



The CpxA/CpxR Two-Component System Affects Biofilm Formation and Virulence in *Actinobacillus pleuropneumoniae*

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Gram-negative bacteria have evolved numerous two-component systems (TCSs) to cope with external environmental changes. The CpxA/CpxR TCS consisting of the kinase CpxA and the regulator CpxR, is known to be involved in the biofilm formation and virulence of *Escherichia coli*. However, the role of CpxA/CpxR remained unclear in *Actinobacillus pleuropneumoniae*, a bacterial pathogen that can cause porcine contagious pleuropneumonia (PCP). In this report, we show that CpxA/CpxR contributes to the biofilm formation ability of *A. pleuropneumoniae*. Furthermore, we demonstrate that CpxA/CpxR plays an important role in the expression of several biofilm-related genes in *A. pleuropneumoniae*, such as *rpoE* and *pgaC*. Furthermore, The results of electrophoretic mobility shift assays (EMSAs) and DNase I footprinting analysis demonstrate that CpxR-P can regulate the expression of the *pgaABCD* operon through *rpoE*. In an experimental infection of mice, the animals infected with a *cpxA/cpxR* mutant exhibited delayed mortality and lower bacterial loads in the lung than those infected with the wildtype bacteria. In conclusion, these results indicate that the CpxA/CpxR TCS plays a contributing role in the biofilm formation and virulence of *A. pleuropneumoniae*.

Keywords: *Actinobacillus pleuropneumoniae*, CpxA/CpxR, biofilm, *rpoE*, virulence

INTRODUCTION

Actinobacillus pleuropneumoniae is a species of Gram-negative bacteria that causes porcine contagious pleuropneumonia (PCP), which is characterized by fibrinous pleurisy and fibrinous pneumonia. Each year this pathogen causes substantial economic damages in the pork industry globally (Zimmerman et al., 2012). Previous studies have discovered many virulence factors of *A. pleuropneumoniae* (Fuller et al., 2000; Sheehan et al., 2003). These virulence factors, such as Apx toxins, capsule, adhesins, transferrin-binding proteins, and outer membrane proteins, play crucial roles in the pathogenicity of *A. pleuropneumoniae* (Chiers et al., 2010).

To increase their adaptability, bacteria generally utilize two-component signal transduction systems (TCSs) to perceive and respond to environmental influences. A representative TCS consists of a histidine kinase (HK) in the inner membrane and a response regulator (RR) in

the cytoplasm (Buelow and Raivio, 2010). After perceiving an environmental signal, the HK subsequently autophosphorylates and transfers the same phosphate group to the RR, after which the RR can influence the transcription level of downstream genes by binding to their promoter regions (Vogt and Raivio, 2012). Whole genome sequencing of *A. pleuropneumoniae* serovar 3 strain JL03 revealed five putative TCSs: ArcA/ArcB, QseB/QseC, CpxR/CpxA, NarP/NarQ, and PhoB/PhoR (Xu et al., 2008).

When it was initially characterized, the TCS CpxA/CpxR was viewed as a newly discovered envelope stress response system. Experimental evidence in *Escherichia coli* then provided a model: when misfolded envelope proteins accumulate, CpxA autophosphorylates and then transfers the phosphate group to CpxR, which causes the upregulation of a series of chaperonins and proteases that can degrade or refold the misfolded proteins, thus alleviating the envelope stress (Vogt and Raivio, 2012). In recent years, the CpxA/CpxR system has been demonstrated to be involved in the virulence of uropathogenic *Escherichia coli* (Debnath et al., 2013), *Salmonella enterica* serotype Typhimurium (Humphreys et al., 2004), and *Vibrio cholerae* (Acosta et al., 2015). The CpxA/CpxR system has also been shown to be involved in the biofilm formation of *E. coli* (Dorel et al., 1999; Ma and Wood, 2009; Dudin et al., 2014). In addition, the CpxA/CpxR system has been implicated in antibiotic resistance (Mahoney and Silhavy, 2013).

Biofilms are aggregated bacterial groups that are embedded in a matrix of extracellular polymeric substances (Donlan and Costerton, 2002). Biofilm formation of *A. pleuropneumoniae* *in vitro* (such as on polystyrene microtiter plates) depends on the lipopolysaccharides (LPS), the capsular polysaccharides (CPS) and the yields of β -1,6-N-acetyl-D-glucosamine (PGA) polymer (Kaplan et al., 2004; Hathroubi et al., 2015b). In *A. pleuropneumoniae*, the production of PGA depends on the expression of the *pgaABCD* operon, which is repressed by the protein H-NS and upregulated by the alternative sigma factor σ^E (Bosse et al., 2010). Kaplan et al. found that only two of the fifteen *A. pleuropneumoniae* reference strains, including 5b and 11, were able to form a pronounced biofilm on polystyrene microtiter plates (Kaplan and Mulks, 2005). However, Hathroubi and colleagues found that when cultured in the presence of sub-minimum inhibitory concentration of penicillin G, many *A. pleuropneumoniae* strains can form pronounced biofilms and this is likely the result of a cell envelope stress sensed by the CpxRA system resulting in an increased production of PGA and other matrix components (Hathroubi et al., 2015a). Several studies have also proposed that CpxA/CpxR may be involved in the mechanism of biofilm formation in *A. pleuropneumoniae* (Tremblay et al., 2013; Hathroubi et al., 2015b). However, these studies did not use a deletion mutant to confirm the phenotype directly.

Although previous work has demonstrated that CpxA/CpxR plays an important role in biofilm formation and virulence in *E. coli* and other bacteria, the role of CpxA/CpxR in *A. pleuropneumoniae* remained unclear. In this study, we found new growth conditions that induce biofilm formation in *A. pleuropneumoniae* (i.e., 42°C, TSB, static). Based on this new system, we investigated the role of CpxA/CpxR in biofilm

formation, focusing on the *rpoE* gene and its relationship to the expression of the *pga* operon. We also assessed whether or not CpxA/CpxR contributes to the virulence of *A. pleuropneumoniae* serotype 1 strain S4074.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The bacterial strains, primers, and plasmids used in this study are listed in **Table 1**. *A. pleuropneumoniae* strains were cultured at 37°C or 42°C in tryptic soy broth (TSB; Difco Laboratories, Detroit, MI, USA) or on tryptic soy agar (TSA; Difco Laboratories) supplemented with nicotinamide adenine dinucleotide (10 $\mu\text{g mL}^{-1}$) and 10% (v/v) newborn calf serum. Additionally, *A. pleuropneumoniae* strains were also cultured in brain heart infusion broth (BHIB; Oxoid Ltd., Hampshire,

TABLE 1 | Bacterial strains and plasmids used in this study.

