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Construction of *Salmonella* Enteritidis and *Salmonella* Typhimurium ghosts isolated from retail chicken meat

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Abstract

Salmonellosis is an important public health problem and causes large economic losses in the poultry industry. The emergence of molecular technology has opened various possibilities for constructing tailor-made proteins, particularly protein E from bacteriophage PhiX174 for the production of bacterial ghosts (BGs) applied in vaccines purposes. In the present study, the plamdaPRcI-Elysis plasmid carrying the PhiX174 lysis gene E and thermo-sensitive lamda PR-cl857 regulatory system was constructed. Two *Salmonella* Enteritidis (SE-2 and SE-4) and one *Salmonella* Typhimurium (ST-4) isolates were able to uptake the lysis plasmid via electrotransformation. Generation of ghosts was enhanced by increasing the incubation temperature up to 42˚C. Cell viability of SE-2, SE-4 and ST-4 decreased ranging in log 2.7 to log 4.1 cycles after lysis induction. Moreover, SE-2 and SE-4 exhibited the earliest reduction of CFU after 3 h of incubation. Our results may provide a promising avenue for the development of *Salmonella* BGs vaccines.

Introduction

Salmonella Enteritidis and *Salmonella* Typhimurium are important cause of food-borne illness. Based on surveillance studies, the main vehicles of *S.* Enteritidis and *S.* Typhimurium infection include raw meat, eggs and poultry products (Kramarenko *et al*., 2014; Thung *et al*., 2016). Due to the frequency of antimicrobial resistance and the number of resistance determinants in *Salmonella* have raised markedly, vaccination plays an important role in the overall bio-security system on animal farms, typically chicken farms to prevent *Salmonella* infections (Jawale *et al*., 2012).

With the advent of molecular technology, the findings of a gene encoding the lysis protein E (with a 91 amino acid polypeptide) from bacteriophage PhiX174 and its expression in Gram-negative bacteria had created an empty bacterial cell envelops lacking cytoplasm and DNA known as bacterial ghosts (BGs) (Muhammad *et al*., 2012). BGs system represents a safe, cost-effective, progressive and multipurpose approach in the development of potent vaccines for the prophylaxis of variety of infectious diseases. Additionally, it has been used as adjuvants and as carriers of foreign protein antigens or biologically active substances as well as DNA vaccines (Langemann *et al*., 2010). Recently, a safety enhanced BGs vaccine provided efficient protection against virulent bacterial challenge in chickens, inducing specific antibodies and cell-mediated immune responses, and reducing challenge strain colonization (Guo *et al*., 2016).

In general, the induction of ghost cell formation by cell lysis was achieved through the expression of lysis gene E coupled with the transcriptional control of the thermo-sensitive lamda PR-cl857 system. Mutations to the lamda PR promoter/operator regions have resulted in new expression systems, which stably repress gene E expression at temperatures of up to 37˚C, but still allowed induction of cell lysis at a temperature range of 39˚C to 42˚C (Jechlinger

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Table 1. *Salmonella* isolates used for production of bacterial ghosts and transformation efficiency with lysis plasmid (plamdaPRcI-Elysis)

No.	Isolates	*Salmonella* serovar	Transformation efficiency[a]
1	SE-1	S. Enteritidis	-
2	SE-2	S. Enteritidis	$(1.07 \pm 0.51) \times 10^8$
3	SE-3	S. Enteritidis	-
4	SE-4	S. Enteritidis	$(8.58 \pm 0.57) \times 10^4$
5	SE-5	S. Enteritidis	-
6	SE-6	S. Enteritidis	-
7	SE-7	S. Enteritidis	-
8	SE-8	S. Enteritidis	-
9	ST-1	S. Typhimurium	-
10	ST-2	S. Typhimurium	-
11	ST-4	S. Typhimurium	$(6.23 \pm 0.43) \times 10^5$

[a]Transformation efficiency was expressed as the number of transformants per micrograms of plasmid DNA. Data are mean values ± standard deviations from triplicates per assay.

