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Salmonella choleraesuis Strains Deficient in O Antigen Remain Fully Virulent for Mice by Parenteral Inoculation but Are Avirulent by Oral Administration

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O-antigen-deficient derivatives of two mouse-virulent strains of *Salmonella choleraesuis* (serogroup C_1; O-6,7) were constructed by transduction of a long deletion of the *rfb* operon. Strains SN36 and SN57 were derived from the smooth ancestor SL2824, while SN37 was derived from the smooth ancestor SL2840. These *rfb* deletion derivatives (*rfb* strains) had typical bacteriophage sensitivity patterns of "rough" *Salmonella* strains and were at least 200,000 times more sensitive to serum than their smooth ancestors. Lipopolysaccharides (LPS) extracted from them consisted only of two low-molecular-weight bands and lacked the ladderlike pattern of bands seen in the LPS of their smooth ancestors. The LPS from the *rfb* strains did not react in an enzyme immunoassay with any of three monoclonal antibodies directed against different epitopes of the O-6,7 antigen but reacted well with at least one of three monoclonal antibodies specific for core epitopes. The data were consistent with inability of these strains to synthesize O-specific chains and showed that the LPS extracted from SN57 was of chemotype Ra and that from SN36 was of chemotype Rb1, while that of SN37 consisted of a mixture of the two chemotypes. The virulence of these strains was tested by various routes in BALB/c mice. All three O-antigen-deficient derivatives were about as virulent as their "smooth" ancestors by the intraperitoneal and intravenous routes (50% lethal dose, 20 to 700 bacteria) but, unlike their ancestors, were avirulent by the oral route (50% lethal dose, $\geq 5 \times 10^9$ bacteria). This suggests that the major role of smooth LPS in the mouse virulence of *S. choleraesuis* is to facilitate survival in the gastrointestinal tract and eventual entry into deeper tissues.

The lipopolysaccharide (LPS) is the outermost component of the cell wall of gram-negative bacteria and is involved in a number of interactions with the environment. It consists of three distinct regions: lipid A; the core; and the O antigen, a polymer of short repeat units. It is well established that LPS is essential for the virulence of many *Salmonella* serotypes and that mutants with LPS defects are much less virulent than their wild-type parents. Lipid A is responsible for the biologic effects of LPS, but its contribution to the virulence of *Salmonella* species is not known. Likewise, the role of the core has not been well examined. By contrast, the O antigen has been the subject of intensive investigation and its contribution to the virulence of several *Salmonella* serotypes has been well characterized. Lack of O antigen causes great reduction in virulence (6, 13, 24), while its chemical composition affects virulence in a more subtle manner (41).

Evidence available at present indicates that the O antigen affects virulence mainly by modulating complement activation and bacterial phagocytosis within host tissue. Strains which lack O antigen, often referred to as "rough," unlike their "smooth" parents are serum sensitive (30, 40) and more readily phagocytosed and killed by macrophages (2, 4, 28). It is known that different kinds of O antigen confer different degrees of virulence to *Salmonella* species (43). This has been shown to be because they activate complement by the alternate pathway at different rates and, as a consequence, promote bacterial phagocytosis by macrophages at different rates (19, 20).

As would be expected from its location at the bacterial surface and role as a virulence factor, the O antigen is the main target of the protective immune response in *Salmonella* infection (25). The dual role of the O antigen as a virulence factor and a protective immunogen has been exploited to advantage in the design of vaccine. A class of live vaccines, the *galE* mutants, lack O antigen but partially synthesize it in media containing galactose. Used as live vaccines, *galE* mutants elicit an O-specific immune response which, presumably, indicates the availability to the bacteria of galactose, though from what source is not known. *galE* mutants of several *Salmonella* serotypes have been found effective as live vaccines because they were not only avirulent but also elicited an O-specific immune response (44, 47). The avirulence of *galE* mutants has been attributed to both galactose sensitivity and defective LPS (10, 34). A number of findings suggest, however, that the O antigen may not be essential for the virulence of some *Salmonella* species. Some *galE* mutants of *Salmonella choleraesuis* remain as virulent as their wild-type parents (34); a virulent *rfb*-type rough mutant of *S. choleraesuis* has been isolated (C. Hormaeche, personal communication as cited in reference 34), and a galactose-sensitive *galE* mutant of *Salmonella typhi* has recently been shown to cause typhoid in human volunteers (15). These facts underscore the need for thorough investigation of the importance of smooth LPS as a *Salmonella* virulence factor.

We have constructed O-antigen-deficient mutants of two mouse-virulent strains of *S. choleraesuis* by transduction of a long deletion of the *rfb* operon. We report testing of the virulence of these strains by various routes of inoculation in BALB/c mice. The results support predictions on the bases of clinical findings (45) and surface properties of intact bacteria (16) that *S. choleraesuis* (and perhaps other members of *Salmonella* serogroup C_1) has pathogenic mecha-

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TABLE 1. Bacterial strains

Strain	Description	Source or reference
SL2824	*S. choleraesuis* 38_1 wild type (smooth)	34
SL2840	*S. choleraesuis* 110 wild type (smooth)	34
SL2847	38_1 *galE710*; Rc[a]	34
SL2853	110 *galE716*; Rc	34
SL5318	*S. typhimurium* M7471 (ColE1) *malB479 leu-1051* Δ*his*(*rfb*)*695 hisD8557*::Tn*10*; Ra	32
SN16	38_1 *galE710* Δ*his*(*rfb*)*695 hisD8557*::Tn*10*	This work
SN17	38_1 *galE710* Δ*his*(*rfb*)*695 hisD8557*::Tn*10*; Rc	
SN18	38_1 *galE710* Δ*his*(*rfb*)*695 hisD8557*::Tn*10*; *rfa*	This work
SN23	110 *galE716* Δ*his*(*rfb*)*695 hisD8557*::Tn*10*; Rc	
SN36	SN18 *gal*$^+$; Rb1	This work
SN37	SN23 *gal*$^+$; Ra	This work
SN57	SN17 *gal*$^+$; Ra	This work

[a] *galE* mutants are switchable; that is, they synthesize smooth LPS when grown in media containing galactose. Thus, a *galE* population contains various amounts of smooth and Rc LPS. Symbol: Δ, deletion mutation.

nisms different from those of other well-investigated *Salmonella* species.

MATERIALS AND METHODS

Media and reagents. The defined medium used was that of Davis and Mingioli (3) which contains both sodium citrate (0.5 g/liter) and glycerol (5 mg/liter) as carbon sources. The complete media used included L broth, L agar, and MacConkey agar (Difco Laboratories, Detroit, Mich.). Anti-O-6,7 rabbit serum for slide agglutination was purchased from the National Bacteriology Laboratory, Stockholm, Sweden. Alkaline phosphatase conjugates against rat and mouse immunoglobulins used in enzyme-linked immunosorbent assays (ELISA) were from Sigma Chemical Co., St. Louis, Mo. Monoclonal antibodies against *Salmonella* antigens were produced in this laboratory.

