Regulated Delayed Expression of rfaH in an Attenuated Salmonella enterica Serovar Typhimurium Vaccine Enhances Immunogenicity of Outer Membrane Proteins and a Heterologous Antigen

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RfaH is a transcriptional antiterminator that reduces the polarity of long operons encoding secreted and surface-associated cell components of Salmonella enterica serovar Typhimurium, including O antigen and lipopolysaccharide core sugars. A ΔrfaH mutant strain is attenuated in mice (50% lethal dose [LD₅₀], >10⁸ CFU). To examine the potential for using rfaH in conjunction with other attenuating mutations, we designed a series of strains in which we replaced the native rfaH promoter with the tightly regulated arabinose-dependent araC P_BAD promoter so that rfaH expression was dependent on exogenously supplied arabinose provided during in vitro growth. Following colonization of host lymphoid tissues, where arabinose was not available, the P_BAD promoter was no longer active and rfaH was not expressed. In the absence of RfaH, O antigen and core sugars were not synthesized. We constructed three mutant strains that expressed different levels of RfaH by altering the ribosome-binding sequence and start codon. One mutation, ΔP_rfaH178, was introduced into the attenuated vaccine strain χ9241 (ΔpabA ΔpabB ΔasdA), expressing the pneumococcal surface protein PspA from an Asd⁺ balanced-lethal plasmid. Mice immunized with this strain and boosted 4 weeks later induced higher levels of serum immunoglobulin G specific for PspA and for outer membrane proteins from other enteric bacteria than either an isogenic ΔfhaH derivative or the isogenic RfaH⁺ parent. Eight weeks after primary oral immunization, mice were challenged with 200 LD₅₀ of virulent Streptococcus pneumoniae WU2. Immunization with ΔP_rfaH178 mutant strains led to increased levels of protection compared to that of the parent χ9241 and of a ΔfhaH derivative of χ9241.

When recombinant attenuated Salmonella enterica serovar Typhimurium vaccines (RASV) are used to deliver heterologous antigens, it may be advantageous to reduce the host immune response against the RASV carrier, thereby enhancing the immune response against the heterologous antigen. The dominant immunogen on the Salmonella cell surface is lipopolysaccharide (LPS) O antigen (41). However, strains with mutations that eliminate LPS O antigen may be less immunogenic due to their failure to colonize the intestinal tract and to invade intestinal mucosal cells (43, 44). We hypothesized that in vivo-programmed downregulation of O-antigen expression, occurring after colonization of host lymphoid tissues, would serve to reduce the immune response against the RASV carrier while triggering a strong immune response against heterologous antigens (10) and outer membrane proteins cross-reactive with other enteric bacteria (28).

The genes for LPS core and O-antigen biosynthesis are clustered into long operons (37, 49) that cannot be fully transcribed if the native promoter is replaced by a heterologous promoter. RfaH, a transcriptional antiterminator, reduces the polarity of long operons by binding to the ops sequence, located in an untranslated S′ region of the transcript, and interacting with the transcription complex (1). RfaH is required for the expression of secreted and surface-associated cell components of S. enterica serovar Typhimurium, including O antigen and core sugars components of LPS (3, 39). rfaH mutant strains produce truncated LPS and reduced amounts of O antigen and core (24), rendering them sensitive to human serum (27), hypersensitive to bile, and attenuated in mice (26, 45). rfaH mutants are immunogenic in mice, inducing a protective immune response against Salmonella challenge (27).

The major immunogenic surface molecules of S. enterica are the O antigen and flagella. Complete LPS is of considerable importance, as rough mutants of Salmonella lacking LPS O-antigen side chains or portions of the core are avirulent, fail to colonize the intestinal tract, and are deficient in invading cells of the intestinal mucosa (43). To circumvent this problem, we and others have explored different ways to achieve regulated O-antigen synthesis so that O antigen is synthesized in vitro but not in vivo, creating vaccine strains that are phenotypically wild type at the time of immunization and become attenuated after colonization of host tissues. We have termed this strategy regulated delayed attenuation (11, 12, 22). One means to achieve regulated delayed attenuation is the deletion of certain genes essential for O-antigen synthesis, such as pmi (manA) (9, 12, 22) or galE (15, 19, 42). Strains with pmi or galE deletions have a reversibly rough phenotype because they are able to synthesize complete O antigen or O antigen and entire core only when grown in the presence of mannose or galactose, respectively. When grown in the presence of their respective sugars,
The bacterial strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. S. Typhimurium cultures were grown at 37°C in LB broth (4) or nutrient broth (Difco) or on LB agar with or without 0.1% arabinose. Selenite broth, with or without supplements, was used for enrichment of Salmonella from mouse tissues. Difco

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Source or reference</th>
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<tbody>
<tr>
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<td>16</td>
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<td>ΔaraBAD23 ΔrelA198::araC ( P_{BAD} ) lacI TT</td>
<td>Laboratory stock</td>
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<td>Laboratory stock</td>
</tr>
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<td><strong>S. pneumoniae WU2</strong></td>
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<tr>
<td>pYA4088</td>
<td>852-bp DNA encoding the α-helical region of PspA</td>
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The Shine-Dalgaro sequence and start codon for each rfaH allele are indicated in uppercase, bold letters.

