Characterization and Evaluation of a *Salmonella enterica* Serotype Senftenberg Mutant Created by Deletion of Virulence-Related Genes for Use as a Live Attenuated Vaccine

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Natural infections of chickens with *Salmonella enterica* subsp. *enterica* serovar Senftenberg (S. Senftenberg) are characterized by low-level intestinal invasiveness and insignificant production of antibodies. In this study, we investigated the potential effects of *lon* and *cpxR* gene deletions on the invasiveness of S. Senftenberg into the intestinal epithelium of chickens and its ability to induce an immune response, conferring protection against S. Senftenberg infection. With the allelic exchange method, we developed JOL1596 (Δ*lon*), JOL1571 (Δ*cpxR*), and JOL1587 (Δ*lon ΔcpxR*) deletion mutants from wild-type S. Senftenberg. Deletion of the *lon* gene from S. Senftenberg produced increased frequency of elongated cells, with significantly greater amounts of exopolysaccharide (EPS) than in the *cpxR*-deleted strain and the wild-type strain. The *in vivo* intestinal loop invasion assay showed a significant increase in epithelial invasiveness for JOL1596 (Δ*lon*) and JOL1587 (Δ*lon ΔcpxR*), compared to JOL1571 (Δ*cpxR*) and the wild-type strain. Furthermore, the S. Senftenberg wild-type and mutant strains were internalized at high levels inside activated abdominal macrophages from chicken. The *in vivo* inoculation of JOL1587 (Δ*lon ΔcpxR*) into chickens led to colonization of the liver, spleen, and cecum for a short time. Chickens inoculated with JOL1587 (Δ*lon ΔcpxR*) showed significant increases in humoral, mucosal, and cellular immune responses specific to S. Senftenberg antigens. Postchallenge, compared to the control group, the JOL1587 (Δ*lon ΔcpxR*)-inoculated chickens showed not only lower persistence but also faster clearance of wild-type S. Senftenberg from the cecum. We conclude that the increased intestinal invasiveness and colonization of internal organs exhibited by JOL1587 (Δ*lon ΔcpxR*) led to the establishment of immunogenicity and conferred protective efficacy against S. Senftenberg infections in chickens.

*Salmonella enterica* subsp. *enterica* serovar Senftenberg (S. Senftenberg) is a nontyphoidal serovar (NTS) of the Gram-negative facultative intracellular pathogen *S. enterica* (1). S. Senftenberg was reported as causing several foodborne outbreaks of gastroenteritis and invasive intestinal infections in humans (2–4). A previously reported nosocomial outbreak of S. Senftenberg in humans was directly related to contaminated turkey supplied to the hospital kitchen, which in turn contaminated the hospital equipment (2, 5). Various *Salmonella* serotypes are frequently isolated from feed mills and poultry houses (6–8). The trademark of S. Senftenberg infections in chicken is heterogeneity regarding persistence in the cecum and fecal shedding (9). Also, S. Senftenberg has been shown to resist stresses such as low pH, heating, desiccation, irradiation, and cleaning and disinfection procedures used on poultry farms (1, 10, 11). To date, there have been no reported vaccine candidates for control of S. Senftenberg in poultry, and the potential risk of transmission of S. Senftenberg from chickens to human populations emphasizes the need for the development of a homologous vaccine strain (12). Routine vaccination with killed vaccines alone or a combination of live and killed *Salmonella* vaccines against S. *enterica* serotype Enteritidis and S. *enterica* serotype Typhimurium was found to be inefficient for cross-protection against different prevalent *Salmonella* serotypes (13). Considering the history of poor cross-protection among different *Salmonella* serotypes, the establishment of preexisting immunity through immunization with a homologous strain of S. Senftenberg offers a valuable means for preventing the spread of S. Senftenberg infections through poultry (14). Immunization with a homologous serotype has been shown to produce a significantly higher rate of protection than immunization with a heterologous serotype (15).

A potential hurdle in the development of a homologous immunization candidate against wild-type (WT) S. Senftenberg involves the intrinsic inability of the various serotypes to establish systemic infections and the subsequent failure to elicit an immune response following natural infections in chickens (9, 16). The poor immune response, especially the development of lower antibody titers after S. Senftenberg infections, has been correlated with lower levels of invasion into the intestinal epithelium and subsequently lower levels of bacterial colonization in the splenic tissue of chickens (9, 16). We hypothesized that, by increasing the intestinal invasiveness and splenic colonization of an attenuated strain of S. Senftenberg for a short period, we might generate an effective immune response against wild-type S. Senftenberg. Optimal attenuation along with augmentation of the invasiveness and colonization can be achieved by deletion of two virulence-associated regulatory genes, i.e., *cpxR* and *lon* genes, from wild-type S. Senftenberg infections in chickens.
The functional inactivation of the lon gene by mutation was shown to increase the invasiveness of S. Typhimurium (17) and deletion of the cpxR gene yielded enhanced ability of the bacteria to adhere to or to invade host cells (18–20). Deletion of the lon and cpxR genes from S. Typhimurium and Salmonella enterica serotype Gallinarum was shown to attenuate the bacteria by impairing replication and enabling faster clearance of the bacteria from macrophages (19–21).

