Roles of the Putative Type IV-like Secretion System Key Component VirD4 and PrsA in Pathogenesis of Streptococcus suis Type 2

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Streptococcus suis type 2 (SS2) is a zoonotic pathogen causing septic infection, meningitis and pneumonia in pigs and humans. SS2 may cause streptococcal toxic shock syndrome (STSS) probably due to excessive release of inflammatory cytokines. A previous study indicated that the virD4 gene in the putative type IV-like secretion system (T4SS) within the 89K pathogenicity island specific for recent epidemic strains contributed to the development of STSS. However, the functional basis of VirD4 in STSS remains unclear. Here we show that deletion of virD4 led to reduced virulence as shown by about 65% higher LD50, lower bacterial load in liver and brain, and lower level of expression of inflammatory cytokines in mice and cell lines than its parent strain. The ΔVirD4 mutant was more easily phagocytosed, suggesting its role as an anti-phagocytic factor. Oxidative stress that mimic bacterial exposure to respiratory burst of phagocytes upregulated expression of virD4. Proteomic analysis identified 10 secreted proteins of significant differences between the parent and mutant strains under oxidative stress, including PrsA, a peptidyl-prolyl isomerase. The SS2 PrsA expressed in E. coli caused a dose-dependent cell death and increased expression of proinflammatory IL-1β, IL-6 and TNF-α in murine macrophage cells. Our data provide novel insights into the contribution of the VirD4 factor to STSS pathogenesis, possibly via its anti-phagocytic activity, upregulation of its expression upon oxidative stress and its involvement in increased secretion of PrsA as a cell death inducer and proinflammatory effector.

Keywords: Streptococcus suis type 2, streptococcal toxic shock syndrome, VirD4, anti-phagocytic, PrsA, inflammatory response

INTRODUCTION

Streptococcus suis type 2 (SS2) is a major swine pathogen and an emerging zoonotic agent, causing meningitis, arthritis, septicemia and pneumonia in humans and pigs (Lun et al., 2007; Gottschalk et al., 2010; Feng et al., 2014). S. suis type 2 was initially seen to cause only sporadic cases of meningitis and sepsis in humans working with pigs or pork-derived products (Wertheim et al., 2009). However, two outbreaks of human SS2 infection in China in 1998 and in 2005 raised considerable concerns among public health professionals (Tang et al., 2006). Infection in these two outbreaks was characterized by acute high fever, clear systemic erythematous blanching rash,
disseminated intravascular coagulation and multiple organ failure and shock as well as short duration of the disease (acute death within hours of infection) and high mortality (Sriskandan and Slater, 2006; Tang et al., 2006).

*Streptococcus suis* type 2 isolates of these two outbreaks exhibit strong invasiveness and high pathogenicity leading to a new disease form as streptococcal toxic shock syndrome (STSS) which was originally referred to *S. pyogenes* (GAS) (Feng et al., 2010). The main virulence factors involved in *S. pyogenes* STSS consist of so-called superantigens or molecules that trigger nonspecific, uncontrolled activation of T cells and massive cytokine release. Although a collection of new virulence factors have been described in SS2, there were no superantigen candidates identified so far, implying that a different mechanism could be involved in the STSS form caused by SS2 variant strains from these two outbreaks (Tang et al., 2006; Lappin and Ferguson, 2009; Feng et al., 2010). Comparative genomics analysis has revealed that a functional pathogenicity island (PAI) of about 89 kb (89K) is exclusively present in the epidemic strains in the two Chinese SS2 outbreaks but not in other clinical isolates (Chen et al., 2007; Feng et al., 2011). Further studies suggested that 89K PAI with a transposon-like essence could undergo GI-type (genomic island) T4SS (type IV secretion system)-mediated horizontal transfer and encodes at least two sets of genetic elements involved in SS2 virulence: a salK-salR two-component regulation system and a type IV- like secretion system (T4SS-like) (Zhao et al., 2011).

Another specific feature for STSS caused by SS2 variant isolates from the outbreaks was an early phase excessive burst of proinflammatory cytokines storm (Ye et al., 2009; Fittipaldi et al., 2012; Lachance et al., 2013). Such an unbalanced immune response could be harmful not only to the pathogens but also to the host. Such excessive inflammation is responsible for high mortality observed in STSS cases. A recent study illustrated that deletion of the key component (VirD4 or VirB4) of the T4SS-like system led to decreased virulence and alleviation of excessive systemic inflammatory responses in mice (Zhao et al., 2011). However, the functional mechanisms of the T4SS-like system (including VirD4) in SS2 infection are still poorly understood.

This study was aimed to investigate the roles of VirD4 in microbe-host interaction and to explore possible effectors contributing to the pathogenicity of *S. suis* type 2 infection.

**MATERIALS AND METHODS**

**Bacterial Strains and Culture Conditions**

*Streptococcus suis* type 2 strain HA9801, kindly donated by Professor C. Lu (Nanjing Agricultural University, China), was an isolate from the Jiangsu outbreak of STSS in 1998. Its isogenic mutant with deletion of *virD4* ORF (ΔVirD4) was constructed using the pSET4s vector kindly provided by D. Takamatsu (National Institute of Animal Health, Japan) (Takamatsu et al., 2001). Deletion of the target gene was confirmed by PCR with flanking, internal and external primers (Figure S1). The SS2 strains were grown in Brain Heart Infusion (BHI) (Oxoid, England) at 37°C with shaking at 180 rpm. *Escherichia coli* DH5α and BL21 (TransGen, Beijing) were grown in Luria broth media (Oxoid, England).