Bacterial strains, bacteriophages, and general genetic methods. Strains used are listed in Table 1. The donor strain used in strain construction was *Salmonella typhimurium* SL5318. It is of FIRN biotype (5) and among other mutations contains a long deletion of the *rfb* operon (32). It is thus unable to synthesize O antigen and has rough LPS of chemotype Ra. Wild-type strains of *S. choleraesuis*, SL2824 and SL2840, and their respective *galE* derivatives, SL2847 and SL2853, have been described previously (34). The somatic antigenic composition of SL2824 is O-6_2,7 and that of SL2840 is O-6_1,7. Strains SN36, SN37, and SN57 were constructed by phage P1::Tn*9* *c*(Ts) cotransduction of the *rfb* deletion in SL5318 and a nearby insertion of Tn*10* in the *hisD* gene with the *galE* strains SL2847 and SL2853 as recipients. The *galE* defects of the transductants were subsequently corrected either by a second P1::Tn*9* *c*(Ts) transduction to *gal*$^+$ character or by selection for *gal*$^+$ revertants. Phage P1::Tn*9* *c*(Ts) is a derivative of P1kc carrying a chloramphenicol resistance transposon, Tn*9* (18), and a temperature-sensitive repressor (39). It cannot lysogenize bacteria or persist as prophage at 37°C but can do so at 30°C. It was propagated on *Salmonella* strains as described below and used for transduction as described previously (35, 36). Other phages were used for characterizing transductants by determination of phage sensitivity pattern (46). These included the smooth LPS-specific phage 14 (active only on strains with O-6_2,7), the complete core LPS-specific phage FO, and the rough LPS-specific phages Br60, 6SR, Ffm, P221, and C21. Phage C21 adsorbs to the core structure with a terminal glucose 1→3 heptose which is specifically exposed when *galE* mutants are grown in the absence of galactose. In the presence of galactose, smooth LPS is made and the structure is no longer exposed. They then become resistant to the phage.

Propagation of phage P1::Tn*9* *c*(Ts) on *Salmonella* strains. The *S. typhimurium* strain used as genetic donor, SL5318, has chemotype Ra LPS; no phages are known to transduce efficiently between this species and *S. choleraesuis*. Rough mutants of *S. typhimurium* of types *rfaG*, *rfaH*, *galU*, and *galE*, however, adsorb P1 (7, 37) and have been used as donors in P1-mediated transduction with *galE* recipients of *S. choleraesuis* (35, 36). For these reasons *galE* mutants of *S. choleraesuis*, SL2847 and SL2853, were chosen for use as recipients in strain construction instead of their wild-type parents. To propagate P1::Tn*9* *c*(Ts) on SL5318, we rendered it P1 sensitive by a rapid procedure which obviated the need to isolate a specific P1-sensitive mutant (35). This involved preselection for resistance to 2-deoxygalactose on defined agar medium (supplemented as necessary), which essentially increases the proportion of *galE* and other galactose-negative mutants in the population and thus increases the chance of lysogenization with P1. An overnight broth culture made from the preselected population can be used to readily isolate P1::Tn*9* *c*(Ts) lysogenic clones by applying a drop of lysate on a lawn of bacteria and selecting for conversion to chloramphenicol resistance (12 μg/ml) at 30°C. A modified version of the above procedure was used to isolate P1::Tn*9* *c*(Ts) lysogens of SL2824 which still retained the *gal*$^+$ character; such lysogens were needed to make transducing lysates for use in *galE* correction. After preselection in the presence of 2-deoxygalactose and lysogenization with P1::Tn*9* *c*(Ts), a pool of lysogens was streaked on a plate of MacConkey base agar containing galactose (5 g/liter) and chloramphenicol (12 μg/ml) and incubated for up to 3 days at 30°C. Several of the resulting galactose-fermenting colonies were pooled and used for lysate production. Lysates were made by heat induction. A lysogen is grown up in broth at 30°C with shaking and shifted to 42°C for 30 min and then to 37°C for 1 h, both with shaking. This treatment induces the lytic cycle. Several drops of chloroform are added and the culture is shaken vigorously for 5 min to complete lysis. Lysed cultures are clarified by centrifugation, sterilized by filtration, and stored at 4°C with a few drops of chloroform added as preservative. For brevity, lysates of P1::Tn*9* *c*(Ts) are hereinafter referred to as P1.

Extraction and electrophoretic analysis of LPS. The bacteria were grown to stationary phase in L broth, using a 12-liter fermentor (LKB, Bromma, Sweden). LPS was extracted from strains SL2824, SN57, and SN36 by hot phenol-water (23) and from SN37 by phenol-chloroform-light petroleum (12). Purified LPS samples were analyzed by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) incorporating 4 M urea (42) in an 18% separating gel. A 10-μg amount of smooth LPS or 5 μg of rough LPS was mixed with an equal volume of sample buffer (0.1 M Tris hydrochloride, pH 6.8, containing 2% [wt/vol] SDS, 20% [wt/vol] sucrose, 1% [wt/vol] 2-mercaptoethanol, 0.001% [wt/vol] bromophenol blue) and immersed for 5 min in boiling water before use for loading wells. After electrophoresis at 30 mA (15°C), the gels were stained with silver as described by Tsai and Frasch (42).

Monoclonal antibodies. O-antigen-6,7-specific monoclonal antibodies MR9 (immunoglobulin M), MR11 (immunoglobulin G2b), and MR15 (immunoglobulin G2b) were generated by fusion of spleen cells from Lou/C rats immunized with heat-killed *Salmonella thompson* (with O antigens 6_1, 6_2, and

7) with the rat myeloma Y3.Ag.1.2.3 (23a). Core-specific monoclonal antibodies MAST60 (Rb1 specific), MAST71 (Ra specific) (S. Lind and A. A. Lindberg, unpublished data), and MM3 (Ra specific) (N. A. Nnalue and A. A. Lindberg, unpublished data) were similarly produced by fusion of spleen cells from BALB/c mice immunized with heat-killed rough *Salmonella* strains with the mouse plasmacytoma Sp2/0-Ag14. MAST60 and MAST71 are both of the immunoglobulin G3 isotype, while MM3 is of the immunoglobulin M isotype.

