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Functional Definition of LuxS, an Autoinducer-2 (AI-2) Synthase and Its Role in Full Virulence of *Streptococcus suis* Serotype 2[§]

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Quorum sensing is a widespread chemical communication in response to fluctuation of bacterial population density, and has been implicated into bacterial biofilm formation and regulation of expression of virulence factors. The *luxS* gene product, S-ribosylhomocysteinase, catalizes the last committed step in biosynthetic pathway of autoinducer 2 (AI-2), a signaling molecule for inter-species quorum sensing. We found a *luxS* homologue in 05ZYH33, an epidemic strain of *Streptococcus suis* serotype 2 (SS2) in China. A *luxS* null mutant (Δ*luxS*) of 05ZYH33 strain was obtained using an approach of homologous recombination. LuxS was determined to be required for AI-2 production in 05ZYH33 strain of *S. suis* 2. Inactivation of *luxS* gene led to a wide range of phenotypic changes including thinner capsular walls, increased tolerance to H_2O_2, reduced adherence capacity to epithelial cells, etc. In particular, loss of LuxS impaired dramatically its full virulence of SS2 in experimental model of piglets, and functional complementation restored it nearly to the level of parent strain. Genome-wide transcriptome analyses suggested that some known virulence factors such as CPS are down-regulated in the Δ*luxS* mutant, which might in part explain virulence attenuation by *luxS* deletion. Similarly, 29 of 71 genes with different expression level were proposed to be targets candidate regulated by LuxS/AI-2-dependent quorum sensing.

***Keywords*:** *Streptococcus suis*, Quorum sensing, Autoinducor 2 (AI-2), LuxS

Among 35 kinds of different serotypes of *Streptococcus suis* (*S. suis*), serotype 2 (SS2) is most frequently isolated from diseased-piglets and also most virulent (Gottschalk *et al.*, 2007; Feng *et al.*, 2010). Although somewhat it was previously neglected pathogen, SS2 has been emerging into an important zoonotic agent (Gottschalk *et al.*, 2010). Since its first discovery of a human meningitis case caused by SS2 in Denmark in 1968 (Staats *et al.*, 1997), human SS2 infections have been reported in above 30 countries/regions in which no less than 700 cases are involved (Feng *et al.*, 2010, Wertheim *et al.*, 2009). The clinical infectious diseases by SS2 infections included meningitis, septicemia, arthritis, etc. (Staats *et al.*, 1997). Moreover, major human infection cases were reported in Southeast Asia, such as Thailand (Takamatsu *et al.*, 2001; Suankratay *et al.*, 2004; Fongcom *et al.*, 2009), Vietnam (Mai *et al.*, 2008; Wertheim *et al.*, 2009; Ho *et al.*, 2011), and China (Iyer *et al.*, 2005; Tang *et al.*, 2006; Feng *et al.*, 2009). The situation of the SS2 endemic in China can be believed as co-existence of outbreaks and sporadic cases (Iyer *et al.*, 2005; Tang *et al.*, 2006; Feng *et al.*, 2009, 2010), while the scenarios in Europe and North America are featured with sporadic infections (Gottschalk *et al.*, 2007). The molecular mechanism underlying *S. suis* infections have been elucidated partially, which included no less than 20 bacterial virulence determinants (Baums and Valentin-Weigand, 2009, Feng *et al.*, 2010), and a series of host cell factors (Dominguez-Punaro *et al.*, 2007; Graveline *et al.*, 2007). Unlike P1/7, an international SS2 strain, the epidemic strain of Chinses SS2, 05ZYH33, possessed a specific putative 89K pathogenicity island (PAI) (Tang *et al.*, 2006; Chen *et al.*, 2007). We have demonstrated that 89K is a functional PAI that carries two virulence-related elements: one is *salK-salR* two component system (Li *et al.*, 2008), and the other is *virD4-virB4,* a type IV-like secretion system (Tang *et al.*, 2006). Recently, we identified that Rgg transcription factor is required for the virulence of *S. suis* 2 (Zheng *et al.*, 2011). In contrast, an orphan regulator CovR was found to negatively regulate virulence manifestation of *S. suis* 2 (Pan *et al.*, 2009).

Quorum sensing is a chemical communication among bacterial populations, which have been confirmed to be involved in a wide variety of biological process, like bioluminance production, biofilm formation, virulence factors expression, etc. (Surette *et al.*, 1999; Schauder *et al.*, 2001; Campbell *et al.*, 2005). Using the model system of marine *V. harveyi*, Bassler and coworkers led to identification of autoinducer-1 (AI-1)-dependent intraspecies communication pathway and AI-2-dependent interspecies communication system (Surette *et al.*, 1999; Campbell *et al.*, 2005). For the latter, *luxS* gene product was determined to catalyze the last committed step of AI-2 biosynthetic pathway (Schauder *et al.*, 2001). No less than 55 bacterial species were suggested to harbor *luxS* orthologs,

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Table 1. Strains and plasmids in this study

Strains/Plasmids	Characteristics/Functions	Source/Reference
Bacterial strains		
05ZYH33	High virulent strain of SS2 isolated from blood of a dead patient due to STSS	(Tang *et al.*, 2006)
Δ*luxS*	Isogenic *luxS* deletion mutant of 05ZYH33, Spc^R	this study
Δ*luxS*+DPD	Δ*luxS* strain added by exogenous DPD (AI-2 precursor)	this study
05HAS68	Avirulent SS2 isolated from the tonsil of a clinically healthy pig	laboratory collection
E. coli DH5α	Cloning host for maintaining the recombinant plasmids	Promega
V. harveyi BB170	*luxN*::Tn*5* (sensor 1- sensor 2+), AI-2 reporter strain	(Bassler *et al.*, 1997)
Plasmids		
pMD-18T	Cloning vector; Amp^R	TaKaRa
pUC::*luxS*	recombinant vector with the background of pUC18, designed to knock out gene *luxS*, Amp^R, Spc^R	This study
pSET2	*E. coli-S. suis* shuttle vector; Spc^R	(Takamatsu *et al.*, 2001)
pVA838	*E. coli-S. suis* shuttle vector; Spc^R, Em^R	(Macrina *et al.*, 1982, 1983)

