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Some *galE* Mutants of *Salmonella choleraesuis* Retain Virulence

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***galE* mutants were isolated from three mouse-virulent strains of *Salmonella choleraesuis* (of group C_1, O antigen 6,7) by selection for resistance to 2-deoxygalactose. The *galE* derivative of strain 38_1 comprised two components: galactose sensitive, thought to be the original mutant; and galactose resistant, presumably by a second mutation reducing *galK* or *galT* function or both. The galactose-sensitive component had an intraperitoneal 50% lethal dose for BALB/c mice of ca. 4×10^6 CFU, whereas the galactose-resistant component was about as virulent as its *gal*$^+$ parent, with a 50% lethal dose of ca. 100 CFU. The *galE* mutant of strain 110 was somewhat sensitive to galactose, as shown by retardation of growth; its 50% lethal dose, ca. 500 CFU, was not much greater than the ca. 200 CFU value for its parent. The *galE* mutant of strain 117 showed the same partial sensitivity to galactose as strain 110 *galE*, but was nonvirulent (50% lethal dose of ca. 10^6 CFU versus ca. 400 CFU for its parent). Growth on galactose-supplemented medium restored the smooth phenotype, as indicated by phage sensitivity to three of the four *galE* strains, but only partially so for the strain 117 *galE* mutant. The retention of parental virulence by *galE* mutants of *S. choleraesuis* which are galactose resistant or somewhat galactose sensitive contrasts with the greatly reduced virulence of galactose-resistant *galE* mutants of *Salmonella typhimurium* and *Salmonella typhi*; this difference may result from the absence of galactose from the O repeat unit in the lipopolysaccharide of group C_1 salmonellae.**

galE mutants are unable to make UDP-glucose-4-epimerase, the enzyme which interconverts UDP-glucose and UDP-galactose (9). All *Salmonella* species contain galactose units in their surface lipopolysaccharide (LPS). Since UDP-galactose is the precursor of the galactose units in LPS, *galE* mutants of *Salmonella* species make incomplete or "rough" LPS of the chemotype Rc (Fig. 1) unless exogenous galactose is supplied. Complete LPS synthesis is possible in the presence of galactose because the *galK* and *galT* gene products, galactokinase and galactose-1-phosphate uridyltransferase, respectively, can synthesize UDP-galactose from galactose via galactose 1-phosphate. Several studies have shown that *galE* mutants of *Salmonella typhimurium* and *Salmonella typhi* are of much reduced mouse virulence and are effective as live vaccines (8–11, 13). Field trials of the *galE* mutant of *S. typhi* designated strain Ty21a, isolated by Germanier and Furer (10), have shown that it is a safe and effective live vaccine in humans (25, 26).

The loss of virulence by *galE* mutants is believed to be due to two causes. The first cause is that typical *galE* mutants are galactose sensitive, and they lyse when incubated in the presence of galactose (6, 7), supposedly because of the accumulation of galactose 1-phosphate and UDP-galactose (6). The second reason for the loss of virulence by *galE* mutants is that they are "rough" mutants, and rough mutants in general are more rapidly phagocytosed and killed by macrophages than their "smooth" parents (3, 5, 18). Galactose-resistant derivatives are readily isolated from galactose-sensitive *galE* strains; they have secondary mutations in the *galK* and *galT* genes. Such galactose-resistant derivatives of *galE* mutants of *S. typhimurium* are somewhat more virulent and less immunogenic than their *galE* parents (9).

Although all members of the genus *Salmonella* contain galactose units in their LPS, the number of galactose units per LPS molecule differs in different O groups (Fig. 1). *S. typhimurium* (O group B) and *S. typhi* (O group D_1) contain two galactose residues in the LPS core and one galactose residue in each O repeat unit, of which there may be up to 40 per LPS molecule (12, 20). In contrast *Salmonella choleraesuis* (O group C_1) contains two galactose residues in the core region but no galactose in the O repeat units. *galE* mutants of *S. choleraesuis* would therefore need much less galactose than *galE* mutants of *S. typhimurium* or *S. typhi* to make complete LPS.

There is currently no approved safe and effective vaccine to combat *S. choleraesuis* infections in livestock and in humans at risk. We have therefore isolated and tested *galE* mutants from three mouse-virulent strains of *S. choleraesuis* var. Kunzendorff to determine the effect of a mutation in this gene on virulence. We found that, in contrast to results obtained with *S. typhimurium*, some of these *S. choleraesuis* *galE* mutants remained as virulent as their parental strains.

