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The QseB/QseC two-component system contributes to virulence of *Actinobacillus pleuropneumoniae* by downregulating *apf* gene cluster transcription

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Abstract

Actinobacillus pleuropneumoniae (APP) is the major pathogen of porcine contagious pleuropneumoniae (PCP). The QseB/QseC two-component system (TCS) consists of the regulator QseB and the kinase QseC, which relates to quorum sensing (QS) and virulence in some bacteria. Here, we investigated the role of QseB/QseC in *apf* gene cluster (*apfABCD*) expression of APP. Our results have showed that QseB/QseC TCS can potentially regulate the expression of *apf* gene cluster. The Δ*qseBC*, Δ*apfA*, Δ*apfB*, Δ*apfC* and Δ*apfD* strains are more sensitive to acidic and osmotic stressful conditions, and exhibite lower biofilm formation ability than wild-type (WT) strain, whereas the complemented strains show similar phenotype to the WT strain. In additon, the mutants have defective anti-phagocytosis, adhesion and invasion when they come into contact with the host cells. In experimental animal models of infection, mice infected with Δ*qseBC*, Δ*apfA*, Δ*apfB*, Δ*apfC* and Δ*apfD* strains showed lower mortality and bacterial loads in the lung and the blood than those infected with WT strain. In conclusion, our results suggest that QseB/QseC TCS contributes to stress resistance, biofilm formation, phagocytosis, adhesion, invasion and virulence by downregulating expression of *apf* gene cluster in *A. pleuropneumoniae*.

Keywords: *A. pleuropneumoniae*, QseB/QseC, Transcriptional regulation, *apf* gene cluster, Virulence

Background

Porcine contagious pleuropneumoniae (PCP) is a highly infectious porcine respiratory disease, which is caused by *Actinobacillus pleuropneumoniae* (APP). This disease is widespread in many countries and has brought great loss to farming and animal husbandry enterprises (González et al. 2017). Clinically, PCP is mainly characterized by acute fibrinous hemorrhagic pleuropneumonia and chronic fibrinous necrotizing pleuropneumonia (Sassu et al. 2018). So far, APP is divided into 2 biotypes and 19 serovars (Bosse et al. 2018; Sassu et al. 2018; To et al. 2021; Stringer et al. 2021). Some factors associated with virulence such as toxins, lipopolysaccharide, adhesion molecules, and outer membrane proteins contribute to the pathogenicity (Chiers et al. 2010).

To improve the adaptability of bacteria, their changing environment can be sensed and responded by the two-component systems (TCSs) (Jacob-Dubuisson et al. 2018). The bacterial TCS consists of a histidine kinase (HK) and a response regulator (RR) (Buelow and Raivio 2010). When the HK transfers a phosphate group to the RR, the RR can directly or indirectly regulate the

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expression levels of downstream genes (Vogt and Raivio 2012). Genomic sequencing has revealed that there are 5 putative TCS in APP: QseB/QseC (YgiX/YgiY), CpxR/CpxA, PhoB/PhoR, NarP/NarQ and ArcA/ArcB (Xu et al. 2008).

Quorum sensing (QS) is identified as a cell density sensing phenomenon, which utilizes autoinducers, or bacterial hormone-like compounds (Mukherjee and Bassler 2019; Li et al. 2021). As autoinducers reach a certain concentration, the signaling molecules can be involved in regulating certain genes expression (Lerat and Moran 2004). According to the sequence similarity, there are two QS systems called LuxS and QseB/QseC in APP. Previous studies have shown that LuxS is closely associated with infection of APP (Li et al. 2011). QseB/QseC of APP is a putative TCS with high homology to the YgiX/YgiY system of *Escherichia coli* and QseB/QseC system of *Erwinia* and *Haemophilus* (Liu et al. 2015). In recent years, QseB/QseC TCS has been linked to the virulence of *Enterobacteriaceae* and *Pasteurellaceae* (Weigel and Demuth 2016). The QseB/QseC TCS has also been confirmed to contribute to the biofilm formation of *Aggregatibacter actinomycetemcomitans* (Novak et al. 2010), *Salmonella enterica* (Ji et al. 2017) and *E. coli* (Li et al. 2020). In addition, the QseB/QseC TCS is related to the stress resistance of *E. coli* (Li et al. 2020).

Previous studies have confirmed that QseB/QseC TCS regulates the expression of *pilM*, which encodes a type IV pili (Tfp) assembly protein (Liu et al. 2015), and contributes to virulence, biofilm formation and stress resistance in some bacteria (Novak et al. 2010; Yang et al. 2021). However, the function of QseB/QseC TCS in APP is not fully revealed. In this study, we have shown that QseB/QseC TCS positively regulates the expression of *apf* gene cluster, which also encodes Tfp assembly protein. RNA-seq, quantitative reverse transcription PCR (RT-qPCR) and electrophoretic mobility shift assay (EMSA) were used to screen downstream genes potentially regulated by QseB/QseC TCS. It was found that the RR QseB could bind to the promoter of *apf* gene cluster. The Δ*qseBC*, Δ*apfA*, Δ*apfB*, Δ*apfC*, and Δ*apfD* strains significantly contribute to stress resistance, biofilm formation, phagocytosis, adhesion, invasion and virulence in APP. Our results provide a basis for further understanding the QseB/QseC TCS and *apf* gene cluster function in bacteria.

Results

QseB/QseC TCS influences transcription of *apf* gene cluster

We constructed Δ*qseBC* mutant and its complemented strain (CΔ*qseBC*) and investigated the expression of *apf* gene cluster in WT, Δ*qseBC* and CΔ*qseBC* strains by RT-qPCR. It was found that the transcription levels of the genes in the *apf* gene cluster were downregulated significantly in Δ*qseBC* strain than that in WT and CΔ*qseBC* strains (Fig. 1).

QseB can bind to the promoter of *apf* gene cluster

SDS-PAGE results showed that the QseB (28.64 KDa) was successfully expressed and purified (Fig. 2). We performed EMSAs using purified QseB protein with DNA fragments containing the putative promoter regions of *apf* gene cluster, *pilM* (positive control), and *glpK* (negative control), respectively. Results revealed that QseB can bind to the promoter regions of *apf* gene cluster (Fig. 3a) and *pilM* (Fig. 3b), however not to that of *glpK* (Fig. 3c). These data suggested that transcription of *apf* gene cluster, and *pilM*, but not *glpK* was regulated by QseB.

Role of *qseBC* and *apf* in environmental stress resistance of APP strains

Next, Δ*apfA*, Δ*apfB*, Δ*apfC*, Δ*apfD* mutants and the corresponding complemented strains were constructed. When the bacteria were exposed to 0.02 M HCl-induced acidic or 0.50 M KCl-induced osmotic stress, the count of live bacteria of the Δ*qseBC*, Δ*apfA*, Δ*apfB*, Δ*apfC* and Δ*apfD* strains were lower compared with those of WT strain at 3 h (Fig. 4).

