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Colonization of the Mouse Intestine by an Avirulent *Salmonella typhimurium* Strain and Its Lipopolysaccharide-Defective Mutants

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For study of the role of lipopolysaccharide (LPS) character in colonization of the mouse large intestine, use was made of *S. typhimurium* strain SL5316, which is streptomycin resistant and smooth (wild-type LPS) but nonvirulent because it is Aro⁻ (aromatic dependent). Several rough variants of different LPS chemotype derived from strain SL5316 comprised: an *rfb* deletion transductant making type Ra (complete core) LPS; an *rfaJ* mutant making incomplete core of type Rb2; and an *rfa-990* mutant making LPS core less complete than chemotype Rb2. We tested these strains for colon-colonizing ability by feeding them to male CD-1 mice receiving streptomycin sulfate (5 g/liter) in their drinking water. Each strain, if fed alone, was found in the feces throughout the 15 days of the experiment at about 10^8 CFU/g for the smooth strain or 10^7 CFU/g for each of its rough derivatives. However, when mice were fed equal numbers of two strains (with differentiating antibiotic resistance characters), the strain with the more complete LPS was found in the feces in great excess, 1000- to 100,000-fold, according to the pair. Thus, when strains were placed in direct competition with one another, their relevant colon-colonizing abilities were found to be wild type > *rfb* >> *rfaJ* > *rfa-990*, showing that the ability of a *Salmonella* strain to colonize the mouse large intestine decreases as its LPS structure becomes more defective.

In general, gram-negative bacteria which initiate infection in the small intestines of mammals do so by means of specific pili which mediate the adhesion of these bacteria to intestinal epithelial cells (9, 11, 15, 16, 19). The pili are protein in nature, and the biochemical structure of the pilus appears to recognize specific receptors in specific animals (5, 22, 29). For example, the K88 pilus recognizes pig intestine preferentially (19, 39), whereas the CFA/I pilus appears to be specific to human intestine (10, 11). In contrast to the information on small intestine colonization, relatively little is known about how gram-negative bacteria colonize the mammalian colon. Recently, lipopolysaccharide (LPS) has been implicated in both adhesion and subsequent colonization of the colon by gram-negative bacteria (3, 4, 17, 30). For example, changes in the abilities of *Escherichia coli* strains of human fecal origin to colonize the mouse colon have been correlated with as yet chemically uncharacterized changes in their LPS (4). Furthermore, the LPS of a strain of *Shigella flexneri* which was isolated from a patient with dysentary completely blocked adhesion of the strain to guinea pig colonic epithelial cells and sections of colonic tissue (17).

In the present investigation, we took advantage of *Salmonella typhimurium* SL5316, which, though smooth, is avirulent because of aromatic auxotrophy (14), and several genetically characterized LPS mutants derived from it to ask whether LPS is involved in *S. typhimurium* colonization of the mouse colon. The colonizing ability of each strain relative to each of the other strains was determined by means of the competitive mouse model which we have previously described (31). The data show that the relative mouse colon colonizing abilities of the *S. typhimurium* strains with respect to LPS type are: wild type > *rfb* >> *rfaJ* > *rfa-990*. Thus, specific alterations in LPS can result in alterations in colonizing ability.

MATERIALS AND METHODS

Bacterial strains, phages, and genetic procedures. The bacterial strains used are listed in Table 1. The starting strain, SL3237, is a streptomycin-resistant transductant derived from SL3235, a *cys leu his aroA* (nonleaky, nonreverting) strain of *S. typhimurium* constructed for use as a live vaccine (14, 40). The Aro⁺ parent of strain SL3235, i.e., strain SL3201, is a genetically marked (ColE1 *malB cysI hisC*) but fully virulent descendant of a wild-type *S. typhimurium* strain, M7471, of biotype FIRN, i.e., rhamnose negative, inositol negative, and permanently nonfimbriate (6). Strain SL3237 was first made Cys⁺ by transduction. Spontaneous mutants resistant to Felix O phage were selected in the cys⁺ transductant strain SL5316 and tested for sensitivity or resistance to a collection of LPS-specific phages (44) and by other tests of smooth or rough character. A derivative of strain SL5316 with a deletion of most of the *rfb* (O-antigen-determining) operon (and of the adjacent *his* operon) was constructed by transduction as described below.

Phage P22 HT105/1 *int* was used for transduction. Cystine-independent or tetracycline-resistant transductants were selected by the drop-on-lawn procedure (25) on appropriate media. The tetracycline resistance caused by transposon Tn*10* inserted in gene *hisD* of the constructed *rfb* (deletion) derivative SL5318 allowed its quantitative recovery when present in feces together with its smooth parent SL5316 or with either of the *rfa* mutants SL5325 and SL5326, all of which are tetracycline sensitive. Spontaneous mutants resistant to nalidixic acid (Nal^r) were selected in SL5325 (resistant to 50 µg/ml) and SL5326 (resistant to 10 µg/ml), the *rfaJ* and *rfa-990* derivatives of SL5316, respectively. In addition, a spontaneous mutant resistant to bicozamycin (100 µg/ml; Bicr), was selected in strain SL5326.

