The quorum sensing system *luxS* gene contributes to the environmental fitness of *Streptococcus suis*

Jinpeng Li

Henan University of Science and Technology

Yuxin Wang

Henan University of Science and Technology

Yanbin Du

Henan University of Science and Technology

Hui Zhang

China Animal Health and Epidemiology Center

Qingyin Fan

Henan University of Science and Technology

Liyun Sun

Henan University of Science and Technology

Li Yi

Luoyang Normal University

Shaohui Wang

Shanghai Veterinary Research Institute

Yang Wang (✉ wangyocan@163.com)

Henan university of science and technology

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Abstract

Background: *Streptococcus suis* type 2 (SS2) is an important zoonotic pathogen. We have previously reported the structure of LuxS protein and found that the *luxS* gene is closely related to biofilm, virulence gene expression and drug resistance of SS2. However, the mechanism of *luxS* mediated SS2 stress response is unclear. Therefore, this experiment performed stress response to *luxS* mutant ($\Delta luxS$) and complement strain ($C\Delta luxS$), overexpression strain (*luxS+*) and wild-type SS2 strain HA9801, and analyzed the differential phenotypes in combination with transcriptome data.

Results: The results indicate that the *luxS* gene causes a wide range of phenotypic changes, including chain length. RNA sequencing identified 278 *luxS*-regulated genes, of which 179 were up-regulated and 99 were down-regulated. Differential genes focus on bacterial growth, stress response, metabolic mechanisms and drug tolerance. Multiple mitotic genes were down-regulated; while the ABC transporter system genes, cobalamin /Fe³⁺-iron carrier ABC transporter ATPase and oxidative stress regulators were up-regulated. The inactivation of the *luxS* gene caused a significant reduction in the growth and survival in the acid and iron stress environments. However, the mutant strain $\Delta luxS$ showed increased antioxidant activity to H₂O₂.

Conclusions: The *luxS* gene in SS2 appears to play roles in iron metabolism and protective responses to acidic and oxidative environmental conditions.

Background

Streptococcus suis (SS) is a major pathogen in pigs, and it is also a zoonotic agent of a variety of diseases for swine and humans. Among the thirty-three serotypes of SS (serotypes 1–32 and serotype 1/2), *Streptococcus suis* serotype 2 (SS2) are generally considered to be the most virulent serotypes found so far. It can cause a variety of life-threatening infections, including meningitis, arthritis and sepsis [1, 2]. The LuxS/AI-2 quorum sensing (QS) system is considered a process by which bacteria communicate using autoinducers 2 (AI-2). It is widespread in Gram-positive and Gram-negative bacteria. LuxS-mediated QS mechanism is based on the production of AI-2 that regulates various important biological properties in different bacteria. In our previous study, we have shown that the loss of *luxS* gene can reduce the biofilm formation ability, hemolytic activity, adhesion to human laryngeal carcinoma (HEp-2) cell line and virulence genes transcription, and the *luxS* gene also related to drug-resistant efflux gene expression of SS [3–6]. Furthermore, by analyzing the crystal structure of LuxS protein, it was found that Zn²⁺ and two important amino acids, Phe80 and His87, can affect the catalytic activity of S-ribose homocysteine (*luxS*) [7]. The ability of bacteria to resist environmental stress is one of the important factors for their survival. Studies have shown that LuxS protein is involved in regulating changes in bacterial resistance to stress[8]. However, the relevant mechanisms remain unclear.