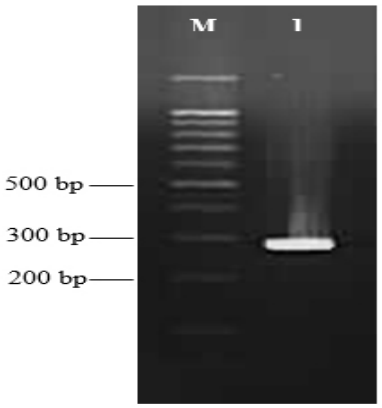

Figure 1. Gel electrophoresis of gene E. Lane M shows the 100-bp DNA ladder. Lane 1 shows the gene E (276 bp) amplified from bacteriophage PhiX174.

et al., 1999). Therefore, the objective of this study was to construct and determine the potential utility of the bacteriophage PhiX174 lysis gene E driven by the lamda PR-cI857 regulatory system for the generation of *S*. Enteritidis and *S*. Typhimurium ghosts.

Materials and Methods

Bacterial strains and culture conditions

S. Enteritidis and *S*. Typhimurium isolates (Table 1), which collected from the previous study (Thung et al., 2016) were grown in tryptic soy broth (TSB) (Merck, Darmstadt, Germany), then inoculated onto tryptic soy agar (TSA) (Merck, Darmstadt, Germany) slants and incubated at 37˚C for 24 h. The agar slants were kept at 4˚C and used as working cultures.

Construction of lysis vector

Two sets of primers were employed for amplification of gene E from bacteriophage PhiX174 (New England BioLabs Inc., USA) and lamda PR-cI857 regulatory system from pLDR20 plasmid DNA (*E. coli* ATCC 8720), respectively. Primers Elysis-F (5'-ATGGT ACGCT GGACT TTGTG-3') and Elysis-R (5'-ACATT ACATC ACTCC TTCCG-3') (Kwon *et al*., 2005) were used for targeting gene E. Amplification of DNA was performed in 50 µL reaction mixtures containing 10 µL 5× PCR buffer, 1 µL 10 mM dNTP, 4 µL 1.2 µM (Elysis-F and Elysis-R) primer, 0.8 µL (2 U) *Taq* DNA polymerase and 1 µL DNA template. The PCR reactions were carried out in a Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) with the following condition: initial denaturation at 95˚C for 2 min, 30 cycles of denaturation at 95˚C for 30 s, annealing at 55˚C for 30 s, extension at 72˚C for 1 min and final extension at 72˚C for 7 min.

Meanwhile, primers LPR-F (5'-CCGCGG CCCTT TAGCT GTCTT GGTTT GC-3') and LcI-R (5'-GGGCCC GACCA GAACA CCTTG CCG-3') (Kwon *et al*., 2005), which contained *Sac*II and *Apa*I restriction sites (underlined), respectively, were used for targeting lamda PR-cI857 gene. PCR amplification using Q5 High-Fidelity DNA polymerase (New England BioLabs Inc., USA) was performed in 50 µL reaction mixtures containing 10 µL 5× Q5 reaction buffer, 1 µL 10 mM dNTP, 5 µL 0.5 µM (LPR-F and LcI-R) primer, 0.5 µL Q5 High-Fidelity DNA polymerase and 1 µL DNA template. A two-step cycling was carried out in Veriti 96-Well Thermal Cycler with the following condition: initial denaturation at 98˚C for 30 s, 30 cycles of denaturation at 98˚C for 10 s, annealing and extension at 72˚C for 30 s, and final extension at 72˚C for 2 min. Each amplified PCR product was visualized on 1.5% (w/v) agarose gel stained with ethidium bromide, purified with a gel extraction kit (Promega, Madison, USA) and cloned into pGEM T east vector (Promega, Madison, USA). After S*ac*II and *Apa*I digestion of both plasmids, the lamda PR-cI857 system was inserted into the plasmid containing the lysis gene E, and the resulting plasmid was designated as plamdaPRcI-Elysis. The generated lysis plasmid was further sequenced to confirm accurate construction.