Mouse colonization experiments. Recently, we described a model that was used to distinguish relative colonizing abilities of *E. coli* strains in mice (31). The same model was used in the present investigation. Briefly, 5- to 8-week-old male

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TABLE 1. Bacterial strains

Strain no.	Genotype and LPS chemotype	Origin or reference
his-695	LT2 ΔhisB (HAFIE gnd rfbMNHGFD) 695; Ra	32
SL3237	M7471 (ColE1) malB leu cysI hisC527 CRR401[aroA554::Tn10 (Tet^s, nonreverting]^a rpsL120; wild type (smooth)	14, 40
SL5261	LT2 Δhis (rfb)695/F's401-his$^+$-D.rfb^{+b}; wild type (smooth; 09,12)	his-695 given F's401-his$^+$-D.rfb$^+$ by conjugational cross
SL5267	As SL5260, but hisD8557::Tn10; wild type (smooth; 09,12)	SL5260 made hisD::Tn10 by transduction
SL5316	As SL3237, but Cys$^+$; wild type (smooth)	SL3237 made Cys$^+$ by transduction
SL5318	As SL5316, but Δhis (rfb) 695 hisD8557::Tn10; Ra	SL5316 made Δhis(rfb) by cotransduction with hisD::Tn10 from SL5267
SL5319	As SL5316, but hisD8557::Tn10; wild type (smooth)	SL5316 made hisD::Tn10 bytransduction from SL5267
SL5325	As SL5316, but rfaJ989; Rb2	Spontaneous mutant of SL5316 selected as resistant to Felix O phage
SL5326	As SL5316, but rfa-990; core less complete than Rb2	Spontaneous mutant of SL5316 selected as resistant to Felix O phage

^{a} Complex rearrangement mutation, either deletion or deletion inversion, affecting an aroA554::Tn10 strain and causing loss of tetracycline resistance and ability to revert to aromatic independence (14).

^{b} This F' plasmid is F's401 with B.rfb$^+$ (specifying enzymes for synthesis of 04 repeat units) (42) replaced by D.rfb$^+$ (for 09 repeat units) (B. Johnson and B. A. D. Stocker, unpublished results).

CD-1 mice were given drinking water containing streptomycin sulfate (5 g/liter). After 1 day of streptomycin treatment, the count of facultative bacteria dropped from about 10^8 to less than 10^2 CFU/g of feces, whereas the count of anaerobic bacteria remained constant at about 10^9 CFU/g of feces. Mice were then held overnight without food or water, and the next morning, groups of three individually housed animals were fed approximately 3×10^{10} CFU of each of the S. typhimurium strains to be tested in 1 ml of sterile 20% (wt/vol) sucrose. The mice drank the bacterial suspension almost immediately and were then returned to and maintained on their normal diet (Charles River Valley Rat, Mouse, Hamster Formula) including sterile drinking water containing streptomycin (5 g/liter). The next morning and at 48-h intervals thereafter, 1 g of feces from each mouse was collected and homogenized in 10 ml of L broth (16). This suspension was then diluted and plated on the appropriate selective media. Plates were incubated at 37°C from 18 to 24 h. Colonizing ability was assessed by the level at which a strain persisted in the feces. Each colonization experiment was performed at least twice with essentially identical results. The results of typical experiments are reported here.

Media and antibiotics. L broth was made as described by Revel (35). L agar is L broth containing 12 g of Bacto-Agar (Difco Laboratories, Detroit, Mich.) per liter. Streptomycin sulfate, nalidixic acid, and tetracycline were purchased from Sigma Chemical Co., St. Louis, Mo. and were used in agar

plates at the concentrations indicated in the figure legends. Bicozamycin (33) was a gift from CIGA-GEIGY Ltd., Basel, Switzerland and was used in agar plates at a concentration of 100 μg/ml. Motility agar is L broth containing 3.5 g of Bacto-Agar per liter (23).

Motility. Strains to be tested for motility were streaked on L agar and incubated for 18 h at 37°C. At that time, samples of each strain were transferred with a sterile toothpick from L agar to motility agar, the plates were incubated for 6 h at 37°C, and the diameters of visible spread were measured (23).

Radioactive labeling of S. typhimurium LPS. Cultures of the S. typhimurium strains (20 ml) were grown in L broth (35) and labled with D-1-[^{3}H]arabinose-5'-monophosphate (1 μCi/per ml, 5 μg/ml) as described by Eidels et al. (8), except that chloramphenicol was not added to the culture during the labeling period. Arabinose-5'-monophosphate, a substrate of the S. typhimurium hexose phosphate transport system, is incorporated specifically into the 2-keto-3-deoxyoctonate (KDO) of LPS (34).

Measurement of KDO per bacterial cell. LPS was phenol extracted (43) from both radioactively labeled 20-ml cultures (see above) and nonradioactive 200-ml cultures of the S. typhimurium strains both grown to 10^9 cells per ml with vigorous aeration in L broth at 37°C. The phenol extracts of each labeled culture (approximately 5×10^5 cpm) and homologous unlabeled culture were combined (10 ml total), dialyzed overnight against 250 volumes of distilled H_2O, and lyophilized. Each combined extract was suspended in 5 ml of distilled H_2O and centrifuged at $100,000 \times g$ for 1 h at 4°C in a Spinco L5-50 ultracentrifuge to pellet the LPS (43). The pellets were suspended in 1 ml of distilled water, assayed for KDO by the method of Karkhanis et al. (21), and counted with Formula 963 (New England Nuclear Corp., Boston, Mass.) in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Rockville, Md.). Radioactivity measurements were used to correct KDO measurements for loss of LPS during the entire purification procedure. Routinely at least 80% of the added radioactive LPS was recovered in pellets. Control experiments showed that only about 5% of each LPS was lost owing to partition in phenol during phenol extraction. Protein and total cell count measurements on each culture were made before phenol extraction, and calculations of KDO per cell were made for each S. typhimurium strain.

