The non-conserved region of MRP is involved in the virulence of *Streptococcus suis* serotype 2

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**ABSTRACT**

Muramidase-released protein (MRP) of *Streptococcus suis* serotype 2 (SS2) is an important epidemic virulence marker with an unclear role in bacterial infection. To investigate the biologic functions of MRP, 3 mutants named Δmrp, Δmrp domain 1 (Δmrp-d1), and Δmrp domain 2 (Δmrp-d2) were constructed to assess the phenotypic changes between the parental strain and the mutant strains. The results indicated that MRP domain 1 (MRP-D1, the non-conserved region of MRP from a virulent strain, a.a. 242–596) played a critical role in adherence of SS2 to host cells, compared with MRP domain 1/C3 (MRP-D1/C3, the non-conserved region of MRP from a low virulent strain, a.a. 239–598) or MRP domain 2 (MRP-D2, the conserved region of MRP, a.a. 848–1222). We found that MRP-D1 but not MRP-D2 could bind specifically to fibronectin (FN), factor H (FH), fibrinogen (FG), and immunoglobulin G (IgG). Additionally, we confirmed that *mrp-d1* mutation significantly inhibited bacteremia and brain invasion in a mouse infection model. The *mrp-d1* mutation also attenuated the intracellular survival of SS2 in RAW264.7 macrophages, shortened the growth ability in pig blood and decreased the virulence of SS2 in BALB/c mice. Furthermore, antiserum against MRP-D1 was found to dramatically impede SS2 survival in pig blood. Finally, immunization with recombinant MRP-D1 efficiently enhanced murine viability after SS2 challenge, indicating its potential use in vaccination strategies. Collectively, these results indicated that MRP-D1 is involved in SS2 virulence and eloquently demonstrate the function of MRP in pathogenesis of infection.

**KEYWORDS**

infection; muramidase-released protein; *Streptococcus suis* serotype 2; vaccine; virulence

**Introduction**

*Streptococcus suis* (SS) is an emerging pathogen that can cause severe invasive infections in both pigs and humans. Septicemia and meningitis are the most common clinical features associated with SS2 infection. It is also a potential public health threat through zoonotic transmission and is responsible for significant economic losses to the porcine industry worldwide. Among the 33 serotypes, SS serotype 2 (SS2) is considered to be the most virulent zoonotic agent. However, the molecular pathogenesis of infection with this serotype remains unclear. Thus far, the virulence factors of SS remain poorly understood and proposed virulence factors, such as muramidase-released protein (MRP), extracellular protein factor (EF) and suilysin (SLY), do not necessarily define the virulence of SS strains. Although some virulence markers (MRP and EF) have been suggested by some researchers, once a SS strain is isolated, it is difficult to predict whether the strain is virulent in vivo. Over the past decade, significant progress has provided valuable insights into SS virulence factors through the use of isogenic mutants. Unfortunately, there is still no universal virulence marker that can be used to assess virulence for all SS strains. Therefore, current putative virulence factors need to be studied in more detail.

MRP is a 136-kDa cell wall-anchored surface protein that was discovered as an important virulence factor released into culture supernatant from virulent SS2 strains after muramidase treatment. A recent study also indicated that MRP was a major fibrinogen-binding protein. The binding of MRP with human fibrinogen increases the viability of SS2 in human blood and promotes the development of SS2 meningitis. However, in North America, a MRP- strain was shown to be virulent, while a MRP+ strain was reported to be avirulent. Therefore, MRP is currently considered to be a virulence marker but not an essential virulence factor for SS2 and...
it is possible that MRP- SS2 strains are less virulent than MRP+ SS2 strains. In this study, we aimed to explore the function of MRP in pathogenicity to augment our understanding of SS2 infection.

Infection of the host by microbial pathogens is a consequence of successful adherence and invasion of host cells, intracellular multiplication, colonization, and dissemination of host tissues, or persistence. In this study, we found that MRP has high amino acid sequence similarity in 8 highly virulent SS2 strains. Similar to the results of the virulent strains, the sequence alignment of MRP is extremely conserved among 4 low virulence SS2 strains. However, amino acid sequence analysis showed that MRP protein has a variable region that differs between highly virulent (designated MRP-D1, a.a. 242–596) and low virulent (designated MRP-D1*, a.a. 239–598) strains. Herein, we found that MRP-D1 can bind specifically to human fibronectin (FN), factor H (FH), fibrinogen (FG) and pig immunoglobulin G (IgG), whereas MRP-D2 failed to bind to all of these host components. Both indirect immunofluorescence assays and pull-down assays confirmed that MRP-D1 but not MRP-D1* or MRP-D2, plays a critical role in the adherence of SS2 to host cells. Previous research indicated that FN-binding proteins usually contribute significantly to the pathogenesis of SS infection and FG-binding proteins often serve as virulence factors that contribute to evasion of innate immunity. Previous studies also demonstrated that FH-binding proteins play critical roles in the ability of the pathogenic bacteria to avoid complement attack resulting in host invasion. Therefore, we concluded that domain 1 may be a major host protein-binding region of the MRP protein, which may contribute to the virulence and pathogenesis of SS2 during infection. Further study indicated that inactivation of mpr-d1 shortened pathogen survival in pig blood, decreased intracellular survival and adherence ability of SS2 in vitro. Mutation of mpr-d1 significantly reduced the pathogenicity of SS2 in an experimental BALB/c mouse model. Immunization experiments suggested that the survival rate of mice immunized with recombinant MRP-D1 was significantly higher compared with recombinant MRP-D2. Taken together, these results suggested that MRP-D1 could be an important virulence marker and improved our understanding of MRP biologic function in the pathogenesis of SS2 infection.

