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Construction and immunization with double mutant Δ*apxIBD* Δ*pnp* forms of *Actinobacillus pleuropneumoniae* serotypes 1 and 5

Hoai Thu Dao[1,†] **Quang Lam Truong**[1,2,†] **Van Tan Do**[1] **Tae-Wook Hahn**[1,*]

[1]College of Veterinary Medicine and Institute of Veterinary Science, Kangwon National University, Chuncheon 24341, Korea
[2]Key Laboratory of Veterinary Medicine, Faculty of Veterinary Medicine, Vietnam National University of Agriculture, Hanoi 100000, Vietnam

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***Corresponding author:**
Tae-Wook Hahn
Department of Veterinary Medicine, College of Veterinary Medicine, Kangwon National University, 1 Kangwondaehak-gil, Chuncheon 24341, Korea.
E-mail: twhahn@kangwon.ac.kr

†These authors distributed equally in this study.

ORCID iDs
Hoai Thu Dao
https://orcid.org/0000-0002-2797-9955
Quang Lam Truong
https://orcid.org/0000-0002-9403-2614
Van Tan Do
https://orcid.org/0000-0003-4512-7622
Tae-Wook Hahn
https://orcid.org/0000-0002-3919-1153

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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 APP1Δ*apxIBD*Δ*pnp* and APP5Δ*apxIBD*Δ*pnp* 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 APP1Δ*apxIBD*Δ*pnp* and APP5Δ*apxIBD*Δ*pnp* mutants were significantly attenuated compared with wild-type ones. However, mice vaccinated intraperitoneally with APP5Δ*apxIBD*Δ*pnp* 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 APP1Δ*apxIBD*Δ*pnp* offered 75% protection against a homologous challenge. The Δ*apxIBD*Δ*pnp* mutants were significantly attenuated and gave different protection rate against homologous virulent wild-type APP challenging.

Keywords: *Actinobacillus pleuropneumoniae*; deletion mutation; *apxIBD* gene; *pnp* gene; virulence

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.

Plasmids 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		
DH5α	Cloning vehicle: *supE44 ΔlacU169 (ϕ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Invitrogen
WM3064	Donor strain for conjugation; *thrB1004 pro thi rpsL hsdS lacZΔM15 RP4-360_(araBAD)567_dapA1341 ::_erm pir(wt)_*	[37]
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
APP1Δ*apxIBD*Δ*pnp*	Mutation in *apxIBD* and *pnp* gene of APP1	This study
APP5Δ*apxIBD*Δ*pnp*	Mutation in *apxIBD* and *pnp* gene of APP5	This study
APP1Δ*apxIBD*	Mutation in *apxIBD* of APP1	This study
APP5Δ*apxIBD*	Mutation in *apxIBD* of APP5	This study
APP1Δ*pnp*	Mutation in *pnp* gene of APP1	This study
APP5Δ*pnp*	Mutation in *pnp* gene of APP5	This study

APP, *Actinobacillus pleuropneumoniae*.

