The Asd⁺-DadB⁺ Dual-Plasmid System Offers a Novel Means To Deliver Multiple Protective Antigens by a Recombinant Attenuated *Salmonella* Vaccine

Wei Xin,^a Soo-Young Wanda,^a Xiangmin Zhang,^a Javier Santander,^a Giorgio Scarpellini,^a Karen Ellis,^a Praveen Alamuri,^a and Roy Curtiss III^{a,b}

Center for Infectious Diseases and Vaccinology, The Biodesign Institute,^a and School of Life Science,^b Arizona State University, Tempe, Arizona, USA

We developed means to deliver multiple heterologous antigens on dual plasmids with non-antibiotic-resistance markers in a single recombinant attenuated vaccine strain of *Salmonella enterica* serotype Typhimurium. The first component of this delivery system is a strain of *S. Typhimurium* carrying genomic deletions in *alr*, *dadB*, and *asd*, resulting in obligate requirements for diaminopimelic acid (DAP) and D-alanine for growth. The second component is the Asd⁺-DadB⁺ plasmid pair carrying wild-type copies of *asdA* and *dadB*, respectively, to complement the mutations. To evaluate the protection efficacy of the dual-plasmid vaccine, *S. Typhimurium* strain χ 9760 (a strain with multiple attenuating mutations: Δ *asd* Δ *alr* Δ *dadB* Δ *recF*) was transformed with Asd⁺ and DadB⁺ plasmids specifying pneumococcal antigens PspA and PspC, respectively. Both plasmids were stable in χ 9760 for 50 generations when grown in nonselective medium. This was significantly ($P < 0.05$) greater than the stability seen in its *recF*⁺ counterpart χ 9590 and could be attributed to reduced interplasmid recombination in χ 9760. Oral immunization of BALB/c mice with 1×10^9 CFU of χ 9760 (carrying Asd⁺-PspA and DadB⁺-PspC plasmids) elicited a dominant Th1-type serum IgG response against both antigens and protected mice against intraperitoneal challenge with 200 50% lethal doses (LD₅₀s) of virulent *Streptococcus pneumoniae* strain WU2 or intravenous challenge with 100 LD₅₀s of virulent *S. pneumoniae* strain L81905 or intranasal challenge with a lethal dose of *S. pneumoniae* A66.1 in a pneumonia model. Protection offered by χ 9760 was superior to that offered by the mixture of two strains, χ 9828 (Asd⁺-PspA) and χ 11026 (DadB⁺-PspC). This novel dual-plasmid system marks a remarkable improvement in the development of live bacterial vaccines.

Recombinant attenuated *Salmonella* vaccines (RASVs) stimulate mucosal, systemic, and cell-mediated immune (CMI) responses, independent of the route of vaccine administration, to self-antigens as well as to heterologous antigens of bacterial, viral, or parasite origin (5, 6, 8, 14, 18, 35). *Salmonella* vaccine strains serve as carriers to deliver a foreign antigen(s) to primary and secondary lymphoid tissues in the host, and the first critical step in construction of such RASVs is introduction of a gene encoding an antigen either in the chromosome or on a self-replicating plasmid capable of inducing a protective immune response. The potency of a RASV strain is determined by its ability to produce high levels of target antigen inside the host, which is in turn is dependent on the stability of the plasmid(s) in the vaccine strain that allows constitutive or regulated delayed expression of antigen genes (13–15, 37, 38). We routinely use balanced-lethal plasmid vector-host combinations to avoid the undesirable use of plasmids with antibiotic resistance (15, 27). They consist of *Salmonella enterica* serotype Typhimurium Δ *asdA* strains that require diaminopimelic acid (DAP) for growth and the plasmid vector providing a wild-type copy of *asd* in *trans* (Asd⁺ plasmid) to complement the mutation (13). Unavailability of DAP in mammalian tissues forces any *Salmonella* strain carrying a Δ *asd* mutation to stably maintain the Asd⁺ plasmid to prevent death by lysis.

To confer complete protection against a pathogen, the *Salmonella* vaccine strain would preferably deliver multiple antigens. For example, a fusion construct of pneumococcal surface protein PspA representing both PspA families (Rx1 and EF5668) constituted an effective antipneumococcal vaccine, extending and enhancing protection against multiple strains of *Streptococcus pneumoniae* (41). It may not be convenient, however, to create fusion

constructs of multiple protective antigens whenever a multivalent vaccine is desired. One potential approach to overcome this limitation is to develop plasmids such as Asd⁺ that carry non-antibiotic-resistance markers and that can be stably maintained in the vaccine strain. To facilitate cloning of multiple antigen genes in a single bacterium, we explored the use of another key enzyme, DadB, as the marker such that, together with an Asd⁺ plasmid, the enzyme can be used to deliver multiple antigens. Genes *dadB* and *alr* encode two alanine racemases that catalyze the interconversion of D-alanine (D-Ala) and L-alanine. DadB is responsible for the formation of the D-enantiomer used in the construction of the peptidoglycan layer of bacterial cell walls (2, 12), and, similar to what has been observed of the Δ *asd* mutation, Δ *dadB* and Δ *alr* together create an obligate requirement for exogenous D-alanine for survival of the mutant (*in vitro* or *in vivo*) or would require complementation with a wild-type copy of the gene on the chromosome or in the form of a DadB⁺ plasmid. The latter component would form the vector part of the *alr* *dadB* balanced-lethal host-vector system. Due to the redundant functions of *Alr* and

Received 7 June 2012 Returned for modification 8 July 2012

Accepted 16 July 2012

Published ahead of print 6 August 2012

Editor: A. Camilli

Address correspondence to Roy Curtiss III, rcurtiss@asu.edu.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

doi:10.1128/IAI.00620-12

DadB, deletion of both responsible genes is required to generate a D-alanine auxotroph.

In this report, we demonstrate multiple-antigen delivery using an *S. Typhimurium* dual-plasmid system consisting of an attenuated *S. Typhimurium* ($\Delta asd \Delta dadB \Delta alr$) triple mutant and Asd^+ and $DadB^+$ plasmids specifying protective antigens PspA and PspC of *S. pneumoniae*. Asd^+ and $DadB^+$ plasmids stably carried PspA and PspC, respectively, for 50 generations. In addition, introducing the $\Delta recF$ mutation into this strain dramatically decreased interplasmid recombination and enhanced the immunogenicity of the *Salmonella* vaccine. Our RASV, with its ability to carry two expression plasmids, provides an ideal platform for the development of multivalent live attenuated vaccines.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *S. Typhimurium* were grown at 37°C in LB broth or on LB agar plates (20). When required, antibiotics were added to culture media at the following concentrations: ampicillin (Amp), 100 µg/ml; kanamycin (Km), 50 µg/ml; and tetracycline (Tet), 12.5 µg/ml. Diaminopimelic acid (DAP) was added (50 µg/ml) for the growth of Δasd strains (not carrying an Asd^+ plasmid) (20). D-Alanine (D-Ala) (50 µg/ml) was added for the growth of $\Delta alr \Delta dadB$ strains. *S. pneumoniae* strains WU2, A66, and L81905 were each cultured on brain heart infusion agar containing 5% sheep blood or in Todd-Hewitt broth plus 0.5% yeast extract in an anaerobic container (3).

Construction and phenotypic characterization of *S. Typhimurium* vaccine strains. P22 transduction routinely used in our laboratory was used to generate mutants (19). Strain $\chi 8901$ ($\Delta dadB \Delta alr$) was constructed by conjugating $\chi 3761$ with *E. coli* donor strain $\chi 7213$ carrying pYA3668 or pYA3667. Suicide vector pYA3668 has a 664-bp-long flanking region (with 357 bp upstream and 307 bp downstream) deleting the 1,106-bp *dadB* gene (from -10 to +1073) (Fig. 1A). Plasmid pYA3667 has a 684-bp flanking region (with 325 bp upstream and 359 bp downstream) deleting the 1,083-bp *alr* gene (from -10 to +1096) (Fig. 1A). The genotypes of both mutants were verified by PCR. To verify the phenotype of $\Delta dadB \Delta alr$ mutants, strains were streaked onto LB agar with or without D-alanine. Deletion mutations $\Delta dadB4$ and $\Delta alr-3$ were sequentially introduced into *S. Typhimurium* $\chi 9558$ strains by allelic exchange (using suicide vectors pYA3668 for $\Delta dadB4$ and pYA3667 for $\Delta alr-3$) to yield $\chi 9590$ ($\chi 9558$ plus $\Delta alr \Delta dadB$). The presence of the 1.1-kb deletion of *dadB* gene was confirmed by PCR with a primer set consisting of P1 (ACATGCATGCGAATGCGAAATTCGCCGACGTG) and P2 (TCCCCCGGGCTTTAATACCGACTTACTGCAACC). The 1.08-kb deletion of the *alr* gene was confirmed by PCR with a primer set consisting of P3 (CGTCGCTTTCTGATAACCGTACTC) and P4 (CCCGTCTTCGTTGT AATACAGGC). For a control vaccine strain, we generated an asd^+ version of $\chi 9590$ by transduction. This was called $\chi 11025$. The presence of the Δasd mutation in *Salmonella* was confirmed by its dependence on DAP for growth (27). Suicide vector pYA3886 was used to introduce a $\Delta recF126$ mutation into strains $\chi 9558$, $\chi 9590$, $\chi 9052$, and $\chi 11025$ to obtain strains $\chi 9828$, $\chi 9760$, $\chi 11018$, and $\chi 11026$, respectively (Table 1). The presence of the $\Delta recF$ mutation was confirmed by PCR with a primer set as described by Zhang et al. (44). Lipopolysaccharide (LPS) profiles of all *S. Typhimurium* mutants were examined as described previously (17).