Results

Sequence analysis of SS2 MRP proteins

Multiple sequence alignments show that the amino acid sequences of MRP are fairly conserved among the 8 highly virulent SS2 strains (Fig. S1A). Similar to the results of the virulent strains, multiple alignment results of MRP sequences revealed 99.98% identity with 4 low virulent SS2 strains (Fig. S1B). Of particular note, the N-terminal portion of the MRP protein has a variable region that differs between highly virulent (MRP-D1, a.a. 242–596) and low virulent (MRP-D1*, a.a. 239–598) strains, whereas the C-terminal portion of the MRP protein is extremely conserved (Fig. 1A and B).

Confirmation and characterization of mrp, mpr-d1, and mpr-d2 knockout mutant strains

Amino acid sequence analysis showed that MRP has a non-conserved region that differs between both high and low virulent strains. It is possible that the variable region of MRP is involved in SS2 virulence. To determine whether MRP-D1 contributes to the virulence of SS2, 3 knockout mutants (Δmrp, Δmpr-d1, and Δmpr-d2) were designed mainly according to the MRP sequence analysis (Fig. 2A). The obtained Δmrp, Δmpr-d1, and Δmpr-d2 strains were confirmed by combined PCR analysis using 3 pairs of primers (Fig. 2B). There were no fragments amplified by PCR in mutant strains with internal primers (E/F), while the flanking primers (A/D) and external primers (X/Y) amplified smaller fragments from the mutant strain templates compared with those amplified from the WT template (Fig. 2B). Western blotting analysis verified that the expression of MRP was detected in the WT, Δmpr-d1, and Δmpr-d2 strains but was not detected in the Δmrp strain (Fig. 2C). Molecular weights of MRP from the Δmpr-d1 and Δmpr-d2 strains were smaller than that of the WT strain (Fig. 2C). These results suggested that Δmrp, Δmpr-d1, and Δmpr-d2 strains were successfully constructed.

We next evaluated the effect of inactive Δmrp, Δmpr-d1, and Δmpr-d2 mutants on the basic growth characteristics of SS2. The growth curves indicated that there was no significant growth differences between the WT strain and the 3 mutant strains (Fig. 2D). Hence, the growth characteristics should not affect subsequent evaluation of SS2 virulence.

MRP-D1 plays a critical role in the adherence of SS2 to host cells

Successful adherence to host cells is a prerequisite to infection of the host by microbial pathogens. To explore the contribution of MRP to the adherence of SS2, an indirect immunofluorescence assay was performed to assess whether recombinant MRP-D1, MRP-D1*, and MRP-D2 could bind to HEp-2 cells and bEnd.3 cells. As shown in Fig. 3A and B, an obvious green fluorescent signal was observed from the cell surface pre-incubated with recombinant MRP-D1 and MRP-D2,
while a weak green fluorescence was observed from cells pre-incubated with recombinant MRP-D1\(^*\). However, positive fluorescence was not detected from the unrelated his-tagged protein (recombinant HP07325), the negative control (BSA) or the blank control (without primary antibody). Qualitatively, we next evaluated the binding of MRP-D1, MRP-D1\(^*\), and MRP-D2 to HEp-2 cells using a pull-down assay. Recombinant HP07325 and BSA were served as negative controls. HEp-2 cells were collected and incubated with purified recombinant proteins or BSA for 2 h. Then, the cells were washed twice in PBS and separated by SDS-PAGE. The Western blotting was probed with his tag monoclonal antibody. As expected, both MRP-D1 and MRP-D2 were pulled down by the HEp-2 cells. Notably, MRP-D1 plays a critical role in MRP adherence ability of SS2, compared with MRP-D2 (Fig. 3C). Neither MPR-D1\(^*\) nor HP07325 could bind the HEp-2 cells (Fig. 3C). As a positive control, specific response of purified recombinant proteins was detected by Western blotting analysis using an anti-His tagged monoclonal antibody (Fig. 3D). Under similar assay conditions, no specific band was observed in the negative control (BSA). Pull-down assays demonstrated that MRP-D1 plays a critical role in the adherence of SS2 to HEp-2 cells, corroborating the results obtained in the indirect immunofluorescence assay. To further investigate the role of MRP adherence ability, we compared adherence of the WT strain and the mutant strains to HEp-2 cells. The results indicated that the adherence rate of the WT strain to HEp-2 cells was significantly higher than that of the 3 mutant strains (Fig. 3E). These results confirmed that both domains 1 and 2 contribute to MRP adherence to host cells and that domain 1 of MRP plays a major role in the adherence ability of SS2.

The FN/FH/FG/IgG-binding region is located in domain 1 of the MRP protein

Surface proteins of bacterial pathogens can facilitate colonization, invasion and evasion of host innate immune defenses through binding to host component factors.\(^{18,21,22}\) To determine whether host components are receptors of MRP, Far-Western blotting analysis of recombinant MRP-D1, MRP-D1\(^*\), and MRP-D2 to human FN, FH, FG and pig IgG was performed as described previously.\(^{16}\) After purification by nickel-chelating chromatography, SDS-PAGE analysis indicated that the recombinant MRP-D1, MRP-D1\(^*\), and MRP-D2 proteins were successfully purified (Fig. 4A). Our results confirmed that both MRP-D1 and MRP-D1\(^*\) can specifically interact with human FN (Fig. 4B), FH (Fig. 4C), FG (Fig. 4D) and pig IgG (Fig. 4E), whereas MRP-D2 did not bind to any of these. On the other hand, the binding ability of MRP-D1 to host components was stronger than MRP-D1\(^*\). The pET-32a series is designed for cloning and high-level expression of peptide sequences fused with the 109 aa Trx-Tag thioredoxin protein. We have