Strains/ plasmids	Characteristics	Source/ references
<i>A. pleuropneumoniae</i>		
S4074	<i>A. pleuropneumoniae</i> reference strain of serovar 1; WT strain	Dr P. Blackall
ΔcpxAR	<i>A. pleuropneumoniae</i> 4074 <i>cpxAR</i> -deletion mutant	This study
$\text{C}\Delta\text{cpxAR}$	Complemented strain of ΔcpxAR ; Cm^r	This study
<i>E. coli</i>		
DH5a	Cloning host for recombinant vector	Takara
BL21(DE3)	The expression host for pET-28a and their derivative	Takara
β 2155	Transconjugation donor for constructing mutant strain	From Prof. Gerald-F. Gerlach
PLASMID		
pMD18-T	T-vector; Amp^r	Takara
pMD18-T- <i>cpxAR</i>	pMD18-T carrying <i>cpxAR</i> genes	This study
pMD19-T	T-vector; Amp^r	Takara
pMD19-T- <i>rpoE</i>	pMD19-T carrying the promoter region of <i>rpoE</i>	This study
pEMOC2	Transconjugation vector: ColE1 ori mob RP4 sacB, Amp^rCm^r	From Prof. Gerald-F. Gerlach
p ΔcpxAR	Up- and down-stream arms of <i>cpxAR</i> were ligated sequentially into pEMOC2, and used as the transconjugation vector for <i>cpxAR</i> gene deletion	This study
pJFF224-XN	<i>E. coli</i> -APP shuttle vector: RSF1010 replicon; mob oriV, Cm^r	Frey, 1992
p ΔcpxAR	pJFF224-XN carrying the intact <i>cpxAR</i>	This study
pET-28a	Expression vector; Kan^r	Novagen
pET- <i>cpxR</i>	pET-28a carrying <i>cpxR</i> gene	This study
pET- <i>rpoE</i>	pET-28a carrying <i>rpoE</i> gene	This study

Cm^r , Chloramphenicol resistance; Amp^r , Ampicillin resistance; Kan^r , Kanamycin resistance.

United Kingdom) or on brain heart infusion agar (BHIA; Oxoid Ltd.) supplemented with $15 \mu\text{g mL}^{-1}$ NAD (Solarbio, Beijing, China) at 37°C . For the selection of *A. pleuropneumoniae* transformants, chloramphenicol ($5 \mu\text{g mL}^{-1}$) was added. When culturing different *E. coli* strains, appropriate antibiotics were added in Luria-Bertani (LB) broth or agar (Haibo, Qingdao, China). For cultivation of *E. coli* β 2155, diaminopimelic acid (dapA; $50 \mu\text{g mL}^{-1}$) (Sigma-Aldrich, St. Louis, USA) was added to LB medium (Yuan et al., 2014).

Construction of the *A. pleuropneumoniae* Mutant ΔcpxAR and Complement $\text{C}\Delta\text{cpxAR}$

To construct a *cpxA/cpxR* (*cpxAR*) gene deletion mutant, the upstream and downstream fragments of these two genes were amplified from the genomic DNA of *A. pleuropneumoniae* strain S4074 (wildtype, WT) using primers *cpxAR*-up F/R or *cpxAR*-down F/R. These PCR products were cloned sequentially into the suicide vector pEMOC2 to generate plasmid pE ΔcpxAR . The mutant ΔcpxAR was then constructed using the plasmid pE ΔcpxAR as previously described (Liu et al., 2015).

For complementation studies, the intact *cpxAR* genes were cloned with their promoters from S4074 using primers *cpxAR*-F and *cpxAR*-R. The entire fragment was then ligated into the T vector pMD18-T, generating plasmid pMD18-T-*cpxAR*. For constructing complementation plasmid pC ΔcpxAR , the 2.3-kb fragment from pMD18-T-*cpxAR* containing the *cpxAR* genes was ligated into *E. coli*-*A. pleuropneumoniae* shuttle vector pJFF224-XN via *Pst*I-*Not*I restriction sites (Yuan et al., 2014). Then, pC ΔcpxAR was electroporated (2.5 KV, 25 μFD , 800 Ω) into the mutant strain ΔcpxAR . The correct C ΔcpxAR was selected via its chloramphenicol resistance. When the C ΔcpxAR strain was cultured, $2 \mu\text{g mL}^{-1}$ chloramphenicol was always added in the culture medium.

The resultant mutant strain ΔcpxAR and complement strain C ΔcpxAR were verified by both PCR amplification with primers *cpxAR*-exterior F/R and *cpxAR*-interior F/R and RT-PCR amplification with primers *cpxA*-F/R and *cpxR*-F/R. The sequence accuracy of each strain was verified by DNA sequencing (data not shown).

Biofilm Assay

The microtiter plate biofilm assay is especially advantageous for examining early processes in biofilm formation (Merritt et al., 2005). Overnight inoculums were balanced to the same optical density (OD) with fresh TSB, and $100 \mu\text{L}$ of the 1:100-diluted balanced inoculums were transferred to each well of a 96-well microtiter plate (Corning, USA). Following an incubation of 36, 48, or 60 h at 37°C or 42°C , each well was washed three times with $200 \mu\text{L}$ of sterile PBS to remove slackly adherent cells. To fix the remaining attached bacteria, $100 \mu\text{L}$ of methanol was added to each well. After air-drying, the wells were then stained with $100 \mu\text{L}$ per well of crystal violet (0.1%) for 10 min at room temperature. After removing the crystal violet solution, the wells were washed under running tap water and dried in a 37°C incubator for 30 min. At this point, the

plates were photographed. Last, $100 \mu\text{L}$ per well of glacial acetic acid (33%, v/v) was added, and the OD_{590nm} of each well was monitored by a Multi-Detection Microplate Reader. Furthermore, we also used this method to detect the biofilm formation of *A. pleuropneumoniae* strains grown in BHIB, since this is the common method used inducing biofilm formation in *A. pleuropneumoniae* (Labrie et al., 2010). Both assays were performed in triplicate.

RNA Extraction, RT-PCR, and Real-Time RT-PCR

The WT (S4074), *cpxAR* mutant, and complement strains were grown in TSB in six-well plates for 4 h at 37°C or 42°C . Ice-cold methanol was used to prevent changes in transcript levels after the planktonic cells of each strain were collected (Subashchandrabose et al., 2013). A total of 1 mL of bacterial cells was blended with 1 mL of 100% ice-cold methanol and centrifuged at $10,000 \times g$ for 3 min at 4°C . For real-time RT-PCR (qRT-PCR), total RNA from the WT, mutant, and complement strains was extracted using the Bacteria Total RNA Isolation Kit (Sangon Biotech, China) and was then reverse-transcribed using the HiScript II Q RT SuperMix for qRT-PCR (+gDNA wiper) (Vazyme, China) following the manufacturer's instructions. The real-time PCR experimental method was based on SYBR-Green dye, and all reactions were performed in triplicate. The $10\text{-}\mu\text{L}$ real-time PCR reaction mixtures contained $5 \mu\text{L}$ of $2\times$ SYBR Green Master Mix (Vazyme, China), $0.2 \mu\text{L}$ of Rox Reference Dye 2, $0.2 \mu\text{L}$ each of the forward and reverse primers ($10 \mu\text{M}$), $1 \mu\text{L}$ of template cDNA, and $3.4 \mu\text{L}$ of ddH₂O. The real-time PCR amplification conditions were as follows: 5 min at 50°C , then 5 min at 95°C , followed by 40 cycles of 10 s at 95°C and 35 s at 60°C . The ViiA™ 7 real-time PCR system was used for qRT-PCR analysis by the $2^{-\Delta\text{Ct}}$ method (Pfaffl, 2001). For normalizing the relative expression of target genes, the 16S rRNA gene was used as an endogenous control (Hathroubi et al., 2015b). The cDNA of WT was also used for RT-PCR by primers *cpxAR*-RT-F/R.