Table 2. Plasmid and primers used in this study

Plasmids, primers	Characteristics	Source
Plasmids		
pBluescriptII SK(+)	*E. coli* cloning vector carrying an ampicillin resistance determinant	Stratagene
pEMOC2	Transconjugation vector: ColE1 *ori mob* RP4 *sacB*, Amp^rCam^r	[38]
pEMOC2Δ*apxIBD*	pEMOC2 including the truncated *apxIBD*	This study
pEMOC2Δ*pnp*	pEMOC2 including the truncated *pnp*	This study
Primers for constructing APP1Δ*apxIBD*Δ*pnp*		
P1-F	5′-ACGCGTCGACCGTTCTCTTAAACTCTCCG-3′ (*Sal*I site underlined)	Amplified an upstream sequence of *apxIB* (892 bp) in APP1
P2-R	5′-CGGGATCCCGCTATTTGATCACCGAGC-3′ (*Bam*HI underlined)	
P3-F	5′-CGGGATCCCGAGGATTATGCGTTTCCAA-3′ (*Bam*HI site underlined)	Amplified a downstream sequence of *apxID* (1,003 bp) in APP1
P4-R	5′-ATGCGCGGCCGCGACGGTGGTGTAGGCAATG-3′ (*Not*I underlined)	
P5-F	5′-GCCAACTCATTTACCGCTT-3′	Amplified 3,759 bp in parent and 1,389 bp in mutant. These primers were used to confirm the deletion of *apxIBD* in APP1
P6-R	5′-GGTAACGGAAACGACCGAT-3′	
P7-F	5′-ATACGTCGACGCCATAACGCTCGGTACG-3′ (*Sal*I site underlined)	Amplified an upstream sequence of *pnp* (1,052 bp) in APP1
P8-R	5′-CATTAATCGGGATCCCGTAAAGGTGGTGCGGTAAT-3′ (*Bam*HI site underlined)	
P9-F	5′-CGGGATCCCGACCTTCACGTTTGAAGA-3′ (*Bam*HI site underlined)	Amplified a downstream sequence of *pnp* (1,142 bp) in APP1
P10-R	5′-ATGCGCGGCCGCACATCACGCTTGCTTCGG-3′ (*Not*I site underlined)	
P11-F	5′-CATTTGTGCCCCAGAACTCA-3′	Amplified 3,885 bp in parent and 2,423 bp in mutant. These primers were used to confirm the deletion of *pnp* in APP1
P12-R	5′-ATTGAGCCTTTGCCTTGCTC-3′	
Primers for constructing APP5Δ*apxIBD*Δ*pnp*		
P1-F	5′-ACGCGTCGACACTTTGGTTGAAAGGCTAT-3′ (*Sal*I site underlined)	Amplified an upstream sequence of *apxIB* in APP5 (922 bp)
P2-R	5′-CGTTACCGGGATCCCGGTACGGTTCAGCAACTT-3′ (*Bam*HI underlined)	
P3-F	5′-ACCGTACCGGGATCCCGGTAACGAGCCTAAAAT-3′ (*Bam*HI site underlined)	Amplified a downstream sequence of *apxID* in APP5 (922 bp)
P4-R	5′-ATGCGCGGCCGCTCGGGAAGAAGACTACGG-3′ (*Not*I site underlined)	
P5-F	5′-GCCTGCCATCACAGGTAA-3′	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′-CGGTCCATTAGCTTACAGC-3′	
P7-F	5′-ACGCGTCGACGCCATAACGCTCGGTACG-3′ (*Sal*I site underlined)	Amplified an upstream sequence of *pnp* in APP5 (1,077 bp)
P8-R	5′-CATTAATCGGGATCCCGTAAAGGTGGTGCGGTAAT-3′ (*Bam*HI underlined)	
P9-F	5′-CACCTTTACGGGATCCCGGATTAATGTTTCGCCTTC-3′ (*Bam*HI site underlined)	Amplified a downstream sequence of *pnp* in APP5 (1,171 bp)
P10-R	5′-ATGCGCGGCCGCCGTTGATATTGTGCGGCG-3′ (*Not*I site underlined)	
P11-F	5′-CGTTTCGGCACGCTTAAT-3′	Amplified 3,828 bp in parent and 2,393 bp in mutant. These primers were used to confirm the deletion of *pnp* in APP5
P12-R	5′-GGCAATATCGGCTTTAGGG-3′	

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Construction of double mutants Δ*apxIBD*Δ*pnp* in APP1 and APP5

The conjugative plasmids pEMOC2Δ*apxIBD* and pEMOC2Δ*pnp* 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 pEMOC2Δ*apxIBD* (or pEMOC2Δ*pnp*) 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 $MgSO_4$. After 6 h incubation, bacteria were washed off with TMN buffer (1 mM Tris-HCl pH 7.2, 10 mM $MgSO_4$, 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 Δ*apxIBD*Δ*pnp* 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 (OD_{600}) of 0.1, the OD_{600} 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 $CaCl_2$ 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**.

