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Regulated delayed expression of *rfc* enhances the immunogenicity and protective efficacy of a heterologous antigen delivered by live attenuated *Salmonella enterica* vaccines

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1. Introduction

The use of attenuated bacteria as vaccine delivery vehicles for heterologous antigens has been studied extensively in both animals and humans. Attenuated *Salmonella* is an attractive choice due to its ability to stimulate both cell and humoral-mediated immunity against a heterologous antigen and thus provide protection against pathogen challenge [1–3] when given orally. An optimal live oral *Salmonella* vaccine would retain its ability to colonize and invade host lymphoid tissues while remaining completely avirulent [4]. Several *Salmonella* strains with various modifications have been created in an attempt to balance safety and immunogenicity (see reviews [1, 3, 5]), however, there is a need for improved strains.

A safe vaccine should carry multiple attenuating mutations. Some target genes for attenuation are those involved in the synthesis of lipopolysaccharide (LPS). LPS is a structural component of the gram-negative outer membrane. Full length (smooth) LPS is comprised of three components – lipid A, also a potent activator of the innate immune response, the core oligosaccharide, and O-antigen, a polymer of repeating sugar units.

Four LPS genes that have been shown previously to be useful deletion targets for *Salmonella* vaccines are *galE*, *pmi*, *rfaH* and *rfc*. Two of these genes, *galE* and *pmi*, are involved in synthesizing sugars required for core and/or O-antigen synthesis. GalE is a UDP-galactose epimerase that inter-converts UDP-glucose and UPD-galactose, an essential part of core sugar and O-antigen. This mutant synthesized core-defective LPS in the absence of galactose but made normal LPS when galactose was available in the growth media [6, 7]. *S*. Typhimurium *galE* strains are avirulent and immunogenic [6]. However, this same mutation, transferred to *S.* Typhi, was not attenuated and was poorly immunogenic in humans [8].

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The *pmi* gene codes for phosphomannose isomerase, which converts fructose-6-P to mannose-6-P [9]. The deletion mutant is unable to synthesize the O-antigen due to unavailability of mannose, an O-antigen sugar. When the mutant is grown in the presence of mannose, the smooth LPS phenotype is exhibited. *S*. Typhimurium strains with a *pmi* deletion are attenuated and immunogenic [10]. We constructed and evaluated a Δ*pmi* mutant in our lab, confirming previous results [10–12]. This mutant was attenuated as expected, but also induced antibodies that cross-reacted with outer membrane proteins from other *Salmonella* serovars and other gram-negative bacteria [12]. A *pmi* deletion in Typhi has not yet been evaluated in humans. Note that both *galE* and *pmi* mutant strains, if grown in the presence of galactose or mannose, respectively, transiently express LPS before colonizing the gut-associated lymphoid tissue (GALT) or organs [11, 13]. Because these mutants are essentially wild-type at the time of immunization and become attenuated in host tissues, we have termed this effect regulated delayed attenuation.

RfaH is a transcriptional anti-terminator required for the synthesis of many virulence determinants including O-antigen, core sugar, capsular polysaccharide, and Vi antigen [14, 15]. An *rfaH* deletion mutant, described as “gently rough”, exhibited some deep-rough characteristics, i.e. lack of O-antigen and outer core, sufficient attenuation, susceptibility to detergents and to some antibiotics, but still proved to be attenuated and immunogenic [14, 16].

Rfc (Wzy) is the O-antigen polymerase that, in conjunction with Wzx (transporter), Wzz (length determinant) and WbaP (O-antigen synthesis initiation), synthesizes, assembles, and transports the O-antigen to the periplasm, where WaaL (ligase) ligates O-antigen to lipid A to form complete LPS [17–19]. *rfc* mutants exhibit a characteristic “semi-rough” phenotype, making lipid A and core capped with a single O-antigen subunit. An *S*. Typhimurium *rfc* mutant administered orally to mice was avirulent and protective against oral challenge with wild-type *S.* Typhimurium [10].

Unlike *pmi* and *galE* mutants, strains with mutations in *rfaH* or *rfc* do not transiently express full length O-antigen and therefore may be impaired in their ability to invade and colonize host tissues effectively. A tightly regulated *araC* P_{BAD} activator-promoter has been used extensively in our lab to regulate gene expression [12, 20]. In a previous study, we replaced the *rfaH* promoter with *araC* P_{BAD} and the resulting strain exhibited arabinose-dependent O-antigen synthesis [21]. The promoter substitution mutation was then introduced into attenuated *S*. Typhimurium strain χ9241 (Δ*pabA* Δ*pabB* Δ*asdA*). The resulting strain was found to be superior to a χ9241 Δ*rfaH* strain for delivery of the pneumococcal protein PspA, eliciting significantly greater protection against challenge with virulent *Streptoccocus pneumoniae* [21].

In this study, we evaluated whether this regulated delayed attenuation approach would provide advantages when used with *rfc*. We replaced the *rfc* promoter with an *araC* P_{BAD} promoter to create arabinose inducible production of Rfc and thus regulate *rfc* expression to mimic transient expression of smooth LPS. We evaluated a series of *rfc* promoter mutations in vitro and introduced one of them into vaccine strain χ9241. The resulting strain was evaluated for its ability to deliver the heterologous protein PspA to mice.

2. Materials and Methods

2.1. 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 [22], nutrient broth (NB) (Difco) or on LB agar with or without 0.1% arabinose. Selenite broth, with or without supplements, was used for

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enrichment of *S.* Typhimurium from mouse tissues. Diaminopimelic acid (DAP) was added (50 μg/ml) for the growth of Δ*asd* strains [23]. LB agar containing 5% sucrose was used for *sacB* gene-based counterselection in allelic exchange experiments. *S. pneumoniae* WU2 was cultured on brain heart infusion agar containing 5% sheep blood or in Todd-Hewitt broth plus 0.5% yeast extract, and was kept at −80°C for future use. MOPS minimal medium [24] with/without 10 μg/ml *p*-aminobenzoic acid (pABA) was used to confirm the phenotype of Δ*pabA* Δ*pabB* mutants.