Expression of His-CpxR Protein in *E. coli*

The *cpxR* gene was amplified via PCR from the genomic DNA of *A. pleuropneumoniae* S4074 using primers P*cpxR*-F and P*cpxR*-R (Table 2) and then cloned into the *Bam*HI and *Hind*III sites of prokaryotic expression plasmid pET-28a, generating the recombinant plasmid pET-*cpxR*; the accuracy of the inserted sequence was confirmed by DNA sequencing. The plasmid pET-*cpxR* was transferred into BL21 (DE3) strain and grown at 37°C with energetic shaking in 1 L of LB broth containing $50 \mu\text{g mL}^{-1}$ kanamycin to an OD₆₀₀ of 0.6–0.8. The *E. coli* BL21 (DE3) cells containing the plasmid pET-*cpxR* were then induced by isopropyl- β -D-thiogalactoside (0.25 mM) at 25°C for 5 h. A Low Temperature Ultra-high Pressure Continuous Flow Homogenizer (JNBIO, China) was used to disrupt cells, and the resulting cellular debris and membranes were removed by centrifugation at $12,000 \times g$ for 20 min at 4°C . The recombinant protein was purified by Ni-NTA resin affinity chromatography, as described in the OIAexpress manual (Qiagen, Germany). The purified protein rCpxR was dissolved by elution buffer and was

TABLE 2 | Primers used in this study.

Primers	Sequence (5'-3') ^a	Source or references
FOR MUTANT CONSTRUCTION		
cpxAR-up F/R	CGTCGACCCGTTTCATAATCGTCATAGT CCGTCTAGAGACCGCTTGTCTACTC	This study
cpxAR-down F/R	GGTCTAGAGTTCGTGCAGAGAGCA TGCGGCCGCTTAATCGTTCTTTGT	This study
cpxAR-exterior F/R	CGAACTTACGCTGACG ATGGCGCAATACCCT	This study
cpxAR-interior F/R	CAGTGTAATGCAAGTAAGATAGCG GTCTCCGGAAGAAAATAGCAA	This study
FOR COMPLEMENT CONSTRUCTION		
cpxAR-F/R	AAACTGCAGCAACCTTGATAAAGTTGT AAATT AAGGAAAAAGCGCCGCTTATTCAATC CATAAAGGTAACCT	This study
FOR RT-PCR		
cpxA-F/R	GATTTTGTTCGGCATCGAAT CGGAATTAACCTCGGATCGAA	This study
cpxR-F/R	CCGGAGACTGGTTGGAATAA ATGAAATTGATCGCGTCCCTC	This study
FOR PROTEIN EXPRESSION		
PcpxR-F/R	CGGGATCCATGCCTAGAAATTTACTCGTTG GGGAAGCTTAAGGACTTATTTTTTCAGTAA CGAG	This study
PrpoE-F/R	CGCCATATGATGAGTGAGCTGGTAGCC GATCAAG CGCCCTCGAGAATCTGTTGCATTAGCGG ATTG	This study
FOR EMSA ASSAY		
rpoE-F/R	TAAAAAGATAAGATAAGCGGTC AGTGTGTAACAAAATGAAAAGT	This study
pga-F/R	TAATTAAGACGTCGACTTGCTTTA GCATTCTTAATCGCATAAAGACTAC	This study
FOR RT-PCR AND REAL-TIME PCR		
16SrRNA-F/R	CCATGCCGCGTGAATGA TTCTCGCTACCGAAAGAACTT	Subashchandrabose et al., 2013
pgaC-F/R	GATTCCGCATTGCTCAATCT CAATACCGCATCACCGTCTATG	This study
rpoE-F/R	TTTGATGTTGGGGTCAACT TGCATCAATCGCTTCTCTTG	This study

^aRestriction sites are underlined.

stored at -80°C until use. The expression of His-RpoE was also performed as above described.

Electrophoretic Mobility Shift Assays

The promoter regions of *rpoE* and *pgaABCD* genes were cloned into the T-vector pMD19-T to generate pMD19-T-*rpoE*

and pMD19-T-*pgaABCD*. The promoter regions of *rpoE* and *pgaABCD* were then amplified via PCR from the plasmid using primers M13F-47 (FAM) and M13R-48, generating fluorescent FAM-labeled probes.

The recombinant protein CpxR was phosphorylated by acetyl phosphate (Sigma, USA) (Pogliano et al., 1997). Electrophoretic mobility shift assays (EMSAs) were carried out in 20 μL of reaction buffer (50 mM Tris-HCl (pH 8.0), 2.5 mM MgCl_2 , 100 mM KCl, 2 μg of salmon sperm DNA, 0.2 mM DTT, and 10% glycerol) that contained 40 ng of probe and varied quantities of CpxR or RpoE proteins. After incubation for 30 min at 30°C , the reaction liquid was loaded onto 4% non-denaturing PAGE gels in $0.5\times$ TBE [5.4 g L^{-1} of Tris base, 2.75 g L^{-1} of boric acid, 2 mL L^{-1} of 0.5 M EDTA (pH 8.0)]. The resulting gel was photographed using an ImageQuant LAS 4000 mini system (GE Healthcare, USA).

