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RESEARCH ARTICLE

# Immune Responses of BALB/c Mice to a Polyvalent *Salmonella typhimurium* Ghost Vaccine Constructed Using a Dual-Regulated *E* Gene and a Foreign Antigen Delivery System

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**A B S T R A C T**

In order to induce stringent regulation of ghost cassette, a new ghost system was designed so that *E* gene is regulated by cI857 of a temperature sensitive regulator and antisense RNA under arabinose system at transcriptional and translational levels, respectively. The constructed system under stringent regulation owing to both regulatory factors was designed as pMMP184 which carries pBR replication origin. A ghost cell obtained from *S. typhimurium* harboring pMMP184 was examined for immune response after injection via intramuscular (IM) route against BALB/c mouse. The immune responses IgG, IgG1, IgG2a and vaginal IgA of the mice against *S. typhimurium* ghost cells were increased suddenly at 4 weeks post injection and maintained until 10 weeks. Additionally, pathogenic *E. coli* antigens, such as FedA, FedF, F41 and intimin, carried in the ghost cells elicited similar immune responses to those of *S. typhimurium* lipopolysaccharide. Proliferations of T and B cells, indicated by elevations of CD3e and CD23, respectively, were observed using FACS analysis. The challenge assays against sublethal dose of virulent *S. typhimurium* were presented by protections of 25~50% more than that of control. Our results suggest that the *S. typhimurium* ghost cell produced from the new ghost system is a potential possibility as a ghost vaccine.



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## INTRODUCTION

The majority of vaccines marketed against pathogenic bacteria have been prepared as killed or live-attenuated vaccines. Ghost vaccines have been evaluated as replacements for the killed vaccines which show low immune response and efficacy (Jawale *et al*., 2012; Chen *et al*., 2014; Eko *et al*., 2014). In order to obtain ghost vaccine, a ghost cassette is cloned into a suitable vector via recombination, which the cassette is adjusted by temperature or other methods such as the *cI*857-$\lambda P_R$-*E* gene and *lacI* (Langemann *et al*., 2010). A strain carrying the ghost cassette *cI*857-$\lambda P_R$-*E* gene is grown under 30°C, whereas ghost formation is induced via cell lysis with expression of *E* gene at 42ºC or higher.

The use of ghost vaccine is advantageous because they show the low suppression of immune response owing to the exclusion of many nonspecific cytosolic components, and because they maintain similar their surface antigenicity to that of the wild-type strain (Langemann *et al*., 2010). When a gene encoding an appropriate antigen is cloned into the ghost cassette, an effective polyvalent vaccine can be prepared against various pathogens at a lower cost, but overcome the limitations of conventional techniques. The conventional killed vaccines are obtained by additional treatments (*e.g*., chemical or heat) after cell growth, whereas ghost vaccine production can easily be scaled-up by increasing the culture cell mass and altering the incubation temperature. A conventional vaccine protects against a single pathogenic bacterium or several pathogenic bacteria simply by mixing different types of killed bacteria

(Meeusen *et al*., 2007; Peng *et al*., 2011). In contrast, the polyvalent ghost vaccine obtained in this study was prepared to protect against various pathogenic bacteria via anchors of various antigens originating from bacteria or viruses onto the ghost-bacterial surface such as *Salmonella*.

In the current study, a ghost cassette was designed so that *E* gene is regulated at transcriptional and translational levels via temperature-sensitive *cI*857-λ$P_R$ and *araC* $P_{araBAD}$, respectively. Furthermore, aspartate semialdehyde dehydrogenase (*asd*) gene was cloned into the vector system as a non-antibiotic selection marker. The constructed ghost vaccine was analyzed for various immunities including BALB/c mice immunoglobulins, T cell and B cell immunities and protection against virulent *S. typhimurium*.

## MATERIALS AND METHODS

**General DNA manipulations:** The ghost cassette was PCR-amplified from pHCE-GAPDH ghost37SDM or pLDR20 (Kwon *et al*., 2006 and 2009). Taq DNA polymerase for PCR amplification was employed by EX-Tag (TaKaRa, Japan) or Eco-Tag (Solgent, South Korea), and *Pfu* DNA polymerase was purchased from Bioneer (South Korea). The recombinant plasmid carrying a target gene was identified by digestion of restriction enzyme, PCR, and nucleotide sequencing (Macrogen, South Korea).