**Virulence in Murine Model**

A mouse infection model was used to compare virulence between ΔVirD4 mutant and its parent strain (or wild-type strain, WT). Bacterial cultures were adjusted to OD₅₆₀ 0.4 with PBS (equivalent to about 3–5 × 10⁸ CFU/ml). 6-week old female BALB/c mice, eight for each strain, were infected by intraperitoneal injection of the two strains in 1.0 ml volume of the PBS dilutions containing 1.8 × 10⁸ to 1.2 × 10⁹ CFU. Mice inoculated with sterile PBS were included as control. Mortality was monitored every day for 7 days post-infection (dpi). The 50% lethal dose (LD₅₀) was calculated using the Reed and Muench method. To compare bacterial load in organs, mice (six for each strain) were inoculated intraperitoneally with 4.3–4.5 × 10⁸ CFU. Mice were humanely euthanized at 12 h post-infection (hpi). Spleen, liver and brain samples were collected, homogenized and diluted for plate counting on BHI agar plates.

**Survival in Murine Blood**

To examine the contribution of VirD4 in survival, Mouse whole blood survival assay was conducted as previously described (Liu et al., 2014). Briefly, 50 µl of the WT and ΔVirD4 strains at logarithmic phase adjusted to 0.4 in PBS were inoculated into sterile Eppendorf tubes pre-filled with 450 µl of fresh anticoagulant whole blood pooled from clinically healthy mice. The blood-bacteria mixtures were incubated with shaking at 180 rpm at 37°C for 1 h. Surviving bacteria were then diluted and plated on BHI agar plates for enumeration. Survival percentage was calculated as (CFU on plate/CFU in initial inoculum) × 100%.

**Phagocytosis Assay**

Phagocytosis assay was performed as previously described (Feng et al., 2012) in four types of cells, i.e., primary bone marrow macrophages (BMDM) from C57BL/6 mice (Hu et al., 2014), mouse macrophage cell lines RAW264.7, porcine alveolar macrophage 3D4/2 (PAM 3D4/2) and human monocyte cell line THP-1. Briefly, cells (2–2.5 × 10⁵ cells) grown in DMEM or 1640 medium (Gibco, Invitrogen, Germany) were infected at a multiplicity of infection (MOI) of 100 for 1 h. The infected monolayers were washed twice with warm PBS and re-incubated for another hour in the medium containing gentamicin (300 µg/ml) (Sigma-Aldrich, Ontario, Canada) to kill extracellular bacteria. Cultures were then washed four times with PBS and replaced with 1 ml of sterile distilled water to lyse cells. Viable intracellular bacteria were determined by plating of serial dilutions of the lysates on BHI agar. This assay was repeated at least four times with each strain tested in triplicate wells in independent experiments. Double immuno-fluorescence was performed to visualize phagocytosis as previously described (Benga et al., 2004). The RAW264.7 or BMDM cells were infected as above and washed three times with PBS. The cells were fixed for 10 min with 4% paraformaldehyde in PBS and blocked for 1 h with 10% inactivated fetal bovine serum in PBS. The macrophage cells were then sequentially probed.
with rabbit anti-S. suis antibody (1:100) for 1 h and TRITC-conjugated goat anti-rabbit IgG antiserum (1:50, Boster, China) for 1 h to stain extracellular bacteria. Afterwards, cells were washed with PBS and permeabilized with 0.2% Triton X-100 for 15 min. Total cell-associated bacteria were identified by staining of permeabilized cells with the same primary antibody followed by FITC-conjugated goat anti-rabbit IgG antibody for 1 h. Samples were analyzed with 600-fold magnification on an IX-81 confocal microscope integrated into the FV-1000 imaging system (Olympus, Hamburg, Germany).

**Analysis of Proinflammatory Cytokines**
BALB/c mice (four mice per strain at each time point) were inoculated intraperitoneally with sublethal dose (2.1–3.3 × 10^8 CFU) of WT and ΔVirD4 strains. Mice injected with sterile PBS were included as control. Spleen, brain and blood samples were collected at 6, 12, and 24 hpi. Total RNA samples from spleens and brains were isolated using animal tissue total RNA isolation kit (TIANGEN, Beijing) and used for cDNA synthesis with AMV reverse transcriptase reaction kit (TOYOBO, Japan) according to the manufacturer's instructions. Quantitative PCR (qPCR) was performed to measure the transcriptional levels of proinflammatory cytokines (IL-β, IL-6, TNF-α and MCP-1) using the SYBR green PCR Kit (TOYOBO) and the Agilent MX3000P qPCR system (Stratagene, USA). Additionally, macrophage cell lines RAW264.7 and 3D4/2 were collected at late logarithmic phase. Total bacterial RNA was extracted for cDNA synthesis with the reverse transcriptase reaction kit (TOYOBO, Japan) according to the manufacturer's instructions. Quantitative PCR (qPCR) was performed to measure the transcriptional levels of proinflammatory cytokines (IL-β, IL-6, TNF-α and MCP-1) using the SYBR green PCR Kit (TOYOBO) and the Agilent MX3000P qPCR system (Stratagene, USA). Additionally, macrophage cell lines RAW264.7 and 3D4/2 were collected at MOI of 100 for 2 h. Total RNA isolation, cDNA synthesis and qPCR were conducted as described above. All primers used for qPCR were listed in Table S1. β-actin was used as the reference gene for normalization. Results were presented as fold changes relative to non-infected cells using the 2^-ΔΔCt method. IL-1β and IL-6 cytokines in serum samples were determination by ELISA kits (ExCell, Shanghai).

