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Comparison of a Regulated Delayed Antigen Synthesis System with *In Vivo*-Inducible Promoters for Antigen Delivery by Live Attenuated *Salmonella* Vaccines[▽]

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Induction of strong immune responses against a vectored antigen in hosts immunized with live attenuated *Salmonella* vaccines is related in part to the amount of antigen delivered and the overall fitness of the *Salmonella* vector in relation to its ability to stimulate the host immune system. Constitutive high-level antigen synthesis causes a metabolic burden to the vaccine vector strain that can reduce the vaccine strain's ability to interact with host lymphoid tissues, resulting in a compromised immune response. A solution to this problem is the use of systems that regulate antigen gene expression, permitting high levels of antigen synthesis only after the vaccine strain has reached its target tissues. *In vivo*-inducible promoters (IVIPs) are often used to accomplish this. We recently developed an alternative strategy, a regulated delayed antigen synthesis (RDAS) system, in which the LacI-repressible P_{trc} promoter controls antigen gene expression by adding arabinose. In this paper, we compared the RDAS system with two commonly used IVIPs, P_{ssaG} and P_{pagC}. Three nearly identical plasmids, differing only in the promoter used to direct transcription of the pneumococcal *pspA* gene, P_{trc}, P_{ssaG}, or P_{pagC}, were constructed and introduced into isogenic *Salmonella* vaccine strains with or without arabinose-inducible LacI synthesis. Mice immunized with the RDAS strain developed slightly higher titers of mucosal and serum anti-PspA antibodies than P_{pagC}-immunized mice, while titers in mice immunized with the P_{ssaG} strain were 100-fold lower. Both the RDAS and P_{pagC} strains conferred similar levels of protection against *Streptococcus pneumoniae* challenge, significantly greater than those for the P_{ssaG} strain or controls. Thus, RDAS provides another choice for inclusion in the live vaccine design to increase immunogenicity.

Attenuated live bacteria have been widely used as vaccine and vaccine vector systems to deliver antigens or plasmids encoding antigen genes for prophylaxis and therapy purposes (56, 65, 93). One of the most important factors that affect the immune response is the level of antigen synthesis (11). To achieve high levels of antigen synthesis, strong promoters driving antigen gene expression from multicopy plasmids have been used. One problem with this strategy is that high levels of antigen synthesis can result in a metabolic burden to the vaccine strain, leading to a number of unwanted effects, including hyperattenuation, loss of viability, loss of plasmid, modified or poorly expressed antigen genes, and reduction in colonizing ability, ultimately resulting in poor immunogenicity (31). To circumvent this problem, a number of different strategies have been proposed, such as reducing the level of protein synthesis by expressing the antigen gene from the vaccine strain chromosome (41, 82) or using a low-copy-number plasmid (34), the use of secretion signals to export the antigen out of the cell (34, 86), and the use of runaway vectors (68, 85). Induction of gene expression from an arabinose-inducible promoter by injecting immunized animals with arabinose has also been explored (56). One of the most popular solutions is using *in vivo*-inducible promoters (IVIPs) to drive antigen gene expression (13). In *Salmonella*, the IVIPs chosen as candidates for vaccine development drive expression genes that are poorly expressed in common laboratory media but exhibit elevated expression levels in cultured macrophages or in host tissues (36).

P_{nirB} was the first *in vivo*-inducible promoter introduced for use in live bacterial vaccines and has been tested in mice and in a phase one clinical trial (13, 58, 83). Subsequently, many IVIPs have been evaluated in live bacterial vaccines, driving antigen gene expression from multicopy plasmids or from the bacterial chromosome (1, 2, 9, 11, 29, 39, 44, 69, 70, 77, 84, 96). Two commonly used IVIPs are P_{pagC} and P_{ssaG}. PagC is an outer membrane protein important for survival in macrophages (64), and SsaG is a component of secretion apparatus in the *Salmonella* pathogenicity island 2 (SPI-2) type III secretion system (12). Synthesis of both proteins is upregulated in macrophages (23, 25, 62, 73, 87). P_{pagC} has been shown to function in different species (88). While P_{nirB} has been used to express a number of different antigen genes in live attenuated *Salmonella* vaccines and other species (15, 28, 43, 57, 63, 90, 94), P_{pagC} has emerged as a favorable choice in several studies, including studies that directly compare the two promoters (9, 10, 14, 22, 39, 79). In studies comparing a number of promoters, P_{pagC} was found to have the greatest activity in murine tissues (11, 22). This attribute, combined with its low *in vitro* activity, has made it an attractive choice for driving antigen expression. The *ssaG* gene is also highly induced during macrophage infection (23, 87) and under *in vitro* conditions when

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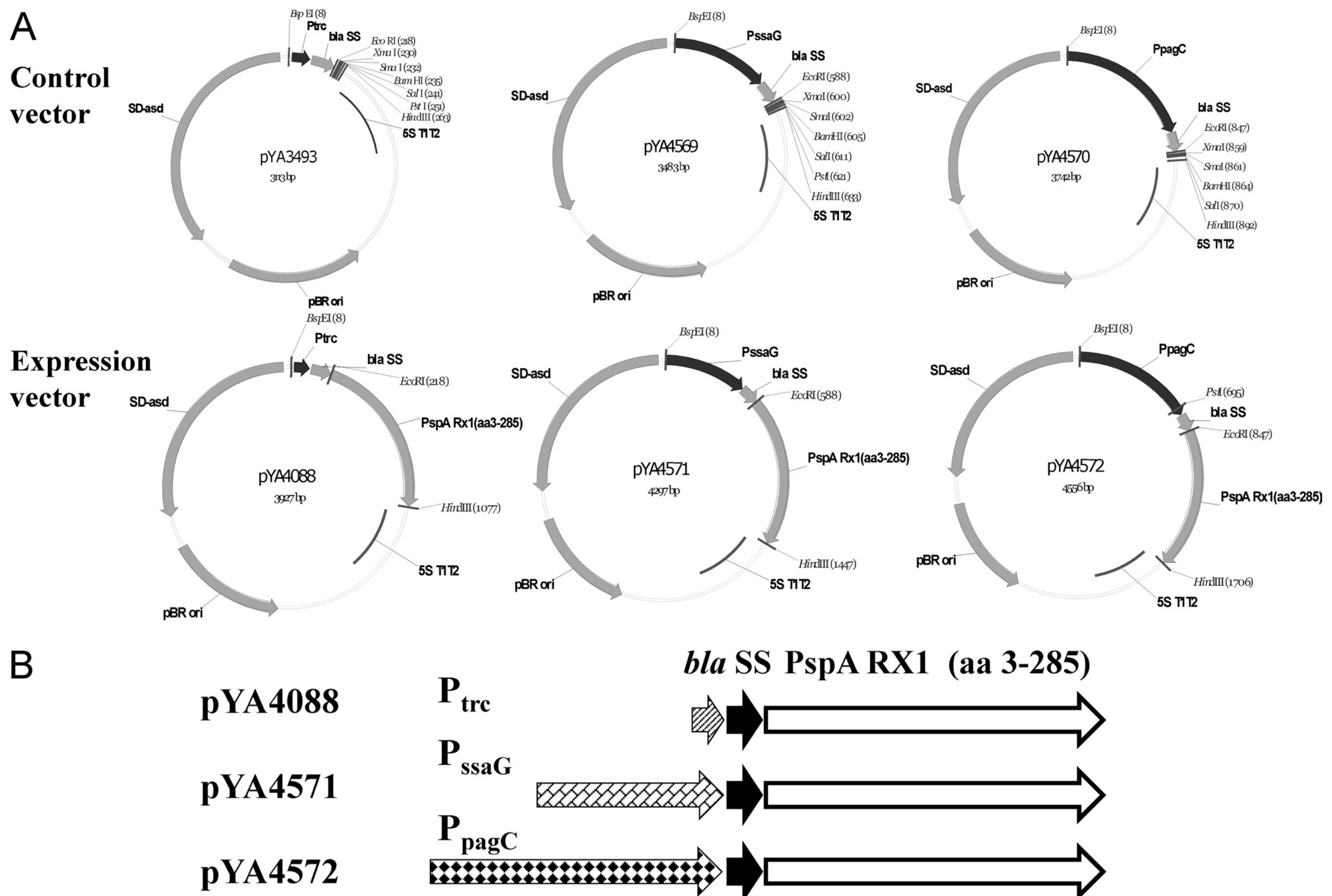

FIG. 1. Recombinant plasmids for *pspA* expression. (A) Maps of recombinant plasmids pYA3493 (P_{trc}), pYA4569 (P_{ssaG}), pYA4570 (P_{pagC}), pYA4088 (P_{trc} *pspA*), pYA4571 (P_{ssaG} *pspA*), and pYA4572 (P_{pagC} *pspA*). (B) Schematic diagram of the promoter, secretion signal, and antigens. Black arrows, β-lactamase secretion signal; white arrows, *pspA* (amino acids 3 to 285); other arrows, indicated promoters.

