The Effect of Defined Lipopolysaccharide Core Defects upon Antibiotic Resistances of *Salmonella typhimurium*

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Antibiotic resistances of two sets of *Salmonella typhimurium* *rfa* transductants (along with those of their smooth *pyrE*⁺ and *cysE*⁺ sister transductants) were measured. One set was derived from a *pyrE* smooth LT2 parent and the other from a *cysE* smooth LT7 parent. Results showed that strains with defects at the *rfa*(R-res-2) level and deeper were more susceptible to bacitracin, novobiocin and polymyxin. Those with defects at the *rfaG* level or deeper were in addition more sensitive to vancomycin, erythromycin, oxacillin and nafcillin. At these levels the presence or absence of galactose I or glucose I from the lipopolysaccharide core made a considerable difference. A heptose-less *rfaE* mutant was the most sensitive of the strains tested to the above named antibiotics. Strains with *rfa* lesions at several levels of defect showed slight increases in resistances to tetracycline, cephalothin, ampicillin and penicillin.

One would expect strains with *galE* mutations to be similar to *rfa*(R-res-2) strains and those with *galU* mutations to be similar to *rfaG* strains if the core defects accounted for the differing antibiotic resistances. They proved to be so except that the *galE* and *galU* strains in the *S. typhimurium* FIRN line were as resistant to novobiocin as were smooth strains.

The results are interpreted in respect to Nikaido's hypothesis that hydrophilic antibiotics with molecular weights of less than about 650 can gain access to the periplasmic space through protein-lined, water-filled pores and that hydrophobic ones can gain access in deep rough strains when phospholipid patches appear on the surface due to absence of polysaccharides and proteins.

INTRODUCTION

This report deals with the effects of defined mutations that control the polysaccharide composition of the rough core region of the lipopolysaccharide (LPS) of *Salmonella typhimurium* upon the sensitivity of strains containing such mutations to a battery of antibiotics. Preliminary results of these experiments have been reported (Roantree *et al.*, 1969*a, b*) and they agree in general with the results reported by Schlecht and his associates who investigated the antibiotic and dye resistances of rough mutant strains of several *Salmonella* species (Schlecht & Schmidt, 1969, 1970, 1972; Schlecht & Westphal, 1970).

The stimulus for this study was the observation that *Salmonella* mutants selected for their resistances to penicillin or cephalothin included a number of mutants belonging to several different rough classes (Nelson & Roantree, 1967). We had available two sets of *S. typhimurium* transductants, one derived from an LT2 (*pyrE*) strain and one from an LT7 (*cysE*) strain. Thus far, most of the genes described as necessary for the attachment to each

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Fig. 1. Main features of *Salmonella* lipopolysaccharide structure and symbols assigned to genes concerned with formation of individual units of the core.

other of the sugar subunits of the LPS core are located in a short region of the *S. typhimurium* linkage map between *cysE* and *pyrE* (Kuo & Stocker, 1972; Sanderson & Saeed, H., 1972; Sanderson & Saeed, Y. A., 1972). Treatment with phage ES18 grown on LT2 strains with different *rfa* mutations yielded LT7 *cysE*⁺ and LT2 *pyrE*⁺ transductants with different *rfa* mutations, all the members of a set being isogenic except for the *cysE rfa* or *pyrE rfa* segments. Genes from the *rfa* segment that can be cotransduced with either *cysE* or *pyrE* (Kuo & Stocker, 1972; Sanderson & Saeed, Y. A., 1972) include *rfaL*, *rfaJ*, *rfa(R-res-2)*, *rfaG* and *rfaF* (Fig. 1). The designation *rfa(R-res-2)* is used rather than the usual upper-case letter symbol because mutants of this phage pattern although lacking galactose I (Hellerqvist & Lindberg, 1971), do possess galactosyl transferase I (Osborn, 1968).

When it became evident from studies with these and other *rfa* strains that changes in the polysaccharide portion of the rough core probably affected resistances to a number of antibiotics in a predictable way, we used other mutants with LPS core defects (*galE*, *galU* and *rfaE*) to test the validity of the concept.

METHODS

Media. Difco brain-heart infusion broth (for overnight cultures) and Oxoid sensitivity test medium (CM215) agar were used for testing antibiotic sensitivities. For the derivation of the *galU* [deficient in UDP-glucose pyrophosphorylase (Nakae & Nikaido, 1971)] and for phage testing, we used Oxoid nutrient extract broth no. 2 (CM67) as liquid medium and Oxoid blood agar base (CM55) as solid medium. The defined medium employed was Davis minimal medium with glycerol (2 ml l⁻¹) instead of glucose as the carbon source; citrate was omitted in tests for ability to utilize galactose.

Bacteria. The *S. typhimurium* strains constituting the two sets (*pyrE*⁺ LT2 or *cysE*⁺ LT7) of transductants, which were isogenic except for their *rfa* genetic character, and their parent smooth strains are listed in Table 1. The core defects associated with each *rfa* locus are indicated in Fig. 1. *galE*, *galU* and *rfaE* mutants, together with their parent and derivative strains, are listed in Table 2.