The quorum sensing system is an important protective mechanism for bacteria to adapt to the environment [9]. Previous studies have shown that *luxS* and AI-2 involved in bacterial regulation of a

series of important stress responses, including heat shock, anti-gamma radiation, H₂O₂ and other oxidative stress responses [10–14]. With the deepening of *luxS* research, researchers found that *luxS* showed different phenotypes in some bacteria after mutation. For example, the *luxS* mutation in *Helicobacter pylori* leads to a decrease in the expression of flagella transcription regulator *flhA* and a decrease in motility [15]. Compared with the wild-type strain, the *luxS* mutant of *Escherichia coli* has increased survival in the environment with pH < 3.2 [13]. In addition, the ABC transporter gene of radiation *Deinococcus radiodrans luxS* mutant is up-regulated under oxidative stress, and ABC transporter participates in the adaptation mechanism of stress environment, transporting damaged nucleotides and polypeptides to vitro. And it's proved that the *luxS* mutant of *Borrelia burgdorferi* has reduced pathogenicity in mice [16]. Moreover, Liu *et al.* found that overexpression of *luxS* gene could improve the stress resistance of *Lactobacillus paraplantarum* L-ZS9 [8]. We have found *luxS* gene as an important regulator in many aspects. However, it is still unknown that the impact of the *luxS* genes on SS growth and stress responses. In order to understand the *luxS* gene more fully, in this study, we wanted to know the differences of wild-type strain, $\Delta luxS$, $C\Delta luxS$ and *luxS*⁺ in growth characteristics and stress responses.

Results

Cell morphology

Through SEM observations, mutant strain $\Delta luxS$ tended to aggregate into clusters without any apparent chain formation, and exhibited abnormal morphology relative to the wild-type strain. In addition, the capsule of $\Delta luxS$ cells was significantly thinner than that of wild-type strain. These morphology phenotypes can be restored in part by $C\Delta luxS$. However, the differences between the overexpression strain *luxS*⁺ and wild-type strain were not significant (Fig. 1). Gram stain results showed that the morphological characteristics of the four strains consistent with the results of SEM (data not shown).

Growth curves

The growth curves of wild-type, $\Delta luxS$, $C\Delta luxS$ and *luxS*⁺ SS2 strains are presented in Fig. 2. Compared with the wild-type strain, the mutant strain $\Delta luxS$ did not show growth defects. For complementary strains, the difference between $C\Delta luxS$ and overexpression strain *luxS*⁺ is not significant.

$\Delta luxS$ mutant and wild-type strain transcriptome analysis

Analysis of transcriptome data shows that *luxS* gene mutation has a wide-ranging effect on the gene expression of SS. There were 1978 identically expressed genes in the SS wild-type strain and the mutant strain $\Delta luxS$. In addition, there were 179 up-regulated genes and 99 down-regulated genes (Fig. 3A). Obviously, the amino acid ABC transporter permease gene expression was up-regulated most and acetyltransferase gene, cell division genes was down-regulated most. Gene Ontology (GO) analysis identified the biological functions of differentially expressed genes, and found that the main enrichment

of differential genes was in biological processing and molecular functions. There was no significant enrichment of differential genes in cellular components, as shown in Fig. 3B.

qRT-PCR to verify changes in gene expression

In order to verify the reliability of the RNA-seq results, 6 differentially expressed genes were randomly selected for verification (Fig. S1). The results showed that SSU05_2024, SSU05_1111, and SSU05_1069 were significantly up-regulated, and SSU05_0050, SSU05_0087, and SSU05_0302 were significantly down-regulated. The above results are consistent with the sequencing results ($P \leq 0.05$).

Acid tolerance of SS

The acid tolerance assay suggested that compared with pH values of 5.0, 6.0, 7.0, the OD_{600nm} values of the four strains (HA9801, $\Delta luxS$, $C\Delta luxS$, and $luxS^+$) at pH = 3.0 and pH = 4.0 decreased significantly. In addition, there is no difference in growth status among the HA9801, $C\Delta luxS$, and $luxS^+$ strains in acidic environments. However, the mutant strain $\Delta luxS$ showed significantly decreased survival in acidic environments ranging from pH 3.0 to pH 5.0 at tested times compared with wild-type strain (Fig. 4). Moreover, complementation of $luxS$ gene restored the acid resistance for the complemented strain. These results indicated that $luxS$ gene contributed to the acid tolerance of SS2.

