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PrsA contributes to *Streptococcus suis* serotype 2 pathogenicity by modulating secretion of selected virulence factors

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ABSTRACT

Streptococcus suis serotype 2 (*S. suis* 2) is a major zoonotic pathogen. Parvulin-type peptidyl-prolyl isomerase (PrsA) in *S. suis* 2 is found surface-associated, pro-inflammatory and cytotoxic. To further explore the roles of PrsA in *S. suis* 2 infection, we constructed a *prsA* deletion mutant (Δ*prsA*) and a complemented strain (CΔ*prsA*). The Δ*prsA* mutant showed increased length of bacterial chains and decreased growth. Deletion of *prsA* increased bacterial adhesion to host epithelial cells but with weakened invasion. The Δ*prsA* mutant had reduced survival in RAW264.7 macrophages and pig whole blood, and significantly attenuated in virulence to mice. All these phenotypes of the mutant could be reversed largely to the levels of its parental strain by gene complementation. Western blotting revealed that suilysin was markedly reduced both in surface-associated (SAP) and secreted fractions (SecP) of Δ*prsA*, which might be responsible for reduced hemolytic activity. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and enolase were significantly increased in both SAP and SecP fractions as a result of *prsA* deletion. Increased adhesion of the Δ*prsA* mutant to bEND.3 cells was prevented using polyclonal antibodies against GAPDH and enolase. Overall, we propose that *S. suis* 2 deploys PrsA to control translocation of important virulence factors, thereby favoring its survival in the host with enhanced pathogenicity by compromising its interactions with the host cells. Further investigation is required to find out how PrsA modulates protein translocation to benefit *S. suis* infection and if there are other *S. suis* 2 substrates of potential virulence regulated by PrsA.

1. Introduction

Streptococcus suis is one of the major swine pathogens in pigs, causing severe infections such as meningitis, septicemia, and endocarditis. Of the 35 serotypes, *S. suis* serotype 2 (*S. suis* 2) is the most virulent and prevalent, responsible for most humans and swine infections worldwide as zoonotic pathogen, and *S. suis* serotype 9 is also often isolated in pig infections in Europe (Goyette-Desjardins et al., 2014). In China, there were two major outbreaks of *S. suis* 2 infections in 1998 and 2005 with severe cases and death in humans and swine (Tang et al., 2006).

Efforts have been made to characterize virulence factors as part of the strategy to reveal the mechanisms of *S. suis* infections (Haas and Grenier, 2018). A number of virulence factors have been reported so far (Fittipaldi et al., 2012; Segura et al., 2017), and some of them are considered critical, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Brassard et al., 2004), suilysin (SLY) (He et al., 2014; Tenenbaum et al., 2016), enolase (Esgleas et al., 2008; Feng et al., 2009), extracellular protein factor (EF) (Smith et al., 1993), muramidase-released protein (MRP) and capsular polysaccharide (CPS) (Smith et al., 1999; Wang et al., 2015). However, pathogenesis of *S. suis* 2 infections is still poorly understood at molecular level.

Bacteria can secrete virulence factors to the extracellular environment to facilitate infection. Parvulin-type peptidyl-prolyl isomerase (PrsA) is ubiquitous among Gram-positive bacteria, which is important for secretion and maturation of extracellular proteins (Cahoon and Freitag, 2014; Unal and Steinert, 2014). In *Listeria monocytogenes*, PrsA2 was proved to play important roles in its pathogenesis by affecting its hemolytic activity, secretion of virulence factors and intracellular survival in murine macrophages (Alonzo and Freitag, 2010; Alonzo et al., 2009, 2011; Chatterjee et al., 2006). In *Staphylococcus aureus*, deletion of *prsA* caused dramatic change of exoproteomes, increased adhesion to human lung epithelial cells and attenuated virulence in mouse model (Lin et al., 2018). In *Streptococcus pneumoniae*, PpmA, a PrsA homolog, was involved in immune evasion and resistant to phagocytosis and contributed to virulence in mice (Cron et al., 2009; Overweg et al.,

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2000). In *Streptococcus equi*, the PrsA homolog PrtM is a potential vaccine candidate and deletion of codons 138–213 of PrtM attenuated virulence to ponies and altered secretion of multiple proteins (Hamilton et al., 2006; Ikolo et al., 2015). These studies suggest that PrsA contributes to pathogenesis of pathogenic Gram-positive bacteria.

Our previous proteomic approach identified PrsA as a potential substrate of type IV-like secretion system (T4SS) of *S. suis* serotype 2 and purified PrsA expressed in *E. coli* could induce release of pro-inflammatory cytokines and was cytotoxic to host cells (Jiang et al., 2016). PrsA is present both on the cell surface and in the supernatant and has been found protective to mice to challenges by *S. suis* serotype 2 and 9 strains (Jiang et al., 2019). However, the specific roles of PrsA in *S. suis* serotype 2 pathogenicity remain unknown. Here we report the functions of PrsA in pathogenicity in cultured cell models and mice, and its effect on translocation of several known virulence factors.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture conditions

Streptococcus suis 2 strain 05ZYH33, kindly donated by Professor Y. Feng (Zhejiang University School of Medicine), is a high virulent isolate from a clinical patient (Tang et al., 2006). The *S. suis* serotype 2 strain and its mutant were grown in Brain Heart Infusion (BHI; Oxoid, England), and the *E. coli* strains were grown in Luria-Bertani (LB) broth. Spectinomycin (Spc) was added for *S. suis* (100 μg/ml) and *E. coli* (50 μg/ml) if necessary. Bacterial strains and plasmids used in this study were listed in Table 1.

