

Escherichia coli Susceptible to Glycopeptide Antibiotics

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Mutants of *Escherichia coli* susceptible to vancomycin were isolated after mutagenesis with nitrosoguanidine. One such mutant was studied extensively. Multiple regression analysis of the relationship between physical properties of 20 glycopeptides and their in vitro activities against the vancomycin-susceptible mutant revealed a significant correlation with molecular mass ($P = 0.007$). pI, hydrophobicity, and affinity of the glycopeptide for the pentapeptide target were not as important for activity. This suggested that a block of access of the antibiotic to its target could be the major factor determining activity. Outer membrane proteins of the vancomycin-susceptible mutant, resistant parent, and revertant strains appeared normal. The mutant exhibited increased susceptibility to both erythromycin and fusidic acid which was lost in single-step revertants to vancomycin resistance. Polymyxin B nonapeptide was synergistic with erythromycin and fusidic acid against the parent and revertant but not against the susceptible mutant. Analysis of the susceptibilities of control strains of *E. coli* and *Salmonella typhimurium* with known defects in lipopolysaccharide (LPS) synthesis revealed that core LPS mutants (Re chemotype) were phenotypically similar to the *E. coli* mutant under study. However, the LPS core of the mutant migrated slightly less rapidly on sodium dodecyl sulfate-polyacrylamide gel electrophoresis than wild-type or revertant core LPS and did not resemble Re chemotype LPS core obtained from *Salmonella rfaC* and *rfaD* mutants. These data suggest that defects in LPS core structure other than loss of heptose moieties may also be important in loss of resistance to large, hydrophilic molecules such as glycopeptides.

Enteric gram-negative bacilli are normally resistant to vancomycin and other glycopeptide antibiotics. The mechanism of this resistance has not been characterized, but since these antibiotics are large molecules (1,449 daltons for vancomycin), one might presume that the complex outer membrane could play a role. Lipopolysaccharide (LPS) has been shown to be important in resistance to large, lipophilic molecules such as the macrolides, as well as to smaller ones such as the aminoglycosides (2, 9, 10-12, 15, 17-19, 22). The outer membrane proteins (OMPs) have been demonstrated to play a role in resistance mainly to smaller, hydrophilic molecules, such as β -lactams, trimethoprim, chloramphenicol and tetracyclines (12). Resistance to some compounds such as quinolones and tetracyclines can be affected by changes in either OMPs or LPS (2, 10).

Vancomycin and other glycopeptides are thought to be beyond the normal size exclusion limit of the porin proteins (11, 12). Even though they are relatively hydrophilic compounds, glycopeptides are more active against *rfaF* and *rfaE* deep rough mutants (Rd2 and Re chemotypes) of *Salmonella typhimurium* lacking core heptose moieties, than their smooth parents (18). These data suggested that the LPS core, especially the heptose-containing area just proximal to the core 2-keto-3-deoxyoctulosonic acid (KDO), might play a role in the normal exclusion of glycopeptides from their peptidoglycan targets in the cell wall. By using a vancomy-

cin-susceptible mutant of *Escherichia coli*, we determined that other, as yet undefined, defects in the LPS core can also result in hypersusceptibility to glycopeptides.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used include the following: *E. coli* DCO and DC2 (16); K802 (a K-12 strain [*hsdR*⁺ *hsdM*⁺ *gal met supE*]) (24), from which a spontaneous nalidixic acid- and rifampin-resistant mutant, K802NR, was derived by sequential selections; a vancomycin-susceptible mutant, designated strain 16, derived from K802NR; the spontaneous vancomycin-resistant derivative of strain 16, 16R1; *S. typhimurium* SGSC 225 (18) with normal LPS; SA1377, *rfaC*; and SGSC 358, *rfaD* (these strains were a gift of K. E. Sanderson, *Salmonella* Genetic Stock Center). *E. coli* D280 is a smooth K27 strain, and F829 is a derivative of D280 lacking both the K27 antigen and the O antigen (strains were received from Hiroshi Nikaido).

