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Role of ClpX and ClpP in *Streptococcus suis* serotype 2 stress tolerance and virulence

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ABSTRACT

Streptococcus suis has received increasing attention for its involvement in severe infections in pigs and humans; however, their pathogenesis remains unclear. ClpX and ClpP, two subunits of the ATP-dependent caseinolytic protease Clp, play key roles in bacterial adaptation to various environmental stresses. In this study, a virulent *S. suis* serotype 2 strain, ZY05719, was employed to construct *clpX* and *clpP* deletion mutants ($\Delta clpX$ and $\Delta clpP$, respectively) and their complementation strains. Both $\Delta clpX$ and $\Delta clpP$ displayed significantly reduced adaptability compared with the wild-type strain, evident through several altered phenotypes: formation of long cell chains, tendency to aggregate in culture, and reduced growth under acidic pH and H₂O₂-induced oxidative stress. ClpP and ClpX were required for the optimal growth during heat and cold stress, respectively. An *in vitro* experiment on RAW264.7 macrophage cells showed significantly increased sensitivity of $\Delta clpX$ and $\Delta clpP$ to phagocytosis compared with the wild-type strain. Mouse infection assays verified the deletion of *clpX* and *clpP* led to not only fewer clinical symptoms and lower mortality but also to a marked attenuation in bacterial colonization. These virulence-related phenotypes were restored by genetic complementation. Furthermore, the deletion of *clpX* or *clpP* caused a significant decrease in the expression of *sodA*, *tpx*, and *apuA* compared with the wild-type strain, suggesting that these genes may be regulated by ClpX and ClpP as downstream response factors to facilitate the bacterial tolerance against various environmental stresses. Taken together, these results suggest that ClpX and ClpP play important roles in stress tolerance for achieving the full virulence of *S. suis* serotype 2 during infection.

1. Introduction

Streptococcus suis is one of the predominant zoonotic pathogens responsible for numerous maladies, including septicemia, meningitis, endocarditis, and, consequently, death in swine and humans (Gottschalk and Segura, 2000; de Greeff et al., 2011; Tan et al., 2017). *S. suis* serotype 2 (SS2) is considered the most prevalent virulent *S. suis* strain associated with acute infections (Du et al., 2014) and is of zoonotic importance (Dupas et al., 1992). Two notable large-scale outbreaks of SS2 in China (Jiangsu Province in 1998 and Sichuan Province in 2005) led to streptococcal toxic shock syndrome in humans (Tang et al., 2006; Feng et al., 2010; Du et al., 2014). Over the past 40 years, several virulence-associated factors, such as capsule polysaccharides, glyceraldehyde-3 phosphate dehydrogenase (GAPDH), sortase A, CiaRH, suilysin (Sly), extracellular factor, enolase, and muramidase-

released protein (MRP), have been reported to be involved in the pathogenesis of SS2 (Fittipaldi et al., 2012; Li et al., 2018). Although *S. suis* is a common inhabitant of the upper respiratory system, especially the nasal epithelium and palatine tonsils, it can opportunistically translocate into the bloodstream by crossing the mucosal epithelium. However, there is a lack of information on the molecular pathogenesis of SS2 infection (Fittipaldi et al., 2012; Feng et al., 2014).

Proteases help maintain cellular homeostasis by clearing short-lived regulatory proteins as well as misfolded and damaged proteins during protein quality control processes (Yu and Houry, 2007). In eukaryotic cells, environmental stresses, such as different temperatures, exposure to ethanol, changes in pH, oxidative (H₂O₂-induced) and osmotic (NaCl-induced) conditions, and nutrient scarcity, induce the expression of a set of heat-shock proteins (HSPs) (Craig et al., 1993), a well-known type of proteases. Many heat shock proteins act as molecular

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chaperones to protect cells against adverse effects, most notably heat, by increasing their survival rate. So, a detailed understanding of the heat shock response could give useful information on adaptation of *S. suis* to hostile environments it encounters. Among prokaryotes, many bacterial species contain four distinct types of ATP-dependent proteases: Clp proteases (ClpAP, ClpBP, ClpCP, ClpEP and ClpXP), single-chain AAA (FtsH), ClpYQ (also called HslUV), and the Lon family of proteases (Robertson et al., 2002; Gottesman, 2003). The Clp Proteases also designated as HSPs, are energy-dependent cytoplasmic proteases that were first identified and most studied in *Escherichia coli* and characterized as two-component systems containing an ATPase specificity factor (ClpA/X in *E. coli* or ClpC in *Bacillus subtilis*) and a proteolytic domain (ClpP), which has a consensus serine protease active site (Gottesman and Maurizi, 1992; Maurizi, 1992; Robertson et al., 2002; Dalia and Weiser, 2011). Even though progress has been achieved in understanding of the mechanisms of action of the Clp family in Gram-negative bacteria such as *E. coli* and *Salmonella typhimurium*, little is known about the Clp family in Gram-positive pathogenic bacteria (Schirmer et al., 1996; Kwon et al., 2003).

The genomic analysis of the virulent SS2 strain ZY05719 has revealed the presence of ClpP and ClpX homologs, along with ClpA. The ATP-dependent proteases ClpX and ClpP are two common HSPs that belong to the Clp/Hsp100 family of molecular chaperones (Rath et al., 2012), which comprise a large group of closely associated proteins that are ubiquitously present in both prokaryotes and eukaryotes (Ibrahim et al., 2005). They are commonly synthesized and polymerized into enzyme complexes under stress (Rath et al., 2012). When the multimers of Clp ATPases and ClpP are assembled, they play important roles in intracellular protein folding, assembly, and degradation during normal cell growth, and especially under stress conditions (Lemos and Burne, 2002).

ClpP proteases are fundamental components in bacterial cells that enable their survival under stress, allowing virulence and the development of diseases in the host, which are mediated by ClpP proteolysis (Robertson et al., 2002). In addition, ClpP proteolytic complexes also display autonomous chaperone activity, and can dynamically reactivate and remodel unfolded or misfolded proteins, in turn helping protein folding during stress conditions (Frees and Ingmer, 1999; Kruger et al., 2000; Singh et al., 2001; Wojtyra et al., 2003; Thibault et al., 2006). ClpP has been reported to modify the exposure of various membrane and secretory proteins implicated in the pathogenesis and biofilm formation in *Staphylococcus aureus* (Frees et al., 2004). ClpX and ClpP also play a role in acid stress resistance in *Streptococcus agalactiae* and *Salmonella enterica* serovar Typhimurium (Hensel et al., 1995; Webb et al., 1999; Nair et al., 2003) and in the expression of virulence factors in *Listeria monocytogenes* and *Yersinia enterocolitica* (Pederson et al., 1997; Nair et al., 2003). Therefore, we hypothesized that homologs of ClpX and ClpP encoded in ZY05719 genome may play important roles in survival of SS2 cells during stress conditions.

In the present study, we identified the ATP-dependent Clp proteases ZY05719_04030 and ZY05719_07365 in the SS2 strain ZY05719 and aimed to investigate their role in the stress tolerance and pathogenicity of *S. suis* to better the understanding of *S. suis* infection.

2. Material and methods

2.1. Bacterial strains, culture conditions, plasmids, and host cell lines

All the *S. suis* 2 strains and plasmids used in this study are available in Table 1. In this study, we used SS2 strain ZY05719, one of the representative Chinese virulent strains that were isolated from a diseased pig during an outbreak in the Sichuan province of China (Yu et al., 2006). The SS2 strains were cultured at 37 °C with shaking at 180 rpm in Todd-Hewitt Broth (THB) or Todd-Hewitt broth agar (THA) (Becton-Dickinson, USA) and harvested at the mid-exponential growth phase (OD_{600nm} = 0.6). *E. coli* strains were cultured on Luria-Bertani (LB)

broth (Sigma, USA) or LB agar plates at 37 °C. According to the requirement of antibiotics, spectinomycin (Spc, sigma, USA) were applied at the dose of 100 µg/ml for *S. suis*, and 50 µg/ml for *E. coli*. DMEM culture medium (Gibco, Invitrogen, USA) including 10% fetal calf serum (Gibco, USA) was used to culture the human laryngeal epithelial cell line (HEp-2) and Raw264.7 macrophage cell line at 37 °C in a 5% CO₂ humidified atmosphere.

2.2. DNA manipulations

Total genomic DNA was extracted by using an E.Z.N.A.[®] Bacterial DNA Kit (OMEGA. bio-tek, Shanghai, China), according to the manufacturer's instructions. Isolation and purification of plasmids were performed by using AxyPrep[™] plasmid Miniprep Kit (AXYGEN[®] A Corning Brand, China) in accordance with manufacturer's instructions.

