Involvement of the *Actinobacillus pleuropneumoniae* ompW Gene in Confrontation of Environmental Pressure

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Actinobacillus pleuropneumoniae causes porcine pleuropneumonia. The function of the outer membrane protein W gene (*ompW*) of *A. pleuropneumoniae* has not been evaluated. Thus a deletion mutant of *ompW*, Δ*ompW*, was constructed to explore the effect of *ompW* gene deletion on bacterial growth, biofilm formation, bacterial morphology, oxidative tolerance, susceptibility to antibiotics, and the expression of ribosome synthesis and ABC transporter related genes. Results showed that the *ompW* gene deletion did not affect biofilm formation and the growth of *A. pleuropneumoniae* but did affect bacterial morphology during steady growth, oxidative tolerance, and bacterial susceptibility to polymyxin B, kanamycin, and penicillin. The *ompW* gene deletion also affected the expression of ribosome synthesis and ABC transporter related genes. These results suggested that *ompW* may regulate the biological phenotype of *A. pleuropneumoniae*.

Keywords: *A. pleuropneumoniae*, *ompW*, susceptibility, phenotype, infections

INTRODUCTION

*Actinobacillus pleuropneumoniae* causes porcine pleuropneumonia and is one of the most important bacterial respiratory pathogens of pigs (1). In large-scale pig breeding farms, economic loss due to *A. pleuropneumoniae* is a serious concern. Based on the growth requirement for nicotinamide adenine dinucleotide (NAD), *A. pleuropneumoniae* are identified as biotype I or biotype II (2). Biotype I strictly requires NAD for growth, while biotype II does not require NAD as a growth supplement (3). Based on the nature of the bacterial capsule and lipopolysaccharide, *A. pleuropneumoniae* is classified into 19 serotypes, with serotypes 1, 5, 9, 10, and 11 highly virulent (4, 5). In China, serotypes 1, 3, and 7 are the dominant serovars (6). Immunological protection does not exist across serotypes (7), hence *A. pleuropneumoniae* infections are difficult clinical problems.

Recently, important virulence related factors have been identified that are involved in the pathogenesis of *A. pleuropneumoniae*. Repeats-in-toxin (RTX) and ApxIIIA toxin have been shown to target host cell β2 integrins killing many types of leukocytes and phagocytic...
cells (8). Deletion of PEP-carboxylase (PEPC) and PEP-carboxykinase (PEPCK) attenuate A. pleuropneumoniae virulence in a pig infection model (9). Disruption of TolC1 significantly reduces A. pleuropneumoniae virulence in a murine intraperitoneal injection model (10). Inactivation of flp1 and tadD genes affected A. pleuropneumoniae biofilm formation, cell adhesion, and resistance to phagocytosis (11). The hfg gene of A. pleuropneumoniae is helpful to regulate the adhesion to biological and abiotic surfaces, resistance to various stress conditions, and virulence (12). Deletion of outer membrane lipoprotein, Lip40, significantly attenuates adherence to St. Jude porcine lung cells and colonization of mouse lung tissue, indicating that Lip40 participates in the virulence of A. pleuropneumoniae (13). CpxA/CpxR affect A. pleuropneumoniae biofilm formation with mutation of cpxA/cpxR reducing mortality and bacterial load in a murine experimental lung infection model (14). Although these virulence factors have been intensively investigated, the mechanistic basis for A. pleuropneumoniae pathogenicity remains unclear.

Outer membrane proteins (OMPs) of bacteria play core roles in bacterial pathogenesis (15). The ompW gene is found in many bacteria, such as Escherichia coli (16), Aeromonas hydrophila (17), and Vibrio harveyi (18). E. coli OmpW knockout contributes to oxidative stress resistance (19). Response to the OmpW protein provides immunity to Aeromonas veronii challenge, with an immunization strategy a potential means by which to control Aeromonas veronii infection (20). OmpW regulates biofilm formation of Cronobacter sakazakii during NaCl stress (21). Deletion of ompW attenuates Vibrio cholerae growth in hypersaline culture conditions (22). Caulobacter crescentus ompW is an outer membrane cation channel (23). It serves as a multidrug resistance transporter involved in the efflux of ethidium multidrug resistance protein E (EmrE) substrates across the outer membrane (24). The function of A. pleuropneumoniae ompW has not been identified.