Characterization of LPS by ELISA. The binding of various core- and O-antigen-6,7-specific monoclonal antibodies to LPS extracted from the various strains was tested by ELISA. The ELISA procedure used has been described previously in detail (1). Briefly, a 96-well microtiter plate (Nunc, Roskilde, Denmark) was coated overnight at 20°C with smooth (10 μg/ml) or rough (25 μg/ml) LPS in 0.05 M carbonate buffer (pH 9.6), overcoated with 1% bovine serum albumin for 1 h at 37°C, and washed three times with washing buffer (0.15 M NaCl, 0.05% Tween 20). Hybridoma culture supernatant containing monoclonal antibody was diluted in phosphate-buffered saline containing 0.05% Tween 20; 100-μl aliquots were added to wells, followed by a 4-h incubation at room temperature and washing as before. Alkaline phosphatase-labeled rabbit antibody to mouse or rat immunoglobulin was diluted as recommended by the manufacturer (Sigma Chemical Co.), and 100 μl was added to each well. The plate was incubated at 20°C and washed again. Substrate solution (100 μl of *p*-nitrophenyl phosphate dissolved at 1 g/liter in 1 M diethanolamine–0.5 mM $MgCl_2$ buffer, pH 9.8) was added, and the plate was incubated for 100 min at 37°C; then the A_{405} was read in a Titertek Multiscan photometer (Flow Laboratories, Irvine, Scotland).

Sugar analyses. Lipid-free polysaccharides were prepared from LPS by treatment with 2% aqueous acetic acid at 100°C for 1 h; this cleaves the acid-labile 3-deoxy-D-manno-octulosonyl linkages. The insoluble lipid was removed by centrifugation at 12,000 × *g* at 4°C for 15 min, and the polysaccharide was purified by gel chromatography on a Bio-Gel P4 column and lyophilized. The polysaccharides were hydrolyzed in 0.5 M trifluoroacetic acid at 100°C for 16 h, and the resulting mixtures of aldoses were reduced to the corresponding alditols with sodium borohydride. The alditols were converted to more volatile alditol acetates by treatment with acetic anhydride-pyridine (1:1, vol/vol) at 100°C for 1 h. After removal of excess acetic anhydride by repeated addition of water and concentration to dryness, the resulting mixture of fully acetylated alditol acetates was dissolved in dichloromethane and analyzed by gas-liquid chromatography in a Hewlett-Packard model 5890A gas chromatograph, using column DB5 when simultaneous determination of all the sugars of LPS was desired and column DB225 when only hexoses were of interest.

Survival and growth of bacteria in human serum. The serum used was pooled from five healthy northern Europeans with no history of salmonellosis. An overnight broth culture of a strain to be tested was diluted fivefold in fresh broth and grown for another 2 h at 37°C to bring the cells to exponential growth phase. A 0.1-ml aliquot of broth culture containing 2.5×10^7 to 4×10^7 CFU was mixed with 0.9 ml of 50% pooled normal human serum (diluted with phosphate-buffered saline), and an aliquot (0.1 ml) was immediately withdrawn and diluted for use in determination of initial viable count. A control was set up in parallel in exactly the same manner as above but with serum that had been heated for 90 min at 56°C and then diluted with an equal volume of phosphate-buffered saline. The mixtures were incubated for 100 min at 37°C and then diluted and plated for determination of the final viable counts. The ability of each strain to survive and grow in human serum is expressed as percentage of final CFU recovered from unheated serum relative to that recovered from heated serum.

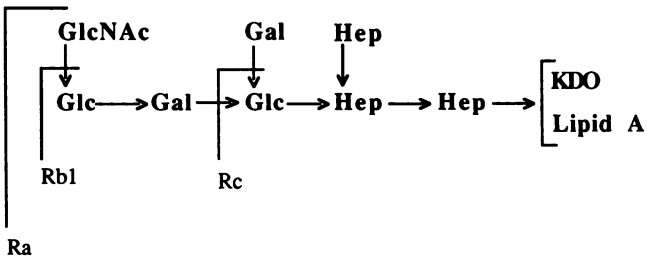

FIG. 1. Simplified structure of the *S. typhimurium* LPS core, showing relevant chemotypes. For the complete structure, see references 11 and 17. KDO, 2-Keto-3-deoxyoctulosonic acid.

Testing of mouse virulence. All tests were done in BALB/c mice purchased from ALAB, Stockholm, Sweden. Overnight, unshaken broth cultures for use in parenteral inoculation were kept at 4°C while their counts were determined by plating. They were then diluted and appropriate doses in 100 μl of saline were injected intraperitoneally (i.p.) or into a lateral tail vein. Bacteria for oral administration were also grown and counted as described above but had to be concentrated by centrifugation and resuspension in saline before inoculation in mice by a modified version of the method of Duguid et al. (5). In brief, a mouse was fully restrained in one palm while 10 μl of inoculum was deposited on its tongue with a micropipette. Preliminary assessment of the virulence of *rfb* strains was done by i.p. inoculation of groups of six mice. Parenteral route 50% lethal dose (LD_{50}) determinations (38) were subsequently done by inoculating groups of 6 to 14 mice with doses of bacteria between 10 and 10^4 CFU in 10-fold dilution steps. Oral route LD_{50} studies were done with six mice per group inoculated with 5×10^7, 5×10^8, or 5×10^9 CFU.

RESULTS

Construction of *rfb* deletion derivatives. Nonleaky *rfb* derivatives of smooth *S. choleraesuis* strains were constructed in two steps, using their *galE* mutants as recipients. SN36 and SN57 were derived from SL2847 by the following steps. The mutation Δ*his(rfb)695*, which is a deletion of part of the *his* operon and of at least six distal genes of the *rfb* operon (33), was cotransduced with a nearby Tn*10* insertion in the *hisD* gene, using phage P1 grown on a 2-deoxygalactose-resistant population of strain SL5318. Selection was on L agar containing tetracycline (25 μg/ml). We expected, on the basis of the widely assumed similarity of LPS core structure (Fig. 1) in all *Salmonella* spp., that in the absence of galactose transductants of SL2847 which inherited the deletion would require histidine and synthesize only an incomplete core of chemotype Rc. Such mutants should make a complete core of chemotype Ra when provided with galactose, but should remain unable to synthesize O antigen. They therefore should be sensitive to phage C21 and resistant to phage FO on L agar but resistant to C21 and sensitive to FO on L agar supplemented with galactose (5 g/liter); in neither case should they agglutinate in anti-O-6,7 serum.

TABLE 2. Phage sensitivity patterns of *S. choleraesuis* strains and their Δ*rfb* derivatives[a]

Strain	Description	Sensitivity to phages						
		14	FO	BR60	P221	6SR	Ffm	C21
SL2824	Wild type	S	S	R	SR	R	R	R
SN36	Δ*rfb*	R	R	S	S	R	S	R
SN57	Δ*rfb*	R	S	S	S	S	S	R
SL2840	Wild type	NR	S	R	SR	R	R	R
SN37	Δ*rfb*	NR	S	S	S	S	S	R

[a] S, Sensitive; R, resistant; SR, slight thinning of the lawn; NR, not relevant. Symbol: Δ, deletion mutation.

