Immunity in Experimental Salmonellosis

II. Basis for the Avirulence and Protective Capacity of *gal* E Mutants of *Salmonella typhimurium*

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Salmonella typhimurium strains which are deficient in uridine diphosphate (UDP)-galactose-4-epimerase (*gal* E mutants) owe their outstanding protective capacity when used as live vaccine to the fact that when galactose is supplied exogenously, such as occurs in vivo, smooth cell wall lipopolysaccharides are synthesized. The mutants lose most of their protective capacity when this phenotypic curing is prevented by a second mutation of the kind found in strains LT_2M_1A (deficient in galactokinase) or E_{32} (deficient in UDP-galactose-lipopolysaccharide transferase). Despite such phenotypic reversion, the *gal* E mutants are rendered avirulent as a result of galactose-induced bacteriolysis. Secondary mutants have been isolated which differ from each other with respect to the extent of galactose-induced lysis. The differences in galactose sensitivity are attributable to different activities of the other Leoloir pathway enzymes, namely, galactokinase and galactose-1-phosphate-uridyl transferase. The influence of these enzymes on lipopolysaccharide composition and galactose sensitivity and thus on virulence and immunogenicity of *gal* E mutants has been studied.

It is generally accepted that living, attenuated strains of *Salmonella* produce a more effective immunity in mice than do killed suspensions of virulent challenge strains (4, 12, 13, 15, 16). Since rough mutants can also be used successfully as the basis for a live vaccine (37), it has been suggested (27) that O-antibodies do not play an essential role in immunity to mouse typhoid. Previous studies (11) have shown that not all rough strains confer the same protective capacity and that only vaccination with rough mutants of the *gal* E type provide mice with a protection equal to that produced by a sublethal infection with virulent smooth strains.

Gal E mutants of *Salmonella* were first isolated by Fukasawa and Nikaido (6, 7) as galactose-sensitive strains. These mutants fail to ferment galactose and undergo lysis when grown in the presence of this sugar. They are characterized by a block in the enzyme uridine diphosphate (UDP)-galactose-4-epimerase. Without an external supply of galactose, these mutants cannot synthesize UDP-galactose, and, because galactose is incorporated in the lipopolysaccharide via UDP-galactose, only incomplete cell wall lipopolysaccharide is formed (29, 33, 34).

In *Salmonella*, as in other gram-negative bacteria, the receptor sites for some phages are provided by the LPS layer of the cell wall. With the smooth-specific phage P22, it is inferred that the LPS component responsible for O-factor 12 specificity functions, at least in part, as the adsorption site. Because *gal* E mutants of *Salmonella* produce an incomplete LPS structure lacking the O-specific oligosaccharide repeat unit, they are resistant to phage P22 and become sensitive to rough-specific phages such as C_{21} (9, 10, 18, 22, 23). By exploiting these properties, it is possible to isolate *gal* E mutants from smooth strains of *Salmonella* (33).

When *gal* E mutants are grown in the presence of galactose, UDP-galactose can again be synthesized, and, under these conditions, the composition of the lipopolysaccharide is similar to that of the wild type (8). However, prolonged contact with galactose brings about a dramatic lysis of the cells (9). Repeated subculturing of the mutants on galactose media permits the selection of galactose-resistant mutants, and these secondary mutants are generally characterized by a low galactokinase activity (28).

The present investigations were designed to elucidate the mechanism responsible for the unique immunizing capacity of these galactose epimerase-negative mutants.

MATERIALS AND METHODS

Bacteria. *S. typhimurium* smooth strains LT2 and 395 MS and their epimerase-negative mutants G 30, LT2 M1, and 395 MR 9 have been described in a preceding paper (11). A series of secondary mutants isolated from G 30 and LT2 M1 is described below. SL 3684 is LT2 M1 hut+; SL 1032 and SL 1033 lack glucosyl and galactosyl-lipopolysaccharide transferase, respectively (39); and SL 1034 and SL 1036 are leaky, semirough mutants (10). The strains were cultivated at 37 C in a rotary shaker (142 rev/min) in Brain Heart Infusion or in the medium described by Lennox (20). Where growth in the presence of galactose was required, Brain Heart Infusion broth supplemented with 0.2% galactose or Endo Agar in which lactose had been replaced by galactose was used. For the production of cell material for lipopolysaccharide extracts, overnight cultures were used; for all other experiments, 6-hr-old cultures were employed.

Phages. Phages P22, P22C2, and FO1, active against smooth strains of *S. typhimurium*, and phages 6SR, Ffm, and C21, active against rough strains, were all received from B. A. D. Stocker (10). They were propagated in suitable bacterial strains by the soft-agar layer method of Adams (1). The susceptibility of *Salmonella* strains to phages was determined by applying a drop of the lysate to bacterial lawns prepared by flooding agar plated with a 6-hr-old broth culture, draining off the excess, and allowing it to dry at room temperature. Lysis was recorded after incubation at 37 C for 16 hr.

Isolation of rough mutants. For the selection of rough mutants, bacterial suspensions were infected with the smooth-specific phage P22, and the surviving bacteria were plated on Brain Heart Infusion agar. For the selection of *gal* E mutants, the colonies were replicated to galactose-Endo Agar. Spontaneously occurring rough mutants as well as mutants induced by *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (NG) were isolated in this way (2).

Virulence test. Female spf mice, F 2 Charles River x BALB C, obtained from Tierfarm AG, Sisseln, were used. Groups of 10 mice (18 to 20 g) were injected intraperitoneally (ip) with 0.2-ml amounts of 10-fold dilutions of 6-hr-old cultures. The animals were observed for 10 days, and the median lethal dose (LD_{50}) was calculated by the method of Reed and Muench (35).