2.2. Deletion of the prsA gene and complementation

The *prsA* deletion mutant was constructed according to a previous study (Yu et al., 2016). Primers used for amplification of the flanking fragments are listed in Table 1. Briefly, the fragments flanking *prsA* were amplified from the genome of the wild-type strain, fused together using overlap-extension PCR and cloned into the *S. suis-E. coli* shuttle vector pSET4s (Dr. Takamatsu, Japan) to generate the knockout vector pSET4s-Δ*prsA*. pSET4s-Δ*prsA* was electro-transformed into strain 05ZYH33. Deletion of *prsA* was initially screened by spectinomycin resistance and verified by PCR assay using two pairs of primers *prsA*-E/F and *prsA*-A/D.

The complementation plasmid was generated by fusing the previous reported *impdh* promoter with *prsA* and cloned into the pSET2 vector (Dr. Takamatsu, Japan) with the primers listed in Table 1 (Zhu et al., 2014). The pSET2-CΔ*prsA* plasmid was electro-transformed into the Δ*prsA* strain. Complementation (CΔ*prsA*) was screened by spectinomycin resistance. Deletion and complementation of *prsA* was further confirmed by Western blotting.

2.3. Growth curves

A single colony from the BHI agar plates inoculated with strains 05ZYH33, Δ*prsA* and CΔ*prsA* was dispensed into 5 ml BHI broth and incubated at 37 °C for 12 h. The 12-h cultures were adjusted to 0.5 at OD_{620nm}. Each calibrated culture (250 μl each) was inoculated into 25 ml fresh BHI. The mixtures were incubated in a 37 °C incubator at 180 rpm. A volume of 200 μl from each culture was transferred, at 1-h interval for 12 h, to a microplate (triplicate wells for each culture) for measurement at OD_{620nm} using a spectrophotometer (TECAN, Switzerland). Values at OD_{620nm} at each time point were averaged from three independent experiments.

2.4. Morphological analysis by scanning electron microscopy and transmission electron microscopy

S. suis 2 wild-type strain 05ZYH33, Δ*prsA* and CΔ*prsA* were harvested at mid-log growth phase, fixed with 2.5% glutaraldehyde in phosphate buffer (PB 0.1 M, pH 7.0) overnight at 4 °C, and postfixed with 1% osmium tetroxide in PB for 1–2 h at room temperature (RT). The samples were first dehydrated by a graded series of ethanol (30%, 50%, 70%, 80%, 90% and 95%) for 15 min at each step, and then re-dehydrated twice with absolute alcohol for 20 min. For scanning electron microscopic (SEM) analysis, the samples were subjected to dehydration in Hitachi Model HCP-2 critical point dryer, coated with gold-palladium in Hitachi Model E-1010 ion sputter for 5 min, and finally observed in Hitachi Model SU-8010 SEM (Hitachi, Japan). For transmission electron microscopic (TEM) analysis, the ethanol-dehydrated samples were transferred into absolute acetone and incubated for

Table 1
Bacterial strains, plasmids, and primers used in this study.

Strains, plasmids, and primers	Descriptions or sequences	Source, reference, or function
Strains		
05ZYH33(WT)	Virulent strain isolated from a dead patient in China, serotype 2	Donated by Pro. Y. Feng
Δ*prsA*	Isogenic *prsA* knockout mutant of WT	This study
CΔ*prsA*	Complemented strain of Δ*prsA*; Spc^R	This study
E. coli DH5a	For cloning the recombinant plasmids	Tsingke
Plasmids		
pSET4s	SS thermosensitive suicide vector; Spc^R	Takamatsu (2001)
pSET2	*E. coli–S. suis* shuttle vector	Takamatsu (2001)
pSET4s:: Δ*prsA*	A recombinant vector with the background of pSET4s, designed to knockout *prsA*; Spc^R	This study
pSET2:: *prsA*	pSET2 containing the intact *prsA* gene and promoter, Spc^R	This study
Primers		
prsA -A	CCGGAATTCAGTCCGTAGCCCGAGTTCATT	Upstream flanking regions of *prsA*
prsA -B	TTTTAAGCTTTCTTAATAAGTAAATCGTCAA	
prsA -C	CTTATTAAGAAAGCTTAAAAACTTCTTAAAT	Downstream flanking regions of *prsA*
prsA -D	CGCGGATCCCAGTAAGATATGGACACGGATAG	
prsA -E	TTCGTCTGCCAACTTGATGACA	Internal regions of *prsA*
prsA -F	ATTACGATGAAAGGTAACACGAT	
C*prsA* -F	AGGAAAGAACTTGTGTTATAATAAAGGGGTTAATG	The complementation sequence of *prsA*
C*prsA* -R	CCGGAATTCTTACTGACCTGATGAACTAGA	
P*impdh-F*	ACATGCATGCATGGAGGCAGGACAGGTA	The promoter sequence of *impdh*
P*impdh-R*	TATAACACAAGTTCTTTCCTTTCTTTTGGG	
prsA -F	ATGAAACAAACTAAAAAAATTCTCG	The ORF of *prsA*
prsA -R	TTACTGACCTGATGAACTAGAA	

[a] Spc^R, spectinomycin resistance.
[b] Underlined nucleotides denote enzyme restriction sites.

20 min at RT. The samples were then embedded in Spurr resin and sectioned in LEICA EM UC7 ultratome. The sections were stained by uranyl acetate (5 min) and alkaline lead citrate (10 min), and observed in Hitachi Model H-7650 TEM (Hitachi, Japan).

