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Salmonella enterica Serovar Typhimurium *surA* Mutants Are Attenuated and Effective Live Oral Vaccines

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A previously described attenuated Tn*phoA* mutant (BRD441) of *Salmonella enterica* serovar Typhimurium C5 (I. Miller, D. Maskell, C. Hormaeche, K. Johnson, D. Pickard, and G. Dougan, Infect. Immun. 57:2758–2763, 1989) was characterized, and the transposon was shown to be inserted in *surA*, a gene which encodes a peptidylprolyl-*cis*,*trans*-isomerase. A defined *surA* deletion mutation was introduced into *S. enterica* serovar Typhimurium C5 and the mutant strain, named *S. enterica* serovar Typhimurium BRD1115, was extensively characterized both in vitro and in vivo. *S. enterica* serovar Typhimurium BRD1115 was found to be defective in the ability to adhere to and invade eukaryotic cells. Furthermore, *S. enterica* serovar Typhimurium BRD1115 was attenuated by at least 3 log units when administered orally or intravenously to BALB/c mice. Complementation of the mutation with a plasmid carrying the intact *surA* gene almost completely restored the virulence of BRD1115. In addition, *S. enterica* serovar Typhimurium BRD1115 demonstrated potential as a vaccine candidate, since mice immunized with BRD1115 were protected against subsequent challenge with *S. enterica* serovar Typhimurium C5. *S. enterica* serovar Typhimurium BRD1115 also showed potential as a vehicle for the effective delivery of heterologous antigens, such as the nontoxic, protective fragment C domain of tetanus toxin, to the murine immune system.

Modern genetic approaches, involving random or gene-targeted mutagenesis, can be used to identify genes required for the survival and replication of bacterial pathogens in infected mammalian hosts. One of the most intensively studied of these pathogens is *Salmonella enterica* serovar Typhimurium. This bacterium is highly amenable to genetic manipulation, and a very well characterized animal model exists which facilitates the analysis of mutant derivatives (6). To date, around 100 *S. enterica* serovar Typhimurium genes have been associated with virulence in various animal and in vitro model systems (12, 13, 22). These mutations can be classified according to their assigned gene functions, such as auxotrophy, regulation of gene expression, or secretion systems (10, 13, 14, 30). *Salmonella* has also attracted interest because of the potent immunogenicity of live vaccines based on these microorganisms.

Any novel live vaccine, including those based on *Salmonella*, should ideally harbor only fully defined, stable mutations which are responsible for the attenuated phenotype (3, 15, 32, 37–39). However, not all mutations that attenuate *Salmonella* result in protective vaccine strains. For example, some are not sufficiently attenuated for safe administration, while others are so attenuated that they are unable to induce protection against virulent *Salmonella* challenge (30, 37). Among the most comprehensively studied attenuating lesions are those in genes encoding enzymes from biosynthetic pathways. Several different auxotrophic mutants, including *aroA* and *purA* mutants, have been evaluated as live *Salmonella* vaccines in various animal species (7, 16, 18, 21, 29), and some have proven to be excellent live oral vaccines (4, 5, 39, 42, 43). In a search for further attenuating lesions, Miller et al. screened a bank of *S. enterica* serovar Typhimurium C5 Tn*phoA* mutants and described six mutants which were highly attenuated in mice after administration by both oral and intravenous routes (24).

In this paper we describe further investigations on one of these mutants, BRD441, which is highly attenuated following oral or intravenous inoculation in mice. The site of the transposon insertion was determined to be in the *surA* gene, located on the *Salmonella* chromosome upstream of *pdxA* (whose product is involved in the biosynthesis of vitamin B_6) and *ksgA* (encoding the modification enzyme 16S rRNA methyltransferase; mutation of this gene confers kasugamycin resistance) (1). We have constructed a defined deletion mutation in *surA* and characterized both in vitro and in vivo phenotypes of the mutant, including the ability to adhere to and invade epithelial cells and the degree of attenuation and persistence in vivo. We also investigated the ability of the mutant to protect against virulent challenge with the parental strain and to deliver a heterologous antigen to the murine immune system.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. enterica* serovar Typhimurium C5 is a fully mouse-virulent strain (17, 24). BRD441 is a previously described attenuated *S. typhimurium* C5::Tn*phoA* derivative (24). Bacteria were routinely cultured at 37°C on Luria-Bertani (LB) agar or in LB broth containing 100 μg of ampicillin per ml or 50 μg of kanamycin per ml where appropriate. All strains used in this study are described in Table 1.

Identification of the Tn*phoA* insertion site in BRD441. Unless otherwise stated, all DNA manipulations, including Southern blotting, were carried out as described by Sambrook et al. (34). Restriction enzymes, T4 DNA ligase, and *Taq* DNA polymerase were purchased from Boehringer Mannheim (Lewes, United Kingdom) and used as specified by the manufacturer. Chromosomal DNA, prepared from BRD441, was partially cleaved using *Sau*3A, and a cosmid library was constructed using the vector pHC79 (34). The cosmid library was screened by colony blotting using DNA probes complementary to the 5′ and 3′ ends of Tn*phoA* transposon (5′GTAATATCGCCCTGAGC; 3′ATATTCTGGTAAATCAATAACCATCTCATC). Cosmids exhibiting homology to the probes were isolated and digested with a number of restriction enzymes. A 3.3-kb *Eco*RI fragment which hybridized to the 5′ probe and a *Pst*I 5.5-kb fragment which

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
C5	Mouse-virulent strain	15
BRD441	Tn*phoA* mutant of C5	21
BRD1115	Δ*surA* mutant of C5	This study
BRD1115 (pLG339/*surA*)	Complemented *surA* strain	This study
LB5010	Intermediate strain	1
Plasmids		
pBluescriptII SK(+)	Commercial cloning vector	Stratagene
pGP704	Suicide vector	22
pGEM-T	Commercial cloning vector	Promega
pGEM-T/212/213	pGEM-T vector containing intact *surA* gene	This study
pGEM-T/Δ*surA*	pGEM-T vector containing *surA* gene with deletion	This study
pLG339	Low-copy-number vector	9
pTET*nir*15	Fragment C under the control of the *nirB* promoter	3
pTET*htrA*	Fragment C under the control of the *htrA* promoter	28

hybridized to the 3′ probe were subcloned into the vector pBluescriptII SK(+) (Stratagene, Maidstone, United Kingdom). Positive clones were then sequenced by double-stranded plasmid sequencing with the Sequenase kit (United States Biochemical Corporation). The nucleotide sequences immediately flanking the transposon insertion were determined using the Tn*phoA*-specific probes above. To complete the sequence of the entire open reading frame, oligonucleotide primers homologous to sequences already obtained were designed. The nucleotide sequence obtained was compared to known sequences in GenBank by using the BlastX search routine.