**Analysis of Secreted Protein Profiles and VirD4 Expression of S. suis Type 2 Exposed to Hydrogen Peroxide Stress or in Infected Macrophages**
Pathogenic bacteria are faced with oxidative stress during infection as a result of respiratory burst of the phagocytic cells. To mimic such oxidative stress, the WT and ΔVirD4 strains were cultured at 37°C in BHI with or without 10 mM H2O2. Bacterial cells were collected at late logarithmic phase. Total bacterial RNA was extracted for cDNA synthesis with the reverse transcriptase reaction kit (TOYOBO). qPCR was performed to measure the mRNA level of the *virD4* gene using specific primers (Table S1). To examine the role of cellular effects in *virD4* expression, RAW264.7 cells were infected with the wild-type strain (MOI 100 for 2 h) in the presence or absence of a reactive oxygen species inhibitor (N-acetyl-l-cystein, NAC, 5 mM). Two parts of the bacterial population were collected for qPCR analysis of *virD4* mRNA: bacteria adhered to and phagocytosed by the macrophages (cell-associated) and unassociated supernatant bacteria (bacteria in culture supernatant). To analyze possible involvement of VirD4 in protein secretion upon H2O2 treatment, culture supernatant samples of both the WT and ΔVirD4 strains were prepared as previously reported (Geng et al., 2008). Briefly, culture supernatants at late logarithmic phase BHI cultures with or without H2O2 treatment were harvested by centrifugation (8000 rpm for 20 min at 4°C), treated with a protease inhibitor PMSF (Phenylmethylsulfonyl fluoride) (Betoyome, Shanghai) and filtered through a 0.22-μm membrane. The secreted proteins were precipitated with 10% trichloroacetic acid (TCA) in acetone for 1 h on ice and collected by centrifugation at 10,000 × g for 20 min at 4°C. The precipitates were washed 3 times with ice-cold acetone containing 0.1% PMSF and air-dried. Proteins were quantified with the BCA kit (Betoyome, Shanghai). The protein samples (30 µg per lane) were subjected to SDS-PAGE followed by Coomassie blue R250 staining or by Western blotting using rabbit anti-SS2 whole cell polyclonal antibodies (1:300) and HRP-conjugated goat anti-rabbit secondary antibodies. Protein bands were visualized using the chemi-luminescence substrate (Thermo, USA) and chemiluminescent imaging system (SageCreation, Beijing).

**Identification of Secreted Proteins by 2D-Page and LC-MS/MS**
The above protein samples were further treated with a 2D Clean-Up kit (Bio-Rad), resuspended in lysis buffer and then quantified for equal loading. Two dimensional electrophoresis (2DE) and identification of proteins by mass spectrometry (Bruker Dalton, Ultraflex III TOF/TOF) were performed as described elsewhere (Jing et al., 2008). Comparison of secreted protein spots was performed with the ImageMaster 2D platinum 5.0 (GE Healthcare, Uppsala, Sweden). Only spots with relative high abundance showing consistent and reproducible changes (>3 folds) in 2D-gels were excised and subjected to mass spectrometry. FlexAnalysis software (Bruker Dalton) was used to remove contaminant peaks (including matrix peaks and solvent peaks). Peptide mass fingerprinting (PMF) data was analyzed using the MASCOT server (http://www.matrixscience.com) to search for target proteins from the *S. suis* protein sequence data in the NCBI database.

**Transcriptional Analysis of the Genes Encoding Differentially Secreted Proteins upon Oxidative Stress**
Procedures for oxidative stress, total RNA extraction from the WT and ΔVirD4 strains and cDNA synthesis were described above. qPCR was conducted using specific primers (Table S1) to evaluate transcriptional levels of the genes encoding significantly upregulated or downregulated proteins between WT and ΔVirD4 strains. The 16S rRNA gene was tested in parallel for normalization.

**Expression of Recombinant PrsA Protein and Antiserum Preparation**
The PrsA gene was PCR-amplified from the genome DNA of the strain HA9801 using specific primers (Table S1), digested with corresponding restriction enzymes, and cloned to pET-30a vector for expression in *E. coli* BL21. The recombinant protein was purified using Ni-NTA columns (Novagen). Lipopolysaccharides in the purified protein were removed using a ToxinEraser™ Endotoxin Removal Kit (Genscript, USA), and tested using a
Chromogenic LAL Endotoxin Assay Kit (GenScript, USA). The protein was then passed through a 0.22-µm filter, concentrated by membrane ultrafiltration (Millipore) and stored at −80°C. The BCA protein assay kit (Beyotime) was used to determine the protein concentration. SDS-PAGE and Western blotting were performed to confirm presence of the target protein with an anti-His monoclonal antibody (Sungene Biotech, China). Hyperimmune sera to PrsA were obtained from New Zealand White rabbits after four times of subcutaneous immunization at a 2-week interval with 200 µg of purified recombinant PrsA emulsified with Freund’s complete (primary immunization) or incomplete adjuvant (booster immunization). Anti-PrsA sera were collected and had titers >1:10000 as measured by ELISA. Preimmune serum samples were collected as negative control. In order to measure the relative abundance of PrsA in the culture supernatants of the WT and ΔVirD4 strains treated with or without hydrogen peroxide, the extracted protein samples (3 µg/well) were coated onto the 96-wells and probed with rabbit anti-PrsA polyclonal antibodies for indirect ELISA.