2.5. Bacterial adherence and invasion assays

Adherence and invasion experiments were performed as previously reported using two cell lines: human laryngeal epithelial cell line Hep-2 and mouse brain micro-vascular endothelial cell line bEnd.3 (Zhang et al., 2015). In brief, the bacterial suspensions were added to confluent cell monolayers in 24-well plates at a multiplicity of infection (MOI) of about 50:1, with triplicate wells for each strain. After 2-h incubation at 37 °C and 5% CO_2, the wells were washed four times to remove non-adherent bacteria. For the adherence assay, 1 ml of sterile distilled water was added to each well and incubated for 10 min to lyse the cells. Appropriate dilutions of the lysates were plated on BHI agar and incubated at 37 °C for 24 h for colony counting. Adhesion is defined as CFU adhered to the cells / CFU in original inoculum × 100%. For the invasion assay, DMEM containing gentamicin (200 μg/ml) was added into the wells after the washing step to kill surface-exposed bacteria. After 1-h incubation, the wells were washed three times to remove gentamicin, and cells lysed as above for plate counting on BHI agar. Invasion is defined as intracellular CFU / adhered CFU × 100%. The experiments were repeated three times.

To examine whether PrsA was directly involved in the adhesion process of 05ZYH33, or indirectly by its regulation of GAPDH and enolase, the antibody inhibition assay was conducted using the specific antisera as preciously described (Zhang et al., 2015). Briefly, the mid-log wild-type 05ZYH33 strain was pre-incubated with 10-fold diluted rabbit anti-PrsA serum for 30 min at 37 °C and 5% CO_2. For GAPDH and enolase, the mid-log Δ*prsA* mutant was pre-incubated with 10-fold diluted rabbit anti-GAPDH serum or anti-enolase serum (prepared in our laboratory). The pre-immune serum was included as negative control. The serum-treated bacteria were added to cell monolayers with MOI at 20:1. The remaining steps were the same as described in the adherence assay.

2.6. Phagocytosis and intracellular survival

Phagocytosis and survival assays were conducted in murine macrophage cell line RAW264.7 according to a reported procedure (Feng et al., 2012). Cells were cultured at 37 °C and 5% CO_2 overnight in DMEM with 10% fetal bovine serum to form monolayers in 24-well plates. The mid exponential bacteria, washed with PBS, were added to the wells at MOI of 30:1. The plates incubated for 1 h at 37 °C and 5% CO_2. The wells were then washed three times to remove non-adherent bacteria. DMEM containing gentamicin (200 μg/ml) was then added to the wells. After 1 h (phagocytosis) or 2 h (intracellular survival) incubation, the wells were washed three times and the cells were lysed as afore-mentioned for serial dilutions and plate counting on BHI agar. Phagocytosis was expressed as CFU at 1 h / CFU in original inoculum × 100%, and intracellular survival, as CFU at 2 h / CFU at 1 h × 100%. All assays were performed in triplicate and repeated three times.

2.7. Survival in swine whole blood

Whole blood survival was performed as previously described (Jiang et al., 2016). Mid-exponential bacteria were adjusted to 0.1 at OD_{620nm} with PBS, and 50 μl of each bacterial suspension was added to 450 μl fresh individual blood samples from three clinically healthy pigs. Each blood sample was tested in triplicate. The mixtures were incubated at 37 °C, and agitated at 15 min interval. At 1 h or 2 h of incubation, the mixtures were serially diluted for plating on BHI agar. Survival was calculated as CFU recovered at 1 h or 2 h / CFU in original inoculum × 100%. Data were expressed as mean ± standard deviation (SD) of individual blood samples from three pigs.

2.8. Virulence in mice

Mouse model was used to investigate the effect of *prsA* deletion on virulence. Mid exponential bacteria were harvested and washed with PBS, and adjusted to the same OD_{620nm} values for all three strains (equivalent to a concentration of 1.5×10^9 CFU/ml). Four groups (10 mice per group) of 4-week old female BALB/c mice were intraperitoneally inoculated with 500 μl of strain 05ZYH33, Δ*prsA*, CΔ*prsA*, or PBS (as control). All mice were observed for one week and death was recorded every 12 h.

2.9. Bacterial protein extraction and Western blotting

The whole bacterial proteins (WCP), surface-associated proteins (SAP) and secreted proteins (SecP) were prepared as previously described with minor modifications (Jiang et al., 2019; Li et al., 2015). Briefly, the mid-exponential bacterial cultures in BHI were calibrated to the same optical density. The same volume of such suspensions was subjected to centrifugation at 12,000 × *g* for 10 min at 4 °C. Bacterial pellets were resuspended using the extraction buffer (30 mM Tris-HCl, 25% sucrose, 2 mM $MgCl_2$, 5 mM protease inhibitor phenylmethanesulfonyl fluoride, and 3 mg/mL lysozyme at pH 7.5) and incubated for 1 h at 37 °C. For SAP extraction, the incubation mixtures were centrifuged at 12,000 × *g* for 10 min at 4 °C, and the supernatants were collected as SAP. For WCP extraction, the incubation mixtures were homogenized using Precellys 24 Homogenizer (Bertin, France), centrifuged at 12,000 × *g* for 10 min at 4 °C, and the supernatants were collected as WCP. For SecP extraction, culture supernatants were harvested after centrifugation, and filtered through a 0.22-μm membrane filter. Pre-chilled trichloroacetic acid (TCA) was added to a concentration of 10% (w/v). After 1 h incubation in ice-water bath, the precipitated SecP were collected by centrifugation at 12,000 × *g* for 10 min at 4 °C, and washed three times with ice-cold acetone to remove residual TCA. The protein concentrations were measured using the BCA kit (Beyotime, Shanghai). For Western blot analysis, the separated proteins on SDS-PAGE gels were electro-blotted onto PVDF membranes. For confirmation of *prsA* deletion and complementation, the WCPs were used in SDS-PAGE and Western blotting with rabbit-anti PrsA serum as the primary antibody. For detection of SLY, GAPDH and enolase, respective rabbit antisera were used as primary antibodies. HRP-conjugated goat anti-rabbit IgG was used as the secondary antibody. The primary antibodies were incubated overnight at 4 °C and 60 rpm. The secondary antibody was incubated for 1 h at 37 °C and 60 rpm. The Protein bands were visualized with Pierce™ ECL Western Blotting Substrate (Thermo) using the chemiluminescent imaging system (Sage Creation, Beijing, China).