Construction of $DadB^+$ plasmids. The coding sequence of the *dadB* gene (encoding alanine racemase) was amplified from *S. Typhimurium* UK-1 by PCR using a primer set consisting of *DadB*-5' (CTCGAGATGACCCGCCCTATACAGGCCAGCCT) and *DadB*-3' (TCTAGAAATTAGGTTACGTTGTCACAAACGGCACAC). The purified PCR product was digested with XhoI and XbaI and ligated into pYA3342, which was linearized with the same restriction enzymes, resulting in plasmid pYA4554 (Fig. 2A). The *Lpp* promoter with *LacO* as the *LacI* binding site

was amplified using PCR from pIN-II-ompA (10) and digested with XhoI and NcoI and cloned into pYA4554 to form $DadB^+$ vector pYA4635. The region encoding the α -helix domain and the proline-rich domain (amino acids [aa] 4 to 444) of the *pspC* gene from *S. pneumoniae* L81905, along with the sequence incorporating the *bla* signal sequence (*bla*-SS) upstream, was amplified from pYA4502 using PCR (42). Purified PCR products were digested with BspHI and SalI and cloned into $DadB^+$ vector pYA4635 to yield pYA4634. Plasmids were transformed into *Salmonella* strain $\chi 8901$ by electroporation (Bio-Rad, Hercules, CA). Transformants containing $DadB^+$ plasmids were selected on LB agar plates. Only clones containing the recombinant plasmids expressing the *dadB* gene were able to grow on LB agar devoid of D-alanine. All constructs were confirmed by DNA sequencing. Nucleotide sequencing reactions were performed by the sequencing facility at Arizona State University using ABI Prism Fluorescent BigDye terminators according to the instructions of the manufacturer (PE Biosystems, Norwalk, CT). *PspC* synthesis was confirmed by immunoblot analysis using anti-PspC serum.

Construction of dual-plasmid system in *S. Typhimurium* strain $\chi 9760$. Plasmids pYA4088 (Asd^+ -PspA) (42) and pYA4634 ($DadB^+$ -PspC) were cotransformed into *S. Typhimurium* strains $\chi 9760$ ($\Delta asd \Delta alr \Delta dadB \Delta recF$) and $\chi 9590$ ($\Delta asd \Delta alr \Delta dadB$) by electroporation. Transformants containing Asd^+ -PspA and $DadB^+$ -PspC plasmids were selected on LB agar plates. Only clones containing both recombinant plasmids were able to grow in the absence of D-alanine and DAP. For vaccine controls, plasmid pYA4088 was transformed into $\chi 9828$ (Δasd) and pYA4634 was transformed into $\chi 11026$ ($\Delta dadB \Delta alr$). Synthesis of the antigens in the respective *S. Typhimurium* vaccine strains was confirmed by immunoblot analyses performed using serum specific for PspA or PspC as described before (42). Lipopolysaccharide (LPS) profiles of *Salmonella* strains were also examined to rule out any pleiotropic effects (17).

Plasmid stability of *Salmonella* vaccine strains. Stability tests were done according to the standard plasmid stability test protocol described by Zhang et al. (44). A 3-µl volume of overnight culture (in LB broth) of *Salmonella* strains carrying $DadB^+$ vector pYA4634 and Asd^+ vector pYA4088 was inoculated into 3 ml of LB medium containing D-Ala and DAP (nonselective conditions) for 12 h of incubation by rotation at 37°C (T_0). At this point, a sample of culture was obtained and serial dilutions of the culture were plated on LB-D-Ala-DAP (LAD) plates. The process described above was repeated 5 times every 12-h period, and the conclusion of the final passage was considered T_5 (~50 generations). Before each passage, a sample from the culture was diluted and spread onto LB agar plates containing D-Ala and DAP. Then, 100 single colonies were picked and patched on LB agar plates without any supplements (selective media) and on LB agar plates containing either D-Ala or DAP (differential selective media). Plasmid stability was determined as the percentage of colonies (of 100 selected) growing on selective media after each of the five passages. The desired plasmid stability at T_5 is >95%. Representative colonies after five passages were evaluated for maintenance of plasmid size and the ability of the isolate to produce PspA or PspC of the expected size.

Determining the extent of intra- or interplasmid recombination. To determine interplasmid recombination events, *S. Typhimurium* strain $\chi 9760$ containing a $\Delta recF$ mutation and its isogenic *recF⁺* $\chi 9590$ version, each carrying $DadB^+$ pYA4634 and Asd^+ pYA4088, were individually inoculated into 3 ml of LB medium at a 1:1,000 dilution for 15 days. The inoculated bacteria were cultured by rotation at 37°C for 12 h per day. After every 12-h period, the process described above was repeated for 15 periods. Using Vector NTI, we found that each plasmid had one unique site for restriction enzymes AflII and BlnI that can be used to identify any intra- or interplasmid recombination events within or between the two plasmids. Plasmid DNA was isolated from the 15-day-old culture and digested with AflII and BlnI for 3 h at 37°C. All the DNA samples were separated by capillary electrophoresis using a PA800 analysis system (Beckman Coulter) at a setting of 4.5 kV for 35 min. The electrophoresis readouts are in the form of peaks; each peak represents either the native form or the recombined form of the plasmids. The value associated with