Construction of an *S. enterica* serovar Typhimurium C5 derivative harboring a defined null deletion in the *surA* gene. To construct a fully defined *surA* deletion mutation in *S. enterica* serovar Typhimurium C5, a DNA fragment encompassing the entire *surA* gene and flanking region was amplified from chromosomal DNA using PCR and cloned into the plasmid pGEM-T; this plasmid was named pGEM-T/212/213. Reactions were carried out with *Taq* DNA polymerase using the GeneAmp kit (Perkin-Elmer Cetus, Oakland, Calif.) as specified by the manufacturer. Cleavage of the resulting plasmid with the enzymes *Hpa*I and *Sma*I generated a 5.5-kb DNA fragment encoding the vector, the remaining *surA*-associated 5′ and 3′ regions, and flanking sequences. This DNA fragment was gel purified and religated, and the resulting plasmid, harboring a 419-bp deletion within the *surA* gene, was designated pGEM-T/Δ*surA*. The plasmid was then cleaved with *Sph*I and *Sal*I, and a 2.6-kb DNA fragment containing the deleted *surA* gene was gel purified and ligated to the suicide replicon pGP704 (27). pGP704/Δ*surA* was then used to transform the *recA S. enterica* serovar Typhimurium strain LB5010 (2). Clones in which successful single-crossover events had occurred were identified by selection with ampicillin. This single-crossover event was moved from LB5010 into *S. enterica* serovar Typhimurium C5 by P22 transduction (25) using ampicillin resistance as a selection marker. To encourage completion of the recombination event, a single ampicillin-resistant clone was selected and this transductant was subcultured for 48 h without antibiotic selection. Aliquots of this culture were then inoculated in parallel onto plates with or without ampicillin. Loss of ampicillin resistance is associated with a successful second homologous recombination event. A single ampicillin-sensitive clone was selected from over 500 colonies plated. The presence of the *surA* deletion on the *S. typhimurium* C5 chromosome was confirmed by Southern blotting using the entire 3-kb coding region for *surA*. This defined mutant was designated BRD1115.

Complementation of the *surA* mutation. A 3-kb fragment of plasmid pGEM-T/212/213, containing the intact *surA* gene and flanking sequences, was cloned into the *Sph*I and *Sal*I sites of the low-copy-number plasmid pLG339 (36) to create plasmid pLG339/*surA*. This plasmid was electroporated into BRD1115. The resultant strain was kanamycin resistant and was named BRD1115(pLG339/*surA*).

Determination of the plasmid stability of BRD1115(pLG339/*surA*). Plasmid stability was determined in vitro by growth and subculture of the bacteria in broth cultures in the absence of antibiotic. After each passage, the number of bacteria retaining the plasmid was determined by plating diluted bacterial cultures on parallel LB agar plates containing or lacking ampicillin. Stability was assessed by calculating the number of bacteria able to grow on ampicillin compared to the total number of bacteria in the culture.

Introduction of genes encoding heterologous antigen into BRD1115. Two plasmids encoding fragment C of tetanus toxin (TETC) were introduced into BRD1115 by electroporation. The plasmids used were pTET*nir*15, in which transcription of the TETC protein is under the control of the *nirB* promoter (4), and pTET*htrA*, in which transcription is under the control of the *htrA* promoter (32). The stability of these plasmids in vitro in the absence of antibiotic selection was determined as above. The expression of TETC from each of these constructs was confirmed by Western blotting.

In vitro characterization of vaccine strains. The structural integrity of the lipopolysaccharide of all strains was determined by several methods including agglutination with typing antisera (O:4 and O:5; Murex Diagnostics, Dartford, United Kingdom) and silver staining (41). Bacteria were assayed for survival of bacteria in the stationary phase. In brief, bacteria were inoculated into LB broth and left at 37°C in continuous culture for 10 days. Bacterial viability (23) and optical density were assessed daily during that time. The kasugamycin sensitivity of the *S. enterica* serovar Typhimurium *surA* mutants was monitored by plating on LB agar containing 100 μg of kasugamycin per ml. The prototrophy of the strain was determined by growth on minimal agar plates.

HEp-2 invasion assay. Adhesion and invasion of HEp-2 cells by *S. enterica* serovar Typhimurium C5, BRD1115, and BRD1115(pLG339/*surA*) were determined using standard gentamicin resistance assays (35). The multiplicity of infection was approximately 300:1, and the results shown represent an average of three experiments in which triplicate wells were infected with each strain.

In brief, shaken overnight cultures were used to infect semiconfluent monolayers of HEp-2 cells. Each well was infected with approximately $2 \times \log_{10}7$ bacteria/well. The bacteria were centrifuged onto the cells at $250 \times g$ for 5 min, and the plates were then incubated at 37°C in 5% CO_2 for 2 h. The monolayers were washed five times with Dulbecco's phosphate-buffered saline (DPBS) to remove extracellular bacteria. For measurement of adhesion, cells were lysed by the addition of 0.5 ml of 0.1% Triton X-100. Viable counts were then calculated by serial dilution of cultures followed by plating for single colonies on LB agar plates. To determine cellular invasion, medium containing gentamicin (100 μg/ml) was added to the wells and cells were incubated for a further 90 min before being subjected to lysis and harvesting of intracellular bacteria. The number of adherent bacteria was determined by subtracting the number of gentamicin-resistant bacteria from the total number of bacteria recovered before the addition of gentamicin. Invasion and adhesion levels were compared with those determined for *S. enterica* serovar Typhimurium C5 within the same assay.

Infection of mice. Female BALB/c mice, 6 to 8 weeks of age, were obtained from Harlan/Olac. Mice were immunized with PBS-washed, overnight statically grown cultures of *S. enterica* serovar Typhimurium. The number of organisms given to the mice was estimated by measurement of optical density, while the actual number of viable bacteria was determined by viable counting. For oral immunizations, the mice were lightly anesthetised with a mixture of halothane and oxygen and the bacteria were administered by gavage in a 0.2-ml volume. For intravenous (i.v.) immunization, 0.2-ml volumes were injected into the tail vein of each mouse through a 27-gauge needle. The oral and i.v. 50% lethal doses (LD_{50}s) of the *S. enterica* serovar Typhimurium strains were calculated using the susceptible mouse strain BALB/c. Five mice per group were inoculated either orally or i.v. with serial 10-fold dilutions of bacteria with doses ranging from 4 to 10 $\log_{10}$ units orally and 1 to 5 log units i.v. Deaths were recorded over 28 days, and LD_{50}s were determined (31).

