

The role of *rpoS*, *hmp*, and *ssrAB* in *Salmonella enterica* Gallinarum and evaluation of a triple-deletion mutant as a live vaccine candidate in Lohmann layer chickens

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Salmonella enterica Gallinarum (SG) causes fowl typhoid (FT), a septicemic disease in avian species. We constructed deletion mutants lacking the stress sigma factor RpoS, the nitric oxide (NO)-detoxifying flavohemoglobin Hmp, and the SsrA/SsrB regulator to confirm the functions of these factors in SG. All gene products were fully functional in wild-type (WT) SG whereas mutants harboring single mutations or a combination of *rpoS*, *hmp*, and *ssrAB* mutations showed hypersusceptibility to H₂O₂, loss of NO metabolism, and absence of *Salmonella* pathogenicity island (SPI)-2 expression, respectively. A triple-deletion mutant, SGΔ3 (SGΔ*rpoS*Δ*hmp*Δ*ssrAB*), was evaluated for attenuated virulence and protection efficacy in two-week-old Lohmann layer chickens. The SGΔ3 mutant did not cause any mortality after inoculation with either 1 × 10⁶ or 1 × 10⁸ colony-forming units (CFUs) of bacteria. Significantly lower numbers of salmonellae were recovered from the liver and spleen of chickens inoculated with the SGΔ3 mutant compared to chickens inoculated with WT SG. Vaccination with the SGΔ3 mutant conferred complete protection against challenge with virulent SG on the chickens comparable to the group vaccinated with a conventional vaccine strain, SG9R. Overall, these results indicate that SGΔ3 could be a promising candidate for a live *Salmonella* vaccine against FT.

Keywords: live attenuated vaccine, oxidant response genes, protection, *Salmonella enterica* Gallinarum, virulence

Introduction

Salmonella enterica serovar Gallinarum (SG) causes fowl typhoid (FT), a severe septicemic disease in poultry. This disease causes significant morbidity and mortality, and is a major concern in the poultry industry [3]. Over recent decades, attenuated SG strain 9 (SG9R) has been used as a live vaccine to control FT [3]. However, several limitations have been reported for this strain, including incomplete protection [4], virulence in certain breeds of chickens [22], a need for multiple injections to provide adequate protection [22], and virulence in newly hatched and young chicks [3]. Moreover, SG9R-like strains have been isolated from chicken field outbreaks [16,24], and the genetic basis for attenuated virulence in SG9R is unknown [23]. For enhanced vaccine safety, it is therefore

recommended that attenuated strains with well-defined genetics be explored.

Mutations of several genes that affect the intraphagosomal survival of *Salmonella enterica* serovar Typhimurium (ST) in host macrophages have been shown to attenuate the virulence of the bacterium. The *rpoS* gene (NC_003197.1) encodes the alternative sigma factor σ^S (RpoS) in *Salmonella*. RpoS promotes adaptation to a range of stress conditions and starvation. It also contributes to *Salmonella* virulence via regulation of the virulence plasmid gene *spv* and chromosomal genes [1,15]. Mutations of the *rpoS* gene attenuate the virulence of ST [9], and it was reported that *rpoS* mutants confer high levels of protection against a virulent ST strain in mice [6].

The *hmp* (NC_016857.1) gene encodes the flavohemoglobin Hmp in *Salmonella*. Hmp provides the principal means of

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protection of *Salmonella* from nitric oxide (NO) and nitrosative stress within professional phagocytes [11]. An ST mutant lacking this enzyme is hypersensitive to nitrosative stress, accumulates S-nitrosylated proteins in its cytoplasm, and shows attenuated virulence in mice [2]. The virulence genes located on *Salmonella* pathogenicity island (SPI)-2 encode a type III secretion system (T3SS) and a set of effector proteins that, through interaction with intracellular traffic in macrophages and dendritic cells, create a safe compartment for the salmonellae inside the phagocyte. A two-component regulatory system consisting of a sensor kinase (SsrA) and a response regulator (SsrB) responds to environmental signals [8,17,25] and controls SPI-2 expression. Mutation of *ssrAB* (NC_003197.1) in ST and SG results in defective intracellular replication, and the virulence of ST mutant was reported to be attenuated in a mouse model [5,13].

It is important to note that the pathogenesis of *Salmonella* infection varies depending on the host-strain interaction [3,18,19]. The roles of *rpoS*, *hmp*, and *ssrAB* in the pathogenesis of avian-specific SG have not been reported. Therefore, the objectives of the present study were: 1) construct deletion mutants *SGΔrpoS*, *SGΔhmp*, and *SGΔssrAB*, and validate the importance of these genes in SG; 2) construct a triple mutant, *SGΔrpoSΔhmpΔssrAB* (designated hereafter as *SGΔ3*), and investigate the effectiveness of this mutant as a live vaccine for FT prevention in chickens.

