Sains Malaysiana 45(6)(2016): 933–940

Development of *Salmonella typhimurium* Ghost Vaccine using *Asd*-Based Vector System with *E* Gene and the Immune Responses Evoked in BALB/c Mice

(Pembangunan Vaksin Kosong *Salmonella typhimurium* menggunakan Sistem Vektor Berasaskan *Asd* dengan Gen *E* dan Meningkatkan Tindak Balas Imun pada Tikus BALB/c)

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ABSTRACT

Salmonella typhimurium *MMP13 harboring pMMP99, a recombinant plasmid derived by cloning a ghost cassette into T-vector, were employed for production of ghost cells. Growth of MMP13 (pMMP99) showed an initial increase after a shift in temperature from 28 to 42°C, and then decreased gradually with ghost formation being complete within 3 h. Ghost yield of MMP13 (pMMP99) were 99.99% less than 10^4 CFU/mL. MMP13 (pMMP101-1) showed a ghost yield similar to MMP13 (pMMP99). Immune responses of BALB/c mice against ghost cells originated from MMP13 (pMMP101-1) were assessed by measuring total IgG, IgG1, IgG2a, and secretory IgA levels in each sample. Levels of total IgG, IgG1 and IgG2a and vaginal IgA, increased abruptly after 4 weeks post vaccination, whereas the fecal IgA level did not induce significant change. Splenocyte proliferation was observed at the cellular level owing to stimulation of ghost cells. Ghost vaccination protected 25-59% of mice against wild-type* S. typhimurium *more than those of controls.*

Keywords: Ghost cassette; ghost vaccine; immune response; non-antibiotic marker; protection; Salmonella typhimurium

ABSTRAK

Salmonella typhimurium *MMP13 melindungi pMMP99, plasmid rekombinan yang diperoleh melalui pengklonan kaset kosong ke dalam T-vektor, telah digunakan untuk pengeluaran sel kosong. Pertumbuhan MMP13 (pMMP99) menunjukkan peningkatan awal selepas berlaku perubahan dalam suhu daripada 28 kepada 42°C dan kemudian menurun secara beransur-ansur dengan pembentukan kosong yang lengkap dalam masa 3 jam. Hasil kosong MMP13 (pMMP99) adalah 99.99% kurang daripada 10^4 CFU/mL. MMP13 (pMMP101-1) menunjukkan hasil kosong adalah sama dengan MMP13 (pMMP99). Tindak balas imun tikus BALB/c terhadap sel kosong daripada MMP13 (pMMP101-1) dinilai dengan mengukur jumlah IgG, IgG1, IgG2a dan tahap IgA rembes dalam setiap sampel. Tahap jumlah IgG, IgG1 dan IgG2a serta IgA faraj meningkat secara mendadak selepas 4 minggu vaksinasi, manakala tahap IgA tahi tidak menunjukkan perubahan ketara. Percambahan splenocyte diperhatikan pada tahap sel kerana rangsangan sel kosong. Vaksinasi kosong melindungi 25-59% tikus daripada* S. typhimurium *jenis liar lebih daripada kawalan.*

Kata kunci: Kaset kosong; penanda bukan antibiotik; perlindungan; Salmonella typhimurium*; tindak balas imun; vaksin kosong*

INTRODUCTION

Diarrhea during weaning period in the pig industry causes death owing to pathogenic *Escherichia coli* and *Salmonella* and is one of the reasons for reducing pig performance (Fairbrother et al. 2005; Szabo et al. 2009). Enterotoxigenic *E. coli* (ETEC) is an important causative agent of diarrhea in neonatal and weaned piglets and the high morbidity and mortality of this disease result in severe economic losses (Moon & Bunn 1993; Sarmiento et al. 1988). *S. typhimurium* is the serovar isolated most commonly from pigs worldwide (Boyen et al. 2008; Brumme et al. 2007). In order to prevent *Salmonella* shedding and their piglets from subsequent infection due to infected sows, antibiotics are often used, but vaccination of pregnant sows can be used as an effective method (Abd El Ghany et al. 2007; Roesler et al. 2006).

