The SapA Protein Is Involved in Resistance to Antimicrobial Peptide PR-39 and Virulence of Actinobacillus pleuropneumoniae

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Antimicrobial peptides are essential to the innate immune defense of the mammal against bacterial infection. However, pathogenic bacteria have evolved multiple strategies to resist and evade antimicrobial peptides, which is vital to bacterial survival and colonization in hosts. PR-39 is a linear porcine antimicrobial peptide containing 39 amino acid residues with a high proline content. Resistance to antimicrobial peptide PR-39 has been observed in Actinobacillus pleuropneumoniae. However, little is known about the factors required for this resistance. In the present study, PR-39 exposure increased the expression of the sapA gene in A. pleuropneumoniae. The sapA gene, which encodes a putative peptide transport periplasmic protein, was deleted from this bacterium. The ΔsapA mutant showed increased sensitivity to PR-39 compared to the wild-type MD12 and complemented PΔsapA strains. However, the ΔsapA mutant did not exhibit any alterations in outer membrane integrity. Scanning electron microscopy showed that the ΔsapA mutant displayed morphological defects, as indicated by a deformed and sunken shape after PR-39 treatment. In addition, disruption of the SapA protein led to reduced colonization and attenuated virulence of A. pleuropneumoniae in the BALB/c mouse model. Collectively, these data suggest that SapA acts as one mechanism for A. pleuropneumoniae to counteract PR-39-mediated killing. To the best of our knowledge, this is the first study to show a mechanism underlying antimicrobial peptide resistance in A. pleuropneumoniae.

Keywords: Actinobacillus pleuropneumoniae, SapA, antimicrobial peptide resistance, PR-39, Virulence

INTRODUCTION

Actinobacillus pleuropneumoniae is a Gram-negative bacterial pathogen responsible for porcine pleuropneumonia, which is a highly contagious respiratory disease that causes major economic losses to the swine industry worldwide (Chiers et al., 2010; Bossé et al., 2017). This pathogen mainly causes damage to respiratory tissue, leading to hemorrhagic, fibrinous and necrotic lung lesions (Bossé et al., 2002; Li et al., 2016). The ability of A. pleuropneumoniae to adhere to, colonize, and invade its host, and host factors such as innate and adaptive immune responses are crucial to the outcome of this disease (Chiers et al., 2010).
Antimicrobial peptides, also known as host defense peptides, are important components of innate immunity as a first line of defense against bacterial infection (Band and Weiss, 2015). Relative to other mammals, the pig has the most diverse set of cathelicidins (Wessely-Szponder et al., 2010). According to their primary amino acid structures, porcine cathelicidins divide into three subgroups: linear proline-rich cathelicidins (including PR-39, Prophenin 1 and 2), disulfide-rich Protegrins 1–5, and α-helix-rich porcine myeloid antimicrobial peptides (PMAP)–23, PMAP-36, and PMAP-37 (Sang and Blecha, 2009). The proline-rich antimicrobial peptide PR-39 contains 39 amino acid residues with high contents of proline (49%) and arginine (26%) (Zhang et al., 2000). PR-39 manifests antibacterial activity against a variety of Gram-negative bacteria and some Gram-positive bacteria, including multidrug-resistant clinical isolates (Linde et al., 2001). Like other proline-rich peptides, PR-39 kills bacteria without inducing lysis through pore-forming mechanisms. Instead, it translocates across the membrane and disrupts DNA and protein synthesis (Pranting et al., 2008). In addition to its antibacterial activity, PR-39 exerts other important functions, including immunomodulation, wound repair, and the prevention of inflammation during tissue injury (Shi et al., 1996; Veldhuizen et al., 2014).

PR-39 is prominent in tissue of the upper and lower respiratory tract of healthy pigs, and of pigs infected with A. pleuropneumoniae, and plays a pivotal role in the innate immune defense of the pig against A. pleuropneumoniae infections (Hennig-Pauka et al., 2012). The concentration of PR-39 has been shown to be significantly elevated in bronchoalveolar lavage fluid (BALF) of pigs chronically infected with A. pleuropneumoniae (Hennig-Pauka et al., 2006). However, the minimal inhibitory concentration (MIC) of PR-39 for A. pleuropneumoniae was 5-fold higher than that of Escherichia coli, suggesting the resistance of A. pleuropneumoniae to PR-39 (Hennig-Pauka et al., 2006). This resistance observed in vitro is consistent with the ability of the pathogen to persist in pig respiratory tissue for long periods. The cause of this resistance is not yet clear. A. pleuropneumoniae may have already evolved several strategies to control or evade killing by PR-39 in vivo, and the ability to adapt to PR-39 exposure is vital to the pathogenicity of A. pleuropneumoniae.

