Vet. Res. (2010) 41:59
DOI: 10.1051/vetres/2010031

www.vetres.org

Original article

Physiology, pathogenicity and immunogenicity of *lon* and/or *cpxR* deleted mutants of *Salmonella* Gallinarum as vaccine candidates for fowl typhoid

Kiku MATSUDA[1], Atul A. CHAUDHARI[1], Sam Woong KIM[2], Kyeong Min LEE[1], John Hwa LEE[1*]

[1] College of Veterinary Medicine and Bio-Safety Research Institute, Chonbuk National University, Jeonju, 561-756, Republic of Korea
[2] Swine Science & Technology Center, Jinju National University, 150 Chilam-dong, Jinju, Gyeongnam, 660-758, Republic of Korea

(Received 8 January 2010; accepted 20 May 2010)

Abstract – To construct a novel live vaccine candidate for fowl typhoid (FT) caused by *Salmonella* Gallinarum (SG), the *lon* and *cpxR* genes that are related to host-pathogen interaction were deleted from a wild type SG using the allelic exchange method. The mutants were grown normally, as was the wild type. The biochemical properties of the mutants remained very similar to those of the wild-type, while JOL914 (Δ*lon*) and JOL916 (Δ*lon*Δ*cpxR*) were mucoid. Extracellular polysaccharide increased 30.6-, 1.3-, and 46.2-fold in JOL914, JOL915 (Δ*cpxR*), and JOL916, respectively. Dot-blot analysis demonstrated significant increases of FimA expression at 6.77-, 2.33-, and 3.90-fold for JOL914, JOL915, and JOL916, respectively. Internalizations of JOL914, JOL915, and JOL916, in chicken abdominal macrophages, were increased at 4.65-, 0.50-, and 2.72-fold, respectively. Virulences of JOL914, JOL915 and JOL916, analyzed by LD_{50} using 1-week-old chickens, were attenuated approximately at 10^1-, 10^1-, and > 10^3-fold, respectively. The oral inoculations of 2×10^7 cfu of the wild type, JOL914, JOL915 and JOL916 caused 55.6, 16.7, 22.2, and 0.0% mortality, respectively. Significantly moderate gross lesions of the liver and spleen were observed in the JOL916 group compared to the other groups. An induced immune response and significant peripheral mononuclear proliferation reaction were observed in the JOL916 group. At the protection against the wild type challenge, JOL916 offered 100% protection. Thus, the results of this study suggest that JOL916 among the mutants studied represented the safest and most effective live vaccine candidate against FT.

***S.* Gallinarum / vaccine / attenuation / virulence gene / immune response**

1. INTRODUCTION

Fowl typhoid (FT) is an acute disease causing septicemia in poultry; it is caused by *Salmonella enterica* serovar Gallinarum biovar Gallinarum (SG) [27]. Even though the causative agent has adapted to its avian host and rarely induces food poisoning in humans, vigilant control of the poultry industry has been instituted worldwide to prevent economic loses due to FT outbreaks; eradication of this disease in some countries has been extremely difficult [1, 3, 42]. Vaccination with a rough mutant strain referred to as 9R, for the control of FT, was initially reported about half a century ago [36]. However, residual virulence has been observed and the protection provided has been

* Corresponding author: johnhlee@jbnu.ac.kr

insufficient [6]. Alternative vaccine strategies including a number of live vaccine candidates have been investigated during the last two decades. In contrast to the genetically undefined 9R strain, genetically defined mutant strains of genes encoding metabolic pathways have been studied, and a degree of attenuation of virulence and protection against FT has been reported. These disrupted mutants include the following: *aroA* mutant [14], *nuoG* mutant [46], *metC* mutant [35], and the *crp* mutant of SG [33]. However, there are no trials evaluating the mutants of host-pathogen interaction-related genes in SG. This is because the genetic basis of SG virulence and the molecular mechanisms involved in systemic infection and the development of FT are relatively unknown, while the pathogenic mechanisms are supposed to be different from non-host adapted serotypes such as *Salmonella* Typhimurium (ST) [16, 17, 34].

In ST, deletion of *lon* and/or *cpxR* genes has been recently reported [20, 23, 37, 38]. The intracellular protease Lon represents four measure energy-dependent proteases, known as stress-induced proteins in bacteria. The importance of Lon has been shown not only for the regulation of various physiological activities but also in the pathogenesis of a number of bacteria [40]. In ST, *lon* has been shown to negatively regulate *Salmonella* pathogenicity island (SPI) 1. In addition, the *lon* mutant of ST has shown satisfactory attenuation of virulence and induction of protective immunity in mice [23, 38]. Recently, the *lon* mutant of ST was shown to increase the synthesis of fimbriae as well as capsular polysaccharides, suggesting alteration of attachment and invasion capabilities [20]. The CpxAR is known as a two-component regulator system that governs one of the major response pathways of changes in the cell envelope of bacteria. Activated CpxR controls part of the envelope stress response system, the pilus assembly, type III secretion system (TTSS), motility and chemotaxis, adherence, and biofilm development [44]. In a number of gram-negative bacteria, this pathway has been shown to be involved in virulence gene expression at the early stage of infection such as with *hilA* in ST [25]. Recent research has shown that the deletion of *cpxR* does not alter extracellular polysaccharide (EPS) production; however, it increases the expression of fimbriae in ST [20].

In this study SG mutants were constructed with deletions of the *lon* and/or *cpxR* genes to determine the efficacy of the mutants for use as live vaccine candidates for the prevention of FT. The physiological features related to attachment and invasion mechanisms were investigated, and the pathogenicity and immunogenicity after inoculation of the mutants in susceptible chickens were studied. The results showed satisfactory attenuation of SG virulence with deletion of *lon*/*cpxR* and significant specific immune responses and protection against wild type challenge.

