수의학석사학위논문

Application of the C-terminal fragment of *Pasteurella multocida* dermonecrotic toxin on the development of vaccines against progressive swine atrophic rhinitis

2013년 7월

서울대학교 대학원
수의학과 수의미생물학전공
김 아 름

Application of the C-terminal fragment of *Pasteurella multocida* dermonecrotic toxin on the development of vaccines against progressive swine atrophic rhinitis

지도교수 우 희 종
이 논문을 수의학석사학위논문으로 제출함

2013년 6월

서울대학교 대학원
수의학과 수의미생물학전공
김 아 름

김아름의 석사학위논문을 인준함
2013년 7월

위 원 장	유 한 상 (인)
부 위 원장	우 희 종 (인)
위 원	이 정 민 (인)

Abstract

Application of the C-terminal fragment of *Pasteurella multocida* dermonecrotic toxin on the development of vaccines against progressive swine atrophic rhinitis

Arum Kim

Department of Veterinary Medicine

(Major: Veterinary Microbiology)

The Graduate School

Seoul National University

Pasteurella multocida is a Gram-negative pathogen that causes respiratory diseases in wild and domestic animals. *P. multocida* D:4-producing *P. multocida* toxin (PMT) is a primary virulence factor in swine atrophic rhinitis and specific antibody against toxin is known to play a major role in protection. It has been reported that the C-terminal large fragment of PMT (PMT2.3) offers protective immunity against *P. multocida* D:4 infection in vaccination and challenge in mice. For investigation of oral-route vaccination effect of PMT2.3, a recombinant *Lactococcus lactis* expressing PMT2.3 was constructed in this study. Mice were immunized with live prepared cells by three day-consecutive oral administrations with a 2-week interval. The mucosal and systemic immune responses were investigated by detection of specific IgA and IgG from feces, and sera in the mice. The protective immunity against homologous challenge in the immunized mice was also examined. In oral

administration, the recombinant *L. lactis* strain expressing PMT2.3 showed no production of sfecific antibodies and protection against homologous challenge. A mild protection against a homologous challenge with *P. multocida* was observed in mice vaccinated with rPMT2.3 and commercial vaccine. The PMT2.3 production process using the recombinant *L. lactis* can provide an approach to efficient production of recombinant protein using a generally recognized as safe (GRAS) lactic acid bacteria as a significant carrier for the oral-route vaccination. However, further study for achieving a functional oral vaccine was needed.

On the other hand, the immune effect of rPMT2.3 was not proven well in cytokine production analysis in from rPMT2.3 vaccinated mice. The levels of IFN-γ cytokines in rPMT2.3-stimulated splenocytes showed significant increase; however, after challenge, the level of IL-4 was increased in rPMT2.3 immunized mice. This results means that the rPMT2.3 was related to inducing Th1 type immune response and switched to Th2 type immune response after challenge. The protective function and cytokine changes of rPMT2.3 have been previously; therefore, the present study determined whether rPMT2.3 has potential application as material that can compensate for the effect of existing vaccine ART-T4. The immunization with atrophic rhinitis commercial vaccine containing rPMT2.3 protein showed a higher level of anti-PMT2.3 IgG and the survival rate was also increased after the challenge compared to immunization with ART-T4 only. As combination of ART-T4 and the rPMT2.3 protein improved antigenicity and protective effect of ART-T4, rPMT2.3 might be used as an effective component in *P. multocida* D:4 vaccine.

Key words: Progressive atrophic rhinitis (PAR), *Pasteurella multocida* D:4, *Pasteurella multocida* toxin, oral vaccine, Lactic acid bacteria (LAB), *Lactococcus*

lactis

Student Number: 2007-21769

Contents

I. Introduction

Pasteurella multocida, belonging to the group of Gram-negative bacteria, can be a primary or secondary agent involved in pneumonia and hemorrhagic septicemia in cattle, sheep, and goats; fowl cholera in chicken; snuffles in rabbits; and progressive atrophic rhinitis (PAR) in swine; in addition, uncommonly, human infections have been observed in a range of sites, commonly following cat or dog bites (Harper et al., 2006; Weber et al., 1984). *P. multocida* strains are classified into four different serogroups (A, B, D, E and F) based on capsule antigens and 16 serotypes (1–16) based primarily on lipopolysaccharide antigens (Carter, 1955; Heddleston et al., 1972). Different serotypes are associated with particular types of disease. The majority of acute fowl cholera cases are caused by serogroup A strains, and acute hemorrhagic septicemia in cattle and buffalo predominantly are caused by serotypes B and E strains of *P. multocida*. Strains of *P. multocida* isolated from porcine pneumonia have generally been serotype A. Atrophic rhinitis is caused by serogroup D strains of *P. multocida* that produce *P. multocida* toxin (PMT) (Harper et al., 2006; Ross, 2006).

In swine, *P. multocida* causes PAR and is thought to play a crucial role in pneumonia. Co-infections with *P. multocida* and various bacteria occurs naturally in cases such as *Hemophilus parasuis*, *Salmonella choleraesuis*, *Streptococcus suis*, *Actinobacillus suis*, *Bordetella bronchiseptica*, and *Mycoplasma hyorhinis* (Gois et al., 1975; L'Ecuyer et al., 1961; Morrison et al., 1985; Ryu, 1954; Straw et al., 1996). Toxin-producing strains associated with PAR are most frequently of capsular type D and less often of capsular type A (Amigot et al., 1998; Bowersock et al., 1992), while strains causing pneumonia are usually non-toxigenic and can be of capsular types A and D,

the proportion of type A strains usually being higher (Choi et al., 2001; Ewers et al., 2006).

The interaction of toxigenic *P. multocida* with *B. bronchiseptica* is the causative factor for atrophic rhinitis (AR). Notably, it was considered that *B. bronchiseptica* causes moderate turbinate lesions whereas *P. multocida*, which by itself poorly colonizes the nasal cavity, establishes a secondary infection only after *B. bronchiseptica*, and exacerbates the disease. The disease exacerbated by the secondary infection by *P. multocida* is referred to as PAR to distinguish it from ‘‘nonprogressive (NP) AR’’ caused by the single infection of *B. bronchiseptica*. *P. multocida* is also recognized as the primary pathogen causing PAR without other agents like *B. bronchiseptica* (de Jong and Nielsen, 1990). PAR is an important upper respiratory tract disease of swine characterized by turbinate atrophy, facial distortion, nasal hemorrhage as a result of frequent sneezing, and in severe cases, difficulty in eating and subsequent growth retardation. This is disease rarely causes death; nevertheless, it is economically important as it causes significant global economic loss in swine production (Liao et al., 2006; Rutter, 1985). Therefore, control of the disease is very important in swine industry.

P. multocida serotype A and D strains synthesize PMT, encoded by the chromosomal *tox*A gene on a lysogenic bacteriophage (Nagai et al., 1994; Pullinger et al., 2004). The dermonecrotic toxin (DNT), PMT, is a large, 146-kDa protein that is known to act intracellularly, binding to cell-surface ganglioside-type receptors and becoming internalized (Dudet et al., 1996; Pettit et al., 1993; Rozengurt et al., 1990). Several studies have indicated that the N-terminal region of the toxin binds to target cells and that the C-terminal region carries the intracellularly-active moiety, the nature of which

remains unknown (Busch et al., 2001; Orth et al., 2003; Pullinger et al., 2001; Ward et al., 1998). *P. multocida* toxin and *B. bronchiseptica* dermonecrotic toxin have been considered to independently or cooperatively disturb the osteogenesis of the turbinate bone by inhibiting osteoblastic differentiation and/or stimulating bone resorption by osteoclasts (Horiguchi, 2012).

PMT is a primary virulence factor in porcine atrophic rhinitis (Chrisp and Foged, 1991; Kamp and Kimman, 1988). Both purified native and recombinant PMT toxin can be used to experimentally induce clinical signs of disease (Lax and Chanter, 1990). Field studies have shown that a recombinant *P. multocida* toxin derivative, missing a segment of the amino-terminal portion of the protein, is non-toxic but immunogenic and has superior efficacy in swine (Bording et al., 1994a; Pejsak et al., 1994a). More recently, full-length recombinant toxoid engineered to contain two amino acid substitutions that eliminate toxigenicity was also found to be highly efficacious. The genetically modified PMT was created by replacing the serine at position 1164 with alanine (S1164A) and the cysteine at position 1165 with serine (C1165S). A nontoxigenic PMT leads to a complete loss of toxic effects of PMT without impairment of the ability to induce protective immunity in pigs (To et al., 2005b). A DNA vaccine encoding a full-length but enzymatically inactive toxoid was shown to be highly immunogenic in pigs; however, it has not been evaluated for efficacy against challenge (Register et al., 2007a). In previous studies, C-terminal large fragment of PMT (PMT2.3) has been found to have protective immunity against *P. multocida* D:4 infection in vaccination and challenge in mice and showed high immunoreactivity to the antisera from infected swine (Lee et al., 2012; Lee and Woo, 2010).

Oral vaccine is an attractive model of immunization because of its acceptability and its simplicity of administration. The most important reason for using a mucosal route of vaccination is that the vast majority of pathogens invade via mucosal surfaces, including those of the intestine and vaccination directly on these surfaces may induce local protective immunity and prevent infection and disease (Foss and Murtaugh, 2000; Holmgren et al., 2003; Pavot et al., 2012). Oral vaccines have the potential to elicit antigen-specific secretory immunoglobulin A (sIgA) responses at mucosal surfaces, which can neutralize viruses or toxins and inhibit colonization by enteric microbes (Lavelle and O'Hagan, 2006; Mannam et al., 2004; Neutra and Kozlowski, 2006).

Lactic acid bacteria (LAB) are a Gram-positive bacteria that are best known for their use in the food industry. *Lactococcus lactis* (*L. lactis*) is a non-pathogenic, non-invasive, noncolonizing Gram-positive LAB that is "generally recognized as safe" (GRAS) and has a long history of widespread use in the food industry for the production of fermented milk products (Rottiers et al., 2009). *L. lactis* is a potential candidate for an antigen delivery vehicle, for the development of live mucosal vaccines. Several delivery systems have been developed to target heterologous proteins to a cytoplasm, cell wall or extracellular medium (Nouaille et al., 2003). *L. lactis* is known to lack the ability to colonize the digestive tract in vivo so there is only a passive transit (persistence time <24 h) through the digestive tract (Gruzza et al., 1994). It has been suggested that antigens expressed by *L. lactis* are presented to the immune system in particulate form and, therefore, may be less likely to induce oral tolerance than soluble antigens (Lee et al., 2001). Furthermore, *L. lactis* is approximately the same size as biodegradable microparticles that are known to be

taken up by M cells and have been shown to be capable of acting as effective oral vaccine vehicles (Challacombe et al., 1992). Recently, a number of research groups have reported that *L. lactis* can be genetically engineered to express antigens, including the Group A Streptococcus (GAS) Shr (Huang et al., 2011), Rotavirus VP8 (Marelli et al., 2011), *Mycobacterium tuberculosis* Hsp65 (Jing et al., 2011), *Streptococcus pneumoniae* PppA (Villena et al., 2010), *Helicobacter pylori* UreB (Gu et al., 2009), HIV-1 V2−V4 loop of gp 120 (Xin et al., 2003), Rotavirus VP7 (Perez et al., 2005), *Brucella abortus* L7/L12 antigen (Pontes et al., 2003), SARS coronavirus nucleocapsid antigen (Pei et al., 2005), and tetanus toxin fragment C (Robinson et al., 2004). Mice fed these recombinant strains develop antigen-specific immune responses or protection (Pontes et al., 2011; Wells and Mercenier, 2008).