2.3. Recombinant DNA techniques

Deletion of *clpX* and *clpP* in ZY05719 strains were performed by using the thermosensitive suicide *S. suis*-*E. coli* vector pSET4s (Takamatsu et al., 2001a). Briefly, we amplified two flanking regions (LA and RA) of the target gene using specific primers scheduled in Table 2. Then, the two flanking regions were integrated together by overlap-extension PCR. The PCR products were purified and digested with respective endonucleases (BamHI and EcoRI). Simultaneously, ligation between temperature-sensitive vector (pSET4s) and digested PCR products was performed to clone the target gene. The recombinant vectors were transformed by electroporation into competent cells SS2 ZY05719. After five time serial allelic passages spectinomycin (Spc) sensitive clones were selected and then mutants were detected by PCR and sequencing.

The complementation strains were constructed by the amplification of the target genes and its putative promoter sequences in PCR from the ZY05719 genome and cloned into the *E. coli*-*S. suis* shuttle vector pSET2 (Takamatsu et al., 2001b) to generate recombinant plasmids. The recombinant plasmids were electroporated into mutants ($\Delta clpX$, and $\Delta clpP$). In addition, the complementation strains ($C\Delta clpX$ and $C\Delta clpP$) were selected with spectinomycin and verified by PCR. Table 2 shows the sequences of primers used in this study.

2.4. Growth characteristics and genetic stability of mutant strains

Individual inoculation of the wild-type (ZY05719), mutant ($\Delta clpX$, and $\Delta clpP$) and the complementation strains ($C\Delta clpX$ and $C\Delta clpP$) (Diluted 1:100) were performed into 100 ml THB to incubate at different temperatures (28 °C, 37 °C and 42 °C) at 180 rpm/min. Culture bacteria were measured at 1 h intervals using a spectrophotometer (Smart Spec Plus, Bio-Rad, USA) at an absorbance of OD_{600 nm}, with quartz cuvettes. Each test was repeated thrice.

2.5. Grams stain assay

The grams stain assay was carried out as previously describe (Zhong et al., 2018). The cells were allowed to grow upto an OD₆₀₀ of 0.6 in THB at 37 °C, and gram stained cells were examined under a light microscope using oil immersion.

2.6. Biofilm plate assay

The capacity for biofilm formation was tested using the protocol as reported previously (Grenier et al., 2009). The overnight culture was diluted and 200 µl of each (strain) diluted culture (at OD₆₀₀ of 0.1) was added in 96-well microplates and incubated for 24 h at 37 °C. We added crystal violet dye after removing free-floating bacteria by aspiration and wells were washed thrice by PBS to remove unbound crystal violet dye. After washing, the wells were dried for 30 min at 37 °C. 100 µl of

Table 1
Summary of bacterial strains and plasmids.

Strains or plasmid	Characteristic or function	Source or reference
Strains		
ZY05719	Virulent strain of SS2 isolated from a dead patient	Stored in our lab
$\Delta clpP$	Isogenic <i>clpP</i> mutant of strain ZY05719	This study
$\Delta clpX$	Isogenic <i>clpX</i> mutant of strain ZY05719	This study
$C\Delta clpP$	Complemented strain of ZY05719 $\Delta clpP$; Spc^R	This study
$C\Delta clpX$	Complemented strain of ZY05719 $\Delta clpX$; Spc^R	This study
DH5 α	Cloning host for maintaining the recombinant plasmids	Invitrogen
Plasmids		
pSET4 _s	Thermo-sensitive <i>S. suis</i> - <i>E. coli</i> shuttle vector; Spc^R	Takamatsu et al., 2001b
pSET4 _s :: <i>clpP</i>	A recombinant vector with the background of pSET4 _s , designed to $\Delta clpP$, Spc^R	This study
pSET4 _s :: <i>clpX</i>	A recombinant vector with the background of pSET4 _s , designed to $\Delta clpX$, Spc^R	This study
pSET2	<i>E. coli</i> - <i>Streptococcus</i> shuttle Cloning vectors; Spc^R	Takamatsu et al., 2001a
pSET2:: <i>clpP</i>	pSET2 containing <i>clpP</i> gene and its promoter	This study
pSET2:: <i>clpX</i>	pSET2 containing the <i>clpX</i> gene and its promoter	This study

Spc^R , Spectinomycin resistant.

95% ethanol were added into each well of a 96-well microplate and the plates were again incubated for 10 min at 37 °C. Absorbance was measured at 550 nm. The wells with a sterile THB culture medium were used as a negative control. Each test was done in triplicate and repeated at least thrice.

2.7. Survival assays of oxidative (H_2O_2), acidic and osmotic (NaCl) condition in SS2

To assess the role of the ClpX and ClpP under risk factors, the strains (WT, $\Delta clpX$, $\Delta clpP$, $C\Delta clpX$, and $C\Delta clpP$) were challenged to various stresses including H_2O_2 and acidic conditions. Bacterial cultures were centrifuged at 5000 × g at 4 °C for 10 min at mid-exponential phase and subsequently washed twice with PBS and resuspended in PBS

containing 30 mM H_2O_2 followed by incubation at 37 °C for 15 min and 30 min gradually. After proper dilution, subsist cells were spread on THB plates for incubation in a 5% CO_2 aerobic atmosphere at 37 °C for 12 h. Following that, the survival rate was measured as the percentage of survival. In acidic conditions, the bacteria were harvested at OD₆₀₀ of 0.6 at 37 °C and washed twice with PBS (pH 7.4) by centrifugation at 5000 × g at 4 °C for 10 min and resuspended in 1 ml PBS of different pH (4, 5, 6 and 7) for 1 h at 37 °C for survival assays in the acidic condition. Survived cells were diluted appropriately, plated on THB agar plates and incubated in a 5% CO_2 aerobic atmosphere at 37 °C for 12 h. The results were counted during pre and post-exposure of stress challenges.

The adaptability of the SS2 strains to osmotic stresses was tested using the protocol as described previously (Zhu et al., 2014). The overnight cultures of ZY05719, $\Delta clpX$, $\Delta clpP$, $C\Delta clpX$, and $C\Delta clpP$ were

Table 2
Primer used in this study.

Primers	Sequences ((5'-3'))	Target gene
ClpX-F ¹	CGCGGATCC AAGA AACTTCGTTCTTGCTATT	LA fragment of <i>clpX</i>
ClpX-R ¹	GTGTTTCCTCTTTCATATC	LA fragment of <i>clpX</i>
ClpX-F ²	AGAGGAAACACAAAAATAAAGGGTGAAACTG	RA fragment of <i>clpX</i>
ClpX-R ²	CGGGAATTCGCAAAATCTCCACGAAATAGTC	RA fragment of <i>clpX</i>
ClpX-F ³	CGCGGATCC AAACCCGTATGATTTTACAATA	<i>clpX</i> and its promoter
ClpX-R ³	CGGGAATTC CAGTTTCTAGTAAAGGCITTTTC	
ClpX-F ⁴	ATGGCTGTTAAGCATAACACAGAG	ORF of <i>clpX</i>
ClpX-R ⁴	TTATGCAGTTTCTAGTAAAGGCTT	ORF of <i>clpX</i>
ClpP-F ¹	CGCGGATCCGTTATTGACTTTTGATTGTCCAT	LA fragment of <i>clpP</i>
ClpP-R ¹	CAGGACCAGTTGAGGACAACATGGC	RA fragment of <i>clpP</i>
ClpP-F ²	CTGGTCTCG AAAATTACTCCTTTTCGAGAT	RA fragment of <i>clpP</i>
ClpP-R ²	CGGAAATTC TGGTATGGTTGATGGTTTGCT	RA fragment of <i>clpP</i>
ClpP-F ³	CGCGGATCC TTATTGACTTTTGATTGT	<i>clpP</i> and its promoter
ClpX-R ³	CGGGAATTC ACGTGGTGCTCAAATATCA	
ClpP-F ⁴	TTATTGACTTTTGATTGTCCAT	ORF of <i>clpP</i>
ClpP-R ⁴	ATGATTCCAGTAGTTATTGAACAA	ORF of <i>clpP</i>
ClpP RT-F	CCTCCTGGCGTATTAACATAGAG	<i>clpP</i> gene for qRT-PCR
ClpP RT-R	GGACCAGTTGAGGACAACAT	
ClpX RT-F	ACCGTGACAGAAAGAGGAATTA	<i>clpX</i> gene for qRT-PCR
ClpX RT-R	CTGGACACCTTCACAGATAC	
GAPDHRT-F	ACACTGAAGACCAACTCGTATC	GAPDH gene for qRT-PCR
GAPDHRT-R	CGCCATCAACTTCGATAACTTTAG	
SodA-F	TGCGAGGAATGCGATGAAT	<i>sodA</i> gene for qRT-PCR
SodA-R	AGGTTGCGTAGGTTGCTTAATA	
Tpx-F	CGACTTGGTGTGGAGATTG	<i>tpx</i> gene for qRT-PCR
Tpx-R	GTGCCAAAGGCATAAAGTCATC	
ApuA-F	GAAGTTCAGCCAGCCTTTAT	<i>apuA</i> gene for qRT-PCR
ApuA-R	GGTGTGTGCCTTGATGTTATTG	
HsDs-F	GAGAAGTTAGCTGATGGGACTG	<i>hsDs</i> gene for qRT-PCR
HsDs-R	GTGTGCCTCCGAAAGTAATA	
MRP-F	GCTCAATGGTCAGGAGATGAA	<i>mnp</i> gene for qRT-PCR
MRP-R	ACCAGCTTGGTCATCAGAATAG	