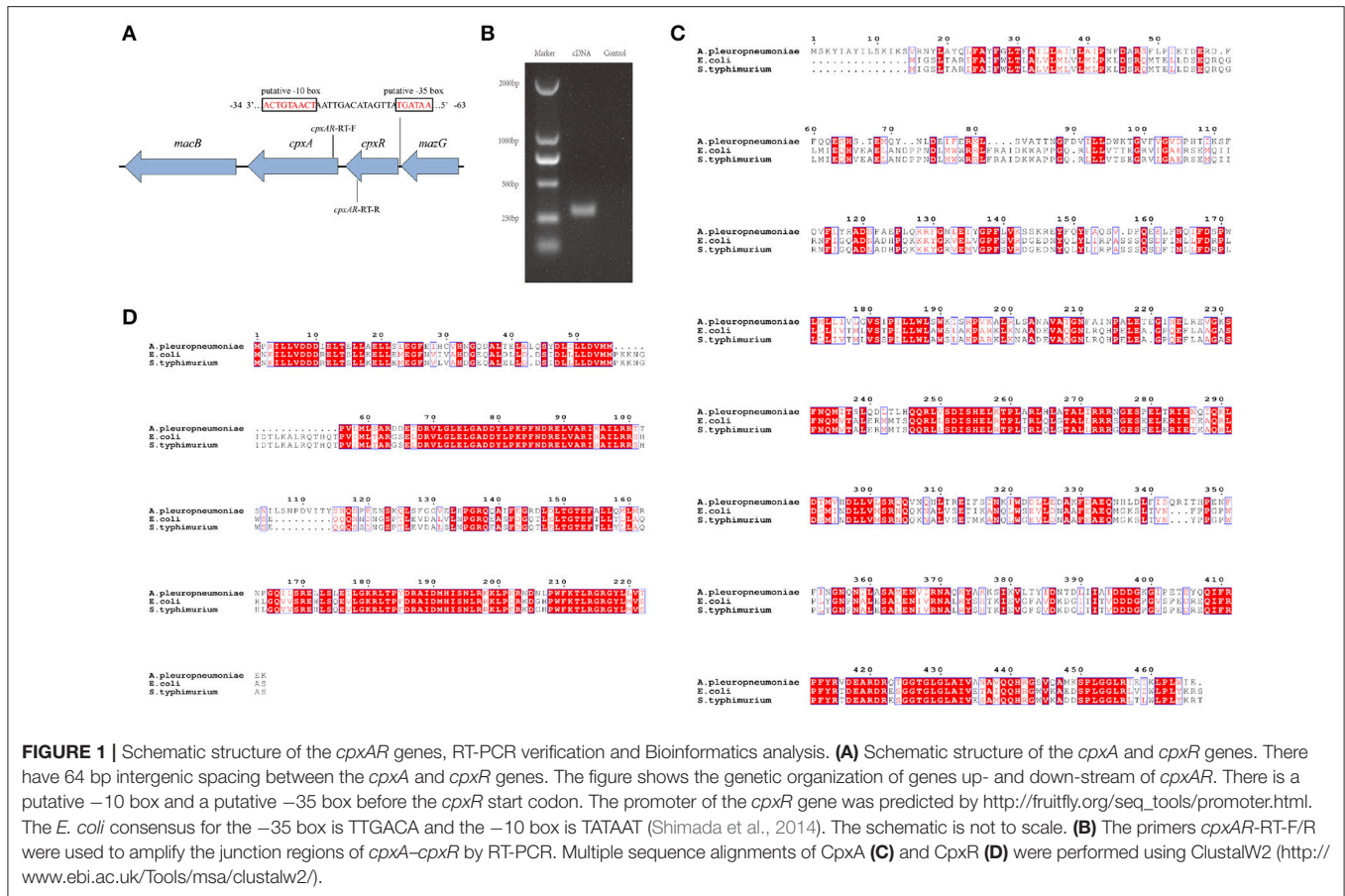
DNase I Footprinting Assay

The DNase I footprinting assay was carried out as previously described (Wang et al., 2012). For each assay, 40 μL of the total reaction mixture contained 400 ng of the probe and different amounts of CpxR. After incubating for 30 min at room temperature, a 10- μL solution [0.015 units of DNase I (Promega, USA) and 100 nmol of freshly prepared CaCl_2] was added to the mixture and incubated for 1 min at room temperature. To stop the reaction, 140 μL of DNase I stop solution, which contained 200 mM unbuffered sodium acetate, 30 mM EDTA, and 0.15% SDS, was added to the mixture. The processing method of samples and the DNA ladder, electrophoresis, and data analysis were all performed as previously described (Wang et al., 2012), except that the GeneScan-LIZ500 size standard (Applied Biosystems, USA) was used in our assay.

Bacterial Virulence *in Vivo*

Six-week-old BALB/c mice were purchased from the Wuhan Institute of Biological Products Co., Ltd. (Wuhan, China). All procedures and handling techniques were approved by the Laboratory Animal Monitoring Committee of Huazhong Agricultural University and were performed accordingly. We equally divided 30 female BALB/c mice into three groups: WT, ΔcpxAR , and $\text{C}\Delta\text{cpxAR}$. Briefly, all strains were cultured in TSB at 37°C , harvested in logarithmic phase ($\text{OD}_{600} = 0.6-0.8$), and washed three times with PBS. For each group of test mice, bacteria at 1×10^7 CFU mouse⁻¹ were injected via the abdominal cavity, while an additional five control mice were injected with same volume of sterile PBS. The clinical signs, such as appetite, dyspnea, and lethargy, and mortality rates for each group were recorded twice daily.

To evaluate the colonization ability of ΔcpxAR in susceptible mouse tissue (lung), another 18 female BALB/c mice were divided into three equal groups (6 mice/group). Groups 1 and 3 were intraperitoneally administered 100 μL of PBS containing the WT strain (5×10^5 CFU mouse⁻¹) or the $\text{C}\Delta\text{cpxAR}$ strain (5×10^5 CFU mouse⁻¹), respectively, while group 2 received a dose of ΔCpxAR (6.5×10^5 CFU mouse⁻¹). After 72 h, the blood of each mouse was removed via cardiac perfusion, and the lung was aseptically removed. Each lung was homogenized (100 mg



weight mL^{-1} of PBS) using a Tissuelyser (Jingxin, Shanghai, China), and 100 μL of the resulting homogenate were processed for determining the CFU counts.

Bioinformatic and Statistical Analyses

The prediction of bacterial promoters was carried out using the tool in http://fruitfly.org/seq_tools/promoter.html. All data are shown as means \pm standard deviation. The statistical analyses of the results were performed using a two-tailed Student's *t*-test or a one- or two-way analysis of variance, and comparisons with a $p < 0.05$ were considered as statistically significant. The data and analyses were graphed using GraphPad Prism.

RESULTS

Bioinformatics Analysis of the *cpxAR* Genes and RT-PCR Verification

To gain a thorough understanding of the role of CpxA/CpxR in *A. pleuropneumoniae*, we first analyzed the genetic organization of the *cpxAR* operon *in silico*. The results indicate that the *cpxA* and *cpxR* genes form an operon (Figures 1A,B). We also found that the CpxA and CpxR proteins of *A. pleuropneumoniae* each have a relatively high amino acid sequence identity with those of *E. coli* and *Salmonella enterica* serovar Typhimurium (Figures 1C,D). BlastP analysis revealed that

the *A. pleuropneumoniae* CpxA has 41% amino acid sequence identity each with the CpxA from *E. coli* and *Salmonella enterica* serovar Typhimurium, while CpxR has 58 and 59% amino acid sequence identity, respectively, with the CpxR of these two species.

Construction of the $\Delta cpxAR$ Mutant and Complemented Strain $C\Delta cpxAR$

An unmarked, in-frame mutant strain $\Delta cpxAR$ was designed based on the genome sequence of *A. pleuropneumoniae* strain S4074, along with a complemented strain $C\Delta cpxAR$ to confirm that there was no polarity effect in the $\Delta cpxAR$ using plasmid pJFF224-XN. The successful constructions of mutant strain $\Delta cpxAR$ and complemented strain $C\Delta cpxAR$ were confirmed by PCR using primers *cpxAR*-exterior F/R and *cpxAR*-interior F/R (Supplementary Figure 1) and by RT-PCR using primers *cpxA*-F/R and *cpxR*-F/R (Supplementary Figure 2). Furthermore, DNA sequencing results confirmed the sequence accuracy of these two strains (data not shown).