Materials and Methods

Bacterial strains and media

The bacterial strains and plasmids used in this study are listed

in Table 1. SG97 was used as the parental SG strain. These bacteria were isolated from a layer chicken in Gangwon-do (Korea) in 2000, and the genetic type was identified by pulsed-field gel electrophoresis. The bacteria were grown at 37°C with shaking in Luria-Bertani (LB) broth (Difco, USA) or minimal E medium (1.66 mM MgSO₄, 9.5 mM citric acid monohydrate, 57 mM K₂HPO₄, and 16.7 mM NaNH₃PO₄) supplemented with 0.2% glucose (EG medium). The LB broth also contained 200 µg/mL ampicillin (AP), 50 µg/mL kanamycin (KM), and 20 µg/mL chloramphenicol (CM). S-nitrosoglutathione (GSNO) was synthesized via the reaction of glutathione and acidified sodium nitrite as previously described [1,12]. All chemicals were purchased from Sigma-Aldrich (USA) unless otherwise stated.

Construction of the SG mutants

The SG mutants were constructed using the λ Red recombinase system [7]. The primers used to generate mutations in the SG cells are listed in Table 2. To generate the deletion mutants *SGΔrpoS*, *SGΔhmp*, and *SGΔssrAB*, the primer pairs *rpoS*-P1/*rpoS*-P2, *hmp*-P1/*hmp*-P2, and *ssrA*-P2/*ssrB*-P1, respectively, were used. The primer pairs *rpoS*-Fw/*rpoS*-Rev, *hmp*-Fw/*hmp*-Rev, and *ssrA*-Fw/*ssrB*-Rev were used to confirm the mutations by PCR. To construct the triple mutant *SGΔ3*, the chloramphenicol resistance cassette in the *ssrAB*::Cm mutant was first removed using flippase (FLP) recombinase encoded in the pCP20 plasmid, resulting in an *ssrAB* mutant lacking chloramphenicol resistance. The *SGΔ3* mutants were then constructed by sequential transduction of *rpoS*::Km and *hmp*::Cm into the *ssrAB* mutant with removal of the antibiotic resistance cassette by FLP recombinase after each

Table 1. Bacterial strains and plasmids

Strain/plasmid	Description	Source or reference
Strains		
SG97	WT SG	[23]
SG97 <i>hmpA</i> ::KM	<i>SGΔhmpA</i>	Present study
SG97 <i>rpoS</i> ::Tn10dcm	<i>SGΔrpoS</i>	Present study
SG97 <i>ssrAB</i> ::CM	<i>SGΔssrAB</i>	Present study
SG97 <i>ssrAB hmpA rpoS</i> ::KM		Present study
SG97 <i>ssrAB hmpA rpoS</i>	<i>SGΔ3</i>	Present study
Plasmids		
pKD3: <i>cat</i> cassette	Template plasmid for amplification of a <i>cat</i> cassette flanked by FLP recognition sites (FRT; Amp ^R Cm ^R)	[7]
pKD4: <i>kan</i> cassette	Template plasmid for amplification of a <i>kan</i> cassette flanked by FLP recognition sites (FRT; Amp ^R Cm ^R)	[7]
pCP20: FLP recombinase	Temperate-sensitive (replicates at 30°C) plasmid bearing the FLP recombinase gene, (Cm ^R Amp ^R)	[7]

SG: *Salmonella enterica* Gallinarum, WT: wild type, KM: kanamycin, CM: chloramphenicol, FLP: flippase, FRT: flippase recognition target.

Table 2. Oligonucleotide primers used in the current study

Primer	Sequence (5'-3')
<i>rpoS</i> -P1	tttcgcgagcagcgccaggcctcaacctgaatctgacgggtaggctggagctgcttc
<i>rpoS</i> -P2	tgatttaaataagacgcggaatttgatgagaacggagtagacatagaatatcctccttag
<i>rpoS</i> -Fw	cagtgtcagcattgtctgta
<i>rpoS</i> -Rev	cagctctacaagcttgctt
<i>hmp</i> -P1	gcttgacgcacaacacatcgctacagtaaaggccaccattgtgtaggctggagctgcttc
<i>hmp</i> -P2	tgggtgcatctggctgcgcccaggctgactctttatgacatagaatatcctccttag
<i>hmp</i> -Fw	cataacgtaaagcagagaag
<i>hmp</i> -Rev	gctcgttaggcatgctttat
<i>ssrA</i> -P2	ttactggcatatttggtgtacacaatattaactgtgctgctgtagctggagctgcttc
<i>ssrB</i> -P1	acctcattctcgggcacagtaagtaactctgtcactttgtgtagctggagctgcttc
<i>ssrA</i> -Fw	caggcattatcattcgg
<i>ssrB</i> -Rev	ggattttgctgacgatgagca
<i>gyrB</i> -RT-Fw	tgactgccgtgctgcttt
<i>gyrB</i> -RT-Rev	agccaactctgaccgaagcc
<i>katE</i> -RT-Fw	ctgagccagcgtgacatcaa
<i>katE</i> -RT-Rev	gcgttcagcttgaactggg
<i>hmp</i> -RT-Fw	tggacgaaatgtcaacccc
<i>hmp</i> -RT-Rev	aatctcggctcccgatgaa
<i>sseA</i> -RT-Fw	gaggggaatgatgataaagaaa
<i>sseA</i> -RT-Rev	ggggcttgagcattaagtt
<i>ssaB</i> -RT-Fw	ggatatcagggccgaaggtg
<i>ssaB</i> -RT-Rev	aaatgcaagttaaagccaggtg
<i>sseJ</i> -RT-Fw	ctttaccaccaccatgcag
<i>sseJ</i> -RT-Rev	tgggcttgggatgtgatta

