The ClpP Protease Is Required for the Stress Tolerance and Biofilm Formation in *Actinobacillus pleuropneumoniae*

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**Abstract**

In the respiratory tract and lung tissue, a balanced physiological response is essential for *Actinobacillus pleuropneumoniae* to survive various types of challenges. ClpP, the catalytic core of the Clp proteolytic complex, is involved in various stresses response and regulation of biofilm formation in many pathogenic bacteria. To investigate the role of ClpP in the virulence of *A. pleuropneumoniae*, the clpP gene was deleted by homologous recombination, resulting in the mutant strain S8 ΔclpP. The reduced growth of S8 ΔclpP mutant at high temperatures and under several other stress conditions suggests that the ClpP protein is required for the stress tolerance of *A. pleuropneumoniae*. Interestingly, we observed that the S8 ΔclpP mutant exhibited an increased ability to take up iron in vitro compared to the wild-type strain. We also found that the cells without ClpP displayed rough and irregular surfaces and increased cell volume relative to the wild-type strain using scanning electron microscopy (SEM). Confocal laser scanning microscopy (CLSM) revealed that the S8 ΔclpP mutant showed decreased biofilm formation compared to the wild-type strain. We examined the transcriptional profiles of the wild type S8 and the S8 ΔclpP mutant strains of *A. pleuropneumoniae* using RNA sequencing. Our analysis revealed that the expression of 16 genes was changed by the deletion of the clpP gene. The data presented in this study illustrate the important role of ClpP protease in the stress response, iron acquisition, cell morphology and biofilm formation related to *A. pleuropneumoniae* and further suggest a putative role of ClpP protease in virulence regulation.

**Introduction**

*Actinobacillus pleuropneumoniae* is the causative agent of porcine pleuropneumonia, a highly contagious and often fatal respiratory disease that affects pigs worldwide [1]. This organism can cause sudden death and can colonize the respiratory tracts, tonsils and lungs of pigs, causing chronic and persistent infections, lung lesions, and reduced growth [2]. The ability of *A. pleuropneumoniae* to persist in host tissues is a major obstacle to the eradication of the organism [1,3,4], which is the primary source for new cases. Moreover, the disease causes serious economic losses for the swine industry [5].

Transitioning between respiratory tract and lung tissue subjects *A. pleuropneumoniae* to environmental stresses. *A. pleuropneumoniae* is well equipped to respond to these stressors through the production of a series of stress-related proteins [6]. Among these proteins, the ClpP protease, which is the member of the Clp (caseinolytic protease, Hsp100) family, has been studied in several pathogenic bacteria and has proved to be an important virulence factor [7–15].

The ClpP protease was first discovered and is best characterized in *Escherichia coli* [16,17]. ClpP protease is important for normal growth and is involved in the stress response and the degradation of misfolded proteins in most bacteria, including *E. coli* and *Salmonella enterica* [18,19]. Clp proteolytic enzymes are also required for full virulence in several pathogenic organisms, including *Listeria monocytogenes*, *Treponema pestis*, *Mycobacterium tuberculosis* and *Helicobacter pylori* [7–10]. Interestingly, the ClpP proteases may affect biofilm formation in some bacteria. Decreased biofilm formation was observed in clpP mutants of *Pseudomonas fluorescens*, *Streptococcus mutans* and *Staphylococcus epidermidis* [11–13], while increased biofilm formation was observed in clpP mutants of *Staphylococcus aureus* and *Pseudomonas aeruginosa* [14,15]. There is, however, no evidence that ClpP protease plays a role in the stress response or biofilm formation related to *A. pleuropneumoniae*.

In the present study, we inactivated the clpP gene in *A. pleuropneumoniae* strain S8 by homologous recombination using a sucrose counter-selectable marker system. We found that the ClpP protease mediates tolerance to multiple stressors, including heat, oxidative stress and osmotic stress. Interestingly, we found that the deletion of the clpP gene improved the iron utilization of *A. pleuropneumoniae*. We also showed that ClpP affects the cell morphology of and biofilms formed by *A. pleuropneumoniae* and that ClpP might play an important role in the regulation of virulence.