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**Figure 1.** Multiple sequence alignments of SS2 MRP. ((A)and B) Multiple sequence alignments of the 12 SS2 MRP proteins at the amino acid level. All the sequence analyses were performed by DNAMAN software. MRP has a variable region that differs between highly virulent and low virulent strains and the C-terminus of MRP protein is extremely conserved, whereas the N-terminus differs considerably.
Figure 2. Construction of Δmrp, Δmrp-d1, and Δmrp-d2 strains and growth curves of the WT and 3 mutant strains. (A) Schematic representation of the 3 knockout mutants and 3 recombinant proteins used in this study. The constructs were designed mainly according to the multiple sequence alignments of SS2 MRP. S: signal sequence; LPxTG: cell wall anchoring motif; Black arrows denote the truncated forms of MRP. (B) PCR confirmation of the knockout mutant strains. Three different primer sets, including internal (E/F), flanking (A/D), and external (X/Y) regions used in the PCR analysis are indicated above the lanes. Genomic DNAs from the WT and mutant strains were used as templates. (C) Western blotting confirmation of the mutant strains. The released MRP in the extracellular proteins of WT and mutant strains were probed with anti-MRP-D1 antibodies or anti-MRP-D2 antibodies. Extracellular protein samples of culture supernatant were prepared as described previously. Western blotting analysis confirmed that the expression of MRP was detected in the WT, Δmrp-d1, and Δmrp-d2 strains but was not detected in the Δmrp strain. (D) Growth characteristics of WT and mutant strains using OD600 values.
confirmed that the 109 aa Trx-Tag thioredoxin part of the fusion proteins does not interfere with the binding properties of recombinant MRP-D1* and MRP-D2 (data not shown). This suggested that MRP-D1 is an important host protein-binding domain of MRP, which may contribute to the virulence and pathogenesis of SS2 by binding to host components during infection.

**MRP-D1 contributes to SS2 survival in RAW246.7 macrophages**

To determine whether MRP-D1 plays an important role in the virulence of SS2, we evaluated the WT and mutant strains in RAW264.7 macrophage-mediated phagocytosis and killing assays *in vitro*. After 1 h of phagocytosis, uptake of the WT strain by RAW264.7 macrophages was not significantly different to any of the mutant strains (Fig. 5A). As shown in Fig. 5B, the intracellular survival of the WT and 3 mutant strains in RAW264.7 macrophages was attenuated as time progressed. However, the survival rate of the \( \Delta \text{mrp} \) and \( \Delta \text{mrp-d1} \) strains was significantly lower than that of the \( \Delta \text{mrp-d2} \) and WT strains (Fig. 5B). The data showed that inactivation of \( \text{mrp-d1} \) attenuated the virulence of SS2 and decreased the intracellular survival ability of the bacteria in RAW264.7 macrophages *in vitro*.

**MRP-D1 contributes to SS2 virulence in a mouse infection model**

The aforementioned results suggested that the \( \text{mrp-d1} \) mutation inhibited intracellular survival ability of SS2.
To evaluate the role of \( \text{mrp-d1} \) in \( \text{SS2} \) \textit{in vivo} infection, we compared the virulence of the WT, \( \Delta \text{mrp} \), \( \Delta \text{mrp-d1} \), and \( \Delta \text{mrp-d2} \) strains in BALB/c mice. Firstly, we investigated the colonization efficiency of the WT and 3 mutant strains in blood and brain tissue of infected mice. Four groups of BALB/c mice received intraperitoneal challenges with a dose of \( 2 \times 10^7 \) CFU WT or mutant SS2. The infected mice from each group were selected for euthanasia and dissection at 24 h and 48 h post-infection. Strikingly, bacterial counts of the \( \Delta \text{mrp-d1} \) strain in

\[ \text{Figure 4. Assessment of the binding region of MRP to host components (FN, FH, FG, and IgG) by Far-western blotting. Recombinant MRP-D1, MRP-D1^*, and MRP-D2 were separated by SDS-PAGE and stained with Coomassie blue (A) or transferred to PVDF membranes and then incubated with FN (B), FH (C), FG (D), or IgG (E). Bound host components were detected using primary antibodies or HRP-conjugated secondary antibodies, respectively.} \]

\[ \text{Figure 5. Effect of WT and mutant strains on phagocytosis and killing of SS2 by RAW246.7 macrophages. (A) Phagocytosis analysis of different SS2 strains by macrophages. The uptake of bacteria by macrophages was monitored for 1 h. At the 0 h time point, antibiotics were added to kill bacteria extracellular to macrophages. Intracellular bacteria in RAW246.7 macrophages were determined by quantitative plating at the 1 h time point. (B) Intracellular survival analysis of SS2 strains at different time points. The numbers of intracellular bacteria were recovered from cell lysates at different time points. The initial number of viable intracellular bacteria at 1 h was designated as 100%. The survival rate of the \( \Delta \text{mrp} \) and \( \Delta \text{mrp-d1} \) strains was significantly lower than that of the \( \Delta \text{mrp-d2} \) and WT strains. Data were expressed as the mean \( \pm \) SEM of 3 independent experiments performed in triplicate. Significant differences are indicated (***p < 0.001; **p < 0.01; *p < 0.05).} \]
blood (Fig. 6A) and brain tissue (Fig. 6B) were significantly lower than other strains at 24 h and 48 h, which indicated that the \textit{mrp-d1} mutation enhanced microbial clearance in blood and brain tissue of the infected mice.

To determine the role of MRP-D1 in SS2 infection, we performed bactericidal assays to compare the growth of the WT, \(\Delta\text{mrp}\), \(\Delta\text{mrp-d1}\), and \(\Delta\text{mrp-d2}\) strains in pig blood. As expected, the survival rate of the \(\Delta\text{mrp}\) and \(\Delta\text{mrp-d1}\) strains was significantly attenuated compared with the WT and \(\Delta\text{mrp-d2}\) strains (Fig. 6C). The results suggest that MRP-D1 contributes to the survival of SS2 in pig blood.