2.2. Plasmids and mutant strain construction

DNA manipulations were carried out as previously described [25]. Electroporation was used to transform *E. coli* and *S. enterica*. Transformants were selected on LB agar plates containing appropriate antibiotics. Strains were grown on LB agar plate without supplement to select for bacteria harboring the Asd^+ plasmid. The primers used in this work are listed in Table 2. A 500-bp DNA fragment containing the region upstream of the *rfc* promoter (P_{rfc}) was PCR amplified using the *S.* Typhimurium χ3761 genome as template with primers Rfc1-FXmaI-pstI and Rfc1-RPstI (Table 2). The amplicon was digested with PstI and ligated into the PstI site of vector pYA3700 (Table 1). This placed the promoter just upstream of *araC*. Primer T4TT-R, which binds to the T4 transcriptional terminator antisense sequence present downstream of the PstI site in pYA3700, and primer Rfc1-RPstI were used to screen plasmid isolates for inserts in the correct orientation. This intermediate plasmid was digested with XhoI and KpnI at restriction sites that lie downstream of the *araC* P_{BAD} cassette. Three different 0.5 kb PCR fragments of the *rfc* gene were amplified from the *S.* Typhimurium χ3761 genome, using three different upstream primers, Rfc2-FXhoI, Rfc2-1, and Rfc2-2, and the same downstream primer Rfc2-RkpnI. Each PCR fragment was engineered to encode a different Shine-Dalgarno (SD) sequence and/or start codon (Table 1) due to the differences in the upstream primers. The PCR fragments were digested with XhoI and KpnI and inserted into the intermediate plasmid described above. The three resulting constructs were confirmed by DNA sequence analysis. Then, 2.5-kb DNA fragments encoding *araC* P_{BAD} *rfc* and *rfc* 5′ and 3′ flanking regions were excised from each of the plasmids by digestion with KpnI and XmaI and inserted into pRE112, resulting in plasmids pYA4297, pYA4298, and pYA4299. To construct the Δ*rfc-48* deletion, two pairs of primers Rfc-1F/Rfc-1R and Rfc-2F/Rfc-2R were used to amplify approximately 300-bp fragments upstream and downstream of *rfc*, respectively, from the χ3761 genome. The two PCR fragments were purified from agarose gels and were used as template at a 1:1 molar ratio for joining by PCR using primers Rfc-1F and Rfc-2R. The resulting PCR product was digested with KpnI and XmaI and ligated into plasmid pRE112, digested with the same two enzymes, resulting in plasmid pYA4717, which carries a deletion of the entire *rfc* gene from ATG to TAA. The mutations were introduced into *S.* Typhimurium χ3761 by allelic exchange using the four suicide vectors pYA4717, pYA4297, pYA4298 and pYA4299 to generate χ9944, χ9659, χ9736, χ9737, respectively. The Δ*rfc-48* and ΔP_{rfc174} mutations were also introduced into *S.* Typhimurium strain χ9241 to yield strains χ9885 and χ9853, respectively. The presence of both Δ*pabA1516* and Δ*pabB232* mutations in *S.* Typhimurium strains χ9241, χ9885 and χ9853 were verified by the inability of the strains to grow in MOPS minimal medium without *p*-aminobenzoate. The presence of Δ*asdA16* mutation was confirmed by PCR and by the strains' inability to grow in media without DAP. The Δ*araBAD23* mutation was verified by PCR and by its white colony phenotype on MacConkey agar supplemented with 1% arabinose. LPS profiles of *Salmonella* strains were examined by the methods of Hitchcock and Brown [26] using cultures standardized based on OD_{600}.

2.3. P22 transduction studies

P22HT *int* [27] was propagated on *S.* Typhimurium strain χ9430 carrying the integrated suicide plasmid pYA4284, which confers chloramphenicol resistance. Strains to be tested

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were grown overnight in NB at 37°C. Cultures were diluted 1:100 into fresh, prewarmed nutrient broth with or without 0.1% arabinose and grown at 37°C to an OD_{600} of 0.9. Then, 10 μl of phage was added to 1 ml of cells (5×10^8 CFU) and the mixture was incubated at room temperature for 30 min, centrifuged and resuspended in 200 μl of buffered saline with gelatin (BSG) [28]. A 100 μl aliquot was spread onto LB agar plates containing 15 μg/ml chloramphenicol and incubated overnight at 37°C. Colonies were counted the following day. We performed this experiment three times. Duplicate samples were plated for each repetition.

2.4. Minimum inhibitory concentration (MIC) test

The MICs of different antimicrobial substances were determined using 96-well microtitre plates [29]. Two-fold serial dilutions of the bile salt deoxycholate (0.3 to 40 mg/ml) and polymyxin B (0.075 to 4.7 μg/ml) were made down the plates. Bacteria were grown until they reached an OD_{600} of 0.8–0.9 in NB with or without 0.1% arabinose and washed in PBS. Cells were diluted to 1.0×10^5 to 1.0×10^6 CFU in NB with or without arabinose. Then, 0.1 ml of the diluted cell suspension was added to each well. The microtitre plates were incubated overnight at 37°C. The optical density of each culture was determined using a SpectraMax M2e (Molecular Devices, CA) plate reader. The threshold of inhibition was 0.1 at OD_{600}. Actual titers were determined by spreading culture dilutions onto LB agar plates followed by overnight incubation at 37°C. Assays were repeated at least three times.

2.5. Determination of virulence in mice

Seven week old, female BALB/c mice were obtained from the Charles River Laboratories. The Arizona State University Animal Care and Use Committees approved all animal procedures. Mice were acclimated for 7 days after arrival before starting the experiments. For determination of the 50% lethal dose (LD_{50}), bacteria were grown statically overnight at 37°C in LB broth, diluted 1:50 into fresh media containing 0.1% arabinose, and grown with aeration (180 rpm) at 37°C. When the cultures reached an OD_{600} = 0.8–0.9, they were harvested by room temperature centrifugation at 4,000 rpm, and normalized to the required inoculum densityin BSG by adjusting the suspension to the appropriate OD_{600} value. Groups of five mice each were infected orally with 20 μl containing various doses of *S.* Typhimurium χ3761 or its derivatives, ranging from 1.0×10^3 CFU to 1.0×10^9 CFU. Animals were observed for 4 weeks post infection, and deaths were recorded daily.

To evaluate colonization, mice were orally inoculated with 20 μl of BSG containing 1.0×10^9 CFU of each strain. At days 4 and 8 after inoculation, three animals per group were euthanized and their spleens and livers were collected. Each sample was aseptically weighed and then homogenized in a total volume of 1 ml BSG. 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. If 0.1ml of homogenized organ yielded no colony on the LB plate, the remaining BSG suspension of each tissue sample was inoculated into selenite cysteine broth the next day. Samples that were positive by enrichment in selenite cysteine broth for 14 h at 37°C were recorded as < 10 CFU/g.

2.6. Immunogenicity of vaccine strains in mice

Recombinant attenuated *Salmonella* vaccine (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 with 0.1% arabinose and grown with aeration at 37°C to an OD_{600} of 0.8 to 0.9. Cells were harvested by room temperature centrifugation at 4,000 rpm for 15 min and the pellet resuspended in 1 ml of BSG. Mice were orally inoculated with 20 μl of BSG containing 1×10^9 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

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intervals. Blood was allowed to coagulate at 37°C for two hours. Following centrifugation, the serum was removed from the whole blood and stored at −20°C.