2. MATERIALS AND METHODS

2.1. Bacterial strains and genetic manipulation

Construction of deletion mutants was performed by the allelic exchange method using the suicide vector pMEG-375 as described previously. The wild-type SG JOL394 chromosomal DNA was used as a template when the left and right arms of the *lon* and *cpxR* genes were amplified by the polymerase chain reactions (PCR) and as host cells of conjugational transfer of recombinant suicide vectors [18, 20]. After conjugational transfer followed by antibiotic and sucrose sensitivity counter selection, the selected colonies were examined by PCR for the *lon* amd *cpxR* gene deletions, and designated as JOL914 and JOL915, respectively. For confirmation of the *lon* and *cpxR* genotype, primer sets of 5′-CAGGAGTTCTTACAGGTAGA-3′/5′-CCACACTCCGCTGTAGGTGA-3′ and 5′-CATCATCTGCGGGTTGCAGC-3′/5′-GATAATTTACCGTTAACGAC-3′ were constructed, respectively. Using the same conjugational procedures and selection, deletion of *cpxR* was introduced into JOL914, and designated as JOL916.

2.2. Phenotype of mutant strains

2.2.1. Colony morphology and growth curves

Growth curves were determined by adding 1/100 volume of overnight culture into 200 mL of LB broth (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and incubating at 37 °C with shaking at 250 rpm. The optical density at 600 nm (OD_{600})

was determined every 1.5 h for 9.5 h. The colony morphologies of each strain streaked on LB agars (Becton, Dickinson and Company) were assessed after 24 h incubation at 37 °C.

2.2.2. Biotype of mutant strains

The biochemical phenotypes were analyzed with the API 20E system (bioMérieux, Rhône, France). The cells were grown for 24 h at 37 °C on LB agar suspended in 0.85% sodium chloride solution and processed as per the instructions provided by the manufacturer.

2.2.3. Extra-cellular polysaccharides

Fluorometric quantification of EPS by the concanavalin A (Con-A) binding assay was performed [32]. Briefly, the cells grown on LB agar for 24 h at 37 °C were suspended in phosphate-buffered saline solution (PBS) at an OD_{600} of 0.5. Fluorescein isothiocyanate-conjugated Con-A (Sigma-Aldrich, St. Louis, MO, USA) was added at 4 μg/mL, incubated for 30 min, and washed twice with PBS. Finally, 200 μL of suspension was transferred into a 96-well microplate (Berthold technologies GmbH & Co. KG, Bad Wildbad, Germany). Fluorescence intensity was recorded with TriStarLB941 (Berthold technologies GmbH & Co. KG). Normalization was performed with the viable cell number obtained by colony count.

2.2.4. Colonies with FimA expression

Relative expression of FimA was compared between strains. The *FimA* gene was cloned from a ST wild isolate to pET28a using a primer set of 5′-GGATCCGCTGATCCTACTCCGGTGAG-3′, 5′-CTCGAGTTCGTATTTCATGATAAAGG-3′ to over-express the 6 × His-tag attached protein, which was purified using Ni-NTA agarose (Peptron, Daejon, South Korea) and inoculated into rabbits to obtain anti-FimA serum. The colonies grown on LB agar for 24 h at 37 °C were suspended in PBS at an OD_{600} of 0.5, and diluted at 1:100. The concentration of each suspension was determined by the colony count method for normalization. A piece of Protran® nitrocellulose membrane with a pore size of 0.2 μm (Schleicher & Schuell, Dassel, Germany), which was immersed in Tris-buffered saline (TBS, 10 mM Tris, 0.9% sodium chloride, pH 7.4) was set in a 96-well Bio-Dot™ apparatus (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's instructions.

2.3. Invasion of chicken macrophages

Sephadex-elicited abdominal exudate cells were harvested from six female Brown Nick layer chickens at 17 weeks of age using the methods reported by Qureshi et al. [28]. Invasion analysis was performed in triplicate with minor modification of the previously reported methods [15, 17]. Overnight cultures of SG strains were inoculated at a multiplicity of infection (m.o.i.) of 10 in RPMI-1640 medium supplemented with 10% fetal calf serum and 2 mM L-glutamine but without antibiotics in a shaking incubator at 37 °C. The cells were incubated in the presence of SG strains for 1 h at 40 °C, followed by incubation in RPMI medium containing 100 μg/mL of gentamicin sulfate for 30 min at 37 °C. The sensitivity of each strain against gentamicin was preliminarily tested. The estimation of bacterial invasion was made by lysing the adherent cells with 100 μL of 1% Triton X in PBS and then plating the lysate onto LB agar at adequate dilutions.

2.4. Determination of 50% lethal doses (LD_{50}) in chickens

The animal experiments in this study were conducted with the approval of the Chonbuk National University Animal Ethics Committee in accordance with the guidelines of the Korean Council on Animal Care. Five hundred female 1-week-old Brown Nick chickens, divided into 25 groups (n = 20), were orally inoculated with bacterial suspensions at adequate concentrations. The mortality rates were monitored every day for 3 weeks. The cause of death was confirmed by evaluation of the gross lesions and bacterial recovery. On the third week, all remaining chickens were sacrificed and bacteriological examination was performed to diagnose bacteremia using a direct stamp of the liver on brilliant green agar (BGA, Becton, Dickinson and Company). The method described by Reed and Muench was used for the LD_{50} [31].

2.5. Pathogenicity and immunogenicity of the mutants in chickens

2.5.1. Bacterial inoculation into animals

Ninety female 1-week-old Brown Nick chickens, divided into five groups (n = 18), provided water and antibiotic-free food *ad libitum*, were inoculated via the oral route with 100 μL of bacterial suspension containing 2×10^7 cfu of the overnight culture in

LB broth with continuous shaking. The control group was inoculated with PBS.

2.5.2. Observations of mortality, gross lesions and recovery after inoculation

After inoculation, the mortality rate was assessed daily for 2 weeks. After the apparent recovery of all remaining animals, on the second week post immunization (wpi), five randomly selected animals, per group, were sacrificed. The gross lesions of enlarged and necrotic foci of the liver and spleen were given scores of: 0, 1, 2, or 3 for each lesion. A score of 0 indicated no lesion and the higher scores were associated with more severe lesions. For bacterial recovery, a sample of the liver and spleen was minced in buffered peptone water (Becton, Dickinson and Company), and the homogenate was inoculated on BGA for enumeration and incubated overnight at 37 °C with continuous shaking, followed by culture in Rappaport-Vassiliadis R10 broth (Becton, Dickinson and Company) for 48 h at 42 °C. A loop of the enrichment broth was streaked onto BGA and the *Salmonella*-type colonies were examined after incubation at 37 °C for 24 h.