TABLE 1 Plasmids and bacterial strains

Plasmid or strain	Description or relevant characteristic(s) ^a	Reference or source
Plasmids		
pRE112	Suicide vector; sacB mobRP4 R6K ori; Cm ^r	19
pYA3493	Asd ⁺ vector (pBR <i>ori</i>) with SD— <i>asd</i> , P _{trc} promoter and <i>bla</i> -SS signal sequence	19
pYA3667	Suicide vector to generate Δ <i>dadB4</i> mutation from pRE112, sacB mobRP4 R6K ori; Cm ^r	This study
pYA3668	Suicide vector to generate Δ <i>alr-3</i> mutation from pRE112, sacB mobRP4 R6K ori; Cm ^r	This study
pYA3886	Suicide vector to generate Δ <i>recF126</i> mutation from pRE112, sacB mobRP4 R6K ori; Cm ^r	44
pYA3994	Gene encoding (0.7-kb) green fluorescent protein cloned into EcoRI and HindIII sites in Asd ⁺ vector pYA3342	This study
pYA4015	<i>DadB</i> ⁺ vector (pBR <i>ori</i>) with P _{<i>dadB</i>} —ATG— <i>dadB</i>	This study
pYA4088	A 0.8-kb DNA fragment encoding the α -helical region of <i>S. pneumoniae</i> protein PspA (Rx1, aa 3–286) cloned in pYA3493	42
pYA4346	<i>DadB</i> ⁺ vector (p15A <i>ori</i>) with SD-GTG— <i>dadB</i> and the P22 P _L promoter—MCS—transcriptional terminator cassette to clone antigen gene of interest was derived from pYA4554 by replacing the pBR <i>ori</i> with p15A <i>ori</i>	This study
pYA4552	<i>DadB</i> ⁺ vector (pUC <i>ori</i>) with SD-GTG— <i>dadB</i> and the P22 P _L promoter—MCS—transcriptional terminator cassette to clone antigen gene of interest was derived from pYA4554 by replacing the pBR <i>ori</i> with pUC <i>ori</i>	This study
pYA4554	<i>DadB</i> ⁺ vector (pBR <i>ori</i>) was derived from pYA4015; contains SD-GTG— <i>dadB</i> and the P22 P _L promoter—MCS—transcriptional terminator cassette to clone antigen gene of interest	This study
pYA4635	<i>DadB</i> ⁺ vector (pBR <i>ori</i>) with SD-GTG— <i>dadB</i> was derived from pYA4554 by replacing P22 P _L promoter with the P _{lpp-lacO} promoter and introducing MCS- <i>bla</i> -SS signal sequence cassette for cloning and expression of antigen gene of interest	This study
pYA4634	A 1.2-kb DNA encoding the α -helical region and the proline-rich domain of <i>S. pneumoniae</i> antigen PspC (L81905, aa 4–477) in <i>DadB</i> ⁺ vector pYA4635	This study
pYA5041	A 0.7-kb DNA encoding mCherry under the control of the P22 P _L promoter in <i>DadB</i> ⁺ vector pYA4552	This study
Strains		
<i>S. Typhimurium</i>		
χ 3761	UK-1 wild-type <i>Salmonella</i> Typhimurium	9
χ 8901	Δ <i>alr-3</i> Δ <i>dadB4</i> in χ 3761	This study
χ 9558	Δ <i>pmi-2426</i> Δ (<i>gmd-fcl</i>)-26 Δ P _{fur81} ::TT <i>araC</i> P _{BAD} <i>fur</i> Δ P _{crp527} ::TT <i>araC</i> P _{BAD} <i>crp</i> Δ <i>asdA27</i> ::TT <i>araC</i> P _{BAD} c2 Δ <i>araE25</i> Δ <i>araBAD23</i> Δ <i>relA198</i> :: <i>araC</i> P _{BAD} <i>lacI</i> TT Δ <i>sopB1925</i> Δ <i>agfBAC811</i>	21
χ 9590 (χ 9558 + Δ <i>alr</i> Δ <i>dadB</i>)	Δ <i>pmi-2426</i> Δ (<i>gmd-fcl</i>)-26 Δ P _{fur81} ::TT <i>araC</i> P _{BAD} <i>fur</i> Δ P _{crp527} ::TT <i>araC</i> P _{BAD} <i>crp</i> Δ <i>asdA27</i> ::TT <i>araC</i> P _{BAD} c2 Δ <i>araE25</i> Δ <i>araBAD23</i> Δ <i>relA198</i> :: <i>araC</i> P _{BAD} <i>lacI</i> TT Δ <i>sopB1925</i> Δ <i>agfBAC811</i> Δ<i>alr-3</i> Δ<i>dadB4</i>	This study
χ 9760 (χ 9590 + Δ <i>recF</i>)	Δ <i>pmi-2426</i> Δ (<i>gmd-fcl</i>)-26 Δ P _{fur81} ::TT <i>araC</i> P _{BAD} <i>fur</i> Δ P _{crp527} ::TT <i>araC</i> P _{BAD} <i>crp</i> Δ <i>asdA27</i> ::TT <i>araC</i> P _{BAD} c2 Δ <i>araE25</i> Δ <i>araBAD23</i> Δ <i>relA198</i> :: <i>araC</i> P _{BAD} <i>lacI</i> TT Δ <i>sopB1925</i> Δ <i>agfBAC811</i> Δ<i>alr-3</i> Δ<i>dadB4</i> Δ<i>recF126</i>	This study
χ 9828 (χ 9558 + Δ <i>recF</i>)	Δ <i>pmi-2426</i> Δ (<i>gmd-fcl</i>)-26 Δ P _{fur81} ::TT <i>araC</i> P _{BAD} <i>fur</i> Δ P _{crp527} ::TT <i>araC</i> P _{BAD} <i>crp</i> Δ <i>asdA27</i> ::TT <i>araC</i> P _{BAD} c2 Δ <i>araE25</i> Δ <i>araBAD23</i> Δ <i>relA198</i> :: <i>araC</i> P _{BAD} <i>lacI</i> TT Δ <i>sopB1925</i> Δ <i>agfBAC811</i> Δ<i>recF126</i>	This study
χ 11025 (<i>asd</i> ⁺ of χ 9590)	Δ <i>pmi-2426</i> Δ (<i>gmd-fcl</i>)-26 Δ P _{fur81} ::TT <i>araC</i> P _{BAD} <i>fur</i> Δ P _{crp527} ::TT <i>araC</i> P _{BAD} <i>crp</i> Δ <i>araE25</i> Δ <i>araBAD23</i> Δ <i>relA198</i> :: <i>araC</i> P _{BAD} <i>lacI</i> TT Δ <i>sopB1925</i> Δ <i>agfBAC811</i> Δ <i>alr-3</i> Δ <i>dadB4</i>	This study
χ 11026 (χ 11025 + Δ <i>recF</i>)	Δ <i>pmi-2426</i> Δ (<i>gmd-fcl</i>)-26 Δ P _{fur81} ::TT <i>araC</i> P _{BAD} <i>fur</i> Δ P _{crp527} ::TT <i>araC</i> P _{BAD} <i>crp</i> Δ <i>araE25</i> Δ <i>araBAD23</i> Δ <i>relA198</i> :: <i>araC</i> P _{BAD} <i>lacI</i> TT Δ <i>sopB1925</i> Δ <i>agfBAC811</i> Δ <i>alr-3</i> Δ <i>dadB4</i> Δ<i>recF126</i>	This study
χ 9052	Δ <i>alr-3</i> Δ <i>dadB4</i> Δ <i>asdA33</i>	Curtiss laboratory Chi collection
χ 11018 (χ 9052 + Δ <i>recF</i>)	Δ <i>alr-3</i> Δ <i>dadB4</i> Δ <i>asdA33</i> Δ<i>recF</i>	This study
<i>S. pneumoniae</i>		
WU2	Wild-type encapsulated type 3, PspA family 1, clade 2	41
L81905	Wild-type encapsulated type 4, PspA family 1, clade 1	41
A66.1	Wild-type encapsulated type 3, PspA family 1, clades 1 and 2	41

^a Cm^r, chloramphenicol resistance. Boldface characters indicate genotype differences from the corresponding parent strains.

FIG 1 Mutations imposing a requirement for D-alanine. (A) Schematic diagram of chromosomal deletion of *dadB* and *alr* genes in *S. Typhimurium*. The numerical designations are based on the nucleotide position with respect to the first nucleotide for the start codon of the gene. (B) PCR-based confirmation of Δ *dadB* and Δ *alr* deletions.

each peak (expressed in relative fluorescence units [RFU]) represents the associated plasmid DNA concentration. When recombination occurred, pYA4088 (3,927 bp) and pYA4634 (4,527 bp) were resolved as two bands of 6,378 bp and 2,073 bp in size upon digestion with the chosen restriction enzymes; when recombination did not occur, the linear sizes of the plasmids corresponded to their theoretical sizes.

Fluorescent imaging of RASV strains. Plasmids pYA3994 (Asd⁺-green fluorescent protein [GFP]) and pYA5041 (DadB⁺-mCherry) were transformed into strain χ 11018 (Δ *alr* Δ *dadB* Δ *asd* Δ *recF*). Strains were grown to mid-logarithmic phase, and cultures were washed and resuspended in phosphate-buffered saline (PBS) at a 1:100 dilution. A smear of the suspension was heat-fixed on a glass slide and then observed under a fluorescence microscope (Nikon TE2000) at different excitation wavelengths of 488 nm and 587 nm. Images were taken at $\times 100$ magnification. Cells displaying green fluorescence contained the Asd⁺-GFP plasmid, and cells with red fluorescence contained the DadB⁺-mCherry plasmid.

Immunization of mice. Inbred 7-week-old female BALB/c mice (10 mice per group) were deprived of food and water for 6 h before oral immunization. The recombinant *Salmonella* strains χ 9828(pYA4088-PspA), χ 9760 (triple mutant with Δ *recF*126) (pYA4088-PspA; pYA4634-PspC), χ 9828(pYA4088-PspA), χ 11026(pYA4634-PspC), χ 9590 (triple mutant with *recF*⁺) (pYA4088-PspA; pYA4634-PspC), χ 11026 (pYA4634), and χ 9828 (empty vector control-pYA3493) were individually grown in LB broth with 0.05% arabinose to an optical density at 600 nm (OD₆₀₀) of 0.8. Cultures were centrifuged at 4,000 $\times$ g at room tem-

FIG 2 *dadB*⁺ plasmid pYA4554 and synthesis of DadB in RASV. (A) Plasmid pYA4554 is a pBR *ori* vector that carries the wild-type copy of the *dadB* gene with a weak ribosome binding site and GTG start codon to achieve reduced expression of *dadB*. The plasmid contains the P22 P_L promoter—MCS—5ST1T2 cassette to facilitate cloning and expression of antigen genes of interest. (B) *dadB*⁺ plasmids with p15A *ori* (pYA4346), pUC *ori* (pYA4552), and pBR *ori* (pYA4554) with various copy numbers were individually transformed into *S. Typhimurium* strain χ 8901 (Δ *alr* Δ *dadB*) or χ 9760 (Δ *asdA* Δ *alr* Δ *dadB* Δ *recF*), and the levels of DadB synthesized in each were determined by immunoblot analysis using anti-DadB serum. The ~40-kDa DadB protein is indicated by an arrow. Whole-cell protein obtained from wild-type *S. Typhimurium* χ 3761 was used as a control. The blot was also stained with anti-GroEL serum to serve as a loading control.

perature and suspended in buffered saline solution containing 0.01% glycerol (BSG) to achieve a final concentration of 5×10^{10} CFU/ml. The cell suspension (20 μ l containing 1×10^9 cells) was orally administered to BALB/c mice (10 mice in each group) on days 1 and 42. RASV strain χ 9558 (pYA3493-empty vector) was used as the control. Food and water were returned to the mice 30 min after immunization. Blood samples were taken by submandibular bleeding at 4 and 8 weeks post-primary immunization. After incubation at 37°C for 60 min, blood was centrifuged at $4,000 \times g$ for 5 min and serum was removed and stored at -70°C . Vaginal secretions (for determination of mucosal IgA responses) were collected in week 8 using a 50- μ l BSG wash and stored at -20°C (20).