Nitrosoguanidine mutagenesis. K802NR was grown to mid-log phase in the presence of 0.5 \times the MIC of nitrosoguanidine in antibiotic medium 3 at 37°C. Cells were diluted and plated on antibiotic-free agar and then were replicated onto agar containing concentrations of vancomycin, erythromycin, or other antibiotics at levels below the MIC for the K802NR parent. Colonies which grew well on antibiotic-free agar and which were completely inhibited at the lowest concentrations of vancomycin were subjected to a second round of mutagenesis performed in a fashion identical to that used for the first round. Again, healthy colonies susceptible to vancomycin were chosen. One such mutant, here called 16, was utilized for the remainder of these studies.

Selection of a spontaneous vancomycin-resistant revertant of mutant 16. Because of the multiple mutagenesis procedures used to isolate strain 16, we selected a spontaneous vancomycin-resistant revertant of the mutant by plating 100

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μ l of an overnight culture on Mueller-Hinton agar plates onto which four disks containing 30 μ g of vancomycin were placed. One of the colonies growing close to the disk was chosen, shown to be vancomycin resistant (see Results), and was used along with the K802 parent and the vancomycin-susceptible mutant 16 in the studies to be described. It was designated strain 16R1.

MICs. MIC assays were performed by agar dilution methods in brain heart infusion agar (Difco Laboratories, Detroit, Mich.). Glycopeptides used were the following: vancomycin, 281980, 197221, 264656, 212556 and 227409 (Eli Lilly & Co., Indianapolis, Ind.); teicoplanin, 62208, 62476 (4 [reported as A40926]), and 62211 (Lepetit Research Center, Milan, Italy); 104662, 104421, 103658, 102010, 104093, 104428, 104214, 105285, 104420, and 101542 (Smith Kline & French Laboratories, Philadelphia, Pa.). These compounds have all been characterized by molecular mass, and for all but the numbered Lilly compounds their charge (pI) has been estimated based on isoelectric focusing. For all but the numbered Lepetit compounds, an estimate of hydrophobicity based on high-pressure liquid chromatography retention times was also provided. All these properties were provided for both vancomycin and teicoplanin, whose precise structures are also known (13). Polymyxin B nonapeptide (PMBN) was kindly provided by M. Vaara (23).

Peptidoglycan synthesis. Bacteria were grown overnight in antibiotic medium 3 broth (Difco) at 37°C with agitation, diluted in the morning 1/20 to 1/50 in fresh antibiotic medium 3, and incubated at 37°C with agitation until an optical density of 0.4 to 0.6 was obtained. The cells were then centrifuged at 5,900 \times g at 4°C for 10 min and washed twice with an equal volume of distilled water. The pellets were suspended in a 20% volume of wall-thickening medium (1, 21) with 50 μ g of chloramphenicol per ml and 0.5 μ Ci of ³H-N-acetyl-D-glucosamine per ml and were incubated for 60 min at 37°C. The cells were centrifuged in microcentrifuge tubes and extracted with 5% (wt/vol) sodium dodecyl sulfate (SDS). The pellets were then collected by centrifugation and suspended in cold 5% (wt/vol) trichloroacetic acid with bovine serum albumin added (final concentration, 24 μ g/ml) as a carrier. The cells were allowed to precipitate for at least 1 h at 4°C and then were filtered through glass fiber filters (Whatman GFA), washed with 10 ml of cold 10% (wt/vol) trichloroacetic acid followed by 10 ml of cold 95% (vol/vol) ethanol, dried, and counted.

