Optimization of *Salmonella enterica* Serovar Typhi ΔaroC ΔssaV Derivatives as Vehicles for Delivering Heterologous Antigens by Chromosomal Integration and In Vivo Inducible Promoters

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Novel candidate live oral vaccines based on a *Salmonella enterica* serovar Typhi ZH9 (Ty2 ΔaroC ΔssaV) derivative that directed the expression of either the B subunit of *Escherichia coli* heat-labile toxin or hepatitis B virus core antigen from the bacterial chromosome using the in vivo inducible *ssaG* promoter were constructed. The levels of attenuation of the two *S. enterica* serovar Typhi ZH9 derivatives were similar to that of the parent as assessed by measuring the replication of bacteria within human macrophage-like U937 cells. The expression of heterologous antigen in the respective *S. enterica* serovar Typhi ZH9 derivatives was up-regulated significantly within U937 cells compared to similar *S. enterica* serovar Typhi ZH9 derivative bacteria grown in modified Luria-Bertani broth supplemented with aromatic amino acids. Immunization of mice with these *S. enterica* serovar Typhi ZH9 derivatives stimulated potent antigen-specific serum immunoglobulin G responses to the heterologous antigens.

Live oral vaccines based on attenuated *Salmonella enterica* serovar Typhi derivatives are able to stimulate humoral, cellular, and local mucosal responses in vaccinees, making them attractive vehicles for delivering heterologous antigens (17). The efficiency of this approach is likely to be dependent on the ability of the immunizing bacteria to optimally present heterologous (non-*Salmonella*) antigen to the human immune system. Unfortunately, synthesis of heterologous antigens in live vaccine vectors can place an additional metabolic burden on the vector, reducing “fitness” and thereby impacting upon the ability to stimulate an immune response (9). This problem is compounded when the heterologous antigen is expressed using plasmid-based systems, as the metabolic burden can favor the selection of vector bacteria that have lost the plasmid encoding the heterologous gene (9). Furthermore, the requirement for antibiotic resistance markers with traditional plasmid-based expression systems raises significant issues associated with the registration of any vaccine based on these derivatives.

There are a number of approaches to reducing the metabolic burden or stabilizing the plasmid loss associated with heterologous antigen expression. One approach relies on inactivating an essential gene, for example, that encoding aspartate β-semialdehyde dehydrogenase (*asd*), in the vector chromosome and inserting a copy of the same gene into the expression plasmid, the so-called balanced-lethal approach (8). Another approach involves the exploitation of promoters (6) that activate heterologous antigen expression in vivo, thereby reducing the metabolic burden on the vector during vaccine production and the early phases of host colonization (2). A further strategy involves integrating the heterologous gene into the vector chromosome. A limitation of chromosomal integration is that only a single copy of the heterologous gene is available to drive antigen expression (1, 7, 16).

One approach for circumventing this limitation has been to combine chromosomal integration with the use of in vivo inducible promoters. Studies comparing the immune responses generated in mice following oral and intraperitoneal immunization with *Salmonella* strains expressing a PagC-alkaline phosphatase fusion protein from the chromosome under the control of either the in vivo inducible *pagC* promoter or a constitutive promoter have shown that only the strain utilizing the in vivo inducible promoter stimulated detectable immune responses against the heterologous PagC-alkaline phosphatase fusion protein (11).

A live attenuated serovar Typhi-based oral typhoid vaccine strain, called serovar Typhi ZH9 (Ty2 ΔaroC ΔssaV), was previously described (10). Serovar Typhi ZH9 harbors independently attenuating deletion mutations in the *aroC* and *ssaV* genes. The *aroC* gene encodes chorismate synthase, an enzyme involved in the biosynthesis of aromatic compounds. Serovar Typhi *aro* derivatives are attenuated but can cause bacteremia in humans (12). The *ssaV* gene encodes a component of the type III secretion system encoded on *Salmonella* pathogenicity island 2 (SPI-2). SPI-2 is required for *S. enterica* survival and growth within macrophages and, unlike serovar Typhi *aro* derivatives, serovar Typhi ZH9 does not cause bacteremia in humans (10).