Contribution of *qseBC* and *apf* to biofilm formation of APP

Previous studies have reported that *A. pleuropneumoniae* S4074 strain can form significant biofilm in BHI (supplemented with NAD) (Labrie et al. 2010). As expected, a robust biofilm was observed in WT strain, however the biofilm formation abilities of Δ*qseBC*, Δ*apfA*, Δ*apfB*, Δ*apfC* and Δ*apfD* were lower than that of WT strain. The quantitative evaluation of these biofilms confirmed that biofilm formation was significantly impaired in Δ*qseBC* and Δ*apfABCD* mutants compared to WT and the complemented strains (Fig. 5).

The *qseBC* and *apf* gene cluster contribute to the resistance to phagocytosis, adhesion and invasion of APP to host cells

To investigate whether the *qseBC* and *apf* gene cluster contribute to the resistance to phagocytosis, adhesion and invasion, Δ*qseBC*, Δ*apfA*, Δ*apfB*, Δ*apfC*, Δ*apfD* and the complemented strains were examined for their phagocytosis of RAW264.7, adhesion and invasion to NPTr cells, and the WT strain was used as a control. It was found that the Δ*qseBC*, Δ*apfA*, Δ*apfB*, Δ*apfC* and Δ*apfD* mutants had defective anti-phagocytosis of RAW264.7 macrophage (Fig. 6a) and the abilities of

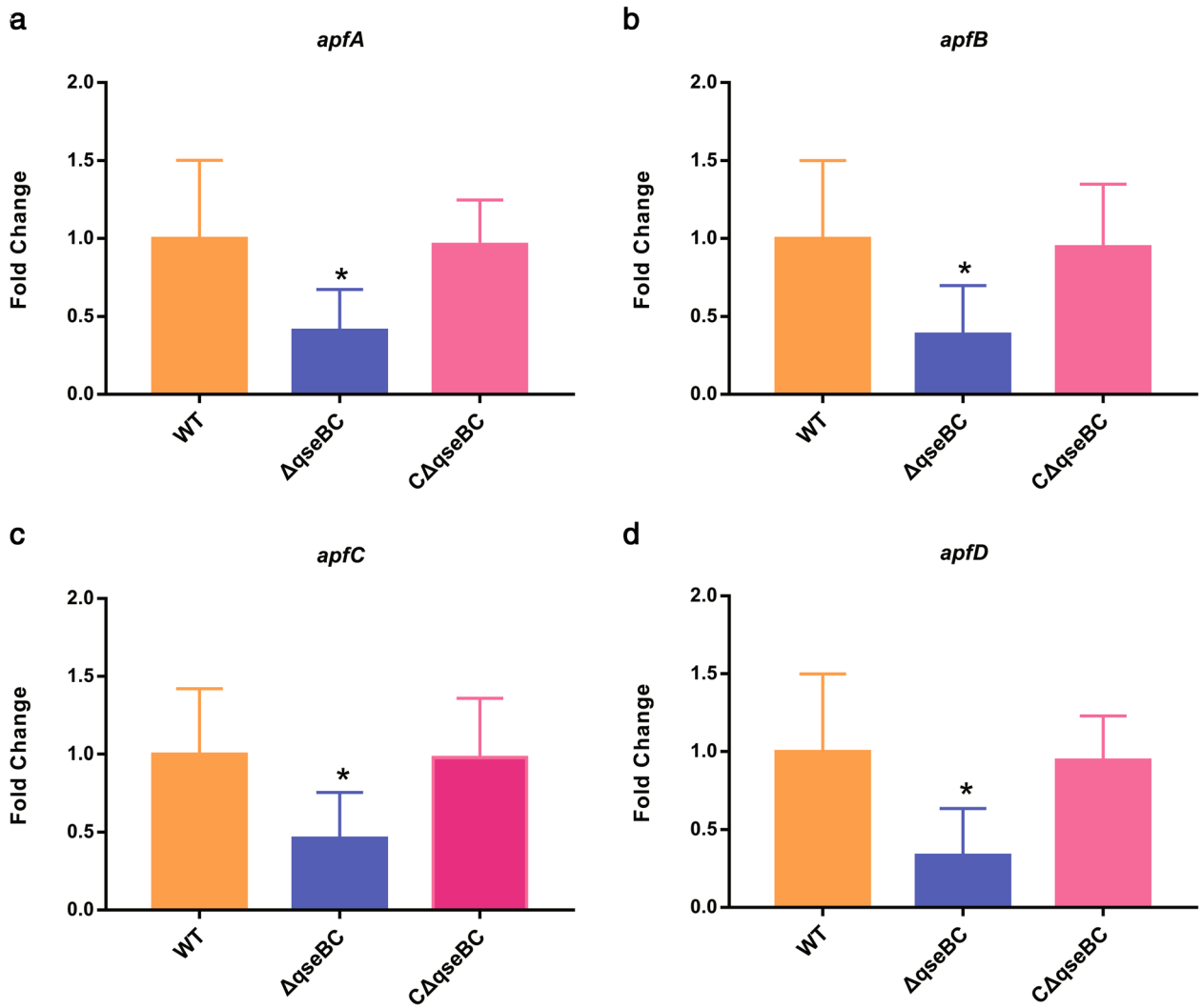

Fig. 1 Effect of *qseBC* deletion on the transcription of *apf* gene cluster. RT-qPCR analysis of transcription levels of genes in the *apf* gene cluster in WT S4074, Δ*qseBC* and CΔ*qseBC* strains. mRNA levels were normalized to 16S *rRNA* gene for each strain. Data are shown as mean ± SD ($N = 3$). *$P < 0.05$

adhesion (Fig. 6b) and invasion to NPTr cells (Fig. 6c) were lower than those of WT and the complemented strains.

Virulence of the Δ*qseBC* and Δ*apf* gene cluster mutants in mice

The virulence of Δ*qseBC* and Δ*apf* gene cluster mutants was investigated by using Balb/c mouse models of APP *in vivo* (Xie et al. 2016). We found that the Survival rates of mice at 120 h were 16.67, 16.67, 83.33, 66.67, 66.67, 66.67, 0, 0, 0, 16.67 and 0 for WT, Δ*qseBC*, Δ*apfA*, Δ*apfB*, Δ*apfC*, Δ*apfD*, CΔ*qseBC*, CΔ*apfA*, CΔ*apfB*, CΔ*apfC* and CΔ*apfD*-infected groups, respectively (Fig. 7a). Although the survival of mice at 120 h were equal in WT and Δ*qseBC* groups, it was interesting that the survival of mice infected with Δ*qseBC* (83.33) was significantly higher than that of WT (50.00) at 6 h post-infection. Furthermore, the bacterial loads of the Δ*qseBC*, Δ*apfA*, Δ*apfB*, Δ*apfC* and Δ*apfD* strains in the lung (Fig. 7b) and the blood (Fig. 7c) were lower than those by WT and the complemented strains at 6 h post-infection ($P < 0.05$). Taken together, our results suggest that QseB/QseC TCS and *apf* gene cluster may contribute to the virulence in the early stage of infection of *A. pleuropneumoniae*.