In the present study, genetically engineered live attenuated mutants of S. Senftenberg were constructed by deleting the lon and cpxR genes via the allelic exchange method. Physiological, morphological, and biochemical properties were characterized for all of the developed mutants. The differences in invasiveness among the mutants were evaluated in the chicken model, in vitro as well as in vivo. Finally, the Δlon ΔcpxR vaccine candidate was further evaluated for safety and immunogenicity as well as protective efficacy against wild-type S. Senftenberg challenge.

### MATERIALS AND METHODS

**Bacterial strains and genetic manipulation.** Table 1 contains the list of bacterial strains and plasmids used in this study. Deletion mutants were constructed by an allelic exchange method using the suicide plasmid pMEG375, as described previously (22). Wild-type S. Senftenberg (strain KVCC-BA0000590) chromosomal DNA was used as the template for amplification of the flanking sequences of the lon and cpxR genes. The amplified DNAs were cloned into a T-vector (Promega) to construct the recombinant connected to the two arms of the flanking regions. The ligated flanking recombinant DNA was then subcloned into the suicide vector pMEG375 (Table 1). The constructed suicide plasmid was finally conjugationally transferred into S. Senftenberg, followed by antibiotic and sucrose sensitivity counter-selection. The selected colonies were examined for lon and cpxR gene deletion by PCR using the specific primer sets of 5′-CAGGAGTCTTACAGGTA-GA-3′ and 5′-CCACACCTCGGCTGTAGGTGA-3′ for lon and 5′-TTTAACCGCGGCGATCTTTCG-3′ and 5′-GATATTACCGTTACAGCAC-3′ for cpxR.

**Growth characteristics, colony morphology, and electron microscopic analysis.** Single colonies of wild-type and mutant strains were inoculated into Luria-Bertani (LB) broth (Becton Dickinson, Sparks, MD, USA) and grown in a shaker incubator at 250 rpm and 37°C. The optical density at 600 nm (OD600) was observed for determination of CFU per milliliter for the wild-type and mutant strains. The calculated CFU per milliliter for each strain was plotted against time to establish a growth curve. The colony morphology was observed after staining and incubation of each mutant on LB agar at 37°C for 24 h. Transmission electron microscopy (TEM) was performed for morphological observation of mutants, as described elsewhere (23). Bacterial samples loaded on the Formvar grid were negatively stained with 1% (wt/vol) aqueous uranyl acetate (pH 4.5) and air dried before examination by TEM.

**Exopolysaccharide production.** Fluorometric quantification of exopolysaccharide (EPS) with the concanavalin A (ConA) binding assay was performed according to a previously described method (24). Briefly, the bacteria were grown on LB agar and suspended in sterile phosphate-buffered saline (PBS) at an OD600 of 0.5. The suspension was stained with fluorescein isothiocyanate (FITC)-conjugated ConA (4 µg/ml; Sigma-Aldrich, St. Louis, MO, USA) and incubated for 30 min. The cells were washed twice with PBS, 200 µl of each strain was transferred to a microtiter plate, and the fluorescence intensity was recorded with a TriStarLB941 microplate reader (Berthold Technologies, Bad Wildbad, Germany). The qualitative abundance of bacterial EPS was also visualized by fluorescence microscopy, on the basis of FITC-conjugated ConA binding to EPS. The bacterial cultures were grown for 16 h on glass coverslips immersed in LB broth. The coverslips were then fixed with 0.5% paraformaldehyde for 1 h. The DNA of the bacteria was counterstained with 4′,6-diamidino-2-phenylindole (DAPI). The fluorescence stains ConA-FITC and DAPI were added to the bacterial culture on coverslips and incubated for 1 h each, followed by gentle washing with PBS. The coverslips were visualized under a fluorescence microscope (Axio Imager.M2; Zeiss, Oberkochen, Germany).

**In vivo intestinal loop invasion assay.** The in vivo intestinal invasion assay was performed in Salmonella-free female Brown Nick chickens (10 to 12 weeks of age), as described elsewhere (25). Chickens that had been fasted overnight were anesthetized by intravenous injection of xylazine (5 mg/kg) and ketamine (20 mg/kg). A transverse incision was made in the shaved and disinfected abdominal area, to expose the jejunal part of the small intestine. The jejunum was carefully freed from the abdominal cavity, and seven intestinal loops (2 cm long) were made surgically. The intestinal loops were separated by 1-cm-long spacers, to avoid leakage and cross-contamination. Then, 5 × 10⁷ CFU of the mutant and wild-type S. Senftenberg strains and the S. Enteritidis reference strain was injected into individual loops. The jejunum was reintroduced into the abdominal cavity and maintained for 2 h. The jejunum was then freed again, injected with PBS containing gentamicin (300 µg/ml), reintroduced into the abdomen, and maintained for 1 h. Finally, the chickens were euthanized, and individual loops were isolated aseptically. Individual loops were opened and washed with sterile PBS to remove excess gentamicin. The isolated loop tissue was homogenized in sterile PBS with 0.5% Triton X-100. The homogenized tissue was then serially diluted in physiological saline and plated and incubated on brilliant green agar (BGA) (Becton Dickinson). The first and seventh loops were injected with sterile PBS and

### TABLE 1 Wild-type and mutant strains and plasmids used for this study

| Strain or plasmid | Description | Reference |
|-------------------|-------------|-----------|
| S. Senftenberg strains |  |  |
| JOL1556 | S. Senftenberg wild-type strain originating from chicken salmonellosis | Strain KVCC-BA0000590 |
| JOL1596 | S. Senftenberg JOL1556 derivative (Δlon) | This study |
| JOL1571 | S. Senftenberg JOL1556 derivative (ΔcpxR) | This study |
| JOL1587 | S. Senftenberg JOL1556 derivative (Δlon ΔcpxR) | This study |
| JOL1196 | Wild-type S. Typhimurium strain |  |
| Plasmids |  |  |
| pMEG375 | Suicide vector to construct derivatives of S. Senftenberg | 53 |
| pBP294 | pMEG375 Δlon | This study |
| pBP210 | pMEG375 ΔcpxR | This study |

*The strains were maintained as frozen glycerol cultures in LB broth at −70°C.*
coverslips at a density of 5H11003nalized inside PECs.