**Investigation of PrsA Protein Functions**

Cell cytotoxicity of PrsA was conducted as previously described (Jiang et al., 2016). The mouse brain microvascular endothelial cell line bEND3.0 cultured in DMEM supplemented with 10% FBS (Gibco, USA) were stimulated with purified PrsA at different concentrations and incubated for 1 or 2 h at 37°C with 5% CO2. PBS and 0.2% Triton X-100 treatments served as negative and positive controls, respectively. Cytotoxicity was qualified by Lactate Dehydrogenase Cytotoxicity Assay Kit (Beyotime, China) or observed directly under fluorescent microscope (×100) after staining with Live/Dead cytotoxic kit (Invitrogen, Eugene, OR).

Hemolysis assay was conducted as elsewhere described (Zheng et al., 2011). Purified PrsA protein was added to 2% sheep red blood cells in PBS at final concentration of 50 and 100 µg per ml. The mixtures were incubated for 1 h at 37°C with 5% CO2. After brief centrifugation at 800 g for 10 min, supernatant samples were measured at OD540 on the SpectraMax M2 Microplate reader (Molecular Devices, USA). Erythrocytes with His-tag, bovine serum albumin or 0.2% Triton-100 and those without any treatment served as controls.

To analyze whether PrsA could induce release of proinflammatory cytokines, RAW264.7 cells (2–2.5 × 10^5 cells) were seeded into 24-well cell culture plates (Corning, USA) and incubated in DMEM with 10 µg/ml of PrsA or 400 ng/ml LPS (positive control) and without treatment (DMEM only as negative control) at 37°C with 5% CO2. Culture supernatants were collected at indicated times for determination of IL-1β and TNF-α using commercially available ELISA Max Deluxe kits (Biolegend, USA). These two cytokines and IL-6 were also analyzed at the transcriptional level by qPCR as described above.

**Animal Ethics**

All animal experiments were conducted following the International Guiding Principles for Biomedical Research Involving Animals-1985 and approved protocols of the Laboratory Animal Management Committee of Zhejiang University (Approval No. 2015016).

**Statistical Analysis**

All experiments were performed in triplicate and repeated at least three times. Experimental results were expressed as mean ± SD unless otherwise stated. Data were subjected to normality tests and analyzed using two-tailed Student’s t-test with P values <0.05 or 0.01 considered as significant.

**RESULTS**

**VirD4 Played a Role in Virulence of S. suis Type 2**

To determine the role of VirD4 in virulence, an isogenic mutant with deletion of virD4-ORF (ΔVirD4) was constructed from the wild-type strain HA9801 (Figure S1). Deletion of virD4 did not affect growth in BHI broth with or without hydrogen peroxide treatment, as compared with its parent strain (Figure 1A and Figure S2). With BALB/c mouse infection model, we found that VirD4 contributed to virulence as shown by about 65% higher LD50 (Figure 1B) and lower bacterial load in liver and brain (P < 0.05; Figure 1C) than its parent strain. The ΔVirD4 mutant was also more susceptible to bactericidal effect of the whole blood than the wild-type strain (P < 0.01, Figure 1D).

**VirD4 Was Anti-Phagocytic**

Phagocytic cells serve as the first line of host defense against invading bacterial pathogens. We investigated whether VirD4 could play roles in phagocytosis using murine BMDM and RAW264.7, porcine PAM 3D4/2 cells as well as human derived THP-1 cells. Surprisingly, deletion of virD4 rendered the bacteria more easily phagocytosed, particularly in BMDM (Figure 2A). Double label immuno-fluorescence assay further verified this phenomenon (Figure 2B). These findings indicated that VirD4 might be an anti-phagocytic factor.

**Deletion of VirD4 Decreased Expression of Inflammatory Cytokines**

To determine whether VirD4 is involved in expression of proinflammatory cytokines, serum or organ samples were collected from intraperitoneally infected mice for analysis of proinflammatory cytokines. Figure 3 showed that S. suis could induce a rapid proinflammatory systemic response in mice with systemic cytokine and chemokine release. The transcriptional levels of IL-6, TNF-a, IL-1β and MCP-1 were remarkably increased in the early stage of infection (within 12 h) and gradually decreased to their basal levels by 24 hpi (Figures 3A–C). IL-6, TNF-a, and MCP-1 levels induced by WT strain were obviously higher than ΔVirD4 strain infection at 12 hpi. Because the bacterial load of the WT strain was higher than that of the virD4 mutant in the mice brain (Figure 1C), the observed differences in mRNA transcripts of cytokines in mice brain could be attributed partly to bacterial load. Serum levels of IL-6 and IL-1β in mice infected with ΔVirD4 strain at 6 hpi were significantly lower than those infected with the WT strain (Figure 3D). Proinflammatory cytokines from RAW264.7 and PAM3D4/2 macrophage cells infected with ΔVirD4 strain were also lower than the WT strain at the transcriptional level, specifically for IL-1β and IL-6 (Figure 3E).
Oxidative Stress Activated VirD4 Factor and Deletion of virD4 Changed Profiles of Secreted Proteins