Seven of eight transductants tested satisfied these criteria. One of them was numbered SN17. One transductant was atypical because, though resistant to tetracycline, it did not require histidine and, while it "switched" from C21 sensitive to resistant when supplied with galactose, it did not simultaneously become sensitive to phage FO. The genetic basis for the properties of this transductant was unclear, but these properties were stable. It was retained and numbered SN18. The phenotype of SN18 suggested that it might contain a duplication of the *hisD* gene and a mutation other than *galE* affecting LPS core structure. Moreover, it was uncertain whether or not it inherited mutation Δ*his(rfb)695*. To characterize this strain further, it was plated on minimal medium containing galactose but no sodium citrate or glycerol for isolation of *gal*$^+$ revertants. One such revertant, SN36, was retained for further investigation. Another strain, SN57, a *gal*$^+$ transductant of SN17 obtained by use of a P1 lysate propagated on its wild-type ancestor, SL2824, was also further investigated. SN37 was constructed similarly to SN57 but in a different genetic background. First, tetracycline-resistant transductants were evoked in SL2853 with the P1 lysate of SL5318. One transductant, SN23, of phenotype expected from acquisition of the Δ*his(rfb)695* mutation in a *galE* background was made *gal*$^+$ by a second transduction with the P1 lysate of SL2824. The resulting strain was SN37.

Properties of *rfb* deletion derivatives. SN37 and SN57 were sensitive to all of a battery of rough-specific phages except C21, whose receptor lies deep within the core (Table 2). This indicates that they made complete LPS core of chemotype Ra. The sensitivity pattern of SN36 differed from this because it was resistant to phage FO as well as to phage 6SR. This pattern suggested that it made core chains longer than Rc but shorter than Ra. SN36 and SN57, both derived from a phage 14-sensitive background, were resistant to this phage and thus did not synthesize O antigen. Overall, the sensitivity of these strains to phages conforms to that published for mutants of *S. typhimurium* devoid of O antigen (46) and suggest that SN37 and SN57 have a complete core of chemotype Ra while SN36 has less than a complete core.

The LPS from the rough *S. choleraesuis* mutants were subsequently extracted and analyzed. SDS-PAGE showed that the LPS from SN36 and SN57 (Fig. 2A, lanes 2 and 3) contained only two or three bands of low molecular weight and did not have a ladderlike pattern like that of their smooth parent, SL2824 (lane 1). In at least three analyses of these LPS samples by SDS-PAGE, the bands in SN36 LPS migrated somewhat farther in the gel than those of SN57 LPS; this is consistent with phage sensitivity data which showed

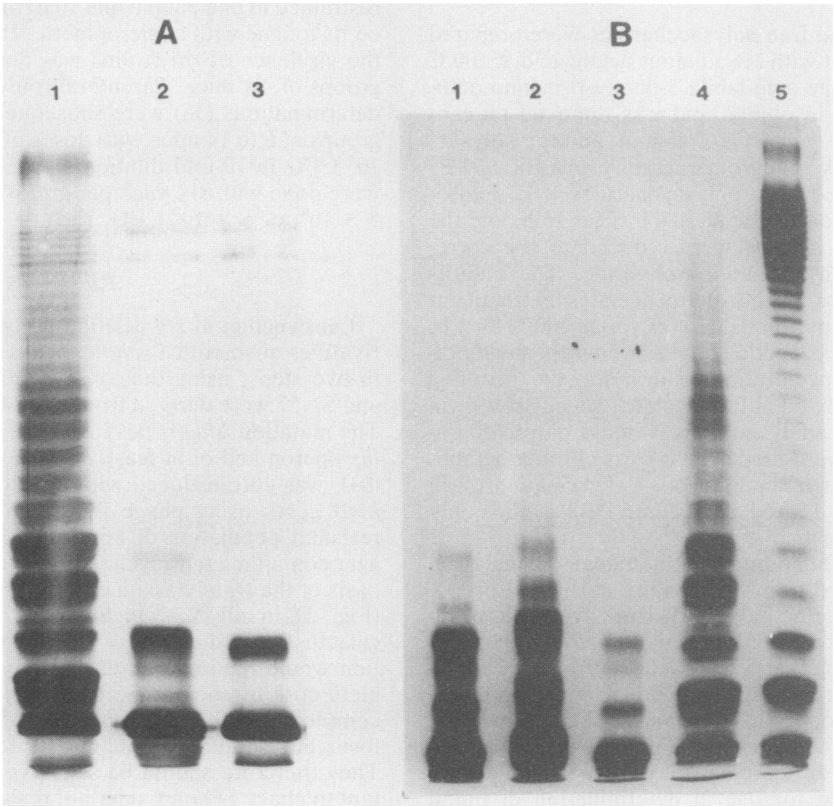

FIG. 2. SDS-PAGE of LPS extracted from *Salmonella* strains: A, SL2824 (lane 1), SN57 (lane 2), SN36 (lane 3); B, SN37 (lane 1), TV119 (lane 2), SN57 (lane 3), SL2824 (lane 4), SL2840 (lane 5).

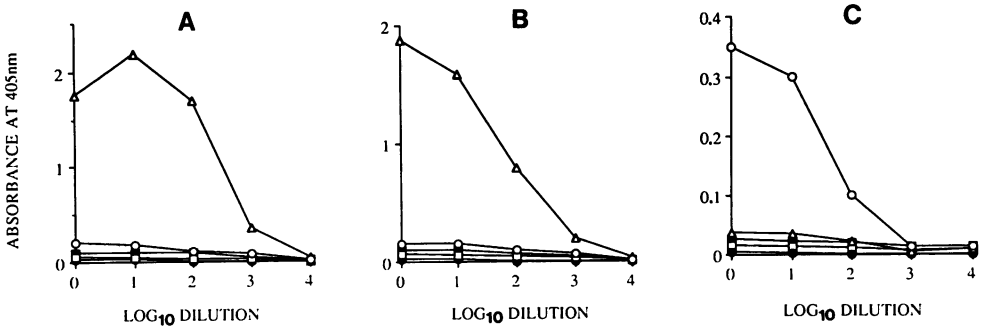

FIG. 3. Titration of three monoclonal antibodies specific for O antigen 6,7 epitopes against bacterial LPS: (A) MR9; (B) MR11; (C) MR15. The LPS used were SL2824 (Δ), SL2840 (○), SN36 (●), SN57 (■), and SN37 (□). When not evident, a curve lies on or close to the horizontal axis.

that SN36 has an incomplete core. In another SDS-PAGE analysis (Fig. 2B), the LPS from SN37 (lane 1) and from its smooth ancestor, SL2840 (lane 5), were examined. LPS from SN57 (lane 3), SL2824 (lane 4), and TV119, a well-characterized *rfb* mutant of *S. typhimurium* known to synthesize only LPS of chemotype Ra (lane 2), were included for comparison. Again, three bands were present in the LPS from SN57, whereas those from TV119 and SN37 had several bands. The presence of several bands in the LPS from the rough *S. choleraesuis* strains is not considered to be due to the presence of O antigen since the same pattern was observed in the LPS from TV119. Rather, it was thought to indicate that the core LPS from these strains (lowest band in each lane) were so hydrophobic that they formed molecular aggregates even in the presence of SDS and 6 M urea.