Persistence of *S. enterica* serovar Typhimurium in murine organs. To determine the persistence of *S. typhimurium* strains in vivo, groups of BALB/c mice were orally immunized with 8 log units of organisms. At various times postinfection, mice were sacrificed by cervical dislocation. Spleens, livers, mesenteric lymph nodes, and Peyer's patches were removed asceptically and homogenized in 10 ml of sterile PBS by using a stomacher (Colworth, Northamptonshire, United Kingdom). Dilutions of these homogenates were plated out to determine viability.

Protection of mice against homologous challenge with virulent *S. enterica* serovar Typhimurium C5. BALB/c mice were orally immunized as described above with 8 log units of BRD1115 and challenged with *S. enterica* serovar Typhimurium C5 at 4 and 10 weeks postimmunization using several different challenge doses from 4 to 10 log units of bacteria; an oral LD_{50} was calculated.

Determination of antibody titers against TETC in mice following immunization with BRD1115 vaccine derivatives. Groups of 10 mice were immunized orally with 8 log units of either *S. typhimurium* BRD1115 alone, BRD1115 (pTET*nirB*), or BRD1115(pTET*htrA*). The immune response to TETC was monitored by sample bleeds taken on days 0 and 28. These samples were analyzed for total antibodies against TETC by enzyme-linked immunosorbent assay as previously described (32), using 96 well enzyme immunoassay/radioimmunoassay plates. Absorbance values were read at 490 nm, and titers were calculated. Preimmune samples were used to determine the background level response. The anti-TETC titers were determined as the highest dilution giving an absorbance of 0.3 unit above that obtained with the preimmune sera.

Tetanus toxin challenge. Mice immunized as above with BRD1115 derivatives were challenged subcutaneously 4 weeks postimmunization with 0.05 μg ($50LD_{50}$) of purified tetanus toxin as previously described (11), and fatalities were recorded for 4 days.

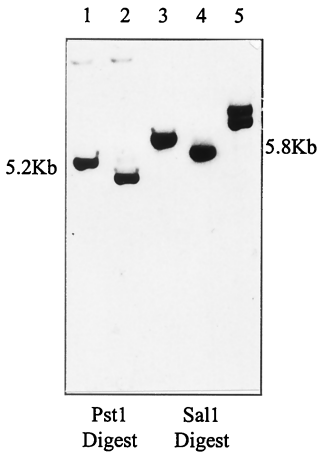

FIG. 1. Southern blot analysis of whole-cell DNA from *S. enterica* serovar Typhimurium C5 and *surA* mutant derivatives probed with a 3-kb DNA fragment encoding the entire *surA* open reading frame. Lanes: 1, *S. enterica* serovar Typhimurium C5 cleaved with *Pst*I; 2, *S. enterica* serovar Typhimurium BRD1115 cleaved with *Pst*I; 3, *S. enterica* serovar Typhimurium C5 cleaved with *Sal*I; 4, *S. enterica* serovar Typhimurium BRD1115 cleaved with *Sal*I; 5, *S. enterica* serovar Typhimurium BRD441 cleaved with *Sal*I.

RESULTS

Identification of the insertion site of Tn*phoA* in BRD441. BRD441 was originally identified as an attenuated Tn*phoA* mutant of *S. enterica* serovar Typhimurium C5 by using an oral screen for virulence in mice (24). Sequencing of the DNA around the site of transposon insertion and comparison with existing databases showed the transposon had inserted into the 3′ end of an open reading frame with 90.2% protein homology to the *Escherichia coli* stationary-phase survival gene, *surA* (20, 28). The putative *S. enterica* serovar Typhimurium C5 *surA* open reading frame was found to be 1,281 bp in length, encoding a protein of 427 amino acids with a predicted molecular mass of 47.2 kDa. The protein contained a potential leader peptidase cleavage site, indicating that it may be a secreted product.

Construction of an *S. enterica* serovar Typhimurium C5 derivative, BRD1115, harboring a null deletion mutation in *surA*. Analysis of the *surA* gene in *S. enterica* serovar Typhimurium C5 revealed the presence of unique *Hpa*I and *Sma*I restriction enzyme sites. These sites were used to generate a defined deletion of 419 bp in the *surA* gene. The presence of the deletion in *surA* was confirmed using Southern blotting, as shown in Fig. 1. *Pst*I and *Sal*I cleaved genomic DNA from *S. enterica* serovar Typhimurium C5, and BRD1115 was probed with a 3-kb DNA fragment containing the intact *surA* gene, with flanking sequences, from *S. enterica* serovar Typhimurium C5. A single DNA fragment from both strains was found to hybridize with this sequence. As expected, the DNA fragment generated by restriction digestion of BRD1115 was approximately 400 bases smaller than that seen in *S. enterica* serovar Typhimurium C5, indicating successful deletion of the internal *surA* sequence. In contrast, genomic DNA from BRD441, when cleaved with *Sal*I, hybridized to two DNA fragments.

Characterization of BRD441 and BRD1115 in vitro. BRD441 and BRD1115 were characterized in a variety of in vitro assays. The presence of intact, smooth LPS was confirmed by agglutination with anti-*Salmonella* O-antigen-specific typing sera and silver staining of sodium dodecyl sulfate-polyacrylamide gels. In *E. coli*, *surA* forms part of an operon with *pdxA* (whose product is involved in biosynthesis of vitamin B_6) and *ksgA* (encoding kasugamycin sensitivity) immediately downstream (40). These genes were also shown to flank the *S. enterica* serovar Typhimurium C5 *surA* gene (our unpublished results). To confirm that the *surA* mutation in BRD441 and BRD1115 was not exerting significant polar effects on expression of genes downstream of *surA*, the phenotypes of BRD441 and BRD1115 were investigated. Both strains were found to be prototrophic and kasugamycin sensitive, suggesting that the polarity of the mutations on these downstream genes was not significant. The growth of BRD441 and BRD1115 in broth cultures appeared consistently slightly slower than that of the wild type by 20 to 40%. However, BRD441 and BRD1115 consistently survived as well as did *S. enterica* serovar Typhimurium C5 in long-term stationary-phase survival assays. This is a different phenotype from that reported for *E. coli surA* mutants, which were described as being unable to survive in stationary phase (40).

