2010

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Clin. Vaccine Immunol. **2010, 17(3):354. DOI: 10.1128/CVI.00412-09.**
Published Ahead of Print 6 January 2010.

CLINICAL AND VACCINE IMMUNOLOGY, Mar. 2010, p. 354–362 Vol. 17, No. 3
1556-6811/10/$12.00 doi:10.1128/CVI.00412-09

Construction of Recombinant Attenuated *Salmonella enterica* Serovar Typhimurium Vaccine Vector Strains for Safety in Newborn and Infant Mice▽†

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Received 16 September 2009/Returned for modification 1 December 2009/Accepted 18 December 2009

Recombinant bacterial vaccines must be safe, efficacious, and well tolerated, especially when administered to newborns and infants to prevent diseases of early childhood. Many means of attenuation have been shown to render vaccine strains susceptible to host defenses or unable to colonize lymphoid tissue effectively, thus decreasing their immunogenicity. We have constructed recombinant attenuated *Salmonella* vaccine strains that display high levels of attenuation while retaining the ability to induce high levels of immunogenicity and are well tolerated in high doses when administered to infant mice as young as 24 h old. The strains contain three means of regulated delayed attenuation, as well as a constellation of additional mutations that aid in enhancing safety, regulate antigen expression, and reduce disease symptoms commonly associated with *Salmonella* infection. The vaccine strains are well tolerated when orally administered to infant mice 24 h old at doses as high as 3.5×10^8 CFU.

Recombinant attenuated *Salmonella* vaccines (RASV) must decrease disease symptoms associated with infection but must also elicit a robust and lasting immune response to the vaccine. Unfortunately, many of the disease symptoms are caused by the same pathogenic strategies of *Salmonella* that are also needed for delivery of the antigen to effector lymphoid tissue. The ability of *Salmonella* to colonize the gut-associated lymphoid tissues (GALT) and adhere to and invade the intestinal epithelium has been implicated in the success of live attenuated vaccines (7); however, many means of attenuation render the bacteria unable to survive and withstand host stresses, and thus, they fail to reach the GALT in sufficient numbers to stimulate robust mucosal, systemic, and cellular immune responses (reviewed by Curtiss et al. [13]). We have developed five means of regulated delayed attenuation (13) in order to retain near-wild-type abilities to reach the GALT, but after several rounds of replication in host tissues, the bacterium loses its ability to survive or withstand host stresses. Such a recombinant *Salmonella* vaccine strain, which contains multiple means of regulated delayed attenuation and is also unable to cause the disease symptoms associated with infection, would hopefully be a highly effective vaccine that could be distributed to a heterogenous population of different ages and various health and nutritional states.

We describe here the construction of attenuated *Salmonella enterica* serovar Typhimurium vaccine strains that display a phenotype of regulated delayed attenuation *in vivo*, that have been further genetically altered for the safe and efficacious delivery of antigens, and that are well tolerated when administered to infant mice. Regulated delayed attenuation is achieved through arabinose-controlled expression of the *fur*, *crp*, and *phoPQ* genes. It has been shown previously that mutations in the *fur*, *crp*, and *phoPQ* genes are attenuating and immunogenic in adult female BALB/c mice (10, 17, 20). The *fur* gene encodes the Fur protein, which regulates iron uptake and acquisition (2); the *crp* gene, encoding the cyclic AMP (cAMP) receptor protein, is required for the transcription of many genes involved in the breakdown and transport of metabolites (23); and the *phoPQ* genes are needed for *Salmonella* survival in macrophages (16, 22, 33). It has been shown previously that mutations in the *pmi* gene, which encodes phosphomannose isomerase, responsible for synthesizing an intact lipopolysaccharide (LPS) O antigen, are also attenuating when strains are grown in the absence of mannose (5, 11). In addition to these attenuating mutations, we have investigated the inclusion of mutations to lessen the inflammation of, and fluid secretion by, the intestinal mucosa in order to enhance vaccine safety and acceptability.

Our vaccine strains are derived from the highly virulent *S.* Typhimurium strain UK-1 (12); the wild-type UK-1 strain χ3761 has a 50% lethal dose (LD_{50}) of 1.2×10^4 CFU when administered orally to adult BALB/c mice. Attenuated UK-1 strains can induce full protective immunity to challenge with *S.* Typhimurium strain SL1344 in mice (46) and strain F98 in chickens (24), but isogenic attenuated strains of SL1344 and F98, though able to induce full protective immunity to their wild-type parents, are unable to induce full protective immunity to the UK-1 strain χ3761. One of our vaccine constructs, χ9558, has essentially the same mutations selected as *Salmonella enterica* serovar Typhi vaccine vectors currently being tested in adult human volunteers.

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† Supplemental material for this article may be found at http://cvi.asm.org/.

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▽ Published ahead of print on 6 January 2010.