The results of ELISAs showed that LPS from strains SN36, SN37, and SN57 did not react with any of the three monoclonal antibodies specific for different epitopes of the O-6,7 antigen (Fig. 3) and thus did not contain O-repeat units. By contrast, these LPS reacted with core-specific monoclonal antibodies. The LPS from two strains, SN57 and SN37, but not that from SN36 reacted well with each of two Ra-specific monoclonal antibodies (Fig. 4A and B). SN57 LPS did not react with an Rb1-specific monoclonal antibody (Fig. 4C), which reacted well with those from SN36 and SN37. These results strongly indicate that the LPS from SN57 was of chemotype Ra and that from SN36 was of chemotype Rb1, while that of SN37 apparently consisted of a mixture of the two chemotypes.

Further evidence for a complete absence of O repeat units in the LPS of these strains, as indicated by their failure to react with O-6,7-specific monoclonal antibodies, was obtained from sugar analyses, the results of which (Table 3) showed that the LPS from each rough strain lacked mannose, a hexose present in the O antigen of their smooth parents and which, on the basis of structure (22), should constitute four of no more than six sugar residues in the O-6,7 repeat unit of *S. thompson*. The molar ratio of sugars in the LPS of SN37 was as would be expected of a strain with a core structure similar to that of *S. typhimurium* (Fig. 1), but that of SN57 was different and contained more galactose and glucosamine than would be expected. SN36 seemed to have a slightly reduced amount of glucosamine and a much increased amount of glucose relative to SN57. When only hexose content was analyzed, it was observed that a galactose/glucose ratio of 3:2 in SN57 had been altered to an approximately 1:1 ratio in SN36. Thus, while immunochemical data indicated that SN36 has Rb1 specificity, the chemical data showed that SN36 LPS contained less galactose than SN57 LPS, contrary to expectation from comparison of Ra and Rb1 LPS of *S. typhimurium* (Fig. 1).

The ability of these strains to survive and multiply in normal human serum was also tested. The numbers of the smooth strains, SL2824 and SL2840, recovered from unheated serum after 100 min of incubation were, respectively, 57 and 88% of the numbers recovered from heat-inactivated serum. With the rough strains SN36, SN37, and SN57, the numbers from unheated serum were between 0.0004 and 0.0002% of the numbers from heat-inactivated serum. Thus, the smooth strains were at least 200,000 times better able than rough strains to survive and multiply in human serum.

Mouse virulence of *Salmonella* strains. A preliminary assessment was done to compare the i.p. virulence of the *rfb* deletion strains (SN36, SN37, and SN57) with those of their

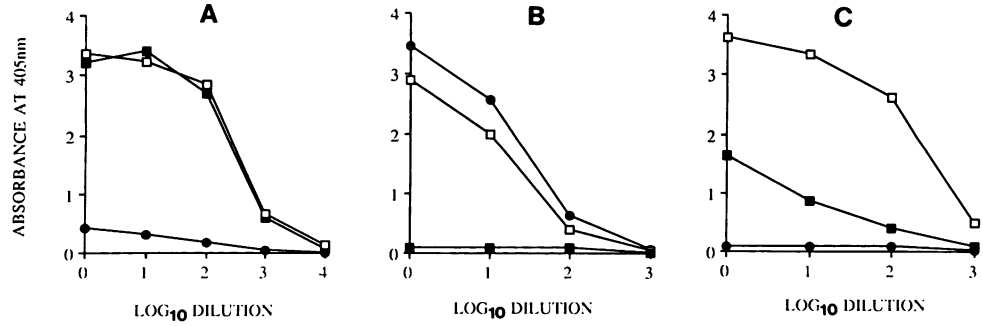

FIG. 4. Titration of three monoclonal antibodies specific for *Salmonella* LPS core chemotypes against bacterial LPS: (A) MM3 (Ra specific); (B) MAST71 (Ra specific); (C) MAST60 (Rb1 specific). The LPS used were SN36 (●), SN37 (□), and SN57 (■).

TABLE 3. Sugar composition of the oligo-/polysaccharide segment of LPS from *Salmonella* strains

Sugar	Amt (M)[a]				
	SN57	SN36	SN37	SL2824	SL2840
D-Galactose	28.7 (3)	33.0 (4)	26.2 (2)	7.2	3.7
D-Glucose	19.2 (2)	29.5 (3.6)	23.6 (2)	12.2	19.6
D-GlcNAc[b]	22.4 (2)	12.7 (1.5)	12.0 (1)	36.3	24.7
L-Glycero-D-manno heptose[b]	29.8 (3)	24.8 (3)[c]	38.2 (3)	9.6	4.5
Mannose	0	0	0	34.7	49.0

[a] Numbers of sugar residues indicated are given in parentheses.
[b] These values have been adjusted by using response factors (GlcNAc, 1.98; heptose, 1.60) found for model compounds (14).
[c] Three heptose residues per core chain were assumed in calculating approximate number of sugar units indicated by molar ratios for SN36.

galE ancestors (SL2847 and SL2853) which have been shown to be virulent by this route. Each strain was tested at two levels, 2×10^3 and 2×10^5 CFU, given to six mice each by i.p. injection. All of the mice challenged with 2×10^5 CFU of each strain died within 8 days, while at least half of those challenged with 2×10^3 CFU died within 2 weeks (data not shown). The mortality data (not shown) suggested that each *rfb* deletion strain was about as virulent by the i.p. route as its *galE* ancestor. We then did two determinations of i.p. LD_{50} with each *rfb* deletion strain, using 8 or 14 mice per group and challenge doses of 10^2, 10^3, and 10^4 CFU. Both determinations consistently showed that the i.p. LD_{50}s of SN36 and SN57 were each ca. 100 CFU, while that of SN37 was ca. 650 CFU. These values are about the same as was found for their *galE* and wild-type ancestors in a previous study (34). We subsequently compared the virulence of the *rfb* deletion strains with those of their wild-type ancestors by the oral and intravenous (i.v.) routes. SN36 and SN57 were tested by the i.v. route on two separate occasions with groups of 6 or 10 mice. The combined results showed that the i.v. LD_{50}s of SN36 and SN57 were ca. 20 and 30 CFU, respectively, and not much different from that (10 CFU) of their wild-type ancestor, SL2424 (Table 4). On the single occasion when SN37 was administered to mice by the i.v. route, it caused 100% mortality in each group of six mice inoculated with 10^2, 10^3, and 10^4 CFU (Table 4) within 10 days. Unlike the results obtained by parenteral administration, the results of testing by the oral route showed that each *rfb* deletion strain was at least 100-fold less virulent than its wild-type ancestor (Table 4).