2.7. Antigen preparation

The rPspA protein was purified as described [30]. The rPspA clone, which encodes the α-helical region of PspA (aa 1–302) in pET20b, was a kind gift from Dr. Susan Hollingshead at the University of Alabama at Birmingham. *S*. Typhimurium LPS was purchased from Sigma. *Salmonella* outer membrane proteins (SOMPs) were prepared as described [30].

2.8. SDS-PAGE and western blot analyses

Protein samples were boiled for 5 min and then separated by SDS-PAGE. For western blotting, proteins separated by SDS-PAGE were transferred electrophoretically to nitrocellulose membranes. The membranes were blocked with 3% skim milk in 10 mM Tris-0.9% NaCl (pH 7.4) and incubated with rabbit polyclonal antibodies specific for PspA [31] or GroEL (Sigma, St. Louis, MO). The secondary antibody was an alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG) (Sigma). Immunoreactive bands were detected by the addition of BCIP/NBT solution (Sigma). The reaction was stopped after 2 min by washing with large volumes of deionized water.

2.9. Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to assay serum antibodies against *S.* Typhimurium LPS, rPspA and SOMPs as previously described [32]. Color development (absorbance) was recorded at 405 nm using a SpectraMax M2e automated ELISA plate reader (Molecular Devices, CA). Absorbance readings 0.1 nm higher than PBS control values was considered positive.

2.10. Pneumococcal challenge

We assessed the protective efficacy of immunization with the attenuated *Salmonella* expressing *pspA* at week 8 by intraperitoneal (i.p.) challenge with 4×10^4 CFU of *S. pneumoniae* WU2 in 200 μl of BSG [33] The LD_{50} of *S. pneumoniae* WU2 in BALB/c mice was 2×10^2 CFU by i.p. administration (data not shown). Challenged mice were monitored daily for 30 days.

2.11. Statistical analysis

Antibody titers were expressed as means ± SEM and the relative immunoreactivity was expressed as an arithmetic mean. The means were evaluated with one or two-way ANOVA and Bonferroni's Multiple Comparison Test for multiple comparisons among groups of antibody titers. Chi square test and the log-rank test were used to analyze the survival rate after challenge by WU2. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Mutant construction and LPS phenotypes

Mutants designed to produce various levels of Rfc were constructed as described in Materials and Methods. Figure 1A illustrates the chromosomal structure of the *araC* P_{BAD} *rfc* mutant strains. Nucleotides (285) upstream from the *rfc* start codon were replaced with *araC* P_{BAD} and either a different Shine-Dalgarno (SD) sequence, a changed ATG/GTG start codon, or both. Table 1 shows the SD and start codon sequence for each mutant strain.

The effects of the *rfc* and arabinose-regulated *rfc* mutations on the LPS phenotypes were revealed by SDS-PAGE with silver staining (Fig. 1B) and by western blotting against *Salmonella* Group B O-antigen (Fig. 1C). Deletion of *rfc* resulted in expected LPS semi-

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rough phenotype, but two of the three promoter-replacement mutants produced low levels of complete O-antigen when grown in NB without arabinose. Growth in the presence of arabinose resulted in synthesis of full-length O-antigen in the *araC* P_{BAD} *rfc* strains, but not in the χ9944 (Δ*rfc-48*) strain. Strain χ9736 (ΔP_{rfc174}::TT *araC* P_{BAD} *rfc*, or ΔP_{rfc174}) and χ9659 (ΔP_{rfc173}::TT *araC* P_{BAD} *rfc*, or ΔP_{rfc173}), when grown with arabinose, produced an LPS pattern similar to the wild-type *Salmonella* UK-1. However, strain χ9659 also produced complete O-antigen when grown in the absence of arabinose, detectable in both silver-stained gels and by western blot (Fig. 1B) indicating leaky expression of *rfc*. Strain χ9737 (ΔP_{rfc175}::TT *araC* P_{BAD} *rfc*, or ΔP_{rfc175}), when grown in the absence of arabinose, did not produce any detectable full-length O-antigen. However, this strain did not produce wild-type amounts of O-antigen when grown in the presence of arabinose. Strain χ9736 (ΔP_{rfc174}::TT *araC* P_{BAD} *rfc*) produced low levels of full-length O-antigen detectable only by western blot.

To determine if the O-side chains are present, albeit at a level non-detectable by silver staining or western blotting, we tested the transduction efficiency of each strain using the *S.* Typhimurium O-antigen specific phage P22, which cannot infect *rfc* mutants [10]. Strains were grown in NB with or without arabinose and used as recipients for transduction assays. Strains χ9659 (ΔP_{rfc173}), χ9736 (ΔP_{rfc174}) and χ9737 (ΔP_{rfc175}) were sensitive to transduction when grown with or without arabinose (Table 3), confirming existence of the complete O-antigen under both conditions. But fewer transductants were obtained for all three promoter mutants when they were grown in NB without arabinose, indicating a decrease in O-antigen synthesis during arabinose-free growth. The number of transductants obtained for each strain roughly mirrored the amount of O-antigen detected for each strain (Fig. 1B, 1C). As expected, no transductants of Δ*rfc-48* mutant were found, confirming that P22 transduction requires multiple O-unit O-antigens to infect.

3.2. Phenotypes of mutant strains

Rough mutants display a pleiotropic phenotype. Three common features are increased susceptibility to environmental factors, loss of cell surface organelles, and decreased ability to colonize the mouse intestine [34]. We began our analysis of these mutants with in vitro assays examining their susceptibility to different environmental factors such as the bile salt deoxycholate and the antimicrobial peptide polymyxin B. The results are summarized in Table 3. The deoxycholate MIC for the wild-type strain, χ3761, was about four-fold higher than that for the Δ*rfc-48* mutant, χ9944 and the arabinose-regulated mutants, χ9659 (ΔP_{rfc173}), χ9736 (ΔP_{rfc174}), χ9737 (ΔP_{rfc175}) when grown in absence of arabinose (Table 3).

The results of the MIC to polymyxin B were consistent with the levels of LPS observed in Figure 1. In the presence of arabinose, the arabinose-regulated *rfc* mutants behaved identically to the wild-type strain (MIC = 0.59 μg/ml); while in the absence of arabinose, the MIC was reduced four-fold compared to the wild-type strain (MIC = 0.15 μg/ml), except for χ9659 (ΔP_{rfc173}). This strain, which appeared to have leaky control over *rfc* expression as shown in Figure 1, had an MIC closer to the wild-type control. The MIC for strain χ9944 (Δ*rfc-48*), in which the *rfc* gene was deleted, is similar to the levels of the promoter mutation strains under non-inducing conditions (MIC = 0.15 μg/ml) regardless of whether arabinose was present.