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Materials and Methods

Bacterial strains, plasmids and growth conditions

The bacterial strains, plasmids and primers used in this work are listed in Table 1. E. coli B2155 (ΔclpP) was cultured in LB medium supplemented with diaminopimelic acid (1 mM; Sigma-Aldrich, St. Louis, MO, USA), 100 μg/mL kanamycin and chloramphenicol (5 μg/mL). E. coli DH5α (E. coli strain carrying the following plasmid: pMD18-T simple A cloning vector Takara, Dalian, China) was used as the recipient strain for conjugative and transformation experiments. Selection of transconjugants was done on minimal medium supplemented with NAD (10 μg/mL; Sigma-Aldrich, St. Louis, MO, USA) and kanamycin (30 μg/mL).

Chromosomal inactivation of the clpP gene

Primers clpPSF/clpPSR and clpPXF/clpPXR (Table 1) were designed to generate a 491 bp internal deletion in the clpP gene by single-overlap extension PCR (SOE-PCR) [20]. The resultant 2449 bp PCR product, containing an internal in-frame deletion of 491 bp in the clpP gene (from nt 44 to 534), was ligated into the conjugal plasmid pEMOC2 to yield plasmid pEMAcclpP. Using E. coli strain B2155 [21], plasmid pEMAcclpP was used to introduce the clpP deletion into A. pleuropneumoniae strain S8 via the single-step transconjugation system, as described previously [22,23], resulting in A. pleuropneumoniae S8ΔclpP. PCR using primers clpPJDF/clpPJDR was used to distinguish between wild-type strains and mutants, and the PCR products were sequenced.

Complementation of A. pleuropneumoniae S8ΔclpP

The pLSclpP plasmid was constructed by cloning the 591-bp PCR product generated by the clpPHBF/clpPHBR primers (Table 1), which contained the entire clpP open reading frame (ORF), into the pLS88 plasmid [24]. The plasmid was then electroporated into A. pleuropneumoniae strain S8ΔclpP. The complemented A. pleuropneumoniae mutants were selected on BHI containing both NAD and kanamycin, and confirmed with PCR using the clpPHBF/clpPHBR primers.

Table 1. Characteristics of bacterial strains, plasmids, and primers used in this study.

| Strains, plasmids, and primers | Characteristics or sequence | Source or reference |
|--------------------------------|-----------------------------|---------------------|
| **Strains**                    |                             |                     |
| E. coli B2155                  | thrB1004 pro thi strA hsdS lacZΔM15 (9” lacZΔM15 lacI proph3 proB”)Δdop : erm (Emr) recA : RPA-2-tet (Tc-) ; Mu-km (Kmr) pIP | [21]                |
| A. pleuropneumoniae S8         | A. pleuropneumoniae serotype 7 clinical isolate from the lung of a diseased pig in northern China | This work           |
| A. pleuropneumoniae S8ΔclpP    | Unmarked ClpP protease-negative knockout mutant of A. pleuropneumoniae S8 | This work           |
| A. pleuropneumoniae S8HB       | The complemented strain of A. pleuropneumoniae S8ΔclpP containing the clpP gene | This work           |
| **Plasmids**                   |                             |                     |
| pMD18-T simple                 | A cloning vector            | Takara              |
| pMD.ΔclpP                      | Cloning vector with a 491 bp deletion in the clpP gene which have a 1.2-kb upstream fragment and 1.2-kb downstream fragment | This work           |
| pEMOC2                         | Conjugative vector based on pBluescript SK with mob RP4, polycloning site, Cm’, and transcriptional fusion of the omA promoter with the sacB gene | Accession no. AJ868288. [22] |
| pEM.ΔclpP                      | Conjugative vector pEMOC2 with a 491 bp deletion in the clpP gene which have a 1.2-kb upstream fragment and 1.2-kb downstream fragment | This work           |
| pLS88                          | Broad-host-range shuttle vector from Haemophilus ducreyi, Stx’ Smr Km’ | [24]                |
| pLSclpP                        | pLS88 with a PCR-derived insert containing the clpP gene | This work           |
| **Primers**                    |                             |                     |
| clpPSF                         | 5’ GCCCTGACGGGGGCGTACTGGATGC 3’, upstream primer with internal Sall site (underlined) comprising positions 1157 to 1141 upstream of the clpP gene start codon | This work           |
| clpPSR                         | 5’ CCACTGCCTTCCGCTTGGAGTTGTC 3’, downstream primer with reverse complement sequence (underlined) of sequence in bold from primer clpPXF, comprising positions 24 to 40 downstream of the clpP gene start codon | This work           |
| clpPXF                         | 5’ TCCAAGGGCAGAAGGCTAGATATCGGTGC 3’, upstream primer with reverse complement sequence (underlined) of sequence in bold from primer clpPSR, comprising positions 54 to 36 upstream of the clpP gene stop codon | This work           |
| clpPXR                         | 5’ TTGGCCCGCTTCTTGTAGTGGCCC 3’, downstream primer with internal NotI site (underlined) comprising positions 1172 to 1192 downstream of the clpP gene stop codon | This work           |
| clpPJDF                        | 5’ CGTGGTGTCGTCGAAACC 3’, upstream primer comprising positions 300 to 281 upstream of the clpP gene start codon | This work           |
| clpPJDR                        | 5’ ATTGACCGCTTCCATCGGC 3’, downstream primer comprising positions 51 to 31 upstream of the clpP gene stop codon | This work           |
| clpPHBF                        | 5’ CGGAATTCTGAGAATAAGTCATCGGT 3’, upstream primer with internal EcoRI site (underlined) comprising positions 1 to 21 downstream of the clpP gene start codon | This work           |
| clpPHBR                        | 5’ CGGAGCTTATAATTTATATCGTGTTGTA 3’, downstream primer with internal Sac site (underlined) comprising positions 23 to 1 upstream of the clpP gene stop codon | This work           |
Role of ClpP in *Actinobacillus pleuropneumoniae*
Growth experiments