Amp^R: Ampicillin resistance, Spc^R: Spectinomycin resistance; Em^R: Erythromycin resistance

some of which exhibited AI-2-like activities (Xavier and Bassler, 2003; Vendeville *et al.*, 2005). The receptor of AI-2 is the kinase of LuxPQ two component system in *Vibrio* species (Bassler *et al.*, 1993), whereas it is a ABC-type transporter, Lsr in *E. coli* (Xavier and Bassler, 2005) and *S. typhimurium* (Taga *et al.*, 2001). Interestingly, LuxS/AI-2 systems seemed to play multiple/varied roles in different bacterial species: 1) it regulates growth of *Neisseria meningitidis* (Heurlier *et al.*, 2009); 2) it is associated with biofilm formation in *Streptococcus gordonii* (McNab *et al.*, 2003), *Streptococcus mutans* (Merritt *et al.*, 2003; Yoshida *et al.*, 2005), *Salmonella enterica* serovar Typhimurium (Prouty *et al.*, 2002), *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans* (Shao *et al.*, 2007), *Vibrio cholerae* (Hammer and Bassler, 2003), *Helicobacter pylor*i (Cole *et al.*, 2004) and *Klebsiella pneumoniae* (Balestrino *et al.*, 2005); 3) it contributed to virulence in *Streptococcus pneumoniae* (Stroeher *et al.*, 2003), *Escherichia coli* 0157:H7 (EHEC) (Sircili *et al.*, 2004) and *Serratia marcescens* ATCC 274 (Coulthurst *et al.*, 2004); 4) it controls antibiotics susceptibility in *Streptococcus anginosus* (Ahmed *et al.*, 2007); and 5) it is involved in motility in *Campylobacter jejuni* (Jeon *et al.*, 2003) and *Helicobacter pylori* (Rader *et al.*, 2007). Recently, presence of a *luxS* homologue was suggested in strain H19801 of *S. suis* 2, and AI-2 activity was also observed (Han and Lu, 2009), while the pleitropic roles of *S. suis luxS* and AI-2 function remained unclear.

Here we reported modeled structure of *S. suis* LuxS protein and luxS-mediated global regulation using the genome-wide microarray analyses. We also demonstrated a variety of phenotypic changes due to *luxS* inactivation. Using the natural hosts, SPF-piglets, we demonstrated that *S. suis luxS* is involved in full virulence.

Materials and Methods

Bacterial strains and growth conditions

Bacterial strains and plasmids used here are listed in Table 1. The *S. suis* serotype 2 strains were grown at 37°C in Todd-Hewitt broth (THB) (Difco Laboratories, USA) and plated on THB agar (Biotrading, Netherland) containing 6% (vol/vol) sheep blood. *E. coli* DH5α was cultured in Luria-Bertani broth (LB) liquid medium at 37°C. Antibiotics were used as follows: 100 μg/ml of spectinomycin (Spc) (Sigma, USA) for *S. suis* transformants, and 50 μg/ml of ampicillin (Amp) (Sigma) for *E. coli*. The commercial pMD18-T vector (TaKaRa, P. R. China) was utilized to clone *luxS* gene for direct sequencing. *V. harveyi* BB170 was grown in autoinducer bioassay (AB) medium at 30°C (Bassler *et al.*, 1993). Assays of functional complementation were conducted in triplicates using the commercial DPD (4,5-dihydroxy-2,3-pentanedione) (Omm Scientific Inc., USA).

Construction of Δ*luxS* mutant and functional complementation

The *luxS* gene in strain 05ZYH33 (WT) was inactivated by allelic replacement with a spectinomycin resistance (Spc^R) cassette. The applified upstream and downstream DNA fragments adjacent to *luxS* (~1 kb) were cloned into pUC18 vector (TaKaRa) (Table 1), and then the intermediate vector was inserted with the spc^R gene (from the *E. coli-S. suis* shuttle vector pSET2), giving the *luxS* knockout vector, designated pUC::*luxS*. The knockout plasmid was electroporated into *S. suis* competent cells as we previously showed with minor improvement (Li *et al.*, 2008). The pulse parameters were described as follows: 25 μF, 22.5 kV/cm, 200 Ω and a time constant of 4.5-4.6 ms. Colony PCR assay was initially applied to screen all the spc^R transformants, and suspected mutants were further verified using southern blotting plus RT-PCR.

For functional complementation, the DNA fragment covering the *luxS* coding region plus its putative upstream promoter and downstream sequence was amplified from the chromosomal DNA of 05ZYH33 and cloned into an *E. coli-S. suis* shuttle vector, pVA838 (Macrina *et al.*, 1983) yielding plasmid pVA838::*luxS*. The resulting plasmid was introduced into the Δ*luxS* mutant to make the complemented strain (C-Δ*luxS*).

AI-2 bioassay

Using reporter strain *V. harveyi* BB170, we carried out the AI-2 assay as Bassler *et al.* (Bassler *et al.*, 1993; Smith *et al.*, 1995) earlier described. In this assay, exogenous AI-2 is supplemented to induce production of bioluminescence by reporter strain BB170. Overnight culture of *S. suis* 2 was inoculated into 200 ml of fresh media at the ratio of 1:100, and 1.5 ml of sample was collected each two hours. The bacterial cells were pelleted by centrifugation, and resulting supernatants were subjected to further clarification by filtering through a 0.22-μm-pore-size filter (Millipore, USA). Eventually the acquired

