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A *galE via* (Vi Antigen-Negative) Mutant of *Salmonella typhi* Ty2 Retains Virulence in Humans

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We have recently described the construction of a *galE* derivative of *Salmonella typhi* Ty2 (Ty2H1) which had a 0.4-kilobase deletion in the *galE* gene and was sensitive to galactose-induced lysis when cultured with ≥0.06 mM galactose (D. M. Hone, R. Morona, S. Attridge, and J. Hackett, J. Infect. Dis. 156:167–174, 1987). We now report the selection of a rifampin-resistant, *via* derivative of Ty2H1, EX462. Compared with the Ty2 parent strain, EX462 was serum sensitive and highly attenuated in the mouse mucin virulence assay. When four human volunteers ingested 7×10^8 viable EX462, two became ill and developed a typhoidlike disease with fever and bacteremia. Blood isolates from these individuals were indistinguishable from the vaccine strain by a variety of criteria. We concluded that, even in a *via* background, the *galE* mutation was not attenuating for *S. typhi* in humans.

Although parenteral vaccines stimulate only a humoral response in mice (5, 7), killed *Salmonella typhi* vaccines given parenterally have nevertheless been able to confer significant protection against typhoid fever in humans, as demonstrated by a series of controlled field trials conducted by the World Health Organization in the 1960s (1, 35, 44). However, the field trials were performed in areas where typhoid fever was endemic, and it has been argued that such vaccines may be effective only in areas where vaccination serves only to boost natural immunity acquired by periodic exposure to subclinical *S. typhi* infections (18). Furthermore, 25% of the vaccinees receiving killed vaccines developed unacceptably severe systemic and local reactions (1, 35, 44). Thus, it is accepted that there is still a need to develop an effective, safe, and nonirritating vaccine against typhoid, which continues to present developing countries with a serious public health problem (10). One way to achieve this is by the construction of attenuated live oral vaccines (18). Unfortunately, there is no good animal model for typhoid fever because *S. typhi* is not naturally virulent for any laboratory animal (4). Because of this, efforts towards attenuation rely heavily on knowledge gained from the experimental host-parasite relationships between *S. typhimurium* or *S. enteritidis* and mice (3–8, 16, 17, 23, 24). The progression of oral infections by these bacteria in mice to bacteremia, spleen involvement, and the development of the carrier state has obvious parallels with the human disease. However the applicability of such models to typhoid in humans remains unclear (18).

Nevertheless, a few attenuated *S. typhi* strains have been developed for use as candidate live oral vaccines against typhoid. These strains can be divided into two classes: those that carry mutations that remove part or all of the somatic antigen, and those that have mutations which either inhibit or preclude growth in the host (10, 18, 20, 28, 29). Until now, only four such vaccines have been tested in humans. A streptomycin-dependent mutant of *S. typhi* 19V (*S. typhi* 27V) was satisfactorily attenuated and immunogenic, but was abandoned when found to be ineffective when given as a lyophilized preparation (28). Strain Ty21a, a *galE* derivative of *S. typhi* Ty2, has been extensively evaluated for its suitability as a live oral vaccine. Both small- and large-scale trials have shown that this strain stimulated moderate to good protection with minimal side reactions (10, 18, 41, 42). Ty21a was made by extensive nonspecific mutagenesis which induced mutations other than *galE*. These include (i) a *via* mutation blocking Vi antigen synthesis (21); (ii) one or more mutations giving Ty21a a growth rate half that of its parent Ty2 (20); (iii) a mutation(s) causing a requirement for isoleucine and valine (our unpublished observation); and (iv) an inability to produce H_2S (21). Some of these mutations, and perhaps others yet to be detected, may contribute to the attenuation of Ty21a. More recently, 541Ty, an *S. typhi*-derived strain bearing deletions in *purA* and *aroA*, and 543Ty, its Vi-negative derivative, were tested for safety and immunogenicity in humans. Both strains were found to be safely attenuated when fed to volunteers in doses as high as 10^9 (543Ty) or 10^{10} (541Ty). Neither strain gave a detectable humoral response in volunteers, but all vaccine recipients developed an anti-*S. typhi* cell-mediated response (29). Since the vaccinees were not challenged with virulent *S. typhi*, the protective efficacy of these strains remains unknown.

We recently described the construction of a *galE* derivative of *S. typhi* Ty2, Ty2H1. This strain lacks 0.4 kilobase of the *galE* gene but is isogenic in all other respects to its parent *S. typhi* Ty2 (24). An *S. typhimurium* strain carrying the same deletion as Ty2H1 was completely avirulent in mice and gave high levels of protection (24). In this communication, a rifampin-resistant, *via* derivative of Ty2H1, EX462, is shown to be markedly attenuated when compared with its Ty2 parent strain in the mouse mucin virulence assay. Its safety and immunogenicity in humans were then assessed.