*Actinobacillus pleuropneumoniae* the wild-type S8 strain, the S8Δ*clpP* mutant and the complemented S8HB strain were first grown in 5 ml of BHI for about 20 h and then diluted to similar optical densities at an OD_{600} value of approximately 0.2. These new cultures were then incubated at 25°C, 37°C and 42°C. OD_{600} was determined using an Eppendorf Biophotometer (Eppendorf, Hamburg, Germany) at various time points. The experiments were carried out in triplicate.

Stress resistance assays

Stress resistance assays were conducted using *Actinobacillus pleuropneumoniae* wild-type S8 strain, the S8Δ*clpP* mutant and the complemented S8HB strain. These strains were grown in BHI at 37°C. At an OD_{600} value of approximately 0.6, cells from 1 ml of broth cultures were centrifuged at 5,000 g for 5 min. For the heat-shock assay, cells were resuspended in BHI and placed in a 52°C water bath for 20 min. For the oxidative tolerance assay, the cells were resuspended in 1 ml of BHI supplemented with 1 mM hydrogen peroxide for 30 min. For the osmotic tolerance assay, the cells were resuspended in 1 ml of BHI supplemented with 0.3 M potassium chloride for 1 h. The control samples of each strain were resuspended in 1 ml of BHI without any treatment. Then, the cultures from each stress resistance assay were serially diluted in BHI, and spread on BHI plates for CFU counting. Stress resistance was calculated as \([\text{pressed sample CFU ml}^{-1}/\text{control sample CFU ml}^{-1}] \times 100\). The experiments were carried out in triplicate.

Iron utilization assays

The iron utilization assay has been described previously [25,26]. Briefly, *Actinobacillus pleuropneumoniae* strains S8, S8Δ*clpP* and S8HB were grown in 5 ml of BHI for about 20 h and then diluted into fresh BHI medium containing 30 μM of the iron chelator ethylenediamine dihydroxypophenylacetic acid (EDDHA) to similar optical densities at an OD_{600} value of approximately 0.2. In the iron supplementation culture, 10 μM FeSO_{4} was added to the iron chelated culture after the addition of EDDHA. OD_{600} was determined using an Eppendorf Biophotometer (Eppendorf, Hamburg, Germany) at various time points. The experiments were carried out in triplicate.

Scanning electron microscopy

For scanning electron microscopy, *Actinobacillus pleuropneumoniae* strains S8, S8Δ*clpP* and S8HB were grown to similar optical densities at the OD_{600} value of approximately 0.5, 2.0 and 2.7, respectively. Then washed 3 times with phosphate-buffered saline (PBS; in mM: 137 NaCl, 2.7 KCl, 1.8 KH_{2}PO_{4} and 10 Na_{2}HPO_{4}, pH 7.4). Cultures were then fixed with 2.5% glutaraldehyde and 1% OsO_{4}, dehydrated in a graded ethanol series and embedded in isoamyl acetate. The cells were dried using a critical point drying method, mounted on aluminum stubs and shadowed with gold. A scanning electron microscope (S-3400N, Hitachi, Japan) at 5 kV was used to visualize cells.