We then evaluated the virulence of the WT, \(\Delta\text{mrp}\), \(\Delta\text{mrp-d1}\), and \(\Delta\text{mrp-d2}\) strains in precipitating mouse mortality. Murine infection assays showed that many of the typical clinical symptoms, including rough coat hair, shivering, limping, lethargy, and swollen eyes could be observed in all mice infected with the WT strain; consequently 70% of these mice died within 2 d of infection. In contrast to the \(\Delta\text{mrp-d2}\) strain, 60% the mice died within the same period. However, most of the mice infected with the \(\Delta\text{mrp-d1}\) and \(\Delta\text{mrp}\) strains survived until the end of the experiment. All mice injected with PBS survived the entire experimental period. As shown in Fig. 6D, the survival rate of mice was dramatically higher in mice infected with the \(\Delta\text{mrp-d1}\) strain compared with the WT strain. Thus, we concluded that inactivation of \textit{mrp-d1} attenuated the virulence of SS2 in a mouse model.

Taken together, the \textit{in vivo} assays assessing colonization efficiency in mouse tissues, bactericidal assays in pig blood, and the survival rates of a mouse infection model confirmed that MRP-D1 is involved in the virulence profile of SS2.

**Figure 6.** Effect of WT and mutant strains on the virulence of SS2. ((A)and B) The elimination of SS2 in mice blood and brain tissue in infection of BALB/c mice. The WT and mutant strains were separately inoculated into mice at \(2 \times 10^7\) CFU/mouse. The asterisk suggested that the bacterial counts of the \(\Delta\text{mrp-d1}\) strain in blood and brain tissue were significantly lower than other strains at 24 h and 48 h time points. Data are expressed as the mean \(\pm\) SEM of 5 infected mice per time point. (C) MRP-D1 promotes the survival of SS2 in pig blood at the measured time points (1 h and 2 h). The survival rate of the \(\Delta\text{mrp}\) and \(\Delta\text{mrp-d1}\) strains was significantly attenuated compared with the WT and \(\Delta\text{mrp-d2}\) strains. Significant differences are indicated ("**" \(p < 0.001\); "*" \(p < 0.01\); "p < 0.05"). (D) Comparative analysis of bacterial virulence of the WT strain with the mutant strains using a mouse model. The WT and mutant strains were separately inoculated into mice at \(2 \times 10^8\) CFU/mouse. Mice challenged with the \(\Delta\text{mrp-d1}\) strain had a higher survival rate than those challenged with the \(\Delta\text{mrp-d2}\) strain or WT strain, indicating that deletion of \textit{mrp-d1} dramatically attenuated the virulence of SS2.
MRP-D1 confers partial protection against SS2 infection

We next evaluated whether administration of recombinant MRP-D1 and MRP-D2 could protect mice against infection with SS2. Four groups of ICR mice were immunized 3 times with MRP-D1 and MRP-D2, and then challenged with the bacteria. Firstly, specific IgG titers against recombinant MRP-D1 and MRP-D2 were evaluated in mice sera on the seventh day after the last injection. The results indicated that antibody titers against recombinant MRP-D1 and MRP-D2 from the immunized group were significantly higher compared with the blank control (PBS) or negative control (adjuvant only) groups (Fig. 7A). Ten days after the third booster, all mice received intraperitoneal challenge with SS2 virulent strain ZY05719. As shown in Fig. 7B, all of the negative and blank control mice died within 48 h after challenge with ZY05719, while immunization with recombinant MRP-D1 or MRP-D2 conferred 50% protection and 12.5% protection to mice, respectively. The results suggested that MRP-D1 confers partial protection in mice.

To further determine the bactericidal activity of anti-MRP-D1/MRP-D2 antibodies, a killing assay was used as described previously using pig blood. As shown in Fig. 7C, the survival rate of SS2 pretreated with
anti-MRP-D2 sera in pig blood was not significantly different compared with that with preimmune sera. In contrast, pre-incubation of SS2 with anti-MRP-D1 sera significantly decreased the survival of SS2 in whole blood, corroborating the results obtained in the vaccine experiment.

In summary, the vaccine protection assays in mice and the bactericidal assays in pig blood confirmed that MRP-D1 could confer protection against SS2 infection.

Discussion

SS is an important zoonotic pathogen that can cause severe and invasive infections in the swine industry worldwide.3 It is also a prominent infectious disease in humans, with deadly outbreaks in several Asian countries.24,25 Over the past decade, significant progress has been made in improving our understanding of the virulence-associated factors of SS infection. Thus far, over 40 confirmed/putative virulence factors have been reported that include capsular polysaccharides (CPS), MRP, EF, SLY, adhesins, and enzymes.3,4 However, the majority of these have not yet been clearly characterized, and most of the identified virulence factors are only considered ‘putative’ virulence factors.3,26 For example, SS strains possessing some of the proposed virulence factors were not necessarily virulent. Despite numerous articles being published in the past decade, the role of virulence factors in the pathogenesis of SS infection remains unclear.26 Therefore, more studies are necessary to investigate the differences in virulence of SS strains.

MRP is often considered as a virulence marker in SS2 strains in European and Asian clinical isolates.27,28 Many studies have indicated that MRP is a virulence marker but not an essential virulence factor for SS2.5,9–11,29,30 Whether MRP is a critical SS2 virulence factor remains to be determined because the roles of MRP have not yet been well characterized. Therefore, exploring the function of MRP in SS pathogenicity will expand our understanding of this infection.