MATERIALS AND METHODS

Media. The complete media used were Oxoid blood agar base (code CM55; Oxoid Ltd., London), Oxoid nutrient broth (code CM67) and MacConkey agar base (Difco Laboratories, Detroit, Mich.) supplemented with galactose (5 g/liter). The defined medium used was the minimal medium of Davis and Mingioli (4), which contains both sodium citrate (0.5 g/liter) and glycerol (5 ml/liter) as the main carbon sources. In this study, redistilled glycerol (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was used in place of regular glycerol because initial tests indicated that batches of the latter may be contaminated with galactose.

Mice. All experiments were performed with BALB/c mice purchased from the Department of Radiology, Stanford University. The mice were from a specific-pathogen-free colony originally derived by caesarian section and given a simplified gut flora. They were housed in groups of four or five per cage and fed and watered ad libitum.

Bacterial strains. Three pig- and mouse-virulent strains of *S. choleraesuis* var. Kunzendorff strains designated 38_1, 110, and 117 were kindly provided by Ron Griffith of The Department of Veterinary Microbiology and Public Health, Iowa

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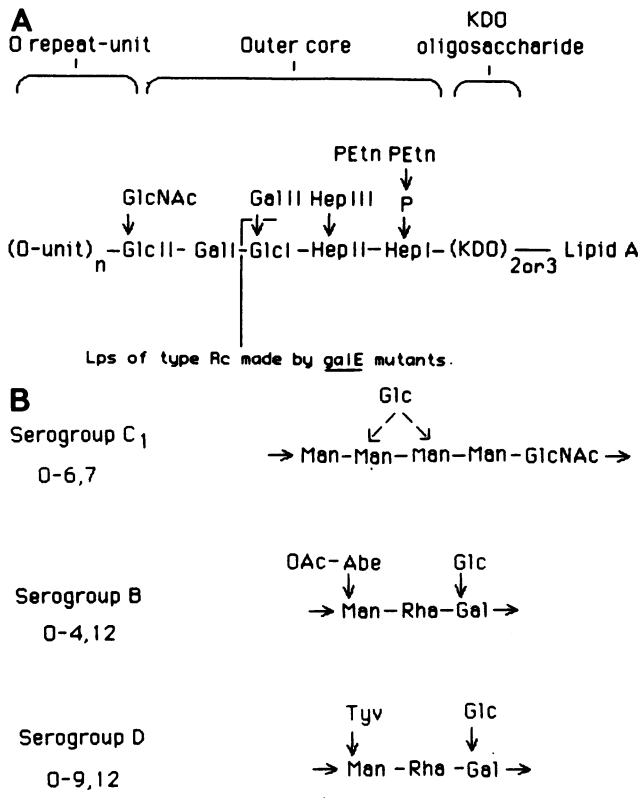

FIG. 1. Structure of *Salmonella* LPS. A, General structure; B, O repeat units. Abbreviations: Gal, galactose; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Hep, heptose; KDO, 3-deoxy-D-mannooctulosonic acid; Man, mannose; OAc-Abe, *O*-acetyl abequose; PEtn, phosphoethanolamine; Rha, rhamnose; Tyv, tyvelose.

State University of Science and Technology, Ames. The somatic antigenic compositions of these bacteria are as follows: strain 38_1, O 6_2,7; strain 110, O 6_1,7; and strain 117, O 6_1,7.

Phages and phage methods. A battery of phages was used to characterize putative *galE* mutants by phage sensitivity pattern (27). These included the smooth LPS-specific phage 14 (active only on strains with O 6_2,7), the complete core LPS-specific phage FO, the rough LPS-specific phages Br60 and 6SR, and phage 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. This ability to switch from C21 sensitive to C21 resistant when supplied with galactose is characteristic of *galE* mutants which retain *galK* and *galT* function, at least in part. The sensitivity patterns of *galE* derivatives and their parents to selected phages are given in Table 2.