The infected PECs were then maintained for 24 h in RPMI 1640 medium with RPMI 1640 medium supplemented with 300H9004.
The infected PECs were washed three times with PBS and incubated for 1

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FIG 1 Characterization of S. Senftenberg mutant strains. (I) Colony morphology of the mutant and WT S. Senftenberg strains in LB broth. (A) JOL1556 (WT). (B) JOL1596 (Δlon). (C) JOL1571 (ΔcpxR). (D) JOL1587 (Δlon ΔcpxR). The lon and cpxR mutants of S. Senftenberg showed mucoid-appearing colonies, compared to the WT strain. (II) Confirmation of lon and cpxR gene deletion from the JOL1556 (WT) strain. Primers specific to the truncated regions of the lon and cpxR genes were used to confirm the deletion of the respective genes. The lon gene primers amplified 250 bp from the region spanning the deleted Δlon region and 2.71 kb from the nondeleted lon gene, whereas the cpxR gene primers amplified 350 bp from the region spanning the deleted ΔcpxR region and 1 kb from the nondeleted cpxR gene. OMP gene-specific primers amplifying 200-bp regions were used to identify S. Senftenberg. M1 and M3, 100-bp markers; M2, 1-kb markers (Elipsbio, South Korea). (III) Growth curve analysis of S. Senftenberg WT and mutant strains. The growth of the bacteria is expressed as log10 CFU per milliliter at different time points. No significant differences in the growth patterns and rates of the mutant and WT strains of S. Senftenberg were found.

considered controls. The enumerated colony counts per centimeter of homogenate were log10 transformed and used to express the invasion of the bacteria.

Gentamicin protection assays for macrophage internalization experiment. Chicken peritoneal macrophages were isolated for the gentamicin protection assay as described elsewhere (26, 27). Six-week-old chickens peritoneal macrophages were isolated for the gentamicin protection assay as described elsewhere (26, 27). Six-week-old chickens were inoculated orally with 100H9004Δlon ΔcpxR) at an MOI of 100. The JOL1587 (Δlon ΔcpxR)-inoculated chickens for assessment of bacterial recovery from internal organs. The collected tissue samples were weighed and homogenized in 2 ml of buffered peptic acid. The BGA plates were then examined for the presence of Salmonella-type colonies. Postenrichment positive samples were scored as log10 CFU/g, while negative samples were scored as 0 log10 CFU/g (29). The
positive colonies were confirmed by PCR using Salmonella-specific primers (OMPFC, 5’-ATCGGTAGTTAGCAATCG-3’; OMPCR, 5’-CGG GTCGTTATAGGGTCG-3’) (30).

**Bacterial isolation from fecal shedding.** The presence of the JOL1587 (Δlon ΔcpxR) strain in feces was monitored to 21 days postinoculation. Fecal samples were collected from five chickens in each group. The collected fecal samples were diluted in BPW (1:10 [wt/vol]) and homogenized with sterile sticks. Colony counting and enrichment culture analysis were performed as described above. Further, wild-type strain- or JOL1587 (Δlon ΔcpxR)-positive colonies on BGA plates were confirmed by strain-specific PCR.

**Lymphocyte proliferation assay.** A peripheral lymphocyte proliferation assay (LPA) was performed to evaluate cell-mediated immunity in the immunized groups. Soluble antigen was prepared from the S. Senftenberg wild-type strain (31). On day 21 postinoculation, peripheral lymphocytes were separated from the blood of five randomly selected chickens per group. Heparinized blood was carefully loaded on Histopaque 1083 (Sigma-Aldrich) and centrifuged at 1,200 × g for 40 min at 23°C. The cell layer at the interface was collected and washed twice with RPMI 1640 medium (HyClone Laboratories, South Logan, UT, USA). Trypan blue staining indicated cell viability, and the final concentration was adjusted to 1 × 10^6 peripheral blood mononuclear cells (PBMCs)/ml in RPMI 1640 medium. The diluted PBMC samples were then added in triplicate to 96-well tissue culture plates and were incubated for 72 h at 40°C, in 5% CO₂, with medium alone or medium containing 4 μg/ml of the soluble antigen of S. Senftenberg. Lymphocyte proliferation in response to the soluble antigen of S. Senftenberg was measured by using thiazolyl blue tetrazolium bromide dye (Sigma-Aldrich), according to the manufacturer’s guidelines. The blastogenic response was expressed as the mean stimulation index (SI), as described elsewhere (31).