These mutants are fully fit to carry out host colonization and invasion of host tissues during the early stages of infection (12, 18). Upon reaching deeper tissues where free mannose and galactose are not available, O antigen is no longer synthesized and the strains become phenotypically rough.

Another strategy for achieving regulated delayed attenuation relies on replacement of the promoter of a gene of interest with the arabinose-regulated araC \( P_{BAD} \) promoter (11, 12). The araC \( P_{BAD} \) promoter has been used to develop regulated delayed attenuation strains in which the expression of a number of Salmonella virulence genes, such as fur, crp, and rpoS, is dependent upon arabinose availability (11). In this work, the rfaH promoter, including sequences for activator or repressor protein binding, was deleted and replaced with an araC \( P_{BAD} \) cassette to yield Salmonella strains in which rfaH transcription was arabinose dependent. By manipulation of translation signals, we constructed a series of strains, each synthesizing different amounts of RfaH. Growth of these strains in the presence of arabinose permitted transcription of rfaH and synthesis of full-length O antigen. We evaluated these strains for virulence, immunogenicity, and the ability to deliver a test antigen, the pneumococcal protein PspA. Immunized mice were challenged with virulent Streptococcus pneumoniae to determine protective efficacy.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, media, and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. S. Typhimurium cultures were grown at 37°C in LB broth (4) or nutrient broth (Difco) or on LB agar with or without 0.1% arabinose. Selenite broth, with or without supplements, was used for enrichment of Salmonella from mouse tissues. Difco
across the plates. Bacteria were grown at an OD600 of 0.8 to 0.9 in nutrient broth. The cells were diluted to 10^5 to 10^6 CFU, and the mixture was incubated at room temperature for 30 min, centrifuged, and resuspended in 200 μl of buffered saline with gelatin (BSG). A 100-μl aliquot was spread onto LB agar plates containing 15 μg/ml chloramphenicol and incubated overnight at 37°C. The colonies were counted the following day. This experiment was performed twice. MIC test. The MICs of different antimicrobial substances were determined by using 96-well tissue culture plates (50). Twofold serial dilutions of the bile salt, deoxycholate (0.1 to 50 mg/ml), and polymyxin B (0.1 to 10 μg/ml) were made across the plates. Bacteria were grown at an OD600 of 0.8 to 0.9 in nutrient broth with or without 0.1% arabinose and washed in phosphate-buffered saline (PBS). The cells were diluted to 10^5 to 10^6 CFU in nutrient broth with or without arabinose, and 0.1 ml of the diluted cell suspension was added to each well. The microtiter plates were incubated overnight at 37°C. The OD of each culture was determined using a SpectraMax M2e (Molecular Devices, CA) plate reader. The threshold of inhibition was 0.1 at OD600. The actual titers were determined by spreading culture dilutions on LB plates, followed by overnight incubation at 37°C. Assays were repeated at least three times.

Swarming. Swarming motility was assessed on LB plates solidified with 0.3% agar and grown overnight at 37°C. Assays were repeated at least three times. Determination of virulence in mice. Seven-week-old female BALB/c mice were obtained from the Charles River Laboratories (Wilmington, MA). All animal procedures were approved by the Arizona State University Animal Care and Use Committees. The mice were acclimated for 7 days after arrival before the experiments were started. For determination of the 50% lethal dose (LD50), bacteria were grown statically overnight at 37°C in LB broth, diluted 1:50 in fresh medium containing 0.1% arabinose, and grown with aeration (180 rpm) at 37°C. When the cultures reached an OD600 of 0.8 to 0.9, they were harvested by room temperature centrifugation at 4,000 rpm, washed once, and normalized to the required inoculum density in BSG by adjusting the suspension to the appropriate OD600 value. Groups of five mice each were infected orally with 20 μl containing various doses of S. Typhimurium or its derivatives, ranging from 1 x 10^5 to 1 x 10^6 CFU. The animals were observed for 4 weeks postinfection, and deaths were recorded daily.