Protection test. Mice were immunized by ip injection of 0.2 ml of a live bacterial suspension. The amount given was approximately one-fiftieth of one LD_{50}. Six weeks after immunization, the mice were challenged by ip injection of 1,000 LD_{50} of the virulent strain 395 MS.

Enumeration of bacteria in liver and spleen. The livers and spleens of five mice from each group were removed separately and homogenized in 10 ml of Brain Heart Infusion by using a Potter-Elvehjem homogenizer. Serial 10-fold dilutions of the homogenate were prepared, and 0.1-ml volumes were plated in duplicate on galactose-Endo Agar. All homogenates were counted separately and then averaged. A minimum of 10 bacteria per mouse could be detected by this procedure.

Isolation of lipopolysaccharide from cell walls. Cell walls were prepared in a Sorvall Omni-Mixer (31), and the lipopolysaccharide was extracted by the phenol-water procedure of Westphal (31, 38), the phenol being removed by repeated extraction with ether. Lipopolysaccharide of the smooth type was purified by fractionated alcohol precipitation and rough-type lipopolysaccharide was purified by $MgCl_2$ precipitation (33).

Sugar analysis of lipopolysaccharide. Five milligrams of lyophilized lipopolysaccharide was dissolved in 1 ml of 1 N sulfuric acid and hydrolyzed in sealed tubes for 5 hr in a boiling-water bath. The hydrolysate was neutralized with barium carbonate. Galactose and glucose were determined by the Worthington "Galactostat" and "Glucostat" special test combination. Thin-layer chromatography was carried out on cellulose layers with pyridine:water:ethyl acetate (1:2:2) as solvent. 1-Glycero-D-manno-heptose was measured by the method of Osborn (30).

Preparation of extracts for the enzyme assays. Two grams (wet weight) of cells suspended in 4 to 5 ml of water with glass beads (10 g, 0.17 to 0.18 mm diameter) was disrupted by treatment for 3 min (three runs of 1 min) at full speed in an ice-cooled Sorvall Omni-Mixer. The mixture was then centrifuged at 40,000 × *g* for 10 min, and the supernatant fluid was used for enzyme assay.

Enzyme assays. UDP-galactose-4-epimerase was determined by the procedure of Nikaido (28).

The procedure for assay of galactose-1-phosphate-uridyl-transferase (25, 28) was slightly modified. A medium with the following composition was used for incubation: cysteine, 30 μliters (33 mg/ml, pH 8.5); $MgCl_2$, 10 μliters (0.1 M); glycine, 60 μliters (1 M, pH 8.7); phosphoglucomutase, 8 μliters (2 mg/ml, Boehringer); glucose-1,6-diphosphate, 8 μliters (1 mg/ml, Boehringer); uridin disphosphoglucose, 20 μliters (10 μmoles/ml); water, 400 μliters; enzyme extract, 5 μliters; and galactose-1-phosphate, 30 μliters (10 μmoles/ml). The reaction was started by the addition of galactose-1-phosphate and then carried on for 15 min at 25 C. It was then stopped by transfer to a boiling-water bath for 1 min. After centrifugation, the supernatant fluid was transferred to a 2-mm quartz cuvette. Ten microliters of nicotinamide adenine dinucleotide phosphate (NADP, 20 mg/ml) and 10 μliters of glucose-6-phosphate-dehydrogenase (1 mg/ml, crystallized, Boehringer) were added, and the increase in extinction at 340 nm was measured. A standard calibration curve for glucose-6-phosphate was prepared for each lot. Based on the definition of the galactokinase unit, the uridyl transferase unit is defined as the amount of enzyme which transforms 1 μmole of galactose-1-phosphate in 1 hr.

Galactokinase was determined by the method of Wilson and Hogness (40).

For the determination of galactose permease, cells in log phase were suspended in Lennox medium containing chloromycetin (50 μg/ml). The transmission at 550 nm was adjusted to 10%, and ^{14}C-galactose was added at a final concentration of 3 × 10^{-5} to 5 × 10^{-5} M (15,000 counts per min per ml). The mixture was then incubated for 15 min at 37 C. After
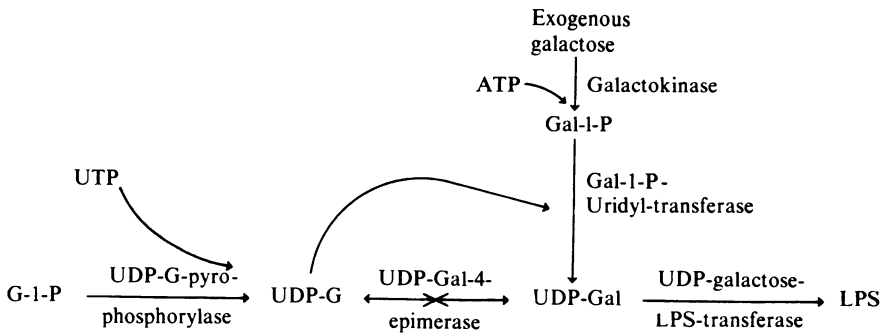
FIG. 1. Schematic representation of the incorporation of exogenous galactose into the lipopolysaccharides of gal E mutants. G-1-P, glucose-1-phosphate; UDP-G, uridine diphosphate-glucose; Gal, galactose; Gal-1-P, galactose-1-phosphate; UDP-Gal, uridine disphosphate-galactose; LPS, lipopolysaccharide.

incubation, the suspension was filtered through a 450-nm membrane filter (Millipore Corp.), which was then placed in a counting vial and dried for 20 min at 60 C. A 15-ml amount of Bray solution [4 g of 2,5-diphenoxazole, 0.2 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene, 20 ml of ethyleneglycol, 100 ml of methanol, and 60 g of naphthalene, made up to 1 liter with dioxane] was added and counted in a Packard liquid scintillation spectrometer.

