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A genetically engineered derivative of *Salmonella* Enteritidis as a novel live vaccine candidate for salmonellosis in chickens

Rahul M. Nandre, Kiku Matsuda, Atul A. Chaudhari, Bumseok Kim, John Hwa Lee *

College of Veterinary Medicine and Bio-Safety Research Institute, Chonbuk National University, Jeonju 561-756, South Korea

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ABSTRACT

To construct a novel live *Salmonella* Enteritidis (SE) vaccine candidate, SE was genetically engineered using the allelic exchange method to delete two virulence genes, *lon* and *cpxR*. The *lon* gene deletion is essential to impair *Salmonella* replication and avoid overwhelming systemic disease in the host. The *cpxR* gene deletion is needed to enhance the ability of bacteria to adhere and invade the host cell. Scanning electron microscopy revealed that the derivatives JOL917 (Δ*lon*), JOL918 (Δ*cpxR*), and JOL919 (Δ*lon*/Δ*cpxR*) had increased surface fimbrial filamentous structures. Significant elevations of extracellular polysaccharide and FimA expression were observed for the derivatives compared to the parental wild type JOL860, while biochemical properties of the derivatives were not altered. In the safety examination by inoculation of the derivatives in chickens, gross lesion scores of the liver, spleen, kidney, small intestine and caecal tonsils were moderate in the JOL917 and JOL918 groups, and significantly lower in the JOL919 group than those of the JOL860. Bacterial counts from the spleen and caeca of the JOL917 and JOL918 groups were moderate, and significantly reduced in the JOL919 group compared to the JOL860 group. In addition, only the JOL919 group showed significantly lower bacterial counts in the faecal samples than those of the JOL860 group. Significant elevations of IgG and secretory IgA levels observed in the derivative groups, while the JOL919 and JOL860 groups showed a potent lymphocyte proliferation response as compared to those of the control group. In the protection efficacy examination, JOL919 immunized group showed significantly lower depression, lower gross lesion in the liver and spleen, and lower number of the SE positive internal organs than those of the control group against a virulent wild type SE challenge.

1. Introduction

Salmonellosis is an important public health problem in many countries and a frequent cause of gastroenteritis and zoonotic infections (Dhanoa and Fatt, 2009; Matheson et al., 2010). The serotype *Salmonella* Enteritidis (SE) may also cause an asymptomatic infection in poultry, which may result in egg contamination with transmission to humans upon consumption of raw or undercooked eggs and their derivatives (Bäumler et al., 2000; Clavijo et al., 2006). Therefore, *Salmonella* control within poultry farms is an urgent issue. Vaccination is a potentially effective tool for the prevention of salmonellosis. Whole-cell killed vaccines and subunit vaccines have been used with variable results to prevent *Salmonella* infection in both humans and animals (Mastroeni et al., 2001). It is thought that live vaccines have advantages over killed vaccines as they stimulate both humoral and cell-mediated immunity (Carvajal et al., 2008). The commercially available live *Salmonella* vaccines of for poultry are either auxotrophic double-marker mutants derived through chemical mutagenesis (Meyer et al., 1993; Springer et al., 2000), or developed on the basis of the principle of metabolic drift mutations (Vielitz et al., 1992; Linde et al., 1997; Hahn, 2000). However, the efficacy of live vaccines is limited depending upon the residual virulence and host clearance, while multiple studies have been carried out to demonstrate the efficacy of live *Salmonella* vaccines (Takaya et al., 2002, 2003; Matsui et al., 2003; Kim et al., 2009).

Lon has been characterized as a powerful negative regulator of the expression of invasion genes encoded on *Salmonella* pathogenicity island 1 (SPI-1), and it also effects macrophage survival and is required for systemic infection (Takaya et al., 2002, 2003). The CpxAR two-component signal transduction pathway consists of a sensor kinase (SK), CpxA, and a cognate response regulator (RR), CpxR. The SK, CpxA, is located in the cytoplasmic membrane, where it senses diverse signals, including alkaline pH, altered membrane lipid composition, interaction with hydrophobic surfaces, and misfolded pilin subunits. In response, CpxA autophosphorylates and donates its phosphoryl group to activate CpxR. Activated CpxR regulates part of the envelope stress response system, pilus assembly, type III secretion, motility and chemotaxis, adherence, and biofilm development (Wolfe et al., 2008). Therefore, the derivative strains constructed with deletions of *lon* and

* Corresponding author. Tel.: +82 63 270 2553; fax: +82 63 270 3780.
E-mail addresses: johnhlee@jbnu.ac.kr, johnhlee@chonbuk.ac.kr (J.H. Lee).

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cpxR are expected to increase capability for adhesion or invasion, but decreased survival and systemic infection in the host cell, resulting in easy eradication from host cells without causing damage.

In this study, the *lon* and/or *cpxR* genes were deleted from SE and the potential of the derivatives for use as an attenuated live vaccine candidate against wild-type SE was examined. For this, their morphology, physiological changes, biochemical properties, safety, and immunogenicity were evaluated.