To evaluate colonization, mice were orally inoculated with 20 μl of BSG containing 1 x 10^8 CFU of each strain. At days 4 and 8 after inoculation, three animals per group were euthanized and spleen and liver samples were collected. Each sample was homogenized in a total volume of 1 ml BSG, and dilutions of 10^-1 to 10^-6 (depending on the tissue) were plated onto MacConkey agar and LB agar, each containing 0.1% arabinose, to determine the number of viable bacteria. A 0.1-ml aliquot of each tissue sample was enriched for Salmonella by incubation into selective cysteine broth and incubated for 14 h at 37°C. Samples that were negative by direct plate and positive by enrichment in selective cysteine broth were recorded as <10 CFU/g.

Immunogenicity of vaccine strains in mice. RASV strains were grown statically overnight in LB broth with 0.1% arabinose at 37°C. The following day, 2 ml of the overnight culture was inoculated into 100 ml of LB broth with 0.1% arabinose and grown with aeration at 37°C to an OD600 of 0.8 to 0.9. The cells were harvested by room temperature centrifugation at 4,000 rpm for 15 min, and the pellet was resuspended in 1 ml of BSG. Mice were orally inoculated with 20 μl of BSG containing 1 x 10^8 CFU of each strain on day 0 and boosted on day 28 with the same dose of the same strain. Blood was obtained by mandibular vein puncture at biweekly intervals. The blood was allowed to coagulate at 37°C for 2 hours. Following centrifugation, the serum was removed from the whole blood and stored at -20°C.

Antigen preparation. Recombinant PspA (rPspA) protein was purified as described previously (20). The rPspA plasmid that encodes the Ω-helical region of PspA (amino acids 1 to 302) in PET20b was a kind gift from Susan Hollingshead at the University of Alabama at Birmingham. S. Typhimurium LPS was purchased from Sigma. Outer membrane proteins were prepared as described previously (20). To prepare whole-cell antigens, various enteric bacteria were grown statically overnight at 37°C. Cells were harvested by room temperature centrifugation at 4,000 rpm for 15 min, and the pellet was resuspended in 1 ml of BSG. Mice were orally inoculated with 20 μl of BSG containing 1 x 10^8 CFU of each strain on day 0 and boosted on day 28 with the same dose of the same strain. Blood was obtained by mandibular vein puncture at biweekly intervals. The blood was allowed to coagulate at 37°C for 2 hours. Following centrifugation, the serum was removed from the whole blood and stored at -20°C.

ELISA. The IgG antibodies against S. Typhimurium LPS, rPspA, and whole-cell bacterial suspensions (1 x 10^8 CFU/ml), as previously described (23). Color development (absorbance) was recorded at 405 nm using a SpectraMax M2e automated ELISA plate reader (Molecular Devices, Menlo Park, CA). Absorbance readings 0.1 higher than PBS control values were considered positive.
Pneumococcal challenge. We assessed the protective efficacy of immunization with the attenuated Salmonella strains expressing pspA at week 8 by intraperitoneal (i.p.) challenge with 4 × 10^4 CFU of S. pneumoniae WU2 in 200 µl of PBS (31). The LD50 of S. pneumoniae WU2 in BALB/c mice was 2 × 10^2 CFU by i.p. administration (data not shown). The challenged mice were monitored daily for 30 days.

Statistical analysis. Antibody titer data were expressed as geometric means, and the relative immunoreactivity was expressed as an arithmetic mean. The means were evaluated by two-way analysis of variance and a chi-square test for multiple comparisons among groups. A P value of <0.05 was considered statistically significant.

RESULTS

Mutant construction and LPS phenotypes. We constructed S. Typhimurium χ3761 derivatives, each designed to synthesize different amounts of RfaH, with rfaH transcribed from the arabinose-regulated araC PBAD promoter. Figure 1A illustrates the chromosomal structures of the araC PBAD rfaH mutant strains. Ninety-eight base pairs upstream of the start codon were replaced with araC PBAD to create the ΔP_rfaH49 mutation. A different SD sequence or a GTG start codon was introduced to create ΔP_rfaH176 and ΔP_rfaH178, respectively. The SD and start codon sequences for each mutant strain are given in Table 1.

The levels of O-antigen synthesis in the mutants were determined by silver staining (Fig. 1B) and by Western blotting using anti-Salmonella group B O-antigen serum (Fig. 1C). Lack of rfaH expression, because of either deletion (ΔrfaH49) or growth of P_BAD rfaH strains in the absence of arabinose, resulted in reduced O-antigen synthesis in all four mutant strains. In the three araC PBAD rfaH mutants, nearly wild-type O-antigen levels were restored by the addition of arabinose to the growth medium. None of the three arabinose-regulated rfaH mutants were completely rough when grown in the absence of arabinose, although the ΔP_rfaH176 strain produced the smallest amount of high-molecular-weight O antigen (Fig. 1B and C). The ΔP_rfaH176 mutant, with a canonical SD sequence and an ATG start codon, would be expected to synthesize the most RfaH, and the ΔP_rfaH178 mutant, with a nonideal SD sequence and a GTG start codon, would be expected to synthesize the least RfaH among the three strains. Although we did not directly measure the amount of RfaH synthesized by each strain, the differing amounts of O antigen produced were consistent with our expectations, with the P_rfaH176 mutant producing the most O antigen and the P_rfaH178 mutant producing the least in the absence of arabinose. The ΔrfaH49 mutant did not produce any detectable high-molecular-weight O antigen with or without arabinose (Fig. 1B and C).