2.5.3. Peripheral lymphocyte proliferation assay

The lymphocyte proliferation assay was carried out according to Rana et al. to evaluate the cell-mediated immunity in the immunized groups [29]. Soluble antigen was prepared from the SG wild-type strain JOL394 [29]. Ten days after the immunization, the peripheral lymphocytes were separated from five randomly selected chickens per group using the gentle swirl technique [13]. After trypan blue dye exclusion testing, 100 μL of viable mononuclear cell suspension at 1×10^5 cfu/mL in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 U/mL of penicillin, 50 μg/mL of streptomycin, and 2 μg/mL of fungizone were incubated in triplicate in 96-well tissue culture plates with 50 μL of medium alone or medium containing 4 μg/mL of soluble antigen at 40 °C, in a humidified 5% CO_2 atmosphere for 72 h. The proliferation of stimulated lymphocytes was measured using adenosine triphosphate (ATP) bioluminescence as the marker of cell viability with the ViaLight® Plus Kit (Lonza Rockland, ME, USA), which provides an estimation of mitochondrial activity [9], according to the product information. The emitted light intensity was measured using a luminometer (TriStarLB941, Berthold thechnologies GmbH & Co.) with an integrated program for one second duration. The blastogenic response against specific antigen was expressed as the mean stimulation index (SI) as previously reported [29].

2.5.4. Plasma IgG concentration

Indirect ELISA was performed with an outer membrane protein fraction (OMP) that was extracted from the JOL394 strain [19]. The plasma sample was separated by centrifugation of the peripheral blood. ELISA was performed using chicken IgG ELISA Quantitation Kits (Bethyl laboratories, Montgomery, TX, USA) according to the product information. Wells of Microlon® ELISA plate (Greiner Bio-One GmbH, Frickenhausen, Germany) were coated with 100 μL of OMP at a concentration of 0.2 mg/mL. The wells were reacted with plasma at 1:250 dilution for 1 h, followed by reaction with goat anti-chicken IgG-horseradish peroxidase (HRP)-conjugate at 1:100 000 dilutions for 1 h. The bound HRP activity was determined using *o*-Phenylenediamine dihydrochloride (Sigma-Aldrich). The OD_{492} was determined with an ELISA reader after the reaction was stopped with 3 M H_2SO_4.

2.6. Protection against challenge

Thirty female 1-week-old Brown Nick chickens, divided into two groups ($n = 15$), provided water and antibiotic-free food *ad libitum*, were inoculated via the oral route with 100 μL of bacterial suspension containing 2×10^7 cfu of the overnight culture of JOL916 in LB broth with continuous shaking. The control group was inoculated with PBS. At the third week, chickens were orally challenged with a wild type SG JOL394 at the dose of 2×10^6 cfu per chicken. Animals were daily observed for depression and mortality during 2 weeks, at which time all the chickens showed symptomatic recovery. Parameters used for evaluation of depression included loss of appetite, closed eyes, and lack of response to external stimuli. Each animal was scored on a scale of 0 (no depression) to 3 (severe depression), and for each group the mean depression score was calculated.

2.7. Statistical analysis

All data are expressed as means ± SEM unless otherwise specified. The analyses were performed with SPSS 16.0 (SPSS Inc., Chicago, IL, USA). The Student's *t*-test was used to analyze the in vitro experimental data of mutant strains compared to JOL394. The Mann–Whitney *U* test was used for analysis of significant differences in the gross lesion

scores and immune responses of the immunized groups compared to the immunized group with JOL394 and the unimmunized control group.

3. RESULTS

3.1. Constructions of mutant strains

Successful deletions of the *lon* gene in JOL914, of the *cpxR* gene in JOL915, and of both genes in JOL916 were confirmed by PCR with the expected amplicon sizes of 3.88 kb and 1.54 kb for the intact wild and deleted mutant of the *lon* gene, respectively, and the 2.71 kb and 2.04 kb for the intact wild and deleted mutant of the *cpxR* gene.

3.2. Phenotypes of mutant strains

The growth conditions of the mutants were examined using growth curves. The growth curves of the mutants in the LB broth under high-oxygen conditions were fundamentally similar to the wild type, while the mutants were slightly impeded at entering the exponential phase (data not shown). Both wild-type and mutant strains reached exponential and stationary phases after 1.5 h and 9.5 h from initiation, respectively. Colony morphology on the LB agar of JOL394 was white, round and small. JOL914 and JOL916 were mucoid compared to the wild type, while the colony appearance of JOL915 did not differ from that of JOL394. The biochemical phenotypes of the mutant strains examined by API 20E were almost consistent with the wild type, except for glucose fermentation of JOL915: the wild type and the other mutants did not perform glucose fermentation, but JOL915 did. Interestingly, the double mutant JOL916 recovered the wild type phenotype to offset the JOL915 property. The EPS per cell of the 24-h culture on LB agar of JOL914, JOL915, and JOL916 showed 30.6-, 1.3-, and 46.2-fold increases, respectively, compared to the wild type JOL394 (Fig. 1A). To investigate the fimbrial expression of the mutants, 24-h cultures of the mutants and wild type were examined on the LB agar by dot-blot analysis. Significant FimA