Antibiotic sensitivity tests. Usually a series of 10 to 18 agar plates containing stepwise concentrations of antibiotics and two control plates without antibiotics were prepared for each antibiotic to be used. The plates were inoculated by use of a 25-pronged replicator, each prong of which had been dipped into 0.75 ml bacterial suspension contained in one of the 25 compartments of a Bertani box (Elesa, Milan, Italy). The bacterial suspensions were prepared from unshaken overnight broth cultures, grown at 37 °C, by 1/2000 dilution in saline. Thus each prong delivered 100 to 200 bacteria. Control plates were inoculated first and last in each series of inoculations. The lowest concentrations of antibiotic yielding no colony or < 5 % of those on the control plate after 24 h at 37 °C was taken as the minimum inhibitory concentration (m.i.c.). This method allows a direct assessment of the growth of each member of a comparison group at each antibiotic concentration. The results in Tables 3 and 4 for each comparison group were obtained from one such run for each antibiotic. Two or three preliminary runs were done for each antibiotic to establish the correct range of concentrations.

Genetic methods. Methods used to derive the two sets of *rfa* transductants listed in Table 1 have been reported in detail (Kuo & Stocker, 1972). The smooth *pyrE*⁺ transductant sL3770 and smooth *cysE*⁺ transductant sL3856 were derived in the same manner but did not receive any *rfa* defect. The *galE*₄₅₆ mutant and its smooth parent tv253 (Table 2) have been described (Krishnapillai, MacPhee & Stocker, 1971). The mutant was given F'*galE* by conjugation with an *Escherichia coli* K12 F'*gal*⁺ donor strain (MacPhee & Stocker, 1969). The *galU*₄₅₅ mutation occurred spontaneously in the tv253 strain and was selected by its

Table 1. *The two rfa transductant series of Salmonella typhimurium and their parent strains*

These strains are the smooth *pyrE* LT2 SL1515 recipient strain and its *pyrE*⁺ transductional derivatives and the smooth *cysE* LT7 SL1547 recipient and its *cysE*⁺ transductional derivatives. The transduced *rfa* loci and *pyrE* and *cysE* loci are indicated. Phage ES18 was used for transduction.

Strain	Genotype	Source of <i>rfa</i> allele	Reference
SL1515 LT2	<i>pyrE125 rfa</i> ⁺ smooth recipient	—	Sanderson & Saeed, Y.A. (1972)
SL3770	like SL1515 but <i>pyrE</i> ⁺	—	see Methods
SL3749	like SL3770 but <i>rfaL446</i>	TV225	Kuo & Stocker (1972)
SL3750	like SL3770 but <i>rfaJ417</i> *	TV161	Kuo & Stocker (1972)
SL3748	like SL3770 but <i>rfa(R-res-2)432</i> †	TV148	Kuo & Stocker (1972)
SL3769	like SL3770 but <i>rfaG471</i>	SL1032	Kuo & Stocker (1972)
SL3789	like SL3770 but <i>rfaF511</i>	SL1181	Kuo & Stocker (1972)
SL1547 LT7	<i>cysE30 gal-851</i> ‡ <i>rfa</i> ⁺ smooth recipient	—	from K. E. Sanderson
SL3856	like SL1547 but <i>cysE</i> ⁺	—	see Methods
SL3766	like SL3856 but <i>rfaL446</i>	TV225	Kuo & Stocker (1972)
SL3848	like SL3856 but <i>rfaL447</i>	TV226	Kuo & Stocker (1972)
SL3849	like SL3856 but <i>rfaJ419</i>	TV166	Kuo & Stocker (1972)
SL3787	like SL3856 but <i>rfa(R-res-2)432</i> †	TV148	Kuo & Stocker (1972)
SL3854	like SL3856 but <i>rfaG489</i>	SL1062	Kuo & Stocker (1972)
SL3790	like SL3856 but <i>rfaF511</i>	SL1181	Kuo & Stocker (1972)
SL3855	like SL3856 but <i>rfaF537</i>	SL1182	Kuo & Stocker (1972)

* Genes *rfaJ* formerly designated *rfa(R-res-1)* are now known to indicate failure to add glucose II (Hellerqvist & Lindberg, 1971).

† The lipopolysaccharide of strain TV148 carrying gene *rfa(R-res-2)432* has been shown by methylation analysis to lack galactose I (Hellerqvist & Lindberg, 1971).

‡ The *gal-851* mutation does not affect the composition of the LPS.