The purpose of this study was to investigate the role of A. pleuropneumoniae ompW in bacterial pathogenicity. To do so we constructed a deletion mutant of ompW, strain ΔompW, and a complemented strain, CΔompW. Deletion of the ompW gene did not affect the growth and biofilm formation of A. pleuropneumoniae, but did influence steady state growth bacterial morphology, as well as bacterial susceptibility to polymyxin B, kanamycin, and penicillin. In addition, ompW gene inactivation modified expression of ribosome synthesis related genes and ABC transporter genes. These results provide a better understanding of A. pleuropneumoniae ompW gene function during infection.

**MATERIALS AND METHODS**

**Bacterial Strains and Growth Conditions**

The A. pleuropneumoniae 4074 strain, serovar 1, is highly virulent and cultured on tryptic soy agar (TSA; Difco Laboratories, USA) or in tryptic soy broth (TSB, Difco Laboratories, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) and 10 µg/mL NAD (Sigma-Aldrich, USA). The ΔompW and CΔompW strains were constructed in this study, and the culture conditions were the same as the A. pleuropneumoniae 4074 strain. The pEMOC2 plasmid was from the laboratory of Prof. gerald-f. Gerlach and the pJFF224-XN plasmid was stored in our laboratory. The pEMΔompW and pJFF-ompW plasmid were constructed in this study. The E. coli β2155 strain was cultured on Luria-Bertani (LB, Oxoid Ltd. UK) agar or in broth. The A. pleuropneumoniae and E. coli strains were cultured at 37°C. If necessary, 2 µg/mL or 50 µg/mL of chloramphenicol (Sigma-Aldrich, USA) was added to the culture medium.

**Construction of the A. pleuropneumoniae ompW Gene Deletion Mutant, ΔompW, and the OmpW Gene Deletion Complemented Mutant, CΔompW**

The method for construction of ΔompW has been reported previously (25). Briefly, a 648 bp internal deletion of the ompW gene was amplified with primers ompWUF/ompWUR and ompWDF/ompWDR utilizing single-overlap extension polymerase chain reaction (SOE PCR). Then a 2073 bp PCR product (pEompWUF/pEompWUR) was cloned into the suicide plasmid vector pEMOC2 to obtain the plasmid pEMΔompW (26), which included the 648 bp deletion fragment in frame. The pEMΔompW plasmid in E. coli β2155 strain was transformed into the wild-type strain of A. pleuropneumoniae with a single-step transconjugation system (27, 28). The ΔompW mutant was obtained by two homologous recombination steps and was identified with ompWMF/ompWMR primers.

The ompW gene was amplified with ompWF/ompWR primers for construction of CΔompW. The ompW gene was then connected to the shuttle vector pJFF224-XN and was electrically transferred into the ΔompW mutant to construct the corresponding complemented strain. Voltage was 2.5 KV, capacitance 25 µFD, and pulse resistance 800 Ω using an electroporation apparatus (Bio-Rad, USA). The complemented strain was screened with 2 µg/mL chloramphenicol and was identified with PompWR/ PompWF primers. The bacterial strains, plasmids used in this study are listed in Table 1 and the primers used in this study are listed in Table 2.

**The Effect of the OmpW Gene Deletion on A. pleuropneumoniae Growth Kinetics**

The effect of the ompW gene deletion on A. pleuropneumoniae growth was assessed as described previously (29). Briefly, the A. pleuropneumoniae, the ΔompW, and CΔompW strains were cultured in TSB supplemented with 10% FBS and 10 µg/mL NAD at 37°C overnight. The bacterial were then transferred to fresh TSB supplemented with 10% FBS and 10 µg/mL NAD at a 1:100 (V/V) dilution for logarithmic growth. The initial OD600 value was adjusted to 0.2 and the OD600 value of the bacterial suspension determined 1 h to 8 h after culture initiation for kinetic analysis.
**TABLE 1 |** Bacterial strains and plasmids used in this study.

