Construction and immunization with double mutant ΔapxIBD Δpnp forms of Actinobacillus pleuropneumoniae serotypes 1 and 5

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ABSTRACT

Actinobacillus pleuropneumoniae (APP) causes a form of porcine pleuropneumonia that leads to significant economic losses in the swine industry worldwide. The apxIBD gene is responsible for the secretion of the ApxI and ApxII toxins and the pnp gene is responsible for the adaptation of bacteria to cold temperature and a virulence factor. The apxIBD and pnp genes were deleted successfully from APP serotype 1 and 5 by transconjugation and sucrose counter-selection. The APP1 ΔapxIBD Δpnp and APP5 ΔapxIBD Δpnp mutants lost hemolytic activity and could not secrete ApxI and ApxII toxins outside the bacteria because both mutants lost the ApxI- and ApxII-secreting proteins by deletion of the apxIBD gene. Besides, the growth of these mutants was defective at low temperatures resulting from the deletion of pnp. The APP1 ΔapxIBD Δpnp and APP5 ΔapxIBD Δpnp mutants lost hemolytic activity and could not secrete ApxI and ApxII toxins outside the bacteria because both mutants lost the ApxI- and ApxII-secreting proteins by deletion of the apxIBD gene. Besides, the growth of these mutants was defective at low temperatures resulting from the deletion of pnp. The APP1 ΔapxIBD Δpnp and APP5 ΔapxIBD Δpnp mutants were significantly attenuated compared with wild-type ones. However, mice vaccinated intraperitoneally with APP5ΔapxIBD Δpnp did not provide any protection when challenged with a 10-times 50% lethal dose of virulent homologous (APP5) and heterologous (APP1) bacterial strains, while mice vaccinated with APP1 ΔapxIBD Δpnp offered 75% protection against a homologous challenge. The ΔapxIBD Δpnp mutants were significantly attenuated and gave different protection rate against homologous virulent wild-type APP challenging.

Introduction

Actinobacillus pleuropneumoniae (APP) causes pleuropneumonia in pigs, with sequelae such as hemorrhagic necrotizing pneumonia, and fibrinous pleuritis. It significantly impairs the swine industry worldwide [1]. To date, 15 serovars of APP have been identified. However, some new proposed ones have been reported based on serological testing and genotypic analysis [2].

APP has multiple virulence factors including outer membrane, capsule, and repeats in toxin (RTX) toxins, proteases, adhesins, transferrin-binding proteins, and lipopolysaccharides [1]. Among these factors, RTX toxins are considered the most important and play predominant
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Conflict of Interest
The authors declare no conflicts of interest.

Author Contributions
Conceptualization: Dao HT, Truong QL, Do VT, Hahn TW; Data curation: Dao HT, Truong QL; Formal analysis: Dao HT, Truong QL, Do VT, Hahn TW; Methodology: Dao HT, Truong QL, Do VT, Hahn TW, Project administration: Hahn TW, Supervision: Hahn TW; Validation: Dao HT, Truong QL, Do VT, Hahn TW, Writing - original draft: Dao HT, Writing - review & editing: Hahn TW, Dao HT.

roles in APP pathogenesis in the host. ApxI is strongly hemolytic and strongly cytotoxic. ApxII is weakly hemolytic and moderately cytotoxic, while ApxIII is not hemolytic but strongly cytotoxic. ApxIV is present in all serovars of APP in vivo, but the functional role regard to haemolytic or cytotoxic activity is still unknown [1]. APP serotype 1 and 5 are the predominant types in Korea [3] and both types are the most virulent serotypes because of the production of both ApxI and ApxII toxins [4,5].

The production and secretion of RTX toxins require at least four functional genes: structural gene A, activator gene C, and secretion factor genes B and D. The inner membrane proteins encoded by apxB and apxD genes are responsible for transferring activated toxins through the cell membrane and secrete outside the cell [6]. In APP, the ApxI and ApxII toxins share the transporter system, encoded by apxIBD genes, to secrete toxins outside the bacterial cell [7]. When the apxIBD genes are inactivated, APP still produces ApxI and ApxII toxins in activated forms, but does not secrete them, so APP cannot affect the host cell by these toxins, leads to attenuation.