Protein determinations were carried out as described by Layne (19). Specific enzyme activities are given as units per milligram of protein.

Serological methods. Bacterial agglutination was performed with heat-inactivated cells of a virulent smooth strain (10^8/ml) by the classical method of Widal.

RESULTS

Origin of the immunogenic capacity of gal E mutants. It seems improbable that the unique immunogenic capacity of the *gal* E mutants is solely due to the structure of their cell wall lipopolysaccharide. It is unlikely that a block in the incorporation of the two galactose molecules into the core of the lipopolysaccharide should uncover some structure with peculiar antigenic properties which could then be important for the virulent smooth strain. Thus strains SL 1032 (glucosyltransferaseless) and SL 1033 (galactosyltransferaseless), with lipopolysaccharide composition most similar to *gal* E mutants, possess poor protective capacity which is not different from that of other rough mutants. It may, however, be assumed that the high efficacy of protection by the epimerase-negative mutants is related to the manner in which the block in lipopolysaccharide synthesis functions. *Gal* E mutants form lipopolysaccharide of the rough type when grown without galactose; when galactose is supplied externally, smooth-type lipopolysaccharide is synthesized (Fig. 1). *Gal* E bacteria used for vaccination were always grown in galactose-free media. It is conceivable,

however, that these bacteria are provided in vivo with galactose or galactose derivatives that may be used for lipopolysaccharide biosynthesis.

To check this assumption, two double mutants were isolated which, in addition to possessing a defect in the epimerase, are also galactosyltransferase I- and glucosyltransferase I-negative, respectively. These secondary mutants were obtained by treating strains SL 1033 (galactosyltransferase I-negative) and SL 1032 (glucosyltransferase I-negative) (39) with NG, followed by plating on Brain Heart Infusion agar and replication on galactose agar. Non-galactose-fermenting colonies with a central lysis area were preselected and tested for epimerase activity. One mutant with no galactase epimerase activity was selected from each parent strain. The double mutant E 33, derived from SL 1033, when grown in a galactose-free medium, should have lipopolysaccharide identical to that of strain G 30. When grown in the presence of galactose, there should only be one additional galactose (Gal II) molecule attached to the glucose I molecule. Lipopolysaccharide of mutant E 32 is always identical to that of strain SL 1032, regardless of the cultural conditions (Fig. 2). Viable cells from these two double mutants and from mutant G 30 were used to vaccinate groups of 20 mice. Six weeks later they were challenged with the virulent strain 395 MS. The degree of protection produced by these mutants was measured by carrying out bacterial counts in liver and spleen. In addition, the agglutination titers of their sera against the challenge bacteria were measured 10 days after vaccination (Fig. 3).

Gal E mutants lose most of their protective capacity if, through a second mutation, the biosynthesis of smooth-type lipopolysaccharide is blocked, even when galactose is supplied externally. This, together with the high agglutination titer against the smooth strain, supports the
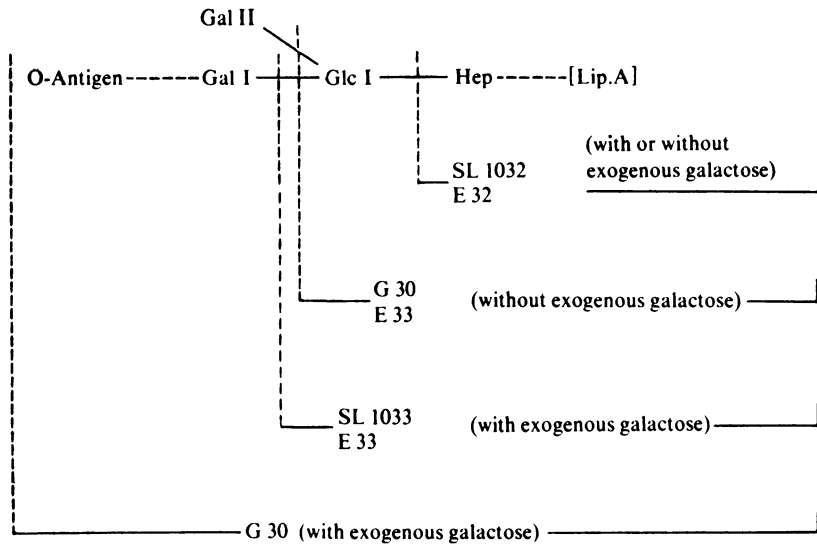
FIG. 2. *Schematic configuration of the lipopolysaccharides of mutant SL 1033 (no galactosyltransferase I), G 30 (no UDP-galactose-4-epimerase), and SL 1032 (no glucosyltransferase I) and of the double mutants E 33 (no galactosyltransferase I and no epimerase) and E 32 (no glucosyltransferase I and no epimerase). Hep, 1-glycero-D-manno-heptose; Glc, glucose; Gal, galactose.*
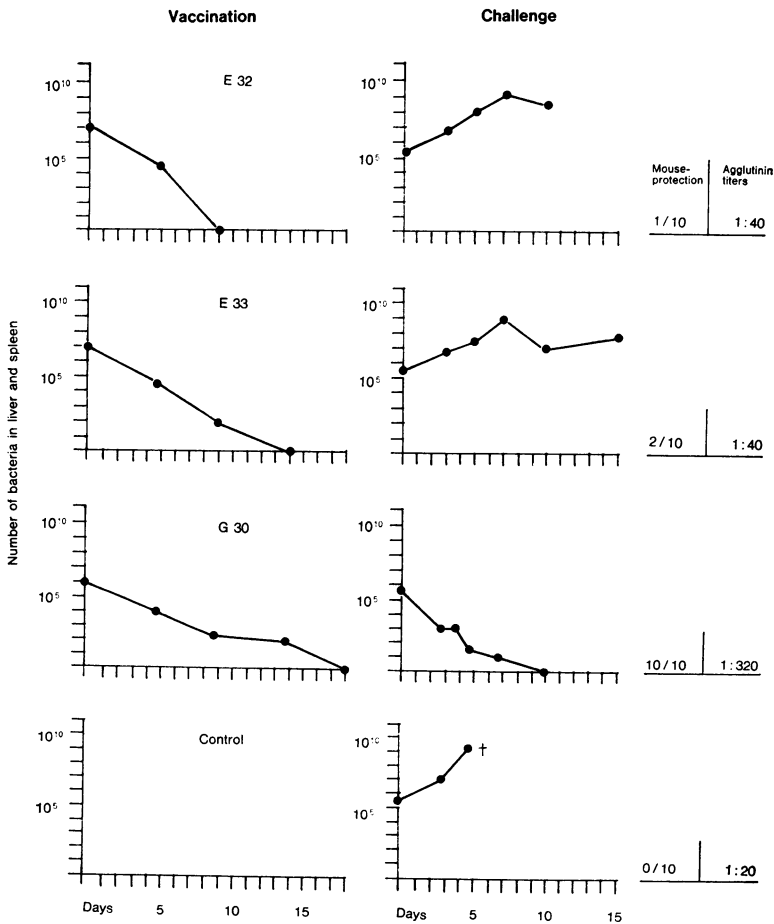

