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Development of a *toxA* Gene Knock-out Mutant of *Pasteurella multocida* and Evaluation of its Protective Effects

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Pasteurella multocida **is an important veterinary and opportunistic human pathogen. In particular, strains of *P. multocida* serogroup D cause progressive atrophic rhinitis, and produce a potent, intracellular, mitogenic toxin known as *P. multocida* toxin (PMT), which is encoded by the *toxA* gene. To further investigate the toxigenic and pathogenic effects of PMT, a *toxA*-deleted mutant was developed by homologous gene recombination. When administrated to mice, the toxigenicity of the *toxA* mutant *P. multocida* was drastically reduced, suggesting that the PMT constributes the major part of the toxigenicity of *P. multocida*. Similar results were obtained in a subsequent experiment, while high mortalities were observed when *toxA*(+) *P. multocida* bacterial culture or culture lysate were administrated. Mice immunized with *toxA*(–) *P. multocida* were not protected (none survived) following challenge with *toxA*(+) *P. multocida* or bacterial culture lysate (toxin). These results suggest that the toxigenicity of *P. multocida* is mainly derived from PMT.**

Keywords: homologous recombination, knock-out, *Pasteurella multocida* toxin (PMT), *toxA*

Pasteurella multocida-induced pneumonia and progressive atrophic rhinitis (PAR) are widespread diseases, causing growth retardation and a reduction in the efficiency of feed utilization among grower-finisher pigs (de Jong, 1999; Pijoan, 1999; Hunt, 2000). *P. multocida* is a common secondary colonizer in swine pneumonia, and is believed to be incapable of invading the lungs unless some predisposing damage has occurred (Ciprian, 1994; Lee, 2004). The *P. multocida* toxin (PMT), which is a monomeric 146 kDa protein that is encoded by the *toxA* gene, is produced by some *P. multocida* serotype A and D strains (Pullinger, 2004). PMT is highly toxic to animals, being lethal to mice after an intraperitoneal inoculation and causing dermonecrotic skin lesions in mice or guinea pigs that had been injected intradermally (Nakai, 1984; Petersen and Foged, 1989). It is the major virulence factor associated with porcine atrophic rhinitis (AR), which is a respiratory infection characterized by the loss of the nasal turbinate bones and a twisting or shortening of the snout (Rutter and Mackenzie, 1984; Chanter, 1986; Hunt, 2000). This toxin is responsible for bone loss in AR. In addition, the intraperitoneal introduction of PMT into pigs causes proliferative changes in the epithelium of the bladder wall and ureter (Rutter and Mackenzie, 1984). A similar effect has also been observed after a nasal infection of gnotobiotic pigs with a toxigenic *P. multocida* strain (Hoskins, 1997). PMT is an intracellularly acting toxin (Rosengurt, 1990; Smyth, 1995; Smyth, 1999; Lax, 2004). It is believed to bind to a ganglioside cell-surface receptor, and enter the cells by endocytosis in order to modify its target (Pettit, 1993). However, the molecular mode of action of PMT as well as its target is unknown. PMT action results in the activation of several intracellular signaling pathways, leading to mitogenesis and a rearrangement of the actin cytoskeleton (Lax, 1990; Lax and Grigoriadis, 2001).

PMT is a highly potent mitogen in various cell types including fibroblasts, osteoblastic cells, and dentritic cells (Rozengurt, 1990; Mullan and Lax, 1996; Lax and Grigoriadis, 2001; Lax, 2004; Bagley, 2005) by modulating the progression of the host cell-cycle (Oswald, 2005). PMT is one of the major contributing factors in the pathogenesis of AR, and inhibits osteoblast differentiation and bone formation (Mullan and Lax, 1996). It is a modular protein, with domains at the N-terminus and C-terminus for cell-binding/internalization and enzymatic activity, re-

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spectively (Busch, 2001; Pullinger, 2001).

Recombinant or purified bacterial PMT has been used in some studies to illuminate its toxigenicity. However, to further investigate the toxigenic and pathogenic effects of PMT, a *toxA*-deleted mutant is required. To do this, we constructed a *P. multocida toxA*-deleted mutant by homologous recombination and assessed its virulence properties in mice.