Early studies identified some S. suis type 2 proteins induced in vivo, including the T4SS-like VirD4 component using the in vivo-induced antigen technology (IVIAT) (Gu et al., 2009; Li et al., 2013). One of the major in vivo factors that the invading bacteria encounter is reactive oxygen species (ROS, including hydrogen peroxide) resulting from respiratory burst (Forman and Torres, 2002; Fittipaldi et al., 2012). We attempted to mimic in vivo environment by exposing the WT and ΔVirD4 strains to H₂O₂ added to the BHI medium at 10 mM with a sub-inhibitory effect on growth in the pilot test (Figure S2). The virD4 mRNA level was about 50 folds higher after H₂O₂ stress (Figure 4A, P < 0.001), indicating that VirD4 could be an in vivo induced gene. Figure 4B shows that virD4 mRNA in cell-associated bacteria was significantly higher than that of mock control (P < 0.05), a finding similar to oxidative stress assay (Figure 4A). Treatment of the cells with ROS inhibitor (N-acetyl-l-cystein, NAC at 5 mM) reduced virD4 expression (P < 0.05). Thus, we believe that up-regulation of virD4 mRNA is mainly due to oxidative stress. There were also significant differences of secreted proteins between WT and ΔVirD4 strains exposed to H₂O₂ as shown by Western blot and SDS-PAGE (Figure 4C). These findings prompted us to use two dimensional gel electrophoresis to separate the supernatant proteins of WT and ΔVirD4 strains exposure to H₂O₂. The differentially expressed proteins with high abundance were marked in 2DE gels for further identification by mass spectrometry (Figure 4D).

Confirmation of the Selected Differentially Secreted Proteins by qPCR

Table 1 showed that 148 protein spots were upregulated in the ΔVirD4 strain exposed to hydrogen peroxide stress while only 33 protein spots with 1.5-fold or higher upregulation in the WT strain. Mass spectrometry was used to identify protein spots of significant upregulation: four spots were identified as parvulin-like peptidyl-prolyl isomerase (PrsA), succinate dehydrogenase (SucD), and glycerate-dependent phosphoglycerate mutase (GpmA) in the WT strain with spots
Type IV secretion system (T4SS) is a versatile system in Gram-negative bacteria but was recently identified in Gram-positive S. suis type 2 strains causing STSS outbreaks in China (Wallden et al., 2010; Trokter et al., 2014). Type IV secretion system (T4SS) is a versatile secretion system important for virulence and even survival of some bacterial species. It was once believed to be present only in Gram-negative bacteria but was recently identified in Gram-positive S. suis type 2 strains causing STSS outbreaks in China (Wallden et al., 2010). This putative SS2-T4SS is contained in the 89K pathogenicity island and proposed as a new T4SS subgroup (Type-IVC secretion system) (Zhang et al., 2012). T4SS contains a channel enabling secretion of proteins and DNA molecules across the cell envelope. This translocation is driven by a number of cytoplasmic ATPases that energize conformational changes in the translocation complex. VirD4 is considered as a key coupling protein that could recruit substrates to T4SS for translocation (Wallden et al., 2010; Trokter et al., 2014). In Helicobacter pylori, VirD4 presumably acts as an adapter protein guiding CagA into the transport channel and may play a role in inducing host pro-inflammatory responses in both VirD4-CagA-dependent and VirD4-CagA-independent mechanisms (Selbach, 2002). Here we show that VirD4 play important roles in SS2 infection, such as evasion from phagocytosis and increased release of proinflammatory cytokines.

**DISCUSSION**

Of 35 S. suis serotypes, SS2 has emerged as an important zoonotic agent and causes unusual outbreak of streptococcal toxic-shock-like syndrome (STSS) (Gottschalk et al., 2010; Feng et al., 2014). Type IV secretion system (T4SS) is a versatile secretion system important for virulence and even survival of some bacterial species. It was once believed to be present only in Gram-negative bacteria but was recently identified in Gram-positive S. suis type 2 strains causing STSS outbreaks in China (Wallden et al., 2010). This putative SS2-T4SS is contained in the 89K pathogenicity island and proposed as a new T4SS subgroup (Type-IVC secretion system) (Zhang et al., 2012). T4SS contains a channel enabling secretion of proteins and DNA molecules across the cell envelope. This translocation is driven by a number of cytoplasmic ATPases that energize conformational changes in the translocation complex. VirD4 is considered as a key coupling protein that could recruit substrates to T4SS for translocation (Wallden et al., 2010; Trokter et al., 2014). In Helicobacter pylori, VirD4 presumably acts as an adapter protein guiding CagA into the transport channel and may play a role in inducing host pro-inflammatory responses in both VirD4-CagA-dependent and VirD4-CagA-independent mechanisms (Selbach, 2002). Here we show that VirD4 play important roles in SS2 infection, such as evasion from phagocytosis and increased release of proinflammatory cytokines.