RESULTS

Construction of nearly isogenic rough derivatives of a streptomycin-resistant, smooth, but nonvirulent strain of S. typhimurium. Strain SL5316, used as a parent of rough derivatives, is a cystine-independent (Cys$^+$) transductant of strain SL3237, which is a genetically marked (Table 1) streptomycin-resistant strain constructed for use as a live vaccine (14, 40) and is nonvirulent because of an aroA defect, causing a requirement for aromatic compounds, including two which are not available in host tissues (p-aminobenzoate [for synthesis of folate] and 2,3-dihydroxybenzoate [for synthesis of enterobactin]). Strain SL3237 efficiently colonized the gut of germ-free chicks (G. H. Snoeyenbos, personal communication) and in preliminary experiments likewise colonized the gut of mice given streptomycin in their drinking water.

A nonleaky rfb derivative of strain SL5316 was obtained by transducing into it mutation his(rfb)695, which is a deletion of part of the his operon and of (at least) six distal genes of the rfb (O-unit-determining) operon (32). The rough
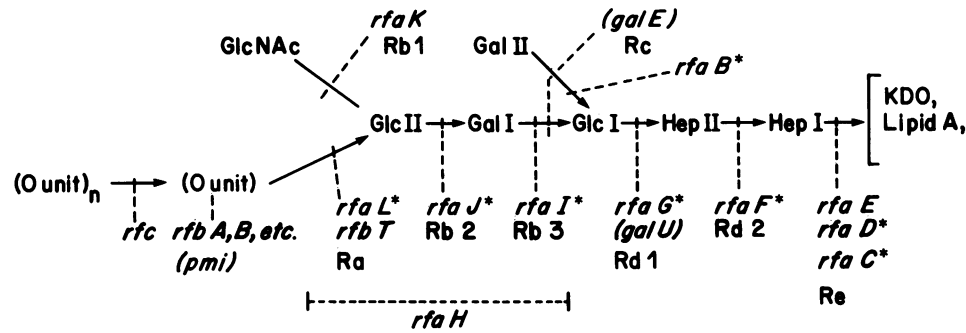
FIG. 1. Structure of the LPS of *S. typhimurium* and genes required for its synthesis (26, 28). Genes are shown in italics. Genes marked with an asterisk are located beween *cysE* and *pyrE* in a cluster of *rfa* genes at 79 min on the linkage map (43). Genes in parentheses have functions other than LPS synthesis. Entries in roman type (Ra, Rb, etc.) are symbols for the chemotype of LPS produced by mutants of the indicated class (i.e., for LPS completed up to the indicated block). The *rfaH* mutation produces LPS of heterogenous composition (26). GlcNAc, N-Acetylglucosamine; Gal, galactose; Glc, glucose; Hep, heptose. Reprinted from Kadam et al. (20) with permission of the authors.

deletion strain TA837 [LT2 *his(rfb)6951* was made phenotypically smooth by transfer to it of F' factor F's401-D, which includes an *his⁺* operon and the *rfb⁺* operon from a strain of *S. enteritidis* specifying O antigen 9,12 (42; B. Johnson and B. A. D. Stocker, unpublished data); a transconjugant was next made *hisD8557*::Tn*10* by transduction. Phage P22 HT105/1 *int* grown on a tetracycline-resistant transductant inferred to have *hisD8557*::Tn*10* inserted into the residual *hisGD* segment of its chromosome (i.e., close to the deletion) was used to evoke tetracycline-resistant transductants from strain SL5316. Of 43 clones tested, 2 were of rough phenotype, with phage sensitivity patterns characteristic of strains making complete core LPS without O chains (Fig. 1; chemotype Ra). One such transductant, SL5318, inferred to have integrated the *his(rfb)* deletion of the donor, was used for colonization tests as a representative of the complete-core LPS class. A sister transductant, SL5319, which retained smooth character, was used as a control strain for test of possible effect of the transposon insertion itself on colonizing ability.

Since previously characterized *rfa* (LPS core defect) mutations could not conveniently be transduced into strain SL5316, mutants resistant to Felix O phage (which adsorbs to complete-core LPS) were instead isolated and tested for phage sensitivities as an indication of type of LPS core defect (44). Two such mutants were chosen for use in colonization experiments because their phenotypes suggested that they had different nonleaky defects in LPS core assembly. Strain SL5325 had the phage sensitivities of strains making incomplete core LPS of type Rb₂, i.e., lacking the glucose II unit (and sugars distal to it) (Fig. 1); its mutation was therefore designated *rfaJ989*. Phage-resistant mutant SL5326, when first tested, was sensitive to phage C21 and, from this and other characters, was inferred to lack ability to form the glucose I unit of the core and so to make LPS of type Rd₁ through mutation at *rfaG* (Fig. 1). However, later, no sensitivity to phage C21 was detectable, and the exact nature of the LPS core defect of this strain remains to be determined; we refer to its mutation as *rfa-990*. The LPS of strain SL5326 travels faster on gel electrophoresis than that of the *rfaJ* strain SL5325 (P. Hitchcock, personal communication), so the site of the core defect in this strain must be deeper than the glucose II unit. For simplicity, the strains used in mouse colonization experiments, their LPS genotypes, and their defects in LPS structure, are listed in Table 2.