2.10. Quantitative real-time polymerase chain reaction (RT-PCR)

Mid-log bacterial cultures were collected, total RNA extracted using bacterial RNA isolation kit (TIANGEN, Beijing, China), and cDNA synthesized using the reverse transcriptase reaction kit (TOYOBO, Japan) according to the manufacturer's protocols. Quantitative PCR (qPCR) was performed to measure the transcriptional levels of *sly*, *gapdh* and *enolase* (with *16S rRNA* as the internal control) using the SYBR green PCR Kit (TOYOBO, Japan) on the Agilent MX3000 P qPCR system (Stratagene, USA). Expression was shown as relative fold changes based on the $2^{-\Delta\Delta}$Ct method.

2.11. Hemolytic activity assays

The hemolytic assay was conducted as described (Wang et al., 2011). Briefly, mid-exponential bacterial cultures were centrifuged at 12,000 × *g* for 10 min. The supernatant samples were collected,

subjected to two-fold serial dilutions in PBS, and mixed with equal volume of 2% sheep red blood cells (prewashed twice with PBS). After 1-h incubation at 37 °C, the mixtures were centrifuged at 1000 × g for 10 min, and 150 μl of the supernatants were transferred into a 96-well polystyrene microplate to measure OD_{540nm}. Hemolytic titer was calculated as the reciprocal of the highest dilution of the samples that induced at least 50% hemolysis. The experiments were performed in triplicate and repeated three times independently.

2.12. Statistical analyses

All data were expressed as means ± standard deviations (SD) of three independent experiments and the mean between the groups were analyzed using paired two-tailed Student's *t*-test using GraphPad Prism 6. For mouse virulence experiments, survival was analyzed using the log-rank test. The P value of 0.05 was considered as the threshold for significance.

3. Results

3.1. Confirmation of prsA deletion and complementation

To investigate the roles of *prsA* in *S. suis* 2, an isogenic *prsA* deletion mutant strain and a corresponding complemented strain were constructed. Deletion was verified by PCR using primers listed in Table **1**. In Δ*prsA*, no fragment was amplified using the internal primers E/F and ORF primers of *prsA*, and fragment in Δ*prsA* using the flanking primers A/D was smaller than that from the parent strain 05ZYH33 (Fig. S1A in the supplemental material). Western blotting showed that PrsA were detected in 05ZYH33 and CΔ*prsA*, but not in Δ*prsA* (Fig. S1B in the supplemental material). These results indicated successful deletion and complementation of *prsA*.

3.2. Characterization of the prsA deletion mutant

Fig. 1A showed that Δ*prsA* exhibited slower growth than the other two stains with $P < 0.05$ from hour 2 post-inoculation. Deletion of *prsA* had apparent effect on streptococcal cell morphology. Light microscopy and SEM revealed increased length of bacterial chains of the Δ*prsA* mutant with swelling at the ends of some chains (Fig. 1B and C), which was similar to what was reported in *Bacillus subtilis* and *Streptococcus mutans* (Crowley and Brady, 2016; Vitikainen et al., 2001), suggesting that *prsA* deletion might have affected cell division of *S. suis* 2. Complementation restored the cell morphology of *S. suis* 2. No significant differences in capsular thickness were observed between the parent and mutant strains (Fig. 1D).

3.3. PrsA deletion promoted adhesion but reduced invasion

Successful colonization to host cells and tissues is important to initiate infection and dissemination of *S. suis* 2. The *prsA* deletion mutant and relevant strains were tested for adherence and invasion in HEp-2 and bEND.3 as the model cell lines. Adherence of Δ*prsA* to both cells was significantly higher than that of the parent or complemented strain ($P < 0.05$, $P < 0.01$ or $P < 0.001$, Fig. 2A). However, the Δ*prsA* deletion mutant had marked reduction in cell invasion as compared with its parental strain ($P < 0.01$ or $P < 0.001$, Fig. 2B). Complementation could largely restore its invasion. To further investigate whether PrsA has anti-adherence effect, we used the rabbit anti-PrsA serum to block PrsA of the wild-type strain 05ZYH33 and tested for adhesion to bEND.3 cells. Bacteria pre-incubated with the pre-immune serum served as negative control. No significant difference was observed between the treatments by the two sera, indicating that blocking of PrsA did not affect adhesion of the strain 05ZYH33 and suggesting that PrsA did not have direct involvement in cell adhesion (Fig. 2C).

3.4. PrsA was involved in resisting phagocytosis and killing in macrophages and surviving in pig whole blood

We further examined the eff ;ect of *prsA* deletion on phagocytosis and intracellular survival in a macrophage cell line RAW264.7. The Δ*prsA* mutant was more readily phagocytosed than its parental and complemented strains ($P < 0.05$, Fig. 3A). However, deletion of *prsA* rendered the bacteria more susceptible to phagocytic killing ($P < 0.01$, compared with the wild-type strain 05ZYH33 or CΔ*prsA*) (Fig. 3B). Survival of Δ*prsA* was also significantly reduced at both time points in fresh blood with $P < 0.05$ in comparison with its parental strain (Fig. 3C). These data suggest that PrsA might be an anti-phagocytic factor.