TABLE 1. Bacterial strains used in this study

Strain no.	Genotype	Reference or strain of origin
E. coli strain χ7213	*thr-1 leuB6 fhuA21 lacY1 glnV44 recA1* Δ*asdA4* Δ(*zhf-2*::Tn*10*) *thi-1* RP4-2-Tc::Mu [λ*pir*]; Km^r	37
S. Typhimurium strains		
χ3761	Wild-type *S.* Typhimurium UK-1	9
χ8477	Δ*araE25*	χ3761
χ8573	Δ*msbB48*	χ3761
χ8606	Δ*agfBAC811*	χ3761
χ8650	Δ*pmi-2426*	χ3761
χ8767	Δ*araBAD1923*	χ3761
χ8831	Δ(*gmd-fcl*)*-26*	χ3761
χ8848	ΔP_{fur33}::TT *araC* P_{BAD} *fur*	χ3761
χ8868	Δ*pmi-2426* Δ(*gmd-fcl*)*-26*	χ8650
χ8882	Δ*relA1123*	χ3761
χ8918	$\Delta P_{phoPQ107}$::TT *araC* P_{BAD} *phoPQ*	χ3761
χ8923	Δ*sopB1925*	χ3761
χ9021	ΔP_{crp527}::TT *araC* P_{BAD} *crp*	χ3761
χ9107	ΔP_{fur33}::TT *araC* P_{BAD} *fur* ΔP_{crp527}::TT *araC* P_{BAD} *crp*	χ8848
χ9108	$\Delta P_{phoPQ107}$::TT *araC* P_{BAD} *phoPQ* ΔP_{crp527}::TT *araC* P_{BAD} *crp*	χ8918
χ9109	ΔP_{fur33}::TT *araC* P_{BAD} *fur* $\Delta P_{phoPQ107}$::TT *araC* P_{BAD} *phoPQ* ΔP_{crp527}::TT *araC* P_{BAD} *crp*	χ9000
χ9135	ΔP_{fur33}::TT *araC* P_{BAD} *fur* ΔP_{crp527}::TT *araC* P_{BAD} *crp* Δ*pmi-2426*	χ9107
χ9241	Δ*pabA1516* Δ*pabB232* Δ*asdA16* Δ*araBAD23* Δ*relA198*::*araC* P_{BAD} *lacI* TT	45
χ9295	Δ*pabA1516* Δ*pabB232* Δ*asdA16* Δ*relA198*::*araC* P_{BAD} *lacI* TT Δ*sopB1925* Δ*msbB48*	χ9241
χ9373	Δ*pmi-2426* Δ(*gmd-fcl*)*-26* ΔP_{fur81}::TT *araC* P_{BAD} *fur* ΔP_{crp527}::TT *araC* P_{BAD} *crp* Δ*asdA21*::TT *araC* P_{BAD} *c2* Δ*araE25* Δ*araBAD23* Δ*relA198*::*araC* P_{BAD} *lacI* TT	χ9352
χ9402	Δ*pmi-2426* Δ(*gmd-fcl*)*-26* ΔP_{fur81}::TT *araC* P_{BAD} *fur* ΔP_{crp527}::TT *araC* P_{BAD} *crp* Δ*asdA21*::TT *araC* P_{BAD} *c2* Δ*araE25* Δ*araBAD23* Δ*relA198*::*araC* P_{BAD} *lacI* TT Δ*sopB1925*	χ9373
χ9513	Δ*pmi-2426* Δ(*gmd-fcl*)*-26* ΔP_{fur81}::TT *araC* P_{BAD} *fur* ΔP_{crp527}::TT *araC* P_{BAD} *crp* Δ*asdA21*::TT *araC* P_{BAD} *c2* Δ*araE25* Δ*araBAD23* Δ*relA198*::*araC* P_{BAD} *lacI* TT Δ*sopB1925* Δ*agfBAC811*	χ9402
χ9554	Δ*pmi-2426* Δ(*gmd-fcl*)*-26* ΔP_{fur81}::TT *araC* P_{BAD} *fur* ΔP_{crp527}::TT *araC* P_{BAD} *crp* Δ*asdA21*::TT *araC* P_{BAD} *c2* Δ*araE25* Δ*araBAD23* Δ*relA198*::*araC* P_{BAD} *lacI* TT Δ*sopB1925* Δ*agfBAC811* Δ*msbB48*	χ9513
χ9558	Δ*pmi-2426* Δ(*gmd-fcl*)*-26* ΔP_{fur81}::TT *araC* P_{BAD} *fur* ΔP_{crp527}::TT *araC* P_{BAD} *crp* Δ*asdA27*::TT *araC* P_{BAD} *c2* Δ*araE25* Δ*araBAD23* Δ*relA198*::*araC* P_{BAD} *lacI* TT Δ*sopB1925* Δ*agfBAC811*	χ9513

It contains three means of achieving regulated delayed attenuation, as well as a constellation of additional mutations that aid in efficacy, safety, and delivery of recombinant antigens. These additional mutations are included in the vaccine vector strain to increase the safety and tolerability of the vaccine for oral administration to infants. The inclusion of these mutations reduces disease symptoms associated with infection, such as diarrhea and inflammation in the gut, as revealed in studies of infection in rabbit ileal loops. We show here the construction of these recombinant attenuated *S.* Typhimurium vaccine vector strains, and we show that the strains are well tolerated by infant mice less than 24 h old at doses of more than 10,000 times the LD_{50} of UK-1 in adult BALB/c mice and 10,000,000 times the LD_{50} in newborn mice. The strain we constructed, *S.* Typhimurium χ9558, has been evaluated by other members of our group for safety (4) and immunogenicity (31) in adult mice, and we describe its colonizing ability and immunogenicity in infant mice in the companion paper (40).

MATERIALS AND METHODS

Bacterial strains, media, and bacterial growth. The vaccine strains used in this study were derived from the *S.* Typhimurium strain UK-1 χ3761 (12). All bacterial strains used are listed in Table 1. LB broth and agar (3) were most often used as the complex rich media for the propagation of bacterial strains. Nutrient broth (Difco), which does not contain any mannose or arabinose, was also used to assess the synthesis of LPS O-antigen side chains and the activation of the *araC* P_{BAD} cassette. Sugar fermentation was confirmed by plating bacterial strains onto MacConkey agar (Difco) containing either 0.2% mannose, 0.2% arabinose, or 0.2% maltose. Bacterial titers from colonization studies were enumerated on MacConkey agar containing 1% lactose to differentiate between *Escherichia coli* and *S.* Typhimurium. Siderophore production was evaluated on CAS plates containing chrome azurol S with Fe^{3+} and hexadecyltrimethyl ammonium bromide (HDTMA) in a morpholinepropanesulfonic acid (MOPS; Sigma-Aldrich, St. Louis, MO) agar (39). The ability of bacteria to form thin aggregate fimbriae was evaluated by plating on Congo red agar.

Construction of bacterial mutant strains. *S.* Typhimurium mutant strains were constructed using either P22HT*int* phage-mediated transduction or conjugation using suicide vectors (27). Transductants were selected by growth on Lennox agar (L agar) (30) plates containing either 25 μg/ml chloramphenicol or 12.5 μg/ml tetracycline. Transductants were resuspended in sterile buffered saline gelatin (BSG) (8) and were streaked onto antibiotic-containing medium for isolated colonies. Isolated colonies were inoculated into fresh Lennox broth (L broth) and were grown to an optical density at 600 nm (OD_{600}) of ≈0.6. Double crossovers were selected by growth on L agar plates without NaCl or dextrose but containing 5% sucrose and were subsequently screened for antibiotic sensitivity. The alternate method of using conjugation to construct the strains was also employed (37). Parental *S.* Typhimurium strains were mated on L agar plates containing 50 μg/ml of diaminopimelic acid (DAP) with the *E. coli* host strain χ7213 harboring the relevant suicide vector. Transconjugants were selected by growth on L agar containing antibiotics without DAP. Conjugations with Δ*asdA*

mutant strains were plated onto antibiotic-containing medium containing DAP and colicin B to inhibit *E. coli* growth. Defined deletion mutations with and without insertions were confirmed by PCR and phenotypic verification (11, 13). Table S1 in the supplemental material lists the steps for the construction of χ9558.