A ghost vaccine is prepared by a ghost cassette including the *E* gene originated from the bacteriophage phiX174 (Witte et al. 1992). The ghost cassette is formed by the fusion between the cI857 P_R or cI P_R promoter of bacteriophage lambda and the *E* gene of phiX174 (Mayr et al. 2005; Walcher et al. 2008). The cI857 protein turns off *E* gene expression under control of the P_R promoter at 30°C or less, whereas the promoter turns on the gene in 42°C. The expressed E protein interacts with the MraY protein of the phospho-MurNAc-pentapeptide translocase to repress the synthesis of lipid I in the conserved peptidoglycan biosynthesis pathway (Zheng et al. 2009, 2008) or forms a transmembrane tunnel structure (Langemann et al. 2010). Finally, E protein causes bacterial cell lysis in a growth-dependent manner.

Most killed vaccines for sale were prepared by formalin treatment. A formalin-killed vaccine elicits a vigorous immune response less than live-attenuated vaccines (Gupta et al. 2009), likely due to decreased immunogenicity owing to separation of appendages or extracellular materials. Although the ghost cells eject all cytosolic components via the cell lysis, the cells maintain intact extracellular components and share functional and antigenic determinants with its live counterparts (Witte et al. 1992). Furthermore, the ghosts stimulate significant increases in the secretion of cytokines TNF-α, IFN-γ, IL-12 and IL-18 and dendritic cells exposed to ghosts exhibit the increased T-cell activation (Haslberger et al. 2000, 1997). The merits of ghosts make them an attractive adjuvant, because they were composed of immunostimulatory compounds such as lipopolysaccharides, lipid A and peptidoglycan (Langemann et al. 2010). Although ghost vaccines might have the immunogenicity slightly lower than live attenuated vaccines, the ghost can be used as a stable vaccine because it is not associated with the dangerous factors of live–attenuated vaccines.

In this study, in order to carry out the preliminary investigation for construction of ghost vaccine to livestock, a ghost cassette was prepared by the fusion between the P_R promoter and *E* gene and a ghost vaccine candidate which constructed using the ghost cassette and a non-antibiotic marker was assessed in terms of its effects on immune responses, splenocyte proliferation and protection against *Salmonella* infection.

MATERIALS AND METHODS

BACTERIAL STRAINS, REAGENTS AND GENERAL DNA MANIPULATIONS

The bacterial strains and plasmids used in this study are listed in Table 1. *S. typhimurium* and *E. coli* were grown at 37°C in Luria-Bertani (LB) or M9 minimal medium supplemented with 1.5% agar (Bertani 1951). Antibiotics were added to culture media at the following concentrations: ampicillin 100 µg/mL and streptomycin 50 µg/mL.

The ghost cassette was PCR-amplified from pLDR20 (Table 1) and an oligonucleotide set (Ghost-F-*Xba*I; TCTAGAGACCAGAACACCTTGCCGATC and Ghost-R-*Xba*I; TCTAGA ACATTACATCACTCCTTCCG). Taq and *Pfu* DNA polymerases employed for PCR were purchased from EX-Taq (TaKaRa), Japan or Eco-Tag (Solgent), South Korea and Invitrogen, USA, respectively. Recombinant plasmids carrying target DNA segment were identified by restriction enzyme digestion, PCR and nucleotide sequencing (Macrogen, South Korea). The

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmids	Descriptions	References
E. coli		
Top10	F-mcrA (mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK rpsL (Str^R) endA1	Invitrogen
DH5α	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Lab stock
χ7213	*hi-1 thr-1 leuB6 supE44 tonA21 lacY1 recA RP4-2-Tc::Mu λpir, Δasd Δzhf-2::Tn10*	Lab stock
χ6212	Φ80d lacZ ΔM15 *deoR Δ(lacZYA-argF)U169 supE44$λ^-$ gyrA96 recA1 relA1 endA1 asdA4 Δzhf-2::Tn10 hsdR17* (R^- M^+)	Lab stock
Salmonella		
S. typhimurium JOL401	an isolate from Korean livestok	Lab stock
MMP13	*S. typhimurium* JOL401 *asd*A16	This study
CK110	*S. typhimurium* JOL401Δ*cpxR*Δ*lon asd*A16	Lab stock
S. typhimurium χ3339	wild-type strain SL1344, hisG rpsL	Lab stock
*S. typhimurium*χ8554	ΔasdA16, a derivative of *S. typhimurium* χ3339	Lab stock
plasmids		
T-vector	a TA cloning vector	promega
pLDR20	a vector carrying cI*857* P_R *E*	Lab stock
pYA3332	a vector containing p15A origin	Kang et al. 2002
pYA3342	a vector containing pBR origin	Kang et al. 2002
pYA3493	a derivative of pYA3342 carrying beta-lactamase signal sequence	Kang et al. 2002
pMMP99	a derivative of T-vector carrying a ghost cassette	this study
pMMP101-1	a derivative of pYA3493 carrying a ghost cassette	this study

ghost cassette cloned into pMMP99 was digested by *Xba*I and ligated with pYA3342, which was digested by the same restriction enzyme and treated with calf intestinal alkaline phosphatase. The resultant recombinant plasmid was named as pMMP101-1.