3.5. PrsA deletion led to attenuated virulence of S. suis 2 in mice

Effect of *PrsA* deletion on virulence was evaluated in BALB/c mice. All mice inoculated with the wild-type strain 05ZYH33 showed signs

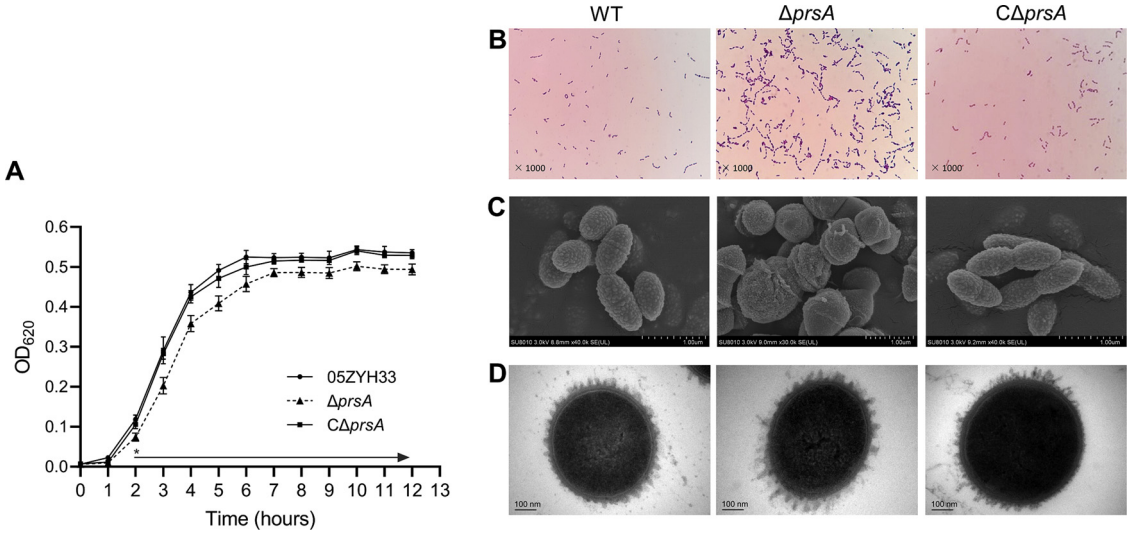

Fig. 1. Growth characteristics and morphology of *Streptococcus suis* serotype 2 strain 05ZYH33 (WT), *prsA* deletion mutant (Δ*prsA*) and its complemented strain CΔ*prsA*. (A) Growth of *S. suis* 2 strains in BHI with '*' means $P < 0.05$ from hour 2 post-inoculation. The data are expressed as the mean ± SD of three independent experiments. (B) Gram staining images of *S. suis* strains under light microscopy. (C) Scanning electron micrographs. (D) Transmission electron micrographs.