MATERIALS AND METHODS

Bacteria and bacteriophage. The bacterial strains used here are listed in Table 1. For short-term storage, strains were kept at −70°C in 32% (vol/vol) glycerol–0.6% (wt/vol) peptone. Long-term storage of lyophilized strains is described elsewhere (24). All experiments were performed with freshly grown bacteria. Bacteriophages P22 and Felix-O were the

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TABLE 1. Bacterial strains

Bacterial strain	Method of derivation	Genotype	Source or reference
S. typhimurium SL1654		*hsdA hsdC trpB2 nml* H1(b) flaA66 H2(e,n,x) *rpsL xylT404 ilvE452 metE551 metA22*	P. Reeves
S. typhi Ty21a[a]		*cys trp ile val galE via*[b]	R. Germanier
S. typhi Ty2		*cys trp* (wild type)	IMVS[c]
Ty2H1	Site-directed mutagenesis	*cys trp galE*-H1	24
Ty2Vi	ViII-resistant mutant of Ty2	*cys trp via*	This paper
Ty2H1Vi	ViII-resistant mutant of Ty2H1	*cys trp galE*-H1 *via*	This paper
EX462	Rifampin-resistant mutant of Ty2H1Vi	*cys trp galE*-H1 *via rif*[b]	This paper
J669	Blood isolate, volunteer A	*cys trp galE*-H1 *via rif*	This paper
J670	Blood isolate, volunteer A	*cys trp galE*-H1 *via rif*	This paper
J671	Blood isolate, volunteer B	*cys trp galE*-H1 *via rif*	This paper
J672	Blood isolate, volunteer B	*cys trp galE*-H1 *via rif*	This paper
EX590	Transduction of Ty2H1 to *gal*$^+$, using SL1654 as donor	*cys trp gal*$^+$	This paper
EX592	Transduction of EX462 to *gal*$^+$, using SL1654 as donor	*cys trp gal*$^+$ *via rif*	This paper

[a] Strain Ty21a may have other uncharacterized mutations.
[b] *via* denotes that the genetic locus affected by mutation is likely to be *viaA* or *viaB*; *rif* denotes a mutation that produces a rifampin-resistant phenotype.
[c] IMVS, Institute of Medical and Veterinary Science, Adelaide, Australia.

gift of P. Reeves, University of Sydney, Sydney, Australia. Bacteriophage ViII (propogated here on *S. typhi* Ty2) was a gift from B. Stocker, Stanford University, Stanford, Calif. Bacteriophage propagation and storage techniques are described elsewhere (31).

Media. Shaking liquid cultures were grown for 16 h at 37°C in NBG (1.6% [wt/vol] nutrient broth [Difco Laboratories, Detroit, Mich.], 0.5% [wt/vol] NaCl, 25 mM glucose) or NBGG (NBG with 6 mM galactose). Nutrient agar (1.6% [wt/vol] blood agar base [Difco]) was normally supplemented with glucose (25 mM). M9 minimal medium is described elsewhere (31); however, sodium citrate was omitted, and glucose was added to a final concentration of 25 mM. Minimal galactose medium was the same modified M9 medium with galactose (6 mM) substituted for glucose as the sole carbon source. For the growth of *S. typhi* Ty2 and its derivatives, M9 medium was supplemented with tryptophan (20 μg/ml) and cystine (20 μg/ml) (21). In addition to these growth factors, Ty21a required isoleucine (20 μg/ml) and valine (20 μg/ml). Selection and growth of rifampin-resistant mutants were effected on nutrient agar plates supplemented with 100 μg rifampin (Sigma Chemical Co., St. Louis, Mo.) per ml (31).

Sensitivity to galactose-induced lysis. To test for sensitivity to galactose-induced lysis (15, 32), various concentrations of galactose (6 mM to 60 nM) were added to nutrient agar (without glucose). Bacteria were grown in NBG to stationary phase and then swabbed across the galactose-containing plates. After 16 h at 37°C, sensitivity to a given galactose concentration was detected by inhibition of growth at that concentration. A nutrient agar plate supplemented with both glucose (25 mM) and galactose (6 mM) served as a positive control.

Bacteriophage sensitivity. Bacteria were grown to stationary phase in NBG and then swabbed across a nutrient agar plate supplemented with 25 mM glucose or onto the same medium also with 6 mM galactose. Bacteriophage P22 and Felix-O were spotted (ca. 10^6 PFU in 5 μl of NBG) onto the bacteria, and the plates were incubated at 37°C for 16 h. Strains were scored as bacteriophage sensitive if a clear zone of lysis appeared. Clearing due to P22 probably occurred as a result of killing by phage infection without lysis, since P22 does not grow in *S. typhi*. To determine the minimum galactose concentration required for bacteriophage sensitivity, galactose was diluted in 10-fold steps from 6 mM to 60 nM. The minimum galactose concentration was the lowest concentration in the presence of which the bacteriophage could effect complete lysis.