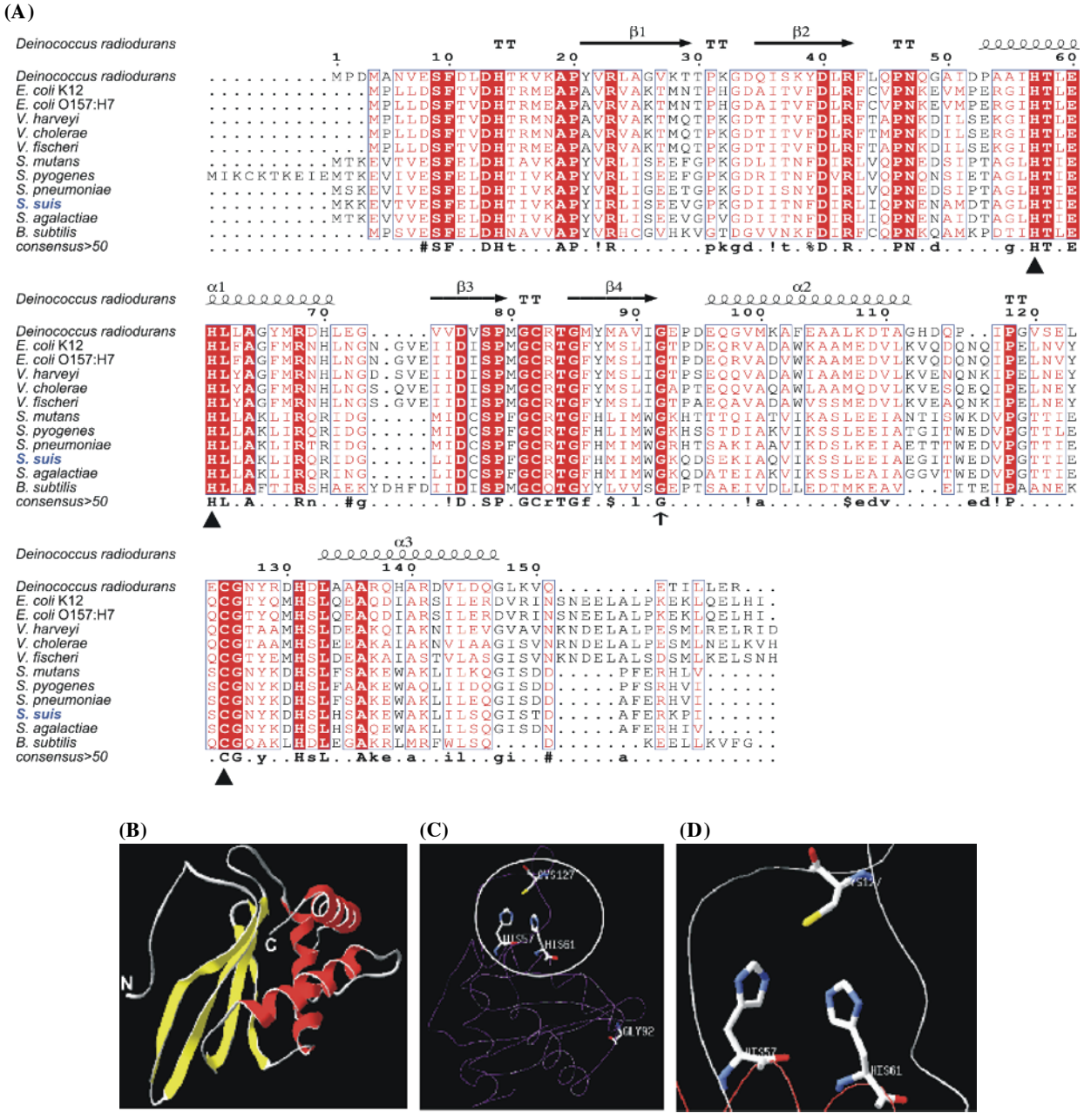

Fig. 1. Bioinformatics analyses of *S. suis* LuxS protein and its modeled structure. (A) Multiple alignments of LuxS homologue from *S. suis* ZYH33 with the related LuxS proteins. As Feng and the coworker described (Feng and Cronan, 2010), the multiple alignment was conducted using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html), and the final output was expressed through processing of program ESPript 2.2 (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi). The protein sequences of LuxS proteins used here are sampled from a collection of selected bacteria, which included *Deinococcus radiodurans* (NP_296108.1), *E. coli* K12 (NP_417172.1), *E. coli* O157:H7 (BAB36972), *V. harveyi* (EDL66900), *V. fischeri* (NC_006840.2), *V. cholerae* (AELJ01000043.1), *B. subtilis* (CAB15045.1), *S. pyogenes* (ACI61556), *S. pneumoniae* (ZP_02714888), *S. agalactiae* (EAO62013), *S. mutans* (AAN58222.1), and *S. suis* 05ZYH33 (YP_001197787). *S. suis* was indicated in blue. Identical residues are in white letters with red background, similar residues are in red letters with white background, varied residues are in black letters, and dots represent gaps. The predicted secondary structure of LuxS protein is shown in top. α: α-helix; β: β-sheet; T: β-turns/coils. Three conserved zinc-binding sites (H57, H61, and H127) are indicated with black triangles (Hilgers and Ludwig, 2001, Ruzheinikov *et al.*, 2001). A critical amino acid G81 was also very conserved (highlighted with an arrow), which was recently found to be an active site for AI-2 production in *Campylobacter jejuni* (Plummer *et al.*, 2011). (B) Ribbon structure modeled for *S. suis* LuxS. α-helix is in red, and β-sheet is yellow. "N" decodes N-terminus, and "C" decodes C-terminus. (C) Four critical amino acids visualized in the LuxS structure, and the pocket of zinc binding sites was enlarged in Panel D. The protein sequence of *S. suis* LuxS was submitted to CPHmodels 3.0 Server (http://www.cbs.dtu.dk/services/CPHmodels), generating a PDB file of the modeled structure, which searches fro a reasonable template of the known structure. The tertiary structure of the modeled LuxS was visualized using Swiss_PDBViewer 4.0.1 software from the Swiss Institute of Bioinformatics (http://spdbv.vital-it.ch/).

cell-free media (CM) were added into wells of the 96-well plate. 180 μl of the log phase *V. harveyi* BB170 culture diluted in 5,000 times was also supplemented into above wells. Luminescence was monitored hourly with Fluoroskan Ascent FL (Labsystems, USA).

Measurement of the capsular sialic acid content

The thiobarbituric acid assay (Warren, 1959; Charland *et al.*, 1996) was applied to determine the content of the capsular sialic acids.

Oxidative stress tests

We used the method described by Verneuil *et al* (Verneuil *et al.*, 2005) to evaluate effects of *S. suis* by oxidative challenge. Briefly, overnight cultures of four species (WT, Δ*luxS*, C-Δ*luxS* and Δ*luxS*+DPD strain) all were inoculated in fresh THB media at the ratio of 1:100. When cells reached an OD600 of 0.8, 100 μl of bacterial culture were sampled and incubated with hydrogen peroxide (H_2O_2) at various concentrations (0, 9, 18, 36, and 72 mM) for 15 min. Subsequently, the challenged bacteria in appropriate dilutions were plated on THB agar plates, and colonies were counted on the next day. The results were expressed as the Bacterial survival percentage was expressed by dividing the number of CFU at different concentration of H_2O_2 with the initial number of CFU prior to H_2O_2 challenge. The assays were repeated for no less than three times.

Cell adherence analyses

Human laryngeal epithelial cell line Hep-2 (CCTCC GDC004) and Human umbilical vein endothelial cells (HUVEC) both were utilized to perform cell adherence experiments (de Greeff *et al.*, 2002; Pan *et al.*, 2009). Cell lines were maintained routinely and *S. suis* cultures (WT, Δ*luxS*, C-Δ*luxS* and Δ*luxS*+DPD strain) were collected when OD600 was around 0.6. Cells were infected at a multiplicity of infection of ten bacteria per cell for 2 h in 37°C incubating oven with 5% CO_2. The monolayer was then washed three times with PBS, and the cells were disrupted by the addition of 0.3 ml of sterile deionized ice-cold water and repeated pipetting. Serial dilutions of the cell lysates were plated onto THB agar to enumerate viable bacteria. The percentage of bacterial adherence was calculated as following formula, (CFU on plate/CFU in original inoculum) ×100%.

Experimental infection of piglets

Three-week-old SPF piglets with average weight of ~3.0 kg randomly allotted to four groups (each consisting of six piglets) were challenged with SS2 (Li *et al.*, 2008). Briefly, piglets were inoculated intravenously with the Δ*luxS* and C-Δ*luxS* at a dose of 10^8 CFU/piglet. The parental strain 05ZYH33 and avirulent strain 05HAS68 separately served as positive control and negative control. Clinical monitoring was performed as previously described (Tang *et al.*, 2006), and recorded for 14 days post-inoculation and moribund pigs were humanely sacrificed in time. Samples were taken at necropsy and processed for histological examinations. Animal experiments were performed in a facility of bio-safety level 3 (BSL-3) and all piglets were treated in accordance with the institutional guidelines for the humane care and treatment of animals.