The aim of this study was to evaluate a combination of chromosomal integration and the SPI-2-associated in vivo in-
ducible ssaG promoter as a basis for heterologous antigen expression and delivery in serovar Typhi ZH9 using the clinically relevant model antigens hepatitis B virus (HBV) core antigen (HBcAg) and the B subunit of Escherichia coli heat-labile toxin (LT-B). The ssaG promoter was selected because it has been shown previously to be up-regulated at least 400-fold in macrophages (18).

**Materials and Methods**

Bacterial strains, plasmids, media, and growth conditions. Bacteria were routinely grown at 37°C with shaking in modified Luria-Bertani broth (tryptone was replaced with soy peptone and supplemented with aromatic amino acids [methionine, histidine, tryptophan, and threonine], 1% glucose, 1% yeast extract, and tryptophan, tyrosine, and tryptophan [amino] as required. Constructions of Salmonella derivatives. (i) Construction of serovar Typhi DTY8 and serovar Typhi ZH9. A human serovar Typhi strain, Ty2, isolated in 1916 from a patient with typhoid fever, was used as the background to create serovar Typhi DTY8 (Ty2 ΔaroC) and serovar Typhi ZH9; their construction has been described previously (10, 13). (ii) Construction of control strains expressing LT-B or HbcAg from multicopy plasmids. Serovar Typhi ZH9 was transformed with either the plasmid pBRDO26 expressing LT-B from the constitutive tac promoter or the plasmid pPN1 expressing HbcAg from the constitutive tac promoter, using standard protocols as described previously (13), to generate the strains ZH9/taceltB and ZH9/tac/HbcAg, respectively. The plasmids were maintained by growth in the presence of 100 μg of ampicillin/ml. Expression of LT-B or HbcAg by the recombinant Salmonella strains was confirmed by capture enzyme-linked immunosorbent assay (ELISA) (see below). (iii) Construction of serovar Typhi RSC5 (Ty2 ΔaroC ΔssaG-HbcAg). (a) Modification of the T-cell epitope sequences in HbcAg and codon optimization for expression in serovar Typhi. The amino acid and nucleotide sequences of HbcAg from a clinical HBV isolate were kindly provided by Peter Karayiannis, Imperial College, London, United Kingdom. This sequence was subsequently modified to maximize the number of human T-cell epitopes encoded that conformed to previously identified T-cell epitope consensus sequences (3, 14), altered to maximize the number of human T-cell epitopes encoded that conformed to previously identified T-cell epitope consensus sequences (3, 14), although all of the CD4+ and CD8+ epitopes relevant to BALB/c (H-2d) mice were preserved. To improve expression of HbcAg in serovar Typhi, codon usage in the gene was modified to replace potentially poorly translated codons. Codons for modification were identified by comparing the average codon usage in the highly expressed flagellin and the previously generated vector pCVD442/aroC. (b) Cloning of the ssaG-HbcAg fusion within an arC6 deletion in the suicide vector pCDV442. The 0.47-kb ssaG promoter sequence was amplified from the chromosomal DNA of Salmonella enterica serovar Typhimurium TML by PCR and cloned into an intermediate vector along with the codon-optimized sequence encoding HbcAg to form a promoter-antigen fusion using standard techniques. The ssaG-HbcAg fusion was subsequently cloned into a modified version of pMAC23, which is a pUC18-based vector containing a 4.8-kb HindIII fragment derived from serovar Typhi Ty2, which carries a defined 0.6-kb deletion in the arC6 gene, to form pMAC23/ssaG-HbcAg. A 5.6-kb fragment containing the ssaG-HbcAg fusion within the arC6 deletion was excised from pMAC23/ssaG-HbcAg and cloned into the suicide vector pCDV442 using standard techniques to form pCVD442/ssaG-HbcAg. (c) Insertion of the ssaG-HbcAg fusion into the chromosome of serovar Typhi ZH9 to generate serovar Typhi RSC5. The ssaG-HbcAg fusion was introduced into the chromosome of serovar Typhi ZH9 at the site of the attenuating arC6 deletion by homologous recombination, using a method described previously (10). Briefly, the pCDV442/ssaG-HbcAg construct was introduced into serovar Typhi ZH9 by electroporation. Ampicillin-resistant transformants were selected and allowed to grow in the absence of ampicillin to allow for loss of the pCDV442 DNA sequences and one copy of the arC6 gene, either the deleted copy or the deleted copy harboring the ssaG-HbcAg fusion. Strains that had undergone this second recombination event were identified as ampicillin-sensitive derivatives that were able to grow in the presence of 10% sucrose. The arC6 gene harboring the ssaG-HbcAg fusion and the ssaG deletion mutation were confirmed by PCR, Southern blotting, and sequence analysis. Several positive clones were analyzed, which led to the identification of serovar Typhi RSC5.