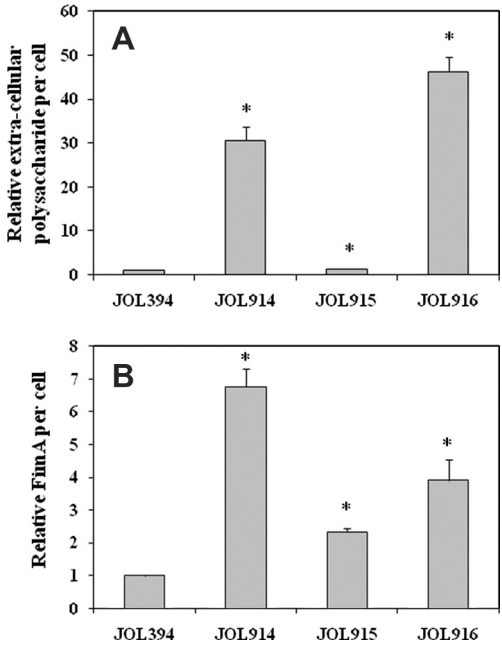

Figure 1. Phenotype examination of *Salmonella* Gallinarum wild type strain (JOL394), Δ*lon* (JOL914), Δ*cpxR* (JOL915), and Δ*lon*Δ*cpxR* (JOL916) mutant strains: A, relative extra-cellular polysaccharide of 24-h culture on LB agar examined by concanavalin-A binding fluorometric assay; B, relative FimA expression of 24-h culture on LB agar by dot-blot analysis. Error bars indicate the SEM. * Significant difference of mutants from the parental strain JOL394 ($p < 0.05$).

expression was detected at 6.77-, 2.33-, and 3.90-fold increases of JOL914, JOL915, and JOL916 compared to the wild type, respectively ($p < 0.05$, Fig. 1B).

3.3. Internalization in macrophages

To examine whether the gene deletions had an effect on chicken macrophage internalization, the SG mutants were inoculated into chicken abdominal macrophages. All of the SG strains were internalized by the chicken abdominal macrophages. Viable cell counts were demonstrated at 3.32 ± 0.03, 4.05 ± 0.02, 3.07 ± 0.09, and 3.81 ± 0.03 cfu/mL on a $\log_{10}$ scale, which were 4.65- ($p < 0.001$), 0.50- ($p = 0.057$), and 2.72-fold ($p < 0.001$)

Table I. Internalization of the *Salmonella* Gallinarum wild type and *lon*, *cpxR*, and *lon*/*cpxR* mutants in the chicken abdominal macrophages determined by the gentamicin protection assay.

Strain	Genotype	Internalization (Log_{10} cfu/mL)[a]
JOL394	Wild type	3.32 ± 0.03
JOL914	Δ*lon*	4.05 ± 0.02[b]
JOL915	Δ*cpxR*	3.07 ± 0.09[c]
JOL916	Δ*lon*Δ*cpxR*	3.81 ± 0.03[b]

[a] Number of intracellular bacteria in chicken abdominal macrophage after exposure with m.o.i. of 10 for 60 min and killing of extracellular bacteria by gentamicin for 30 min (mean ± SEM).
[b] Significant difference between JOL394 ($p < 0.001$).
[c] Not significant difference between JOL394 ($p = 0.057$).

increases for JOL914, JOL915, and JOL916, respectively, compared to JOL394 (Tab. I).

3.4. Attenuation of the virulence of mutants in chickens

In order to validate the safety of the mutants as live vaccines, the attenuation of virulence was examined by the LD_{50} using systemic FT-susceptible Brown Nick female chicks at 1 week of age. By the third week of inoculation, all remaining animals were in good general condition. The postmortem bacteriological examination showed no bacteremia in all chickens regardless of the dose and strains inoculated. As shown in Table II, the LD_{50} of JOL914 and JOL915 were similar, 2.3×10^8 cfu and 2.1×10^8 cfu, respectively, approximately a 10-fold attenuation compared to 3.0×10^7 cfu with the wild type JOL394. By contrast, the LD_{50} of JOL916 was more than 2.8×10^{10} cfu, which was at least a 10^3-fold attenuation.

3.5. Pathogenicity and immunogenicity of mutant strains in chickens

3.5.1. Mortality, gross lesions, and bacterial recovery after inoculation

Following oral inoculation with 2×10^7 cfu of JOL394, JOL914, and JOL915, 55.6% (10/18), 16.7% (3/18), and 22.2% (4/18) mortality, respectively, was observed by the second week post inoculation (wpi) (Tab. III). There was no mortality in the JOL916 group and the unimmunized control group. At the second wpi, five randomly selected chickens from each group were euthanized for postmortem examination to evaluate the safety of the mutants. The findings of enlarged white spots of the liver and spleen were scored from 0 to 3 as mentioned above; the means of the organ lesion scores for the mutant groups were compared to the wild type group. As demonstrated in Table III, the JOL394 group was scored as 1.40 ± 0.80 and 0.80 ± 0.40 for the liver and spleen, respectively. The JOL914 group was statistically similar to the JOL394 group, with a score of 1.20 ± 0.40 and 1.00 ± 0.63 for the liver and spleen, respectively. The scores of the JOL915 group were 1.20 ± 0.00 and 0.60 ± 0.49 for the liver and spleen, respectively, where the spleen lesions scored significantly lower than the JOL394 group. The liver and spleen lesion scores for the JOL916 group were 1.00 ± 0.89 and 0.40 ± 0.20, respectively; significantly lower than the JOL394 group for both organs. The persistence of the mutants in the tissues of the liver and spleen was also examined. By both direct colony count of homogenized tissue on the BGA and enrichment culture of the five chickens from the JOL394 and JOL914 groups, no colony was observed from the liver and spleen samples (Tab. III). In the JOL915 group, no colony was found from either organ by the direct colony count method, and bacteria were recovered from only one out of five spleens by the enrichment culture method. In the JOL916 group, 3 out of 5 livers and 3 out of 5 spleens

Table II. Chicken LD_{50} for the wild type and mutant strains.

Strain	LD_{50} (cfu)
JOL394	3.0×10^7
JOL914	2.3×10^8
JOL915	2.1×10^8
JOL916	$> 2.8 \times 10^{10}$

Table III. Mortality, gross lesion, and bacterial recovery of *Salmonella* in the chickens inoculated with the wild type and mutants.

