Galactose Epimeraseless Mutants of *Salmonella typhimurium* as Live Vaccines for Calves

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ABSTRACT

The purpose of the study was to evaluate the safety and efficacy of a galactose epimeraseless mutant of *Salmonella typhimurium* administered as an oral vaccine to one week old calves and to investigate properties of galactose epimeraseless mutants which affect their virulence and immunogenicity. The galactose epimeraseless mutant *S. typhimurium* strain G30D caused diarrhea and fever in three calves to which it was administered orally at a dose of 10^{10} organisms; all three calves died following challenge with virulent *S. typhimurium* ten days postvaccination. Mild illness developed in four calves vaccinated with a dose of 9×10^6 organisms and one of these calves survived challenge. Three unvaccinated calves died following challenge. The vaccine organism persisted in tissues and was shed for a prolonged period by calves which received 10^{10} organisms. Studies of characteristics of galactose epimeraseless mutants of *S. typhimurium* showed that, in the presence of galactose, there is selection for secondary mutants which are galactose resistant. The studies indicate that galactose epimeraseless mutants of *S. typhimurium* are not good candidate live vaccine organisms for use in calves.

Key words: Galactose epimeraseless mutants, *Salmonella typhimurium*, calves, vaccine.

RÉSUMÉ

Cette expérience consistait à évaluer l'innocuité et l'efficacité de l'utilisation d'un mutant de *Salmonella typhimurium*, dépourvu de l'enzyme uridine-diphosphate-galactose-épimérase, comme vaccin buccal, chez des veaux âgés d'une semaine; elle visait aussi à étudier quelles propriétés de ces mutants affectent leur virulence et leur pouvoir immunogène. Le mutant de la souche G30D de *S. typhimurium* causa de la diarrhée et de la fièvre, chez les trois veaux auxquels on en avait administré 10^{10} organismes, par la voie buccale. Ces trois veaux moururent à la suite d'une infection de défi avec une souche virulente de *S. typhimurium*, dix jours après leur vaccination. Une salmonellose mitigée se développa, chez quatre veaux vaccinés avec une dose de 9×10^6 organismes; l'un d'eux survécut à une infection de défi, tandis que trois veaux témoins y succombèrent. L'organisme du vaccin persista dans les tissus des veaux qui en avaient reçu une dose de 10^{10} et ils l'éliminèrent pendant longtemps. L'étude des caractéristiques des mutants de *S. typhimurium* dépourvus de l'enzyme précité révéla qu'en présence de galactose, il se produit une sélection pour des mutants secondaires qui sont résitants au galactose. Cette étude indique par conséquent que les mutants précités de *S. typhimurium* se prêtent mal à la préparation d'un vaccin atténué, pour les veaux.

Mots clés: mutants dépourvus de l'enzyme uridine-diphosphate-galactose-épimérase, *Salmonella typhimurium*, veaux, vaccins.

INTRODUCTION

In recent years interest has developed in the use of galactose epimeraseless (*galE*) mutants as live vaccines against salmonellosis (1,2,3,4). These mutants are deficient in the enzyme uridine diphosphate (UDP)-galactose-4-epimerase, and in the absence of exogenous galactose, they are unable to synthesize UDP-galactose, an essential precursor in the formation of smooth-type cell wall lipopolysaccharide (LPS) (2). When galactose is supplied exogenously, UDP-galactose can be formed by an alternate pathway, resulting in the synthesis of smooth-type LPS. Uptake of galactose by this alternate pathway also leads to the accumulation of toxic levels of galactose-1-phosphate and UDP galactose which results in bacterial lysis (2). Since galactose is available *in vivo* it has been speculated that the avirulence of *galE* mutants is due to galactose-induced lysis as well as the inability to form complete cell wall LPS (2). Studies in mice (1) have shown that *galE* mutants of *S. typhimurium* stimulate better protection in mice than other types of rough mutants. This superior immunizing capability is thought to be due to the unique ability of *galE* mutants to synthesize smooth-type LPS when supplied with galactose *in vivo* (2).

One *galE* mutant of *S. typhimurium* (G30D) has been the subject of extensive studies in mice (1,2,3) in which it has been shown to be very effective in inducing protective immunity. Limited trials have been carried out in calves by Wray and coworkers (3) who reported that vaccination of one to two week old calves by the subcutaneous route with *S. typhimurium* strain G30D induced significant protection against lethal

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Part of a dissertation submitted by the senior author in partial fulfillment of the requirements for the Ph.D. degree, University of Guelph.

Submitted August 13, 1985.

challenge with *S. typhimurium* but also resulted in fever, swelling at the site of injection, and lesions of pyemic nephritis.

For a live *Salmonella* vaccine to be of value in the prevention of salmonellosis in calves it must be free of serious adverse effects. The undesirable features associated with the subcutaneous inoculation of calves with *S. typhimurium* G30D (3) might be avoided by oral administration of the organisms. Accordingly, an initial experiment was conducted to evaluate the virulence of *S. typhimurium* strain G30D administered orally to calves and to assess the protection achieved in orally vaccinated calves challenged with a virulent strain of *S. typhimurium*.

The results of the initial experiment using the *galE* mutant *S. typhimurium* G30D indicated that this organism, administered orally in high doses, was virulent for calves. Previous studies (1,2,4) had shown that the reduced virulence and enhanced immunogenicity of *galE* mutants were dependent on their ability to switch from rough to smooth cell wall LPS and to undergo lysis, when galactose is supplied exogenously. Furthermore, *galE* mutants were unstable and tended to develop secondary mutations which affected the rough-smooth LPS switch and resulted in decreased immunogenicity. These studies indicated that a delicate balance must exist between stability, the rough-smooth LPS switch, and galactose-induced lysis in order for *galE* mutants of *S. typhimurium* to elicit optimum protection and maintain reduced virulence. A second experiment was therefore designed to compare the *S. typhimurium* G30D used in this study with other *galE* mutants with respect to stability, ability to switch from rough to smooth LPS, and galactose-induced lysis.