GROWTH CURVE OF GHOST BACTERIA, GHOST FORMATION AND MEASUREMENT OF VIABLE CELLS

Pre-cultured ghost bacteria were inoculated in 100 mL of LB medium and incubated at 28°C until the optical density (OD) 0.2-0.4 at 600 nm in wavelength. The ghost cells were generated by shifting the temperature from 28 to 42°C. The ghost formation was stopped when the incubation reached at the indicated time. The ghost cells were collected by centrifugation for 30 min at 6000 rpm and then directly employed for vaccination or when required, the concentrated ghost cells were dried by freeze-drying. The concentrated ghost cells were administered into mice after appropriate dilution using BSG buffer (PBS buffer containing 0.1% gelatin).

OBSERVATION OF GHOST CELLS BY SCANNING ELECTRON MICROSCOPY (SEM)

Strains carrying ghost cassette were grown at 28°C until 0.2-0.4 at 600 nm in wavelength. The ghost cells were generated by shifting the temperature from 28 to 42°C. The prepared cells were precipitated by centrifugation for 30 min at 6000 rpm and were then fixed for 24 h at 4°C in 2.5% glutaraldehyde/0.1 M sodium cacodylate (pH7.4). The fixed ghost cells were dehydrated by incubation in 20% ethyl alcohol for 15 min, 50% ethyl alcohol for 15 min, 70% ethyl alcohol for 15 min, 90% ethyl alcohol for 15 min and 100% ethyl alcohol for 30 min. The dehydrated ghost cells were observed by SEM after electronic ion coating.

MOUSE VACCINATION WITH GHOST CELLS

The concentrated or lyophilized ghost cells were serially diluted tenfold and were then injected or administered intramuscularly or orally to BALB/c mice acclimatized for 1 week. The intramuscularly or orally administered dosages were 5×10^8 and 1×10^9 CFU/mL, respectively. Food and water were prohibited for 4 h before administration of the ghost cells and then supplied with food and water at 1 h after administration *ad libitum*. The vaccinated mice were observed for 10 weeks after administration. Peripheral blood, vaginal lavage and fecal extract were collected at 0-10 weeks after vaccination with ghost cells for the measurement of IgG and IgA levels, respectively.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

ELISA was performed with lipopolysaccharides (LPS) extracted from *S. Typhimurium* (Kang et al. 2002). LPS (0.2 mg/mL) was dissolved in 0.05 M carbonate buffer (pH9.6) and then coated on a microtiter plate for overnight at 4°C. The coating solution was removed from the treated microtiter plate (SPL), which was then washed 4-6 times with PBS buffer (pH7.4). The treated plate was blocked for 1 h at 37°C with PBS buffer containing 0.1% skim milk. The wells were reacted with plasma (dilution 1:100), fecal extract (dilution 1:4) and vaginal washing solution (dilution 1:4) for 2 h at 37°C. The reactions were followed by treatment with goat anti-mouse IgG-, IgG1-, IgG2a- and IgA-horseradish peroxidase (HRP) conjugates (Southern Biotech) (dilution 1:5,000) for 2 h. The bound HRP was reacted for 10-90 min using 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS, Sigma-Aldrich). The reaction was stopped by 0.1% SDS and then antibody titers were determined at 405 nm in wavelength by an ELISA reader (Dynex, USA).

SPLENOCYTE PROLIFERATION ASSAY

Spleens of BALB/c mice were collected from mice of 3 weeks after intramuscular injection of 1×10^8, 1×10^9 and 1×10^{10} CFU/mL ghost cells. Red blood cells (RBCs) were lysed using a RBC lysis solution and then washed twice with RPMI solution. After dissolving the precipitated pellet in RPMI1640 solution, the final splenocyte density was adjusted to 5×10^6 cells/mL in RPMI1640 medium (Zhong et al. 2010). Splenocytes in RPMI medium supplemented with *S. typhimurium* ghost cells (4 μg/mL) were incubated for 1, 2 and 3 days at 37°C in 5% CO_2. The proliferated cells were assessed for splenocyte proliferation using cell lysis reagent (ViaLight Plus kit, Lonza) according to manual's instructions.