Polystyrene microtiter plate biofilm assay

The microtiter plate biofilm assay is a static assay that is particularly useful for examining biofilm formation [27]. The wells of a sterile, 96-well microtiter plate (Costar® 3599, Corning, NY, USA) were filled in triplicate with a dilution (1/100) of an overnight bacterial culture. Following an incubation period of 5–48 h at 37°C, the wells were washed with water and stained with crystal violet, as previously described [11]. The bound dye was solubilized in 100 μL of 70% ethanol, and the optical density of the ethanol-dye solution was measured using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA).

Confocal laser scanning microscopy

The same biofilm assay protocol was used as mentioned above. After 16 h of incubation, the wells were washed with water to remove non-adherent bacteria. Cells were then stained with LIVE/DEAD® BacLight™ Bacterial Viability Kit solution (Molecular Probes, Eugene, Oregon, USA), incubated for 20 min at room temperature in the dark, and washed with water. The plate was examined with a confocal microscope (TCS SP5, Leica Microsystems, Hamburg, Germany). SYTO 9 nucleic acid stain was excited at 485 nm and detected using 520 nm filters. Propidium iodide was excited at 488 nm and detected using 572 nm filters.

RNA isolation, cDNA library construction and sequencing

*Actinobacillus pleuropneumoniae* strains S8 and S8Δ*clpP* were grown to early log phase (OD_{600} nm = 0.5) in BHI. The cells were collected at 4°C, and the Ribopure-Bacteria kit (Ambion) was used according to the manufacturer’s instructions to isolate RNA. Both of the samples were quantified and examined for protein and reagent contamination with a Nanodrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). The RNA samples exhibiting a 23S/16S rRNA band intensity of 2:1, a spectroscopic A260/A280 nm ratio of 1.8–2.0, and an A260/A230 nm ratio greater than 1.5 were selected for analysis. A total of 20 μg of RNA was equally pooled from the S8 and S8Δ*clpP* strains for cDNA library preparation.

Illumina sequencing was performed at the Beijing Genomics Institute (BGI)-Shenzhen in Shenzhen, China [http://www.genomics.cn/index] according to the manufacturer’s instructions (Illumina, San Diego, CA). The cDNA libraries were prepared according to Illumina’s protocols and sequenced using the Illumina HiSeq 2000.

Differential expression analysis

The raw sequence reads were filtered by the Illumina pipeline. All of the low-quality reads, reads with adapter contamination, and reads with a copy number of one were excluded from the analysis, while the clean reads were mapped to the *Actinobacillus pleuropneumoniae* serotype 7 strain S8 reference sequences (Genbank accession No. ALYN00000000.1). To identify the genes affected by the deletion of the *clpP* gene, the libraries were initially compared. To complete this procedure, the number of reads for each coding region was determined, the number of total reads was normalized between the libraries and the ratio of S8 reads to S8Δ*clpP* reads was calculated. The differentially expressed genes were detected as previously described [28], with the false discovery rate (FDR) being set below 0.01 [29]. An FDR≤0.001 and a log2Ratio absolute value ≥1 was set as the threshold for significant differences in gene expression.
Role of ClpP in Actinobacillus pleuropneumoniae
The Blast2GO program [30] was used to obtain GO annotations for molecular functions, biological processes and cellular component ontologies (http://www.geneontology.org).

The Kyoto Encyclopedia of Genes and Genomes pathway database [31] (http://www.genome.jp/kegg) was used to make pathway assignments. The BlastN program (http://blast.ncbi.nlm.nih.gov)

Figure 2. Impaired stress tolerance of the *A. pleuropneumoniae* S8ΔclpP mutant. Overnight cultures were inoculated into fresh medium and grown to an OD<sub>600</sub> value of approximately 0.6. Cells were then treated with (A) 1 mM H<sub>2</sub>O<sub>2</sub> for 30 min, * p<0.01, (B) 52 C heat shock for 20 min, * p<0.01, or (C) 0.3 M KCl for 1 hour, * p<0.05. * denotes P values (t test) for comparison to S8ΔclpP.