Molecular and phenotypic characterization of χ9558 and its ancestor χ9373. All deletions and deletion-insertion mutations were confirmed by PCR amplification. (See Table S2 in the supplemental material for oligonucleotide sequences.) Diagrams of deletion and deletion-insertion mutations are available elsewhere (13, 31). The ΔP_{fur81}::TT *araC* P_{BAD} *fur* and ΔP_{crp527}::TT *araC* P_{BAD} *crp* regulated delayed attenuation deletion-insertion mutations were verified as described by Curtiss et al. in 2009 (13). The Δ*pmi-2426* mutation results in a loss of O-antigen side chain synthesis when strains are grown in nutrient broth containing no mannose, but the mutant is able to synthesize the side chains in the presence of 0.2% mannose (11). The LPS profiles of χ9373, χ9554, and χ9558 grown in nutrient broth in the presence or absence of 0.2% mannose were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining. The Δ*araBAD23* and Δ*araE25* mutations were confirmed by lack of fermentation of arabinose (by plating onto MacConkey plates containing 0.2% arabinose), in comparison with wild-type UK-1 χ3761, which is able to utilize and ferment arabinose, and by inability to grow on minimal agar supplemented with threonine, methionine, and DAP and containing arabinose as the sole carbon source. The Δ*asdA27*::TT *araC* P_{BAD} *c2* deletion-insertion mutation was confirmed by the inability to grow in LB broth in the absence of 50 μg/ml of DAP. C2 synthesis was confirmed by Western blot analysis of C2 protein when strains were grown in nutrient broth in the presence or absence of 0.2% arabinose. LacI synthesis due to the presence of the Δ*relA198*::*araC* P_{BAD} *lacI* TT deletion-insertion mutation was confirmed by Western blot analysis of LacI protein when strains were grown in nutrient broth in the presence or absence of 0.2% arabinose. When the Asd^+ balanced-lethal (18, 34) vaccine vector plasmid pYA4088 was introduced into χ9373, χ9554, and χ9558, PspA synthesis under the control of the P_{trc} promoter was correlated with a decrease in LacI synthesis (31, 45). The presence of PspA was determined by Western blot analysis. The Δ*sopB1925*, Δ*msbB48*, and Δ(*gmd-fcl*)*-26* mutations were confirmed through PCR amplification. The Δ*agfBAC811* mutation was confirmed by the absence of Agf fimbriae as detected by growth on Congo red agar.

Electroporation of *Salmonella* strains with plasmids pYA3802 and pYA4088. The balanced-lethal (18, 34) Asd^+ plasmid vectors pYA3802 and pYA4088 were constructed by cloning the DNA sequence encoding amino acids (aa) 3 to 285 of pneumococcal surface protein A (PspA) from *Streptococcus pneumoniae* strain Rx1 into the EcoRI and HindIII restriction enzyme sites of pYA3620 (11) and pYA3493 (28) as previously described (45). *Salmonella* strains were grown aerobically in LB broth containing 50 μg/ml DAP and 0.2% mannose to an OD_{600} of ≈0.6 and were prepared for electroporation using standard methods. Strains containing the Asd^+ plasmid were selected on LB agar containing 0.2% mannose without DAP. After transformation with pYA4088, the *S.* Typhimurium vaccine vector strains were grown in LB broth with 0.2% mannose and 0.2% arabinose. Strains were verified for synthesis of complete LPS by silver staining of 10% SDS-PAGE gels (25) to screen against the inadvertent selection of rough variants. Synthesis of PspA protein was verified using an anti-PspA antibody raised in rabbits for Western blot analysis against whole-cell lysates. Regulation of PspA synthesis by the arabinose-regulated *lacI* gene was verified by Western blot analysis against whole-cell lysates grown in nutrient broth with and without 0.2% arabinose.

Measurement of fluid secretion using rabbit ileal loops. New Zealand White rabbits were fasted overnight and then anesthetized with isoflurane through an endotracheal tube, and the ileum was exposed and then ligated into several loops 5 to 6 cm long using 1-cm spacers. *S.* Typhimurium strains were injected into separate loops in a volume of 1 ml at a titer of 1×10^9 CFU. LB broth was injected into one of the loops as a control. The abdominal musculature was closed using 3-0 chromic gut sutures, and the skin was closed with 3-0 ethilon sutures. Rabbits were maintained in a thermal blanket at 37°C. After 8 h, each rabbit was euthanized with an overdose of sodium pentobarbital. The abdomen was reopened, and the fluid within the ligated loops was collected, the volume measured, and the bacterial content enumerated. The loops were fixed in 10% formalin and subjected to histopathological examination.

Animal experimentation. Six-week-old female and male BALB/c mice to be used as breeders were purchased from Charles River Laboratories (Wilmington, MA) and housed according to Arizona State University IACUC-approved caging and protocols. For immunization studies with adult mice, we purchased 6- to 7-week-old female BALB/c mice and held them for 1 week so that they could acclimate prior to use. All animals were housed in biosafety level 2 (BSL-2) containment with filter bonnet-covered cages. To produce infant mice, two female mice were placed in the same cage with one male mouse for breeding. Pregnant female mice were placed in separate cages prior to the birth of pups. The bacterial inocula were prepared as follows: 16- by 150-mm tubes with 5 ml of LB broth containing 0.2% mannose and 0.2% arabinose were inoculated with bacterial strains and incubated statically at 37°C for 18 h. The cultures were diluted 1:50 into fresh prewarmed LB broth with 0.2% mannose and 0.2% arabinose and were grown with aeration (180 rpm) at 37°C to an OD_{600} of 0.8 to 0.85. Cultures were pelleted at $4,500 \times g$ and were resuspended in BSG at the densities required to produce the desired dose in the appropriate volume. Adult mice were deprived of food and water for 6 h before oral inoculation, and food and water were returned 30 min after oral inoculation. Adult mice were orally inoculated with 20 μl of the appropriate dose. To orally inoculate infant mice for the evaluation of the various *S.* Typhimurium strains, the mother mouse was placed in a separate cage from the pups, and the pups were inoculated with 2.5 μl to 10 μl of culture. Pups were monitored until the culture was swallowed. Pups inoculated on the day of birth received 2.5 μl of bacteria; 1- to 3-day-old pups received 5 μl of bacteria; and pups 3 days to 1 week old were inoculated with 10 μl of bacteria. Pups were returned to the original position in the nest. The mother was held in the investigator's hands for a few moments so that she would gain the smell of the gloves. Then she was placed back in the cage with her pups and was monitored for abnormal and suspicious activity, such as removing certain pups from the nest. In other cases, we left a glove in the cage in order to acclimate the mother to the glove odor. Once the mother began to eat and drink, the cage was placed back in the rack. Pups were monitored for death every 24 h for 30 days. Sometimes, with large litters, the mother cannibalized some mice to reduce the litter size.