TABLE 4. Virulence of smooth, *galE*, and Δ*rfb* derivatives of *S. choleraesuis*

Strain	LPS chemotype or phenotype	LD_{50} in BALB/c mice[a]		
		i.p.	i.v.	Oral
SL2824	Smooth	(100)	10	$<5 \times 10^7$ (10^7)
SL2847	Rc[b]	$<2 \times 10^3$ (100)	ND	ND
SN36	Rb1	ca. 100	20	5×10^9
SN57	Ra	ca. 100	30	$>5 \times 10^9$
SL2840	Smooth	(200)	ND	10^7
SL2853	Rc	$<2 \times 10^3$ (500)	ND	ND
SN37	Ra	700	<100	$>5 \times 10^9$

[a] LD_{50}s in parentheses have been determined previously (see reference 34). ND, Not done.
[b] Rc strains are switchable and synthesize various amounts of smooth LPS when provided with galactose.

DISCUSSION

We have constructed rough derivatives of two mouse-virulent strains of *S. choleraesuis* and shown by various methods that they lacked O antigen and synthesized only core LPS of chemotype Ra or Rb1. As would be expected from their lack of O antigen, these strains were much less virulent than their smooth parents when administered to mice by the oral route. However, contrary to dogma, they remained as virulent for mice as their smooth parents when administered by the i.v. or i.p. route. This raises many questions on the pathogenesis of mouse salmonellosis.

It is accepted that death from mouse infection with *Salmonella* spp. requires penetration of a virulent strain deep into host tissues and its subsequent multiplication to very high numbers. The outcome of an infection therefore depends on the interaction of host defense mechanisms which prevent systemic invasion or limit multiplication and those of the bacterium which antagonize such defenses. The relevant protective mechanisms in a nonimmunized host are the phagocytic system which engulfs and kills bacteria and the complement system which opsonizes bacteria and consequently promotes phagocytosis. As *S. choleraesuis* does not make a Vi antigen or any other kind of capsule, its main antagonist to host defenses should be smooth LPS. Although the role of smooth LPS in virulence is not well understood (21), possession of it seems to be a general and basic requirement for the survival and multiplication of noncapsulate gram-negative bacteria in a mammalian host. Recent work has analyzed the role of different kinds of smooth *Salmonella* LPS as antiphagocytic factors. It was found that the O-6,7 LPS of serogroup C_1 (e.g., *S. choleraesuis*), unlike that of serogroup B (e.g., *S. typhimurium*) or D (e.g., *Salmonella enteritidis*), activated complement by the alternate pathway at a rapid rate (19, 20), and this caused a 10- to 100-fold more rapid phagocytosis of O-6,7 than O-4,12 or O-9,12 bacteria in vitro (20). Much faster phagocytosis and killing of O-6,7 than O-4,12 strains also occur in the mouse peritoneal cavity (35, 41), and this has been shown to be primarily a result of the difference in the rates of complement activation by the two kinds of smooth LPS (41).

A consequence of the difference in the rates of phagocytosis of O-6,7 and O-4,12 organisms is that an inoculum of an O-6,7 strain (up to 5×10^6 CFU) given as challenge to mice by i.p. injection is almost completely killed by 24 h so that <1% of the CFU injected can be recovered in the liver and spleen compared with at least 30% of similarly inoculated O-4,12 strains (35). When placed in an *S. typhimurium* background, the O-6,7 antigen of *S. choleraesuis* caused a 10,000-fold increase in i.p. LD_{50} (36), at least as much as can be expected from loss of O antigen in *S. typhimurium* (13, 24). It is therefore evident that, while the O-4,12 LPS can antagonize phagocytosis and consequently promote bacterial survival in vivo, the O-6,7 LPS has a limited capacity to do so. One may deduce from this apparent lack of O-antigen-mediated antiphagocytic function that smooth O-6,7 bacteria are as readily phagocytosed as rough salmonellae and that virulent serogroup C_1 salmonellae would rely little, if at all, on extracellular multiplication to survive in vivo. Mutation from smooth to Ra or Rb1 chemotype in *S. typhimurium* results in a 6,000- to 40,000-fold reduction in mouse virulence by the i.p. route (13). Our finding that mutations resulting in similar phenotypic changes in *S. choleraesuis* caused only at most a two- to three-fold reduction in virulence by the i.p. or i.v. route indicates a profound difference in the interaction of the two bacterial species with

the mechanisms of host defense in mammalian tissues. Our results therefore signify two things. First, they demonstrate that a gram-negative organism which lacks both a capsule and O antigen can survive, multiply, and cause disease within a normal mammalian host. Second, it indicates that the critical events that determine the outcome of *S. choleraesuis* infection occur intracellularly after phagocytosis or invasion.

Though unlikely, it remains possible that rough mutants of *S. choleraesuis*, unlike rough mutants of other *Salmonella* serotypes, are less readily phagocytosed than their smooth parents in vivo and consequently killed mice mainly from extracellular multiplication. The results we have obtained from tests of the survival and growth of the strains in human serum would, however, argue against this possibility. Our data showed that all of the rough strains of *S. choleraesuis* were several orders of magnitude more sensitive to serum than their smooth ancestors. While mouse serum is poorly bactericidal, serum sensitivity could be relevant to mouse salmonellosis because it reflects the rate of stable deposition of complement components at the bacterial surface. The rate at which different LPS of *Salmonella* spp. activate complement by the alternate pathway has been shown to determine the rate at which they are phagocytosed in the peritoneal cavity (19, 20) and consequently their virulence for mice (41). Based on our data, we would expect that the rough strains of *S. choleraesuis* would be phagocytosed even more readily than their smooth ancestors in vivo and that their mouse virulence would not be due primarily to extracellular multiplication as a consequence of an ability to resist phagocytosis. Two other possibilities we have not excluded include that rough strains are in some way better able to survive intracellularly than smooth strains or are able to find a niche within host tissue inaccessible to phagocytic cells. In these regards, it may be of significance that the sugar compositions of SN36 and SN57 are different from those expected of similar *S. typhimurium* mutants. We consider it likely that the cores of at least SN36 and SN57 do differ in structure from the well-studied *S. typhimurium* core and surmise that an LPS structure-function relationship might exist which explains the virulence of these mutants. The assumption that all *Salmonella* strains have the same core structure is in fact based on structural studies of a few rough mutants (14, 17). A difference in core structure, as suggested by our data, might cause rough bacteria of the two species to interact differently with factors of host defense or host cells. We plan to test this possibility by construction of genetic hybrids of *S. choleraesuis* and *S. typhimurium* and will also attempt to determine the LPS structure of the three rough mutants of *S. choleraesuis*.