Transduction. Bacteriophage P22 was propagated on SL1654, and the lysate was used to transduce *galE*-H1 strains (grown in NBGG) to *gal*$^+$ by selection on minimal galactose. All transductants were tested for sensitivity to P22 to ensure that no lysogens had been selected (31).

Enzyme assays. The enzymes of the Leloir pathway catabolize galactose to UDP-glucose (27). Strains were grown in shaking liquid culture (NBG) at 37°C overnight. The overnight culture was then subcultured 1:100 in nutrient broth with galactose (6 μM) and grown, with aeration, to late log phase. Enzyme extracts were prepared by the method of Saito et al. (39). Galactokinase (EC 2.7.1.6; *galK*) was assayed as described elsewhere (39). The galactose-1-phosphate uridylyltransferase (EC 2.7.7.9; *galT*) and UDP-glucose-4-epimerase (EC 5.1.3.2; *galE*) assays are also described elsewhere (27).

HA inhibition assay. Hemagglutination (HA) inhibition assays were generally performed with suspensions of Formalin-killed bacteria. The bacterial strains were grown in either NBG or NBGG overnight with shaking at 37°C. They were then subcultured 1:10 into fresh medium of the same type and incubated at 37°C with shaking until the cultures reached late log phase. Bacteria were harvested by centrifugation, washed once in 0.9% (wt/vol) NaCl (saline), and suspended to ca. 10^{10}/ml in saline with 1% (wt/vol) formaldehyde (Univar). After incubation at 37°C for 60 min, the bacteria were centrifuged, washed four times in saline, and suspended to 10^{10}/ml (by microscopic count) in saline. The HA inhibition system involved the agglutination of sheep erythrocytes, which had been sensitized with *S. typhi* lipopolysaccharide (LPS; Sigma) (9) by limiting concentrations (4 HA units/ml) of a rabbit antiserum prepared against Ty2Vi bacteria, a *via* derivative of *S. typhi* Ty2. Twofold falling dilutions of the suspensions of inactivated bacteria were tested for their capacities to inhibit HA, the endpoints being expressed as the number of wells showing clear inhibition (control wells received saline diluent). Formalin-killed Ty2Vi was used as a bacterial standard, and a suspension of *S. typhi* LPS indicated that the level of detection of O antigen was typically 0.1 μg/ml.

ELISA. Enzyme-linked immunosorbent assays (ELISAs) were performed as described elsewhere (2), using trays (Costar, Cambridge, Mass.) sensitized with *S. typhi* LPS. Goat anti-human immunoglobulins A and G, each conjugated with alkaline phosphatase, were obtained from KPL. Color development after 2 h at 37°C was read (at 405 nm) with a Titertek Multiskan (Flow Laboratories, Inc., McLean, Va.). ELISA values were expressed as absorption units per milligram of total immunoglobulin of the given class.

Bactericidal assay. Fourfold serial dilutions of heat-inactivated (56°C for 30 min) sera were prepared in 0.1% (wt/vol) peptone in saline and mixed with equal volumes (0.4 ml) of a suspension of the indicator bacteria, Ty2Vi (ca. 4×10^3/ml), in the same diluent containing 20% (vol/vol) guinea pig serum as a complement source. After 60 min at 37°C, the tubes were transferred to an ice-water bath and 0.1-ml aliquots were spread on nutrient agar plates to determine residual viability. By plotting the viability as a function of reciprocal serum dilutions, bactericidal titers were obtained by interpolation and expressed as the reciprocal serum dilution killing 50% of the added bacteria.

Serum sensitivity. Normal human serum, obtained from workers in our laboratories who had no history of prior exposure to *S. typhi* or to vaccination against *S. typhi*, was adsorbed with Ty2 (10^{10}/ml for 1 h at 4°C) before filtration and use. Bacteria were grown as described in the HA inhibition assay; they were diluted to ca. 2,500 CFU/ml in saline, and 0.1-ml aliquots were mixed with 0.9 ml of various concentrations (90, 30, 10, 3, and 1%, (vol/vol) of the adsorbed human serum, in saline. The mixtures were incubated at 37°C for 1 h and plated on nutrient agar for determination of residual viability. From a plot of viability as a function of serum concentration, the serum resistance of a bacterial strain is expressed as the concentration of serum which reduced viability by 50% (relative to viability in saline control tubes).

Selection of spontaneous *via* mutants. The *via* mutants of Ty2 and Ty2H1 (Ty2Vi and Ty2H1Vi, respectively) were selected as follows. An overnight liquid culture (NBG) was divided into 0.1-ml aliquots (ca. 5×10^8 CFU) which were mixed with 0.1 ml of ViII phage (ca. 5×10^9 PFU). After absorption (standing at 37°C, 10 min), the mixture was spread evenly on a nutrient agar plate. Typically, about 50 to 100 colonies were detected after overnight incubation at 37°C. Phage-resistant mutants that showed no agglutinating activity with anti-Vi serum, and increased sensitivity to the bactericidal action of serum, were stored.