Genome-wide microarray analyses

The whole-genome microarray of strain 05ZYH33 contained 2,163 oligonucleotides probing SS2 open reading frames specifically at the genomic level. Design and synthesis of the oligonucleotides were performed by Agilent Technologies, Inc., Santa Clara, CA. Each probe is 60-mer oligonucleotide. Six replicates were immobilized randomly per oligonucleotide and six arrays (three for transcriptional comparison between WT strain, 05ZYH33 and Δ*luxS* mutant, the other three for comparison between Δ*luxS* mutant and the Δ*luxS* mutant complemented with DPD were placed on one slide). cRNA (the complementary sequences of mRNA) was synthesized and labeled according to the optimized protocol for use with Agilent oligonucleotide microarrays (Agilent Technologies, Inc., USA). The microarray hybridization, washing and scanning were carried out according to the manufacturer's manual. Statistically significant differences were defined as those with a *t* test *P* value of less than 0.05 and a ratio (Δ*luxS*/WT

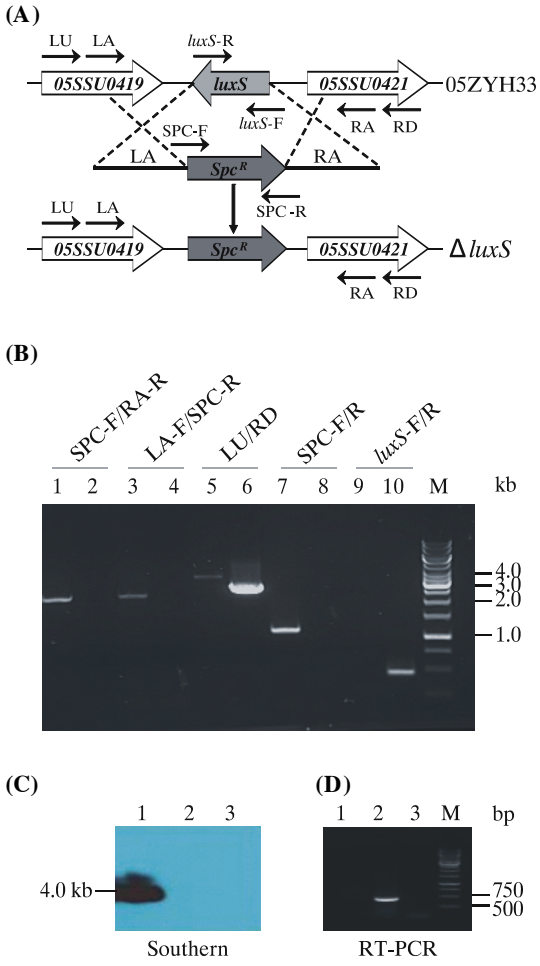

Fig. 2. Construction of an isogenic *luxS* mutant of *S. suis* 05ZYH33. (A) Strategy for knock out of *luxS* from *S. suis* 05ZYH33.The spectinomycin resistance gene, indicated by a thick black arrow, replaced *luxS* by allelic exchange. LA, RA, LU, and RU indicate the primers used for the construction and identification of the *luxS* knockout mutant. (B) PCR-based verification of the Δ*luxS* mutant. The primer pairs used in the PCR analysis are indicated above the lanes. Lanes 1, 3, 5, 7, and 9 correspond to the Δ*luxS* mutant, whereas lanes 2, 4, 6, 8, and 10 are specific to and the WT strain, 05ZYH33. (C) Sourthern blotting analyses of the Δ*luxS* mutant. Bacterial genomic DNA was digested with *Eco*RI, and the labeled luxS gene was used as a probe for hybridization. Lanes 1, 2, and 3 separately decode wild type, the Δ*luxS* mutant, and the complemented strain Δ*luxS*+DPD. (D) RT-PCR identification of the Δ*luxS* mutant. Lanes 1, 2, and 3 separately decode wild type, the Δ*luxS* mutant, and the complemented strain Δ*luxS*+DPD.

or Δ*luxS*/Δ*luxS*+DPD) of change threshold of at least 2.0 standard deviations compared to the median ratio for each strain.

RNA isolation and RT-qPCR

SS2 cultures (WT, Δ*luxS* and Δ*luxS*+DPD) in log phases were collected for bacterial RNA preparation with SV Total RNA Isolation System (Promega, USA). Absence of contaminating DNA was confirmed by PCR, and qualified RNA was visualized by electrophoresis analysis plus assessment with an Agilent 2100 bioanalyzer (Agilent Technologies, USA).

To validate the data from microarray analysis, 10 genes from the comparison array were selected, and real-time quantitative PCR asays were performed with method of SYBR Green detection (Pan *et al.*, 2009) in triplicate. cDNA samples were synthesized using RNA species collected from three independent cultures of each strain (WT, Δ*luxS* and Δ*luxS*+DPD). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH,) served as the internal control gene (Schmittgen and Zakrajsek, 2000). The mean fold changes in target gene expression were calculated as described by Livak *et al* (Livak and Schmittgen, 2001).

Results

Bioinformatic analyses of a LuxS homolog in *S. suis* 05ZYH33

A *luxS* ortholog encoding 160 aa of polypeptide (05SSU0420) was found to locate on the reverse strand of *S. suis* 05ZYH33, a Chinese epidemic strain causing human streptococcal toxic shock-like syndrome. This LuxS homologue exhibits highly similar to those known LuxS proteins from seleceted organisms (e.g., 97% for *S. pneumoniae* and 56% for *V. harveyi*) (Fig. 1A). Not only were three highly conserved sites critical for zinc binding (His58, His61, and Cys127) observed clearly in this suspected LuxS specie from 05ZYH33 (Fig. 1C, D), but also a recently-reported amino acid (Gly82) was present that is required for AI-2 production (Plummer *et al.*, 2011). Structural modeling showed that LuxS homologue adopt similar folding mode of tetranary structure, i.e., four β-sheets plus three α-helixs (Fig. 1B), indicating its possibility of being a functional member of LuxS family protein. In addition, bacterial genome annotation-based analyses showed that this putative *luxS* gene is opposite to its two neighboring genes (Fig. 2A), suggesting that it might be transcribed in an independent operon.

Extensive alterations of bacterial phenotypes by inactivation of *S. suis luxS* homologue

To test its physiological role of *luxS* ortholog in the context of *S. suis* 05ZYH33, we attempted to make an isogenic *luxS* knockout mutant (Δ*luxS*) using the strategy of alleic exchange (Fig. 2A). The expected mutant in which a double-crossover event has been undergone was confirmed by series of approaches including clony PCR (Fig. 2B), Southern bloting (Fig. 2C), RT-PCR (Fig. 2D) as well as direct DNA sequencing (data not shown).