Deletion of the virD4 gene decreased SS2 virulence in mice, as shown by higher LD_{50} value, lower bacterial load in organs and reduced survival in fresh mouse blood. These virulence phenotypes were similar to the previous findings of another SS2 strain (05ZYH33) with virD4 deletion (Zhao et al., 2011). However, we found that SS2-VirD4 had a novel antiphagocytic
function as evidenced by increased number of bacterial cells in phagocytic cell lines upon virD4 deletion. In H. pylori, putative T4SS core components virB7 and virB11 (ATPase essentially similar to VirD4) were antiphagocytic (Ramarao et al., 2000; Tegtmeyer et al., 2011). Thus, we assume that VirD4 is involved in S. suis virulence possibly by evasion from phagocytosis.

Once S. suis colonization and invasion of epithelial cells of the respiratory tract and entry to the systemic circulation or deep tissues, SS2 faces phagocytosis by neutrophils and macrophages,
Jiang et al. VirD4 in S. suis Pathogenesis

FIGURE 4 | The virD4 gene was induced by hydrogen peroxide treatment and its deletion could induce differentially secreted protein profiles between wild-type (WT) S. suis type 2 and ΔVirD4 strains subjected to oxidative stress. (A) The virD4 mRNA level from the wild-type S. suis type 2 strain subjected to hydrogen peroxide treatment. **Indicated significant at $P < 0.01$. (B) The virD4 mRNA transcripts of the cell-associated bacteria and unassociated supernatant bacteria (bacteria in supernatant) after 2 hs infection in RAW264.7 cells in the presence or absence of N-acetyl-l-cystein (NAC), a reactive oxygen species inhibitor. *Indicated significant at $P < 0.05$. (C) Differentially secreted proteins were found in regular SDS-PAGE gels and confirmed by Western blotting. (D) Differentially secreted supernatant protein profiles detected by 2DE. Red and Green arrows indicate some upregulated or down-regulated proteins in the wild-type or ΔVirD4 strains compared between the two gels that were used for LC/MS.

TABLE 1 | Summarization of differentially secreted proteins between wild-type and ΔVirD4 strains separated on the two-dimensional PAGE gels.

| Cells   | No. of protein spots showing changes | 1.5- to 3-fold changes | 3- to 5-fold changes | >5 fold changes | >10000* fold change |
|---------|-------------------------------------|------------------------|---------------------|-----------------|-------------------|
| WT      | 33                                  | 14                     | 5                   | 3               | 11                |
| ΔVirD4  | 148                                 | 91                     | 29                  | 12              | 16                |

* indicates presence of the protein spots only in the wild-type or ΔVirD4 strain.

TABLE 1 shows the number of protein spots showing changes and their fold changes for both WT and ΔVirD4 strains.

the first line of host defense. Phagocytosis-associated respiratory burst would generate antimicrobial reactive oxygen species (ROS) including superoxide anion ($\text{O}_2^*$), hydrogen peroxide ($\text{H}_2\text{O}_2$), and hydroxyl radical ($^\cdot\text{OH}$) (Forman and Torres, 2002; Fang et al., 2015; Zheng et al., 2016). We attempted an in vitro oxidative stress assay using non-lethal dose of hydrogen peroxide to mimic host-bacteria interaction in vivo to examine if such exposure would affect expression of virD4 itself and hence the release of secreted proteins. We did find significantly higher expression of virD4 mRNA in SS2 not only upon H$_2$O$_2$ stress but also upon phagocytosis, suggesting that it might be an in vivo induced gene. Such oxidative stress also led to differential expression of secreted proteins between WT and ΔVirD4 strains. The protein spots with a >3-fold abundance change due to H$_2$O$_2$ stress were subjected to MALDI-TOF/MS analysis. Results showed that ten proteins identified are involved in DNA damage repair, nucleotide biosynthesis, carbohydrate metabolism and

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TABLE 2 | Identification and qualification of differentially secreted proteins between wild-type and ∆VirD4 strains selected for LC/MS and qPCR.

| Spotsa | Proteins identified | Score | % coverage | Matched Peptides | Locus tag (05ZYH33) | Protein change in abundanceb | Change in mRNA level |
|-------|---------------------|-------|------------|-----------------|-----------------|----------------------------|---------------------|
| B13   | Parvulin-like peptidyl-prolyl isomerase (PrsA) | 102   | 18         | 6               | SSU05_1238      | 8.8                        | 1.5                 |
| B14   | Succinate dehydrogenase/fumarate reductase (SucD) | 115   | 21         | 7               | SSU05_2153      | 3.8                        | 1.7                 |
| B31   | Glycerate-dependent phosphoglycerate mutase (GpmA) | 295   | 33         | 8               | SSU05_1638      | 9.7                        | 1.4                 |
| A20   | Ribonucleoside-diphosphate reductase (RNR) | 541   | 32         | 25              | SSU05_1207      | 7.8                        | 27                  |
| A39   | 6-phospho-beta-galactosidase (LacG) | 464   | 27         | 13              | SSU05_1036      | 10000                      | 30                  |
| A120  | Uracil phosphoribosyltransferase (UPP) | 354   | 57         | 14              | SSU05_1553      | 12.2                       | 13                  |
| A132  | Galactose-6-phosphate isomerase subunit (LacB) | 218   | 50         | 10              | SSU05_1042      | 10000                      | 68                  |
| A143  | Arginine deiminase (arcA) | 514   | 50         | 26              | SSU05_0624      | 3.7                        | 1.6                 |
| A146  | Ornithine carbamoyltransferase (arcB) | 348   | 56         | 24              | SSU05_0626      | 5.0                        | 2.2                 |
| A148  | Tagatose-bisphosphate aldolase (LacD) | 422   | 49         | 12              | SSU05_1040      | 10000                      | 102                 |

a B or A means these spot numbers were from differential expressed proteins in the wild-type strain or ∆VirD4 mutant, respectively as shown in Figure 4C.
b The number “10000” means that these proteins were present only in wild-type or ∆VirD4 strain.