To further evaluate arabinose-regulated O-antigen synthesis, we performed infection studies with the O-antigen-specific phage P22. The strains were grown in nutrient broth with or without arabinose and used as recipients for transduction assays. When strains χ9660 (ΔP_rfaH176), χ9734 (ΔP_rfaH177), and

![FIG. 1. Arabinose regulation of rfaH. (A) Map of deletion-insertion mutations resulting in arabinose-regulated rfaH expression. (B) LPS phenotypes of wild-type S. Typhimurium χ3761 and the indicated isogenic derivatives. LPSs from different mutant strains grown in nutrient broth with (+) or without (−) 0.1% arabinose were silver stained after separation by 12% SDS-PAGE. (C) Western blots of LPS preparations from panel B. The blots were probed with anti-Salmonella group B antibodies.](http://iai.asm.org/)

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χ9735 (ΔP<sub>rfaH176</sub>) were grown with arabinose, the number of transductants obtained was similar to that obtained with the wild-type parent strain, χ3761 (Table 3). In the absence of arabinose, the number of transductants was reduced about 10-fold, which was still about 3- to 4-fold more than that obtained with arabinose, the number of transductants was reduced about 10-fold, which was still about 3- to 4-fold more than that obtained without arabinose. Swarming was restored to wild-type levels in the presence of arabinose in all three arabinose-regulated rfaH strains.

Virulence of ΔP<sub>rfaH</sub> and ΔrfaH49 mutant strains in mice. To assess the virulence of the rfaH mutants, we determined their LD<sub>50</sub> in BALB/c mice (Table 3). The ΔrfaH49 mutant was highly attenuated, with no death occurring at the maximum dose tested, 1 × 10<sup>6</sup> CFU, in agreement with previous results (27). The parent strain, χ3761, was highly virulent, with an LD<sub>50</sub> of 1 × 10<sup>4</sup> CFU. The arabinose-regulated mutant strains χ9734 and χ9735 were both attenuated, with LD<sub>50</sub> of 1 × 10<sup>4</sup> CFU, despite the fact that they were grown in the presence of arabinose prior to inoculation. Strain χ9660, the ΔP<sub>rfaH176</sub> mutant, was not tested, since our earlier phenotypic analyses indicated that rfaH expression was not tightly regulated (Fig. 1 and Table 3).

Expression of the pneumococcal gene pspA in RASV strain χ9241 derivatives carrying different rfaH mutations. S. Typhimurium strain χ9241 is an attenuated vaccine strain that has been successfully used to deliver the pneumococcal surface protein PspA and to induce protective immunity against S. pneumoniae challenge (23, 51). To evaluate the effects of rfaH mutations on the efficacy of this vaccine strain, the ΔrfaH49 and ΔP<sub>rfaH176</sub> mutations were introduced into χ9241 to yield strains χ9884 and χ9852, respectively. Subsequently, the Asd<sup>+</sup> recombinant plasmid pYA4088, which carries a recombinant pspA gene fused to DNA encoding the β-lactamase signal sequence (51), was introduced into the new strains. Expression of pspA is driven by the P<sub>rec</sub> promoter, and the bla signal sequence directs periplasmic secretion of PspA. Note that strain χ9241 carries the ΔrelA198::zarC PBAD lacI TT deletion/insertion (TT stands for transcriptional terminator). When this strain is grown in the presence of arabinose, lacI is expressed. The LacI protein binds to the P<sub>rec</sub> promoter on pYA4088, preventing pspA expression. Once the strain invades and colonizes host tissues, where arabinose is not available, LacI is no longer synthesized and pspA is expressed. This feature has been termed regulated delayed antigen synthesis (51).