Selection of rifampin-resistant mutants. To assist with monitoring of the vaccine strain in humans, a rifampin resistance marker was introduced into Ty2H1Vi as previously described (31). Care was taken to check that the rifampin-resistant strain (EX462) had a growth rate, in NBG, comparable to that of Ty2H1Vi.

Animal experiments. (i) Mice. Female LACA mice of an average weight of 25 g were used in this study. They were bred in specific-pathogen-free conditions at the Waite Agricultural Institute, Adelaide, Australia, and were allowed to acclimatize in our conventional animal house for 24 to 96 h with free access to food and water.

(ii) In vivo bacterial persistence. Mice were injected intraperitoneally (i.p.) with bacteria that had been harvested from a mid-log-phase culture grown in NBGG (see below). On days 1, 3, 6, 10, and 15, groups of five mice were sacrificed and bacteria persisting in the peritoneal cavities and spleens were enumerated (23).

(iii) Mouse mucin virulence assay. Bacteria were grown for 16 h in NBG, subcultured (1:10) into NBGG, and grown to mid-log phase (optical density at 650 nm, ca. 0.5 to 1.0). Cells were harvested by centrifugation (3,500 × *g* for 7 min), washed in saline, and suspended in saline. Bacterial concentrations were estimated by applying typical viability indices to total microscopic count. After appropriate initial dilutions in saline, each suspension was diluted 1:40 into iron-supplemented hog gastric mucin (5%, wt/vol). Doses (0.5 ml) of this final suspension were injected i.p. into mice and 50% lethal doses (LD_{50}s), based on the number of survivors after 72 h, were calculated by the method of Reed and Muench (7). Hog gastric mucin (Koch-Light) was a generous gift from David McKay, Australian National University, Canberra. A 5% (wt/vol) suspension in saline was heated at 56°C for 1 h with occasional shaking and boiled for 6 min (no contaminants were detected after such treatment). After boiling, the mucin was supplemented with ammonium ferric citrate (BDH, Poole, England) so that each mouse eventually received 5 mg of Fe^{3+} per kg of body weight (36). Finally, the mucin was neutralized (pH 7.0) with 10 M NaOH.

(iv) Comparison of vaccine potential. Fivefold serial dilutions of the strain of interest were prepared in saline and used to immunize (i.p.) groups of eight mice. Control mice received 0.5 ml of saline only. Fourteen days later, all mice were challenged with Ty2 (ca. 12,000 CFU) in Fe^{3+}-supplemented mucin, as described above. Survival data at 72 h were used to calculate the number of organisms that constituted an effective immunizing dose for 50% of the mice. This value was obtained by interpolation from a plot of cumulative percent mortality (calculated according to Reed and Muench [37]) versus immunizing dose. The LD_{50} obtained for Ty2 in this experiment was inexplicably high; survival of the control mice indicated that in this experiment the challenge dose represented 40 LD_{50}s.

Volunteer trial. (i) Volunteers. With the approval of the Ethics Committee of the Royal Adelaide Hospital, four volunteers, 24 to 34 years of age, gave their informed consent and took part in a phase 1 trial of the vaccine, designed to examine its safety and immunogenicity. Each volunteer was assessed as being normal on physical examination with no contraindication to participation being suggested by their past medical history. Hematological indices and serum biochemistry were within normal limits. As part of the information provided to the volunteers before they consented to the study, they were asked to contact one of the clinical investigators if they noticed any untoward symptoms such as fever or disturbance in bowel habit.

(ii) Administration of vaccine. Each volunteer fasted overnight and the next morning ingested 1 g of $NaHCO_3$, in 50 ml of water, followed within 10 min by 7.0×10^8 CFU of the vaccine organism, EX462, in saline. The bacteria were harvested from an exponential-phase culture in NBGG and, hence, were phenotypically smooth.

(iii) Sampling of serum and intestinal fluid. In the week preceding the trial, and on day 22 after vaccine administration, samples of serum and intestinal fluid were obtained from the volunteers to determine the antibody response to the vaccine (2). Intestinal fluid was aspirated through an intestinal tube positioned just before the ligament of Treitz, its position being confirmed by fluoroscopy. Only samples of intestinal fluid with a pH of >6.5 were retained. Samples were stored on ice until collection was complete and centrifuged at 10,000 × *g* for 30 min at 4°C, and the supernatants were stored as aliquots at −70°C until assayed.

(iv) Stool samples. Stool samples were collected daily from

each volunteer from 7 days before until 10 days after the vaccine had been ingested.

(v) Blood cultures. Blood samples (20 ml) from the febrile volunteers were mixed with an equal volume of medium (0.2% [wt/vol] glucose, 0.5% [wt/vol] peptone [Difco], 1.5% [wt/vol] tryptone [Difco], 0.5% [wt/vol] yeast extract [Difco], 0.4% [wt/vol] NaCl, 0.25% [wt/vol] NaH_2PO_4, *p*-aminobenzoic acid [50 μg/ml]) and incubated standing at 37°C for 16 h, after which culture aliquots were Gram stained or spread on nutrient agar plates with 25 mM glucose for colony isolation.

