

Generation of an attenuated *Salmonella*-delivery strains expressing adhesin and toxin antigens for progressive atrophic rhinitis, and evaluation of its immune responses in a murine model



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ABSTRACT

An expression/secretion plasmid containing genes encoding the FimA, CP39, PtfA, ToxA and F1P2 antigens associated with porcine pneumonic pasteurellosis and progressive atrophic rhinitis (PAR) was constructed and harbored in an attenuated *Salmonella* Typhimurium, which was used as the vaccine candidate. The immune responses induced by this delivery strain were investigated in a murine model. Each antigen secreted from the delivery strain was confirmed by Western blot analysis. Thirty BALB/c mice were divided equally into two groups; group A were intranasally inoculated with the mixture of the five delivery strains, and group B were inoculated with sterile PBS. In group A, all antigen-specific serum IgG were significantly increased compared to those of group B from the 2nd week post-inoculation (WPI) till the 8th WPI. All antigen-specific mucosal IgA in group A were also significantly greater than those of group B. In addition, the significant splenic lymphocyte proliferative responses, the elevations of CD3⁺CD4⁺, CD3⁺CD8⁺ and B-cell populations, and the induction of IFN- γ expression in group A were observed. In conclusion, the mixture of five delivery strains expressing specific antigen for these diseases was found to be capable of inducing significant humoral and cellular immune responses.

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1. Introduction

Pneumonic pasteurellosis, caused by both toxigenic and non-toxicogenic strains of *Pasteurella multocida* serotypes A and D, which is responsible for significant losses in the swine industry [1]. In addition, progressive atrophic rhinitis (PAR) is an important upper respiratory disease is caused by *Bordetella bronchiseptica* and *P. multocida* serotype D, which is characterized by the shortening and twisting of the snout, dark tear staining, and pneumonia in swine [2,3]. Potential virulence factors of *P. multocida* and *B. bronchiseptica*, which are involved in bacterial attachment and internalization, include hemagglutinating molecules, capsule, fimbriae, and toxin [4,5]. These virulence factors can be targeted for the prevention of *P. multocida* and *B. bronchiseptica* infection [6]. The *fimA* gene encodes a subunit of fimbriae, fimbriin (FimA) of *P. multocida* strains, which abundantly expressed bacterial surface protein [7]. This protein has been shown to be a potent target for host immunity [8]. Cp39 is a capsule-associated adhesin, and a cross-protective antigen among

P. multocida strains [9,10]. Its properties were found to be identical to OmpH, which stimulates significant protection against heterologous challenge in chickens [10]. The PtfA, one among colonization factors of *P. multocida* strains, showed variation in their potency as a vaccine based on the ability of this fimbrial antigen to cause a heterologous, and capsule- and serotype-independent protection [11]. In addition, *P. multocida* toxin (PMT), a monomeric 146 kDa protein encoded by the *toxA* gene, is produced by *P. multocida* serotype A and D strains [12,13]. A nontoxic but immunogenic PMT derivative, such as the N-terminal truncated fragment of PMT may be advantageous in the development of efficient vaccines against PAR [13,14]. An adhesin protein, F1P2 of *B. bronchiseptica*, which consists of the immunodominant protective type I domain (F1) of filamentous hemagglutinin and the immunogenic region II domain (P2) of pertactin may serve as a protective antigen against porcine bordetellosis in pigs [15,16].

Live vaccine vehicles can powerfully induce both mucosal and systemic immune responses against pathogenic microorganisms [17]. In recently, many protection studies using delivery systems to express the immunogenic antigens of *P. multocida* [18–20] or *B. bronchiseptica* [15,16] have been performed to prevent *P. multocida* infections or *B. bronchiseptica* infections. In particular, live,

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attenuated *Salmonella* strains have been manipulated to express a wide range of antigens from bacterial, parasitic, and viral sources [17,21]. *Salmonella* Typhimurium colonizes the gastrointestinal system, and the respiratory system [22]. The intranasal inoculation of mice with the *Salmonella* systems was particularly efficient at inducing antibody responses both in serum and respiratory tracts [23,24]. A balanced-lethal host-vector system based on the essential bacterial gene for aspartate β -semialdehyde dehydrogenase (*asd*) has been used to specify recombinant antigens from Asd + plasmids that are retained in vivo in *Salmonella* strains with the *asd* gene deleted [25]. The objective of this study was to construct a live *Salmonella* delivery system expressing FimA, CP39, PtfA ToxA and F1P2 antigens associated with pneumonic pasteurellosis and PAR for young pigs in a murine model. Immune responses induced via intranasal inoculation with this delivery system were examined using a murine model. Mice inoculated with the delivery system not only showed robust systemic and mucosal immune responses, but also demonstrated powerful cellular immune responses.

2. Materials and methods

2.1. Experimental animals

Five-week-old, 30 female BALB/c mice were equally divided into two groups. Mice were maintained in 12 h light/dark cycles and allowed free access to a standard rodent diet and water. The animal experiments mentioned in this study were conducted under ethics approval (CBU 2011-0017) from the Chonbuk National University Animal Ethics Committee in accordance with the guidelines of the Korean Council on Animal Care.