Test for possible effect of antibiotic resistance characters on

colonizing ability. Strain SL5318, used as a representative of the LPS Ra class, differs from its smooth parent not only by presence of the *his(rfb)* deletion but also by the presence of the tetracycline resistance transposon Tn*10* inserted in gene *hisD*. To test for any possible effect of the transposon insertion on colonizing ability, we administered the smooth parent strain SL5316 and a derivative, SL5319, obtained from it by transducing in the *hisD*::Tn*10* insertion but still smooth, to mice as a mixture. The two strains cocolonized the mouse intestine equally (data not shown). Thus, the presence of transposon Tn*10* in a smooth strain had no effect on its colonizing ability. It was, therefore, assumed that the presence of Tn*10* in strain SL5318, the *his(rfb)* deletion strain making LPS of chemotype Ra, would likewise have no effect on the colonizing ability of the strain. Similarly before strain SL5325-Nal', SL5326-Nal', or SL5326-Bic' was used in cofeeding experiments with strains of different LPS types, the ability of each to colonize the mouse colon in competition with its immediate antibiotic-sensitive parent was tested. Neither nalidixic acid resistance nor bicozamycin resistance altered the colonizing abilities of the strains (data not shown). The selective markers of the strains used in the cocolonization experiments are listed in the legends to the figures.

Individual colonizing abilities of the *S. typhimurium* strains. Each strain was fed individually to a set of nine streptomycin-treated mice, and the number of CFU/g of feces was monitored for each mouse over a 15-day period. In general, by day 5 postfeeding, CFU per g of feces stabilized in each mouse and remained at the 5-day level throughout the course of the experiment. The three rough strains all gave similar counts, about 10⁷ CFU/g of feces; the smooth parent strain, SL5316, gave counts nearly 1 log unit higher (Table 3). None

TABLE 2. Strains used in colonization experiments

Strain	LPS genotype	Defect in LPS structure[a]
SL5316	Wild type	None
SL5318	*rfb*	Lacks O side chain
SL5325	*rfaJ*	Lacks O side chain, glcNac, and glc II
SL5326	*rfa-990*	Lacks O side chain, glcNac, glc II, and one or both gal units

[a] See Fig. 1 for specific structural defects. See the text for uncertainty as to the exact LPS core defect in the *rfa-990* strain. glcNac, N-Acetylglucosamine; glc, glucose; gal, galactose.

TABLE 3. Individual colonizing abilities of S. typhimurium SL5316 and its LPS mutants[a]

Strain	LPS genotype	$\log_{10}$ CFU/of feces (mean ± SEM)
SL5316	Wild type	7.88 ± 0.38
SL5318	rfb	7.09 ± 0.46
SL5325	rfaJ	7.02 ± 0.36
SL5326	rfa-990	6.93 ± 0.46

[a] Eleven-day postfeeding data are shown for each strain fed individually to nine streptomycin-treated mice.

of the mice developed diarrhea, and all appeared as healthy as untreated controls.

Location of S. typhimurium in the mouse intestine. A set of three streptomycin-treated mice were fed SL5316, the wild-type LPS strain, and a second set were fed SL5325, the rfaJ strain. The mice were sacrificed at 7 days postfeeding, and their intestines were removed and cleaned of debris and fecal material. Bacteria were then released from the intestinal wall with trypsin (1 mg/ml) and assayed for viable counts as described previously (31). The S. typhimurium strains were found in the large intestine and in the small intestine immediately proximal to the cecum. However, in the large intestine SL5316 and SL5325 were found in far greater numbers (an average of 3,160 and 6,310 CFU/mg of tissue, respectively) than in the small intestine (an average of 15.8 and 16.0 CFU/mg of tissue, respectively).

Relative colonizing abilities of SL5316 and SL5318. Strains SL5316 and SL5318 were fed simultaneously to streptomycin-treated mice (Fig. 2). Both strains persisted in the feces for the duration of the experiment. SL5316, the wild-type LPS strain, colonized at a level of about 10^8 CFU/g of feces, i.e., at the same level as when fed to mice individually. In contrast, SL5318, the rfb strain, colonized at about 10^5 CFU/g of feces, i.e., about 2 orders of magnitude less than when it was fed to mice alone (compare Table 3 and Fig. 2).

Relative colonizing abilities of SL5316 and SL5325 and of SL5316 and SL5326. Strain SL5316, the wild-type LPS strain, colonized the mouse colon at a level of about 10^8 CFU/g of feces, i.e., the same as when fed to mice individually, when competed with SL5325, the rfaJ strain (Fig. 3). However, SL5325, colonized at a level of between 5×10^3 and 5×10^2 CFU/g of feces (Fig. 3), i.e., at least 3 orders of magnitude less than when fed individually to mice (Table 3). Therefore, the rfaJ strain did not compete effectively with the wild-type LPS strain for colon colonization sites. The same was true for SL5326, the rfa-990 strain, when competed with SL5316, the wild-type LPS strain (Fig. 4).