Table 2. *Second set of Salmonella typhimurium strains including galE, galU and rfaE mutants and their parent and derivative strains*

Strain	Genotype	Origin and description	Reference
TV253	M7471 (ColE1-30) <i>leu-1051 malB479 hisC1150 cysG1175</i>	From M7471*; smooth parent of SL4510 and SL4507	Krishnapillai <i>et al.</i> (1971)
SL4510	like TV253 but <i>galE456</i> †	Mutant of TV253 (selection: FO + P22.c2 phages)	Krishnapillai <i>et al.</i> (1971)
SL4521	like SL4510 but F' <i>gal</i> ⁺	SL4510 given F'-1 <i>gal</i> ⁺ from SL4516‡	MacPhee & Stocker (1969)
SL4507	like TV253 but <i>galU455</i>	Spontaneous mutant of TV253 (selection: FO + P22.c2 phages)	see Methods
SL4591	like SL4507 but <i>galU</i> ⁺	Transductant of SL4507 by phage ES18 grown on SL1183	see Methods
SL3622	SL1027 (see below) lysogenized by P22	P22-lysogenic SL1027	see Methods
SL4593	like SL3622 but <i>trpB</i> ⁺ <i>galU455</i>	Transductant of SL3622 by ES18.h1 grown on SL4507	see Methods
SL4596	like SL3622 but <i>trpB</i> ⁺	Transductant of SL3622 by ES18.h1 grown on SL4507	see Methods
SL1027	LT2 <i>metA22 trpB2 H1-b H2-e, n, x</i> 'cured of Fels 2' <i>flaA66 strA120 metE551</i>	Parent of SL3622	Wilkinson <i>et al.</i> (1972)
SL1102	like SL1027 but <i>rfaE543</i>	Heptose-less mutant of SL1027	Wilkinson <i>et al.</i> (1972)

* M7471 is an *S. typhimurium* strain of the FIRN wild type (lacks fimbriae, fails to ferment rhamnose and inositol; Morgenroth & Duguid, 1968).

† Strains with *galE456* produce very low levels (1 to 5 % of normal) of all three galactose enzymes but neither ferment nor utilize galactose to a detectable extent (Krishnapillai *et al.*, 1971).

‡ SL4516 is an *Escherichia coli* K12 *gal*⁻ strain containing F'-1*gal*⁺, obtained from J. Roth.

Table 3. *Minimum inhibitory concentrations of antibiotics for rfa transductants relative to those for smooth strains*

Minimum inhibitory concentrations (see Methods) for the *rfa* transductants are expressed relative to the m.i.c. (taken as 1.0) for smooth strains (generally SL1515, but SL1547 for Meth). Absolute m.i.c. values for the comparison strains (expressed in $\mu\text{g ml}^{-1}$ for bacitracin and penicillin and $\mu\text{g ml}^{-1}$ for all other antibiotics) are given at the head of each column.

Strain*	Bac m.i.c. (125)	Vanco (175)	Novo (500)	Ery (75)	P-m (0.7)	N-m (4.4)	Tet (1.0)	Ceph (1.25)	Pen (6)	Amp (0.6)	Oxa (200)	Naf (350)	Meth (400)
Smooth													
SL1515 <i>pyr</i> ⁻	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.8
SL3770 <i>pyr</i> ⁺	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.2
SL1547 <i>cys</i> ⁻	1.0	1.0	0.9	1.0	0.9	1.0	0.9	1.0	1.0	1.0	1.0	1.0	1.0
SL3856 <i>cys</i> ⁺	1.0	1.0	0.9	1.0	0.9	0.9	0.9	1.0	0.8	1.0	1.0	0.8	1.0
<i>rfaL</i>													
SL3749 <i>pyr</i> ⁺	1.2	1.0	1.4	1.0	0.9	1.3	1.0	1.2	1.3	1.3	1.0	1.1	1.4
SL3766 <i>cys</i> ⁺	1.4	1.0	1.4	1.0	0.9	1.1	1.2	1.4	1.3	1.3	1.0	1.0	1.2
SL3848 <i>cys</i> ⁺	1.4	1.0	1.4	1.0	1.0	1.0	1.2	1.6	1.3	1.3	1.2	1.1	1.5
<i>rfaJ</i>													
SL3750 <i>pyr</i> ⁺	1.0	1.0	1.4	1.0	0.6	1.1	1.2	1.2	1.3	1.3	1.0	1.1	0.9
SL3849 <i>cys</i> ⁺	1.2	1.0	>1.4	1.0	0.6	1.1	1.4	1.6	1.6	—	1.2	1.3	1.5
<i>rfa(R-res-2)</i>													
SL3748 <i>pyr</i> ⁺	0.4	1.0	<0.05	1.0	0.1	1.5	1.4	1.6	1.6	1.3	0.7	1.0	1.1
SL3787 <i>cys</i> ⁺	0.6	1.1	0.05	1.0	0.1	1.5	1.4	2.4	2.0	2.0	1.0	1.0	1.5
<i>rfaG</i>													
SL3769 <i>pyr</i> ⁺	0.2	0.4	0.05	0.5	0.1	1.1	1.4	1.2	1.0	1.0	0.2	0.14	0.5
SL3854 <i>cys</i> ⁺	0.4	0.4	0.05	0.4	0.1	1.1	1.4	1.6	1.6	1.3	0.2	0.3	1.1
<i>rfaF</i>													
SL3789 <i>pyr</i> ⁺	0.2	0.3	<0.05	0.12	0.1	1.1	1.8	1.4	1.0	0.7	0.2	0.14	0.5
SL3790 <i>cys</i> ⁺	0.2	0.3	<0.05	0.08	0.1	1.0	2.0	2.4	1.0	1.0	0.2	0.08	0.7
SL3855 <i>cys</i> ⁺	0.2	0.3	<0.05	0.16	0.1	1.0	2.0	2.4	1.3	1.3	0.2	0.5	0.8