MATERIALS AND METHODS

EXPERIMENT I

Calves

Ten colostrum-fed Holstein-Friesian calves between one and three days of age were divided into groups (A,B,C) of three, four and three, respectively. The calves in each group were kept in separate isolation rooms, with each calf in a separate elevated pen and with free access to water and calf starter. Each calf was provided with 2 L of milk replacer at each of three feedings daily. Two fecal samples were taken prior to vaccination; one on each of the first two days after arrival of the calves at the isolation facility. The feces were checked for *Salmonella* sp. and for serogroups of enterotoxigenic *Escherichia coli*.

Bacteria

The strains of *S. typhimurium* used in this experiment were *S. typhimurium* strain G30D (2,3), a *galE* mutant of *S. typhimurium* LT2 received from Dr. C. Wray, Central Veterinary Laboratory, Weybridge, England and *S. typhimurium* 3860C, a nalidixic acid resistant, virulent strain that had been passaged in a calf.

Experimental Design

The experimental design is summarized in Table I. Briefly, calves were vaccinated once orally between three and six days of age. A lyophilized culture of *S. typhimurium* G30D was resuspended and grown overnight in brain heart infusion broth (BHIB) containing 0.2% galactose at 37°C in a shaking water bath at 140 rpm. [The galactose was added to promote production of smooth LPS, which has been shown to result in better protective immunity (5)]. The culture was centrifuged at 8,000 x *g* for 20 minutes at 5°C and the pellet was resuspended in an equal volume of sterile phosphate-buffered saline, pH 7.2, (PBS). Calves in group A were vaccinated orally after an overnight fast by allowing them to suckle 20 mL of milk replacer containing 5×10^{10} organisms. Calves in group B were vaccinated in the same manner with 9×10^6 organisms. Calves in group C were not vaccinated.

All calves were challenged ten days after vaccination. *Salmonella typhimurium* strain 3860C was grown overnight in BHIB in a shaking water bath at 140 rpm. After an overnight fast each calf was allowed to suckle 10^{10} organisms in 30 mL of milk replacer.

Enumeration of Salmonella

Plate counts for the vaccine strain *S. typhimurium* G30D were made on MacConkey base agar (Difco) with 0.5% galactose (Mac/gal). Challenge strain *S. typhimurium* 3860C was plated on MacConkey agar (Difco) with 50 μg/mL of nalidixic acid (Mac/nal). For both the vaccine and challenge organisms, dilutions of the inoculum were plated by means of a spiral plater (Spiral Systems, Bethesda, Maryland) in order to determine the dose given. Following vaccination, fecal samples were examined at two day intervals for the presence of the vaccine strain (G30D) by plating on Mac/gal agar before and after enrichment in tetrathionate broth. The number of viable challenge organisms (3860C) in feces and tissues was determined as follows. One gram of sample was macerated in 5 mL of PBS in a 15 mL Potter Elvenjheim tissue grinder with teflon resin pestle, then 0.2 mL of the suspension was

TABLE I. Fecal Shedding of *S. typhimurium* G30D by Vaccinated Calves

Group[a]	Calf No.	Days after Vaccination 2	4	6	8	10
A	11	+[b]	+	+	NT[c]	+
	12	–[d]	+	+	NT	+
	13	+	+	+	NT	+
B	14	–	–	–	–	–
	15	–	+	–	+	–
	16	–	–	–	–	–
	17	–	–	–	–	–

[a]Calves in both groups were vaccinated orally with *S. typhimurium* G30D. Those in group A received 5×10^{10} organisms and those in group B received 9×10^6 organisms
[b]+ *S. typhimurium* G30D detected
[c]NT = Not tested
[d]– None detected

spread over the surface of a Mac/nal plate. Dilutions of the suspension were plated by means of a spiral plater.

Postmortem Examination

Following challenge, each calf was watched closely for signs of illness. When death appeared to be imminent, or on the tenth day postchallenge, the calf was euthanized by intravenous injection of sodium pentobarbitol (Euthanyl forte, MTC Pharmaceuticals, Mississauga, Ontario). Calves which developed signs of severe salmonellosis and were euthanized when they appeared to be dying were treated as though they had died in response to the *Salmonella* organisms. Samples of the abomasum, duodenum, jejunum, ileum, cecum and colon were removed immediately and fixed in Bouin's fluid. Portions of liver, spleen, kidney and lung were fixed in 10% formalin. The concentrations of challenge organisms in the ileal mucosa, an ileal lymph node, liver and spleen were determined by plating a one gram sample of tissue as described previously.

Direct Microagglutination Test for Detection of Salmonella Antibodies

The test was an adaptation of the method described for *Brucella* by Brown and associates (6). A suspension of a washed overnight broth culture of *S. typhimurium* 3860C was made in 1% formalized PBS and held at 4°C overnight. The cells were centrifuged and resuspended in 0.1% formalized PBS, then used as the antigen preparation after adjustment to optical density 1.8 at 525 nm (Spectronic 20, Bausch and Lomb, Rochester, New York). Phosphate-buffered saline containing 0.005% safranin 0 (25 μL) was added to each well of a sterile microtitration plate with 96 U-shaped wells (Flow Laboratories, McLean, Virginia). Serum (25 μL) was added to the first well in each row of 12, then doubling dilutions were made. Antigen (25 μL) was then added to each well. Each plate was incubated for two hours at 37°C then overnight at 4°C. The titers were expressed as the $\log_2$ of the reciprocal of the highest dilution which caused agglutination.