2.2. Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Wild-type *P. multocida* type A, JOL976, isolated from a piglet was used to amplify gene encoding FimA adhesin antigen. In addition, JOL977, *P. multocida* type D isolated from a piglet, was used to amplify genes encoding CP39 and PtfA adhesin antigens, and the ToxA toxin antigen. *B. bronchiseptica* isolate from a piglet, JOL978, was used to amplify the gene encoding the F1P2 adhesin antigen (Table 1). The JOL977 was inoculated in mice and subsequently isolated from internal organs. In this way, the strain was passaged three times to increase the virulence of JOL977. After three passages, this strain was renamed as JOL1080 and was used as the virulent challenge strain. These strains were kindly supplied by the National Veterinary Research and Quarantine Service, Korea. The attenuated *Salmonella* Typhimurium ($\Delta lon \Delta cpxR \Delta asd$) mutant strain, JOL912 [26], and the pBP244 plasmid [27] were used as a host and a vector for the delivery of individual antigens.

2.3. Cloning of individual recombinant fimbrial antigens

CP39, FimA, PtfA, ToxA and F1P2 proteins were purified from JOL1136, JOL1234, JOL1214, JOL1135 and JOL991, respectively, (Table 1) for enzyme-linked immunosorbent assay (ELISA), and splenocyte stimulation in flow cytometry and quantitative real-time polymerase chain reaction (PCR). Briefly, genes for CP39, FimA, PtfA, ToxA and F1P2 proteins were amplified by PCR using specific primer sets (Table 2). The PCR fragments of each gene were digested with restriction enzymes, and were subsequently cloned into pQE31 or pET28a. These plasmids were then transformed into *Escherichia coli* TOP10 or *E. coli* BL21 in order to create JOL1136, JOL1234, JOL1214, JOL1135 and JOL991 strains. The recombinant CP39, FimA, PtfA, ToxA and F1P2 antigens were prepared from JOL1136, JOL1234, JOL1214, JOL1135 and

Table 1
Bacterial strains and plasmids used in this study.

Strain/plasmid	Description	Source
Strains		
<i>E. coli</i>		
Top10	F-mcrA (mrr-hsdRMS-mcrBC) 80lacZM15 lacX74 recA1 ara139 (ara-leu)7697 galUgalKrrpSL (Str ^R)endA1nupG F ⁻ , ompT, hsdS _B (r _B ⁻ , m _B ⁻), dcm, gal, λ (DE3), pLysS, Cm ^r	Lab stock
BL21(DE3)pLysS	BL21 with pET-CP39	Lab stock
JOL1136	BL21 with pET-FimA	This study
JOL1234	Top10 with pQE-PtfA	This study
JOL1214	BL21 with pET-ToxA	This study
JOL1135	BL21 with pET-F1P2	This study
JOL991		
<i>Salmonella</i> Typhimurium		
JOL401	<i>Salmonella</i> Typhimurium wild type	Lab stock
JOL911	<i>Salmonella</i> Typhimurium JOL401 derivative $\Delta lon \Delta cpxR$	Lab stock
JOL912	<i>Salmonella</i> Typhimurium JOL911 derivative Δasd	Lab stock
JOL932	JOL912 with pBP244	This study
JOL1240	JOL912 with pBP244-CP39	This study
JOL1251	JOL912 with pBP244-FimA	This study
JOL1247	JOL912 with pBP244-PtfA	This study
JOL1244	JOL912 with pBP244-ToxA	This study
JOL1074	JOL912 with pBP244-F1P2	This study
<i>P. multocida</i>		
JOL976	<i>Pasteurella multocida</i> sero type A wild type (PmA037)	Lab stock
JOL977	<i>P. multocida</i> (PDNT)sero type D wild type	Lab stock
<i>B. bronchiseptica</i>		
JOL978	<i>Bordetella bronchiseptica</i> wild type	Lab stock
Plasmids		
pQE31	IPTG-inducible expression vector; Am ^r	Qiagen
pET28a	IPTG-inducible expression vector; Km ^r	Novagen
pBP244	pYA3493 derivative containing <i>lepB</i> , <i>secA</i> and <i>secB</i> genes	Lab stock

JOL991, respectively, using an affinity purification process with nickel-nitrilotriacetic acid-agarose (Qiagen, Valencia, CA, USA). The identities of the purified antigens were confirmed via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). All purified antigens were stored at -70°C until use.

2.4. Purification of fimbria-specific antisera

Preparation of specific antibodies against the individual adhesin or toxin antigens was carried out via subcutaneous injection of an emulsion containing approximately 250 μg of each purified recombinant antigen in 1 ml of sterile phosphate buffered saline (PBS) and 1 ml of complete Freund adjuvant (Sigma-Aldrich, St. Louis, MO, USA) into New Zealand white rabbits. Two boosters with the same antigen quantity in incomplete Freund adjuvant (Sigma-Aldrich, St. Louis, MO, USA) were administered at 14 and 28 days post-prime immunization. Blood was collected for the preparation of antisera at 14 days after the final injection. The each antigen-specific antibody was stored at -70°C until use.