RESULTS

Construction of *S.* Typhimurium strains with individual mutations and combinations of mutations. With the ultimate objective of constructing a vaccine strain that would be safe and nonreactogenic in newborn and infant mice, we generated a number of derivatives of the wild-type UK-1 strain χ3761 (Table 1) with the Δ*msbB48* mutation, to render lipid A nontoxic yet able to be an agonist for TLR4 in mice (41), and with the Δ*sopB1925* mutation, to decrease fluid secretion in the gut of the mammalian host, with the effects of lessening both the frequency and severity of diarrhea and decreasing neutrophil accumulation in the intestine, thereby reducing inflammation (19). We also constructed strains with mutations that conferred a phenotype of regulated delayed attenuation *in vivo* (13). These include strains with the Δ*pmi-2426* mutation, which eliminates phosphomannose isomerase so that LPS O-antigen synthesis ceases *in vivo*, due to the lack of availability of free nonphosphorylated mannose. This renders strains more sensitive to complement-mediated cytotoxicity and to killing by phagocytosis (11). Three other types of regulated delayed attenuation have been conferred on some strains by deletion of the promoters for the *fur*, *crp*, and *phoPQ* virulence genes and their replacement by a tightly regulated *araC* P_{BAD} cassette (29). The resulting ΔP_{fur81}::TT *araC* P_{BAD} *fur*, ΔP_{crp527}::TT *araC* P_{BAD} *crp*, and $\Delta P_{phoPQ107}$::TT *araC* P_{BAD} *phoPQ* deletion-insertion mutations have been diagrammed in other publications (13, 31) and have been introduced into a number of strains (Table 1).

The *sopB* and *msbB* mutations decrease fluid secretion and inflammation in rabbit ileal loops. The *Salmonella sopB* gene encodes an inositol phosphate phosphatase that is secreted by the bacteria into host intestinal epithelial cells via the type III secretion system encoded by *Salmonella* pathogenicity island 1 (SPI-1), causing an increase in chloride secretion from the cell, resulting in a water efflux, fluid secretion, and subsequent diarrhea that is commonly associated

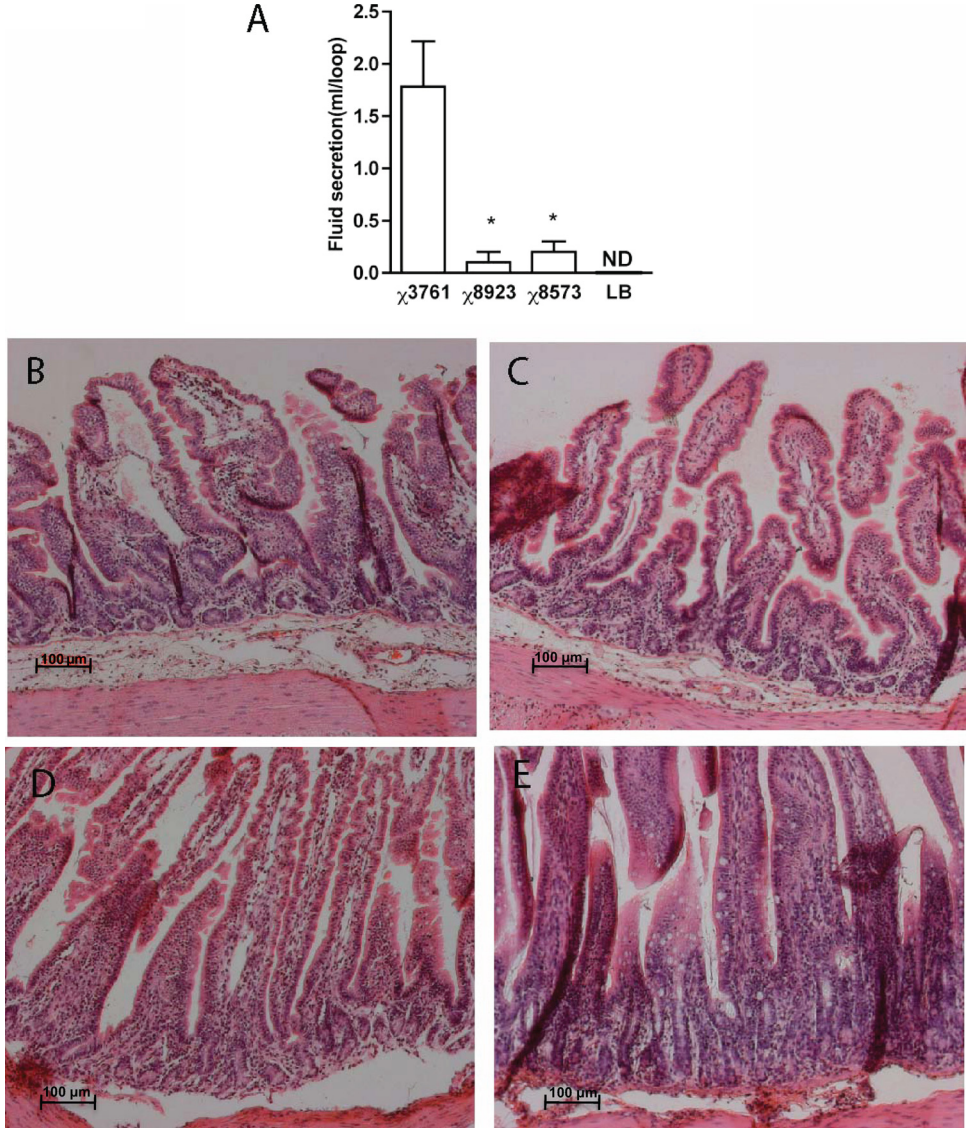

FIG. 1. *S.* Typhimurium Δ*sopB1925* and Δ*msbB48* mutants decrease fluid secretion and inflammation in rabbit ileal loops. Five- to 6-cm-long rabbit ileal loops were injected with 10^9 CFU of *S.* Typhimurium strains for 8 h, and the fluid secretion and histopathological changes in the ileum were examined. (A) Fluid secretion induced by *S.* Typhimurium strains in rabbit ileal loops. ND, not detectable. Asterisks, $P < 0.05$. (B to E) Histopathological staining of rabbit ileal loops injected with *S.* Typhimurium strains or the L broth control. (B) Wild-type χ3761 UK-1; (C) χ8923 Δ*sopB1925*; (D) χ8573 Δ*msbB48*; (E) L broth. Bar, 100 μm.

