The two-component regulatory system CiaRH contributes to the virulence of *Streptococcus suis* 2

Jinquan Li, Chen Tan, Yang Zhou, Shulin Fu, Linlin Hu, Jin Hu, Huanchun Chen, Weicheng Bei

**1. Introduction**

Among the known 33 serotypes, *Streptococcus suis* serotype 2 (*S. suis* 2) is considered a major swine pathogen and an important zoonotic disease causing a variety of life-threatening infections that include meningitis, arthritis, septicaemia and even sudden death in pigs and humans (Lun et al., 2007). *S. suis* infections cause great economic loss to the pig industry worldwide (Staats et al., 1997). In addition, reported outbreaks from different countries have raised considerable international concerns among public health professionals in recent years (Gottschalk et al., 2007; Yu et al., 2006).

Many results have implicated two-component regulatory systems (TCS) as playing an important role in the regulation of a variety of essential processes including cell-cycle progression, pathogenicity, and development. The CiaRH has been identified as the first two-component signal transduction system for *Streptococcus pneumoniae* and deletion of the CiaRH also contributed to a high degree of attenuation in a murine model. However, only the CiaRH was necessary for the process of nasopharyngeal colonization in nine pneumococcal two-component signal transduction systems examined (Kowalko and Sebert, 2008).

In *S. suis* 2, only the SalK/SalR (Li et al., 2008) and two orphan response regulators have been described (Pan et al., 2009). CiaRH was found to be highly conserved in all the sequenced strains of *S. suis* 2 by bioinformatics. However, functions of CiaRH in *S. suis* 2 have not been reported before. In this work, we investigated the role of CiaRH of *S. suis* 2 in virulence in vivo and in vitro by constructing CiaRH deleted gene mutations. The results demonstrate...
that the CiaRH is important for pathogenesis of this pathogen.

2. Materials and methods

2.1. Bacterial strains, primers and plasmids

The bacterial strains, primers and plasmids used in this study are described in Table 1.

2.2. Construction of the S. suis 2 ΔCiaRH deletion mutant

The procedures for selection of mutants by allelic exchange via double cross-over were described previously (Takamatsu et al., 2001a,b). The resultant mutant strain was verified by PCR (Fig. 1B) using two pair primers T1/T2 (flanking CiaRH), T3/T4 (between CiaRH) and direct DNA sequencing of the mutation sites using genomic DNA preparations.

2.3. Cell adherence assay and bactericidal assay

The cell adherence assay was performed as previously described (Segura and Gottschalk, 2002). Bacteria were collected, washed twice with PBS, and resuspended in RPMI 1640. Confluent monolayers of the human laryngeal cancer cell line Hep-2 and porcine iliac artery endothelial cell (PIEC) grown in 24-well cell culture plates were infected at a multiplicity of infection of 10 bacteria per cell. The bactericidal assay was also performed as previously described (Tsao et al., 2009).

2.4. Experimental infections of CD1 mice

In survival and mortality studies, 30 female CD1 (six-week-old mice, Beijing Vital River Laboratory Animal Co., Ltd. whose colonies were all introduced from Charles River Laboratories) mice were infected by intraperitoneal injection with 1 ml of either WT or the ΔCiaRH mutant at approximately 1.5 × 10⁶ CFU in TSB. TSB were used as controls. To determination of viable bacteria in blood, 6 CD1 mice were infected with two strains at approximately 1.5 × 10⁷ CFU respectively. At each designated time point, blood samples were collected from the tail vein and used to evaluate the bacterial load by plating onto TSA plates as described before (Fittipaldi et al., 2008). At day 10 post-challenge, all surviving mice were euthanized. All animal experiments were approved by the local ethical committee.