The importance of smooth LPS for the virulence of *Salmonella* spp. by the oral route has been shown previously (9). It is evident that for a *Salmonella* strain to establish systemic infection by the oral route it must survive passage through the stomach, withstand intestinal motility, colonize the mucosa, and subsequently penetrate the epithelium. It is not clear at present whether the nature of the LPS is important in all or only some of the above events, but there is evidence that smooth LPS is of importance in invasion and colonization. The data of Mintz and Deibel (27) suggest that smooth LPS is required for invasiveness of *S. typhimurium* in a rabbit ileal loop model, while data of Finlay et al. (9) show that it is required for the passage of *S. choleraesuis* through polarized monolayers of MDCK cells in vitro. Nevola et al. (32) have shown that the ability of a *Salmonella* strain to colonize the large intestine of streptomycin-treated mice decreased as its LPS structure became more defective. Under direct competition, a smooth strain colonized 1,000- to 100,000-fold better than its nearly isogenic descendants which lacked O antigen or had core defects. In subsequent reports, Nevola et al. (31) and McCormick et al. (26) related the colonization ability of *Salmonella* strains to their ability to penetrate the mucus layer on intestinal wall and showed that not only were rough strains less able than smooth strains to penetrate mucus but also they adhered more strongly to it and consequently should be more easily discharged into the lumen by sloughing. As the mice we have used were not pretreated with antibiotics, our *S. choleraesuis* challenge strains must compete with gut flora for colonization sites on the mucosa. The results we have obtained by oral challenge can therefore be readily explained by the observations of the above workers.

S. choleraesuis and *S. typhi* have several properties related to pathogenesis in common. Both organisms are highly invasive in humans, regularly produce systemic disease, and cause infections that have the highest mortality rates of human salmonellosis. That rough mutants of *S. choleraesuis* are highly virulent for mice may therefore have significance for human infections caused by *S. typhi*. We have reported that some *galE* mutants of *S. choleraesuis* were as virulent for mice as their wild-type parents (34). More recently, a virulent typical *galE* mutant of *S. typhi* was described (15). A paradox surrounding the pathogenesis of *Salmonella* infections, including those caused by *S. typhi*, is that protective immunity does not correlate with humoral response to smooth LPS. Another unanswered question is the fact that, while some strains of a serotype remain confined to the gut, others can invade and cause systemic disease. These observations underscore the need for continued effort to identify virulence factors other than the O antigen which are better correlated with the pathogenic properties of the different strains.

We plan to continue our efforts in the present system with a view to further characterizing the properties of the virulent rough mutants of *S. choleraesuis* in vivo and identifying components of bacterial structure that confer such properties. There are reports which show that *S. choleraesuis* and *S. typhi*, unlike *S. typhimurium*, can multiply within mammalian cells other than "professional" phagocytes in vitro (8, 29). It remains to be investigated whether this property is of any significance to their common ability to cause systemic disease regularly in humans or to the observed virulence of rough mutants of *S. choleraesuis*. It is considered an attractive possibility that rough mutants of *S. choleraesuis* remain virulent because they can invade and multiply within host cells not proficient in killing bacteria and hence escape normal antimicrobial defenses.

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LITERATURE CITED

1. **Carlin, N. I. A., and A. A. Lindberg.** 1983. Monoclonal antibodies specific for O-antigenic polysaccharides of *Shigella flexneri*: clones binding to II, II:3,4, and 7,8 epitopes. J. Clin. Microbiol. **18**:1183–1189.
2. **Chedid, L., M. Parant, F. Parant, and F. Boyer.** 1968. A proposed mechanism for natural immunity in enterobacterial pathogens. J. Immunol. **100**:292–301.
3. **Davis, B. D., and E. S. Mingioli.** 1950. Mutants of *Escherichia coli* requiring methionine or vitamin B12. J. Bacteriol. **60**:17–28.

4. **Dlabac, V.** 1968. The sensitivity of smooth and rough mutants of Salmonella typhimurium to bactericidal and bacteriolytic action of serum, lysozyme and to phagocytosis. Folia Microbiol. **13**:439–449.
5. **Duguid, J. P., M. R. Darekar, and D. W. F. Wheater.** 1976. Fimbriae and infectivity in *Salmonella typhimurium*. J. Med. Microbiol. **9**:459–473.
6. **Edebo, L. B., and B. Normann.** 1970. Virulence and immunogenicity of mutant strains of *Salmonella typhimurium*. Acta Pathol. Microbiol. Scand. Sect. B **78**:75–84.
7. **Enomoto, M., and B. A. D. Stocker.** 1974. Transduction of phage P1kc in *Salmonella typhimurium*. Virology **60**:503–514.
8. **Finlay, B. B., and S. Falkow.** 1988. Comparison of the invasion strategies used by *Salmonella choleraesuis*, *Shigella flexneri*, and *Yersinia enterocolitica* to enter cultured animal cells. Biochimie **70**:1089–1099.
9. **Finlay, B. B., M. N. Starnbach, C. L. Francis, B. A. D. Stocker, S. Chatfield, G. Dougan, and S. Falkow.** 1988. Identification and characterisation of Tn*phoA* mutants of Salmonella that are unable to pass through a polarized MDCK epithelial cell monolayer. Mol. Microbiol. **2**:757–766.
10. **Fukasawa, T., and H. Nikaido.** 1961. Galactose-sensitive mutants of *Salmonella*. II. Bacteriolysis induced by galactose. Biochim. Biophys. Acta **48**:470–483.
11. **Galanos, C., O. Lüderitz, E. T. Rietschel, and O. Westphal.** 1977. Newer aspects of the chemistry and biology of bacterial lipopolysaccharides with special reference to their lipid A component. Int. Rev. Biochem. **14**:239–335.
12. **Galanos, B., O. Lüderitz, and O. Westphal.** 1969. A new method for extraction of R lipopolysaccharides. Eur. J. Biochem. **9**: 245–249.
13. **Germanier, R.** 1970. Immunity in experimental salmonellosis. I. Protection induced by rough mutants of *Salmonella typhimurium*. Infect. Immun. **2**:309–315.
14. **Hellerqvist, C. G., and A. A. Lindberg.** 1971. Structural studies of the common core polysaccharide of the cell-wall lipopolysaccharide from Salmonella typhimurium. Carbohydr. Res. **16**: 39–48.
15. **Hone, D. M., S. R. Attridge, B. Forrest, R. Morona, D. Daniels, J. T. LaBrooy, R. C. A. Bartholomeusz, D. J. C. Shearman, and J. Hackett.** 1988. A *galE via* (Vi antigen-negative) mutant of *Salmonella typhi* Ty2 retains virulence in humans. Infect. Immun. **56**:1326–1333.
16. **Huixiu, J., O. Stendahl, and L. Edebo.** 1984. Activation of polymorphonuclear leucocytes by *Salmonella*. Acta Pathol. Microbiol. Immunol. Scand. Sect B **92**:107–113.
17. **Jansson, P.-E., A. A. Lindberg, B. Lindberg, and R. Wollin.** 1981. Structural studies on the hexose region of the core lipopolysaccharides from enterobacteria. Eur. J. Biochem. **115**: 571–577.
18. **Kondo, E., and S. Mitsuhashi.** 1964. Drug resistance of enteric bacteria. IV. Active transducing bacteriophage P1CM produced by combination of R factor with bacteriophage P1. J. Bacteriol. **88**:1266–1276.
19. **Liang-Takasaki, C., N. Grossman, and L. Leive.** 1983. Salmonellae activate complement via the alternate pathway depending on the structure of their lipopolysaccharride O-antigen. J. Immunol. **130**:1867–1870.
20. **Liang-Takasaki, C., P. H. Makela, and L. Leive.** 1982. Phagocytosis of bacteria by macrophages: changing the carbohydrate of lipopolysaccharide alters the interaction with complement and macrophages. J. Immunol. **128**:1229–1235.
21. **Lindberg, A. A.** 1980. Bacterial virulence factors with particular reference to *Salmonella* bacteria. Scand. J. Infect. Dis. Suppl. **24**:86–92.
22. **Lindberg, B., K. Leontein, U. Lindquist, S. B. Svenson, G. Wrangsell, A. Dell, and M. Rogers.** 1988. Structural studies of the O-antigenic polysaccharide of *Salmonella thompson*, serogroup C1 (6,7). Carbohydr. Res. **174**:313–322.
23. **Lüderitz, O., O. Westphal, A. M. Staub, and H. Nikaido.** 1971. Isolation and chemical and immunological characterization of bacterial lipopolysaccharides, p. 145–233. *In* G. Weinbaum, S. Kadis, and S. J. Ajl (ed.), Microbial toxins, vol. 4. Academic Press, Inc., New York.