Isolation of *galE* mutants. *galE* mutants were isolated by selection for resistance to 2-deoxygalactose (1). Samples (0.1 ml) of overnight unshaken nutrient broth cultures of the bacterial strains were spread on minimal agar plates which also contained 0.1% (wt/vol) 2-deoxygalactose. This compound is toxic for cells having a normal ability to metabolize galactose. Only clones that have suffered a mutation in one or more of the genes of the galactose operon or are defective in galactose uptake can grow in the presence of the compound. Colonies appearing on plates after 2 to 3 days of incubation at 37°C were further tested to identify *galE* mutants. Among such colonies only *galE*, *galU*, and *galETK* deletion mutants are sensitive to phage C21. *galE* mutants were subsequently readily distinguished from *galU* and *galETK* deletion mutants by phage sensitivity pattern determination. *galE* but not *galU* and *galETK* deletion mutants can switch LPS phenotype from rough to smooth when provided with exogenous galactose. Hence the addition of galactose to blood agar base plates induces a change of phage sensitivity pattern from $C21^s$ FO^r to $C21^r$ FO^s.

Galactose sensitivity tests. When streaked out on MacConkey-galactose agar plates, colonies of *galE* mutants after 24 h of incubation have a central depression consisting of lysed cells. On continued incubation these colonies acquire a stippled appearance due to the development of galactose-resistant subclones within the colonies. The colonies of strains that are already partially galactose resistant or somewhat galactose sensitive do not develop a central depression after 24 h but still acquire a stippled appearance on continued incubation due to the development of subclones that are even more resistant to galactose. The colonies of strains that are totally galactose resistant develop neither a central depression nor a stippled appearance.

A second method used in testing galactose sensitivity (Fig. 2) involves plating out the *galE* mutants and their wild-type parents on two types of minimal agar plates: one type containing redistilled glycerol (5 ml/liter) as the carbon source and the other type containing both redistilled glycerol (5 ml/liter) and galactose (5 g/liter). Glucose is unsuitable for this test because it induces catabolite repression of the galactose operon (7, 24). Six well-isolated colonies were marked on each plate, and their sizes were measured at intervals over a period of 4 days at 37°C by using a magnifying comparator (Laboratory Supplies Co. Inc., Hicksville, N.Y.).

Test for ability to switch LPS phenotype. The sensitivities of *galE* mutants to both rough and smooth LPS-specific phages (27) in the presence and absence of galactose in blood agar base plates were determined. Some batches of blood agar base appear to already contain enough galactose to enable some of the *galE* mutants to switch to the smooth phenotype. Some glucose was therefore added to such media to catabolically repress the galactose operon or uptake of galactose by permease or both. For this purpose blood agar base plates containing glucose (0.7 g/liter) were prepared by spreading 0.1 ml of a 20% stock solution of glucose on the

TABLE 1. Tests of the virulence of *galE S. choleraesuis* mutants for BALB/c mice

Strain	Genotype	Galactose sensitivity	i.p. LD_{50} (CFU)
SL2824	38_1 (wild type)	NA[a]	100
SL2805	38_1 *galE710*[b]	Mixture Gal^s and Gal^r	100
SL2829	38_1 *galE710*[c]	Gal^s	4,000,000
SL2847	38_1 *galE710*[d]	Gal^r	100
SL2840	110 (wild type)	NA	200
SL2853	100 *galE716*	Gal^r	500
SL2839	117 (wild type)	NA	400
SL2852	117 *galE717*	Gal^r	1,000,000

[a] NA, Not applicable.
[b] This strain is heterogenous; one component has probably acquired a second mutation causing galactose resistance.
[c] Galactose-sensitive component of SL2805.
[d] Galactose-resistant component of SL2805.

surface of 30 ml of solidified blood agar base. Blood agar base plates containing galactose (0.7 g/liter) were similarly prepared by using a 20% galactose stock solution.

Virulence tests. Mice were inoculated intraperitoneally (i.p.) with bacterial strains and observed for deaths over a period of 21 days. The 50% lethal doses (LD_{50}) were estimated from mortality data (21). Usually bacterial strains to be tested were grown unshaken overnight at 37°C and then stored overnight at 4°C, and counts were determined by plating. Dilutions in 0.85% NaCl were made as desired, and 0.1-ml samples of appropriate dilutions were injected i.p.

Correction of *galE* defect by reversion and by F′ *gal* factor. Revertants were isolated by plating 0.5-ml samples of overnight broth cultures of each *galE* mutant (ca. 10^8 CFU) in triplicate on MacConkey-galactose agar plates. The plates were incubated for up to 1 week at 37°C and checked periodically for galactose-fermenting colonies or papillae.