^aThe underlined sequences are restriction enzyme sites. GGATCC, BamH I; and GAATTC, EcoRI.

diluted in fresh medium THY containing 400 mM NaCl to obtain at OD₆₀₀ of 0.2. Samples were inoculated individually at 37 °C in a shaking incubator for 10 h at 180 rpm/min. At 1 h interval, bacterial growth was monitored by measuring the OD₆₀₀ using a spectrophotometer (Smart Spec Plus, Bio-Rad, USA). Each test was repeated thrice.

2.8. Adhesion and invasion assays

The human laryngeal cancer epithelial cells (HEp-2) were used to perform adhesion and invasion assays of SS2. For the adherence assay, the strains were harvested by centrifugation at mid-exponential growth phase (OD₆₀₀ = 0.6) and washed twice with PBS. The bacteria were suspended with DMEM culture medium without antibiotics to a density of 5×10^7 CFU/ml and then were poured into 24-well tissue culture plates containing the HEp-2 cell. The plates were centrifuged at $800 \times g$ for 15 min and incubated at 37 °C for 2 h. The infected cells were washed five times with PBS. The numbers of cell adherent bacteria were ascertained by plating on 10 fold dilutions. These adherent bacteria were cultivated on the THB agar plates and incubated at 37 °C for 12 h to enumerate the number of bacteria that had adhered to cells. The invasion assay has the similarity with the adhesion assay except that the extracellular and surface bacteria were destroyed by using gentamicin (100 µg/ml) and penicillin (5 µg/ml). Each assay was performed thrice independently.

2.9. Phagocytosis assay

For the phagocytosis assays, all strains were harvested by centrifugation at mid-exponential growth phase (OD₆₀₀ = 0.6) and washed twice with PBS. The strains were incubated with Raw264.7 macrophages cell at a bacteria-to-cell ratio of 100: 1 at 37 °C for 1 h. Then the infected cells were washed thrice with PBS and incubated for 2 h after adding gentamicin (100 µg/ml) and penicillin (5 µg/ml) into DMEM to kill the extracellular bacteria. Subsequently, the macrophage was lysed with water after thrice times washing with PBS. Twofold dilution of the cell lysate was plated on THB agar plates. Each assay was carried out in triplicate wells and repeated thrice.

2.10. Animal infection model

To investigate the survival curves of mice, specific pathogen-free (SPF) BALB/c 4-weeks old female mice with each group of ten (10) members were challenged with the strains of interest at a dose of 5×10^8 CFU/mouse intraperitoneally. Another set of 10 SPF BALB/c mice treated with equal volume of intraperitoneal sterile (PBS) injections was considered a negative control. Survival rates were monitored twice a day for one week of post-infection.

Another animal study was performed to detect the colonization and invasion ability of different strains in systemic organs as described previously (Wang et al., 2011). In this case, we used 6 specific pathogen-free BALB/c 4-weeks old female mice in each group and 3×10^8 CFU strain/mouse was injected intraperitoneally. The mice by euthanasia were sacrificed and anatomized to collect the tissues (liver, brain, spleen, and blood) 8 h post-infection. The samples were isolated from differential organs and homogenized in PBS after weighing. The presence of each colonized bacterium was determined by plating 10-fold serial dilutions on THB agar plates. Colonies were recorded as CFU/ml for blood samples and CFU/g for organ samples.

2.11. RNA extraction and qRT-PCR analysis

Bacterial cultures were grown to the logarithmic phase at an OD₆₀₀ of 0.6. Total RNA was extracted by using Total RNA Extraction Reagent (Vazyme BioTech Co. Ltd. Nanjing, China) and treated with RNase-free DNase I to encounter any contaminated genomic DNA from the samples. In addition, cDNA synthesis was carried out using HiScript II 1 st

Strand cDNA Synthesis Kit (Vazyme BioTech Co. Ltd. Nanjing, China) based on the manufacturers order. The QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to perform quantitative PCR. Thermal cycling parameters consisted of initial denaturation at 95 °C for 30 s, followed by 40 cycles of annealing at 95 °C for 10 s and primer extension at 60 °C for 30 s. The target segment of cDNA was amplified by using ChamQ™ Universal SYBR qPCR Master Mix ((Vazyme BioTech Co. Ltd. Nanjing, China), and a single specific PCR product for each gene was ensured by melting curve performance. The primers of the desirable genes were designed from known sequences using Primer Quest Tool (Integrated DNA Technologies, Inc, Skokie, Illinois, USA) software. The sequences of primers used for qRT-PCR are shown in Table 2. The data was standardized to the housekeeping gene (GAPDH) transcript and relative fold change was enumerated by using the threshold cycle ($2^{-\Delta\Delta CT}$) method (Chang et al., 2009).

2.12. Bioinformatics and statistical data analysis

We used online BLAST program in the National Center for Biotechnology Information (NCBI) and DNASTAR Lasergene 7 software (Madison, WI, USA) to analyze the DNA and amino acid sequences. The BP program (Softberry, Inc. Mount Kisco, NY, and USA) was operated for the prediction of the promoter region. Multiple Sequence alignment for the ClpX and ClpP proteins from *S. suis* and some other *Streptococcus* sp. was performed using <https://www.ebi.ac.uk/tools/msa/clustalo/>. The phylogenetic tree was performed using ExPASy software (SIB, Lausanne, Switzerland). The statistical data was performed using Graph-Pad Prism, Version 5 for windows (La Jolla, CA, USA). The differences among groups were evaluated by using one-way analysis of variance (ANOVA) in Graph-Pad Prism software. For *in vivo* mice infection experiments, survival data were measured using log-rank (Mantel-Cox) test. All of the results were expressed as the mean \pm SEM. * indicates $P \leq 0.05$, ** indicates $P \leq 0.01$, and *** indicates $P \leq 0.001$.

2.13. Ethics statement

Six-week-old female germfree BALB/c mice were purchased from the Comparative Medicine Center of Yangzhou University. Laboratory Animal Center situated in the Nanjing Agricultural University was used to perform all the animal experiments with the permission of the Laboratory Animal Monitoring Committee of Jiangsu Province, China. Permit number: [SYXK (SU) 2017-0007].

3. Results

3.1. Identification of ClpX and ClpP homologs in SS2

In the SS2 strain ZY05719 genome, we annotated the proteins ZY05719_04030 and ZY05719_07365, which were ATPase subunits of Clp, as ATP-dependent ClpX and ClpP proteases, respectively. Further phylogenetic analysis confirmed that ZY05719_04030 is a homolog of ClpX, with a conserved ATP-binding domain, and that ZY05719_07365 is more homologous with ClpP. Thus, we re-designated ZY05719_04030 and ZY05719_07365 proteins as ClpX and ClpP, respectively.