PROTECTION ASSAY OF MICE IMMUNIZED WITH SALMONELLA GHOST CELLS AGAINST VIRULENT *S. TYPHIMURIUM*

A challenge test was performed by virulent *S. typhimurium* to assess protection of BALB/c mice induced by immunization with the *Salmonella* ghost cells. The ghost cells were administered orally or intramuscularly (dosage 1×10^9 CFU/mL) and boosting was performed 2 weeks later using the same dosage. The challenge test was performed by oral administration of 1.8×10^6 CFU/mL *S. typhimurium* χ3339 at 4 weeks after primary ghost administration. Food and water were prohibited for 4 h before administration of bacteria and then supplied with food and water at 1 h after administration *ad libitum*. The treated mice were observed for 4 weeks. The animal experiments in this study were conducted under approval from the Gyeongnam National University of Science and Technology Animal Ethics Committee in accordance with the guidelines of the Korean Council on Animal Care (AEC-20100730-0002).

RESULTS AND DISCUSSION

THE GHOST CASSETTE PLAYS A FUNCTIONAL ROLE IN *SALMONELLA TYPHIMURIUM* MMP13

In order to examine the function in *S. typhimurium* by generally well-known ghost cassette *cI*857 P_R *E*, where *cI*857 plays a role as a transcriptional level repressor by

temperature-dependent manner, the cassette was cloned into a plasmid vector carrying ColE1 replication origin. PCR-amplification of the ghost cassette was done from pLDR20 as a template and then cloned into T- easy vector (Promega), designed as pMMP99 (Figure 1(a)). The *E* gene is encoded into a product to induce bacterial cell lysis and is expressed in a temperature-dependent manner owing to the *cI*857 P_R promoter. E protein inhibits biosynthesis of bacterial envelope via interaction with the integral membrane protein MraY, a translocase involved in the transfer of phospho-MurNac-Pep5 (Bernhardt et al. 2002) or forms lysis tunnel in membrane (Langemann et al. 2010). Therefore, E-protein-mediated bacterial lysis was dependent on the growth.

In order to form bacterial ghost cells, a strain carrying the ghost cassette was grown at 28°C until it reaches the number of specific population and then was transferred to 42°C to induce the ghost cells. Bacterial growth curve at 42°C was increased until 1 h and then decreased dramatically, showing a minimal level at 3 h (Figure 1(c)). Thereafter, the growth curve was maintained at a constant level. During this period, the bacterial viability was changed to a pattern similar to that of the OD value, which exhibited maximum value and minimum value of 10^9 and 10^3 CFU/mL in 1 and 3 h, respectively (Figure 1(d)). However, viable cell numbers were not decreased to 10^3 CFU/mL or less. It was suggested that the bacterial cells survived after ghost induction cause point mutations in MraY and the mutated MraYs were impossible to interact with E protein, or only weakly interact with it (Zheng et al. 2008). These phenomena were always induced to survive a certain number of population in the batch culture.