EXPERIMENT II

Experimental Design

Four *galE* mutants were examined for ability to switch from rough to smooth LPS, and for galactose-induced lysis. These mutants were strains G30D, SGSC163, RC201 and RC267. Strain G30D was described in experiment I; SGSC163 was a *galE* mutant of *S. typhimurium* LT2, received from Dr. Ken Sanderson, at the *Salmonella* Genetic Stock Centre, University of Calgary; RC201 was a freshly isolated mutant of *S. typhimurium* LT2, produced in this study; and RC267 was a galactose resistant derivative of RC201.

Phages

Bacteriophages were used to determine the presence of smooth or rough LPS as described by Wilkinson *et al* (7). Bacteriophages FO-1, Ffm, Br60, P221 and C21 were received from Dr. Ken Sanderson. Bacteriophage P22*c*2, a virulent clear plaque mutant of P22, was received from Dr. John Roth, the University of Utah. Phages were reisolated from single plaques and grown in soft-agar overlays (8). Bacteriophages FO-1 and P22*c*2 were grown on *S. typhimurium* LT2; phages C21, Ffm, Br60 and P221 were grown on *S. typhimurium* SGSC163. Phage stocks were titered by putting 10 μL drops of serial tenfold dilutions on a lawn of susceptible bacteria.

Isolation of galE *Mutants*

Salmonella typhimurium LT2 was grown overnight in eight tubes of L broth at 37°C. A 0.1 mL volume of each culture was incubated with 1 x 10^8 plaque forming units (pfu) of FO-1 phage in each of eight small glass tubes at 37°C for ten minutes. To each tube was added 3 mL of L soft-top agar which was quickly spread over the surface of a fresh L plate and then incubated overnight at 37°C. Soft agar overlay of bacteriophage with no bacteria and *S. typhimurium* LT2 with no bacteriophage were used as controls. After incubation, 50 colonies from each plate that received the mixture of phage and bacteria were picked with sterile toothpicks onto L plates and incubated for 6 h at 37°C. These master plates were replicated onto MacConkey agar with 0.5% galactose, L agar with 0.4% sodium desoxycholate (Fisher Scientific, Fair Lawn, New Jersey) and control L agar. Colonies which grew on L agar with sodium desoxycholate but did not grow or were nongalactose fermenters on Mac/gal were picked from the master L agar plate and examined further. The Gal$^-$ strains isolated were checked for their ability to switch from rough to smooth LPS by cross-streaking against phages FO-1, P22*c*2, Br60, Ffm, C21 and P221 on plates of L, L + galactose (0.5%) and L + galactose (0.5%) + glucose (0.5%). Strains that were sensitive to galactose and switched from the rough to the smooth LPS phage sensitive to galactose and switched from the rough to the smooth LPS phage pattern when galactose was present in the medium were classed as *galE* mutants.

Galactose-induced Lysis

Strains of *S. typhimurium* were grown overnight in tubes containing 5 mL of L broth at 37°C, then centrifuged at 8,000 x *g* for 20 minutes. The pellet from each culture was resuspended in 1 mL sterile PBS, and 0.5 mL of the suspension was added to each of two 300 mL Nephlo culture flasks containing 50 mL of 1% peptone broth (Difco). Spectrophotometer readings were taken at 550 nm (Spectronic 20, Bausch and Lomb). The cultures were grown at 37°C in a shaking water bath at 140 rpm and in the mid-log phase, sterile galactose was added to one of the flasks to make a final concentration of 1%.

Stability of galE *Mutants in Galactose Broth*

In order to assess the effects of galactose on the selection for galactose resistant and galactose fermenting mutants, *S. typhimurium* RC201 was grown as described for galactose-induced lysis except that heart infusion broth (Difco) was used and the duration of the experiment was longer. Spectrophotometer readings were taken as before and, as well, aliquots were removed at various times to determine the total number of organisms per mL, the number of Gal$^+$ organisms per mL and the number of Gal$^-$ resistant organisms per mL. This

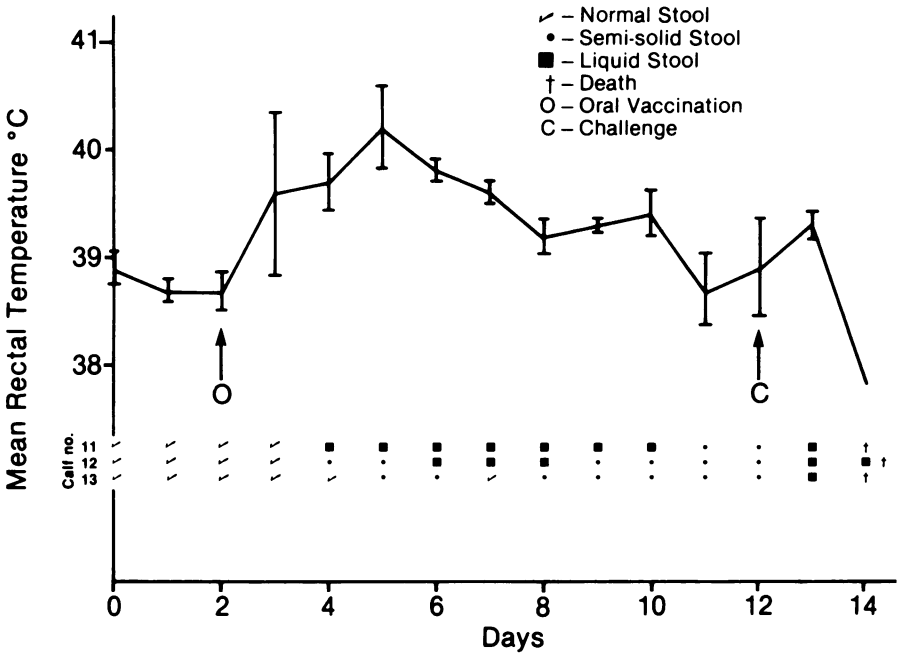

Fig. 1. Mean rectal temperatures (°C ± standard error) and stool consistency for three calves (group A) vaccinated with 5 x 10^{10} *S. typhimurium* G30D orally. Ten days after vaccination calves were challenged with 4 x 10^{10} *S. typhimurium* 3860C given orally. Stool consistency was classified daily as normal, semi-solid or liquid.

was accomplished by plating samples onto L agar and Mac/gal agar either directly or with the spiral plater.