FIG. 3. *Growth curves of the vaccinating strains (left) and the challenge strain (right). Vaccination was done intraperitoneally with mutant G 30 (2 × 10⁶ viable cells/mouse) and with the double mutants E 32 and E 33 (10⁷ viable cells/mouse). Challenge was 6 weeks later with the virulent strain 395 MS (5 × 10⁵ viable cells/mouse). Survival rate: number of survivors/number of challenged mice 10 days after challenge. Agglutination titer: serum in dilutions 1:20 to 1:640; bacterial suspension: 10⁸ 395 MS/ml.*

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TABLE 1. *Relationship between galactose sensitivity and virulence of gal E mutants*

Strains	Degree of sensitivity to galactose[a]	Virulence[b] (LD$_{50}$)	Mouse protection[c] (survivors/ challenged mice)
G 30 E	++++	5×10^7	10/10
G 30 D	+++	5×10^7	10/10
G 30 C	++	2×10^7	10/10
G 30 B	+	5×10^5	10/10
G 30 A	−	1×10^5	10/10
SL 3684	++++	1×10^6	5/10
LT2 M1 C	++	5×10^7	7/10
LT2 M1 B	+	6×10^6	10/10
LT2 M1 A	−	5×10^7	1/10
395 MR9	−	5×10^4	10/10
GE 7	++++	5×10^7	10/10

[a] Galactose sensitivity was estimated from the proportion of the lysis area in colonies grown on galactose-Endo Agar. Symbols: ++++, strong lysis; −, no lysis.

[b] Virulence was calculated from mortality occurring within 10 days after intraperitoneal injection of live bacteria.

[c] Mouse protection: intraperitoneal vaccination with live bacteria (0.05 of the LD$_{50}$) challenge 6 weeks later with 1,000 LD$_{50}$ of the virulent strain 395 MS.

view that in vivo *gal* E bacteria are presented with a medium which enables the biosynthesis of O-specific lipopolysaccharide side chains.

Relationship between galactose resistance and virulence. *Gal* E mutants of *S. typhimurium* can easily be identified on galactose differential medium; they do not ferment galactose and grow as typical flat colonies which have a small outer wall and a concave center. This central area consists mostly of lysed cells. The size of the central area is dependent upon the degree of sensitivity to galactose. Prolonged cultivation on galactose favors the recovery of resistant strains. The latter appear as papillae or, more often, as small secondary colonies in the central lysis area. They grow as ordinary convex colonies and are unable to ferment galactose. Intermediate forms varying between extreme sensitivity and complete resistance also exist. Starting from the *gal* E mutants G 30 and LT 2 M1, many such secondary mutants were isolated. We have placed them in 5 groups ranging from resistant (A) to extremely sensitive (E), depending on the level of galactose sensitivity. Representatives of each group were tested for virulence and immunogenicity. As will be seen later, the parent strains used (G 30 and LT M1) were already secondary mutants. For this reason, a new pure *gal* E mutant was isolated.

This strain, GE 7, was lyophilized immediately to avoid unnecessary subcultivation. Only bacteria freshly grown from lyophilized ampoules were used. There exists a clear relationship between the degree of galactose sensitivity and virulence (Table 1). Thus, virulence of *gal* E mutants increases with increasing resistance to galactose. The exception in the properties of strain LT 2 M1 A are readily explained and will be discussed in a subsequent section; we shall emphasize here only that this strain is also very weakly immunogenic. No valid explanation could, however, be found for the somewhat divergent results obtained with strain SL 3684, which is as sensitive to galactose as the pure *gal* E mutant GE 7 but, nevertheless, more virulent than expected.