23a. **Luk, J. M. C., N. A. Nnalve, and A. A. Lindberg.** 1990. Efficient production of mouse and rat monoclonal antibodies against the O antigens of *Salmonella* serogroup C_1, using LPS-coated bacteria as immunogen. J. Immunol. Methods **129**:243–250.

24. **Lyman, M. B., J. P. Steward, and R. J. Roantree.** 1976. Characterization of the virulence and antigenic structure of *Salmonella typhimurium* strains with lipopolysaccharide core defects. Infect. Immun. **13**:1539–1542.
25. **Lyman, M. B., B. A. D. Stocker, and R. J. Roantree.** 1979. Evaluation of the immune response directed against *Salmonella* antigenic factors O4,5 and O9. Infect. Immun. **26**:956–965.
26. **McCormick, B. A., B. A. D. Stocker, D. C. Laux, and P. S. Cohen.** 1988. Roles of motility, chemotaxis, and penetration through and growth in intestinal mucus in the ability of an avirulent strain of *Salmonella typhimurium* to colonize the large intestine of streptomycin-treated mice. Infect. Immun. **56**:2209–2217.
27. **Mintz, C. S., and R. H. Deibel.** 1983. Effect of lipopolysaccharide mutations on the pathogenesis of experimental *Salmonella* gastroenteritis. Infect. Immun. **40**:236–244.
28. **Mitsuhashi, S., I. Sato, and T. Tanaka.** 1961. Experimental salmonellosis: intracellular growth of *Salmonella enteritidis* ingested in mononuclear phagocytes and cellular basis of immunity. J. Bacteriol. **81**:863–868.
29. **Mroczenski-Wildey, M. J., J. L. DiFabio, and F. C. Cabello.** 1989. Invasion and lysis of HeLa cell monolayers by *Salmonella typhi*: the role of lipopolysaccharide. Microb. Pathogen. **6**: 143–152.
30. **Nelson, B. W., and R. J. Roantree.** 1967. Analysis of lipopolysaccharides extracted from penicillin-resistant serum-sensitive *Salmonella* mutants. J. Gen. Microbiol. **48**:179–188.
31. **Nevola, J. J., D. C. Laux, and P. S. Cohen.** 1987. In vivo colonization of the mouse large intestine and in vitro penetration of intestinal mucus by an avirulent smooth strain of *Salmonella typhimurium* and its lipopolysaccharide-deficient mutant. Infect. Immun. **55**:2884–2890.
32. **Nevola, J. J., B. A. D. Stocker, D. C. Laux, and P. S. Cohen.** 1985. Colonization of the mouse intestine by an avirulent *Salmonella typhimurium* strain and its lipopolysaccharide-defective mutants. Infect. Immun. **50**:152–159.
33. **Nikaido, H., M. Levinthal, K. Nikaido, and K. Nakane.** 1967. Extended deletions in the histidine-rough-B-region of the *Salmonella* chromosome. Proc. Natl. Acad. Sci. USA **57**:1825–1832.
34. **Nnalue, N. A., and B. A. D. Stocker.** 1986. Some *galE* mutants of *Salmonella choleraesuis* retain virulence. Infect. Immun. **54**:635–640.
35. **Nnalue, N. A., and B. A. D. Stocker.** 1987. The effects of O-antigen character and enterobacterial common antigen content on the *in vivo* persistence of aromatic-dependent *Salmonella* sp. live-vaccine strains. Microb. Pathogen. **3**:31–44.
36. **Nnalue, N. A., and B. A. D. Stocker.** 1987. Tests of the virulence and live-vaccine efficacy of auxotrophic and *galE* derivatives of *Salmonella choleraesuis*. Infect. Immun. **55**:955–962.
37. **Ornellas, E. P., and B. A. D. Stocker.** 1974. Relation of lipopolysaccharide character to P1 sensitivity in *Salmonella typhimurium*. Virology **60**:491–502.
38. **Reed, L. J., and J. Muench.** 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. **27**:493–497.
39. **Rosner, J. L.** 1972. Formation, induction and curing of bacteriophage P1 lysogens. Virology **49**:678–689.
40. **Rowley, D.** 1968. Sensitivity of rough gram-negative bacteria to the bactericidal action of serum. J. Bacteriol. **95**:179–188.
41. **Saxen, H., I. Reima, and P. H. Mäkelä.** 1987. Alternative complement pathway activation by Salmonella O polysaccharide as a virulence determinant in the mouse. Microb. Pathogen. **2**:15–28.
42. **Tsai, C.-M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119**:115–119.
43. **Valtonen, M. V., M. Plosila, V. V. Valtonen, and P. H. Mäkelä.** 1975. Effect of the quality of the lilopolysacharide on mouse virulence of *Salmonella enteritidis*. Infect. Immun. **12**:828–832.

44. **Wahdan, M. H., C. Serie, Y. Cerisier, S. Sallam, and R. Germanier.** 1982. A controlled field trial of live *S. typhi* Ty21a oral vaccine against typhoid: three year results. J. Infect. Dis. **145**:292–295.
45. **Weissbluth, M., S. T. Shulman, B. Holson, and C. Lerner.** 1981. *Salmonella choleraesuis*: a distinctive bacterial pathogen. J. Pediatr. **98**:423–426.
46. **Wilkinson, R. G., P. Gemski, and B. A. D. Stocker.** 1972. Non-smooth mutants of *Salmonella typhimurium*: differentiation by phage-sensitivity and genetic mapping. J. Gen. Microbiol. **70**:527–554.
47. **Wray, C., W. J. Soyka, J. A. Morris, and W. J. B. Morgan.** 1977. The immunization of mice and calves with *galE* mutants of *Salmonella typhimurium*. J. Hyg. **79**:17–24.