Salmonella encounters an acidic pH combined with low levels of phosphate and magnesium (21, 76). The P_{ssaG} promoter has been used successfully to drive antigen gene transcription from the bacterial chromosome in both *Salmonella enterica* serovar Typhimurium and *S. enterica* serovar Typhi, generating anti-antigen antibody responses in immunized mice (62, 82). An *S.* Typhi strain, in which P_{ssaG} drives transcription of *eltB*, encoding the *Escherichia coli* heat-labile toxin subunit B, has been evaluated in a phase 1 clinical trial (48). Thus, these two promoters were chosen for comparison with our regulated delayed antigen synthesis (RDAS) system (Fig. 1).

In our laboratory, we typically use the LacI-repressible P_{trc} promoter directing transcription of antigen genes (46, 66, 78). Transcription from P_{trc} is constitutive due to the absence of LacI in *Salmonella*. The P_{trc} promoter is more transcriptionally active both anaerobically and aerobically than the *nirB* promoter (13, 22). In one study comparing P_{trc}, P_{pagC}, and P_{nirB} driving *tetC* expression in an attenuated *Salmonella* strain, all mice immunized with the P_{pagC} strain developed high anti-TetC serum IgG titers (22). Four of five mice immunized with the P_{trc} strain developed high anti-TetC IgG titers, although the titers in these mice were lower than those of the mice immunized with the P_{pagC} strain. Mice immunized with the P_{nirB} strain did not develop detectable anti-TetC IgG serum antibody. The mice in all three groups developed similar anti-lipopolysaccharide (LPS) IgG titers, indicating that each of the vaccine strains were capable of stimulating the host immune system. The reason for the differences observed between P_{trc} and P_{pagC} in this study may be a reflection of the inability of strains with unregulated antigen gene expression from the P_{trc} promoter to reliably induce a robust immune response in immunized animals.

In an effort to reduce the metabolic burden imposed by constitutive antigen gene expression and to permit the initiation of antigen synthesis when the bacterium reaches an immunocompetent site in the host, we developed an RDAS system (91). In this system, LacI represses transcription from P_{trc} during *in vitro* cultivation, with gradual derepression as a consequence of cell divisions occurring during colonization of internal lymphoid tissues following immunization. The arabinose-regulated promoter P_{BAD} drives *lacI* transcription such that when a vaccine strain is grown *in vitro* in the presence of arabinose, the LacI synthesized binds to P_{trc}, resulting in a reduction in antigen gene expression. Once the *Salmonella* vaccine invades host tissues, where free arabinose is not available, *lacI* expression ceases and antigen gene expression increases (91). In our initial analysis using a plasmid carrying the pneumococcal gene *pspA* under transcriptional control of P_{trc},

TABLE 1. Strains and plasmids used in this research

Strain or plasmid	Relevant characteristic(s)/genotype	Source or reference
Strains		
S. enterica serovar Typhimurium UK-1		
χ3761	Wild-type UK-1	19
χ8276	Δ*asdA16*	46
χ8916	Δ*asdA16* Δ*phoP233*	50
χ9555	Δ*pabA1516* Δ*pabB232* Δ*asdA16* Δ*araBAD23* Δ*relA1123*	91
χ9241	Δ*pabA1516* Δ*pabB232* Δ*asdA16* Δ*araBAD23* Δ*relA198*::*araC* P_{BAD} *lacI* TT	92
S. pneumoniae WU2	Wild-type virulent, encapsulated type 3	8
Plasmids		
pYA3342	Asd^+ vector, P_{trc}, pBR *ori*	46
pYA3493	Asd^+ vector with β-lactamase N-terminal signal sequence, P_{trc}, pBR *ori*	46
pYA4088	Asd^+ vector with β-lactamase N-terminal secretion signal specifying PspA Rx1 (amino acids 3-285) in pYA3493, P_{trc}, pBR *ori*	91
pYA4569	Asd^+ vector with β-lactamase N-terminal secretion signal, P_{ssaG}, pBR *ori*	pYA3342
pYA4570	Asd^+ vector with β-lactamase N-terminal secretion signal, P_{pagC}, pBR *ori*	pYA3342
pYA4571	Asd^+ vector with β-lactamase N-terminal secretion signal specifying PspA Rx1 (amino acids 3-285) in pYA4569, P_{ssaG}, pBR *ori*	pYA4569
pYA4572	Asd^+ vector with β-lactamase N-terminal secretion signal specifying PspA Rx1 (amino acids 3-285) in pYA4570, P_{pagC}, pBR *ori*	pYA4570

mice immunized with RDAS strains developed higher anti-PspA IgG and mucosal IgA titers and provided significantly greater protection against pneumococcal challenge than mice immunized with an isogenic strain without the RDAS system (no *lacI*) (91).

In this study, we compared strains with the RDAS system expressing *pspA* from P_{trc} to strains with and without the RDAS system expressing *pspA* from the IVIPs P_{pagC} and P_{ssaG}. Our results show that RDAS strains colonized spleen tissues and induced anti-PspA antibody responses as well or better than strains expressing *pspA* from P_{pagC} or P_{ssaG}. Immunization with either the RDAS or P_{pagC} strain elicited similar levels of protective immunity that were significantly greater than that of the P_{ssaG} strain or controls.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Salmonella* Typhimurium vaccine strains were derived from the highly virulent parent strain UK-1 (19). The attenuated strains all carry Δ*pabA* Δ*pabB* mutations. *pabA* and *pabB* are two unlinked genes that encode 4-amino-4-deoxy-chorismate synthase, required for the production of folic acid in *Salmonella*. Mutations in either of these genes are attenuating, due to the fact that *Salmonella* cannot assimilate folic acid from the environment (81). Growth of strains with *araC* P_{BAD}-regulated genes in the presence of arabinose results in acid production that can cause cessation of growth. We have therefore included the Δ*araBAD23* mutation, which prevents use of arabinose. The Δ*asdA16* mutation was introduced to support the use of the *asd* balanced-lethal plasmid maintenance system (66). *S.* Typhimurium cultures were grown at 37°C in LB broth (5) or on LB agar with or without 0.05% arabinose. The MgM medium was used as an induction medium (6, 16). Bacterial growth curves were obtained using optical density measurements with a Genesys 10 spectrophotometer (Thermo Scientific) and by plating serial dilutions of bacterial culture on LB agar. Diaminopimelic acid (DAP) was added (50 μg/ml) for the growth of Δ*asd* mutant strains (66). *Streptococcus pneumoniae* WU2 was cultured on brain heart infusion agar containing 5% sheep blood or in Todd-Hewitt broth plus 0.5% yeast extract (8).

Construction of plasmids and strains. To construct plasmid pYA4569, the *ssaG* promoter was amplified by primer pairs PssaG-*Bsp*EI-S (5′-ATATTCCG GATATTGCCATCGCGGATGTC-3′) and PssaG-*Nco*IEcoRI-a (5′-GGAATT CCCATGGTGCTTTTCCTTAAAATAAATACATC-3′) using *S.* Typhimurium strain χ3761 genomic DNA as a template. The *bla* N-terminal sequence was amplified by primer pairs BlaN-S (5′-AGTATTCAACATTTCCGTGTCGC-3′) and BlaN-(C)-EcoRI-a (5′-GCGCGCGAATTCTTCAGCATCTTTTACTTTC-3′) by using plasmid pYA3493 as a template. The two resulting PCR fragments were cloned into plasmid pYA3342 cut with BspEI/EcoRI to generate plasmid pYA4569. The *pspA* gene encoding amino acids 3 to 285 was cloned from plasmid pYA4088 using EcoRI/HindIII and ligated into pYA4569 cut with the same enzymes to yield plasmid pYA4571.