3.3. Virulence in BALB/c mice

Groups of female BALB/c mice received graded doses of various strains orally and were monitored for 30 days after inoculation. The LD_{50}s for the strains were shown in Table 3. The LD_{50} of χ3761 (1.0×10^4) in mice was similar to that previously observed [21]. The mutant strain χ9944 (Δ*rfc-48*) was avirulent by the oral route, as previously reported [10].

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The LD_{50} of χ9659 (ΔP_{rfc173}), which exhibited leaky O-antigen synthesis (Fig. 1), was 50-fold higher than that of χ3761 when it was grown in arabinose-containing LB prior to feeding. However, strain χ9736 (ΔP_{rfc174}) was totally avirulent ($LD_{50} > 10^9$ CFU), even when grown with arabinose prior to oral administration to mice.

Mice that survived infection with the different mutants were challenged orally with 10^5 LD_{50} (1.0×10^9 CFU) of wild-type *S*. Typhimurium 30 days after administration of the attenuated strains. All five mice immunized with 1.0×10^9 χ9736 (ΔP_{rfc174}) were resistant to challenge. However, only two of five mice immunized with 1.0×10^9 χ9944 (Δ*rfc-48*) survived after challenge by wild-type *S*. Typhimurium. These data indicate that χ9736 (ΔP_{rfc174}) was sufficiently attenuated but still retained its immunogenic characteristics.

3.4. Expression of the pneumococcal gene *pspA* in RASV strain χ9241 derivatives carrying different *rfc* mutations

Based on the above results, we decided to focus on the ΔP_{rfc174}::TT *araC* P_{BAD} *rfc* mutant. We wanted to evaluate the ability of strains carrying the Δ*rfc-48* and ΔP_{rfc174}::TT *araC* P_{BAD} *rfc* mutations, in a genetic background that included additional attenuating mutations, to elicit an effective immune response against a vectored antigen in mice. Mutations were introduced into attenuated *S.* Typhimurium strain χ9241 (Δ*pabA1516* Δ*pabB232* Δ*asdA16* Δ*araBAD23* Δ*relA198::araC* P_{BAD} *lacI* TT) [31, 32]. In addition to the *pabA* and *pabB* mutations that confer attenuation, strain χ9241 also encodes an arabinose-regulated *lacI* gene. Synthesis of LacI represses transcription from promoters such as P_{trc} that carry a *lacO* sequence. This system was designed to prevent the stress of high-level expression of heterologous antigens transcribed from P_{trc} during *in vitro* growth and the early stages of host infection. Once inside the host, where free arabinose is not available, the antigen gene is expressed [31].

The χ9241 derivatives χ9885 (Δ*rfc-48*) and χ9853 (ΔP_{rfc174}) were constructed by using suicide plasmids pYA4717 and pYA4298, respectively, as described in Materials and Methods. The pYA4088 plasmid (Asd^+ plasmid that encodes *pspA* under transcriptional control of the LacI-repressible P_{trc} promoter) or the empty vector pYA3493, was electroporated into both mutants and the parent strain. We then confirmed arabinose-regulated PspA and LacI synthesis. We found that all strains carrying plasmid pYA4088 synthesized LacI, but no PspA when arabinose was present and synthesized PspA, but no LacI, when grown in the absence of arabinose (supplementary Figure 1), as we have previously observed [21]. All strains synthesized similar amounts of PspA and LacI (supplementary Fig. 1). No PspA was detected in χ9241(pYA3493), which does not carry the *pspA* gene.

3.5. Colonization of mouse tissues and immune responses in mice after oral immunization with RASV expressing PspA

The observation that the χ9736 (ΔP_{rfc174}) was avirulent when given orally yet was capable of immunizing mice against a χ3761 challenge suggests that the strain retained a limited ability to colonize either or both the intestinal or systemic immune tissues of the mice. To confirm that these mutations in the χ9241 background still retained the ability to colonize systemic immune tissues of the mice, groups of mice were orally inoculated with 1.0×10^9 cells of various strains harboring pYA4088, and bacterial colonization of spleen and liver was enumerated at 4 and 8 days post inoculation (Fig. 2).

Strains χ9241(pYA4088) and χ9853(pYA4088) (ΔP_{rfc174}) colonized the spleen and liver, and reached high numbers in both tissues (approximately 10^6 CFU per gram of tissue), with a slight reduction at eight days. The titers in spleen and liver for strain χ9885(pYA4088)

(Δ*rfc-48*) were significantly lower than the other two strains at both time-points tested. There was a slight reduction in tissue colonization by χ9853(pYA4088) (ΔP_{rfc174}) compared to its parent strain χ9241(pYA4088), but the difference was not statistically significant.

3.6. Effect of Δ*rfc* and ΔP_{rfc174} mutations on immunogenicity of RASV strains

Mice were inoculated orally (1.0×10^9 CFU) with one of four strains harboring either pYA4088 or the empty vector pYA3493. Mice were boosted with a similar dose of the same strain 4 weeks later. This experiment was performed twice, 5 mice per group were involved in the first experiment, and 6–8 mice per group were used in the second experiment. The results from both experiments were similar and have been pooled for analysis.

High serum IgG titers against rPspA (Fig. 3A) were observed 2 weeks after the primary immunization in mice inoculated with χ9241(pYA4088), χ9885(pYA4088) (Δ*rfc-48*) and 9853(pYA4088) (ΔP_{rfc174}). There was a significant difference between the serum IgG titers from the mice immunized with χ9241(pYA4088) and χ9853(pYA4088) (ΔP_{rfc174}). Anti-LPS titers were low, but detectable for all strains at 2 weeks, and reached a maximum by 8 weeks (Fig. 3B). Anti-LPS titers from mice immunized by χ9885(pYA4088) (Δ*rfc-48*) and χ9853(pYA4088) (ΔP_{rfc174}) were significantly lower than those of mice immunized by χ9241(pYA4088 or pYA3493) during all time points measured. The anti-SOMP responses developed faster for the ΔP_{rfc174} strain than that for the other groups, with significantly higher titers by week 2 (Fig. 3C).

By week 4, the serum anti-rPspA IgG antibody levels of mice immunized with χ9885(pYA4088) (Δ*rfc-48*) was significantly higher than mice immunized with χ9241(pYA4088). The titers were slightly lower than those in mice immunized with χ9853(pYA4088) (ΔP_{rfc174}), but the difference was not significant. The anti-LPS IgG titers in χ9853(pYA4088) (ΔP_{rfc174}) immunized mice were significantly lower than that in mice immunized with χ9241(pYA4088). All groups except those immunized with the Δ*rfc* strain developed similar, high titers against SOMPs. No anti-PspA IgG was detected in mice immunized with strain χ9241(pYA3493).