doi:10.1371/journal.pone.0053600.g002

Figure 3. The growth curves of the *A. pleuropneumoniae* in iron-restricted and iron supplemented conditions. Overnight cultures of the S8 (□), S8ΔclpP (○) and S8HB (△) strains were diluted into fresh medium and then incubated in (A) BHI containing 30 μM of the iron chelator EDDHA or (B) BHI containing 30 μM of the iron chelator EDDHA and 10 μM FeSO<sub>4</sub>. Growth was monitored by OD<sub>600</sub> at various time points. Points indicate the mean values, and error bars indicate standard deviations.

doi:10.1371/journal.pone.0053600.g003
nih.gov) was also used to compare these sequences to the *A. pleuropneumoniae* serotype 7 strain AP76 reference sequences (Genbank accession No. CP001091.1) and obtain a historical annotation.

**Statistical analysis**

Basic statistical analyses were conducted using the SPSS software (SPSS, Inc., Chicago, IL, USA). The Student’s t test was used to determine the significance of the differences in the means between multiple experimental groups. The data were expressed as the mean ± standard deviation, and values of *P* < 0.05 were considered to be significant.

**Results**

**Construction of S8ΔclpP mutant strain**

In order to determine whether the function of ClpP protease was crucial for the stress tolerance and biofilm formation related to *A. pleuropneumoniae*, we constructed an isogenic *clpP* deletion mutant of *A. pleuropneumoniae* S8 with plasmid pEMOC2. The *clpP* deletion mutant was constructed by the allelic exchange of the wild-type *clpP* gene with an in-frame deletion lacking 491 bp at position 44–534 of the *clpP* ORF (Figure S1 and Figure S2). The resulting *A. pleuropneumoniae* mutant was designated as S8ΔclpP.

**Growth experiments**

We first examined the impact of ClpP protease on growth. As shown in Figure 1, the growth curves of the wild-type S8 strain, the S8ΔclpP mutant and the complemented S8HB strain were similar at 25°C and 37°C (Figure 1A and 1B), demonstrating that ClpP protease is not required for optimal growth at lower temperatures. However, the S8ΔclpP mutant strain exhibited impaired growth at 42°C (Figure 1C), indicating an important role for ClpP protease in the optimal growth of *A. pleuropneumoniae* at high temperatures.

**ClpP Protease is required for the stress tolerance of *A. pleuropneumoniae***

The wild-type S8 strain, the S8ΔclpP mutant and the complemented S8HB strain were exposed to various stress conditions. When the cells were treated with 1 mM hydrogen peroxide for 30 min, the S8ΔclpP mutant cell survival rate was 36.8%, which was much lower than that of the S8 cells (64.9%) and the S8HB cells (58.7%; Figure 2A). These results suggest that ClpP has a role in the tolerance of *A. pleuropneumoniae* to oxidative stress. Similar results were obtained in the heat shock assay. Wild-type cells incubated in a 52°C water bath for 20 min exhibited an 81.4% survival rate, and the complemented S8HB strain exhibited a 76.2% survival rate; however, only 50.5% of the S8ΔclpP mutant cells survived (Figure 2B). These results indicate that the deletion of *clpP* impairs the ability of *A. pleuropneumoniae* to successfully respond to heat shock. Similarly, when cells were treated with 0.3 M potassium chloride for 1 hour, the survival rate of S8ΔclpP mutant cells (50.6%) was lower than that of the S8 cells (70.6%) and the S8HB cells (67.8%; Figure 2C), indicating that ClpP protease is also important for the response of *A. pleuropneumoniae* to osmotic stress. Collectively, these results indicate that ClpP protease is involved in the tolerance of multiple stresses.

**ClpP Protease affects the iron acquisition ability of *A. pleuropneumoniae***

The ability of the S8, S8ΔclpP and S8HB strains to utilize iron was analyzed using iron-restricted medium (BHI, 30 μM EDDH) and iron supplementation medium (BHI, 30 μM EDDHA and 10 μM FeSO₄). As shown in Figure 3A, the growth of the S8,
S8ΔclpP and S8HB strains was significantly inhibited in low-iron, BHI medium with the addition of EDDHA. However, the S8ΔclpP mutant strain exhibited slightly increased growth as compared with the S8 and S8HB strains in these conditions. In the iron supplementation culture, the growth capacity of all strains was largely restored, but the growth ability of the S8ΔclpP mutant strain was still slightly increased relative to the S8 and S8HB strains (Figure 3B). These results suggest that the deletion of the clpP gene might improve the iron utilization of *A. pleuropneumoniae*.