The plasmid pYA4570 was constructed essentially as described above, except that the P_{pagC} promoter was substituted for the P_{ssaG} promoter. The P_{pagC} promoter was amplified by primer pairs PpagC-*Bsp*EI-S (5′-AGTGTCCGGAG TTAACCACTCTTAATAATAATG-3′) and PpagC-*Nco*IEcoRI-a (5′-GGAAT TCCCATGGCAACTCCTTAATACTAC-3′) using χ3761 genomic DNA as a template. The *bla* N-terminal sequence was amplified as described above, and the resulting two PCR fragments were cloned into pYA3342 cut with BspEI/EcoRI to generate plasmid pYA4570. The *pspA* gene was inserted as described above to generate plasmid pYA4572. All inserts were verified by DNA sequencing. Plasmids pYA4569, pYA4570, pYA4571, and pYA4572 were then transferred into *S.* Typhimurium strains χ9555 and χ9241.

SDS-PAGE and immunoblot analyses. Vaccine strains from static cultures grown overnight at 37°C in LB broth without (for IVIPs) or with (for RDAS) 0.05% arabinose were diluted 1:100 into the same medium. The culture was grown with aeration at 37°C to an optical density at 600 nm (OD_{600}) of 0.85 to 0.9. Equal numbers of cells were collected as preinduction samples. The cultures were washed three times with MgM medium, and growth was continued in MgM medium for 4 h to induce the IVIPs (6, 16). Equal numbers of cells were collected from each culture. All samples were subjected to SDS-PAGE and Western blot analysis as previously described (91).

Immunization of mice. All animal procedures were approved by the Arizona State University Animal Care and Use Committees. Female BALB/c mice, 6 to 8 weeks old, were obtained from Charles River Laboratories (Wilmington, MA). Mice were acclimated for 7 days after arrival before starting the experiments.

Static overnight cultures of vaccine strains were diluted 1:100 into LB broth at 37°C. LB was supplemented with 0.05% arabinose for growth of RDAS strains. Each culture was grown with aeration at 37°C to an OD_{600} of 0.85 to 0.9. Procedures for cell collection, immunization, and blood and vaginal-wash sample collection and storage have been described (91). Groups of mice were orally inoculated with approximately 1×10^9 CFU of vaccine strains on day 0 and again on day 42.

To evaluate colonization, five animals per group were euthanized on days 3 and 7 after inoculation. Spleen, liver, Peyer's patches (PPs), and mesenteric lymph nodes (MLNs) were collected and weighed, and BSG buffer (33) was added to a final volume of 1 ml. Samples were homogenized and plated onto MacConkey agar and LB agar to determine the number of viable bacteria. The

detection limit was 2 CFU. For representation in graphic and statistical analysis, $\log_{10}$ was applied to the values, and recovery of 0 CFU was reported as 1 CFU/g.

Antigen preparation and ELISA. Recombinant PspA (rPspA) protein and *Salmonella* serovar Typhimurium outer membrane proteins (SOMPs) were purified as described previously (46). *S.* Typhimurium LPS was obtained from Sigma. The rPspA clone was a kind gift from Susan Hollingshead at the University of Alabama at Birmingham. An enzyme-linked immunosorbent assay (ELISA) was used to assay antibodies to *S.* Typhimurium LPS and rPspA in serum and to rPspA in vaginal washes as described previously (91).

IL-4 and IFN-γ ELISPOT assays. On days 7, 14, and 49, spleen and MLN cells from three mice were harvested from each group. Enzyme-linked immunospot (ELISPOT) assays were performed as previously described (44). Briefly, polyvinylidene difluoride membrane plates (Millipore, Bedford, MA) were coated with 100 μl of anti-interleukin-4 (IL-4) or anti-gamma interferon (IFN-γ) monoclonal antibodies (MAb) (BD Pharmingen, San Diego, CA) at 5 μg/ml in phosphate-buffered saline (PBS) overnight at 4°C. The wells were washed with PBS and blocked with RPMI medium with 10% fetal calf serum (FCS). Next, 100 μl of RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 IU/ml streptomycin, and 1% HEPES, with or without rPspA at 5 μg/ml, and approximately 1,000,000 spleen cells in 100 μl of cell medium were added per well in duplicate and incubated overnight in 5% CO_2 at 37°C. The next day, the cell suspensions were discarded and the plates washed with PBS. Biotinylated anti-IL-4 or anti-IFN-γ MAb (BD Pharmingen) at 0.5 μg/ml in PBS with 1% FCS was added and incubated at room temperature for 2 h. After the plates were washed with PBS, 100 μl/well of avidin peroxidase diluted 1:1,000 (vol/vol) in PBS-Tween 20 containing 1% FCS was added and was followed by incubation for 1 h at room temperature. 3-Amino-9-ethylcarbazole substrate (Vector Laboratories, Burlingame, CA) was prepared according to the manufacturer's specifications, and 100 μl of substrate was added per well. Spots were developed for 15 min at room temperature. Plates were dried and analyzed by using an automated CTL ELISPOT reader system (Cellular Technology Ltd., Cleveland, OH).

Pneumococcal challenge. At week 10, mice were challenged by intraperitoneal injection with 2×10^4 CFU of *S. pneumoniae* WU2 in 100 μl of BSG buffer (33), equivalent to 100 times the 50% lethal dose (LD_{50}). Challenged mice were monitored daily for 30 days.

Statistical analysis. Statistical analyses were performed by using the GraphPad Prism 5 software package (Graph Software, San Diego, CA). Antibody titers were expressed as means ± standard deviations. The methods for comparison are shown in the figure legends. Differences were considered significant at P values of <0.05. The survival data were analyzed using the Kaplan-Meier method, and survival comparisons were made using the Mantel-Cox test method.

RESULTS

Systems for comparison of IVIPs and RDAS. Different combinations of strains and plasmids were set up to compare the IVIP and RDAS systems. For P_{pagC} and P_{ssaG} plasmids, we cloned long promoter sequences to ensure inclusion of all the elements required for regulation (67, 74, 89). Two empty vector plasmids, pYA4569 (P_{ssaG}) and pYA4570 (P_{pagC}), were generated to include the same β-lactamase N-terminal secretion signal as that present in P_{trc} plasmid pYA3493 (46) (Fig. 1A). The *pspA* gene encoding amino acids 3 to 285 was cloned into pYA4569 and pYA4570 to generate IVIP expression plasmids pYA4571 (P_{ssaG} *pspA*) and pYA4572 (P_{pagC} *pspA*) (Fig. 1). Plasmid pYA4088, a derivative of plasmid pYA3493, carries the same *pspA* fragment under transcriptional control of the P_{trc} promoter (91) (Fig. 1A). Plasmid pYA4571 carries a 467-bp fragment encoding P_{ssaG}, plasmid pYA4572 carries a 726-bp fragment encoding P_{pagC} (67), and plasmid pYA4088 carries an 82-bp fragment encoding P_{trc} (Fig. 1B). The only differences among plasmids pYA4571, pYA4572, and pYA4088 are the promoters driving transcription of *pspA* (Fig. 1B). Strain χ9555, which does not express *lacI*, was used as the vector for IVIP plasmids pYA4571 and pYA4572. RDAS strain χ9241, isogenic to χ9555 except for the presence of the Δ*relA198*::*araC* P_{BAD} *lacI* TT cassette that directs arabinose-regulated LacI synthesis, was used as the bacterial host for plasmid pYA4088 (91). Thus, we have the direct comparison of RDAS vaccine strain χ9241(pYA4088) to IVIP strains χ9555(pYA4071) and χ9555(pYA4072). We also included strains χ9241(pYA4071) and χ9241(pYA4072) in our analysis to determine the effect of LacI on the efficacy of the IVIP strains.

Synthesis of rPspA in *Salmonella*. We evaluated PspA synthesis in RDAS vaccine strain χ9241(pYA4088) and IVIP strains χ9555(pYA4071), χ9555(pYA4072), χ9241(pYA4071), and χ9241(pYA4072), grown in LB broth and in MgM medium, to mimic *in vivo* conditions for induction of P_{pagC} and P_{ssaG}. Although strain χ9241(pYA4088) was grown in LB with arabinose, some PspA synthesis was observed in the culture (Fig. 2A). When the χ9241 (*lacI*) and χ9555 (no *lacI*) strains were grown in LB, there was detectable PspA synthesis in the P_{pagC} constructs (pYA4572) but not in the P_{ssaG} constructs (pYA4571), consistent with previous reports (62) (Fig. 2A). However, after induction in MgM medium, which lacks arabinose, strain χ9241(pYA4088) produced levels of PspA similar to the two IVIP constructs in both χ9241 and χ9555 backgrounds (Fig. 2A). These results demonstrate that all three promoters can drive similar levels of *pspA* expression under MgM-inducing conditions. The presence of *lacI* in the chromosome of χ9241 had no effect on PspA synthesis directed by the IVIPs.