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Virulence in mice

To investigate the virulence of the mutants, the 50% lethal dose (LD_{50}) 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 OD_{600} of 0.1 and incubated at 37°C with shaking until the OD_{600} reached 0.8. Different doses ranging from 1×10^6 to 5×10^9 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 LD_{50} 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 previous described [21-23]. Seventy-two 6-week-old female BALB/c mice used in this experiment were divided randomly into three equal groups: an APP1Δ*apxIBD*Δ*pnp*-vaccinated group, an APP5Δ*apxIBD*Δ*pnp*-vaccinated group, and a negative control group (injected with TSB). An injection dose of 1×10^7 CFU of APP1Δ*apxIBD*Δ*pnp* or APP5Δ*apxIBD*Δ*pnp* 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 LD_{50} 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 APP1Δ*apxIBD*Δ*pnp*, 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 APP1Δ*apxIBD*Δ*pnp*, which produced a 2,423 bp product compared with the 3,885 bp product in wild-type APP1 (**Fig. 1A**). APP5Δ*apxIBD*Δ*pnp* 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 Δ*apxIBD*Δ*pnp* mutants in APP1 and APP5

Hemolytic activity was analyzed for APP1Δ*apxIBD*Δ*pnp* and APP5Δ*apxIBD*Δ*pnp* 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.

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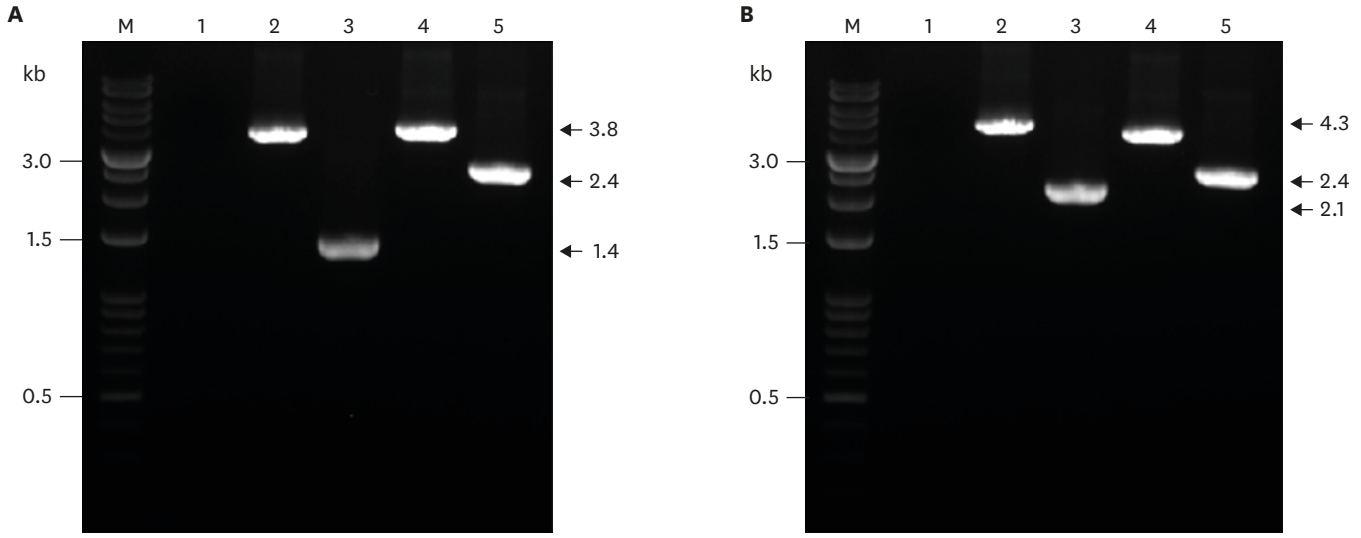

Fig. 1. PCR analysis of the wild-type and *apxIBD*, *pnp*-deleted mutant of APP1 (A) and APP5 (B) using primer pairs P5–P6 and P11–P12. Lane M, DNA molecular weight ladder 1 kb; lane 1, negative control; lane 2, *apxIBD* gene amplified from genomic DNA of wild-type APP; lane 3, *apxIBD* gene amplified from genomic DNA of the Δ*apxIBD*Δ*pnp* mutant; lane 4, *pnp* gene amplified from genomic DNA of the wild-type APP; lane 5, *pnp* gene amplified from genomic DNA of Δ*apxIBD*Δ*pnp* mutant. PCR, polymerase chain reaction; APP, *Actinobacillus pleuropneumoniae*.