transduction.

Measurement of SG susceptibility to H₂O₂ and GSNO

Overnight cultures were added to the wells of a microtiter plate containing LB or EG medium (OD₆₀₀ = 0.02). H₂O₂ was added to LB medium as an oxidative agent, and the NO congener GSNO was added to EG medium. Bacterial growth at 37°C with shaking was monitored by measuring the OD₆₀₀ at 30-min intervals.

Quantification of SPI-2 gene transcription by real-time reverse transcription (RT)-PCR

The transcription of SPI-2 in SG was induced by reducing the extracellular Mg²⁺ concentration as previously described [8]. Briefly, *Salmonella* cells grown overnight in LB broth were washed once with high-Mg²⁺ N salts medium [5 mM KCl, 7.5 mM (NH₄)₂SO₄, 0.5 mM K₂SO₄, 1 mM KH₂PO₄, 38 mM glycerol, 0.2% casamino acid, 10 mM MgCl₂, and 100 mM Tris-HCl at pH 7.6], subcultured in fresh high-Mg²⁺ N salts

medium, and grown at 37°C to early log phase (OD₆₀₀ = 0.5). Next, the bacterial cells were harvested, resuspended in a low-Mg²⁺ (8 μM MgCl₂) N salts medium (pH 6.9) to induce SPI-2 expression or high-Mg²⁺ N salts medium as a control, and incubated for another 3 h. Transcription was stopped by adding a one-fifth volume of ice-cold phenol/ethanol (5% phenol in 95% ethanol) solution before harvesting the cells. Total RNA was extracted from the bacteria using RNAiso Plus reagent (Takara Bio, Japan). To measure the transcription levels of SPI-2, quantitative real-time RT-PCR was performed using a QuantiTect SYBR Green RT-PCR kit (Qiagen, Germany), as previously described [1]. DNA sequences of the primers used for this analysis are listed in Table 2.

Measurement of NO consumption rate

Bacteria were grown in 10 mL LB broth at 37°C with shaking to OD₆₀₀ ~ 1.0. The harvested cells were washed once with phosphate-buffered saline (PBS) and resuspended in 10 mL PBS prewarmed to 37°C. The bacterial culture was continuously stirred with a magnetic stirring bar. The rate of NO consumption was determined by measuring the NO remaining after addition of 2 μM PROLI NONOate (Cayman Chemical, USA) to the bacterial solution. The concentration of NO in the solution was measured using a NO-sensitive electrode with a 2-mm diameter tip (ISO-NOP sensor; World Precision Instruments, USA) connected to a free radical analyzer (TBR4100; World Precision Instruments). The data signal was obtained using the LabChart program and expressed in picoamperes.

Assay for the triple mutant virulence in chickens

Three groups of 2-week-old Lohmann layer chickens were used for protocols approved by the Kangwon University Institutional Animal Care and Use Committee (permit no. KW-130924-2). To determine virulence of the triple mutant, groups of five chickens were housed separately and inoculated orally with 500 μL containing 1 × 10⁶ or 1 × 10⁸ colony-forming units (CFUs) of SGΔ3 or 500 μL containing 1 × 10⁸ CFUs of wild-type (WT) SG. Each group of chickens was monitored for 2 weeks to measure mortality. At the end of each experiment, all the surviving chickens were humanely sacrificed, and the livers and spleens were collected. The tissue samples were homogenized with a tissue lyser (Qiagen) in peptone-buffered saline. Diluted homogenates were used to inoculate *Shigella-Salmonella* agar. After incubating at 37°C for 24 h, the CFUs were counted.