One of the important strategies for bacterial evasion of antimicrobial peptides involves the aid of transporter systems (Band and Weiss, 2015). The Sap transporter system is important for resistance to antimicrobial peptides in several Gram-negative pathogens, including Haemophilus ducreyi, non-typeable Haemophilus influenzae, and Salmonella enterica serovar Typhimurium (Parra-Lopez et al., 1993; Mason et al., 2005; Mount et al., 2010). In general, the Sap transporter consists of five proteins: SapA is a periplasmic solute binding protein, SapB and SapC are permease subunits of the transporter, and SapD and SapF function as ATPase proteins for providing energy to translocate the substrate(s) across the bacterial inner membrane (Parra-Lopez et al., 1993; Mount et al., 2010).

Analysis of the A. pleuropneumoniae serovar 5 strain L20 genome sequence (NC_009053.1) reveals the presence of a predicted intact sap operon. The nucleotide sequence of sapA of A. pleuropneumoniae L20 is similar to that of the sapA genes of H. ducreyi strain 35000HP and non-typeable H. influenzae strain 86-028NP, with 68.1 and 53.6% identity, respectively. The amino acid sequence of SapA exhibited 71.1 and 44.4% sequence identity with the SapA protein of H. ducreyi strain 35000HP and non-typeable H. influenzae strain 86-028NP, respectively. The SapA protein of A. pleuropneumoniae has been shown to be expressed in vivo during the chronic stage of the disease and is responsible for persistence of A. pleuropneumoniae (Baltes et al., 2007). In addition, the sapF gene has been reported to be up-regulated in BALF (Lone et al., 2009). Interestingly, PR-39 translocates across the cellular membrane of bacteria via some sort of transport system (Li et al., 2014). It is therefore here hypothesized that the Sap transporter is involved in the resistance of A. pleuropneumoniae to PR-39. To test this hypothesis, a nonpolar, unmarked deletion mutation in the sapA gene was constructed to investigate the role of SapA protein in PR-39 resistance of A. pleuropneumoniae. This is the first demonstration of the mechanism underlying antimicrobial peptide resistance in A. pleuropneumoniae and may improve comprehension of the role of the SapA protein in the persistence and pathogenicity of A. pleuropneumoniae.

MATERIALS AND METHODS

Ethics Statement

Animal experiments were approved by Animal Ethics Committee of Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences (CAAS) and carried out in strict accordance with the recommendations of the Animal Ethics Procedures and Guidelines of the People’s Republic of China. All efforts were made to minimize animal suffering.

Bacterial Strains and Growth Conditions

The bacterial strains and plasmids used for this study are described in Table 1. The A. pleuropneumoniae strains were cultured in a brain heart infusion (BHI, Difco Laboratories, Detroit, MI, USA) medium supplemented with 10 µg/ml nicotinamide adenine dinucleotide (NAD) (Sigma-Aldrich, U.S.). For culture of A. pleuropneumoniae transconjugants (single crossovers), BHI medium was supplemented with 10 µg/ml of NAD and 7 µg/ml of chloramphenicol. E. coli ATCC 25922 strain and S. enterica ATCC 51741 strain (American Type Culture Collection, ATCC) were cultured in a Luria-Bertani (LB, Difco Laboratories, Detroit, MI, USA) medium. E. coli β2155 was grown in LB medium supplemented with 1 mM diaminopimelic acid (DAP) (Sigma-Aldrich, U.S.). All strains were routinely grown at 37°C.