After the second immunization at week 4, no significant boosting of serum antibody responses to either rPspA or LPS or SOMP was observed for any group of immunized mice, except in the case of the anti-SOMP titers for the Δ*rfc-48* strain χ9885(pYA4088) (Δ*rfc-48*). This strain achieved anti-SOMP titers similar to all other strains by week 6 (Fig. 3C). The serum immune responses peaked at 6 weeks with no significant changes observed at week 8.

3.7. IgG isotype analyses

The serum immune responses to rPspA were further examined by measuring the levels of IgG isotype subclasses IgG1 and IgG2a. Th1-biased immune responses are typically observed after immunization with attenuated *Salmonella* strains. Although the IgG2a levels were always higher than IgG1 levels during each time period checked, the levels of anti-rPspA IgG1 and IgG2a isotypes antibodies gradually increased (Fig. 4). The ratio of IgG2a to IgG1 was 16:1 for χ9885(pYA4088) (Δ*rfc-48*)-immunized mice and 2–4:1 for χ9241(pYA4088) or χ9853(pYA4088) (ΔP_{rfc174})-immunized mice.

3.8. Evaluation of protective immunity

To examine the ability of RASV-rPspA vaccines to protect against pneumococcal infection, mice were challenged i.p. with 4.0×10^4 CFU (200 LD_{50}) of *S. pneumoniae* WU2 4 weeks after they were boosted. The *pspA* gene carried by plasmid pYA4088 was derived from *S. pneumoniae* strain RX1 and is cross-reactive with PspA produced by *S. pneumoniae* WU2. Immunization with any of the *pspA*-expressing strains provided significant protection

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against challenge compared with the control group receiving χ9241(pYA3493) (empty vector) (Table 4). However, the protection afforded by χ9885(pYA4088) (Δ*rfc-48*) and χ9853(pYA4088) (ΔP_{rfc174}) was significantly higher than χ9241(pYA4088). There was no significant difference in protection afforded by strains χ9885(pYA4088) (Δ*rfc-48*) and χ9853(pYA4088) (ΔP_{rfc174}). All of the mice that died in these experiments succumbed within 4 days of the challenge.

4. Discussion

The greatest challenge in the development of live *Salmonella* vaccines is to maintain a balance between safety and immunogenicity [3, 35]. After oral immunization, a RASV must be able to withstand acidic, osmotic, and enzymatic stresses as well as surviving the host defenses of bile, antimicrobial peptides, and innate immunity. The ideal RASV strain will be capable of tolerating the aforementioned stressors while remaining avirulent and capable of eliciting a robust immune response. Smooth LPS provides *Salmonella* with a covalently attached, protective envelope conferring resistance to a variety of environmental stresses encountered both within and outside a mammalian host thereby enhancing virulence. Strains with mutations that eliminate LPS O-antigen are typically less immunogenic, a phenotype associated with their failure to colonize the intestinal tract and invade intestinal mucosal cells [36, 37]. It is therefore advantageous for vaccine strains to regulate the O-antigen synthesis, producing the full-length LPS during immunization, when it will encounter stresses imposed by the host, and then shutting off O-antigen polymerization *in vivo* after the bacteria have colonized the host lymphoid tissues [3].

Structural rough mutants have been considered to be inappropriate live vaccine carriers due to their hyperattenuation and poor colonization [38, 39]. In this work, we applied the regulated delayed attenuation approach to produce strains with arabinose-regulated synthesis of Rfc, the polymerase required for production of full-length O-antigen, and compared arabinose-regulated *rfc* expression strains to a Δ*rfc* strain and wild-type strain in *in vitro* assays. As previously observed in other arabinose regulated expression systems [21], the SD sequence and start codon greatly affected the tight control of expression of *rfc* under the *araC* P_{BAD} promoter. In the case of arabinose-regulated *rfc* expression, based on the LPS profile silver staining and western blot analysis using *Salmonella* anti-LPS sera, only the strain containing the ΔP_{rfc174}::TT *araC* P_{BAD} *rfc* construct was unable to produce complete O-antigen in the absence of arabinose, and able to produce the full-length O-antigen in the presence of arabinose (Fig. 1). The strain containing ΔP_{rfc173} was leaky for expression of *rfc*, leading to some polymerization of O-antigen without arabinose, and ΔP_{rfc175} exhibited tight control of *rfc* expression (Fig. 1). When grown without arabinose, production of full length O-antigen in the strain carrying the ΔP_{rfc175} mutation, undetectable by gel or western blot (Fig. 1), was revealed by the P22 transduction assay (Table 3). However the number of P22 transductants obtained for all three promoter mutants was reduced when they were grown in the absence of arabinose compared to the number obtained when the strains were grown with arabinose.

As mentioned above, an ideal *Salmonella* vaccine strain should exhibit wild-type abilities to withstand stresses and host defenses encountered after mucosal immunization while remaining avirulent. When the ΔP_{rfc174} mutant was grown in the presence of arabinose, its phenotype was similar to that of its wild-type parent χ3761 (Fig. 1 and 2 and Table 3), though it was totally attenuated ($LD_{50} > 10^9$) for virulence in mice (Table 3). In the absence of arabinose, this mutant was more susceptible to deoxycholate and polymyxin B than the wild-type parent χ3761.

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It is also important that live RASV strains carry multiple attenuating mutations. Therefore, we evaluated the effect of the ΔP_{rfc174} mutation in the context of an attenuated vaccine strain, χ9241, and introduced plasmid pYA4088 to allow expression of the heterologous antigen gene, *pspA*. The ΔP_{rfc174} strain χ9853(pYA4088) colonized host lymphoid tissues as well as the parent strain χ9241(pYA4088) (Fig. 2), while the Δ*rfc* strain, χ9885(pYA4088), colonized poorly. It is interesting to note that the numbers we obtained in the spleen for χ9885(pYA4088) (Δ*rfc-48*) are similar to numbers previously reported for an *rfc* mutant strain with no additional attenuating mutations [10].

The most surprising result we obtained in this study was the observation that the Δ*rfc* mutant χ9885(pYA4088) performed as well as the (ΔP_{rfc174}) strain χ9853(pYA4088) and the Rfc^+ strain χ9241(pYA4088) Despite the fact that χ9885(pYA4088) (Δ*rfc-48*) was a poor colonizer of spleen and liver tissues compared to the other two strains (Fig. 2), immunized mice developed high titers against PspA and achieved levels of protection against *S. pneumoniae* challenge similar to the other two strains (Table 4). This is in contrast to our previous study in which we introduced Δ*rfaH* and *araC* P_{BAD} *rfaH* mutations into the χ9241(pYA4088) background [21]. In that study, the Δ*rfaH* mutant colonized poorly, induced significantly lower anti-PspA titers and significantly less protection, although some protection was observed.