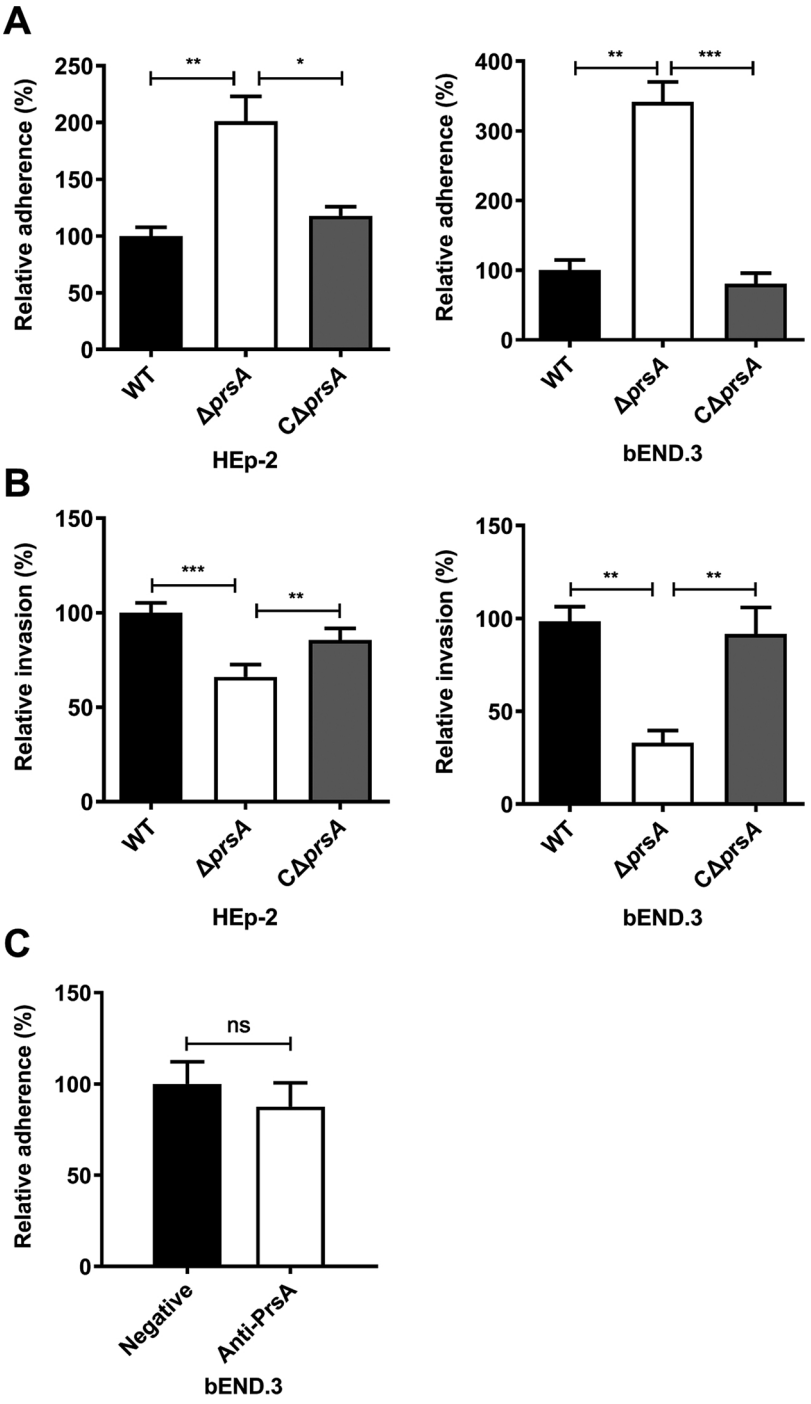

Fig. 2. Adhesion and invasion to HEp-2 and bEND.3 cells of *Streptococcus suis* serotype 2 wild-type strain (WT), *prsA* deletion mutant (Δ*prsA*) and its complemented strain CΔ*prsA*. (A) Adhesion. (B) Invasion. (C) Effect of pre-treatment of the wild-type strain 05ZYH33 with rabbit anti-PrsA antibodies (Anti-PrsA) on bacterial adhesion, and pre-immune serum served as negative control. Data were shown as mean ± SD of three independent experiments with significant differences: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

such as ruffled hair coat, dropping and moist eyes, slow response to stimuli, and died within the first 12 h, and those receiving complemented strain also died in the first day. However, most of the mice inoculated with Δ*prsA* did not show clinical symptoms and stayed alive with only two deaths during the first two days (Fig. 4), suggesting the important role that PrsA played in *S. suis* 2 virulence.

3.6. PrsA affected secretion or translocation of SLY, GAPDH and enolase

In *L. monocytogenes*, *prsA2* deletion caused changes of secretion of some proteins, including listeriolysin-O (LLO), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and enolase (Alonzo and Freitag, 2010; Zemansky et al., 2009). *Streptococcus suis* 2 contains homologs of these molecules. We attempted to investigate whether PrsA could modulate secretion or translocation of selected proteins in the surface-associated and secreted fractions of *S. suis* 2 cultures using SDS-PAGE/Western blotting. SLY was dramatically reduced in both fractions of SAP and SecP of the Δ*prsA* mutant as compared with its parental strain (Fig. 5A). However, GAPDH and enolase were significantly increased in the SAP fraction of the Δ*prsA* culture (Fig. 5A). Both GAPDH and enolase were

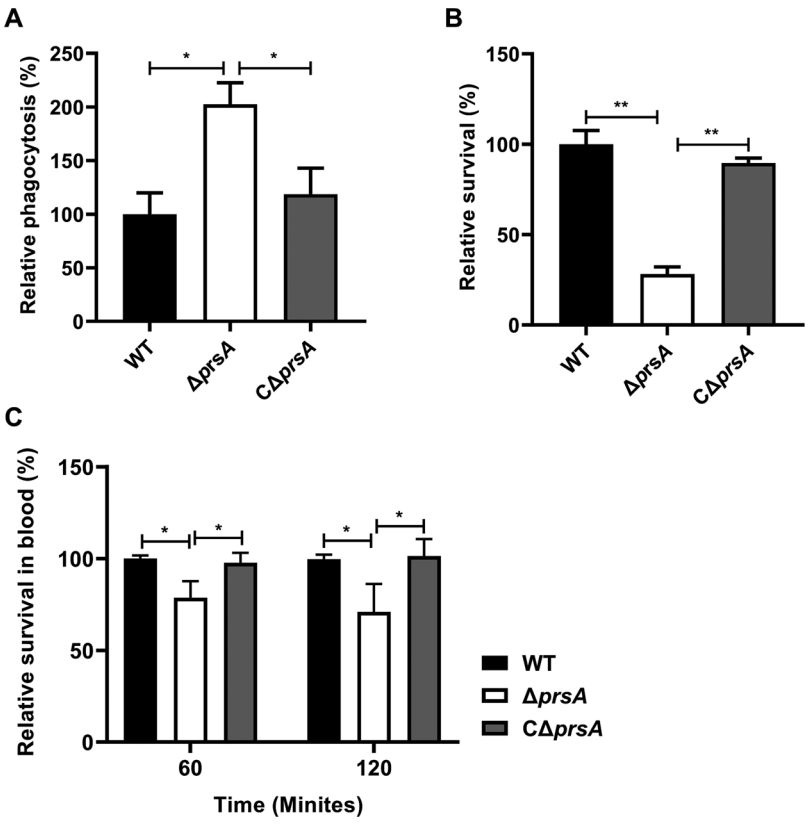

Fig. 3. Phagocytosis and survival in RAW264.7 macrophages or pig blood of *Streptococcus suis* serotype 2 wild-type strain (WT), *prsA* deletion mutant (Δ*prsA*) and its complemented strain CΔ*prsA*. (A) Phagocytosis. (B) Survival in macrophages. (C) Survival in fresh pig blood. Data were shown as mean ± SD of three independent experiments (A) and (B) or of three pig blood samples (C) with significant differences: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