**Loss of clpP leads to aberrant cell morphology of *A. pleuropneumoniae***

Samples of the S8, S8ΔclpP and S8HB strains were processed using standard procedures and examined under a scanning electron microscope. A significant morphological variation was observed. Notably, the morphology of the S8ΔclpP strain showed an increase in volume (1.8-fold) compared to the wild-type S8 strain (Figure 4). Furthermore, the cells of the S8ΔclpP strain showed rougher, more irregular surfaces than the wild-type cells (Figure 4). However, the morphology of the complemented S8HB strain is similar to the wild-type S8 strain. These results indicate that the ClpP protease plays an important role in maintaining cell morphology related to *A. pleuropneumoniae*.

**ClpP Protease affects the biofilm formation by *A. pleuropneumoniae***

The biofilm formation phenotype of the S8, S8ΔclpP and S8HB strains was examined in polystyrene microtiter plates using crystal violet staining (Figure 5A) and was quantitatively analyzed using a microplate reader (Figure 5B). The S8ΔclpP mutant exhibited weak biofilm formation, while the biofilm formation phenotypes of the S8 and S8HB strains were stronger than the S8ΔclpP

![Figure 5. Polystyrene microtiter plate biofilm assay.](image-url)
phenotype. The biofilm formation process was also observed under a confocal scanning laser microscope (Figure 6). Overall, the biofilm formation was significantly decreased during the middle to late exponential phases in the S8(clpP mutant strain compared to the S8 and S8HB strains under each culture condition (Figure 5 and 6). The clpP mutation attenuates biofilm formation in this strain, indicating that ClpP protease is required for biofilm formation in A. pleuropneumoniae.

Differential expression analysis
To identify the A. pleuropneumoniae genes affected by the deletion of the clpP gene, the S8ΔclpP and S8 strains were transcriptionally profiled using RNA sequencing. A total of 13,694,332 and 12,883,314 reads were obtained for each library (“S8ΔclpP” and “S8”, respectively). Of these reads, 13,340,847 (S8ΔclpP) and 12,589,286 (S8) reads mapped to the genome, and 13,316,197 (S8ΔclpP) and 12,562,203 (S8) mapped uniquely. One thousand two hundred thirteen of the unique reads in the S8 sample aligned to the clpP gene, while none of the reads from the S8ΔclpP sample aligned to this gene. This finding proved that the clpP gene was not expressed in the S8ΔclpP mutant. The differential expression analysis of the S8ΔclpP and S8 strains revealed that 16 A. pleuropneumoniae genes exhibited an FDR < 0.001 and an absolute value of log2Ratio absolute value ≥1, proving that these genes were affected by the deletion of the clpP gene (Table 2). Of these 16 genes, 4 were upregulated, including 2 genes involved in the iron acquisition system, and 12 were downregulated.

Discussion
The ClpP protease is a conserved protein that is present in many bacteria [32]. In this study, we initially determined the nucleotide level similarities in the clpP gene sequences with a BLASTN analysis. Currently, only the complete genomes of A. pleuropneumoniae serotypes 3, 5 and 7 have been sequenced. We found that the clpP gene was present in all of these genomes and that the identity of these nucleotide sequences was more than 94%. We also used PCR to confirm that the clpP gene is present in A. pleuropneumoniae isolates available in our laboratory (data not shown). These results indicated that the clpP gene is conserved in several A. pleuropneumoniae serotypes. In the present study, the clpP mutant was constructed and challenged with a variety of stressors and growth conditions to examine the physiological role of the ClpP protease in the stress tolerance, cell morphology, and biofilm formation related to A. pleuropneumoniae. To confirm that the characteristics of the S8ΔclpP mutant were not due to polar effects and were truly caused by the clpP gene deletion, we also constructed a complemented strain S8HB. In addition, the RNA sequencing results indicated that the expression levels of the 10 genes upstream of the clpP gene and the 10 genes downstream of the clpP gene did not change. All of these results proved that the characteristics of the S8ΔclpP mutant were truly caused by the deletion of the clpP gene and not by polar effects.