Materials and Methods

Escherichia coli *and plasmids*

E. coli JM109, BL21(DE3)pLysS and pCR® Blunt vector were purchased from Invitrogen (USA). The pGEM-T easy vector (Promega, USA) was used for cloning procedures. *E. coli* manipulations were performed according to the manufacturer's instructions. Standard DNA and protein manipulations were carried out as described by Ausubel *et al.* (1996) and Sambrook *et al.* (1989). A Red helper plasmid pKD46 (Datsenko and Wanner, 2000), which uses the pBAD promoter to express *red* and *gam* from a low copy number temperature-sensitive replicon was used. This plasmid contains λ Red recombinase, which allows recombination in linear DNA in bacteria to occur successfully, and is a temperature sensitive replicon to allow for its easy elimination at 43°C.

Cloning of the toxA *gene and kanamycin-resistance (kan^r) gene*

The *P. multocida* type D strain used was originally obtained from the National Veterinary Research & Quarantine Service, Korea. The PCR primers were designed using the Gene Runner software program (Hastings Software, USA) from the nucleotide sequence in the GenBank database (AF240778) (*toxA*-F: 5'-ATAT ATGAAAACAAAACATTTTTT-3', *toxA*-R: 5'-TGTG TTATAGTGCTCTTGTTAAGC-3'). The whole *toxA* gene was amplified by PCR using *P. multocida* genomic DNA as a template. The PCR conditions consisted of 5 μl (50 ng/μl) DNA and 1 μl of each primer (50 pM) in a 5 μl 10 × reaction buffer, 5 μl 25 mM $MgCl_2$, 5 μl 10 mM dNTP (each 2.5 mM) mixture and 1 μl 5U Ex Taq™ DNA polymerase (TaKaRa, Japan) in a final volume of 50 μl in a thermal cycler (PTC-100™, MJ Research, USA). PCR was initiated with an incubation step at 94°C for 5 min, followed by 30 cycles of 94°C / 1 min, 55°C / 1 min, and 72°C / 4 min, with a final extension step at 72°C for 7 min. The amplified DNA product was electrophoresed on a 0.8% (w/v) agarose gel. The PCR product was purified using a PCR purification kit (Qiagen, Germany) according to the manufacturer's instructions, and was then cloned into pGEM-T easy, generating pGEM- *toxA*. The construct was transformed into chemically competent *E. coli* JM109 cells, which were then used to propagate the plasmid construct. The transformants were selected on Luria-Bertani (LB) plates containing 100 μg/ml ampicillin, 0.5 mM IPTG and 80 μg/ml X-Gal. The white colonies were selected and a mini-scale isolation of the plasmid DNA was used to prepare the recombinant plasmid for sequencing using the plasmid DNA QIAprep™ Spin Mini Kit (Qiagen, USA). Restriction enzyme analysis using *Eco*RI and DNA sequencing confirmed the presence of *toxA*. The DNA sequencing reaction was performed using an automated DNA sequencer (ABI PRISM® 3100 Genetic Analyzer, Applied Biosystems, USA).

Cloning of kan^r gene

For the selection of knock-out colonies, a kanamycin resistance (*kan*^{r}) gene was used for the transformant-selection. Briefly, the *kan*^{r} gene was amplified by PCR using the pCR® Blunt vector (Invitrogen, USA) as a template with the 5`-primer containing an *Eco*RV restriction site (kan-F: 5'-GCGC GATATC ATGATTG AACAAGATGGATT-3') and 3`-primer containing a *Hind*III restriction site (kan-R: 5'-CGCG AAGCTT TCAGAAGAACTCGTCAAGAA-3'). The PCR conditions were the same as those for the *toxA* gene. PCR was initiated with an incubation step at 94°C for 5 min, followed by 30 cycles of 94°C / 30 s, 55°C / 30 s, and 72°C / 1 min, with a final extension step at 72°C for 7 min. Following cloning procedures were the same with that of *toxA* gene. The PCR product was cloned into pGEM-T easy, generating pGEM-*kan^r*.

Preparation of an insert for homologous recombination

Two constructs (pGEM-*toxA* and pGEM-*kan^r*) were digested with two restriction enzymes, *Eco*RV and *Hind*III, and the *kan^r* gene was inserted into the *Eco*RV + *Hind*III-site of pGEM-*toxA* (2.4 kb fragment of *toxA* gene was exchanged with 0.8 kb *kan^r* gene), generating pGEM-*toxA-kan^r*. A linear DNA fragment (*toxA-kan^r-toxA*: 1.8 kb) was produced by digestion of pGEM-*toxA-kan^r* with *Eco*RI restriction enzyme and used as an insert (100 ng/μl) for homologous recombination. The cloning strategy is represented as a diagram in Fig. 1.