Vaccination with SGΔ3 and protection against SG challenge

To evaluate the protection conferred by the triple mutant against SG infection, 2-week-old Lohmann layer chickens were divided into five groups. Two groups (n = five birds in each group) were primed and boosted at 2 and 4 weeks of age either orally or subcutaneously (SC) with 500 μL of a suspension

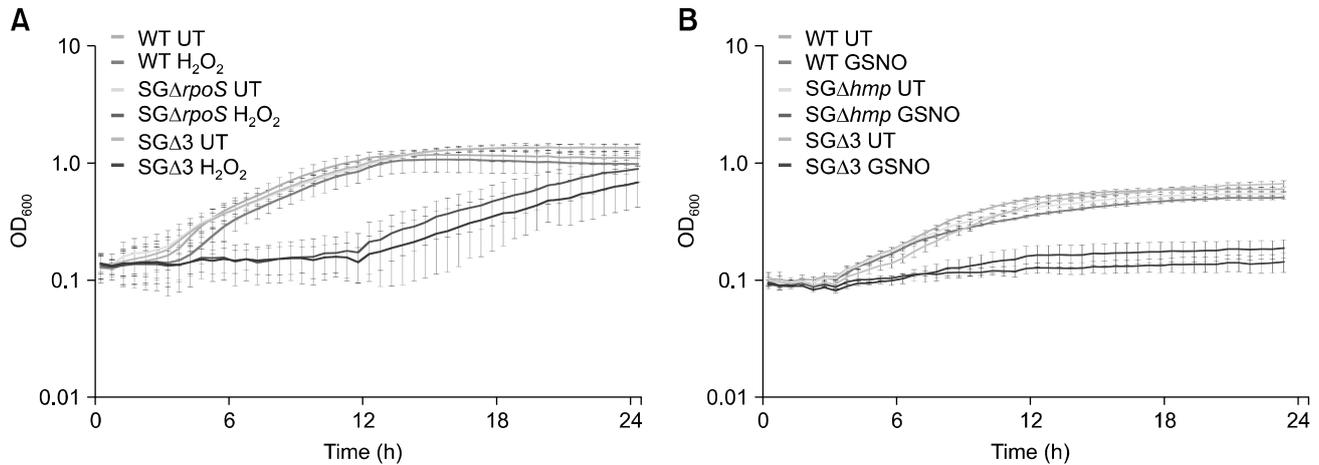


Fig. 1. Susceptibility of SG strains to H_2O_2 or GSNO. Bacterial cells were (A) grown in microtiter plates containing LB broth in the presence (closed symbols) or absence (open symbols) of $250 \mu M H_2O_2$, and (B) cultured in a microtiter plate containing EG media with (closed symbols) or without (open symbols) $1 mM GSNO$. OD_{600} of the bacteria cultured in the microtiter plates was measured for 24 h at $37^\circ C$ with vigorous agitation. Data shown are the mean values \pm standard deviation (SD) of three independent cultures. UT: untreated, H_2O_2 : H_2O_2 -treated, GSNO: S-nitrosoglutathione-treated.

containing 1×10^8 CFUs of SG $\Delta 3$. Another two groups ($n =$ five birds each group) were primed and boosted at 2 and 4 weeks of age either orally or SC with $500 \mu L$ of a suspension containing 1×10^8 CFUs of the SG9R vaccine strain. The negative control group was not vaccinated. At 6 weeks of age, all groups were challenged orally with $500 \mu L$ of a suspension containing 4×10^8 CFUs of WT SG. Mortality was assessed daily for 14 days post-challenge (dpc). All surviving chickens were euthanized at 14 dpc, and the livers and spleens were collected to measure the CFU counts.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, USA). Differences were analyzed using an unpaired two-tailed Student's *t*-test. *P* values < 0.05 were considered significant.

Results

RpoS is required for SG resistance against oxidative stress

To examine the role of RpoS in the resistance of SG to oxidative stress, we compared the growth of SG $\Delta rpoS$ and SG $\Delta 3$ mutants to that of parental WT SG in LB media containing H_2O_2 . As an uncharged molecule, H_2O_2 can freely diffuse through the bacterial cell membrane and damage various macromolecules. Because high iron levels further increase the cytotoxicity of H_2O_2 by producing hydroxyl radicals via Fenton chemistry, we used iron-rich LB media. Growth of the SG mutant strains lacking RpoS was temporally inhibited by H_2O_2 while that of the WT SG was slightly delayed (panel A in Fig. 1). When the SG genome was sequenced, it was found to contain a