We noted that colonies of Δ*luxS* mutants are little bit smaller than thoses of parental strain when growing on THB paltes (data not shown). Gram staining analyses showed that the Δ*luxS* mutant tends to aggregate into clusters without apparent formation of chains, and exhibit abnormal morphology relative to the wild type 05ZYH33 (Fig. 3A). Transmission electron microscopy-based observation revealed that the capsule of Δ*luxS* is significantly thinner than that of its parental strain (Fig. 3B). These phenotypes can be restored in part by functional complementation (C-Δ*luxS*) (Fig. 3). Intriguingly, the microscopic phenotype alteration above cannot be restored by the addition of DPD, a precursor for AI-2 production (Fig. 3), indicating that these phenotypes are related to some other unknown function of LuxS rather than its putative role in quorum sensing of *Streptococcus suis*. In much consistence with growth defect observed for deletion of *luxS* gene from *S. pyogenes* (Lyon *et al.*, 2001), *Bacillus anthracis* (Prouty *et al.,* 2002; Jones and Blaser, 2003), and *N. meningitides* (Heurlier *et al.*, 2009), we also noted this scenario in the Δ*luxS* mutant of *S. suis* (Fig. 4). In contrast, disruption of luxS led to faster growth in enterohemorrhagic *E. coli* O157:H7 (Jordan *et al.*, 2005). In this case, the Δ*luxS* mutant exhibited the lagged logarithmic phase relative to the wild type (Fig. 4). Moreover, this growth defect of Δ*luxS* mutant strain can not be restored by an exogenous addition of DPD (or the supernant from

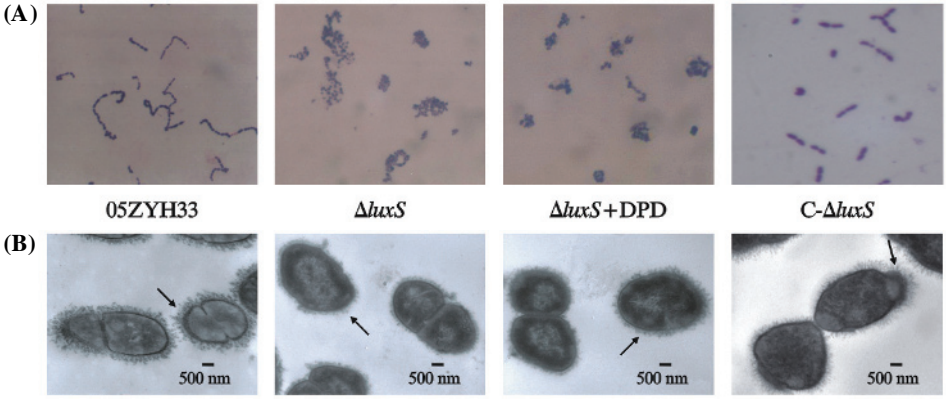

Fig. 3. Microbiological characterization of WT, Δ*luxS* and the chemical complementary strain Δ*luxS*+DPD. (A) Gram-stained images of bacteria under light microscopy (×1000) (B) Transmission electron analyses of bacterial species (×50,000). The bars mean to 500 nm. Bacteria were cultured in THB containing 10% FBS. The capsule is indicated by the arrows.

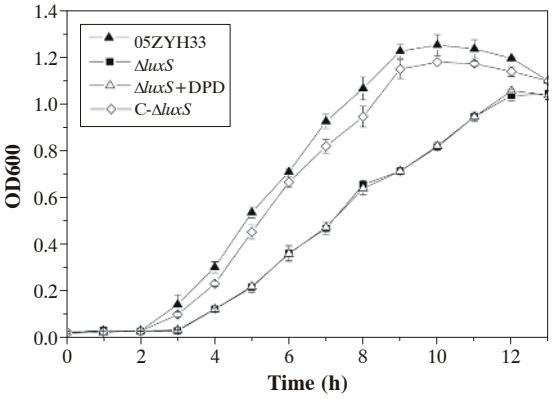

Fig. 4. Growth curves of the WT strain 05ZYH33, the Δ*luxS* mutant, Δ*luxS*+DPD and C-Δ*luxS*. The cell density is measured spectrometrically at 600 nm, and the records are collected at the indicated times. The results shown are representative of three independent experiments.

WT strain), but can be re-covered significantly by functional complementation (C-Δ*luxS*) to the level observed in the wild type (Fig. 4). It seemed that effects exerted on bacterial growth by LuxS are divergent or species-specific.

Role of *S. suis luxS* ortholog in AI-2 production

V. harveyi BB170, an indicator strain developed by Bashler *et al.* (Surette *et al.*, 1999), was subjected to test the ability of 05ZYH33, a human isolate of Chinese SS2 to produce AI-2. In much consistence with performence of strain HA9801 described by Han *et al.* (Han and Lu, 2009), we noted that AI-2 activity was apparently present in strain 05ZYH33 (Fig. 5A), indicating existence of a functional pathway for AI-2 production. Briefly, the dynamic activity of AI-2 behaved as follows: ascending during the logarithmic phase, peaking in the middle- to late-exponential phase and decreasing in the stationary phase gradually (data not shown). In contrast to around 800-fold AI-2 activity in the indicator strain *V. harveyi* BB170, the maximum AI-2 activity of strain 05ZYH33 was