A previous study revealed that the 136-kDa MRP could be detected in SS serotypes 1, 2, 1/2, 14, and 15.30 The larger and smaller size variations of MRP were observed among SS of almost all serotypes.30,31 Fittipaldi et al. had reported that MRP has a variable region with 50–60% homology to 92 sequenced SS strains.32 Similar to the results of this study, we observed that MRP contains a non-conserved region between the highly virulent and low virulent SS2 strains. We assume that the non-conserved region of MRP could be involved in the virulence of SS2 infection. Bacterial adhesion to host epithelial cells is usually associated with the necessary early steps in the process of infection.3,20 MRP is a cell wall-anchored surface protein containing an LPxTG motif at the C-terminal region. Our results showed that MRP contributes to the adherence of SS2 to HEp-2 cells. We compared the results obtained with MRP-D1 from the highly virulent strain ZY05719 with MRP-D1* from the low virulent strain SS26035. Both indirect immunofluorescence assays and pull-down assays confirmed that MRP-D1, rather than MRP-D1*, plays a critical role in the adherence of SS2 to host cells, which may contribute to the virulence of SS2 during infection.

Previous studies have demonstrated that SS surface proteins can bind different host components of human extracellular matrix or serum proteins, including FN,13–16 FH,19,34 FG,8 and IgG.35,36 These proteins play various roles in the adhesion, invasion, physiology, and immune escape of the bacteria via different mechanisms, which cooperatively contribute to the full virulence of SS.37 As we expected, the binding ability of MRP-D1 to host components including FN, FH, FG, and IgG was stronger compared with MRP-D1*. All the results indicated that the non-conserved region of MRP (MRP-D1/MPR-D1*) is involved in the virulence and pathogenesis of SS2 during infection. The non-conserved region of MRP-D1 from highly virulent strains might be a major host protein-binding domain of MRP, which may contribute to the pathogenicity of SS2 through binding to host components during infection. Of particular note, the FN-binding activity of MRP was not detected by Far-western blotting in our previous study.16 Similar to these data, MRP was also not identified by Smith et al.,6 probably due to its low abundance in cell wall samples. Furthermore, our previous study showed that a recombinant part of MRP (a.a. 860–1227) that was nearly identical to MRP-D2 (a.a. 848–1222), does not bind to FN. In contrast, recombinant MRP-D1 (a.a. 242–596) bound specifically to FN, which suggested that MRP-D1 is a critical FN-binding region of the MRP protein.

To probe whether MRP-D1 could independently affect the virulence of SS2, 3 knockout mutants (Δmrp, Δmrp-d1, and Δmrp-d2) were constructed. The intracellular survival of bacteria in RAW246.7 macrophages should be a major event associated with SS2 virulence. Our results demonstrated that the mrp-d1 mutation significantly decreased the intracellular survival of SS2 in RAW246.7 macrophages. Survival ability in the different tissues or in the bloodstream is also considered to be a major factor in the pathogenesis of SS2 infection.38,39 As expected, the survival rate of the Δmrp and Δmrp-d1 strains was significantly lower than that of the Δmrp-d2 and WT strains in pig blood. Furthermore, the survival rate was significantly higher of mice injected with the Δmrp-d1 strain compared with the WT strain ZY05719. Given these results, we concluded that MRP-D1 is involved in full virulence of SS2.
To further evaluate the role of \textit{mrp-d1} in \textit{SS2} \textit{in vivo} infection, a mouse infection model was generated. Strikingly, deletion of \textit{mrp-d1} enhanced bacterial clearance in blood and brain tissue of infected mice but deletion of the full length \textit{mrp} is not strongly associated with clearance in blood and brain tissue. This observation is difficult to understand. To improve our understanding of the structures and biologic functions of MRP, future research will consider the following 3 aspects: 1) \textit{in vivo} assays in pigs will be performed; 2) an exchange of the non-conserved regions between high and low virulent strains will be investigated; 3) we plan to resolve the 3D crystal structures of MRP and/or its domains by using X-ray crystallography of the protein.

Immunogenic surface proteins are considered to be potential candidates for vaccine development. MRP is a cell wall-anchored surface protein and is highly immunoreactive to convalescent sera from pigs, which suggests that MRP is expressed \textit{in vivo} during infection. In the present study, we confirmed that both domains 1 and 2 of MRP could elicit strong immune responses in mice. Herein, the protection assays in mice and the bactericidal assays in pig whole blood revealed that domain 1 of MRP could confer partial protection against SS2 infection.

In summary, both \textit{in vitro} and \textit{in vivo} results demonstrated that the presence of domain 1 in MRP could be an important virulence marker of SS2. These results expanded our understanding of the role of MRP in the pathogenesis of SS2 infection.

**Materials and methods**

**Bacterial strains, plasmids, and culture conditions**

The SS2 strain ZY05719, verified as a virulent strain, was originally isolated from a diseased pig during an outbreak in Ziyang, China. The bacteria were grown in Todd Hewitt Broth (THB; pH 7.8; BD) or agar medium at 37°C and collected by centrifugation at the late stage of exponential phase. DH5a and BL21 (DE3) strains of \textit{E. coli} were cultured in Luria-Bertani (LB) broth or on LB agar plates at 37°C. The pET-32a and pET-28a vectors were used for protein expression. A total of 100 μg/ml gentamicin and 5 μg/ml penicillin G (Sigma, USA) were used to kill extracellular bacteria. When required, appropriate antibiotics were used to screen transformants as follows: 100 μg/ml spectinomycin (Spc, Sigma) for SS; 100 μg/ml ampicillin (Amp, Sigma), 50 μg/ml kanamycin (Kan; Sigma), and 50 μg/ml spectinomycin for \textit{E. coli}. Bacterial strains and plasmids used in this study are listed in Table 1.