We have derived for another use a strain of *S. choleraesuis* 38_1 (SL2812) which is aromatic dependent (requires six aromatic compounds) and also carries an F′ factor, F′ 8-*gal*. F′ 8-*gal* carries the entire wild-type galactose operon of *Escherichia coli* K-12. We used the plate-mating technique to transfer F′ 8-*gal* by conjugation to *galE* mutants.

Curing of F′ 8-*gal*. It became necessary to eliminate the F′ 8-*gal* factor from strain 117 *galE* (F′ 8-*gal*), into which it was introduced to correct a chromosomal *galE* defect. In theory any *galE* derivative of this strain must have either lost F′ 8-gal, which masks the chromosomal *galE* defect, or acquired a mutation in the *galE* gene of the plasmid and thus be doubly mutant in two *galE* genes, one on the plasmid and the other on the chromosome. To isolate such a strain without exposure to galactose, pools of mutants selected as resistant to Felix O phage were diluted and spread on nutrient agar so as to obtain about 500 colonies per plate. Velvet replication (15) to MacConkey-galactose plates allowed identification of clones which had become resistant to Felix O phage by loss of the F′ *gal* factor rather than by mutation at *rfa*. Six Gal$^-$ isolates thus obtained lacked the F′ 8-*gal* band when examined for plasmid content (2).

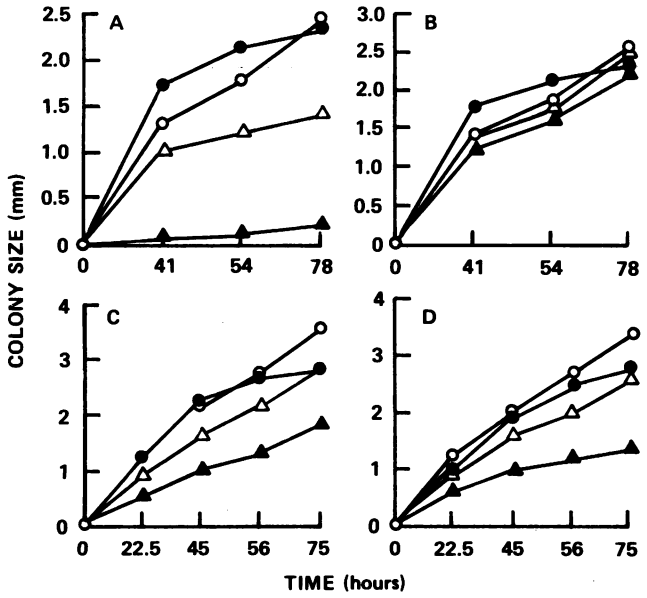

FIG. 2. Growth of *S. choleraesuis* strains and their *galE* derivatives determined as average colony size in minimal agar with and without galactose. A, Strain 38_1 and its *galE* Gal^s derivative; B, strain 38_1 and its *galE* Gal^r derivative; C, strain 117 and its *galE* derivative; D, strain 110 and its *galE* derivative. Symbols: △, *galE* mutant on glycerol agar; ▲, *galE* mutant on glycerol-galactose agar; ○, *gal*$^+$ parent on glycerol agar; ●, *gal*$^+$ parent on glycerol-galactose agar.

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

Strain	Test medium	Sensitivity to phages					
		FO	14	C21	BR60	6SR	Ffm
38_1	BB	S	S	R	R	R	R
	BB+gal	S	S	R	R	R	R
38_1 *galE* Gal^s	BB	SR	SR	SR	SR	SR	SR
	BB+gal	S	S	R	R	R	R
	BB+glu	R	R	S	S	S	S
38_1 *galE* Gal^r	BB	SR	SR	SR	SR	SR	SR
	BB+gal	S	S	R	R	R	R
	BB+glu	R	R	S	S	S	S
110	BB	S	NA	R	R	R	R
	BB+gal	S	NA	R	R	R	R
110 *galE*	BB	R	NA	S	S	S	S
	BB+gal	S	NA	R	R	R	R
117	BB	S	NA	R	R	R	R
	BB+gal	S	NA	R	R	R	R
117 *galE*	BB	R	NA	S	S	S	S
	BB+gal	S*	NA	R*	R*	R*	R*

[a] Abbreviations: BB, blood agar base; BB+gal, blood agar base plus galactose; BB+glu, blood agar base plus glucose; S, sensitive; R, resistant; SR, strain is sensitive or resistant in different batches of medium; NA, means that test is not applicable since strain is already resistant to phage 14 because it is lysogenic for phage 6_1; R*, partial resistance; S*, partial sensitivity.