Affinity column chromatography. Affinity columns were prepared by using Affi-Gel-202, 1-ethyl-3 (3-dimethylamino-propyl) carbodiimide hydrochloride (Bio-Rad Laboratories, Vitry-Sur-Seine, France) according to the instructions provided by the manufacturer. A 100-mg sample of either teicoplanin or vancomycin was used with 5 ml of Affi-Gel beads, and the reaction proceeded for at least 2 h at room temperature. Beads were stored at 4°C. Columns were poured into Pasteur pipettes using 0.5 ml of beads in a 1-ml volume. The beads were washed three times with 1 to 2 ml of water. Approximately 2,000 cpm of ¹⁴C-UDP-muramyl pentapeptide purified from *E. coli* was applied in 0.5 ml of distilled water for each assay. The column was then washed with 0.5 ml of 0.01 M Tris hydrochloride at pH levels of 8, 9, and 10, and it was washed finally with 0.5 ml of 1 N NaOH. Fractions were collected directly into scintillation vials and were counted. These methods are similar to those used by DePedro and Schwartz (3).

Affinity of glycopeptides for pentapeptide. Approximately 1,000 cpm of ¹⁴C-UDP-muramyl pentapeptide was added to 20 μ l of teicoplanin-Affi-Gel bead suspension plus differing

TABLE 1. Physical properties of glycopeptides versus in vitro activity against *E. coli* 16

Glycopeptide	M_r	pI	Log ₂ MIC (μ g/ml) for strain:	
			16	K802
62208	1,283	8.00	3	5
104421	1,290	7.90	3	6
103658	1,294	7.10	3	5
102010	1,295	5.00	5	7
104093	1,337	3.90	7	8
104428	1,338	7.10	5	6
Vancomycin	1,449	8.10	3	8
104662	1,452	7.90	3	8
281980	1,529	NA ^a	4	NA
197221	1,589	NA	5	NA
264656	1,595	NA	4	NA
212556	1,632	NA	6	NA
227409	1,668	NA	4	NA
104214	1,728	7.30	8	8
105285	1,730	3.80	7	8
62476	1,733	4.10	8	8
104420	1,872	7.30	5	8
101542	1,874	3.80	7	8
Teicoplanin	1,880	5.10	8	8
62211	1,970	8.70	8	8

^a NA, Not available.

amounts of various glycopeptides. The final volume was adjusted to 50 μ l with distilled water in 500- μ l microcentrifuge tubes. The mixtures were incubated at 37°C for 30 min. The beads were centrifuged for 1 min at 13,000 rpm in an Eppendorf microcentrifuge and 40 μ l of supernatant was removed and counted directly in Liquisol scintillation fluid. The percentage of total counts bound to beads that had been released into the supernatant was calculated.

Visualization of OMPs. SDS-polyacrylamide gel electrophoresis (PAGE) for OMPs was performed as described by Laemmli (8).

Characterization of LPS. The LPS of *E. coli* and *S. typhimurium* were solubilized and separated by SDS-PAGE in 6 M urea and stained as previously described (20).

Statistical methods. For multivariate analysis, the variables included log₂ MIC, molecular mass, pI, high-pressure liquid chromatography retention time, and relative affinity of the glycopeptide for pentapeptide (1 to 100% displacement of labeled pentapeptide from Affi-Gel-teicoplanin beads). First, analysis of variance was performed. If a significant *F* value was obtained, the data were subjected to multiple linear regressions. Microstat software and an Amstrad 1512 micro-computer were utilized for these analyses.

RESULTS

MICs. Log₂ MICs of various glycopeptide antibiotics for *E. coli* K802 and strain 16, as well as the molecular masses and pIs of the compounds used, are shown in Table 1. MICs of the listed antibiotics for 16R1 were also measured and were always within one dilution of the values shown for K802 (data not shown). The *E. coli* mutant was more susceptible to the glycopeptides than either its parent or the resistant revertant, although the magnitude of the difference varied somewhat with the compound.