2. Materials and methods

2.1. Bacterial strains and genetic manipulation

Genetic manipulation of the derivatives was accomplished by deletion of the genes using the allelic exchange method. The left and right arms of the *lon* and *cpxR* genes were amplified by polymerase chain reaction (PCR) with *lon* and *cpxR* primers to construct Δ*lon*, Δ*cpxR* and Δ*lon*/Δ*cpxR* derivatives, which consisted of flanking DNA sequences excluded in the *lon* and/or *cpxR* genes, using wild-type SE JOL860 (Table 1). The amplified DNA segments were cloned into a T-vector (Promega, Madison, WI, USA). The cloned left and right arms were directly ligated between the two fragments, and the recombinant connected by two arms was completed. The recombinant DNA fragment was digested with the appropriate restriction enzymes and then cloned into suicide vector pMEG375 (Table 1). The transfer of recombinant suicide plasmids into *Salmonella* was accomplished by conjugation using pMEG375 as the suicide vector. The electroporation method was used for the plasmid transformation into *Salmonella* competent cells (Sambrook et al., 1989). The recombinant suicide plasmid pBP294 was obtained by cloning the ligated fragment of *lon* gene into pMEG375. The plasmid pBP210 was generated by the same method as in the two flanking PCR amplicons of the *cpxR* gene into pMEG375. To induce the deletion of the *lon* gene, the plasmid pBP294 was conjugationally transferred into SE JOL860. The selected colonies were confirmed by PCR using the specific primer sets of 5′-CAGGAGTTCTTACAGGTAGA-3′/5′-CCACACTCCGCTGTAGG TGA-3′ (*lon*). By conjugational transfer of pBP210 into JOL860, the deletion of the *cpxR* gene was introduced. The selected colonies were confirmed by PCR using the specific primer sets of 5′-CATCATCTGCGGGTTGCAGC-3′/5′-GATAATTTACCGTTAACGAC-3′ (*cpxR*).

2.2. Growth character, colony morphology and scanning electron microscopy

Growth character was studied by adding a 1/100 volume of an overnight culture of wild type and derivative strains into 200 mL of Luria–Bertani (LB, Becton, Dickinson and Company, Sparks, MD, USA) broth and incubating at 37 °C while shaking at 250 rpm. The optical density at 600 nm (OD_{600}) was determined every 1.5 h for 9.5 h. For colony morphology observation, each strain was streaked on LB agar and kept for incubation at 37 °C for 24 h. These colonies, grown on LB agar, were gently collected and fixed in 1.5% glutaraldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.4). After fixation, they were placed in 1% aqueous osmium tetroxide phosphate buffer and serially dehydrated in acetone. Samples were critical point dried and coated with platinum-palladium alloy for scanning electron microscopy (JSM-6400, JEOL, Japan).

Table 1
Bacterial strains and plasmids used in this study.

Strains/ plasmid	Description	Reference
S. Enteritidis		
JOL860	*S.* Enteritidis wild type, originated from chicken salmonellosis	This study
JOL917	*S.* Enteritidis JOL860 derivative Δ*lon*	This study
JOL918	*S.* Enteritidis JOL860 derivative Δ*cpxR*	This study
JOL919	*S.* Enteritidis JOL918 derivative Δ*lon*	This study
JOL1182	*S.* Enteritidis virulent strain, originated from chicken salmonellosis	This study
Plasmid		
pMEG375	Suicide vector to construct derivatives of *S.* Enteritidis	(Dozois et al., 2003)
pBP294	pMEG375 Δ*lon*	This study
pBP210	pMEG375 Δ*cpxR*	This study

2.3. Biotype

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

2.4. FimA expression

To compare relative expression of FimA between strains, the *FimA* gene was cloned from a *Salmonella* Typhimurium (ST) wild isolate to pET28a using a primer set of 5′-GGATCCGCTGATCCTA CTCCGGTGAG-3′, 5′-CTCGAGTTCGTATTTCATGATA AAGG-3′ for over-expression of the 6× His-tag attached protein, which was purified using Ni–NTA Agarose (Peptron, South Korea) and inoculated into rabbits to obtain anti-FimA serum. Colonies grown on LB agar for 24 h at 37 °C were suspended in PBS at an OD_{600} of 0.5, and diluted 1:100. The diluted suspension of each strain was divided into triplicate samples. The concentration of each suspension was determined by the colony count method after spreading of these triplicate samples on LB agar plates for normalization. A piece of Protran® nitrocellulose membrane with a pore size of 0.2 μM (Schleicher and Schuell, Dassel, Germany), was immersed in Tris-buffered saline (TBS, 10 mM Tris, 0.9% sodium chloride, pH 7.4) and set in a 96-well Bio-Dot™ apparatus (Bio-Rad Laboratories, USA), according to the manufacturer's instructions. The result was expressed as mean ratio ± standard error of the mean (SEM) of the normalized value after division by the average of the normalized value for JOL860. This experiment was repeated for four times.

2.5. Extra-cellular polysaccharides (EPS)

Fluorometric quantification of EPS by the Concanavalin A (Con-A) binding assay was performed (Robitaille et al., 2006). Briefly, cells grown on LB agar were suspended in 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. Then, 200 μL of suspension of each strain was transferred to 5 wells of a 96-well microplate and fluorescence intensity was recorded by TriStarLB941 (Berthold Technologies GmbH and Company, Bad Wildbad, Germany). This experiment was repeated for four times.

2.6. Preparation of mutant and wild strains

The strains were maintained as glycerol-frozen cultures in LB broth at −70 °C. The frozen cultures were streaked on LB agars and incubated at 37 °C for 16 h. A colony per strain was inoculated in the LB broth for overnight incubation. For inoculation to chickens, the overnight culture was inoculated in fresh LB broth at 1:20 dilution, incubated at 37 °C to an optical density $(OD)_{600}$ of 0.6. Cells were harvested by centrifugation at 13,200 rpm for 5 min. The pellets were washed and re-suspended in sterile PBS,