**Sequence analysis of MRP**

The amino acid sequences of MRP proteins of 8 virulent reference SS2 strains were retrieved from NCBI

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**Table 1.** Characteristics of the bacterial strains and plasmids used in this study.

| Strains or Plasmids | General Characteristics | Sources or References |
|---------------------|-------------------------|-----------------------|
| ZY05719             | Isolated from a diseased pig in Sichuan Province in China in 2005; Virulent SS2 strain | Stored in our lab |
| \(\Delta\text{mrp}\)  | Isogenic \textit{mrp} mutant of strain ZY05719 | This study |
| \(\Delta\text{mrp-d1}\) | Isogenic \textit{mrp-d1} mutant of strain ZY05719 | This study |
| \(\Delta\text{mrp-d2}\) | Isogenic \textit{mrp-d2} mutant of strain ZY05719 | This study |
| \textit{E. coli} DH5a | For cloning the recombinant plasmids | Invitrogen |
| \textit{E. coli} BL21 | For expression the recombinant plasmids | Invitrogen |
| HA9801              | Isolated from a diseased pig in Jiangsu Province of China; Virulent SS2 strain | Stored in our lab |
| 0SZYH33             | Virulent SS2 strain | 46 |
| A7                  | Virulent SS2 strain | 47 |
| GZ1                 | Virulent SS2 strain | 48 |
| P1/7                | Virulent SS2 strain | 49 |
| S735                | Virulent SS2 strain | 50 |
| SC84                | Virulent SS2 strain | 51 |
| ZJUX0908005         | Isolated from a healthy pig in Zhejiang Province of China; Low virulent SS2 strain | Stored in our lab |
| ZJUX0908008         | Isolated from a healthy pig in Zhejiang Province of China; Low virulent SS2 strain | Stored in our lab |
| S5,6035             | Unknown source; Low virulent SS2 strain | Stored in our lab |
| pET28a-MRP-D1       | A recombinant expression vector containing \textit{mrp-d1}; Kan\(^b\) | This study |
| pET32a-MRP-D2       | A recombinant expression vector containing \textit{mrp-d2}; Amp\(^b\) | This study |
| pET32a-MRP-D1\(^c\) | A recombinant expression vector containing \textit{mrp-d1}; Amp\(^b\) | This study |
| pET32a-hp07325      | A recombinant expression vector containing \textit{hp07325}; Amp\(^b\) | This study |
| PMD19-T             | Cloning vector; Amp\(^b\) | Takara |
| pSET4s              | S. suis thermosensitive suicide vector; Spc\(^b\) | This study |
| \textit{mp-d1-4s}   | A recombinant vector with the background of pSET4s, designed to knockout \textit{mrp}; Spc\(^b\) | This study |
| \textit{mp-d2-4s}   | A recombinant vector with the background of pSET4s, designed to knockout \textit{mrp-d2}; Spc\(^b\) | This study |

\(^a\) Kan\(^b\), kanamycin resistance; Amp\(^b\), Ampicillin resistance; Spc\(^b\), spectinomycin resistance.

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Note. \(\triangle\text{mrp}\), \textit{mrp} deletion; \(\triangle\text{mrp-d1}\), \textit{mrp-d1} deletion; \(\triangle\text{mrp-d2}\), \textit{mrp-d2} deletion; Kan\(^b\), kanamycin resistance; Amp\(^b\), ampicillin resistance; Spc\(^b\), spectinomycin resistance.
databases (https://www.ncbi.nlm.nih.gov/), including ZY05719 (ABG91742.1), HA9801 (ABG91743.1), 05ZHY33 (ABP89719.1), A7 (AER44031.1), GZ1 (ADE31200.1), P1/7 (CAR45511.1), S735 (AFR00193.1), and SC84 (CAZ51449.1). The complete genomes of 4 low virulence SS2 strains, including ZJX0908005, HN01104001, ZJX0908008, and SS6035 were sequenced by our laboratory. The virulence of these 4 SS2 strains has been tested in a previous study. Multiple alignment analyses of these 12 MRP proteins were performed using DNAMAN software.

**Construction of mutant strains**

The Δmrp, Δmrp-d1, and Δmrp-d2 strains were constructed according to our previous report. Primers for the amplification of DNA fragments corresponding to the upstream (A/B) and downstream regions (C/D) of the target genes are listed in Table 2. Briefly, the 2 flanking regions were fused as an intact fragment by overlap-extension PCR using 3 pairs of primers (A/D) and cloned into vector PMD19-T. The correct fusion of the flanking fragment was verified by sequencing. The resulting fragment was cloned into the temperature-sensitive SS-E. coli shuttle vector pSET4s to generate recombinant knockout vectors. The single-crossover mutants were obtained by culturing the cells at 37°C on Todd-Hewitt agar (THA) with spectinomycin, while the double-crossover mutants were screened by repeated passaging on THA at 28°C without spectinomycin. The mutation of the 3 target genes was confirmed by PCR with 3 different primer sets for flanking (A/D), internal (E/F) and external (X/Y) regions, and by sequencing (A/D).

**Expression and purification of recombinant proteins and preparation of antiserum**

To express recombinant MRP-D1 (the non-conserved region of MRP, a.a. 242–596) and MRP-D2 (the conserved region of MRP, a.a. 848–1222) in E. coli, the genomic DNA of ZY05719 was used for PCR with the 2 pairs of primers described in Table 2 and Phanta HS Super-Fidelity DNA Polymerase (Vazyme). The recombinant MRP domain 1° low virulence strain SS6035 (MRP-