Minimum Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) Analysis

A microdilution broth method was performed to determine the minimal inhibitory concentration (MIC) of antimicrobial peptide PR-39 according to the broth micro dilution guideline of the Clinical and Laboratory Standards Institute (CLSI, 2013). PR-39 was purchased from AnaSpec (San Jose, CA). Standardized
### Table 1 | Characteristics of bacterial strains and plasmids used in this study.

| Strains, plasmids, and primers | Characteristics or sequence | Source or references |
|--------------------------------|----------------------------|---------------------|
| **STRAINS**                   |                            |                     |
| E. coli β2155                  | thrB1004 pro thi strA hasdS lacZDΔM15 (P’ lacZDΔM15 lacP) traD36 proA+ proB+ Δdaps: erm (Erm’) recA4:: RP4-Z-tet(Tc’); Mu- Km (Km’) λpir | Dehio and Meyer, 1997 |
| E. coli ATCC 25922             | E. coli serovar O6 isolate | ATCC                |
| S. enterica ATCC 51741         | S. enterica serovar Infantis isolate | ATCC |
| A. pleuropneumoniae ATCC 27090 | A. pleuropneumoniae serovar 3 isolate | ATCC |
| A. pleuropneumoniae S-8        | A. pleuropneumoniae serovar 7 clinical isolate from the lung of a dead pig with pleuropneumonia in Heilongjiang province | Lab stock |
| A. pleuropneumoniae MD12       | A. pleuropneumoniae serovar 5 clinical isolate from the lung of a dead pig with pleuropneumonia in Heilongjiang province | Lab stock |
| A. pleuropneumoniae ΔsapA      | Unmarked sapA gene knockout mutant of A. pleuropneumoniae MD12 | This work |
| A. pleuropneumoniae PΔsapA     | The complemented strain of A. pleuropneumoniae ΔsapA containing the sapA ORF and 675 bp of the upstream region | This work |
| A. pleuropneumoniae ΔvacJ      | Unmarked vacJ gene knockout mutant of A. pleuropneumoniae MD12 | Xie et al., 2016 |
| **PLASMIDS**                  |                            |                     |
| pEMOC2                        | Conjugal vector based on pBluescript SK with mob RP4, polycloning site, Camel, and transcriptional fusion of the ornA promoter with the sacB gene | Accession no. AY868288 (Bai et al., 2003) |
| pEMΔsapA                      | Conjugal vector pEMOC2 with a 570 bp deletion in the sapA gene which have a 1.3-kb upstream fragment and 1.3-kb downstream fragment | This work |
| pGZRS-19                      | A. pleuropneumoniae-E. coli shuttle vector; ApI | West et al., 1995 |
| pGZRS-sapA                    | pGZRS-19 with a PCR-derived insert containing the sapA gene | This work |

bacterial suspensions of log-phase cultures of E. coli ATCC 25922, S. enterica ATCC 51741, A. pleuropneumoniae strains ATCC 27090, S-8 and MD12 were prepared and diluted to a concentration of 1 × 10^6 CFU/ml. MIC determinations were performed using commercially sterile 96-well microtiter plates (Costar 3599, U.S.A.). The MIC value was determined as the lowest concentration of PR-39 that prevented visible growth. Then 20 µl of each bacteria-peptide suspension in the 96-well microtiter plates was plated onto LB or BHI agar plates and incubated for 20 h at 37°C. The MBC value was determined as the lowest concentration of PR-39 that showing no visible growth on the plates (Hu et al., 2016).

**In vitro Growth Assays**

The A. pleuropneumoniae wild-type strain MD12 was grown in 5 ml of BHI medium for 15 h, and then diluted to an optical density at 600 nm (OD600) of 0.1. Fresh cultures in 5 ml of BHI medium were supplemented with PR-39 (concentration range 0–0.2 µM) and incubated while shaking at 37°C. Growth was monitored by measuring the OD600 values at an interval of 1 h using the Eppendorf BioPhotometer (Eppendorf, Germany).

**RNA Isolation and qRT-PCR**

For RNA isolation, A. pleuropneumoniae MD12 strain was grown to mid-logarithmic phase in 3 ml of BHI medium supplemented with PR-39 (concentration range 0–0.2 µM). The cultures were harvested by centrifugation at 10,000 g at 4°C. Total RNA was extracted using RNeasy kit (Qiangen) and cDNA was synthesized using the PrimeScript RT reagent kit (TaKaRa, Japan) according to the manufacturer’s instructions. The primers used for analysis of sapA expression are listed in Table S1. The cDNA samples were amplified using SYBR Green I (TakaRa). Quantitative real-time polymerase chain reactions (qRT-PCR) were performed in a MicroAmp Optical 96-well reaction plate using a Stratagene Mx3000P system (Agilent Technologies, Germany). Amplification efficiency was evaluated using a standard curve generated by qRT-PCR using the cDNA dilution series with three replicates. The stability of the six housekeeping genes recF, glyA, rho, tpiA, pykA (Nielsen and Boye, 2005) and sup (Lone et al., 2009) was examined using the program geNorm (Vandesompele et al., 2002). The geometric mean of the best-scoring reference genes glyA, tpiA, and sup was used to normalize the target gene expression levels. The qRT-PCR experiments were performed in triplicate with three independent biological replicates. Relative expression levels were analyzed by a threshold cycle (ΔΔCt) method to calculate the fold change in gene expression (Pfaffl, 2001).