In this study, we observed that the S8ΔclpP mutant exhibited a reduced growth rate at high temperatures (Figure 1C) and an impaired resistance to heat shock (Figure 2B) compared to the wild-type S8 strain and the complemented S8HB strain. Several previous studies have demonstrated growth deficits resulting from ClpP deficiency over a broad range of temperatures [7,33,34]. However, there was no significant difference between the growth curves of the S8ΔclpP mutant, the wild-type S8 strain and the complemented S8HB strain when these strains were cultured at a lower temperature (Figure 1A). The observations in our study

![Figure 6. Microscopy analysis of the biofilm formation. Biofilm development was monitored by confocal scanning laser microscopy (CSLM) after 12, 18, 24, and 30 h. The cells were stained with LIVE/DEAD® BacLight™ Bacterial Viability Kit solution. The S8ΔclpP strain shows a reduction in biofilm formation compared to the wild-type S8 strain and the complemented S8HB strain. doi:10.1371/journal.pone.0053600.g006](https://www.plosone.org/doi/abs/10.1371/journal.pone.0053600.g006)
suggest a more restricted role for ClpP in *A. pleuropneumoniae*, independent of cold stress.

Iron is an essential factor for the growth of *A. pleuropneumoniae*, and low iron availability in the host represents a major stress for the pathogen. *A. pleuropneumoniae* has evolved a highly sophisticated system for iron acquisition that includes transferrin receptor complexes TbpAV/TbpB, TonB-ExbB-ExbD, Afu, ABC transporter and so on [26]. In the current study, the S8ΔclpP mutant was shown to exhibit a faster growth compared to the wild-type S8 strain and the complemented S8HB strain. In contrast, this result was not reported in studies on the role of ClpP in other bacteria. Based on this result, we hypothesize that the ClpP protease might regulate the expression of some of the genes involved in iron uptake, thus regulating virulence. Therefore, we also transcriptionally profiled the effects of the deletion of the *clpP* gene in *A. pleuropneumoniae*. The results of this analysis showed that 2 genes encoding hypothetical proteins was upregulated or down-regulated. These changes might also affect biofilm formation in *A. pleuropneumoniae*. However, these findings should also be confirmed in future studies.

### Supporting Information

**Figure S1** Schematic representation of the *A. pleuropneumoniae clpP* locus. The figure shows the binding locations for the oligonucleotide primers used to amplify the two flanking regions (1249 bp and 1200 bp, respectively) used in the construction of the pEMΔclpP plasmid and the diagnostic PCR analysis of the *clpP*-deleted mutant (367 bp) and wild type *A. pleuropneumoniae* strains (858 bp). The S8ΔclpP mutant contains a 491 bp in-frame deletion (shadowed domain) in the *clpP* gene. (TIF)

**Figure S2** PCR identification of the S8ΔclpP mutant. PCR identification of the S8ΔclpP mutant using the paired primers clpPJD/clpPJDR. For lanes 8, the identified S8ΔclpP mutant (367 bp); for lane M, DL2000 DNA marker was used (from top to bottom: 2000, 1000, 750, 500, 250, and 100 bp); for other lanes, the wild-type S8 strain. (TIF)

### Acknowledgments

We thank Dr. Gerald-F. Gerlach (Institute for Microbiology, Department of Infectious Diseases, University of Veterinary Medicine Hannover, Germany) for the generous donation of *E. coli* β2155 strain and vector PAM7_0616 1.037253 hypothetical protein

**Table 2.** The regulated genes in *A. pleuropneumoniae* by the deletion of the *clpP* gene.

| Gene         | Log2 Ratio | Annotation                                           |
|--------------|------------|-----------------------------------------------------|
| **Up-regulated genes** |            |                                                     |
| APP7_1881    | 1.46581    | putative periplasmic iron/siderophore binding protein |
| APP7_1156    | 1.198833   | hypothetical protein                                 |
| APP7_1879    | 1.051793   | Fe(III) dicitrate ABC transporter, ATP-binding protein |
| APP7_0104    | 1.037253   | hypothetical protein                                 |
| **Down-regulated genes** |          |                                                     |
| APP7_0616    | −2.30679   | pyridoxal biosynthesis lyase PdxS                    |
| APP7_2064    | −2.12199   | tRNA 2-thioadenosine synthesizing protein A          |
| APP7_1497    | −1.78015   | hypothetical protein                                 |
| APP7_0617    | −1.5762    | glutamine amidotransferase subunit PdxT              |
| APP7_0418    | −1.44626   | RNA polymerase sigma-70 factor                       |
| APP7_0419    | −1.33961   | putative sigma E factor negative regulatory protein  |
| APP7_1517    | −1.26523   | hypothetical protein                                 |
| APP7_1695    | −1.25314   | hypothetical protein                                 |
| APP7_1286    | −1.1338    | maltose/maltodextrin import ATP-binding protein MalK|
| APP7_1284    | −1.0894    | maltose operon periplasmic protein                   |
| APP7_0747    | −1.05714   | putative methylation subunit, type III restriction-modification system |
| APP7_1152    | −1.04195   | hexosaminidase                                       |