Polynucleotide phosphorylase (PNPase), encoded by the pnp gene, is a 3′–5′ exoribonuclease, a component of RNA degradosomes relevant to cold shock induction and mRNA degradation [8-10] that is highly conserved among plants and bacteria [11,12]. Deletion of the pnp gene was found to influence the virulence of Pasteurella multocida [13], Neisseria meningitidis [14], Salmonella enterica and Yersinia sp. [10]. Interestingly, a transposon mutant form of APP, in which the pnp gene was disrupted, was significantly attenuated using a competition challenge experiment. In addition, this mutant form showed significant lower clinical signs, lung lesion scores and protected all pigs against homologous challenge [15].

In order to make highly attenuated APP mutants, we constructed markerless double mutants by deleting the apxIBD and pnp genes in the APP1 and APP5 using allelic replacement. These mutants were characterized to confirm deletion of target genes based on differences in phenotype between wild-type and mutant APP. The virulence and protection efficacy of the double mutants were evaluated in a mouse model.

MATERIALS AND METHODS

Strains, growth conditions, plasmids, and primers
The bacterial strains used in this study are listed in Table 1. Escherichia coli was cultured in Luria-Bertani (LB) broth, supplemented with appropriate antibiotics (100 µg/mL ampicillin; 25 µg/mL chloramphenicol). For culture of E. coli strain WM3064, 1 mM diaminopimelic acid (Merck, Germany) was added. APP strains were cultured in tryptic soy broth (TSB) or brain heart infusion (BHI) agar, supplemented with nicotinamide adenine dinucleotide (NAD; 10 µg/mL; Merck). Chloramphenicol (2.5 µg/mL) was added for culture of APP transconjugants. Sucrose (10% w/v; Duchefa Biochemie b.v, The Netherlands), and horse serum (10% v/v; Thermo Fisher Scientific, New Zealand) were added during the sucrose counter-selection procedure. All bacterial strains were grown at 37°C for 18 h to 24 h.

Plasmid and polymerase chain reaction (PCR) primers used in this study are listed in Table 2. Plasmid pEMOC2 was kindly provided by Dr. Janine T. Bossé (Section of Paediatrics, Department of Medicine, Imperial College London, London, UK).
Table 1. Bacterial strains used in this study

| Strain                  | Relevant characteristics | Source  |
|-------------------------|--------------------------|---------|
| Escherichia coli        |                          |         |
| DH5x                    | Cloning vehicle: supE44 ΔlacU169 (Δφ80 lacZΔM15) hisDR17 recA1 endA1 gyrA96 thi-1 relA1 | Invitrogen |
| WM3064                  | Donor strain for conjugation; thrB1004 pro thi rpsL | hsD5 lacZΔM15 R43-360 (araBAD567 ΔapxA341 __ermA pir(wr)__) |

| Actinobacillus pleuropneumoniae |                         |         |
| APP1                      | Serovar 1, an isolate from infected pig in Korea | This study |
| APP5                      | Serovar 5, an isolate from infected pig in Korea | This study |
| APP1ΔapxIBDΔpnp           | Mutation in apxIBD and pnp gene of APP1 | This study |
| APP5ΔapxIBDΔpnp           | Mutation in apxIBD and pnp gene of APP5 | This study |
| APP1ΔapxIBD               | Mutation in apxIBD | This study |
| APP5ΔapxIBD               | Mutation in apxIBD of APP5 | This study |
| APP1Δpnp                  | Mutation in pnp gene of APP1 | This study |
| APP5Δpnp                  | Mutation in pnp gene of APP5 | This study |

APP. Actinobacillus pleuropneumoniae.

Table 2. Plasmids and primers used in this study

| Plasmids                  | Characteristics | Source |
|---------------------------|-----------------|--------|
| pBluescriptII SK(+)       | E. coli cloning vector carrying an ampicillin resistance determinant | Stratagene |
| pEMOC2                   | Transconjugation vector: ColE1 ori mob | RPM 4 sacB, Amp Cam |
| pEMOC2ΔapxIBD            | pEMOC2 including the truncated apxIBD | This study |
| pEMOC2Δpnp               | pEMOC2 including the truncated pnp | This study |