with *Salmonella* infection (15, 35). Other work has shown that *sopB* has a role in neutrophil accumulation in the calf intestine and thus leads to a population of inflammatory cells in the ileum (19). The *msbB* gene encodes an acyltransferase that is responsible for the secondary acyl substitution onto lipid A and has been shown to play a significant role in the inflammation and endotoxicity in the gut that are associated with infection (42). Patients infected with *Salmonella* who have inflamed intestines report pain, and since the vaccines we are developing are to be administered to and tolerated by infants, the roles of both Δ*sopB* and Δ*msbB* mutations in potentially decreasing fluid secretion, inflammation, and endotoxicity in the gut would be advantageous. We therefore evaluated the effects of the Δ*sopB1925* and Δ*msbB48* mutations on fluid secretion and inflammation in rabbit ileal loops. The volume of fluid was decreased in ileal loops injected with the Δ*sopB1925* (χ8923) and Δ*msbB48* (χ8573) mutants in comparison to those injected with the wild-type parent strain, as shown in Fig. 1A. Hematoxylin-and-eosin staining of sections of the ileal loops showed a decrease in the presence of inflammatory infiltrates, as well as a decrease in the level of destruction of the intestinal epithelium, in the loop injected with χ8923 Δ*sopB1925* (Fig. 1C) or χ8573 Δ*msbB48* (Fig. 1D) in comparison with the loop injected with wild-type χ3761 UK-1 (Fig. 1B). It is thus desirable to add the Δ*sopB1925* mutation and mutations like the Δ*msbB48* mutation to future vaccine strains. Since the *msbB* mutation modifies lipid A to render it an antagonist for human TLR4 (21, 42), we will add other genetic modifications to render lipid A noninflammatory while allowing it to serve as an agonist for human TLR4.

TABLE 2. Attenuation of orally administered *S.* Typhimurium strains with single mutations displaying regulated delayed attenuation in mice of different ages[a]

Strain	Genotype	Sugar in medium (%)	Age of mice	Inoculating dose (CFU)	No. of survivors/ total no.
χ8650	Δ*pmi-2426*	Mannose (0.5)	7–8 wk	1.1×10^6	5/5
		Mannose (0.1)	3 wk	1.0×10^4	3/4
			2 wk	7.0×10^5	3/3
			4 days	1.0×10^5	0/4
χ8848	ΔP_{fur33}::TT *araC* P_{BAD} *fur*	Arabinose (0.2)	7–8 wk	9.0×10^8	5/5
			3 wk	1.0×10^4	4/4
			2 wk	5.0×10^5	2/5
			1 wk	7.0×10^5	0/5
			4 days	1.0×10^5	0/4
χ8918	$\Delta P_{phoPQ107}$::TT *araC* P_{BAD} *phoPQ*	Arabinose (0.2)	7–8 wk	1.0×10^9	5/5
		Arabinose (0.1)	3 wk	1.0×10^4	5/5
			2 wk	6.0×10^5	3/5
			1 wk	7.0×10^5	0/5
			4 days	1.0×10^5	0/5
χ9021	ΔP_{crp527}::TT *araC* P_{BAD} *crp*	Arabinose (0.2)	7–8 wk	1.0×10^9	5/5
		Arabinose (0.1)	2 wk	6.0×10^5	1/5
			1 wk	7.0×10^5	0/4
			4 days	1.0×10^5	0/3

[a] Strains were grown in LB broth containing the percentage of sugar indicated. Mice were orally inoculated as described in Materials and Methods and were observed for 30 days postinoculation.

Mutant attenuation for infant and newborn mice. We initiated studies with low doses of strains administered to 3-week-old weaned mice so as to minimize animal deaths and reveal which mutations would confer maximal safety and attenuation. As we established levels of attenuation at different ages, we increased the doses and decreased the ages of mice orally infected. We also constructed and evaluated strains with multiple mutations. As revealed by the data in Table 2, strains with single Δ*pmi-2426*, ΔP_{fur33}::TT *araC* P_{BAD} *fur*, ΔP_{crp527}::TT *araC* P_{BAD} *crp*, or $\Delta P_{phoPQ107}$::TT *araC* P_{BAD} *phoPQ* mutations are fully attenuated in 7- to 8-week-old BALB/c mice. However, they are only partially attenuated at moderate doses in 3-week old weaned mice and are less attenuated in younger mice (Table 2). Strains with two or three mutations conferring regulated delayed attenuation led to complete attenuation in 3-week-old mice (data not shown) and increasingly better attenuation in younger mice (Table 3). We used the χ9241 Δ*pabA1516* Δ*pabB232* vaccine strain as the parental strain for addition of the Δ*sopB1925* and Δ*msbB48* mutations, since these attenuating *pab* mutations are not fully attenuating in infant mice, as they are in adult mice (45). Inclusion of the Δ*sopB1925* and Δ*msbB48* mutations in χ9241 (χ9295) also contributed improved tolerability for infant mice (Table 3).