2.5. Preparation of *Salmonella* delivery strain formulation

The delivery strain formulations were prepared as previously described with a slight modification [27]. The genes for CP39, FimA, PtfA and F1P2 adhesin, and ToxA toxin antigen were prepared by digestion from JOL1136, JOL1234, JOL1214, JOL1135 and JOL991 strains, respectively. And then each gene was inserted in pBP244 for

Table 2

PCR primers used in this study and their product sizes.

Primer	Sequence	Size (bp)	Enzyme site	Primer coordinates	Accession number	Reference
CP39	CP39-F CCGCGAATTCGCAACAGTTTACAATCAAGACCG	963	EcoRI	61–81	JX473022.1	This study
	CP39-R CCGCGTCGACTTAGAAGTGTACGCGTAAACC		Sall	1003–1023		
fimA	FimA-F CCGCGAATTCGATGGGGTAACAGACACT	960	EcoRI	254878–254895	CP03328.1	This study
	FimA-R CCGCGTCGACTTACTTGCTTAAGCAAGC		Sall	255820–255837		
toxA	toxA-F CCGCGGATCCAAACATTTTTTAATCAGAT	591	BamHI	10–30	FN398147.1	This study
	toxA-R CCGCAAGCTTATAAAGCTGAGCATATTTT		HindIII	581–600		
ptfA	ptfA-F CCGCGGATCCGCCATTTTCTTTTCGATTAA	426	BamHI	10–29	JX139092.1	This study
	ptfA-R CCGCAAGCTTTTATGCGCAAAATCCTGCTGG		HindIII	415–435		
F1	F1-F TTAAAGAATTCCTGACTGCCCTGGACAAT	465	EcoRI	843888–843905	HE965806.1	[15]
	F1-R TTAAAGTCGACTCGCAGATCCGCGGCAAA		Sall	843441–843458		
P2	P2-F TAATTCTCGACAACACCATGCTGCTGGTG	279	Sall	1453–1470	HQ259258.1	[15]
	P2-R TTAACTCGAGGCGGACAACCTCCCTGCC		HindIII	1714–1731		

Underlines indicate the sites of restriction enzymes, such as BamHI, EcoRI, Sall and HindIII.

the construction of vaccine strains. Subsequently, these plasmids were electroporated into JOL912 and the results were designated JOL1240 for CP39, JOL1251 for FimA, JOL1247 for PtfA, JOL1244 for ToxA, and JOL1074 for F1P2.

2.6. Western blot analysis

Western blot analysis was performed to examine the secretions of the individual recombinant FimA, CP39, PtfA, ToxA and F1P2 proteins from JOL1251, JOL1240, JOL1247, JOL1244 and JOL1074, respectively. In addition, JOL932, JOL912 containing only pBP244, was used as a control. The individual strains were cultured in LB broth at 37 °C, and the cultures were harvested at an optical density (OD) of 0.8 at 600 nm (OD₆₀₀). Culture supernatants were harvested via centrifugation at 3400 × g for 20 min. The supernatants were passed through a filter with 0.22 μm pores and precipitated overnight with 20% trichloroacetic acid. The precipitates were re-suspended in cold PBS. The antigens were separated using 12% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). After overnight blocking at 4 °C with 5% skim milk in PBS containing 0.1% Tween-20, individual recombinant antigens were incubated with each protein-specific polyclonal antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG (Southern Biotech, Birmingham, AL, USA). Immunoreactive bands were detected using the Westone™ Western Blot Detection System (iNtRON, Seongnam, South Korea) and the multi-wavelength KODAK Image Station 4000MM illumination system (Kodak, New Haven, CT, USA).

2.7. Inoculation in mice, and sample collection

All mice were inoculated at 6 weeks of age. Group A mice were intranasally inoculated with approximately 1×10^5 colony-forming units (CFU)/ml in 10 μl of an equal-volume mixture of the five live delivery strains (consisting of approximately 0.2×10^5 CFU per each strain in 10 μl). Group B mice were intranasally inoculated with 10 μl sterile PBS as the control [28]. Inoculation was performed by instilling a total of 20 μl of the mixture suspension into both nostrils. Serum and vaginal samples were collected at 0, 2, 4, 6, and 8 weeks post inoculation (WPI).

For investigating immune responses of broncho-alveolar lavage samples, 60 female-6 weeks aged BALB/c mice were equally divided into two groups mice. Group A mice were intranasally inoculated with approximately 1×10^5 CFU/ml in 10 μl of the vaccine mixture. Group B mice were intranasally inoculated with approximately 1×10^5 CFU/ml in 10 μl of JOL932 as the control. Broncho-alveolar lavage samples were collected at the 0, 2, 4 and 6th WPI by inserting a suitable cannula toward the lungs into the exposed trachea. A 3 ml syringe was attached to the cannula and the lungs were washed with 1 ml of sterile PBS containing 1.0% bovine serum albumin by

flushing the solution in-out of the lungs several times. The samples were centrifuged at 3000 rpm to remove particulate matter [29,30]. All samples were prepared and stored as previously described [26].