RESULTS

Isolation and characterization of *galE* mutants. Mutants resistant to 2-deoxygalactose were selected from the three wild-type mouse-virulent strains. Testing such mutants for sensitivity to phage C21 (which attacks *Salmonella* mutants making incomplete LPS core of type Rc [galactose deficient] or type Rd1 [glucose deficient]; Fig. 1) allowed recognition of one putative *galE* mutant in each of the three strains (Table 2). As described below the original culture of the C21-sensitive mutant of strain 38_1 was found to comprise two components that differed in galactose sensitivity. Each of the C21-sensitive mutants, including each component of that derived from strain 38_1, was inferred to be deficient of *galE* function, but to retain at least partial *galT* and *galK* function, because of the following properties: (i) failure to ferment galactose, as tested by lack of acid reaction after incubation on MacConkey base agar with galactose (5 g/liter) or during overnight incubation in phenol-red broth with galactose (10 g/liter); (ii) sensitivity to the rough LPS-specific phages Br60, 6SR, and Ffm, and resistance or partial resistance to Felix O phage, which attacks only strains making complete core LPS when tested on unsupplemented blood agar base; (iii) restoration (*galE* mutants of strains 38_1 and 110) or partial restoration (*galE* mutant of strain 117) of the wild-type phage sensitivity pattern (sensitivity to Felix O phage, resistance to the three rough LPS-specific phages) when tested on blood agar base medium with galactose (0.7 g/liter); (iv) sensitivity or partial sensitivity to phage C21 when tested

TABLE 3. Virulence of Gal$^+$ (F′ 8-*gal*) transconjugants of strains 38_1 *galE710* Gal^s and 117 *galE717*

Strain	i.p. LD_{50} in BALB/c mice (CFU)
117 *galE717* Gal$^+$ (F′ 8-*gal*)	400
117 *galE717*	1,000,000
117	400
38_1 *galE710* Gal^s Gal$^+$ (F′ 8-*gal*)	100
38_1 *galE710* Gal^s	4,000,000
38_1	100

on blood agar base, but resistance (or in one case much reduced sensitivity) when tested on galactose-supplemented blood agar base medium. Of the three parent strains only strain 38_1 was sensitive to phage 14, an O antigen-converting phage which attacks only strains with O antigen 6,7 (16); the *galE* mutant of strain 38_1 was resistant to phage 14 when tested on blood agar base but sensitive on the same medium supplemented with galactose. (The failure of phage 14 to attack the other two parent strains, 110 and 117, reflects their lysogeny for the O antigen-converting phage, 6_1, which confers immunity to phage 14.) The only defect in carbohydrate metabolism known to cause inability to ferment galactose and rough character with sensitivity to phage C21, but restoration of smooth character by provision of galactose, is a lack of UDP-galactose-epimerase, the consequence of loss of function of gene *galE* (17). The inference that the LPS defect of all three mutants resulted only from loss of *galE* function was later confirmed by the restoration of the smooth character by introduction of an F′ *gal*$^+$ factor.

In a preliminary test, strain SL2805, identified as a *galE* mutant of strain 38_1, proved to be as virulent as its parent strain (LD_{50} by the i.p. route in BALB/c mice, ca. 100 CFU; Table 1). Streaking of strain SL2805 on MacConkey galactose agar showed that it (and so the inoculum for the virulence test) comprised two components in about equal proportions: (i) galactose negative and galatose sensitive and (ii) galactose negative but galactose resistant. We infer that a mutation causing loss of sensitivity to galactose, probably by loss or reduction of function of gene *galK* or gene *galT* or both, had occurred in a clone derived from an initially galactose-sensitive *galE* mutant (even though the strain had never been exposed to galactose, to minimize the probability of selection). A galactose-sensitive subclone, SL2829, was isolated from strain SL2805 by testing individual colonies from a blood agar base streak plate for galactose sensitivity. A galactose-resistant subclone, SL2847, was isolated from the liver of a mouse dying from infection with SL2805. For clarity, we hereafter refer to the galactose-sensitive subclone, SL2829, as strain 38_1 *galE* Gal^s, and to the galactose-resistant subclone, SL2847, as strain 38_1 *galE* Gal^r. Similarly strain SL2853, the *galE* mutant of strain 110, is referred to below as strain 110 *galE*, and SL2852, the *galE* mutant of strain 117, is referred to as strain 117 *galE*.