Relative colonizing abilities of SL5318 and SL5325 and of SL5318 and SL5326. When mice were fed SL5318, the rfb strain, and SL5325, the rfaJ strain, simultaneously, the rfb strain colonized at a level of about 2×10^7 CFU/g of feces, i.e., the same as when fed to mice individually (Table 3), and the rfaJ strain colonized at 10^3/g of feces (Fig. 5), i.e., at about 4 orders of magnitude less than when fed individually to mice (Table 3). Clearly, the rfaJ strain was unable to compete effectively with the rfb strain for mouse colon colonization sites. The same was true for SL5326, the rfa-990 strain, when competed with SL5318, the rfb strain (Fig. 6).

Relative colonizing abilities of SL5325 and SL5326. SL5325, the rfaJ strain, colonized at about 10^7 CFU/g of feces and SL5326, the rfa-990 strain, colonized at about 10^4 CFU/g of feces (Fig. 7), about 3 orders of magnitude less than its

individual colonizing ability when the strains were fed to mice together (Table 3). Therefore, the rfa-990 strain did not compete well with the rfaJ strain for colon colonization sites.

Motility. Since LPS mutants have been reported to be less motile than strains making wild-type LPS (13, 23), it was thought possible that the decreased ability of S. typhimurium LPS mutants to colonize the mouse colon, at least when in competition, was due to reduced motility or chemotactic ability or both rather than to changes in LPS structure. To test this possibility, we measured the rate of spreading of the strains in semisolid medium. Such spreading depends both on motility and on chemotactic ability. The rfb strain, SL5318, spread less rapidly than SL5316, its wild-type parent; however, the rfb strain and SL5325, the rfaJ strain, had almost identical rates of spreading (Table 4). Strain SL5326, rfa-990 making LPS less complete than chemotype Rb2, spread considerably less rapidly than the rfaJ and rfb strains.

In addition to differences in the chemical composition of LPS among the S. typhimurium strains, it was possible that as the S. typhimurium strains lost more core oligosaccharide they also contained fewer LPS molecules per bacterial cell (38). Such differences among the strains might explain their different relative colonizing abilities. Each S. typhimurium LPS molecule has three KDO molecules (27), and KDO is easily assayed (21). Therefore, a measure of the number of LPS molecules per bacterial cell was determined for each strain (see above). SL5316, the wild-type LPS, had about
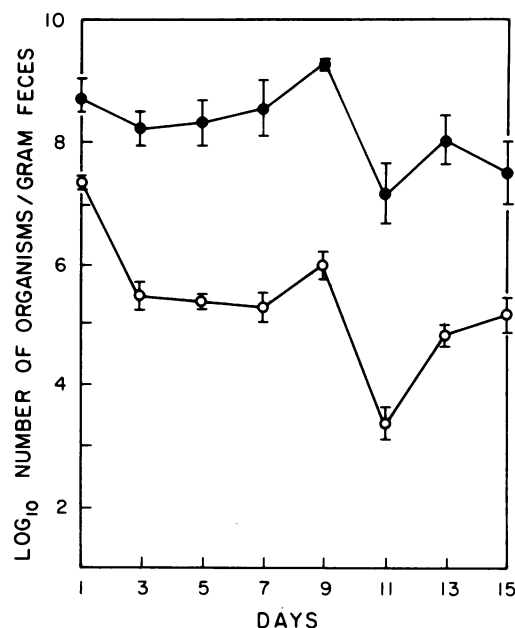

FIG. 2. Relative colonizing abilities of SL5316 (wild-type LPS) and SL5318 Tn10 (rfb). Both strains were fed simultaneously to a set of three streptomycin-treated mice as described in the text. Fecal samples were diluted and plated on L agar containing 100 µg of streptomycin sulfate per ml for total Salmonella viable counts and on L agar containing both 100 µg of streptomycin sulfate and 10 µg of tetracycline per ml for SL5318 Tn10 (rfb) viable counts. The number of SL5316 (wild-type LPS) was determined by subtracting the number of SL5318 Tn10 (rfb) CFU per g of feces from the total number of Salmonella CFU per g of feces. Bars representing standard errors of the mean are presented for each data point. Symbols: ●, SL5316 (wild-type LPS); ○, SL5318 Tn10 (rfb).

four times as many LPS molecules per cell as either SL5318, the *rfb* strain, or SL5325, the *rfa-990* strain, and six times as many LPS molecules per cell as SL5326, the *rfa-990* strain (Table 5).