(iv) Construction of serovar Typhi TS7 (Ty2 ΔaroC ΔssaV ssaG-eltB). (a) Cloning of the ssaG-eltB fusion within an arC6 deletion into the suicide vector pCDV442. The E. coli eltB open reading frame, which encodes the LT-B subunit, and the 0.47-kb ssaG promoter sequence (see above) were amplified by PCR and cloned into an intermediate cloning vector using standard techniques to form a promoter-antigen fusion. The final step in the generation of pCDV442/ssaG-eltB was to insert the ssaG-eltB fusion into the mutated arC6 gene of serovar Typhi cloned in a pCDV442-based suicide vector. This was achieved by using the previously generated vector pCDV442/ssaG-HbcAg (see above). The suicide construct pCDV442/ssaG-eltB was generated by simply replacing the ssaG-HbcAg insert in pCDV442/ssaG-HbcAg with the ssaG-eltB insert from the intermediate vector using standard techniques to form pCDV442/ssaG-eltB. (b) Insertion of the ssaG-eltB fusion into the chromosome of serovar Typhi ZH9 to generate serovar Typhi TS7. The ssaG-eltB fusion was introduced into the chromosome of serovar Typhi ZH9 at the site of the attenuating arC6 deletion by transformation with the suicide vector construct pCDV442/ssaG-eltB using a method identical to that described above for the construction of serovar Typhi RSC5 using pCDV442/ssaG-HbcAg. One strain, called serovar Typhi TS7, was selected for further study. The insertion of the ssaG-eltB fusion into the arC6 deletion of serovar Typhi ZH9 and the integrity of the arC6 deletion were confirmed by PCR, Southern blotting, and sequence analysis.