**Construction of Gene Deletion Mutant**

The primers used for the construction of the deletion mutant ΔsapA are listed in Table S1. Primers AUF/AUR, and ADF/ADR were used to amplify the two segments flanking the sapA gene. Using single-overlap extension PCR (SOE PCR), the fragment with a 570 bp internal in-frame deletion in the sapA gene (from nt 24 to 593) was generated, and cloned into the conjugal vector pEMOC2 (Bai et al., 2003) to produce the plasmid pEMΔsapA. Using E. coli β2155 and a single-step transconjugation system (Dehio and Meyer, 1997; Oswald et al., 1999), plasmid pEMΔsapA was used to introduce the sapA mutation into the wild-type strain MD12. After two homologous recombination steps, the A. pleuropneumoniae ΔsapA mutant
was verified by sequencing and PCR analyses using AJDF/AJDR primers.

Complementation of the A. pleuropneumoniae ΔsapA Mutant
The 2,471 bp PCR product including the entire sapA open reading frame (ORF) and 675 bp of the upstream region containing the native promoter was amplified with the primers AHBF/AHBR (Table S1). The PCR reaction was performed under the following conditions: 95°C for 3 min, 30 cycles with 94°C for 30 s, 52 °C for 30 s and 72°C for 2 min, the final extension at 72°C for 8 min. The PCR product was digested with Sall/Sacl and ligated to Sall/Sacl-digested pGZRS-19 plasmid (West et al., 1995), yielding plasmid pGZRS-sapA. The recombined plasmid pGZRS-sapA was confirmed by DNA sequencing. Specific-pathogen-free, 6-week-old female BALB/c mice (Beijing Vital River Laboratory Animal Co., Ltd.) were purchased from the Vital River Laboratories (VRL, Beijing, China). A. pleuropneumoniae strains MD12 and ΔsapA were cultured in BHI medium at 37°C to mid-logarithmic growth phase. Cells of each strain from the broth cultures (10⁶ CFU/ml) were incubated with 4 µM PR-39 for 1 h, and harvested by centrifugation. The cells were washed three times with PBS, and fixed overnight using 2.5% glutaraldehyde at 4°C. Dehydration was performed in upgraded ethanol (washed once with each of 50, 70, 85, 95%, and three times with 100%). Then the samples were dried using a critical point drying method and sputter-coated with gold. The cell morphology of all samples was visualized using a scanning electron microscope (JSM-7500F, JEOL, Japan).

Mouse In vivo Experiments
The BALB/c mouse model has been acknowledged as an appropriate one to assess A. pleuropneumoniae infection (Chiang et al., 2009; Seo et al., 2013). Specific-pathogen-free, 6-week-old female BALB/c mice (Beijing Vital River Laboratory Animal Co., Ltd.) were purchased from the Vital River Laboratories (VRL, Beijing, China). A. pleuropneumoniae strains MD12 and ΔsapA were cultured in BHI medium at 37°C, and harvested during the mid-exponential phase and washed three times with sterile PBS. A total of 50 mice were randomly divided into 5 groups (n = 10/group). Group 1 and group 2 were respectively inoculated intraperitoneally with 100 µl of PBS containing 10⁶ and 10⁷ CFU of MD12. Group 3 and group 4 were respectively inoculated intraperitoneally with 100 µl of PBS containing 10⁸ and 10⁹ CFU of ΔsapA. Non-infected mice in the control group were inoculated with 100 µl of sterile PBS (pH 7.4). The health status and the weight of the mice were monitored twice daily for a 14-day period and humane endpoints used to determine if the mice met criteria to be euthanized (Nemzek et al., 2004). These criteria included weight loss >10–15%, lethargy, inability to stand, anorexia or flocked together for more than 6 h. Mice meeting criteria were euthanized by cervical dislocation under isoflurane anesthesia.