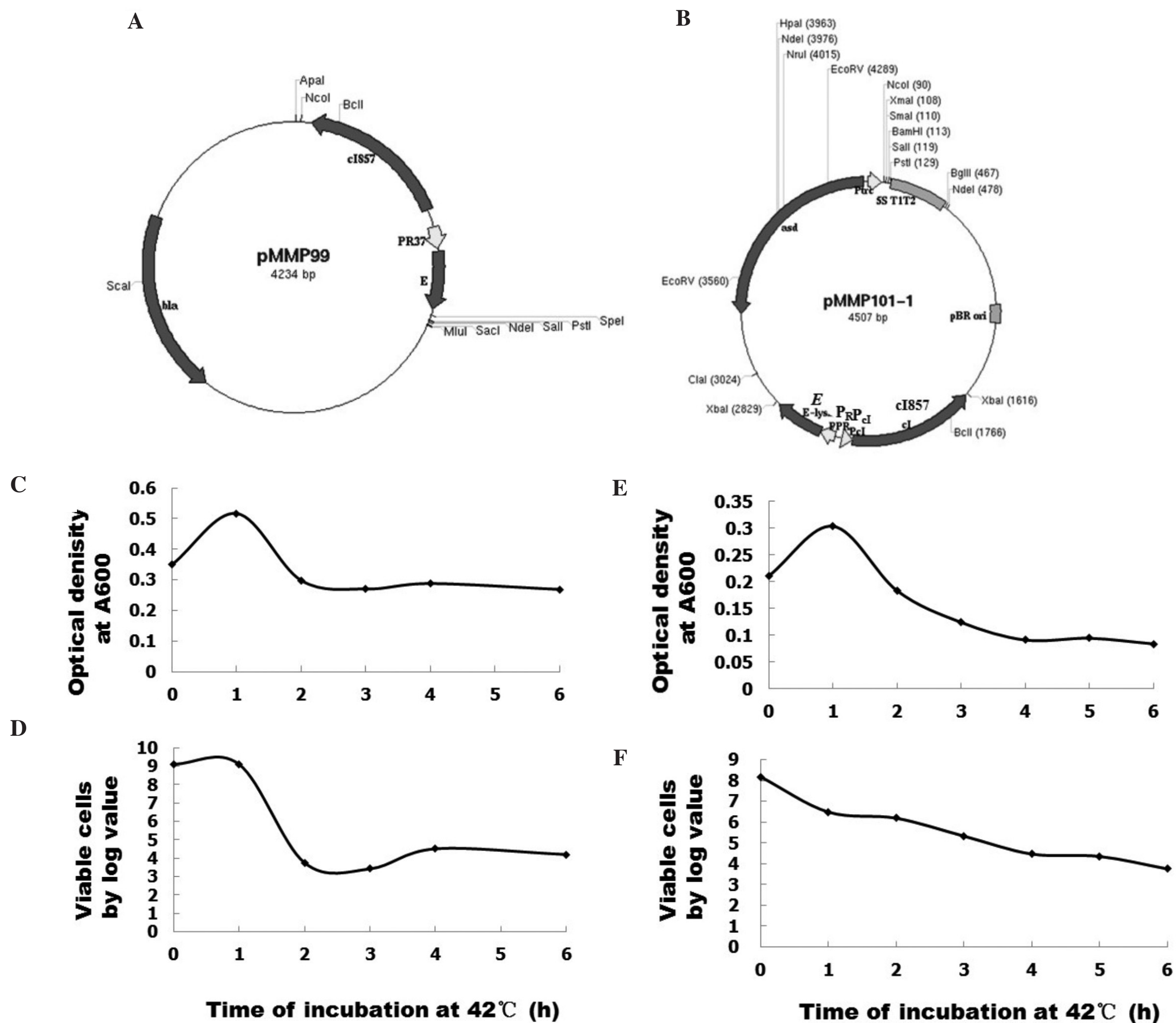

FIGURE 1. Genetic maps of pMMP99 and analysis of ghost formation. Genetic maps of pMMP99 (A) and pMMP101-1 (B), growth curves (C) and (E), and ghost formations (D) and (F) in *Salmonella* Typhimurium MMP13. Plasmid pMMP99 consists of beta-lactamase as a selection marker, whereas pMMP101-1 maintains *asd* gene as a selection marker. Growth curves were measured by optical density at 600 nm in wavelength. Viable cells were inoculated on LB agar plates with serial 10 fold-dilution of withdrawn broths from each cultural time and then counted after incubation at 28℃. cI857, bacteriophage rambda repressor *cI*857 gene; P_R, bacteriophage rambda P_R promoter; E, bacteriophage ΦX174 *E* gene

EFFICIENT GHOST FORMATION IS POSSIBLE IN AN *ASD*-DEFICIENT STRAIN TRANSFORMED WITH A RECOMBINANT PLASMID CARRYING THE GHOST CASSETTE AND ASD GENE

One goal of this study was the construction of an environmentally friendly system using a non-antibiotic marker. Therefore, plasmid pMMP101-1 carrying a non-antibiotic marker was prepared using pYA3342 as a backbone plasmid (Figure 1(b)). MMP13 carrying the recombinant plasmid pMMP101-1 showed a similar frequency or a little low of ghost formation to *E. coli* MMP13 (pMMP99) (Figure 1(d) and 1(e)). Regarding final cell survival, MMP13 (pMMP101-1) was less than 4.8×10^3 CFU/mL. MMP13 (pMMP101-1) were observed by mutational accumulation similar to MMP13 (pMMP99). The ghost cells formed due to the presence of E protein were identified by holes on the cell surfaces upon SEM examination (Figure 2). Therefore, it was suggested that these cells induce normal ghost formation. In a summary, since MMP13 (pMMP101-1) carrying the *asd* gene exhibits a frequency of ghost formation similar to MMP13 (pMMP99) carrying the antibiotic marker, the system is evaluated to have enough value for ghost production.