The aims of the present study were to determine whether rPMT2.3 region functions as an AR vaccine in two parts: oral AR vaccine and role of additives in commercial AR vaccine. First, as an oral vaccine, the gene encoding PMT2.3 region was cloned into *L. lactis*, and recombinant *L. lactis* expressing PMT2.3 were orally administrated in mice. The mucosal and systemic immune responses were measured. In addition, protectivity was investigated in mice orally immunized with the *L. lactis* expressing PMT2.3. Second, the rPMT2.3 was used as additive to commercial AR vaccine. The immune response and protectivity were investigated in mice intraperitoneally vaccinated with a combination of commercial AR vaccine and the rPMT2.3 protein.

II. Materials and methods

Strains, culture media, and growth conditions

1) *Pasteurella multocida*

The pathogenic *P. multocida* D:4 was received from the Animal and Plant Quarantine Agency of Korea and grown on sheep blood agar plate or Bacto™ brain-heart infusion (BHI) (Difco Laboratories, Detroit, MI, U.S.A.) broth at 37˚C.

2) *Lactococcus lactis*

Lactococcus lactis NZ9000 strain was used as host for the lactic acid expression shuttle vector pNZ8148. *L. lactis* was grown in M17 medium (Difco, Sparks, Maryland, USA) supplemented with 0.5% (w/v) glucose at 30℃ under anaerobic conditions.

3) *Escherichia coli*

E. coli ER2738 was used in cloning of pPNZ8148 vector. *E. coli* BL21(DE3) and DH5a was used in a bacterial host which required protein expression and plasmid cloning respectively. *E. coli* was grown in Luria-Bertani (LB) agar or broth at 37˚C.

Expression of rPMT2.3 in *L. lactis*

1) Construction of the rPMT2.3 expression vector.

The truncated PMT2.3 gene (2.3 kbp) was amplified from the vector pRSET/PMT2.3 (which carries PMT2.3 gene deletion of N-terminal sequence of *P. multocida* D:4, Genebank number AY603962) using specific primers: PMT2.3-F (5'-

GATAGGTACCATGCGAGAAATTACTGACGAA-3') and PMT2.3-R (5'-GCCCGAGCTCTCTAAGGCTAAAACTCAATG-3'). The recombinant vector pRSET/PMT2.3 was obtained from the Department of Genetic Engineering of Sungkyunkwan University in Korea. Polymerase chain reaction (PCR) was performed as follows: 5 min at 95˚C; 30 cycles of 1 min at 95˚C, 30 sec at 66˚C and 1 min 30 sec at 72˚C; and 7 min at 72˚C. The PCR products were separated by 1% agarose gel-electrophoresis and purified using a Accuprep® PCR purification kit (Bioneer, Daejeon, Korea). The PCR products were then digested with restriction enzyme and inserted at *Kpn* I and *Sac* I sites in pNZ8148 vector.

The recombinant plasmid pNZ8148/PMT2.3 was transformed into *E. coli* ER2738 using a standard heat shock method (Sambrook et al., 1989). Transformants ER2738 were selected from LB agar plate containing 100 ug/ml ampicillin, followed by plasmid extraction using the Accuprep® nano-plus plasmid mini extraction kit (Bioneer, Daejeon, Korea). The constructed pNZ8148/PMT2.3 vector was confirmed by enzyme digestion and colony PCR.

2) *L. lactis* transformation

The constructed vector was tansformated into *L. lactis* NZ9000 using Gene Pulser (Bio-Rad, Hercules, CA, USA). Briefly, 1 μl of pNZ8148/PMT2.3 plasmid DNA was added to 40 μl of *L. lactis* strain NZ9000, gently mixed in a pre-chilled cuvette, and subjected to a single electric pulse (25 μF , 2.5 kV/cm, 200 Ω). The mixture was then incubated in GM17 medium at 30◦C for 1 h and selected on GM17-agar plate containing 10 μg/ml of chloramphenicol at 30°C for 2 days. Colonies were picked at random and analysed by colony PCR using PMT2.3-F and PMT2.3-R primers to test

the integrity of the pNZ8148/PMT2.3 plasmid in the NZ9000.

3) Heterologous expression of rPMT2.3 protein in *L. lactis*

L. lactis NZ9000-pNZ8148/PMT2.3 and *L. lactis* NZ9000-pNZ8148 were grown overnight at 30℃, inoculated (3% v/v) in fresh medium and grown until OD_{600}=0.4~0.5. The strains were induced with 80 ng/ml of nisin (Sigma-Aldrich, St. Louis, Missouri, USA) for 3 h, and cells were harvested by centrifugation at 4000 g, 20 min, 4℃, resuspended in 10 mM Tris-HCl (pH 8.0) buffer containing 1 mg/ml lysozyme, and incubated at 37℃ for 30 min. After washing twice with 10 mM Tris-HCl (pH 8.0), the cells were lysed with lysis buffer (10% glycerol, 2% sodium dodecyl sulfate, 375 mM Tris-HCl, pH 7.6). The 20 μl aliquots of cell extracts were analyzed by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred onto a nitrocellulose membrane (NC; Millipore, Bedford, MA, USA) and blocked with TBS-T (Tris-buffered saline, 0.05% Tween-20) containing 5% skimmed milk at 4℃ overnight, and then washed with TBS-T for 10 min three times. The washed NC membrane was and incubated with 1 : 5000 dilution of HRP-conjugated anti-His Ab (ABcam, Cambridge, MA, USA) for 2 h at room temperature, then washed for 10 min three times. Western blot detection was performed using the ECL Western blotting system (Amersham Biosciences UK Ltd.) according to the manufacturer's protocol. The positive clones were preserved in glycerol stocks at -70℃.

To maximize yields of rPMT2.3 protein, the incubation time after induction and the amount of nisin were optimized. And to quantify the expression level of PMT2.3, the bands of western blot were quantified using the ImageJ program. Titration curves

were generated using purified rPMT2.3 protein standards, and concentrations of the expression level of PMT2.3 in *L. lactis* were calculated by reference to these standard curves.

4) Preparation of *L. lactis* for immunization

Cultures of *L. lactis* NZ9000-pNZ8148/PMT2.3 cells were induced for 5 h with nisin A to express rPMT2.3; level of expression was checked using Western blot. The recombinant NZ9000-pNZ8148/PMT2.3 cells were then harvested by centrifugation at 3000 g at 4℃ and the pellets were washed twice with sterile PBS. For immunization, cell pellets of the recombinant *L. lactis* expressing PMT2.3 were resuspended in PBS to a final concentration as described inTtable.1.

Expression and purification of rPMT2.3 in *E.coli*

1) Bacterial transformation

The recombinant vector pRSET C/ PMT2.3 which has a C-terminal fragment of PMT sequence (505–1285 amino acids) was obtained from the Department of Genetic Engineering of Sungkyunkwan University in Korea. *E. coli* DH5a and BL21(DE3) were transformated using a standard heat shock method (Hanahan, 1983) and selected from LB agar plate containing 100 ug/ml ampicillin (Sambrook, 1989).

2) Purification of rPMT2.3 from *E. coli*

The rPMT2.3 protein was produced as inclusion bodies and purified under denaturing conditions. An overnight culture of *E. coli* BL21 (DE3) harboring the recombinant plasmids grown in LB medium with ampicillin (100 μg/ml) was inoculated into 1 L of

LB medium. Isopropyl-β-*D*thiogalactopyranoside (IPTG, 1 mM) was added to induce gene expression at OD_{600} 0.8. The cells were further cultured for 12 h at 37℃ with vigorous shaking, after which they were collected and resuspended in a lysis buffer [50 mM Tris-HCl (pH 8.0), 1% SDS, 10 mM EDTA; 5 ml/g of wet cells]. Subsequently, the cells were sonicated and then centrifuged (12,000 ×g, 4℃, 30 min). Insoluble materials were resuspended in a denaturing buffer (100 mM NaH2PO4, 10 mM Tris-HCl, 8M urea; pH 8.0) and sonicated. The supernatant was recovered by centrifugation (10,000 ×g, 4℃, 30 min) and used for the purification of the recombinant proteins. About 1 ml of 50% Ni-NTA slurry (QIAGEN, Hilden, Germany) was added to 5 ml of the supernatant solution and mixed gently overnight at 4^{o}C and the mixture was then loaded onto an empty column. The column was washed with 25 ml of wash buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea; pH 6.3). The recombinant protein was eluted with elution buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea; pH 5.3) and further elution buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea; pH 4.5). Fractions were collected and analyzed by SDS-PAGE. For Western blots, proteins were fractionated by SDS-PAGE and transferred to a nitrocellulose. After transfer, membranes were blocked in Tris-base saline (TBS) containing 5% drymilk and 0.05% Tween-20 (TBS-T) for 2 h at room temperature, washed three times in TBS-T, and incubated with anti-His-tag antibody (Invitrogen, Carlsbad, CA, USA) diluted in TBS-T containing 1% drymilk overnight at 4◦C. After additional washes, the blots were incubated for 1 h at room temperature with anti-mouse IgG HRP conjugated (cell signaling, Danvers, MA, USA). Blots were developed with DAB substrate. The purified fractions were serially diluted with PBS for refolding of the protein and further analysis.

Vaccination and challenge studies in mice

For animal experiments, specific pathogen-free 6-week-old female BALB/c mice were purchased from Laboratory Animal Co., Ltd., Hallym. Experiments were performed under the control of the animal welfare committee of Seoul National University Institutional Animal Care and Use Committee (SNUIACUC) in accordance with the laboratory's animal ethics guidelines. At the end of the experiments, the animals were euthanized.