| Strains/plasmids | Relevant characteristics | Source of references |
|------------------|--------------------------|----------------------|
| A. pleuropneumoniae 4074 | high toxic strain | Laboratory stock |
| ΔompW | ompW gene knockout mutant | This work |
| CΔompW | Complemented strain of ΔompW | This work |
| p215S | thrB1004 pro thi strA hsdS lacZΔM15 (F- lacZΔM15 lacIq traD36 proA+ proB+ ΔompW. erm (Emr) : recA : RPA-2-tet[ThC] : Mu- Δ (Km) Δpir | From Prof. Gerald-F. Gerlach |
| pEMOC2 | Conjugal vector based on pBluescript SK with mobR P4, polycloning site, Cm’, and transcriptional fusion of the omvA promoter with the ompW gene | This work |
| pEΔompW | Conjugal vector pEMOC2 with a 648bp deletion in the ompW gene which have a 1065 bp upstream fragment and 1008 bp downstream fragment | This work |
| pJFF224-XN | E. coli-APP shuttle vector: RSF1010 replicon; mob orV, Cm’ | Laboratory stock |
| pJFF-ompW | pJFF224-XN carrying the intact ompW | This work |

**Determination of the Effect of the OmpW Gene Deletion on A. pleuropneumoniae Biofilm Formation**

The A. pleuropneumoniae and the ΔompW biofilm formation were as described previously with minor modifications (30). Briefly, an overnight bacterial culture was transferred to fresh TSB supplemented with 10% FBS and 10 µg/mL NAD at a 1:100 (V/V) dilution. Then a 100 µL bacterial suspension was added to the wells of a 96-well microplate and incubated for 12 h, 24 h, 36 h, and 48 h at 37°C. The bacterial culture liquid was removed, each well washed with PBS, and 100 µL of Crystal Violet dye solution (0.1%) added for 30 min. The wells were washed with PBS to remove unbound crystal violet dye and dried for 5 h at 37°C. Then 100 µL of a glacial acetic acid solution (33%, v/v) was added to each well and the microplate was shaken for 1 h. The absorbance at 590 nm was measured.

**Oxidative Tolerance Test**

Stress resistance tests were conducted using the A. pleuropneumoniae, the ΔompW, and CΔompW strains. These strains were grown in TSA supplemented with 10% FBS and 10 µg/mL NAD at 37°C overnight. The bacterial were then transferred to fresh TSB supplemented with 10% FBS and 10 µg/mL NAD at a 1:100 (V/V) dilution for logarithmic growth. At an OD600 value of approximately 0.6, cells from 1 ml of broth cultures were centrifuged at 5000 g for 5 min. For the oxidative tolerance test, the cells were resuspended in 1 ml of TSB supplemented with 10% FBS, 10 µg/mL NAD and 1 mM hydrogen peroxide for 10 min or 20 min, respectively. The control samples of each strain were resuspended in 1 ml of TSB supplemented with 10% FBS and 10 µg/mL NAD without any treatment. Then, the cultures from oxidative stress resistance test were serially diluted in TSB, and spread on TSB plates supplemented with 10% FBS and 10 µg/mL NAD for CFU counting. Oxidative stress resistance was calculated as ((oxidative stressed sample CFU mL⁻¹)/(control sample CFU mL⁻¹)) × 100. The experiments were carried out in triplicate (20).

**Determination of the Effect of the OmpW Gene Deletion on the Bacterial Morphology of A. pleuropneumoniae by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)**

The bacterial morphologies of the A. pleuropneumoniae and the ΔompW strains were examined by SEM and TEM as described previously with minor modifications (31, 32). Briefly, the strains were cultured to logarithmic and steady state phases of growth. The bacteria were collected, washed three times with PBS, and fixed with 4% glutaraldehyde. Specimens were dehydrated, dried, and sprayed with gold. Bacterial morphologies were observed with a HITACHI SU8010 scanning electron microscope (Hitachi, Japan). For TEM, the strains were fixed with 0.1 M cacodylate buffer containing 5% glutaraldehyde and 0.15% ruthenium red at 37°C for 4 h and then treated with 1 mg/mL polycationic ferritin. Thin sections were prepared with a Tecnai G² 20 TWIN transmission electron microscope (FEI, Portland, OR, USA).