P_{ssaG} and P_{pagC} are affected by *phoP*. To confirm that expression from P_{ssaG} and P_{pagC} in our constructs is regulated by *phoP* (4, 7, 74), we evaluated PspA synthesis in isogenic strains χ8276 and χ8916, differing only at the *phoP* locus (Table 1). Strain χ8916 is a Δ*phoP* mutant. Before induction, there was no detectable PspA synthesis in either strain carrying plasmids pYA4571 (P_{ssaG} *pspA*) or pYA4572 (P_{pagC} *pspA*) (Fig. 2B, lane 1). After induction, high levels of PspA synthesis were detected in *phoP*$^+$ strains χ8276(pYA4571) and χ8276(pYA4572). Low-level PspA synthesis was detected in Δ*phoP233* strain χ8916(pYA4571) (P_{ssaG} *pspA*) but not in strain χ8916(pYA4572) (P_{pagC} *pspA*) (Fig. 2B). Our results indicate that *phoP* regulates expression from both promoters but has a more profound influence on P_{pagC} than it does on P_{ssaG}.

Colonization of mouse tissues after oral immunization with recombinant attenuated *Salmonella* vaccines (RASVs) expressing *pspA*. The abilities of the vaccine strains to colonize mouse PPs, MLNs, spleens, and livers were analyzed on days 3 and 7 after oral inoculation. All strains colonized PPs equally well on day 3 except the strains carrying the P_{ssaG} *pspA* promoter, which colonized less well (Fig. 3A). In particular, we were unable to isolate the strain expressing *lacI*, χ9241(pYA4571) (P_{ssaG} *pspA*), from four of the five mice examined on day 3. On day 7, we were unable to recover χ9241(pYA4571) from any of the five mice examined, although this did not reach statistical significance due to the wide variations in numbers of bacteria recovered from the tissues of mice inoculated with other strains expressing *pspA* from IVIPs. The poor colonization of this strain was also reflected in the MLN (Fig. 3B), spleen (Fig. 3C), and liver (Fig. 3D). By day 7, the numbers of the RDAS strain χ9241(pYA4088) (P_{trc} *pspA*) CFU recovered from MLNs (Fig. 3B) and spleens (Fig. 3C) were significantly greater than those of all other strains ($P < 0.01$), although no differences were observed between strains for liver coloniza-

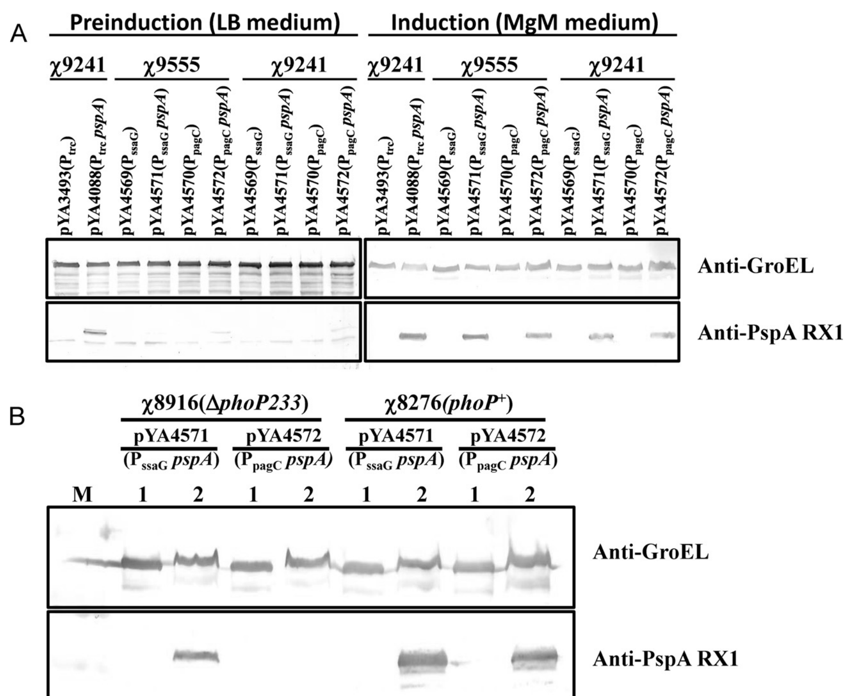

FIG. 2. PspA synthesis in *S.* Typhimurium vaccine strains. Sample preparation and the Western blot procedure are described in Materials and Methods. Nitrocellulose membranes were probed with polyclonal antibodies specific for either PspA or GroEL. GroEL was used as a standardization marker. Relevant portions of each blot are shown. (A) Western blots showing PspA and GroEL synthesis in strains χ9241 and χ9555 harboring the indicated plasmids. (B) PspA synthesis in *S.* Typhimurium vaccine strains with or without *phoP* mutation. The Western blot shows PspA and GroEL synthesis in Δ*phoP* strains χ8916(pYA4571) (P_{ssaG} *pspA*) and χ8916(pYA4572) (P_{pagC} *pspA*) and $PhoP^+$ strains χ8276(pYA4571) (P_{ssaG} *pspA*) and χ8276(pYA4572) (P_{pagC} *pspA*). M, protein marker. Lane 1, cells grown in LB medium (preinduction sample); lane 2, cells grown in MgM medium (induction sample).

tion (Fig. 3D). Of note is the fact that, even in the absence of statistical significance, the two strains expressing *lacI* (χ9241 derivatives) and carrying IVIP-driven *pspA* appeared to be at a disadvantage with regard to tissue colonization based on the fact that the strain was not recovered from a majority of the mice inoculated with those strains. The only exception was for day 7 recovery from the liver for strain χ9241(pYA4572) (P_{pagC} *pspA*) (Fig. 3D).

We also evaluated the *in vivo* stability of the RDAS and IVIP strains using the bacteria recovered from spleens on day 7. Six colonies from each vaccine strain were randomly chosen and grown overnight in MgM medium. Each culture was analyzed by Western blot analysis using antiserum against PspA. In each case, all six isolates synthesized PspA at levels identical to those seen in the starting cultures (data not shown). These results demonstrate that *pspA* expression in both the RDAS and IVIP strains is stable *in vivo*.

Antibody responses in mice immunized with χ9555 and χ9241 harboring *pspA* expression plasmids. We compared the antibody responses induced by three plasmids delivered by strain χ9241 (Δ*relA198*::*araC* P_{BAD} *lacI* TT) and the two IVIP plasmids delivered by strain χ9555 (no *lacI*). The two strains are isogenic, differing only in the presence of an arabinose-regulated *lacI* gene. The *lacI* gene in χ9241 was codon optimized to increase LacI synthesis and provide tighter control over the P_{trc} promoter present in pYA4088 (91). All mice immunized with RASVs expressing *pspA* developed titers of anti-rPspA serum IgG (Fig. 4A). The anti-PspA serum IgG titers in mice immunized with RDAS strain χ9241(pYA4088) were higher than titers in mice immunized with either strain carrying the P_{ssaG} plasmid pYA4571. The difference in titers was greater than 100-fold by week 4 (Fig. 4A). The anti-PspA titers were about 2-fold higher in the serum of RDAS-immunized mice than in the serum of mice immunized with χ9555(pYA4572) (P_{pagC} *pspA*). The presence of *lacI* had a negative impact on antibody responses generated by χ9241(pYA4572) compared to χ9555(pYA4572) at all times and χ9241(pYA4571) compared to χ9555(pYA4571) at 6 and 8 weeks, although the differences were less than 4-fold in most cases. By week 8, identical strains carrying P_{pagC} plasmid pYA4572 elicited anti-PspA serum IgG titers 100-fold greater than those of strains carrying P_{ssaG} plasmid pYA4571, regardless of whether the strains expressed *lacI*. No anti-PspA IgG was detected in mice immunized with strains carrying control plasmid pYA4569, pYA4570, or pYA3493 (data not shown).