Discussion

The QseB/QseC TCS is considered as a regulatory system that relates to quorum sensing. Previous studies have revealed that QseB/QseC TCS contributes to interboundary signal transduction and regulation of virulence gene expression, as well as toxin production (Clarke

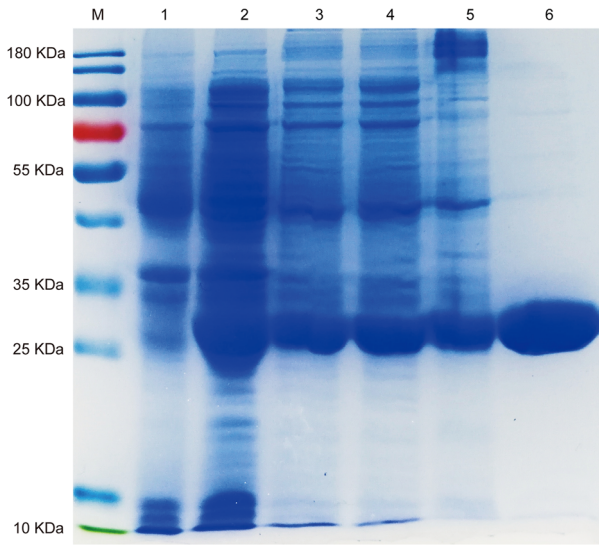

Fig. 2 Analysis of QseB protein. Lane 1, cell lysate of BL21 (DE3) containing plasmid pET-30a; Lane 2, lysate of BL21 (DE3) containing plasmid pET-*qseB* without isopropyl-β-D-thiogalactoside (IPTG) induction; Lane 3, cell lysate of BL21 (DE3) containing plasmid pET-*qseB* with IPTG induction at 25 °C for 4 h; Lane 4 and 5, supernatant (lane 4) and inclusion body (lane 5) of cell lysate of BL21 (DE3) containing plasmid pET-*qseB* with IPTG induction at 25 °C for 4 h; Lane 6, purified QseB protein

et al. 2006). Khajanchi et al. have demonstrated that QseB/QseC TCS can regulate virulence of APP *in vitro* and *in vivo* (Khajanchi et al. 2012). It has been reported that the inactivation of QseB/QseC leads to a decrease in biofilm formation of *E. coli* (Li et al. 2020). Bearson and Bearson have also found that QseB/QseC is involved in *S. typhimurium* colonization of swine (Bearson and Bearson 2008).

In this study, the EMSAs were performed to detect the regulatory relationships between QseB and *apf* gene cluster. Initially, purified QseB protein was directly used for EMSAs, but it was found that QseB could not bind to the promoter of *apf* gene cluster and *pilM* (positive control). Yan et al. demonstrated that phosphorylated CpxR protein could bind to the promoter of *wecA* in APP (Yan et al. 2020). Then we phosphorylated the QseB protein and found that phosphorylated QseB could bind to the promoter of and *apf* gene cluster and *pilM* (positive control). The results suggest that phosphorylated QseB might regulate the transcription of *apf* gene cluster.

Our results presented here demonstrate that the Δ*qseBC* and Δ*apf* gene cluster mutants are more sensitive to acidic and osmotic stressful conditions than WT the and complemented strains, which are consistent with earlier studies (Li et al. 2020). The Δ*qseBC* and Δ*apf* gene cluster mutants also exhibite lower biofilm formation ability than WT strain and the complemented mutants. Biofilm is an extracellular polymer formed on the surface of bacterial colonies, which can cause self-agglutination and adhesion of bacteria. Biofilms of many strains of APP have been detected and are thought to relate to bacterial colonization (Kaplan et al. 2004; Kaplan and Mulks 2005). Taken together, our results suggest that QseB/QseC affects stress resistance and biofilm formation by regulating the expression of *apf* gene cluster.

In addition, this research suggests that the virulence of Δ*qseBC* and Δ*apf* gene cluster mutants were more attenuated than that of WT and the complemented strains in the mouse infection models. Liu et al. used a pig infection model to evaluate the virulence of Δ*qseBC* of *A. pleuropneumonia*, and measured the clinical signs such as appetite, dyspnea, lethargy and fever of the infected pigs at 12, 24, 36, 48 and 60 h. They found that there was no significant difference between the clinical scores of the pigs inoculated with Δ*qseBC* mutant and WT, indicating that QseB/QseC had no significant effect on the virulence

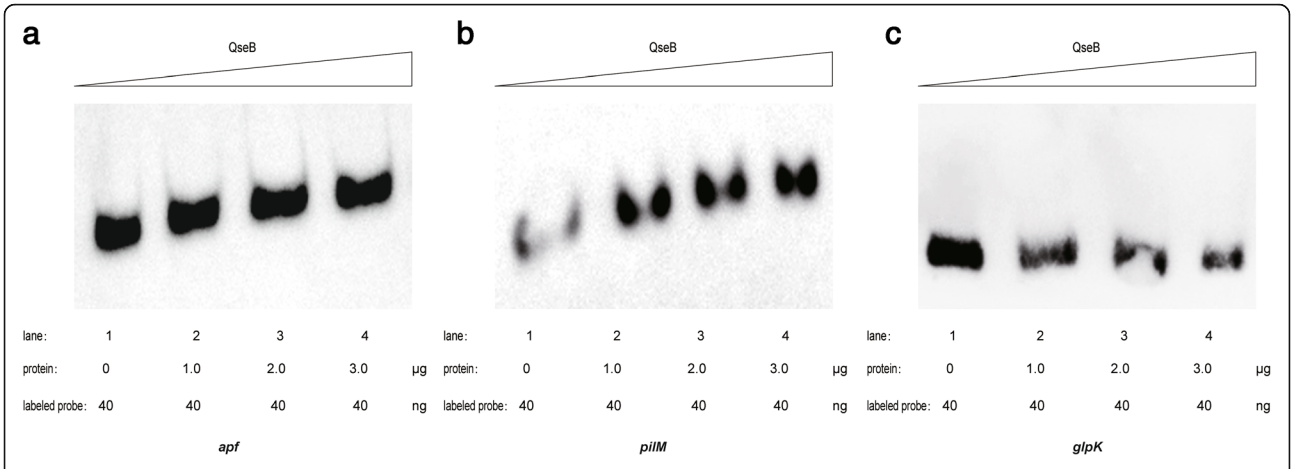

Fig. 3 Electrophoretic mobility shift assay of interaction between QseB and the promoter regions. Different amount of purified QseB protein was incubated with DNA fragment of promoter region of *apf* (**a**), *pilM* (positive control) (**b**) and *glpK* (negative control) (**c**)