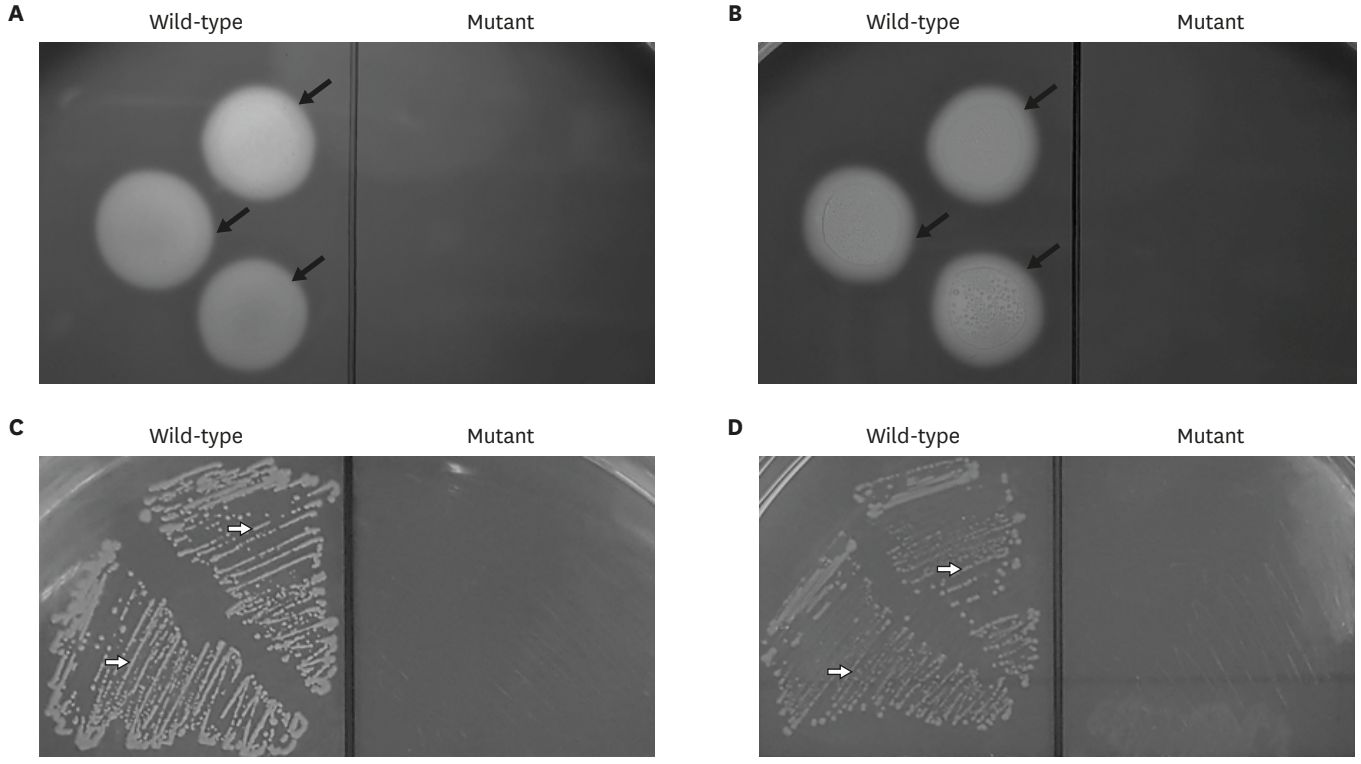

Fig. 2. Phenotypes of the APP mutants. Hemolytic activity of wild-type and mutants of APP1 (A) and APP5 (B). The black arrow indicates the clear zone caused by the hemolytic activity surrounding bacteria on blood agar. Cold shock adaptive ability of wild-type and mutant APP1 (C) and APP5 (D). Open arrow indicates colony growth on BHI agar containing a final amount of 10 μg/mL NAD. APP, *Actinobacillus pleuropneumoniae*; BHI, brain heart infusion; NAD, nicotinamide adenine dinucleotide.