Virulence of *galE* mutants. The i.p. LD_{50} of the various *galE* mutants together with those of their respective parents are shown in Table 1. The finding that SL2805 (38_1 *galE*), although it consists of a mixture Gal^s and Gal^r clones, was about as virulent as its parent suggested that, contrary to expectation, some *galE* defects may not have any effect on the virulence of *S. choleraesuis* strains. All mice dying from SL2805 (38_1 *galE*) were determined at autopsy to have died from salmonellosis both from the gross lesions and from the presence of high numbers of non-lactose-fermenting bacteria in their livers. Since only Gal^r colonies were recovered at autopsy, we suspected that it was this subpopulation of strain 38_1 *galE* that was virulent. Subsequent tests confirmed that strain 38_1 *galE* Gal^s was of much reduced virulence, with an LD_{50} of $>4 \times 10^6$ CFU for BALB/c mice; whereas strain 38_1 *galE* Gal^r was as virulent as the strain 38_1 wild type. The two strains presumably have the same genetic lesion in the *galE* gene and differ only with respect to the subsequent genetic event which occurred either in the galactose operon promoter or the *galK* or the *galT* gene or both to make the strain galactose resistant. The galactose sensitivity of strain 38_1 *galE* Gal^s was shown both by colony morphology on MacConkey-galactose agar plates (its colonies on this medium had central depressions after overnight incubation at 37°C) and by the slower rate of the enlargement of its colonies on minimal agar with galactose as compared with minimal agar without galactose (Fig. 2). By contrast, strain 38_1 *galE* Gal^r was completely resistant, since its colonies on MacConkey-galactose agar did not show central depressions or papillae after several days of incubation at 37°C and also enlarged at the same rate in minimal media with or without galactose (Fig. 2). Galactose sensitivity therefore appears to be responsible for the about 400,000-fold reduction in virulence of strain 38_1 *galE* Gal^s.

To determine whether such high virulence is a general property of *galE* mutants of *S. choleraesuis* that are not galactose sensitive, strains 110 *galE* and 117 *galE* were also tested for mouse virulence. Like strain 38_1 *galE* Gal^r, strain 110 *galE* proved to be about as mouse virulent (LD_{50}, 500 CFU) as its *gal*$^+$ parent, strain 110 (LD_{50}, 200 CFU). This strain was somewhat sensitive to galactose as determined by tests of reduction of the rate of colony size enlargement by galactose (Fig. 2D) and by the appearance of papillae on its colonies after several days of incubation on MacConkey-galactose agar. Such papillae, which have the same color as the parent colony, indicate that the strain could mutate to even greater galactose resistance. It was especially important with this strain to determine that the mice did not die from revertants, since with continued incubation on MacConkey-galactose agar the strain sometimes gives rise to red papillae (galactose-fermenting revertants). Plating at autopsies was therefore done so that at least 50 colonies appeared on each of duplicate MacConkey-galactose plates. Two mice dying from 500 CFU and four dying from 5,000 CFU were autopsied. None of the 150 to 800 clones tested from each mouse was galactose fermenting. This rules out reversion to the wild type as being responsible for the virulence of this strain.

In contrast to strains 38_1 *galE* Gal^r and 110 *galE*, strain 117 *galE* turned out to be of greatly reduced virulence with an i.p. LD_{50} for BALB/c mice of 10^6 CFU. It also differs from all of the *galE* mutants examined in this study in being only partially restored to the smooth character by the addition of galactose to the medium (even when the galactose concentration was increased to 2.8 g/liter). However, it was somewhat galactose sensitive as determined by the criteria of reduction by galactose of colony size enlargement on minimal media (Fig. 2C) and the development of papillae on its colonies on MacConkey-galactose agar after several days of incubation at 37°C.

Correction of the *galE* defect by F′ *gal* transfer and by reversion. Galactose-fermenting colonies were readily isolated from strain 110 *galE* by plating on MacConkey-galactose agar plates. Continued incubation for up to 1 week was necessary to isolate revertants from strains 38_1 *galE*

Gal^s and 38_1 *galE* Gal^r. No revertants were obtained from strain 117 *galE*. However, galactose-fermenting transconjugants were readily isolated from all *galE* strains, including strain 117 *galE*, by plate mating with SL2812, the aromatic compound-dependent derivative of *S. choleraesuis* 38_1 which carries an F′ *gal* factor (F′ 8-*gal*).