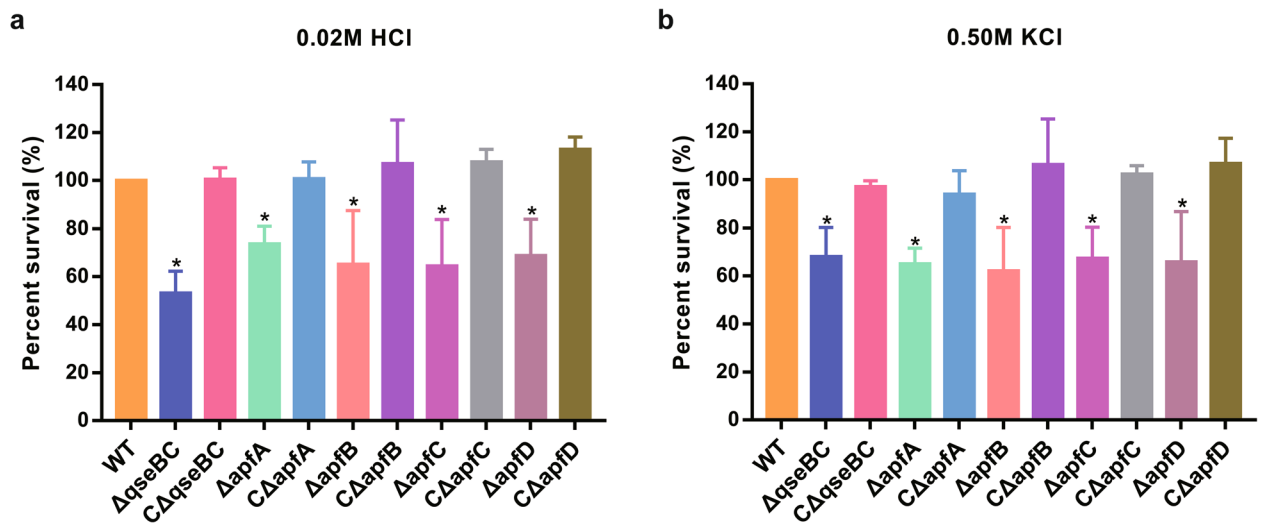

Fig. 4 Acidic and osmotic stress resistance assays. WT S4074, Δ*qseBC*, Δ*apfA*, Δ*apfB*, Δ*apfC* and Δ*apfD* were treated with 0.02 M HCl (**a**) and 0.50 M KCl (**b**) or tryptic soy broth and incubated for 3 h at 37 °C. Then the bacterial survival rates were calculated. Data presented are the mean ± SD ($N = 3$). *$P < 0.05$

of APP (Liu et al. 2015). We also found that the survival rates were similar in mice infected with WT or the Δ*qseBC*, however, it was interesting that the survival rate of mice infected with Δ*qseBC* (83.33) was significantly higher than that of WT (50.00) at 6 h . In order to further verify our result, we analyzed the bacterial colonization ability in mice tissues, and found that the amount of colonization by the Δ*qseBC* in the lung and the blood were both lower than those by the WT at 6 h post-infection. These results suggested that QseB/QseC TCS might contribute to the virulence in the early stages of infection.

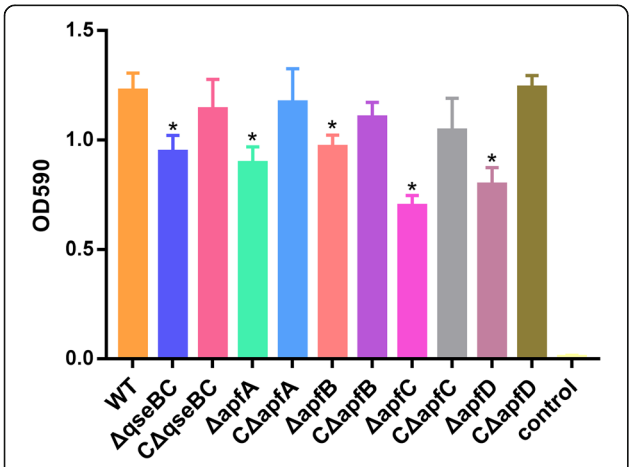

Fig. 5 Quantitative determination of biofilm formation. WT S4074, Δ*qseBC*, Δ*apfA*, Δ*apfB*, Δ*apfC* and Δ*apfD* strains were grown in BHI at 37 °C. OD_{590} of the bacterial biofilm were measured after 72 h of incubation. Data are shown as mean ± SD ($N = 3$). *$P < 0.05$

Besides, the Δ*qseBC* and Δ*apf* gene cluster mutants contribute to the resistance to phagocytosis. Bacteria are phagocytic and form phagosomes in phagocytes. Lysosomes fuse with phagosomes to form phagolysosomes. A variety of bactericidal substances and hydrolases in lysosomes kill and digest bacteria. The thallus residue is expelled from the cell (Cao et al. 2019). By knocking out the *apf* genes, the mutant strains could not synthesize the Tfp assembly protein normally and the virulence of these mutant strains were significantly weakened, which made the mutants more easier to be phagocytic by macrophage.

At the same time, the abilities of the Δ*qseBC* and Δ*apf* gene cluster strains to adhesion and invasion of cells were lower than that of the WT strain. Adhesion colonization is a key step for pathogen infection and pathogenesis after pathogen invasion. APP specifically colonizes the lower respiratory tract of pigs, adhering to bronchial cilia and alveolar epithelial cells (Dom et al. 1994). The results suggest that the QseB/QseC TCS can affect the expression of *apf* gene clusters, mediating the adhesion and invasion of APP, and thus establishing infection. To sum up, this research indicate that QseB/QseC TCS and *apf* gene cluster could contribute to virulence in the early stage of infection of APP *in vivo*. The data in this study will provide theoretical basis for the prevention of infection with APP.

Conclusions

In summary, we confirm that QseB/QseC TCS contributes to the stress resistance, biofilm formation, phagocytosis, adhesion, invasion and virulence of APP by downregulating the expression of *apf* gene cluster.