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

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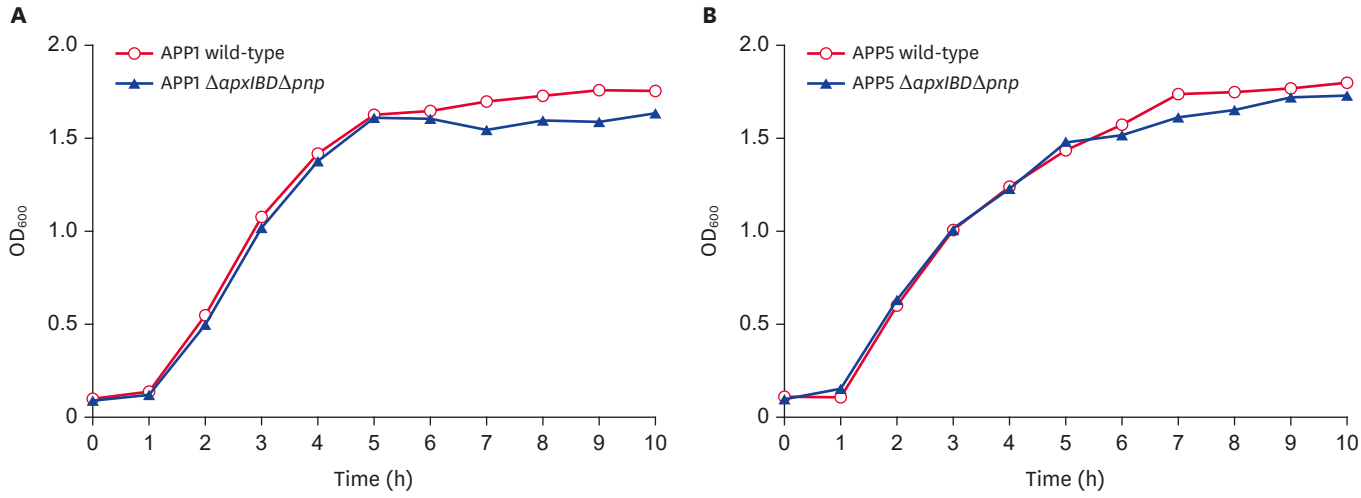

Fig. 3. Growth curves of wild-type and mutants of APP1 (A) and APP5 (B). Symbols: o, wild-type APP; ▲, mutant APP Δ*apxIBD*Δ*pnp*. APP, *Actinobacillus pleuropneumoniae*; OD_{600}, optical density at 600 nm.

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 *apxIBD* and *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 Ca^{2+} cations [24]. Therefore, these toxins could be secreted in the supernatant culture, could bind 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 *apxIBD* and *pnp* genes by PCR using primer pairs P5–P6 and P11–P12, respectively. PCR fragments of expected sizes for the deleted *apxIBD* and *pnp* genes were amplified using APP1Δ*apxIBD*Δ*pnp* and APP5Δ*apxIBD*Δ*pnp* genomes (**Fig. 5**). The amplified target genes in mutants were approximately 2.2–2.4 kb shorter for the *apxIBD* gene and 1.4 kb shorter for *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 APP1Δ*apxIBD*Δ*pnp* and wild-type APP1 were 1.5×10^8 CFU and 1.6×10^6 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 *apxIBD* and *pnp* genes lowered virulence significantly. The LD_{50} of APP5Δ*apxIBD*Δ*pnp* (3.1×10^8 CFU) was increased by 48-fold compared with that of the wild-type (6.5×10^6 CFU) (**Table 3**). As with APP1Δ*apxIBD*Δ*pnp*, the virulence of APP5 deleted with the *apxIBD* and *pnp* genes was significantly attenuated when evaluated in our mouse model. In contrast to the markedly decreased virulence in Δ*apxIBD*Δ*pnp* mutants, the virulence of APP1Δ*pnp* and APP5Δ*pnp* were only slightly decreased by 3-fold and 2-fold, respectively (**Table 3**). It means the *pnp* mutants in the APP1 and APP5 were less attenuated and that deletion of the *pnp* gene did not have much effect on the virulence of APP.