Primers for constructing APP1ΔapxIBDΔpnp

| Primers for constructing APP1ΔapxIBDΔpnp | Characteristics | Source |
|-----------------------------------------|-----------------|--------|
| P1-F                                    | 5'-ACGCCTGACGACGGTCTTCTAATCCTCCG-3' (SalI site underlined) | Amplified an upstream sequence of apxIB (892 bp) in APP1 |
| P2-R                                    | 5'-ACCGATCCGGAGATCCAGGGGTTCGCAACATCTTTCG-3' (SalI site underlined) | Amplified a downstream sequence of apxID (1,003 bp) in APP1 |
| P3-F                                    | 5'-CGGAATCCTTTACCGCTCCG-3' (NotI site underlined) | Amplified 3,759 bp in parent and 1,389 bp in mutant. These primers were used to confirm the deletion of apxIBD in APP1 |
| P4-R                                    | 5'-GGCACTCTTTACCGCTCCG-3' (BamHI site underlined) | Amplified 3,885 bp in parent and 2,423 bp in mutant. These primers were used to confirm the deletion of pnp in APP1 |
| P5-F                                    | 5'-CATTAATCGAGATGGAGTGTCCGTAATG-3' (BamHI site underlined) | Amplified a downstream sequence of pnp (1,142 bp) in APP1 |
| P6-R                                    | 5'-ATAGCTGATCCGGATCCG-3' (NotI site underlined) | Amplified a downstream sequence of pnp (2,080 bp) in APP1 |
| P7-F                                    | 5'-CCGGACGTCACCATACCTGCTCCG-3' (SalI site underlined) | Amplified 4,308 bp in parent and 2,080 bp in mutant. These primers were used to confirm the deletion of apxIBD in APP1 |
| P8-R                                    | 5'-ATAGCTGATCCGGATCCG-3' (BamHI site underlined) | Amplified a downstream sequence of pnp (1,077 bp) in APP1 |

Primers for constructing APP5ΔapxIBDΔpnp

| Primers for constructing APP5ΔapxIBDΔpnp | Characteristics | Source |
|-----------------------------------------|-----------------|--------|
| P1-F                                    | 5'-ACGCCTGACGACGGTCTTCTAATCCTCCG-3' (SalI site underlined) | Amplified an upstream sequence of apxIB in APP5s (922 bp) |
| P2-R                                    | 5'-CCGTACATCCGGATCCG-3' (SalI site underlined) | Amplified a downstream sequence of apxID in APP5s (922 bp) |
| P3-F                                    | 5'-ACCGTACACGCTCCG-3' (BamHI site underlined) | Amplified 4,308 bp in parent and 2,080 bp in mutant. These primers were used to confirm the deletion of apxIBD in APP5 |
| P4-R                                    | 5'-ATAGCTGATCCGGATCCG-3' (SalI site underlined) | Amplified a downstream sequence of pnp in APP5s (1,171 bp) |
| P5-F                                    | 5'-CCGGACGTCACCATACCTGCTCCG-3' (SalI site underlined) | Amplified 4,308 bp in parent and 2,080 bp in mutant. These primers were used to confirm the deletion of apxIBD in APP5 |
| P6-R                                    | 5'-ATAGCTGATCCGGATCCG-3' (BamHI site underlined) | Amplified a downstream sequence of pnp in APP5s (1,171 bp) |
| P7-F                                    | 5'-CCGGACGTCACCATACCTGCTCCG-3' (SalI site underlined) | Amplified 4,308 bp in parent and 2,080 bp in mutant. These primers were used to confirm the deletion of apxIBD in APP5 |
| P8-R                                    | 5'-ATAGCTGATCCGGATCCG-3' (BamHI site underlined) | Amplified a downstream sequence of pnp in APP5s (1,171 bp) |
| P9-F                                    | 5'-ACCGTACACGCTCCG-3' (BamHI site underlined) | Amplified 4,308 bp in parent and 2,080 bp in mutant. These primers were used to confirm the deletion of apxIBD in APP5 |
| P10-R                                   | 5'-ATAGCTGATCCGGATCCG-3' (BamHI site underlined) | Amplified a downstream sequence of pnp in APP5s (1,171 bp) |
| P11-F                                   | 5'-CCGGACGTCACCATACCTGCTCCG-3' (SalI site underlined) | Amplified 4,308 bp in parent and 2,080 bp in mutant. These primers were used to confirm the deletion of apxIBD in APP5 |
| P12-R                                   | 5'-ATAGCTGATCCGGATCCG-3' (BamHI site underlined) | Amplified a downstream sequence of pnp in APP5s (1,171 bp) |
Construction of double mutants ΔapxIBDΔpnp in APP1 and APP5