**Determination of the Effect of the OmpW Gene Deletion on A. pleuropneumoniae Antibiotic Susceptibility**

The minimal inhibitory concentration (MIC) of the A. pleuropneumoniae, ΔompW, and CΔompW strains for 19 antibiotics (rifampicin, Ofloxacin, polymyxin B, vancomycin, acriflavine, cefalexin, lincomycin, gentamicin, kanamycin, neomycin, penicillin, amoxicillin, tetracycline, streptomycin, vibramycin, enroflaxacin, florfenicol, sulmethoxazole, and ceftiofur sodium; Yuanye biology Co., Ltd, China) was determined as described previously with minor modifications (33). Briefly, 50 µL of a TSB culture supplemented with 10% FBS and 10 µg/mL NAD of each strain were added to 96-well plates. The appropriate concentration of antibiotics was diluted 12 times continuously, and then 50 µL of diluted antibiotics were added to the 96 well plate to make the final concentration of 0.125 to 256 µg/mL or 0.015625 to 32 µg/mL. Bacterial concentration was adjusted to a 0.5 Macbeth turbidity using 5 mL of sterile broth.
normal saline. Then 100 µL of the bacterial suspension was added to 10 mL TSB supplemented with 10% FBS and 10 µg/mL NAD for measurement. The positive control (with bacteria and without drugs) and the negative control (without bacteria and drugs) were assessed by adding 50 µL of the tested bacterial solution to each antibiotic well. The tested bacterial solution was cultured at 37°C for 24 h. The highest concentration without bacterial growth was observed as the MIC of the antibiotic and recorded.

### RT-PCR Determination of the Effect of the *ompW* Gene Deletion on Genes Involved in the Ribosome Synthesis Pathway and the ABC Transporter Pathway

To explore the effect of the *ompW* gene deletion on the expression levels of chosen genes, total RNA of bacteria was extracted by TRIzol reagent (Invitrogen, USA) (34). The strains cultured in TSB supplemented with 10% fetal bovine serum and

### TABLE 2 | Primers used in this study.

| Gene   | Primer          | Sequence                     |
|--------|-----------------|------------------------------|
| kdsB   | kdsB-F          | 5’-CAATCCGAATGCCGTCAAA-3’    |
|        | kdsB-R          | 5’-CGGCACAGAGAAATAGG-3’      |
| rpmA   | rpmA-F          | 5’-GGTTCAACTGTTAOGTGG-3’     |
|        | rpmA-R          | 5’-TTTCTGGGCTTTGCTTAAA-3’    |
| rpmB   | rpmB-F          | 5’-AGAGTTGCGCAAGTACCCGG-3’   |
|        | rpmB-R          | 5’-ACGCTACCTTCCGCGTTA-3’     |
| xyIG   | xyIG-F          | 5’-TCGTTTATCCGCTGATACCC-3’   |
|        | xyIG-R          | 5’-AACCGAAGATCATTGCCGC-3’    |
| modA   | modA-F          | 5’-CCGCGACCTTCAAATTTGC-3’    |
|        | modA-R          | 5’-GTTTGGACACCGGATTGGA-3’    |
| rpmT   | rpmT-F          | 5’-AGAGCAAGGCATAAGAAAGTG-3’  |
|        | rpmT-R          | 5’-AGCCGTGGATATTCTGT-3’      |
| rpsA   | rpsA-F          | 5’-GCTCTTCTCACTGCGATT-3’     |
|        | rpsA-R          | 5’-GAACACCTCGACGACTCTCA-3’   |
| malK_1 | malK_1-F        | 5’-CGCTTAATAAGTCCTGCC-3’     |
|        | malK_1-R        | 5’-TAATCTGGCGACGCCAACG-3’    |
| phnS_2 | phnS_2-F        | 5’-TCGTTATTGCCCTTCTTTC-3’    |
|        | phnS_2-R        | 5’-TTACGGTTCTGCTTCGACC-3’    |
| rpsC   | rpsC-F          | 5’-AATCTTAGACACGCGTTC-3’     |
|        | rpsC-R          | 5’-CCGACGGTCAAGTTGAAAC-3’    |
| rplU   | rplU-F          | 5’-AACCTTAGGCCACTGCTT-3’     |
|        | rplU-R          | 5’-ACCACCTGGATGACCTTCTT-3’   |
| fhuB   | fhuB-F          | 5’-GGATTTTCAGCCTGGCACC-3’    |
|        | fhuB-R          | 5’-AATCTGGCGACGCCAACG-3’     |
| PROKKA_00896 | PROKKA_00896-F | 5’-TTACTGGCGAAACTGAGCG-3’   |
|        | PROKKA_00896-R  | 5’-ACGTACCTCGCCGAAATAT-3’    |
| rplC   | rplC-F          | 5’-TCCGTACCAGAGTGGCTTACC-3’  |
|        | rplC-R          | 5’-CGCTAGCATCTACAAGAAC-3’    |
| ompW   | ompW-U         | 5’-TTTCTCTGGTTGCTAATTCCG-3’  |
|        | ompW-R          | 5’-TCTATAAACACTTTGCTAATTTGCTA-3’ |
| ompWDF | ompWDF-F       | 5’-GAAAAATTAAGGCTGAATATTTTGGAAACTGTTGAGAA-3’ |
|        | ompWDF-R       | 5’-TATTAGCCGTGGCTATCCAGG-3’  |
| pEompWU | pEompWU-F     | 5’-ATTGCCGTCGGCCTTCTCTCTTGGCTATCCAGG-3’ (Not I) |
|        | pEompWU-R      | 5’-ACGCGTCAGTATTAGGCCTCTGTCCGA-3’ (Bam I) |
| ompWMF | ompWMF-F       | 5’-GGCTTCTGACATTTGG-3’       |
|        | ompWMF-R       | 5’-GGCTCCCTTCTCTCTTACAGG-3’  |
| ompWMR | ompWMR-F       | 5’-GCCTTCTCTTTGGTGCTCCAA-3’  |
|        | ompWMR-R       | 5’-GCCTTCTCTTTGGTGCTCCAA-3’  |
| ompW   | ompW-F         | 5’-GCCTGCGATCTGCGAAAACGATTAAGG-3’ (BamHI) |
|        | ompW-R         | 5’-CTAGCTAGATTTAGTATGCTAAATCCCTG-3’ (Bam I) |
| PJompWF | PJompWF-F   | 5’-AGACCTCCAGAGTATCGG-3’     |
|        | PJompWF-R      | 5’-CATTTTGCTGATTTGGC-3’      |
10 µg/mL NAD. The RNA was reverse-transcribed to cDNA with a PrimeScript™ II 1st Strand cDNA kit (Takara, Dalian, China). Amplification of cDNA was carried out using the SYBR Premix Ex Taq kit (Takara, Dalian, China). The rpmA, rpmB, xylG, modA, rplT, rpsA, malK_1, phnS_2, rpsC, rplU, fhuB, prokka_00896, rplC genes were analyzed and the kdsB gene encoding 3-deoxy-manno-octulosonate cytidylyltransferase was used as the internal control (35). The primers used for RT-PCR are found in Table 2.