Analysis of serum IgG and mucosal IgA by ELISA. Antigen-specific (PspA or PspC) antibody titers were determined by enzyme-linked immunosorbent assay (ELISA) as described by Kang et al. (20).

Pneumococcal challenge. To assess the ability of the RASVs to protect the immunized mice against different strains of *S. pneumoniae*, immunized and control mice (10 mice per group) were challenged intraperitoneally (i.p.) with 2×10^4 CFU of *S. pneumoniae* strain WU2 (200 50% lethal doses [LD_{50}]) (22, 41) or intravenously (i.v.) with 1×10^6 CFU of *S. pneumoniae* strain L81905 ($100 \times \text{LD}_{50}$) (30) in 200 μ l of BSG. To evaluate protection against intranasal (i.n.) challenge, 1×10^8 CFU of *S. pneumoniae* strain A66.1 ($20 \times \text{LD}_{50}$) (1, 40) in 20 μ l of BSG was administered. All challenges were done 2 weeks after the final boost (week 8). Mortality was monitored for 3 weeks following pneumococcal challenge.

Statistical analysis. An analysis of variance (ANOVA) (SPSS Software), followed by the use of Tukey's method, was used to evaluate differences in antibody titers (discerned to 95% confidence intervals) between various groups of immunized and naïve mice. The Kaplan-Meier method (GraphPad Prism; GraphPad Software) was applied to quantify the survival fractions following i.p., i.v., or i.n. challenge of orally immunized mice (43).

RESULTS

D-Alanine-dependent Δ *dadB* Δ *alr* mutants of *S. Typhimurium*. In *S. Typhimurium*, two separate genes (*dadB* and *alr*) encode alanine racemases, which are key enzymes in D-alanine metabolism (11, 39). D-Alanine is an essential building block of Gram-negative peptidoglycan (2, 12). We took advantage of the obligate requirement of the enzyme alanine racemase to develop an *S. Typhimurium* balanced-lethal DadB⁺ vector system. We first constructed the host strain containing Δ *alr* Δ *dadB* mutations using suicide vectors pYA3668 and pYA3667, respectively, (Table 1) (Fig. 1A). The Δ *alr* Δ *dadB* double mutant of *S. Typhimurium* was generated by sequential conjugation using *E. coli* χ 7213 as the donor strain. The resulting strain was designated χ 8901 (Δ *alr* Δ *dadB*) (Table 1). χ 8901 required D-alanine for growth and was totally avirulent in BALB/c mice, which survived doses in excess of 10^9 CFU (see below).

Our goal is to develop an *S. Typhimurium* vaccine strain that can carry two plasmids (Asd⁺ and DadB⁺) and deliver multiple antigens in the host. Using the approach described above, we generated Δ *alr* Δ *dadB* derivatives of the well-studied *S. Typhimurium* χ 9558 vaccine strain (16, 21) to obtain χ 9590 (Δ *asdA* Δ *alr* Δ *dadB*). The presence of the Δ *asdA* mutation in this strain ensures maintenance of the Asd⁺ balanced-lethal vector for survival (Table 1). As a control vaccine strain, we generated an *asdA*⁺ version of χ 9590 by transduction to Asd⁺ χ 11025 (*asdA*⁺ Δ *alr* Δ *dadB*) (Table 1). Strain χ 9052 from the Curtiss laboratory Chi collection was used to evaluate our dual-plasmid system in a strain with a simple genotype. Strain χ 9052 is a triple mutant (Δ *asdA* Δ *alr* Δ *dadB*) and can maintain both Asd⁺ and DadB⁺ plasmids.

RecF in *S. Typhimurium* mediates intra- and interplasmid recombination, and deletion of the gene substantially reduces the frequency of such events (44). Since our strains were designed to

carry both Asd⁺ and DadB⁺ plasmids, we introduced the Δ *recF126* mutation by allelic exchange in strains χ 9052, χ 9558, χ 9590, and χ 11025 to generate χ 11018, χ 9828, 9760, and χ 11026, respectively (Table 1).

DadB⁺ plasmid pYA4554 and its copy-number variants. Our goal was to construct a DadB⁺ plasmid carrying the wild-type copy of the *dadB* gene to complement Δ *alr* Δ *dadB* mutations such that strain χ 8901 would not require D-alanine in the medium for growth. Based on our experience with Asd⁺ vectors (20), the virulence of *Salmonella* vaccine strains carrying the DadB⁺ vector and the stability of the DadB⁺ plasmid are related to the amount of alanine racemase synthesized. Originally, we introduced the native *dadB* gene promoter and Shine-Dalgarno (SD) sequence from wild-type strain χ 3761 to construct DadB⁺ vector pYA4015 (pBR *ori*) (Table 1). Plasmid pYA4015 was introduced into *S. Typhimurium* strain χ 8901 (Δ *alr* Δ *dadB*) to determine if the wild-type copy of the *dadB* gene (on pYA4015) complements the genomic deletions (Δ *alr* Δ *dadB*). To our surprise, strain χ 8901 still had a 4-log-higher LD_{50} ($>10^8$) (unpublished data), suggesting that the mutation was either over- or undercomplemented. We postulated that alanine racemase synthesized from plasmid pYA4015 at levels higher than those seen in wild-type χ 3761 may have put additional stress on the cell, resulting in hyperattenuation and/or decreased plasmid stability *in vivo*. Two alterations were made to decrease the level of alanine racemase in *Salmonella*: (i) we modified the upstream region of the native *dadB* gene by increasing the space between the ribosome binding site (SD sequence) and start codon (by placing additional nucleotides in the sequence), and (ii) we changed the start codon from ATG to GTG, which affected the level of *dadB* expression on the plasmid. After several rounds of optimization, we finally obtained *dadB*⁺ expression-cloning vector pYA4554 (pBR *ori*) (Fig. 2A). This plasmid complemented the two mutations *in vitro* such that χ 8901 (pYA4554) did not require D-alanine for growth. The DadB⁺ expression-cloning vector depicted in Fig. 2A has the P22 P_L promoter immediately followed by a modified ribosome binding site (SD), a multiple-cloning site (MCS) to facilitate insertion of the (antigen) gene of interest, a 5ST1T2 transcription terminator, and the pBR *ori*. Expression of a gene encoding an antigen is controlled by P22 P_L. We constructed variants of pYA4554 by replacing the pBR *ori* with the p15A *ori* sequence to yield pYA4346 (DadB⁺-p15A *ori*) or with the pUC *ori* sequence to obtain pYA4552 (DadB⁺-pUC *ori*), all producing various amounts of the 40-kDa DadB protein in *S. Typhimurium* (Fig. 2B). However, only the strain carrying plasmid pYA4346 (p15A *ori*) produced DadB at levels similar to that seen in wild-type χ 3761 (Fig. 2B).

We made two additional modifications to the DadB⁺ pYA4554 plasmid. First, the P22 P_L promoter was replaced with a weaker P_{1pp} promoter such that the level of antigen gene expression was lower and the toxicity of the foreign protein was minimal. Second, we introduced the nucleotide sequence encoding the beta-lactamase signal sequence such that the fusion protein product could be secreted to the periplasm and to the exterior of the cell via the type II secretion system. The resulting plasmid was called pYA4635. Gene *pspC*, derived from *S. pneumoniae* L81905, was cloned into pYA4635 to be expressed under the control of P_{1pp} to yield pYA4634 (Table 1).

DadB⁺ plasmids restore the virulence of χ 8901. To determine if introduction of DadB⁺ vectors into χ 8901 can fully restore its virulence in mice, we determined the 50% lethal dose (LD_{50}) of

FIG 3 Determination of virulence of RASV strains complemented with *DadB*⁺ plasmids. The LD₅₀ of *S. Typhimurium* wild-type strain χ3761 (WT) or χ8901 ($\Delta dadB \Delta alr$) or the mutant complemented by transformation with *DadB*⁺ plasmids p15A *ori* (pYA4346), pBR *ori* (pYA4554), and pUC *ori* (pYA4552) with various copy numbers was determined by oral inoculation of various doses of each strain in BALB/c mice.

strain χ8901 and of derivatives carrying each of the three *DadB*⁺ plasmids. The LD₅₀ of wild-type *S. Typhimurium* UK-1 strain χ3761 is about 1.2×10^4 CFU, with 9 days as the mean time to death (16). We inoculated BALB/c mice orally with 10⁴, 10⁵, 10⁶, 10⁷, 10⁸, or 10⁹ cells of χ8901 ($\Delta dadB \Delta alr$) with or without a *DadB*⁺ vector. Strain χ8901 was completely attenuated, with no clinical symptoms in mice even at 10⁹ CFU (Fig. 3); however, χ8901 carrying *DadB*⁺ plasmid pYA4346 (p15A *ori*) regained virulence. The LD₅₀ of this strain was reduced to 1.2×10^5 CFU, with the mean time to death around 12 days. Similarly, introduction of *DadB*⁺ plasmid pYA4554 (pBR *ori*) also complemented the mutation *in vivo*; however, the LD₅₀ was still higher at 7.6×10^6 , with the mean time to death around 12 days. On the other hand, *DadB*⁺ plasmid pYA4552 (pUC *ori*) had no effect on the virulence of strain χ8901 (Fig. 3), suggesting that the increased levels of *DadB* produced in this strain may have rendered this plasmid unstable or may have imposed a metabolic burden to compromise its function *in vivo*. We observed an inverse correlation between the amount of *DadB* produced in χ8901 and its virulence.