### Table 2. Primers used for PCR amplification and detection.

| Primers         | Sequences (5’–3’)     | Function                        | Length (bp) and PCR temperature | Restriction enzyme |
|-----------------|-----------------------|---------------------------------|----------------------------------|--------------------|
| mrp-A           | CGCCGATCCGCTTGAAGAATTCGAGACGAGAC | Upstream flanking regions of mrp | 702 bp; 50°C | BamHI |
| mrp-B           | CCCCAATTTAGCTTGGTACGAG | Downstream flanking regions of mrp | 669 bp; 54°C | SmaI |
| mrp-C           | ATCTGTAAGATCCTTTGCTTGGTACGAG | Internal regions of mrp | 630 bp; 51°C | EcoRI |
| mrp-D           | TCCGCCGCTCAATTGCTGATCGAG | External regions of mrp | 5247 bp or 1641 bp; 54°C | XhoI |
| mrp-E           | GCATTGAAATTCTCTGCTAAT | Upstream flanking regions of mrp-d1 | 657 bp; 55°C | BamHI |
| mrp-F           | GCATTGAAATTCTCTGCTAAT | Downstream flanking regions of mrp-d1 | 711 bp; 51°C | EcoRI |
| mrp-G           | CCGGATCTCATATCCTTTGCTTGGTACGAG | Internal regions of mrp-d2 | 630 bp; 51°C | XhoI |
| mrp-H           | CCGGATCTCATATCCTTTGCTTGGTACGAG | External regions of mrp-d2 | 2571 bp or 1506 bp; 51°C | EcoRI |
| mrp-I           | CCAATTTTGGTCTGTTGAA | Internal regions of mrp-d2 | 714 bp; 52°C | EcoRI |
| mrp-J           | TACATTGGACCTTCTGACGATGAG | External regions of mrp-d2 | 732 bp; 53°C | EcoRI |
| mrp-K           | CCGGATCTCATATCCTTTGCTTGGTACGAG | Internal regions of mrp-d2 | 963 bp; 53°C | EcoRI |
| mrp-L           | CCGGATCTCATATCCTTTGCTTGGTACGAG | External regions of mrp-d2 | 2994 bp or 1869 bp; 54°C | EcoRI |
| mrp-M           | CGCCGATCCGCTTGAAGAATTCGAGACGAGAC | The ORF of MRP-D1 | 1065 bp; 49°C | BamHI |
| mrp-N           | CGCCGATCCGCTTGAAGAATTCGAGACGAGAC | The ORF of MRP-D2 | 1125 bp; 52°C | BamHI |
| mrp-O           | CCGGATCTCATATCCTTTGCTTGGTACGAG | The ORF of MRP-D1 | 1080 bp; 52°C | EcoRI |
| mrp-P           | CCGGATCTCATATCCTTTGCTTGGTACGAG | The ORF of MRP-D2 | 459 bp; 51°C | XhoI |
| mrp-Q           | CCGGATCTCATATCCTTTGCTTGGTACGAG | The ORF of MRP-D2 | 459 bp; 51°C | XhoI |

Note: Underlined nucleotides denote enzyme restriction sites.
D1*, a.a. 239–598) and recombinant HP07325 of ZY05719 were expressed as controls. The expression experiment was performed as described previously.16 PCR products were cloned into the pET-32a or pET-28a vectors and transformed into BL21 (DE3) for expression. The cloned sequences were verified by DNA sequencing. Protein purification was performed using nickel-chelating chromatography (GE Healthcare), according to the instruction manual.

The antiserum for recombinant MRP-D1 and MRP-D2 was generated as described previously.16 The purified recombinant proteins were emulsified with Freund’s complete adjuvant (FCA) and injected subcutaneously into rabbits. After 2 weeks, the rabbits were booster-immunized with the same antigen concentration emulsified with Freund’s incomplete adjuvant (FIA). Ten days after the second immunization, serum samples from the rabbits were collected.

Indirect immunofluorescence assays

To evaluate whether recombinant MRP-D1, MRP-D1*, MRP-D2 and HP07325 could specifically adhere to the human laryngeal epithelial cell line HEp-2 and the mouse brain microvascular endothelial cell line bEnd.3, immunofluorescence assays were performed as described previously.16 Briefly, the cells in 24-well plate cells were treated as the customary procedure: coated with 2% BSA, washed twice with PBS, and fixed for 20 min with cold methanol at −20°C. The fixed cells were incubated with 100 μg/ml of purified recombinant proteins or BSA (negative control) for 1 h at 37°C, and then incubated with the His tag monoclonal antibody (Boster; 1:1,000 dilution) and goat anti-mouse IgG-FITC (Boster; 1:2,000 dilution) at 37°C for 30 min, respectively. The samples without the primary antibody were used as a blank control. After a final wash, the fixed cells were examined using a fluorescence microscope (ZEISS, Germany). We acquired the images using an emission wavelength of 495 nm and a detection wavelength of 525 nm.

Bacterial adherence assays

Adherence assays of wild-type (WT), Δmrp, Δmrp-d1, and Δmrp-d2 strains to HEp-2 cells were performed as described previously.41 Briefly, SS2 strains including WT and the mutant strains were harvested by centrifugation when the OD600 was between 0.6–0.8, washed twice in PBS, and resuspended in DMEM culture medium. HEp-2 cells were seeded into 24-well cell plates and cultivated overnight in DMEM culture medium with 10% fetal bovine serum (FBS) (Gibco). The cell monolayers were washed 3 times with PBS, and then the bacteria (WT, Δmrp, Δmrp-d1 and Δmrp-d2) were added to the plates at a ratio of 50:1. Inoculated wells were centrifuged at 800 × g for 10 min and then incubated at 37°C in 5% CO2 for 2 h. The plates were washed 5 times to remove unbound SS2 and treated with 100 μl lysis buffer containing 0.025% (v/v) Triton X-100 and 0.1% trypsin at 37°C for 10 min, followed by THB agar plating. Assays for each sample were performed in triplicate and repeated independently 5 times.