RESULTS

Comparison of *S. typhi* with its *galE* and *via* derivatives. (i) Biochemical reactions. All strains (Ty2, Ty2H1, Ty2Vi, and EX462) were tested in Microbact 12E biotyping trays (Disposable Products Pty. Ltd., Adelaide, South Australia). Their biochemical profiles were identical. When grown on M9 minimal media, Ty2 had growth requirements for cystine and tryptophan (21), as had all of its derivatives. No strain showed any additional growth requirements. The activities of the enzymes of the Leloir pathway (galactokinase, galactose-1-phosphate uridylyltransferase, and UDP-glucose-4-epimerase) were assayed (Table 2), confirming that no detectable UDP-glucose-4-epimerase activity was found in the strains carrying the *galE*-H1 mutation (Ty2H1 and EX462). This result reflects the fact that no functional *galE* gene product could be made in these strains. Also, the levels of galactokinase and galactose-1-phosphate uridylyltransferase, the gene products of *galK* and *galT*, respectively, were lower in strains Ty2H1 and EX462 than in Ty2 and Ty2Vi. Strains Ty2H1 and EX462 exhibited identical sensitivity to galactose-induced lysis, which was seen at 0.06 mM galactose, reflecting the similar expression levels of the *galT* and *galK* genes in these strains.

(ii) Outer membrane properties. The ability of the *galE* and *via* mutants to synthesize smooth LPS in the presence or absence of exogenous galactose was examined. All strains showed sensitivity to bacteriophage specific for O antigen (P22) and LPS core (Felix-O) when grown on nutrient agar supplemented with 25 mM glucose and 6 mM galactose (Table 2). If galactose was omitted from the above medium, the *galE*-H1 strains, Ty2H1 and EX462, became resistant to both P22 and Felix-O. This confirmed that Ty2H1 and EX462 were able to make smooth LPS, but only when exogenous galactose was supplied, a phenotype typical of *galE* mutants (15, 32). The minimum level of galactose required for Ty2H1 and EX462 to become sensitive to P22 was 6 μm. To quantitate the level of O-antigen expression, HA inhibition assays were performed. These data (Table 2), together with the phage resistance patterns, demonstrated clearly that the *galE*-H1 strains synthesized amounts of smooth LPS comparable to those made by the parent strain, Ty2, when exogenous galactose was supplied (Table 2).

(iii) Resistance to serum and in vivo killing. The bactericidal action of normal serum on Ty2 and its *galE* and *via* derivatives was determined following growth in the presence or absence of galactose. As previously described (11–14, 34, 38), Vi antigen played an important role in conferring resistance to the killing effect of serum (Table 2). The *galE*-H1 mutation in both Ty2H1 and EX462 also reduced the ability to survive in serum but only if the organisms were cultured in the absence of exogenous galactose; in its presence, resistance equal to that of the gal^+ equivalents (Ty2 and Ty2Vi, respectively) was obtained (Table 2). Each of the four strains (Ty2, Ty2H1, Ty2Vi, and EX462) was injected i.p. into mice, and bacterial counts were enumerated in the peritoneal cavities and spleens to day 15 postinjection (Fig. 1).

The in vivo survival of these strains demonstrated that the two mutations (*galE*-H1 and *via*) acted synergistically to decrease the ability of *S. typhi* to persist in vivo. Ty2H1 and Ty2Vi survived in the spleen as did the parental strain, Ty2, but EX462 was never recovered from this organ. Strain Ty2H1 was rapidly cleared from the peritoneum; Ty2Vi persisted longer but was cleared more rapidly than Ty2. EX462, however, was rapidly cleared in vivo, and even on day 1 postinjection no viable bacteria could be detected in the peritoneal washouts (Fig. 1). The behavior of EX462 in vivo was comparable to that of Ty21a (data not shown).

Virulence and immunogenicity in mice. (i) Virulence. Ty2 and the *galE*-H1 *via* derivative, EX462, were compared for virulence in mice when injected i.p. in 5% (wt/vol) mucin supplemented with iron (Table 3). The importance of Vi to the virulence of *S. typhi* in mice was consistent with results reported previously (11–14, 34, 38). By removing this com-

TABLE 2. Characteristics of *S. typhi* Ty2 and its derivatives[a]

Bacterial strain	Leloir enzyme activity[b]			Galactose sensitivity (mM)	Bacteriophage sensitivity			HA inhibition[c]		Serum resistance[d]	
					ViII	P22 and F-O					
	galE	*galT*	*galK*			Gal^-	Gal^+	Gal^-	Gal^+	Gal^-	Gal^+
S. typhi Ty2	45.0	13.7	21.6	R	S	S	S	7	7	25	22
Ty2H1	U	5.6	11.5	0.06	S	R	S	<1	6	2	23
Ty2Vi	42.0	15.8	18.4	R	R	S	S	7	7	4	5
EX462	U	5.4	12.3	0.06	R	R	S	<1	7	<1	3
Ty21a	U	2.0	1.2	6	R	R	S^R	<1	7	<1	<1
EX590	ND	ND	ND	R	S	S	S	7	7	22	22
EX592	ND	ND	ND	R	R	S	S	7	7	3	5
J670	U	5.4	10.8	0.06	R	R	S	<1	6	<1	<1
J671	U	5.6	13.1	0.06	R	R	S	<1	6	<1	<1

[a] F-O, Felix-O; S, senstitive; R, resistant; S^R, partial sensitivity; Gal^+ or Gal^-, presence or absence of galactose in the base medium. Glucose (25 mM) was always present.