Macrophage assays. (i) Preparation of bacteria and U937 monolayers. The survival of the vaccine strains serovar Typhi TS7 and serovar Typhi RSC5 within human macrophage-like U937 cells was compared with those of the recipient strain ZH9 and the single arC6 mutant DTY8. Salmonella organisms were grown to stationary phase in Luria broth containing 0.3 M NaCl. U937 cells, obtained from the European Cell and Culture Collection, were grown in RPMI 1640 supplemented with 10% fetal bovine serum (Sigma) supplemented with gentamicin (Sigma GlaxoSmithKline) and 10% nonessential amino acids (Sigma) and 1% sodium bicarbonate (Sigma). The cells were incubated at 37°C in 5% CO2, and air. Cells were seeded into 24-well tissue culture plates at a concentration of 2 × 104 to 3 × 105/well and activated with 100 nM phorbol 12-myristate 13-acetate (Sigma). The cells became adherent once they had been activated. The cells were incubated for 4 to 5 days until a confluent monolayer was formed. (ii) Intracellular replication assay. Stationary-phase bacteria were opsonized by adding an equal volume of human serum minus immunoglobulin A (IgA) (Sigma) at a concentration of 50 mg/ml. The mixture was subsequently vortexed and incubated for 20 min at room temperature. The opsonized bacteria were added to each well at a multiplicity of infection of 50:1. Each strain was tested in triplicate. After incubation at 37°C and 5% CO2 for 30 min, the medium was removed from the infected monolayers and replaced with complete medium containing 200 μg of gentamicin (Gibco-BRL)/ml so that any remaining extracellular bacteria were killed. After a further 1-h incubation, this medium was replaced with complete medium containing 16 μg of gentamicin/ml for the remainder of the incubation period. At different time points following infection, the infected U937 monolayers were washed three times with phosphate-buffered saline (PBS; Gibco-BRL). The salmonellae were harvested by lysing the macrophages with PBS plus 1% Triton X-100 for 20 min. The lysates were collected and diluted in maximal recovery diluent (0.85% NaCl and 0.1% soy peptone), and aliquots were spread on brain heart infusion plus aromatic amino acid mixture plates to assess viability. (iii) Intracellular expression. To induce expression of the heterologous antigen, U937 cells were grown in 75-cm2 tissue culture flasks as described above. Similar to the replication assay, the cells were washed with complete medium prior to bacterial infection. Stationary-phase Salmonella organisms were opsonized, and the bacteria were added to each flask at a multiplicity of infection of ~50:1. The flask were incubated for 60 min at 37°C and 5% CO2. Extracellular bacteria were removed by the addition of complete medium containing 200 μg of gentamicin/ml. After a further incubation of 60 min, the medium was replaced with complete medium containing 16 μg of gentamicin/ml, and the cultures were incubated at 37°C and 5% CO2 for 4 h. After this period, the cells were washed repeatedly with PBS and lysed with PBS plus 1% Triton X-100 for 20 min to release the salmonellae. The bacteria were harvested by centrifugation, resuspended in PBS, and split into aliquots of equal volume. One aliquot was resuspended in B-PER lysis buffer (Pierce), and the levels of expression in these lysates were quantified by ELISA as outlined below. The other aliquot was serially diluted in maximal recovery diluent, and aliquots were spread on brain heart infusion plus aromatic amino acid mixture plates to assess viability.