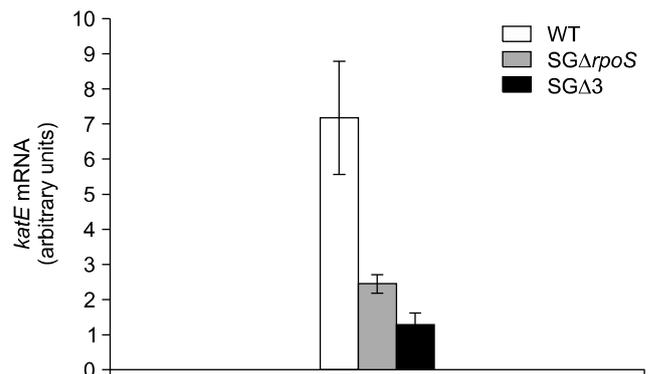


Fig. 2. RpoS-dependent *katE* transcription in SG. Bacterial cells cultured to log phase in LB media were treated with H_2O_2 ($1 mM$) for 30 min. The mRNA levels of *katE* were measured by quantitative real-time RT-PCR. The values were normalized relative to mRNA levels of a housekeeping gene, *gyrB*. Data shown are the mean values \pm SD of three independent experiments.

conserved *katE* gene encoding a catalase. Quantitative real-time RT-PCR analysis showed that *katE* transcription was reduced by 3- to 7-fold in SG strains containing the *rpoS* mutation (Fig. 2). These data clearly indicate that SG has conserved RpoS-dependent gene regulation that plays an important role in the antioxidant defense of the bacteria.

Flavo-hemoglobin Hmp of SG metabolizes NO and promotes SG replication under nitrosative stress

To evaluate the role of flavo-hemoglobin Hmp in the resistance of SG to nitrosative stress, we measured the NO consumption

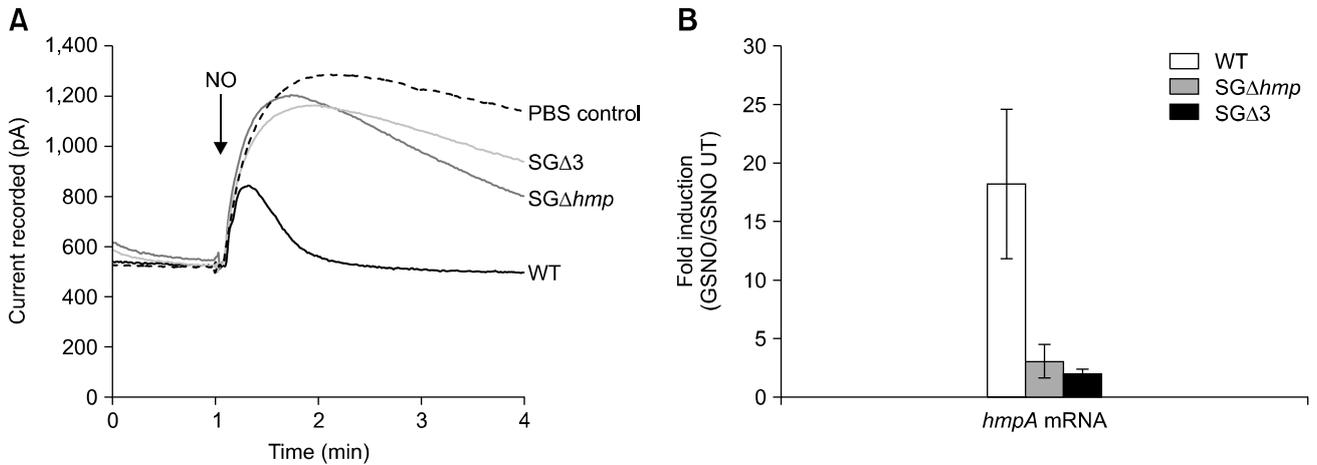


Fig. 3. Role of Hmp in nitric oxide (NO) detoxification of SG. (A) NO consumption of the SG strains was recorded by measuring the NO remaining in PBS containing bacteria after the addition of a fast-releasing NO donor, ProliNONOate (2 μ M). A NO-sensitive electrode connected to a free radical analyzer was used to measure NO levels as described in the Materials and Methods section. Data are representative of three independent experiments. (B) WT SG cells were cultured in EG media until OD₆₀₀ = 0.5. Bacterial transcription was then stopped by adding phenol/ethanol solution to the culture before and 1 h after treatment with GSNO (1 mM). Measured values were normalized relative to mRNA levels of the housekeeping gene *gyrB*. Data shown are mean fold increases \pm SD calculated as the ratio of *hmpA* mRNA levels before and after GSNO treatment for three independent experiments.

rate of WT and *hmp* mutant SG. After NO was added to the bacterial cultures, WT SG consumed almost all the NO within 1 min. In contrast, the *hmp* mutant strains barely metabolized NO, maintaining NO levels comparable to that of the buffer control. These data indicate that flavohemoglobin Hmp metabolizes almost all NO that enters into the SG cell (panel A in Fig. 3). Real-time RT-PCR showed that NO treatment increased the level of *hmp* transcription in WT SG up to 20-fold (panel B in Fig. 3), suggesting that SG has a regulatory system that controls Hmp expression in response to NO. Moreover, the growth rate of WT and *hmp* mutant SG in NO-producing cultures also indicated the importance of Hmp for resistance to nitrosative stress (panel B in Fig. 1). Collectively, these observations demonstrated that flavohemoglobin Hmp is required for SG defense against NO-mediated nitrosative stress.