* The *pyr*⁺ transductants are LT2 strains and the *cys*⁺ transductants are LT7 strains.

resistance to phages FO and P22.c2. The *galU455* gene was then transferred by phage ES18.h1 into the smooth SL3622 LT2 by cotransduction with *trpB*⁺, resulting in strain SL4593; SL4596 is a sister transductant receiving only the *trpB*⁺ gene. The derivation of the heptose-less *rfaE* mutant strain SL1102 from its smooth parent strain SL1027 has been described (Wilkinson, Gemski & Stocker, 1972).

Phages and phage methods. The phages used to select resistant mutants and to determine LPS by phage pattern tests were described by Wilkinson *et al.* (1972). Phage sensitivities were determined by the 'drop on lawn' method on nutrient agar or nutrient agar with galactose and glucose (each at 5 g l⁻¹). For transduction we used two phages: phage ES18, active on both smooth and non-smooth *S. typhimurium*, and its variant ES18.h1 which, unlike ES18 itself, is fully active on strains lysogenic for Fels 2, carried by strain LT2 (Kuo & Stocker, 1970). Lysogeny for P22 makes strains better recipients for ES18 transduction (Kuo & Stocker, 1970) and strains not already lysogenic for P22 or a similar phage were made lysogenic before use as recipients.

RESULTS

The relative antibiotic resistances of the two sets of *rfa* transductants together with those of their smooth parent and sister transductants are listed in Table 3. The *pyr*⁺ transductants are derived from the SL1515 *pyrE* LT2 smooth parent and the *cys*⁺ transductants from the SL1547 *cysE* LT7 smooth parent. A value of 1.0 is assigned to the resistance (m.i.c.) of SL1515, except for methicillin where the *cys* parent and its *cys*⁺ smooth transductant gave a more consistent reading; the corresponding absolute m.i.c. values are indicated. Values obtained for the smooth parents and their smooth transductants were either identical or nearly so for all antibiotics tested (Table 3).

Bacitracin (Bac). The *rfaL* transductants showed slight (but consistent) increases in resistance; *rfaJ* transductants were as resistant as the smooth strains and *rfa(R-res-2)* were

about half as resistant. The *rfaG* and *rfaF* strains were more sensitive (relative m.i.c., 0.2 to 0.4 of control).

Vancomycin (Vanco). The *rfa* transductants were as resistant as the smooth strains, except for the *rfaG* and *rfaF* strains which were considerably more sensitive (relative m.i.c., 0.3 to 0.4).

Novobiocin (Novo). The two more superficial *rfa* classes (*L* and *J*) showed small but consistent increases in resistance, but there was a drastic decrease in resistance in the three deeper classes (*R-res-2*, *G* and *F*) (relative m.i.c., 0.05 or less).

Erythromycin (Ery). The three more superficial classes were as resistant as the smooth strains; the *rfaG* transductants were about one-half and the *rfaF* about one-tenth as resistant.

Polymyxin B sulphate (P-m). The *rfaJ* transductants showed an increase in sensitivity (relative m.i.c., 0.6) and the three deeper classes a further more marked increase (relative m.i.c., 0.1).

Neomycin (N-m). Except for slight increases (relative m.i.c., 1.5) in resistance exhibited by the *rfa(R-res-2)* transductants, the whole set had similar resistance to the smooth strains.

Tetracycline (Tet). All the *rfa* transductants showed slight but very consistent increases in resistance (relative m.i.c., 1.4 to 2.0) compared with the smooth strains; *rfaF* strains were the most resistant (2.0).

Cephalothin (Ceph). All classes of *rfa* transductants were more resistant than the smooth strains with the *rfa(R-res-2)* *cys*⁺ and *rfaF cys*⁺ strains being the most resistant (relative m.i.c., 2.4).

Penicillin (Pen) and *Ampicillin* (Amp). Most classes showed slight increases in resistance, although the *rfaF* strains were about as resistant as the smooth ones.