RESULTS

EXPERIMENT I

Within 24-60 h of vaccination all calves which had received the high dose of vaccine organism (Group A) developed elevated temperatures and remained febrile for most of the vaccination period (Fig. 1). Although their condition improved with time, the calves were still weak and had semi-solid stools by the end of the ten day postvaccination period. Fecal shedding of the vaccine strain was detected in all group A calves throughout the period between vaccination and challenge (Table I). Following challenge all calves developed severe diarrhea within 12 h and became critically ill by 30-52 h (Table II).

Calves in group B received a lower dose of the vaccine (9 x 10^6 organisms) and, except for the occasional transient mild fever or soft stool, these calves remained normal and ate well prior to challenge (Fig. 2). One calf in group B was found to shed the vaccine strain intermittently. Within 12-54 h after challenge all four calves were febrile and had diarrhea. Three calves became critically ill and were euthanized at 33, 51 and 73 h postchallenge. The surviving calf was febrile for three days and had diarrhea for eight days. His condition returned to normal and he was euthanized ten days postchallenge.

All three unvaccinated control calves in group C were clinically normal during the prechallenge period. Following challenge all three calves rapidly developed fever and diarrhea and became critically ill (Fig. 3). These calves were euthanized at 33, 37 and 50 h postchallenge.

Most calves had a low initial anti-*S. typhimurium* titer which decreased following vaccination (Table III). Only the surviving calf (#16 of group B) showed an increased titer at ten days postchallenge.

All vaccinated and control calves that died exhibited similar gross and microscopic lesions, which were consistent with acute salmonellosis (9). At the time of death all animals were severely dehydrated, depressed and in lateral recumbency. They had rapid shallow respirations, cold mucous membranes and a rapid, weak pulse. Postmortem examination revealed a moderate to severe fibrinous enteritis which was most pronounced in the ileum. The contents of the small and large bowel were watery and sometimes bloody. Peyer's patches and mesenteric lymph nodes were at least two times normal size.

The numbers of challenge organisms recovered from tissue samples taken from the calves at necropsy are shown in Table IV. The highest counts were in the ileal mucosa and the next

TABLE II. Survival of Calves Vaccinated Orally with *S. typhimurium galE* Mutant, G30D, and Challenged with Virulent *S. typhimurium* 3860C

Group	Vaccine Dose	Vaccine Reactions	Challenge[a] Dose	No. Survivors[b] / No. Challenged	Time of Euthanasia[c] (Hours)
A	5 x 10^{10}	High fever, diarrhea, depression	4 x 10^{10}	0/3	30 32 52
B	9 x 10^6	Transient fever, occasional soft stool	2 x 10^{10}	1/4[d]	33 51 73
C	0	NA	2 x 10^{10}	0/3	33 37 50

[a]At ten days postvaccination
[b]At ten days postchallenge
[c]Calves were euthanized when their condition deteriorated and it appeared they would not recover
[d]The one surviving calf was euthanized ten days postchallenge
NA = Not applicable

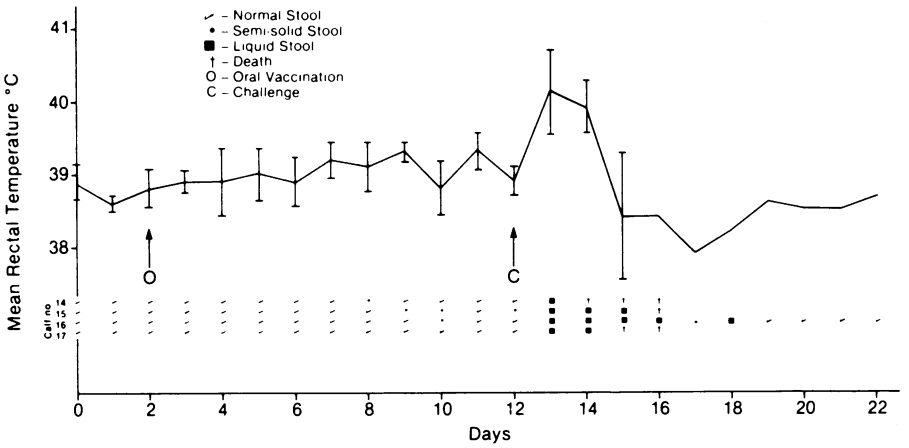

Fig. 2. Mean rectal temperatures (° C ± standard error) and stool consistency for four calves (group B) vaccinated with 9 x 10^6 *S. typhimurium* G30D orally. Ten days after vaccination calves were challenged with 2 x 10^{10} *S. typhimurium* 3860C given orally. Stool consistency was classified daily as normal, semi-solid or liquid.

highest counts were in the ileal lymph nodes. The number of organisms isolated from the liver and spleen was low even though these animals were terminally ill.