In the oral administration experiments, 10 mice each were immunized five times by intragastric route using an oral zonde needle. Each group was immunized with PBS, NZ9000, 1 or 10 doses of NZ9000-pNZ8148/rPMT2.3, respectively. Commercial vaccine ART-T4 (Dae sung Microbiological Labs, Seoul, Korea) and purified rPMT2.3 protein (50 ug/mouse) were injected into mice intraperitoneally as positive control. On the other hand, to determine a complementary effect of the commercial vaccine, 6 mice each were immunized twice with PBS, ART-T4 commercial AR vaccine, purified rPMT2.3 protein (50 ug/mouse) and ART-T4 supplemented with rPMT2.3. The rPMT2.3 proteins were emulsified (1:1) in Freund's incomplete adjuvant or Freund's complete adjuvant (Sigma-Aldrich, St. Louis, Missouri, USA) and intraperitoneally injected in mice in a total volume of 200 ul per animal.

For the protection study, the BHI broth-grown cultured *P. multocida* D:4 were harvested and resuspended in PBS, and the immunized mice were intraperitoneally challenged with 2 or 5-median lethal dose (LD50) of virulent *P. multocida* D:4 (1.45×10^5 or 3.63×10^5 CFU/mouse) at 2 weeks after the last immunization. This dose was based on the present author's previous mice challenge studies and determined by standard plate count. Protection was determined by mouse survival about 3 d post

challenge. Survival rate was calculated by dividing the number of live mice by the total number of mice in each group.

Immune responses to vaccination

1) rPMT2.3 specific antibodies

For antibody titers, sera were collected at 2-week intervals (at days 7, 21, 35, 49, 63 and 77) and stored at -80 ℃ until required. Fresh mice feces were collected at the same time. Each 0.1 g of fecal pellets was suspended in 1 ml of PBS containing 1% BSA and 1 mM phenylmethylsulphonyl fluoride (PMSF) (Sigma-Aldrich, St. Louis, Missouri, USA) and incubated at 4℃ overnight. Fecal samples were vortexed and centrifuged for 5 min at 13,000 rpm. Supernatants were stored at -80 ℃ until required.

rPMT2.3-specific IgG and IgA levels were determined by ELISA. Briefly, 96-well plates were coated with rPMT2.3 in 0.1M carbonate/bicarbonate buffer, pH 9.6 (100 ul/well), incubated overnight at 4 ℃, and washed three times with PBS containing 0.05% Tween 20 (PBS-T). The plates were blocked with PBS-T containing 5% non-fat dry milk for 2 h at room temperature and washed with PBS-T as indicated above. Dilutions of sera or feces samples in PBS-T containing 1% non-fat dry milk were added to wells, the plates were incubated for 1 h at room temperature, washed three times with PBS-T, and binding detected with anti-mouse IgG or IgA horseradish peroxidase (HRP)-conjugated secondary antibodies. Wells were washed three times in PBS-T, and o-Phenylenediamine-Dihydrochloride (OPD) (Sigma-Aldrich, St. Louis, Missouri, USA) was diluted in Phosphate-Citrate Buffer, pH 5.0 (Sigma-Aldrich, St. Louis, Missouri, USA) and 100 ul/well was added. The reaction was allowed to develop for 30 min then stopped with 2 N H_2SO_4. The absorbance was read as optical

density (OD) at 490 nm in a Microplate Reader (Bio-Rad Laboratories Inc., CA, USA).

2) Production of cytokines in splenocytes

Spleen cells of each group were cultured in 1.5 ml of complete medium in 24-well plates, with each well containing 5 x 10^6 cells/ml for 48 h. Splenocytes were stimulated with 10 ug of rPMT2.3 protein or PBS was added to negative wells. Culture supernatants were assayed for IL-4 and IFN-γ using an ELISA. Briefly, 96-well plates were coated with 100 ul per well of anti-IL-4 or anti-IFN-γ Ab in carbonate-bicarbonate buffer, pH 9.6, overnight at 4℃. Plates were washed between each step with PBS-T, and then the wells were blocked with PBS-T containing 5% non-fat dry milk. Serial twofold dilutions of murine IL-4 and IFN-γ standards or test supernatants were added to triplicate wells. The detection stages were HRP-conjugated anti-IL-4 or anti-IFN-γ Ab was used at 1:1000 dilution, and wells were developed with o-Phenylenediamine-Dihydrochloride (OPD) (Sigma-Aldrich, St. Louis, Missouri, USA). The reaction was allowed to develop for 30 min then stopped with 2 N H_2SO_4. The absorbance was read as optical density (OD) at 490 nm in a Microplate Reader (Bio-Rad Laboratories Inc., CA, USA). Titration curves were generated using IL-4 and IFN-γ standards, and concentrations of cytokines in test supernatants were calculated by reference to these standard curves.

Statistical analysis

All statistical analyses were performed using SPSS 20 software 20.0 (SPSS, Inc.).The statistical significance of variation among different groups was determined by one-

way analysis of variation (ANOVA). A p-value of < 0.05 was considered significant in all cases.

III. Result

Expression of rPMT2.3 in *L. lactis*

The gene encoding the PMT2.3 (2.3 Kbp; 781 amino acids) was PCR-amplified and cloned as a *Kpn* I – *Sac* I fragment in the expression plasmid pNZ8148 (Fig. 1A). The constructed plasmid pNZ8148/ rPMT2.3 was transformed into *L. lactis* NZ9000. Series of construction processes was confirmed at the level of the gene (Fig 1B). To determine whether rPMT2.3 could be expressed in *L. lactis*, whole-cell lysates of *L. lactis* NZ9000-pNZ8148/ rPMT2.3 were analyzed by Western blot after SDS-PAGE (Fig. 1C). In addition, optimization of nisin induction dose and time was performed for maximization of rPMT2.3 expression in *L. lactis*. Conditions of nisin induction dose and time were selected for 80 ng/ml and 4 h (Fig 2 A,B).

Expression and purification of the rPMT2.3

pRSET expression vectors containing C-terminal of PMT (2.3 Kbp; 781 amino acids) regions were donated by the Department of Genetic Engineering of Sungkyunkwan University in Korea. (Fig.3A) The sequence of this gene is identical to the registered sequence of PMT (GenBank Accession No. X51512). The recombinant expression vectors for the partial PMT fragments were transformed into the expression host *E. coli* BL21 (DE3), and a transformants were selected on LB agar plates. The colony showing the highest expression of the several colonies were chosen and used for the purification of the rPMT2.3 proteins. The recombinant proteins with N-terminal 6×His supplemented by the expression vector were produced as insoluble proteins by using the *E. coli* BL21 (DE3) expression system. The recombinant proteins in

insoluble state were successfully purified by Ni-NTA affinity column chromatography under denaturing conditions. The expression yield of each recombinant protein was estimated to be about 14.5% of the total cellular proteins (data not shown). The molecular masses of PMT2.3 were ca. 84 kDa, as determined by SDS-PAGE and Coomassie blue staining after purification of the proteins by Ni-NTA affinity column chromatography (Fig.3B). The molecular mass of recombinant protein was considered to agree with the respective amino acid compositions and lengths of the proteins.

Specific antibody responses

To examine the humoral responses, the levels of anti-rPMT2.3 IgG antibodies in the immunized mice were determined by ELISA, and the results are shown in Fig 4. Antibody levels are presented as relative units (RU) (absorbance values after subtraction of background levels and multiplied by 100). Relative units over 0 were considered as a positive antibody levels. The IgG antibody levels were measured in the sera from immunized mice. In all groups immunized with i.p. injection, significantly higher levels of anti-rPMT2.3 IgG had been induced compared to those in the orally-administrated groups. The highest antibody levels were detected in the rPMT2.3 immunized group on day 35 after the third immunization, and IgG levels were sufficient even at the second immunization. The IgG levels of commercial vaccine were increased according to the flow of time; however, the rPMT2.3 group IgG levels were increased until 35 days and then maintained in the reduced state.

For an indicator of mucosal immune response, rPMT2.3-specific sIgA levels in feces were determined by ELISA (Fig 5). Antibody levels are presented as relative units (RU). As with the above results, there were no significant levels in all oral immunized

groups and a mild increase in the rPMT2.3-immunized. No antibody RUs in orally immunized groups were over 0.

Production of cytokines in splenocyte

The level of IFN-γ and IL-4 in supernatants of splenocytes stimulated with rPMT2.3 were measured using sandwich ELISA, and the concentration was calculated by standard curve of cytokines (Fig. 6). The immunized group with rPMT2.3 showed a significant rise in the levels of IFN-γ cytokines in rPMT2.3-stimulated splenocytes; however, no significant difference was observed in the level of IL-4. After challenge, level of cytokines changes that IFN-γ induced was decreased and IL-4 was increased in rPMT2.3-immunized groups.

Challenge study

The immunized mice were intraperitoneally injected with 2 or 5 LD_{50} of pathogenic *P. multocida* D:4 at two weeks after the last immunization, and were monitored for 3 d for calculation of survival rates (Table 2). The results of the protection experiment are summarized in Table 2. The mice in the orally-administrated groups were not protected (0% survival) against the challenge, whereas those immunized with commercial vaccine ART-T4 and rPMT2.3 protein were protected to some extent against the challenge (28.5% and 57.1% respectively).

The function of rPMT2.3 in ART-T4

Immunization was performed to investigate function of the rPMT2.3 as additional material to the existing vaccine, as described in Table 3. The atrophic rhinitis

commercial vaccine ART-T4 containing rPMT2.3 protein were immunized to mice and compared with control groups. The levels of anti-rPMT2.3 antibodies were determined by ELISA. The highest antibody titers (mean ΔA_{450} values were 2.188) were detected in ART-T4 containing rPMT2.3 protein immunized group after challenge.

The results of protection studies are summarized in Table 4. As in the results of the preceding experiments, the negative control group were not protected against the challenge, while commercial vaccine and rPMT2.3 protein immunized groups were more protected upon challenge after two immunizations. The highest survival rate was detected in the commercial vaccine containing rPMT2.3 protein immunized group.