stress modulation reported in S. suis or other bacteria. These secreted proteins with currently unknown mechanisms could be termed as moonlighting proteins that function not only in catalytic or metabolic activities but also act as modulators in bacterial virulence (Henderson and Martin, 2011, 2013).

The PrsA protein secreted abundantly in WT strain, but not in ∆VirD4 mutant, was selected for further evaluation of its functions. Because prokaryotes and eukaryotes are known to have three ubiquitously distributed enzymes known as cyclophilins, FK506 binding proteins (FKBPs), and parvulins, which catalyze the cis/trans isomerization of peptide bonds preceding prolyl residues, thereby assisting protein folding at the post-translational level (Ünal and Steinert, 2014; Nath and Isakov, 2015). We found that SS2-PrsA expressed in E. coli showed dose-dependent cytotoxicity with significant cell death at concentrations above 75 µg/ml. At a non-cytotoxic dose of 10
μg/ml, PrsA induced significant expression of IL-1β and TNF-α in murine macrophage cell line RAW264.7. These results suggest that PrsA may contribute to the pathogenicity of *S. suis* type 2, as its counterparts in other bacterial species that function in a number of biological processes, such as colonization or invasion, protease exportation, immune activation and cells apoptosis (Ünal and Steinert, 2014). In gram-positive bacteria, PrsA is the only general factor mediating folding of secreted proteins essential for bacterial pathogenicity and cell wall biosynthesis (Sarvas et al., 2004). In *S. pneumoniae*, PpmA, a surface-associated homology of the parvulin protein, was demonstrated to contribute to pneumococcal pathogenesis since *PpmA* gene deletion reduced bacterial persistence in mice nasopharynx and enhanced bacterial uptake by macrophages (Cron et al., 2009). In *Listeria monocytogenes*, PrsA2 plays a critical role in folding of virulence factors, cell wall biosynthesis and resistance to osmotic stress (Alonzo and Freitag, 2010; Cahoon and Freitag, 2014). The parvulin peptidyl-prolyl isomerase HP0175 in gram-negative *Helicobacter pylori* induces apoptosis of gastric epithelial cells and modulates the inflammatory response during infection (Basak et al., 2005; Kundu, 2013).

Taken together, we demonstrate that VirD4 is antiphagocytic and its expression increases upon oxidative stress or phagocytosis. PrsA is cytotoxic and proinflammatory, and its expression is dependent on VirD4. We suppose that there might be complex relationship between PrsA and VirD4 and...
their expression upon oxidative stress during respiratory burst, between VirD4 and phagocytosis, and between PrsA and cytotoxicity in in vivo conditions. Further research is required to examine if VirD4 has direct effects as an antiphagocytic protein or results from increased expression of PrsA that exerts cytotoxic effects to debilitate the phagocytic function. The roles of putative SS2-T4SS in PrsA secretion and the possible mechanisms of PrsA in inducing cells death and proinflammatory response are also worth investigation.

AUTHOR CONTRIBUTIONS

WF and XJ designed the experiments. XJ, YY, and JZ performed the experimental work and data collection. Other authors contributed equally to animal test, literature search, data analysis and interpretation. XJ and WF wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcimb.2016.00172/full#supplementary-material
REFERENCES

Alonso, F. III, and Freitag, N. E. (2010). Listeria monocytogenes PmpA2 is required for virulence factor secretion and bacterial viability within the host cell cytosol. *Infect. Immun.* 78, 4944–4957. doi: 10.1128/IAI.00532-10

Basak, C., Pathak, S. K., Bhattacharyya, A., Pathak, S., Basu, J., and Kundu, M. (2005). The Secreted Peptidyl Prolyl cis-trans-Isomerase HP0175 of Helicobacter pylori Induces Apoptosis of Gastric Epithelial Cells in a TLR4- and Apoptosis Signal-Regulating Kinase 1-Dependent Manner. *J. Immunol.* 174, 5672–5680. doi: 10.4049/jimmunol.174.9.5672

Beng, L., Goethe, R., Rohde, M., and Valentijn-Weigand, P. (2004). Non-encapsulated strains reveal novel insights in invasion and survival of *Streptococcus suis* in epithelial cells. *Cell. Microbiol.* 6, 867–881. doi: 10.1111/j.1462-5822.2004.00409.x

Cahoon, L. A., and Freitag, N. E. (2014). *Listeria monocytogenes* virulence factor modulators: a modulator of the host immune response, “in Moonlighting Cell Stress Proteins in Microbial Infections, ed B. Henderson (Dordrecht: Springer Netherlands), 81–91.