To evaluate PspA synthesis, whole-cell lysates from strain

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**Table 3. MICs of antibiotic substances, swarming motilities, transduction efficiencies, and virulences of S. Typhimurium strain χ3761 and its rfaH mutant derivatives**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Presence of 0.1% arabinose&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of P22 transductants&lt;sup&gt;b&lt;/sup&gt;</th>
<th>MIC (mg/ml)</th>
<th>Polymyxin B (μg/ml)</th>
<th>Swarming motility (mm)</th>
<th>Oral LD&lt;sub&gt;50&lt;/sub&gt; (CFU)</th>
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<tbody>
<tr>
<td>χ9660 (ΔP&lt;sub&gt;rfaH176&lt;/sub&gt;)</td>
<td>–</td>
<td>305</td>
<td>3.2</td>
<td>0.25</td>
<td>14.5</td>
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<td>3,270</td>
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<td>3,980</td>
<td>3.2</td>
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<td>1.0 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>+</td>
<td>ND</td>
<td>&lt;1.6</td>
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<tr>
<td></td>
<td>–</td>
<td>3,180</td>
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<td></td>
<td>+</td>
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<td>0.60</td>
<td>21.0</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
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</table>

<sup>a</sup> +, present; –, absent.

<sup>b</sup> The phage lysate used for transduction was grown on a chloramphenicol-resistant strain. Transduction was performed as described in Materials and Methods. The results reflect the numbers of chloramphenicol-resistant colonies obtained after transduction.

<sup>c</sup> DOC, deoxycholate.

<sup>d</sup> Not determined.
The Western blots show the synthesis of PspA in S. Typhimurium strains χ9884(pYA4088) (ΔrfaH49), χ9852(pYA4088) (ΔPrfaH178), χ9241(pYA4088) (RfaH+), and χ9241(pYA3493). The bacteria were grown in LB broth with (+) or without (−) 0.1% arabinose overnight at 37°C. Equal numbers of cells from each culture were pelleted, suspended in loading buffer, and boiled. After centrifugation, equal volumes were subjected to SDS-PAGE in triplicate gels. Each gel was transferred to nitrocellulose and probed with a different polyclonal antibody specific for either PspA, LacI, or GroEL. GroEL was used as a standardization marker. Relevant portions of each blot are shown.

FIG. 2. PspA and LacI synthesis is regulated by 0.1% arabinose. The horizontal lines represent the means, and the error bars represent standard errors of the means.

Colonization of mouse tissues and immune responses in mice after oral immunization with RASV expressing PspA. To evaluate the effect of arabinose-regulated rfaH expression on colonization of mouse tissues, strains χ9241(pYA4088), χ9884 (pYA4088), and χ9852(pYA4088) were grown in the presence of arabinose and used to inoculate groups of BALB/c mice. On days 4 and 8, three mice from each group were euthanized, and spleen and liver samples were harvested, homogenized, and plated on MacConkey and LB plates, each containing 0.1% arabinose. We found significant differences between the strains in their abilities to colonize the liver and spleen (Fig. 3). Strain χ9852(pYA4088) (ΔPrfaH178) and its parent strain, χ9241 (pYA4088), colonized the spleen and liver in significantly higher numbers than χ9884(pYA4088) (ΔrfaH49) (P < 0.0001). There was a slight reduction in tissue colonization by χ9852 (pYA4088) compared to its parent strain, χ9241(pYA4088), but the difference was not statistically significant (P > 0.05).

In a separate experiment, we evaluated the in vivo stability of the ΔPrfaH178 mutation in χ9852(pYA4088). A group of three mice were inoculated with 1 × 10⁹ CFU of χ9852(pYA4088). Five days later, the mice were euthanized, and vaccine was recovered from the spleens and livers. Seven colonies were chosen at random and evaluated for arabinose-regulated LPS synthesis by growing each isolate in LB with or without arabinose. Each culture was analyzed on LPS gels. All seven isolates exhibited the same arabinose-regulated LPS phenotype as the parent strain (data not shown).

Effects of rfaH mutations on the immunogenicity and protective efficacy of RASV strains. We orally inoculated groups of mice with 1 × 10⁹ to 2 × 10⁹ CFU of either χ9241(pYA4088), χ9884(pYA4088) (ΔrfaH49), χ9852(pYA4088) (ΔPrfaH178), or the control strain, χ9241(pYA3493), which does not express pspA. The mice were boosted with a similar dose of the same strain 4 weeks later. The antibody responses to rPspA and Salmonella LPS in the sera of immunized mice were measured (Fig. 4). This experiment was performed twice; 5 mice per group were used in the first experiment, and 8 to 11 mice per group were used in the second experiment. The results from the two experiments were similar and were pooled for analysis. High serum IgG titers against PspA were observed by 2 weeks after the primary immunization in mice inoculated with χ9241(pYA4088) and χ9852(pYA4088) (Fig. 4A). Anti-PspA titers in mice immunized with χ9884(pYA4088) (ΔrfaH) were slower to develop and by 6 weeks had reached high titers, although they never achieved titers comparable to those in mice immunized with χ9241(pYA4088). However, mice immunized with χ9852(pYA4088) (ΔPrfaH178) achieved significantly higher titers than the other two groups. No anti-PspA IgG was
detected in sera from mice inoculated with the control strain, \( \chi_{9241}(pYA3493) \).