For the evaluation of the distribution of ClpX and ClpP among *S. suis* strains, a BlastN search in the National Centre for Biotechnology Information (NCBI) database showed availability of 36 complete genomes of *S. suis* (for ClpX; Supplemental Table S1) and 37 complete genomes of *S. suis* (for ClpP; Supplemental Table S2), indicating that these two Clp proteases are highly conserved among *S. suis* strains. Multiple sequence alignment of amino acids showed that ClpP and ClpX encoded in *S. suis* are similar to the homologs in *S. mutans* (83% identity of ClpX and 90% identity of ClpP), *S. pneumoniae* (88% identity of ClpX and 89% identity of ClpP), *S. pyogenes* (82% identity of ClpX and 92%

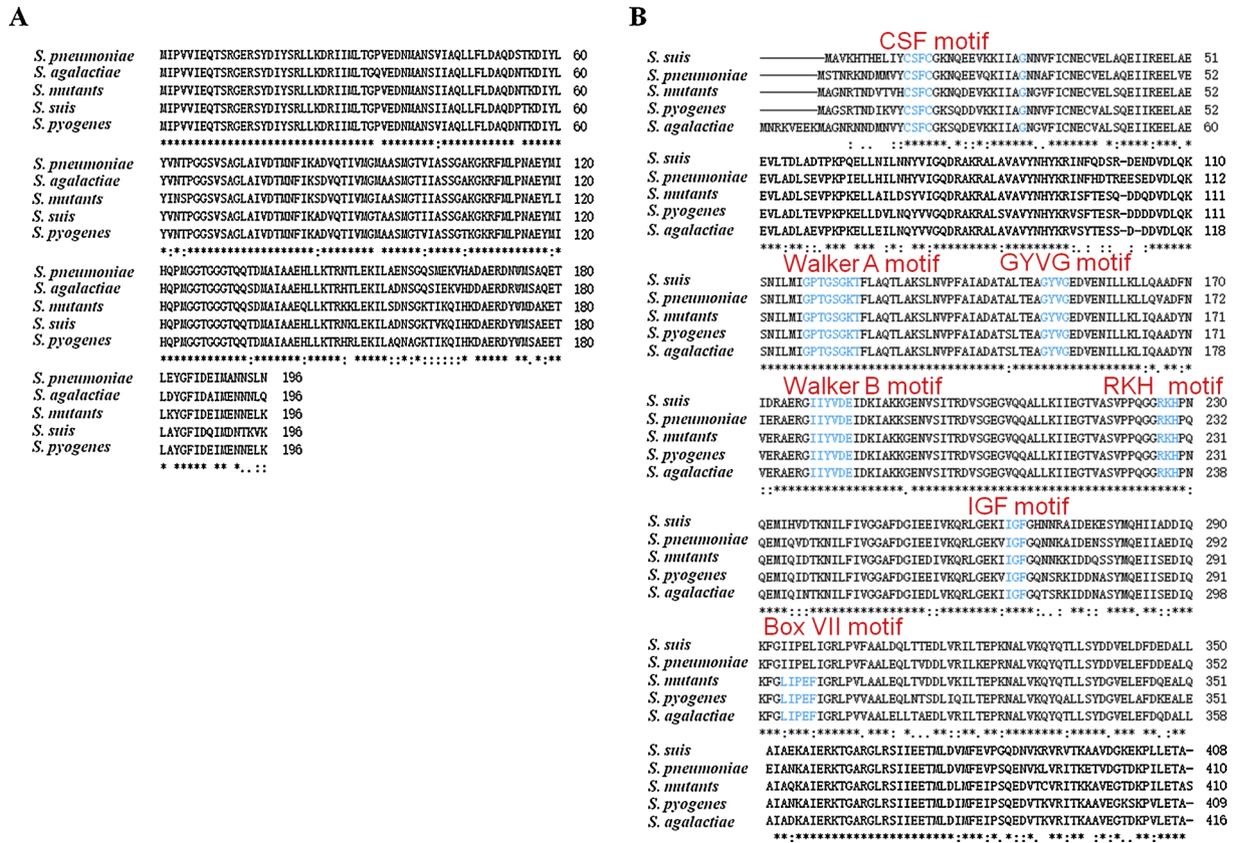


Fig. 1. (A) Multiple sequence alignment of *S. suis* ClpP with related homologs from *Streptococcus* species. The amino acid sequence of *S. suis* ClpP (AKG40801.1) was aligned with that of *S. Pneumoniae* (ABJ54512.1), *S. mutants* (AAN59311.1), *S. agalactiae* (ABA44910.1), *S. pyogenes* (AAM78894.1), and *S. mitis* (CBJ22657.1) by the CLUSTAL program (B) Multiple sequence alignment of *S. suis* ClpX with related homologs from *Streptococcus* species. The amino acid sequence of *S. suis* ClpX (AKG40165.1) was aligned with that of *S. Pneumoniae* (ABJ55423.1), *S. mutants* (AAN58653.1), *S. agalactiae* (AKI95676.1), and *S. pyogenes* (AAM79211.1) by the CLUSTAL program. The blue-colored represents those conserved in all sequences; identical residues and homologs are marked with stars and double dots, respectively; drastic changes are marked with a single dot.

identity of ClpP), and *S. agalactiae* (84% identity of ClpX and 88% identity of ClpP) (Fig. 1A–B).

Further bioinformatics analysis for ZY05719_04030 was performed to identify conserved motifs that are widely present in well-known ClpX family members (Fig. 1B). ZY05719_04030 contains an additional strand, catalytic E (ASCE) motif, which belongs to conserved group of ATPases. The AAA + ATPases function as molecular chaperones, ATPase subunits of proteases, helicases, or nucleic-acid-stimulated ATPases. In fact, ZY05719_04030 also contains several distinct features of AAA + proteins, such as a conserved alpha-beta-alpha core domain structure, the Walker A motif GxxxxGK (S/T) and Walker B motifs hhhh (D/E), and the P-loop NTPases. Taken together, these observations suggest that ZY05719_04030 may function as a ClpX homolog in SS2.

To provide more genetic information, mutant and complementation *clpX* and *clpP* strains were constructed to explore the biological functions of ClpX and ClpP. Phylogenetic trees were constructed to clarify their potential evolutionary relationship with other *Streptococcus* species and Gram-negative bacteria based on their amino acids sequences (Fig. 2A–D).

3.2. ClpP is required for the optimal growth of SS2 during heat stress, while ClpX is required during cold stress

During the *in vitro* infection of host tissues, *S. suis* was exposed to different stress factors such as reduced and elevated temperature. The growth of $\Delta clpX$ and $\Delta clpP$ mutants at different temperatures (28 °C, 37 °C, and 42 °C) was recorded at their OD₆₀₀ values at 1 h intervals (Fig. 3A–F). Both $\Delta clpX$ and $\Delta clpP$ mutants showed growth curves

similar to those of the wild-type strain at 37 °C. Unexpectedly, the growth of $\Delta clpP$ significantly decreased at 42 °C compared with that of the wild-type and complementation strains, while there were no significant differences between the strains at 28 °C. In contrast, $\Delta clpX$ showed normal growth at 42 °C, but growth was significantly inhibited at 28 °C. In fact, growth at elevated temperatures leads to accumulation of misfolded proteins in the cell (Goff and Goldberg, 1985; Wickner et al., 1999), which causes premature abortion of protein translation (Goldberg, 1972). Notably, a study has reported that the synthesis of Clp proteases is increased during cold-shock in *Listeria monocytogenes* cells (Liu et al., 2002). Our results showed that two Clp protease homologs contributed differently, where ClpP and ClpX were required for SS2 growth at 42 °C (heat stress) and at 28 °C (cold stress), respectively. However, the underlying mechanisms of these dissimilar functions of Clp proteases require further research.

3.3. Deletion of *clpX* and *clpP* caused a significant deficiency in bacterial chain and biofilm formation

In culture, both *clpX* and *clpP* mutant strains always showed maximum sedimentation after overnight growth compared with the wild-type strains (Fig. 3A–B), and this deficiency was restored in the complementation strains, suggesting that bacterial cell morphology may be changed in the mutants. Indeed, morphological examination by Gram staining showed that both mutants formed longer chains than the wild-type and complementation strains (Fig. 4C–G), suggesting that the lack of ClpX and ClpP affects the bacterial growth and morphology. Moreover, the lack of ClpP but not of ClpX significantly attenuated the