SsrAB activates SPI-2 gene transcription in SG

To explore the regulation of SPI-2 gene expression in SG, we measured the transcription of *sseJ* and *ssaB*, which encode effector proteins, and *sseA* that encodes chaperones of the SPI-2 T3SS. Transcription levels of *sseA*, *ssaB*, and *sseJ* in SG cultures shifted from high to low Mg concentrations were 3.5, 6, and 5 times higher, respectively, compared to levels observed in SG cultured continuously grown in high-Mg media (Fig. 4). The transcription of *sseA*, *ssaB*, and *sseJ* was clearly abolished in the *ssrAB* mutant, demonstrating that existence of the SPI-2 T3SS in SG is subject to SsrA/SsrB-dependent regulation.

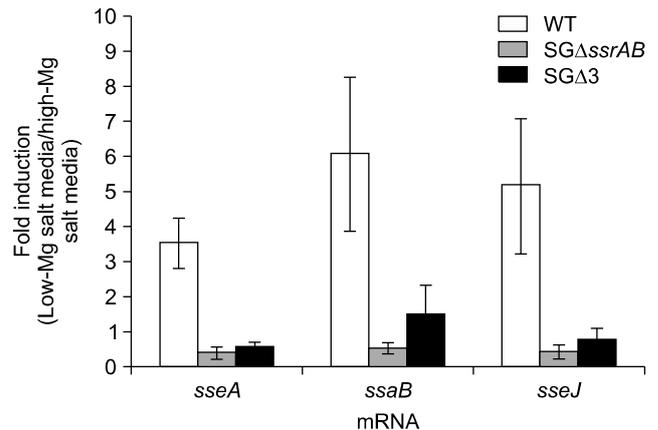


Fig. 4. SPI-2 gene transcription in SG. mRNA levels of *sseJ*, *ssaB*, and *sseA* were measured using total RNA isolated from SG strains cultured under SPI-2-inducing conditions as described in the Materials and Methods section. Measured values were normalized relative to mRNA levels of the housekeeping gene *gyrB*. Data shown are mean fold increases \pm SD calculated as the ratio of mRNA levels before and after the media change for three independent experiments.

Mutant phenotypes

As shown in Fig. 1, the growth curves of SG mutants cultured without any additional stressors were very similar to that of WT SG. All strains showed a 2-h lag phase and reached a stationary phase within 12 h in LB broth. These results indicated that the growth of SG $\Delta 3$ was not defective. All mutants containing an *rpoS* mutation had almost the same level of susceptibility to

H₂O₂. Additionally, all *hmp* mutants were unable to metabolize NO and showed the same susceptibility to nitrosative stress. Moreover, *SsrAB*-dependent SPI-2 gene regulation in SGΔ3

cells was comparable to that in the SGΔ*SsrAB* mutant. These data demonstrate that the phenotypes of each deletion mutant were observed in SGΔ3 cells.

Assay of the triple mutant virulence in chickens

Virulence testing was performed by inoculating Lohmann layer chickens orally with SGΔ3 and WT SG. One chicken infected with WT SG died 7 days post-inoculation while no deaths occurred in the SGΔ3 group. All the surviving birds were sacrificed 2 weeks post-inoculation, and the numbers of viable bacteria in the liver and spleen were determined (Fig. 5). The number of CFUs isolated from the liver and spleen of chickens inoculated with SGΔ3 (low and high doses) was significantly lower than that isolated from chickens inoculated with WT SG. Bacterial counts for the liver and spleen were also significantly different between chickens inoculated with low and high doses of SGΔ3. The mutant did not induce splenomegaly that was evident in the group inoculated with WT SG (data not shown). These results indicate that virulence of the SGΔ3 mutant was attenuated compared to WT SG.

Level of protection conferred by the SG mutants against challenge with highly virulent SG

Vaccinated chickens were challenged with SG and observed for 2 weeks. In nonvaccinated chickens, 60% mortality was observed from 7 ~ 14 dpc (Fig. 6). Chickens immunized (both