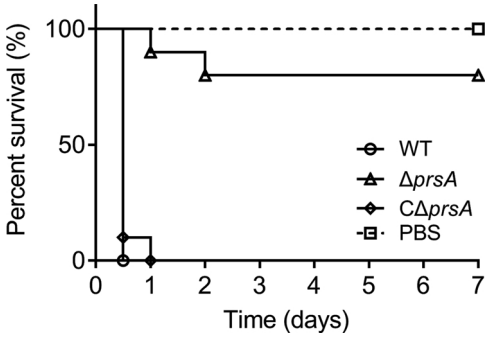

Fig. 4. Survival of the mice challenged with *Streptococcus suis* serotype 2 wild-type strain (WT), *prsA* deletion mutant (Δ*prsA*) and complemented strain CΔ*prsA* with PBS as negative control. Survival data were analyzed using the log-rank test.

found in the SecP fraction of the Δ*prsA* strain, but not detectable in supernatant samples of strains 05ZYH33 and CΔ*prsA* (Fig. 5A). qRT-PCR analysis showed that there was no significant difference of transcription of these genes between the parental strain and *prsA* deletion mutant (Fig. 5B).

3.7. PrsA contributed to hemolytic activity of S. suis 2

It has been reported that *prsA* deletion can cause significant loss in hemolytic activity in Gram-positive bacteria. Here, we found that the culture supernatant of the mutant Δ*prsA* strain had significantly lower hemolytic activity than its parental strain 05ZYH33 ($P < 0.001$), which could be restored by *prsA* complementation (Fig. 5C). This could be due to reduced secretion of hemolytic suilysin in the Δ*prsA* strain (Fig. 5A).

3.8. Enhanced translocation of GAPDH and enolase contributed to increased adhesion to bEND.3 cells

To prove that increased abundance of GAPDH and enolase in the SAP fraction is involved in increased adhesion, we conducted the antibody inhibition assay using anti-GAPDH and anti-enolase sera. Fig. 5D shows that blocking of the surface-associated GAPDH and enolase with specific antisera markedly reduced adherence of the Δ*prsA* mutant to bEND.3 cells with $P < 0.05$ (GAPDH) or $P < 0.01$ (enolase) as compared to the pre-immune serum control.

4. Discussion

PrsA is a chaperone protein ubiquitously distributed in Gram-positive bacteria, such as *L. monocytogenes*, *S. pneumoniae*, *S. aureus*, *B. subtilis*, *S. mutans* and *Lactococcus lactis*, and plays important roles in regulation of protein secretion and virulence (Cahoon and Freitag, 2014; Cron et al., 2009; Hamilton et al., 2006; Ikolo et al., 2015; Lin et al., 2018; Overweg et al., 2000). The specific role of PrsA in *S. suis* 2 pathogenicity has not been explored in detail. We have previously reported that PrsA is conserved in *S. suis* strains, and is a potential substrate of type IV secretory system (T4SS) of *S. suis* 2. Purified PrsA expressed in *E. coli* is able to induce pro-inflammatory cytokines release and has cytotoxicity (Jiang et al., 2019, 2016). Here we provide further evidence that *S. suis* 2 PrsA is involved in secretion and translocation of several important virulence factors, favoring its survival in the host for increased pathogenicity.

Colonization of bacteria to host cells is central to initiate the infection process (Pizarro-Cerda and Cossart, 2006). Involvement of PrsA in bacterial adhesion might vary with species or even strains. In *S. aureus*, deletion of *prsA* caused enhanced adhesion to host cells (Lin et al., 2018). In *S. pneumoniae*, its PrsA homolog PpmA impacted adhesion in a strain-specific manner, and deletion of *ppmA* did not affect the interaction of pneumococcal surface proteins with some host ECM proteins and several other cellular receptors (Cron et al., 2009).