doi:10.1371/journal.pone.0053600.t002
References

1. Haesebrook F, Chiers K, Van Overbeke I, Ducatelle R (1997) Actinobacillus pleuropneumoniae infections in pigs: the role of virulence factors in pathogenesis and protection. Vet Microbiol 50: 239–249.

2. Bosse JT, Janson H, Sheehan BJ, Beddek AJ, Rycroft AN, et al. (2002) Actinobacillus pleuropneumoniae: pathobiology and pathogenesis of infection. Micr Infect 4: 225–235.

3. Frenwicz B, Henny S (1994) Porcine pleuropneumonia. J Am Vet Med Assoc 204: 1334–1340.

4. Chiers K, van Overbeke I, De Laender P, Ducatelle R, Carel S, et al. (1998) Effects of endobronchial challenge with Actinobacillus pleuropneumoniae serotype 9 of pigs vaccinated with inactivated vaccines containing the Apx toxins. Vet Q 20: 63–69.

5. Rycroft AN, Garide LH (2000) Actinobacillus species and their role in animal disease. Vet J 159: 18–36.

6. Chiers K, De Waele T, Pasmans F, Ducatelle R, Haesebrock F (2010) Virulence factors of Actinobacillus pleuropneumoniae involved in colonization, persistence, and induction of lesions in its porcine host. Vet Res 41: 65.

7. Guillot O, Pellegrini E, Bregenhoit S, Nair S, Berche P (2000) The ClpP serine protease is essential for the intracellular parasitism and virulence of Listeria monocytogenes. Mol Microbiol 35: 1296–1304.

8. Jackson MW, Silva-Herzog E, Plan G (2004) The ATP-dependent ClpXP and Lon proteases regulate expression of the Termini type III secretion system via regulated proteolysis of YnoA, a small histone-like protein. Mol Microbiol 54: 1364–1378.

9. Raju RM, Unnikrishnan M, Rubin DH, Krishnamoorthy V, Kandror O, et al. (2012) Atybacterium tuberculosis ClpP1 and ClpP2 function together in protein degradation and are required for viability in vitro and during infection. PLoS Pathog 8: e1002511.

10. Loughlin MF, Arandhara V, Okolie C, Aldsworth TG, Jenks PJ (2009) Maintenance of broad-host-range incompatibility materials/analysis tools: YZ SL. Wrote the paper: FX CW.

11. Lemos JA, Burne RA (2002) Regulation and Physiological Significance of ClpC and ClpP in Staphylococcus mutans. Proc Natl Acad Sci U S A 99: 5505–5509.

12. Pruteanu M, Baker TA (2009) Controlled degradation by ClxpP protease tunes the levels of the excision repair protein UvrA to the extent of DNA damage. Mol Microbiol 71: 912–924.

13. Thomsen LE, Olsen JE, Foster JW, Ingmer H (2002) ClpP is involved in the stress response and degradation of misfolded proteins in Salmonella enterica serovar Typhimurium. Microbiology 148: 2727–2735.

14. Ser'yan-Arshavsky SD, Brian DA (1995) Precise large deletions by the PCR-based overlap extension method. Mol Biotechnol 3: 15–18.

Author Contributions

Conceived and designed the experiments: FX CW. Performed the experiments: FX LZ. Analyzed the data: FX GL. Contributed reagents/materials/analysis tools: YZ SL. Wrote the paper: FX CW.

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pEMOC2. We also thank Dr. Wang and Dr. Shen [Basic Condition and Technology Services Center, Harbin Veterinary Research Institute of Chinese Academy of Agricultural Sciences, China] for technical assistance with the SEM experiments.