2.8. Immune response measurement by ELISA

Modified ELISA was used to determine individual protein-specific IgG antibody titers in serum and mucosal IgA titers in vaginal wash samples similar to a previous study [31]. Briefly, Greiner ELISA-plates (Microton, 96W, flat-bot; Greiner Bio-One, Frickenhausen, Germany) were coated with each purified antigen (500 ng/well) overnight at 4 °C. Sera were diluted 1:200 in PBS, and vaginal wash samples were diluted 1:3 in PBS. The plates were treated with horseradish peroxidase-conjugated goat anti-mouse IgG or IgA antibody (Southern Biotechnology Associates, Birmingham, AL, USA). Enzymatic reactions were produced through the addition of substrate containing o-phenylenediamine (Sigma-Aldrich, St. Louis, MO, USA) and were measured using an automated ELISA spectrophotometer (TECAN, Salzburg, Austria) at 492 nm. A standard curve was generated to represent the relationship between the concentrations of the standards and their absorbance as described previously [31], and the concentration of antibodies in each sample was determined using this curve. The antibody concentrations are expressed as mean ± standard deviation.

2.9. Splenocyte proliferation assay

Spleens from the each group mice were collected on 4 WPI as previously described [32,33]. The splenocyte proliferation assay (SPA) was performed with specific antigen as previously described [34,35], with a slight modification. Briefly, 100 μl of cell suspension at 5×10^6 cells/ml in RPMI-1640 medium were incubated in 96-well tissue culture plates with 50 μl of medium or medium containing 4 μg/ml of FimA, CP39, PtfA, or F1P2 antigen at 37 °C, in a humidified 5% CO₂ atmosphere for 48 h. The blastogenic response for the assay was expressed as the mean stimulation index (SI). The % difference between stimulated and unstimulated splenocytes of each antigen was calculated. To analyze the significance of group A to B, these % differences were compared.

2.10. Flow cytometry

To analyze changes in the T-lymphocyte subsets in spleen post-inoculation, the splenocytes from 5 mice in each group were collected on 4 WPI following the method described above. Subsequently, the cells were stimulated with the individual antigens for 12 h [32]. The cells were incubated with monoclonal antibodies against CD3, CD4, CD8, and CD45R (eBioscience, San Diego, CA) in the dark at 4 °C for 30 min, as previously described [33]. The analysis was carried out using a FACS Calibur™ (Becton Dickinson, San

Jose, CA) instrument equipped with a 15 mW, 488 nm air-cooled argon laser. Data analysis was performed using FlowJo software (Tree Star, Ashland, OR).

2.11. Real-time quantitative RT-PCR analysis for cytokine expression

Splenocytes from the mice on 4 WPI were harvested, and 5×10^6 cells per well were cultured in 24-well tissue culture plates at 37°C with 5% CO_2 for 72 h in the presence of $4 \mu\text{g/ml}$ CP39- or PtfA-specific antigen. Total RNA was extracted from the splenocytes using an RNeasy Plus mini kit, (Qiagen, GmbH, Hilden, Germany) and subsequent cDNA synthesis was performed using a High Capacity cDNA Reverse Transcription Kit. (Applied Biosystems, Foster, USA) Levels of cytokine interleukin (IL)-4 and interferon (IFN)- γ mRNA expression were quantified with real-time RT-PCR as previously described with a slight modification [33]. PCR was performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster, USA) with the following cycle profile: 1 cycle at 95°C for 15 min, and 40 cycles at 94°C for 15 s, 55°C for 30 s and 72°C for 30 s. The primer sets, (Qiagen, GmbH, Hilden, Germany) QT00160678 for IL-4, QT01038821 for IFN- γ , and QT01658692 for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), were used for the PCR. The threshold method was used to quantify the mRNA expression levels, and ΔCT values were calculated based on the internal standard, GAPDH. Results are expressed as $2^{-(\Delta\Delta\text{CT})}$ (n-fold change compared to the non-treated control group) [33].

2.12. Histology

Lung tissue samples from mice that were euthanized at 4 WPI were fixed in 10% neutral phosphate-buffered formalin. The sections were stained with hematoxylin and eosin and examined. Photomicrographs were taken using a light microscope (BX-51, Olympus Corp., Tokyo, Japan) and analyzed using digital imaging software (Analysis TS, Olympus Corp., Tokyo, Japan).

2.13. Statistical analysis

Results are expressed as mean $\pm$ standard deviation (SD). The Mann-Whitney U-test (SPSS 16.0 program, SPSS, Chicago, IL) was

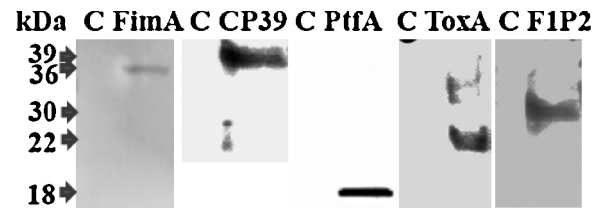


Fig. 1. Identification of secreted recombinant FimA, CP39, PtfA, ToxA, and F1P2 adhesin antigens using Western blot analysis. Recombinant FimA, CP39, PtfA, ToxA, and F1P2 adhesin antigens expressed and secreted by JOL912 were detected via Western blot using the appropriate antigen-specific antibodies. *Salmonella* Typhimurium $\Delta\text{lon}\Delta\text{cpxR}\Delta\text{asd}$ containing only pPB244, JOL932, was used as a control. Lanes: C, control; FimA, recombinant FimA adhesin antigen secreted by JOL1251; CP39, recombinant CP39 adhesin antigen secreted by JOL1240; PtfA, recombinant PtfA adhesin antigen secreted by JOL1247; ToxA, recombinant ToxA toxin antigen secreted by JOL1244; F1P2, recombinant F1P2 adhesin antigen secreted by JOL1074.

used to determine the significant differences in serum and vaginal antibody titers between the groups A and B mice. A P value ≤ 0.05 was regarded as statistically significant.