The common mucosal system in mice (61) facilitates the production of antigen-specific antibody responses at mucosal sites distant from the site of mucosal immunization, including both the upper respiratory and genital tract (42). Therefore, vaginal washes can be used as a surrogate for nasal or lung secretions. This approach also provided a convenient way to obtain multiple mucosal samples from the same animal and allowed us to keep the animals alive for challenge studies. There were no differences in mucosal anti-PspA IgA responses at 2 weeks, but there were clear

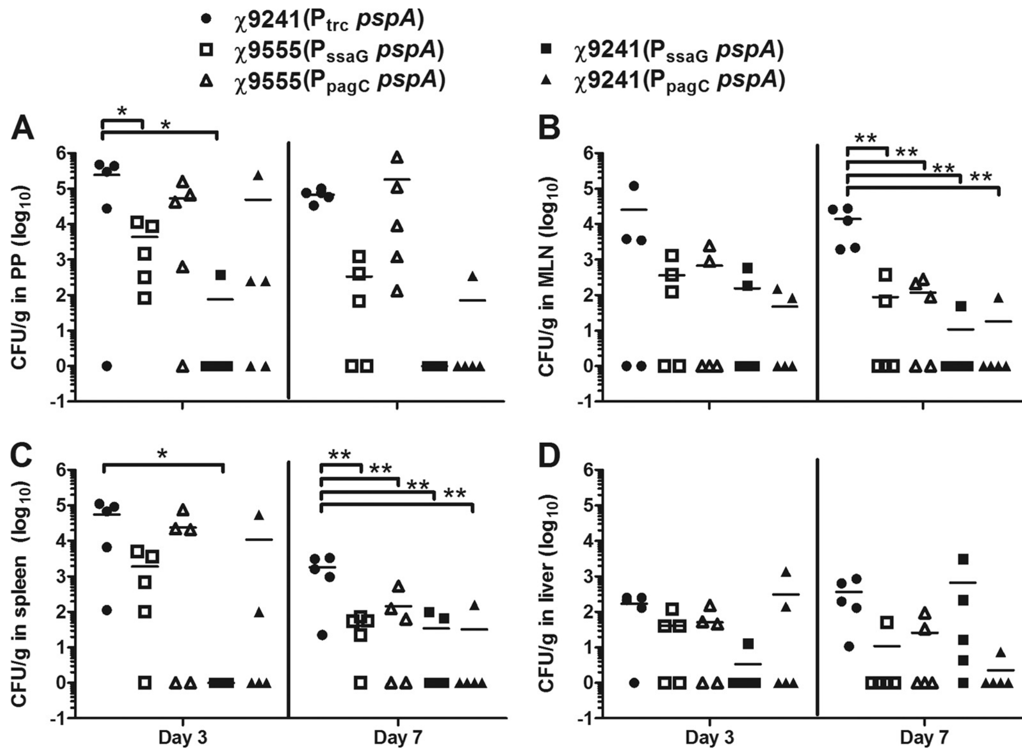

FIG. 3. Colonization of BALB/c mice by attenuated *S.* Typhimurium vaccine strains. Bacterial numbers shown are recovered from PPs (A), MLNs (B), spleens (C), and livers (D) at 3 and 7 days postinoculation. For representation in graphic and statistical analysis, $\log_{10}$ was applied to the values. For graphic and statistical analysis, an entry of 1 CFU/g was listed in cases where no bacteria were recovered from a given tissue. The horizontal lines represent the means of each data set. Significant differences between groups are indicated and were determined using one-way analysis of variance (ANOVA) and Tukey's tests (*, $P < 0.05$; **, $P < 0.01$).

differences by 4 weeks. RDAS strain χ9241(pYA4088) and strain χ9555(pYA4571) (P_{pagC} *pspA*) induced detectable anti-PspA IgA titers, while titers of mucosal samples from mice immunized with the other three strains were below the limits of detection (Fig. 4B). By week 8, mice immunized with either the RDAS strain or χ9555 carrying the P_{pagC} *pspA* plasmid had developed mucosal IgA titers >100-fold or >50-fold greater than the titers in mice immunized with χ9555(pYA4572) (P_{ssaG} *pspA*), respectively. The negative effect of *lacI* on the immunogenicity of the P_{pagC} strains was similar to the effect observed for the serum IgG responses (Fig. 4A and B). The absence of any detectable effect of *lacI* on the IgA titers elicited by the P_{ssaG} strains was probably due to the low magnitude of the overall response.

The anti-LPS serum IgG responses were similar for RDAS strain χ9241(pYA4088), IVIP strains χ9555(pYA4571) (P_{ssaG} *pspA*) and χ9555(pYA4572) (P_{pagC} *pspA*), and their matched control strains carrying empty plasmid vectors (Fig. 4C). In fact, all strains induced similar responses except strain χ9241(pYA4571) (P_{ssaG} *pspA*), which elicited anti-LPS titers 80-fold lower than those of its matched control strain χ9241(pYA4569) (P_{ssaG}) by 8 weeks. The LPS response for χ9241(pYA4569) (P_{ssaG}) at 2 weeks lagged behind the rest, and the titers increased to levels comparable with those of the other strains by 4 weeks. These results indicate that, with the exception of χ9241(pYA4571) (P_{ssaG} *pspA*), all the strains were able to interact with the mouse immune system in similar fashions.

We then evaluated the anti-PspA IgG isotype subclasses IgG1 and IgG2a in these five strains (Fig. 5). Immunization with *lacI* strains χ9241(pYA4088) (P_{trc} *pspA*) or χ9241(pYA4572) (P_{pagC} *pspA*) or the non-LacI-producing strain χ9555(pYA4572) (P_{pagC} *pspA*) induced a Th1-biased response, based on the higher titers of IgG2a than of IgG1 in these mice. Immunization with either strain harboring plasmid pYA4571 (P_{ssaG} *pspA*) resulted in a mixed Th1-Th2 response, except the week 8 samples from mice immunized with strain χ9241(pYA4571), which indicated a Th1-biased response.

IFN-γ and IL-4 responses in immunized mice. The ELISPOT assay is widely used to detect the function and frequency of antigen-specific T cell responses. Using the ELISPOT assay, we evaluated PspA-specific IFN-γ and IL-4 stimulation in splenocytes and MLN-derived cells harvested from immunized mice on days 7, 14, and 49 postimmunization (Fig. 6). IFN-γ production reached its peak by day 14 and was greatly reduced on day 49, 1 week after boosting (Fig. 6A). IL-4 responses peaked by day 14 and were still strong for some strains on day 49 (Fig. 6B). In mice immunized with χ9241(pYA4088) (P_{trc} *pspA*), χ9555(pYA4571) (P_{ssaG} *pspA*), and χ9555(pYA4572) (P_{pagC} *pspA*), IL-4-secreting cells were detected at all time points, and IFN-γ-secreting cells were detected at days 7 and 14. On days 7 and 14, we detected significantly greater numbers of IL-4-secreting cells in the spleens of mice immunized with RDAS strain χ9241(pYA4088) than in those of mice immunized with other strains except χ9555(pYA4571) at day 14 ($P < 0.05$). The RDAS vaccine strain also generated significantly greater num-