Stability of gal E mutants. The stability of the different *gal* E mutants was examined by plating NG-treated and -untreated cells in the exponential growth phase on galactose-Endo Agar. Galactose-fermenting revertants are easily seen as dark-red colonies on a pale bacterial lawn. The frequency of secondary mutations to altered galactose resistance was measured at the same time (Table 2). Only the mutants 395 MR 9 and GE 7 revert to the wild type. We do not believe, however, that this is the reason for the relatively high virulence of mutant 395 MR 9 since such a revertant could never be demonstrated in liver and spleen homogenates from mice infected with cells of this strain. Moreover, the mutant GE 7 which possesses approximately the same frequency of reversion is completely avirulent. This leads to the unexpected conclusion that the virulence of *gal* E mutants is not altered by reversions to wild type. After NG treatment, the mutation rate is increased by a factor of 10. No such reversions were observed for other than GE 7 and 395 MR 9 *gal* E mutants, even after mutagenic treatment. On the other hand, mutations that are responsible for changes in the degree of galactose sensitivity are fairly common. This is particularly true for the extremely sensitive strains. Strains that are partly galactose-resistant are more stable. Since a shift in the level of galactose sensitivity may bring about an important change in virulence, it appeared necessary to investigate its basis. This was done by measuring the enzymes of the Leloir pathway.

Enzyme activities of the Leloir pathway. *Gal* E mutants are lysed by growth on galactose because of the intracellular accumulation of galactose-1-phosphate or UDP-galactose (or both). All of the *gal* E mutants, including the strongly galactose-resistant strains, are completely epimerase-negative at the level of sensitivity of the assay. It was, therefore, obvious that differences in the activities

TABLE 2. Stability of different gal E mutants[a]

Strains	Without mutagenic treatment[b]				After NG treatment			
	Reversion to wild type	Mutation to type A	Mutation to type C	Mutation to type E	Reversion to wild type	Mutation to type A	Mutation to type C	Mutation to type E
G 30 E	0	9.5×10^{-5}	0	—	0	1.8×10^{-2}	8.8×10^{-3}	—
G 30 D	0	0	—	10^{-7}	0	1.2×10^{-5}	—	2×10^{-4}
G 30 C	0	0	—	0	0	8×10^{-4}	—	3×10^{-3}
G 30 B	0	—	—	0	0	—	—	5×10^{-3}
G 30 A	0	—	0	0	0	—	2×10^{-3}	2×10^{-6}
395 MR 9	2×10^{-6}	—	0	0	1.7×10^{-5}	—	1.7×10^{-3}	2×10^{-6}
SL 3684	0	1.2×10^{-6}	0	—	0	1.7×10^{-2}	8×10^{-2}	—
LT2 M1 C	0	0	—	0	0	3×10^{-2}	—	9×10^{-2}
LT2 M1 B	0	0	—	0	0	1.5×10^{-2}	—	3×10^{-2}
LT2 M1 A	0	—	0	0	0	—	4×10^{-3}	0
GE 7	5×10^{-7}	3×10^{-5}	—	10^{-7}	4×10^{-6}	1.5×10^{-2}	—	2×10^{-2}

[a] Cells in the exponential growth phase were plated directly or after treatment with 300 µg of N-methyl-N'-nitro-N-nitrosoguanidine (NG) per ml of Tris buffer (pH 6) for 30 min on galactose Endo Agar. Readings 24 hr later (untreated samples) or 40 hr later (NG-treated samples).

[b] Symbols: 0, $\leqslant 5 \times 10^{-9}$; —, not measured.

of the other galactose enzymes (kinase, galactose-1-P-uridyl transferase, permease, UDP-galactose-lipopolysaccharide transferase) might be responsible for the different level of galactose resistance. The activity of these enzymes was therefore determined before and after induction by galactose. The principal differences in galactose resistance, virulence, and immunogenicity of the gal E mutants are explained by these results (Table 3). Thus the basis for the absolute galactose resistance of mutant LT 2 M1 A is a defect in the galactokinase. The lack of this enzyme prevents the accumulation of galactose-1-phosphate and UDP-galactose. Since, for the same reason, the biosynthesis of the O-specific side chains is blocked, it is obvious why this mutant is so weakly immunogenic and why it is not more virulent, despite complete galactose resistance. Indeed, this double mutant gal E/gal K does not differ in any way from other rough mutants such as SL 1032 or SL 1033 which have similar lipopolysaccharide composition.

The strain G 30 E, which is at the other end of the lysis scale, owes its extremely high galactose sensitivity to the constitutivity of its galactose enzymes. These enzymes exhibit in uninduced cells of this mutant a similarly high activity, as in induced cells of the wild strain. When galactose is supplied exogenously, the high activities of these enzymes lead to a rapid accumulation of galactose-1-phosphate.

The different degrees of galactose resistance of the other mutants (G 30 A, B, C, and D; LT 2 M1 B and C; as well as 395 MR 9) can certainly be attributed to the low activity of the galactokinase

and galactose-1-phosphate-uridyl transferase, which in addition are only weakly inducible. Permease plays a less important role, since it is not the rate-limiting factor in the formation of galactose-1-phosphate and UDP-galactose. Of the three permease-negative mutants, only strain G 30 B shows a markedly reduced galactose uptake. The increased galactose uptake by the pure gal E mutant GE 7 indicates that the high activity of the galactokinase may influence the utilization of galactose (Fig. 4).