Strain	Mortality		Gross lesion[a]			Bacterial recovery							
						Liver		Spleen		Ileum		Ceca[d]	
	n	Death rate (%)	*n*	Liver	Spleen	Count[e]	No positive[f]	Count	No positive	Count	No positive	Count	No positive
JOL394	18	55.6	5	1.40 ± 0.80[b]	0.80 ± 0.40	< 1	0/5	< 1	0/5	< 1	0/5	< 1	0/5
JOL914	18	16.7	5	1.20 ± 0.40	1.00 ± 0.63	< 1	0/5	< 1	0/5	< 1	0/5	< 1	0/5
JOL915	18	22.2	5	1.20 ± 0.00	0.60 ± 0.49[c]	< 1	0/5	< 1	1/5	< 1	0/5	< 1	0/5
JOL916	18	0	5	1.00 ± 0.89[c]	0.40 ± 0.20[c]	1.03 ± 0.92	3/5	2.14 ± 1.75	3/5	< 1	1/5	< 1	1/5
Non	18	0	5	0.00 ± 0.00[c]	0.00 ± 0.00[c]	< 1	0/5	< 1	0/5	< 1	0/5	< 1	0/5

[a] Gross lesion was observed after sacrifice of randomly selected five animals per group at 2 weeks post inoculation.
[b] Group lesion score (mean ± SEM).
[c] The gross lesion score of the group with mutant is significantly different from the group with wild type SG ($p < 0.05$).
[d] Cecum wall and cecal content.
[e] Mean ± SEM Log_{10} cfu/g.
[f] Number of positive sample after enrichment culture.
n, numbers of the birds used.

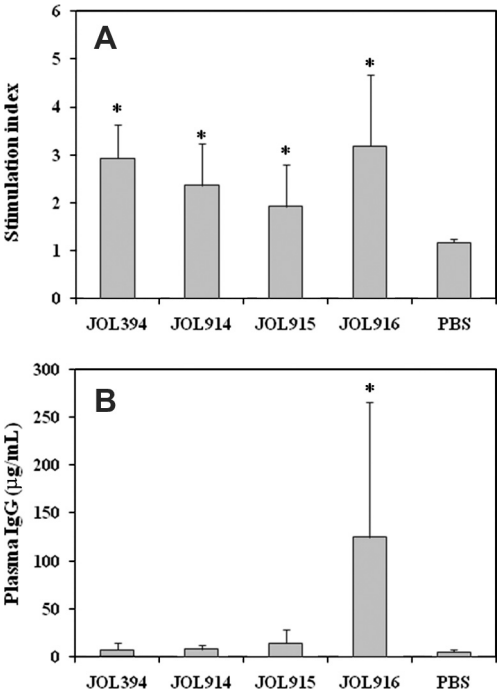

Figure 2. Antigen-specific immune responses in immunized chickens with *Salmonella* Gallinarum wild type strain (JOL394), JOL914, JOL915, and JOL916: A, SI of lymphocyte sample from the chickens by peripheral lymphocyte proliferation assay using soluble antigen at 10th day post inoculation; B, plasma IgG concentration (μg/mL) by indirect-ELISA at second week post immunization. Error bars indicate the SEM (n = 5). * Immunized chickens showed the significant immune responses compared to the control group that was inoculated with PBS ($p < 0.05$).

Table IV. Mortality and depression score after challenge with wild type SG.

Strain	Mortality		Depression score[a]
	n	Death rate (%)	
JOL916	15	0 (0)	0.2 ± 0.5
Control	15	9 (60)	2.5 ± 0.6

[a] Group depression score (mean ± SEM).
Control represents the unimmunized group.

were positive for bacterial recovery, and the mean number per gram was 1.03 ± 0.92 and 2.14 ± 1.75 on the $\log_{10}$ scale for the liver and spleen, respectively.

3.5.2. Immune response

Cellular immune responses induced by the strains in the chickens were examined by the peripheral mononuclear cell proliferation assay using soluble antigen extracted from the wild type SG strain. The SI values were calculated as mentioned above. On the 10th day post immunization, all immunized groups showed significantly elevated SI values compared to the unimmunized group. The SI values of 2.93 ± 0.71, 2.37 ± 0.86, 1.92 ± 0.88, and 3.18 ± 1.50 were observed in the JOL394, JOL914, JOL915 and JOL916 immunized groups, respectively; while the SI value was 1.17 ± 0.07 for the unimmunized group (Fig. 2A). The systemic IgG reaction was analyzed by determining the specific IgG in plasma by indirect ELISA using OMP antigen extracted from the wild type SG. On the second wpi, only 3 out of 5 chickens from the JOL916 group showed a considerable reaction resulting in a significantly elevated value, 124.97 ± 140.65 μg/mL of IgG concentration compared to 4.65 ± 2.61 μg/mL in the unimmunized group (Fig. 2B). However, in the chickens from the JOL394, JOL914, and JOL915 groups, only negligible reactions, if any, were observed and the mean values were 7.36 ± 6.53, 7.70 ± 4.34, and 14.30 ± 13.92 μg/mL, respectively.

3.6. Protection against challenge

To evaluate the protective effect of JOL 916, the birds were challenged with the SG virulent strain and were observed for depression and mortality. The mortality and depression scores after challenge with the SG virulent strain are indicated in Table IV. In the immunized group, two chickens showed slight and temporal depression followed by recovery while in the un-immunized group all the chickens showed prolonged depression. No mortality was observed in the immunized group with JOL916 while 9 of 15 birds died in the un-immunized group.

4. DISCUSSION

Although SG is one of the most important bacterial pathogens found in poultry, the genetic basis of SG virulence and molecular mechanisms of FT have not been fully elucidated [7, 16, 29, 42]. Recent research has shown the importance of SPI-2 clusters in the virulence of SG for systemic infections by analysis of various gene-deficient mutants [16, 17]. SG is thought to invade the mononuclear phagocyte system within macrophages of the liver and spleen [2]. Although bacterial clearance of the attenuated strains of SG have been reported to correlate with the immune response peak in the chicken [42], unlimited proliferation of fully virulent strains in the host leads to bacteremia, acute illness and death [7]. In this study, the effects of deletions of *lon* and/or *cpxR* genes of SG that are related to attachment and invasion of pathogens to host cells were examined. ATP-dependent protease Lon and the signaling pathway Cpx system in pathogenic bacteria have been discussed in the context of their relationship to pathogenesis. Several decades ago their role along with *Escherichia coli* were in part initially reported; the contribution of Lon in EPS production and repression by the Cpx pathway of adherence structures such as curli, P pilil and F pili were initially discussed [11, 22, 39]. In ST, significant involvement of these genes in the pathogenic mechanisms has been recently suggested in studies reporting on gene-disrupted mutants, and the possibility of target genes, for the construction of live vaccine, has been proposed [20, 23, 38].