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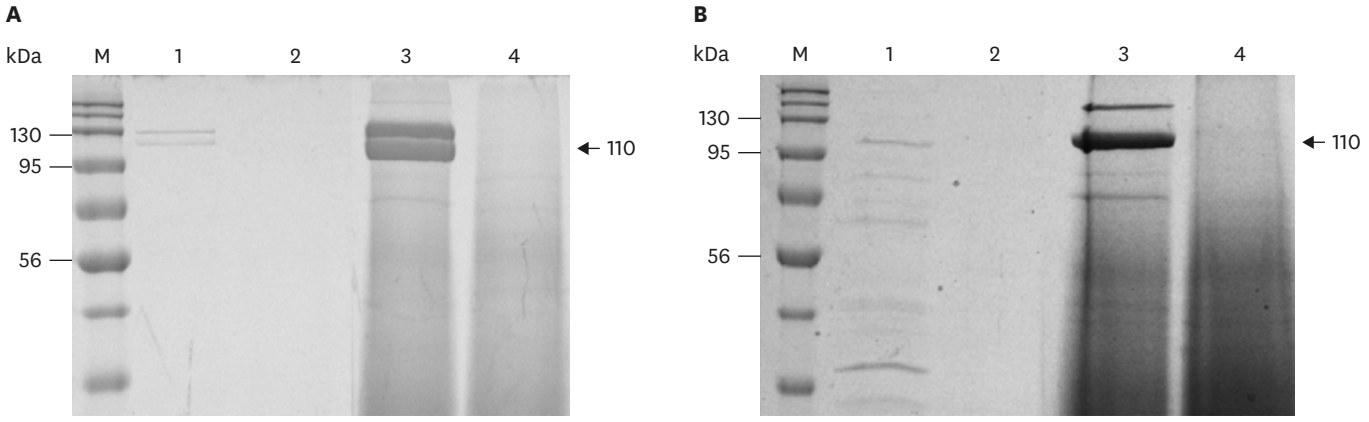

Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of Apx toxins in wild-type and the mutants of APP1 (A) and APP5 (B). Lane M, Xpert Prestained Protein marker; lane 1, culture supernatant of the wild-type APP before precipitating; lane 2, culture supernatant of the mutants before precipitating;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