Two Gal$^+$ revertants from each *galE* strain (except strain 117 *galE*, from which no revertants were isolated) and two transconjugants derived from each *galE* strain including strain 117 *galE* were tested and found sensitive to phage FO but resistant to phages C21, Br60, Ffm, and 6SR. This shows that these galactose-fermenting derivatives synthesized smooth LPS and proves that their *galE* parents have no further genetic lesions affecting LPS character. Since 117 *galE* does not make smooth LPS when supplied with galactose, a second experiment was done to confirm the absence of such secondary genetic lesions. Its *gal*$^+$ derivative, strain 117 *galE* Gal$^+$ (F′ 8-*gal*), was cured of F′ 8-*gal* by selection for resistance to phage Felix O. The phage sensitivity patterns of six independently selected cured strains were tested and found to be the same as that of their 117 *galE* ancestor. Complete phenotypic cure by F′ 8-*gal* of 117 *galE*, a strain which could neither revert to Gal$^+$ nor completely switch its LPS in the presence of galactose, could mean that this strain has a polar mutation (such as a frameshift or a deletion mutation) in the *galE* gene. Thus the properties of all of the *galE* mutants are due only to events occurring in the galactose operon.

Virulence of *galE* strains corrected by reversion and by F′ 8-*gal*. (F′ 8-*gal*) transconjugants of the two nonvirulent *galE* mutants, 38_1 *galE* Gal^s and 117 *galE*, were as virulent in BALB/c mice as their Gal$^+$ parental ancestors (Table 3). Restoration of full virulence by the introduction of the F′ 8-*gal* factor is evidence that these *galE* strains have no mutations affecting virulence except in the galactose operon.

DISCUSSION

The surface lipopolysaccharide is a major virulence determinant for all *Salmonella* species so far tested. Various kinds of rough mutants are known to be less virulent than their smooth parents. The proposed explanations for such loss of virulence are that rough mutants are sensitive to serum bactericidal activity (19, 22, 23) and are also more readily phagocytosed and killed intracellularly by macrophages (3, 5, 18). Although nonvirulent, most rough mutants are not useful as vaccines because the distal portion of the LPS molecule (the O repeat units) is either absent or much reduced. *galE* mutants are special among rough mutants and in theory should combine the properties of reduced virulence with high immunogenicity. This is because they are rough only when cultivated in the absence of galactose. When exogenous galactose is available, as is probably the case in vivo, they synthesize smooth LPS and so stimulate the O-specific immune response.

galE mutants are galactose sensitive, and this factor is believed to contribute to loss of virulence. Germanier (8) and Krishnapillai et al. (14) have shown that *galE* mutants of *S. typhimurium* are of much reduced virulence for mice. Germanier and Furer (10) have also isolated a *galE* mutant of *S. typhi* which they designated strain Ty21a; they showed in a mouse i.p. hog gastric mucin model that it was nonvirulent. Various field trials have confirmed strain Ty21a to be nonvirulent for humans (11, 25, 26).

Galactose sensitivity is believed to be the main reason why *galE* mutants remain nonvirulent despite being able to regain the smooth phenotype in vivo. The importance of galactose sensitivity as a factor in the loss of virulence of *galE* mutants of *S. typhimurium* was demonstrated by Germanier and Furer (9). They found that when *galE* mutants of *S. typhimurium* became galactose-resistant by a secondary mutation that drastically reduced the level of galactokinase, they become somewhat more virulent for mice (about 500-fold), because galactose sensitivity was alleviated whereas smooth LPS could still be made. They also found that a *galE* mutant of *S. typhimurium*, strain LT2M1A, which was totally galactose resistant as a result of complete loss of galactokinase activity, was also nonvirulent; they inferred this was because it could not make O side chains even when supplied with galactose.