The growth conditions and biochemical properties were not altered by deletions of the *lon*/*cpxR* genes from SG; these findings suggest that these genes are not central to the related metabolic pathways of SG. The mucoid colony morphology of the Δ*lon* mutant, JOL914 and the Δ*lon*Δ*cpxR* mutant, JOL916 was consistent with the results of fluorometric quantification of EPS using the Con-A binding assay; whereas the wild type and Δ*cpxR* mutant, JOL915 showed a little elevation of the EPS. While SG is considered a *Salmonella* serovar without a capsule [27], the colanic acid is thought to be an important EPS component that plays a role in biofilm formation of *E. coli* on abiotic surfaces. The responsible gene clusters are known to be well conserved among Gram negative species such as *E. coli*, *Klebsiella*, and *Salmonella* [10, 21, 26, 30].

FimA is the major structural subunit of *Salmonella* type I fimbriae that is expressed by most *Salmonella* serovars; its role in the SG attachment or invasion into chicken cells is not fully understood [43]. The amino acid sequence of this protein is well conserved among serovars, while type I fimbriae of SG does not exhibit mannose-sensitive hemagglutination [8, 12]. However, ST has been reported to have the ability to adhere to and form a biofilm on chicken intestinal epithelium in a type I fimbriae-dependent manner, followed by EPS secretion, suggesting involvement of both type I fimbriae and EPS on attachment and invasion of ST in chicken cells [4, 5, 21]. In this study, 6.77-, 2.33-, and 3.90-fold elevations of FimA in the JOL914, JOL915 and JOL916 groups were compared to the wild type, respectively (Fig. 1B). The biological features of the mutants including the elevated expression of EPS and FimA observed in this study indicated that these strains may increase their attachment and invasiveness into host cells. In addition, since polysaccharides and FimA are important antigenic components, these mutants may be expected to induce an effective immune response for protection against pathogens.

Internalization of SG mutants into chicken abdominal macrophages was analyzed to examine whether gene deletions altered invasiveness into chicken macrophages. The bacteria-mediated cytotoxicity was not determined in this study; SG is reported to cause little or no cytotoxicity in chicken macrophages in contrast to the serovars Enteritidis and Typhimurium associated with higher levels of cytotoxicity [41]. As shown in Table I, the intracellular number of bacteria in chicken macrophages of the JOL914 group was significantly increased by 4.65-fold compared to the wild type as expected from the activity of Lon that negatively regulates SPI-1 expression [4, 38]. By contrast, the number was decreased in the JOL915 group. The Cpx pathway related to physiological responses has been extensively studied

[22, 24, 25, 44]; more than 20 target genes have been identified that are involved in the pathway of persistence in the host environment [45]. Therefore, the decreased internalization of the mutant was probably due to impaired adaptability to the environment, resulting in decreased persistence in the macrophages.

The effects of the gene deletions on virulence were examined by LD_{50} using 1-week-old chickens. As shown in Table II, the virulence of JOL914 was only attenuated10-fold, compared to the wild type. This result was unexpected since the *lon*-mutant of ST in the mouse model was more than 10^3-fold attenuated [20, 37]. The modest degree of attenuation of the *lon* mutant in this study implies that *lon* is less responsible for attenuation in SG. JOL915 also showed an approximately 10-fold attenuation. The mutant with both genes deleted, JOL916, showed considerable attenuation of virulence, which indicated that deletions of these two genes may have synergistic effects on the attenuation of SG.

To evaluate the safety of the SG mutants as live vaccines, mortality, gross lesions, and bacterial persistence were evaluated after inoculation of these strains into chickens. The observed mortalities were 55.6, 16.7, 22.2 and 0.0% in the wild type, JOL914, JOL915 and JOL016 groups, respectively, consistent with the LD_{50} results (Tabs. II and III). The gross lesions found in the JOL916 chickens were scored as moderate compared to the other groups; this indicated satisfactory attenuation of the virulence caused by the double deletions of the two genes (Tab. III). Among all strains, JOL916 demonstrated a long bacterial persistence in the organs of the chicken (Tab. III). These data suggest that this strain resides longer in the organs without any significant virulence. However, it cannot be excluded that this long persistence may result in undesirable complications when immunity is suppressed by any immunosuppressive factors including pathogens, medicines and vaccines.

Cell-mediated immunity induced by the vaccine is one of the most important indices of protection; it has been correlated with a high rate of survival in chickens after challenge with virulent SG [29]. In this study, ATP bioluminescence was used as a marker of cell viability, and clear responses were detected 10 days after immunization in the groups (Fig. 2A). In addition, a specific IgG response was exclusively detected in the immunized JOL916 group of chickens (Fig. 2B). The high rate of immune responses was very likely due to the long persistence of the attenuated *Salmonella* in reticuloendothelial organs such as the spleen and liver. These data suggest that this strain may elicit a long induction of immunity and may efficiently protect against FT.

In the protection assay, the immunization with JOL916 offered efficient protection against the wild type challenge, while 60% mortality was observed in the control group, indicating that JOL916 is a potential live vaccine candidate. However, more studies dealing with the possible side effects induced by this mutant strain are necessary. The possibility of egg contamination with the vaccine candidate strain might also be a point of concern.

In conclusion, the *lon* mutant JOL914 was shown to increase significantly in its invasiveness of chicken macrophages with moderate but not satisfactory attenuation. The deletion of *cpxR* decreased its persistence in macrophages with unsatisfactory attenuation. These two mutants did not induce a high degree of protective immune responses. By contrast, the mutant with both genes deleted, JOL916, had increased invasiveness with satisfactory attenuation, resulting in successful induction of immunity that could safely and effectively protect against FT.