Peptidoglycan synthesis in *E. coli*. The inhibition of peptidoglycan synthesis by increasing concentrations of vancomycin for the vancomycin-susceptible mutant and its resis-

TABLE 2. Inhibition of peptidoglycan synthesis in *E. coli* by vancomycin

Strain (MIC in $\mu\text{g/ml}$)	% Inhibition at vancomycin concn ($\mu\text{g/ml}$) of:		
	0	64	128
16 (16)	0	82	81
16R1 (128)	0	0	0

tant revertant strain is shown in Table 2. K802, in a separate experiment, also demonstrated increased resistance to inhibition of peptidoglycan synthesis by vancomycin compared with strain 16 (data not shown).

Pentapeptide affinity for glycopeptides. To show that *E. coli* pentapeptide is capable of binding glycopeptides and to get a rough idea of the relative binding affinities of teicoplanin and vancomycin for *E. coli* pentapeptide, the experiments shown in Fig. 1 were performed. For these experiments, vancomycin and teicoplanin affinity columns were prepared as outlined in Materials and Methods. Purified ^{14}C -UDP-muramyl pentapeptide was layered onto these small affinity columns prepared without glycopeptide, or with either vancomycin or teicoplanin, and the columns were then washed with pH steps. The results of these assays are shown in Fig. 1. Pentapeptide binds tightly to both vancomycin and teicoplanin columns but not to the blank column. In addition, that the affinity of the labeled pentapeptide precursor for teicoplanin was stronger than that for vancomycin was indicated, since label was only recoverable from the teicoplanin column after washing with 0.1 N NaOH, compared with pH 10 to pH 11 buffer for vancomycin. Our results for the vancomycin column were essentially identical to those of DePedro and Schwartz (3).

Glycopeptide affinity for purified pentapeptide precursor. This was measured as indicated in Materials and Methods. Basically, labeled and purified pentapeptide precursor was added to Affi-Gel beads with teicoplanin covalently attached. Various glycopeptides were then added to displace the pentapeptide from the teicoplanin beads. Initially, for selected compounds, a dose-response curve was constructed (Fig. 2). On this basis, 100 μg of each of 14 glycopeptides was used in the competition assay, and the percentage of radioactivity released was used to analyze the relationship

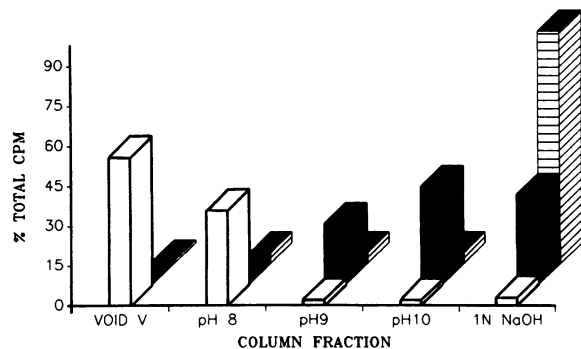


FIG. 1. Affinity column chromatography of purified *E. coli* ^{14}C -UDP-muramyl pentapeptide precursor on glycopeptide columns. Symbols: □, a blank column with no attached glycopeptide; ■, a column prepared with vancomycin; ▨, a column prepared with teicoplanin. The x axis represents the pH steps used to elute the pentapeptide, with VOID V as the void volume fraction. The y axis represents total counts per minute released.

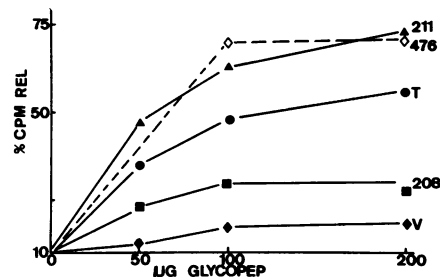


FIG. 2. Relative binding affinities of glycopeptides. A competition experiment was performed (see text). Percent counts per minute released from teicoplanin-Affi-Gel beads by various glycopeptides is shown along the y axis while the micrograms of glycopeptide added to the reaction mixture is shown along the x axis. Abbreviations: T, teicoplanin; V, vancomycin; 211, 62211; 476, 62476; and 208, 62208.

between the affinity of glycopeptides for pentapeptide and their *in vitro* activity.