Oxacillin (Oxa), *Nafcillin* (Naf) and *Methicillin* (Meth). The three more superficial classes of *rfa* strains showed similar or slightly increased resistances to these three antibiotics when compared with the smooth strains. The *rfaG* and *rfaF* transductants were considerably more sensitive to Oxa (relative m.i.c., 0.2) and Naf (0.08 to 0.5). This sensitivity of the two deeper *rfa* classes was less evident with Meth (relative m.i.c., 0.5 to 1.1).

The above results indicated that LPS defects including absence of O side-chains caused moderate increases in resistance to some antibiotics (1.1-fold to 2.4-fold) compared with the resistances of the smooth strains. They also showed that defects in the deeper part of the core [*rfa(R-res-2)* and, more often and to a greater extent, *rfaG* and *rfaF*] caused considerably greater sensitivity to those antibiotics which are less effective against Gram-negative than against Gram-positive bacteria (Bac, Vanco, Novo, Ery, Oxa, Naf) and also to polymyxin.

Strains with LPS core-defects similar to those of the *rfa(R-res-2)* and *rfaG* transductants could be investigated by testing *galE* or *galU* mutants. The effect of a deeper defect than those of the *rfaF* strains could be investigated by comparing the antibiotic resistances of an *rfaE* heptose-less mutant (see Fig. 1) with those of its parent smooth strain. The descriptions of these strains are shown in Table 2.

The polysaccharide core of a *galE* mutant differs from that of a *rfa(R-res-2)* mutant in that it contains neither galactose unit whereas the latter has galactose II (see Fig. 1). The parent strain of our *galE* mutant was TV253, a smooth virulent auxotrophically marked strain in an *S. typhimurium* FIRN line (lacks fimbriae, fails to ferment inositol and rhamnose; see Morgenroth & Duguid, 1968). Results from testing this mutant along with its parent and its derivative strain given an F'*gal* which 'cures' the *galE* defect are listed in Table 4, which shows absolute m.i.c. values. The *rfa(R-res-2)* transductants (Table 3) in LT2 and LT7 lines showed increased sensitivity to Bac, Novo and P-m. The *galE* mutant showed similar increases in sensitivity to Bac and P-m but was more resistant than its comparable smooth strains to Novo. The *rfa(R-res-2)* strains were more resistant than the smooth strains to Tet, Ceph, Pen and Amp as was the *galE* mutant, although its increase in resistance to Tet was

Table 4. Antibiotic resistances of *S. typhimurium* smooth strains and their rough and smooth derivatives

Resistances are expressed as minimum inhibitory concentrations (see Methods) in $\mu\text{g ml}^{-1}$ except for bacitracin and penicillin which are in u. ml^{-1} .														
Strain	Character	Bac	Vanco	Novo	Ery	P-m	Tet	Ceph	Pen	Amp	Oxa	Naf	Meth	Core ends at:
TV253	smooth parent	175	150	250	50	0.6	0.8	1.0	6	1.0	140	350		
SL4510	<i>galE</i> mutant	75	100	450	50	0.2	1	1.6	10	1.6	80	250		GlcI
SL4521	F' <i>galE</i> ⁺ conjugant	200	150	200	50	0.6	0.8	1.0	6	1.0	120	250		
SL4507	<i>galU</i> mutant of TV253	50	15	200	10	0.1	1.4	2.25	2	1.2	20	25	400	HepII
SL4591	<i>galU</i> ⁺ transductant of SL4507	100	100	200	100	0.4	0.8	0.5	4	0.8	250	400	500	
SL1027	smooth	75	50	50	100	0.3	0.6	1.0	4	1.2	180	350	400	
SL4593	<i>galU</i> transductant, SL1027 line	10	15	2	15	0.1	0.8	1.5	2	0.8	40	75	500	HepII
SL4596	<i>galU</i> ⁺ transductant, SL1027 line	75	50	25	100	0.3	0.4	1.0	2	0.8	160	350	400	
SL1027	smooth	100	100	50	75	0.3	0.6	1.5	4	1.2	100	250	350	KDO
SL1102	<i>rfaE</i> mutant of SL1027	<1	<5	0.5	2	0.1	1.0	1.5	2	0.4	<5	5	50	

Table 5. Comparison of the relative minimum inhibitory concentrations of the LT2 and LT7 *rfaG* transductants, the LT2 *galU* transductant and the *galU* FIRN mutant

The minimum inhibitory concentrations of rough strains are expressed relative to the m.i.c. (taken as 1.0) of their smooth comparison strains. Smooth comparison strains for *rfaG* transductants are as in Table 3; those for the *galU* strains are their *galU*⁺ transductants (Table 4).