Fe stress response

The effects of exogenous Fe^{2+} and Fe^{3+} on the growth of SS were determined. It was observed that wild-type and $luxS^+$ strains, in the presence of 3 mmol/L iron chelator were not significantly impacted by the metabolic stressor ($P > 0.05$). However, mutant strain $\Delta luxS$ was observed to have a significantly reduced OD_{600nm} value ($P < 0.001$) compared with wild-type strain. Moreover, the growth capacity of $C\Delta luxS$ was restored by the $luxS$ gene complementation. The effect of Fe^{2+} on the growth of $\Delta luxS$ was more pronounced than that of Fe^{3+} (Fig. 5).

Oxidative stress response

To assess the capability of the $luxS$ gene to manage oxidative stresses, survival of the SS2 wild-type, $\Delta luxS$, $C\Delta luxS$ and $luxS^+$ strains were measured after 1 h, 2 h, 3 h or 4 h of H_2O_2 treatment. The results indicated that the wild-type strain were more susceptible to H_2O_2 treatment (58.8 mmol/L) than the mutant strain $\Delta luxS$. Survival rate of the $C\Delta luxS$ and wild-type strains were significantly different from that of $\Delta luxS$ ($P \leq 0.05$), and it was very significant with $luxS^+$ strain ($P \leq 0.01$). Therefore, it can be concluded that $luxS$ is associated with the antioxidant activity of SS2 (Fig. 6).

Discussion

The highly conserved $luxS$ gene has been extensively studied in recent years due to its involvement in the regulation of the expression of various growth and virulence-related genes [17, 18]. The related AI-2 is a

compound that plays a key role in bacterial cell-to-cell communication [19]. In this study, we compared the cell morphology and response of mutant strain $\Delta luxS$ and wild-type strain HA9801 to different stress conditions. The transcriptome differences between HA9801 and $\Delta luxS$ were also determined. Our results showed that mutant strain $\Delta luxS$ showed a large transcriptional difference and significantly different tolerance to stress environments.

We found that the growth rate of mutant strain $\Delta luxS$ was similar with those of wild-type, $C\Delta luxS$ and $luxS+$ strains. This experimental results corroborate the findings of Zhang *et al.* [20] and Van *et al.* [21], in which the absence or overexpression of the *luxS* gene no affecting the expression of other downstream genes important for bacterial growth. Previous studies also reported that *luxS+* did not increase the level of AI-2 production, and no affected the growth of SS [1].

Acid resistance is a necessary for bacterial survival in acidic environments and during infection of the host through the digestive tract. In the present experiment, the acid stress test revealed an overall downward trend in vitality in acidic environmental conditions. The downward trend of $\Delta luxS$ was more pronounced than that of wild-type strain, and the viability decreased precipitously when the pH < 5.0. Acid stress on *luxS+* showed slightly stronger acid resistance than wild-type strain. The loss of the *luxS* gene results in many altered traits, including thinning of the bacterial capsule, which may be a cause of the altered acid resistance. In addition, with the down-regulated of the cell division protein genes SSU05_0760 and SSU05_0761, the $\Delta luxS$ strain showed a thin capsule structure. The results were similar to Cao *et al* [22]. At same, previous studies have also found that *luxS* gene is involved in biofilm formation [9]. At pH = 3.0, the wild-type and $\Delta luxS$ strains had not yet been completely killed, suggesting that the *luxS* gene is part of a redundant system through which SS2 creates its acid resistance. These observation suggest that acid regulation is a complex process that may be influenced through a variety of regulatory pathways [23], the SS2 *luxS* gene appears to be part of one such regulatory pathway.

