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Immune Response Induced by ppGpp-Defective *Salmonella enterica* serovar Gallinarum in Chickens

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To protect chickens from typhoid caused by *Salmonella enterica* serovar Gallinarum (*S*. Gallinarum), the attenuated 9R strain has been used in the field as a vaccine. However, safety concerns have been raised because the mutations in 9R are undefined while its efficacy is still a question under debate. A global regulator, ppGpp, synthesized by RelA and SpoT, has been shown to induce various virulence genes in *S*. Gallinarum (Jeong *et al*., 2008). In this study, two mutant strains defective in ppGpp-synthesis were constructed in wild-type *S*. Gallinarum (ΔppGpp) and 9R strain (9R-ΔppGpp) backgrounds and tested as live vaccines in chickens. After oral inoculation, the LD_{50} values of ΔppGpp and 9R-ΔppGpp were approximately 5×10^{10} colony forming unit (CFU) similarly as 9R strain, which was ~10^5-fold higher than that of the wild-type *S*. Gallinarum strain. Immunological analyses revealed immunization with either of the two attenuated ppGpp-defective strains induced significant antibody responses, the production of antibody-secreting B cells in blood, proliferation of CD4+ and CD8+ T cells in the spleen, and splenic expression of proinflammatory cytokines, such as IFN-γ and TGF-β4, at levels comparable to the 9R strain. Chickens immunized with the mutants (1×10^8 CFU) were 80% protected against oral challenge with 1×10^9 wild-type virulent bacteria (4,000-fold LD_{50} dose), similar to the level of protection achieved by 9R immunization. Based on these data, live attenuated ΔppGpp-defective strains may serve as novel vaccines to control fowl typhoid in chickens.

***Keywords*:** *Salmonella enterica* serovar Gallinarum, ppGpp, *hilA*, *ssrAB*, 9R, immune response, vaccine

The non-motile, Gram-negative bacterium *Salmonella enterica* serovar Gallinarum (*S*. Gallinarum) is the causative agent of fowl typhoid, a severe systemic disease of chickens that causes high mortality (Shivaprasad, 2000). *S*. Gallinarum is host-restricted for poultry, and zoonotic transmission to man rarely occurs. Infection in chickens occurs at all ages and induces severe hepatosplenomegaly accompanied by characteristic liver bronzing, anemia and septicemia (Shivaprasad, 2000). Fowl typhoid has been nearly eradicated in Australia, North America and most European countries. However, it is an important chicken disease of considerable economic impact in many countries in Africa, Asia, the Middle East, and Central and South America (Silva *et al*., 1981; Kim *et al*., 1991). A number of strategies aimed at reducing chicken infection by this bacterium have been employed, including establishment of hygiene standards, husbandry improvement, and usage of antibiotics and vaccines (Zhang-Barber *et al.*, 1999; Beal *et al.*, 2004b). Amongst these strategies, vaccination best fulfils the requirement for practical eradication of fowl typhoid without the risks or difficulties associated with other control measures. Various killed or live attenuated *Salmonella* strains have been tested in fowl model systems as potential vaccines (Barrow *et al*., 1990; Feberwee *et al*., 2000; Woodward *et al*., 2002). Killed vaccines stimulate strong immune responses, but offer a relatively low degree of protection compared to live attenuated vaccines (Barrow *et al.*, 1990) and are considered to be relatively expensive and difficult to apply to large flocks.

The cellular and humoral responses to various *Salmonella enterica* serovar strains, such as Typhimurium, Gallinarum and Pullorum, have been previously studied in chickens (Beal *et al.*, 2004a; Wigley *et al.*, 2005; Lee *et al.*, 2007). *Salmonella* infection begins after passage of the bacteria through the intestinal lining and requires various genes clustered in a region of the chromosome called the Salmonella pathogenicity island (SPI) (Marcus *et al*., 2000). Among the five SPIs identified to date, the roles of SPI-1 and SPI-2 in the infection process have been most extensively studied. In *S*. Typhimurium, SPI-1 and SPI-2 encoded genes are required for host cell invasion and intracellular proliferation, respectively. These genes are thought to be coordinately regulated to ensure optimum survival and propagation within the host (Altier, 2005). Previously, we reported that SPI-1 and SPI-2 encoded genes are expressed at the entry of stationary phase in a manner dependent on the stringent signaling molecule, ppGpp (Song *et al*., 2004; Jeong *et al.*, 2008). ppGpp is synthesized by two enzymes encoded by the *relA* and *spoT* genes, and is a global regulatory signaling molecule that accumulates in unfavorable growth conditions (Cashel *et al*.,

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1996; Pizarro-Cerda and Tedin, 2004). Interestingly, however, although SPI-1 is present in *S.* Gallinarum, it does not play any role in host cell invasion in this strain (Jeong *et al.*, 2008). The genetic element(s) responsible for *S.* Gallinarum host cell invasion have not been identified, but the ppGpp null mutant was shown to be defective in host cell invasion as well as intracellular proliferation. Thus, *S.* Gallinarum entry into animal cells seems to occur by a SPI-1 independent, but ppGpp-dependent, mechanism. As in *S.* Typhimurium, intracellular growth of *S.* Gallinarum requires ppGpp for transcription of genes encoded in SPI-2.