The conjugative plasmids pEMOC2ΔapxIBD and pEMOC2Δpnp containing upstream and downstream fragments of target genes were constructed. These plasmids were introduced into APP using allelic replacement [16] with modifications. Briefly, overnight cultures of E. coli WM3064 containing the transconjugative plasmids pEMOC2ΔapxIBD (or pEMOC2Δpnp) and APP1 were mixed together at a proportion of 1:10 and dropped onto a Whatman cellulose nitrate membrane filter (GE Healthcare, Germany) on BHI agar containing 10 µg/mL NAD, 1 mM diaminopimelic acid, and 10 mM MgSO4. After 6 h incubation, bacteria were washed off with TMN buffer (1 mM Tris-HCl pH 7.2, 10 mM MgSO4, and 100 mM NaCl) and plated on BHI agar supplemented with 2.5 µg/mL chloramphenicol. Chloramphenicol-resistant transconjugants were then incubated in salt-free LB broth for 6 h and plated onto LB-HSN agar (10% sucrose, 10% horse serum, and 10 µg/mL NAD). Sucrose-resistant and chloramphenicol-sensitive colonies were selected and screened by PCR using primer pairs P5–P6 for the apxIBD and P11–P12 for the pnp deletion gene. Double mutants in APP5 were constructed in the same approach.

Characterization of ΔapxIBDΔpnp double mutants

Growth curves

To obtain growth curve, wild-type APP1, APP5, and their double mutants were cultured overnight into TSB supplemented with NAD. After sub-inoculation into a fresh medium at a starting optical density at 600 nm (OD600) of 0.1, the OD600 of the cultures was measured at 1 h intervals [17].

Hemolytic activity analysis

The apxIBD deletion mutants were tested for hemolytic activity previously [17]. Briefly, the wild-type and double mutants were cultured overnight and then 10 µL aliquots of each culture were dropped onto blood agar plate containing 10 µg/mL NAD. The hemolytic activity of wild-type and the mutants was assessed based on clear zones around the colonies after 24 h incubation at 37°C.

Cold shock adaptation

The pnp deletion mutants were characterized by cold shock adaptation ability testing [11,18]. The wild-type and the mutants were inoculated onto BHI agar containing 10 µg/mL NAD, incubated at 19°C for 72 h, and the growth of bacterial strains was recorded. Cold shock adaptation was assessed by the appearance of APP colonies.

Expression of RTX toxins

Apx toxins from the wild-type APP and the mutants were analyzed in a previous report [19]. Briefly, the wild-type APP and the mutants were inoculated into fresh Columbia broth media containing 25 mM CaCl2 and incubated for 6 h. After centrifugation at 500 × g for 10 min at 4°C, the supernatants were collected, mixed with 55% saturated ammonium sulfate, and incubated overnight at 4°C. The precipitated toxins were harvested by centrifugation as above, dissolved in phosphate-buffered saline (PBS) and analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to evaluate the expressions of Apx toxins in the wild-type APP and mutants.

Stability of double mutants

To test the stability of the mutants, they were passaged ten times in TSB containing 10 µg/mL NAD [20], and the target genes were analyzed by PCR at each passage using primer pairs P5–P6 and P11–P12, as shown in Table 2.
Virulence in mice
To investigate the virulence of the mutants, the 50% lethal dose (LD₅₀) was measured using 6-week old female BALB/c mice. Overnight cultures of the wild-type APP and mutants were inoculated into fresh TSB supplemented with 10 µg/mL NAD at a starting OD₆₀₀ of 0.1 and incubated at 37°C with shaking until the OD₆₀₀ reached 0.8. Different doses ranging from 1 × 10⁶ to 5 × 10⁹ colony-forming unit (CFU) of wild-type APP and the mutants in 200 µL TSB were prepared and injected in mice via the intraperitoneal route. Survival of the mice was recorded 5 days after inoculation. The LD₅₀ values of the wild-type APP and mutants were calculated by Reed and Muench’s method.