DISCUSSION

The LPS of strain SL5318, given an *his(rfb)* deletion by transduction, could be confidently stated to be of type Ra, complete core without O chains, because the effect of deletion on the part of the *rfb* gene cluster has been thoroughly investigated in the donor strain (32). The identification of mutant SL5325 as an *rfaJ* mutant, therefore making LPS of chemotype Rb2, rested on the characteristic phage sensitivity pattern of such mutants; however,the identifications, both of site of mutation and of LPS chemotype, have recently been confirmed by complementation tests with cloned fragments of the *rfa* cluster (K. Sanderson, personal communication) and the electrophoretic mobility of its LPS (P. Hitchcock, personal communication). The site of the *rfa* mutation of strain SL5326 and the precise nature of its LPS core defect are not known with certainty; however, the increased electrophoretic mobility of its LPS makes it clear that it consists of LPS core less complete than chemotype Rb2.

S. typhimurium SL5316, the avirulent strain containing wild-type LPS colonized the streptomycin-treated mouse colon at a level of about 10^8/g of feces, whereas the *rfb*, *rfaJ*, and *rfa-990* strains derived from SL5316 all colonized at levels of about 10^7 CFU/g of feces when fed individually to mice (Table 3). It might be argued that the observed differ-

FIG. 4. Relative colonizing abilities of SL5316 (wild-type LPS) and SL5326-Nal^r (*rfa-990*). As described in the legend to Fig. 2, fecal samples were plated on L agar containing 100 μg of streptomycin sulfate per ml for total *Salmonella* viable counts and on L agar containing 100 μg of streptomycin sulfate and 10 μg of nalidixic acid per ml for SL5326-Nal^r (*rfa-990*). Calculations were as described in the legend to Fig. 2. Symbols: ●, SL5316 (wild-type LPS), ○, SL5326-Nal^r (*rfa-990*).
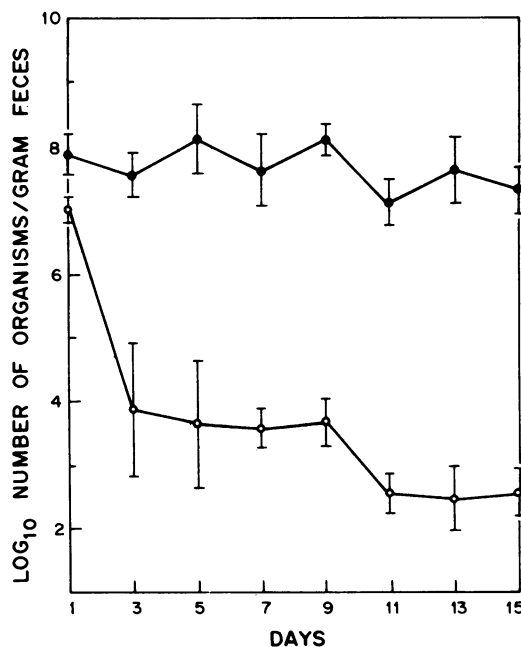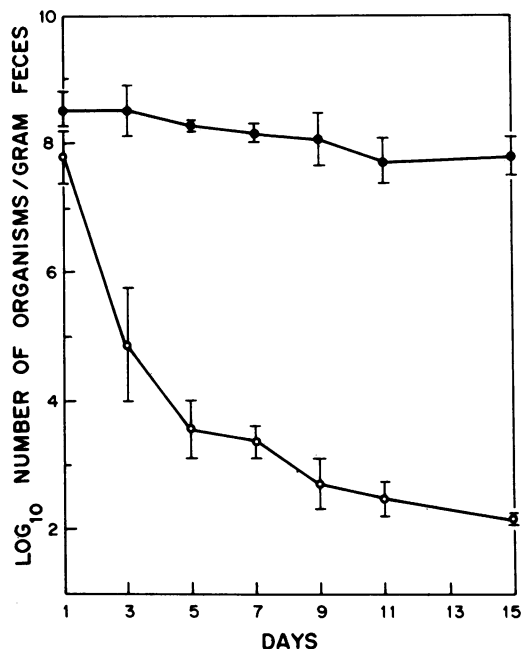

FIG. 3. Relative colonizing abilities of SL5316 (wild-type LPS) and SL5325-Nal^r (*rfaJ*). Both strains were fed to a set of three streptomycin-treated mice and plated on L agar containing 100 μg of streptomycin sulfate per ml for total (*rfaJ*) viable counts and on L agar containing 100 μg of streptomycin sulfate and 50 μg of nalidixic acid per ml for SL5325-Nal^r viable counts. Calculations were as described in the legend to Fig. 2. Symbols: ○, SL5316 (wild-type LPS); ●, SL5325-Nal^r (*rfaJ*).

ences in individual colonizing abilities reflect differences in in vivo replication rates between strain SL5316 and its LPS mutants and thereby explain why SL5316 is a better colonizer than its LPS mutants in competition experiments. This is unlikely, since each of the LPS mutant strains reached a steady-state number of CFU per gram of feces in competition with SL5316 and did not disappear completely (Fig. 2–4). This is especially evident in the case of competition between SL5316, the wild-type LPS strain, and SL5318, the *rfb* strain (Fig. 2). It seems more likely that the LPS mutants do not compete well for intestinal colonization sites with SL5316 but replicate as well as SL5316 from the small fraction of intestinal sites they occupy. Moreover, SL5318, SL5325, and SL5326, the *rfb*, *rfaJ*, and *rfa-990* strains, respectively, had almost identical individual colonizing abilities (Table 3) but, when competed in mice two at a time, had widely disparate relative colonizing abilities in the order *rfb* >> *rfaJ* > *rfa-990* (Fig. 5–7). Therefore, the data indicate that the ability of a *Salmonella* strain to colonize the mouse large intestine decreases as its LPS structure becomes more defective (Fig. 2–7; Table 3).