The basis for the ability of χ9885(pYA4088) (Δ*rfc-48*) to elicit a protective response while being impaired for colonization is not clear. However, this result is not without precedent. For example, *rpoS* and *relA spoT* mutants of *S*. Typhimurium are immunogenic and protective against challenge with virulent *S*. Typhimurium and yet these mutants colonize no better than χ9885(pYA4088) (Δ*rfc-48*) [40, 41]. What is not clear, either from this study or the studies just cited, is what impact poor colonization will have on the duration of immunity where it is likely that a strong cellular response is required. We did not include measurement of cellular responses in our experimental design because antibody responses to PspA are adequate for protection against *S. pneumoniae* [42]. With regard to the observed protection, it is possible that while colonization was not robust, presentation of PspA may have been enhanced in the Δ*rfc* mutant due to its lack of full-length O-antigen. In addition, the robust immune response to PspA may have been due, at least in part, to the fact that PspA is a highly immunogenic protein [43]. It is not clear whether or not our results would have been different if we had used a less immunogenic test protein. Alternatively, it was recently shown that mesenteric lymph nodes are an important barrier to systemic disease caused by *Salmonella* [44] and, along with the spleen, can serve as a site for induction of systemic immunity [45]. In light of our current findings that the Δ*rfc* mutant strain χ9885(pYA4088) was a poor colonizer of spleen and liver (Fig. 2), yet was able to induce systemic and protective immunity (Fig. 3, Table 4), it is of interest for us to examine the ability of our vaccine strains to colonize the mesenteric lymph nodes in future studies. If χ9885(pYA4088) can colonize the mesenteric lymph nodes as well or better than χ9241(pYA4088) and χ9853(pYA4088), this may provide a clear understanding of our results with respect to the relationship between colonization and induction of the immune response which would aid greatly in the design of future vaccine strains.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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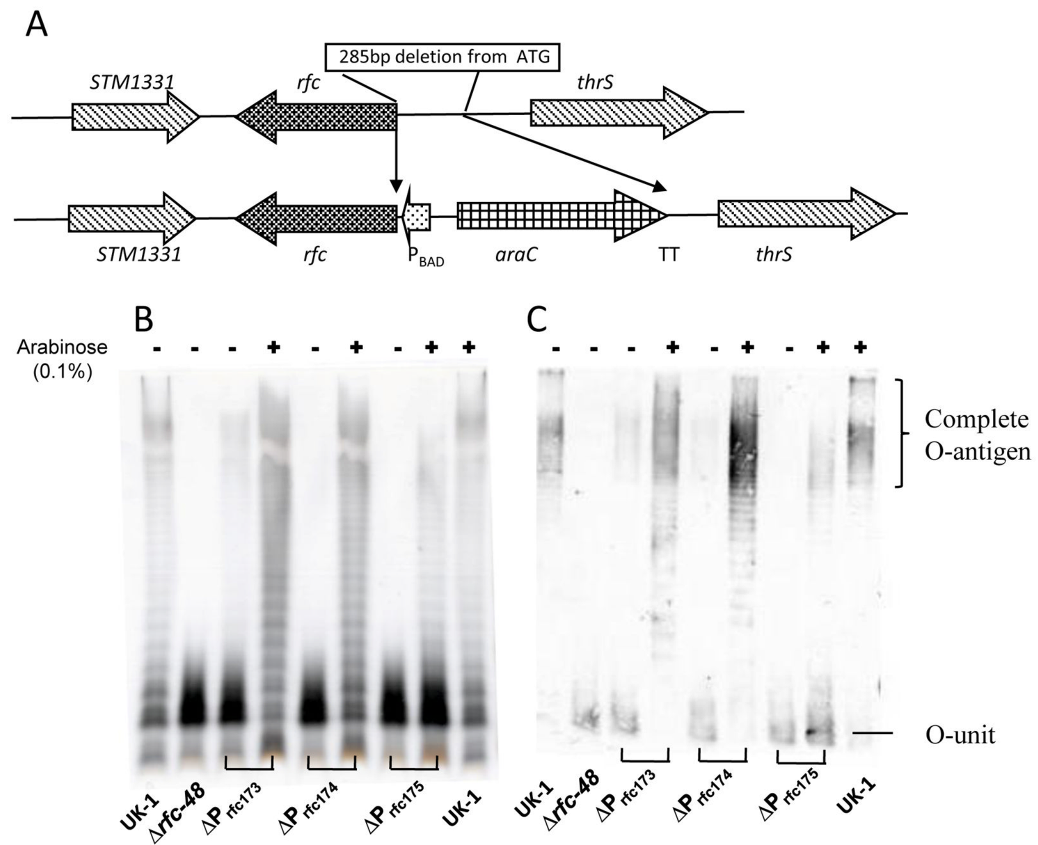

Fig. 1.

Arabinose regulation of *rfc*. (A) Map of deletion-insertion mutations resulting in arabinose-regulated *rfc* 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.

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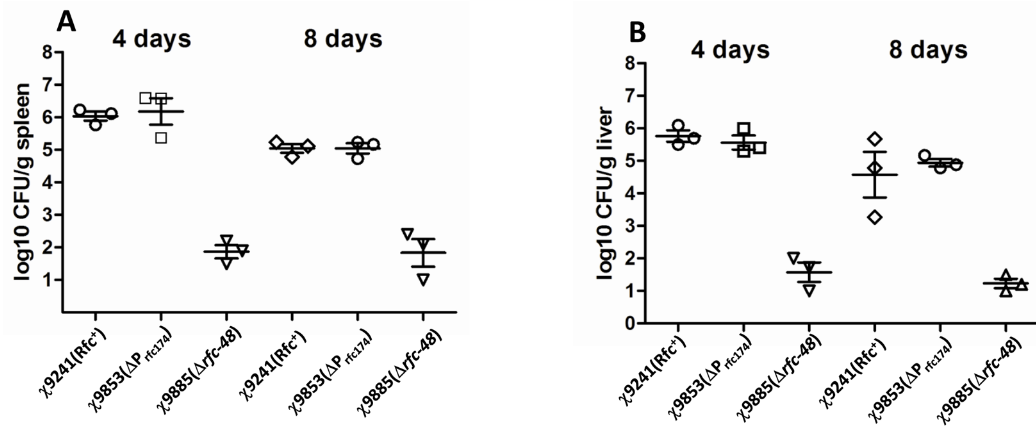

Fig. 2.

Colonization of mouse spleens and livers by attenuated *S*. Typhimurium harboring plasmid pYA4088 growing in LB broth containing 0.1% arabinose. Shown is spleen (A) and liver (B) colonization by the indicated strains in BALB/c mice at 4 and 8 days post-inoculation. The horizontal lines represent the means, and the error bars represent standard errors of the means.