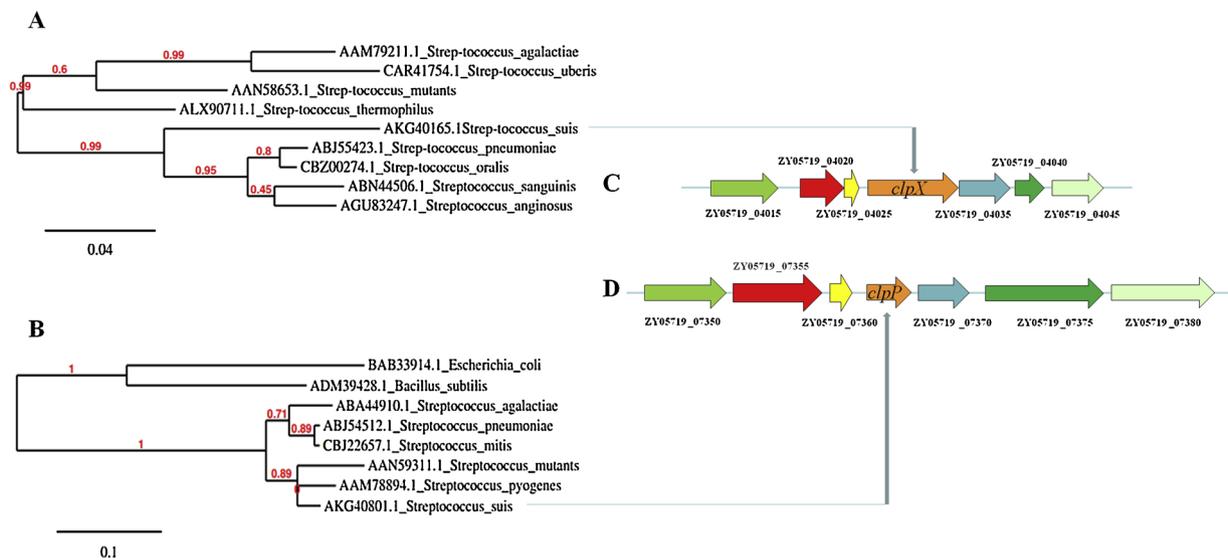


Fig. 2. Phylogenetic relationships of ClpX (A) and ClpP (B) from several *Streptococcus* species and several Gram-negative bacteria. The tree was constructed with ExPASy: SIB. Bootstrapping values are marked in red. The genetic neighborhoods of the *clpX* (C) and *clpP* (D) genes in SS2 strain ZY05719. The locus tags of the predicted ORF number in the *S. suis* ZY05719 genome are indicated. Arrows indicate the transcriptional direction but do not represent the exact length.

biofilm formation (Fig. 3H), and complementation completely restored this deficiency, which further confirmed that ClpP plays an important role in maintaining morphological stability in response to external stimuli.

3.4. Deletion of *clpX* and *clpP* attenuated oxidative and acidic tolerance, but not sensitivity to osmotic stress

During infection, *S. suis* needs to adapt to an unfavorable environmental condition, such as the host's temperature, acidic pH, and oxidative stress. In case of oxidative (H_2O_2) tolerance study, the survival rate of $\Delta clpX$ and $\Delta clpP$ mutants their complementation strains, and the wild-type strains showed no significant difference in response to H_2O_2

(30 mM) after 15 min of incubation at 37 °C, whereas there was a significant decrease in both mutant strains after 30 min of incubation compared to the complementation and the wild-type strain (Fig. 5A), suggesting that ClpX and ClpP play a key in the resistance to oxidative stress in SS2.

Under acidic conditions, the survival rate of the deletion strains exhibited significant differences at diverse pH values (4, 5, 6, and 7). When the pH value of the medium was at 4, the survival rate of $\Delta clpX$ and $\Delta clpP$ significantly decreased compared with that of the wild-type (Fig. 5B), and this deficiency was restored in their complementation strains. Furthermore, the survival rate of the wild-type strain was over 80%, whereas that of $\Delta clpX$ and $\Delta clpP$ was less than 55% at pH 5. When the pH of the medium was neutral, the survival rates were similar

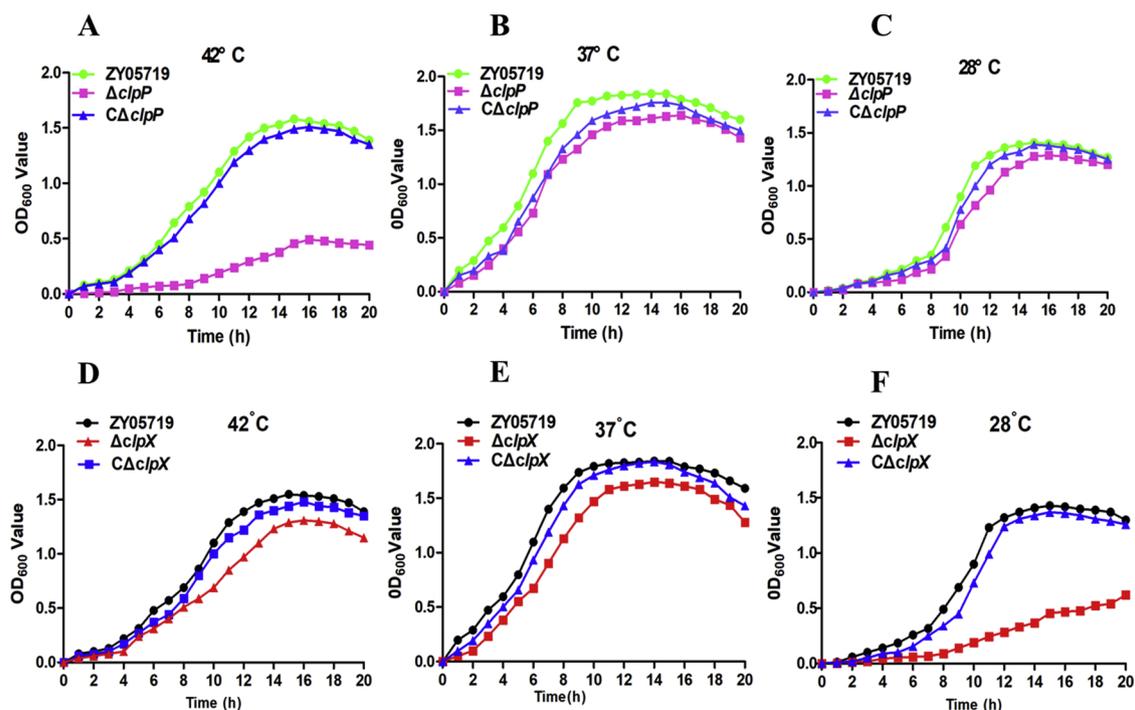


Fig. 3. Effect of ClpX and ClpP on the growth of the SS2 strain in response to temperature stress. Samples of the same optical density (OD₆₀₀) were withdrawn. Representative graphs of *in vivo* growth of wild-type (ZY05719), $\Delta clpX$, $\Delta clpP$, $C\Delta clpX$ and $C\Delta clpP$ strains at 37 °C, 42 °C, and 28 °C.