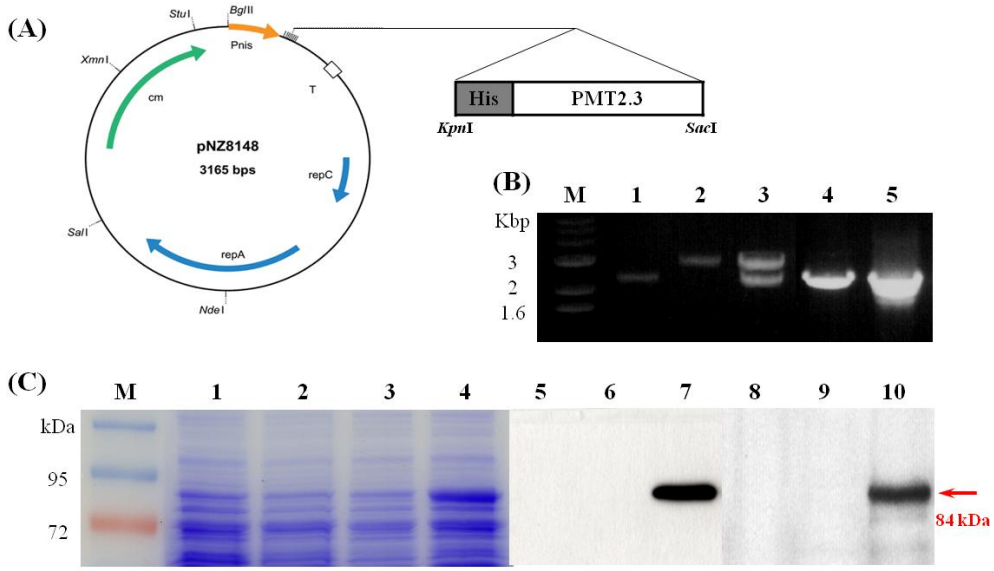


Figure 1. The construction of vector and expression of rPMT2.3 in *L. lactis* NZ9000. The 2.3-kb fragment encoding PMT2.3 with 6 X His-tag was cloned into pNZ8148 vector (A). The steps of construction were confirmed by electrophoresis in 1.5% agarose gel (B). M: size marker, lane 1: pNZ8148 vector, lane 2: PMT2.3 gene, lane 3: pNZ8148-PMT2.3 vector with enzyme cutting, lane 4: purified pNZ8148-PMT2.3 vector from *E. coli*., lane 5: colony PCR of *L. lactis* NZ9000 transformants. SDS-PAGE and Western blot analysis (C). M: Molecular weight marker, lane 1: cell lysate of NZ 9000 strain, lane 2: cell lysate of pNZ8148/NZ9000, lanes 3 and 4: cell lysate of pre- and post-induction of pNZ8148-PMT2.3/NZ9000, respectively. Lanes 5_7 and 8_10: Western blot using anti-6 X His monoclonal antibody and anti-rPMT2.3 antisera, respectively. Lanes 5 and 8: pPNZ8148/NZ9000, lanes 6 and 9: pre-induction of pNZ8148-PMT2.3/NZ9000, lanes 7 and 10: post-induction of pNZ8148-PMT2.3/NZ9000.

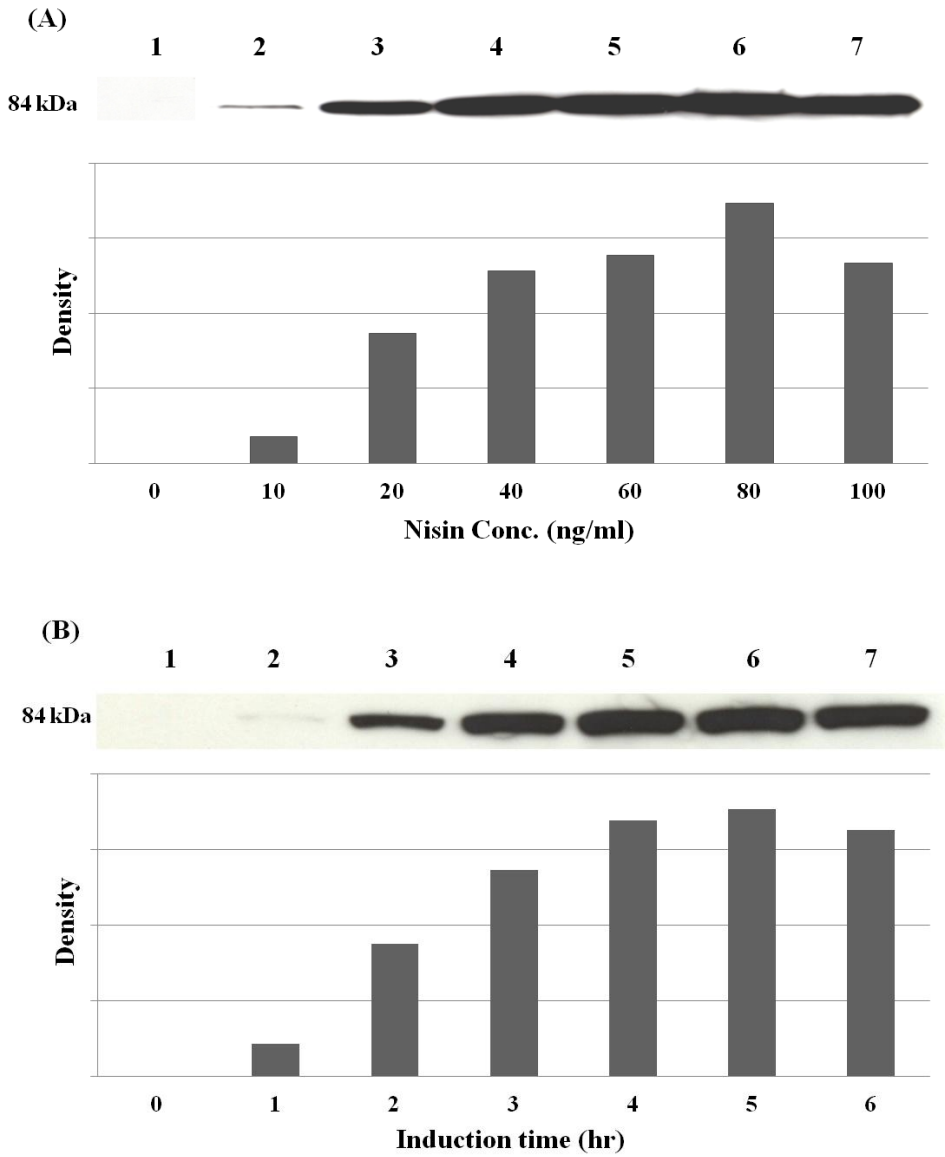


Figure 2. Optimal condition of nisin concentration and induction time for recombinant *L. lactis* NZ9000 expressing PMT2.3. Quantitative analyses were performed by Western blot using anti-6 X His monoclonal antibody. The nisin concentration for induction (0_100 ng/ml) (A). Time-course expression of rPMT2.3 (0_6 h culture at 80 ng/ml nisin) (B).

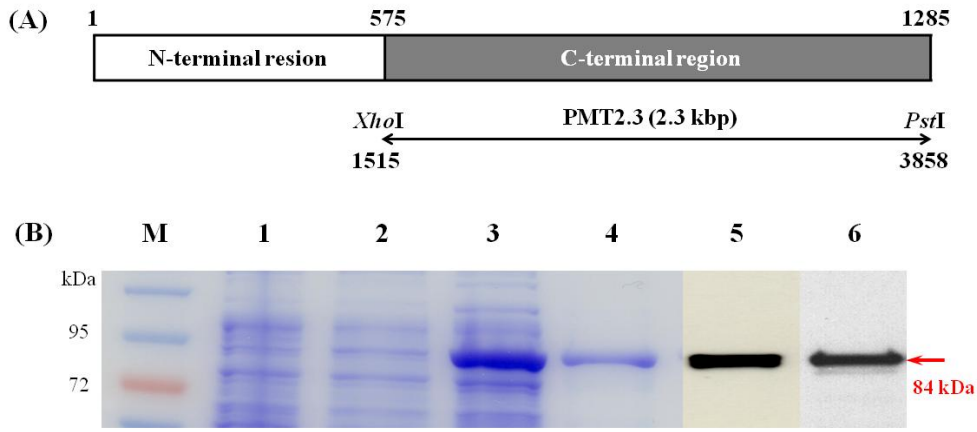
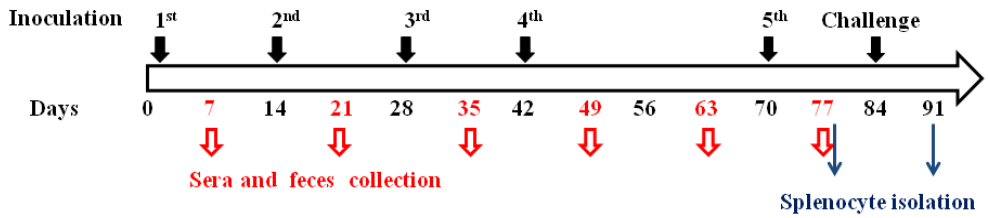


Figure 3. Physical map of *Pasteurella multocida* toxin (PMT) gene and expression of recombinant PMT2.3 protein in *E. coli* BL21(DE3). The C-terminal part of a PMT gene (3858 nucleotide encoding 1285 amino acids) was used to generate recombinant PMT2.3 protein (A). The purified rPMT2.3 protein was confirmed by SDS-PAGE and immunoblot (B). M: Molecular weight marker; lane 1: *E. coli* BL21(DE3); lanes 2 and 3: pre- and post-IPTG induction, respectively; lane 4: rPMT2.3 purified by Ni-NTA affinity column chromatography; lanes 5 and 6: detection of purified rPMT2.3 by western blot using anti-His-tag polyclonal antibody and anti-rPMT2.3 antisera, respectively.

Table 1. Immunization schedule and the different groups



Groups	Route	Dose	Population
1. PBS	orally	200 ul	10
2. NZ9000	orally	1 X 10^{11} CFU/200 ul	10
3. rPMT2.3/NZ9000-1	orally	1 X 10^{10} CFU/200 ul	10
4. rPMT2.3/NZ9000-10	orally	1 X 10^{11} CFU/200 ul	10
5. ART-T4	i.p.	100 ul	10
6. rPMT 2.3 protein	i.p.	50 ug/200 ul	10

Mice were immunized with PBS and NZ9000 for the negative control (groups 1 and 2), 1 or 10 doses of NZ9000 expressing rPMT2.3 (groups 3 or 4, respectively), commercial vaccine ART-T4 and rPMT2.3 for the positive control (groups 5 and 6). The rPMT2.3 proteins were emulsified (1:1) in Freund's incomplete adjuvant or Freund's complete adjuvant at 1^{st} or 2^{nd}_5^{th} immunization, respectively. Six groups of 10 mice each were immunized five times orally or intraperitoneally in mice.