Structure-function relationships among glycopeptides. The 20 glycopeptides were compared for physical properties, molecular mass, pI, and affinity for pentapeptide versus activity (Table 1) against either the *E. coli* mutant or the resistant parent or revertant strains. For the vancomycin-susceptible mutant, multiple linear regressions using the 14 compounds that could be compared for all these properties revealed the following: a regression coefficient (r) of 0.0048, $P = 0.00714$ for molecular mass; $r = -0.3854$, $P = 0.0836$ for pI; and $r = 0.024$, $P = 0.315$ for relative affinity of labeled pentapeptide for the glycopeptides. Hydrophobicity was not related in several other statistical models and was not included in this analysis. Thus molecular mass appeared to be the most important factor, with perhaps charge playing some role. Neither pentapeptide affinity nor hydrophobicity appeared to be as important as molecular mass and charge for glycopeptide activity against *E. coli*. The molecular mass was also important for glycopeptide activity against the parent strain ($r = -0.72$, $P = 0.00036$; univariate regression). These observations further support the idea that there is a barrier or obstacle to glycopeptide entrance to its pentapeptide target in *E. coli*, which can be overcome to a certain extent by reducing the size of the glycopeptide or by increasing its pI.

Nature of the barrier to glycopeptide access. On the basis of the experiments described above, we concluded that vancomycin resistance is normally caused by a block of antibiotic access to its target. Separation of OMPs derived from the vancomycin-susceptible mutant and its resistant revertant derivative were performed in 12% polyacrylamide-SDS gels. The results are shown in Fig. 3. No difference in the OMP profiles could be discerned. A simultaneous analysis of OMPs from the parent yielded findings identical to those from the mutant and revertant (data not shown).

The *E. coli* mutant was also more susceptible to erythromycin and fusidic acid than was the vancomycin-resistant parent or revertant (Table 3). The addition of 3 μg of PMBN per ml, which is known to interact with the LPS of *E. coli* (23), to erythromycin and fusidic acid resulted in a decrease in the MICs of these latter compounds for the parent and revertant strains but not in those for the vancomycin-susceptible mutant. The addition of PMBN to vancomycin or teicoplanin had little effect (if anything, the MICs of these compounds were increased by PMBN [data not shown]). This suggested that the resistance to erythromycin and

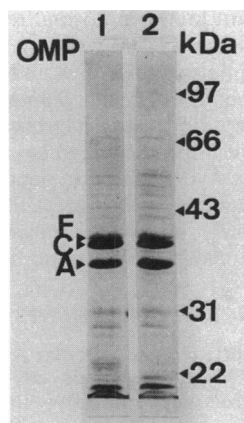


FIG. 3. Separation of OMPs of *E. coli* by 12% SDS-PAGE. Lanes: 1, strain 16; 2, strain 16R1. The position of molecular mass markers are indicated on the right. The positions of OMPs A, C, and F are indicated on the left.

fusidic acid were linked to vancomycin resistance in *E. coli* and that the basis of this resistance may be related to some aspect of LPS structure.

Since K802, the parent strain, was a K-12 derivative and was therefore *rfb* and therefore lacking the O antigen (6, 7), we also tested strain D280, a K27 strain with a complete O antigen, and its mutant derivative F829 which lacks both the K27 and the O antigen (strains obtained from H. Nikaido). Both these strains were resistant to all the glycopeptides tested (data not shown), suggesting that O antigen is not involved in resistance to glycopeptides. This was consistent with the observations of Roantree et al. (18). *E. coli* DCO and DC2 (16, 17), a wild type and LPS mutant pair, were also examined (Table 3). DC2 carries a known defect in LPS core relating to the level of esterification of the lipid A (17). The profile of susceptibility of DC2 to the glycopeptides (normal levels of resistance), ampicillin, chloramphenicol, and erythromycin, as well as erythromycin plus PMBN, was different from that of the mutant strain. Thus, it was unlikely that the defect in vancomycin-susceptible *E. coli* resulted from a defect identical to that present in DC2. However, the *rfaC* and *rfaD* deep rough mutants of *S. typhimurium* which lack the heptose portion of their LPS core structure (Re chemo-