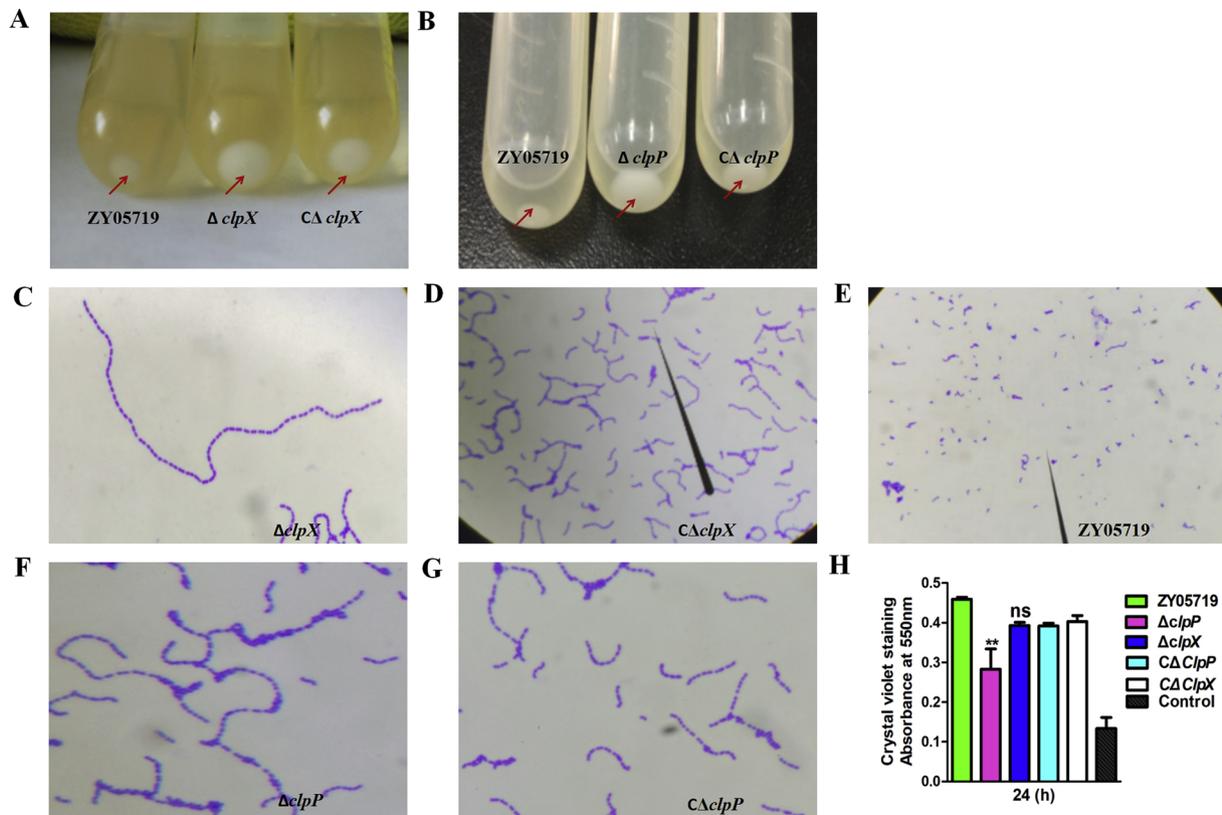


Fig. 4. Sedimentation and morphology of bacteria cultured in THB for 16 h. The bacterial sedimentations of *clpX* (A) and *clpP* (B) mutant, wild-type and complementation strains are shown. The arrows indicate the sedimented cells at the bottom of the tubes. The bacterial cells at log-phase were Gram stained and examined by light microscopy: $\Delta clpX$, (C) $C\Delta clpX$, (D) wild-type (ZY05719), (E) $\Delta clpP$ (F), and $C\Delta clpP$ (G). Biofilm formation by SS2 strains (H). The growth was monitored from an initial OD_{600} of 0.2. Strains were incubated (each well 200 μ l) in 96-well microplates for 24 h at 37 °C. The wells with THB medium served as a negative control. Significant differences are indicated (** $P < 0.01$; ** $P < 0.01$; * $P < 0.05$).

between the strains. Given that ClpX and ClpP contribute to the stress tolerance under oxidative and acidic conditions, we evaluated whether these two Clp proteases also are involved in osmotic stress by incubating them in THY broth containing 400 mM NaCl. The growth curves of $\Delta clpX$ and $\Delta clpP$ mutants showed no significant difference compared to the wild-type strain, indicating that they did not affect the response of SS2 to osmotic stress (Fig. 5C).

3.5. *ClpX* and *ClpP* in SS2 contribute to antiphagocytosis against RAW264.7 cells and adhesion to HEp-2 cells

It is well known that oxidative and acidic stresses are two important manners in which macrophages kill pathogenic bacteria. Thus, we performed a phagocytosis assay using RAW264.7 cells to detect the role of ClpX and ClpP on the antiphagocytosis of SS2. The host cells' capacity for phagocytosis of the wild-type, mutant, and complementation strains was assessed equal conditions. The rate of phagocytosis of $\Delta clpX$ and $\Delta clpP$ mutants was significantly higher than that of the phagocytosis of wild-type strain, and this deficiency was restored in the complementation strains (Fig. 6A) indicating that antiphagocytosis was significantly attenuated in SS2 by the lack of ClpX and ClpP. These results suggest that ClpX and ClpP may mediate antiphagocytosis, enabling survival of SS2 via oxidative and acidic tolerance.

Adhesion is the primary step in colonization of tissues by pathogens (Klemm et al., 2010). To verify whether *clpX* and *clpP* affect the proficiency of SS2 to adhere to and invade host cells, we used host cell lines HEp-2 to identify the pathogen host interaction. The adhesion ability of the wild-type strain was significantly higher than that of the $\Delta clpX$ and $\Delta clpP$ (relative 43%) strains (Fig. 6B). In contrast, there was no significant difference in invasion between the different strains (Fig. 6C).

These results indicate that the ClpX and ClpP may be involved in the adhesion of SS2 to epithelial cells.

3.6. Effect of *clpX* and *clpP* deletion during systemic infection in vivo

Mice were empirically infected to explore the role of *clpX* and *clpP* mutants in SS2 virulence. Seventy 4-weeks-old female BALB/c mice were randomly divided into 7 groups with 10 animals per group. Each strain was injected intraperitoneally at a dose of 5×10^8 CFU/mouse separately. The last group was injected with an equal volume of sterile phosphate-buffered saline (PBS) intraperitoneally. All mice (10/10) challenged by wild-type strain showed clinical signs such as drowsiness, shivering, and meningitis and subsequently died on the 1st and 2nd days post-infection. In contrast, the group of mice challenged with $\Delta clpX$ and $\Delta clpP$ strains showed fewer clinical symptoms and lower mortality (3 out of 10) within 36 h. Eight mice out of 10 died in the complementation $\Delta clpX$ group, and, nine mice out of 10 died in the complementation $\Delta clpP$ group within 36 h (Fig. 7A–B), indicating that the deficiency was restored in the complementation strains. These results suggest that ClpX and ClpP are required for the full virulence of SS2.

Furthermore, we observed *in vivo* systemic infection in the mouse model. Thirty-six female BALB/c mice were assigned into six groups of six animals per group and inoculated with the strains intraperitoneally at 5×10^8 CFU/mouse. Bacteria were isolated from the brain, blood, liver, and spleen at 8 h post-infection. The number of recovered $\Delta clpX$ and $\Delta clpP$ bacteria was significantly lower than that of the wild-type strain (Fig. 8A–D). The bacterial burden in the organs of mice challenged with the complementation strains was similar to that recovered from the wild-type strain. In summary, the infection and colonization ability of the $\Delta clpX$ and $\Delta clpP$ strains was significantly impaired in the

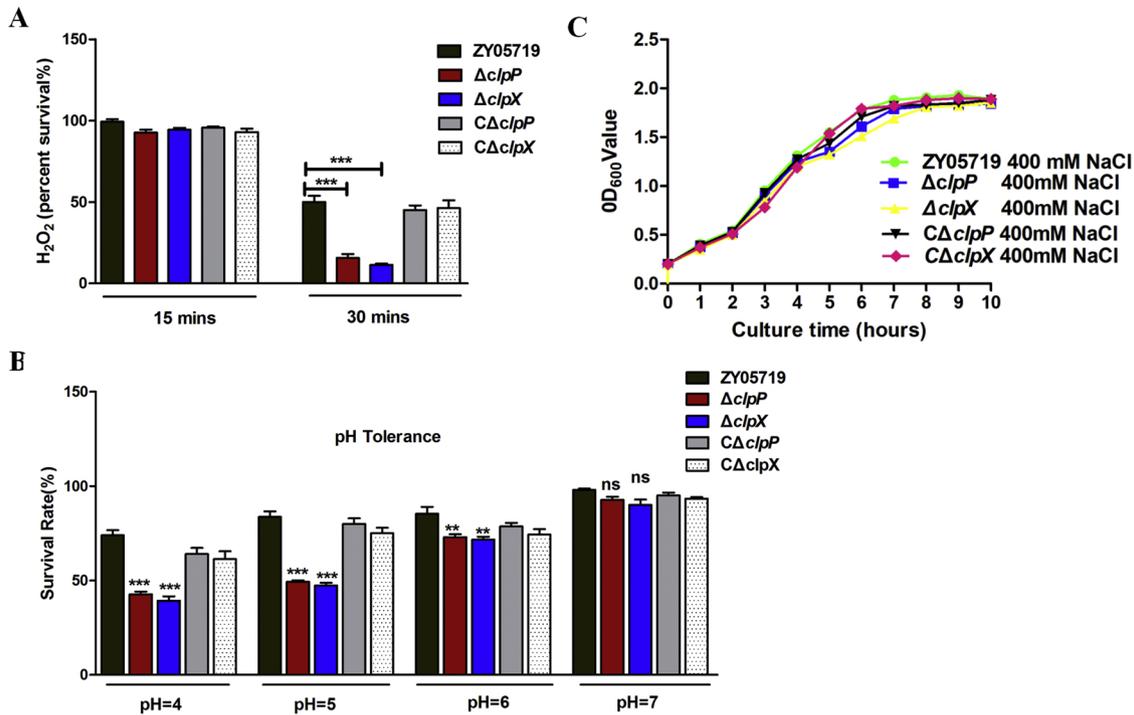


Fig. 5. Assessment of the role of ClpX and ClpP in stress tolerance to oxidative, acidic, and osmotic conditions. Bacterial cells of ZY05719, $\Delta clpX$, $\Delta clpP$, $C\Delta clpX$ and $C\Delta clpP$ strains grown to the mid-exponential phase were harvested and used to test the survival ability in a variety of stress challenges. (A) Bacteria were incubated at 37 °C for 15 min and 30 min gradually with 30 mM of H₂O₂. (B) The strains were resuspended in 1 mL PBS and incubated at 37 °C for 1 h at pH values of 4–7 for survival assays. Viable bacteria were determined by dilution plating on THB agar plates before and after exposure to stress challenges. (C) Growth curves were measured by the OD₆₀₀ values of the ZY05719, $\Delta clpX$, $\Delta clpP$, $C\Delta clpX$ and $C\Delta clpP$ strains grown in THY containing 400 mM NaCl. The growth of cultures was monitored from an initial OD₆₀₀ of 0.2. Data are representative of three independent experiments (****P* < 0.001; ***P* < 0.01; **P* < 0.05).

murine model of infection, suggesting that ClpX and ClpP are important virulence factors in systemic SS2 infection.