**Statistical Analysis**

Experimental data are presented as means ± SD. The difference between two groups was analyzed by the two-tailed Student's t-test. P values of < 0.05 were considered significant, *p < 0.05 and **p < 0.01.

**RESULTS**

**Construction of the OmpW Gene Deletion Mutant, ΔompW, and the Complemented Strain of the OmpW Gene Deletion Mutant, CΔompW**

The ompW gene deletion mutant, ΔompW, was constructed by homologous recombination and verified by PCR (Figure 1A). The CΔompW strain was confirmed with PJompW/PompWR (Figure 1A).

The OmpW Gene Deletion Did Not Affect the Growth of A. pleuropneumoniae in vitro

The in vitro effect of the ompW gene deletion on 8h growth was assessed for the A. pleuropneumoniae strain, the ΔompW mutant strain, and the CΔompW strain. Results showed the growth kinetics of the A. pleuropneumoniae, the ΔompW mutant strain, and the CΔompW strain to not differ significantly for this incubation period (Figure 1B).

**Deletion of the OmpW Gene Did Not Affected Biofilm Formation by A. pleuropneumoniae in vitro**

We explored the effect of ompW gene deletion on biofilm formation of A. pleuropneumoniae and ΔompW using a quantitative assay. The results showed that deletion of ompW gene reduced the biofilm formation ability of A. pleuropneumoniae at 12h to 48h, but the difference is not significant (Figure 2).
The Effect of the OmpW Gene Deletion on the Bacterial Morphology of *A. pleuropneumoniae* During Logarithmic and Steady State Growth

SEM and TEM morphologies were assessed for the *A. pleuropneumoniae* strain, the ΔompW mutant strain, and the CΔompW strain during logarithmic and steady state growth. There were no significant morphological differences for any of the strains during the logarithmic growth phase as judged by SEM (Figure 3A). However, for steady growth, the cell surface of the ΔompW mutant became rough, while the surfaces of the *A. pleuropneumoniae* strain and the CΔompW strain were smooth by SEM (Figure 3A), which indicated absence of the *ompW* gene resulted in a rough surface for *A. pleuropneumoniae* during steady growth. These surface morphologies were confirmed by TEM (Figure 3B).