**Plasma IgY and secretory IgA concentrations.** The humoral immune response was studied by determining the levels of specific IgY in plasma and secretory IgA (sIgA) in intestinal wash fluid by indirect enzymelinked immunosorbent assay (ELISA), using the outer membrane protein (OMP) antigen and whole-cell lysate prepared from wild-type S. Senftenberg (32). For quantification of sIgA levels in the intestinal mucosa, the intestinal lavage samples were collected by using a pilocarpine-based lavage procedure, as described elsewhere (33). Wells of Microlon ELISA plates (Greiner Bio-One GmbH, Frickenhausen, Germany) were coated overnight with 100 μl of the extracted OMP antigen (500 ng/well) and whole-cell lysate (300 ng/well) at 4°C. After unbound antigen was washed away, the wells were blocked with 5% bovine serum albumin (BSA) solution for 1 h at 37°C. The plasma and intestinal wash samples were added to separate ELISA plates at 1:100 and 1:5 dilution in PBS, respectively. The IgY and sIgA ELISA plates were incubated for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-chicken IgY and IgA secondary antibodies (Bethyl Laboratories, Montgomery, TX, USA), respectively, at 1:100,000 dilution. The HRP activity of the secondary antibodies was determined by absorbance at 490 nm, using the substrate o-phenylenediamine dihydrochloride (Sigma-Aldrich). Quantification of the plasma IgY and intestinal sIgA responses against S. Senftenberg was performed by plotting a standard curve of OD₄₉₀ values versus antibody concentrations using a chicken IgY and IgA ELISA quantitation kit (Bethyl Laboratories), according to the manufacturer’s instructions and as described elsewhere (34).

**Protection against wild-type challenge.** At the fourth week, chickens from all groups were orally challenged with the virulent wild-type (WT) S. Senftenberg strain JOL1556 (WT), at 1 × 10⁸ CFU per chicken. Challenge strain recovery was performed on days 1, 5, 12, 19, 26, and 33 postchallenge. For challenge strain recovery, liver, spleen, and cecum samples were homogenized in preenrichment BPW and plated on BGA plates after 1:10 (vol/vol) dilution. The negative samples from preenrichment and direct plating were further processed for enrichment culture in RV broth, as described above. The challenge strain was confirmed by PCR using S. Senftenberg serotype-specific primers. The challenge strain was distinguished from inoculated JOL1587 (Δlon ΔcpxR) by using primer sets specific for lon and cpxR genes.

**Statistical analysis.** The results of this study are expressed as mean ± SEM unless otherwise specified. One-way analysis of variance (ANOVA) with Bonferroni correction was employed to analyze statistical significance for plasma IgY, intestinal sIgA, LPA, and fecal shedding data. The values for the individual groups were considered to be statistically significant if the P values were ≤0.05 or ≤0.01. All analyses were performed by using SPSS 16.0 (SPSS Inc.).

**RESULTS**

**Construction of mutant strains.** Deletions of the lon and cpxR genes from S. Senftenberg JOL1556 (WT) by the allelic exchange method led to the development of the JOL1596 (Δlon), JOL1571 (ΔcpxR), and JOL1587 (Δlon ΔcpxR) mutant strains. PCR confirmed the successful deletion of the lon and cpxR genes, with decreases in amplicon sizes from 1 kb for JOL1556 (WT) to 350 bp...
TABLE 2 Analysis of biochemical characteristics of S. Senftenberg mutant and wild-type strains

| Biochemical phenotype | Strain characteristic |
|-----------------------|-----------------------|
|                       | JOL1556 (WT) | JOL1596 (Δlon) | JOL1571 (ΔcpxR) | JOL1587 (Δlon ΔcpxR) |
| Acetoin production    | –            | –              | –              | –                      |
| Amygdalin fermentation | –            | –              | –              | –                      |
| Arabinose fermentation | +           | +              | +              | +                      |
| Arginine dihydrolase  | +            | +              | +              | +                      |
| β-Galactosidase       | –            | –              | –              | –                      |
| Citrate utilization   | –            | –              | –              | –                      |
| Gelatinase            | –            | –              | –              | –                      |
| Glucose fermentation  | +            | +              | +              | +                      |
| H₂S production        | +            | +              | +              | +                      |
| Indole production     | –            | –              | –              | –                      |
| Inositol fermentation | –            | –              | –              | –                      |
| Lysine decarboxylase  | –            | –              | –              | –                      |
| Mannitol fermentation | +            | +              | +              | +                      |
| Melibiose fermentation| –            | –              | –              | –                      |
| Ornithine decarboxylase| –      | –              | –              | –                      |
| Rhamnose fermentation | +            | +              | +              | +                      |
| Sorbitol fermentation | +            | +              | +              | +                      |
| Sucrose fermentation  | –            | –              | –              | –                      |
| Tryptophan deaminase  | –            | –              | –              | –                      |
| Urease                | –            | –              | –              | –                      |