HEp-2 invasion assay. The ability of BRD1115 and BRD441 to invade the epithelial cell line HEp-2 in vitro was assessed (Fig. 2). There was a consistent 10-fold reduction in the invasion levels of both BRD441 and BRD1115 compared to *S. enterica* serovar Typhimurium C5. This reduction in invasion may in part be explained by the reduced ability of BRD441 and BRD1115 to adhere to these cells.

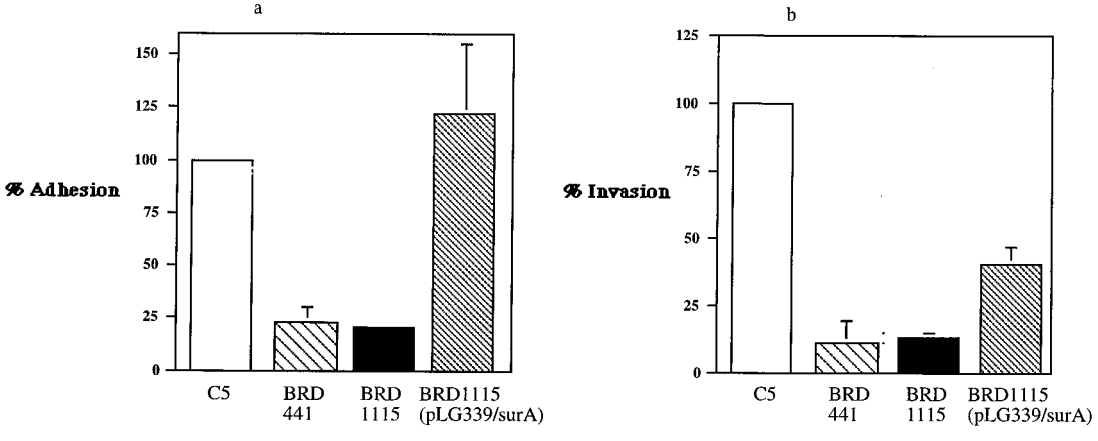

FIG. 2. Adhesion and invasion of HEp-2 cells by mutants of *S. enterica* serovar Typhimurium C5. (a) Percent adhesion of *S. enterica* serovar Typhimurium C5 (□), BRD441 (▨), BRD1115 (■), and BRD1115(pLG339) (▨). (b) Percent invasion of *S. enterica* serovar Typhimurium C5 (□), BRD441 (▨), BRD1115 (■), and BRD1115(pLG339/*surA*) (▨). Error bars represent the standard deviation of the mean percent adhesion or invasion between three independent experiments.

TABLE 2. LD_{50}s determined following oral and i.v. challenge of BALB/c mice with *S. enterica* serovar Typhimurium C5, *surA* mutants of C5, or the *surA* mutant strain complemented with an intact copy of the gene

Strain	Oral LD_{50} (log_{10} CFU)	i.v. LD_{50} (log_{10} CFU)
C5	4.16	<1.87
BRD441	8.62	2.46
BRD1115	8.98	5.22
BRD1115(pLG339/*surA*)	4.17	ND[a]

[a] ND, not determined.

Complementation analysis of the *surA* mutation in BRD1115. In an effort to assess the ability of a wild-type *surA* gene to complement the *surA* mutation in BRD1115, a full-length copy of the gene, carried on plasmid pLG339/*surA*, was introduced into BRD1115. pLG339/*surA* was relatively stable (cultures were over 80% plasmid positive after 72 h) even in the absence of antibiotic selection. BRD1115(pLG339/*surA*) was equally as adherent to HEp-2 cells as was *S. enterica* serovar Typhimurium C5, while invasiveness was restored to only 40% of that observed with the wild-type strain.

Properties of *S. enterica* serovar Typhimurium BRD441 and BRD1115 in mice. The oral and i.v. LD_{50}s of *S. enterica* serovar Typhimurium C5, BRD441, and BRD1115 in BALB/c mice were calculated (Table 2). Both BRD441 and BRD1115 were highly attenuated in vivo compared to *S. enterica* serovar Typhimurium C5. BRD1115 was attenuated by nearly 5 log units orally and almost 3.5 log units i.v. compared to *S. enterica* serovar Typhimurium C5. BRD441 showed 4.5 log units of attenuation orally. In contrast, the complemented strain BRD1115 (pLG339/*surA*) was as virulent as *S. enterica* serovar Typhimurium C5 was for mice (Table 2).

Unlike *S. enterica* serovar Typhimurium C5, which colonizes internal organs in large numbers, resulting in death within a few days, mice challenged orally with 10^8 CFU of BRD1115 or BRD441 survived. Bacteria were found persisting at low levels within all tissues monitored, including Peyer's patches, mesenteric lymph nodes, livers, and spleens, for up to 42 days after initial oral infection (Fig. 3).

Protection of mice immunized with BRD1115 against virulent *Salmonella* challenge. To determine whether BRD1115 might be an effective vaccine strain, groups of BALB/c mice were orally immunized with 8 log units of BRD1115 and challenged with *S. enterica* serovar Typhimurium C5 at 4 and 10 weeks postinoculation. The mice were challenged with be-