2.5. Experimental infections of piglets and colonization ability analysis

Prior to the infection studies, 30 high-health-status pigs (4–5 weeks) were tested negative by ELISA kit for S. suis (Wuhan Keqian biological products Co., Ltd., China). 18 piglets were divided into 3 groups (n = 6). Animals were inoculated by intravenous injection of 1 ml of 2 × 10⁵ CFU of WT strain, ΔCiaRH mutant or saline (sham inoculation), respectively. The infected-piglets were monitored for clinical signs, and their survival times were recorded. At day 14 post-challenge, all surviving pigs were euthanized. All animal experiments were approved by the local ethical committee. 12 piglets were used for colonization analysis to further distinguish virulence between the two strains. Piglets were divided into two groups (n = 6) and intravenously injected with the WT and ΔCiaRH at the dose of 2 × 10⁵ CFU. Animals were sacrificed at 24 h and tissues were weighed and homogenized in sterile PBS using a tissue homogenizer. 100 μl homogenates were serially diluted in PBS and plated onto TSA agar to determine the bacteria recovered.

3. Results

3.1. Identification of CiaRH in S. suis 2 and characteristics of the ΔCiaRH mutant

In the genome of 05ZYH33, SSU05_1095 exhibits 87% and 90% amino acid sequence identity with response...
Fig. 1. Genomic organization of CiaRH in *S. suis* 05ZYH33 and confirmation analysis of the knockout mutant strain ΔCiaRH. (A) Genomic organization of the CiaRH locus in *S. suis* 05ZYH33. (B) Confirmation of ΔCiaRH mutant strain by PCR using primer pairs T1/T2 (flanking CiaRH), T3/T4 (between CiaRH). Marker is shown to the left (M). (C) Growth curves of WT and ΔCiaRH. (D) Transmission electron micrographs of bacteria. The bar indicates the magnification size.
Fig. 2. Interactions of the two strains with cells and animals. (A) The ΔciaRH mutant showed reduced levels of adherence to Hep-2 and PIEC. (B) Macrophage bactericidal activity. (C) Bacterial loads were monitored in the blood of CD1 mice. The results of three experiments are presented and expressed as the mean ± standard deviation. The asterisks indicate significant differences (P < 0.05, t-test). (D) Survival curves for CD1 mice in experimented infection. (E) Survival curves for piglets in experimented infection.
regulators CiaR of Streptococcus pneumoniae R6. Therefore, SSU05_1094 and SSU05_1095 were defined as CiaH and CiaR, respectively and they consist of CiaRH two-component regulatory system.

The resulting ΔCiaRH mutant was verified by PCR (Fig. 1B). According to the growth curves, inactivation of CiaRH led to a lower growth rate compared with the parent strain (Fig. 1C). The mean chain length of the ΔCiaRH was found to be shorter than that of the WT strain under the same growth conditions by transmission electron microscopy, but the capsule and the division manner of the ΔCiaRH strain showed no differences with its parental strain (Fig. 1D).

3.2. Contribution of CiaRH to in vitro adhesion and bactericidal assay

The adherence efficiencies of the WT and the ΔCiaRH mutant to Hep-2 cells and PIEC were compared. As shown in Fig. 2A, WT showed significantly more adhesion to both Hep-2 cells and PIEC than the ΔCiaRH mutant, indicating the role of CiaRH as an important mediator in the cellular-adhesion process. The WT strain can still multiply when incubated with RAW264.7 macrophage cells, indicating that CiaRH is able to enhance the bactericidal activity of RAW264.7 cells (Fig. 2B).

3.3. The virulence of the ΔciaRH mutant is impaired in the CD1 mouse model

The morbidity and mortality analysis showed that almost all mice in the WT group presented severe clinical signs of sepsis, such as depression, rough hair coat, swollen eyes, weakness and prostration during the first three days post-infection. Eight mice died from septicemia in the WT group during 3 days post-inoculation (Fig. 2D). In contrast, only two mice died in the ΔCiaRH group during the 24 h post-inoculation and all of remaining eight mice did not present severe clinical signs of sepsis associated with S. suis 2 infection during the trial, recovering after 4 days post-inoculation.

To further confirm the clinical signs of ΔCiaRH mutant in vivo, bacteria loads of blood were monitored. Results showed that bacteria loads in blood following inoculation were lower in the ΔCiaRH mutant group, suggesting that the mutant could not multiply effectively in blood compared with WT (Fig. 2C).