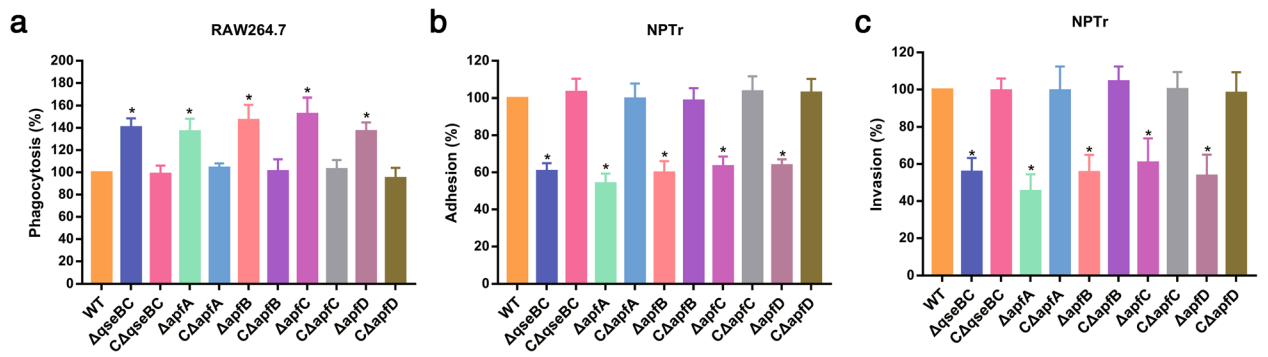

Fig. 6 Cell phagocytosis, adhesion and invasion assays. **a** Phagocytosis of Δ*qseBC*, Δ*apfA*, Δ*apfB*, Δ*apfC* and Δ*apfD* strains. The strains were incubated with RAW264.7 cells at the multiplicity of infection (MOI) of 100 at 37 °C for 2 h. **b**, **c** Adhesion (**b**) and invasion (**c**) of Δ*qseBC*, Δ*apfA*, Δ*apfB*, Δ*apfC* and Δ*apfD* strains. The strains were incubated with NPTr cells at the MOI of 100 for 2 h of incubation at 37 °C. After washing out the unbound bacteria, the NPTr cells were lysed and the bacterial counts in the lysates were determined. Data are shown as mean ± SD ($N = 3$). *$P < 0.05$

Methods

Strains, plasmids, primers and culture conditions

The experimental materials are listed in Tables 1 and 2. S4074 was used as WT strain of *A. pleuropneumoniae* (Donà and Perreten 2018). APP strains were inoculated on tryptic soy agar (TSA; Difco Laboratories, USA) containing 10% (*v*/*v*) inactivated newborn bovine serum and 10 μg/mL nicotinamide adenine dinucleotide (NAD; Solarbio, China), then, a single colony was selected and inoculated into tryptic soy broth (TSB; Difco Laboratories, USA). *E. coli* strains were cultured in Luria-Bertani (LB; Haibo, China), and the cultivation of *E. coli* β2155 requires the addition of diaminopimelic acid (dapA; Sigma-Aldrich, USA) (Yuan et al. 2014). Chloramphenicol was added to the culture medium as needed, where the final concentration was 25 μg/mL for *E. coli* screening, 4 μg/mL for APP transformants screening, and 2 μg/mL for APP complemented strains screening. All strains were oscillated in a 37 °C incubator at 180 r/min. RAW264.7 mouse macrophage cell line and NPTr (newborn piglet tracheal cell line) were cultured in Dulbecco's modified eagle medium (DMEM) (Gibco, USA) containing 10% (*v*/*v*) foetal bovine serum (FBS; Gibco, USA) (Liu et al. 2017) with 5% (*v*/*v*) CO_2 at 37 °C.

Construction of mutant and complemented strains

The mutant strains Δ*qseBC*, Δ*apfA*, Δ*apfB*, Δ*apfC*, Δ*apfD* and the complemented strains CΔ*qseBC*, CΔ*apfA*, CΔ*apfB*, CΔ*apfC* and CΔ*apfD* were constructed as described earlier (Li et al. 2018). In Brief, the upstream and downstream fragments of *qseBC*, *apfA*, *apfB*, *apfC and apfD* were amplified, respectively. And the fragments were combined *via* overlapping polymerase chain reaction (PCR). These products were purified and cloned into the vector pEMOC2 (Oswald et al. 1999) to generate the recombinant plasmids of pEΔ-*qseBC*, pEΔ*apfA*, pEΔ*apfB*, pEΔ*apfC* and pEΔ*apfD*, respectively. These plasmids were used to construct Δ*qseBC*, Δ*apfA*, Δ*apfB*, Δ*apfC* and Δ*apfD* mutants by conjugational transfer. The *qseBC* and *apf* gene cluster were amplified and PCR products were cloned into vector pJFF224-XN (Frey 1992), respectively. Then, the plasmids pJFF-*qseBC*, pJFF-Δ*apfA*, pJFF-Δ*apfB*, pJFF-Δ*apfC* and pJFF-Δ*apfD* were transferred into the corresponding mutant strains by electric transformation (2.5 KV, 25 μFD, 800 Ω). These mutants were screened on TSA (supplemented with chloramphenicol, NAD, and bovine serum) and verified by PCR and DNA sequencing (data not shown).

RNA extraction and RT-qPCR

WT and Δ*qseBC* strains were cultured in TSB (supplemented with NAD and bovine serum) overnight, then diluted with fresh medium at a ratio of 1:100 and grown to the OD_{600} of 0.6. The Bacteria Total RNA Isolation Kit (Sangon Biotech, China) was used to extract total RNA. The HiScript II Q RT SuperMix (+gDNA wiper) (Vazyme, China) was used to synthesize the first strand cDNA. AceQ qPCR SYBR Green Master Mix (Vazyme, China) was used for quantitative PCR (qPCR), which performed by a one-step reaction (Walters et al. 2006). The inverted cDNA and 16S rRNA gene were used as template and endogenous control, respectively. Specific procedure, reaction system and conditions were as instructed by these kits. Then, we used the $2^{-\Delta\Delta Ct}$ method to quantitatively analyze the expression level of target genes (Livak and Schmittgen 2001).

Expression of QseB

Primers P*qseB*-F and P*qseB*-R were used to amplify *qseB* gene for PCR, and plasmid pET-*qseB* was transferred