OmpW Is Required for *A. Pleuropneumoniae* Oxidative Stress Tolerance

The *A. pleuropneumoniae*, ΔompW, and CΔompW strains were exposed to oxidative stress conditions. When the strains were treated with 1 mM hydrogen peroxide for 10 min, the ΔompW strain survival rate was 0.21%, which was much lower than that of the *A. pleuropneumoniae* strain (0.64%) and the CΔompW strain (0.58%; Figure 4). When the strains were treated with 1 mM hydrogen peroxide for 20 min, the ΔompW strain survival rate was 0.18%, which was lower than that of the *A. pleuropneumoniae* strain (0.46%) and the CΔompW strain.
Deletion of OmpW

**Deletion of OmpW Affected Susceptibility for Polymyxin B, Kanamycin, and Penicillin**

The *A. pleuropneumoniae*, ΔompW, and CΔompW strains were assessed for bacterial susceptibility to; rifampicin, ofloxacin, polymyxin B, vancomycin, acriflavin, cefalexin, lincomycin, gentamicin, kanamycin, neomycin, penicillin, amoxicillin, tetracycline, streptomycin, vibramycin, enrofloxacin, florenchol, sulfamethoxazole, and ceftiofur sodium. As judged by MIC, deletion of the ΔompW gene did not affect bacterial susceptibility to most assessed antibiotics (Table 3). However the deletion resulted in two times sensitive to polymyxin B, kanamycin, and penicillin (Table 3).

**Effect of the OmpW Gene Deletion on Ribosome Synthesis Related and ABC Transporter Gene Expression**

The effect of the *A. pleuropneumoniae* and ΔompW strains on ribosome synthesis related genes and ABC transporter genes at the mRNA level was determined by RT-PCR. Three genes, rpmA, rpmB, and rplT were significantly upregulated and three genes, rpsC, rplU, and rplC were significantly downregulated within the ribosome synthesis pathway (Figure 5). For the ABC transporter pathway, deletion of the ompW gene upregulated xylG, and modA, while phnS_2, PROKKA_00896, and flhuB were downregulated (Figure 5).

**DISCUSSION**

*A. pleuropneumoniae* is considered one of the most important bacterial respiratory pathogens of pigs with epidemic disease resulting in serious economic loss (36). There are many studies on the infection mechanism and the resistance to environmental pressure of *A. pleuropneumoniae*, but there are still many unknowns to be researched. In this study, we explored the role of the *A. pleuropneumoniae* ompW gene in biofilm formation, resistance to environmental pressure and gene regulation, with the concept that this exploration may provide a novel new strategy for the control of *A. pleuropneumoniae* infections.

Biofilms are a complex community of microorganisms thought to be a sessile mode of life that permits attachment and growth of microorganisms on surfaces (37). Microorganisms comprising biofilms are highly resistant to antibiotics and are capable of prolonged persistence within a host (38), and are involved in virulence and pathogenicity (39). Device-related and chronic infections are often associated with biofilm formation (40). *A. pleuropneumoniae* can produce biofilm that confers bacterial resistance to antibiotics and increased pathogenicity (41–43). Interestingly, the transcription of ompW gene and the biofilm formation of *A. pleuropneumoniae* were inhibited by zinc (44). The transcription of ompW gene of *A. pleuropneumoniae* is down regulated under iron restriction (45). Studies have shown that ompW gene is involved in the protection of bacteria against various forms of environmental pressure. The OmpW was related to oxidative stress and that ompW gene transcription and expression were up-regulated in cultures containing high NaCl concentrations. Deletion of ompW gene did not affect the cell morphology of *V. cholerae* (22). Furthermore, iron regulates OmpW by binding to Fur and SoxS, a transcriptional factor involved in oxidative stress, was found to negatively regulate OmpW of *E. coli*