3. Results

3.1. Secretion of recombinant FimA, Cp39, PtfA, ToxA, and F1P2 antigens from vaccine constructs

Western blot analysis was performed using the culture supernatants of the delivery constructs to confirm the secretion of FimA, CP39, PtfA, ToxA, and F1P2 antigens. JOL932, *Salmonella* Typhimurium $\Delta\text{lon}\Delta\text{cpxR}\Delta\text{asd}$ containing pPB244, was used as a control. The expected sizes, 36 kDa for FimA, 39 kDa for CP39, 18 kDa for PtfA, 22 kDa for ToxA, and 30 kDa for F1P2, were observed in the precipitated culture supernatants from the individual constructs (Fig. 1), and no bands of these sizes were observed in the control lane.

3.2. Humoral immune response in immunized mice

Antibody responses against each antigen in the sera and vaginal samples, and bronchio-alveolar lavage samples are presented in Figs. 2 and 3, respectively. Serum IgG concentrations against

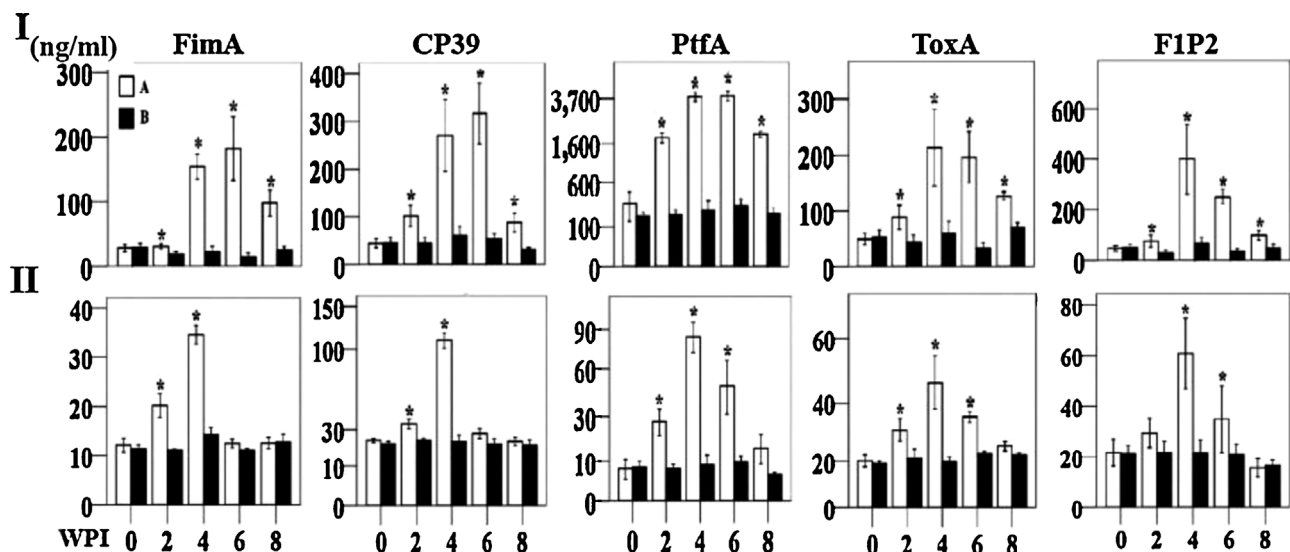


Fig. 2. Antibody responses against the recombinant CP39, FimA, PtfA, ToxA, and F1P2 antigens: (I) serum IgG (ng/ml) concentrations and (II) secretory IgA (ng/ml) concentrations; A, vaccine strain-inoculated group; B, control group. WPI: weeks post-inoculation. Data are the means of all mice in each group, and error bars show the standard deviations (SD). Asterisks indicate a significant difference between the values of the group inoculated with the delivery strain ($P \leq 0.05$) and those of the control group.