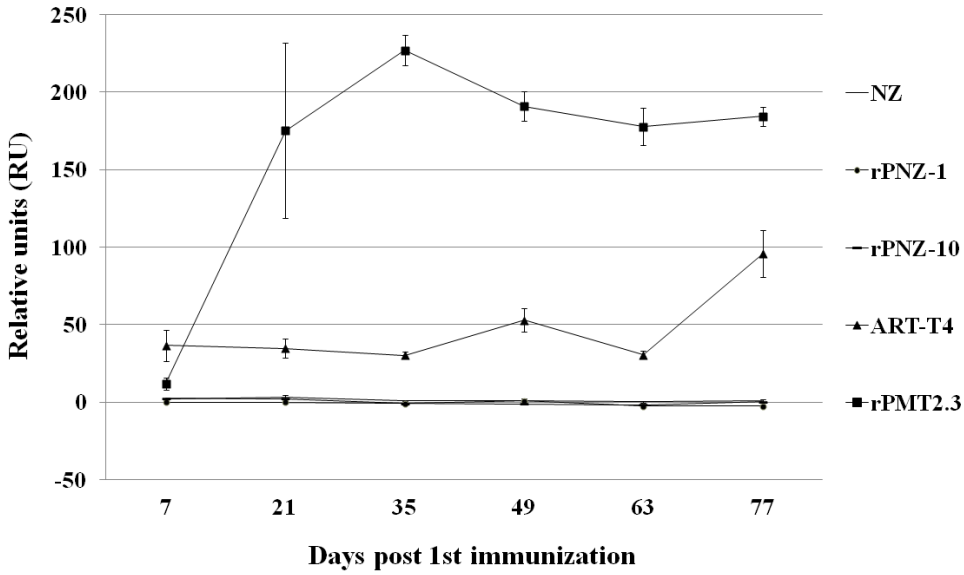


Figure 4. Relative units of specific anti-rPMT2.3 IgG responses of mice after immunization. Immunizations were performed five times in two-week intervals, and the antisera were collected at two-week intervals starting 7 d after 1st immunization. Serum IgG titers were measured by ELISA with purified rPMT2.3 as antigens. Relative units were calculated by the following equation: (OD value of experiment – OD value of PBS group; negative control) X 100. Relative units over 0 were considered as having a positive antibody level.

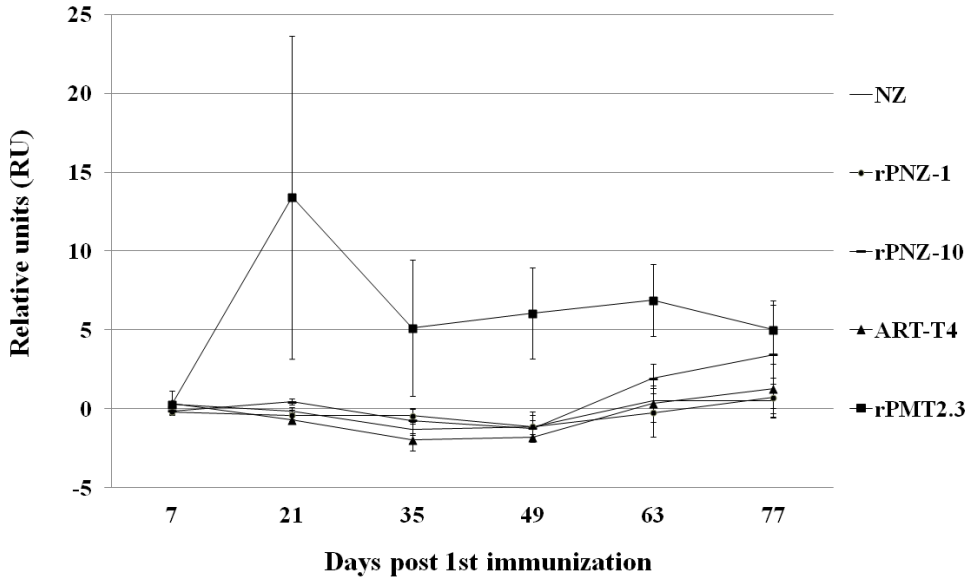


Figure 5. Relative units of specific anti-rPMT2.3 IgA responses of mice after immunization. The titers of PMT2.3-specific fecal IgA were measured by ELISA with purified rPMT2.3 as antigens. Relative units were calculated by the following equation: (OD value of experiment – OD value of PBS group; negative control) X 100. Relative units over 0 were considered as having a positive antibody level.

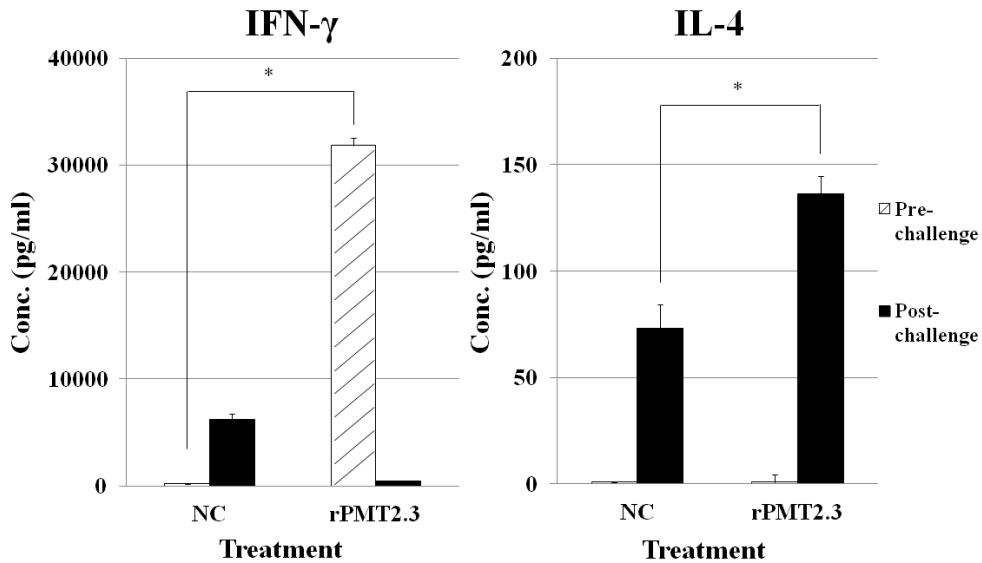


Figure 6. Production of cytokines in splenocytes from rPMT2.3-immunized mice pre- and post-challenge. Splenocytes were stimulated with purified rPMT2.3 proteins and cytokine levels measured using ELISA. Culture supernatants were assayed for IFN-γ (A) and IL-4 (B). Titration curves were generated using IFN- γ and IL-4 standards, and concentrations of cytokines in test supernatants were calculated by reference to these standard curves.

Table 2. Evaluation of protection conferred on immunized mice against live *P. multocida* challenge

Groups	Challenge dose (CFU)	Number of dead mice/ number of challenged mice	Survival rate (%)
1. PBS	$1.45 \text{ X } 10^5$ ($2LD_{50}$)	7/7	0
2. NZ9000	$1.45 \text{ X } 10^5$ ($2LD_{50}$)	7/7	0
3. rPMT2.3/NZ9000-1	$1.45 \text{ X } 10^5$ ($2LD_{50}$)	7/7	0
4. rPMT2.3/NZ9000-10	$1.45 \text{ X } 10^5$ ($2LD_{50}$)	7/7	0
5. ART-T4	$3.63 \text{X } 10^5$ ($5LD_{50}$)	5/7	28.5
6. rPMT 2.3 protein	$3.63 \text{ X } 10^5$ ($5LD_{50}$)	3/7	57.1

Immunized mice were intraperitoneally challenged with two or five median lethal doses (LD50) of live homologous virulent *P. multocida* D:4 (1.45×10^5 or 3.63×10^5 CFU/mouse) 2 weeks after the last immunization. The survival rate was calculated as the ratio of living mice to total mice challenged in a group 3 d after the challenge.

Table 3. Immunization schedule for the different groups

Groups	Route	Dose	Population
1. PBS	i.p.	200 ul	6
2. ART-T4	i.p.	100 ul	6
3. rPMT 2.3 protein	i.p.	50 ug/200 ul	6
4. rPMT 2.3 protein + ART-T4	i.p.	50 ug + 100 ul	6

Mice were immunized with PBS for the negative control (group 1), commercial vaccine ART-T4 (group 2), rPMT-2.3 (group 3) and ART-T4 with rPMT2.3 (group 4). The rPMT2.3 proteins were emulsified (1:1) in Freund's incomplete adjuvant or Freund's complete adjuvant at 1^{st} or 2^{nd} immunization, respectively. Four groups of 10 mice each were immunized twice by intraperitoneal injection.

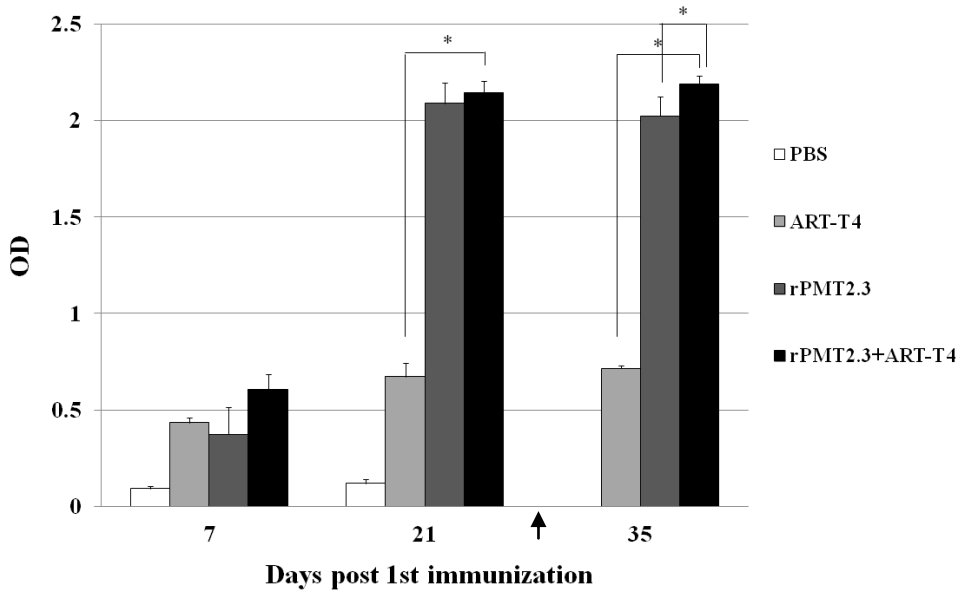


Figure 7. Specific anti-rPMT2.3 responses of mice after immunization. Immunizations were performed twice at two-week intervals and the sera were collected in two-week intervals from 7 d after immunization. Serum IgG titers were measured by ELISA with purified rPMT2.3 as antigens. Arrows means that the challenge occurred at 7 d after last immunization.