for JOL1596 (Δlon) and JOL1587 (Δlon ΔcpxR) for the lon gene and from 2.71 kb for JOL1556 (WT) to 250 bp for JOL1571 (ΔcpxR) and JOL1587 (Δlon ΔcpxR) for the cpxR gene (Fig. 1III). Characterization of S. Senftenberg mutants. The mutants were evaluated for the effects of lon and cpxR deletions on their physiological, phenotypic, and biochemical characteristics. Physiological properties such as growth conditions for the mutants were examined using growth curves. The growth curves for the JOL1596 (Δlon), JOL1571 (ΔcpxR), and JOL1587 (Δlon ΔcpxR) mutants and JOL1556 (WT) were similar, with both wild-type and mutant strains reaching the exponential and stationary phases at 1.5 h and 9.0 h postinoculation, respectively (Fig. 1III). The colony morphologies of JOL1596 (Δlon), JOL1571 (ΔcpxR), and JOL1587 (Δlon ΔcpxR) strains appeared mucoid, while the JOL1556 (WT) strain was nonmucoid and slightly larger in size (Fig. 1I). TEM results showed the increased frequency of elongated bacteria in both JOL1596 (Δlon) and JOL1587 (Δlon ΔcpxR), compared with the JOL1571 (ΔcpxR) and wild-type strains (Fig. 2I). For each mutant, 10 fields were viewed and counted for the frequency of elongated bacteria. Both JOL1596 (Δlon) and JOL1587 (Δlon ΔcpxR) showed 30% increases in the incidence of elongated bacteria (data not shown). ConA-FITC-EPS fluorescence microscopy examined the effects of Δlon and ΔcpxR deletions on EPS contents. JOL1556 (WT) and JOL1571 (ΔcpxR) showed very little or negligible green fluorescence, indicating less EPS, whereas JOL1596 (Δlon) and JOL1587 (Δlon ΔcpxR) showed bright green fluorescence, suggesting very high EPS levels, compared to the wild-type strain (Fig. 2II). Quantitative estimation of the EPS contents of JOL1596 (Δlon) and JOL1587 (Δlon ΔcpxR) showed significant increases of 8.3- and 12.2-fold (P < 0.05), respectively, above the JOL1556 (WT) level (data not shown).

Biochemical characteristics of mutant strains. A total of 20 biochemical phenotypes of the S. Senftenberg mutant strains were evaluated with an API 20E system. Except for melibiose fermentation, all of the biochemical phenotypes were similar for the S. Senftenberg mutant and JOL1556 (WT) strains (Table 2). All of the S. Senftenberg mutant strains exhibited melibiose fermentation, which the JOL1556 (WT) strain did not perform.

Invasiveness of S. Senftenberg mutant and wild-type strains. The S. Senftenberg mutant and wild-type strains were placed in direct contact with intestinal epithelium through the construction of intestinal loops, for the estimation of in vivo invasiveness. The variations in intestinal epithelial CFU counts among the mutant, S. Senftenberg wild-type, and S. Typhimurium strains are shown in Fig. 3A. The S. Typhimurium reference strain showed significantly higher (P < 0.05) intestinal epithelial CFU counts than the S. Senftenberg mutant and wild-type strains. Although all of the S. Senftenberg mutant and wild-type strains showed intestinal invasion, JOL1596 (Δlon) and JOL1587 (Δlon ΔcpxR) showed significantly greater invasiveness (P < 0.05) than did JOL1571 (ΔcpxR) and JOL1556 (WT) (Fig. 3A). JOL1596 (Δlon) and JOL1587 (Δlon ΔcpxR) showed 10-fold increases in intestinal epithelial invasiveness, compared with JOL1556 (WT). JOL1571 (ΔcpxR) and JOL1556 (WT) showed no significant difference in intestinal epithelial invasiveness (Fig. 3A).

Internalization of S. Senftenberg in macrophages. After infection of abdominal macrophages, gentamicin protection assay results showed that S. Typhimurium, S. Senftenberg mutant, and S. Senftenberg wild-type strains were internalized in the abdominal macrophages. All of the mutant and wild-type S. Senftenberg strains showed similar counts for recovery of internalized bacteria from macrophages (Fig. 3B), and no significant differences among the strains were observed. Compared to S. Senftenberg mutant and wild-type strains, the S. Typhimurium reference strain showed significantly (P < 0.01) greater recovery of viable internalized bacteria from macrophages (Fig. 3B). Further, the internalization of JOL1587 (Δlon ΔcpxR) inside macrophages was visualized by staining of the infected macrophages with anti-Salmonella polyclonal antibodies. Fluorescence microscopy showed that JOL1587 (Δlon...
JOL1587 (\(\Delta lon\) \(\Delta cpxR\)) was internalized inside the macrophages, as indicated by the presence of green fluorescence surrounding the blue-stained nuclei of macrophages (Fig. 4).

**Recovery of JOL1587 (\(\Delta lon\) \(\Delta cpxR\)) from internal organs and feces.** For evaluation of *in vivo* invasion, safety, and clearance of JOL1587 (\(\Delta lon\) \(\Delta cpxR\)), the spleens, livers, and ceca of the inoculated chickens were examined. All spleen, liver, and cecum samples from chickens in the control group were negative for JOL1587 (\(\Delta lon\) \(\Delta cpxR\)) strain recovery (Table 3). After enrichment, the JOL1587 (\(\Delta lon\) \(\Delta cpxR\)) strain was isolated from the spleen only on days 3 and 7 postinoculation (Table 3). As shown in Table 3, bacterial recovery from the spleen, liver, and cecum samples harvested from orally inoculated chickens on days 1, 3, and 7 postinoculation, whereas the JOL1587 (\(\Delta lon\) \(\Delta cpxR\)) strain was isolated from the spleen only on days 3 and 7 postinoculation (Table 3). In contrast, the intramuscularly inoculated group showed JOL1587 (\(\Delta lon\) \(\Delta cpxR\)) strain recovery from the spleen on days 1, 3, and 7 postinoculation. Recovery from the liver was observed only on days 1 and 3, with recovery from the cecum on days 3 and 7 postinoculation. Both inoculated groups showed no bacterial recovery from the spleen, liver, and cecum from day 7 to day 21 postinoculation (Table 3).