Plasmids *Asd*⁺ and *DadB*⁺ restore the phenotypes of $\Delta asdA$ and $\Delta dadB$ mutants, respectively. To facilitate the use of two plasmids in a vaccine strain, it is important that the plasmids are stably maintained in the strain and complement the respective genomic mutations. The phenotypes of strains χ8901, χ9590, χ9760, and χ11026 with $\Delta asdA$ and/or $\Delta alr \Delta dadB$ were first tested by growing the strains on LB agar with or without DAP and D-alanine. As expected, none of these strains grew on LB-arabinose or even when DAP was added the medium (Fig. 4). Addition of D-alanine, however, restored growth of the χ8901 and χ11026 strains that carried $\Delta alr \Delta dadB$ mutations (Fig. 4), and addition of DAP together with D-alanine rescued strains χ9590 and χ9760 (Fig. 4), as they lack *asdA*, *alr*, and *dadB* genes. These observations together confirmed that individual deletion mutations had resulted in the desired phenotypic defect.

To check the ability of the *DadB*⁺ and *Asd*⁺ plasmids to com-

FIG 4 Phenotype determination of various mutants of *S. Typhimurium* with-out and with complementing plasmids. RASV strains χ8901 ($\Delta alr \Delta dadB$), χ9590 ($\Delta asdA \Delta alr \Delta dadB$), χ9760 ($\Delta asdA \Delta alr \Delta dadB \Delta recF$), and χ11026 ($\Delta alr \Delta dadB \Delta recF$) with or without *Asd*⁺ plasmid (pYA4088-*pspA*) or *DadB*⁺ plasmid (pYA4634-*pspC*) were streaked on LB agar with or without DAP or D-alanine and incubated at 37°C for 18 h.

plement the corresponding mutation, single or double mutants of *S. Typhimurium* were individually transformed with *Asd*⁺ vector pYA4088 (encoding the α -helical domain of the *pspA* gene from *S. pneumoniae* RX1) (41) or *DadB*⁺ vector pYA4634 (encoding the α -helical domain of the *pspC* gene from *S. pneumoniae* L81905) or both in the cases of χ9590 ($\Delta asdA \Delta alr \Delta dadB$) and χ9760 ($\Delta asdA \Delta alr \Delta dadB \Delta recF$). *DadB*⁺ vector pYA4634 complemented $\Delta alr \Delta dadB$ mutations in both strains χ8901 and χ11026 such that they did not require an exogenous supply of D-alanine for growth (Fig. 4). Similarly, pYA4088 (*Asd*⁺) and pYA4634 (*DadB*⁺) sufficiently complemented the respective mutations in strains χ9590 and χ9760 (Fig. 4).

Determination of recombination frequency of *DadB*⁺ and *Asd*⁺ vectors in *S. Typhimurium*. We used *RecF*⁺ (χ9590) and *RecF*⁻ (χ9760) versions of the vaccine strains that can potentially carry both balanced-lethal vectors *Asd*⁺ and *DadB*⁺ (Table 1) to understand the significance of the *RecF* enzyme in promoting recombination between homologous sequences on the two plasmids. We took advantage of unique restriction sites for *Afl*III and *Blp*I on plasmids pYA4088 and pYA4634 and compared the restriction profiles of the two plasmids in *recF*⁺ and *ΔrecF* genotypes. As show in Fig. 5 (upper panel), possible recombination between the two plasmids pYA4088 (3,927 bp) and pYA4634 (4,527 bp) in strain χ9590 (*recF*⁺) resulted in a larger plasmid and digestion with the above-described enzymes resulted in two distinct peaks corresponding to 6,378 bp and 2,073 bp, whereas peaks corresponding to the native sizes were significantly reduced. In contrast, the restriction profile of plasmids obtained from χ9760 (*ΔrecF*) (Fig. 5, lower panel) contained high-intensity peaks corresponding to the native sizes of 3,927 bp (pYA4088) and 4,527 bp (pYA4634) and those resulting from possible recombination (2,073 bp and 6,378 bp) were substantially reduced. Together, these results indicate that the absence of *RecF* can decrease plasmid recombination in *S. Typhimurium*.

Antigen synthesis and plasmid stability of the two-plasmid system. To ensure that the two antigen genes were expressed in the

FIG 5 Effect of RecF on plasmid recombination. Plasmids pYA4088 (Asd⁺) and pYA4634 (DadB⁺) recovered from χ 9590 (Δ asdA Δ alr Δ dadB) or χ 9760 (Δ asdA Δ alr Δ dadB Δ recF) were digested with AflIII and BlnI and separated by electrophoresis. Relative fluorescent units (RFU) were used as an indirect measure to determine the intensity of linearized DNA fragments of various sizes.

dual-plasmid system, production of both PspA (from pYA4088; Asd⁺-PspA) and PspC (from pYA4634; DadB⁺-PspC) in strain χ 9760 (Δ alr Δ dadB Δ asdA) was confirmed by immunoblot analysis (Fig. 6A). Levels of PspA and PspC in χ 9760 were similar to the level of PspA in χ 9828 (Δ asdA) and the level of PspC in χ 11026 (Δ alr Δ dadB), used as controls (Fig. 6A). This confirms that the presence of two plasmids in a single vaccine strain did not decrease individual antigen synthesis.

We used fluorescence imaging to further confirm the coexistence of two plasmids in a vaccine strain by tracking the expression of *gfp* and mCherry markers on plasmids pYA3994 (Asd⁺-gfp) and/or pYA5401 (DadB⁺-mCherry), respectively, in strain χ 11018 (Δ asdA, Δ dadB, Δ alr, Δ recF) (Fig. 6B). The amount of GFP (Asd⁺-gfp) or mCherry (DadB⁺-mcherry) produced in isolation was not significantly different than when both plasmids were provided in the single strain, thus ensuring the coexistence of different plasmids.

In addition to synthesis of multiple antigens, stable maintenance of the plasmids over several generations is an essential requirement to ensure efficacy of RASVs. The coexistence of plasmids pYA4088 (Asd⁺-PspA) and pYA4634 (DadB⁺-PspC) in RASV strains χ 11018 (Δ asdA Δ alr Δ dadB Δ recF), χ 9590 (Δ asdA Δ alr Δ dadB Δ recF⁺), and χ 9760 (Δ asdA Δ alr Δ dadB Δ recF) was determined by plating actively growing cultures on both selective and nonselective medium for 50 generations (Fig. 7). In general, the Asd⁺ plasmid was more stable than the DadB⁺ plasmid in

all three strains and the stability of both plasmids was ~95% in strain χ 11018, which carries relatively fewer mutations (Fig. 7). Strains χ 9590 (Δ recF⁺) and χ 9760 (Δ recF), however, make an interesting comparison. The Asd⁺ plasmid was stable in both strains, thus negating the need for the Δ recF mutation. However, the stability of the DadB⁺ plasmid dropped significantly ($P < 0.01$) to 50% in strain χ 9590 and reverted to about 75% in χ 9760 (Δ recF126) (Fig. 7), suggesting that RecF-mediated intraplasmid recombination in the former may have affected plasmid stability.

Immune responses to heterologous antigens PspA and PspC.

To investigate the immunogenicity of PspA and PspC proteins codelivered by Asd⁺ and DadB⁺ vectors in RASV, we orally inoculated groups of BALB/c mice with two doses of various *S. Typhimurium* vaccine strains on days 0 and 42. Serum IgG responses to PspA and PspC were measured by ELISA at weeks 4 and 8 (Fig. 8). Antigen-specific IgG titers in mice immunized with vaccine strains were significantly ($P < 0.05$) higher (at 8 weeks) than the titers obtained from mice receiving the vector control strain, χ 9828 (pYA3493). Immunization with strains χ 9828 (pYA4088-PspA) and χ 9760 (pYA4088-PspA plus pYA4634-PspC) resulted in higher anti-PspA IgG titers compared to the vector control results ($P < 0.05$), and the results seen with the two strains did not differ significantly, suggesting that antigen synthesis and delivery *in vivo* were not compromised in the χ 9760 strain in spite of its carrying two plasmids.