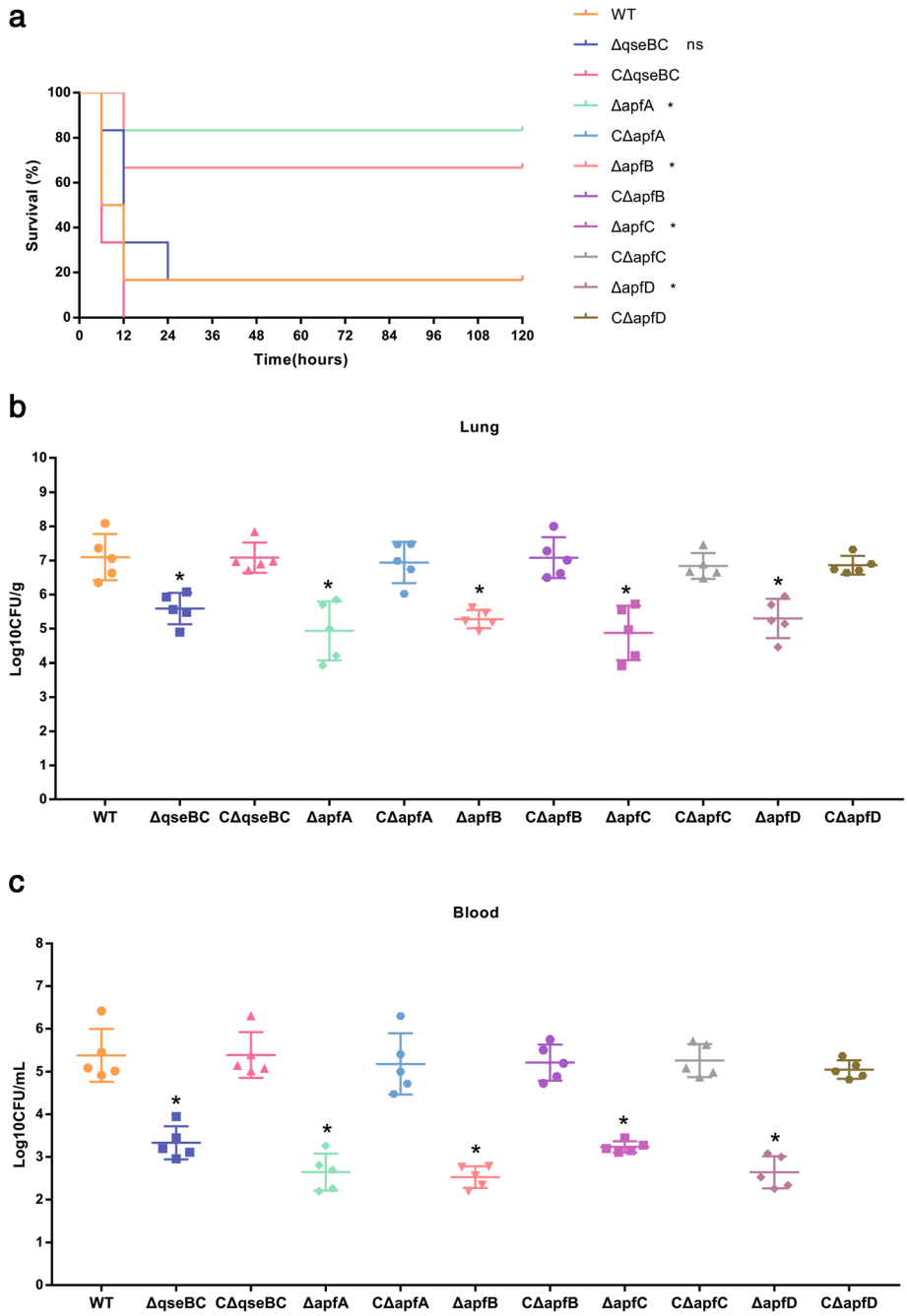

Fig. 7 Survival curves and bacteria loads of mice infected with *A. pleuropneumoniae*. **a** Survival curves for Balb/c mouse infected with WT S4074, Δ*qseBC*, Δ*apfA*, Δ*apfB*, Δ*apfC* and Δ*apfD* strains. Six-week-old Balb/c mice were inoculated WT S4074, Δ*qseBC*, Δ*apfA*, Δ*apfB*, Δ*apfC* and Δ*apfD* strains with 5.00×10^6 CFU by intraperitoneal injection, and the survival of mice was monitored for 120 h post-infection. Data were analyzed by log-rank test. **b**, **c** The *A. pleuropneumoniae* load in lung (**b**) and blood (**c**) tissues of infected mouse. Each mice was inoculated WT S4074, Δ*qseBC*, Δ*apfA*, Δ*apfB*, Δ*apfC* and Δ*apfD* strains with 5.00×10^6 CFU by intraperitoneal injection. *$P < 0.05$

into *E. coli* BL21 (DE3) competent cells and grown in LB to OD_{600} of 0.6. QseB protein was induced with 1.00 mM isopropyl-β-D-thiogalactoside (IPTG) at 25 °C for 4 h. After suspension, cells were crushed by high-pressure cell crusher and centrifuged at 4 °C. QseB was then purified by the Ni-NTA resin affinity chromatography. The purified QseB protein was analyzed by SDS-PAGE and Western Blot, then stored at − 80 °C.

Electrophoretic mobility shift assays

Primers were used to amplify DNA probes containing *apf*, *pilM* and *glpK* promoter region for PCR,

Table 1 Strains, plasmids and protein used in this study

Strains or plasmids	Characteristics
	A. pleuropneumoniae
S4074	*A. pleuropneumoniae* reference strain of serovar 1; WT strain
Δ*qseBC*	*A. pleuropneumoniae* S4074 *qseBC*-deletion mutant
Δ*apfA*	*A. pleuropneumoniae* S4074 *apfA*-deletion mutant
Δ*apfB*	*A. pleuropneumoniae* S4074 *apfB*-deletion mutant
Δ*apfC*	*A. pleuropneumoniae* S4074 *apfC*-deletion mutant
Δ*apfD*	*A. pleuropneumoniae* S4074 *apfD*-deletion mutant
CΔ*qseBC*	Complemented strain of Δ*qseBC*; Cm^r
CΔ*apfA*	Complemented strain of Δ*apfA*; Cm^r
CΔ*apfB*	Complemented strain of Δ*apfB*; Cm^r
CΔ*apfC*	Complemented strain of Δ*apfC*; Cm^r
CΔ*apfD*	Complemented strain of Δ*apfD*; Cm^r
	E. coli
DH5α	Cloning host for recombinant vector
β2155	Transconjugation donor for constructing mutant strain
BL21 (DE3)	The expression host for pET-30a and their derivative
	Plasmids
pEMOC2	Transconjugation vector: ColE1 ori mob RP4 sacB, Amp^r Cm^r
pEΔ*qseBC*	Up- and down-stream arms of *qseBC* were ligated sequentially into pEMOC2, and used as the transconjugation vector for *qseBC* gene deletion
pEΔ*apfA*	Up- and down-stream arms of *apfA* were ligated sequentially into pEMOC2, and used as the transconjugation vector for *apfA* gene deletion
pEΔ*apfB*	Up- and down-stream arms of *apfB* were ligated sequentially into pEMOC2, and used as the transconjugation vector for *apfB* gene deletion
pEΔ*apfC*	Up- and down-stream arms of *apfC* were ligated sequentially into pEMOC2, and used as the transconjugation vector for *apfC* gene deletion
pEΔ*apfD*	Up- and down-stream arms of *apfD* were ligated sequentially into pEMOC2, and used as the transconjugation vector for *apfD* gene deletion
pJFF224-XN	*E. coli*-APP shuttle vector: RSF1010 replicon; mob oriV, Cm^r
pJFF-*qseBC*	pJFF224-XN carrying the intact *qseBC*
pJFF-*apfA*	pJFF224-XN carrying the intact *apfA*
pJFF-*apfB*	pJFF224-XN carrying the intact *apfB*
pJFF-*apfC*	pJFF224-XN carrying the intact *apfC*
pJFF-*apfD*	pJFF224-XN carrying the intact *apfD*
pET-30a	Expression vector; Kan^r
pET-*qseB*	pET-30a carrying *qseB* gene