[b] Expressed as micromoles of substrate converted per milligram of protein per hour. Galactose to a final concentration of 6 μM was used to induce the *gal* operon. U, Undetectable; ND, not done.

[c] Expressed as number of wells of HA inhibition relative to the saline control. Twofold serial dilutions of bacteria were used. Erythrocytes were coated with *S. typhi* LPS.

[d] Expressed as the percent (vol/vol) serum that caused 50% killing of the bacterial strain relative to the saline control.

ponent from Ty2 (Ty2Vi), the LD_{50} increased by a factor of 10^4 in the mouse mucin virulence assay. The *galE*-H1 mutation alone caused only a small increase (18-fold; Table 3) in LD_{50}. However, there appeared to be a synergistic increase when *S. typhi* Ty2 carried both the *galE*-H1 and *via* mutations. This synergy can be seen by comparing EX462 with its gal^+ equivalent, Ty2Vi; here the *galE*-H1 mutation resulted in a 100-fold increase in LD_{50}. That is, *galE* was five times more attenuating in mice when the Vi component was absent than when it was present. Strain Ty21a was commonly only 30% viable after growth in NBGG; consequently, the upper limit that could be assigned to its LD_{50} was restricted by the presence of nonviable bacteria which contributed to the total "endotoxin load." To determine whether the *galE*-H1 mutation was the factor responsible for increasing the LD_{50} of strains Ty2H1 and EX462, we transduced the strains to gal^+ (EX590 and EX592, respectively). Both EX590 and EX592 reverted to the full virulence of the isogenic strains Ty2 and Ty2Vi, respectively (Table 3), confirming that the *galE*-H1 mutation was the sole factor causing the LD_{50} differences seen between Ty2H1 and EX462 and their gal^+ counterparts Ty2 and Ty2Vi, respectively.

(ii) Immunogenicity. To compare the vaccine potential of Ty2 and its derivatives, an experiment was performed to determine the 50% effective immunizing dose (Table 3). The *via* derivative of Ty2, Ty2Vi, showed a significant decrease in its ability to immunize. This finding was consistent with

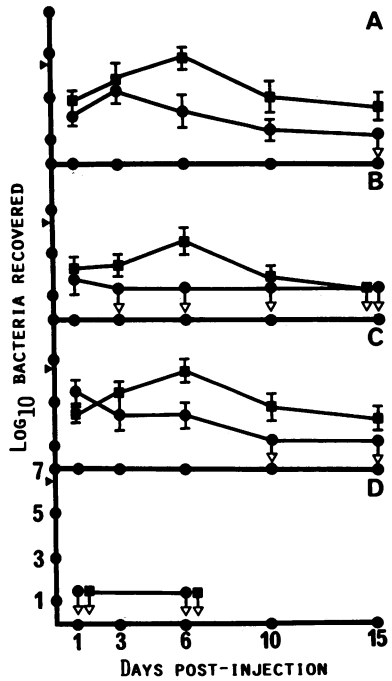

FIG. 1. Persistence of Ty2, and derived strains, in the peritoneal cavities and spleens of mice after i.p. injection. Mice (groups of 25) were injected with bacteria, grown in NBGG, and suspended in 0.9% (wt/vol) NaCl, at the dose levels per mouse indicated by arrows on the left. At each time point shown, five mice per group were sacrificed and bacterial counts in the peritoneal cavities and spleens were enumerated. Geometric mean values, and standard error bars, are shown. The limit of detection in this assay was 25 bacteria ($10^{1.39}$). Symbols: ●, peritoneal cavities; ■, spleens. The levels of bacteria per mouse injected were as follows: Ty2, 6.1×10^4; Ty2H1, 5.0×10^4, Ty2V1, 8.2×10^4, EX462, 4.7×10^6. (A) Ty2; (B) Ty2H1; (C) Ty2Vi; (D) EX462.

TABLE 3. Virulence and vaccine potential of Ty2 and its derivatives in mice

Bacterial strain	LD_{50}[a]	EID_{50}[b]
S. typhi Ty2	15	$<1.2 \times 10^3$
Ty2H1	260	$<9.0 \times 10^3$
Ty2Vi	1.8×10^5	1.0×10^5
EX462	1.6×10^7	6.5×10^5
Ty2la	$>2.2 \times 10^7$	1.5×10^7
EX590	19	ND
EX592	3.4×10^5	ND
J670	1.6×10^7	ND
J671	2.8×10^7	ND

[a] Calculated by the method of Reed and Muench (37) based on the number of survivors after 72 h.