It appears from the present investigations that, since galactose is normally provided in vivo, the virulence and immunogenicity of the gal E mutants are strongly influenced by the activities of galactokinase and galactose-1-phosphate-uridyl transferase. Strains with full kinase and uridyl transferase activity synthesize galactose-1-phosphate and UDP-galactose at a normal rate, thus providing the basis for the biosynthesis of the virulence- and immunogenicity-promoting smooth-type lipopolysaccharides. Because of the epimerase defect, these intermediary products cannot be metabolized and are accumulated, thus resulting in bacteriolysis and concomitant reduction in virulence. Such pure gal E mutants are highly immunogenic but nevertheless virtually avirulent. Strains with reduced kinase and uridyl transferase activity are still able to synthesize lipopolysaccharide with O-side chains, but there will be only a small or no accumulation of galactose intermediary products and no lysis will take place. Such strains elicit good immunity but are relatively virulent. In strains with negative kinase, neither O-side chain biosynthesis nor

TABLE 3. *Activities of the enzymes of the Leloir galactose pathway[a]*

Strains	Galactokinase		Uridyl-transferase		Permease: not induced
	Not induced	Induced	Not induced	Induced	
LT 2	10.8	100	16.2	100	100
G 30 E	100	70	80	50	284
G 30 D	3.5	13.2	8.1	15.1	123
G 30 C	3.4	6.3	10.7	16.8	107
G 30 B	5.4	8.6	7.7	16.2	9
G 30 A	2.0	2.2	3.7	5.7	77
SL 3684	6.8	52.5	7.0	32	7
LT2 M1 C	6.1	9.5	5.3	7.8	54
LT2 M1 B	4.1	10.3	8.7	11.9	123
LT2 M1 A	<0.5	<0.5	7.4	10.0	7
395 MR 9	0.6	8.1	3.5	9.9	100
GE 7	19.5	102	17.4	70	123

[a] Induction was done by supplying of 0.1% galactose 2 hr before centrifugation. The activities of kinase and transferase in induced cells and of permease in noninduced cells of strain LT 2 were taken as 100. All other activities are expressed in terms of these values. Galactokinase: $100 = 5.13$ μmoles of substrate per mg of protein per hr. Gal-1-phosphate-uridyl transferase: $100 = 21.5$ μmoles of substrate per mg of protein per hr. Galactose-permease: $100 = 20\%$ of the supplied galactose/15 min.

bacteriolysis occurs. Such strains are no more virulent and no better immunizing strains than any ordinary rough mutant.

The UDP-galactose-lipopolysaccharide transferase is another enzyme that plays an important role in the establishment of the degree of virulence and immunogenicity of *gal* E mutants. The activity of this enzyme, which is responsible for the incorporation of galactose from UDP-galactose into lipopolysaccharides, was not measured in this investigation. Insight into the activity of this enzyme in the different strains was indirectly obtained by means of an analysis of the lipopolysaccharide composition.

Synthesis of O-specific phage receptors. In *Salmonella*, cell wall lipopolysaccharide provides receptor sites for different phages. The relationship between receptor and phage is highly specific. Thus phage P22 is only adsorbed by smooth *S. typhimurium* strains. Rough mutants, such as *gal* E, with incomplete lipopolysaccharide are resistant to this phage. Growth in the presence of galactose enables the biosynthesis of normal smooth-type lipopolysaccharides to take place, thus allowing adsorption of phage P22. Measurements of the number of P22 phages adsorbed by *Salmonella* cells under defined conditions should
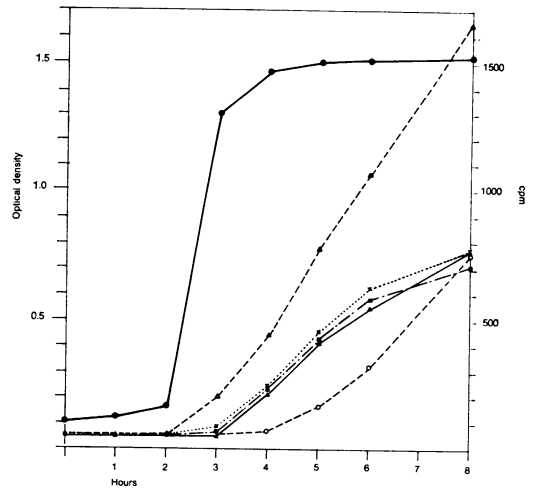

FIG. 4. *Galactose uptake by different gal E mutants during growth. (●) Optical density at 550 nm (the growth curves of all mutants were almost identical; thus, only one representative curve was drawn for the others). Galactose uptake from Brain Heart Infusion with 0.1% galactose and ^{14}C-galactose (15,500 counts per min per ml). Symbols: △, GE 7; ✕, LT2 M1 C; □, SL 3684; ▲, G 30 C; ○, G 30 B.*

allow a quantitative assessment of the O-specific side chains of the cell wall lipopolysaccharide (36). For this purpose, heat-inactivated cells of different *gal* E strains grown with and without galactose were mixed with P22 phages in a proportion of $10^4:1$ and shaken for 15 min in a water bath at 37 C. The remaining free phages were then titrated on the indicator strain LT 2. The leaky rough mutant SL 1026 was included for comparative purposes.

Low levels of possible adsorption were detected even with *gal* E mutants grown in galactose-free medium (Table 4). These results do not allow a decision between specific phage adsorption due to leakiness of the *gal* E lesion or due to non-specific trapping by strains totally devoid of receptor. However, since galactose epimerase activity could never be detected in our *gal* E mutants and since no traces of galactose could be detected in lipopolysaccharide hydrolysates of these mutants either chromatographically or by enzymatic assay with galactose oxidase, we assume that our *gal* E mutants are not leaky and that the low level of phage adsorption represents a high background due to the high proportion of bacterial cells used in our mixture.