Anti-LPS titers were low but detectable at 2 weeks (Fig. 4B). The titers remained low in mice immunized with the \( \chi_{9004} \) strain, \( \chi_{9273} \), throughout the course of the experiment, while anti-LPS serum titers increased in mice immunized with the other strains. Before week 8, the anti-LPS titers were significantly lower in mice immunized with the \( \chi_{9004} \) strain, \( \chi_{9273} \), than in either group immunized with a \( \chi_{9241} \) derivative (\( P < 0.001 \)), and they were significantly higher than those of mice immunized with the \( \Delta \text{fah} \) strain (\( P < 0.001 \)). By week 8, mice immunized with either of the \( \chi_{9241} \) derivatives or with \( \chi_{9273} \) had similar anti-LPS IgG titers. Anti-SOMP titers were similar for all strains except \( \chi_{9273} \), which was statistically different (\( P < 0.001 \)) (Fig. 4C).

**IgG isotype analyses.** We evaluated the responses of the IgG isotype subclasses IgG1 and IgG2a (Fig. 5) to rPspA. Th1 helper cells direct cell-mediated immunity and promote class switching to IgG2a, and Th2 cells provide potent help for B-cell antibody production and promote class switching to IgG1. Immunization with strain \( \chi_{9884}(pYA4088) \) induced a strong Th2 response, since the levels of anti-PspA IgG1 were higher than those of IgG2a. Conversely, immunization with strains \( \chi_{9852}(pYA4088) \) and \( \chi_{9241}(pYA4088) \) induced primarily a Th1-type response against PspA.

**Evaluation of protective immunity.** To evaluate the effects of the \( \Delta \text{fah} \) and \( \Delta \text{fahH} \) mutations on the ability of the RASV expressing PspA to induce protective immunity, mice were challenged by the i.p. route with \( 4 \times 10^{4} \) CFU (200 LD\(_{50}\)) of *S. pneumoniae* WU2 4 weeks after the boost. Immunization with any of the pspA-expressing strains provided significant protection against challenge compared to immunization with the control strain, \( \chi_{9241}(pYA3493) \) (Fig. 6; \( P < 0.001 \)). Immunization with \( \chi_{9273} \) induced significantly greater protection than did immunization with \( \chi_{9004} \) (\( P < 0.05 \)). There was no significant difference between the protective efficacy of \( \chi_{9273} \) and that of the other strains expressing pspA (\( P > 0.05 \)). All of the mice that died in these experiments succumbed within 4 days after challenge.

**Increased immunogenicity of conserved antigens against different enteric bacteria.** Previous work had shown that a \( \Delta \text{fah} \) mutation increased the immunogenic reactivities of conserved minor epitopes from other enteric bacteria (29). Therefore, we examined the cross-reactive antibodies elicited by the \( \Delta \text{fahH} \) vaccine strain, \( \chi_{9884}(pYA4088) \), with those elicited by the arabinose-regulated \( \Delta \text{fahH} \) strain, \( \chi_{9852}(pYA4088) \),
and the RfaH strain, χ9241(pYA4088). The reactivities of pooled immune sera (11 to 16 mice per group) from groups of mice inoculated in the previous experiment taken 2 weeks after boosting was evaluated by ELISA against a panel of homologous and heterologous wild-type strains, as well as their outer membrane proteins (Fig. 7). In each case, immunization with the arabinose-regulated rfaH strain, χ9852(pYA4088), generated higher titers against both whole cells and OMPs isolated from a diverse group of gram-negative organisms, including S. Typhimurium, S. enterica serovars Typhi and Enteritidis, Shigella flexneri, E. coli, and Yersinia pestis, than the χ9004 (rfaH49) strain, χ9884(pYA4088).

DISCUSSION

Recombinant attenuated S. Typhimurium strains have been used extensively as multivalent vectors expressing heterologous antigens. An ideal RASV should be able to invade and transiently persist in lymphoid tissues (Peyer’s patch, spleen, and liver) to stimulate both strong primary and lasting memory immune responses, cause no disease symptoms, and be susceptible to all clinically useful antibiotics (2, 7, 8). Achieving this balance between adequate attenuation/safety and ability to elicit long-lasting protective immunity is not always easy or straightforward. We have developed several strategies to address this issue, including regulated delayed attenuation (11, 12, 22), whereby the live-vaccine strain displays abilities similar to those of a wild-type virulent parental pathogen to successfully colonize effector lymphoid tissues before display and imposition of the fully attenuated phenotype, and regulated delayed antigen synthesis, in which the expression of a heterologous antigen gene is delayed until the vaccine strain has colonized host tissues (51).