**Bacterial growth, measurement of viable cell, and formation of ghost bacteria:** Bacterial strains carrying the ghost cassette were inoculated with 1% pre-cultured cells in 100 mL LB medium containing 0.2% arabinose and then incubated at 28°C until an optical density at 600 nm ($OD_{600}$) of >1.5 was reached. Ghost cells were then obtained after incubation at 42°C. When the surviving cells were reduced to $\leq 10^3$ CFU/mL, the ghost formation was terminated forcibly, washed with distilled water, and resuspended to an appropriate cell mass. The resuspended ghost cells were directly used for vaccination, or freeze-dried and then stored until use.

**Mouse vaccination using ghost cells:** The concentrated or freeze-dried ghost cells were serially diluted 10-fold and injected by IM route into BALB/c mice that had been acclimatized for 1 week. The inoculated dosage was adjusted to $5\times10^8$ CFU/mL, and boosting was done in the same dose and route at 2 week post 1st injection. The vaccinated mice were observed for 10 weeks post-injection. The animal experiments in this study were approved by 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).

**Fluorescence activated cell sorting (FACS):** The ghost cells were injected via IM route of mice, the spleen was removed at 5 weeks post-injection, and the splenocytes were separated. The separated splenocytes ($1.0\times10^6$) were transferred into an E-tube, mixed with fluorescent dye-conjugated antibodies (BD Pharmingen), and incubated for 30 min at 4℃. The antibody cocktail used for B lymphocytes contained fluorescently labeled PE-CD45R/B220, PE CD23 (FcεRII), and APC sIgM. The antibody cocktail used for T lymphocytes contained PE-CD3e, PE CD4, and FITC CD8 (BD Pharmingen, USA). The treated solutions were centrifuged at 3,000 × *g* for 10 min at 4℃. The supernatant was removed, and the precipitated pellet was dissolved in 2 mL PBS buffer, pH 7.4, transferred into a FACS tube, and observed by FACS (BD Biosciences, USA). FACS analysis of 20,000 events was performed using CellQuest software (BD Biosciences, USA).

**Protection of mice immunized with *S. typhimurium* ghost cells against virulent *S. typhimurium*:** The ghost cells carrying each antigen were administrated to IM route by $5 \times 10^8$ CFU/ml dose, and boosting was done in the same dose and route at 2 week post 1st injection. The challenge test was performed to oral route by $1.8 \times 10^6$ CFU/ml dose of *S. typhimurium* χ3339 at 2 week post the ghost boosting. Food and water was prohibited for four hours before administration of ghost cells or bacteria. An hour after administration, the mice were supplied with food and water *ad libitum*. The treated mice were observed for 4 weeks.

**Statistical analysis:** All data are expressed as mean±SD unless otherwise specified. An independent sample *t* test was used to analyze statistical differences in immune responses between the immunized groups and non-immunized control group. The value of significance adopted were $P<0.05$ or $P<0.01$.

## RESULTS

**A new ghost system is composed of *E* gene under stringent control via a dual regulatory factors and a foreign antigen delivery system:** The pMMP184 plasmid carrying the stringently regulated *E* gene was originated from the backbone of pYA3342, which includes pBR ori, multicloning site (MCS), and *asd* gene (Kang *et al*., 2002) (Fig. 1A). To analyze function of temperature-sensitive *cI*857 and anti-sense RNA under *ara* system, a strain MMP13 carrying pMMP184 was grown in presence of arabinose until reaching stationary phase and then the cells supplemented with water and M9 medium of the same volume with initial culture were additionally incubated at 42℃ for the times indicated in Figure 1B. As shown in Figure 1B, when the strain was incubated in M9 medium, ghost formation was complete within 28 h. The results demonstrated that the newly constructed system is capable of proper ghost formation.

In order to detect the expression of foreign antigens fused with signal peptides from the constructed vectors, open reading frames of F18 (*fedA* and *fedF*), *f41*, and intimin digested by *Eco*RI and *Hin*dIII were ligated with pMMP184 treated by the same restriction enzymes. Expression of FedA, FedF, F41, and intimin in samples obtained from the transformants was analyzed by immunoblotting. The results showed that FedA, FedF, F41, and intimin were normally expressed in the transformants (data not shown).