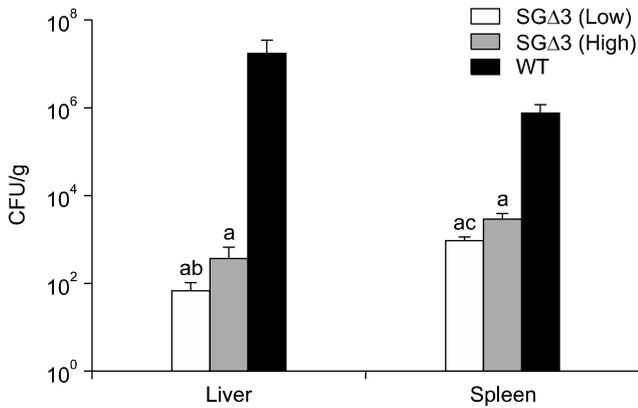


Fig. 5. Attenuation of SGΔ3 in Lohmann layer chickens. The birds were inoculated orally with 1 × 10⁶ (low) or 1 × 10⁸ (high) CFUs of SGΔ3 or 1 × 10⁸ CFUs of WT SG at 2 weeks of age. Bacterial counts in the livers and spleens at 2 weeks post-inoculation are shown as mean values ± SDs of log₁₀ CFU/g for each organ. ^aIndicates a significant difference (*p* < 0.0001) between the mutant and WT SG. ^{b,c}Indicate a significant difference (*p* = 0.0008 and *p* = 0.008) between low and high doses of the SGΔ3 mutant.

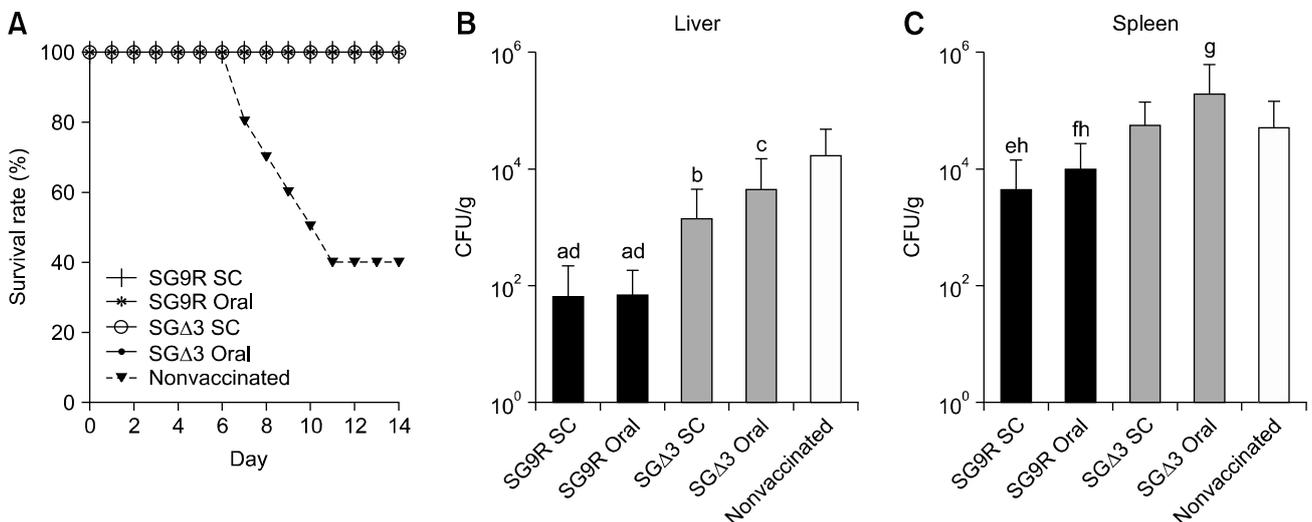


Fig. 6. Protection conferred by SGΔ3 in Lohmann layer chickens. (A) Survival curve for the groups of chickens vaccinated as described in the Materials and Methods section. The chickens were challenged orally at 6 weeks of age with 4 × 10⁸ CFUs of WT SG and observed for 14 days post-challenge (dpc). Bacterial counts in the livers (B) or spleens (C) at 14 dpc are shown as the mean values ± SD of log₁₀ CFU/g for each organ. In the nonvaccinated group, the CFU count was determined for the surviving chickens at 14 dpc. ^aIndicates a significant difference (*p* < 0.0001) between SG9R-vaccinated and nonvaccinated chickens. ^{b,c}Indicate a significant difference (*p* = 0.0005 and *p* = 0.03) between SGΔ3-vaccinated and nonvaccinated chickens. ^dIndicates a significant difference (*p* < 0.0001) between SG9R-vaccinated and SGΔ3-vaccinated chickens. ^{e,f}Indicate a significant difference (*p* = 0.0008 and *p* < 0.01) between SG9R-vaccinated and nonvaccinated chickens. ^gIndicates a significant difference (*p* = 0.02) between SGΔ3-vaccinated and nonvaccinated chickens. ^hIndicates a significant difference (*p* < 0.001) between SG9R-vaccinated and SGΔ3-vaccinated chickens.

SC and orally) with SG Δ 3 and SG9R were completely protected from subsequent WT SG challenge. No deaths were observed in these groups. This finding suggests that the SG Δ 3 mutant conferred a level of protection similar to that conferred by SG9R.