FIG 6 Synthesis of PspA and PspC by *S. Typhimurium* vaccine strain. (A) PspA (Asd⁺-PspA) and PspC (DadB⁺-PspC) transformed individually or together into strains of *S. Typhimurium* and the synthesis of target antigen were determined by Western blot analyses of cell extracts. GroEL was used as the loading control. The expected molecular mass of PspA is 40 kDa and of PspC is 65 kDa. Genotypes: χ9828 ($\Delta asdA \Delta recF$); χ9590 ($\Delta asdA \Delta alr \Delta dadB$); χ9760 ($\Delta asdA \Delta alr \Delta dadB \Delta recF$); χ11026 ($\Delta alr \Delta dadB \Delta recF$). (B) Qualitative analysis of synthesis of GFP and mCherry specified by Asd⁺ and DadB⁺ vectors pYA3994 and pYA5401, respectively, in RASV strains of χ11018 ($\Delta asdA \Delta alr \Delta dadB \Delta recF$). Cells collected from the mid-logarithmic phase of growth and suspended in PBS were imaged. Magnification, $\times 100$.

Similarly, all PspC-vaccinated mice produced antibody that reacted with PspC (Fig. 8). The anti-PspC titers in mice immunized with χ9760(pYA4088-PspA plus pYA4634-PspC), χ11026 (pYA4634-PspC), or χ9590 (pYA4088-PspA plus pYA4634-PspC) or with a mixture of the strains were not significantly different but were significantly ($P < 0.05$) higher than the titers in mice immunized with χ9828 (pYA4088-PspA) and χ9828 (empty vector-YA3493). Interestingly, the immunogenicity of RecF⁺ strain χ9590 was not significantly different from that of χ9760 ($\Delta recF$). Anti-LPS IgG responses in all groups, including the vector control, were similar, in both kinetics and titer, at 8 weeks (data not shown).

To determine if the vaccine strains differed in the level of mucosal antibody responses they stimulated, IgA titers in vaginal washes were analyzed in week 8 (Fig. 8C). Here too, the highest anti-PspA or -PspC IgA titers were seen in mice immunized with χ9760(pYA4088-PspA plus pYA4634-PspC). The group receiving

FIG 7 Plasmid stability of Asd⁺ and DadB⁺ plasmids specifying PspA and PspC. RASV strains χ9590 ($\Delta asdA \Delta alr \Delta dadB$), χ9760 ($\Delta asdA \Delta alr \Delta dadB \Delta recF$), and χ11026 ($\Delta alr \Delta dadB \Delta recF$) carrying both pYA4088 (Asd⁺-PspA) and pYA4634 (DadB⁺-PspC) were subjected to passage for ~50 generations in nonselective medium, and percent plasmid retention of both plasmids was determined by CFU enumeration upon plating on both nonselective medium and selective medium after the final passage. Plasmid stability was determined as the percentage of colonies (out of 100 selected) growing on selective media. *, $P < 0.05$; **, $P < 0.01$.

χ9828 (empty vector-YA3493) had no detectable titers at all, and mice immunized with χ11026 (pYA4634-PspC) showed a log₂ endpoint titer of 10 against PspC only, as was expected. Taking the results together, we observed the induction of both serum and secretory protective antibody responses by these vaccine strains.

RASVs delivering PspA and PspC induce a Th1 response. Th1-helper cells direct cell-mediated immunity and promote IgG class switching to IgG2a, and Th2 cells provide potent help for B-cell antibody production and promote IgG class switching to IgG1 (29, 34). We measured the levels of IgG isotype subclasses IgG2a and IgG1 at 8 weeks post-primary immunization (Fig. 9) and observed that IgG2a titers to PspA and PspC in all groups were higher than IgG1 titers, indicating that all of the *Salmonella* vaccines induced a stronger Th1 response (Fig. 9). These observations were consistent with the notation that Th1-type dominant immune responses are frequently observed after immunization with attenuated *Salmonella* (24).

Evaluation of protective immunity. To determine if immunization with RASV strains delivering the two pneumococcal antigens PspA and PspC provides protection against challenge with different serotypes of *S. pneumoniae*, we conducted three independent challenges with *S. pneumoniae* strains that were used before in our laboratory. In the first study, immunized mice were challenged via the intraperitoneal route with 200 LD₅₀s of *S. pneumoniae* WU2 (serotype 3) (Fig. 10A). All RASV strains synthesizing PspA provided a significantly ($P < 0.05$) greater level of protection against WU2 than the control vaccine strain carrying the empty vector (χ9828-pYA4432) (Fig. 10A). Percent protection ranged from 75% in the case of χ9760 (Asd⁺-PspA plus DadB⁺-PspC) to 25% in the case of χ11026 (DadB⁺-PspC), which was the least efficacious of the vaccine strains tested. Protection conferred by χ9760 delivering both PspA and PspC was significantly greater ($P < 0.05$) than that offered by χ9828 delivering PspA (Asd⁺-PspA) alone; thus, the difference can be attributed to the presence of PspC.

In the second experiment, immunized mice were challenged intravenously with 100 LD₅₀s of *S. pneumoniae* L81905 (Fig. 10B). All mice immunized with RASV χ9760 ($\Delta asdA \Delta alr \Delta dadB$) de-

FIG 8 Anti-PspA and anti-PspC antibody responses in mice. Serum IgG (A and B) and mucosal IgA (C) responses to PspA and PspC were determined by ELISA post-oral immunization with 1×10^9 CFU of *S. Typhimurium* strain χ 9828 (DadB⁺-PspC), χ 9760 (Asd⁺-PspA plus DadB⁺-PspC), χ 9558 (Asd⁺-PspA), χ 11026 (DadB⁺-PspC), or χ 9558 (Asd⁺-empty vector). Purified PspA or PspC were used to coat the ELISA plates. Statistical significance was determined at week 8. Titer values represent the log₂ geometric mean titers of the results determined for eight mice $\pm$ standard deviations. *, $P < 0.05$. (4W, week 4 after primary immunization; 8W, week 8 after primary immunization).

livering both Asd⁺-PspA and DadB⁺-PspC survived (100% protection); vaccine χ 9590 (Asd⁺-PspA and DadB⁺-PspC) and the mixture of χ 9828 (Asd⁺-PspA) and χ 11026 (DadB⁺-PspC) showed the next highest level of efficacy, with both χ 9590 and the mixture of χ 9828 and χ 11026 offering 80% protection. Interestingly, strain χ 9828 (pYA4088-PspA) offered cross-protection against *S. pneumoniae* L81905, although the level was only around 50%.

Vaccine strains were also evaluated for inducing protection against *S. pneumoniae* A66.1 in a pneumonia model. All vaccine strains delivering antigens offered some degree of protection in this challenge and were significantly more efficacious than the vector control ($P < 0.01$), and RASV χ 9760 (Asd⁺-PspA plus DadB⁺-PspC), offering 80% protection, was the most efficacious (Fig. 10C).

Taken together, these results demonstrate that χ 9760 (Δ asdA Δ alr Δ dadB), delivering PspA and PspC, offered superior protection against challenge with different serotypes of *S. pneumoniae*.

DISCUSSION

Most live vector-based vaccines, such as the *Salmonella* delivery system, utilize a single plasmid for expression of a single antigen or a fusion protein carrying epitopes from two or more antigens (14). A multivalent *Salmonella* vaccine that can deliver multiple protective antigens by expressing two or more heterologous antigen genes facilitates maximal protection against a target pathogen. However, the major limitation complicating efforts to achieve this is that a recombinant vaccine strain is required that is capable of stably maintaining two or more expression vectors, with each carrying a unique selectable marker.

FIG 9 Determination of IgG isotypes. The IgG2a and IgG1 titers were determined in sera from BALB/c mice 8 weeks after oral immunization with 1×10^9 CFU of *S. Typhimurium* strain $\chi 9828$ (DadB⁺-PspC), $\chi 9760$ (Asd⁺-PspA plus DadB⁺-PspC), $\chi 9558$ (Asd⁺-PspA), $\chi 11026$ (DadB⁺-PspC), or $\chi 9558$ (Asd⁺-empty vector). Purified PspA or PspC was used to coat the ELISA plates. The IgG2a/IgG1 ratio was determined. Titers represent the log₂ geometric mean titers of the results determined for eight mice $\pm$ standard deviations. *, $P < 0.05$.

Twenty years ago, our group developed the balanced-lethal Asd⁺ expression plasmid and used it successfully with live attenuated *Salmonella* vaccine in both animals and humans (4, 5, 7, 13, 32, 41). In this study, we focused on DadB, another key enzyme for cell wall synthesis in *S. Typhimurium*, and developed a DadB⁺ balanced-lethal vector that can be used either alone (in a $\Delta alr \Delta dadB$ mutant) or in conjunction with an Asd⁺ plasmid in an $\Delta asdA \Delta alr \Delta dadB$ triple-mutant strain of *S. Typhimurium* to

deliver two pneumococcal antigens. The key aspects of the DadB⁺ vector system are as follows. (i) The plasmid possesses the *dadB* gene of *Salmonella* as the marker that complements the *dadB* *alr* mutations of *E. coli* and *S. Typhimurium*. The vaccine strain containing these mutations does not survive in the medium devoid of D-alanine without the DadB⁺ plasmid; this ensures both stable maintenance of the plasmid and biocontainment of the vaccine strain. (ii) Introduction of the DadB⁺ vector into a $\Delta dadB \Delta alr$ mutant restores the virulence of the mutant to the wild-type levels (partially in the case of pBR *ori* and pUC *ori* derivatives), which ensures efficient invasion into deep tissues, a critical requisite for increased stimulation of immune responses. (iii) The multiple cloning site (MCS) downstream of the P22 P_L or P_{lpp}-*lacO* promoter enables convenient cloning and expression of the gene of interest, followed by a strong 5ST1T2 transcription terminator that prevents a read-through. (iv) Availability of the plasmid with various copy numbers provides flexibility to design the expression vector based on the nature of the heterologous antigen. (v) Finally, it is compatible with Asd⁺ vector, an important feature in efforts to develop a dual-plasmid delivery system.