It has been suggested that the ability of *Vibrio cholerae* to colonize germ-free mice is correlated with its ability to migrate up a concentration gradient of a chemotactic attractant in intestinal mucous gel, the relatively thick layer which covers intestinal epithelial cells (1, 12). Furthermore, mutants of *S. typhimurium* and *E. coli* strains with deep defects in LPS core structure are reported to be nonmotile or at least less motile than strains making wild-type LPS, in consequence of the absence of or a reduced number of flagella (13, 23). Indeed, the *rfb*, *rfaJ*, and *rfa-990* strains
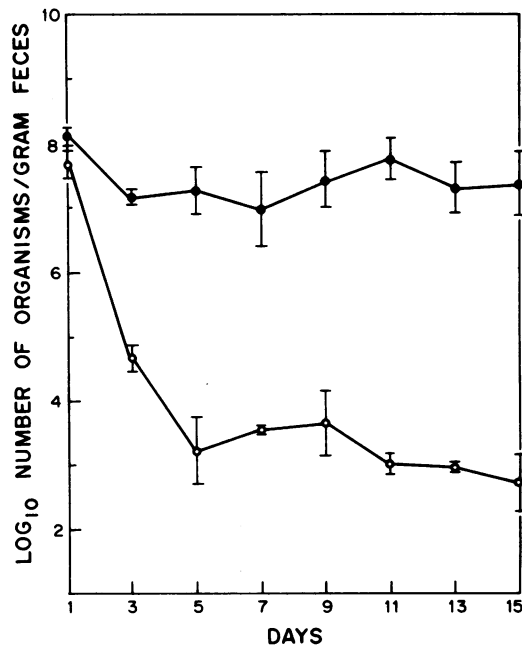
FIG. 5. Relative colonizing abilities of SL5318 Tn*10* (*rfb*) and SL5325-Nal^r (*rfaJ*). As described in the legend to Fig. 2, SL5318 Tn*10* (*rfb*) viable counts were made on L agar containing 100 μg of streptomycin sulfate and 10 μg of tetracycline per ml. SL5325-Nal^r (*rfaJ*) viable counts were made on L agar containing 100 μg of streptomycin sulfate and 50 μg of nalidixic acid per ml. Symbols: ●, SL5318 Tn*10* (*rfb*); ○, SL5325-Nal^r (*rfaJ*).
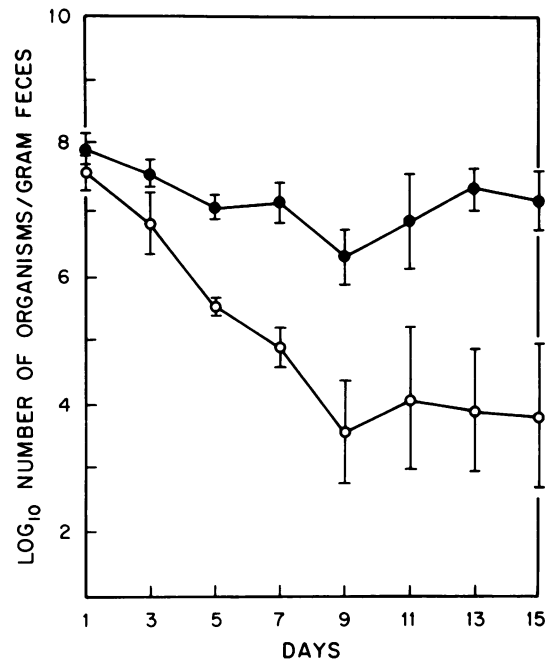

FIG. 7. Relative colonizing abilities of SL5325-Nal^r (*rfaJ*) and SL5326-Bic^r (*rfa-990*). As described in the legend to Fig. 2, fecal samples were plated on L agar containing 100 μg of streptomycin sulfate and 50 μg of naladixic acid per ml for SL5325-Nal^r (*rfaJ*) viable counts and on L agar containing 100 μg each of streptomycin sulfate and bicozamycin per ml for SL5326-Bic^r (*rfa-990*) viable counts. Symbols: ●, SL5325-Nal^r (*rfaJ*); ○, SL5326-Bic^r (*rfa-990*).
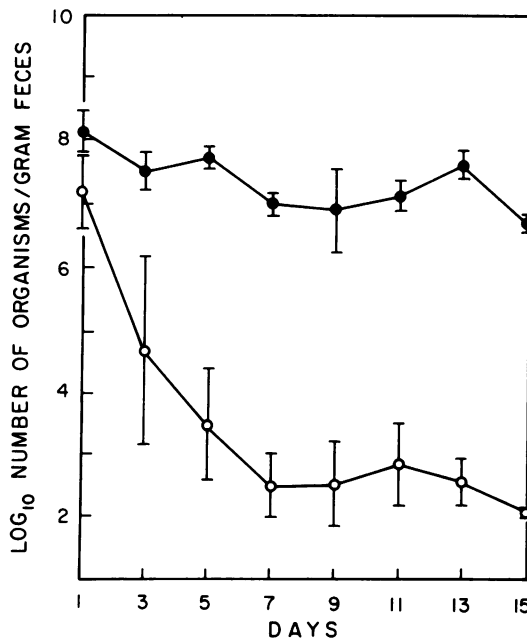