Gene disruption by homologous recombination

Five ml of a fresh overnight *P. multocida* culture was inoculated into 500 ml BHI broth (BBL™, Becton, Dickinson and Company, USA). Cells were grown to an $O.D._{600}$ of approximately 0.5, chilled on ice for 20 min, centrifuged at 4000 × g for 15 min at 4°C. The supernatant was removed and the pellet was concentrated 100-fold and washed three times with ice-cold 10% glycerol and used as a source of

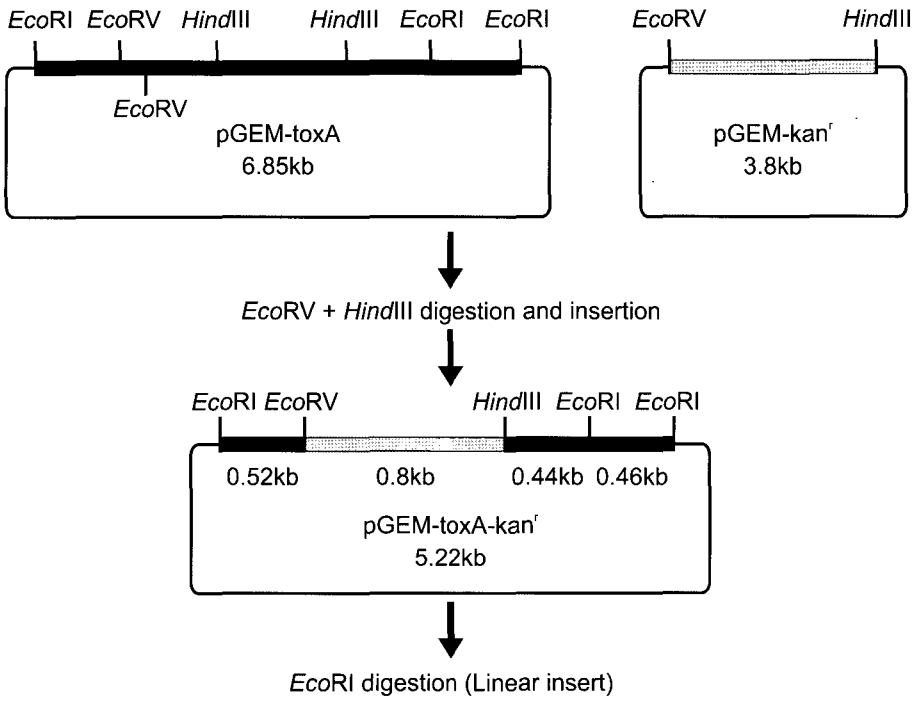

Fig. 1. Diagram of cloning strategy. A restriction fragment (*Eco*RV + *Hind*III) from pGEM-*kan*^{r}, which harboring *kan*^{r} gene was prepared and inserted into the *Eco*RV + *Hind*III-restricted pGEM-*toxA*, generating pGEM-*toxA-kan*^{r}. An insert was prepared by digesting pGEM-*tox*A-*kan*^{r} with *Eco*RI restriction enzyme.

competent cells.

For the induction of λ Red recombinase, competent cells were transformed with pKD46 using a Gene Pulser Xcell™ electroporation system (Bio-Rad, USA) according to the manufacturer's instructions. Transformants carrying a pKD46 insert were grown in 5 ml SOB cultures with ampicillin and L-arabinose (final 10 mM) at 30°C to an $O.D._{600}$ of approximately 0.5, and then made electro-competent by concentrating 100-fold and washing three times with ice-cold 10% glycerol.

Forty μl of electro-competent cells were transformed with 1 μl (100 ng) of insert DNA (*toxA- kan*^{r}*-toxA*) by electroporation according to the manufacturer's instructions. Knock-out mutants were selected on LB plates containing 50 μg/ml of kanamycin. After primary selection, mutants were maintained on medium without an antibiotic. They were colony purified once at 37°C and then tested for ampicillin sensitivity to test for loss of the helper plasmid. If it was not lost, then a few were colony-purified once at 43°C to eliminate the helper plasmid, and similarly tested.