Preparation of Salmonella *competent cells and transformation*

S. Enteritidis and *S*. Typhimurium isolates were grown in LB broth at 35˚C on an orbital shaker until it reached mid-log phase ($OD_{600\ nm}$ = 0.4-0.5). Bacterial cells were rendered electro-competent by

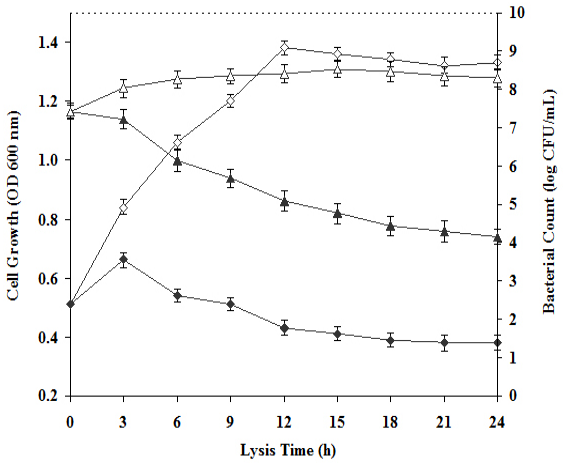

Figure 2. Growth and lysis curve of *S.* Enteritidis (SE-2) after induction of gene E mediated lysis. At time zero, the cultures were shifted from 35˚C to 42˚C. Cell growth was determined at $OD_{600\ nm}$ for naive SE-2 (♦) and ghost SE-2 (♦). Numbers of surviving cells (CFU) were enumerated for naive SE-2 (Δ) and ghost SE-2 (▲). The error bars indicate standard deviations.

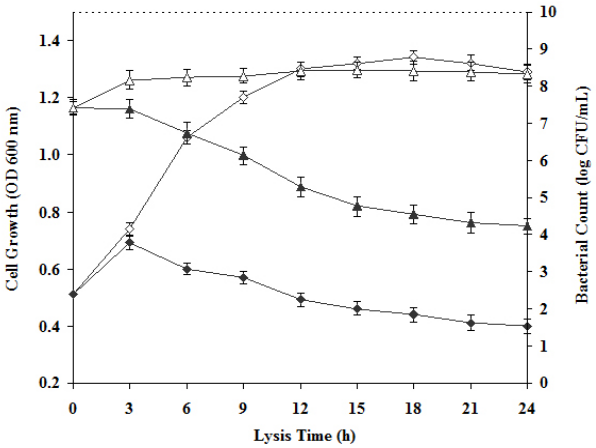

Figure 3. Growth and lysis curve of *S.* Enteritidis (SE-4) after induction of gene E mediated lysis. At time zero, the cultures were shifted from 35˚C to 42˚C. Cell growth was determined at $OD_{600\ nm}$ for naive SE-4 (♦) and ghost SE-4 (♦). Numbers of surviving cells (CFU) were enumerated for naive SE-4 (Δ) and ghost SE-4 (▲). The error bars indicate standard deviations.

washing three times with 10% ice-cold glycerol and resuspended in 200 µL of 10% ice-cold glycerol. The cells suspension (50 µL) was then mixed with pre-cooled plamdaPRcI-Elysis plasmids (2 µL) in a 2.0 mm gap width electroporation cuvette. After applying a pulse with setting of 25 µF; 200 Ω; 2.5 kV on a GenePulserXcell (Bio-Rad Laboratories, Hercules, USA), competent cells were transferred into 250 µL of antibiotic-free LB broth and incubated at 35˚C for 1.5 h. After incubation, 100 µL of the cells suspensions were plated onto LB agar plates supplemented with ampicillin (100 µg/mL).

Generation of Salmonella *ghost*

A single colony of *Salmonella* carrying plamdaPRcI-Elysis was inoculated in 100 mL of LB broth containing ampicillin (50 µg/mL) and incubated at 35˚C. When the cultures reached the mid-log phase ($OD_{600\ nm}$ = 0.4-0.5), the expression of the gene E was induced by a temperature upshift from 35˚C to 42˚C. The induction of gene E-mediated bacteriolysis was monitored by measuring the optical density (OD 600 nm) and surviving bacteria colonies (CFU) at different time points (0, 3, 6, 9, 12, 15, 18, 21 and 24 h).