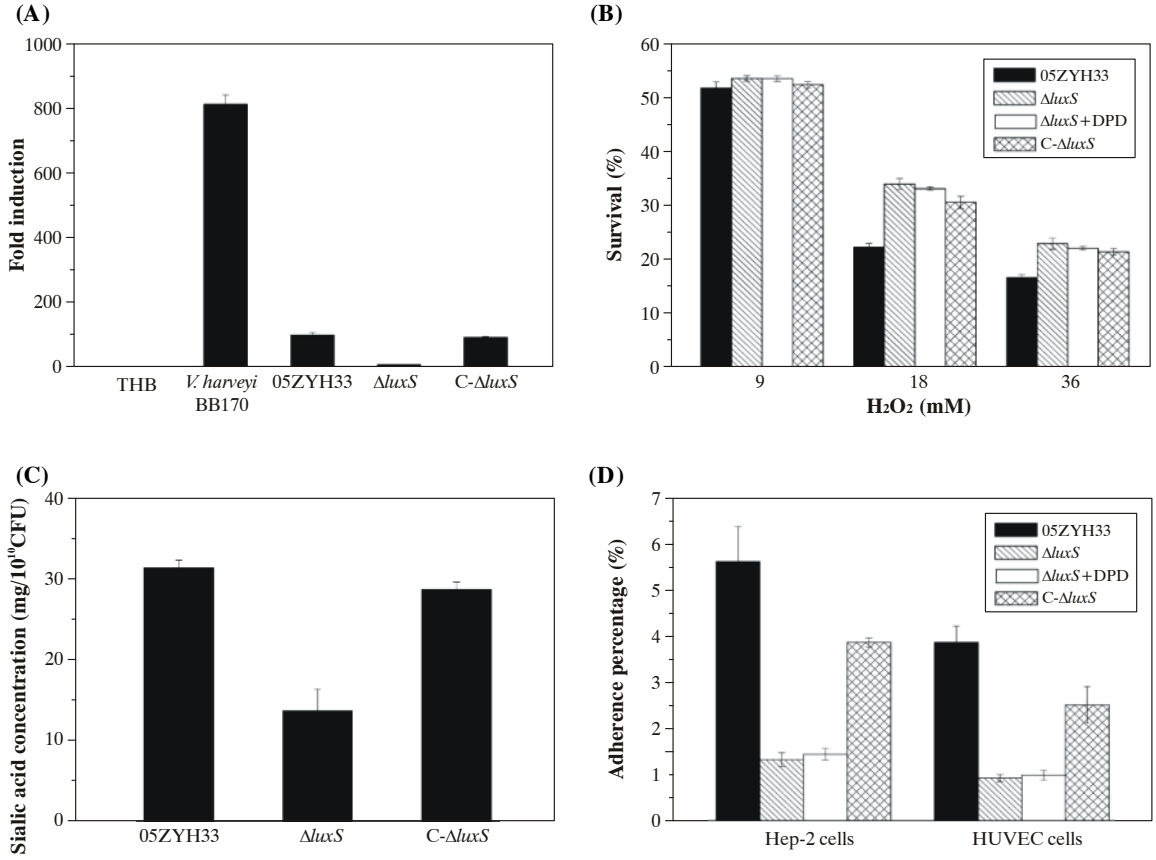

Fig. 5. Inactivation of *luxS* gene led to a wide range of phenotypic changes. (A) Loss of AI-2 activity in bacterial cell-free supernatants of ΔluxS mutant THB liquid medium served as negative control. All experiments were performed in triplicate. AI-2 activity was expressed as the n-fold induction of luminescence light units relative to that of negative control alone. (B) Survival percentage of *S. suis* strains (WT, Δ*luxS*, Δ*luxS*+DPD strain and C-Δ*luxS)* challenged with H_2O_2 at different concentrations. This experiment was repeated two times in triplicate. Results were expressed as the percentage of survival by dividing the number of CFU at different concentration of H_2O_2 with the initial number of CFU before the H_2O_2 challenge (concentration zero). Statistic analysis showed that $p<0.05$ at 9 mM H2O2, $p<0.01$ at 18 mM H_2O_2 and 36 mM H_2O_2, indicating that *luxS* mutation affects *S. suis* resistance to oxidative stress. No difference was seen among Δ*luxS* supplemented with DPD and C-Δ*luxS* ($p>0.05$). (C) Determination of effect of bacterial sialic acid contents by loss of LuxS. Difference the concentration of sialic acid between the wild type and the Δ*luxS* mutant is significant ($p<0.01$). (D) Decreased capability of bacterial adherence to mammalian cells caused by functional impairment of LuxS.

only about 80-fold (Fig. 5A), which is also greatly lower than the reported levels (>1,000 fold) in both *E. coli* and *S. enterica* (Surette *et al.*, 1999). We favored the bellowed two possibilities that might explain this scenario: 1) low expression level of AI-2 synthesis pathway-specific genes in *S. suis*, 2) inefficiency of SS2-producing autoinducer molecule recognized by *V. harveyi* BB170, somehow due to its unique chemical structure. Although it is also probable that some unknown inhibitors in the culture medium might interfere with the binding of the autoinducer to the receptor and in turn cause an antagonistic effect (Hjelmgaard *et al.*, 2003), we are not ameanale to address this question thus far. As we anticipated, the culture supernatant from the Δ*luxS* strain can only induce a luminescence signal 10-fold greater than that of the negative control, which is much lower than that of wild type (80-fold) (Fig. 5A). Also, the complemention strain C-Δ*luxS* can restore the AI-2 activity to the level of wild type strain (Fig. 5A). Definitely, it demonstrated that *S. suis luxS* is functional member responsible for AI-2 generations. Similar to those of *S. pyogenes* (Lyon *et al.*, 2001) and *P. gingivalis* (Yuan *et al.*, 2005), *S. suis luxS* did not thoroughly deprive the ability of luminescence production, indicating possible existence of a *luxS*-independent pathway in SS2. Retrospectively, Tavender and coauthors have ever suggested that LuxS-independent AI-2 formation might begin from ribulose-5-phosphate (Tavender *et al.*, 2008).

Correlation of reduced adherence ability of the Δ*luxS* mutant to its decreased bacterial sialic acid contents

In our trials, we failed to observe any obvious effect on the antibiotic susceptibility, acid-stress and haemolytic activity excerted by removal of *luxS* gene (data not shown). In contrast, divergent effects caused by *luxS* deletion have ever been reported in some other species, which included that 1) reduced antibiotic susceptibility in *S. anginosus* (Ahmed *et al.*, 2007); 2) increased sensitivity to acid killing in *S. mutans* (Fong *et al.*, 2001), while reduced acid tolerance in *S. pyogenes* (Siller *et al.*, 2008); 3) increased haemolytic activity in *S. pyogenes* (Lyon *et al.*, 2001),but decreased haemolytic activity in *V. vulnificus* (Kim *et al.*, 2003) and *S. marcescens* (Coulthurst *et al.,* 2004). In addition, the disruption of *luxS* gene from *S. suis* seemed to improve slightly its resistance to H_2O_2 challenge (18-36 mM) (Fig. 5B), which is somewhat in agreement with an observation in its closely relative, *S. mutans* (Lyon *et al.*, 2001). Intriguingly, the thiobarbituric acid tests clearly showed that the concentration of sialic acid in the Δ*luxS* mutant were decreased to half of wild type strain, and this deficiency in sialic acid synthesis can be complemented by introduction of plasmid-borne *luxS* gene into this mutant (Fig. 5C). It implied that the regulatory network of sialic acid biosynthesis pathway can be negotiated by AI-2 dependent quorum sensing. As we knew that sialic acids are major compoents of lipopolysacchrides localized on bacterial cell surface, and play possible roles in interface between pathogens and its infected host cells, we thereby attempted to test this possibility in this case. Cellular adhesion assays using two different cell lines [human epithelial (Hep-2) and endothelial (HUVEC) cells] demonstrated that the deletion of *luxS* significantly weakened the capability of *S. suis* adherence on its host cells, suggesting an involvement of LuxS into *S. suis* adherence, an important step prior to its successful invasion (Fig. 5D). Given that the attachment ability of Δ*luxS* strain to epidermal cells can be restored only by reintroducting a wild type copy of *luxS* gene, not an addition of DPD, a precursor of AI-2 production (Fig. 5D), we thereby anticipated that *S. suis* adherence is related to some unknown function of *luxS* gene rather than its general role in quorum sensing system. In light that sialic acids are required for efficient adhenrence of human pathogens like *Brucella* (Castaneda-Roldan *et al.*, 2004) and *Salmonella* (Sakarya *et al.*, 2010), it is reasonable to that *S. suis* LuxS control its adherence capability through modifying the contents of sialic acids on bacterial cell surface, as well as regulating expression of three major virulence-associated proteins, MRP (muramidase-released protein), FBP (Fibronectin-binding protein) and EPF (extra-cellular protein factor).