In contrast, lower numbers of challenge organisms were recovered from the surviving calf in group B. The numbers of challenge organisms per gram of feces decreased from 1 x 10^8 on the fifth day to 3 x 10^6 on the seventh day and finally to 8 x 10^4 on the tenth day after challenge. At the time of euthanasia the calf appeared clinically normal, but had still not completely eliminated the challenge strain. Although there was a dramatic reduction in the numbers of organisms in the feces, ileal mucosa and lymph node, the counts in the liver and spleen were still of the same magnitude as those in most of the calves that died (Table IV).

EXPERIMENT II

Bacteriophage FO-1 selection was used to obtain a *galE* mutant of *S. typhimurium* LT2 which was lyophilized immediately in order to prevent the development of secondary mutations. Of 400 rough mutants that were selected, 16 were either unable to grow on galactose medium or did not ferment galactose. These strains showed a variety of sensitivites to galactose, and variable phage patterns and reversion to Gal$^+$. One strain, *S. typhimurium* RC201, which was very sensitive to galactose and underwent the rough-smooth LPS transition was selected for further study. When RC201 was streaked on L galactose (0.5%) medium, the majority of organisms died, but a few galactose resistant mutants grew and one (RC267) was selected for comparative purposes.

Table V summarizes the patterns of phage susceptibility of the strains tested on media without galactose, with galactose only, or with glucose and galactose. Galactose sensitivity for each strain was determined by observing the extent of growth on L galactose medium. The smooth strain *S. typhimurium* LT2 is Gal$^+$, grows well on medium containing galactose and shows no change in susceptibility to phages when galactose is present in the medium. The *galE* mutant *S. typhimurium* SGSC163 shows characteristics typical of this type of mutant in that it is sensitive to galactose and switches from the rough to the smooth LPS phage pattern when galactose is supplied. *Salmonella typhimurium* RC201 is very sensitive to the presence of galactose and is also switchable. *Salmonella typhimurium* RC267, the galactose resistant mutant of RC201, displays a rough LPS phage pattern and is only partially switchable when galactose is present. The vaccine strain *S. typhimurium* G30D grows well on galactose medium but is switchable only on the L galactose media.

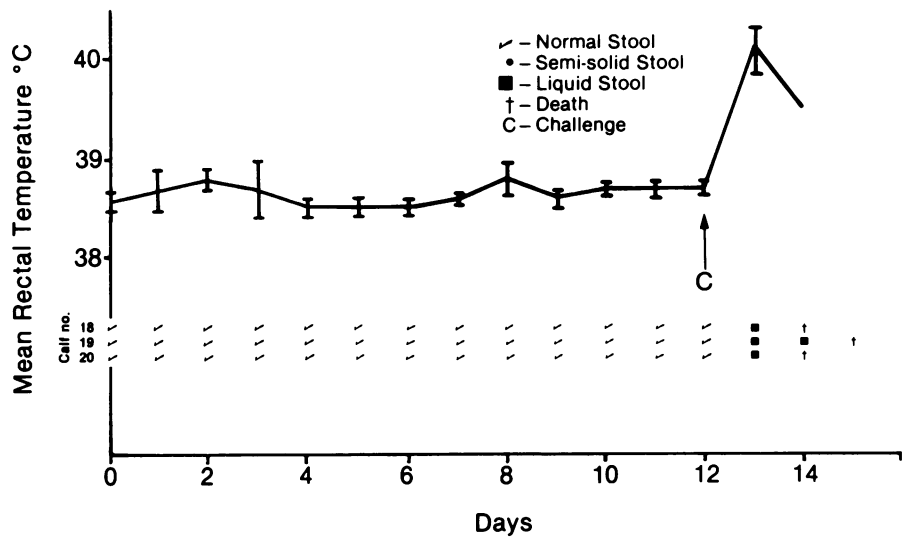

Fig. 3. Mean rectal temperatures (° C ± standard error) and stool consistency of three unvaccinated calves (group C) which were challenged with 2 x 10^{10} *S. typhimurium* 3860C given orally. Stool consistency was classified daily as normal, semi-solid or liquid.

GALACTOSE-INDUCED LYSIS

Galactose sensitivity of the strains was determined by examination of growth curves before and after the addition of galactose to the medium. Changes in optical density correlated well with the number of live organisms present so only the optical density readings are presented. The rates of growth and final concentrations were similar for all strains when they were cultured in the absence of galactose.

TABLE III. Titers[a] of Agglutinating Antibodies to *S. typhimurium* in Sera of Calves Before and After Vaccination

Group[b]	Calf No.	Titer: Pre-vaccination	Titer: Days after Vaccination: 4	Titer: Days after Vaccination: 10	Titer: Post-challenge[c]
A	11	3	—[d]	2	3
	12	1	—	—	—
	13	2	—	1	1
B	14	1	—	—	—
	15	—	—	—	—
	16	1	—	1	4[e]
	17	1	—	—	—
C	18	2	NA[f]	NA	—
	19	—	NA	NA	—
	20	3	NA	NA	—

[a]Calves in groups A and B were vaccinated orally with *S. typhimurium* G30D. Those in group A received 5 x 10^{10} organisms and those in group B received 9 x 10^{6} organisms. Calves in group C were unvaccinated controls

[b]Titers are $\log_2$ of the reciprocal of the highest dilution of serum which caused agglutination of formalin-killed *S. typhimurium*

[c]All calves were challenged on day 10 postvaccination. Samples were taken at time of euthanasia (1.5-3 days postchallenge for all calves except #16)

[d]— no agglutination detected

[e]Sample was taken ten days postchallenge

[f]NA = not applicable

TABLE IV. Numbers of Challenge Organisms (*S. typhimurium* 3860C Nal^R) Recovered at Necropsy from Selected Tissues of Vaccinated and Control Calves