Construction of the *S.* Typhimurium vaccine strain χ9558. Based on the results described above and on those of other studies with additional mutant strains, we designed an *S.* Ty-

TABLE 3. Attenuation of orally administered *S.* Typhimurium strains with multiple mutations displaying regulated delayed attenuation in mice of decreasing ages[a]

Strain	Genotype	Age of mice	Inoculating dose (CFU)	No. of survivors/ total no.
χ9107	ΔP_{fur33}::TT *araC* P_{BAD} *fur* ΔP_{crp527}::TT *araC* P_{BAD} *crp*	2 wk	1.3×10^8	5/5
χ9108	$\Delta P_{phoPQ107}$::TT *araC* P_{BAD} *phoPQ* ΔP_{crp527}::TT *araC* P_{BAD} *crp*	1 wk	1.1×10^6	3/3
χ9109	ΔP_{fur33}::TT *araC* P_{BAD} *fur* $\Delta P_{phoPQ107}$::TT *araC* P_{BAD} *phoPQ* ΔP_{crp527}::TT *araC* P_{BAD} *crp*	1 wk	1.2×10^8	4/4
		4 days	5.7×10^7	5/5
χ9135	ΔP_{fur33}::TT *araC* P_{BAD} *fur* ΔP_{crp527}::TT *araC* P_{BAD} *crp* Δ*pmi-2426*	2 wk	2.4×10^9	7/7
		1 wk	1.0×10^8	2/4
			1.0×10^7	4/4
		2 days	1.5×10^4	3/3
χ9241 harboring pYA3802	Δ*pabA1516* Δ*pabB232* Δ*asdA16* Δ*araBAD23* Δ*relA198*::*araC* P_{BAD} *lacI* TT	2 days	6.1×10^8	0/2
			6.1×10^7	0/2
			6.1×10^6	1/3
χ9295 harboring pYA3802	Δ*pabA1516* Δ*pabB232* Δ*asdA16* Δ*araBAD23* Δ*relA198*::*araC* P_{BAD} *lacI* TT Δ*sopB1925* Δ*msbB48*	2 days	8.9×10^8	0/2
			8.9×10^7	2/3
			8.9×10^6	2/2

[a] χ9107, χ9108, and χ9109 were grown in LB broth with 0.2% arabinose, and χ9135 was grown in LB broth with 0.2% mannose. Mice were orally inoculated as described in Materials and Methods and were observed for 30 days postinoculation.

TABLE 4. Virulence (LD_{50}) of *S.* Typhimurium strains with deletion mutations[a]

Strain	Genotype	Inoculating dose (CFU)	No. of survivors/ total no.
χ8878	Δ*agfBAC811*	7.0×10^5	0/5
		7.0×10^4	0/5
		7.0×10^3	2/5
χ8767	Δ*araBAD23*	5.0×10^5	5/10
		5.0×10^4	5/10
		5.0×10^3	10/10
χ8477	Δ*araE25*	1.1×10^8	0/4
		1.1×10^7	1/4
		1.1×10^6	1/4
		1.1×10^5	2/4
χ8831	Δ(*gmd-fcl*)*-26*	7.0×10^5	1/8
		7.0×10^4	4/8
		7.0×10^3	5/8
χ8573	Δ*msbB48*	8.0×10^5	4/14
		8.0×10^4	13/14
		8.0×10^3	13/14
χ8882	Δ*relA1123*	9.0×10^6	2/9
		9.0×10^5	4/9
		9.0×10^4	7/8
χ8923	Δ*sopB1925*	9.0×10^5	7/10
		9.0×10^4	8/10
		9.0×10^3	10/10

[a] Female BALB/c mice 6 to 7 weeks of age were orally immunized with the indicated strains as described in Materials and Methods. Mice were observed for 30 days after inoculation.

phimurium vaccine strain to serve as a standard for comparison with *S.* Typhi vaccine strains to be evaluated in human volunteers. This strain, χ9558, would then be used to obtain safety and efficacy data to justify human clinical trials. The construction lineage of χ9558 is described in Table S1 in the supplemental material. χ9558 contains three means for achieving regulated delayed attenuation *in vivo*, as described previously (11, 13). χ9558 also contains a constellation of gene deletions and deletion-insertion mutations that enhance the safety and efficacy of the vaccine strain. Data not previously reported on the virulence in adult mice of strains with individual deletion mutations that are present in the various multiple mutant strains are provided in Table 4. Findings on the attenuation and immunogenicity in adult mice of strains with other individual deletion and deletion-insertion mutations present in χ9558 have been provided in other articles (11, 13). The Δ*pmi-2426* mutation and the deletion-insertion mutations ΔP_{fur81}::TT *araC* P_{BAD} *fur* and ΔP_{crp527}::TT *araC* P_{BAD} *crp* achieve regulated delayed attenuation such that growth in a medium with 0.1% mannose and 0.05 to 0.2% arabinose yields a strain that can withstand the host-imposed stresses encountered after oral delivery and enables maximal invasion and colonization of lymphoid effector tissues prior to display of the attenuated phenotype as a consequence of cell division *in vivo* (11, 13). It should be noted that we are using the ΔP_{fur81}::TT *araC* P_{BAD} *fur* deletion-insertion mutation rather than the ΔP_{fur33}::TT *araC* P_{BAD} *fur* construction because the latter causes the synthesis of too much Fur protein when the strain is grown in LB broth with 0.2% arabinose, and that interferes with initial vaccine effectiveness (13). The Δ*araBAD23* mutation prevents the bacteria from using arabinose or breaking down arabinose in the cytoplasm, and the Δ*araE25* mutation allows for the retention of supplied arabinose in the cytoplasm (26, 32). Both mutations thus enable the activation of the *araC* P_{BAD} cassette in strains with *araC* P_{BAD}-regulated deletion-insertion mutations for another cell division or so, which increases colonization levels to further enhance the levels of induced immunity. In addition, the inability to metabolize arabinose precludes an undesirable pH drop during growth in a medium with added arabinose. The deletion-insertion mutation Δ*asdA27*::TT *araC* P_{BAD} *c2* (an improvement over the Δ*asdA18*::TT *araC* P_{BAD} *c2* and Δ*asdA21*::TT *araC* P_{BAD} *c2* versions present in early χ9558 ancestor strains) provides the balanced-lethal host-vector system as previously described (18, 34), and the arabinose-controlled synthesis of the C2 repressor (38) can be used to achieve regulated delayed synthesis of antigens controlled by the bacteriophage P22 P_R or P_L promoter. Deletion of the *asd* gene imposes a requirement for exogenously supplied diaminopimelic acid, an essential component of the peptidoglycan layer of the bacterial cell wall. The vaccine plasmid vector contains a wild-type copy of the *asd* gene, thus complementing the deletion mutation in the host strain, which allows the bacteria to grow without exogenous DAP and ensures that the vector is maintained within the host bacterial strain *in vivo*. This eliminates the need for antibiotic resistance to maintain the plasmid, an important attribute, since live bacterial vaccines should be susceptible to all antibiotics that might be used to control infection. The Δ(*gmd-fcl*)*-26* mutation deletes the genes responsible for the enzymes required for GDP-fucose synthesis, thus blocking the conversion of GDP-mannose to GDP-fucose, and prevents colanic acid production (1, 43). Stress conditions induced by cell wall damage lead to the production of colanic acid and can allow for the survival of *asdA* mutants. The presence of this mutation also reduces the potential of the vaccine strain to form biofilms (14). The deletion-insertion mutation Δ*relA198*::*araC* P_{BAD} *lacI* TT inactivates the *relA* gene, which uncouples the occurrence of cell wall-less death from dependence on protein synthesis (44). The arabinose-controlled synthesis of the LacI repressor is used to achieve regulated delayed synthesis of antigens under the control of the P_{trc} promoter (S. Wang, personal communication). The transcription terminators (TT) present in several constructions prevent readthrough into adjacent genes, which sometimes can interfere with normal functions of adjacent genes. The Δ*sopB1925* mutation is included in order to decrease fluid secretion in the gut of the mammalian host so as to lessen the frequency of diarrhea and also serves to decrease neutrophil accumulation in the intestine and thus reduce inflammation. The Δ*agfBAC811* mutation reduces the bacterium's ability to form thin aggregative fimbriae (6) and precludes the formation of biofilms on gallstones, which are necessary for the establishment and maintenance of chronic infection and for persistence in the gallbladder (36). The Δ*araBAD23*, Δ*relA*, and Δ*sopB1925* mutations each contribute a low level of attenuation to vaccine strains (Table 4) but have no apparent adverse effect on the colonization of internal effector lymphoid tissues. The Δ*araE25*, Δ(*gmd-fcl*)*-26*, and Δ*ag*