When grown on galactose, receptor sites for the smooth-specific phage P22 are synthesized by all *gal* E mutants with the exception of the epimerase- and kinase-negative double mutant LT 2 M1 A. In the presence of an optimal supply of galactose,

TABLE 4. *Adsorption of smooth-specific phage P22 on bacterial cells grown in Brain Heart Infusion with and without galactose*[a]

Strains	Amt of adsorbed P22 phages (%)		
	0.1% Glucose	0.1% Galactose	0.01% Galactose
Control	0	0	0
LT 2	86	88	88
G 30 E	4	79	78
G 30 D	1	83	28
G 30 C	6	71	14
G 30 B	3	39	0
G 30 A	0	58	8
SL 3684	2	33	4
LT2 M1 C	4	62	16
LT2 M1 B	5	90	34
LT2 M1 A	3	0	0
395 MR 9	7	81	38
SL 1036	9	5	4

[a] Heat-inactivated cells were mixed with P22 phages in a ratio 10^4:1, shaken for 15 min in a water bath at 37 C, and plated with the indicator strain LT 2. Plaques formed by the unadsorbed phages were counted 14 hr later.

the concentration of these receptors on cells of strain LT 2 M1 B, G 30 D, 395 MR 9, and G 30 E is approximately the same as for the wild-type strain LT 2. For strain 395 MR 9 with its low kinase and transferase activity, this represents a somewhat surprising result, which nevertheless is in good agreement to the relatively high virulence of the strain. The lowest level of P22 adsorption is shown by strains G 30 B and SL 3684. They also have, together with strain LT 2 M1 A, the lowest permease activity. Nevertheless, we do not believe that this is the reason for the low galactose utilization. Galactose uptake during growth is not very much affected by permease activity (Fig. 4). Thus it can be reasonably assumed that the crucial role in lipopolysaccharide biosynthesis is in this case played by the UDP-galactose-lipopolysaccharide transferase.

A reduction in galactose concentration in the medium from 0.1 to 0.01 % brings about a marked reduction in the level of P22 adsorption. Cells from strains G 30 B, G 30 A, and SL 3684 grown under these conditions can no longer be distinguished from corresponding cells grown without galactose. Only strain G 30 E remains unaffected by the reduction in galactose. Owing to the constitutivity of its galactose enzymes, this mutant can synthesize complete smooth-type lipopolysaccharide even in the presence of a low concentration of galactose.

Composition of the cell wall lipopolysaccharide.

In epimerase-negative mutants, the other enzymes of the galactose pathway exert a regulatory effect on galactose-induced lysis. By controlling together with UDP-galactosyl-lipopolysaccharide transferase the synthesis of the O-specific side chains, they have a decisive influence on the virulence and immunogenicity of these mutants. The influence of their different activities on the cell wall lipopolysaccharide composition was investigated next.

In preliminary experiments, it was confirmed by thin-layer chromatography that the lipopolysaccharide of mutant G 30 grown in galactose-free medium contains only glucose and that, after growth in galactose broth, it contains also galactose, rhamnose, and mannose. It is therefore qualitatively identical to the lipopolysaccharide of the smooth strain.

We were also interested in the molar ratios of the lipopolysaccharide sugars. The structure of the lipopolysaccharide can be characterized conveniently by means of quantitative determinations of glucose, galactose, and L-glycero-D-manno-heptose. By calculating the molar ratios of galactose to glucose and heptose, the average number of tetrasaccharide units per O-specific side chain can easily be estimated. From our results (Table 5) it can be deduced that the O-antigen side chains of the smooth strain LT 2 consist of an average of 7 or 8 repeating units. The lipopolysaccharide of *gal* E mutants grown in galactose-free medium contains no measurable galactose; the cell-wall lipopolysaccharide of this strain is only composed of part of the core and has no tetrasaccharide unit (chemotype C; reference 40). When grown in the presence of galactose, the mutants G 30 E, LT 2 M1 B, and 395 MR 9 can utilize this sugar in such an efficient way that their lipopolysaccharide is no longer distinguishable from that of the smooth parent strains. Lipopolysaccharides of the other strains contain O-side chains, possessing an average of two tetrasaccharide units. From these data, it cannot, of course, be decided whether all side chains are built up of two repeating units or if, in addition to some normal side chains, there are side chains without the O-specific part. The biosynthesis of the O-antigen side chains in these strains is dependent on the concentration of the galactose supplied. A mere increase of its concentration from 0.1 to 0.2% results in the formation of lipopolysaccharides with a higher galactose content. As might be expected, the lipopolysaccharide of mutant LT 2 M1 A, which is also kinase-negative, contains no measurable galactose. Strain SL 3684 is another poor galactose utilizer. Since its kinase and uridyl transferase activities are rather higher than average, it can be assumed that this mutant

TABLE 5. *Quantitative sugar composition of cell-wall lipopolysaccharides of different S. typhimurium mutants and of parent strain LT2*

Strain	Growth on glucose[a]			Growth on 0.1% galactose[a]			Growth on 0.2% galactose[a]		
	Heptose	Galactose	Glucose	Heptose	Galactose	Glucose	Heptose	Galactose	Glucose
LT 2	2	9.8	2.0	2	9.0	2.0	2	9.8	1.8
G 30 E				2	8.2	2.4			
G 30 D	2	0.1	2.2	2	4.4	3.4	2	7.8	1.8
G 30 C	2	0.1	2.6	2	4.0	3.4	2	6.8	1.8
G 30 B				2	4.0	3.0			
G 30 A	2	0.1	2.4	2	3.6	3.0	2	5.6	1.8
SL 3684				2	1.4	3.6			
LT2 M1 C	2	0.1	1.6	2	5.6	3.4			
LT2 M1 B				2	8.2	2.2			
LT2 M1 A	2	0.1	1.8	2	0.1	1.4			
395 MR 9	2	0.1	1.8	2	9.4	2.0	2	10.6	2.6
E 32				2	0.1	0.1			

[a] Molar ratios of glucose and galactose are given relative to heptose taken as 2.

has an extremely weak UDP-galactosyl-lipopolysaccharide transferase.