The attenuated *S*. Gallinarum strain, 9R, has been used as a commercial vaccine to control fowl typhoid (Lee *et al.*, 2007). The 9R vaccine strain was developed in the 1950s and has a semi-rough lipopolysaccharide structure, but the nature of its attenuation is not known (Smith, 1956). Although highly attenuated compared to the parental *S*. Gallinarum strain 9, the 9R vaccine strain still causes systemic disease and induces pathology in the liver and spleen where the bacteria can persist for several weeks (Silva *et al*., 1981). In an attempt to develop an alternative vaccine, *S.* Gallinarum mutants defective in ppGpp synthesis were constructed in this study using wild-type and 9R parental strains, and tested for their potential as attenuated live vaccines.

Materials and Methods

Bacterial strains, media, and growth conditions

The wild-type *S.* Gallinarum SG3001 strain was originally isolated from an infected chicken and was used as the parent strain for subsequent generic modifications (Jeong *et al.*, 2008). The attenuated strain 9R was obtained from Intervet (The Netherlands). All bacterial mutant strains were constructed as described (Datsenko and Wanner, 2000). The 9R-ΔppGpp mutant was constructed from the 9R strain by disrupting both the *relA* and *spoT* genes with homologous recombination using the λred system as described (Jeong *et al.*, 2008). Wild-type and 9R strains were cultured in Luria-Bertani (LB) broth (Becton-Dickinson Labware, UK) at 37°C in a shaking incubator at 150 rpm. Bacterial stocks were stored at -70°C in LB broth supplemented with 30% glycerol. The mutant strains were cultured in LB broth in the presence of 15 μg/ml chloramphenicol (Sigma Chemical, USA) or/and 30 μg/ml kanamycin (Sigma Chemical) to identify their phenotypes.

Experimental animals

The eggs from *Salmonella*-free broiler chickens were purchased from the Institute for Animal Health (Compton, UK). The chickens were hatched and raised in wire cages at a temperature of 30°C (College of Veterinary Medicine, Chonnam National University) for one week, and thereafter maintained at 21°C. Animals were given *ad libitum* access to water and a vegetable protein-based diet (SDS, UK). Prior to the experiments, PCR was used to check for the presence of *Salmonella* in chicken stool samples. All experiments were conducted within the ethical research guidelines of Chonnam National University and according to national legislation.

Determination of 50% lethal dose (LD_{50})

The chickens were fasted overnight prior to oral inoculation. To determine the LD_{50} values of wild-type, 9R and the mutant strains, 1-week-old chickens were orally challenged with bacterial doses from 1×10^1 to 1×10^{10} CFU in 500 μl PBS. Ten chickens were used for each bacterial dose. LD_{50} values were estimated by the Reed and Muench method (Reed and Muench, 1938).

Preparation of Salmonella lysate antigen

To measure immune responses, bacterial lysate was prepared from the wild-type *S*. Gallinarum strain. 3 ml bacteria cultures were grown in LB broth overnight, and this was used to inoculate 100 ml of LB broth. Following overnight incubation at 37°C in a shaking incubator, bacterial pellets were collected by centrifugation at 2,000×g for 10 min at 4°C and washed twice with an equal volume of PBS. The bacteria were resuspended in 10 ml PBS, subjected to three freeze-thaw cycles in liquid nitrogen, lysed using a Soniprep 150 sonicator (MSE Scientific Instruments, UK) with 9/20 sec bursts, and cooled for 1 min in an ice bath. To remove the insoluble fraction, the suspension was centrifuged twice at 3,000×g for 10 min at 4°C followed by centrifugation at 3,000×g for 20 min at 4°C. The protein concentration of the lysate was measured via Bradford assay (Merck, UK) and stored at -20°C until further use.

Chicken immunization

Mutant bacterial strains were administered orally to twenty-five 1-week-old chickens per group in an equal volume of sterile PBS, and animals were maintained for 14 days. Five immunized chickens (10^8 CFU) were sacrificed to obtain blood and tissues for immune response analysis. To measure protection against wild-type bacteria, ten chickens (10^4 or 10^8 CFU) were orally challenged with wild-type *S*. Gallinarum (10^9 CFU) 4 weeks after immunization. The number of surviving chickens was monitored for 30 days.

Anti-Salmonella antibody assay

The levels of anti-*Salmonella* antibodies were determined using an enzyme-linked immunosorbent assay (ELISA). Sera were obtained from immunized chickens by jugular venipuncture every week. For the ELISAs, 96-well plates were coated with wild-type *S.* Gallinarum lysate [5 μl/ml in carbonate bicarbonate buffer (pH 9.6), 50 μl per well] overnight and washed three times with PBS containing 0.05% Tween-20 (PBS-T). The plates were incubated with 3% skim milk (Merck, Germany) in PBS-T for 1 h at 37°C and followed by washing with PBS-T. The serum samples were diluted with 3% skim milk in PBS-T and applied to the coated plates for 1 h at 37°C. The starting serum dilutions were 1:25 for IgA or 1:100 for IgG and IgM. Horseradish peroxidase-conjugated goat anti-chicken IgA (1:2,000) (Serotec, UK), goat anti-chicken IgG (1:20,000) (Serotec, UK), and goat anti-chicken IgM (1:20,000) (Serotec) were used as secondary antibodies. The plates were developed with *o*-phenylenediamine dihydrochloride (OPD) (Sigma, USA) substrate in 0.05 M phosphate citrate buffer (Kemeny and Challacombe, 1988). The reaction was stopped with 2 M H_2SO_4 after 30 min, and the absorbance was measured at 405 nm using a Benchmark microplate reader (Bio-Rad, UK).