In this work, we applied the regulated delayed attenuation approach to produce strains with arabinose-regulated rfaH synthesis of RfaH, a transcriptional antiterminator required for the production of complete LPS (26, 38) and for transcription of other...
virulence-associated genes, including the *siu* operon, which contributes to the intestinal phase of infection (26). We compared arabinose-regulated *rfaH* expression strains to a Δ*rfaH* strain. Based on P22 transduction assays, the Δ*rfaH* mutant produced some full-length O antigen (Table 3) undetectable on silver-stained gels (Fig. 1), consistent with a previous report (29). When grown without arabinose, the regulated delayed *rfaH* strains produced O antigen detectable by silver staining, although the amount was reduced compared to that produced by the same strains grown with arabinose or by strains expressing wild-type *rfaH* (Fig. 1 and Table 3).

A Δ*rfaH* mutant of *S. Typhimurium* has been shown to induce protection against challenge with wild-type *S. Typhimurium* (27). However, a potential drawback of this strain is its lack of O antigen, which contributes to the intestinal phase of infection (26). We compared arabinose-regulated *rfaH* expression strains to a Δ*rfaH* strain. Based on P22 transduction assays, the Δ*rfaH* mutant produced some full-length O antigen (Table 3) undetectable on silver-stained gels (Fig. 1), consistent with a previous report (29). When grown without arabinose, the regulated delayed *rfaH* strains produced O antigen detectable by silver staining, although the amount was reduced compared to that produced by the same strains grown with arabinose or by strains expressing wild-type *rfaH* (Fig. 1 and Table 3).

A Δ*rfaH* mutant of *S. Typhimurium* was attenuated, with an LD₅₀ 100 times greater than that of Δ*rfaH* (Table 3). This in addition to the lack of complete O antigen, could lead to reduced colonization of lymphoid tissues (Fig. 3), an important criterion for stimulation of a strong, lasting immune response. When the Δ*rfaH* mutant was grown in the presence of arabinose, its phenotype was similar to that of its wild-type parent, *χ₃⁷₆₁* (Fig. 1 and Table 3). In the absence of arabinose, this mutant was nearly as susceptible as the Δ*rfaH* mutant to deoxycholate and polynixin B. In addition, even when grown in the presence of arabinose, the Δ*rfaH* mutant was attenuated, with an LD₅₀ 100 times greater than that of Δ*rfaH*, although not as attenuated as the Δ*rfaH* mutant (Table 3).

To ensure the safety of live *Salmonella* vaccines, it is critical that the strain carry at least two genetically unlinked attenuating mutations (13, 35, 48). Therefore, we evaluated the Δ*rfaH* mutation and the arabinose-regulated *rfaH* mutation Δ*rfaH₄₁₇₈ in combination with the Δ*pabA* Δ*pabB* mutations in strain *χ₉₂₄₁* and introduced plasmid pYA4088, which directs the synthesis of a heterologous antigen, the pneumococcal protective antigen PspA (5, 25). The Δ*rfaH* derivative *χ₉₈₈₄* (pYA4088) colonized mouse tissues poorly compared to its parent, *χ₉₂₄₁* (pYA4088), typical of strains lacking full-length O antigen (33, 38). However, the strain with the Δ*rfaH* mutation, *χ₉₈₅₂* (pYA4088), colonized the host spleen and liver nearly as well as *χ₉₂₄₁* (pYA4088). Further, mice immunized with two doses of *χ₉₈₅₂* (pYA4088) elicited an anti-PspA IgG response equal to or greater than that induced by *χ₉₂₄₁* (pYA4088) (Fig. 4). Mice immunized with the Δ*rfaH* mutant, *χ₉₈₈₄* (pYA4088), also developed anti-PspA serum IgG antibodies, although the titers were significantly lower than those in mice immunized with *χ₉₂₄₁* (pYA4088) or *χ₉₈₅₂* (pYA4088). In addition, mice immunized with either *χ₉₂₄₁* (pYA4088) or *χ₉₈₅₂* (pYA4088) developed much higher titers of IgG2a than those immunized with the Δ*rfaH* strain, *χ₉₈₈₄* (pYA4088) (Fig. 5). IgG2a antibody is the most potent isofrom for directing complement deposition, an important host mechanism for clearing *S. pneumoniae* (6).

When challenged i.p. with virulent *S. pneumoniae*, all groups immunized with strains expressing *pspA* were protected (Fig. 6). Consistent with the colonization and serum antibody data, immunization with the regulated *rfaH* expression strain *χ₉₈₅₂* (pYA4088) provided significantly greater protection than immunization with the Δ*rfaH* strain, *χ₉₈₈₄* (pYA4088) (*P < 0.05*). These results support the notion that delaying expression of an attenuation phenotype increases protective efficacy (11, 12, 22).