Cm^r, chloramphenicol resistance, *Amp^r*, ampicillin resistance, *Kan^r*, kanamycin resistance

respectively. After the PCR products were purified, biotin labeling of EMSA probes were carried out using the EMSA Probe Biotin Labeling Kit (Beyotime, China). QseB protein was phosphorylated by Sigma Acetate Kinase from *E. coli* (Sigma, USA). The *pilM* probe that can bind to QseB protein was used as a positive control, and *glpK* probe that can not bind to QseB protein was used as a negative control (Liu et al. 2015). EMSAs were performed with the Chemiluminescent EMSA Kit (Beyotime, `China).

Acidic and osmotic stress resistance assays

WT, Δ*qseBC*, Δ*apfA*, Δ*apfB*, Δ*apfC* and Δ*apfD* strains were cultured in TSB (supplemented with NAD and bovine serum) overnight, then diluted with fresh medium at a ratio of 1:100 and grown to the mid-logarithmic phase. All strains were resuspended in TSB (supplemented with NAD and bovine serum) containing 0.02 M HCl (Rode et al. 2010) and 0.50 M KCl (Yin and Mimura 2020), respectively, and incubated for 3 h, followed by acidic and osmotic stress resistance assays. Bacteria cultured in TSB without any addition were used as control. The incubated samples were serially diluted and selected the appropriate dilution gradient samples to culture in TSA (supplemented with NAD and bovine serum). The bacterial survival rate of each group was determined by dividing CFU of the experimental group by that of the control group.

Biofilm assay

All strains were cultured overnight, then diluted with fresh brain heart infusion broth (BHIB; Oxoid Ltd., UK) (supplemented with NAD) at a ratio of 1:100. Totally 100 μL of the inocula was added to 96-well microtiter plates (Corning, USA) in triplicat. After incubated for 72 h, the bacterial inocula was sucked away with a syringe, and then removed unattached bacteria. Placed the plates in a warm oven to dry and added 100 μL 0.1% (*v*/*v*) crystal violet into the well. The plates were carefully washed with tap water. After drying, 33% (*v*/*v*) glacial acetic acid was used to dissolve the biofilm. Each well of the plates was measured OD_{590} with a Multi-Detection Microplate Reader (BMG Labtech, Germany).

Cell phagocytosis assay

RAW264.7 cells were cultured in 24-well plates with DMEM (supplemented with FBS) to analyze the phagocytosis ability (Carreras-Gonzalez et al. 2019). Briefly, all strains were added to RAW264.7 cells in the plates at the multiplicity of infection (MOI) of 100, respectively. After incubation for 2 h, the mixture were treated with 100 μg/mL of gentamicin to kill any extracellular bacteria. Following an incubation for 45 min, 1 mL precooled 0.025% (*v*/*v*) Triton X-100 was used to lyse those

Table 2 Primers used in this study

Primers	Sequence (5′-3′)[a]
	For mutant construction
qseBC-up-F/R	TTGTCGACCTATGGCTTTAACTTCCTTCG
	GGGAATTCATTATTGTCCGATAAAGCGAC
qseBC-down-F/R	GGGAATTCTTAAGCCGAAGAGAAAAAGACG
	GGTCTAGAGTCGTATCGTTTAATGCGATC
apfA-up-F/R	CTGTCGACTTTGATACCGAGCTGCAATAAGCGT
	GCGTTTACTTTAATTGAATTGATGAGGAAA CGAAATGTAAAGGGGAGGAC
apfA-down-F/R	GTCCTCCCCTTTACATTTCGTTTCCTCATCAATT CAATTAAAGTAAACGC
	ATGCGGCCGCCGCTACGGCTGAGGAAAATC
apfB-up-F/R	CTGTCGACCGATAATCAGCATTAAGATCGGCTC
	GAAATACCTGCTCGGGCAATTAATATTGTTAT CACGGCTACAGAGGC
apfB-down-F/R	GCCTCTGTAGCCGTGATAACAATATTAATT GCCCGAGCAGGTATTTC
	ATGCGGCCGCTACATTCTTTGCATTGCCCCAT
apfC-up-F/R	CTGTCGACGGCTTGTATTTGGCTTAACATCTCT
	ACAACTTCAGAAAGGAAAAGCGTATTATCGGT ACGGTTTTGATCGGAT
apfC-down-F/R	ATCCGATCAAAACCGTACCGATAATACGCTTT TCCTTTCTGAAGTTGT
	ATGCGGCCGCCGAGATTTTTGCTTTTCTTACGC
apfD-up-F/R	CTGTCGACCATTTGCTTTCCTTATCTTTTGCTA
	GTGGTGTAAGCGAAGTATTCCTCAATTTTCGA CTTTCGTGCTAATTATTG
apfD-down-F/R	CAATAATTAGCACGAAAGTCGAAAATTGAG GAATACTTCGCTTACACCAC
	ATGCGGCCGCGCTGAATGATTTAAAC CGTTTGATA
apxIVA-F/R	GGCTACCCATTTCCCTTCG
	GAGCAACAACGTCGCACA
qseBC-exterior-F/R	GTTTATCGGTAGGTCCACAGG
	TGATCGCAGCAAATACCAAA
qseBC-interior-F/R	CGGAACGACTCGCCTCTT
	AAATCGCCAAAATCAATAGCGGTAG
apfA-exterior-F/R	AATACCGGCTCAATTCGCTTA
	ACCAGTCACGTAAAGGGTCGGC
apfA-interior-F/R	GGTGTCATCGTATAACCGTATT
	TTGGCGGCATCGGCTTCTT
apfB-exterior-F/R	GTTGGATACGGATCTGTGAATAG
	TTACGGTGGAAGGCGGAACGATTAC
apfB-interior-F/R	CGCATCATTGGTATGTAAGGTC
	GGATTTCCGTGTTTCGAGTTT
apfC-exterior-F/R	AGGAATACTTCGCTTACACCAC
	GTTATCACGGCTACAGAGGC
apfC-interior-F/R	CTAATCGCCCGCTTTGTT