**Quantification of heterologous antigen expression.** (i) Quantification of LT-B expression. To quantify the levels of LT-B produced by serovar Typhi TS7, a GM1 capture ELISA was employed as outlined previously (13). Briefly, flat-bottomed 96-well Immulon-4 plates (Dynex) were coated overnight at 4°C with 1.5 μg of GM1 gangliosides (Sigma)/well, blocked for 1 h at 37°C with 3% bovine serum albumin (BSA) in PBS–0.05% Tween 20 (PBST), and then incubated with
whole-cell lysates of Salmonella in PBST for 1 h at 37°C. A standard curve was created by serially diluting purified LT from 80 to 0.039 ng/ml in PBS-Tween. These dilutions were then added to the cell lysates, and the plate was incubated for 1 h at 37°C. Following extensive washing, the LT-B was detected by using a goat anti-LT polyclonal antibody (Bioscience Ltd.), followed by biotinylated rabbit anti-goat (Dako, Oxford, United Kingdom) and streptavidin peroxidase (Dako) with o-phenylenediamine dihydrochloride (OPD) substrate (1 mg/ml). After 30 min at 37°C, the reaction was stopped by the addition of 30 μl of 2 M H2SO4/well, and the absorbance was read at 492 nm using an automated Dynex MRX ELISA plate reader. The concentration of LT-B in a sample was read from the standard curve using the optical density at 492 nm (OD492). The results were calculated using Revelation software (Dynex). The concentrations of LT-B produced by attenuated Salmonella were expressed as nanograms of LT-B per 10^9 CFU of Salmonella. (ii) Quantification of HBcAg expression. To quantify the levels of HBcAg produced by serovar Typhi RSC5, a capture ELISA was developed. Flat-bottom 96-well Immulon-4 plates were coated overnight at 4°C with 50 μl of an anti-hepatitis B core antigen monoclonal antibody (Europa Bioproducts)/well diluted 1 in 1,000 in PBS (Sigma). The plates were washed in PBST and then blocked for 1 h at 37°C with 200 μl of 3% BSA well in PBS-0.05% Tween 20 solution. The plates were subsequently washed and then incubated for 60 min with whole-cell lysates of Salmonella resuspended in PBST and shaken at 37°C. A standard curve was created by serially diluting purified HBcAg (Aalto Bio Reagents Ltd.) in PBST, generating a concentration range between 20 and 0.5 ng/ml. Following extensive washing, 50 μl of a rabbit anti-HBcAg polyclonal antibody (Europa Bioproducts)/well diluted 1 in 2,000 in PBST plus 1% BSA was added to each well, and the plates were incubated for 1 h at 37°C with constant shaking. The plates were then washed in PBST before the addition of 50 μl of a goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated antibody (Cambridge Bioscience Ltd.)/well diluted 1 in 8,000 in PBST plus 1% BSA. Following washing, bound HBcAg was detected by the addition of OPD substrate (1 mg/ml; Sigma). After 15 min at 37°C, the reaction was stopped by the addition of 30 μl of 2 M H2SO4/well, and the absorbance was read at 492 nm using an automated Dynex MRX ELISA plate reader. The concentration of HBcAg in a sample was read from the standard curve using the OD492 value. The results were calculated using Revelation software. The concentrations of HBcAg produced by attenuated Salmonella were expressed as nanograms of HBcAg per 10^9 CFU of Salmonella. Mice. Female BALB/c (H-2b) mice aged 6 to 8 weeks were obtained from Harlan-Olac, Bicester, United Kingdom, and housed at the Central Biological Services at the Imperial College of Science, Technology and Medicine, London, United Kingdom. All procedures were performed according to the Home Office guidelines for animals in medical research. Immunization of mice. Mice were immunized either intranasally or subcutaneously. The mice were anesthetized with halothane prior to intranasal inoculation. The anesthetized mice were inoculated with a total volume of 20 μl of a 5 × 10^9 CFU per ml bacterial suspension (10^9 CFU per mouse) into the left and right nares separately. The mice were then washed in PBST before the addition of 50 μl of a prebleed well. LT-B was detected by using a 1:1 mixture of HRP-conjugated goat anti-mouse IgG1 and IgG2a/well, each diluted 1:4,000 in PBST (Southern Biotechnologies Inc.). Specific IgG1 or IgG2a responses were measured by using the respective conjugates separately. The plates were washed as outlined above, and bound IgG was detected by the addition of 100 μl of OPD substrate/well (1 mg/ml; made using Sigmafast OPD two-tablet sets). After 15 min at 37°C, the reaction was stopped by the addition of 30 μl of 2 M H2SO4/well, and the absorbance was read at 492 nm using an automated Dynex MRX ELISA plate reader. The absorbance values were expressed as end point titers as defined above. Statistical method. Statistical significance was calculated using a two-sample t test assuming equal variance between populations with a confidence level of 95% (P < 0.05). RESULTS Survival of different serovar Typhi vaccine derivatives in human macrophage-like U937 cells. The levels of replication of different candidate serovar Typhi vaccine derivatives within human macrophage-like U937 cells have been shown to correlate with their degrees of attenuation in humans (4, 5). Therefore, a reduction in replication of serovar Typhi TSB7 and serovar Typhi RSC5 in human macrophages would be strong evidence supporting the safety of these strains in humans. Replication of the vaccine candidates serovar Typhi TSB7 and serovar Typhi RSC5 was compared with replication of the parent strain, serovar Typhi ZH9, and a Ty2 aroC derivative, serovar Typhi DTY8. The bacteria were opsonized and added to the activated U937 cells, and uptake was allowed to occur for 30 min, after which any extracellular bacteria were killed by the addition of gentamicin. Macrophages were lysed, and the numbers of bacteria were determined at 2 and 48 h so that the amount of bacterial replication or killing could be assessed. The results are shown in Fig. 1. There was no significant difference among the survival rates of serovar Typhi TSB7, serovar Typhi RSC5, and serovar Typhi ZH9 after 48 h, suggesting that the insertion of a promoter-antigen fusion into the chromosome did not affect the replication-deficient phenotype of the vaccine candidates in this system. In contrast, the single aroC mutant serovar Typhi DTY8 demonstrated significantly higher levels of replication. Up-regulation of heterologous antigen expression in human macrophage-like U937 cells. The expression of heterologous antigen in the serovar Typhi vaccine derivatives is directed by
the in vivo up-regulated promoter \( ssaG \). To induce expression of heterologous antigen in vitro, we infected a human macrophage-like U937 cell line with serovar Typhi TSB7, serovar Typhi RSC5, or serovar Typhi ZH9 (negative control) as described above, except that the bacteria were incubated for a 24-h period. The macrophages were subsequently lysed, the bacteria were isolated, and heterologous antigen expression was measured by ELISA. In addition, the viability of the bacteria was evaluated so that the amount of heterologous antigen synthesized could be expressed as a function of the number of viable bacteria. The results outlined in Fig. 2 show that both serovar Typhi TSB7 (Fig. 2A) and serovar Typhi RSC5 (Fig. 2B) can direct the expression of heterologous antigen, demonstrating the functionality of the integrated \( ssaG \)-antigen fusions. Furthermore, a comparison of the level of heterologous antigen expression in U937 cells with that in the same derivatives grown overnight in mod LB aro broth demonstrated the tight in vivo regulation of the \( ssaG \) promoter. Serovar Typhi ZH9-infected U937 cells and uninfected U937 cells were included as negative controls. The amounts of heterologous antigen were measured by ELISA and are expressed as functions of the number of CFU. The error bars indicate the standard deviations.