In our laboratory, we are interested in developing vaccines against enteric pathogens, including pathogenic *E. coli*, enteric *Yersinia* species, and *Shigella*. This is an area in which *S. Typhimurium* Δ*rfaH* mutants may be valuable by inducing cross-protective antibodies against other enteric bacteria (29). Downregulating the production of O antigen, the major immunodominant antigen in *Salmonella*, has been shown to enhance the immunogenicity of conserved antigens (12). A recent study showed that mice immunized with a Δ*rfaH* mutant produced higher titers of serum antibodies against conserved antigens from different *S. enterica* serovars and from other enteric organisms than mice immunized with an attenuated smooth strain (27, 29). The Δ*rfaH* Δ*pabA* Δ*pabB* strain, *χₙ₈₈₄* (pYA4088), induced an enhancement of cross-reactive anti-
bodies to only itself and the avian-pathogenic E. coli strain χ7122, but not to smooth, wild-type S. Typhimurium, S. enterica serovar Typhi, S. enterica serovar Enteritidis, S. flexneri, or Y. pestis (Fig. 7A) or to outer membrane proteins derived from these strains (Fig. 7B). The differences between our results and those previously reported could possibly be due to differences in the time of serum collection, the number of boosts, or strain background and/or the presence in our strains of additional attenuating mutations and plasmid pYA4088, directing the synthesis of a heterologous antigen. In addition, we used χ9241(pYA4088) as the base strain to develop our ratios, while a ΔaraA strain was used in the previous study. Importantly, immunization with the arabinoose-regulated rfaH strain χ9852(pYA4088) enhanced production of cross-reactive antibodies to all strains tested, including strong responses against S. Enteritidis, Shigella, avian-pathogenic E. coli, and Yersinia. These results are most likely due to the overall higher immunogenicity of χ9852(pYA4088) than χ9884(pYA4088) (Fig. 4), which in turn is probably related to the poor colonization of host tissues observed for χ9884(pYA4088) (Fig. 3). These results indicate that inclusion of the ΔP<sub>rfaH</sub> mutation in an RASV expressing pathogen-specific antigens designed to protect against Shigella, E. coli, and/or Yersinia could enhance the protective efficacy against these pathogens.

We stated previously that it would be advantageous to reduce the host immune response against the RASV carrier, thereby enhancing the immune response against the heterologous antigen. One of our goals for this study was to determine whether in vivo downregulation of rfaH would suppress the immune response against carrier-specific O antigen. For this application, the ΔP<sub>rfaH</sub> mutation was not as useful as we had hoped. Although the immune response against LPS O antigen was delayed in mice immunized with χ9852(pYA4088) compared to those immunized with χ9241(pYA4088) (Fig. 4), by week 8, mice immunized with either strain had developed nearly identical anti-LPS titers. There are several possible explanations for this result. One is that a mutation arose in vivo that allowed arabinoise-independent rfaH expression in χ9852(pYA4088). This is unlikely, as we did not observe any such mutants in the spleen isolates we tested. While the araC<sub>Pbad</sub> promoter cassette used in this study is tightly regulated in vivo, as we established in a previous study (21), it is possible that low levels of nonphosphorylated arabinoise in the mouse diet may have been sufficient to allow enough rfaH expression to permit full-length O-antigen synthesis. In addition, χ9852 (pYA4088) was grown in the presence of arabinoise, conditions permissive for rfaH expression, and given as a boost on day 28, which may also have played a role in generating the observed high anti-LPS antibody titers. A final possible explanation is that our ELISA coating antigen was complete LPS, which includes lipid A, core, and O antigen. One might expect better presentation of LPS core to the mouse immune system by the rfaH mutant strains than by χ9241(pYA4088), leading to an increase in anti-core antibody titers. Our ELISA was not designed to distinguish between anti-core and anti-O-antigen antibodies. This will be investigated in a future study to answer this question. It is an interesting question, since these anti-core antibodies may be cross-reactive with and potentially cross-protective against other enteric bacteria that share the same core structure.

In summary, we have shown that rfaH mutations can be combined with other attenuating mutations to produce an RASV capable of delivering a protective antigen to induce protective immunity. A strain with the delayed regulated mutation ΔP<sub>rfaH</sub> in combination with ΔpabA ΔpabB mutations was superior to a ΔpabAΔpabBΔrfaH strain in colonizing lymphoid tissues, eliciting serum antibodies to a heterologous antigen, and inducing protective immunity against S. pneumoniae challenge. Notably, the ΔP<sub>rfaH</sub> vaccine was more effective than the isogenic ΔrfaH strain at inducing antibodies cross-reactive with a number of other enteric pathogens, making it suitable for inclusion in vaccines to protect against enteric diseases.

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REFERENCES