Table 2 Primers used in this study *(Continued)*

Primers	Sequence (5′-3′)[a]
	CTGAATGCCGCAGTCCCT
apfD-exterior-F/R	CATCATCGGCATAACGCAGTC
	AGGCGGATTAGTCGGAGGC
apfD-interior-F/R	GCATAAACCCAGCATACCA
	TTCCTCTGCTTGCCTTTG
	For complement construction
qseBC-F/R	AACTGCAGTTGCAAAATTTCAGCAAATTTAGAC
	TTGCGGCCGCTTAGAAGATAATTTCCGCTTTTAAA
apfA-F/R	CCCTCGAGATGCAAAAACTAAGTCTTATTCGAC
	TTGCGGCCGCTTAATTTGATGCGCAGAAATTTGCC
apfB-F/R	CCCTCGAGATGCAGTATTCCGTATGTGATGTAA
	TTGCGGCCGCTCATACACTTTCAACATGACCTAAC
apfC-F/R	CCCTCGAGATGTTGAAAGTGTATGAATTTCATT
	TTGCGGCCGCCTAACCGCCTCCGACTAATCCGCCT
apfD-F/R	CCCTCGAGATGAGTTCTCCGATTTTCGCATGGT
	TTGCGGCCGCTTAGATATAAATTTTATCAATAATT
	For RT-qPCR
16S rRNA-F/R	CCATGCCGCGTGAATGA
	TTCCTCGCTACCGAAAGAACTT
apfA-qPCR-F/R	GACCGCCGCTACAGTTTTTA
	TTGCCATTTTAGCTACGGTTG
apfB-qPCR-F/R	CGAAGGCGCATCATTGGTAT
	CAAACGCAAATAAACCCGGC
apfC-qPCR-F/R	AAATTTCGCCTAATCGCCCG
	GCCGCAGTCCCTTTAAAACA
apfD-qPCR-F/R	TGTCCCCGCTTCCCAATATT
	TGCTTGCCTTTGGAATTGCT
	For protein expression
PqseB-F/R	CGCCCATATGTTGCAAAATTTCAGCAAATTTAGAC
	CGCCCTCGAGAGCATCTGAATTTCCTATTTTA
	For EMSAs
apf-EMSA-F/R	ATCCCTCTCCTTATCATATCTAAGA
	TTTGACCTCTGAATTTATTAAAACG
pilM-EMSA-F/R	CGAAAAATTCTCTTCTGGTAAATGA
	AATTTTAGTTCCTTAAATCAACTTT
glpK-EMSA-F/R	CGTTTCCCTTTTTTACGGACTAAAC
	ATAGTCCTCCTACGGATAACGAAAA

[a]Restricton sites are underlined

cells for 10 min at 4 °C or on ice. The lysates were serially diluted and selected the appropriate dilution gradient cells to plate on TSA (supplemented with NAD and bovine serum) overnight to determine bacterial counts.

Cell adhesion and invasion assays

NPTr cells were used to investigate the abilities of adhesion and invasion (Zhou et al. 2013). Briefly, all strains were added to NPTr cells at the MOI of 100 and incubated for 2 h. For the adhesion assays, each well was lysed by using 0.025% (*v*/*v*) Triton X-100 after the culture supernatant removed. The cell lysates were serially diluted to determine bacterial counts, which may contain adherent and invasive cells. For invasion assays, gentamicin was also added to each well after washing with PBS and further cultured for 45 min. Then, the cells were lysed and diluted in the appropriate dilution gradient for bacterial count.

Bacterial virulence *in vivo*

Six-week-old female Balb/c mice were purchased from Experimental Animal Center of Three Gorges University (Yichang, China). The animal ecperiments were performed as described previously, with some modifications (Li et al. 2018). To determine the survival rates, mouse were randomly divided into 11 groups (6 per group): WT, Δ*qseBC*, CΔ*qseBC*, Δ*apfA*, CΔ*apfA*, Δ*apfB*, CΔ*apfB*, Δ*apfC*, CΔ*apfC*, Δ*apfD* and CΔ*apfD*. Briefly, all strains were grown to the OD_{600} of 0.6. Each mice was inoculated with 5.00×10^6 CFU by intraperitoneal injection. Clinical symptoms and mortality rates of mice were observed and recorded every day. The surviving mice were euthanized a week later. To determine the bacterial colonization ability of mice tissues, each mice was inoculated with 1.00×10^6 CFU by intraperitoneal injection. At 6 h post-infection, blood samples were collected and anticoagulated by heparin. Then the mice were humanely-euthanized and lung tissue samples were taken out about 0.1 g for homogenization by using a Tissuelyser (Jingxin, China). 100 μL of each blood and lung sample was used for gradient dilution. CFU was calculated by appropriate dilution gradient tissue fluid cultured on TSA (supplemented with NAD and bovine serum).

Statistical analysis

Statistical analysis was performed *via* GraphPad Prism 7 software (San Diego, USA). The results were presented as mean ± SD. The survival rate of mice was analyzed by log-rank (Mantel-Cox) test. The bacterial load in mouse tissues was analyzed by two-tail Mann-Whitney test. Student´s *t* test was used to compare differences between groups, where $P < 0.05$ was considered significant.

Abbreviations

APP: *Actinobacillus pleuropneumoniae*; BHI: Brain heart infusion; bp: Base pair; cDNA: Complementary DNA; CFU: Colony forming units; DAP: 2,6-Diaminopimelic acid; ddH_2O: Double distilled H_2O; DMEM: Dulbecco's modified eagle medium; DNA: Deoxyribonucleic acid; EMSA: Electrophoretic mobility shift assay; FBS: Foetal bovine serum; g: Gram; h: Hour; IPTG: Isopropyl-β-D-thiogalactopyranoside; kDa: Kilodalton; LB: Luria bertani; min: Minute; MOI: Multiplicity of infection; mol: Mole; NAD: Nicotinamide adenine dinucleotide; OD: Optical density; PAGE: Polyacrylamide gel electrophoresis; PBS: Phosphate buffered saline; PCP: Porcine contagious pleuropneumonia; PCR: Polymerase chain reaction; RT-qPCR: Quantitative reverse transcription PCR; r/min: Rotation per minute; RNA: Ribonucleic acid; sec: Second; SDS: Sodium dodecylsulphate; SPF: Specific pathogen free; TCS: Two-component system; TSA: Tryptic soy agar; TSB: Tryptic soy broth; μL: Microliter; °C: Degree centigrade

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Authors' contributions

BD and WB conceived of the study, and participated in its design and coordination. BD and WP constructed the mutant and complemented strains. BD, JT and FenY participated in the animal assays. BD, KY and FL performed the statistical analysis. BD drafted the manuscript. WB, FanY and HC directed the project. All authors have read and approved the final version of the manuscript.

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Availability of data and materials

Data will be shared upon request by the readers.

Declarations

Ethics approval and consent to participate

All animal assays were performed according to the guidelines of the Laboratory Animal Monitoring Committee of Huazhong Agricultural University (HZAUMO-2020-083).

Consent for publication

Not applicable.

Competing interests

Author Huanchun Chen was not involved in the journal's review or decisions related to this manuscript. The authors declare no other conflict of interest.

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