Protective efficacy of the mutants in mice
Protection efficacy test was carried out as previously described [21-23]. Seventy-two 6-week-old female BALB/c mice used in this experiment were divided randomly into three equal groups: an APP1ΔapxIBDΔpnp-vaccinated group, an APP5ΔapxIBDΔpnp-vaccinated group, and a negative control group (injected with TSB). An injection dose of 1 × 10⁷ CFU of APP1ΔapxIBDΔpnp or APP5ΔapxIBDΔpnp was injected intraperitoneally into the mice twice at 2-week intervals.

On day 28 after the first immunization, the mice in each group were further divided into 2 subgroups for challenge and injected intraperitoneally with 10-fold doses of the LD₅₀ of virulent APP1, or APP5. Mouse survival was observed and recorded daily for up to 10 days.

Animal experimentation approval
All animal experiments were conducted with the approval of our Institutional Animal Care and Use Committee (approval number: KW-161010-3) and performed at the Center for Animal Experiments, Kangwon National University, Korea.

RESULTS

Construction of ΔapxIBDΔpnp mutants in APP1 and APP5
The apxIBD and pnp genes were deleted from the APP1 genome using 2 sequential homologous recombinant crossovers, as described in Section 2.2. The original target genes from the wild-type APP were exchanged allelically with unmarked, truncated apxIBD and pnp genes. These deletions were confirmed by PCR using primer pairs P5–P6 and P11–P12, respectively. In APP1ΔapxIBDΔpnp, the amplified apxIBD gene product was 1,389 bp, whereas the product from wild-type APP was 3,759 bp, indicating that a part of the apxIBD gene was deleted from the APP1 genome. The pnp gene was truncated in APP1ΔapxIBDΔpnp, which produced a 2,423 bp product compared with the 3,885 bp product in wild-type APP1 (Fig. 1A). APP5ΔapxIBDΔpnp was constructed in the same way. The apxIBD and pnp genes in APP5 were deleted successfully, as observed by the lack of 2,228 and 1,435 bp, respectively (Fig. 1B).

Characterization of the ΔapxIBDΔpnp mutants in APP1 and APP5
Hemolytic activity was analyzed for APP1ΔapxIBDΔpnp and APP5ΔapxIBDΔpnp mutants. Both mutants showed no hemolytic activity, while the wild-type APP showed significant hemolysis because of the secretion of active ApxI and ApxII toxins, as observed by obvious clear zones surrounding bacteria on the blood agar (Fig. 2A and B). This result indicated that hemolytic activity was abolished by deletion of the apxIBD gene and the mutants were unable to secrete Apx toxins outside the bacterial cell membrane, so they did not lyse blood cells in the medium.
When the pnp mutants and wild-type APP were inoculated on BHI agar containing NAD and incubated at 19°C for up to 72 h, the wild-type APP grew well while the mutant did not (Fig. 2C and D). This result indicated that deletion of the pnp gene of APP could affect its cold
shock adaptive ability. However, the growth curves of the mutants were similar to those of the wild-type APP at 37°C (Fig. 3). This result demonstrated that deletions of the \textit{apxIBD} and \textit{pnp} genes did not affect the growth of APP at 37°C.

Both ApxI and ApxII contain glycine-rich nanopeptides that have a strong ability to bind \(\text{Ca}^{2+}\) cations [24]. Therefore, these toxins could be secreted in the supernatant culture, could bind \(\text{Ca}^{2+}\) in the medium, and then be precipitated with ammonium sulfate. These precipitated toxins were then collected, resuspended in PBS, and indicated by a band with a molecular weight of up to 110 kDa in SDS-PAGE gels (Fig. 4). In contrast, these toxins were not observed in supernatant and precipitated cultures of the mutants. Thus, these mutants lost the ability to secrete active toxins outside the bacterial membrane by using the ApxIBD secretion system.

To access the stability of mutants, genomic DNA was extracted after 10 passages and used to amplify the \textit{apxIBD} and \textit{pnp} genes by PCR using primer pairs P5–P6 and P11–P12, respectively. PCR fragments of expected sizes for the deleted \textit{apxIBD} and \textit{pnp} genes were amplified using APP1\(\Delta\text{apxIBD}\Delta\text{pnp}\) and APP5\(\Delta\text{apxIBD}\Delta\text{pnp}\) genomes (Fig. 5). The amplified target genes in mutants were approximately 2.2–2.4 kb shorter for the \textit{apxIBD} gene and 1.4 kb shorter for \textit{pnp} gene when compared with those of wild-type APP, implying the stability of the deleted genes in these mutants.