[b] EID_{50}, 50% effective immunizing dose (see Materials and Methods). The challenge dose was 40 LD_{50}s of Ty2. ND, Not done.

previous reports that the Vi antigen contributes a significant proportion of the vaccine potential of *S. typhi* in mice (38). The 50% effective immunizing dose of EX462 was 6.5 times higher than that of Ty2Vi but was about 20 times lower than that of Ty21a. Thus, the vaccine potential of EX462 was high compared with Ty21a, a useful standard because of the known efficacy of Ty21a in humans (10, 41, 42).

Safety and immunogenicity in humans. (i) Safety. Two of the four volunteers developed an illness clinically indistinguishable from typhoid within 1 week of ingesting a single dose of 7×10^8 viable EX462 organisms. On day 4, volunteer A and, on day 5, volunteer B reported headaches which became increasingly severe along with nausea, anorexia, fever, and rigors. Both volunteers were admitted to the Royal Adelaide Hospital. Blood cultures (volunteer A, six of seven cultures tested; volunteer B, four of six cultures tested) were positive for a gram-negative bacillus identified as *S. typhi* by virtue of being positive for O groups 9 and 12, when grown on nutrient agar with glucose (25 mM) and galactose (6 mM), and H1-d^+. The isolates were Vi^- (as judged by absence of agglutination with anti-Vi antisera) and resistant to rifampin and did not grow on M9 minimal medium unless it was supplemented with cystine and tryptophan. Other investigations, including leukocyte count, hemaglobin, platelet count, serum biochemistry, and urinary microscopy, remained normal. Both patients were commenced on amoxicillin intravenously (2 g every 6 h); the fever and other symptoms responded rapidly to treatment, and on day 12 the two volunteers were discharged from hospital on oral amoxicillin (a 10-day course of 0.5 g every 6 h). Neither volunteer C nor D complained of any illness, and on day 9 physical examination was unremarkable. Twelve months later, no illness has been noted in either volunteer.

(ii) Immunogenicity. Serum and small-intestinal fluid were obtained from each of the four volunteers on day 22. These and the corresponding prevaccination samples were assayed for anti-*S. typhi* LPS antibodies by ELISA (serum and intestinal fluids) and for their bactericidal capacity against Ty2Vi (serum only; Table 4). Volunteers A and B showed marked rises in anti-*S. typhi* LPS and bactericidal activity. Subjects C and D, neither of whom was ill, also showed anti-*S. typhi* LPS responses: the most marked rises were in serum immunoglobulin A levels and bactericidal activity. Volunteer D showed a 34.8-fold rise in intestinal anti-*S. typhi* LPS immunoglobulin A.

Characteristics of bacteria isolated from blood culture. As reported above, blood cultures from each febrile patient supported growth of *S. typhi*. Four such isolates (J669 to

TABLE 4. Human immunogenicity of EX462 given orally[a]

Volunteer	Stool counts on given day[b]			Fever[c]	Serum bactericidal activity		Serum ELISA				Intestinal ELISA, IgA	
							IgA		IgG			
	1	2	3–10		Pre	Post	Pre	Post	Pre	Post	Pre	Post
A	10^5	U	U	+ (5)	760	34,000	200	10,350	42	126	60	767
B	10^6	10^3	U	+ (6)	2,300	32,000	276	4,080	71	300	87	575
C	10^6	10^4	U	−	630	9,600	503	1,760	17	161	320	1,120
D	U	U	U	−	490	5,900	5,375	33,125	33	71	200	6,960
Mean fold rise[d]					18.4		11.3		2.3		10.0	

[a] Volunteers received 1 g of $NaHCO_3$ in 50 ml of water prior to ingestion of the 7×10^8 viable EX462; fluids for assay of immune responses were obtained 22 days later. All assays were performed as described in Materials and Methods. Pre and post are pre- and postvaccination. IgA, Immunoglobulin A.

[b] Bacterial recoveries per gram of stool. U, Undetectable. The limit of detection is 100 bacteria per g of stool.

[c] + indicates that the volunteer became febrile after ingesting EX462. Number in parentheses indicates the day postvaccination on which the volunteer became ill.

[d] Geometric means of the fold rises of individual volunteers.