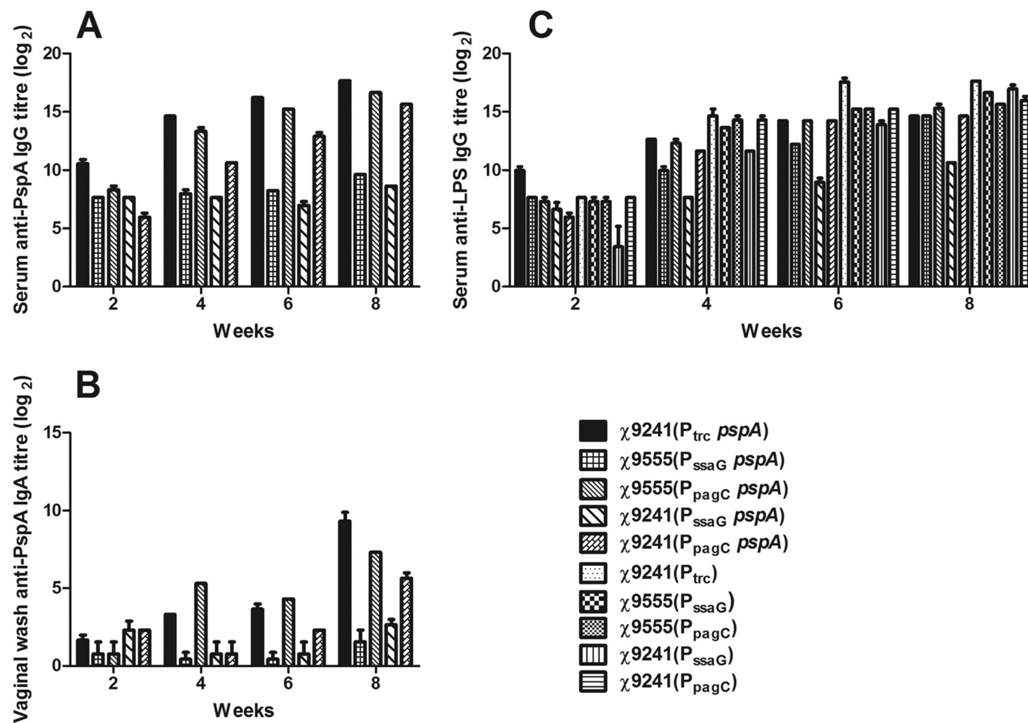

FIG. 4. Reciprocal antibody titers in immunized mice. (A) Serum IgG against rPspA; (B) mucosal IgA against rPspA; (C) serum IgG against *S.* Typhimurium LPS. Mice were orally immunized with approximately 1×10^9 CFU of the indicated strains. Serum and mucosal antibody titers in pooled samples were determined by ELISA. The data represent antibody in pooled sera from mice orally immunized with attenuated *Salmonella* harboring either control vector plasmids or *pspA* expression plasmids. The error bars represent the standard deviations.

bers of IFN-γ-secreting cells than χ9555(pYA4572) (P_{pagC} *pspA*), χ9241(pYA4571) (P_{ssaG} *pspA*), and χ9241(pYA4572) (P_{pagC} *pspA*) on day 14 (Fig. 6A) ($P < 0.01$). At day 49, 1 week after boosting, we detected greater numbers of IFN-γ-secreting cells in mice immunized with strain χ9555(pYA4572) (P_{pagC} *pspA*) than in any of the other strains (Fig. 6A). The numbers of IL-4-secreting cells detected in mice immunized with strains χ9241(pYA4088) (RDAS, P_{trc} *pspA*), χ9555(pYA4571) (P_{ssaG} *pspA*), and χ9555(pYA4572) (P_{pagC} *pspA*) were significantly greater than those of control groups ($P < 0.01$). After the boost, the IL-4 responses were significantly higher in mice immunized with χ9555(pYA4572) (P_{pagC} *pspA*) than in mice immunized with χ9555(pYA4571) (P_{ssaG} *pspA*) ($P < 0.01$) and χ9241(pYA4088) (P_{trc} *pspA*) ($P < 0.01$). We detected background levels of IFN-γ- and IL-4-secreting cells only from MLNs at day 7 and day 14 (data not shown).

Protection of mice immunized with RDAS and IVIP vaccine strains against *S. pneumoniae* challenge. When immunized mice were challenged intraperitoneally with 100 times the LD_{50} of *S. pneumoniae* WU2, all groups that received PspA-producing strains were significantly protected compared with control strains that did not express *pspA* ($P < 0.05$) (Table 2), with the exception of the groups immunized with χ9241(pYA4571) (P_{ssaG} *pspA*). There was no significant difference in protection between mice immunized with χ9241(pYA4088) (P_{trc} *pspA*), χ9555(pYA4571) (P_{ssaG} *pspA*), χ9555(pYA4572) (P_{pagC} *pspA*), or χ9241(pYA4572) (P_{pagC} *pspA*). Although 15% of mice immunized with χ9241(pYA4571) (P_{ssaG} *pspA*) survived challenge, the level of protection was not significant compared to that for controls.

DISCUSSION

Live attenuated *Salmonella* vaccines hold great potential for delivering heterologous antigens or DNAs to a variety of animal hosts to elicit protection against a number of viral, bacterial, and parasite pathogens (18, 20, 33, 52, 80). While there appears to be a need for the vaccine strain to produce high levels of antigen to elicit protection, there is a metabolic flux readjustment and redirection imposed by the diversion of the cell's resources to antigen synthesis (31, 32, 37, 52). This can lead to a reduction in plasmid and/or antigen stability and the ability of the vaccine strain to interact with the host immune system. In this work, we evaluated two strategies for reducing the metabolic burden on the cell, the use of IVIPs and the use of our recently developed RDAS system (91). Our results show that in many ways, the two systems, in particular RDAS and P_{pagC}, are comparable.

Both systems tested were capable of directing similar amounts of the test antigen PspA under *in vitro* induction conditions (Fig. 2). The RDAS system, which relies on the P_{trc} promoter, was induced in MgM medium. The basis for this induction is probably related to the absence of arabinose in MgM medium, as we have previously shown that RDAS strains are induced in LB broth without arabinose (91). Vaccine isolates recovered from mouse tissues could synthesize PspA,

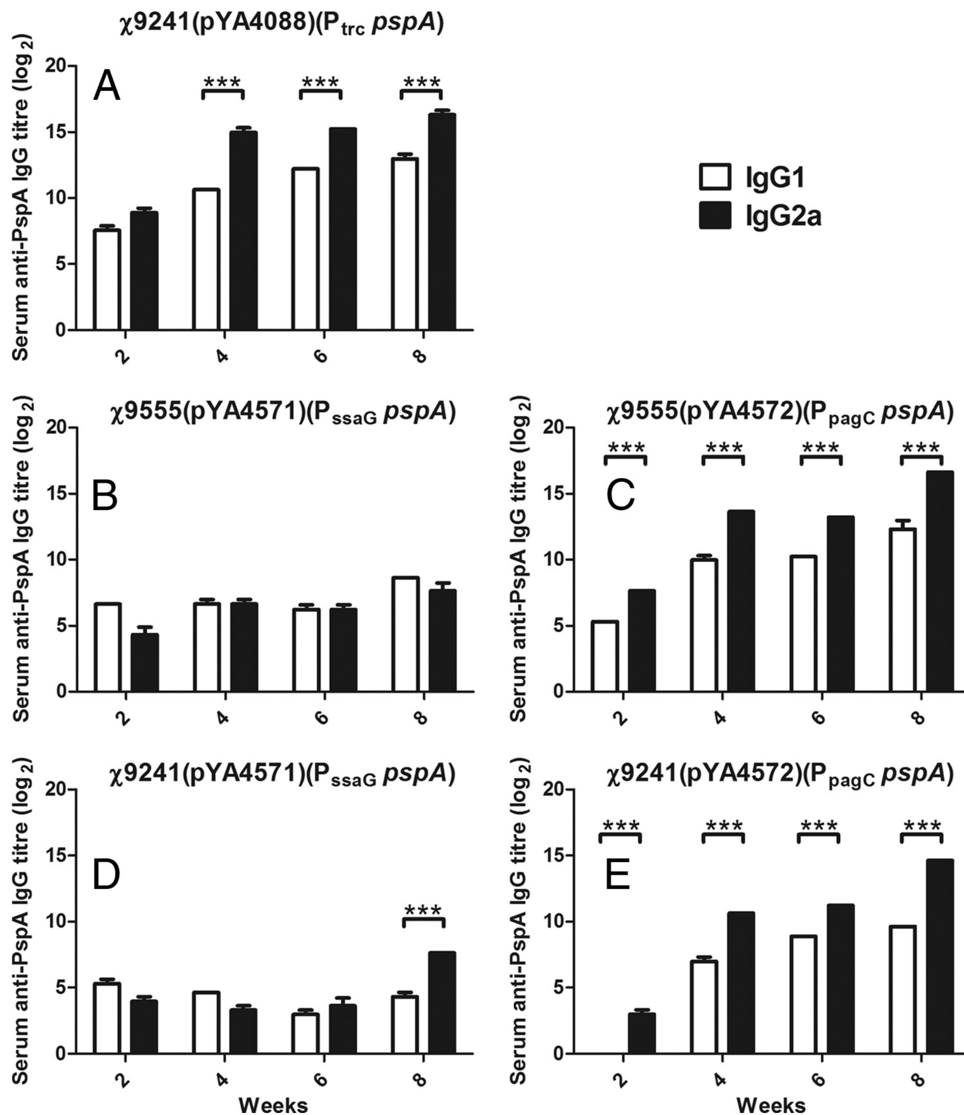

FIG. 5. Serum IgG1 and IgG2a responses to rPspA in mice orally immunized with χ9241(pYA4088) (A), χ9555(pYA4571) (B), χ9555(pYA4572) (C), χ9241(pYA4571) (D), or χ9241(pYA4572) (E). Levels of IgG1 and IgG2a were determined in pooled serum samples by ELISA. The error bars represent the standard deviations. ***, $P < 0.001$.

indicating that PspA production in both RDAS and IVIP strains was stable *in vivo*. Although all strains effectively colonized gut-associated lymphatic tissue (Peyer's patches) and systemic sites (spleens, livers, and MLNs), the RDAS strain χ9241(pYA4088) colonized mouse tissues as well or better than the IVIP strains on days 3 and 7 (Fig. 3). Three strains, χ9241(pYA4088), χ9555(pYA4571), and χ9555(pYA4572), were able to induce serum IgG responses against PspA in immunized mice, although the titers induced by the P_{ssaG} strain were much lower than those of the other two strains (Fig. 4A). The deficiency in the P_{ssaG} strain also extended to the titers of mucosal IgA (Fig. 4B). The RDAS strain χ9241(pYA4088) and the P_{pagC} strain χ9555(pYA4572) induced similar levels of mucosal IgA, a result that correlated well with their comparable abilities to colonize host tissues (Fig. 3).