FIG. 6. Relative colonizing abilities of SL5318 Tn*10* (*rfb*) and SL5326-Nal^r (*rfa-990*). As described in the legend to Fig. 5, except that SL5326-Nal^r viable counts were made on L agar containing 100 μg of streptomycin sulfate and 10 μg nalidixic acid per ml. Symbols: ●, SL5318 Tn*10* (*rfb*); ○, SL5326-Nal^r (*rfa-990*).

were all less motile (or at least spread less rapidly) than their wild-type LPS parent (Table 4). Therefore, it is possible that SL5316 outcompeted the LPS mutant strains for colon-colonizing sites by migrating more rapidly to those sites and filling them preferentially. Both SL5318 and SL5325 spread equally fast on semisolid medium (Table 4), yet SL5325, the *rfaJ* strain, was a far worse colonizer than SL5318, the *rfb* strain, when both strains were fed simultaneously to mice (Fig. 5). Although this result argues that motility does not play a role in the differences observed in relative colonizing abilities beween SL5318 and SL5325, since spreading depends both on motility and on chemotactic ability, it is still possible that differences in one or the other of these properties are responsible for the observed results. Experiments designed to resolve this issue are presently in progress.

There is controversy as to whether type 1 pili are involved in *S. typhimurium* virulence (7, 18, 41), and it might be argued that adhesins involved in virulence could also play a

TABLE 4. Rate of spreading in motility medium of *S. typhimurium* SL5316 and its LPS mutants[a]

Strain	LPS genotype	Spread (mean ± SEM)[b]
SL5316	Wild type	15.75 ± 0.85
SL5318	*rfb*	5.75 ± 0.25
SL5325	*rfaJ*	5.25 ± 0.25
SL5326	*rfa-990*	3.50 ± 0.29

[a] Motility assays were performed in quadruplicate as described in the text.
[b] Measurements are diameter of spread in millimeters after 6 h of incubation at 37°C.

TABLE 5. Micrograms of KDO per cell of *S. typhimurium* SL5316 and its LPS mutants

Strain	LPS genotype	Expt 1			Expt 2[a]
		μg of KDO/mg of protein	No. of cells × 10⁻⁹/mg of protein	μg of KDO × 10⁹/cell	μg of KDO × 10⁹/cell
SL5316	Wild type	59.3	1.7	34.9	37.2
SL5318	*rfb*	8.0	1.0	8.0	9.3
SL5325	*rfaJ*	10.0	1.2	8.3	8.7
SL5326	*rfa-990*	5.5	1.1	5.0	6.4

[a] The values for micrograms of KDO per mg of protein and number of cells × 10^{-9} per mg of protein were very similar to those shown in experiment 1.

role in colon colonization. However, the *S. typhimurium* strains used here are of the FIRN biotype, which does not have type 1 pili (6). This rules out the possibility that the observed differences in relative colon-colonizing abilities were in some way due to changes in type 1 pili associated with changes in LPS.

SL5316, the wild-type LPS strain, has about four times as many LPS molecules per cell as either SL5318, the *rfb* strain, or SL5325, the *rfaJ* strain, and about six times as many LPS molecules per cell as SL5326, the *rfa-990* strain (Table 5). These data might explain why SL5316 outcompeted its LPS mutants in the mouse colon (Fig. 2–4) and why either SL5318 or SL5325 outcompeted SL5326. However, SL5318, the *rfb* strain, and SL5325, the *rfaJ* strain, have the same number of LPS molecules per bacterial cell and the same number of cells per mg of protein (Table 5), yet SL5318 was a far better colonizer than SL5325 when both were fed to mice simultaneously (Fig. 5). These data rule out the possibility that the great difference in relative colonizing abilities between SL5318 and SL5325 was due to a difference between them in the number of LPS molecules per bacterial cell.

Deep rough mutants of both *E. coli* and *S. typhimurium* deficient in LPS core polysaccharide have reduced amounts of several major proteins present in their outer membranes (2, 24); however, very little change was seen in protein levels of outer membrane of the Ra, Rb, or Rc mutants (24, 38), i.e., chemotypes used in this study. Still, at the present time we cannot rule out the possibility that changes in the amounts of specific outer membrane proteins among the strains contribute to the observed differences in their relative colonizing abilities. However, we favor the hypothesis that LPS itself plays an important role in the ability of *S. typhimurium* to colonize the mouse colon, perhaps by mediating bacterial adhesion to colonic tissue. In support of this view, it is known that purified *Shigella flexneri* 1b LPS specifically blocks adhesion of the strain to both guinea pig colon and guinea pig colonic epithelial cells (17). Moreover, the ability of a human fecal *E. coli* strain to colonize the mouse colon has been correlated with the ability of its LPS to bind mouse colonic mucus (3, 4). It is, therefore, possible that *S. typhimurium* LPS is involved in the adhesion of bacterial cells to either mucus or the underlying colonic epithelial cells or both as requisite to the colonization process. Experiments to test this possibility are presently in progress.

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