J672, two from each patient) were purified on nutrient agar with 25 mM glucose and stored at −70°C. Each had an identical serotype to the vaccine strain EX462. Furthermore, each displayed the same sensitivity to galactose-induced lysis (at ≥0.06 mM) and displayed an unaltered bacteriophage P22 and Felix-O sensitivity pattern (Table 2). The minimum galactose concentration required to produce a smooth phenotype was also unaltered (6 μM). The isolates were sent to the Microbiological Diagnostic Unit, Department of Microbiology, University of Melbourne, Melbourne, Australia, which confirmed that they were untypable in the Vi phage-typing system as was EX462, hence providing further evidence that these strains had not reverted to *via*$^+$. Two of the isolates (J670 and J671, one from each volunteer) were characterized further. The activities of the Leloir enzymes, serum sensitivity (Table 2), and virulence in mice (Table 3) were found to be comparable to EX462, indicating that the virulence of EX462 in humans could not be ascribed to a reversion to *gal*$^+$ or to any other detectable phenotypic change.

DISCUSSION

The only natural host of *S. typhi* is humans (4, 18). For ethical reasons, knowledge of the pathogenesis of *S. typhi* and methods of its attenuation are heavily reliant on murine models of *Salmonella* infections (18). These models have led to the belief that attenuation of *Salmonella* spp. should not be so severe as to limit its ability to invade and undergo limited growth in the gut-associated lymphoid tissue, as this capacity correlates with vaccine potential (5, 7, 16–19, 23, 30, 40). *S. typhimurium* carrying mutations in the *galE* gene evidently display the required level of attenuation in mice and other animals, being avirulent and yet protective (6, 19, 24, 43), although a *galE* strain caused fatal infections when the mice were given cyclophosphamide (43). This indicated that cellular immunity played an important role, by controling the growth of *galE* strains in vivo (43). Strain Ty21a, a *galE* mutant of *S. typhi* Ty2 made by extensive mutagenesis, was demonstrably safe and immunogenic in humans (10, 18, 41, 42).

We recently reported the construction of a defined *galE* deletion in *S. typhi* Ty2, Ty2H1 (24). This strain was only slightly attenuated in the mouse mucin virulence assay (Table 3). We sought to further attenuate Ty2H1 by removing the Vi antigen. The rifampin-resistant derivative of this Vi-deficient strain, EX462, was highly attenuated in mice (Table 3). In the presence of the Vi capsule, the *galE*-H1 mutation increased the LD_{50} only 18-fold (Table 3). However, if Vi was absent, *galE*-H1 increased the LD_{50} 100-fold (Table 3). Thus, the *galE*-H1 mutation appeared to be more attenuating when the Vi antigen was absent, suggesting that the *galE*-H1 and *via* mutations acted in synergy to reduce virulence. The synergism of the double mutation *galE*-H1 *via* was also demonstrated by the inability of EX462 to persist in vivo in mice (Fig. 1). Strain EX462 was also highly sensitive to serum (Table 2).

Ty21a was demonstrably safe at doses as high as 10^{11} (2), and since EX462 was comparable to Ty21a by all criteria we could apply (persistence and virulence in mice and serum sensitivity), we decided to assess its safety and immunogenicity in humans. Although only small numbers of volunteers were used, EX462 showed no detectable attenuation in humans. The incubation period was similar to that of wild-type *S. typhi* at a dose of 10^9 per volunteer (10, 22, 25, 28), implying that growth of EX462 is not inhibited in vivo, nor was it likely that it was being rapidly cleared. It seems unlikely that a rough Vi-negative strain could grow in vivo because of evidence (in mice) that suggests that strains of this phenotype would be rapidly cleared (16, 19, 24). Smooth Vi-negative strains have been shown previously to be virulent in humans (25), leading us to the hypothesis that EX462 was able to scavenge sufficient exogenous galactose in human tissue to synthesize smooth LPS. The organisms isolated from the febrile volunteers were shown to be identical to EX462 with respect to phenotype and mouse virulence (Tables 2 and 3). We concluded that EX462 had not reverted in vivo and that mutations other than *galE* contributed to the attenuation of *S. typhi* Ty2 in strain Ty21a.

Strains of *Salmonella cholerae-suis* and *S. typhimurium* carrying the *galE* mutation have been shown to revert to virulence if they became resistant to galactose (19, 33). However, the levels of the Leloir enzymes, and the sensitivities to galactose-induced lysis, were unaltered in the blood isolates from the febrile patients, as were the minimum levels of galactose required for the production of smooth LPS. This galactose sensitivity level was comparable to that effecting lysis of C5H1 and LT2H1, *galE*-H1 derivatives of *S. typhimurium* C5 and LT2, respectively, both of which were found to be avirulent and immunogenic in mice (24).

The lack of galactose-induced lysis in vivo in humans was attributed to either the absence of sufficient exogenous

galactose to cause lysis of EX462 or the existence of sufficient exogenous catabolites (e.g., glucose) that repressed the *gal* operon and in the presence of which EX462 became galactose tolerant.

This paper demonstrates some important points. First, models of *Salmonella* infection in mice have their limitations when being used to assist vaccine development. Second, we have confirmed earlier observations that the Vi antigen does not play a critical role in the pathogenesis of *S. typhi* in humans (25). Finally, our observations here showed that the *galE* mutation effected no detectable reduction on the pathogenic potential of *S. typhi* in humans.

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