The need to develop plasmids with non-antibiotic-resistance markers and the invention of the Asd⁺ vector encouraged identification of several other alternative strategies. Many of these were introduced in food-grade cloning systems developed for microbes such as *Lactococcus lactis* used in the food biotechnology industry. Some examples include *purA* (purine and pyrimidine biosynthesis), *thyA* (thymidylate synthase gene), and *supD* (amber suppressor gene). Although these plasmids were tested successfully, they either suffered from limited areas of application (food or industrial applications) or encountered certain technical impediments (23, 31). For example, use of *supD* on plasmids and *pyrF* in the chromosome of *L. lactis* allows selection and maintenance of plasmids in pyrimidine-free medium, but it additionally suppresses expression of approximately 10% of *L. lactis* genes, which is an undesirable effect (33). *Alr*⁺ plasmid was also used in *Corynebacterium glutamicum* to overproduce D-pantothenic acid (36). In several cases, however, poor plasmid stability or the incompatibility of the markers for use in other bacteria has limited use of these tools in wide-ranging applications. Hence, constructing expression plasmids with essential genes from *Salmonella* as markers becomes necessary.

The Ssb⁺ plasmid that complements genomic deletion of *ssb* of *Salmonella* is the first expression plasmid with a non-enzyme-based permissible marker (28). Gene *ssb* encodes a single-stranded DNA binding protein and is essential for bacterial survival. An *ssb* mutant of *S. Typhi* strain CVD908 successfully delivered *Bacillus anthracis* toxin antigen PA83 on an Ssb⁺ plasmid and is stable for up to 41 generations under nonselective conditions. Consistent with our experience with high-copy-number DadB⁺ vectors (pUC *ori*-pYA4552), Galen et al. note that low-copy-number plasmids are relatively more stable and induce greater levels of antigen-specific immune responses than their high-copy-number versions (15). Indeed, the stability of the Ssb⁺ plasmid starts to diminish as the plasmid copy number reaches around 15 (p15A *ori*), suggesting a possible further decline if a higher-copy-number version, such as the pBR *ori*, were to be used. The study provides some valuable insights into alternative permissible markers and the importance of the copy number of expression plasmids. However, the stability of the plasmid and synthesis of the antigen in vaccine strains having a complex genotype remain to be deter-

FIG 10 Protective efficacy of attenuated *S. Typhimurium* vaccine strains delivering PspA and PspC. BALB/c mice (12 per group) were orally immunized with 1×10^9 CFU of *S. Typhimurium* strains synthesizing PspA and/or PspC proteins. Mice were challenged with 200 LD₅₀s of virulent *S. pneumoniae* WU2 (2×10^4 CFU) intraperitoneally (A) or 100 LD₅₀s of virulent *S. pneumoniae* L81905 (1×10^6 CFU) intravenously (B) or 20 LD₅₀s of virulent *S. pneumoniae* A66.1 (1×10^8 CFU) intranasally (C). Mortality was monitored for 3 weeks. All vaccine group results were significantly different from the χ9828 (pYA3493) (vector control [ctrl]) results ($P < 0.05$). χ9828, Δ*asdA* Δ*recF* (pYA4088-Asd⁺-PspA); χ9760, Δ*asdA* Δ*alr* Δ*dadB* Δ*recF*; χ11026, Δ*alr* Δ*dadB* Δ*recF* (9pYA4634-DadB⁺-PspC); pYA3493, Asd⁺-empty vector; pYA4088, Asd⁺-PspA; pYA4634, DadB⁺-PspC.

mined, and the stability of the plasmid in the presence of another expression vector (for use as a dual-plasmid vaccine system) was not tested. In this report, we have attempted to address these issues and also recommend critical mutations required for developing a stable and effective live *Salmonella* vaccine.

Our DadB⁺ vector with pBR *ori* (pYA4634/4635) is stably maintained under nonselective conditions for ~50 generations in a vaccine strain containing a complex genotype and in the presence of an additional vector (Asd⁺). We did observe that the stability of the DadB⁺ vector was relatively inferior to that of the Asd⁺ vector in the three *S. Typhimurium* vaccine strains tested (χ11018, χ9590, and χ9760). Apart from the metabolic burden on the cell (to produce both the DadB and the pneumococcal antigen) proposed by Galen et al. (15), we speculate that possible intra- or interplasmid recombination promotes instability. This

was tested by comparing levels of plasmid stability in two isogenic strains, χ9590 and χ9760, which differ only in the *recF* gene. The absence of RecF significantly ($P < 0.05$) increases the stability of the DadB⁺ plasmid in strain χ9760 (*recF*) compared to its *recF*⁺ counterpart χ9590, suggesting recombination as a possible cause for this anomaly. Expression plasmids, in general, contain commonly used promoter sequences, transcriptional terminator sequences, and other plasmid backbone elements that are good substrates for recombination. In some cases, researchers may intentionally construct the vector such that it harbors multiple copies of the same DNA sequence, which may lead to plasmid instability. In *E. coli*, *recA*-dependent homologous recombination relies on the RecBCD pathway, the RecFOR pathway (originally designated the RecF pathway), and the RecE pathway. Strains devoid of RecF demonstrate a frequency of recombination between

the plasmids at least 10 times lower without any decrease in the virulence of the bacterium (44). In this report, we demonstrate the critical need for reducing or abolishing recombination of expression plasmids as a means to achieve enhanced stability and protection *in vivo*.

We used antibody titers as a measure of immune responses elicited by each of the vaccine strains. Strain χ 9760 (Asd⁺-PspA plus DadB⁺-PspC) induced the highest titers of serum IgG or mucosal IgA, which directly correlates with the level of protection conferred by this strain. There is increasing evidence of the importance of CD4⁺ T-cell responses, in particular, the role of interleukin-17 (IL-17)-producing Th17 cells, in mediating protection against pneumococcal challenge in mice (25, 26). We, however, restricted this study to demonstration of the ability of *Salmonella* to deliver multiple antigens on two stable plasmids and to evaluation of humoral responses induced by these vaccine strains and did not extend our studies to detection of other immunological markers.

Conclusion. We showed that a *Salmonella* vaccine strain can stably carry two balanced-lethal vectors, DadB⁺ and Asd⁺, each expressing an antigen gene. Deletion of *recF* further stabilizes the plasmids by significantly minimizing any possible recombination events. The *Salmonella* vaccine strain carrying PspA and PspC by Asd⁺ and DadB⁺ vectors, respectively, induced higher antibody response and protection than the strain delivering a single antigen. The DadB⁺-Asd⁺ system thus represents an important tool to develop multivalent live recombinant vaccines.

ACKNOWLEDGMENTS

We thank David Briles and Susan Hollingshead (University of Alabama at Birmingham) for providing the *S. pneumoniae* strain; Heather Matthies for her assistance in constructing *S. Typhimurium* strains χ 11025, χ 11026, and χ 9760; Anita Frevert for strain χ 9052; Wei Kong and Yuhua Li for assistance with animal studies; and Qing Liu for providing the purified antigens PspA and PspC.

This research project was supported by grants from the National Institutes of Health (R01 AI056289) and the Bill and Melinda Gates Foundation (37863).