**A ghost vaccine candidate injected via the IM route elicits immune responses against *S. typhimurium* and each antigen:** Total IgG, IgG1, and IgG2a were evaluated for analyzes of total serum immunoglobulin G, humoral and cellular immune response, respectively. As shown in Figure 2A-C, total IgG, IgG1 and IgG2a levels in plasma

treated with ghost cells apparently increased when compared with control group at 4 week post-administration. Although induction of secretory IgA (sIgA) from stool samples did not show an apparent pattern, sIgAs against FedA and FedF increased after 4 and 8 weeks, respectively (data not shown). The sIgAs from vaginal lavage was induced after 4 weeks, except that for FedA which was elicited after 6 weeks (Fig. 2D). These results indicate that IM vaccination of *S. typhimurium* ghost cells is a potential for eliciting partial mucosal immunity and complete humoral and cellular immunities.

Immune responses against *E. coli* antigens were detected in various amounts. Total IgGs against the antigens showed similar trends to those of *Salmonella* LPS (Fig. 3A). IgG1 against FedA, FedF and F41 and IgG2a against FedA and FedF exhibited slightly delayed production at 6 week (Fig. 3B and C). A small increase in sIgA against F41 and intimin was observed in vaginal lavage samples (Fig. 3D). The mucosal immunity against each antigen by the ghost vaccine from the results of sIgA of vaginal lavage elicited a slightly weaker response when compared with that of LPS. There was no distinct increase in sIgA production in the fecal samples against any of the antigens (data not shown).

The differentiation of T and B cells from mice injected by the ghost vaccine was determined using FACS. As shown in Figure 4A and B, CD3e T cells were elevated in mice injected with the ghost cells containing FedA, FedF, F41 and intimin, when compared with those of the isotype control group. CD23 B-cell was increased by FedA and FedF, but decreased by F41 and intimin (Fig. 4C and D).

**A ghost vaccine administered via IM route is capable of protecting against virulent *S. typhimurium:*** A challenge test using virulent wild type *S. typhimurium* was performed to assess the protection of mice treated with the ghost vaccine. Virulent wild type *S. typhimurium* χ3339 was inoculated in LB broth, grown until an $OD_{600}$ of 0.8 was reached, and then administered orally at a dose of $1.8 \times 10^6$ CFU/ml. As illustrated in Figure 5, the control mice started to die at 10 day post-challenge, and all mice died 14 days. The mice treated with intimin began to die from 9 day and showed a survival rate of 25%. However, the remaining groups began to die from 9 or 11 day and showed a final viability of 50%.

## DISCUSSION

When *E* gene is simultaneously expressed at the same time during ghost cell development, bacteria harboring *E* gene are prevented from premature death, and the ghost cells are formed in excellent. Therefore, to stringently regulate expression of *E* gene, *cI*857 and anti-sense RNA were introduced into the ghost cassette system (Fig. 1). *E* gene in this system is under control of cI857, which acts as a temperature-dependent repressor at transcriptional level. The anti-sense RNA is expressed under ara system, which interacts with E mRNA to regulate its translation. In contrast to a previously reported conditional lethal system using *araC* $P_{araBAD}$::*asd* (Kim *et al*., 2011), *E* gene is repressed by anti-sense RNA when arabinose is supplemented into medium.

Antigens delivered specifically to pathogenic *E. coli* were investigated for application of the constructed ghost system as a polyvalent vaccine. Among pathogenic *E. coli* associated with diarrhea in digestive system, pathogenic *E. coli* in pig carries specific fimbriae. Infection by pathogenic *E. coli* harboring F4 fimbriae causes diarrhea in piglets in 5 or fewer days, F5, F6 and F41 fimbriae are detected at 5~14 days post-delivery, and F4 and F18 (FedA and FedF) at 3~4 weeks (Kim *et al*., 2010; Stahl *et al*., 2011). Furthermore, enterohemorrhagic *E. coli* (EHEC) O157:H7 requires intimin, encoded by *eae* gene, to establish adhesion in host animal (Vidovic *et al*., 2013). Among various adhesion factors carrying pathogenic *E. coli*, F41, F18 and intimin, which are associated with diarrhea in piglets from late lactation to weaning, were employed in this study.

Productions of IgG1 and IgG2a are induced by Th2 and Th1 cells, which elicit humoral and cell-mediated immune responses, respectively (Gor *et al*., 2003; Li *et al*., 2008). IM vaccination in this study strongly elicited total IgG, IgG1 and IgG2a (Figs. 2 and 3), suggesting that IM injection of the developed *Salmonella* ghost vaccine induces humoral and cellular immune responses.