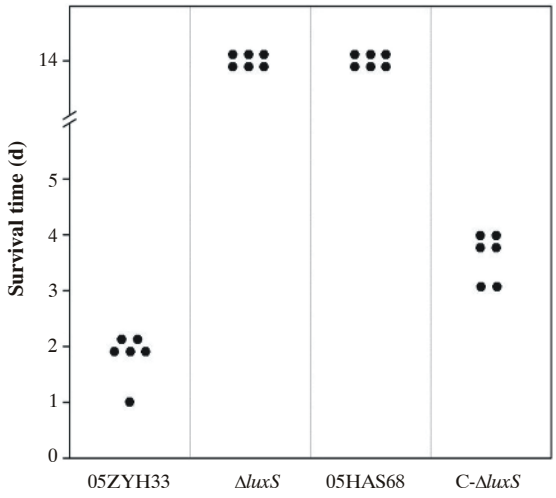

Fig. 6. Evaluation of contribution of LuxS to bacterial virulence using pig infection experiments. Groups of six SPF piglets were challenged intravenously with approximately 10^8 CFU of the indicated strains. The survival time for each piglet is indicated. Each datum point represents one piglet.

Requirement of LuxS for full virulence of *S. suis* 05ZYH33

To further evaluate possible role of LuxS in *S. suis* pathogenesis, experimental infection model of piglets, its natural host, was applied here. We observed that the six SPF-piglets inoculated with wild type virulent strain developed most of the typical disease symptoms (high fever, limping, swollen joints, shivering, central nervous system failure, and respiratory failure within 24 h). One of them died on day 1 and the other five were dead on day 2 post-infection (Fig. 6). Additionally, postmortem examination showed pathological changes in multiple organs and bacteria were recovered from all of the examined tissue specimens (data not shown). In the negative control group, the piglets infected with an avirulent strain 05HAS68, all survived (Fig. 6) and nearly did not exhibit any apparent clinical signs during the entire experiment. Not only were no bacteria detected in the specific tissues (except for tonsil) of these animals, but also no deaths occurred over a 14-day period of observation. In the Δ*luxS* mutant inoculated group, although the typical disease symptoms such

Table 2. DNA Primers in this study

Primers	Primer sequences	Primer Characteristics/ PCR product length	Functions
Primers for general PCR & gene cloning			
LA-F	5'-*GAATTC*ATTCGGACAGTTATACCAAAGC-3′	*Eco*RI	LA, upstream border of *luxS* (1,068 bp)
LA-R	5′-*GGATCC*CCAATCTAACAAAAAAAGC-3′	*Bam*HI	
RA-F	5′-*GTCGAC*GTGATGAATTTATTATACCA-3′	*Sal*I	RA, downstream border of *luxS* (1,125 bp)
RA-R	5′-*GCATGC*TAATCAAGTCTGGATGAAGG-3′	*sph*I	
luxS-F	5′-AGGCATCTGTAGAGATTCCC-3′	/	*luxS* gene (454 bp)
luxS-R	5′-GAAGTCACTGTCGAAAGCT-3′	/	
Spc-F	5′-*GGATCC*GTTCGTGAATACATGTTATA-3′	*Bam*HI	Spc^R gene (1,130 bp)
Spc-R	5′-*GTCGAC*TTTTCTAAAATCTGAT-3′	*Sal*I	
c-*luxS*1	5′-*GAATTC*CGCCGAAATATCAATGTTTCTA-3′	*Eco*RI	*luxS* gene with its putative upstream promoter and downstream sequence (730 bp).
c-*luxS*2	5′-*GGATCC*ATTTCTACCTCTTTTTTTATAA-3′	*Bam*HI	
LU	5′-GCAGAACTGAAAGTGCTG-3′	/	Spc^R gene together with LA and RA segments (2,751 bp)
RD	5′-GAGGTACGGAAATGCAAACG-3′	/	
qPCR primers			
0155-F	5′-GTGACCAAATGGTTCTTGAC-3′	261 bp	*gapdh*
0155-R	5′-ATTCAGTAGCAGCAGCTTTC-3′		
0390-F	5′-GCTGGCTACATTGGCTACGAT-3′	269 bp	*clp*
0390-R	5′-TGCGGTCCATGATGTCTTGAA-3′		
0394-F	5′-TATCACTTTCCGCCGTTGGTT-3′	285 bp	*glgP*
0394-R	5′-GCATTCATTTGTTGGCGTTTGTA-3′		
0565-F	5′-CGGAATTGAATCTGAAAGAG-3′	262 bp	*cps2B*
0565-R	5′-CCAGCTAATAAACCAAGCAA-3′		
0573-F	5′-GGAATACGCAGAGCAAGATG-3′	205 bp	*cps2J*
0573-R	5′-AAGTAACCCTCCCGACAAAT-3′		
0581-F	5′-CCACGTTGTTAGCTTTACCA-3′	237 bp	*neuA*
0581-R	5′-ATCGAATCTTCCTTCGTCAT-3′		
0613-F	5-TTGCGGCTATGAGTAAGATTGTCG-3′	242 bp	*aspB*
0613-R	5′-GCCCTTCATTTCCATAGCCTTTTT-3′		
0753-F	5′-TATAAGGGTGAAGTAGATT-3′	273 bp	*mrp*
0753-R	5′-CCTGAGTAGCGATATAGATTTTTC-3′		
1046-F	5′-TGGCCACTAGTCAAGAGGAGGAAG-3′	261 bp	hypothetical protein
1046-F	5′-TGTCGAGCGAGATGCCAACTAACT-3′		
1491-F	5′-TTGCCAAATATTATAAAGAGGAG-3′	258 bp	*licT*
1491-R	5′-GAATCGGTAGTAATCAATAGAATC-3′		
2173-F	5′-CCTTTTAACTGCGGGTGTAG-3′	236 bp	*lysM* repeat
2173-R	5′-CTGTTGCGGTAGAAGTTGATG-3′		

The underlined italic sequences are the restriction sites.
/, Absence of the restriction sites.

as fever, limp were developed, symptoms were less serious compared with the wild type 05ZYH33 and all the six piglets survived until the end of the experiment (Fig. 6). However, all of the piglets infected with the complemented strain C-Δ*luxS* died within 3 to 4 days, nearly to the median survival time observed in the WT group (Fig. 6). Collectively, we can anticipate that the deletion of *luxS* gene in *S. suis* impairs bacterial adherence ability which might contribute somewhat to attenuation of its virulence.