Table 4. Evaluation of protection conferred on immunized mice against live *P. multocida* challenge

Groups	Challenge dose (CFU)	Number of dead mice/ number of challenged mice	Survival rate (%)
1. PBS	3.63×10^5 ($5LD_{50}$)	6/6	0
2. ART-T4	3.63×10^5 ($5LD_{50}$)	4/6	33.3
3. rPMT 2.3 protein	3.63×10^5 ($5LD_{50}$)	1/6	83.3
4. rPMT 2.3 protein + ART-T4	3.63×10^5 ($5LD_{50}$)	0/6	100

Immunized mice were intraperitoneally challenged with five median lethal doses (LD50) of live homologous virulent *P. multocida* D:4 (3.63×10^5 CFU/mouse) at 2 weeks after the last immunization. The survival rate was calculated as the ratio of living mice to total mice challenged in a group 3 d after the challenge.

IV. Discussion

Progressive atrophic rhinitis (PAR) diseases are widespread in swine, causing reduction in feed ingestion and growth retardation. Strains of *P. multocida* serogroup D produce a dermonecrotic toxin (*P. multocida* toxin; PMT), which is the principle virulence factor in atrophic rhinitis (Chrisp and Foged, 1991; Kamp and Kimman, 1988). There are several commercially available vaccines that contain whole-cell bacterins of *B. bronchiseptica* and a mixture of *P. multocida*, or its toxoid. Vaccines based on a *P. multocida* toxoid offer specific protection against the action of the toxin (Foged, 1992). The level of toxin produced by *P. multocida* is relatively low; thus, the toxin-specific antibody response induced by bacterin-only vaccines may not be optimal. As an alternative, use of a purified toxoid also has difficulty due to the expense of large-scale purification. This could prevent routine incorporation of purified toxoid into vaccines.

As another alternative, recombinant toxins have been used as vaccine candidates (Nielsen et al., 1991; Petersen et al., 1991). Field studies have shown that a recombinant *P. multocida* toxin derivative, missing a segment of the amino-terminal portion of the protein, is non-toxic but immunogenic and has superior efficacy in swine (Bording et al., 1994b; Pejsak et al., 1994b). The full-length recombinant toxin engineered to contain two amino acid substitutions that eliminate toxigenicity was also found to be highly efficacious (To et al., 2005a). A DNA vaccine encoding a full-length but enzymatically inactive toxin was shown to be highly immunogenic in pigs; however, it is yet to be evaluated for efficacy against challenge (Register et al., 2007b). The vaccination with the short fragments of recombinant subunit PMT proteins

containing either the N-terminal or the C-terminal portions resulted in high levels of neutralizing antibody and a specific cellular immune response against PMT in swine (Liao et al., 2006). Vaccine composed of the three rsPMT derivatives, N-terminal (Tox1: aa 1–487), middle (Tox2: aa 485–987), and C-terminal (Tox7: aa 986–1282) portions of PMT in combination with a *P. multocida* bacterin (rsPMT–PM) induced neutralizing antibody and protective immunity in a field study (Hsuan et al., 2009). The short N-terminal fragment (residues 1-390) was immunogenic to produce protective antibody in mice and pig (Seo et al., 2009).

On the other hand, Lee et al. (2010) suggest that the N-terminal region of PMT (residues 1_483) has relatively poor immunoreactivity to the antisera from mice immunized with PMT or to the antisera from an infected swine. However, vaccination of mice with a large portion of the C-terminus, including the intracellular activity of PMT (PMT2.3), could induce protective immunity against homologous challenge, and it conferred a effective protection in swine by passive transfer of maternal antibodies through colostrum (Lee et al., 2012). In the present study,it was determined whether the rPMT2.3 could be applied as an AR vaccine beyond the scope identified than previous studies.

Oral vaccines have the distinct advantage of being able to potentially reach the highly concentrated gut-associated lymphoid tissue, and induce both systemic and mucosal immune responses (McGhee et al., 1992; Villena et al., 2010). They can elicit antigen-specific secretory immunoglobulin A (sIgA) responses at mucosal surfaces, which can neutralize viruses or toxins and inhibit colonization by enteric microbes (Wells, 2011). Because mucosal immunization induces not only Ag-specific mucosal sIgA but also systemic IgG, there are many studies concerning whether a mucosal

delivery system can elicit both antigen-specific sIgA and effective systemic immune responses (Lavelle and O'Hagan, 2006; Neutra and Kozlowski, 2006).

In particular, lactic acid bacteria *L. lactis* is generally recognized as safe (GRAS). There are several advantages to using *L. lactis* with the exception of safty. It can be taken up by M cells in gut-associated immune tissues because it is sized as biodegradable microparticles and can be less likely to induce oral tolerance for its characteristics of passive transit through the digestive tract allowing it to act as an effective oral vaccine vehicle (Challacombe et al., 1992; Lee et al., 2001). The target diseases of the present study, PAR, are caused by *P. multocida* pathogen, which initially colonizes and then produces toxins at mucosal surfaces. Therefore, oral vaccines were selected to enhance the mucosal immunity. As mentioned previously, due to the many benefits, *L. lactis* was chosen as a carrier expressing target protein. A recombinant *L. lactis*-expressing rPMT2.3 protein was successfully constructed.

In oral vaccination experiments with recombinant *L. lactis* expressing rPMT2.3, specific IgG and IgA responses and protection effects were not found. There are different potential explanations as to why this did not induced specific immune responses. First of all, one reasonably predictable problem is a wrong expression of target protein in *L. lactis.* However, this is not relevant to the absence of specific immune response because whether the expression of rPMT2.3 protein has occurred was confirmed through Western blot just before every administration.

Another possibility is induction of oral tolerance for unknown reasons, such as interval and concentration. It was reported that the frequency and interval of oral administration is a key factor for unresponsive state (Weiner, 1994). The same protocol in the present study, three doses daily with one or two weeks intervals, failed

to induce specific antibody responses, and that results could be explained by the induction of oral tolerance. On the other hand, the protocol of one dose with one week intervals successfully induced an immune response. It is suggests that unresponsiveness was induced by primary antigen challenge given too frequently over too short a period (Lee et al., 2001). Induction of a tolerant state by oral administration of an antigen depends not only on the dose, but also on the frequency and interval of administration (Weiner, 1994). However, the relationship between oral tolerance and administration method has not yet been established. In a recent study, a similar finding was reported (Ahmed et al., 2013) that no specific antibody responses were detected in the serum or the feces following oral immunization. However, a specific serum IgG and fecal IgA were detected after intraperitoneal boosting with recombinant target protein. These results show that oral administration resulted in mucosal priming rather than induction of oral tolerance. This will be a useful way to check whether induced oral tolerance.

In addition, immunological adjuvants that can enhance immunity are substances used in combination with a specific antigen. Co-administration of high dose recombinant *L. lactis* with CTB enhanced immune responses (Liu et al., 2010). CTB is the B subunit of cholera toxin and is used as a mucosal adjuvant by being simply mixed with an antigen or chemically and genetically conjugated (Matoba et al., 2006; Price et al., 2005). Co-administration of a major subunit of Enterotoxigenic *E. coli* (ETEC) with CT or CTB in pigs has been reported to promote antigen-specific immune responses and to reduce ETEC excretion (Foss and Murtaugh, 1999; Verdonck et al., 2005). The rack of specific Ab response and protection by oral administration in the present study could be improved with reference to these previous studies. Therefore, further study

into a functional oral vaccine that elicits rPMT2.3-specific Abs and protective immune responses is needed. Future research should consider factors from several points such as dose and intervals of oral administration, and the use and type of mucosal adjuvants for producing mucosal immune responses.

Unlike oral administration, the intraperitoneally injected groups induced rPMT2.3 specific IgG levels effectively. In particular, the rPMT2.3 immunized group had higher levels of rPMT2.3-specific Ab induced than the ART-T4 immunized group did. The IgA level was induced at insignificant levels in only the rPMT2.3 immunized group, and titers were not maintained. This result means that rPMT2.3 has antigenicity and a role as a vaccine candidate, as in previous research. In addition, it could enhance the effectiveness of existing vaccines by application as an alternative to PMT toxoid, which has risk of side effects.

In addition, the production of IFN-γ was significantly increased in the rPMT2.3-immunized group, and after the challenge, more IL-4 was secreted in splenocyte from the rPMT2.3-immunized group. Cytokines actively participate in the immune response. The antigen-stimulated-$CD4^+$ T cell can differentiate to Th cells, which secrete many cytokines, including IL-4 and IFN-γ. IL-4 is a B-cell growth and differentiation factor, and might indicate the status of activation and proliferation of B cells. Thus, IL-4 is considered to be a feature of the humoral immune response and it may enhance B cell production of neutralization antibodies. CD8+ T cell can be activated and differentiated to cytotoxic T lymphocyte (CTL), and IFN-γ is considered to be an accelerant of the cellular immune response (Huang et al., 1993). Thus, rPMT2.3 is related to induction of Th1- and Th2-type immune response.

So far, based on the experimental results that rPMT2.3 protein induced significant

levels of rPMT2.3 specific Ab responses, protective effect, and its relevance for IFN-r and IL-4 secretion before and after challenge, its applicability as an additive for commercial vaccine is demonstrated. As expected, The immunization with atrophic rhinitis commercial vaccine ART-T4 containing rPMT2.3 protein showed a higher level of anti-PMT2.3 IgG after 2nd immunization than when immunized with ART-T4 only. At challenge study, the survival rate in the rPMT2.3-immunized group was also increased compared to the ART-T4 group. ART-T4 commercial vaccine contains *B. bronchiseptica, P. multocide* serotype D and its toxoid. This can explain the lower levels of rPMT2.3-specific Abs and protection to *P. multocida* homologous challenge than immunization with rPMT2.3 protein. As the combination of ART-T4 and the rPMT2.3 protein improved antigenicity and protective effect of ART-T4, rPMT2.3 show great promise for alternatives in promoting protective effects in *P. multocida* D:4 vaccine.

VI. Conclusion

The rPMT2.3 protein was successfully produced in *L. lactis*. This is the first report of production of rPMT2.3 using lactic acid bacteria-based expression system by nisin induction. However, further study is needed for development of a functional oral vaccine. On the other hand, immunization with the purified rPMT2.3 induces strong antibody response that was significantly maintained by a subsequent injection. In addition, rPMT2.3 induced production of IFN-γ and switched to production of IL-4 after challenge. Therefore, a safe rPMT2.3 protein that induces immunoreactivity is available as additional material in AR vaccines. As the combination of ART-T4 and the rPMT2.3 protein significantly improved antigenicity and protective effect of ART-T4, the rPMT2.3 might be used as a component in an effective *P. multocida* D:4 vaccine.

VII. References

Ahmed, B., Loos, M., Vanrompay, D., Cox, E., 2013. Mucosal priming of the murine immune system against enterohemorrhagic *Escherichia coli* O157:H7 using *Lactococcus lactis* expressing the type III secretion system protein EspB. Veterinary immunology and immunopathology 152, 141-145.