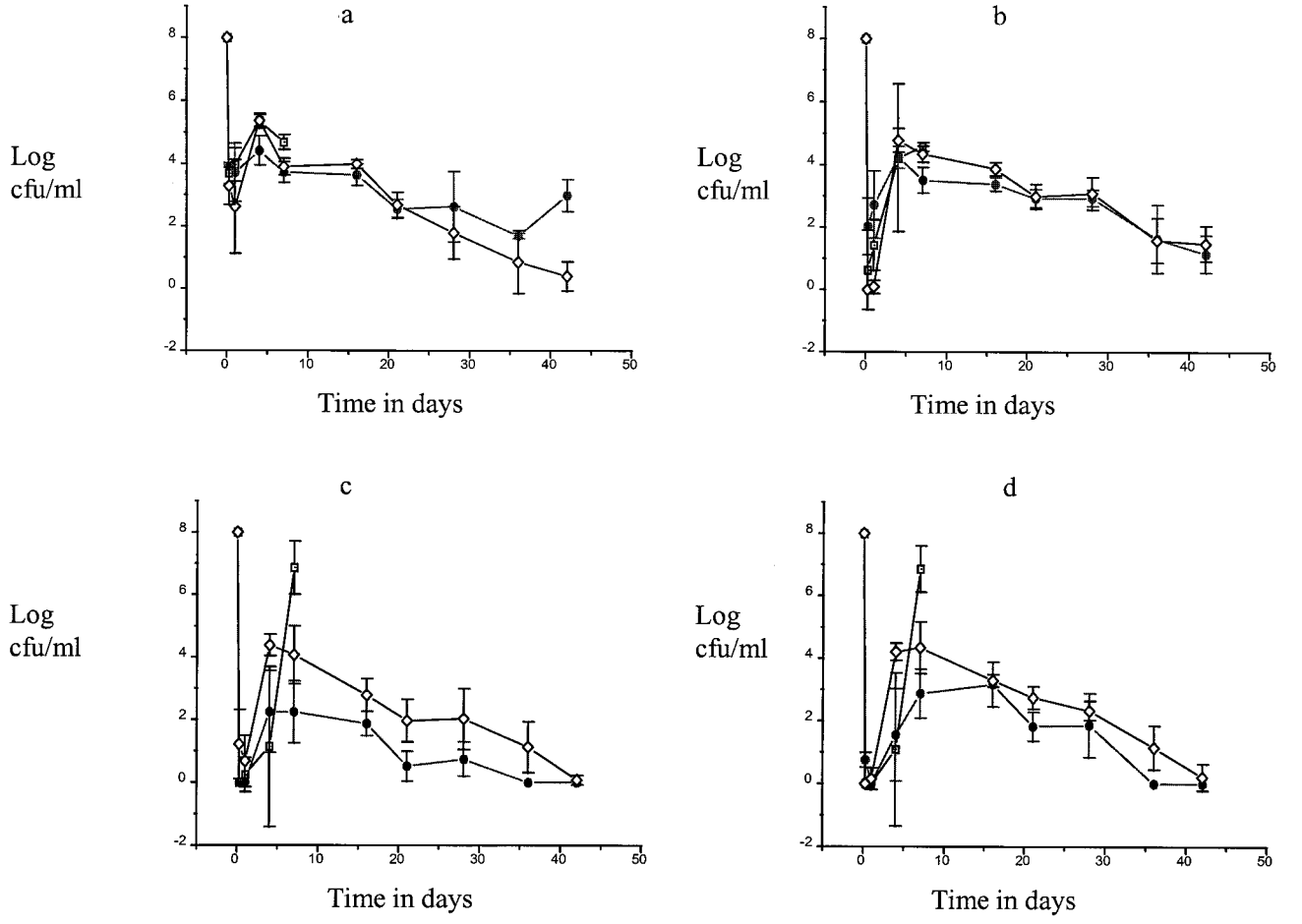

FIG. 3. Persistence of *S. enterica* serovar Typhimurium strains in the tissues of animals following oral challenge with $log_{10}8$ CFU/dose. Mice were challenged with *S. enterica* serovar Typhimurium C5 (□), BRD1115 (●), or BRD441 (◇). Each time point indicates the mean number of bacteria recovered from the organs of four mice. The errors indicate the standard deviation of the mean of these counts. (a) Bacterial counts in Peyer's patches; (b) counts in mesenteric lymph nodes; (c) counts in the liver; (d) counts in the spleens.

TABLE 3. Protection conferred by the *S. enterica* serovar Typhimurium *surA* deletion mutant against subsequent challenge with the virulent parental C5 strain

Strain used for immunization	Log_{10} oral LD_{50}s following C5 challenge	
	4 wk postimmunization	10 wk postimmunization
BRD1115	8.58	9.51
None (mock infected)	4.74	4.68
Protection	3.8	4.8

tween 4 and 10 log units of viable bacteria, and an oral LD_{50} of protection was calculated. The levels of protection are presented in Table 3. These results show that even 10 weeks postvaccination, mice were resistant to challenge with the equivalent of nearly 5 log_{10} LD_{50}s of the virulent parental *S. typhimurium* C5 strain.

Ability of BRD1115 to deliver heterologous antigen to the murine immune system. The potential of BRD1115 to act as a carrier strain for heterologous antigen was determined using TETC as a model antigen. Two plasmids encoding TETC (pTET*nir*15 [4] and pTET*htrA* [32]) were independently introduced into BRD1115. In these plasmids, TETC expression is under the control of promoters activated by either anaerobiosis (*nirB*) or heat shock (*htrA*). The stability of the plasmids in vitro was determined, and both plasmids were maintained in vitro at levels greater than 90% over a period of continual growth in the absence of antibiotic selection (a period equivalent to over 30 generations). Expression of antigen was monitored by Western blot analysis. Figure 4 shows that the highest level of TETC expression was observed in BRD1115 (pTET*nir*15) under anaerobic conditions and in BRD1115 (pTET*htrA*) at 42°C. These promoters do show some leakiness, since low levels of expression of TETC were also observed under noninducing conditions. No TETC polypeptide was detected when BRD1115 was included as a negative control.

To investigate the immunogenicity of these strains, groups of 10 BALB/c mice were orally immunized with 8 log units of BRD1115, BRD1115(pTET*nir*15), or BRD1115(pTET*htrA*). The titers of anti-TETC antibodies present in the serum of each animal was determined on day 28 by ELISA (Fig. 5).

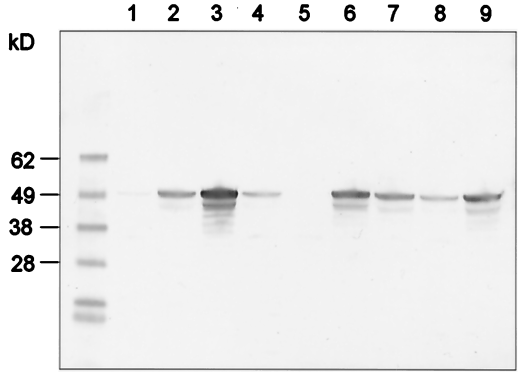

FIG. 4. Expression of TETC in BRD1115(pTET*nir*15) and BRD1115 (pTET*htrA*). Lanes 1 to 5 show induction of fragment C expression by strain BRD1115(pTET*nir*15) at 30°C (lane 1), at 42°C (lane 2), under anaerobic conditions (lane 3), and under aerobic conditions (lane 4) and induction by BRD1115 control. Lanes 6 to 9 show induction of TETC expression by BRD1115 (pTET*htrA*) under anaerobic conditions (lane 6), under aerobic conditions (lane 7), at 30°C (lane 8), and at 42°C (lane 9).