Analysis of antibody-secreting B lymphocytes

ELISPOT assays were used to quantify antibody-secreting B lymphocytes as described (Yuan *et al*., 1996; Salerno-Goncalves *et al*., 2002). In brief, peripheral blood mononuclear cells (PBMC) isolated from heparinized whole blood by Ficoll gradient centrifugation were cultured in RPMI1640 (Gibco, USA) containing 10% fetal calf serum (FCS) (Hyclone, USA) and used to measure *Salmonella*-specific antibody secreting cells (ASCs). Ninety-six-well MAIPN4510 plates

(Milipore, USA) were coated with 5 μg/ml *Salmonella* lysate. After overnight incubation at 4°C and washing with PBS-T, isolated splenocytes (2×10^5/ml) were added to each well, and the plates were incubated for 4 h or longer at 37°C in 5% CO_2. Purified goat anti-chicken IgM, IgG and IgA (2.5 μg/ml, KPL, USA) were added to each well, and the plates were incubated overnight. After washing, horseradish peroxidase-conjugated rabbit anti-goat IgG (2 μg/ml, Sigma) was used as secondary antibody. Plates were then washed with PBS and developed with an AEC substrate kit for peroxidase (Vector, USA) and a blue alkaline phosphatase substrate kit (Vector), which stained cells red or blue, respectively. The number of ASCs per well was determined by counting the spots with a CTL-ImmunoSpotH S5 Macro Analyzer (Cellular Technology Ltd., USA). The number of nonspecific spots detected in negative control wells incubated only with PBS was subtracted from the total number of *Salmonella*-specific ASCs.

Analysis of CD4+ and CD8+ T cells by flow cytometry

PBMCs were separated and stained with monoclonal antibodies against CD4 (Serotec) and CD8 (Serotec) as described (Bohls *et al*., 2006). Flow cytometry was carried out using a FACSCalibur (Becton Dickinson, USA) according to the manufacturer's protocol.

Quantitative analysis of cytokine mRNA

Cytokine levels in spleens from immunized animals were quantified by a real-time reverse transcription polymerase chain reaction (RT-PCR) analysis. Spleen samples stored in liquid nitrogen were thawed and total RNA was purified using an RNeasy Mini kit (QIAGEN, UK) according to the manufacturer's instructions. PCR primer sets for both the cytokines and 28S RNA have been described previously (Kaiser *et al*., 2000; Kogut *et al*., 2003). cDNA synthesis was carried out in a 40-μl reaction volume using a TaqMan Reverse Transcription Reagent kit (Applied Biosystems, UK), and DNA was amplified and quantified in the ABI Prism 7300 Real-time PCR System (Applied Biosystems) after mixing with SYBR GREEN PCR Master Mix (Applied Biosystems). PCRs were performed for 35 cycles of 95°C (30 sec), 60°C (30 sec), and 72°C (30 sec).

Statistics

Statistical analysis was performed either using Microsoft Excel or Minitab for Windows. Comparisons between immunized and control groups were performed using ANOVA. Values of $P<0.05$ were considered significant.

Results

Virulence of *S*. Gallinarum mutants

The LD_{50} values of mutant strains defective in ppGpp synthesis (Δ*relA*, Δ*spoT*; SG3003), the SPI1 gene *hilA* (SG3004), and the SPI2 gene *ssrAB* (SG3005) were determined using chickens to examine their virulence (Table 1). The expression of the SPI-1 secretion system and the expression of its secreted effectors are known to be coordinately regulated by HilA, a member of the OmpR/ToxR family of transcriptional regulators, in *S*. Typhimurium (Lucas and Lee, 2000) and the expression of genes encoding the type III secretion system of SPI-2 is dependent on a sensor-kinase system, SsrA-SsrB (Lee *et al*., 2000).

The LD_{50} of wild-type bacteria and the Δ*hilA* mutant were 2.5×10^5 and 1×10^6 CFU following oral administration, respectively. In contrast, the LD_{50} of the Δ*ssrAB* mutant was 5×10^{10} CFU. This difference is consistent with a previous report in which a SPI-2 mutant was attenuated while a SPI-1 mutant was fully virulent in chickens (Jones *et al*., 2001). The LD_{50} of the ppGpp-defective mutant was 5×10^{10} CFU. Correspondingly, ppGpp has been shown to be critical for animal cell invasion and intracellular proliferation of *S.* Gallinarum (Jeong *et al*., 2008). The LD_{50} of 9R and a 9R strain defective in ppGpp synthesis (9R-ΔppGpp) was 5×10^{10} CFU, the same as the LD_{50} of a ppGpp defective strain in the wild-type background (ΔppGpp). All chickens that survived following mutant inoculation showed no obvious signs of illness and remained healthy for the duration of the experiment (at least 30 days). These results indicate that ppGpp deficiency attenuates virulence $>10^5$ relative to wild-type bacteria. Since the same LD50 (5×10^{10} CFU) was obtained for mutant strains including SPI2 knock-out (ΔssrAB), ΔppGpp, 9R, and 9R-ΔppGpp strains, 5×10^{10} CFU may be the LD_{50} for the most highly attenuated *S*. Gallinarum strains.