PCR verification

Three PCRs were used to show that all mutants have the correct structure. Genomic DNA from a mutant colony was prepared (50 ng/μl) and used as a template. The expected sizes and targets of three

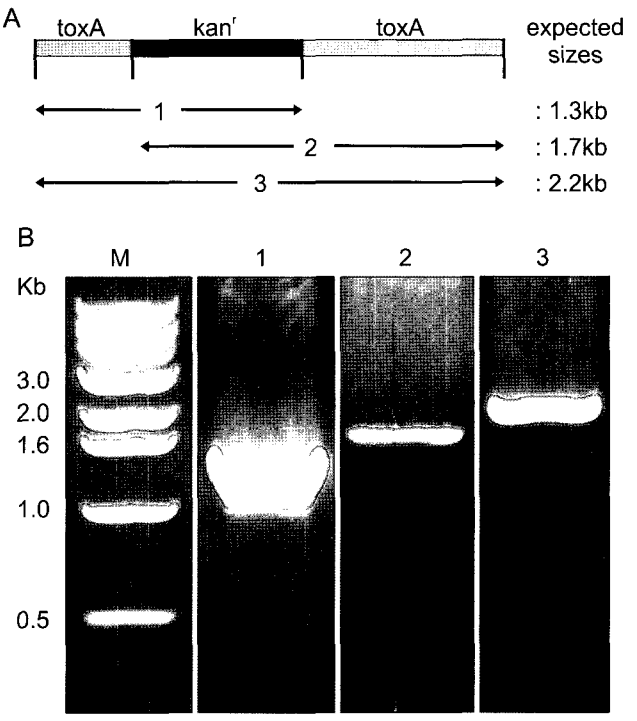

Fig. 2. (A) Diagram of PCR verification for genomic structure. The expected sizes and targets of three PCRs (1-3) were indicated. (B) The result of the PCR verification. One 1.3 kb (PCR 1: lane 1), one 1.7 kb (PCR 2: lane 2), and one 2.2 kb size (PCR 3: lane 3) of PCR products were produced.

Table 1. Experimental design and the results for toxigenicity tests and protection studies. Groups 1-4 were inoculated with live *toxA*(±) *P. multocida*, while groups 5-6 received bacterial culture lysate supernatant. Groups 2-3 were immunized with *toxA*(–) *P. multocida* once more and challenged as indicated

Group	Toxigenicity test (Dosage/ mouse)	Trial number	Challenge with	Survivors
1	*toxA*(+) *P. multocida* (1×10^7 cells)	10	N/P[a]	N/P[a]
2	*toxA*(–) *P. multocida* (1×10^7 cells)	10	*toxA*(+) *P. multocida* (1×10^7 cells)	0/10
3	*toxA*(–) *P. multocida* (1×10^7 cells)	10	*toxA*(+) lysate (500 μl[b])	0/10
4	*toxA*(–) *P. multocida* (1×10^7 cells)	15	N/P[a]	N/P[a]
5	*toxA*(+) lysate (500 μl[b])	10	N/P[a]	N/P[a]
6	*toxA*(–) lysate (500 μl[b])	10	N/P[a]	N/P[a]

a: not performed, b: from 10 ml lysate, which was prepared with 1×10^8 cells

PCRs are represented in Fig. 2A. Primers, *toxA*-F and kan-R were used for PCR 1, kan-F and *toxA*-R for PCR 2, and *toxA*-F and *toxA*-R for PCR 3.

Intraperitoneal inoculation of bacteria, bacterial toxin, and bacterial recovery

To evaluate the toxigenicity of PMT itself, bacterial and toxin challenge studies were undertaken using *P. multocida* wild-type *toxA*(+) and mutant *toxA*(–) and the bacterial culture lysates from them (Table 1).

The bacterial cultures were prepared in BHI broth at a concentration of 1×10^8 cells/ml. For the preparation of bacterial lysate supernatants, 50 ml overnight bacterial cultures were washed twice with 10 ml PBS, sonicated, and the supernatants filtered through a 0.2 μm membrane filter.

Sixty-five 6-week-old ICR mice of both sexes (Charles River, Yokohama, Japan) were divided into 6 groups, as described in Table 1. Group 1, 2, 3 and 4 were inoculated intraperitoneally with freshly prepared 100 μl (1×10^7 cells/mouse, which corresponds to LD100) of the bacterial cultures. The mortality rate (group 1-3) was recorded for 15 days after the inoculation. At 3 days intervals, 3 surviving mice from group 4 were sacrificed randomly, and the peritoneal fluid was prepared by injecting 1 ml PBS into the peritoneum, and 100 μl of aseptically collected peritoneal fluid was inoculated on chocolate agar (with kanamycin) for bacterial recovery. The number of bacteria recovered was determined by culturing serially diluted peritoneal fluid on chocolate agar, and the data were expressed as the mean of the number of CFU (± SD). The isolated colonies were confirmed by *kan*^{r}-specific PCR. Group 5 and 6 were inoculated with 500 μl of bacterial lysate supernatant (corresponds to LD100) and the mortality rate was recorded.