Strain	Bac	Vanco	Novo	Ery	P-m	Tet	Ceph	Pen	Amp	Oxa	Naf	Meth
LT2 <i>rfaG</i> (SL3769)	0.2	0.4	0.05	0.5	0.1	1.4	1.2	1.0	1.0	0.2	0.14	0.5
LT7 <i>rfaG</i> (SL3854)	0.4	0.4	0.05	0.4	0.1	1.4	1.6	1.6	1.3	0.2	0.3	1.1
LT2 <i>galU</i> (SL4593)	0.13	0.3	0.08	0.15	0.3	2.0	1.5	1.0	1.0	0.2	0.2	1.2
FIRN <i>galU</i> (SL4507)	0.5	0.15	1.0	0.1	0.25	1.7	4.5	0.5	1.5	0.06	0.08	0.8

Table 6. Comparison of the relative minimum inhibitory concentrations of the *rfaF* transductants and the *rfaE* mutant

The minimum inhibitory concentrations of rough strains are expressed relative to the m.i.c. (taken as 1.0) of their smooth comparison strains. Smooth comparison strains for *rfaF* transductants are as in Table 3; that for the *rfaE* mutant is its parent smooth strain SL1027 (Table 4).

Strain	Bac	Vanco	Novo	Ery	P-m	Tet	Ceph	Pen	Amp	Oxa	Naf	Meth
<i>rfaF</i> (SL3789)	0.2	0.3	<0.05	0.12	0.1	1.8	1.4	1.0	0.7	0.2	0.14	0.5
<i>rfaF</i> (SL3790)	0.2	0.3	<0.05	0.08	0.1	2.0	2.4	1.0	1.0	0.2	0.08	0.7
<i>rfaF</i> (SL3855)	0.2	0.3	<0.05	0.16	0.1	2.0	2.4	1.3	1.3	0.5	0.2	0.8
<i>rfaE</i> (SL1102)	<0.01	<0.05	0.01	0.03	0.3	1.7	1.0	0.5	0.3	<0.05	0.02	0.15

slight. Against the other antibiotics tested (Vanco, Ery, Naf and Oxa), *rfa*(R-res-2) strains showed about the same sensitivities as the smooth controls; the *galE* mutant behaved similarly except that it was slightly more sensitive to Vanco and Oxa than the smooth strains. These results can probably be attributed to the effect of the mutation because the presence of F'*gal*⁺ results in a strain with antibiotic resistances very similar or identical to those of the smooth parent.

The *galU* mutants are unable to synthesize UDPglucose and hence do not incorporate glucose into the rough core (Nakae & Nikaido, 1971). Their core, then, should be the same as that of *rfaG* mutants that cannot attach glucose I (Fig. 1). Our *galU*455 mutant (SL4507) was derived in the same line (FIRN) as the *galE* mutant. Its antibiotic resistances are compared in Table 4 with those of its derivative (SL4591) made *galU*⁺ by transduction and with those of a smooth *trpB* LT2 strain (SL1027), and its *galU*455 cotransductant (*galU*455 *trpB*⁺) and its *trpB*⁺ only transductant derivatives (SL4593 and SL4596, respectively).

The m.i.c. values of the *rfaG* LT2 and LT7 transductants, the *galU* LT2 transductant (SL4593) and the *galU* FIRN mutant relative to those of their smooth transductant counterparts (taken as 1.0) are shown in Table 5. The *galU* defect appears to cause qualitative changes in antibiotic resistances similar to those shown by the *rfaG* strains. Except for Novo resistance the *galU* defect exerts quite similar effects on the other resistances whether expressed in the LT2 or FIRN background. As in the *galE* mutants, it is evident that the mutation is responsible for the observed change since otherwise isogenic smooth strains possess the smooth phenotype in regard to antibiotic resistances.

The deepest rough mutation we could test was *rfaE*. The resistances of the *rfaE* mutant and of the *rfaF* transductants relative to those of their smooth counterparts (1.0) are shown in Table 6. The *rfaE* mutant was more sensitive to all antibiotics tested (except P-m) than the *rfaF* strains that contain heptose in their core (Fig. 1). But even with this deep defect, the *rfaE* mutant is as resistant to Ceph as its smooth parent and is more resistant to Tet.

DISCUSSION

The results from tests with the two sets of *rfa* transductants, the *galU* strains and the *rfaE* mutant agree in that strains belonging to the three deepest levels tested (*rfaG-galU*, *rfaF*, *rfaE*) were substantially more sensitive than their smooth comparison strains to certain antibiotics relatively inactive against Gram-negative enteric bacilli (Bac, Vanco, Ery, Naf, Oxa). They were also more sensitive to polymyxin. Strains belonging to the *rfa*(R-res-2) class were more sensitive than the smooth strains to Bac, Novo and P-m; the *galE* mutant (FIRN line), which might be expected to be similar to the *rfa*(R-res-2) class, was also sensitive to Bac and P-m, but showed increased resistance to Novo rather than the expected greater sensitivity. Except for resistance to Novo, the *galU* strains resembled the *rfaG* strains in their resistances. The *galU* FIRN mutant was no more sensitive to Novo than its smooth *galU*⁺ counterpart (relative m.i.c., 1.0), but the *galU* transductant in the LT2 line was considerably more sensitive (relative m.i.c., 0.08) than its *galU*⁺ sister transductant and, in this, resembled the *rfaG* LT2 and LT7 strains (relative m.i.c., 0.05). At least in sensitivity to Novo, the *galE* and *galU* strains in the FIRN line appear to differ from the transductants in the LT2 line. This may indicate some unknown difference in the rough core at this level of these two distinct *S. typhimurium* lines.

