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LuxS/AI-2 system is involved in fluoroquinolones susceptibility in *Streptococcus suis* through overexpression of efflux pump SatAB

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ABSTRACT

Increasing resistance to fluoroquinolones (FQs), such as norfloxacin and enrofloxacin, supports the need for the discovery of novel molecules and alternative approaches in antimicrobial therapy. Quorum sensing (QS) is a promising target for next-generation anti-infective agents designed to address the evolving drug resistance in bacterial pathogens. Given that the LuxS/autoinducer-2 (AI-2) quorum-sensing system regulates microbial group behaviors, we hypothesized that this system influences the FQ susceptibility in *Streptococcus suis*. It was found that a *luxS* mutant (Δ*luxS*) of *S. suis* possesses an increased susceptibility to FQs compared to the wild type strain. When grown in the presence of sub-MIC of antibiotics, the Δ*luxS* strain showed a significant decrease in growth rate and biofilm formation. These results suggest that the FQ resistance in *S. suis* could involve a signaling mechanism associated with the LuxS/AI-2 quorum-sensing system. HPLC (High Performance Liquid Chromatography) analyses showed a significant increase in the intracellular accumulation of enrofloxacin in the Δ*luxS* strain compared to the wild type strain. This increase was less pronounced in the presence of exogenous AI-2. Moreover, the expression of *satA* and *satB* genes was decreased in the Δ*luxS* strain. Exogenous AI-2 reversed the down-regulated gene expression observed in the Δ*luxS* strain. Our study brought strong evidence that the LuxS/AI-2 system in *S. suis* is involved in FQ susceptibility by regulating the efflux pump SatAB. LuxS is highly conserved among Gram-positive bacteria and may therefore represent a novel antimicrobial target for an alternative approach in antimicrobial therapy.

1. Introduction

Streptococcus suis is a major swine bacterial pathogen and an emerging zoonotic agent resulted in large economic losses to the swine industry worldwide (Fulde and Valentin-Weigand, 2013; Gruszynski et al., 2015). The number of human and pigs cases of *S. suis* infections has significantly increased worldwide in recent years (Dutkiewicz et al., 2017; Gomez-Torres et al., 2017; Rajahram et al., 2017; Yanase et al., 2018). One of the main causes of persistent infection in *S. suis* is the appearance of multi-drug resistant (MDR) strains, showing resistance to several key antibiotics, including fluoroquinolones, tetracyclines, and aminoglycosides (Bojarska et al., 2016; Zheng et al., 2018). Fluoroquinolones (FQs), such as norfloxacin and enrofloxacin, are among the most effective antibiotics to treat of *S. suis* infections, consequently, this has contributed to the increase in FQ resistance in *S. suis* (Haenni et al., 2018).

Antimicrobial resistance is a major threat for the effective control of infectious diseases. FQ resistance mechanisms in *S. suis* have been associated with point mutations in specific regions of the GyrA subunit of the DNA gyrase and ParC subunit of DNA topoisomerase IV (Escudero et al., 2007). In addition, some reports have demonstrated that the SatAB, ABC transporter homologous of the pneumococcal FQ efflux pumps PatA and PatB, may also contribute to FQ resistance in *S. suis* (Escudero et al., 2011). Lastly, SmrA, a major facilitator superfamily transporter homologous to FQ efflux pump PmrA, may also be involved in FQ resistance (Al-Hamad et al., 2009). Taking into account that FQs are the most widely used antibiotics worldwide for treating *S. suis* human infections (Galbarro et al., 2009), there is a need to explore alternative approaches in antimicrobial therapy.