Examination of the serum IgG isotypes revealed that both the RDAS strain and the P_{pagC} strain elicited a Th1-biased response typical of those induced by *Salmonella* vaccines (Fig. 5) (46, 71, 72, 75, 91). The P_{ssaG} strain elicited a more balanced Th1-Th2 response, although the overall anti-PspA serum IgG titers were very low. Despite the results suggested by analysis of IgG1/IgG2a, the RDAS strain induced a strong IFN-γ response on days 7 and 14 but not on day 49 (Fig. 6A). The P_{ssaG} strain induced strong IFN-γ and IL-4 responses on day 14, consistent with the mixed Th1-Th2 response we observed based on the IgG1/IgG2a ratios (Fig. 5). Conversely, the P_{pagC} strain χ9555(pYA4572) elicited a significant number of IFN-γ-secreting spleen cells at all time points examined, suggesting that the P_{pagC} promoter system may have an advantage for inducing strong cellular immunity compared to either the RDAS system or the P_{ssaG} promoter (Fig. 6B). Interestingly, all three strains elicited PspA-responsive IL-4-secreting cells. Induction of IL-4 by the P_{pagC} strain was low on day 14 but significantly increased after the boost.

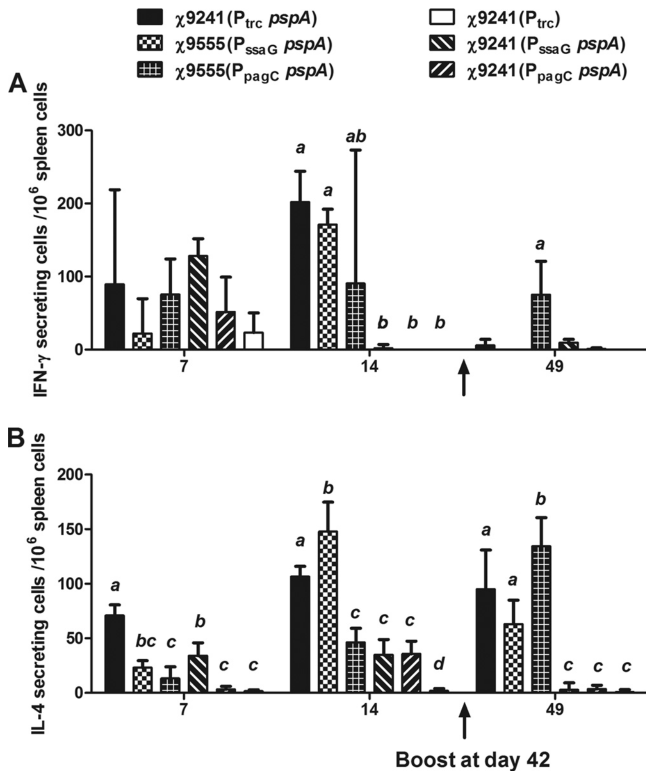

FIG. 6. PspA-specific cytokine stimulation in mice immunized with χ9241(pYA3493) (P_{trc}), χ9241(pYA4088) (P_{trc} *pspA*), χ9555(pYA4571) (P_{ssaG} *pspA*), χ9555(pYA4572) (P_{pagC} *pspA*), χ9241(pYA4571), and χ9241(pYA4572). Numbers of IFN-γ-producing (A) and IL-4-producing (B) cells were determined by ELISPOT assay. Splenectomies were performed on euthanized mice 7, 14, and 49 days after the first immunization. Splenocytes were harvested from three mice per group, and cells from each spleen were assayed in duplicate. The results from each well are expressed as ELISPOTs per million splenocytes minus the background (typically approximately 15 spots) from unpulsed mock controls. Significant differences between groups are indicated and were determined using one-way ANOVA and Tukey's tests. Groups with different letters are statistically different ($P < 0.05$). Groups with no letters or the same letters were not different.

Ultimately, the most important test of a vaccine antigen delivery system is the ability to elicit protective immunity. Surprisingly, despite the differences in immune responses among strains, all three strains, χ9241(pYA4088), χ9555(pYA5471), and χ9555(pYA4572), protected immunized mice equally well from challenge with virulent *S. pneumoniae* (Table 2). Although the antibody responses against *S. pneumoniae* are important for protection, other factors, including T cell responses and production of cytokines such as IL-17, are also important (59). We found that spleen cells harvested from mice 14 days postimmunization with χ9241(pYA4088) produced less IL-17 than those from mice immunized with χ9555(pYA4571) and χ9555(pYA4572) when restimulated with PspA (data not shown). It was also reported that P_{ssaG} can induce high levels of antigen-specific T cell responses (95). Thus, the RDAS and IVIPs may stimulate different aspects of the immune system; the former can induce a stronger antibody response, while the latter provides for better induction of cellular immunity.

Our findings show that each system works in our test scenario. The concept of regulated antigen synthesis to reduce the effect of antigen production on the carrier is especially helpful for toxic antigens. In a previous study, we found that an RDAS-synthesizing LacI strain, but not a strain lacking LacI, could support the growth of a high-copy-number pUC-based plasmid expressing *pspA* from the P_{trc} promoter (91). The RDAS system may have certain advantages over the IVIP system. Expression from IVIPs can be influenced by the genetic background of the host. IVIPs are regulated by native *Salmonella* proteins that are themselves virulence genes that can be targeted for their ability to attenuate virulence. For example, the P_{pagC} and P_{ssaG} promoters are regulated by *phoP* (Fig. 2B) (4, 7, 74). Thus, while *phoP Salmonella* mutants are attenuated and immunogenic (3, 38, 40), these two promoters cannot be used in *phoP* strains. Therefore, selection of an IVIP is limited by the mode of attenuation used for the *Salmonella* delivery strain. The P_{ssaG} promoter is also regulated by the master sensor-kinase system SsrA-SsrB from the *Salmonella*

TABLE 2. Oral immunization with PspA-expressing *Salmonella* strains protects BALB/c mice against intraperitoneal challenge with capsular type 3 *S. pneumoniae* WU2

Vaccine strain[a]	Promoter	Presence of *lacI*/*pspA*[b]	No. of challenged mice	No. of days to death (no. of mice)[c]	% survival[d]
χ9241(pYA4088)	P_{trc}	+/+	22	3 (4), 4 (8), >15 (10)	45.5[e]
χ9555(pYA4571)	P_{ssaG}	−/+	23	2 (2), 3 (8), 4 (3), 5 (1), >15 (9)	39.1[f]
χ9555(pYA4572)	P_{pagC}	−/+	22	3 (7), 4 (7), >15 (8)	36.4[g]
χ9241(pYA4571)	P_{ssaG}	+/+	20	2 (4), 3 (11), 4 (1), 5 (1), >15 (3)	15
χ9241(pYA4572)	P_{pagC}	+/+	20	2 (2), 3 (6), 4 (2), 5 (1), >15 (9)	45[h]
χ9241(pYA3493)	P_{trc}	+/−	8	2 (5), 3 (3)	0
χ9555(pYA4569)	P_{ssaG}	−/−	8	2 (5), 3 (3)	0
χ9555(pYA4570)	P_{pagC}	−/−	8	2 (4), 3 (4)	0
χ9241(pYA4569)	P_{ssaG}	+/−	3	2 (2), 3 (1)	0
χ9241(pYA4570)	P_{pagC}	+/−	3	3 (3)	0