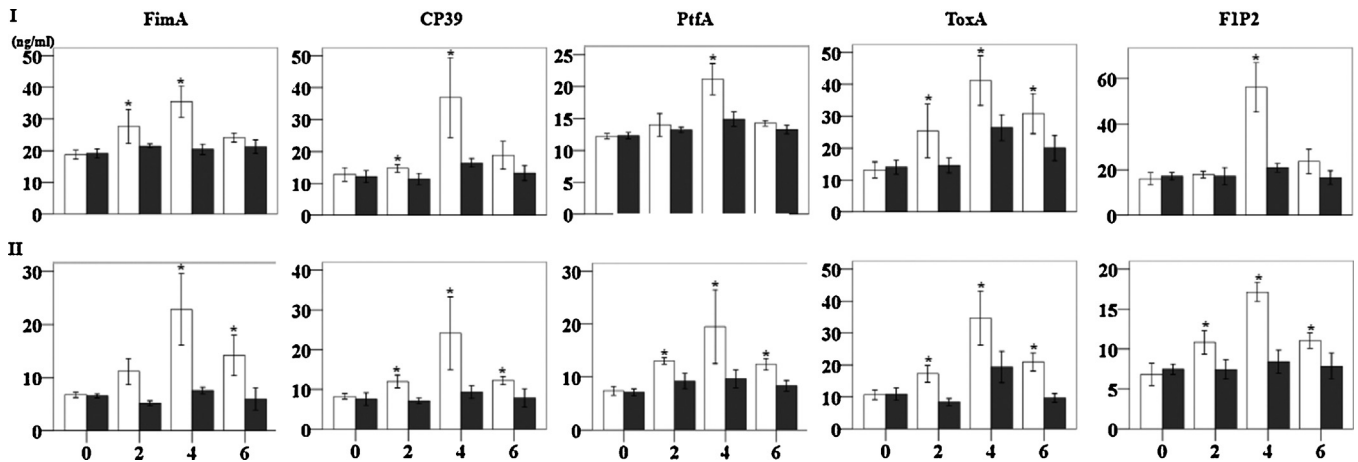


Fig. 3. Antibody responses against the recombinant CP39, FimA, PtfA, ToxA, and F1P2 antigens: (I) broncho-alveolar IgG (ng/ml) concentrations and (II) broncho-alveolar IgA (ng/ml) concentrations; (□), vaccine strain-inoculated group; (■), JOL932-inoculated group. WPI: weeks post-inoculation. Data are the means of all mice in each group, and error bars show SD. Asterisks indicate a significant difference between the values of the group inoculated with the vaccine strain ($P \leq 0.05$) and those of the control group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the individual antigens in group A were significantly increased compared to those of group B from 2 WPI until the end of the study ($P \leq 0.05$) (Fig. 2I). In addition, the vaginal sIgA concentrations against the individual antigens in group A were significantly higher than those in group B on 4 WPI ($P \leq 0.05$) (Fig. 2II). Broncho-alveolar IgG concentrations against all the antigens in group A were significantly increased compared to those of group B at the 4th WPI ($P \leq 0.05$) (Fig. 3I). In addition, the specific broncho-alveolar lavage IgA concentrations in group A were also significantly higher than those in group B from the 2 WPI to 6th WPI ($P \leq 0.05$) (Fig. 3II).

3.3. Cell-mediated immune response

Cell-mediated immune responses were examined by SPA using the CP39-, PtfA-, or F1P2-specific antigens on 4 WPI. Splenocyte proliferation responses against each antigen revealed a significant increase in the SIs in all mice of the group A compared to the control group ($P < 0.05$) (Fig. 4). The SI values against FimA, CP39, PtfA, and F1P2 in group A were 1.12 ± 0.03 , 1.19 ± 0.04 , 1.23 ± 0.15 and 1.35 ± 0.25 , respectively, while the values in group B were 0.93 ± 0.12 , 0.94 ± 0.09 , 0.89 ± 0.12 and 0.95 ± 0.10 , respectively.

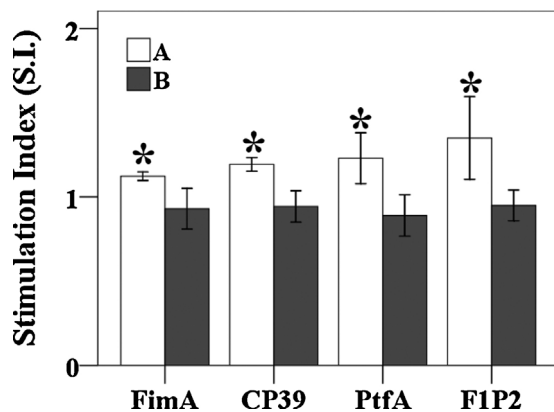


Fig. 4. Splenocyte proliferative responses against FimA, CP39, PtfA, and F1P2 antigens in immunized and nonimmunized mice at week 4 post-immunization. The antigen-specific splenocyte proliferative response is expressed as the stimulation index (SI); (A) delivery strain-inoculated group; (B) control group. Data shown are the means of all mice in each group and error bars show SD. Asterisks indicate significant differences between the values of the immunized and control groups ($P \leq 0.05$).

3.4. Proportion of circulating T- and B-cells according to flow cytometry analysis

In the groups A and B mice, T- and B-lymphocyte subsets in the spleen were analyzed by flow cytometry. Analysis of the T-cell populations against FimA, CP39, ToxA, and F1P2 in group A showed a significantly increased number of CD3⁺CD4⁺ and CD3⁺CD8⁺ lymphocytes compared to those of the control group ($P < 0.05$). In addition, the B-lymphocytes (CD45R⁺) against CP39, FimA, PtfA, and F1P2 in group A were significantly increased compared to those in the control group ($P < 0.05$) (Fig. 5).