3.4. CiaRH contributes to S. suis 2 virulence in piglet infection

All six piglets inoculated with WT strain at the dose of $1.5 \times 10^5$ CFU/piglet developed hyperthermia and depression within 48 h, including two of which died within 24 h. Later, most of the typical disease symptoms, including limping, swollen joints, shivering, central nervous system failure and respiratory failure were observed. All the piglets infected with WT strain died or were sacrificed for ethical reasons within 3 days post-infection (Fig. 2E). In contrast, none of the piglets infected with the ΔCiaRH mutant developed any of the clinical signs mentioned above within the first 24 h with exception of one which was slightly depressed. However, one piglet died within 48 h and another by 72 h. The remaining four recovered from fever showing none of other typical disease symptoms and remained healthy throughout the 14 day experiment.

Almost all the bacterial counts recovered from different tissues of ΔCiaRH mutant infected-piglets were significantly lower compared with WT-infected-piglets (Table 2). Notably, ΔCiaRH could not be isolated from the brain within the first 24 h while the WT strain was recovered from brains all of WT-infected-piglets.

4. Discussion

S. suis 2 is one of the most important swine pathogens, and an emerging, life-threatening zoonotic agent in both pigs and humans. Development of the disease requires temporal and coordinated expression of a series of genes that allow the prospective pathogen to shift to its pathogenic state and adapt to a hostile environment in the host (Mahan et al., 1993). TCS plays important roles in bacterial gene expression (including that of many virulence-related genes) in response to a variety of environmental stimuli (Fabret et al., 1999).

In the present study, CiaRH were found to be highly conserved in all the sequenced strains of S. suis 2 by bioinformatics. CiaRH deletion mutant was constructed via allelic exchange and the impact of this deletion on pathogenesis of S. suis 2 was assessed.

Colonization of the nasopharyngeal epithelium, and the interaction of S. suis 2 with respiratory tract epithelial cells is considered to be an essential step in the infectious process (Lalonde et al., 2000). Compared with the WT strain, ΔCiaRH displayed a significant

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<th>Tissue</th>
<th>WT</th>
<th>ΔciaRH</th>
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<tr>
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<td>Blood</td>
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Table 2
Colonization analysis in various tissues of piglets (CFU/g tissue).
decrease in adhesion to Hep-2 and PIEC. Bactericidal assay was also performed with RAW264.7 cells and the results indicate that the CiaRH is able to enhance the bactericidal activity of RAW264.7. Coincident with the result of the bactericidal activity, ΔCiaRH showed inability to persist in the blood (bacteraemia) which is important for infection. Overall, the outcome in the CD1 murine model clearly indicates the ability to inflict damage to the host within 48 h post-infection, is the CD1 murine model clearly indicates the ability to inflict damage to the host within 48 h post-infection, is attenuated with the deletion of CiaRH. In the piglet model, the ΔCiaRH has been shown to significantly reduce virulence compared to the WT strain, including the survival, clinical symptoms and colonization abilities in specific organs. Outcome of colonization assays indicated that the capacity of ΔCiaRH to move across host barriers was impaired.

In conclusion, this study has provided initial insight that the CiaRH contributes to the virulence of S. suis 2 in both the murine and porcine models of infection. The results of this study strongly suggest that the CiaRH TCS may coordinate and regulate some important factors involved in the pathogenesis of S. suis 2, especially in the early stage of infection. However, which factors and how the CiaRH TCS regulate these factors are still unknown. Therefore, further studies are necessary to define the regulation network of CiaRH system including the downstream genes regulated and direct binding site of response regulator CiaR. Determining the CiaRH signalling pathway may help our understanding of the pathogenesis of S. suis serotype 2 and some other related pathogens.

Acknowledgments

This study was supported by 973 Program (2006CB504404), National Nature Science Foundation of China (30970109), 863 Program (2006AA10A206) and Innovation Teams of Ministry of Education (IRT0726). We are grateful to Dr Sekizaki (National Institute of Animal Health, Japan) for supplying plasmid pSET4s.

References