Enumeration of Bacterial Load in Organs
A total of 10 specific-pathogen-free, 6 week-old female BALB/c mice were randomly divided into 2 groups (n = 5), and each group was intraperitoneally administered with 5.0 × 10⁸ CFU of the MD12 strain or the ΔsapA mutant. Three days post-infection, mice from each group were humanely euthanized and the organs of lung, liver, and kidney were removed aseptically. Samples were weighed, and homogenized using a tissue homogenizer (100 mg weight/ml of PBS). Viable counts in serial dilutions of homogenates were determined following culture on BHI agar plates for 24 h at 37°C.
Statistical Analysis
Statistical analyses were performed using GraphPad Prism version 5.01 (GraphPad Software Inc., U.S.A.). The data are expressed as the means ± standard deviation. The statistical analysis of the data was performed using one-way ANOVA, two-way ANOVA, or the Student’s t-test. P-values less than 0.05 were considered statistically significant.

RESULTS

Antibacterial Activity of PR-39
To explore the antibacterial activity of PR-39, the MICs of PR-39 for A. pleuropneumoniae strains ATCC 27090, S-8, MD12, E. coli ATCC 25922 and S. enterica ATCC 51741 were measured (Table S2). For E. coli and S. enterica, the MICs of PR-39 ranged from 0.5 to 1 µM, the MBCs of PR-39 were 1 µM. However, PR-39 had higher MICs for A. pleuropneumoniae strains than E. coli and S. enterica, ranging from 4 to 8 µM. Similarly, the MBCs of PR-39 for A. pleuropneumoniae strains were 8 µM, much higher than those of E. coli and S. enterica. These results showed that A. pleuropneumoniae exhibited a certain resistance to PR-39 compared to E. coli and S. enterica.

A. pleuropneumoniae Exposure to PR-39
Upregulated Expression of the sapA Gene
The expression of the sapA gene was analyzed using qRT-PCR in A. pleuropneumoniae MD12 when exposed to PR-39. Three housekeeping genes glyA, tpiA, and syp were selected to normalize sapA gene expression levels, and PCR efficiency for each gene was not less than 1.92. When the MD12 strain was exposed to sublethal concentrations of PR39, growth curves were similar to that of untreated bacteria (Figure 1A), but the transcription levels of sapA were higher than that of untreated bacteria (Figure 1B). In the presence of increasing concentrations of PR-39, the expression of sapA was upregulated in a dose-dependent manner, suggesting sapA may contribute to a resistance mechanism in A. pleuropneumoniae MD12 against PR-39.

Construction of A. pleuropneumoniae ΔsapA Mutant and Its Complemented Strain
Analysis of the A. pleuropneumoniae L20 genome sequence revealed the presence of an intact sap operon (Figure 2A). This operon consists of four genes: sapA (APL_RS04160), sapB (APL_RS04165), sapC (APL_RS04160), sapD (APL_RS04155). However, sapF (APL_RS06520) is not linked to the sapABCD locus.

To investigate the function of the SapA protein, an in-frame-deletion mutant of sapA in A. pleuropneumoniae was constructed using double-crossover homologous recombination and confirmed by PCR and DNA sequencing (Figures 2A,B, Supplementary Materials). PCR with primers AJDF/AJDR was used to amplify the 925 bp amplicon from the wild-type MD12 strain, and the 355 bp amplicon from the sapA deletion mutant ΔsapA (Figure 2B). The ΔsapA mutant contains a 570 bp in-frame deletion in the sapA gene. The results of qRT-PCR showed that the transcription levels of the downstream genes sapB, sapC, and sapD were unaffected, confirming that the mutation in ΔsapA was nonpolar (Figure S1). The complemented mutant strain PΔsapA was generated using the plasmid pGZRS-sapA, with transformants selected on plates containing ampicillin, and confirmed by PCR (Figure 2C).

Mutation in sapA Enhanced Sensitivity of A. pleuropneumoniae to PR-39
To determine whether the SapA protein has a role in the survival of A. pleuropneumoniae upon exposure to the antimicrobial peptide PR-39, the MD12, ΔsapA, and PΔsapA strains were tested in a bactericidal assay. Cells of each strain were incubated with specific concentrations of PR-39 for 3 h. The results showed that the mutant devoid of sapA was significantly more sensitive to PR-39 over a concentration range of 0.5–4 µM than was the isogenic wild type strain (Figure 3A). In addition, following the elongation of treatment time, the sensitivity of ΔsapA to PR-39 was increased (Figure 3B). Trans-complementation with...
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A. pleuropneumoniae SapA-Mediated PR-39 Resistance