Antibody responses and induction of antibody-secreting B cells by *S*. Gallinarum mutants

SPI2 mutant *S.* Gallinarum and the 9R strain are attenuated and induce protective immunity in chickens (Jones *et al.*, 2001; Lee *et al.*, 2007). Our results indicated that the ΔppGpp and 9R-ΔppGpp strains were also attenuated and thus may induce protective immunity as well. Consequently, we analyzed the immune responses induced by the highly attenuated ppGpp defective strains and compared these responses with those mediated by the SPI2-defective Δ*ssrAB* mutant and the 9R strain. Chickens were immunized with 1×10^8 CFU through an oral route, and serum samples were collected every week after immunization. *Salmonella*-specific IgM, IgG, and IgA antibody levels in the sera were measured by ELISA using *Salmonella* lysates (Fig. 1). The serum IgM levels in all animals immunized with the test strains increased up to 3 weeks post infection (wpi), and subsequently decreased (Fig. 1A). Serum IgG (Fig. 1B) and IgA (Fig. 1C) peaked at 3-4 wpi. While ΔppGpp and 9R-ΔppGpp induced significant antibody responses, antibody titers after ΔppGpp immunization were somewhat lower than those induced by 9R-ΔppGpp: ~60% vs. 30% for IgG, and ~80% vs. 60% for IgA. Interestingly, the *Salmonella*-specific IgG and IgA antibody levels in sera collected from ΔppGpp and 9R-ΔppGpp immunized animals were lower than the

Table 1. *S.* Gallinarum strains used in this study and their LD_{50} values[a]

Strains	Description[b]	LD_{50} (CFU)
Wild type (SG3001)	Wild-type isolate	2.5×10^5
Δ*hilA* (SG3004)	SG3001 Δ*hilA*::*kan*	1×10^6
ΔppGpp (SG3003)	SG3001 Δ*spoT*::*cat* Δ*relA*::*kan*	5×10^{10}
Δ*ssrAB* (SG3005)	SG3001 Δ*ssrAB*::Kan	5×10^{10}
9R	Avirulent	5×10^{10}
9R-ΔppGpp	9R Δ*spoT*::*cat* Δ*relA*::*kan*	5×10^{10}

[a] Chickens were inoculated perorally with the indicated strains at 1×10^1-1×10^{10} CFU/500 μl. Morbidity and mortality were observed for 30 days.
[b] The ΔppGpp-9R strain was constructed in this study. Construction of the other mutants has been previously described (Smith, 1956; Jeong *et al.*, 2008).