### TABLE 3 Bacterial persistence and clearance in internal organs of chickens inoculated with JOL1587

| Group and organ | No. of positive samples/total no. of samples<sup>a</sup> |
|----------------|---------------------------------------------------------|
|                | 1 day<sup>b</sup> | 3 days | 7 days | 14 days | 21 days |
| Group A        | Liver            | 0/5    | 0/5    | 0/5    | 0/5    | 0/5    |
|                | Spleen           | 0/5    | 0/5    | 0/5    | 0/5    | 0/5    |
|                | Cecum            | 0/5    | 0/5    | 0/5    | 0/5    | 0/5    |
| Group B        | Liver            | 3/5<sup>d</sup> | 4/5<sup>d</sup> | 0/5    | 0/5    | 0/5    |
|                | Spleen           | 0/5    | 3/5<sup>d</sup> | 2/5<sup>d</sup> | 0/5    | 0/5    |
|                | Cecum            | 5/5<sup>d</sup> | 5/5<sup>d</sup> | 2/5<sup>d</sup> | 0/5    | 0/5    |
| Group C        | Liver            | 3/5<sup>d</sup> | 2/5<sup>d</sup> | 0/5    | 0/5    | 0/5    |
|                | Spleen           | 3/5<sup>d</sup> | 4/5<sup>d</sup> | 2/5<sup>d</sup> | 0/5    | 0/5    |
|                | Cecum            | 5/5<sup>d</sup> | 4/5<sup>d</sup> | 2/5<sup>d</sup> | 0/5    | 0/5    |

<sup>a</sup> Group A, PBS-treated control group; group B, chickens orally inoculated with JOL1587 (\(\Delta lon\) \(\Delta cpxR\)); group C, chickens intramuscularly inoculated with JOL1587 (\(\Delta lon\) \(\Delta cpxR\)).

<sup>b</sup> Number of positive samples per total number of samples observed with the enrichment culture method.

<sup>c</sup> Days postinoculation.

<sup>d</sup> Significantly greater number of S. Senftenberg-positive chickens in the immunized group, compared to the control group, postinoculation (\(P < 0.05\)).

![FIG 4 Fluorescence microscopy for visualization of S. Senftenberg internalized inside PECs. (A) Morphology of adherent PECs in culture under bright-field microscopy. Magnification, \(\times 400\). Black arrowhead, morphological appearance of cultured adherent PECs. Inset, morphological structures of PECs at 20 \(\mu m\). (B) Internalization of JOL1587 (\(\Delta lon\) \(\Delta cpxR\)) inside PECs. S. Senftenberg-infected PECs were fixed at 24 h postinfection and analyzed for immunofluorescence by fluorescence microscopy. Total magnification, \(\times 1,000\). DAPI was used as a nuclear stain and FITC as a stain for localization of S. Senftenberg. White arrowheads, presence of green fluorescence surrounding the blue-stained nuclei of PECs, indicating internalized S. Senftenberg inside the PECs.](http://cvi.asm.org/Downloaded from http://cvi.asm.org/)

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**FIG 3** Gentamicin protection assay for evaluation of *in vivo* intestinal loop invasion and *in vitro* macrophage internalization. The variations in the intracellular recovery counts of the S. Senftenberg mutant and wild-type strains in the *in vivo* intestinal loop assay (A) and the *in vitro* macrophage internalization assay (B), relative to the reference strain S. Typhimurium, are depicted graphically. The colony counts for the directly recovered bacteria on BGA plates are expressed as \(\log_{10}\) CFU per gram of intestinal tissue and \(\log_{10}\) CFU per milliliter of abdominal macrophage lysate. An increase of 1 \(\log_{10}\) unit in invasiveness indicates a 10-fold increase in invasiveness. The differences in colony counts were calculated with 95% confidence intervals. Error bars, SEM. *+, significant increase in the invasiveness and internalization of mutant and reference strains in the intestinal epithelium and macrophages, compared to the wild-type S. Senftenberg strain (\(P < 0.05\)).
**Tables 4**

**Fecal shedding of *S. Senftenberg* from chickens inoculated with JOL1587**

| Recovery (log_{10} CFU/g) | Group | 1 day | 3 days | 7 days | 9 days | 14 days | 21 days |
|---------------------------|-------|-------|--------|--------|--------|---------|---------|
|                          |       |       |        |        |        |         |         |
| A                         |       | 0.00  | 0.00   | 0.00   | 0.00   | 0.00    | 0.00    |
| B                         |       | 1.0   | 1.0    | 1.0    | 1.0    | 1.0     | 1.0     |
| C                         |       | 0.2   | 0.2    | 0.2    | 0.2    | 0.2     | 0.2     |

*Group A, PBS-treated control group; group B, chickens orally inoculated with JOL1587 (Δlon ΔcpxR); group C, chickens intramuscularly inoculated with JOL1587 (Δlon ΔcpxR).*

### Discussion

In this study, the allelic exchange method was successfully employed for deletion of the *lon* and *cpxR* genes from *S. Senftenberg* (Fig. 1I). On morphological characterization, deletion of the *lon* gene from wild-type *S. Senftenberg* produced a significant increase in EPS production and elongation of the bacterial cells, whereas *cpxR* deletion produced neither of those morphological changes (Fig. 2I and II). The Lon protease is reported to negatively regulate RcsA, which is a positive transcriptional accessory factor for RcsB and is responsible for excess EPS production in *Esche-
Although the function of the Lon protease in cell division and impaired cell cycle control in perfectly viable cells is replication and cell division (Fig. 2I). Furthermore, the Salmonella lon gene mutants indicates a possible role of the lon gene in Salmonella replication and cell division (Fig. 21). Furthermore, the results of the biochemical characterization showed that all three mutants acquired the additional biochemical property of melibiose fermentation, in contrast to the wild-type strain (Table 2).