** FIG. 1.** Replication of serovar Typhi TSB7 (TSB7) (A), serovar Typhi RSC5 (RSC5) (B), and other control strains of serovar Typhi in U937 cells 48 h postinfection in the absence of supplementary aromatic compounds. The values represent the number of bacteria recovered 48 h postinfection as a ratio of the number of bacteria recovered after 2 h. A replication value of \( >1.0 \) represents a number of bacteria recovered at 48 h larger than the number of bacteria recovered at 2 h. A replication value of \( <1.0 \) equates to the serovar Typhi derivative’s inability to survive. The error bars indicate the standard deviations.

** FIG. 2.** LT-B expression from serovar Typhi TSB7 (TSB7) (A) and HBcAg expression from serovar Typhi RSC5 (RSC5) (Fig. 2B) were compared following a 24-h intracellular incubation in U937 cells and overnight incubation in mod LB aro broth to demonstrate the tight in vivo regulation of the \( ssaG \) promoter. Serovar Typhi ZH9-infected U937 cells and uninfected U937 cells were included as negative controls. The amounts of heterologous antigen were measured by ELISA and are expressed as functions of the number of CFU. The error bars indicate the standard deviations.

**Serovar Typhi TSB7 elicits potent serum antibody responses in mice following subcutaneous inoculation.** To evaluate the ability of serovar Typhi TSB7 to drive anti-LT responses in vivo, mice were immunized subcutaneously with a single dose of \( 5 \times 10^7 \) CFU. The subcutaneous route of administration was chosen because serovar Typhi has been shown to be poorly immunogenic in mice when delivered orally (15). In addition, mice were immunized with the parent strain, serovar Typhi ZH9, and serovar Typhi ZH9 expressing LT-B from a multicopy plasmid under the control of a \( tac \) promoter (ZH9/tac/eltB) as negative and positive controls, respectively. ZH9/tac/eltB had been shown previously to elicit potent anti-LT responses in mice (13). Blood was collected by tail bleeding on days 28 and 42, and anti-LT responses were measured by ELISA. Mice inoculated subcutaneously with ZH9/tac/eltB and serovar Typhi TSB7 exhibited potent LT-specific serum IgG responses on both days 28 and 42, as shown in Fig. 3. There was no significant difference \( (P > 0.05) \) between the responses generated by the two vaccines on either day 28 or 42, demonstrating the utility of the serovar Typhi strain TSB7 as a vehicle for stimulating anti-LT responses in vivo. Interestingly,
similar responses were observed when serovar Typhi TSB7 was administered intranasally (data not shown).