The *rfa* transductants of several classes showed rather small but consistent increases in resistance to certain antibiotics (notably Tet, Ceph, Amp, Pen) in comparison with the smooth strains. Such results are in accordance with the observation that mutants selected for resistance to cephalothin or penicillin are often rough strains of various classes (Nelson & Roantree, 1967).

These results are in essential agreement with those of Schlecht and his colleagues who tested mutants of different rough chemotypes in *Salmonella riuu*, *S. typhimurium* and *Salmonella minnesota* (Schlecht & Schmidt, 1970, 1972; Schlecht & Westphal, 1970). They found that the rough strains, and particularly the deeper rough strains, were more sensitive than smooth control strains to rifamycin and actinomycin as well as to Bac, Vanco and Ery. The increases in resistance of some classes to Tet and Pen were also noted. In general, their results show somewhat more variation within rough classes than we found. The effect of the presence or absence of a particular sugar (e.g. glucose I for Vanco or Ery resistance) although evident, is not as clearcut as in our series. This may be partly because their methods of measuring antibiotic sensitivities by streaking a loopful of each test strain on to a gradient plate and measuring the length of the streak of growth allows less precise quantification than ours. In addition the test strains at their disposal were less genetically homogeneous than ours. For example, some *S. minnesota* deep rough strains in their set have been shown to contain mutations in addition to the suspected *rfa* mutation. Some contain an additional *rfaP* mutation in a gene which governs the phosphorylation of heptose in the rough core (Mühlradt *et al.*, 1968; Jousimies & Mäkelä, 1974) and some contain an additional *rfe* mutation in a gene necessary for the synthesis of O side-chains and the common antigen in this species (Mäkelä *et al.*, 1974). The effect of the *rfaP* mutation on the deep core could conceivably influence antibiotic sensitivities. Our use of sets of transductants should largely avoid the problem of multiple mutations.

The *rfaE* heptose-less mutant is much more sensitive to most of the antibiotics more effective against deep rough strains than the heptose-containing *rfaF* transductants (Table 6). Even at the *rfaE* level, though, penicillin-sensitivity is only moderately greater (relative m.i.c., 0.5) than that of the smooth strain. Since a 10 times greater concentration of penicillin is necessary to inhibit growth of *Escherichia coli* than to inhibit transpeptidation in a cell-free system (Strominger *et al.*, 1967), one might have expected a lower relative m.i.c. than 0.5 with this very defective LPS unless the outer membrane contains a further barrier to penicillin penetration.

We believe the most attractive hypothesis put forward to explain the greater sensitivity

of the deep rough strains to certain antibiotics is that of Nikaido (1976). He postulates that hydrophilic molecules of less than 650 daltons gain entrance to the periplasmic space through water-filled, protein-lined pores in the outer membrane. Thus the outer membrane should not be the barrier to such molecules that it would be to hydrophobic molecules or hydrophilic ones of greater molecular weight. He measured the partition coefficient (1-octanol/water system) of a number of the antibiotics and dyes used by Schlecht and his colleagues (Schlecht & Schmidt, 1970; Schlecht & Westphal, 1970; Schmidt, Schlecht & Westphal, 1969) and of antibiotics used in our study. Nearly all of the antibiotics tested which were more active against the deep rough strains fell into the hydrophobic class or had molecular weights greater than 650. Polymyxin is amphiphilic but has a molecular weight of about 1200. It reacts with LPS (Rifkind, 1967) as well as the phospholipids and may create its own holes. The fact that treatment with polymyxin rendered *Proteus mirabilis* sensitive to killing by deoxycholate (Sud & Feingold, 1972) lends credence to such a concept. Almost all of the antibiotics which were equally active against smooth and rough strains were hydrophilic and less than 650 daltons. Chloramphenicol and tetracycline were exceptions in that they were hydrophobic, but Nikaido notes that tetracycline probably penetrates as a magnesium complex and that chloramphenicol, perhaps, penetrates by means of a specific mechanism. Previous work (Ames, Spudich & Nikaido, 1974) had shown that the outer membranes of mutants lacking the glucose I unit and those with deeper defects lacked considerable amounts of the proteins making up four major bands (33K, 34K, 35K, 36K) on sodium dodecyl sulphate-polyacrylamide electrophoretic analysis. Three of these proteins (34K, 35K, 36K) are those necessary to form aqueous pores in reconstituted LPS-phospholipid bilayers (Nakae, 1976). Thus Nikaido believes that the loss of such proteins leads to a rearrangement in the structure of the outer membrane allowing areas of phospholipid to be present at the surface (Smit, Kamio & Nikaido, 1975). Hence hydrophobic molecules could gain access to the periplasmic space by dissolving in the lipid bilayer.