Further investigation is needed to correlate the effects of lon and cpxR deletion on melibiose fermentation gain of function in S. Senftenberg.

Deletion of the lon gene from wild-type S. Senftenberg produced significant increases in the in vivo intestinal epithelial invasiveness and splenic colonization of chickens (Fig. 3A and B). The invasion of intestinal epithelium in Salmonella is controlled by Salmonella pathogenicity island-1 (SPI-1) and by the hilA gene product, which is a central regulatory transcriptional activator for expression of the SPI-1 invasion genes (40). As observed for S. Typhimurium, the product of the lon gene, Lon protease, has a negative regulatory effect on the invasion-associated gene hilA, and deletion of the lon gene led to increased invasiveness for the mutant strain (17, 38). The function of the Lon protease in different S. Senftenberg strains has not been investigated; moreover, contradicting reports suggesting a role for hilA and heterogeneity regarding the role of the SPI-1-associated gene in S. Senftenberg invasion, the data for the lon gene mutants indirectly indicate a possible role for the hilA gene product in S. Senftenberg invasion in chickens. Furthermore, the lon gene influences the survival and proliferation of Salmonella inside phagocytic cells through upregulation of SPI-2 genes (41). The effects of lon and cpxR gene deletion on macrophage internalization were studied, and the data from the experiment showed that all of the mutant strains were internalized by activated chicken abdominal macrophages (Fig. 3B and 4). The macrophage internalization suggests that lon gene deletion does not affect the internalization potential of Salmonella but might prevent the persistence of S. Senftenberg inside antigen-presenting cells, as observed previously with S. Gallinarum (42). In addition to deletion of the lon gene, deletion of the cpxR gene had an attenuating effect on S. Senftenberg, as the cpxR gene product is part of the virulence-associated regulatory system, involving the type III secretion system, motility, chemotaxis, and adherence (18).

**FIG 5** Humoral and mucosal immune responses to S. Senftenberg antigens. The humoral and mucosal immune responses were assessed by indirect ELISA for measurement of systemic plasma IgY levels and mucosal sIgA levels in intestinal lavage fluid from JOL1587 (Δlon ΔcpxR)-inoculated chickens. (A and B) Systemic IgY responses to the whole-cell lysate (A) and OMP antigen (B) of S. Senftenberg. (C and D) Mucosal sIgA responses to the whole-cell lysate (C) and OMP antigen (D) of S. Senftenberg. Oral, orally JOL1587 (Δlon ΔcpxR)-inoculated group; IM, intramuscularly JOL1587 (Δlon ΔcpxR)-inoculated group; Control, PBS-inoculated group. Error bars, SEM. *, presence of significantly higher antibody titers in the JOL1587 (Δlon ΔcpxR)-inoculated chickens, compared to the PBS-treated control chickens (P < 0.05).

**FIG 6** Cell-mediated immunogenicity and protective efficacy of JOL1587 (Δlon ΔcpxR) in inoculated chickens. (A) SI values from the lymphocyte proliferation assay at week 3 postinoculation for chickens inoculated with the JOL1587 (Δlon ΔcpxR) strain. *, significant differences of values for orally and intramuscularly inoculated groups, compared to the control group (P < 0.05). (B) Rate and extent of clearance and colonization of wild-type S. Senftenberg in the cecum of inoculated and control chickens. *, significant increase in colonization and persistence of S. Senftenberg in the cecum of control chickens, compared to the intramuscularly inoculated group (P < 0.05). Error bars, SEM. Oral, orally JOL1587 (Δlon ΔcpxR)-inoculated group; IM, intramuscularly JOL1587 (Δlon ΔcpxR)-inoculated group; Control, PBS-inoculated group.
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Cecal persistence and clearance of wild-type S. Senftenberg from JOL1587- and PBS-inoculated chickens postchallenge

| No. of positive samples/total no. of samples | Recovery (log10 CFU/g) (mean ± SEM) |
|--------------------------------------------|-------------------------------------|
| **1 day**                                  |                                     |
| A 5/5                                      | 6.5 ± 0.0                           |
| B 3/5                                      | 7.0 ± 0.0                           |
| C 2/5                                      | 7.4 ± 0.0                           |
| **7 days**                                  |                                     |
| A 5/5                                      | 6.4 ± 0.0                           |
| B 5/5                                      | 6.4 ± 0.0                           |
| C 5/5                                      | 6.5 ± 0.0                           |
| **14 days**                                 |                                     |
| A 5/5                                      | 5.0 ± 0.0                           |
| B 5/5                                      | 5.9 ± 0.0                           |
| C 5/5                                      | 6.0 ± 0.0                           |
| **28 days**                                 |                                     |
| A 5/5                                      | 2.8 ± 0.0                           |
| B 5/5                                      | 3.1 ± 0.0                           |
| C 5/5                                      | 3.4 ± 0.0                           |

*Number of positive samples per total number of samples observed with the enrichment culture method.