**Table 1:** Bacterial strains and plasmids used in this study

| Strains and plasmids | Descriptions | References |
|---|---|---|
| *E.coli* | | |
| Top10 | F-mcrA (mrr-hsdRMS-mcrBC) Φ80lacZ⊿M15 ⊿lacX74 nupG recA1 araD139 ⊿(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 |
| χ6212 | *Φ*80d lacZ ΔM15 *deoR ⊿(lacZYA-argF)U169 supE44λ⁻ gyrA96 recA1 relA1 endA1 asdA4 ⊿zhf-2::Tn10 hsdR17* ($R^- M^+$) | Lab stock |
| ***Salmonella*** | | |
| MMP13 | *S. typhimurium* JOL401 *asd*A16 | This study |
| *S.typhimurium* χ3339 | wild-type strain SL1344, hisG rpsL | Lab stock |
| **Plasmids** | | |
| T-vector | A TA cloning vector | Promega |
| pET28a | An overexpression vector containing pBR322 origin | Novogen |
| pHCE37SDM | A cloning vector carrying ghost cassette | From Pukyong national University |
| pLDR20 | A vector carrying *cI*857 $P_R$::*E* | Pukyong National University |
| pYA3332 | A vector containing p15A origin | Kang *et al.* (2002) |
| pYA3342 | A vector containing pBR origin | Kang *et al.* (2002) |
| pMMP184 | A derivative of pYA3342 carrying *cI*857 $P_R$ *E* ara and PR *ompA* TM | This study |
| pMMP187 | A derivative of pYA3332 carrying *cI*857 $P_R$ *E* ara and PR *ompA* TM | This study |
| pMMP258 | A derivative of pMMP184 carrying *fedA* gene | This study |
| pMMP264 | A derivative of pMMP184 carrying *fedF* gene | This study |
| pMMP272 | A derivative of pMMP184 carrying *f41* gene | This study |
| pMMP273 | A derivative of pMMP184 carrying *intimin* gene | This study |

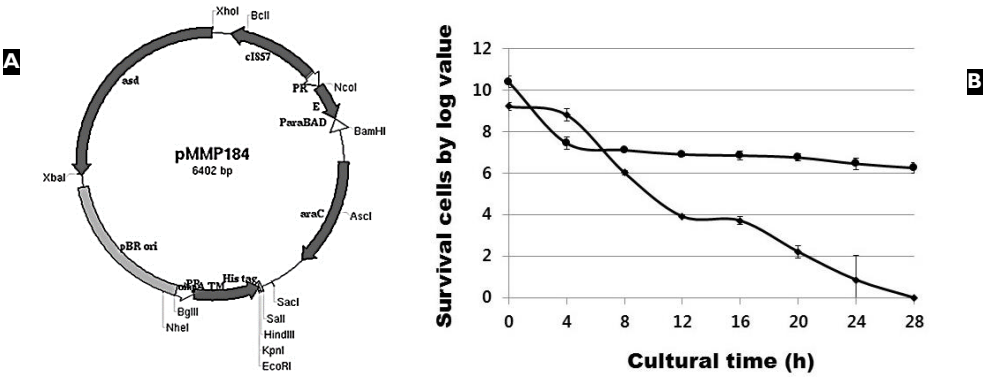


**Fig. 1:** Construction of the ghost system containing a dual regulatory element. Genetic maps of pMMP184 (A) and surviving cell counts for MMP13 [pMMP184] (B). A) pMMP184 contains pBR origin. *asd*, aspartate semialdehyde dehydrogenase gene; cI857, bacteriophage lambda repressor *cI*857 gene; PR, bacteriophage lambda PR promoter; E, bacteriophage ΦX174 *E* gene; ParaBAD, araBAD promoter; *araC*, *araC* gene. B) Filled circular and rectangular markers indicate incubation with PBS buffer, pH 7.4, or M9 medium, respectively, after washing with PBS buffer.