**Virulence in mice**

The calculated LD\(_{50}\) values of APP1\(\Delta\text{apxIBD}\Delta\text{pnp}\) and wild-type APP1 were 1.5 \(\times\) 10\(^6\) CFU and 1.6 \(\times\) 10\(^6\) CFU, respectively. Compared with the wild-type, virulence of the mutant was attenuated by 93-fold (Table 3), showing that mutants with deletions of the \textit{apxIBD} and \textit{pnp} genes lowered virulence significantly. The LD\(_{50}\) of APP5\(\Delta\text{apxIBD}\Delta\text{pnp}\) (3.1 \(\times\) 10\(^6\) CFU) was increased by 48-fold compared with that of the wild-type (6.5 \(\times\) 10\(^6\) CFU) (Table 3). As with APP1\(\Delta\text{apxIBD}\Delta\text{pnp}\), the virulence of APP5 deleted with the \textit{apxIBD} and \textit{pnp} genes was significantly attenuated when evaluated in our mouse model. In contrast to the markedly decreased virulence in \(\Delta\text{apxIBD}\Delta\text{pnp}\) mutants, the virulence of APP1\(\Delta\text{pnp}\) and APP5\(\Delta\text{pnp}\) were only slightly decreased by 3-fold and 2-fold, respectively (Table 3). It means the \textit{pnp} mutants in the APP1 and APP5 were less attenuated and that deletion of the \textit{pnp} gene did not have much effect on the virulence of APP.
Protection efficacy of the mutants in mice

To evaluate the protective effect of mutant strains, mice were immunized intraperitoneally twice with $1 \times 10^7$ CFU of mutant (or medium as a negative control) and challenged at 2 weeks after a booster dose with homologous or heterologous virulent strains. The APP1ΔapxIBDΔpnp mutant provided 75% protection in mice when challenged homologously with 10-fold of the LD$_{50}$ of wild-type APP1. There was no cross-protection when challenged with the wild-type APP5. The APP5ΔapxIBDΔpnp mutant did not protect the mice against homologous and heterologous challenge (Table 4).

Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of Apx toxins in wild-type and the mutants of APP1 (A) and APP5 (B). Lane M, Xpert Prestained Protein marker; lane 1, culture supernatant of the wild-type APP before precipitating; lane 2, culture supernatant of the mutants before precipitating; lane 3, RTX toxins of wild-type after precipitating with ammonium sulfate; lane 4, RTX toxins of the mutants after precipitating with ammonium sulfate. APP, Actinobacillus pleuropneumoniae; RTX, repeats in toxin.

Fig. 5. Evaluation of the stability deletion gene in the genome of APP1ΔapxIBDΔpnp and APP5ΔapxIBDΔpnp over 10 passages by PCR using primer pairs P5–P6 and P11–P12 for amplifying apxIBD and pnp genes, respectively. Amplified apxIBD gene (A) and pnp gene (B) from APP1ΔapxIBDΔpnp. Amplified apxIBD gene (C) and pnp gene (D) from APP5ΔapxIBDΔpnp. Lane M, DNA Ladder 1kb; lane 1, negative control; lane 2, target gene amplified from genomic DNA of wild-type APP, lanes 3–12, deletion gene amplified from genome DNA of mutant through 10 continuous passages. APP, Actinobacillus pleuropneumoniae; PCR, polymerase chain reaction.
DISCUSSION

Vaccination is one of the most effective ways to prevent porcine pleuropneumonia, and live attenuated vaccines are considered to offer the best cross-serotype protection [25]. Therefore, many mutants that mutated in virulence factors, such as RTX toxins, metabolic genes [26], stress response genes [27], and transporters [28], have been constructed. Among these factors, RTX toxins are considered as predominant virulence factors [6]. Thus, many studies on mutations in genes encoding RTX toxins, mainly in structural genes [20,22,29] or their activator genes [17,21,22,29], have been reported. In contrast, there are very few reports for deletions in the \( \text{apxIB} \) and \( \text{apxD} \) genes and evaluation for candidate living attenuated vaccines.