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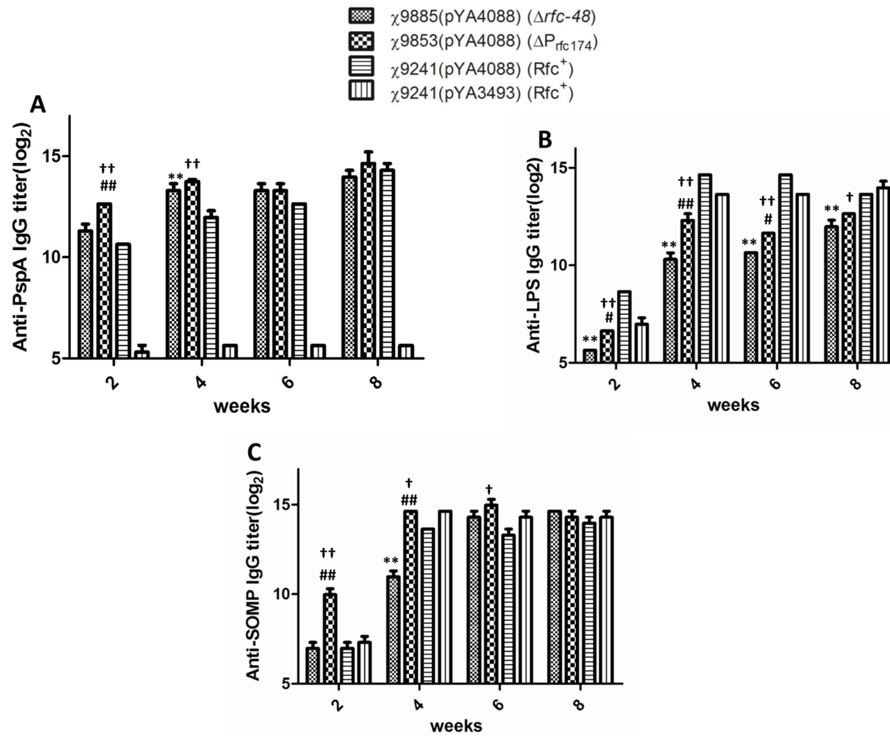

Fig. 3.

Serum IgG responses in immunized and control mice. Total serum IgGs specific for rPspA (A), *S*. Typhimurium LPS (B), and SOMP (C) were measured by ELISA. The data represent reciprocal anti-IgG antibody levels in pooled sera from mice orally immunized with attenuated *Salmonella* carrying either pYA4088 (*pspA*) or pYA3493 (control) the indicated number of weeks after immunization. The error bars represent variations between triplicate wells. The mice were boosted at week 4. **: χ9885(pYA4088) vs. χ9241(pYA4088), $P < 0.001$; #: χ9885(pYA4088) vs. χ9853(pYA4088), $P < 0.01$; ##: χ9885(pYA4088) vs. χ9853(pYA4088), $P < 0.001$; ††: χ9853(pYA4088) vs. χ9241(pYA4088), $P < 0.001$; †: χ9853(pYA4088) vs.χ9241(pYA4088), $P < 0.01$.

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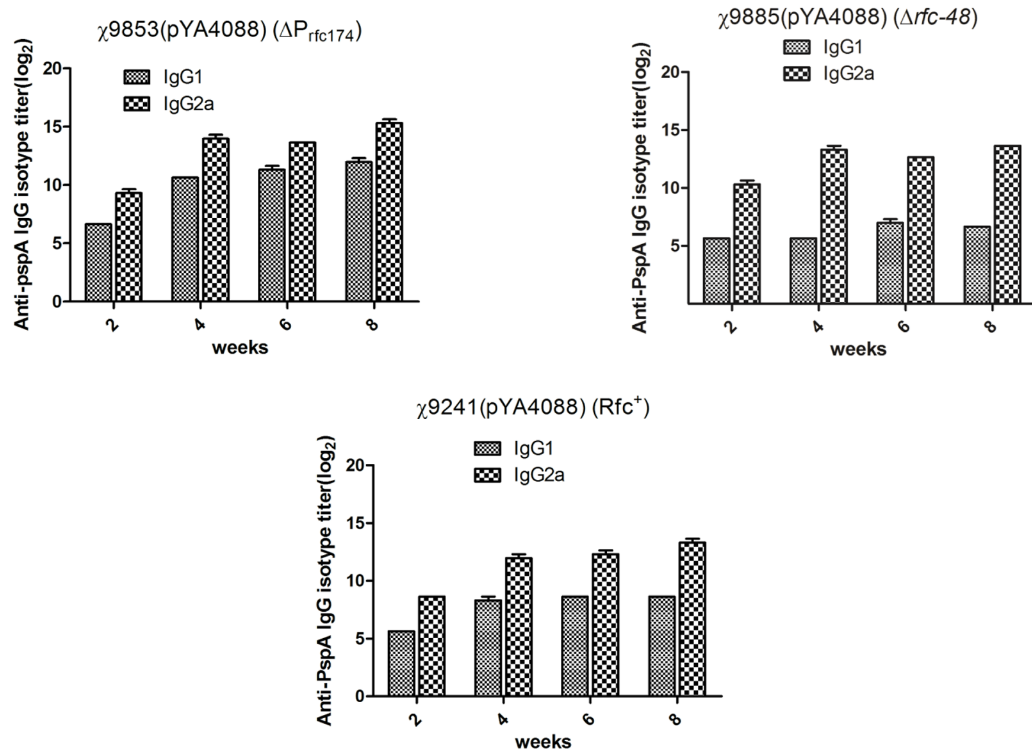

Fig. 4.

Serum IgG1 and IgG2a responses to rPspA. The data represent ELISA results determining the level of IgG1 and IgG2a subclass antibody to rPspA in the sera of BALB/c mice orally immunized with χ9241(pYA4088), χ9885(pYA4088) (Δ*rfc-48*), or χ9853(pYA4088) (ΔP_{rfc174}) the indicated number of weeks after immunization. The error bars represent the standard deviations.