3.5. Cytokine IL-4 and IFN- γ expression measured by real-time quantitative RT-PCR analysis

The levels of IL-4 and IFN- γ mRNA against FimA, CP39, PtfA and F1P2 in the splenocytes of mice were measured using real-time quantitative RT-PCR analysis on 4 WPI. The levels of IFN- γ were significantly elevated in the group A compared to those in the control group ($P < 0.05$) (Fig. 5). However, following stimulation with the CP39- and PtfA-specific antigens, the levels of IL-4 against FimA, CP39, PtfA and F1P2 in group A were not increased compared to those in the control group (Fig. 6).

3.6. Histological analysis of lung post-immunization

The histopathological changes of lung such as congestion and accumulation of polymorphonuclear neutrophils (PMN) in the alveolar sac and blood vessels of the delivery strain inoculated mice were examined. Fewer or no histopathological changes were found in the lungs of the delivery strain inoculated group on 4 WPI compared to those of control group.

4. Discussion

Recombinant live *Salmonella* strains have been used as vectors to deliver pathogen-specific antigens to induce mucosal and systemic immune responses against both vector and foreign antigens [15,26]. In a previous report, intranasal inoculation with a recombinant *Salmonella* Typhimurium strain expressing the Hepatitis B nucleotide induced higher antibody concentrations in the serum and vaginal secretions than those induced by other mucosal routes of inoculation [23]. Currently, clinical PAR is usually controlled by a combined *B. bronchiseptica*–*P. multocida* vaccination. The vaccines

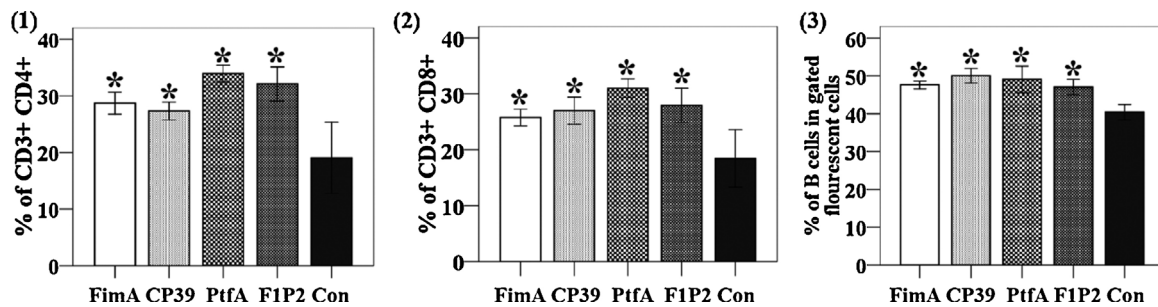


Fig. 5. Flow cytometric analysis of T-lymphocyte and B-lymphocyte subpopulations in spleen at week 4 post-inoculation; (1) Bar-graphs represent CD3⁺CD4⁺, (2) CD3⁺CD8⁺, and (3) CD3⁺CD45R⁺ splenocyte subpopulations in the mice of groups A and B. All values of bar-graphs are shown as means $\pm$ SD. Asterisks indicate significant differences between the values of the delivery strain-inoculated and control groups (* $P \leq 0.05$).

consist mainly of toxoid and/or somatic antigens of both *B. bronchiseptica* and *P. multocida* [36]. Although immunization with the current vaccines has shown increased serum antibody titers and somewhat beneficial protection effects, they are not able to fully eliminate the bacteria [16,36]. They are composed of either killed wild-type bacterial strains that are administered parenterally [36]. Therefore, many new vaccine approaches have been attempted to control *P. multocida* or *B. bronchiseptica* infections and to produce neutralizing antibodies against PMT using live attenuated vaccine strains [16,37–39] and/or recombinant proteins such outer membrane proteins [19,20], adhesins [40] and PMT [41]. Since the basis of attenuation of the commercial vaccine is unknown, there is the potential that the strains may revert to full or partial virulence [16], which can cause an adverse effect. In addition, obtaining abundant amount of authentic PMT for toxoid preparation needs the cultivation of bacteria at a scale that is not economically efficient because PMT constitutes only 0.6% and less of the total bacterial proteins [14]. Thus, recent efforts have been directed at recombinant PMT derivatives that can be produced at lower cost [41].

In the present study, the attenuated *Salmonella* Typhimurium $\Delta lon \Delta cpxR \Delta asd$ strain and the pBP244 plasmid encoding the *asd* gene [27] was used as a delivery system to deliver CP39, FimA, PtfA, and ToxA antigens of *P. multocida*, and F1P2 antigen of *B. bronchiseptica*. Secretion of the individual adhesin and toxin antigens from the delivery strains were demonstrated in the present study by Western blot analysis. Results of this analysis indicated that the plasmids containing the genes for FimA, CP39, PtfA, and ToxA antigens were stably preserved in the constructs, and that the expressed FimA, CP39, PtfA, and ToxA antigens were effectively secreted.

At the mucosal surface in the respiratory tract, a vaccine inducing protective immunity would be an ideal vaccine to prevent the entry and multiplication of pathogens, and ultimately to prevent the diseases [42]. Therefore, an ideal delivery strain should be capable of inducing mucosal immunity such as secretory IgA, while

humoral immunity is generally more important to prevent infection by pathogens [42]. In this study, immune response such as serum IgG and mucosal IgA were investigated in mice intranasally inoculated with the delivery system. The serum IgG, vaginal IgA, and broncho-alveolar IgG and IgA concentrations in the vaccine strain-inoculated group were significantly increased compared to those of the control group.