Bacterial and toxin challenge

To examine the protective effect of *toxA*(–) *P. multocida*, 20 mice in groups 2 and 3 (Table 1) were vaccinated with *toxA*(–) *P. multocida* after 15 days observation (no mortality occurred). Fifteen days after vaccination, they were challenged with freshly prepared 100 μl (1×10^7 cells/mouse) of *toxA*(+) *P. multocida* bacterial cultures (group 2) or 500 μl of *toxA*(+) bacterial lysate supernatant (group 3) intraperitoneally to examine the protective effect of *toxA*(–) *P. multocida* vaccination.

Results

Disruption of the toxA gene by homologous recombination

Fig. 3 shows the results of the PCR amplification and restriction study of the whole *toxA* (lane 1, 2) and *kan*^{r} gene (3, 4). The *kan*^{r} gene was inserted into the *Eco*RV + *Hind*III-site of pGEM-*toxA*, which was confirmed by restriction analysis (Fig. 3, lane 5), following digestion of pGEM-*toxA*-*kan*^{r} with *Eco*RI restriction enzyme (Fig. 3, lane 6) generating an 1.8 kb fragment, which was eluted from the gel and used as an insert.

In the three PCRs for the verification of mutated genomic structure, three (1.3 kb, 1.7 kb, and 2.2 kb size) PCR products were produced (Fig. 2B), indicating the correct structure was generated.

Mortality and bacterial recovery

The toxigenicity of PMT in mice was examined using bacterial and toxin challenge studies. None of mice in groups 1 and 5 survived. All mice in group 1 died within 2 days after inoculation and group 5 within 24 h, whereas groups 2, 3, 4, and 6 showed no mortality during the 15 day observation period ($P < 0.01$). The surviving mice from group 4 were sacrificed, and the number of CFU recovered from peritoneal fluid was measured. No bacterial recovery test could be performed in groups 1 and 5.

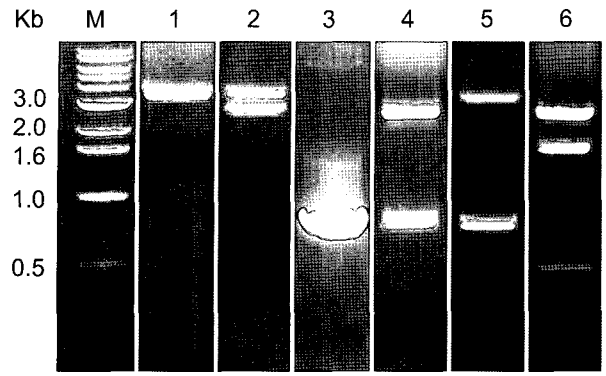

Fig. 3. The *toxA* (lane 1: 3.9 kb) and *kan*^{r} (lane 3: 0.8 kb) genes were amplified from the template DNA of *P. multocida* type D and pCR-blunt vector, respectively. pGEM-*toxA* and pGEM-*kan*^{r} were digested with *Eco*RI restriction enzyme generating two fragments, 3.9 kb (*toxA*: lane 2) and 0.8 kb (*kan*^{r}: lane 4) with 3 kb (pGEM-T easy). The presence of *kan*^{r} gene, which was inserted into pGEM-*toxA*, was confirmed by restriction analysis (lane 5) generating two fragments, 4 kb (0.52 kb of former *toxA* + 3.0 kb of pGEM-T easy + 0.44 kb of latter *toxA*) and 0.8 kb (*kan*^{r}). The insert (1.8 kb) was generated by digesting pGEM-*toxA*-*kan*^{r} with *Eco*RI restriction enzyme (lane 6) and used for homologous recombination. M: 1 kb size marker.

From the first recovery (day 3), 53 cells (SD ±75.6) / 100 μl / mouse of *P. multocida* were recovered. However, from the second recovery (day 6), the number of bacterial colonies was decreased drastically and none of the peritoneal fluids from the surviving mice tested positive for *P. multocida*, indicating that the inoculated bacteria were all cleared. These findings also strongly suggested that the toxigenicity was drastically reduced in *toxA*(–) *P. multocida* when compared to that of the wild-type.