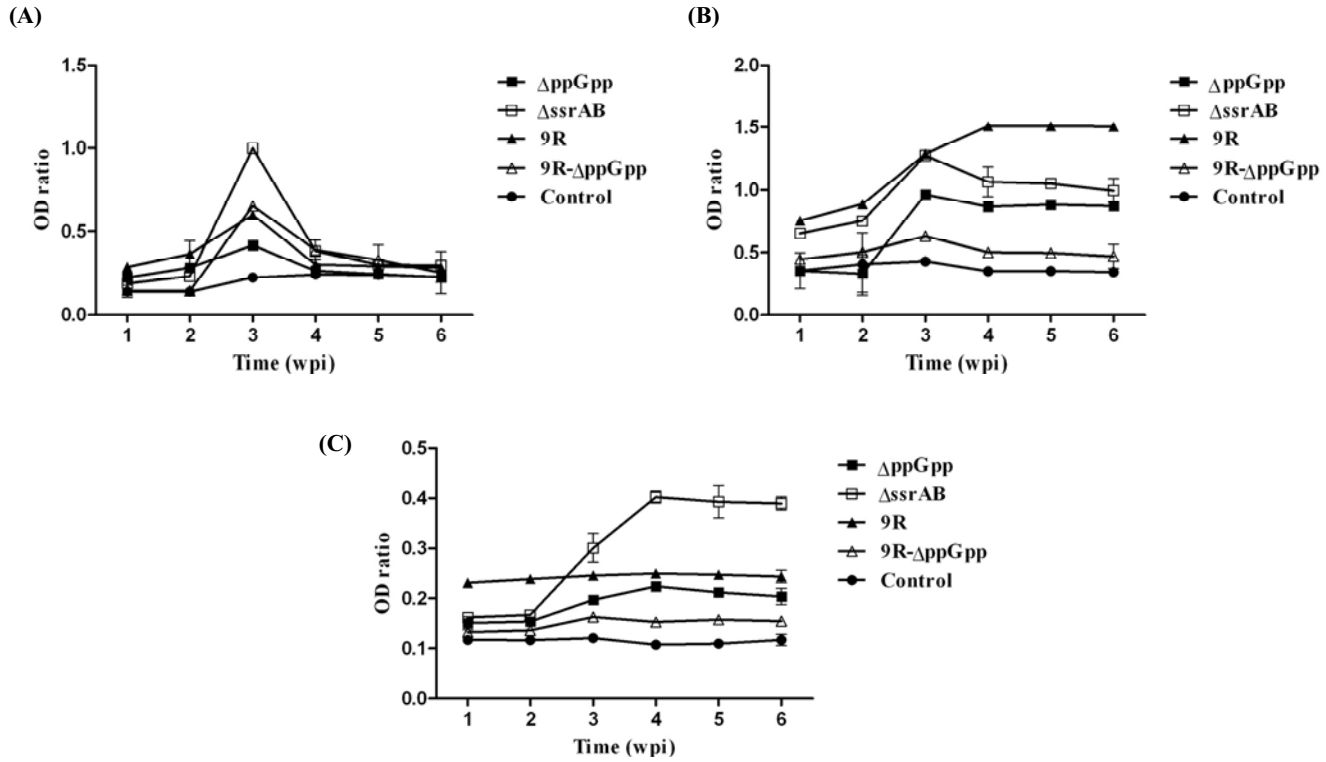

Fig. 1. Serum IgM (A), IgG (B), and IgA (C) titers in chickens immunized with wild-type and mutant *S.* Gallinarum strains. One-week-old chickens were orally immunized with bacteria (1×10^{10} CFU). Samples were collected from the immunized chickens (n=5 per group) at the indicated weeks post infection (wpi). Unimmunized chickens were used as controls. Data are expressed as the geometric mean with the standard deviation. Error bars represent the standard error of the mean.

levels induced by the Δ*ssrAB* mutant and the 9R strain.

Induction of antibody-secreting B cells (ASC)

Using the same group of immunized chickens shown in Fig. 1, we subsequently determined the number of *Salmonella*-specific ASCs by ELISPOT assay using PBMCs collected from immunized chickens at the indicated wpi (Fig. 2). Before immunization, *Salmonella*-specific IgM, IgG, and IgA ASCs were not detected in the samples. ASCs increased at 2 wpi and peaked at 4 wpi. All samples showed similar ASC induction profiles except Δ*ssrAB* and 9R immunization, which resulted in approximately half of the level of IgA ASCs compared to ΔppGpp and 9R-ΔppGpp. These data indicate that the IgM, IgG, and IgA effector B-cell responses induced by immunezation with ΔppGpp and 9R-ΔppGpp were similar to or greater than those induced by immunization with Δ*ssrAB* and 9R.

Measurement of CD4+ and CD8+ T cells in immunezed chicken spleens

The population of CD4+ and CD8+ T lymphocytes is one indicator of pathogen-host immune interaction in immune organs, including the spleen (Lundin *et al*., 2002; Salerno-Goncalves *et al.*, 2002). Changes in T-cell populations after immunization have been documented to be comparable to changes in serum antibody levels (Lundin *et al.*, 2002). In this study, we analyzed the relative ratio of CD4+ and CD8+ T cells in immunized chicken spleens by flow cytometry (Fig. 3). The number of both CD4+ and CD8+ T cells increased at 2 wpi and peaked at 3-4 wpi, indicating vigorous proliferation after immunization. The percentage of CD4+ T cells (23-33%) was >2-fold greater than the percentage of CD8+ T cells (6-12%) at peak proliferation. At 6 wpi, the percent of CD4+ T cells was slightly lower after immunization with ΔppGpp (25%) and 9R-ΔppGpp (22%) compared to Δ*ssrAB* (32%), but was similar to the percent induced by 9R (22%) (Fig. 3A). The percentage of CD8+ T cells was similar after immunization with ΔppGpp (8%), Δ*ssrAB* (10%), and 9R (8%), while 9R-ΔppGpp induced a lower level of CD8+ T cells (5%) (Fig. 3B). These results were consistent with the antibody production and antibody-secreting B cell data (Figs. 1 and 2) and indicate that both B and T cell responses were highly induced following immunization with all of the test strains.

Interferon-γ (IFN-γ) and transforming growth factor-ß4 (TGF-ß4) are cytokines produced by many immune cells during inflammation (Beal *et al.*, 2004a, 2004b). The levels of these cytokines were thus measured in the spleens of immunized chickens using real-time RT-PCR analysis (Fig. 4) and expressed as the fold change relative to the average level in the uninfected group. IFN-γ expression increased in all the immunized animals and was >20-fold higher than the unimmunized control even at 2 wpi (Fig. 4A). Immunization with ΔppGpp, 9R-ΔppGpp, and Δ*ssrAB* induced similar levels of IFN-γ expression, while levels induced by 9R were lower. Levels of splenic TGF-ß4 mRNA increased at least 6-fold following immunization and plateaued at 4 wpi (Fig. 4B).