TABLE 5. Virulence of RASV strains in infant mice[a]

Strain	Genotype	Age of mice	CFU/dose	No. of survivors/ total no.
χ9373 harboring pYA4088	Δ*pmi-2426* Δ(*gmd-fcl*)*-26* ΔP_{fur81}::TT *araC* P_{BAD} *fur* ΔP_{crp527}::TT *araC* P_{BAD} *crp* Δ*asdA21*::TT *araC* P_{BAD} *c2* Δ*araE25* Δ*araBAD23* Δ*relA198*::*araC* P_{BAD} *lacI* TT	6 days	8.8×10^8	4/4
			8.8×10^8	4/4
			8.8×10^7	4/4
		3 days	4.4×10^7	4/4
		2 days	5.4×10^7	6/6
			5.7×10^8	5/6
		Day of birth (1–2 h)	2.2×10^6	5/6
χ9554 harboring pYA4088	Δ*pmi-2426* Δ(*gmd-fcl*)*-26* ΔP_{fur81}::TT *araC* P_{BAD} *fur* ΔP_{crp527}::TT *araC* P_{BAD} *crp* Δ*asdA21*::TT *araC* P_{BAD} *c2* Δ*araE25* Δ*araBAD23* Δ*relA198*::*araC* P_{BAD} *lacI* TT Δ*sopB1925* Δ*agfBAC811* Δ*msbB48*	7 days	9.9×10^8	5/5
		2 days	4.7×10^7	5/5
		Day of birth	2.5×10^7	7/9[b]
χ9558 harboring pYA4088	Δ*pmi-2426* Δ(*gmd-fcl*)*-26* ΔP_{fur81}::TT *araC* P_{BAD} *fur* ΔP_{crp527}::TT *araC* P_{BAD} *crp* Δ*asdA27*::TT *araC* P_{BAD} *c2* Δ*araE25* Δ*araBAD23* Δ*relA198*::*araC* P_{BAD} *lacI* TT Δ*sopB1925* Δ*agfBAC811*	7 days	9.9×10^8	11/11
		4 days	3.0×10^8	6/6
		2 days	3.3×10^8	11/11
		Day of birth	3.5×10^8	13/15[b]

[a] All strains were grown in LB broth with 0.2% arabinose and 0.2% mannose and were orally inoculated into mice as described in Materials and Methods. Mice were observed for 30 days postinoculation.
[b] The mother may have cannibalized two infants due to the large litter size.

fBAC811 mutations have no effect on virulence (Table 4) or colonization (data not shown).

χ9558 was constructed through the sequential deletion of genes with or without insertions, as described in Table S1 in the supplemental material. Two of the mutations have been altered several times, due to discoveries affecting the virulence and survival of the host strain *in vivo* (13) and the expression of the repressor proteins, to achieve regulated delayed synthesis of protective recombinant antigen *in vivo*. The ΔP_{fur81}::TT *araC* P_{BAD} *fur* mutation was substituted for the ΔP_{fur33}::TT *araC* P_{BAD} *fur* mutation after the discovery that overexpression of Fur protein presumably resulted in iron starvation in the intestinal tract so as to actually reduce colonization of the GALT and internal lymphoid organs. Thus, reduced synthesis of Fur by strains with the ΔP_{fur81}::TT *araC* P_{BAD} *fur* insertion-deletion mutation resulted in improved colonization of lymphoid organs when strains were grown in LB broth containing 0.2% arabinose (13). The presence of the ΔP_{fur81}::TT *araC* P_{BAD} *fur* construction, when bacteria were grown in the presence of 0.2% arabinose, resulted in an attenuated strain that conferred complete protection upon challenge with the virulent wild-type strain χ3761 (13). The *asdA* mutation has also undergone several alterations during the course of χ9558 construction. The strain originally contained a defined deletion of the *asdA* gene (Δ*asdA33*) that would serve in the balanced-lethal host-vector system, but this was replaced with the deletion-insertion mutation Δ*asdA18*::TT *araC* P_{BAD} *c2*, which would allow for the regulated delayed expression of antigens under the control of the P22 P_L or P_R promoter. The synthesis of C2 was optimized for complete repression of the antigen in the presence of 0.2% arabinose, and the resulting deletion-insertion mutation is Δ*asdA27*::TT *araC* P_{BAD} *c2*.