Acknowledgements. This study was supported by Technology Development Program for Agriculture and Forestry, Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea.

REFERENCES

[1] Barrow P.A., Immunity to experimental fowl typhoid in chickens induced by a virulence plasmid-cured derivative of *Salmonella gallinarum,* Infect. Immun. (1990) 58:2283–2288.

[2] Barrow P.A., Huggins M.B., Lovell M.A., Host specificity of *Salmonella* infection in chickens and mice is expressed in vivo primarily at the level of the

reticuloendothelial system, Infect. Immun. (1994) 62:4602–4610.

[3] Basnet H.B., Kwon H.J., Cho S.H., Kim S.J., Yoo H.S., Park Y.H., et al., Reproduction of fowl typhoid by respiratory challenge with *Salmonella* Gallinarum, Avian Dis. (2008) 52:156–159.

[4] Boddicker J.D., Ledeboer N.A., Jagnow J., Jones B.D., Clegg S., Differential binding to and biofilm formation on, HEp-2 cells by *Salmonella enterica* serovar Typhimurium is dependent upon allelic variation in the *fimH* gene of the *fim* gene cluster, Mol. Microbiol. (2002) 45:1255–1265.

[5] Boddicker J.D., Jones B.D., Lon protease activity causes down-regulation of *Salmonella* pathogenicity island 1 invasion gene expression after infection of epithelial cells, Infect. Immun. (2004) 72:2002–2013.

[6] Bouzoubaa K., Nagaraja K.V., Kabbaj F.Z., Newman J.A., Pomeroy B.S., Feasibility of using proteins from *Salmonella gallinarum* vs. 9R live vaccine for the prevention of fowl typhoid in chickens, Avian Dis. (1989) 33:385–391.

[7] Christensen J.P., Barrow P.A., Olsen J.E., Poulsen J.S., Bisgaard M., Correlation between viable counts of *Salmonella* Gallinarum in spleen and liver and the development of anaemia in chickens as seen in experimental fowl typhoid, Avian Pathol. (1996) 25:769–783.

[8] Crichton P.B., Yakubu D.E., Old D.C., Clegg S., Immunological and genetical relatedness of type-1 and type-2 fimbriae in salmonellas of serotypes Gallinarum Pullorum and Typhimurium, J. Appl. Bacteriol. (1989) 67:283–291.

[9] Crouch S.P., Kozlowski R., Slater K.J., Fletcher J., The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity, J. Immunol. Methods (1993) 160:81–88.

[10] Danese P.N., Pratt L.A., Kolter R., Exopolysaccharide production is required for development of *Escherichia coli* K-12 biofilm architecture, J. Bacteriol. (2000) 182:3593–3596.

[11] Dorel C., Lejeune P., Rodrigue A., The Cpx system of *Escherichia coli*, a strategic signaling pathway for confronting adverse conditions and for settling biofilm communities?, Res. Microbiol. (2006) 157:306–314.

[12] Duguid J.P., Anderson E.S., Campbell I., Fimbriae and adhesive properties in *Salmonellae,* J. Pathol. Bacteriol. (1966) 92:107–138.

[13] Gogal R.M. Jr, Ahmed S.A., Larsen C.T., Analysis of avian lymphocyte proliferation by a new, simple, nonradioactive assay (lympho-pro), Avian Dis. (1997) 41:714–725.

[14] Griffin H.G., Barrow P.A., Construction of an *aroA* mutant of *Salmonella* serotype Gallinarum: its effectiveness in immunization against experimental fowl typhoid, Vaccine (1993) 11:457–462.

[15] Higgins S.E., Erf G.F., Higgins J.P., Henderson S.N., Wolfenden A.D., Gaona-Ramirez G., Hargis B.M., Effect of probiotic treatment in broiler chicks on intestinal macrophage numbers and phagocytosis of *Salmonella enteritidis* by abdominal exudate cells, Poult. Sci. (2007) 86:2315–2321.

[16] Jeong J.H., Song M., Park S.I., Cho K.O., Rhee J.H., Choy H.E., *Salmonella enterica* serovar Gallinarum requires ppGpp for internalization and survival in animal cells, J. Bacteriol. (2008) 190:6340–6350.

[17] Jones M.A., Wigley P., Page K.L., Hulme S.D., Barrow P.A., *Salmonella enterica* serovar Gallinarum requires the *Salmonella* pathogenicity island 2 type III secretion system but not the *Salmonella* pathogenicity island 1 type III secretion system for virulence in chickens, Infect. Immun. (2001) 69:5471–5476.

[18] Kang H.Y., Dozois C.M., Tinge S.A., Lee T.H., Curtiss R. 3rd, Transduction-mediated transfer of unmarked deletion and point mutations through use of counterselectable suicide vectors, J. Bacteriol. (2002) 184:307–312.

[19] Kang H.Y., Srinivasan J., Curtiss R. 3rd, Immune responses to recombinant pneumococcal PspA antigen delivered by live attenuated *Salmonella enterica* serovar Typhimurium vaccine, Infect. Immun. (2002) 70:1739–1749.

[20] Kim S.W., Moon K.H., Baik H.S., Kang H.Y., Kim S.K., Bahk J.D., et al., Changes of physiological and biochemical properties of *Salmonella enterica* serovar Typhimurium by deletion of *cpxR* and *lon* genes using allelic exchange method, J. Microbiol. Methods (2009) 79:314–320.

[21] Ledeboer N.A., Jones B.D., Exopolysaccharide sugars contribute to biofilm formation by *Salmonella enterica* serovar Typhimurium on HEp-2 cells and chicken intestinal epithelium, J. Bacteriol. (2005) 187:3214–3226.

[22] McEwen J., Silverman P., Genetic analysis of *Escherichia coli* K-12 chromosomal mutants defective in expression of F-plasmid functions: identification of genes *cpxA* and *cpxB,* J. Bacteriol. (1980) 144:60–67.