Group[a]	Calf	Log_{10} Nal^R Organisms/Gram of Tissue: Ileal Mucosa	Ileal Lymph Node	Liver	Spleen
A	11	9.6	6.6	4.3	3.6
	12	8.0	4.8	3.5	3.5
	13	7.9	4.9	2.8	2.6
B	14	9.0	7.7	3.7	3.0
	15	8.8	7.3	4.3	3.6
	16[b]	3.0	2.5	2.7	2.7
	17	8.6	6.7	2.0	3.0
C	18	9.3	7.5	3.6	2.9
	19	8.3	7.7	1.7	3.0
	20	8.9	7.3	2.8	1.4

[a]Calves in groups A and B were vaccinated orally with *S. typhimurium* G30D. Those in group A received 5 x 10^{10} organisms and those in group B received 9 x 10^{6} organisms. Calves in group C were unvaccinated controls

[b]Calf #16 survived and was euthanized at ten days postchallenge

TABLE V. Phage Sensitivity Patterns of *S. typhimurium* LT2 and Derivative Strains

Strain	Reaction with Phages[a]: Smooth Specific P22*c*2	Smooth-Rough FO	Rough Specific Br60	Ffm	C21	P221	Sensitivity to Galactose[b]	Ability to Ferment Galactose
LT2	+++	+++	---	---	---	---	R	+
SGSC163	-++	-++	+--	+--	+--	+--	S	-
RC201	-N+	-N+	+N-	+N-	+N-	+N-	S	-
RC267	---	---	+±-	+++	+±-	+++	R	-
G30D	-+-	-+-	+-±	+-+	+-±	+++	R	-

[a]The patterns listed represent the phage sensitivity of the strain on plates of L, L +0.5% galactose; and L + 0.5% galactose + 0.5% glucose

+ lysis
± thinning
- no lysis
N no growth of bacteria

[b]R = resistant
S = sensitive

The growth of the smooth strain *S. typhimurium* LT2 was enhanced by the addition of galactose to the peptone broth. The *galE* mutant *S. typhimurium* SGSC163, however, was sensitive to the addition of galactose and the net growth of the culture decreased. *Salmonella typhimurium* RC201 was very sensitive to the effects of galactose and there was a dramatic decrease in the numbers of live organisms. These results are depicted in Figure 4. *Salmonella typhimurium* strains G30D and RC267 were only slightly sensitive to the addition of galactose and produced similar patterns of growth (Fig. 4).

The effects of prolonged incubation of a *galE* mutant exposed to galactose are depicted in Figure 5, which shows the changes in optical density of a heart infusion broth culture of RC201 before and after the addition of galactose. The initial decline in O.D. following addition of galactose was short-lived and by 23 h both populations had similar O.D. values. In the absence of galactose, *S. typhimurium* RC201 grew in an almost identical manner as its parent *S. typhimurium* LT2. The types of organisms present at various times during incubation were monitored. When galactose was not present, the proportions of Gal^- sensitive, Gal^- resistant and Gal^+ organisms were roughly constant throughout the growth cycle. When galactose was present, however, there was a dramatic decline in the number of Gal^- sensitive bacteria over time (Fig. 6). This process continued until all the Gal^- sensitive bacteria died and only the Gal^- resistant and Gal^+ organisms remained.

DISCUSSION

At high oral doses, *S. typhimurium* G30D produced clinical signs identical to those observed in calves with natural disease due to *S. typhimurium* and was shed in the feces for at least ten days. Other studies have shown that calves vaccinated orally with *galE* mutants of *S. typhimurium* developed an unacceptable level of prolonged diarrhea and fever (10) and that adult sheep vaccinated orally with *S. typhimurium* G30D became febrile and had depressed appetite (11). The