REFERENCES

- Arulanandam BP, Lynch JM, Briles DE, Hollingshead S, Metzger DW. 2001. Intranasal vaccination with pneumococcal surface protein A and interleukin-12 augments antibody-mediated opsonization and protective immunity against *Streptococcus pneumoniae* infection. *Infect. Immun.* 69:6718–6724.
- Badet B, Roise D, Walsh CT. 1984. Inactivation of the dadB *Salmonella typhimurium* alanine racemase by D and L isomers of beta-substituted alanines: kinetics, stoichiometry, active site peptide sequencing, and reaction mechanism. *Biochemistry* 23:5188–5194.
- Briles DE, et al. 1996. PspA, a protection-eliciting pneumococcal protein: immunogenicity of isolated native PspA in mice. *Vaccine* 14:858–867.
- Curtiss R, III, Goldschmidt RM, Fletchall NB, Kelly SM. 1988. Avirulent *Salmonella typhimurium* delta cya delta crp oral vaccine strains expressing a streptococcal colonization and virulence antigen. *Vaccine* 6:155–160.
- Curtiss R, III, Kelly SM, Gulig PA, Nakayama K. 1989. Stable recombinant avirulent *Salmonella* vaccine strains. *Adv. Exp. Med. Biol.* 251:33–47.
- Curtiss R, III, et al. 1994. Recombinant *Salmonella* vectors in vaccine development. *Dev. Biol. Stand.* 82:23–33.
- Curtiss R, III, Nakayama K, Kelly SM. 1989. Recombinant avirulent *Salmonella* vaccine strains with stable maintenance and high level expression of cloned genes in vivo. *Immunol. Invest.* 18:583–596.
- Curtiss R, III, et al. 2010. New technologies in using recombinant attenuated *Salmonella* vaccine vectors. *Crit. Rev. Immunol.* 30:255–270.
- Curtiss R, et al. 1991. Nonrecombinant and recombinant avirulent *Salmonella* live vaccines for poultry, p 169–198. In *Colonization control of human bacterial enteropathogens in poultry*. Academic Press, Inc., New York, NY.
- Fauconner A, Van Elsen A, Bollen A. 1998. Construction and sequencing of new derivatives of the pIN-III-ompA secretion vector. *Genet. Anal.* 14:129–131.
- Galakatos NG, Daub E, Botstein D, Walsh CT. 1986. Biosynthetic alanine racemase from *Salmonella typhimurium*: DNA and protein sequence determination. *Biochemistry* 25:3255–3260.
- Galakatos NG, Walsh CT. 1989. Mutations at the interdomain hinge region of the DadB alanine racemase: effects of length and conformational constraint of the linker sequence on catalytic efficiency. *Biochemistry* 28:8167–8174.
- Galán JE, Nakayama K, Curtiss R III. 1990. Cloning and characterization of the asd gene of *Salmonella typhimurium*: use in stable maintenance of recombinant plasmids in *Salmonella* vaccine strains. *Gene* 94:29–35.
- Galen JE, et al. 2009. *Salmonella enterica* serovar Typhi live vector vaccines finally come of age. *Immunol. Cell Biol.* 87:400–412.
- Galen JE, et al. 2010. A new generation of stable, nonantibiotic, low-copy-number plasmids improves immune responses to foreign antigens in *Salmonella enterica* serovar Typhi live vectors. *Infect. Immun.* 78:337–347.
- Gunn BM, Wanda SY, Burshell D, Wang C, Curtiss R III. 2010. Construction of recombinant attenuated *Salmonella enterica* serovar typhimurium vaccine vector strains for safety in newborn and infant mice. *Clin. Vaccine Immunol.* 17:354–362.
- Hitchcock PJ, Brown TM. 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *J. Bacteriol.* 154:269–277.
- Hopkins S, et al. 1995. A recombinant *Salmonella typhimurium* vaccine induces local immunity by four different routes of immunization. *Infect. Immun.* 63:3279–3286.
- Kang HY, Dozois CM, Tinge SA, Lee TH, Curtiss R III. 2002. Transduction-mediated transfer of unmarked deletion and point mutations through use of counterselectable suicide vectors. *J. Bacteriol.* 184:307–312.
- Kang HY, Srinivasan J, Curtiss R III. 2002. Immune responses to recombinant pneumococcal PspA antigen delivered by live attenuated *Salmonella enterica* serovar Typhimurium vaccine. *Infect. Immun.* 70:1739–1749.
- Li Y, et al. 2009. Evaluation of new generation *Salmonella enterica* serovar Typhimurium vaccines with regulated delayed attenuation to induce immune responses against PspA. *Proc. Natl. Acad. Sci. U. S. A.* 106:593–598.
- Li Y, et al. 2008. A sopB deletion mutation enhances the immunogenicity and protective efficacy of a heterologous antigen delivered by live attenuated *Salmonella enterica* vaccines. *Infect. Immun.* 76:5238–5246.
- Mahan MJ, Schlauch JM, Mekalanos JJ. 1993. Selection of bacterial virulence genes that are specifically induced in host tissues. *Science* 259:686–688.
- Meli R, et al. 2003. Th1-Th2 response in hyperprolactinemic mice infected with *Salmonella enterica* serovar Typhimurium. *Eur. Cytokine Netw.* 14:186–191.
- Moffitt KL, et al. 2011. T(H)17-based vaccine design for prevention of *Streptococcus pneumoniae* colonization. *Cell Host Microbe* 9:158–165.
- Moffitt KL, Yadav P, Weinberger DM, Anderson PW, Malley R. 2012. Broad antibody and T cell reactivity induced by a pneumococcal whole-cell vaccine. *Vaccine* 30:4316–4322.
- Nakayama K, Kelly SM, Curtiss R III. 1988. Construction of an Asd⁺ expression-cloning vector: stable maintenance and high level expression of cloned genes in a *Salmonella* vaccine strain. *Bio/Technology* 6:693–697.
- Niemann GS, et al. 2011. Discovery of novel secreted virulence factors from *Salmonella enterica* serovar Typhimurium by proteomic analysis of culture supernatants. *Infect. Immun.* 79:33–43.
- O'Garra A, Arai N. 2000. The molecular basis of T helper 1 and T helper 2 cell differentiation. *Trends Cell Biol.* 10:542–550.
- Roche H, Ren B, McDaniel LS, Hakansson A, Briles DE. 2003. Relative roles of genetic background and variation in PspA in the ability of antibodies to PspA to protect against capsular type 3 and 4 strains of *Streptococcus pneumoniae*. *Infect. Immun.* 71:4498–4505.
- Ross P, O'Garra F, Condon S. 1990. Thymidylate synthase gene from *Lactococcus lactis* as a genetic marker: an alternative to antibiotic resistance genes. *Appl. Environ. Microbiol.* 56:2164–2169.
- Santander J, Xin W, Yang Z, Curtiss R. 2010. The aspartate-semialdehyde

- dehydrogenase of *Edwardsiella ictaluri* and its use as balanced-lethal system in fish vaccinology. *PLoS One* 5:e15944. doi:10.1371/journal.pone.0015944.
33. Sørensen KI, Larsen R, Kibenich A, Junge MP, Johansen E. 2000. A food-grade cloning system for industrial strains of *Lactococcus lactis*. *Appl. Environ. Microbiol.* 66:1253–1258.
 34. Spellberg B, Edwards JE, Jr. 2001. Type 1/type 2 immunity in infectious diseases. *Clin. Infect. Dis.* 32:76–102.
 35. Srinivasan J, Nayak A, Curtiss R III, Rubino S. 1995. Effect of the route of immunization using recombinant *Salmonella* on mucosal and humoral immune responses, p 273–280. *In* Brown F, Chanock R, Ginsberg H, Norrby E (ed), *Vaccines*. Cold Spring Harbor Laboratory Press, Plainview, NY.
 36. Tauch A, Gotker S, Puhler A, Kalinowski J, Thierbach G. 2002. The alanine racemase gene *alr* is an alternative to antibiotic resistance genes in cloning systems for industrial *Corynebacterium glutamicum* strains. *J. Biotechnol.* 99:79–91.
 37. Wang S, et al. 2010. *Salmonella* vaccine vectors displaying delayed antigen synthesis in vivo to enhance immunogenicity. *Infect. Immun.* 78:3969–3980.
 38. Wang S, et al. 2010. Immune responses to recombinant pneumococcal PsaA antigen delivered by a live attenuated *Salmonella* vaccine. *Infect. Immun.* 78:3258–3271.
 39. Wasserman SA, Walsh CT, Botstein D. 1983. Two alanine racemase genes in *Salmonella typhimurium* that differ in structure and function. *J. Bacteriol.* 153:1439–1450.
 40. Wu HY, Nahm MH, Guo Y, Russell MW, Briles DE. 1997. Intranasal immunization of mice with PspA (pneumococcal surface protein A) can prevent intranasal carriage, pulmonary infection, and sepsis with *Streptococcus pneumoniae*. *J. Infect. Dis.* 175:839–846.
 41. Xin W, Li Y, Mo H, Roland KL, Curtiss R III. 2009. PspA family fusion proteins delivered by attenuated *Salmonella enterica* serovar Typhimurium extend and enhance protection against *Streptococcus pneumoniae*. *Infect. Immun.* 77:4518–4528.
 42. Xin W, et al. 2008. Analysis of type II secretion of recombinant pneumococcal PspA and PspC in a *Salmonella enterica* serovar Typhimurium vaccine with regulated delayed antigen synthesis. *Infect. Immun.* 76:3241–3254.
 43. Yang X, et al. 2007. Oral vaccination with *salmonella* simultaneously expressing *Yersinia pestis* F1 and V antigens protects against bubonic and pneumonic plague. *J. Immunol.* 178:1059–1067.
 44. Zhang X, Wanda SY, Brennenman K, Kong W, Roland K, Curtiss R III. 2011. Improving *Salmonella* vector with rec mutation to stabilize the DNA cargoes. *BMC Microbiol.* 11:31. doi:10.1186/1471-2180-11-31.