3.7. Expression profiling of virulence gene expression via qRT-PCR

To determine the potential downstream genes regulated by ClpX and ClpP, several reported oxidative resistance, capsule synthesis, and virulence genes were quantified by qRT-PCR. The expression levels of the virulence factors *sodA* and *tpx* were significantly reduced in the *clpX* deletion mutants (Fig. 9A–B). However, the lack of ClpP caused significant downregulation of the transcriptional levels of *tpx* and *apuA*

compared with the wild-type and complementation strains. These results suggest that ClpX and ClpP may regulate different downstream genes to facilitate bacterial tolerance against diverse environmental stresses.

4. Discussion

The potential roles of ClpX and ClpP in bacterial pathogenesis have been recently reported to contribute to stress tolerance and virulence in *Streptococcus pneumoniae*, *S. agalactiae*, *S. mutants*, *Bacillus subtilis*, *Yersinia enterocolitica*, and *Listeria monocytogenes* (Pederson et al., 1997;

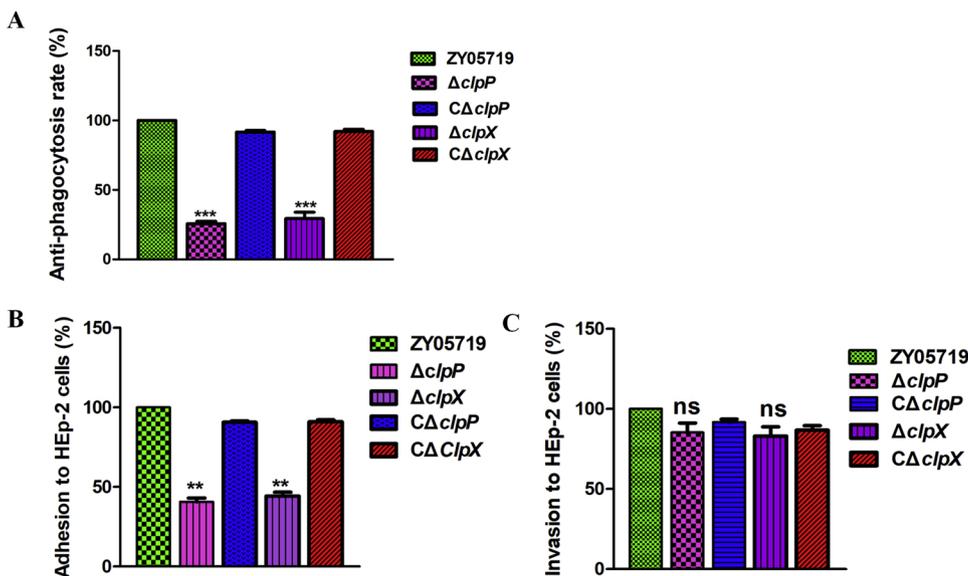


Fig. 6. (A) Phagocytosis assays on RAW264.7 cells. Phagocytized bacteria were recovered after antibiotic treatment. (B) The $\Delta clpP$ strain showed significantly reduced adherence to HEP-2 cells compared with the wild type (ZY05719) strain (*P* < 0.01). (C) Bacterial invasion into HEP-2 cells. Extracellular bacteria were eradicated through using antibiotic treatment. Significant differences are indicated (****P* < 0.001; ***P* < 0.01; **P* < 0.05).

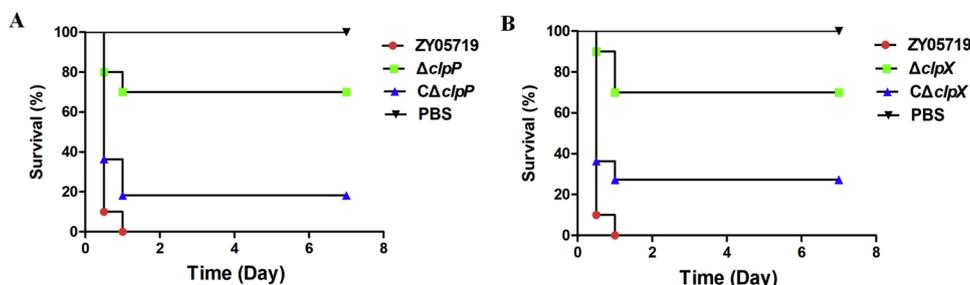


Fig. 7. (A) Survival rates of mice challenged with wild-type (ZY05719), $\Delta clpP$, and $C\Delta clpP$ strains (B) Survival rates of mice challenged with wild-type (ZY05719), $\Delta clpX$, and $C\Delta clpX$ strains. Each mouse was intraperitoneally inoculated with 5×10^8 CFU of the strains. The mice inoculated with phosphate-buffered saline (PBS) served as controls. Ten mice were used in each group. Survival data were analyzed using the log-rank (Mantel-Cox) test. Significant differences are indicated (** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$).

Gaillot et al., 2000; Robertson et al., 2002; Kajfasz et al., 2011; Rath et al., 2012), in all of which the underlying mechanisms remain unclear. In the whole-genome search, we found that the virulent SS2 strain ZY05719 encoded two conserved proteins, ClpX (ZY05719_04030) and ClpP (ZY05719_07365), which showed high sequence identity with the well-known ClpX and ClpP homologs from other *Streptococcus* species. Further analysis confirmed that these homologs were widely encoded in most virulent *S. suis* strains, suggesting that they may be potential virulence factors in *S. suis*.

Bacteria have different genes that provide survival benefits in response to different environmental stresses (Zheng et al., 2014). The expression of many proteins in bacteria is restrained at the transcription initiation step in response to different environmental stresses (Dong et al., 2013). Thus, we aimed to assess whether the ClpX and ClpP proteins are important factors that help SS2 tolerate different environmental stresses. In fact, several previous studies have reported that the deletion mutants of *clpX* or *clpP* in other bacteria showed a significant decrease in stress tolerance due to the lack of ATPase specificity factors that bind to Clp proteases (Kruger et al., 1994; Gerth et al., 1998; Robertson et al., 2002; Frees et al., 2003). In the present study, we found that $\Delta clpX$ and $\Delta clpP$ derived from the virulent *S. suis* strain ZY05719 exhibited several important deficiencies, such as slower growth rates, formation of longer chains, a stronger tendency to aggregate in broth culture, and higher sensitivity to oxidative and acidic stress than the wild-type strain. Previous studies have shown that deletion mutants such as $\Delta comC$, $\Delta brpA$, $\Delta hr11$, and $\Delta rr1$ showed a significant deficiency in biofilm formation (Li et al., 2002a, b; Wen and

Burne, 2002), possibly due to altered structures of bacterial cells, which tended to form longer chains. The ability to form biofilms during infection is indeed beneficial for the resistance of the bacteria to the host's defense systems (Tan et al., 2017).

Chain length is an important factor in the adherence of bacteria to epithelial cells *in vitro* and modulates the colonization capacity in the host tissue (Rodriguez et al., 2012). Our Gram staining results showed that the bacterial chain length of $\Delta clpX$ and $\Delta clpP$ strains significantly increased, and their adhesion to HEp-2 cells significantly decreased in comparison with the wild-type strain. The difference in the length of chains may have also affected bacterial biofilm formation, which normally helps the bacteria to survive and injure in the host tissue by building a defense against the host and antibiotic therapy (Boles and Horswill, 2011; Watters et al., 2016). However, further studies are required to clarify these results. On the other hand, longer length of the bacterial chains may be one of the causes of decreased resistance of $\Delta clpX$ and $\Delta clpP$ to phagocytosis (Dalia and Weiser, 2011; Tan et al., 2017).