Iron is actually an essential element in all cells because iron is a cofactor in many enzymes, especially central metabolism and respiratory enzymes, so it is a coenzyme. Wen *et al.* [24] found that the growth inhibition of $\Delta luxS$ in the medium containing 0.3 mmol/L 2,2'-bipyridine was alleviated by adding exogenous iron and culture supernatant regulated by wild-type strains. In addition, Lee *et al.* [25] used to the gene chip to analyze and compare the transcription data of wild-type and mutant $\Delta luxS$ strains of *Streptococcus pneumoniae* at 4 different growth phages. It was found that two TonB systems are involved in iron absorption. Furthermore, tonB1-exbB1-exbD1 and tonB2-exbB2-exbD2 are affected by the *luxS* gene in all four growth stages [26]. In the present study, the results showed that the addition of exogenous Fe^{2+} resulted in reduced growth of the wild-type strain with increasing ion concentration at 6 h and 12 h. The effects caused by Fe^{3+} were more pronounced than those of Fe^{2+} on growth promotion of the $\Delta luxS$ mutant strain, although the difference was not significant ($P > 0.05$). There was no significant difference observed in the growth status between wild-type and *luxS+* strains ($P > 0.05$). In comparison to the wild-type and *luxS+* strains, the growth of the $\Delta luxS$ mutant was observed to be significantly decreased ($P < 0.001$). The growth ability $C\Delta luxS$ did recover, albeit not to the level of the wild-type strain. Besides, in the mutant strain, the expression of the cobalamin / Fe^{3+} -iron carrier ABC transporter ATPase

gene SSU05_0650 was up-regulated, up to 1.976 times. We speculate that the reason why the mutant strain is more sensitive to Fe^{2+} than Fe^{3+} in the experiment may be due to the enhanced ability of the mutant strain to transport Fe^{3+} ions [27]. Taken together, the data suggest that the *luxS* gene is involved in SS iron absorption, and the regulation of bacterial growth.

Oxidative stress is one defense mechanism of the host against an invading pathogen. Bacterial infections must first overcome the host's stress defense mechanism. Yu *et al.* [28] knocked out the *luxS* gene of *Yersinia pestis*, and observed that the mutant exhibited a reduced resistance to H_2O_2 . However, the *Porphyromonas gingivalis* $\Delta luxS$ strain exhibited increased survival in the presence of H_2O_2 [29], this finding is similar to what was previously reported by Cao *et al.* [30] with respect to a H_2O_2 stress test of $\Delta luxS$ of SS2 strain 05ZYH33. The results of this experiment showed that mutant strain $\Delta luxS$ were more tolerant to H_2O_2 than the wild-type strain. This suggests that *luxS* genes have different roles in different bacterial species. After analyzing comprehensive transcriptome data, we believe that the differences in stress resistance of $\Delta luxS$ and wild-type strains may result in phenotypic differences due to the abnormal transcription of some genes after the *luxS* gene is mutant. Transcriptome data showed that the SS *spxA* transcriptional regulator SSU05_1111 and the possible oxidative stress-related gene SSU05_1069 were found in $\Delta luxS$ strains, and their expressions were up-regulated by 2.5 and 2.1 fold, respectively. It has a certain tolerance to H_2O_2 compared with the wild strain; research shows that *spxA* transcriptional regulators play an important role in oxidative stress response of SS. *spxA* mutant strains are more sensitive to the oxidative environment of SS [31, 32].

Conclusion

In summary, we compared the transcriptome differences and tolerance to various stress environments between mutant strain $\Delta luxS$ and wild-type strain. It has been indicated that quorum sensing system *luxS* gene is of great significance to the morphological structure as well as stress resistance of SS2.

Materials And Methods

Bacterial strains and culture conditions

The SS2 virulent wild-type strain HA9801 was isolated from pigs in the Jiangsu Province in 1998. The mutant strain $\Delta luxS$, complemented strain $C\Delta luxS$ and overexpression strain *luxS*⁺ of HA9801 was constructed previously [1, 2]. The SS strains were grown at 37 °C in Todd Hewitt broth (THB) (Becton, Dickinson and Company, USA) medium or plated on THB agar with 5% (vol/vol) sheep blood.