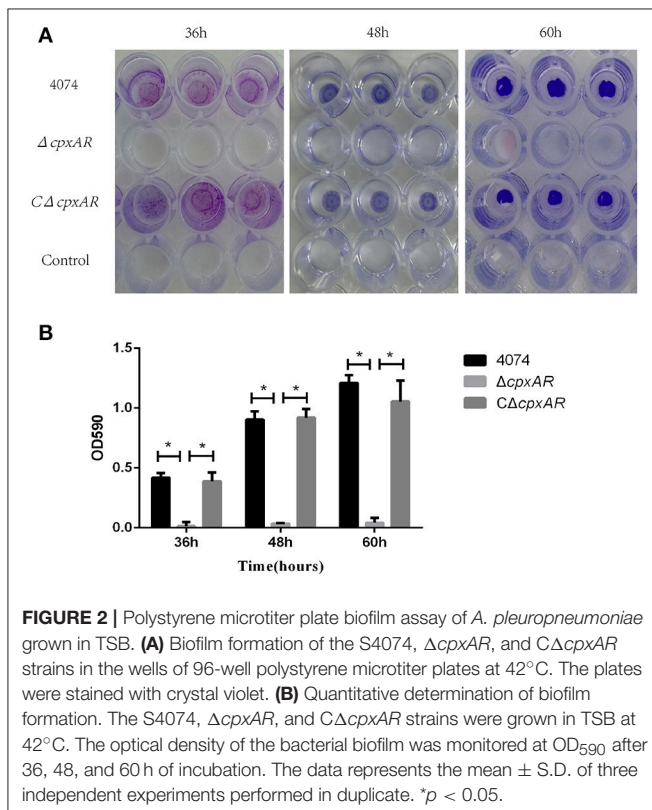
CpxA/CpxR Is Required for Biofilm Formation

No biofilm growth was observed when the WT S4074 strain was grown in TSB at 37°C (Supplementary Figure 3), which confirms the results of previous studies (Kaplan and Mulks,

2005). **Figure 2** illustrates the biofilm formation of WT, $\Delta cpxAR$, and $C\Delta cpxAR$ at 42°C. At this temperature, the WT and $C\Delta cpxAR$ strains formed a pronounced biofilm that increased over time, whereas the mutant strain $\Delta cpxAR$ was unable to form any visible biofilm at 42°C at any time point (**Figure 2A**). A quantitative assay, which monitored the OD₅₉₀, also revealed a significant difference between the $\Delta cpxAR$ mutant strain and the WT and $C\Delta cpxAR$ strains in their abilities to form a biofilm (**Figure 2B**). Additionally, we also compared biofilm formation by the WT and $cpxAR$ mutant strain when the bacteria were grown for 24 h in BHIB at 37°C, which is the most frequently-used culturing condition. The results similarly show that the biofilm formed by the $\Delta cpxAR$ mutant was significantly smaller than those formed by the WT and $C\Delta cpxAR$ strains (**Supplementary Figure 4**).

Growth Analysis of the WT, $\Delta cpxAR$, and $C\Delta cpxAR$ Strains

The growth rates of the WT, $\Delta cpxAR$, and $C\Delta cpxAR$ strains in TSB were analyzed at 37°C and 42°C under shaking and static conditions (**Figure 3**). A lower growth rate was observed with mutant strain $\Delta cpxAR$ compared with the WT and complemented mutant strains at both temperatures. The lack of a growth defect in $C\Delta cpxAR$ demonstrates that CpxA/CpxR slightly affects the growth rate and indicates that there likely were not any polar effects of the mutations or undetected mutations at other genetic loci.



CpxA/CpxR Regulates the mRNA Levels of Some Important Biofilm-Related Genes

Many biofilm-related genes have been identified in recent years, and selected important genes, such as *pgaC* (encoded within the *pgaABCD* operon; Kaplan et al., 2004) and *rpoE* (Bosse et al., 2010) were subsequently studied further. Our results indicate that the *pgaC* and *rpoE* genes were each expressed at lower levels in the mutant strain $\Delta cpxAR$ when grown at 42°C (**Figure 4**). Interestingly, there were also remarkably similar fold-changes in these genes at 37°C, even though $\Delta cpxAR$ was not able to form a biofilm at this temperature. Additionally, these results show that the expression levels of the *pgaC* and *rpoE* genes were each upregulated ($p < 0.05$) in the parental strain S4074 grown at 42°C compared with their expression levels when the bacteria were grown at 37°C.

CpxR Binds to the Promoter Region of the *rpoE* Gene in *A. pleuropneumoniae*

The recognition site of phosphorylated CpxR (CpxR-P) has a conserved sequence GTAAA-(N)₄₋₈-GTAAA (Yamamoto and Ishihama, 2006; Srinivasan et al., 2012; Bernal-Cabas et al., 2015). SDS-PAGE and Western blot results show that the His-CpxR and His-RpoE were successfully expressed in supernatant (**Supplementary Figure 5**). EMSAs revealed that CpxR-P and RpoE clearly bound to the promoter region of the *rpoE* gene and the *pga* operon respectively in a dose-dependent manner, and these binding were abolished by adding excess unlabeled competitor DNA (**Figure 5**). Furthermore, a DNase I footprinting analysis also revealed that the promoter region of *rpoE* had a CpxR-P-binding site (5'-AGTATTTGTAAATAAT-3'), which was 25 bp upstream of the putative transcription start site (**Figure 6A** and **Supplementary Figure 7**). The CpxR-P-binding site partially coincided with the conserved sequence GTAAA-(N)₄₋₈-GTAAA, and overlapped the promoter -35 region (**Figure 6B**). These data show that CpxR-P binds directly to the promoter region of *rpoE*. Taken together, these data support that under this biofilm formation condition (42°C, TSB, static), CpxR-P can regulate the expression of the *pga* operon through *rpoE*.

Virulence of the *cpxAR* Mutant in Mice

The role of CpxA/CpxR in the virulence of *A. pleuropneumoniae* was evaluated *in vivo* in BALB/c mice, which serve as an appropriate model for *A. pleuropneumoniae* infection (Chiang et al., 2009; Seo et al., 2013; Yuan et al., 2014; Wang et al., 2015; Xie et al., 2016). As shown in **Figure 7A**, 90% of the mice in the WT and $C\Delta cpxAR$ groups died within 7 days, but only two mice in the $\Delta cpxAR$ group died within this time frame. Overall, the survival rate of the $\Delta cpxAR$ group was significantly higher than those of the WT and $C\Delta cpxAR$ groups ($p < 0.05$).