**FIGURE 2** | Chromosomal inactivation of *A. pleuropneumoniae* sapA gene. (A) Schematic representation of the *A. pleuropneumoniae* sap operon locus. The sapA gene was inactivated using double-crossover homologous recombination. Binding locations for the primers AUF/AUR and ADF/ADR used to amplify the two flanking regions (1,336 and 1,359 bp, respectively) of the sapA gene are shown in the schematic, and primers AJDF/AJDR were here used to identify the sapA-deleted mutant (355 bp) and wild-type MD12 strain (925 bp). (B) PCR identification of the ΔsapA mutant using the primers AJDF/AJDR. (C) PCR identification of the complemented strain PΔsapA using the primers AJDF/AJDR.

sapA expressed on pGZRS-19 partially restored resistance to PR-39. These findings indicate that the *A. pleuropneumoniae* SapA protein is required for the bacterium's resistance to the PR-39.

To exclude the possibility of impaired outer membrane integrity due to the deletion of the sapA gene, the sensitivity of the MD12, ΔsapA, and PΔsapA strains to SDS-EDTA was analyzed. As shown in **Figure S2A**, all these strains did not exhibit sensitivity to SDS-EDTA. In addition, the outer membrane integrity of each strain was further evaluated using the fluorescent probe NPN, which exhibits fluorescence weakly in aqueous but strongly in hydrophobic environments (Lee et al., 2015). In **Figure S2B**, no significant difference in uptake of NPN was observed between MD12, ΔsapA, and PΔsapA, while NPN fluorescence was significantly higher in the ΔvacJ mutant whose membrane permeability was increased as described previously (Xie et al., 2016). These data indicated that the sapA gene deletion did not cause alterations in the outer membrane integrity in *A. pleuropneumoniae*.

**Morphology of *A. pleuropneumoniae* ΔsapA upon Exposure to PR-39**

To further confirm the increased sensitivity of the ΔsapA strain to PR-39, the morphology of the MD12, ΔsapA, and PΔsapA strains treated with PR-39 was assessed using scanning electron microscopy. After PR-39 treatment, a significant morphological variation was observed among the MD12, ΔsapA, and PΔsapA strains (Figure 4). MD12 displayed a smooth surface, which is typical of this *A. pleuropneumoniae* strain (Figure 4). However, when exposed to PR-39, cells of the ΔsapA mutant had an irregular and crinkled appearance and a sunken shape compared to that of the wild-type MD12 and complemented PΔsapA strains (Figure 4). These data indicated that the *A. pleuropneumoniae* SapA protein functions, at least to some extent, to protect this pathogen from the lethal effects of PR-39.

**Loss of sapA Attenuates the Virulence of *A. pleuropneumoniae* in the BALB/c Mouse Model**

To address whether sapA deletion affected the virulence of *A. pleuropneumoniae*, BALB/c mice were inoculated intraperitoneally with wild type strain MD12 and the ΔsapA mutant at various doses. The MD12 strain gave rise to a higher mortality rate than ΔsapA (Figure 5), which suggested that the deletion of sapA attenuates the virulence of *A. pleuropneumoniae*.

The capacity of the MD12 and ΔsapA strains to colonize mice was then tested. The *A. pleuropneumoniae* load in tissues
The results of the present study showed that the putative peptide transport periplasmic protein SapA of *A. pleuropneumoniae* is involved in resistance to PR-39-mediated killing. This is the first study to show an antimicrobial peptide resistance mechanism in *A. pleuropneumoniae*.