Some of the results we have obtained with *galE* mutants of *S. choleraesuis* resembled those obtained with *galE* mutants of *S. typhimurium*. Strain 38_1 *galE* Gal^s was of much reduced virulence, as expected of a typical *galE* strain. The difference between our result and those obtained with *S. typhimurium* was the very high virulence of some of the *galE* but Gal^r mutants tested. Strain 38_1 *galE* Gal^r, which presumably is a Gal^r derivative of strain 38_1 *galE* Gal^s, had the same LD_{50} in BALB/c mice as its fully virulent ancestor, strain 38_1. The galactose resistance of this strain was complete, since its average colony size on agar was not affected by galactose (Fig. 2). Strain 110 *galE* was somewhat galactose sensitive, since galactose reduced the average size of its colonies on agar. Yet this strain also was about as virulent for BALB/c mice as its parent, strain 110. On the other hand strain 117 *galE*, another somewhat galactose-sensitive *galE* mutant, was of much reduced virulence (i.p. LD_{50} for BALB/c mice, 10^6 CFU). The introduction of an F′ *gal* factor to correct the *galE* defect present in this strain fully restored its ability to ferment galactose and to synthesize complete LPS. The F′ *gal* transconjugants were also fully virulent. Strain 117 *galE*, since it is unable to make smooth LPS when supplied with galactose, may be nonvirulent because it cannot regain the smooth phenotype in host tissues. A mutation causing nearly complete loss of either or both of galactokinase and galactose-1-phosphate uridyltransferase activities as well as of *galE* function would explain the properties of this mutant. Partial sensitivity of the strain to galactose would suggest some degree of galactokinase function. Some ability to switch would indicate that the strain also has galactose-1-phosphate uridyltransferase activity.

The results of Germanier and Furer (8) show that galactose-resistant *galE* mutants of *S. typhimurium* have LD_{50}s in mice that are at least 500-fold greater than those of their *gal*$^+$ parents. The reason that two of the galactose-resistant *galE* strains we derived from *S. choleraesuis* strains remain virulent is not known. A plausible explanation could be adduced from consideration of the structure of the LPS molecule of this species (Fig. 1). *S. choleraesuis* strains have two galactose residues in the core region of each LPS molecule but none in the O repeat units. This means that even under low galactose concentrations or low levels of galactokinase activity (or both) their *galE* mutants can, more easily than those of *S. typhimurium* strains, switch to a fully smooth character. In fact *S. choleraesuis* 38_1 *galE* Gal^r was fully smooth, as determined by phage pattern on some batches of Oxoid blood agar base (CM55) unless glucose was added to repress the galactose operon and galactose uptake. It therefore appears that strain 38_1 *galE* Gal^r and strain 110 *galE* are galactose resistant enough not to lyse in vivo, but still retain enough galactokinase and galactose-1-phosphate

uridyl-transferase activities to regain the wild-type phenotype at the galactose concentration in the host environment. *S. typhimurium* strains have galactose residues not only in their LPS core but also in their O repeat units (Fig. 1). Since *Salmonella* species may have up to 40 O repeat units in their LPS (12, 20), it could be deduced that *galE* mutants of *S. typhimurium* would in general need much more (up to 21 times as much) galactose as their *S. choleraesuis* counterparts to switch their LPS characters from rough to smooth. We suspect that enough galactose may not be available in vivo for *galE* but Gal^r mutants of *S. typhimurium* to do this. Hence they do not regain full parental virulence.

The much reduced virulence of strain 117 *galE* is probably the result of its inability to synthesize smooth LPS in vivo. This view is supported by in vitro evidence which shows that the addition of galactose (2.8 g/liter) to the medium does not restore the smooth LPS phenotype to this strain. Since strain 117 *galE* retains partial galactose sensitivity, we suspect that it has suffered a nearly complete loss of one or both of the enzymes galactokinase and galactose-1-phosphate uridyltransferase.

It has been suggested that *S. choleraesuis* may possess pathogenic mechanisms different from those of other *Salmonella* species. It is therefore quite possible that some *galE* mutants of *S. choleraesuis* are highly virulent because of such differences in pathogenic mechanisms. Recent results (personal communication from Carlos Hormaeche) showing that a rough mutant of *S. choleraesuis* suspected to be of type *rfb* is highly virulent for mice would indicate that smooth LPS is not very important in the virulence of this organism. This would support the idea of the organism having different pathogenic mechanisms from those of other *Salmonella* species. The fact that strains 38_1 *galE* Gal^s and 38_1 *galE* Gal^r, which are nonvirulent and virulent, respectively, are derived from the same parent and presumably carry the same mutation in the *galE* gene makes such a possibility an unlikely explanation for our results. A low requirement for galactose to achieve a smooth character in vivo seems to be the more plausible explanation at present for the differences between the *galE* mutants of this organism and those of *S. typhimurium*.

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