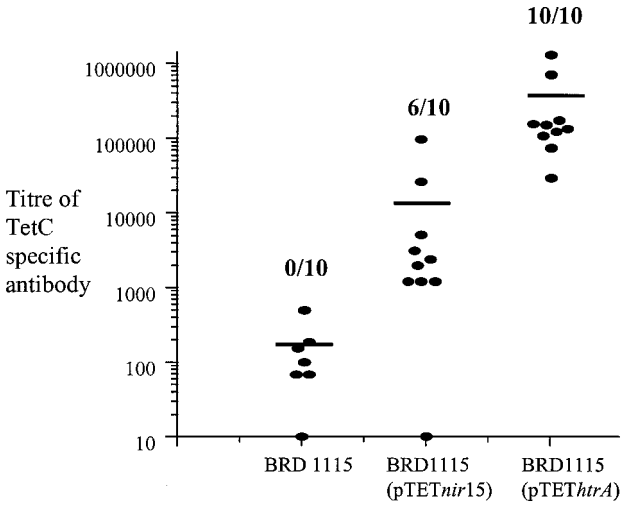

FIG. 5. Titer of TETC-specific antibody by ELISA in mice immunized with strain BRD1115, BRD1115(pTET*nir*15) or BRD1115(pTET*htrA*). Titers of anti-TETC immunoglobulin detected in individual immunized mice are represented by a single spot. The numbers given above each group indicate the number of mice surviving challenge with $50LD_{50}$s of tetanus toxin as a fraction of the total number.

Mice immunized with either BRD1115(pTET*nir*15) or BRD1115(pTET*htrA*) showed significant levels of anti-TETC antibodies in their serum. These titers were 2 to 3 log units higher than those seen in mice immunized with BRD1115 alone. Interestingly, mice immunized with BRD1115(pTET*htrA*) had, in general, a higher and more consistent titer of anti-TETC antibodies in their sera than did mice immunized with BRD1115(pTET*nir*15). At 4 weeks postimmunization, these mice were challenged subcutaneously with the equivalent of $50LD_{50}$ of tetanus toxin, and deaths were noted. Immunization with BRD1115(pTET*htrA*) conferred 100% protection against tetanus, whereas mice immunized with BRD1115 alone were unable to survive the challenge. In contrast 6 of 10 mice immunized with BRD1115 (pTET*nir*15) survived challenge.

DISCUSSION

Salmonella pathogenicity depends on the coordinated expression in vivo of a vast array of virulence genes and their products, many of which are exported from the bacterial cell. Exported proteins may be involved in interactions with host cells in vivo. To identify genes in this class, we previously employed the Tn*phoA* transposon to identify *S. enterica* serovar Typhimurium C5 derivatives which are substantially attenuated in mice by the oral route of infection (24). In this study, we have identified the interrupted gene for one of these transposon mutants, BRD441, as *surA*, a homologue of which has already been described in *E. coli* (20, 28, 33). Since we were interested in investigating the possibility of using novel attenuating lesions in *Salmonella* live vaccine strains, we characterized the properties of *S. enterica* serovar Typhimurium *surA* mutants in vitro and in vivo. An *S. enterica* serovar Typhimurium C5 derivative harboring a defined *surA* deletion mutation, named BRD1115, was characterized alongside the original transposon insertion mutant, BRD441. Both BRD441 and BRD1115 displayed similar phenotypes in all in vivo and in vitro assays employed. The observed attenuated phenotype of BRD441 and BRD1115 could be due to polar effects on the expression of downstream genes, including *pdxA* and *ksgA*.

However, the expression of *pdxA* and *ksgA* was not detectably affected in phenotypic assays. Furthermore, we were able to restore the virulence of BRD1115 by complementation with the wild-type *surA* gene.

Early reports on *E. coli surA* defined an essential role for the protein in survival of the bacterium in late-stationary-phase cultures (40). This growth phase has often been approximated to growth in vivo, where nutrient is limiting and the oxygen tension around the bacteria is low. Neither BRD441 nor BRD1115 showed any defects in stationary-phase survival. However, it was later reported that a second mutation within the *rpoS* gene existed in the original *surA* strain. It is possible that the combination of the mutations was responsible for the inability of the bacteria to survive in stationary-phase culture (19). *E. coli surA* has now been shown to belong to a third family of peptidylprolyl-*cis*,*trans*-isomerases (9, 33). These enzymes catalyze isomerization around the omega angle of prolyl bonds in nascent proteins and thus contribute to their correct protein folding. It is possible that the *surA* gene product plays a critical role in folding key proteins directly involved in host-pathogen interactions during the infection process.

The influence of *surA* mutations on *S. enterica* serovar Typhimurium invasion of epithelial cells in vitro was investigated. The invasiveness of BRD441 and BRD1115 for HEp-2 cells was consistently 10-fold lower than that of *S. enterica* serovar Typhimurium C5. There was also a comparable decrease in adhesiveness of BRD1115 to HEp-2 cells, but at this stage we do not know which other steps in the invasion process are affected. Although in vitro assays can offer some insights into possible effects of the *surA* mutation on loss of virulence in vivo, they may not fully explain the degree of attenuation observed. For example, BRD1115 was highly attenuated via both the oral and i.v. routes of challenge. We were able to complement loss of virulence associated with BRD1115 by using the wild-type *surA* gene carried on a low-copy-number plasmid, again confirming that the attenuated phenotype was associated with the lesion in *surA*. In most cases, including studies on in vitro growth rates and LD_{50}s, the complementation was complete. However, although the adhesion of complemented strains to epithelial cells was fully restored to wild-type levels, the invasiveness of complemented strains was only 40% of the level seen with the parental strain. At present we do not know why this is the case, but it could be linked to the increased copy number of *surA* in the complemented strains.

Preliminary studies with BRD441 identified this strain as a promising live vaccine candidate, since it was both highly attenuated and persistent in vivo following oral inoculation. In this study, we have confirmed these initial observations with BRD441 and extended them to the defined strain BRD1115. Both BRD1115 and BRD441 have LD_{50}s which are more than 4.5 log units higher than the wild-type parent following oral inoculation. Following an initial dose of 8 log units of organisms, bacteria persisted for about 40 days in vivo but were eventually cleared. Furthermore, BRD1115 was able to effectively immunize and protect mice against oral challenge with virulent *Salmonella*; we recorded almost 5 log units of protection 10 weeks after immunization.