As shown in **Figure 7B**, the amount of colonization by the $\Delta cpxAR$ strain in the lungs was significantly lower compared with those by the WT and $C\Delta cpxAR$ strains ($p < 0.001$). This result reveals that the CpxA/CpxR TCS is necessary for *A. pleuropneumoniae* colonization *in vivo*. Taken together, these results indicate that the CpxA/CpxR TCS contributes to the virulence of *A. pleuropneumoniae*.

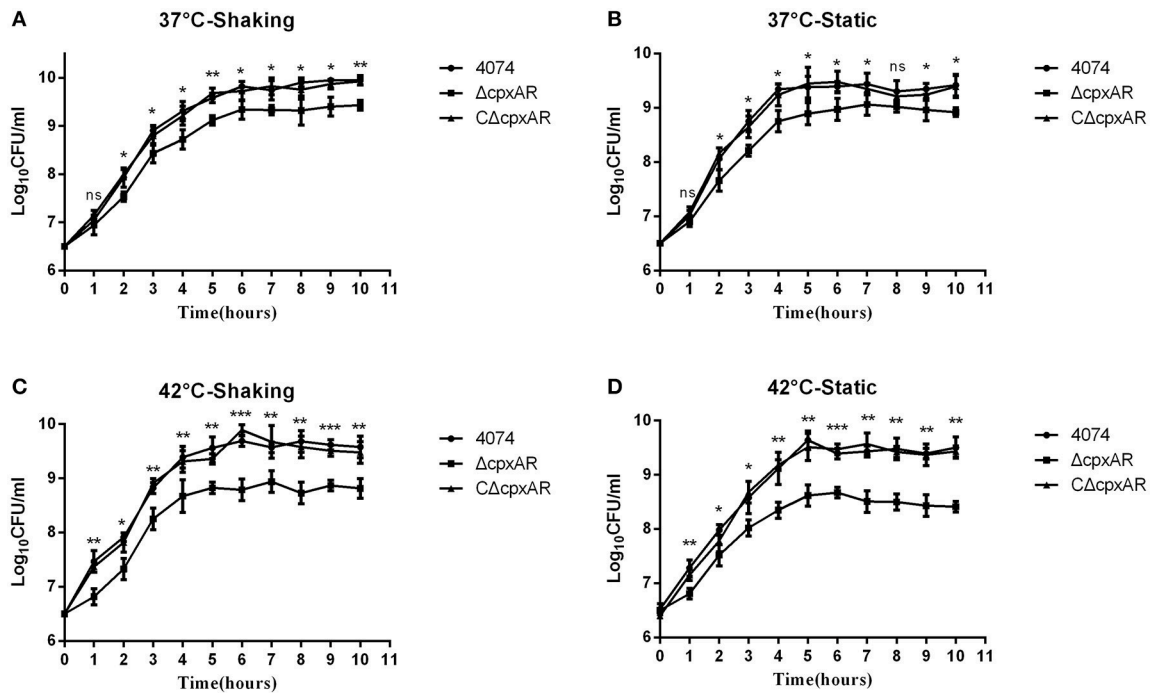


FIGURE 3 | Growth curves of the WT, mutant, and complemented strains of *A. pleuropneumoniae*. Growth curves of the WT (S4074), mutant (Δ cpxAR), and complemented ($C\Delta$ cpxAR) strains of *A. pleuropneumoniae* at 37°C under shaking (A) or static (B) conditions or at 42°C under shaking (C) or static (D) conditions are shown. Bacterial growth was monitored by measurement of viable cell counts expressed as LogCFU/mL. The data represents the mean \pm S.D. of three independent experiments performed in duplicate. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