Lachance, C., Gottschalk, M., Gerber, P. P., Lemire, P., Xu, J., and Segura, M. (2013). Exacerbated type II interferon response drives hyperinflammation and toxic shock by an emergent epidemic strain of *Streptococcus suis*. *Infect. Immun.* 81, 1928–1939. doi: 10.1128/LA01317-12

Lappin, E., and Ferguson, A. J. (2009). Gram-positive toxic shock syndromes. *Lancet Infect. Dis.* 9, 281–290. doi: 10.1016/S1473-3099(09)70066-0

Li, S., Song, J., Huang, H., Chen, W., Li, M., Zhao, Y., et al. (2013). Identification of *in vivo* induced genes of *Streptococcus suis* serotype 2 specially expressed in infected human. *Microb. Pathog.* 63, 8–15. doi: 10.1016/j.mpath.2013.05.011

Liu, P., Pian, Y., Li, X., Liu, R., Xie, W., Zhang, C., et al. (2014). *Streptococcus suis* adenosine synthase functions as an effector in evasion of PMN-mediated innate immunity. *J. Infect. Dis.* 210, 35–45. doi: 10.1093/infdis/jiu050

Lun, Z. R., Wang, Q. P., Chen, X. G., Li, A. X., and Zhu, X. Q. (2007). *Streptococcus suis*: an emerging zoonotic pathogen. *Lancet Infect. Dis.* 7, 201–209. doi: 10.1016/S1473-3099(07)70001-4

Nath, P. R., and Isakov, N. (2015). Insights into peptidyl-prolyl cis-trans isomerase structure and function in immunocytes. *Immunol. Lett.* 163, 120–131. doi: 10.1016/j.imlet.2014.11.002

Ramarao, N., Gray-Owen, S. D., Backert, S., Meyer, T. F. (2000). Helicobacter pylori inhibits phagocytosis by professional phagocytes involving type IV secretion components. *Mol. Microbiol.* 37, 1389–1404. doi: 10.1046/j.1365-2958.2000.02089.x

Selbach, M. (2002). Functional analysis of the *Helicobacter pylori* cag pathogenicity island reveals both VirD4-CagA-Dependent and VirD4-CagA-Independent Mechanisms. *Infect. Immun.* 70, 665–671. doi: 10.1128/iai.70.2.665-671.2002

Sririskandan, S., and Slater, J. D. (2006). Invasive disease and toxic shock due to zoonotic *Streptococcus suis*: an emerging infection in the east? *PLoS Med.* 3:e187. doi: 10.1371/journal.pmed.0030187.g001

Takamatsu, D., Osaki, M., and Sekizaki, T. (2001). Thermosensitive suicide vectors for gene replacement in *Streptococcus suis*. *Plasmid* 46, 140–148. doi: 10.1006/plas.2001.1532

Tak, J., Wang, C., Feng, Y., Yang, W., Song, H., Chen, Z., et al. (2006). Streptococcal toxic shock syndrome caused by *Streptococcus suis* serotype 2. *PLoS Med.* 3:e151. doi: 10.1371/journal.pmed.0030151.001

Tegtmeyer, N., Wessler, S., and Backert, S. (2011). Role of the cag pathogenicity island encoded type IV secretion systems. *Mol. Microbiol.* 80, 9–201

Unal, C. M., and Steinert, M. (2014). Microbial peptidyl-prolyl cis-trans isomerases (PPIases): virulence factors and potential alternative drug targets. *Microbiol. Mol. Biol. Rev.* 78, 544–571. doi: 10.1128/MMBR.00015-14

Walden, K., Rivera-Calzada, A., and Waksman, G. (2010). Type IV secretion systems: versatility and diversity in function. *Cell. Microbiol.* 12, 1203–1212. doi: 10.1111/j.1462-5822.2010.01499.x

Wertheim, H. F., Njieha, H. D., Taylor, W., and Schultz, C. (2009). *Streptococcus suis*: an emerging human pathogen. *Clin. Infect. Dis.* 48, 617–625. doi: 10.1086/596763
Ye, C., Zheng, H., Zhang, J., Jing, H., Wang, L., Xiong, Y., et al. (2009). Clinical, experimental, and genomic differences between intermediately pathogenic, highly pathogenic, and epidemic Streptococcus suis. J. Infect. Dis. 199, 97–107. doi: 10.1086/594370

Zhang, W., Rong, C., Chen, C., and Gao, G. F. (2012). Type-IVC secretion system: a novel subclass of type IV secretion system (T4SS) common existing in gram-positive genus Streptococcus. PLoS ONE 7:e46390. doi: 10.1371/journal.pone.0046390

Zhao, Y., Liu, G., Li, S., Wang, M., Song, J., Wang, J., et al. (2011). Role of a type IV-like secretion system of Streptococcus suis 2 in the development of streptococcal toxic shock syndrome. J. Infect. Dis. 204, 274–281. doi: 10.1093/infdis/jir261

Zheng, C., Ren, S., Xu, J., Zhao, X., Shi, G., Wu, J., et al. (2016). Contribution of NADH oxidase to oxidative stress tolerance and virulence of Streptococcus suis serotype 2. Virulence. doi: 10.1080/21505594.2016.1201256. [Epub ahead of print].

Zheng, F., Ji, H., Cao, M., Wang, C., Feng, Y., Li, M., et al. (2011). Contribution of the Rgg transcription regulator to metabolism and virulence of Streptococcus suis serotype 2. Infect. Immun. 79, 1319–1328. doi: 10.1128/IAI.00193-10

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

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