At 14 dpc, all the surviving chickens were sacrificed, and the SG counts for the liver and spleen were determined. In both organs, the number of CFUs in chickens vaccinated (either SC or orally) with SG Δ 3 or SG9R was significantly lower than in the nonvaccinated group. However, the CFU counts for the SG Δ 3-vaccinated groups were lower than those for the nonvaccinated group, but higher than that for the SG9R-vaccinated group.

Discussion

In the present study, deletion mutants were constructed to determine the contribution of *rpoS*, *hmp*, and *ssrAB* to the pathogenesis of SG. We also evaluated the ability of the SG Δ 3 mutant (SG Δ *rpoS* Δ *hmp* Δ *ssrAB*) to protect layer chickens against SG infection. Different *Salmonella* serovars have evolved to survive and adapt to host-specific niches through either the acquisition or loss of genes and gene functions (pseudogenes) [10]. Since different virulence factors are utilized depending on the host, pathogenesis and disease manifestation also vary among serovars. Thus, the functions of virulence genes cannot necessarily be extrapolated across different serovars. RpoS is known to act as an alternative sigma factor for genes associated with general stress resistance and virulence [15]. The *katE* gene (encoding catalase hydroperoxidase II [HPHII]), which is involved in the resistance to H₂O₂, is under the control of RpoS. Consistent with previous reports, the SG Δ *rpoS* mutant in this study appeared susceptible to H₂O₂ stress [1,20]. Furthermore, real-time RT-PCR results revealed that the H₂O₂-sensitive phenotype resulting from the *rpoS* deletion was associated with a failure to induce the expression of KatE catalase. In host phagocytic cells, NO is generated by enzyme-inducible NO synthase. NO is a precursor for a range of reactive nitrogen species that have potent cytotoxic and/or cytostatic activities against bacteria [11]. Flavohemoglobin Hmp has been reported to play a crucial role in the protection of ST against toxic effects of NO. As observed in a previous study on ST [2], the SG Δ *hmp* mutant failed to decompose NO and its growth was severely impaired in NO-producing media compared to WT SG. Nitrosative stress also upregulated *hmp* expression in WT SG.

The SPI chromosomal regions encode important *Salmonella* virulence factors [25]. Among these, SPI-2 has been reported to be essential for the systemic virulence of *Salmonella*. The T3SS encoded by SPI-2 mediates the translocation of a large number of effector proteins into eukaryotic target cells. The translocated effector proteins modify normal host cell functions and permit

intracellular *Salmonella* proliferation. Consistent with previous studies, findings from the present investigation showed that the expression of SPI-2 virulence genes in SG is regulated by *ssrAB* [5,17,25]. Overall, these results establish the roles of *rpoS*, *hmp*, and *ssrAB* in the pathogenesis of SG infection.

To develop a highly attenuated and safe SG vaccine strain, an SG Δ 3 mutant with three gene deletions (SG Δ *rpoS* Δ *hmp* Δ *ssrAB*) was constructed. This strain should 1) be highly susceptible to the toxic effects of oxidative and nitrosative stresses, 2) have defective production of virulence factors, 3) less likely than SG9R to revert to a virulent phenotype, and 4) be highly susceptible to various environmental stresses. SG Δ 3 retained the defects observed for each individual gene deletion. Virulence of the SG Δ 3 mutant was successfully attenuated as it was unable to cause mortality in chickens even when the birds were inoculated with 10⁸ bacteria, and significantly fewer *Salmonella* CFUs were recovered from the liver and spleen compared to WT SG. This observation is in accordance with previous reports on the deletion of *rpoS*, *hmp*, and *ssrAB* genes in ST [2,5,6,9]. In another previous study of a gene mutation affecting the structure of the SPI-2 T3SS (Δ *ssaU*), failure to recover bacteria from the liver and spleen was reported [14]. The discrepant results observed were possibly due to 1) a difference in the virulence of SG Δ *ssaU*, 2) different breed of chicken used, 3) different age at the time of inoculation, or 4) a weaker effect of the *ssrAB* mutation on reducing SPI-2 expression than the combined effect of the mutations in individual genes of SPI-2. Immunization with SG Δ 3 offered efficient *in vivo* protection against challenge with WT SG while 60% mortality was observed in the nonvaccinated group, indicating that SG Δ 3 is an attractive live vaccine candidate. However, more studies on the safety, efficacy, and immunogenicity of the SG Δ 3 mutant in different breeds and/or age groups of chickens are necessary.

In conclusion, in the present study we validated the importance of *rpoS*, *hmp*, and *ssrAB* in SG. The ability of the SG Δ 3 mutant to confer protection against SG infection was evaluated in layer chickens. The results showed that SG Δ 3 is attenuated and provides efficient protection against WT SG challenge.

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Conflict of Interest

There is no conflict of interest.

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