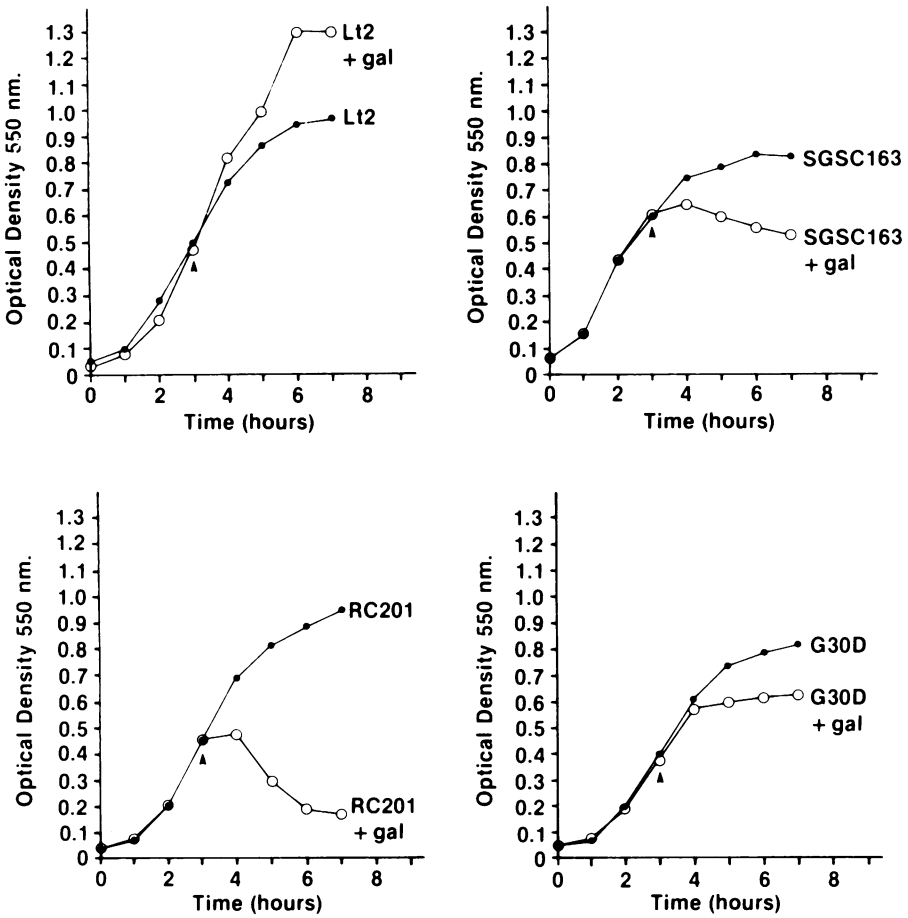

Fig. 4. Growth curves of selected strains of *S. typhimurium* grown in 1% peptone broth without galactose and with galactose added after three hours incubation (↑). A) *S. typhimurium* LT2. There is increased growth of the strain when galactose is added to the medium. B) *S. typhimurium* SGSC163. Shortly after addition of galactose to the medium, this *galE* mutant underwent a moderate degree of galactose-induced lysis. C) *S. typhimurium* RC201. Addition of galactose to the medium resulted in dramatic galactose-induced lysis typical of pure *galE* mutants. D) *S. typhimurium* G30D. This strain was galactose-resistant and did not show the pattern of galactose-induced lysis typical of pure *galE* mutants.

persistence of *S. typhimurium* G30D *in vivo* observed in the present study is consistent with the reports of Wray and associates (12) who isolated this organism from the injection site of two calves six weeks after subcutaneous vaccination and of Germanier (1), who found that the organism survived in the liver and spleen of vaccinated mice for up to five weeks. It appears that, despite an increased susceptibility to phagocytosis (13), *S. typhimurium* G30D can resist the host defences and persist for long periods when given in high doses. This survival may be beneficial for the stimulation of protective immunity (4,14,15), but can result in adverse effects on the health of the vaccinated animal.

The failure of *S. typhimurium* G30D to protect calves in this experiment may have been due to the debilitating effects of the high vaccine dose and the inadequacy of the lower dose. Baljer and associates (10) reported that two week old calves vaccinated orally with 10^{10} organisms of a *galE* strain were protected against death when challenged with a 10^9 dose of a virulent organism, but that doses of less than 10^8 of the vaccine organism were inadequate. The young age of the calves at the time of vaccination may have contributed to the failure of the vaccine in the present study. In a previous study it was found that calves injected parenterally with formalin-killed *Salmonella* did not exhibit antibody responsiveness to *Salmonella* O antigens before two weeks of age (16).

The behaviour of *galE* mutants of *S. typhimurium* in calves reported in this study and by Baljer and associates (10) differs from that of the *galE* mutant *S. typhi* Ty21a in humans. Oral vaccinaton of humans with 10^9 - 10^{10} *S. typhi* Ty21a produces only mild abdominal cramps and diarrhea in a small percentage of subjects and the vaccine strain can be detected in the feces for only one to two days after vaccination (5,17). The dramatic difference in virulence and shedding in the feces observed for *galE* mutants of *S. typhi* in humans and of *S. typhimurium* in calves may be related to differences in the diseases caused by the parent organisms. The data obtained in this study, combined with those of others (3,10), indicate that *galE* mutants of *S. typhimurium*, administered orally or parenterally, are unsuitable as vaccine organisms for use in calves.

Experiment II confirmed an earlier report on instability of *galE* mutants (2) but demonstrated differences in galactose-induced lysis of the *S. typhimurium* strains in this study compared with that of the *galE* mutant vaccine strain *S. typhi* Ty21a (18). Addition of galactose to a mid-log culture of *S. typhi* Ty21a resulted in complete lysis of the culture within two hours (18). In the present study, however, complete lysis of the *galE* strain *S. typhimurium* RC201 did not occur (Figs. 5 and 6), due to selection for galactose resistant mutants. Germanier and Fuerer(18) noted that in media without galactose the *S. typhi galE* mutant Ty21a grew more slowly than its parent. In contrast, experiment II showed that, in the absence of galactose, the *galE* mutants of *S. typhimurium* LT2 (G30D, SGSC163 and RC201) grew at the same rate as the parent. The difference in growth rates between the parent *S. typhi* and its *galE* mutant Ty21a could be due to an unknown growth-limiting mutation induced by the initial mutagenesis procedure. The slower rate of growth of *S. typhi* Ty21a may contribute to the reduced virulence of this strain and the low prevalence of side effects.