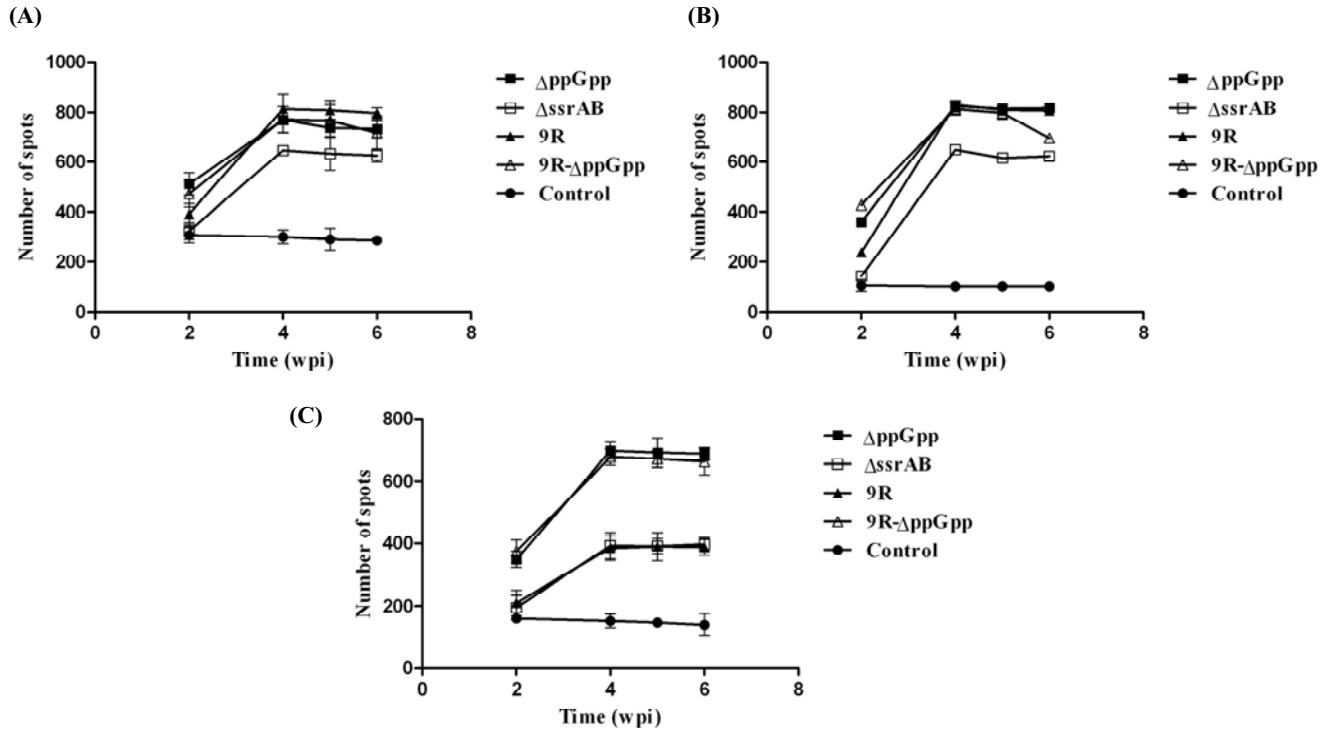

Fig. 2. The numbers of IgM (A), IgG (B), and IgA (C) antibody-secreting cells in chickens immunized with *S.* Gallinarum mutant strains. One-week-old chickens were orally immunized with bacteria (1×10^8 CFU). PBMCs were collected from chicken blood (n=5 per group) at the indicated weeks post infection (wpi). Blood from unimmunized chickens was used as a control. Data are expressed as the geometric mean with the standard deviation. Error bars represent the standard error of the mean.

TGF-ß4 was upregulated most by Δ*ssrAB* (11-fold), ΔppGpp, and 9R induced similar levels of TGF-ß4 (8-fold), while TGF-ß4 induction was lowest following immunization with 9R-ΔppGpp (6-fold). Taken together, these immunological analyses suggest that the ΔppGpp mutant, as well as Δ*ssrAB* and 9R, induced significant immune responses in chickens, while the 9R-ΔppGpp mutant induced a slightly lower response. It was noted that most of values possessed statistically significances, although their deviations were shown to be large. Compared to ones in 9R cytokines at the same wpi, all of *p* values in IFN-γ samples were <0.005 in *t*-test, except 9R-ΔppGpp at 6wpi ($p=0.23$). In contrast, all of *p* values in TGF-ß4 samples were <0.01, except 9R-ΔppGpp at 2, 4, and 5 wpis ($p=0.35$, 0.44 and 0.27, respectively) and Δ*ssrAB* ($p=0.30$).