**Challenge strain recovery was determined by direct and enrichment cultures and the enrichment culture method.*

Note: Significantly greater numbers of S. Senftenberg-positive chickens in the control group, compared to the orally and intramuscularly inoculated groups (Table 4).

We also evaluated the double-gene mutant JOL1587 (Δlon ΔcpxR) for safety, immunogenicity, and efficacy against a wild-type S. Senftenberg challenge in a chicken model. For environmental safety, fecal shedding of genetically manipulated microorganisms is considered undesirable (43). Moreover, due to the property of S. Senftenberg to resist desiccation and disinfection, persistent fecal shedding of the vaccine strain is undesirable (1). In contrast to the previously reported aroA mutant S. Typhimurium live vaccine candidate, which showed fecal excretion up to 26 days postinoculation (44), the fecal shedding of JOL1587 (Δlon ΔcpxR) in this study was found to be significantly reduced and was observed only up to day 7 postinoculation in both the orally and intramuscularly inoculated groups (Table 4). The organ bacterial recovery results from this study are in agreement with those of previous studies involving S. Gallinarum (Δlon ΔcpxR) and S. Enteritidis (Δlon ΔcpxR) (20, 45). The results suggest that the JOL1587 (Δlon ΔcpxR) strain efficiently colonized the chicken spleen, liver, and cecum, proliferated in a controlled manner, and was mostly cleared by day 7 postinoculation.

The immunogenicity of the JOL1587 (Δlon ΔcpxR) strain was also evaluated in chickens up to 28 day postinoculation. The circulatory IgY and mucosal sIgA immune responses are essential for protection from and clearance of Salmonella infections systemically and from intestinal epithelium, respectively (46). Furthermore, due to the ability of the S. Senftenberg strains to colonize the cecum of chickens, the generation of mucosal immunity is important for protection against S. Senftenberg infections in chickens (9). Immunization of chickens with the JOL1587 (Δlon ΔcpxR) strain stimulated significant induction of humoral and cell-mediated immune responses (Fig. 5). Inoculation of chickens with the JOL1587 (Δlon ΔcpxR) strain induced not only significantly higher levels of plasma IgY but also intestinal sIgA responses against S. Senftenberg (Fig. 5). Salmonella-specific plasma IgY mediates humoral immunity against Salmonella by enhancing opsonization and receptor-mediated endocytosis by macrophages (47). In contrast, mucosal immunity acts as the first line of defense and is important for clearance of Salmonella infections during the entry phase at the mucosal level (48, 49). As Salmonella survives and replicates within professional antigen-presenting cells, cell-mediated immunity plays a significant role in the clearance of intracellular Salmonella infections from chickens (50, 51). The JOL1587 (Δlon ΔcpxR)-inoculated chickens showed significantly greater lymphocyte proliferation responses (Fig. 6A). A possible reason for the observed increase in immunogenicity might involve the increased production of EPS, which forms a major antigenic component of bacteria (52). Here we showed that the humoral and mucosal immune responses against the OMP antigen peaked at week 3 postinoculation and started declining thereafter, whereas the immune response against whole-cell lysate, which included EPS and lipopolysaccharide (LPS) components, maintained significantly higher and steady levels until week 5 postinoculation. The results of the ELISA experiment suggested that, apart from the major antigenic OMP fraction, other antigenic determinants of S. Senftenberg, such as LPS and EPS, might be essential for mounting significantly greater immune responses. Our collective results revealed that JOL1587 (Δlon ΔcpxR) is a...
strong inducer of the humoral and cellular arms of immunity in chickens.

Systemic infection with S. Senftenberg is self-limiting and internal organ colonization is at a low level (9), but the S. Senftenberg strain is known to persist in the cecum of chickens for a longer time, with an asymptomatic carrier state (9, 16). For protection of chickens against S. Senftenberg infections, prevention of mucosal infection and cecal colonization with S. Senftenberg is essential. In this study, the inoculated chickens showed significantly (P < 0.05) faster clearance and lower persistence of the challenge strain in the cecum, whereas the control chickens showed greater persistence and colonization of the challenge strain up to 28 day postchallenge (Table 5 and Fig. 6B). In contrast to the results of bacterial recovery from the cecum, the challenge strain failed to colonize the spleen and liver in all groups. Overall, the inoculated chickens showed faster efficient clearance of S. Senftenberg from the cecum, compared to control chickens.

In summary, the present study demonstrated that increasing intestinal invasiveness and splenic colonization for a short time through deletion of lon and cpxR from wild-type S. Senftenberg induced significantly greater immunogenicity in response to JOL1587 (Δlon ΔcpxR) immunization. Furthermore, the lon and cpxR gene mutations together enhanced the biological and environmental safety of the vaccine strain. The immunogenicity that developed in chickens after JOL1587 (Δlon ΔcpxR) immunization conferred protection by decreasing colonization of the challenge strain in the cecum. Therefore, the live vaccine candidate developed, JOL1587 (Δlon ΔcpxR), is a promising vaccine candidate against infection with S. Senftenberg and can be used for routine vaccination in chickens.

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