In this study, *S. typhimurium* G30D was not very sensitive to galactose and

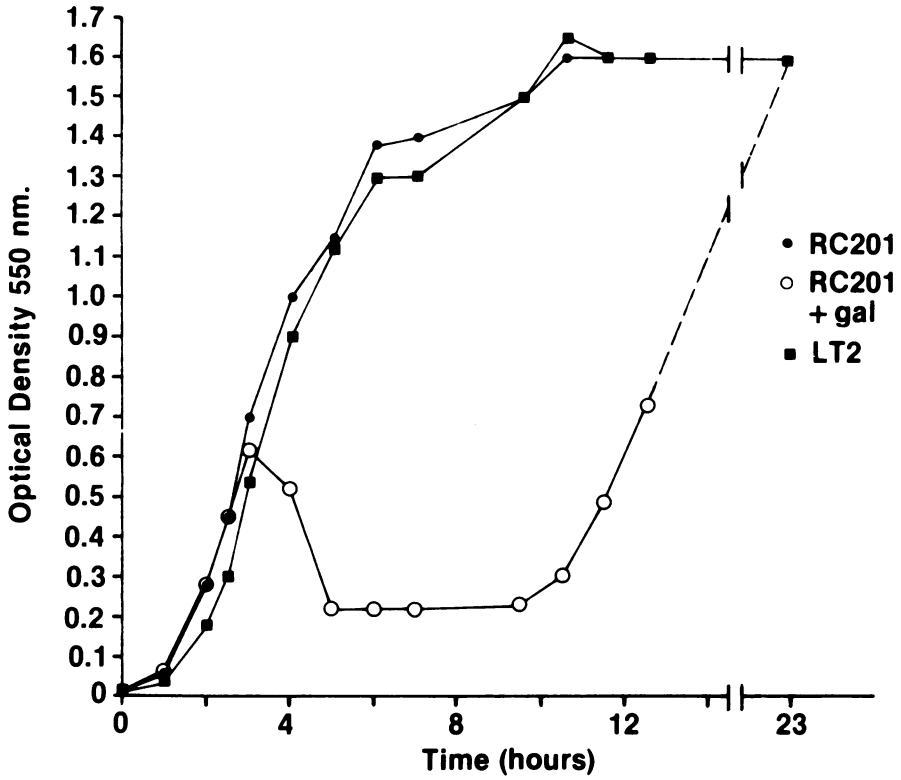

Fig. 5. Growth curves of *S. typhimurium* RC201 in heart infusion broth with and without galactose. Sterile galactose was added at 2.5 hours incubation. *Salmonella typhimurium* LT2 was included for comparison. Strains RC201 and LT2 grew at similar rates in heart infusion broth. On addition of galactose to the medium, strain RC201 showed marked lysis but surviving organisms subsequently grew to the same final concentration as the culture without galactose.

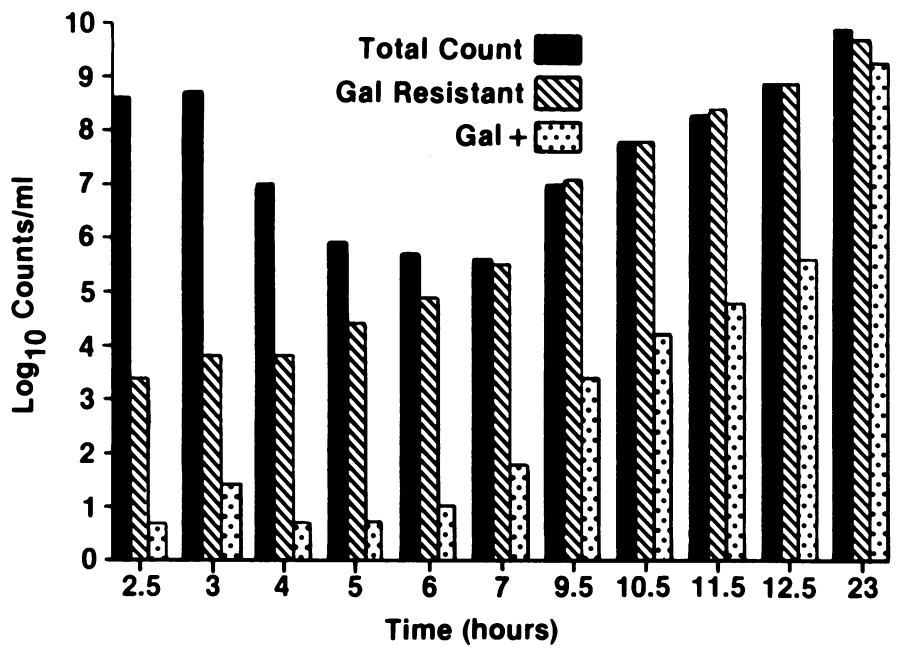

Fig. 6. Galactose phenotypes found at various incubation times during the growth curve depicted in Fig. 5 for *S. typhimurium* RC201 in the presence of galactose. Galactose-induced lysis selected for galactose-resistant and Gal$^+$ phenotypes.

did not switch from rough to smooth LPS in media which contained both glucose and galactose. Germanier and Fuerer (2) reported that the parent strain, *S. typhimurium* G30, was sensitive to galactose but had secondary mutations in the galactose operon affecting galactokinase and galactose-1-phosphate-uridyl transferase. Mutants of *S. typhimurium* G30 were identified which had varying degrees of sensitivity to galactose, ranging from very sensitive (G30E) to very resistant (G30A). *Salmonella typhimurium* G30D was selected for further study because it was still sensitive to galactose but not as unstable as the most sensitive strain, G30E. The *S. typhimurium* G30D used in this study may have developed more galactose resistance than previously described (2). Increased galactose resistance has been associated with increased virulence of the strain for mice (2), but there have been no similar studies for calves. In any event, the fact that *S. typhimurium* G30D seems to have developed increased galactose resistance demonstrates the difficulty in maintaining the galactose sensitivity of *galE* strains, despite the presence of stabilizing secondary mutations.

In order to develop safe live vaccines, mutations should be selected that have complete nonreverting blocks in genes that cannot be influenced by spontaneously occurring mutations in other genetic loci. When mutants that have a complete nonreverting block in the *galE* gene are selected, it is possible to prevent reversion to the Gal$^+$ phenotype. It is impossible, however, to completely prevent the spontaneous occurrence of secondary mutations in other galactose genes. Mutations useful for live vaccines should also impose a self-limited survival or growth of the vaccine strain *in vivo* that is independent of the host's conditon or response (19). Unfortunately, the limitation of survival of *galE* mutants *in vivo* is largely dependent on normal host defense mechanisms and possibly the presence of galactose.

ACKNOWLEDGMENTS

The authors gratefully acknowledge

the technical assistance of Richard Clark and Donna McEvoy-McQuay. The research was supported by Agriculture Canada and the Ontario Ministry of Agriculutre and Foods. The senior author was an employee of the Animal Pathology Laboratory, Agriculture Canada, Guelph, Ontario.

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