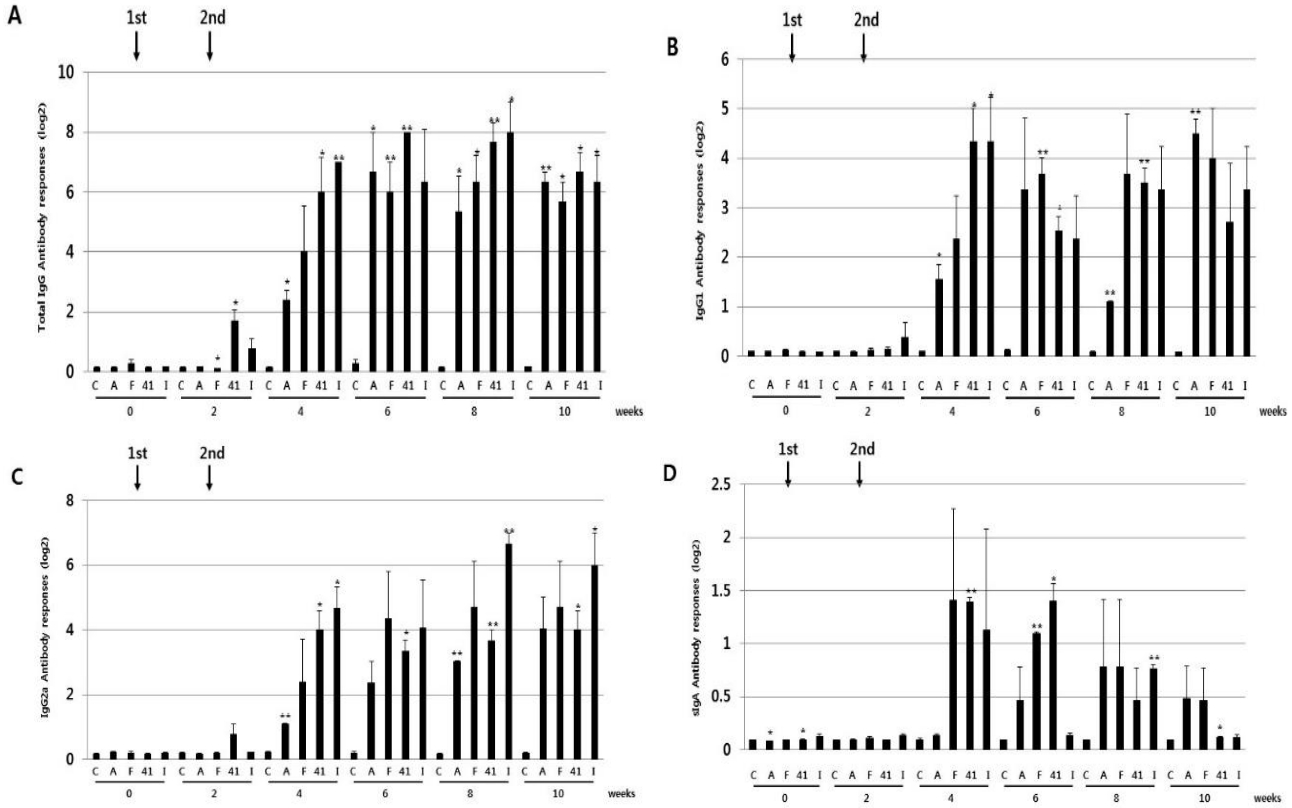


**Fig. 2:** Analyses of total IgG (A), IgG1 (B), IgG2a (C), and vaginal secretory IgA (D) against LPS (lipopolysaccharides) from serum following IM administration of ghost vaccines. LPS (2μg/mL)-treated solutions were measured at 405nm using an ELISA reader. The x- and y-axes indicate a number of weeks post-injection and the $\log_2$ value of the immune response, respectively. Antibody levels are expressed as means ± S.D. Statistical significance was defined as a P<0.05 or P<0.01. *P<0.05, **P<0.01 vs. non-vaccinated control. C, only PBS; A, fedA; F, fedF; 41, f41; I, intimin.

T and B cells of mice treated with the ghost vaccine displayed the increased differentiation into CD3e-T cells and partial differentiation into CD23-B cells, respectively (Fig. 4). CD3e is a component of T cell receptor-CD3 complex, which plays an important role in coupling antigen recognition to several intracellular signal transduction pathways (Kuhns and Badgandi, 2012; Zhu *et al*., 2013). The epsilon polypeptide plays an essential role in T cell development (Fischer *et al*., 2005). Our results suggest that vaccination of ghost cells promotes T cell development through the increased CD3e (Fig. 4A and B).

LPS induces transcriptional activation of CD23, which is regulated by a number of cytokines including IL-4, IL-5, IL-9, IL-13, GM-CSF, INF-γ and CD40 (Lapa e Silva *et al*., 2000; Rosenwasser and Meng, 2005; Jackson *et al*., 2009). It is assumed that CD23 is enhanced by LPS from the vaccinated *Salmonella* ghost cells and additional functions of FedA and FedF as antigens injected via the IM route (Fig. 4C and D).