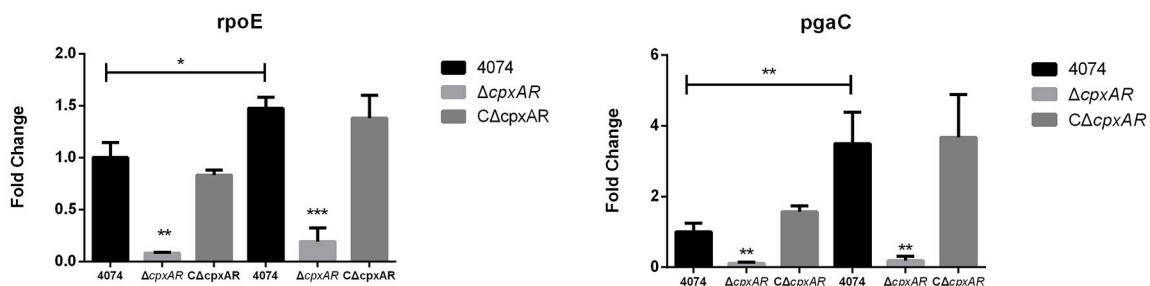


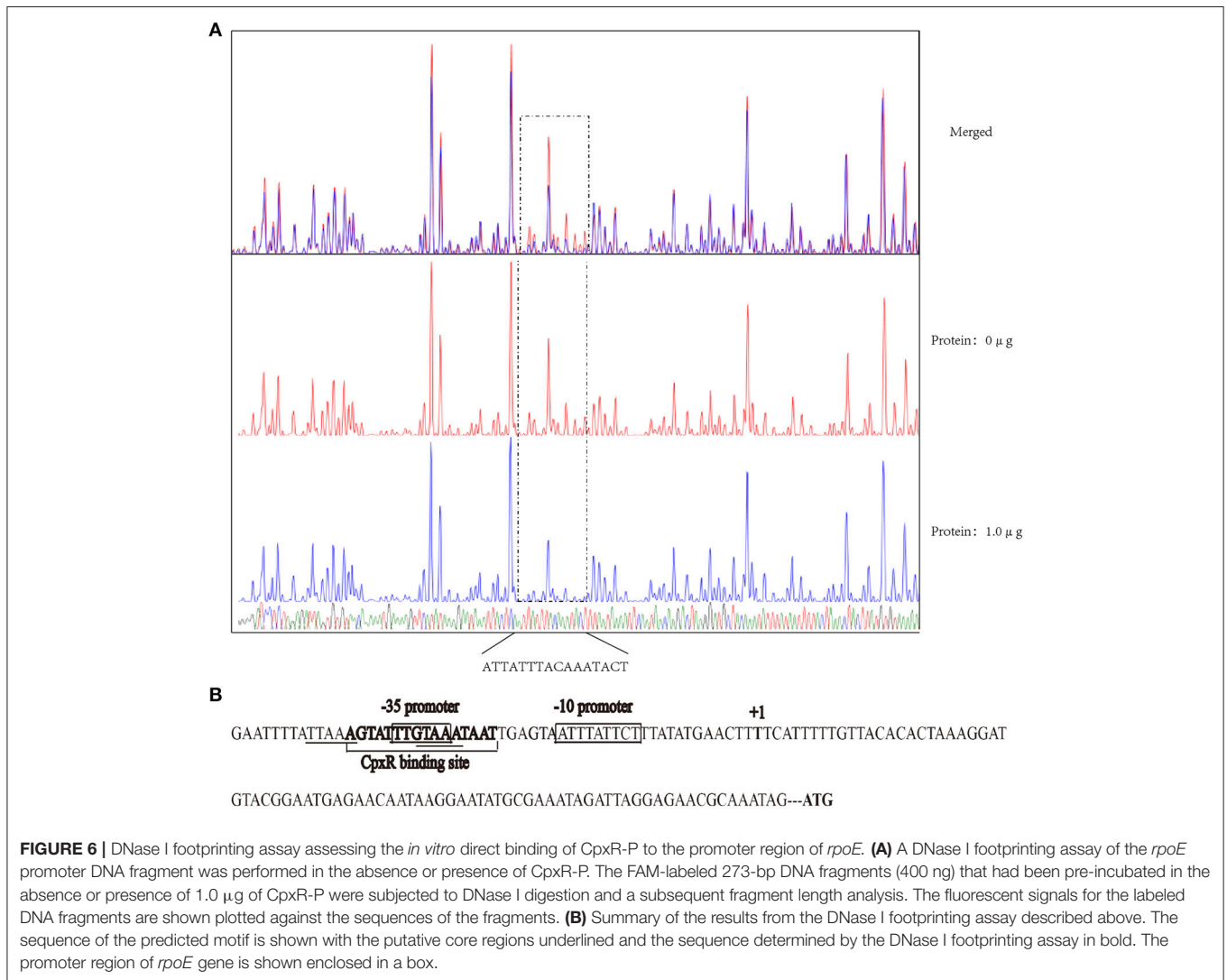
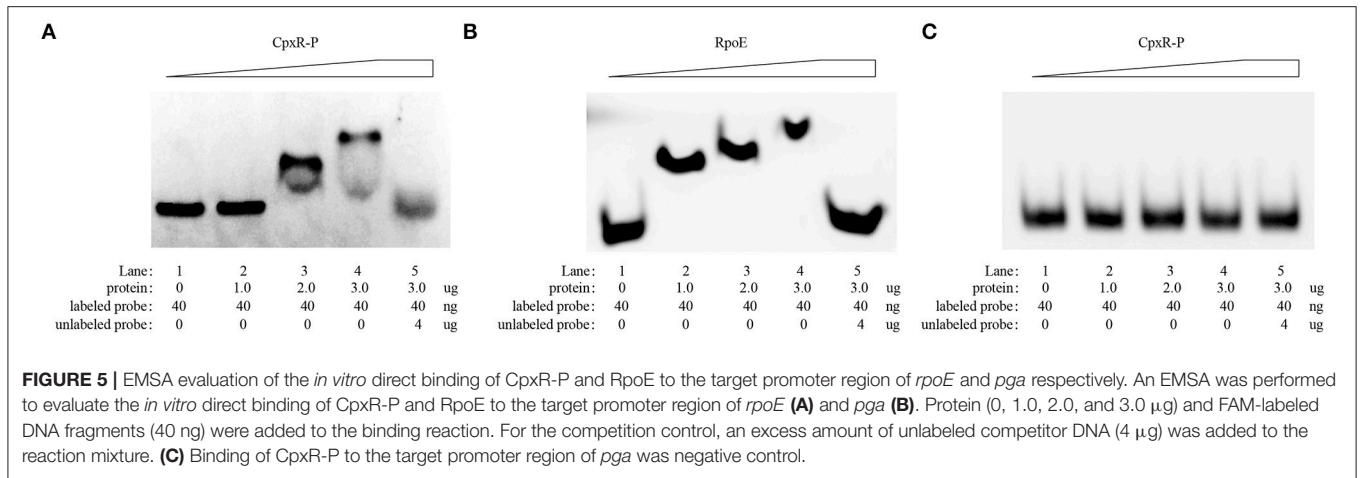
FIGURE 4 | Effects of the *cpxA/cpxR* deletion on the transcription of biofilm-related genes. QRT-PCR analysis of the transcription of important biofilm-related genes (*pgaC* and *rpoE*) in the WT S4074, Δ cpxAR, and $C\Delta$ cpxAR strains at different temperatures. The mRNA levels of biofilm-related genes were normalized to those of the *16S RNA* gene for each strain. Data presented are the mean \pm S.D. from three independent experiments performed in duplicate. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

DISCUSSION

Although many studies regarding biofilm formation in *A. pleuropneumoniae* have cited *cpxAR* and consider *cpxAR* to be a biofilm-related factor, no reports have directly validated the function of *cpxAR* in *A. pleuropneumoniae* (Tremblay et al., 2013; Hathroubi et al., 2015a,b). A mutation of *cpxAR* in *A. pleuropneumoniae* resulted in a lower growth rate than observed for the corresponding WT strain.

Previous work showed that *A. pleuropneumoniae* strain S4074 can produce a pronounced biofilm in BHI broth that had lower than normal levels of zinc, but this condition is inconvenient for use in research (Labrie et al., 2010).

In this study, our microtiter plate biofilm assays revealed that although S4074 cannot produce a detectable biofilm at 37°C, it does produce a pronounced biofilm at 42°C. Furthermore, the qRT-PCR results revealed that the expression levels of three biofilm-related genes (*rpoE* and *pgaC*) in S4074 were significantly upregulated at 42°C compared with their expression levels at 37°C. Thus, our qRT-PCR results were consistent with the results of the biofilm assays. In addition, the expression of *cpxR* was upregulated in S4074 at 42°C compared with at 37°C (Supplementary Figure 6). Together, these results indicate that high temperature can induce a pronounced biofilm through upregulating the *cpxR* gene in S4074.



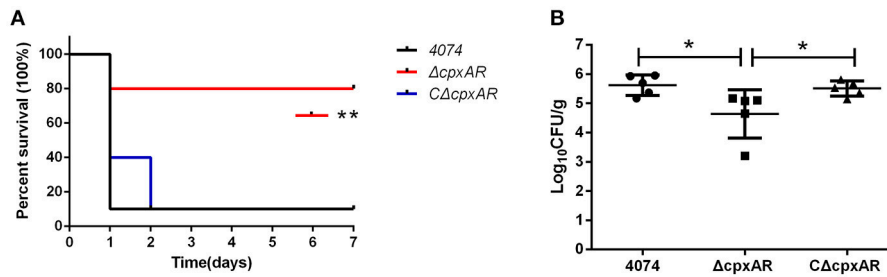


FIGURE 7 | Survival curves and lung bacteria loads of mice infected with *A. pleuropneumoniae*. **(A)** Survival curves for Balb/c mice infected with the WT, $\Delta cpxAR$, and $C\Delta cpxAR$ strains. Six-week-old Balb/c mice were inoculated intraperitoneally with 1×10^7 CFU of bacteria, and their survival was monitored over a 7 day period. Data were analyzed using the log rank test. **(B)** The *A. pleuropneumoniae* load in the lungs of infected mice. The horizontal lines denote the median number of bacteria in each group of 6 mice. The data is representative of one of three independent experiments. ** $p < 0.01$, * $p < 0.05$.