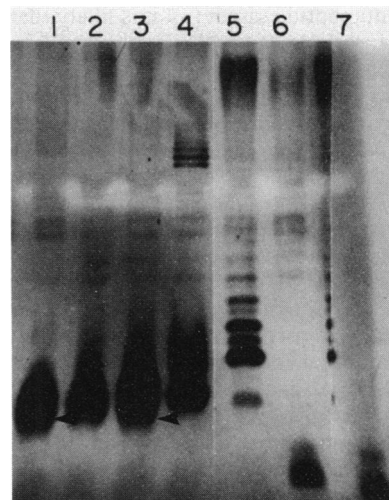


FIG. 4. Separation of LPS by SDS-PAGE. Lanes: 1, strain 16R1; 2, strain 16; 3, K802; 4, D280; 5, SGSC 225 (wild-type *S. typhimurium*); 6, SA1377 (*rfaC*); 7, SGSC 358 (*rfaD*). Arrows indicate the position of the faster moving portion of LPS present in K802 and 16R1 but missing in strain 16.

type) were, in fact, more susceptible to the glycopeptides than their wild-type parents, as noted by Roantree et al. (18), and shared all the other phenotypic features of our vancomycin-susceptible *E. coli* strain.

We next attempted to visualize the core LPS component of the outer membrane of *E. coli* 16. Figure 4 shows the results of a separation of LPS on an 18% polyacrylamide-0.6% bisacrylamide gel containing SDS and 6 M urea. Whereas the K802 parent and 16R1 revertant strains produced a large, poorly resolved band, the vancomycin-susceptible mutant produced a sharper band of core LPS, missing the more rapidly moving component. The difference between strains 16 and 16R1 has always been more apparent (in three experiments [data not shown] loading progressively lower amounts of LPS) than the difference between 16 and K802. It is also clear, however, that mutant 16 apparently did not display Re chemotype LPS when its LPS core mobility was compared with that of the Re chemotype controls shown in lanes 6 and 7 of Fig. 4 (The bands that appear cut off between lanes 6 and 7 are the result of cutting the photograph of a gel with lanes running on a slight diagonal). Both Re and Rd2 chemotype LPS would be expected to move more rapidly than our K802 control, as opposed to less rapidly, as shown (Fig. 4) for the mutant 16. These data were consistent with the hypothesis that a defect in normal LPS core structure accounted for the hypersusceptibility of strain 16, and that this defect was not at the level of the core heptose substituents.

DISCUSSION

Glycopeptides are large, hydrophilic molecules. Their molecular masses vary between approximately 1,200 and 1,900 daltons. At maximum, their octanol-water diffusion coefficient for the octanol phase is 10% (13). However, these molecules have very strong binding affinities for the *E. coli* pentapeptide. Their mechanism of action is due to binding of the terminal D-Ala-D-Ala of the pentapeptide with resultant steric hindrance of further addition to the growing peptidoglycan chain (14). Since these compounds can bind puri-

TABLE 3. MICs of glycopeptides against *E. coli* and *S. typhimurium* with mutations affecting LPS

Strain	MIC ($\mu\text{g/ml}$) ^a					
	TEICO	VANCO	ERY	ERY + PMBN	CHL	AMP
<i>E. coli</i>						
DCO	>512	512	256	32	5.12	2.56
DC2	>512	128	4	<1	1.28	0.64
16	64	8	4	4	2.56	2.56
16R1	>512	128	16	4	2.56	1.28
<i>S. typhimurium</i>						
WT ^b	>512	512	256	16	5.12	1.28
<i>rfaC</i>	64	32	4	8	2.56	1.28
<i>rfaD</i>	64	64	8	4	1.28	1.28

^a VANCO, Vancomycin; TEICO, teicoplanin; ERY, erythromycin; CHL, chloramphenicol; AMP, ampicillin. PMBN was added to erythromycin at 3 $\mu\text{g/ml}$.