Previously, when non-living *Salmonella* vaccines, which were prepared by acetone, heat, and phenol treatments, were administrated parenterally into mice, the treated mice showed 0~100% protection against various inoculums of virulent *Salmonella* (Harrison *et al*., 1997; Jazani *et al*., 2005). The mice treated with the ghost vaccine in this study displayed 25~50% protection against sublethal doses of virulent *S. typhimurium*. Thus, the results of this study are in good agreement with previous reports on non-living *Salmonella* vaccines.

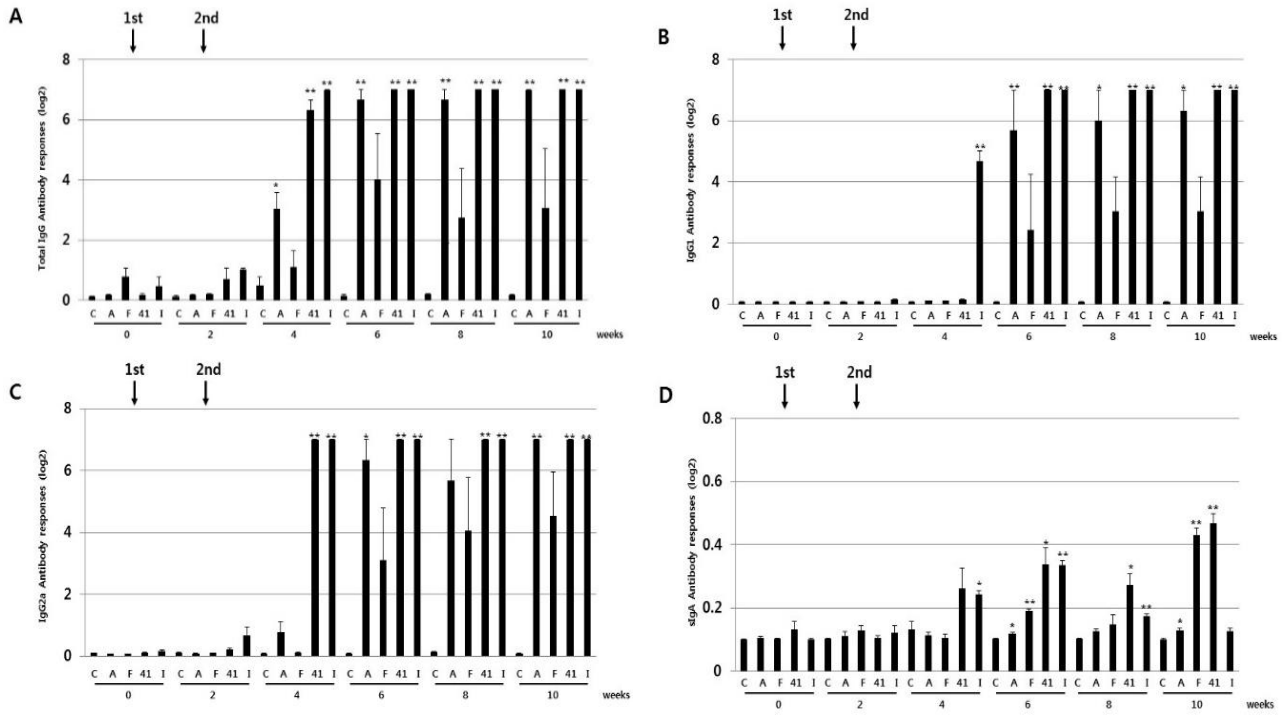


**Fig. 3:** Analyses of total IgG (A), IgG1 (B), IgG2a (C), and vaginal secretory IgA (D) against each antigen from serum after IM administration of ghost vaccines. Solutions treated with each antigen (2μg/mL) were measured at 405nm using an ELISA reader. The x- and y-axes indicate a number of weeks post-injection and the log value of the immune response, respectively. Antibody levels are expressed as means ± S.D. Statistical significance was defined as a P<0.05 or P<0.01. *P<0.05, **P<0.01 vs. non-vaccinated control. C, only PBS; A, fedA; F, fedF; 41, f41; I, intimin.