Morphological characteristics

The morphological differences were determined by scanning electron microscopy according to the method previously described [33]. Briefly, coverslips with SS2 cultures were rinsed 3 times with a phosphate buffered solution. The samples were then post-fixed for 90 minutes with 1% (w/v) osmium

tetroxide in a 0.1 M sodium cacodylate buffer. After staining, the specimens were dehydrated in increasing concentrations of acetone (10%, 30%, 50%, 70%, 90%, and 100%). The specimens were then air-dried for 60 minutes, and were then adhered to metal holders with double-sided tape for coating with gold and palladium in an evaporator. All specimens were positioned with the apices facing up for proper visualization by scanning electron microscopy (SEM) in a vacuum at 5 kV electron beam energy (Hitachi S4700 FESEM; Hitachi Ltd, Tokyo, Japan). The bacterial morphology was also observed by gram staining and optical microscope (OM).

Growth curve

The logarithmic growth phase SS2 cultures were diluted 1: 200 to achieve an optical density at 600 nm ($OD_{600\text{ nm}}$) of approximately 0.05. These cultures were incubated at a constant temperature shaking incubator at 37 °C, shaking at 120 rpm. The $OD_{600\text{ nm}}$ values of the cultures were measured at 1 h interval using a spectrophotometer.

RNA-seq analysis

The experimental operation was performed as previously described with minor modifications [34]. The strains SS HA9801 and Δ/uxS were cultured in THB medium for 6 h, and harvested at 8000×g at 4 °C for 10 min. Then, the total RNA was extracted with the Trizol Reagent kit. Three biological replicates were set for each sample, and all samples were sent to Beijing Novogene Co., Ltd. for sequencing by Illumina HiSeq platform. Quality control was performed on the clean reads, and mapping was performed with reference to the SS 05ZYH33 genome. Gene function annotation was performed through the orthologous group (COG) database [35].

Validation of mRNA-Seq by qRT-PCR

The qRT-qPCR method was used to verify the expression results of mRNA-Seq in the transcriptome. Use Total RNA Extraction Kit (Solarbio, R1200) to extract total RNA, RNase-free DNase I to remove genomic DNA. The cDNA was amplified using MagicSYBR mix (CoWin Biosciences Co., Ltd., China). The volume of the amplification mixture was set to 20 μl (2× MagicSYBR Mixture 10 μl , each primer of 0.5 μM , cDNA 1 μg , finally add RNase-free water to 20 μl). The PCR reaction conditions were as follows: at 95 °C for 30 s, then at 95 °C for 5 s, and then at 60 °C for 30 s for 40 cycles. Randomly select 6 genes, and use 16S rRNA as internal reference to verify the original data. Table S1 lists all primers.

Acid stress assay

To assess the sensitivity of SS strains to acid stress conditions, we carried out an acid stress assay as previously described [36], with slight modification. Liquid THB media was prepared with pH values ranging from 3.0 to 7.0 (adjusted with 6N HCl). Approximately $1\text{--}3\times 10^6$ CFU SS2 bacterial suspension were inoculated at a ratio of 1:10 (v/v) at 37 °C for 12 h or 24 h under aerobic conditions. Growth kinetics of each strain was measured by monitoring $OD_{600\text{ nm}}$ values under various conditions.

Fe stress assay

The Fe stress response assay was performed as previously described [37], with some modifications. All strains were sub-cultured at the same original cell density from two subculture in iron restricted THB medium (containing 100 mM iron chelator 2,2'-dipyridyl). Then, transfer all the strains (HA9801, $\Delta luxS$, $C\Delta luxS$, $+luxS$) grown in iron-restricted THB medium to the same volume of fresh THB medium. Then all the strains cultures were diluted 1: 200 (v/v), and different concentrations of iron ions were added to THB broth, of which Fe^{2+} (0.1, 0.2 and 0.3 mmol/L) and Fe^{3+} (0.1, 0.2 and 0.3 mmol/L). The cultures were incubated at 37 °C with aeration. The above cultures were incubated for 6 h (exponential phase) or 12 h (stable period), and centrifuged at 3000 rpm for 10 min at 4 °C. The bacterial cell pellets were resuspended in the same volume of PBS buffer, and 250 μ L of the mixture was added to a 96-well microtiter plate. The assays were performed in triplicate and the OD_{600nm} values were measured.