S. TYPHIMURIUM GHOST CELLS ELICIT HUMORAL AND CELLULAR IMMUNE RESPONSES

In order to assess immune responses of mice, the *Salmonella* ghost vaccine was intramuscularly injected at doses of 5×10^8 cells. Specimens for analysis of immune responses were collected from peripheral blood and vaginal lavage at 0-10 weeks post-vaccination. Total IgG, IgG1 and IgG2a levels were assayed in peripheral blood and secretory IgA (sIgA) was analyzed in fecal extracts and vaginal lavage fluid. As shown in Figure 3(a), 3(b) and 3(c), serum IgG, IgG1 and IgG2a levels increased from 4 weeks post-intramuscular (IM) injection of the ghost vaccine when compared with controls. Th1 cells were known to elicit cell-mediated immune responses and promote IgG2a propagation, whereas Th2 cells elicit production of IgG1 antibodies by B-cells to mediate humoral immune responses (DeKruyff et al. 1993; Gor et al. 2003; Li et al. 2008). Our results suggested that cellular and humoral immune responses were elicited via IgG2a and IgG1, respectively, after IM administration of the ghost vaccine. However, we also assumed that the humoral immune response was stronger than the cell-mediated response via comparison between serum titers of IgG1 and IgG2a.

Immune responses in vaginal lavage samples increased abruptly at 4 weeks and peaked at 6 weeks. However, vaginal sIgA levels decreased gradually after 6 weeks (Figure 3(d)). As shown in Figure 3(e), secretory IgA in fecal samples exhibited a nonspecific pattern. Fecal sIgA seems to be rapidly degraded by proteases originated from fecal microbes, which resulted in reduced sIgA levels in fecal samples.

LYMPHOCYTES FROM GHOST-CELL-TREATED MICE SHOW AN INCREASED PROLIFERATION

In order to analyze the proliferation of lymphocytes by the ghost cells, we assessed the proliferation of lymphocytes from splenocytes of mice immunized with *Salmonella* ghost cells. Lymphocyte proliferation was increased in all used doses of the ghost cells; the greatest increase was induced by administration of 1×10^{10} ghost cells (Figure 4). Mice that received doses of 1×10^8 and 1×10^9 cells showed a proliferation of markedly increased lymphocyte in 2 days when compared with those of 1 day and the treated mice with 1×10^{10} cells showed a twofold decrease in numbers when compared with 1 day. After 3 days of incubation, the numbers of lymphocytes were lower than those at 1 and 2 days in all cases. When live-attenuated *S. typhimurium gidA* mutant was injected intraperitoneally, splenocyte proliferation *in vitro* was observed by the mutant lysate (Shippy & Fadl 2012). This result indicated that the splenocyte proliferation involved essentially in the process of inducing an immune response. Therefore, our results suggest that splenocyte proliferation *in vitro*

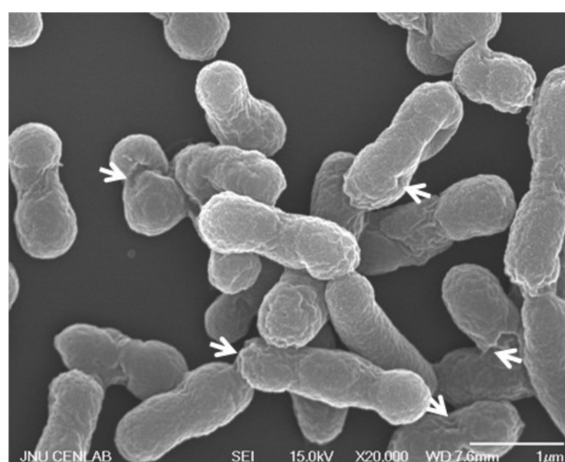

FIGURE 2. Result of observation of cell surface by SEM (Scanning Electron Microscopy). Photographic image was magnified by x 20,000 folds. Allows indicate holes made from function of E protein