**TABLE 3 | Determination of MICs for the three bacterial strains.**

| Antibiotic (μg/mL) | APP | ΔompW | CΔompW |
|--------------------|-----|-------|--------|
| Rifampicin         | 0.5 | 0.5   | 0.5    |
| Ofloxacin          | 0.03125 | 0.03125 | 0.03125 |
| Polymyxin B        | 8   | 4     | 4      |
| Vancomycin         | 128 | 128   | 128    |
| Acriflavin         | 2   | 2     | 2      |
| Cefalexin          | 2   | 2     | 2      |
| Lincomycin         | 16  | 16    | 16     |
| Gentamicin         | 32  | 32    | 32     |
| Kanamycin          | 64  | 32    | 64     |
| Neomycin           | 128 | 128   | 128    |
| Penicillin         | 0.5 | 0.25  | 0.5    |
| Amoxicillin        | 1   | 1     | 1      |
| Tetracycline       | 0.5 | 0.5   | 0.5    |
| Streptomycin       | 128 | 128   | 128    |
| Vibramycin         | 0.5 | 0.5   | 0.5    |
| Enrofloxacin       | 0.015625 | 0.015625 | 0.015625 |
| Florenchol         | 1   | 1     | 1      |
| Sulfamethoxazole   | 256 | 256   | 256    |
| Ceftiofur sodium   | 0.03125 | 0.03125 | 0.03125 |

| Figure 4 | Impaired oxidative stress tolerance of the A. pleuropneumoniae strain, the ΔompW mutant strain, and the CΔompW strain. Overnight cultures were inoculated into fresh TSB supplemented with 10% FBS and 10 μg/mL NAD and grown to an OD600 value of approximately 0.6. Cells were then treated with 1 mM hydrogen peroxide for 10 min or 20 min, respectively. The experiment has at least three biological repetitions. Results are expressed as means ± SD of three independent experiments. ** indicates significance at p < 0.05 and *** indicates significance at p < 0.01.

(0.44%). These results suggest that OmpW has a role in the tolerance of *A. pleuropneumoniae* to oxidative stress.
FIGURE 5 | RT-PCR determination of the expression levels of ribosome synthesis related genes and ABC transporter genes following ompW gene deletion. Total RNA was extracted from bacterial strains and reverse-transcribed to cDNA. Amplification of cDNA was carried out using SYBR Premix Ex Taq kit. The experiment has at least three biological repetitions. Results are expressed as means ± SD of three independent experiments. ‘*’ indicates significance at $p < 0.05$ and ‘**’ indicates significance at $p < 0.01$. 

In this study, the ompW gene was demonstrated to protect A. pleuropneumoniae from oxidative stress and affect the cell morphology during steady growth. Whether the ompW gene is involved in A. pleuropneumoniae virulence requires further study.

Ribosomes are essential to cellular protein production (46). Impairment or abnormalities in the ribosome biogenesis pathways have been related to disease (47). ATP-binding cassette (ABC) transporters use energy derived from ATP hydrolysis for molecular transport across membranes (48) and are involved in many physiological and pathological processes (49). In this study, the ribosome synthesis and ABC transporter pathways were found to have significant involvement. The genes rpmA, rpmB, and rplT were significantly upregulated and rpsC, rpuU, and rplC were significantly downregulated for the ribosome synthesis pathway. The genes xylG, and modA were upregulated and phnS_2, PROKKA_00896, fhuB were downregulated for the ABC transporter pathway. These genes were chose by our previous RNA-seq results (not mentioned in this study). Research have been shown that these genes have important roles involved in the pathogenicity of A. pleuropneumoniae. Thus, we speculate that deletion of the ompW gene affected protein synthesis and ion transport, which may affect the toxicity and drug sensitivity of A. pleuropneumoniae to a certain extent. ompW gene deletion did affect bacterial susceptibility to polymyxin B, kanamycin, and penicillin. The effect on bacterial susceptibility to polymyxin B, kanamycin, and penicillin may also be due to the change of cell membrane permeability. However, the mechanistic basis for these observations requires further investigation.

In summary, data suggested that ompW gene deletion did not affect the growth of A. pleuropneumoniae, but did affect; morphology during steady growth, oxidative tolerance, and bacterial susceptibility to polymyxin B, kanamycin, and penicillin. The ompW gene deletion also altered the expression of ribosome synthesis and ABC transporter related genes. Thus, the ompW gene may regulate the biological phenotype of A. pleuropneumoniae during A. pleuropneumoniae infections.

DATA AVAILABILITY STATEMENT

The authors acknowledge that the data presented in this study must be deposited and made publicly available in an acceptable repository, prior to publication. Frontiers cannot accept a manuscript that does not adhere to our open data policies.

AUTHOR CONTRIBUTIONS

XC, WL, and RZ: designed the experiments. XC, ZS, and LW: performed the experiments. XC, BH, WY, JC, EJ, QH, LL, JX, HL, HZ, YW, WL, and RZ: analyzed the data. XC: wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.846322/full#supplementary-material
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