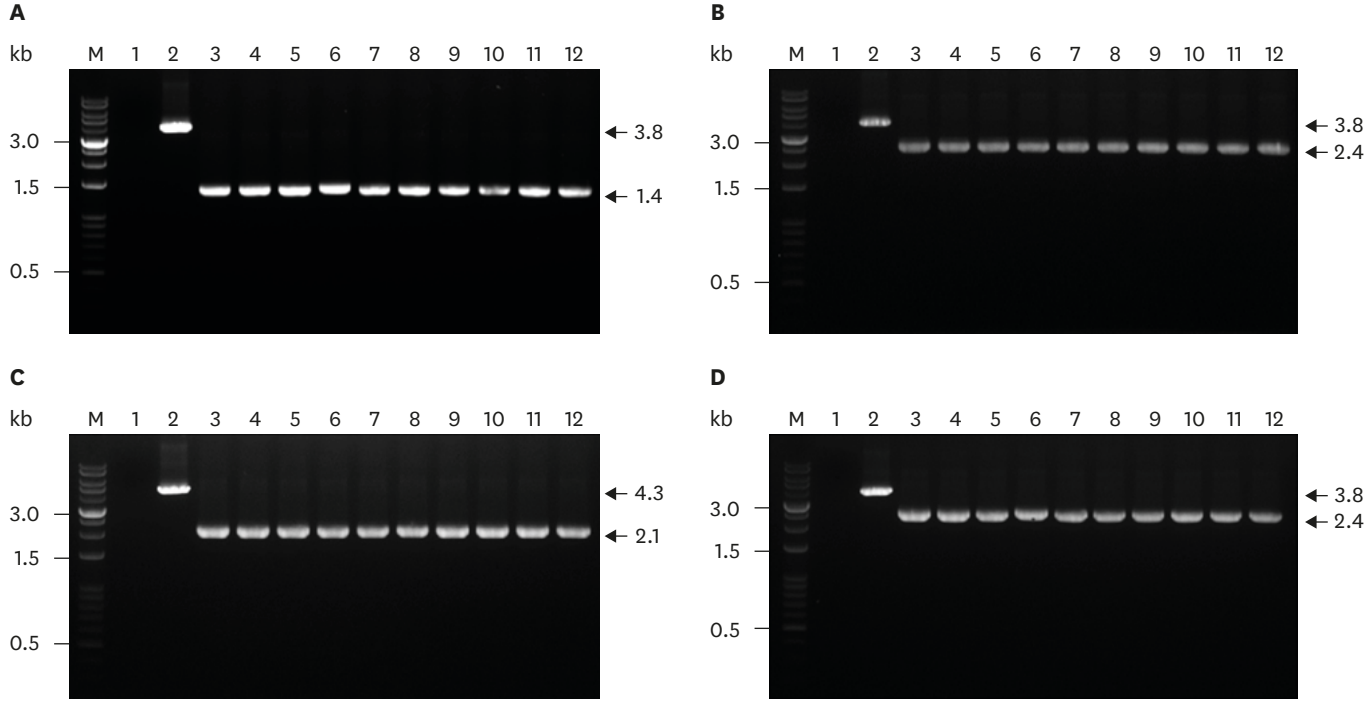

Fig. 5. Evaluation of the stability deletion gene in the genome of APP1Δ*apxIBD*Δ*pnp* and APP5Δ*apxIBD*Δ*pnp* over 10 passages by PCR using primer pairs P5–P6 and P11–P12 for amplifying *apxIBD* and *pnp* genes, respectively. Amplified *apxIBD* gene (A) and *pnp* gene (B) from APP1Δ*apxIBD*Δ*pnp*. Amplified *apxIBD* gene (C) and *pnp* gene (D) from APP5Δ*apxIBD*Δ*pnp*. 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.

Protection efficacy of the mutants in mice

To evaluate the protective effect of mutant strains, mice were immunized intraperitoneally twice with 1×10^7 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 APP1Δ*apxIBD*Δ*pnp* 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 APP5Δ*apxIBD*Δ*pnp* mutant did not protect the mice against homologous and heterologous challenge (**Table 4**).

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

Strain tested	LD_{50}† (CFU)	Fold attenuation‡
APP1 wild-type	1.6×10^6	1
APP1Δ*apxIBD*	6.3×10^7	39
APP1Δ*pnp*	4.4×10^6	3
APP1Δ*apxIBD*Δ*pnp*	1.5×10^8	93
APP5 wild-type	6.5×10^6	1
APP5Δ*apxIBD*	1.4×10^8	21
APP5Δ*pnp*	1.6×10^7	2
APP5Δ*apxIBD*Δ*pnp*	3.1×10^8	48

APP, *Actinobacillus pleuropneumoniae*; 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 APP. The numbers of surviving mice were recorded 10 days after inoculation. †LD_{50} was calculated by the Reed-Muench method; ‡Fold attenuation was normalized to the wild-type 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 (10 × LD_{50})	Number of survival/number of tested (%)
APP1Δ*apxIBD*Δ*pnp*	1×10^7	APP1	6/8 (75%)
		APP5	0/8 (0%)
APP5Δ*apxIBD*Δ*pnp*	1×10^7	APP1	0/8 (0%)
		APP5	0/8 (0%)
Control	TSB	APP1	0/8 (0%)
		APP5	0/8 (0%)

APP, *Actinobacillus pleuropneumoniae*; CFU, colony-forming unit; 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.

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 *apxB* and *apxD* genes and evaluation for candidate living attenuated vaccines. We expect that the mutant with deletions of the *apxIB* and *apxID* 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 *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 *apxIBD* gene in the APP1 and APP5, with an evaluation of the virulence and protective efficacy in mice.

The *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 APP2Δ*apxIIIBD* decreased only about 1.7-fold compared

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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 1.0×10^{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*apxIBD*Δ*pnp* and APP5Δ*apxIBD*Δ*pnp* were attenuated 93-fold and 48-fold, respectively. The LD_{50} values of 1.5×10^8 CFU for APP1Δ*apxIBD*Δ*pnp* and 3.1×10^8 CFU for APP5Δ*apxIBD*Δ*pnp* were similar to the LD_{50} of APP7Δ*apxIIC*Δ*apxIVA* (1.5×10^8 CFU) [22] and the LD_{50} of APP1Δ*apxIC*Δ*apxIIC* (3.1×10^8 CFU) [17]. However, these mutants could not protect mice when they were challenged with ten times the LD_{50} of heterologous virulent strains. APP5Δ*apxIBD*Δ*pnp* 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*apxIBD*Δ*pnp* in this study with the same construction as APP5Δ*apxIBD*Δ*pnp*, 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.

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In conclusion, we constructed and characterized Δ*apxIBD*Δ*pnp* double-unmarked deletion genes from APP1 and APP5. APP1Δ*apxIBD*Δ*pnp* and APP5Δ*apxIBD*Δ*pnp* 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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