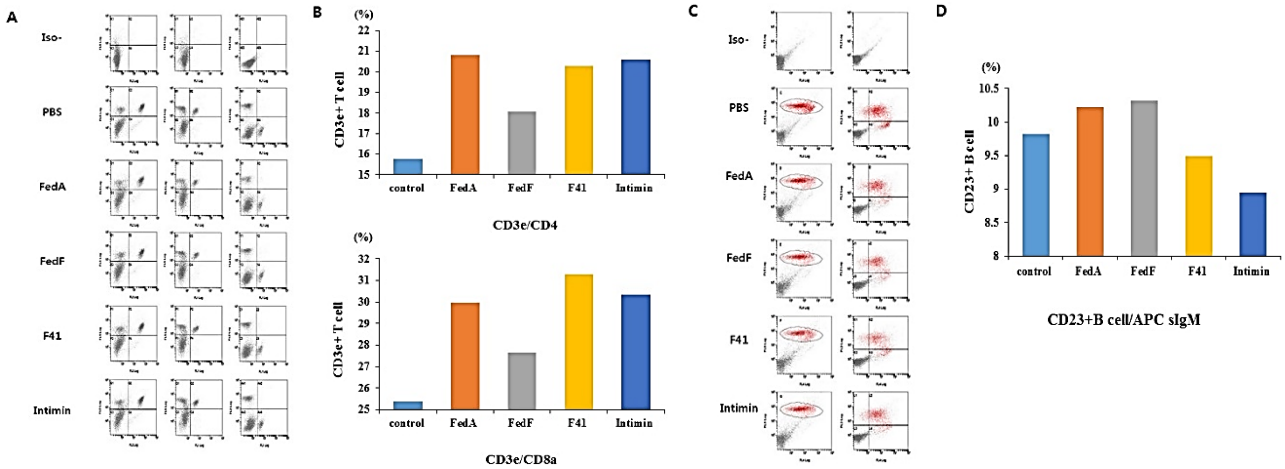


**Fig. 4:** Distributions of T and B cells isolated from splenocytes of mice after IM administration of ghost vaccines. FACS results for T and B cells (A and C) and the associated histograms (B and D). Splenocytes were isolated from mice at 5 weeks post-injection of ghost vaccines. In the left panels in A, X- and Y-axes indicate PE CD4 and PE-Cy7 CD3e, respectively. In the middle panels, X- and Y-axes indicate FITC CD8a and PE-Cy7 CD3e, respectively. In the right panels, X- and Y-axes indicate FITC CD8a and PE CD4, respectively. In the left panels in C, X- and Y-axes indicate APC sIgM and PE-Cy7 CD45R/B220, respectively. In the right panels, X- and Y-axes indicate APC sIgM and PE CD23, respectively. Iso-; isotype control.

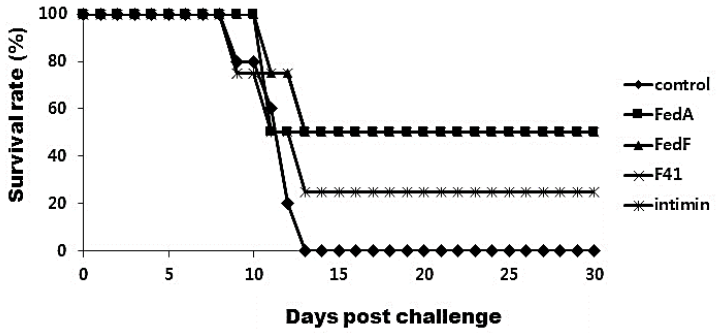


**Fig. 5:** Challenge tests to vaccination by ghost cells. X- and Y-axes indicate days post-challenge by wild type *S. typhimurium* χ3339 and survival rate, respectively. Control=PBS administration; each antigen=vaccination by twice injection of each antigen (FedA, FedF, F41 and Intimin) @ $1 \times 10^8$ CFU/ml.

**Conclusions:** A new ghost vector system based on the *E* gene, which was regulated at transcriptional and translational levels by cI857 and anti-sense RNA, respectively, was developed. We prepared *S. typhimurium* ghost cells using this system and analyzed their immunity-inducing abilities in BALB/c mice. As a result, productions of total IgG, IgG1, IgG2a and sIgA increased significantly by vaccination of the ghost cells. It is estimated that the ghost cells induce the proliferation of CD3e-T and CD23-B cells. Taken together, the current findings suggest that *S. typhimurium* ghost cells possess appropriate level of functionality for use as a vaccine candidate.

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