CpxR was previously shown to bind to the promoter region of the regulator σ^E in *E. coli* (De Wulf et al., 2002). In this study, qRT-PCR revealed that CpxA/CpxR positively influences transcription of the *rpoE* gene in *A. pleuropneumoniae*. Additionally, the sequence GTAAA-(N)₄₋₈-GTAAA is the CpxR-binding site in many other bacteria (Feldheim et al., 2016; Tian et al., 2016). Here, an *in silico* assessment identified a putative CpxR-binding sequence in the vicinity of the promoter region of sigma factor *rpoE*. We also demonstrated via EMSA that CpxR can directly bind to the promoter region of the *rpoE* gene. Furthermore, our DNase I footprinting analysis found that the CpxR-binding site was partially consistent with the putative sequence and was 25 bp away from the putative transcription start site. The CpxR-binding site is generally within 100 bp in the 5' direction from the transcriptional start site, which is essential for CpxR-dependent activation of the target promoters in *E. coli* (De Wulf and Lin, 2000; Raffa and Raivio, 2002). Previous studies demonstrated that CpxR primarily functions as a class I factor binding upstream of the promoter -35 region in *E. coli* (Yamamoto and Ishihama, 2006). In this study, we observed that CpxR activates *rpoE*, as the CpxR binding site overlaps the -35 box sequence in *A. pleuropneumoniae*.

Notably, in TSB, the *cpxAR* mutant showed a remarkable fold-change in the expression of the *pgaC* gene, which was lower than that in the WT strain. Previous studies found that RpoE positively regulates the transcription of the *pga* operon, and the *rpoE* promoter sequence (GAACTT-n16-TCAAA) is 468 bp upstream from the *pgaA* translational start in *A. pleuropneumoniae* (Bosse et al., 2010). PgaC synthesizes the major biofilm matrix polysaccharide PGA, which is a component of *A. pleuropneumoniae* biofilm colonies and may prevent the access of antimicrobial agents to the bacterial cells within the biofilm (Costerton et al., 1999; Kaplan et al., 2004). In this study, we also demonstrated via EMSA that RpoE can directly bind to the promoter region of the *pga* operon. In light of these previous findings and those in the present study, we demonstrated that the CpxA/CpxR TCS in *A. pleuropneumoniae* is able to regulate the expression of the *pga* operon through *rpoE* to facilitate biofilm formation.

Our experimental results suggest that *cpxAR* could be a virulence-associated gene and, therefore, may be a potential

therapeutic target (Wassenaar and Gaastra, 2001). Many major virulence factors reside in the cell envelope, so it is reasonable that the Cpx response is involved in the development of host infection caused by various bacterial pathogens (Vogt and Raivio, 2012). Additionally, RpoE plays an important role in the virulence of *Salmonella* Typhimurium, *Burkholderia pseudomallei*, *Vibrio cholera*, and *Vibrio harveyi* (Humphreys et al., 1999; Kovacicova and Skorupski, 2002; Thongboonkerd et al., 2007; Rattanama et al., 2012). Furthermore, it has been observed that the *pgaBC* genes were upregulated in *A. pleuropneumoniae* following contact with St. Jude porcine lung cells (Auger et al., 2009). In this study, the colonization assay and survival results demonstrate that CpxA/CpxR contributes to the virulence of *A. pleuropneumoniae*. Taken together, these results indicate that the regulation of biofilm formation by CpxA/CpxR may be involved in the pathogenesis of *A. pleuropneumoniae*.

Biofilm formation is reportedly involved in escaping from the host immune system, thereby playing an important role in the pathogenicity of *A. pleuropneumoniae in vivo* (Bosse et al., 2010; Hathroubi et al., 2017). The predominant sign of acute pleuropneumonia is high fever that body temperature can elevated up to 41.8°C (Menzel et al., 2014; Sassu et al., 2017). Here we demonstrate that the fever temperature contribute to biofilm formation in *A. pleuropneumoniae*. Therefore, the mechanism may promote the understanding of the pathogenesis of *A. pleuropneumoniae*-induced infection.

AUTHOR CONTRIBUTIONS

WB, HL, FL conceived and designed the experiments. HL, KY, WP, HZ, HuiC, and TL performed the experiments. FL, HL, PC analyzed the data. FY, HuaC contributed reagents, materials and analysis tools.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcimb.2018.00072/full#supplementary-material>

Supplementary Figure 1 | Identification of the *A. pleuropneumoniae* mutant strain and the complemented strain by PCR. Identification of the Δ cpxAR mutant strain and the C Δ cpxAR complemented strain by PCR, using PCR primer pairs cpxAR-exterior F/R (2,935 bp for WT, 573 bp for Δ cpxAR and C Δ cpxAR) and cpxAR-interior F/R (506 bp for WT and C Δ cpxAR, no fragment for Δ cpxAR).

Supplementary Figure 2 | RT-PCR analysis of cpxAR transcripts. Total RNA was extracted from the WT, Δ cpxAR, and C Δ cpxAR strains. cDNA generated from these RNA samples was subjected to RT-PCR analysis with primer pairs cpxA-F/R

and cpxR-F/R. The products were analyzed by electrophoresis. The DL 2000 DNA Marker is shown on the left (lane M).

Supplementary Figure 3 | Polystyrene microtiter plate biofilm assay of *A. pleuropneumoniae* grown in TSB at 37°C. The S4074, Δ cpxAR, and C Δ cpxAR strains were grown in TSB at 37°C. The resulting bacterial biofilms were photographed (A) and measured (B) at OD₅₉₀ after 36, 48, and 60 h of incubation. Each data point represents the mean \pm S.D. from three independent experiments performed in duplicate.

Supplementary Figure 4 | Polystyrene microtiter plate biofilm assay of *A. pleuropneumoniae* grown in BHI at 37°C for 24 h. The S4074, Δ cpxAR, and C Δ cpxAR strains were grown in BHI at 37°C. The optical density of the bacterial biofilm was monitored at OD₅₉₀ after 24 h of incubation. Each data point represents the mean \pm S.D. from three independent experiments performed in duplicate. **p* < 0.05.

Supplementary Figure 5 | Identification of purified His-CpxR protein and His-RpoE protein. Sodium dodecyl sulfate polyacrylamide gel electrophoresis behavior (A,C) and Western blot analyses (B,D) of the purified His-CpxR protein and His-RpoE protein respectively.

Supplementary Figure 6 | Effects of temperature on the transcription of cpxAR genes. A qRT-PCR analysis of cpxAR gene transcriptions in the WT S4074 strain at different temperatures is shown. The mRNA levels of cpxAR genes were normalized to those of the 16S RNA gene. Data presented are the mean \pm S.D. from three independent experiments performed in duplicate. **p* < 0.05.

Supplementary Figure 7 | DNase I footprinting assay. The FAM-labeled 273-bp DNA fragments (400 ng) that had been pre-incubated with 1.0 μ g (A) or 2.0 μ g (B) of CpxR-P were subjected to DNase I digestion and a subsequent fragment length analysis.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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