^b WT, Wild type.

fied *E. coli* pentapeptide target (3), it is likely that the normal mechanism of resistance in this organism is related to a permeability barrier.

We have confirmed the strong binding of purified *E. coli* pentapeptide to vancomycin and teicoplanin by using affinity column chromatography. Our results indicated a stronger binding affinity of *E. coli* pentapeptide to teicoplanin than to vancomycin, whereas the latter was the more active compound against the mutant strain. The lack of correlation between pentapeptide binding affinity and glycopeptide activity was confirmed by direct comparison of relative binding affinities for pentapeptide using competition assays. In addition, by multiple linear regression analysis, we have shown a significant correlation for molecular mass and activity, both for the resistant strains and for the susceptible mutant, whereas other factors, such as pI, hydrophobicity, or even target affinity, appeared less important in determining activity. These data could suggest directions for synthetic modification of glycopeptides to increase their activities against gram-negative bacilli. These observations also suggested that there was a barrier to glycopeptide access to the pentapeptide target normally present in *E. coli*, which was reduced in the susceptible mutant.

We thought it likely that the normal barrier to glycopeptide penetration would be the complex outer membrane of gram-negative bacilli. By direct examination of OMPs, however, it appeared that no OMP (A, C, or F) was reduced or changed in the vancomycin-susceptible mutant. This is hardly surprising, given the estimated exclusion size of the porin proteins of 600 to 800 daltons (10, 11) and the molecular weight of vancomycin of 1,449 daltons.

LPS is a complex molecule of phosphorylated sugars, bound cations, and lipid A (5). Changes in any of these structural features could potentially modify the rate of diffusion of substances across the LPS. Increases of negative charge of the LPS core mediated by decreased esterification of the lipid A can result in higher affinity of the LPS for polycationic antibiotics such as aminoglycosides and polymyxin (18, 19). Such strains are hypersusceptible to these compounds. One such strain, DC2, is resistant to vancomycin and retains the phenotype of erythromycin-PMBN synergy, thus differentiating this sort of LPS mutation from that in *E. coli* 16.

However, increased esterification of LPS results in decreased charge, with increased susceptibility to hydrophobic compounds (11, 15, 22). The apparent genetic linkage of erythromycin and fusidic acid susceptibility to vancomycin susceptibility, the loss of PMBN synergy with erythromycin and fusidic acid in our vancomycin-susceptible mutant, and the similarity of phenotype between the *E. coli* mutant and *rfaC* and *rfaD* mutants (Re chemotype) of *S. typhimurium* suggested the possibility that the mutant carried a similar defect at the level of the LPS core. Direct examination of the LPS core of parent, mutant, and revertant strains on 18% polyacrylamide gels, however, revealed a different story. Clearly the *rfaC* and *rfaD* core LPS were much smaller in size, and therefore migrated more rapidly on SDS-PAGE, compared with the LPS core of the K802 parent, mutant 16, or its vancomycin-resistant revertant, 16R1. However, a difference in core LPS mobility between the vancomycin-susceptible mutant and its resistant controls could be discerned. A faster moving portion of core LPS was apparently lacking in the mutant. This could indicate the lack of a low-molecular-weight component which might affect the LPS charge. Since the glycopeptides are all relatively hydrophilic compounds, the configuration of the LPS core charge

might be an important factor in normal resistance to vancomycin. The facts that the mutant was also resistant to PMBN synergy with hydrophobic antibiotics and that erythromycin and fusidic acid hypersusceptibility appeared linked to vancomycin hypersusceptibility suggested that the slight difference in the LPS core observed may also have played a role in resistance to these hydrophobic molecules. Further study of the molecular structure of the core LPS in mutant 16 will be required to identify this defect more precisely and to understand its relationship to antibiotic hypersusceptibility.

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