Results and Discussion

The PCR-amplified gene E fragment with the size of 276 bp was obtained clearly by agarose gel electrophoresis (Figure 1). Our findings were consistent with the observation of Yu *et al.* (2011)

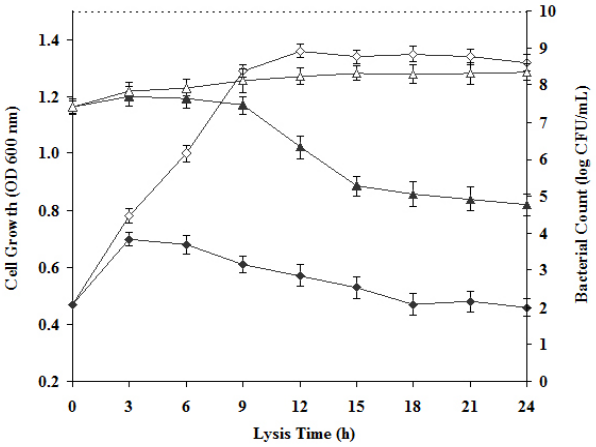

Figure 4. Growth and lysis curve of *S.* Typhimurium (ST-4) after induction of gene E mediated lysis. At time zero, the cultures were shifted from 35˚C to 42˚C. Cell growth was determined at $OD_{600\ nm}$ for naive ST-4 (♦) and ghost ST-4 (♦). Numbers of surviving cells (CFU) were enumerated for naive ST-4 (Δ) and ghost ST-4 (▲). The error bars indicate standard deviations.

who showed that the size of the amplified gene E product by using different pairs of primers (with *Eco*R I and *Bam*H I restriction site) was determined at 276 bp.

Prior to the production of *Salmonella* ghosts, the transformation efficiency of the electroparated *Salmonella* isolates were determined (Table 1). Out of 11 *Salmonella* isolates examined, 3 isolates were able to uptake the lysis plasmid DNA (plamdaPRcI-Elysis). Among the isolates, SE-2 exhibited the highest transformation efficiency of about 1.07×10^6. Electrotransformation (1 to 5 attempts, depending on

the isolate) with the same plasmid did not produce colonies on LB agar, suggesting that size has no impact on transformation efficiency. This finding was consistent with that of Löfblom *et al*. (2007) who reported that the electroporation method for transformation of *Staphylococcus carnosus* yielded in approximately 106 transformats. On the contrary, Binotto *et al*. (1991) had found that high efficiency of 10^8-10^9 electrotransformants per microgram of plasmid DNA were observed in the transformation of *S*. Typhimurium LT2 via electroporation. They concluded that bacterial cell envelope properties are less crucial in electrotransformation than in Ca^{2+} shock methods. However, low frequency of electrotransformation was observed in erythromycin-resistant *Lactobacillus crispatus* strains, with a transformation efficiency of less than 102 transformants per microgram of DNA (Beasley *et al*., 2004).

Production of BGs was carried out in *S*. Enteritidis and *S*. Typhimurium. Growth and lysis curve of the isolates SE-2, SE-4 and ST-4 were shown in Figure 2, Figure 3 and Figure 4, respectively. The OD of the transformed SE-2, SE-4, and ST-4 decreased after 6 h incubation. Cell viability also decreased after lysis induction, where the number of CFU were decreased ranging in log 2.7-log 4.1 cycles. Among the isolates, *S*. Enteritidis SE-2 and SE-4 exhibited the earliest reduction of CFU after 3 h of incubation. The production of BGs studies indicated that the lysis time of the bacterial cells is strain dependence.

Indeed, the lytic activity of protein E depends on the growth phase of the host bacteria. When bacterial cultures reach the mid-log phase, with optical density values of 0.2-0.6 taken at wavelengths 600 nm, bacterial lysis is induced by a temperature elevation in many Gram-negative bacteria with gene E-mediated lysis system (Zhu *et al*., 2015). Although the E-mediated cell killing activity is the same, but the induced lysis time may vary for different bacterial cells. Previously, Kwon *et al*. (2005) reported that the lysis of *Edwardsiella tarda* was determined after 2 h. Besides, a short lysis time with approximately 1 h was reported in a wild strain of *Yersinia enterocolitica* (Cai *et al*., 2013). On the contrary, the induced lysis time was observed at about 36 h for *Brucella suis* strain S2 (Liu *et al*., 2015). Similar with our *S*. Enteritidis ghosts, lysis was induced at 3 to 6 h at 42˚C for *S*. Gallinarum ghost (Jawale *et al*., 2014) and *S*. Enteritidis ghost (Jawale *et al*., 2012).

Conclusion

Our results reveal that through genetic engineering, we have succeeded in electrotransforming a constructed lysis plasmid into several *Salmonella* isolates to produce ghost cells. The functional test of *Salmonella* ghost vaccines to determine the protective immunity against virulent challenge should be objective of future studies.

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