**RSC5 elicits potent serum antibody responses with a strong IgG2a bias in mice following nasal inoculation.** To evaluate the ability of serovar Typhi RSC5 to drive anti-HBcAg responses in vivo, mice were immunized intranasally with a single dose of $10^9$ CFU of serovar Typhi RSC5. In addition, mice were immunized with the parent strain, serovar Typhi ZH9, and serovar Typhi ZH9 expressing HBcAg from a multicopy plasmid under the control of the constitutive tac promoter (ZH9/tac/HBcAg), or the candidate strain serovar Typhi TSB7 (TSB7). Titers are expressed as end point titers obtained with sera from individual mice.

bly represent nonspecific “noise” resulting from cross-reactivity between the anti-serovar Typhi lipopolysaccharide antibody raised in these mice and the *E. coli* lipopolysaccharide contamination present in the recombinant HBcAg used in the ELISA. However, the IgG responses measured in mice immunized with ZH9/tac/HBcAg and serovar Typhi RSC5 were significantly higher than the background responses measured in control mice immunized with serovar Typhi ZH9 ($P < 0.05$). There was no significant difference between the magnitudes of the anti-HBcAg antibody responses in mice immunized with either ZH9/tac/HBcAg or serovar Typhi RSC5 ($P > 0.05$) on day 28 or 70. Interestingly, although immunization with ZH9/tac/HBcAg and serovar Typhi RSC5 stimulated similar levels of anti-HBcAg IgG2a in mice, the level of IgG1 was significantly lower ($P = 0.02$) in animals immunized with serovar Typhi RSC5 on both days 28 and 70, as shown in Fig. 5. This suggests that serovar Typhi RSC5 preferentially stimulates IgG2a production, indicative of a Th1-biased response against the heterologous antigen. Interestingly, similar responses were observed when serovar Typhi RSC5 was administered subcutaneously (data not shown).
macrophage-like cells, suggesting that antigen expression is significantly up-regulated in these cells, which is consistent with previous findings (18). Therefore, it is reasonable to presume that expression of the heterologous antigen is minimal during fermentation, eliminating any metabolic burden during vaccine manufacture. In support of this hypothesis is the observation that both serovar Typhi TSB7 and serovar Typhi RSC5 demonstrate growth kinetics very similar to those of the recipient, serovar Typhi ZH9 (data not shown). Furthermore, insertion of the heterologous gene into the chromosome eliminates the requirement for antibiotics in the growth medium during production.

The ability of the ssaG promoter to drive immunologically relevant levels of heterologous antigen from a single chromosomally located copy of the heterologous gene was evaluated in vivo. Mice were inoculated either intranasally with 10^9 CFU or subcutaneously with 5 × 10^7 CFU of serovar Typhi RSC5 and serovar Typhi TSB7, respectively, and the antigen-specific IgG responses were measured by ELISA. Both strains stimulated potent antibody responses, demonstrating the utility of the ssaG promoter in driving immunologically relevant levels of antigen. Interestingly, no response to the heterologous antigen was observed when the ssaG promoter was replaced with the constitutive promoter trc (data not shown), which is consistent with previous findings (11). This probably reflects the ability of the ssaG promoter to direct high levels of antigen expression in a manner both temporally and physically ideal for generating an immune response. Analysis of the antigen-specific IgG isoforms demonstrated a predominance of IgG2a over IgG1 for both of the antigens, which is indicative of a Th1-biased response (although the bias against the LT-B antigen was not as marked [data not shown] as that observed for HBcAg), suggesting that the vector may be ideal for stimulating these types of responses. These studies outline the value of combining ssaG-driven antigen expression with chromosomal integration in the serovar Typhi ZH9 background as a strategy for developing clinically acceptable, stable, and highly immunogenic recombinant Salmonella-based vaccines.

**REFERENCES**


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