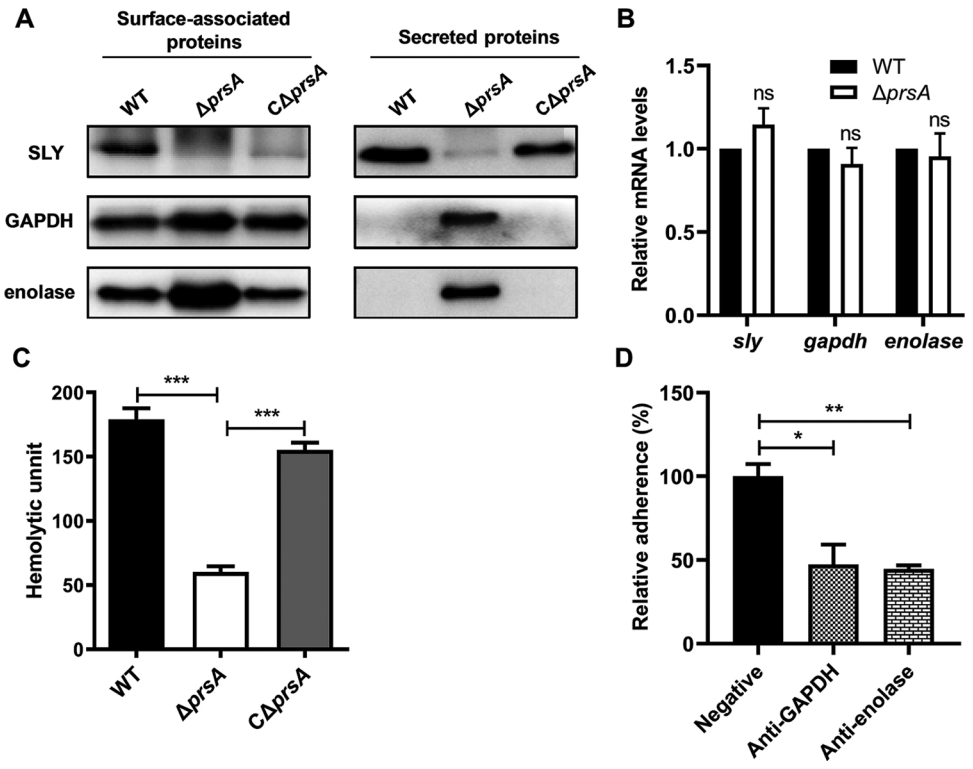

Fig. 5. Effects of *prsA* deletion on translocation, transcription and biological activity of suilysin (SLY), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and enolase. (A) Identification of target molecules in the surface-associated and culture supernatant fractions of *Streptococcus suis* serotype 2 wild-type strain (WT), *prsA* deletion mutant (Δ*prsA*) and its complemented strain CΔ*prsA*. (B) Transcription of target molecules. (C) Hemolytic activity of bacterial culture supernatants. (D) Adhesion to bEND.3 cells of the *prsA* deletion mutant inhibited by anti-GAPDH and anti-enolase sera. Pre-immune serum was used as negative control. For B, C and D, data were shown as mean ± SD of three independent experiments with significant differences: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Antibody inhibition test revealed that increased adhesion of the wild-type strain 05ZYH33 to the cells was not affected by pre-treatment of the bacterial cells with anti-PrsA serum, suggesting that PrsA was not directly involved in adhesion (Fig. 2C).

We found that the abundance of two important adhesins, enolase and GAPDH, which can bind to host ECM proteins and mediate *S. suis* 2 adhesion (Brassard et al., 2004; Esgleas et al., 2008; Li et al., 2015), were significantly increased on the bacterial surface and in the culture supernatant of the Δ*prsA* strain, while secretion and translocation of SLY was markedly reduced (Fig. 5A). This is similar to what have been found in *L. monocytogenes* that *prsA2* deletion led to changed patterns of translocation or secretion of these homologous molecules (Alonzo and Freitag, 2010). Because pre-treatment of the Δ*prsA* mutant with antiserum against GAPDH or enolase significantly attenuated its adhesion to bEnd.3 cells (Fig. 5D), we suggest that increased translocation of GAPDH or enolase in the Δ*prsA* mutant is responsible for enhanced adhesion of *S. suis* 2 to host cells (Fig. 2A). Such enhanced adhesion might also favor bacterial interaction with the macrophages with increased phagocytosis (Fig. 3A).

What is noteworthy here is the significant decrease of SLY secretion in the *prsA* deletion mutant, which corresponds to marked reduction of hemolytic activity in the culture supernatant of the Δ*prsA* mutant (Fig. 5A and C). This means that *prsA* promotes the hemolytic activity of *S. suis* 2 by facilitating SLY secretion. SLY, as a thiol-activated cytolysin, has been considered to play multiple critical roles in *S. suis* 2 pathogenesis. Enhanced SLY secretion can facilitate bacterial invasion into the epithelial cells and promote bacterial survival in the blood circulation, thus contributing to invasive infection of *S. suis* 2 (Chabot-Roy et al., 2006; He et al., 2014; Takeuchi et al., 2014; Tenenbaum et al., 2016). Because *S. suis* 2 became less invasive due to *prsA* deletion (Fig. 2B), we speculate that reduced release of SLY might account for such decreased invasion although the exact mechanism requires further investigation.

Once penetrating the host mucosal and skin barriers, *S. suis* 2 is able to escape killing by macrophages and polymorphonuclear neutrophils, and survive in blood circulation to induce further infection (Fittipaldi et al., 2012). Pneumococcal PpmA was reported to contribute to immune evasion (Overweg et al., 2000), and *Listeria* PrsA2 was found to enhance its survival in murine macrophages (Chatterjee et al., 2006). Several studies indicated that SLY could help *S. suis* 2 resist phagocytosis and killing by neutrophils, macrophages and dendritic cells (Chabot-Roy et al., 2006; Lecours et al., 2011). Here we found that *prsA* deletion led to increased uptake and killing by mouse macrophage RAW264.7 (Fig. 3A and B) and reduced survival in pig whole blood (Fig. 3C). We suppose that marked decrease of SLY secretion and increased translocation of surface-associated GAPDH and enolase of *S. suis* 2 due to *prsA* deletion (Δ*prsA*) might have tilted bacteria-host interaction towards its clearance in the macrophages and whole blood, as well as low pathogenicity in the mouse model (Fig. 4). In addition to the virulence factors tested in this study, some other virulence factors of *S. suis* 2, such as MnmE, FlpS and ArcD of the arginine deiminase system that are reportedly involved in *S. suis* 2 virulence (Fulde et al., 2014; Gao et al., 2019; Willenborg et al., 2016), might also be impacted by prsA deletion that requires further investigation.

In summary, the present study demonstrates that PrsA plays multiple roles in *S. suis* 2 pathogenesis not only by its pro-inflammatory and cytotoxic effects as previously reported, but also by modulating secretion and translocation of suilysin and other important adhesion molecules such as GAPDH and enolase in favor of its survival in the host with enhanced pathogenicity. Further investigation is required to find out how PrsA modulates protein translocation to benefit *S. suis* infection and if there are other *S. suis* 2 substrates of potential virulence regulated by PrsA.

Ethics statement

Animal experiments were performed following the International Guiding Principles for Biomedical Research Involving Animals and approved by the Laboratory Animal Management Committee of Zhejiang University (Approval No. 2018108).

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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.vetmic.2019.07.027.

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