Analysis of recombinant proteins binding to human FN, FH, FG and pig IgG

The binding ability of the recombinant MRP-D1, MRP-D1*, and MRP-D2 to host proteins (FN, FH, FG and IgG) was performed by Far-western blotting according to our previous report.16 Recombinant MRP-D1, MRP-D1*, and MRP-D2 were separated by SDS-PAGE, and the gels were transferred to PVDF membranes (Merck Millipore) and then blocked with 5% (w/v) skimmed milk for 12 h at 4°C. Subsequently, the membrane was washed 3 times and incubated with human FN (10 μg/ml, Sigma), human FH (5 μg/ml, Hycult), human FG (10 μg/ml, Sigma) or pig IgG (10 μg/ml, Sigma) dissolved in TBST for 24 h at 4°C. After 3 washes, the membranes were incubated with primary antibodies (rabbit anti-human FN (Boster, 1:2,000), rabbit anti-human FH (Abcam, 1:2,000) or goat anti-human FG (Sigma, 1:2,000) polyclonal antibodies) followed by HRP conjugated goat anti-rabbit polyclonal antibodies (Boster, 1:2,000), HRP conjugated donkey anti-goat polyclonal antibodies (Boster, 1:2,000) or HRP conjugated goat anti-swine polyclonal antibodies (Boster, 1:2,000) at 37°C for 1 h. The positive bands were detected using 3,3’-diaminobenzidine.

HEp-2 cell pull-down assays

HEp-2 cell pull-down assays were performed as described previously with minor modification.33 Briefly, the cells were collected and washed twice with PBS, and then incubated with 100 μg/ml of purified recombinant MRP-D1, MRP-D1*, MRP-D2, HP07325 or BSA for 2 h at 37°C. The cells were harvested by centrifugation and washed twice in PBS. Then, the cell pellets were boiled and separated by SDS-PAGE, and the gels were transferred to PVDF membranes for Western blotting with an anti–His tagged monoclonal antibody as described above. The purified recombinant proteins and BSA for SDS-PAGE and Western blotting were used as controls.

Phagocytosis and intracellular survival assays

Phagocytosis and intracellular survival assays were performed as described previously.42
macrophages were cultivated overnight in DMEM culture medium with 10% FBS (Gibco). The cell monolayers were washed 3 times with PBS, and then the bacteria (WT, Δmrp, Δmrp-d1, and Δmrp-d2) were added to the plates at a ratio of 30:1. Inoculated wells were centrifuged at 800 × g for 10 min and then incubated at 37°C in 5% CO2 for 1 h. After 3 washes, DMEM culture medium containing 100 μg/ml gentamicin and 5 μg/ml penicillin G was added, when the time point is 0 h, to kill the bacteria outside of macrophages. After 1 h, 2 h, 3 h and 4 h of incubation, the plates were washed 3 times and treated with lysis buffer, followed by THB agar plating. The number of SS2 were assessed in each group at 1 h, 2 h, 3 h and 4 h. The initial number of viable intracellular bacteria at 1 h was considered to be 100%. Assays for each sample were performed in triplicate and repeated independently 3 times.

**Experimental infections using a mouse model**

All animal experiments were approved by the Department of Science and Technology of Jiangsu Province, China. The license number was SCXK (SU) 2012-0004. All experimental procedures conformed to institutional guidelines in accordance with international law. To examine the effects of deletion of mrp, mrp-d1 or mrp-d2 on the virulence of WT, specific pathogen free (SPF) BALB/c mice (female, 4-weeks-old) were challenged with the WT, Δmrp, Δmrp-d1 or Δmrp-d2 strains. Ninety BALB/c mice were randomly divided into 9 groups (10 mice/group). To investigate the survival rate of mice, 4 infection groups received intraperitoneal challenges with WT or 3 mutant strains (200 μl, 2 × 10⁸ CFU/mouse). Ten mice were injected with PBS as the control group (200 μl/mouse). Mice were monitored once a day for up to one week to determine survival rate.

To further evaluate the elimination of SS2 in mice blood and brain tissue, we inoculated 4 groups with 2 × 10⁵ CFU of the WT, Δmrp, Δmrp-d1 or Δmrp-d2 strains. The infected mice from each group were selected for euthanasia and dissection at 24 h and 48 h post-infection. Bacteria were isolated from blood and brain homogenates by plating diluted samples on THB agar. The number of bacteria colonizing the tissues of the mice was recorded as described previously.

**Vaccine protection assays**

Protection assays of recombinant MRP-D1 and MRP-D2 were performed as described previously. Thirty-two SPF ICR mice (female, 4-weeks-old) were randomly divided into 4 groups. Each mouse was immunized 3 times at 2-week intervals with purified recombinant proteins (50 μg) emulsified with ISA 206 adjuvant (Seppic, France). PBS emulsified with ISA 206 adjuvant served as a negative control, and mice were immunized with PBS alone as a blank control. Seven days after the third injection, the antibody titers were measured according to a previous study. Ten days after the third booster, all mice received intraperitoneal challenges with 1 × 10⁸ CFU of SS2 strain ZY05719 in 200 μl PBS. Mice were monitored once a day for one week to determine the survival rate.

**Bactericidal assays using pig blood**

To identify whether the antiserum of recombinant MRP-D1 and MRP-D2 influences the viability of WT strain ZY05719, bacterial growth was measured in pig blood. The bactericidal assays were performed as described previously. Fresh blood samples (800 μl) from different pigs were infected with 100 μl SS2 (2 × 10⁵ CFU/ml) and pre-incubated with 100 μl preimmune sera, anti-MRP-D1 sera, or anti-MRP-D2 sera. The bactericidal assays were also used to compare the growth of the WT, Δmrp, Δmrp-d1, and Δmrp-d2 strains in pig blood. Briefly, fresh blood samples (900 μl) from different pigs were infected with 100 μl SS2 (2 × 10⁵ CFU/ml) and then the mixtures were centrifuged at 37°C. Samples were taken at the measured time points, followed by THB agar plating and bacterial enumeration. The percentage of live SS2 bacteria was measured as (CFU on plate /CFU in original inoculum) × 100%.

**Statistical analysis**

The data were analyzed with GraphPad Prism 5 software using unpaired 2-tailed Student’s t test. For infection experiments, survival was analyzed with the log rank test. For all tests, differences with a P value < 0.05 were considered significant. All the experiments were independently repeated at least 3 times, and the data were expressed as the mean ± SEM.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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