine head group level, presumably with a concomitant increase in charge in the core oligosaccharide; a decrease in charge at this level would result in decreased charge repulsion and hydration of the lipid A head group, allowing closer packing in the aggregate. It is known, for instance, that the head group of R_e LPS from *E. coli*, which contains only two or three phosphate groups, is significantly more rigid than that of R_a LPS, which contains approximately seven phosphate groups (5).

In contrast, the LPS from strain DC1 contained the same level and types of phosphate groups as the parental sample, although the magnesium salt of the LPS of the mutant bound

lower levels of monovalent cations. The phosphates in the magnesium salt were not as charge neutralized as in the magnesium salt from the parental strain, and this resulted in increased head group charge, head group hydration, and thus head group mobility in the DC1 sample. The structural basis for the altered cation binding is not known. Small changes in the phosphomonoester peaks at 1 and 5.5 ppm in the ^{31}P NMR spectrum were observed for the DC1 isolate, and such changes may affect cation binding.

We have no evidence that either the DC1 or DC2 isolate has increased levels of amine-containing groups, as has been reported (3). In contrast, our results indicate that the DC2 isolate has fewer phosphodiester-linked moieties, some of which could be amine-containing groups such as ethanolamine. The lack of a phosphoester-linked group in the KDO region of the LPS from strain DC2 could also explain the higher level of KDO reactivity of the DC2 sample in the thiobarbituric acid assay.

Whatever the structural alterations in the LPSs from strains DC1 and DC2, the changes result in an increased affinity for spermine and gentamicin. The increased affinity of the DC2 sample was due, in part, to the higher acidity of the phosphate groups. The higher affinity of the DC1 sample for the polycations compared with that of the parental sample may have resulted from the lower level of bound cations neutralizing the negative charges in the magnesium salt of the LPS. Thus, for both mutant isolates, the higher effective overall or local negative charge caused an increased affinity for polycations in the aggregates compared with that of the parental sample. The only exception was the binding of low concentrations of polymyxin. In this range of added polycation, the UB1005 samples had a higher affinity for polymyxin than did the samples from the mutants. Binding of polymyxin to LPS involves both ionic and hydrophobic interactions. Thus, differences in the packing of the hydrophobic domain in the LPSs of the mutant strains may decrease polymyxin binding at low concentrations. Upon further addition of polycation, disruption of the acyl chain packing would reduce the contribution of the hydrophobic interactions in controlling further polymyxin binding.

The chemical alterations in the LPS of the DC2 isolates are the opposite to those observed in the LPSs of polymyxin-resistant (*pmr*) mutant strains of *E. coli* (18). In these *pmr* mutants, the LPSs, compared with the LPS of the parent strain, had decreased phosphomonoester and increased phosphodiester groups which resulted in a decrease in LPS acidity, decreased divalent metal ion binding, and decreased polymyxin binding at high antibiotic concentrations. Interestingly, compared with the LPS of the parental strain, the LPSs of the *pmr* mutants had increased polymyxin binding at low polymyxin concentrations, the opposite of what was seen with the DC2 sample.

The alterations in the LPS of the DC1 strain are unique and are not a result of significant changes in phosphate esterification. The change in the structure of the LPS in the DC1 strain results in an increased affinity for polycations. The alteration may be similar to that of the antibiotic-susceptible mutants of *S. typhimurium* (25). The LPSs from these mutant strains also did not appear to have altered phosphate levels, although the fatty acid analyses of the LPSs appeared to show decreased levels of hydroxy-fatty acids. The hydroxyl or carboxyl groups of fatty acids ester linked to the hydroxy-fatty acids may be involved in extensive hydrogen bonding at or below the lipid A head group. Thus, a decrease in hydroxy-fatty acids would result in decreased LPS-LPS hydrogen bonding and a more permeable outer membrane. However, the LPS from *E. coli* DC1 did not have a significantly different level of hydroxy-fatty acids. Thus, the structural basis for the increased cation affinity for the LPS from the mutant strain DC1 has yet to be identified.

The relationship between the increase in cation affinity for the mutant LPS isolates and increased antibiotic susceptibility of the intact cell is not obvious. First, the susceptibilities of these mutant strains to polycationic antibiotics are not dramatically increased. Strains UB1005 and DC1 are equally susceptible to polymyxin, and the DC2 strain shows only a twofold increase in polymyxin susceptibility (3). Also, the susceptibilities of the three strains to aminoglycoside antibiotics are not dramatically different. However, dramatic differences in susceptibilities to β-lactams and hydrophobic compounds have been reported (22). Compared with strain UB1005, strain DC1 is approximately 10-fold more susceptible to spiramycin, methicillin, and penicillin V, and DC2 is approximately 100-fold more susceptible to the same compounds (3). Thus, the mutant strains are not altered in susceptibility to cationic antibiotics, which in some instances can promote their own uptake (16). In contrast, the alterations in the LPSs of the mutant isolates appear to facilitate permeability either through the hydrophobic pathway of the outer membrane, by decreased LPS interaction within the outer membrane, or through the porin (16). Alterations in interactions between the aberrant LPS and porin may modulate the porin channel. Further studies are needed to determine which permeation pathway is altered.

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