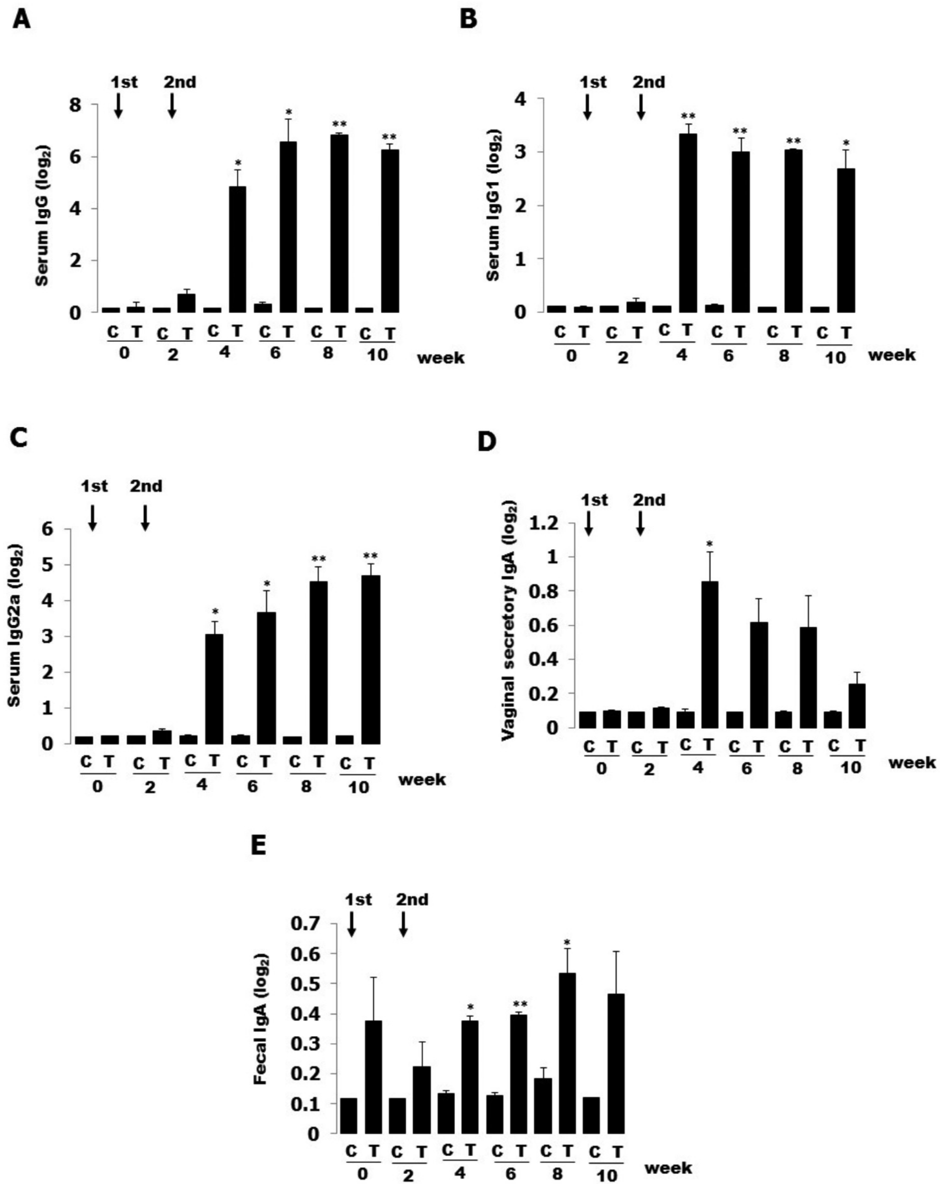

FIGURE 3. Analyses of immune responses to LPS from serum and secretory organs after administration of ghost vaccines into IM. Serum total IgG (A), serum IgG1 (B), serum IgG2a (C), vaginal sIgA (D), and fecal sIgA (E). LPS was treated by 2 ug/mL, serially diluted sera and goat anti-mouse IgG-horseradish peroxidase (HRP)-conjugate. The treated solutions were measured at 405 nm by ELISA reader. X- and Y-axes indicate weeks post injection and immune responses by log_2 value, respectively.
C, group treated with only PBS; T, group treated with *Salmonella* ghost