Attenuation of χ9558 and parent strains in infant mice. The attenuation of χ9373, χ9554, and χ9558 harboring pYA4088 was evaluated through oral inoculation of infant BALB/c mice of various ages, ranging from the day of birth to 7 days (Table 5). The bacterial inoculum varied depending on the age of the mice; however, all inocula administered were above the LD_{50} of χ3761 in adult BALB/c mice. The strains were grown in LB broth in the presence of 0.2% mannose and 0.2% arabinose. The volume of inoculum administered to infant mice depended on their age and ranged from 2.5 μl to 10 μl. At the ages of 6 to 7 days, RASV strains χ9373 and χ9558 displayed safety up to a dose of 10^9 CFU. In 2- to 4-day-old mice, χ9558 displayed safety up to 3.0×10^8 CFU. χ9558 displayed safety in 2-day-old infant mice at a dose roughly 27,000 times the LD_{50} of χ3761 in adult BALB/c mice. It should be noted that large litters of pups can result in cannibalization of some of the pups by a first-time mother mouse. The addition of the Δ*sopB1925* and Δ*agfBAC811* mutations increases the safety of the strain, as seen in the survival of mice inoculated on the day of birth with χ9373 at 2.2×10^6 CFU compared to that of mice of the same age inoculated with χ9558 at 3.5×10^8 CFU. Thus, the infant mice are able to tolerate a higher dose of RASV inoculum when the Δ*sopB1925* and Δ*agfBAC811* mutations are included, as in χ9558. We also evaluated χ9554, which has the Δ*msbB48* mutation rather than the Δ*agfBAC811* mutation present in χ9558, and the strain was well tolerated in 2-day-old and day-of-birth mice at doses of 4.7×10^7 CFU and 2.5×10^7 CFU, respectively. We also assayed infected pups for colonization of the GALT, and all strains showed colonization of the spleen, liver, and intestine and persisted in tissues for at least 7 days (data not shown). The vaccine vector strain χ9558 is thus capable of colonizing infant mice, as reported by Shi et al. (40), who also report on immunogenicity and the induction of protection against *S. pneumoniae* challenges.

DISCUSSION

We describe here the analysis of means of attenuation of *S.* Typhimurium in addition to other mutational alterations to enhance the safety and acceptability of vaccine strains designed

for the immunization of infant and newborn mice. Based on data collected, we designed a vaccine strain that would satisfy these safety objectives and allow the collection of data to justify the use of recombinant attenuated *S*. Typhi strains of the same genotype for the conduct of human clinical trials. The data collected served as the basis for the construction and genetic composition of χ9558 and show that this strain is well tolerated and safe in infant mice and newborn mice less than 24 h old. The three means of achieving regulated delayed attenuation (Δ*pmi-2426*, ΔP_{fur81}::TT *araC* P_{BAD} *fur*, and ΔP_{crp527}::TT *araC* P_{BAD} *crp*) increase the safety of the vaccine by allowing a near-wild-type ability to withstand host stresses and colonize the GALT following oral inoculation yet rendering the bacteria susceptible to host defenses after several rounds of replication in host tissue, due to reduced expression of the *fur* and *crp* genes and loss of LPS O-antigen side chains (11, 13). These methods of attenuation, in combination with mutations that (i) reduce disease symptoms through reduction of fluid secretion and inflammation in the gut (Δ*sopB1925* and Δ*msbB48*), at least in rabbits, (ii) allow bacteria to colonize effector lymphoid tissues (but prevent them from establishing a persistent state in the host) [Δ(*gmd-fcl*)*-26*, Δ*agfBAC811*], (iii) allow regulated delayed synthesis of a recombinant protective antigen in lymphoid tissues (Δ*relA198*::*araC* P_{BAD} *lacI* TT), and (iv) enable the establishment of a drug-sensitive balanced-lethal vector-host system (Δ*asdA27*::TT *araC* P_{BAD} *c2*), yield a vaccine strain that is safe, nonreactogenic, and hopefully effective in inducing protective immunity in mice. In this regard, Li et al. (31) have demonstrated that χ9558 harboring pYA4088 is superior to other attenuated *S.* Typhimurium vaccine strains in inducing antibodies against the PspA antigen and protective immunity to *S. pneumoniae* challenge in adult mice. In another study, Bollen et al. (4) have demonstrated that the genotype of χ9558 harboring pYA4088 very much lessens, if it does not eliminate, the ability of *S.* Typhimurium to induce symptoms of meningitis when administered at high doses to adult mice by intranasal, oral, and intraperitoneal routes.

Additional mutations that further enhance the safety, efficacy, and acceptability of vaccine derivatives of χ9558 are currently under evaluation. These include mutations and special vectors that will achieve total biocontainment through regulated delayed lysis *in vivo* of the vaccine strains such that there is no persistence *in vivo* and no survivors if the bacterium is shed into the environment (29). Most notable is the replacement of the Δ*msbB48* mutation. While the Δ*msbB48* mutation acts as an agonist for TRL4 in mice (41), it serves as an antagonist in humans (21, 42); thus, alternate genes are currently being evaluated in order to eliminate the toxicity of lipid A yet enable lipid A to serve as an agonist for human TLR4. Still other mutations to enhance lymphoid tissue colonization levels and to synthesize recombinant antigens to higher levels are being evaluated. In this regard, Xin et al. (45) have reported multiple means to enhance type II secretion of recombinant protective antigens from *S*. Typhimurium vaccine strains displaying regulated delayed synthesis of recombinant antigens. It should be noted that the same mutations that are present in χ9558 have been constructed in parallel *S*. Typhi strains for evaluation in humans. Since *S*. Typhi is host specific, and *S.* Typhimurium infection results in a typhoid-like disease in mice, we use *S.* Typhimurium in mice to simulate an *S*. Typhi infection in humans. Nevertheless, we are cognizant of the potential that results obtained with *S.* Typhimurium constructs in mice may be misleading in predicting results in other animal species or with similar *S.* Typhi strains in humans. The *in vitro* and *in vivo* (in mice) characterizations of the *S*. Typhi strains will be presented in a separate report. Further studies involving more-detailed analysis of the immune response of infant mice orally immunized with χ9558 harboring pYA4088, as well as the role of maternal immunization, have been conducted and are presented by Shi et al. (40).

ACKNOWLEDGMENTS

This research was supported by grant 37863 from the Bill & Melinda Gates Foundation.

We thank our colleagues in the Curtiss lab for advice and help throughout these studies. We wish to thank Paula Carmody, Niecey Hinkle, and Suellen Greco in the Department of Comparative Medicine at Washington University for their expert assistance in performing the surgeries.

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