Our findings are generally consistent with Nikaido's hypothesis in that for most antibiotics more effective against deep rough strains a definite change occurs at the *rfaG* level. The finding that *rfa*(R-res-2) and the *galE* strains were more sensitive to some of the antibiotics than strains with more superficial lesions may not be at odds with the hypothesis since the outer membrane of *galE* mutants lacks measurable amounts of the 34K and 35K proteins (Ames *et al.*, 1974).

A number of mutants with defects in the outer membrane and changes in permeability to a variety of agents have been reported. *Salmonella typhimurium* strains with reduced amounts of 34K to 36K proteins and presumed to be outer membrane protein mutants (Ames *et al.*, 1974) allow rapid penetration of hydrophobic molecules. Several *S. typhimurium* mutants (FOR) selected for resistance to Felix-O phage appear smooth by other criteria but are sensitive to those antibiotics to which the deep rough strains are susceptible (MacPhee *et al.*, 1975). Felix-O resistance mapped in the *rfa* region and it is thought that the FOR mutations affect the assembly of the inner part of the LPS core causing incomplete blocks in glycosyl transferase reactions. *Escherichia coli* mutants supersensitive to novobiocin and with increased sensitivity to such antibiotics as bacitracin and actinomycin have outer membranes very deficient in heptose, glucose and phosphate (Tamaki, Sato & Matsushashi, 1971; Tamaki & Matsushashi, 1973). An *E. coli* mutant defective in an outer membrane protein was found to be sensitive to dyes and detergents (Wu, 1972). In *E. coli* K12 the *envA* mutation results in incomplete cell division, a relative decrease in the content of phosphatidyl ethanolamine in the outer membrane, and greatly increased sensitivity to penicillin, ampicillin and chloramphenicol as well as to a number of the antibiotics that we found to be more effective against deep rough strains (Boman, Nordström & Normark, 1974). This may indicate that the phospholipid composition of the outer membrane is important in the penetration of penicillin. *Escherichia coli* mutants sensitive to synergistin A are sensitive to many of the antibiotics that are more effective against the deep rough strains (Ennis, 1971).

Increased sensitivities to those antibiotics more effective against the deep rough strains have been found when *E. coli* or *Salmonella* have been treated with EDTA. Thus treatment with EDTA and lysozyme to produce protoplasts allowed access of actinomycin D into *E. coli* (Mach & Tatum, 1963) and, indeed, EDTA treatment alone can increase the permeability to this antibiotic (Leive, 1968). Muschel & Gustafson (1968) showed that EDTA treatment rendered *Salmonella typhi* more sensitive to polymyxin, novobiocin and certain detergents and, to a lesser extent, more sensitive to bacitracin and penicillin. However, such treatment had little or no effect on the sensitivity to streptomycin, neomycin, kanamycin, chloramphenicol or chlortetracycline. In their resistances to these agents, EDTA-treated coliforms are much like deep rough strains. Treatment with EDTA results in the release of 30 to 50% of the LPS from the outer membrane (Leive, 1965) presumably through the interaction of EDTA with Ca^{2+} and/or Mg^{2+} ions (Leive, 1974). The released material contains 5 to 10% protein (Leive, Shovlin & Mergenhagen, 1968). Perhaps phospholipid patches are created or uncovered by such treatment to allow penetration of hydrophobic molecules.

The increase in resistance to certain antibiotics, particularly Tet, Ceph, Amp and Pen, of many of the *rfa* classes is perhaps more difficult to explain than the increased sensitivities. As noted above, both our results and those of Schlecht and his colleagues agree that this phenomenon is real. In addition, it has been shown that among ampicillin-resistant mutants of *E. coli* K12 are a number with outer membrane defects (Eriksson-Grennberg, Nordström & Englund, 1971; Monner, Jonsson & Boman, 1971) including various deficiencies of the polysaccharide portion of the LPS core (Boman & Monner, 1975; Mayer *et al.*, 1976). Since the antibiotics listed above are, according to the Nikaido hypothesis, those likely to penetrate through the water-filled pores, perhaps loss of polysaccharide distorts these channels so as to hinder the access of the antibiotics. If the increases in resistance were limited to those classes with defects deeper than the glucose I (*rfaG*) level one might postulate a quantitative loss of the protein-lined pores, but our data do not allow this interpretation since some more superficial *rfa* strains show the increases in resistances.

The results of our experiments emphasize the importance of the outer membrane of *Salmonella* in preventing access of certain antibiotics, notably those less active against Gram-negative than against Gram-positive organisms. The presence or absence of single sugars from the polysaccharide of the LPS can make a considerable difference. Paradoxically some rough classes are more resistant to antibiotics which are effective against Gram-negative bacteria.

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