The cell-mediated immune response is also a critically important factor in the prevention of infection by bacterial pathogens [14,43]. Activated CD4⁺ T-helper (Th) lymphocytes provide co-stimulation for B cells [43] and B cells produce antibodies, which function to neutralize viruses and bacterial toxins, to block attachment of microorganisms to host cells in Th2-type immune response, and to mediate phagocytosis of invading microorganisms [43]. In addition, activated CD4⁺ T-helper lymphocytes activate phagocytes to eliminate intracellular pathogens in the Th1-type immune response, whereas CD3⁺CD8⁺ T cells are the effectors in cytotoxic responses, killing infected target cells [43]. The cell-mediated immune responses can be numerically expressed as a measurement of lymphocyte proliferation activity (LPA) [34]. The LPA indicated that significant cell-mediated immune responses were elevated in the delivery strain-inoculated group compared to those in the control group. The lymphocyte populations in splenocytes stimulated with FimA, CP39, PtfA, or F1P2 antigens were examined to gain more insight into the immunological effect of intranasal inoculation with the delivery system. The proportions of CD3⁺CD4⁺, CD3⁺CD8⁺ lymphocytes and B cells in the stimulated splenocytes were significantly higher in the mice inoculated with the delivery system than in the control mice. Generally, Th2 cells produce IL-4, IL-5, IL-6, and IL-10 cytokines, which can induce IgA expression [44]. Although IL-4 is important for mucosal IgA induction, IL-5, IL-6, and IL-10 can act as a counterbalance to IL-4 in the presence of a strong T-helper 1 (Th1) response induced by inoculation of *Salmonella* [45]. Our data, also, showed that the levels of

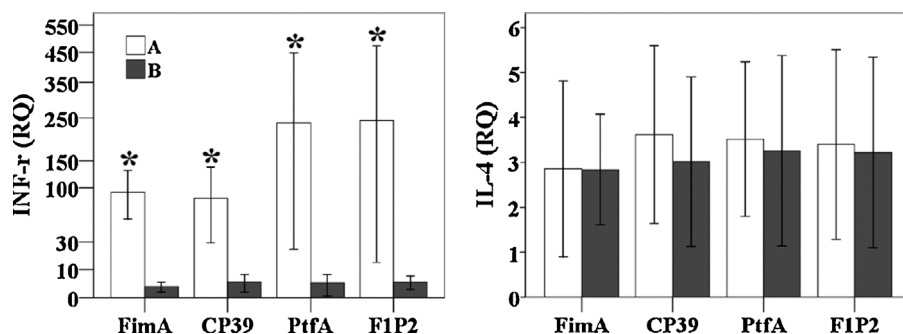


Fig. 6. Relative quantification (RQ) of interferon-gamma (IFN- γ) and IL-4 expression in mice splenocytes using real-time PCR. (A) delivery strain-inoculated group; (B) control group. Data are the means of all mice in each group and error bars show SD. Asterisks indicate significant differences between the values of the delivery strain-inoculated and control groups (* $P \leq 0.05$).

IL-4 against CP39 and PtfA in the vaccine strain-inoculated group were similarly increased when compared to those in control group, while the proliferation of B-cells in splenocytes and vaginal IgA concentrations were significantly increased in the mice of the delivery strain-inoculated group. These results imply that a single dose of intranasal inoculation with the delivery system may strongly induce the Th1-type response against the delivered antigens. However, Th2-type response against the delivery antigens was not sufficiently increased by the single intranasal inoculation with the delivery system. Thus, in order to further analyze the induction of the Th2-type response against the recombinant antigens secreted from the delivery system, a subsequent study involving multiple doses of the intranasal inoculations is necessary. *Salmonella* delivery systems have been known to induce Th1-type cell-mediated immunity as evidenced by increased IFN- γ , which is involved in the recognition of pathogen-associated molecular patterns [15,46]. The measurement of IFN- γ levels in splenocytes stimulated with antigen has provided convincing evidence that the immune cells are stimulated with the antigen [47]. The current study revealed that expression levels of IFN- γ in the antigen-stimulated splenocytes of the delivery strain-inoculated group were significantly elevated, which indicates that the increase in IFN- γ titers was antigen-specific.

Following intranasal inoculation, the histopathological changes of lung, such as congestion and accumulation of PMN in the alveolar sac and blood vessel of delivery strain-inoculated mice, were examined. Fewer or no histopathological changes were found in the lungs of the delivery strain-inoculated mice on 4 WPI compared to the lungs of the control group mice. This finding implies that the recombinant antigen secreted from the delivery system caused little or no damage to the respiratory system following intranasal inoculation, and could therefore be a safe construct to use in animals.

In conclusion, the mixture of five delivery strains expressing specific antigen for prevention of porcine pneumonic pasteurellosis and progressive atrophic rhinitis was found to be capable of inducing significant humoral, especially mucosal, and cellular immune responses.

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