Global regulation of LuxS

Genome-wide transcriptome of *S. suis* was revealed using microarray analyses (Tables 3, 4, and S1) (Feng *et al.*, 2008), and the reliability of microarray data was verified by qPCR assays (Table 3). We observed that 71 genes in the Δ*luxS* mutant are differentially expressed in response to the addition of DPD (Table S2). Among them, 29 genes was restored by DPD supplementation in the Δ*luxS* mutant (Table S2), which is associated with cell division, capsular polysaccharide synthesis, DNA modifications, *etc*. Totally, 14.5% of all the putative genes encoded in SS2 genome are affected by the *luxS* mutation, of which 144 genes are up-regulated, and 168 are down-regulated (Table 4 and S1). These genes were categorized into the following various functions: metabolism, transcriptional regulators, virulence-related factors etc. (Table 4). First we noted decreased expression of known virulence-related determinants: CPS biosynthesis locus (05SSU0564–05SSU0573), sialic acid synthase (05SSU0581); MRP (05SSU0753); EPF (05SSU0177) are less abundant in *luxS* mutant, which is somewhat consistent with the attenuation of virulence by *luxS*

Table 3. qPCR-based validation of microarray data

Gene code	Functional annotation	Average ratio[a] Δ*luxS*/WT	
		Micro-array	RT-PCR
05SSU0390	ATPases with chaperone activity	3.007	3.440
05SSU0394	glucan phosphorylase	5.937	7.068
05SSU0565	capsular polysaccharide biosynthesis protein Cps2B	0.193	0.171
05SSU0573	capsular polysaccharide biosynthesis protein Cps2J	0.161	0.137
05SSU0581	CMP-N-acetylneuraminic acid synthetase	0.470	0.34
05SSU0613	aspartate/tyrosine/aromatic aminotransferase	0.116	0.104
05SSU0753	MRP	0.478	0.385
05SSU1046	an integrase-like protein with N-terminal SAM-like domain	21.430	25.517
05SSU1491	transcriptional antiterminator	7.614	8.382
05SSU2173	LysM repeat	11.018	10.498

[a] The GAPDH gene (a housekeeping gene) served as the reference gene for real-time quantitative RT-PCR experiments. For the 10 randomly selected genes, the quantitative RT-PCR results are relatively in accordance with the micro-array data. These data represents the means of triplicate values.

mutation. Second, we identified three transcription factors down-regulated in the Δ*luxS* mutant, which have ever been determined to be related with pathogenicity of *S. suis* and other pathogens. These regulators included RevS orphan response regulator (de Greeff *et al.*, 2002); *05SSU1630*, a homolog of PadR regulator of phenolic acid metabolism in *Lactobacillus plantarum* (Gury *et al.*, 2004) and *V. cholerae* (Kovacikova *et al.*, 2003) [Of note, PadR is regulated by quorum sensing in *Vibrio* (Kovacikova and Skorupski, 2002)]; plus *05SSU1372*, a catabolite control protein A (CcpA) (Willenborg *et al.*, 2011). Third, a conserved cell division related FtsA homologue (*05SSU0480*) (Paradis-Bleau *et al.*, 2005; Geissler *et al.*, 2007), and *05SSU0019,* a cell shape-determining protein (MreC) (Divakaruni *et al.*, 2007) both were activated by LuxS, which validates the morphological changes observed with the Δ*luxS* mutant (Fig. 3). Finally, expression of a collection of heat shock proteins (HSP)-encoding genes are elevated significantly due to *luxS* disruption, such as Hsp33 (05SSU0617), ClpL (05SSU0389), and ClpE (05SSU0492) (Table S1). The activation of Hsp33 (05SSU0617), a molecular chaperone protecting cells against oxidative protein aggregation (Kumsta and Jakob, 2009) is believed to agree with the increased oxidative tolerance of the *luxS* mutant strain (Fig. 5B). ClpL is essential for virulence of *S. mutans* (Kajfasz *et al.*, 2009) and

Table 4. Summary of gene categories with differential transcription in the Δ*luxS* mutant relative to its parental strain 05ZYH33

	No. of genes	
	Up-regulation	Down-regulation
1. Enzymes		
1.1 Cellular processes and signaling	3	13
1.2 Metabolism	38	25
1.3 Information storage and processing	12	14
1.4 Poorly characterized	1	4
2. ABC-type transport systems		
2.1 Cell processes and signaling	1	4
2.2 Metabolism	6	12
2.3 Poorly characterized	1	1
3. Transcriptional regulators	6	10
4. Virulence-related factors		
4.1 Capsular polysaccharide-related protein	-	8
4.2 Virulence related membrane proteins	-	3
4.3 CMP-N-acetylneuraminic acid synthetase	-	1
4.4 Chaperonin	8	1
5. Other proteins		
5.1 Cell processes and signaling	3	9
5.2 Metabolism	12	5
5.3 Information storage and processing	7	29
5.4 Poorly characterized	21	11
5.5 Unknown or hypothetical protein	25	18
Total	144	168

The data are from DNA microarray-based analyses.

also modulates the adherence, invasion, and tumor necrosis factor alpha secretion during the early stages of infection of *S. pneumoniae* (Tu le *et al.*, 2007). ClpE is necessary for the virulence of the ClpE mutant of *S. pneumoniae* strain D39 (Zhang *et al.*, 2009). It seemed that the positive control of virulence by ClpE in *S. pneumoniae* contradicts our results that ClpE is upregulated while the virulence is attenuated. It also makes sense that ClpE may function differently in different bacteria. Although we lacked solid experimental evidence to explain the exact role of the HSPs in SS2 virulence, the fact that inactivation of *luxS* led to disorder of HSP expression level might provide us clues to follow-up functional studies on the issue of SS2 pathogenesis.

Discussion

Using integrated approaches from bioinformatics, bacterial genetics, to cells/animals-based assays, we systematically define biological roles of luxS gene in the context of *S. suis* 05ZYH33, which is somehow complemented/or validated by partial evidence from the other strain HA9801 described by Wang *et al.* (Wang *et al.*, 2011) nearly at the same time. In our study, we showed the modeled structure of *S. suis* luxS as direct physical evidence (Fig. 1). In particular, we demonstrated that the Δ*luxS* mutant exhibited reduced pathogenicity in a piglet-challenge model (Fig. 6) and the infected piglets showed less serious clinical symptoms and pathological damage (data not shown). In contrast, that Lu.'s group reported evidence for role of luxS in SS2 virulence is mainly dependent on model of zebrafish (Wang *et al.*, 2011), which can not fully represent or replace studies conducted with its natural host piglets. For the first time, we presented a genome-wide glimpse of LuxS-mediated regulation esp. in virulence manifestation of *S. suis* 05ZYH33, providing an useful regulatory network linking luxS and a variety of target genes with known virulence factors included (Table 4). It is of much interest to evaluate the potential of these target genes as subunit vaccines, as well as its implications for development of new antibacterial drug targets. According to our data obtained at different levels in this work combined with informations gained by Lu's group (Wang *et al.*, 2011), we are quite certain that requirement of LuxS for virulecence of *S. suis* 2 might in part depend on its roles in biofilm formation with associated with sialic acid metabolism.

Apparently, the interference of expressions of virulence factors by inactivation of LuxS also contributed significantly to virulence attenuation in the the Δ*luxS* mutant (Fig. 6). However we are still lacking insightful knowledge on luxS/AI-2 quorum sensing system in the specie of *S. suis* 05ZYH33, i.e., a bunch of questions on this topic is remaining open. What receptor responds to chemical signal outside of *S. suis*? How about the chemical structure of AI-2 used for inter-species-communication amongst SS2 populations? We also know nothing about intra-species cell-density signaling for SS2. It also could be a challenge to obtain structural insights into LuxS/AI-2 interaction or AI-2 complexed with its receptor. It is interest to test the possibility of post-transcriptional regulation of LuxS/AI-2 in *S. suis*. Definitely, it might represent a new research direction of *S. suis*, and contributed greatly to its physiology and pathogenesis.

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