Protection of immunized chickens against virulent *S.* Gallinarum challenge

Lastly, we determined whether immunization with the ΔppGpp or 9R-ΔppGpp mutants provided protective immunity in chickens against infection by wild-type *S.* Gallinarum

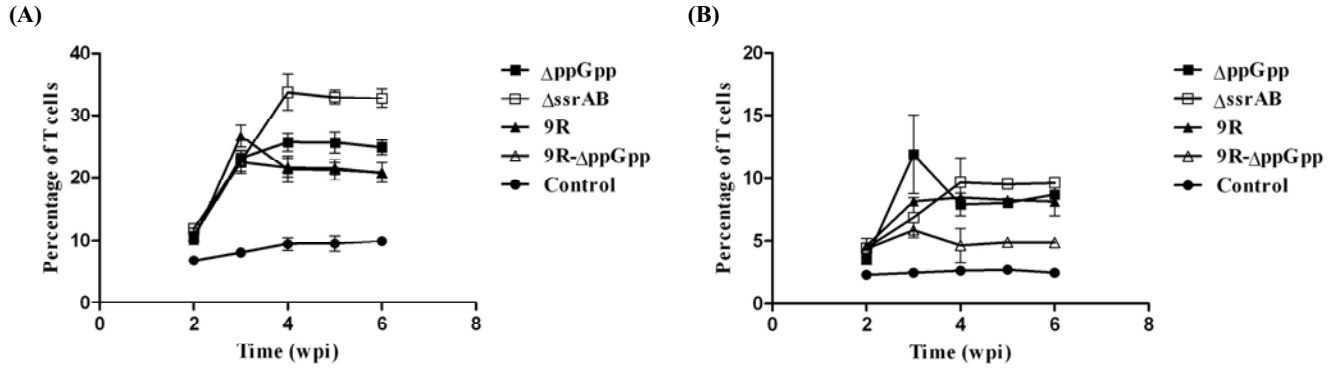

Fig. 3. The kinetics of CD4+ (A) and CD8+ (B) T cell population expansion in peripheral blood from chickens immunized with *S.* Gallinarum mutant strains. One-week old chickens were orally immunized with bacteria (1×10^8 CFU). The PBMCs were collected from blood (n=5 per group) at the indicated weeks post infection (wpi). Blood from unimmunized chickens was used as a control. Data are expressed as geometric mean with the standard deviation. Error bars represent the standard error of the mean.

Table 2. Efficacy of oral immunization with *S.* Gallinarum mutants in protecting *Salmonella*-free chickens against challenge with wild-type bacteria[a]

Strain	Immunization (CFU)	Challenge (CFU)	Survivor/total[b]
ΔppGpp	1×10^4	1×10^9	3/10
	1×10^8	1×10^9	8/10
Δ*ssrAB*	1×10^4	1×10^9	3/10
	1×10^8	1×10^9	8/10
9R	1×10^4	1×10^9	3/10
	1×10^8	1×10^9	7/10
9R-ΔppGpp	1×10^4	1×10^9	2/10
	1×10^8	1×10^9	8/10

[a] *Salmonella*-free chickens were orally immunized with the indicated strains and 4 weeks later were challenged with the wild-type strain.
[b] The numbers of survivors relative to the total number of chickens was recorded 30 days after the challenge.

compared to the protection afforded by the established 9R and Δ*ssrAB* vaccine strains (Silva *et al.*, 1981; Jeong *et al.*, 2008). One-week-old chickens were immunized via an oral route with the test strains. Four weeks later, the immunized chickens were orally challenged with 1×10^9 (4,000-fold LD_{50}) wild-type bacteria. Survival was recorded for 30 days (Table 2). All of the test strains were protective against wild-type *S.* Gallinarum (Table 2), while all of the unimmunized control chickens expired within 5 days. The protection level depended on the immunization dose: ~80% of the chickens survived when immunized with 1×10^8 CFU (9R was 70% protective at this dose), but only ~30% survived following immunization with 1×10^4 CFU. Together, these data show that chickens immunized with ΔppGpp or 9R-ΔppGpp were protected against wild-type bacteria challenge as well as those immunized with 9R or Δ*ssrAB.* Thus, the ΔppGpp *S.* Gallinarum strain could be developed as a live attenuated vaccine to protect chickens from fowl typhoid.

Discussion

This study indicates that mutants deficient in ppGpp synthesis are as highly attenuated as the 9R strain and that immunezation with ppGpp synthesis mutants protects chickens against virulent *S.* Gallinarum infection.

In *Salmonella* Typhimurium, the stringent signaling molecule ppGpp plays a key role in the cessation of ribosome production as a bacterial culture enters stationary phase (Sands and Roberts, 1952; Cashel and Gallant, 1968; Jones *et al*., 1994; Na *et al*., 2006). Recently, ppGpp has been implicated in stationary phase induction of SPI-1 genes by activating HilA, a master transcriptional regulator of SPI-1 and SPI-2 encoded genes (Song *et al.*, 2004). In *S.* Typhimurium, the SPI-1 genes are involved in animal cell invasion, but these genes are not implicated in *S.* Gallinarum cell entry (Jeong *et al.*, 2008). In this study, we also found that Δ*ssrAB* mutants were attenuated ~10^5-fold compared to the wild-type strain, but a Δ*hilA* mutant was not. Mutants defective in ppGpp synthesis, ΔppGpp and 9R-ΔppGpp were as highly attenuated as the 9R and Δ*ssrAB* strains, likely due to decreases in invasion and intracellular proliferation (Jones *et al.*, 2001; Jeong *et al.*, 2008). It is interesting why ppGpp mutation added in 9R strain (ΔppGpp) did not cause any further decrease in virulence, while the same mutation in wild-type strain (9R-ΔppGpp) resulted in significant virulence attenuation. Therefore, it should be further studied whether 9R strain is defective in ppGpp metabolism or whether the virulence genes controlled by ppGpp and those by undefined mutation(s) are correlated or overlapped.