PR-39 has been shown to be essential to the innate immune defense of the pig against *A. pleuropneumoniae* infection (Hennig-Pauka et al., 2012). In this study, the MICs of *E. coli* and *A. pleuropneumoniae* isolates were measured in the laboratory, and an MIC of 1 µM was determined for *E. coli*, but 4–8 µM for *A. pleuropneumoniae* strains. This finding is in accordance with the previous report by Hennig-Pauka et al. suggesting innate resistance of *A. pleuropneumoniae* to PR-39 (Hennig-Pauka et al., 2006). This resistance contributes to the promotion of *A. pleuropneumoniae* survival and colonization in the host for extended periods of time. Most notably, the mode of action of PR-39 killing bacteria does not involve the formation of pores, but translocation across the membrane via some sort of transport system and the targeting of intracellular molecules (Li et al., 2014). Thus, we hypothesized that certain peptide transport systems would be required for resistance of *A. pleuropneumoniae* to PR-39. Analysis of the *A. pleuropneumoniae* L20 genome sequence revealed the presence of an intact sap operon (Figure 2A). This operon consists of four genes, but does not contain the sapF gene: sapA (APL_RS04170), which encodes a putative periplasmic binding protein; sapB (APL_RS04165) and sapC (APL_RS04160), which encode putative permease components; and sapD (APL_RS04155) which encodes the ATPase components. The unlinked sapF (APL_RS06520) is predicted to encode the ATPase component of this transporter. The peptide transport periplasmic protein SapA, which has been found to be expressed in *vivo* during the chronic stage of *A. pleuropneumoniae* infection (Baltes et al., 2007), has drawn considerable attention. Additionally, the sapF gene has been reported to be up-regulated in *A. pleuropneumoniae* when grown in pig BALF (Lone et al., 2009). The results of the present study...

**DISCUSSION**

Antimicrobial peptides are an essential part of innate immune defenses that inhibit pathogen infection and contribute to clearance of bacterial colonization (Band and Weiss, 2015). Upon encountering invasive pathogens, hosts can generate the specific innate immune signaling events to induce production of specific antimicrobial peptides in response to the invasion of pathogens (Plichta et al., 2012). However, to adapt to the environments of elevated antimicrobial peptides, bacteria have evolved multiple countermeasures to resist and evade antimicrobial peptide-mediated killing (Band and Weiss, 2015). Resistance to porcine antimicrobial peptides is vital to survival and colonization of *A. pleuropneumoniae* in host environments (Hennig-Pauka et al., 2006). However, little is known about the factors required for this resistance. This study demonstrated that the putative peptide transport periplasmic protein SapA of *A. pleuropneumoniae* is involved in resistance to PR-39-mediated killing. This is the first study to show an antimicrobial peptide resistance mechanism in *A. pleuropneumoniae*.

![Image of bacterial cultures](https://example.com/image.jpg)

**FIGURE 5** | Survival of mice following intraperitoneally challenge with *A. pleuropneumoniae* strains. Percentage of surviving mice after challenged with 1×10⁷ or 1×10⁶ CFU of MD12 or ΔsapA strain.

![Image of scanning electron microscopy](https://example.com/image.jpg)

**FIGURE 4** | Scanning electron microscopy of *A. pleuropneumoniae* strains treated with PR-39. MD12, ΔsapA, and PΔsapA in the mid-logarithmic phase were harvested and treated with 4 µM PR-39 for 30 min. Scale bar, 100 nm. The cells of ΔsapA displayed a relatively deformed cell shape compared to that of the wild-type MD12 strain and the complemented strain PΔsapA.
showed that inactivation of the sapA gene significantly enhanced sensitivity of A. pleuropneumoniae to PR-39, indicating that the SapA protein is required for A. pleuropneumoniae resistance to the PR-39 (Figures 3, 4).

Until now, several transporter systems have been shown to mediate resistance against antimicrobial peptides. Mutation in the yejF gene of S. enterica from the yejABEF operon encoding an ATP-binding cassette (ABC) peptide import system, reduced resistance to polymyxin B, human defensin (HBD)-1 and HBD-2 (Eswarappa et al., 2008). Additionally, in the pathogens Neisseria meningitidis and H. ducreyi, a periplasmic membrane fusion protein MtrC actively transports cathelicidin LL-37 out of the bacterial membrane to promote resistance to LL-37 (Tzeng et al., 2005; Rinker et al., 2011). Furthermore, the Sap transporter was also demonstrated to contribute to antimicrobial peptide resistance in other Gram-negative species. In non-typeable H. influenzae, mutation in the sapA gene led to reduced resistance to the chinchilla beta defensin 1 (cBD-1) (Mason et al., 2005). The Sap transporter in H. ducreyi confers resistance to LL-37, but not to α- or β-defensins (Mount et al., 2010). In Vibrio fischeri, SapA did not confer resistance to any of the eight tested antimicrobial peptides but was implicated in in vitro growth and in vivo colonization (Lupp et al., 2002). The present study showed that the SapA protein rendered A. pleuropneumoniae resistant to the porcine antimicrobial peptide PR-39 (Figures 3, 4). Taken together, these studies suggested that the SapA protein served multiple functions to satisfy the requirements of different bacterial species over the course of infection.