Bacterial and toxin challenge

In the bacterial challenge experiment, which was performed to examine the protective effect against *toxA*(+) *P. multocida* challenge, mice were not protected by immunization with *toxA*(–) *P. multocida*. One died within 3 days, 5 died at 5 days, 2 at 6 days, and the last two at 7 days after bacterial challenge. Similarly, none of the mice survived (100% mortality within 24 h) when challenged with bacterial lysate supernatant (toxin).

Discussion

PMT is an essential virulence factor for PAR. Isolates of either of the common capsule types, A or D, can be toxigenic. The toxin induces turbinate atrophy and poor weight gain in pigs (Lichtensteiger, 1996). PMT is also a potent mitogen, which induces DNA synthesis and cell proliferation in fibroblasts and osteoblastic cells at picomolar concentrations (Rozengurt, 1990). Other virulence factors, such as fimbriae, capsule, outer membrane proteins A and H, and iron transport proteins (Adler, 1999; Davies, 2003) are also associated with virulence and immunity, although their roles are not yet clear.

In this study, none of the mice vaccinated with wild-type *P. multocida* type D (harbouring *toxA*) survived, indicating this bacterium shows very strong toxigenicity in mice, supporting previous observations (Nakai, 1984; Petersen and Foged, 1989; Hoskins, 1997). In contrast, mice inoculated with *toxA*(–) *P. multocida* showed no mortality and the bacteria inoculated intraperitoneally were cleared by the peritoneal immune system. So, the toxigenicity of this bacterium seemed to be derived mainly from PMT, which can also be assumed from the experiment using bacterial culture lysates (toxin). Other virulence factors also could be responsible for the high lethality (100%) shown in this study.

In the following bacterial challenge experiment with *toxA*(+) *P. multocida*, mice immunized with *toxA*(–) *P. multocida* were not protected, and neither were mice challenged with toxin (100% mortality). This indicates that protective immunity against *toxA*(+) *P. multocida* can be acquired only when protection against PMT has been established (To, 2005).

In the primary toxigenicity tests, all mice in group 1 died within 2 days after inoculation, and group 5 within 24 h. However, in the protection studies, mice with bacterial challenge began to die from 3 days (mainly after 5 days) after challenge, while they died within 24 h when challenged with cell culture lysate (toxin). It seems that bacterial clearance could happen in the peritoneum after bacterial administration (before the mass production of PMT within bacteria), with the help of antibodies induced against other factors, not PMT. However, subsequently toxigenic bacteria would begin to propagate and/or produce PMT, which is lethal to mice, as there would be no neutralizing Abs and/or proper protection against this toxin.

Currently, clinical PAR is usually controlled by combined *Bordetella bronchiseptica*/*P. multocida* vaccinations, which consist mainly of toxoid and/or somatic antigens of both bacteria. Using these vaccines, pathogenic lesions, the excretion of bacteria, and the time to market under experimental and field conditions is decreased. Some vaccines also have been shown to protect pigs against the development of lung lesions when administrated properly. Although vaccination has unquestionable beneficial effects, current vaccines are not able to eliminate the bacteria (Haesebrouck, 2004).

Previously, the vaccination of pigs by exposure to an aerosol of a live temperature-sensitive mutant

strain of *P. multocida* did not produce statistically significant changes in serum IgG levels compared with unvaccinated controls, but produced a significant increase in the levels of the IgA and IgG antibodies in the lung lavage fluid (Mülle, 2001). Live *B. bronchiseptica* expressing the C-terminal 685 amino acids region of PMT was used as a vaccine vehicle previously. However, detectable antibodies (IgM, IgA, and IgG) to PMT were not successfully induced (Rajeev, 2003).

Recently, an *aroA* mutant strain of *P. multocida*, administered via the intramuscular route, successfully protected calves against a challenge with the virulent parent strain (Hodgson, 2005). Furthermore, a genetically modified PMT toxin produced by replacing the serine at position 1164 with alanine, and the cysteine at position 1165 with serine lead to a complete loss of toxic effects of PMT without impairment of the ability to induce protective immunity in pigs (To, 2005). These also suggest that the induction of protective immunity (IgG and/or IgA) against pasteurellosis can be achieved when the appropriate immunogens and administration routes are employed. For these reasons, the *toxA*-deleted mutant was developed here could be useful to investigate immunogenic responses against PMT itself by comparing differences in gene profiles between the *toxA*(–) mutant and pathogenic (harbouring *toxA*) *P. multocida* following their administration to pigs.

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