We expect that the mutant with deletions of the \( \text{apxIB} \) and \( \text{apxD} \) genes cannot secrete ApxI or ApxII toxins outside the bacteria, and cannot cause hemolysis that affects the lung tissues, and the mutants would become attenuated. Furthermore, ApxI and ApxII produced by APP1 and APP5 are considered as strong cytotoxic that cause lethal effects on alveolar macrophages and neutrophils [30]. The \( \text{apxIBD} \) mutants would also decrease the cytotoxicity to macrophages and neutrophils; therefore, it becomes easier to be captured and killed by these phagocytes. To our best knowledge, this is the first report about the mutation of the \( \text{apxIBD} \) gene in the APP1 and APP5, with an evaluation of the virulence and protective efficacy in mice.

The \( \text{apxIBD} \) gene was deleted successfully in APP1 and APP5, as shown in Fig. 1A and B, respectively. This mutation caused the loss of hemolysis activity (Fig. 2A and B), the loss of ApxI and ApxII toxins in supernatant culture (Fig. 4), and significantly reduced virulence by 39- and 21-fold compared with the virulence of the wild-type APP1 and APP5, respectively (Table 3), while the virulence of APP2\( \Delta \text{apxIBD} \) decreased only about 1.7-fold compared with that of the wild-type APP2 (Table 3).

### Table 3. Virulence of the wild-type and mutant of APP in mice*

| Strain tested | \( \text{LD}_{50} \) (CFU) | Fold attenuation† |
|---------------|-----------------|------------------|
| APP1 wild-type | \( 1.6 \times 10^6 \) | 1                |
| APP1\( \Delta \text{apxIBD} \) | \( 6.3 \times 10^4 \) | 39               |
| APP1\( \Delta \text{pnp} \) | \( 4.4 \times 10^4 \) | 3                |
| APP1\( \Delta \text{apxIBD}\Delta \text{pnp} \) | \( 1.5 \times 10^4 \) | 93               |
| APP5 wild-type | \( 6.5 \times 10^6 \) | 1                |
| APP5\( \Delta \text{apxIBD} \) | \( 1.4 \times 10^4 \) | 21               |
| APP5\( \Delta \text{pnp} \) | \( 1.6 \times 10^4 \) | 2                |
| APP5\( \Delta \text{apxIBD}\Delta \text{pnp} \) | \( 3.1 \times 10^4 \) | 48               |

\( \text{APP} \), \text{Actinobacillus pleuropneumoniae}; \( \text{LD}_{50} \), 50% lethal dose; CFU, colony-forming unit.

*Groups of five mice were injected intraperitoneally with 200 mL of bacterial suspension containing various doses of \( \text{APP} \). The numbers of surviving mice were recorded 10 days after inoculation. †\( \text{LD}_{50} \) was calculated by the Reed-Muench method; fold attenuation was normalized to the wild-type \( \text{APP} \).

### Table 4. Protection of mice immunized with APP1 and APP5 mutants challenged with homologous and heterologous serotypes of APP*

| Group | Injection dose (CFU) | Challenge to \( \text{LD}_{50} \) | Number of survival/number of tested (%) |
|-------|---------------------|------------------|----------------------------------------|
| APP1\( \Delta \text{apxIBD}\Delta \text{pnp} \) | \( 1 \times 10^7 \) | APP1 \( 6/8 \) (75%) | APP5 \( 0/8 \) (0%) |
| APP5\( \Delta \text{apxIBD}\Delta \text{pnp} \) | \( 1 \times 10^7 \) | APP1 \( 0/8 \) (0%) | APP5 \( 0/8 \) (0%) |
| Control | TSB | APP1 \( 0/8 \) (0%) | APP5 \( 0/8 \) (0%) |

\( \text{APP} \), \text{Actinobacillus pleuropneumoniae}; CFU, colony-forming unit; \( \text{LD}_{50} \), 50% lethal dose.

*Mice were immunized twice intraperitoneally with the mutant or culture media (controls) and challenged at 2 weeks after vaccination with homologous or heterologous virulent strains. Surviving mice were recorded at 10 days after challenge.
with the parental strain [31]. Thus, our mutants were highly attenuated, and these results confirmed the important roles of the ApxI and ApxII toxins in the virulence of wild-type APP1 and APP5.

