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

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A previously described attenuated TnphoA 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 extensively 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 araA 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 Tpho4 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 pdcA (whose product is involved in the biosynthesis of vitamin B6) 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::Tpho4 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 prepared from BRD441, was partially cleaved using EcoRI. 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 Sau3AI, and a cosmid library was constructed using the vector pHCT79 (34). The cosmid library was screened by colony blotting using DNA probes complementary to the 5′ and 3′ ends of Tpho4 transposon (5′ TATAATATGCCCTTGACC; 3′ ATATCTCGTAGAAT CAAATAACCATCTCATC). Cosmids exhibiting homology to the probes were isolated and digested with a number of restriction enzymes. A 3.3-kb EcoRI fragment which hybridized to the 5′ probe and a PstI 5.5-kb fragment which...

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TABLE 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td>Strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C5</td>
<td>Mouse-virulent strain</td>
<td>15</td>
</tr>
<tr>
<td>BRD441</td>
<td>TphA4 mutant of C5</td>
<td>21</td>
</tr>
<tr>
<td>BRD1115</td>
<td>ΔsurA mutant of C5</td>
<td>This study</td>
</tr>
<tr>
<td>BRD1115 (pLG339/surA)</td>
<td>Complemented surA strain</td>
<td>This study</td>
</tr>
<tr>
<td>LB5010</td>
<td>Intermediate strain</td>
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<tr>
<td>Plasmids</td>
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<td>pBluescriptII SK (+)</td>
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<td>Stratagene</td>
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<td>pPG704</td>
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<td>Promega</td>
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<td>pGEM-T</td>
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<td>This study</td>
</tr>
<tr>
<td>pGEM-T/T212/213</td>
<td>pGEM-T vector containing surA gene</td>
<td>This study</td>
</tr>
<tr>
<td>pLG339</td>
<td>Low-copy-number vector fragment C under the control of the nirB promoter</td>
<td>9</td>
</tr>
<tr>
<td>pTETnirA</td>
<td>Fragment C under the control of the hctA promoter</td>
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<tr>
<td>pTETnirA</td>
<td>Fragment C under the control of the hctA promoter</td>
<td></td>
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</tbody>
</table>

hybridized to the 3′ probe were subcloned into the vector pBluescriptII SK (+) (Stratagene, Maldonine, 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 TphA4-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/T212/213. 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 HpaI and SmaI 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/S′ΔsurA. The plasmid was then cleaved with SphI and SalI, and a 2.6-kb DNA fragment containing the deleted surA gene was gel purified and ligated to the suicide replon pG704 (27). pG704/S′ΔsurA 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 clone 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′-koding region for surA. This defined mutant was designated BRD1115.

Complementation of the surA mutation. A 3-kb fragment of plasmid pGEM-T/T212/213, containing the intact surA gene and flanking sequences, was cloned into the SphI and SalI 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 pTETnirA, in which transcription of the TETC protein is under the control of the nirB promoter and pTETnirA, in which transcription is under the control of the hctA 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 this 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, shaked overnight cultures were used to infect semiconfluent monolayers of HEP-2 cells. Each well was infected with approximately 2 × 10^7 bacteria/well. The bacteria were centrifuged onto the cells at 250 × 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 serially diluting 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 (LD₅₀s) 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 units of bacteria; an oral LD₅₀ was calculated.

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 each orally immunized with 8 log units of organisms. At the time of infection, mice were sacrificed by cervical dislocation. Spleens, livers, mesenteric lymph nodes, and Peyer's patches were removed aseptically 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 LD₅₀ 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 BRD1115(pLG339/surA) or BRD1115(pTETnirB), or BRD1115(pTETnirA). 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 immunosorbant assay as previously described (32), using 96 well enzyme immunosassay/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 (50LD₅₀) of purified tetanus toxin as previously described (11), and fatalities were recorded for 4 days.
Identification of the insertion site of Tphoa in BRD441.
BRD441 was originally identified as an attenuated Tphoa 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 HpaI and SmaI 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. PstI and SalI 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 SalI, 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 B₆) 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 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.

**RESULTS**

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 PstI; 2, S. enterica serovar Typhimurium BRD1115 cleaved with PstI; 3, S. enterica serovar Typhimurium C5 cleaved with SalI; 4, S. enterica serovar Typhimurium BRD1115 cleaved with SalI; 5, S. enterica serovar Typhimurium BRD441 cleaved with SalI.

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.
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$_{10}$ 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.](http://iai.asm.org/)

**TABLE 2.** LD$_{50}$ 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.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Oral LD$<em>{50}$ (log$</em>{10}$ CFU)</th>
<th>i.v. LD$<em>{50}$ (log$</em>{10}$ CFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C5</td>
<td>4.16</td>
<td>&lt;1.87</td>
</tr>
<tr>
<td>BRD441</td>
<td>8.62</td>
<td>2.46</td>
</tr>
<tr>
<td>BRD1115</td>
<td>8.98</td>
<td>5.22</td>
</tr>
<tr>
<td>BRD1115(pLG339/surA)</td>
<td>4.17</td>
<td>ND*</td>
</tr>
</tbody>
</table>

* ND, not determined.
between 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 post-vaccination, mice were resistant to challenge with the equivalent of nearly 5 log$_{10}$ LD$_{50}$ of the virulent parental S. typhimurium C5 strain.

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

<table>
<thead>
<tr>
<th>Strain used for immunization</th>
<th>Log$<em>{10}$ oral LD$</em>{50}$ following C5 challenge</th>
</tr>
</thead>
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<tr>
<td></td>
<td>4 wk postimmunization</td>
</tr>
<tr>
<td>BRD1115</td>
<td>8.58</td>
</tr>
<tr>
<td>None (mock infected)</td>
<td>4.74</td>
</tr>
<tr>
<td>Protection</td>
<td>3.8</td>
</tr>
</tbody>
</table>

Mice immunized with either BRD1115(pTETnir15) or BRD1115(pTEThtrA) 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(pTEThtrA) had, in general, a higher and more consistent titer of anti-TETC antibodies in their sera than did mice immunized with BRD1115(pTETnir15). 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(pTEThtrA) 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 (pTETnir15) survived challenge.

**FIG. 5. Titer of TETC-specific antibody by ELISA in mice immunized with strain BRD1115, BRD1115(pTETnir15) or BRD1115(pTETHttrA).** 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$_{50}$ of tetanus toxin as a fraction of the total number.

**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 kogA.
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 CS. There was also a comparable decrease in adherence 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}, 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 *S. enterica* serovar Typhimurium 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**