[a] Mice were orally immunized with two doses of the indicated vaccine strains at 0 and 6 weeks.
[b] +, presence; −, absence.
[c] Ten weeks after the primary oral immunization, mice were challenged with approximately 2×10^4 CFU of *S. pneumoniae* WU2. The LD_{50} of WU2 in nonimmunized BALB/c mice is 2×10^2 CFU (53).
[d] The survival data were analyzed using the Kaplan-Meier method, and survival comparisons were done by the Mantel-Cox test method.
[e] Compared to χ9241(pYA4571)-immunized mice, $P = 0.002$. Compared to χ9241(pYA3493)-immunized mice, $P < 0.001$.
[f] Compared to χ9241(pYA4571)-immunized mice, $P = 0.046$. Compared to χ9555(pYA4569)-immunized mice, $P < 0.001$.
[g] Compared to χ9555(pYA4570)-immunized mice, $P < 0.001$.
[h] Compared to χ9241(pYA4571)-immunized mice, $P = 0.03$.

pathogenicity island 2 (SPI-2) (51, 87). Expression of SPI-2 type III secretion system apparatus genes, including *ssaG*, is also controlled by a regulatory hierarchy that includes the two-component systems OmpR-EnvZ and PhoP-PhoQ, the MarR homologue SlyA, and nucleoid-associated proteins Fis, H-NS, and YdgT in response to several environmental signals, such as low osmolarity, acidic pH, and cation concentration (7, 17, 26, 35, 47, 49, 51, 55, 60). The regulatory proteins Fis, SsrB, and H-NS bind directly to P_{ssaG} (27, 47, 54, 89). Other IVIPs evaluated for antigen delivery may also be controlled by different environment factors and/or global regulators, a factor which needs to be considered when selecting an IVIP. Even a group of promoters regulated by the same regulator may show differences in strength of expression, efficiency, and genetic background (95). Another disadvantage of IVIPs may be that if they are used to direct antigen expression from multicopy plasmids, as we have done here, the presence of multiple copies of the promoter may interfere with its regulation or the stability of the plasmid due to titration of effector proteins. The strain harboring the P_{pagC} plasmid was not as stable as P_{trc} *in vivo* in MLNs and PPs (Fig. 3) (22). Inserting the IVIP into the bacterial chromosome, as has been done with P_{ssaG} driving *eltB* (41, 82), may be a better choice to ensure proper regulation. Previous work that directly compares different IVIPs showed that the most effective promoter to achieve the desired immune response varied for different antigens (9, 14, 22, 29, 63, 77); in some cases, a constitutive promoter may be optimal (30, 45, 83). In addition, for IVIPs, measures of *in vitro* expression may have limited correlation with their performance *in vivo* (95). Thus, selection of the IVIPs may require an empirical analysis for each antigen, strain, and bacterial species. Modular design of expression cassettes and systematic comparisons are needed to select optimal IVIPs (95). Currently, the RDAS system relies on the P_{trc} promoter and the *lacI* gene, neither of which are naturally occurring in most *Salmonella* species. Therefore, theoretically, this system can be used with any attenuation strategy and should provide a greater degree of flexibility with respect to various antigens and attenuation strategies. In our case, the regulatable repression element is arabinose-regulated *lacI*, and the regulatable promoter is LacI-regulated P_{trc}. Other repressor/promoter pairs, such as C2 from phage P22, which can repress promoter P_L or P_R from phage P22, can also be applied in this system and are currently being evaluated in our laboratory. In other cases, the phage λ *c*I repressor and λ promoters P_L or P_R can be used. Adopting these combinations will provide more choices for optimizing RDAS in different bacterial species.

One potential disadvantage of the RDAS system is its reliance on *lacI*. It was recently shown that LacI is an antivirulence factor and that overexpression of *lacI* in *S*. Typhimurium can impair the organism's ability to invade host tissues (24). However, in that study, *lacI* was expressed from a high-copy-number plasmid, while in our system, *lacI* is expressed from a single copy in the chromosome. Our data show that the level of LacI synthesized in our system had little effect on the ability of χ9241(pYA4572) (P_{pagC} *pspA*) to elicit an anti-PspA serum IgG or mucosal IgA response (Fig. 4A), although the IFN-γ and IL-4 responses were impaired compared to that of χ9555(pYA4572) at day 49 (Fig. 6). On the other hand, while there was no difference in anti-PspA serum IgG titers, we did observe a reduction in anti-LPS IgG titers when comparing the *lacI* strain χ9241(pYA4571) (P_{ssaG} *pspA*) with strain χ9555(pYA4571), which does not express *lacI* (Fig. 4C). Strain χ9555(pYA4571) induced higher numbers of IL-4-secreting cells than χ9241(pYA4571), indicating that the presence of *lacI* had a negative influence on the strain's ability to induce cellular immunity. Finally, there was a significant reduction in protection from challenge in mice immunized with χ9241(pYA4571) (P_{ssaG} *pspA*) compared to that in mice immunized with χ9555(pYA4571). One explanation for why the impact of LacI was greater for the P_{ssaG} strain than the P_{pagC} strain is that LacI interfered with the regulation of *pspA* expression from P_{ssaG} *in vivo*. This hypothesis is supported by our observation that there are comparable levels of PspA synthesis in strains with or without LacI carrying the P_{ssaG} *pspA* and P_{pagC} *pspA* plasmids grown under inducing conditions in MgM medium (Fig. 2). Further support for this explanation is that *ssrA* expression is downregulated about 2-fold in strains overexpressing *lacI* from a multicopy plasmid, as determined by microarray analysis and reverse transcription (RT)-PCR (24). Since SsrA-SsrB constitute the primary regulators of expression from P_{ssaG}, downregulation of *ssrA* may interfere with the regulation of *pspA* expression in strain χ9241(pYA4571). While *phoP* was also shown to be downregulated in minimal medium in the same study, there was no reduction of *phoP* expression in macrophages as determined by RT-PCR. Taken together, the results indicate that LacI can have a negative impact on vaccine strains that rely on the two IVIPs examined in this study. This impact was greater in the P_{ssaG} promoter strain, which did not perform as well even in the absence of *lacI*, than in the P_{pagC} promoter strain.

LacI was not necessary for expression from IVIPs, and its presence reduced colonization and cytokine induction in the strain carrying the P_{ssaG} plasmid (Fig. 3 and 6). In contrast, we have shown previously that when the P_{trc} promoter was used to drive antigen gene expression, inclusion of the Δ*relA198*::*araC* P_{BAD} *lacI* TT cassette improved the performance of the vaccine, including a significant increase in protection from challenge (91). In addition, the RDAS strain induced higher anti-PspA IgG and IgA responses than the P_{ssaG} strain regardless of whether the P_{ssaG} host expressed *lacI* (Fig. 4). Our system also provides suitable flexibility to optimize the synthesis of a given antigen. We have constructed two other *relA*::*araC* P_{BAD} *lacI* TT alleles, *relA196* and *relA197*, which were engineered to direct the synthesis of less LacI than the *relA198* allele (91). These may be useful in situations where antigen genes are expressed from low-copy-number plasmids or from the chromosome. The choice of arabinose concentration can be adjusted to reduce or increase the level of LacI synthesis, which will reciprocally control the levels of antigen synthesis *in vitro* and the time course of antigen gene derepression *in vivo* (91). Thus, there are a number of ways to optimize the RDAS system that require less work and expense than when choosing the optimal IVIP.

The RDAS strain elicited antibody titers and IL-4 responses equal to or greater than those of any of the IVIP strains, suggesting that the levels of LacI in our system did not impair the vaccine's ability to induce a robust immune response. This may be due to the fact that the level of LacI synthesized did not compromise the ability of the strain to invade host tissues. This

notion is supported by the fact that the plasmid carrying the P_{pagC} promoter driving expression of *pspA* stimulated similar levels of protective immunity regardless of whether *lacI* was present (Table 2).

In summary, we have made a direct comparison of the new RDAS system and two IVIPs, each driving the expression of *pspA*. Overall, both systems worked well to provide protection against challenge with virulent *S. pneumoniae*. This work, combined with previous work (91), establishes the RDAS system as a practical alternative to IVIPs to overcome the metabolic burden imposed by constitutive antigen gene expression.

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