In vivo colonization by A. pleuropneumoniae is a complicated process, during which the evasion from the host innate immune plays an important role. Another goal of this study was to clarify whether SapA is essential for A. pleuropneumoniae colonization and pathogenicity in a mouse model. The data presented in this study showed that the ΔsapA mutant displayed an attenuated virulence and reduced bacterial colonization, compared with wild type strain (Figures 5, 6). These results may be explained by the decreased ability of the ΔsapA mutant to resist PR-39 mediated killing in vivo. This is highly consistent with the data presented by a previous study which showed that the SapA protein was required for colonization and virulence of non-typeable H. influenzae in a chinchilla model (Mason et al., 2005). It is clear from these data that the sapA gene product is involved in colonization and virulence in A. pleuropneumoniae infection.

The lung is the major target of A. pleuropneumoniae infection (Bossé et al., 2002; Chiers et al., 2010). PR-39 expression has been reported to be increased in BALF and epithelial lining fluid (ELF) after A. pleuropneumoniae infection of pigs (Hennig-Pauka et al., 2006). The concentration of PR-39 in BALF ranged from 0.4 to 75.9 nM and concentrations in ELF are approximately 6- to 40-fold higher than in BALF in infected pigs, which are lower than the MICs of A. pleuropneumoniae strains. At sub-inhibitory concentrations, PR-39 in combination with other antimicrobial factors in the lower respiratory tract may result in a synergistic antimicrobial effect against A. pleuropneumoniae. The sub-inhibitory concentrations of PR-39 might also let A. pleuropneumoniae adapt to the host environment and is in accordance with the in vivo observation that A. pleuropneumoniae persists in respiratory epithelium of pigs for extended periods. In addition to its antimicrobial properties, PR-39 is also involved in many other biological processes, such as chemotaxis of neutrophils, promotion of wound healing, and inhibition of apoptosis (Shi et al., 1996; Veldhuizen et al., 2014). In A. pleuropneumoniae chronic infection, the involvement of PR-39 in these biological processes might be its primary role.

In conclusion, this study demonstrates that the SapA protein in A. pleuropneumoniae promotes resistance to antimicrobial peptide PR-39, and it is the first mechanism of antimicrobial peptide resistance identified in A. pleuropneumoniae. In addition, disruption of the SapA protein led to reduced colonization and attenuated virulence of A. pleuropneumoniae in the BALB/c mouse model. This has shed light on the role of SapA protein in the pathogenicity of A. pleuropneumoniae. Of note, though the sapA deletion increased sensitivity of A. pleuropneumoniae to PR-39, the MIC of ΔsapA was still more than that of E. coli (data not shown), indicating the presence of other PR39 resistance mechanisms in A. pleuropneumoniae. Future studies should include attempts to determine whether other proteins, such as secreted proteases or two-component systems, participate in the
resistance to PR39, and unravel the mechanism of detection and signal transduction that takes place when *A. pleuropneumoniae* encounters PR-39.

**AUTHOR CONTRIBUTIONS**

FX and CW designed the experiments, FX and GL conducted experiments, YW, ShL, and NC performed the experiments, FX and YW analyzed the data and drafted the manuscript, CW, PL, and SiL finalized the manuscript. All authors read and approved the final manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00811/full#supplementary-material

**Figure S1 | Transcriptional levels of downstream genes of sapA in MD12 and ΔsapA strains.** Transcriptional levels of sapB, sapC, sapD genes were examined by qRT-PCR. Values represent two independent assays in triplicate ±SD.

**Figure S2 | The outer membrane integrity of *A. pleuropneumoniae*. (A) SDS-EDTA sensitivity assay. Cultures were grown until mid-log phase, and 2 μl of each dilution, indicated on the left, was spotted in triplicate onto BHI agar plate supplemented with 0.1% SDS and 0.5 mM EDTA. (B) NPN uptake assay. Changes in fluorescence following the addition of the hydrophobic fluorescent probe NPN for the MD12, ΔsapA, PΔsap, and ΔvacJ strains are shown. Values represent two independent assays in triplicate ±SD, n.s. = not significant, **p < 0.01.

**Table S1 | Primers used in this study.**

**Table S2 | Minimum inhibitory concentrations and minimal bactericidal concentration of PR-39 for *E. coli*, *S. enterica*, and *A. pleuropneumoniae* strains.**

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