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Table 1

Strains and plasmids used in this work

Strain or plasmid	Description	Source
χ3761	*S.* Typhimurium UK-1, Wild-type	[46]
χ9241	Δ*pabA1516* Δ*pabB232* Δ*asdA16* Δ*araBAD23* Δ*relA198::araC* P_{BAD} *lacI* TT	[31]
χ9659	ΔP_{rfc173}::TT *ara*C P_{BAD} *rfc*	χ3761
χ9736	ΔP_{rfc174}::TT *ara*C P_{BAD} *rfc*	χ3761
χ9737	ΔP_{rfc175}::TT *ara*C P_{BAD} *rfc*	χ3761
χ9944	Δ*rfc-48*	χ3761
χ9430	Δ*pagL7*	χ3761
χ9885	Δ*pabA1516* Δ*pabB232* Δ*asdA16* Δ*araBAD23* Δ*relA198::araC* P_{BAD} *lacI* TT Δ*rfc-48*	χ9241
χ9853	Δ*pabA1516* Δ*pabB232* Δ*asdA16* Δ*araBAD23* Δ*relA198::araC* P_{BAD} *lacI* TT ΔP_{rfc174}::TT *araC* P_{BAD} *rfc*	χ9241
χ9761	Δ(*galE-uvrB*)*-1005* Δ*msbB48* Δ*fliC2426* Δ*pefA1225* Δ*fimA2119* Δ*fimH1019* Δ*agfBAC811*	Lab stock
E. coli K-12		
χ7213	*thi-1 thr-1 leuB6 glnV44 fhuA21 lacY1 recA1* RP4-2-Tc::Mu[*λ pir*] Δ*asdA4* Δ(*zhf-2*::Tn*10*)	[47]
χ7232	*endA1 hsdR17* (r_K^- m_K^+) *glnV44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*)*U169 λ pir deoR* (*φ*80d*lac* Δ(*lacZ*)M15)	[47]
S. pneumoniae WU2	Wild-type virulent, encapsulated type 3	[48]
Suicide vectors		
pRE112	*sacB mobRP4* R6K *ori* Cm^+	[49]
pYA4284	pRE112, Δ*pagL7*	Lab stock
pYA4717	Constructed for *rfc-48* deletion	pRE112
pYA4297	Constructed for P_{rfc173} promoter deletion and *araC* P_{BAD} promoter insertion ctcgag **AGGA** gtcatt**ATG**[a]	pRE112
pYA4298	Constructed for P_{rfc174} promoter deletion and *araC* P_{BAD} promoter insertion ctcgag **AGGA** gtcatt**GTG**	pRE112
pYA4299	Constructed for P_{rfc175} promoter deletion and *araC* P_{BAD} promoter insertion ctcgag **GAGG** gtcatt**GTG**	pRE112
Recombinant Plasmid		
pYA3700	TT *araC* P_{BAD} cassette plasmid; Ap^r	[50]
pYA3493	Plasmid Asd^+; pBR*ori* β-lactamase signal sequence-based periplasmic secretion plasmid	[30]
pYA4088	852 bp DNA encoding the α-helical region of PspA from aa 3–285 in pYA3493	[31]

[a]The Shine-Dalgarno sequence and start codon for each *rfc* allele are indicated in uppercase, bold letters.

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Table 2

Primers used in this work

Primer name	Sequence 5′-3′
Rfc1-FXmaI-PstI	ACTGCCTGCAGCCCGGGTCTTTCTGTTCTACAGAACC
Rfc1-RPstI	ACTGCCTGCAGAGATTCATCATGAGGTTCCC
Rfc2-FXhoI	ATGCACTCGAG AGGACTCTATATGCTTATAATTTC
Rfc2-kpnI	ACGGAGGTACCCTCTCTGAACTCCATCAACAC
Rfc2-1	ATGCACTCGAGAGGACTCTATGTGCTTATAATTTC
Rfc2-2	ATGCACTCGAGAGGCTCTATGTGCTTATAATTTC
T4TT-R	ATCACAATTCTAGGATAGAAT
Rfc-1F	CTGCCTGCAGAAGTATGTCGCGGCACGATG
Rfc-1R	ATAACTTACCTGCAGGATAGAGCCTTTAGAAAAAATG
Rfc-2F	GGCTCTATCCTGCAGGTAAGTTATACGGCGGCAATGC
Rfc-2R	ATCTCCCGGG GTTCGTTTAAACCTGTTTCAC

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Table 3

Minimum inhibitory concentrations (MIC) of antibiotic substances, transduction efficiency and virulence of *S*. Typhimurium strain χ3761 and its *rfc* mutant derivatives

Strain	0.1% arabinose	P22 transductants[a] (M±SEM)	MIC DOC[b] Bile (mg/ml)	MIC Polymyxin B (μg/ml)	LD_{50} (CFU)
χ9659 (ΔP_{rfc173})	−	2292±229	2.5	0.3	ND[c]
	+	3250±357	10	0.59	5.0×10^5
χ9736 (ΔP_{rfc174})	−	1892±191	2.5	0.15	$>1.0 \times 10^9$
	+	3425±135	10	0.59	$>1.0 \times 10^9$
χ9737 (ΔP_{rfc175})	−	600±113	2.5	0.15	ND[c]
	+	1908±196	5	0.59	
χ9944 (Δ*rfc-48*)	−	0	2.5	0.15	$>1.0 \times 10^9$
	+	0	2.5	0.15	$>1.0 \times 10^9$
χ3761 (*rfc*$^+$)	−	4430±352	10	0.59	1.0×10^4
	+	4300±306	10	0.59	

[a]The phage lysate used for transduction was grown on a chloramphenicol-resistant strain. Transduction was performed as described in the Materials and Methods section. The results reflect the number of chloramphenicol resistant colonies obtained after transduction.

[b]deoxycholate.

[c]Not determined.

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Table 4

Oral immunization with rPspA-expressing *S. enterica* serovar Typhimurium χ9241(pYA4088) vaccine and its derivatives (pYA4088) protect BALB/c mice against challenge with virulent *S. pneumoniae* strain WU2

Vaccine	Genotype	*pspA* expression[a]	Days to death	Survival/total[b]	Percent protection[b]
χ9853	ΔP_{rfc174}	+	2, 3, 4, 4, 4	6/11	55%
χ9885	Δ*rfc-48*	+	2, 3, 4	10/13	77%
χ9241	Rfc^{+}	+	2, 2, 3, 3, 4, 4, 4	4/11	36%
χ9241	Rfc^{+}	−	1, 1, 1, 2, 2, 2, 2, 2, 2, 2, 3	0/11	0

[a] +, rPspA expressed; -, rPspA not expressed.

[b] Mice were challenged with 4×10^4 CFU of *S. pneumoniae* WU2 (200 times LD_{50}) 4 weeks after the second oral immunization. Mortality was monitored for 2 weeks after pneumococcal challenge. Differences between groups were determined using Chi square test and the log-rank test. All vaccine groups were significantly different from the χ9241(pYA3493) control ($P < 0.002$) by two methods. Chi square test: χ9885(pYA4088) vs. χ9853(pYA4088), $P = 0.1219$; χ9885(pYA4088) vs. χ9241(pYA4088), $P < 0.0001$; χ9853(pYA4088) vs. χ9241(pYA4088), $P = 0.005$. The log-rank test: no significant difference between vaccine strains (pYA4088).