A number of live bacterial vaccines have been used as carriers of heterologous antigens, thereby potentially providing protection against more than one disease organism. We wanted to determine whether the *surA* mutant might prove a useful basis for a multivalent vaccine. We expressed the protective antigen, TETC, in BRD1115 under the control of two different, differentially regulated promoters, *htrA* and *nirB*. This antigen has previously been shown to protect against lethal challenge with tetanus toxin (11). We found that the protein was expressed in BRD1115 from both promoters, with optimal expression under inducing conditions from the *htrA* promoter. *S. typhimurium surA* harboring either plasmid elicited good systemic antibody responses from immunized mice, although again we obtained better titers after immunization with the *htrA* promoter plasmid, perhaps reflecting enhanced expression of the protein in vivo. We were encouraged to find that following lethal challenge, all of the mice immunized with the *htrA* construct were fully protected.

The current literature on the identification of potential attenuating lesions in *Salmonella* has become extensive. A number of different attenuating mutations have been identified, including those involved with aromatic amino acid biosynthesis (*aroA*, *aroC*, and *aroD*) (14, 15, 37), purine metabolism enzymes (*purA*, *purE*) (30), regulatory proteins (*ompR*, *crp*/*cya*) (8, 10), heat shock proteins (*htrA*) (39), and two-component regulator systems (*phoP*/*phoQ*) (26). However, of all the mutations tested to date, the *aro*-based attenuations still provide the "gold standard," since these have proved to be the most successful when tested in humans. Interestingly, the level of protection against challenge with the wild-type strain, following a single oral immunization with the *surA* mutant, was comparable to levels we have reported for *S. enterica* serovar Typhimurium *aro* vaccines (30). In addition, the immune response to the carried antigen was similar to that observed when a double *aroA aroD* mutant of *S. enterica* serovar Typhimurium was used as the carrier strain (32).

In conclusion, *S. enterica* serovar Typhimurium *surA* mutants are clearly promising single-dose, oral *Salmonella* vaccines that may also prove useful for delivery of heterologous antigens to the mammalian immune system. As such, they may provide useful alternative attenuating lesions in the development of new and improved human and veterinary vaccines.

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REFERENCES

1. **Andresson, O. S., and J. E. Davies.** 1980. Isolation and characterisation of lambda transducing phages for the *E. coli* genes *ksgA* and *pdxA*. Mol. Gen. Genet. **179:**201–209.
2. **Bullas, L. R., and J.-I. Ryu.** 1983. *Salmonella typhimurium* LT2 strains which are r^- m^+ for all three chromosomally located systems of DNA restriction and modification. J. Bacteriol. **156:**471–474.
3. **Charles, I., and G. Dougan.** 1990. Gene expression and the development of live enteric vaccines. Trends Biotechnol. **8:**117–121.
4. **Chatfield, S. N., I. G. Charles, A. J. Makoff, M. D. Oxer, G. Dougan, D. Pickard, D. Slater, and N. F. Fairweather.** 1992. Use of the *nir*B promoter to direct the stable expression of heterologous antigens in *Salmonella* oral vaccine strains: development of a single-dose oral tetanus vaccine. Bio/Technology **10:**888–892.
5. **Chatfield, S. N., K. Strahan, D. Pickard, I. G. Charles, C. E. Hormaeche, and G. Dougan.** 1992. Evaluation of *Salmonella typhimurium* strains harbouring defined mutations in *htrA* and *aroA* in the murine salmonellosis model. Microb. Pathog. **12:**145–151.
6. **Collins, F. M.** 1974. Vaccines and cell-mediated immunity. Bacteriol. Rev. **38:**371–402.
7. **Cooper, G. L., L. M. Venables, R. A. Nicholas, G. A. Cullen, and C. E. Hormaeche.** 1992. Vaccination of chickens with chicken-derived *Salmonella enteritidis* phage type 4 *aroA* live oral *Salmonella* vaccines. Vaccine **10:**247–254.
8. **Curtiss, R., III, and S. M. Kelly.** 1987. *Salmonella typhimurium* deletion mutants lacking adenylate cyclase and cyclic AMP receptor protein are avirulent and immunogenic. Infect. Immun. **55:**3035–3043.
9. **Dartigalongue, C., and S. Raina.** 1998. A new heat-shock gene, ppiD, encodes a peptidyl-prolyl isomerase required for folding of outer membrane proteins in *Escherichia coli*. EMBO J. **17:**3968–3980.
10. **Dorman, C. J., S. Chatfield, C. F. Higgins, C. Hayward, and G. Dougan.** 1989. Characterisation of porin and *ompR* mutants of a virulent strain of *Salmonella typhimurium*: *ompR* mutants are attenuated in vivo. Infect. Immun. **57:**2136–2140.