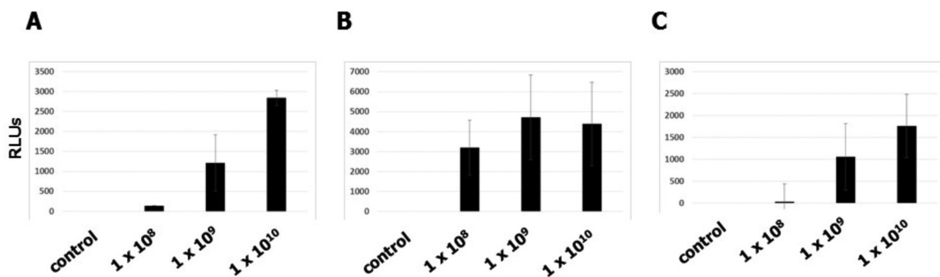

FIGURE 4. Assay of splenocyte proliferation during 1 day (A), 2 days (B) and 3 days (C). Splenocytes were isolated from 5 mice of days post administration of *Salmonella* ghost cells. The isolated splenocytes were stimulated by ghost cells for 3 days, treated by cell lysis reagent (ViaLight Plus kit, Lonza) and then measured by Luminometer (Spectra max GEMINI XPS, Molecular Devices). X- and Y-axes indicate ghost cell amounts with CFU/mL and luciferase activity, respectively

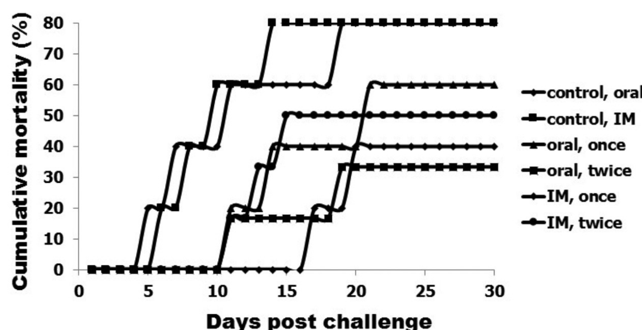

FIGURE 5. Result of challenge test by virulent *Salmonella typhimurium* χ3339 to mice vaccinated by *Salmonella* ghost. X and Y axes are day post challenge and cumulative death rate, respectively. Control administration with PBS buffer; Oral once, orally once administration by x CFU/mL dose; Oral twice, orally twice administration by x CFU/mL; IM once, once injection into intramuscle by x CFU/mL; IM twice, twice injection into intramuscle by x CFU/mL

induced via the ghost cells played important roles for immune responses.

GHOST CELLS INCREASE PROTECTION OF MICE AGAINST VIRULENT *S. TYPHIMURIUM*

S. typhimurium ghost cells were administered once or twice by the oral and IM routes. In order to assess the protection against virulent *S. typhimurium* to mice induced by immunization as the ghost cells, wt *S. typhimurium* χ3339 (1.8×10^6), which is higher dose of 10-fold than that of LD50 (Kim et al. 2011, 2009), was administered orally at 4 weeks post-immunization. The challenge test results were compared with control groups (treated only with PBS buffer) and with the treated groups according to route and number of administrations (Figure 5). Mice in the control group began to die from 4 days post-challenge; the mortality rate at 17 days was 80%. The mice to be treated once orally started to die from 11 days and exhibited a mortality rate of 60% at 30 days post-challenge. In contrast, the mice to be treated twice orally started to die from 17 days and showed a 33% final mortality rate. In mice inoculated by the IM route, those to be vaccinated once started to die at 16 days, while those to be vaccinated twice started to die at 10 days. Final mortalities for mice vaccinated once and twice via IM route were 40% and 50%, respectively. Boosting with the ghost vaccine via IM did not result in additional protection.

CONCLUSION

In order to improve cI857 P_R::*E* system carrying an antibiotic marker, we have developed a new vector system carrying *asd* gene to employ the host-balanced lethal system. *S. typhimurium* MMP13 carrying this new ghost system showed the efficiency of ghost formation of 99.999% or more. A ghost vaccine carrying a non-antibiotic marker increased production of total IgG, IgG1, IgG2a and sIgA antibodies and protected the vaccinated mice from infection with wild-type *S. typhimurium* more than those of control groups.

ACKNOWLEDGMENTS

This research was supported by the Technology Development Program for ('Agriculture and Forestry' or 'Food' or 'Fisheries'), Ministry for Food, Agriculture, Forestry and Fisheries (110050033HD110), by grants from the Priority Research Centers Program (no. 2011-0022965) through the National Research Foundation of Korea (NRF) funded by the Ministry of Education and from the Export Promotion Technology Development Program (No. 313012.05) of Ministry of Food, Agriculture, Forestry, and Fisheries, Republic of Korea.

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Received: 13 November 2015
Accepted: 21 Januari 2016