Amigot, J.A., Torremorell, M., Pijoan, C., 1998. Evaluation of techniques for the detection of toxigenic *Pasteurella multocida* strains from pigs. Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc 10, 169-173.

Bording, A., Nymark, K., Smidt, E., 1994. Field trials with a new genetically engineered vaccine for protection against progressive atrophic rhinitis in pigs. Acta veterinaria Scandinavica 35, 155-163.

Bowersock, T.L., Hooper, T., Pottenger, R., 1992. Use of ELISA to detect toxigenic *Pasteurella multocida* in atrophic rhinitis in swine. Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc 4, 419-422.

Busch, C., Orth, J., Djouder, N., Aktories, K., 2001. Biological activity of a C-terminal fragment of *Pasteurella multocida* toxin. Infection and immunity 69, 3628-3634.

Carter, G.R., 1955. Studies on *Pasteurella multocida*. I. A hemagglutination test for the identification of serological types. American journal of veterinary research 16, 481-484.

Challacombe, S.J., Rahman, D., Jeffery, H., Davis, S.S., O'Hagan, D.T., 1992. Enhanced secretory IgA and systemic IgG antibody responses after oral immunization with biodegradable microparticles containing antigen. Immunology 76, 164-168.

Choi, C., Kim, B., Cho, W., Kim, J., Kwon, D., Cheon, D., Chae, C., 2001. Capsular serotype, *tox*A gene, and antimicrobial susceptibility profiles of *Pasteurella multocida* isolated from pigs with pneumonia in Korea. Veterinary Record 149, 210-212.

Chrisp, C.E., Foged, N.T., 1991. Induction of pneumonia in rabbits by use of a purified protein toxin from *Pasteurella multocida*. American journal of veterinary research 52, 56-61.

de Jong, M.F., Nielsen, J.P., 1990. Definition of progressive atrophic rhinitis. The Veterinary record 126, 93.

Dudet, L.I., Chailler, P., Dubreuil, J.D., Martineau-Doize, B., 1996. *Pasteurella multocida* toxin stimulates mitogenesis and cytoskeleton reorganization in Swiss 3T3 fibroblasts. Journal of cellular physiology 168, 173-182.

Ewers, C., Lübke-Becker, A., Bethe, A., Kießling, S., Filter, M., Wieler, L.H., 2006. Virulence genotype of *Pasteurella multocida* strains isolated from different hosts with various disease status. Veterinary microbiology 114, 304-317.

Foged, N.T., 1992. *Pasteurella multocida* toxin. The characterisation of the toxin and its significance in the diagnosis and prevention of progressive atrophic rhinitis in pigs. APMIS. Supplementum 25, 1.

Foss, D.L., Murtaugh, M.P., 1999. Mucosal immunogenicity and adjuvanticity of cholera toxin in swine. Vaccine 17, 788-801.

Foss, D.L., Murtaugh, M.P., 2000. Mechanisms of vaccine adjuvanticity at mucosal surfaces. Animal health research reviews / Conference of Research Workers in Animal Diseases 1, 3-24.

Gois, M., Sisak, F., Kuksa, F., Sovadina, M., 1975. Incidence and evaluation of the microbial flora in the lungs of pigs with enzootic pneumonia. Zentralblatt für Veterinärmedizin Reihe B 22, 205-219.

Gruzza, M., Fons, M., Ouriet, M.F., Duval-Iflah, Y., Ducluzeau, R., 1994. Study of gene transfer in vitro and in the digestive tract of gnotobiotic mice from *Lactococcus lactis* strains to various strains belonging to human intestinal flora. Microbial releases : viruses, bacteria, fungi 2, 183-189.

Gu, Q., Song, D., Zhu, M., 2009. Oral vaccination of mice *against Helicobacter pylori* with recombinant *Lactococcus lactis* expressing urease subunit B. FEMS immunology and medical microbiology 56, 197-203.

Hanahan, D., 1983. Studies on transformation of *Escherichia coli* with plasmids. Journal of molecular biology 166, 557-580.

Harper, M., Boyce, J.D., Adler, B., 2006. *Pasteurella multocida* pathogenesis: 125 years after Pasteur. FEMS microbiology letters 265, 1-10.

Heddleston, K.L., Gallagher, J.E., Rebers, P.A., 1972. Fowl cholera: gel diffusion precipitin test for serotyping *Pasteruella multocida* from avian species. Avian diseases 16, 925-936.

Holmgren, J., Czerkinsky, C., Eriksson, K., Mharandi, A., 2003. Mucosal immunisation and adjuvants: a brief overview of recent advances and challenges. Vaccine 21 Suppl 2, S89-95.

Horiguchi, Y., 2012. Swine atrophic rhinitis caused by *pasteurella multocida* toxin and bordetella dermonecrotic toxin. Current topics in microbiology and immunology 361, 113-129.

Hsuan, S.L., Liao, C.M., Huang, C., Winton, J.R., Chen, Z.W., Lee, W.C., Liao, J.W., Chen, T.H., Chiou, C.J., Yeh, K.S., Chien, M.S., 2009. Efficacy of a novel *Pasteurella*

multocida vaccine against progressive atrophic rhinitis of swine. Vaccine 27, 2923-2929.

Huang, S., Hendriks, W., Althage, A., Hemmi, S., Bluethmann, H., Kamijo, R., Vilcek, J., Zinkernagel, R.M., Aguet, M., 1993. Immune response in mice that lack the interferon-gamma receptor. Science (New York, N.Y.) 259, 1742-1745.

Huang, Y.S., Fisher, M., Nasrawi, Z., Eichenbaum, Z., 2011. Defense from the Group A *Streptococcus* by active and passive vaccination with the streptococcal hemoprotein receptor. The Journal of infectious diseases 203, 1595-1601.

Jing, H., Yong, L., Haiyan, L., Yanjun, M., Yun, X., Yu, Z., Taiming, L., Rongyue, C., Liang, J., Jie, W., Li, Z., Jingjing, L., 2011. Oral administration of *Lactococcus lactis* delivered heat shock protein 65 attenuates atherosclerosis in low-density lipoprotein receptor-deficient mice. Vaccine 29, 4102-4109.

Kamp, E.M., Kimman, T.G., 1988. Induction of nasal turbinate atrophy in germ-free pigs, using *Pasteurella multocida* as well as bacterium-free crude and purified dermonecrotic toxin of *P. multocida.* American journal of veterinary research 49, 1844-1849.

Lavelle, E.C., O'Hagan, D.T., 2006. Delivery systems and adjuvants for oral vaccines. Expert opinion on drug delivery 3, 747-762.

Lax, A.J., Chanter, N., 1990. Cloning of the toxin gene from *Pasteurella multocida* and its role in atrophic rhinitis. Journal of general microbiology 136, 81-87.

L'Ecuyer, C., Switzer, W.P., Roberts, E.D., 1961. Microbiologic survey of pneumonic and normal swine lungs. American journal of veterinary research 22, 1020-1025.

Lee, J., Kang, H.-E., Woo, H.-J., 2012. Protective Immunity Conferred by the C-Terminal Fragment of Recombinant *Pasteurella multocida* Toxin. Clinical and Vaccine Immunology 19, 1526-1531.

Lee, J., Woo, H.J., 2010. Antigenicity of partial fragments of recombinant *Pasteurella multocida* toxin. Journal of microbiology and biotechnology 20, 1756-1763.

Lee, M.H., Roussel, Y., Wilks, M., Tabaqchali, S., 2001. Expression of *Helicobacter pylori* urease subunit B gene in *Lactococcus lactis* MG1363 and its use as a vaccine delivery system against *H. pylori* infection in mice. Vaccine 19, 3927-3935.

Liao, C.M., Huang, C., Hsuan, S.L., Chen, Z.W., Lee, W.C., Liu, C.I., Winton, J.R., Chien, M.S., 2006. Immunogenicity and efficacy of three recombinant subunit *Pasteurella multocida* toxin vaccines against progressive atrophic rhinitis in pigs. Vaccine 24, 27-35.

Liu, S., Li, Y., Xu, Z., Wang, Y., 2010. Immune responses elicited in mice with recombinant *Lactococcus lactis* expressing F4 fimbrial adhesin FaeG by oral

immunization. Veterinary research communications 34, 491-502.

Mannam, P., Jones, K.F., Geller, B.L., 2004. Mucosal vaccine made from live, recombinant *Lactococcus lactis* protects mice against pharyngeal infection with Streptococcus pyogenes. Infection and immunity 72, 3444-3450.

Marelli, B., Perez, A.R., Banchio, C., de Mendoza, D., Magni, C., 2011. Oral immunization with live *Lactococcus lactis* expressing rotavirus VP8 subunit induces specific immune response in mice. Journal of virological methods 175, 28-37.

Matoba, N., Geyer, B.C., Kilbourne, J., Alfsen, A., Bomsel, M., Mor, T.S., 2006. Humoral immune responses by prime-boost heterologous route immunizations with CTB-MPR(649-684), a mucosal subunit HIV/AIDS vaccine candidate. Vaccine 24, 5047-5055.

McGhee, J.R., Mestecky, J., Dertzbaugh, M.T., Eldridge, J.H., Hirasawa, M., Kiyono, H., 1992. The mucosal immune system: from fundamental concepts to vaccine development. Vaccine 10, 75-88.

Morrison, R., Pijoan, C., Hilley, H., Rapp, V., 1985. Microorganisms associated with pneumonia in slaughter weight swine. Canadian journal of comparative medicine 49, 129.

Nagai, S., Someno, S., Yagihashi, T., 1994. Differentiation of toxigenic from

nontoxigenic isolates of *Pasteurella multocida* by PCR. Journal of clinical microbiology 32, 1004-1010.

Neutra, M.R., Kozlowski, P.A., 2006. Mucosal vaccines: the promise and the challenge. Nature reviews. Immunology 6, 148-158.

Nielsen, J.P., Foged, N.T., Sorensen, V., Barfod, K., Bording, A., Petersen, S.K., 1991. Vaccination against progressive atrophic rhinitis with a recombinant *Pasteurella multocida* toxin derivative. Canadian journal of veterinary research 55, 128-138.

Nouaille, S., Ribeiro, L.A., Miyoshi, A., Pontes, D., Le Loir, Y., Oliveira, S.C., Langella, P., Azevedo, V., 2003. Heterologous protein production and delivery systems for *Lactococcus lactis*. Genetics and molecular research : GMR 2, 102-111.

Orth, J.H., Blocker, D., Aktories, K., 2003. His1205 and His1223 are essential for the activity of the mitogenic *Pasteurella multocida* toxin. Biochemistry 42, 4971-4977.

Pavot, V., Rochereau, N., Genin, C., Verrier, B., Paul, S., 2012. New insights in mucosal vaccine development. Vaccine 30, 142-154.