11. **Fairweather, N. F., S. N. Chatfield, A. J. Makoff, R. A. Strugnell, J. Bester, D. J. Maskell, and G. Dougan.** 1990. Oral vaccination of mice against tetanus by use of a live attenuated *Salmonella* carrier. Infect. Immun. **58:**1323–1329.
12. **Finlay, B. B.** 1994. Molecular and cellular mechanisms of *Salmonella* pathogenesis. Curr. Top. Microbiol. **192:**163–185.
13. **Hensel, M., J. E. Shea, C. Gleeson, M. D. Jones, E. Dalton, and D. W. Holden.** 1995. Simultaneous identification of bacterial virulence genes by negative selection. Science **269:**400–403.
14. **Hoiseth, S. K., and B. A. D. Stocker.** 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature **291:**238–240.
15. **Hone, D. M., A. M. Harris, S. Chatfield, G. Dougan, and M. M. Levine.** 1991. Construction of genetically defined double *aro* mutants of *Salmonella typhi*. Vaccine **9:**810–816.
16. **Hone, D. M., C. O. Tacket, A. M. Harris, B. Kay, G. Losonsky, and M. M. Levine.** 1992. Evaluation in volunteers of a candidate live oral attenuated *Salmonella typhi* vector vaccine. J. Clin. Investig. **90:**412–420.
17. **Hormaeche, C. E.** 1979. Natural resistance to *Salmonella typhimurium* in different inbred mouse strains. Immunology **37:**311–318.
18. **Jones, P. W., G. Dougan, C. Hayward, N. Mackensie, P. Collins, and S. N. Chatfield.** 1990. Oral vaccination of calves against experimental salmonellosis using a double *aro* mutant of *Salmonella typhimurium*. Vaccine **9:**29–36.
19. **Lazar, S. W., M. Almiron, A. Tormo, and R. Kolter.** 1998. Role of the *Escherichia coli* SurA protein in stationary-phase survival. J. Bacteriol. **180:**5704–5711.
20. **Lazar, S. W., and R. Kolter.** 1996. SurA assists the folding of *Escherichia coli* outer membrane proteins. J. Bacteriol. **178:**1770–1773.
21. **Levine, M. M., D. Herrington, J. R. Murphy, J. G. Morris, G. Losonsky, B. Tall, A. A. Lindberg, S. Svenson, S. Baqar, M. F. Edwards, and B. Stocker.** 1987. Safety, infectivity, immunogenicity, and *in vivo* stability of two attenuate auxotrophic mutant strains of *Salmonella typhi*, 541Ty and 543Ty, as live oral vaccines in humans. J. Clin. Investig. **79:**888–902.
22. **Mahan, M. J., J. M. Slauch, and J. J. Mekalanos.** 1993. Selection of bacterial virulence genes that are specifically induced in hose tissues. Science **259:**686–688.
23. **Miles, A. A., S. S. Misra, and J. O. Irwin.** 1938. The estimation of the bactericidal power of the blood. J. Hyg. **39:**732–749.
24. **Miller, I., D. Maskell, C. Hormaeche, K. Johnson, D. Pickard, and G. Dougan.** 1989. Isolation of orally attenuated *Salmonella typhimurium* following Tn*phoA* mutagenesis. Infect. Immun. **57:**2758–2763.
25. **Miller, I. A., S. Chatfield, G. Dougan, L. DeSilva, H. S. Joysey, and C. Hormaeche.** 1989. Bacteriophage P22 as a vehicle for transducing cosmid gene banks between smooth strains of *Salmonella typhimurium*: use in identifying a role for *aroD* in attenuating virulent *Salmonella* strains. Mol. Gen. Genet. **215:**312–316.
26. **Miller, S. I., J. J. Mekalanos, and W. S. Pulkkinen.** 1990. *Salmonella* vaccines with mutations in the *phoP* virulence regulon. Res. Microbiol. **141:**817–821.
27. **Miller, V. L., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins an virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. **170:**2575–2583.
28. **Missiakes, D., J. M. Betton, and S. Raina.** 1996. New components of protein folding in extracytoplasmid compartments of *Escherichia coli surA*, FkpA and Skp/OmpH. Mol. Microbiol. **21:**871–884.
29. **Mukkur, T. K. S., G. N. McDowell, B. A. D. Stocker, and A. K. Lascelles.** 1987. Protection against salmonellosis in mice and sheep by immunisation with aromatic-dependent *Salmonella typhimurium*. J. Med. Microbiol. **24:**11–19.
30. **O'Callaghan, D., D. Maskell, F. Y. Liew, C. S. F. Easmon, and G. Dougan.** 1988. Characterization of aromatic- and purine-dependent *Salmonella typhimurium*: attenuation, persistence, and ability to induce protective immunity in BALB/c mice. Infect. Immun. **56:**419–426.
31. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. **27:**493–497.
32. **Roberts, M., L. J. Li, A. Bacon, and S. N. Chatfield.** 1998. Oral vaccination against tetanus: comparison of the immunogenicities of *Salmonella* strains expressing fragment C from the *nirB* and *htrA* promoters. Infect. Immun. **66:**3080–3087.
33. **Rouviere, P. E., and C. A. Gross.** 1996. SurA, a periplasmic protein with peptidyl-prolyl isomerase activity, participates in the assembly of outer membrane proteins. Genes Dev. **10:**3170–3182.
34. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
35. **Small, P. L. C., R. R. Isberg, and S. Falkow.** 1987. Comparison of the ability of enteroinvasive *Escherichia coli*, *Salmonella typhimurium*, *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica* to enter and replicate within HEp-2 cells. Infect. Immun. **55:**1674–1679.
36. **Stoker, N. G., N. F. Fairweather, and B. G. Spratt.** 1982. Versatile low-copy-number plasmid vectors for cloning in *Escherichia coli*. Gene **18:**335–341.
37. **Tacket, C. O., D. M. Hone, R. Curtiss III, S. M. Kelly, G. Losonsky, L. Guers, A. M. Harris, R. Edelman, and M. M. Levine.** 1992. Comparison of the safety and immunogenicity of delta *aroC* delta *aroD* and delta *cya* delta *crp Salmonella typhi* strains in adult volunteers. Infect. Immun. **60:**536–541.
38. **Tacket, C. O., D. M. Hone, G. A. Losoonsky, L. Guers, R. Edelman, and M. M. Levine.** 1992. Clinical acceptability and immunogenicity of CVD 908 *Salmonella typhi* vaccine strain. Vaccine **10:**443–450.
39. **Tacket, C. O., M. B. Sztein, G. A. Losonsky, S. S. Wasserman, J. P. Nataro, R. Edelman, D. Pickard, G. Dougan, S. N. Chatfield, and M. M. Levine.** 1997. Safety of live oral *Salmonella typhi* vaccine strains with deletions in *htrA* and *aroC aroD* and immune response in humans. Infect. Immun. **65:**452–456.
40. **Tormo, A., M. Almiron, and R. Kolter.** 1990. *surA*, an *Escherichia coli* gene essential for survival in stationary phase. J. Bacteriol. **172:**4339–4347.
41. **Tsai, C. M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Ann. Biochem. **119:**115–119.
42. **Valentine, P. J., B. P. Devore, and F. Heffron.** 1998. Identification of three highly attenuated *Salmonella typhimurium* mutants that are more immunogenic and protective in mice than in a prototypical *aroA* mutant. Infect. Immun. **66:**3378–3383.
43. **Vancott, J. L., H. F. Staats, D. W. Pascual, M. Roberts, S. N. Chatfield, M. Yamamoto, M. Coste, P. B. Carter, H. Kiyono, and J. R. McGhee.** 1996. Regulation of mucosal and systemic antibody responses by T helper subsets, macrophages, and derived cytokines following oral immunisation with live recombinant *Salmonella*. J. Immunol. **156:**1504–1514.

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