[23] Matsui H., Suzuki M., Isshiki Y., Kodama C., Eguchi M., Kikuchi Y., et al., Oral immunization with ATP-dependent protease-deficient mutants protects mice against subsequent oral challenge with virulent *Salmonella enterica* serovar Typhimurium, Infect. Immun. (2003) 71:30–39.

[24] Nakayama S., Watanabe H., Identification of *cpxR* as a positive regulator essential for expression of

the *Shigella sonnei virF* gene, J. Bacteriol. (1998) 180:3522–3528.

[25] Nakayama S., Kushiro A., Asahara T., Tanaka R., Hu L., Kopecko D.J., Watanabe H., Activation of *hilA* expression at low pH requires the signal sensor *CpxA,* but not the cognate response regulator *CpxR*, in *Salmonella enterica* serovar Typhimurium, Microbiology (2003) 149:2809–2817.

[26] Paulsen I.T., Beness A.M., Saier M.H. Jr, Computer-based analyses of the protein constituents of transport systems catalysing export of complex carbohydrates in bacteria, Microbiology (1997) 143:2685–2699.

[27] Pomeroy B.S., Nagaraja K.V., Fowl Typhoid, in: Calnek B.W., Barnes H.J., Beard C.W., Reid W.M., Yoder H.W. Jr (Eds.), Diseases of Poultry, 1991, Ames, Iowa, Iowa State University Press, pp. 87–99.

[28] Qureschi M.A., Dietert R.R., Bacon L.D., Genetic variation in the recruitment and activation of chicken peritoneal macrophages, Proc. Soc. Exp. Biol. Med. (1986) 181:560–568.

[29] Rana N., Kulshreshtha R.C., Cell-mediated and humoral immune responses to a virulent plasmid-cured mutant strain of *Salmonella enterica* serotype Gallinarum in broiler chickens, Vet. Microbiol. (2006) 115:156–162.

[30] Rahn A., Drummelsmith J., Whitfield C., Conserved organization in the *cps* gene clusters for expression of *Escherichia coli* group 1 K antigens: relationship to the colanic acid biosynthesis locus and the *cps* genes from *Klebsiella pneumoniae,* J. Bacteriol. (1999) 181:2307–2313.

[31] Reed I.J., Muench H., A simple method of estimating fifty percent endpoints, Am. J. Hyg. (1938) 27:493–497.

[32] Robitaille G., Moineau S., St-Gelais D., Vadeboncoeur C., Britten M., Detection and quantification of capsular exopolysaccharides from *Streptococcus thermophilus* using lectin probes, J. Dairy Sci. (2006) 89:4156–4162.

[33] Rosu V., Chadfield M.S., Santona A., Christensen J.P., Thomsen L.E., Rubino S., Olsen J.E., Effects of *crp* deletion in *Salmonella enterica* serotype Gallinarum, Acta Vet. Scand. (2007) 49:14.

[34] Shah D.H., Lee M.J., Park J.H., Lee J.H., Eo S.K., Kwon J.T., Chae J.S., Identification of *Salmonella gallinarum* virulence genes in a chicken infection model using PCR-based signature-tagged mutagenesis, Microbiology (2005) 151:3957–3968.

[35] Shah D.H., Shringi S., Desai A.R., Heo E.J., Park J.H., Chae J.S., Effect of *metC* mutation on *Salmonella* Gallinarum virulence and invasiveness in 1-day-old White Leghorn chickens, Vet. Microbiol. (2007) 119:352–357.

[36] Smith H.W., The use of live vaccines in experimental *Salmonella gallinarum* infection in chickens with observation on their interference effect, J. Hyg. (1956) 54:419–432.

[37] Takaya A., Suzuki M., Matsui H., Tomoyasu T., Sashinami H., Nakane A., Yamamoto T., Lona, stress-induced ATP-dependent protease, is critically important for systemic *Salmonella enterica* serovar typhimurium infection of mice, Infect. Immun. (2003) 71:690–696.

[38] Takaya A., Tomoyasu T., Tokumitsu A., Morioka M., Yamamoto T., The ATP-dependent lon protease of *Salmonella enterica* serovar Typhimurium regulates invasion and expression of genes carried on *Salmonella* pathogenicity island 1, J. Bacteriol. (2002) 184:224–232.

[39] Torres-Cabassa A.S., Gottesman S., Capsule synthesis in *Escherichia coli* K-12 is regulated by proteolysis, J. Bacteriol. (1987) 169:981–989.

[40] Tsilibaris V., Maenhaut-Michel G., Van Melderen L., Biological roles of the Lon ATP-dependent protease, Res. Microbiol. (2006) 157:701–713.

[41] Wigley P., Hulme S.D., Bumstead N., Barrow P.A., In vivo and in vitro studies of genetic resistance to systemic salmonellosis in the chicken encoded by the *SAL1* locus, Microbes Infect. (2002) 4:1111–1120.

[42] Wigley P., Hulme S., Powers C., Beal R., Smith A., Barrow P., Oral infection with the *Salmonella enterica* serovar Gallinarum 9R attenuated live vaccine as a model to characterise immunity to fowl typhoid in the chicken, BMC Vet. Res. (2005) 1:2.

[43] Wilson R.L., Elthon J., Clegg S., Jones B.D., *Salmonella enterica* serovars gallinarum and pullorum expressing *Salmonella enterica* serovar typhimurium type 1 fimbriae exhibit increased invasiveness for mammalian cells, Infect. Immun. (2000) 68:4782–4785.

[44] Wolfe A.J., Parikh N., Lima B.P., Zemaitaitis B., Signal integration by the two-component signal transduction response regulator CpxR, J. Bacteriol. (2008) 190:2314–2322.

[45] Yamamoto K., Hirao K., Oshima T., Aiba H., Utsumi R., Ishihama A., Functional characterization in vitro of all two-component signal transduction systems from *Escherichia coli,* J. Biol. Chem. (2005) 280:1448–1456.

[46] Zhang-Barber L., Turner A.K., Dougan G., Barrow P.A., Protection of chickens against experimental fowl typhoid using a *nuoG* mutant of *Salmonella* serotype Gallinarum, Vaccine (1998) 16:899–903.