Oxidative stress assay

Assessment of the bacterial cells abilities to withstand H₂O₂ challenges was determined as previously described [38]. Briefly, SS2 cultures were incubated in THB media until mid-exponential phase (OD_{600nm} $\approx$ 0.8). For H₂O₂ challenge, bacterial cells were prepared similarly, and then incubated in THB containing 58.8 mmol/L H₂O₂ for 1 h, 2 h, 3 h or 4 h. After exponentially reasonable dilution, 10 μ L of the dilution was spread on THA medium, incubated at 37 °C for 24 h and counted, the bacterial concentration was calculated, and the survival rate was calculated.

Statistical analysis

The Graphad Prism software was used to perform statistical analyses for all data. All data points for the experiments, performed in triplicate, were analyzed using the single factor analysis of variance (One-Way ANOVA), where $P < 0.05$ was considered to be statistically significant.

Declarations

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Authors' contributions

Conceived and designed the experiments: YW and SHW. Performed the experiments: YXW, LY, QYF and JPL. Analyzed the data: LY, LYS, SHW, HZ. Contributed reagents/materials/analysis tools: YBD, LYS, LY and JPL. Wrote the paper: YXW, PJL, SHW and YW.

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Availability of data and materials

The data sets used during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Conflict of Interest

The authors declare no conflict of interest.

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Figures

Figure 1

The morphology of SS2 wild-type strain HA9801, mutant strain $\Delta luxS$ complemented strain $C\Delta luxS$ and overexpression strain $luxS^+$ under SEM.

Figure 2

The growth curve of SS2 wild-type strain HA9801, mutant strain $\Delta luxS$, complemented strain $C\Delta luxS$ and overexpression strain $luxS+$ at 37°C. Growth was assessed by determination of OD_{600nm} values at the time points indicated. Each time point represents three independent tests.

Figure 3

Transcriptome analysis of mutant strain $\Delta luxS$ compared to wild-type strain. (A) Venn diagram showing the number of genes that express differently between $\Delta luxS$ mutant and wild type of SS. Among the 2256 genes identified, 179 genes were up-regulated and 99 genes were down-regulated when the cells were in

the logarithmic growth phase. The remaining 1978 genes were not differentially expressed. (B) Gene ontology analysis of differentially expressed genes. The red and black bars represent down-regulated and up-regulated genes, respectively, and the digital tags represent the number of genes associated with this pathway.

Figure 4

Acid resistance trends of SS2 strains at 12 and 24 h. The SS2 bacterial suspensions were inoculated at medium with various pH values, and the growth of each strain was measured at 12 h and 24 h. The growth and survival of mutant strain $\Delta luxS$ was significantly decreased in pH 3.0, 4.0 and 5.0 acidic environments compared with wild-type and complemented strain.

Figure 5

Effect of exogenous Fe^{2+} and Fe^{3+} on SS2 growth. The SS2 strains were inoculated at medium with different concentrations of iron ions, and the growth of each strain was measured at 6 h and 12 h. The addition of exogenous Fe^{2+} and Fe^{3+} exerted a significant growth reducing effect on the mutant strain compared with wild-type, complemented and overexpression strains.

Figure 6

Survival rates of SS2 strains under oxidative stress. The SS2 strains were inoculated at medium containing 58.8 mmol/L H₂O₂, and the survival of each strain was measured at indicated time. The survival rate of mutant strain $\Delta luxS$ was higher than those of the wild-type strain, $C\Delta luxS$ and $luxS^+$ at 1 h, 2 h, 3 h or 4 h.

Supplementary Files

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- [TableS1.docx](#)
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