The pnp gene is involved in regulating bacterial sRNA and is necessary for the growth of *E. coli* [11,18] and *Yersinia* sp. [10]. In this study, the *pnp* gene controlled the growth of APP1 (Fig. 2C) and APP5 (Fig. 2D) at the low temperatures as described for other bacteria. To our knowledge, this is the first study characterizing the role of *pnp* gene in the growth of APP at the cold temperatures. In addition, the *pnp* gene also takes part in the virulence of some related organisms, such as *P. multocida* [13] and *Neisseria meningitidis* [14]. Upon deletion of the *pnp* gene, the *P. multocida* mutant became avirulent in a mouse model [13]. An APP *pnp* insertion transposon mutant confirmed the *pnp* gene as a virulent factor by calculating *in vitro* and *in vivo* competitive indexes. This mutant was at least 100-fold attenuated and caused no mortality and only mild clinical signs in pigs immunized with $1.0 \times 10^{10}$ CFU via the intranasal route and protected pigs against challenge [15]. However, in the present study, APP1Δ*pnp* and APP5Δ*pnp* were only attenuated 3-fold and 2-fold compared with wild-type APP1 and APP5, respectively, in our mouse model (Table 3).

It is still controversial about using mice or piglets as a model to evaluate the pathogenicity of APP in animal experiments. However, mice with lots of advantages compared with pigs, for example, cheaper and easier to handling [32], were being used largely in recent reports to study virulence [33,34], protection effect of a virulence factor. Besides, the mouse model is very helpful and acts as the first step to screen whether a candidate strain is suitable to be continuing to evaluate in the natural host or not [35]. Therefore, in this study, we used a mouse model as an alternative animal to evaluate the virulence and protection efficacy of APP mutants instead of pigs. And it may explain the difference in attenuation rate of *pnp* mutant among this study and previous reports. The fact that the injection route and the method to make the mutation in *pnp* gene also may contribute to this difference. Further experiments on the natural host should be taken with *pnp* mutant to carry out exactly the role of *pnp* gene in virulence of APP.

In this study, we constructed *apxIBD* and *pnp* double-deletion genes in APP1 and APP5 and expected that these mutants would be more attenuated than single mutants. As shown in Table 3, APP1Δ*apxIBDΔpnp* and APP5Δ*apxIBDΔpnp* were attenuated 93-fold and 48-fold, respectively. The LD$_{50}$ values of $1.5 \times 10^6$ CFU for APP1Δ*apxIBDΔpnp* and $3.1 \times 10^6$ CFU for APP5Δ*apxIBDΔpnp* were similar to the LD$_{50}$ of APP7Δ*apxICΔapxIVA* ($1.5 \times 10^6$ CFU) [22] and the LD$_{50}$ of APP1Δ*apxICΔapxIVA* ($3.1 \times 10^6$ CFU) [17]. However, these mutants could not protect mice when they were challenged with ten times the LD$_{50}$ of heterologous virulent strains. APP5Δ*apxIBDΔpnp* did not give any protection in mice when challenged either with 10-fold LD$_{50}$ or with the actual LD$_{50}$ (data not shown). This result can be explained by the loss of secretion of ApxI and ApxII toxins in APP5, which are highly immunogenic in APP5. In a previous study, a chemical mutagenesis-induced mutant in APP5, which lost hemolytic activity and corresponding band in SDS–PAGE, did not protect mice or pigs against challenge [36]. Interestingly, APP1Δ*apxIBDΔpnp* in this study with the same construction as APP5Δ*apxIBDΔpnp*, could provide 75% protection in mice against a challenge with a 10-fold LD$_{50}$ of virulent wild-type APP1. This result may be explained by the difference in pathogenicity between APP1 and APP5. In addition to RTX toxins, there are other factors involved in the protection of APP1 which are only present in APP1 and absent in APP5. Further studies should be conducted for the exact pathogenicity mechanism among serotypes.
In conclusion, we constructed and characterized ΔapxIBDΔpnp double-unmarked deletion genes from APP1 and APP5. APP1ΔapxIBDΔpnp and APP5ΔapxIBDΔpnp had significantly lower virulence, but these 2 mutants had different protection efficacy in mice against challenge with virulent homologous wild-type APP even if the same target genes were deleted.

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