Pei, H., Liu, J., Cheng, Y., Sun, C., Wang, C., Lu, Y., Ding, J., Zhou, J., Xiang, H., 2005. Expression of SARS-coronavirus nucleocapsid protein in *Escherichia coli* and *Lactococcus lactis* for serodiagnosis and mucosal vaccination. Applied microbiology and biotechnology 68, 220-227.

Pejsak, Z., Wasinska, B., Markowska-Daniel, I., Hogg, A., 1994. Field evaluation of thirteen regimens for the control of progressive atrophic rhinitis. Comparative immunology, microbiology and infectious diseases 17, 125-132.

Perez, C.A., Eichwald, C., Burrone, O., Mendoza, D., 2005. Rotavirus vp7 antigen produced by *Lactococcus lactis* induces neutralizing antibodies in mice. Journal of applied microbiology 99, 1158-1164.

Petersen, S.K., Foged, N.T., Bording, A., Nielsen, J.P., Riemann, H.K., Frandsen, P.L., 1991. Recombinant derivatives of *Pasteurella multocida* toxin: candidates for a vaccine against progressive atrophic rhinitis. Infection and immunity 59, 1387-1393.

Pettit, R.K., Ackermann, M.R., Rimler, R.B., 1993. Receptor-mediated binding of *Pasteurella multocida* dermonecrotic toxin to canine osteosarcoma and monkey kidney (vero) cells. Laboratory investigation; a journal of technical methods and pathology 69, 94-100.

Pontes, D.S., de Azevedo, M.S., Chatel, J.M., Langella, P., Azevedo, V., Miyoshi, A., 2011. *Lactococcus lactis* as a live vector: heterologous protein production and DNA delivery systems. Protein expression and purification 79, 165-175.

Pontes, D.S., Dorella, F.A., Ribeiro, L.A., Miyoshi, A., Le Loir, Y., Gruss, A., Oliveira, S.C., Langella, P., Azevedo, V., 2003. Induction of partial protection in mice after oral

administration of *Lactococcus lactis* producing *Brucella abortus* L7/L12 antigen. Journal of drug targeting 11, 489-493.

Price, G.A., Russell, M.W., Cornelissen, C.N., 2005. Intranasal Administration of Recombinant *Neisseria gonorrhoeae* Transferrin Binding Proteins A and B Conjugated to the Cholera Toxin B Subunit Induces Systemic and Vaginal Antibodies in Mice. Infection and immunity 73, 3945-3953.

Pullinger, G.D., Bevir, T., Lax, A.J., 2004. The *Pasteurella multocida* toxin is encoded within a lysogenic bacteriophage. Molecular microbiology 51, 255-269.

Pullinger, G.D., Sowdhamini, R., Lax, A.J., 2001. Localization of functional domains of the mitogenic toxin of *Pasteurella multocida*. Infection and immunity 69, 7839-7850.

Register, K.B., Sacco, R.E., Brockmeier, S.L., 2007. Immune response in mice and swine to DNA vaccines derived from the *Pasteurella multocida* toxin gene. Vaccine 25, 6118-6128.

Robinson, K., Chamberlain, L.M., Lopez, M.C., Rush, C.M., Marcotte, H., Le Page, R.W., Wells, J.M., 2004. Mucosal and cellular immune responses elicited by recombinant *Lactococcus lactis* strains expressing tetanus toxin fragment C. Infection and immunity 72, 2753-2761.

Ross, R.F., 2006. Pasteurella multocida and its role in porcine pneumonia. Animal health research reviews / Conference of Research Workers in Animal Diseases 7, 13-29.

Rottiers, P., De Smedt, T., Steidler, L., 2009. Modulation of Gut-Associated Lymphoid Tissue Functions with Genetically Modified *Lactococcus lactis*. International Reviews of Immunology 28, 465-486.

Rozengurt, E., Higgins, T., Chanter, N., Lax, A.J., Staddon, J.M., 1990. *Pasteurella multocida* toxin: potent mitogen for cultured fibroblasts. Proceedings of the National Academy of Sciences of the United States of America 87, 123-127.

Rutter, J.M., 1985. Atrophic rhinitis in swine. Advances in veterinary science and comparative medicine 29, 239-279.

Ryu, E., 1954. A bacteriological study on swine infectious pneumonia: general bacteriological examination and experiments on artificial infection. Memoirs College of Agriculture, National Taiwan University 3, 1-20.

Seo, J., Pyo, H., Lee, S., Lee, J., Kim, T., 2009. Expression of 4 truncated fragments of *Pasteurella multocida* toxin and their immunogenicity. Canadian journal of veterinary research 73, 184-189.

Straw, B.E., Dewey, C.E., Erickson, E.D., 1996. Interpreting culture reports from

swine lungs. Swine health and production: the official journal of the American Association of Swine Practitioners 4.

To, H., Someno, S., Nagai, S., 2005. Development of a genetically modified nontoxigenic *Pasteurella multocida* toxin as a candidate for use in vaccines against progressive atrophic rhinitis in pigs. American journal of veterinary research 66, 113-118.

Verdonck, F., Snoeck, V., Goddeeris, B.M., Cox, E., 2005. Cholera toxin improves the F4(K88)-specific immune response following oral immunization of pigs with recombinant FaeG. Veterinary immunology and immunopathology 103, 21-29.

Villena, J., Medina, M., Racedo, S., Alvarez, S., 2010. Resistance of young mice to pneumococcal infection can be improved by oral vaccination with recombinant *Lactococcus lactis*. Journal of microbiology, immunology, and infection 43, 1-10.

Ward, P.N., Miles, A.J., Sumner, I.G., Thomas, L.H., Lax, A.J., 1998. Activity of the mitogenic *Pasteurella multocida* toxin requires an essential C-terminal residue. Infection and immunity 66, 5636-5642.

Weber, D.J., Wolfson, J.S., Swartz, M.N., Hooper, D.C., 1984. *Pasteurella multocida* infections. Report of 34 cases and review of the literature. Medicine 63, 133-154.

Weiner, H.L., 1994. Oral tolerance. Proceedings of the National Academy of Sciences

of the United States of America 91, 10762.

Wells, J., 2011. Mucosal vaccination and therapy with genetically modified lactic acid bacteria. Annual review of food science and technology 2, 423-445.

Wells, J.M., Mercenier, A., 2008. Mucosal delivery of therapeutic and prophylactic molecules using lactic acid bacteria. Nature reviews. Microbiology 6, 349-362.

Xin, K.Q., Hoshino, Y., Toda, Y., Igimi, S., Kojima, Y., Jounai, N., Ohba, K., Kushiro, A., Kiwaki, M., Hamajima, K., Klinman, D., Okuda, K., 2003. Immunogenicity and protective efficacy of orally administered recombinant *Lactococcus lactis* expressing surface-bound HIV Env. Blood 102, 223-228.

VIII. 국문초록

Pasteurella multocida dermonecrotic toxin의 C-terminal 부분을 응용한 돼지의 진행성 위축성 비염 백신 개발

김 아 름
수의학과 수의미생물학 전공
서울대학교 대학원

*Pasteurella multocida*는 그람 음성의 병원성 미생물로서 다양한 종류의 야생 또는 가축 동물에 호흡기 질환을 일으킨다. *P. multocida* D형이 생산하는 *P. multocida* toxin (PMT)은 돼지의 진행성 위축성 비염을 일으키는 주요 감염인자이며, PMT 특이 항체는 질병의 보호에 기여한다고 알려져 있다. 선행연구에서 PMT의 C-terminal 부분 (PMT2.3)을 면역화 하였을 때 *P. multocida* D형 감염에 대하여 방어면역 (protective immunity)을 가진다는 것이 밝혀져 있다. 본 연구에서는 경구백신에 있어서 rPMT2.3의 효과를 입증하고자 PMT2.3을 발현하는 재조합 유산균주 *Lactococcus lactis*를 제조하였다. 이 재조합 유산균주의 백신효과를 보기 위해 실험용 마우스에 연속 3일간 2주 간격으로 경구투여 하였으며, 점막 면역 및 전신 면역 반응을 조사하기 위하여 분변과 혈액으로부터 rPMT2.3 특이 IgA와 IgG 항체가를 측정하였다. 동종의 공격접종에 대한 방어면역성 또한 측정하였다. PMT2.3을 발현하는 재조합 *L. lactis*종을 경구 투여한 경우, rPMT2.3 특이 IgA와 IgG 항체 및 동종 병원균의 공격접종에 대한 방어면역성은 관찰되

지 않았다. 반면, rPMT2.3 단백질과 상용 위축성 비염 백신인 ART-T4를 복강 주사한 그룹의 마우스에서는 rPMT2.3 특이 IgA와 IgG 항체가 유효한 수준으로 유도되었고, 공격접종에 대한 방어면역성 또한 보였다. *L. lactis*종은 generally recognized as safe (GRAS) 등급으로 분류된 안전한 유산균주로서 가지고 있는 다양한 장점으로 인하여 경구백신의 carrier로 이용되고 있어 본 연구에서는 PMT2.3 단백질을 *L.lactis* 종에 성공적으로 발현시켰으나 필드에 적용될 수 있는 기능적인 경구백신을 만들기까지는 추후 연구가 필요하다.

다음으로, 지금까지 알려지지 않은 rPMT2.3 부분에 대한 immune effect를 더 연구하고자 rPMT2.3을 면역화시킨 마우스로부터 비장세포를 분리하여 cytokine의 분비양상을 비교하였다. 공격접종을 하기 전의 rPMT2.3에 의하여 감작된 비장세포에서는 IFN-γ의 분비가 유효하게 증가하였고, 공격접종 후 IL-4의 분비가 증가함으로써 cross-regulation 되었음을 확인하였다. 이것은 rPMT2.3의 면역화에 의해 Th1 과 Th2 타입 면역 반응의 유도에 관여하고 있음을 의미한다. PMT2.3의 이와 같은 효능은 백신제재로서의 용용 가능성이 충분함을 시사한다. 따라서, 상용 위축성 비염 백신인 ART-T4의 효능을 증진시키는 대체물로서 PMT2.3의 기능을 확인하고자 하였다. ART-T4에 rPMT2.3 단백질을 첨가하여 함께 접종한 경우 단일의 백신만을 접종한 경우보다 유효한 항체가와 방어성 면역이 현저히 증가하였으므로, ART-T4 상용백신의 효능을 증진시키는데 PMT2.3을 적용할 수 있을 것이라 생각된다.

주요어: 진행성 위축성 비염, *Pasteurella multocida* D:4, *Pasteurella multocida* toxin, 경구백신, 유산균, *Lactococcus lactis*

학 번: 2007-21769