Interestingly, we found that ClpP and ClpX play different roles in biofilm formation and heat/cold stress in SS2. In fact, the Clp proteases consist of a serine-type peptidase ClpP subunit and a regulatory ATPase subunit (ClpA/ClpB/ClpC/ClpE/ClpX/ClpY). In the Clp proteolytic system, ClpP must unite with a Clp ATPase subunit to form a functional complex (Kwon et al., 2003). Therefore, attenuation of biofilm formation by deletion of *clpP* may indicate that ClpP in SS2 is a major subunit of proteases involved in biofilm formation, whereas the finding that biofilm formation was unaffected by deletion of *clpX* may suggest that

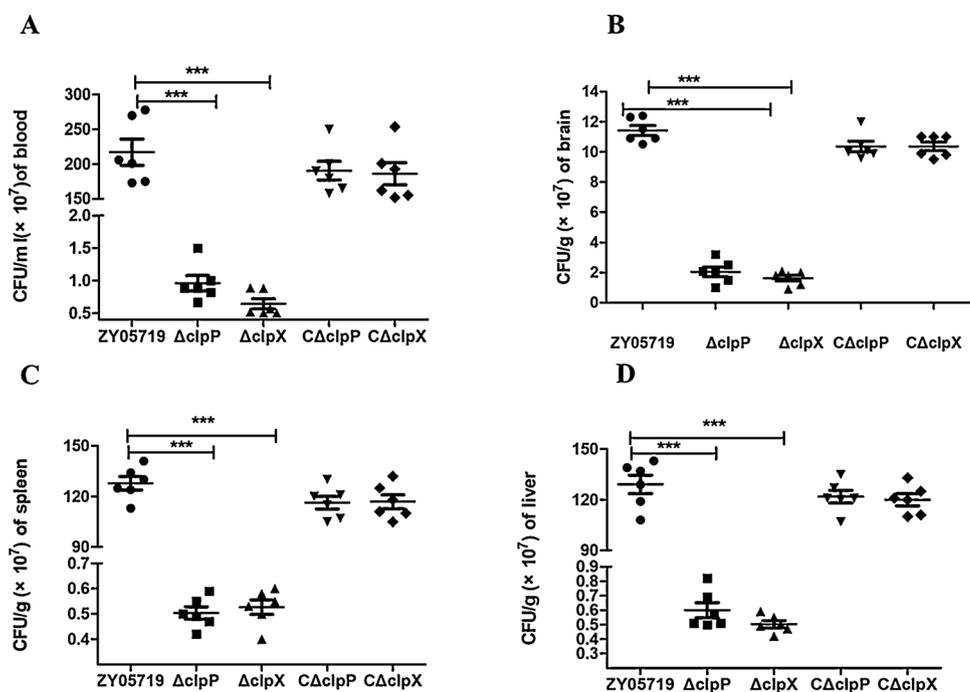


Fig. 8. Colonization of the wild-type (ZY05719), $\Delta clpX$, $\Delta clpP$, $C\Delta clpX$, and $C\Delta clpP$ strains in various tissues of mice. Groups of six female BALB/c mice were inoculated intraperitoneally with 3×10^8 CFU/mice. Bacterial counts in the blood (A), brain (B), spleen (C), and liver (D) were examined at 8 h post-infection. Statistical analysis was performed by one-way ANOVA. Significant differences were found between both mutant challenged groups and the ZY05719 challenged group. Significant differences are indicated (** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$).

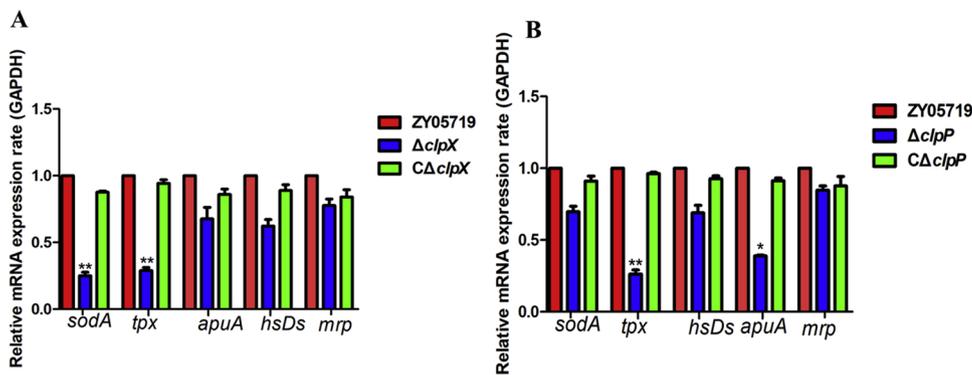


Fig. 9. Quantification of the expression of potential virulence genes. Expression levels of *sodA*, *tpx*, *apuA*, *hsDs*, and *mrp* in wild-type (ZY05719) and mutant strains were measured by qRT-PCR. Data were normalized to the house-keeping gene *GAPDH*. Results are shown as relative expression ratios compared to the expression in the wild-type strain. Significant differences are indicated (***) $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

Clp ATPases other than ClpX may associate with the ClpP subunit during biofilm formation. Similarly, ClpP may co-operate with other Clp ATPases, but not with ClpX, to form proteolytic complexes for optimal degradation of misfolded proteins induced by heat stress. In the absence of ClpX, other Clp ATPases that are capable of recognizing non-native proteins may have easier access to ClpP, thereby increasing the rate of degradation of damaged proteins, leading to maintained growth (Frees et al., 2003). Otherwise, ClpX may associate with other unknown Clp peptidases in SS2 to form functional complexes, resulting in roles different from the well-known ClpP ATPase that facilitate bacterial growth under cold stress. The exact underlying mechanism of ClpX in SS2 stress response ought to be further explored.

Invasion into deeper tissues, presence in the blood stream, and adaptation to suboptimal environmental conditions, such as heat or cold stress, acidic pH, oxidative stress, and high osmolarity, are important factors in the pathogenesis of *S. suis* infection. In a previous report, $\Delta clpP$ and $\Delta clpX$ of *Staphylococcus aureus* were shown to be sensitive to oxidative stress (Frees et al., 2003). Our results confirmed that both mutant strains in SS2 displayed significant deficiency in oxidative and acidic stress tolerance, had strong sedimentation tendency in broth culture, and impaired growth at low pH, all of which are similar to effects observed in *Streptococcus mutants* (Kajfasz et al., 2009). One of the potential reasons why both $\Delta clpX$ and $\Delta clpP$ strains are more sensitive to these adverse stresses are the effects of the proteolytic control of regulatory proteins targeted by ClpXP on transcriptional regulation of downstream genes. For example, the inactivation of Spx, a global transcriptional regulator targeted by ClpXP proteolysis, suppressed all phenotypes supported by ClpX and ClpP in *B. subtilis* (Nakano et al., 2001). Similar observations were reported in *L. lactis* and *S. aureus* (Frees et al., 2001; Pamp et al., 2006), because many bacterial stress responses are actuated by the inhibition of the degradation of stress-induced transcriptional regulators or sigma factors or the degradation of gene repressors (Jenal and Hengge-Aronis, 2003; Frees et al., 2007).

The underlying mechanism of virulence attenuation by deletion of *clpX* and *clpP* remains unknown in SS2. During the critical steps of infection, *S. suis* is able to survive in the bloodstream after its transmission via the respiratory tract (Gottschalk and Segura, 2000). The lower tolerance of the $\Delta clpX$ and $\Delta clpP$ mutants to several environmental stresses might be the main contributor to the attenuated virulence of SS2, as such mutant cells would be less likely to survive in the host (Zhu et al., 2014). The mouse infection model revealed that the $\Delta clpX$ and $\Delta clpP$ strain-challenged mice had increased survival longer time (Fig. 7A–B) than wild-type and complementation strain-challenged mice. The bacterial burden retrieved from mice infected with $\Delta clpX$ or $\Delta clpP$ was significantly lower in the blood, brain, liver, and spleen compared with that of the wild-type strain. Similar results have been reported in *Listeria monocytogenes* (Gaillot et al., 2000), *Salmonella enterica* serovar Typhimurium (Hensel et al., 1995; Webb et al., 1999), *Yersinia enterocolitica* (Pederson et al., 1997), and *Staphylococcus aureus* (Frees et al., 2003). Further qRT-PCR results indicated that *sodA*

(superoxide dismutase), *tpx* (thiol-peroxidase), and *apuA* (amylopullulanase) of the mutant strains were significantly downregulated compared with the wild-type strain. The *sodA* and *tpx* genes have been linked to antiphagocytosis and virulence in *Streptococcus pneumoniae* and *Schistosoma mansoni* (Kwatia et al., 2000; Yesilkaya et al., 2000; Robertson et al., 2002), and *apuA* has been linked to adhesion and virulence in *S. suis* (Ferrando et al., 2010). Taken together, these data imply that the attenuation in virulence of $\Delta clpX$ and $\Delta clpP$ mutants is a complex phenotype with many contributing factors. Thus, it is reasonable to infer that ClpX and ClpP may be important stress response factors in the virulence of SS2.

In conclusion, the present study demonstrated that ClpX and ClpP play key roles in the virulence and stress tolerance of SS2. These results enrich the understanding of the function of Clp protease homologs in the pathogenesis of SS2 infection. Future studies should further explore the exact mechanisms of virulence regulation by Clp proteases in SS2.

Conflict of interest

The authors declared no competing financial interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.micres.2019.04.003>.

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