The immune responses induced by various *Salmonella* strains, such as *S.* Typhimurium, *S.* Enteritidis, and *S.* Gallinarum (avian salmonellosis), have been examined, and bacterial were generally cleared by 3-4 wpi (Berndt and Methner, 2001; Dueger *et al.*, 2001; Jones *et al.*, 2001; Babu *et al.*, 2003, 2004; Beal *et al.*, 2004a, 2004b; Wigley *et al.*, 2005). Immunization of chickens with *S.* Gallinarum ΔppGpp mutants induced both cellular and humoral responses that peaked at 3-4 wpi (Figs. 1 to 4). Although immunization with ΔppGpp and 9R-ΔppGpp induced comparably lower levels of serum IgG and IgA than

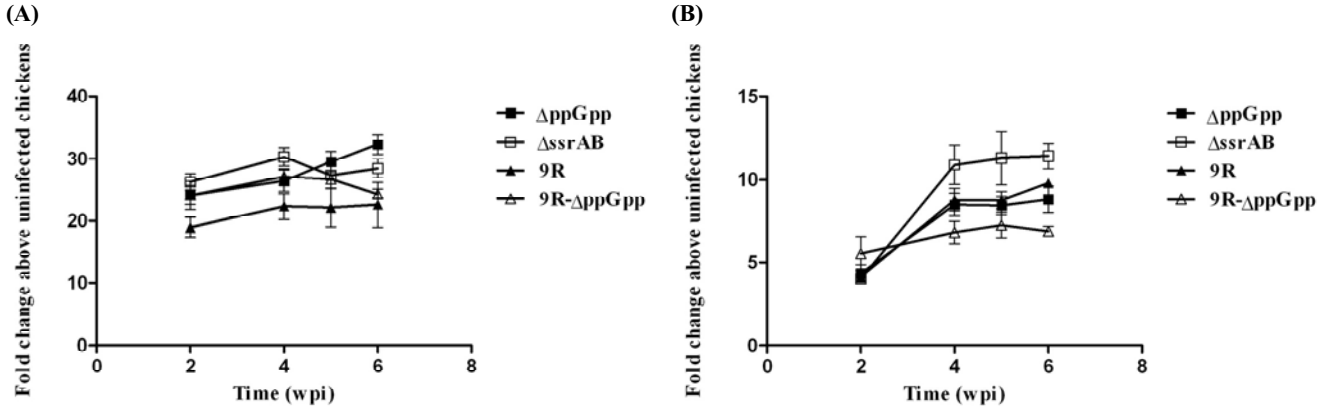

Fig. 4. The kinetics of cytokine mRNA production in spleens from chickens immunized with *S.* Gallinarum mutant strains. The mRNA amounts of IFN-γ (A) and TGF-β_4 (B) were measured by SYBR green-based real-time RT-PCR. One-week-old chickens were orally immunized with bacteria (1×10^8 CFU). Total RNA was isolated from the spleens of immunized chickens (n=5 per group) and was collected at the indicated weeks post infection (wpi). Data are expressed as geometric mean with the standard deviation. Error bars represent the standard error of the mean.

immunization with 9R (Fig. 1), these antibody levels seem sufficient to protect chickens from infection with a virulent strain (Table 2). ΔppGpp, 9R-ΔppGpp and 9R all induced comparable numbers of ASCs, in line with previous results (Berndt and Methner, 2001; Dueger *et al.*, 2001; Jones *et al.*, 2001; Babu *et al.*, 2003, 2004; Beal *et al.*, 2004a, 2004b; Wigley *et al.*, 2005).

Immunization-induced cellular immune responses have been shown to correlate with protection of chickens against fowl typhoid (Alvarez *et al*., 2003). The induction of Th1 cytokines, such as IFN-γ, leads to the development of T cell responses that help clear *Salmonella* from infected animals (Barrow *et al*., 1994; Jones *et al.*, 2001; Wigley *et al*., 2002). In the present study, we demonstrated that oral immunization with ΔppGpp or 9R-ΔppGpp induced the production of large amounts of pro-inflammatory cytokines, such as IFN-γ and TGF-ß4, and increased CD4+ and CD8+ T cell populations in the spleen to levels observed following immunization with 9R (Figs. 3 and 4). It would be interesting to see whether the antibody response mirrored the cytokine responses (i.e., IFN-γ production). Therefore, the experiment to measure the relative ratio of IgG2a (Th1 response) to IgG1 (Th2 response) would provide a better insight into the pattern of immune responses.

In summary, the genetically well defined ppGpp-defective *S.* Gallinarum strain described here could be added to the list of live vaccine candidates for fowl typhoid even though further experiments, including clinical tests in farms and of the effects on egg laying, are required.

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