For control purposes the same sugar analysis was also performed on the double mutant E 32. As expected, the lipopolysaccharide of this strain contains neither galactose nor glucose in measurable amount.

These results are in good agreement with those obtained from the phage-receptor experiments (Table 4). The only discrepancy with mutant G 30 B is probably due to the fact that, for the measurement of the receptors, cells from a 6-hr-old culture were used, whereas the lipopolysaccharide analysis was performed on cells from overnight cultures. With this strain the initially impaired galactose uptake becomes normal after 8 hr of cultivation (Fig. 4).

The results of our sugar analysis contain some anomalies for which no valid explanation could be found. According to the generally accepted structure of cell-wall lipopolysaccharide (25), it is anticipated that in strains with blocked galactose incorporation the incorporation of the glucose molecule II is also blocked. In galactose-free lipopolysaccharides, the molar ratio of heptose to glucose should be 2:1. Nevertheless, an analysis of lipopolysaccharides from *gal* E mutants grown in galactose-free medium consistently shows heptose-glucose ratios of 1:1. Similar results were also described (without comment) by Holme et al. (14) and Lindberg and Holme (21) for their epimeraseless mutant 395 MR 9. In smooth lipopolysaccharide, the ratio of heptose-glucose should be 1:1. This is in agreement with our analytical results on the smooth strain and on *gal* E mutants which, when galactose is supplied exogenously, synthesize smooth-type lipopolysac-

charides (G 30 E, LT 2 M1 B, and 395 MR 9). Lipopolysaccharides with incomplete O-specific side chains, however, show a molar ratio of up to 1.8:1 (SL 3684). This ratio increases correspondingly when less galactose is incorporated. We presume that these two experimental deviations from theory (i.e., the values for glucose are too high or those for heptose are too low, or both) have a common origin. More experimental data are required to find an explanation.

DISCUSSION

Previous experiments with different rough mutants of *Salmonella typhimurium* (11) have shown that, owing to their incomplete cell wall lipopolysaccharide, all of these mutants are more or less avirulent. The reason for this reduced virulence seems to be an increased sensitivity of cells of these mutants to phagocytosis and intracellular killing (26). Concomitant with the loss of virulence, there is a decrease in immunogenic capacity. Only *gal* E mutants represent an exception to this rule. They lack the enzyme UDP-galactose-4-epimerase, which is responsible for the normal synthesis of UDP-galactose from UDP-glucose, and as a consequence no UDP-galactose is available for incorporation into the lipopolysaccharide. The lipopolysaccharide of these mutants is incomplete; it has only short side-chain stubs without O antigens. However, when galactose is supplied in the medium, UDP-galactose can be synthesized via galactose-1-phosphate and again normal smooth-type lipopolysaccharide can be synthesized. It is concluded that such a phenotypic reversion occurring in vivo endowed the *gal* E mutants with their outstanding protective capacity. When this phenotypic modification is

prevented by a second mutation, as in strain E 32 (defect in the UDP-galactose-lipopolysaccharide transferase) or LT 2 M1 A (defect in the galacto-kinase), these double mutants are no longer more immunogenic than any other rough mutant.

If *gal* E mutants possess in vivo smooth-type lipopolysaccharide, then they should be much more virulent than they, in fact, are. Thus, the semirough mutants SL 1034 and SL 1036 (10), with a lipopolysaccharide structure intermediate between smooth and rough, are definitely more virulent than any of the epimerase-negative mutants used in this investigation (*unpublished data*). However, the avirulence of the *gal* E mutants can be explained by an additional property, namely the galactose sensitivity of these strains. As a result of the defect in the epimerase, galactose cannot be metabolised and is accumulated in the form of galactose-1-phosphate and UDP-galactose which causes lysis of the growing cells.

Thus, the properties of the *gal* E mutants in vivo are dependent upon two mechanisms acting in opposite directions: a virulence- and immunogenicity-increasing biosynthesis of cell wall lipopolysaccharide and a virulence-lowering galactose-induced bacteriolysis. Virulence and immunogenicity of these mutants are thus dependent on the activities of the two other enzymes involved in the Leloir pathway, galactokinase and galactose-1-phosphate-uridyl transferase, and on UDP-galactose-lipopolysaccharide transferase Fig. 1). If there is a defect in one of these enzymes, the block in the synthesis of O-specific lipopolysaccharide cannot be by-passed by supplying galactose exogenously. Such a double mutant (for example, LT 2 M1 A) is completely avirulent but also no more immunogenic than any other rough mutant. In strains with a defect in one of the two transferases there will be, in addition, lysis, but in this case it will have no further influence on the properties of the strain. Pure *gal* E mutants on the other hand are very potent immunogenic strains but nevertheless completely avirulent (i.e., GE 7). One disadvantage associated with these mutants is their high tendency to change their properties by secondary mutation in the structural or regulator genes of the galactose operon. The direction in which this shift takes place depends on the enzymes that become the limiting factor in the pathway of galactose to lipopolysaccharide. If it is the activity of the kinase that is strongly reduced (but not abolished), there is no accumulation of the intermediary products, but the galactose taken up is incorporated directly into the lipopolysaccharide. Such strains are highly immunogenic but also somewhat more virulent (for exam-

ple, 395 MR 9). On the contrary, a reduction in the activity of one of the transferases leads to an essentially avirulent, but also only weakly protecting strain (for example, LT 2 M1 C). Thus, on the basis of the activities of the Leloir enzymes, the composition of the cell wall lipopolysaccharide and the degree of galactose sensitivity, reasonable assumptions can be drawn concerning virulence and immunogenicity of *gal* E mutants.

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