Cell-cell communication among bacteria plays vital roles for their

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adaption and survival in the environment (Fuqua et al., 1994; Waters and Bassler, 2005). Quorum sensing (QS) is a mode of cell-cell communication that allows bacteria to monitor the density of the population and regulate their gene expression accordingly (Bassler and Losick, 2006; Orchard and Goodrich-Blair, 2004). One of the QS signaling molecules is autoinducer-2 (AI-2), which is produced through the S-adenosylmethionine metabolism. LuxS, a key enzyme in AI-2 production, is highly conserved and widely distributed in bacteria. Our research group showed that *S. suis* possesses a LuxS homologue and that inactivation of *luxS* gene resulted in a mutant with a significantly reduced ability to form biofilm, to adhere, and to cause infections in an animal model (Wang et al., 2013, 2014; Wang et al., 2011a, b). Recently, the presence of a quorum-sensing system in bacteria has been associated with antibiotic resistance (Asfour, 2018; Garcia-Contreras et al., 2016; Zhang et al., 2018). Therefore, the aim of the present study was to evaluate whether the LuxS/AI-2 system may be involved in the antibiotic susceptibility of *S. suis*.

2. Material and methods

2.1. Bacterial strains and antibiotics

Two *S. suis* strains were used in this study; a wild-type (WT) strain isolated from an infected pig in the Jiangsu Province and confirmed to be a virulent strain (Yao et al., 1999), and its *luxS* mutant (Δ*luxS*) prepared in a previous study (Wang et al., 2011b). Bacterial cells were stored at −20 °C. Before each experiment, colonies were used to inoculate tryptone soy broth (TSB) which was incubated overnight. The AI-2 precursor molecule, DPD, was purchased from Omm Scientific Inc. (Dallas, TX) and performed at 3.9 μM (Our previous research data) (Xue et al., 2013). Norfloxacin and enrofloxacin were purchased from China Institute of Veterinary Drugs Control (Beijing, China). Stock solutions (1280 mg/L) of antibiotics were stored at −20 °C.

2.2. MIC and MBC determinations

Minimal inhibitory concentrations (MICs) of norfloxacin and enrofloxacin were determined with a microbroth dilution technique as described by the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2015). Briefly, an overnight culture of bacteria was diluted in fresh TSB to obtain a concentration of 10^5 CFU/mL. Serial dilutions of antibiotics (640 to 0.0625 mg/L) were prepared in culture medium and added (100 μL) into wells of a 96-well microplate (Corning/Costar, NY, USA). One hundred μL of the bacterial suspension were then added. To verify the relationship between AI-2 signaling and altered antibiotic susceptibility, asssays using the culture medium supplemented with exogenous AI-2 were also performed (Xue et al., 2013). The plates were incubated at 37 °C for 24 h. The MIC was defined as the lowest concentration of antibiotic showing no visible growth. Ten μL from each well showing no visible growth was subcultured on culture plates. Resultant colonies were counted after an overnight incubation at 37 °C. The minimal bactericidal concentration (MBC) was defined as the lowest concentration of antibiotic producing at least a 99.9% killing of the initial inoculum.

2.3. Growth curves

The effect of an antibiotic pressure on growth rate of *S. suis* was determined. A single colony of the *S. suis* WT and Δ*luxS* strains were picked and inoculated into 3 mL of TSB medium. Following an overnight incubation, these cultures were used to inoculate TSB medium containing norfloxacin or enrofloxacin at sub-MICs (1/2 MIC, 1/4 MIC, 1/8 MIC, or 1/16 MIC). The control sample was cultured without antibiotics. The optical density at 600 nm (OD_{600}) of the cultures was monitored every h to produce a growth curve.

2.4. Biofilm assay

Biofilm formation in the presence of antibiotics at sub-MICs was determined by the microtiter plate assay based on the method described in our previous report (Wang et al., 2011b). TSB medium without or with norfloxacin or enrofloxacin at concentrations ranging from of 3.5 to 0.5 mg/L was dispensed into wells of 96-well microtiter plates. The control sample was cultured without antibiotics. Overnight cultures of *S. suis* WT and Δ*luxS* strains were diluted 1:100 in fresh TSB medium at a final concentration of 10^6 CFU/mL and used to inoculate wells of the microplates. The effect of adding exogenous AI-2 on biofilm formation was also evaluated. Crystal violet staining was performed to quantify biofilm. The absorbance of each well was determined at 570 nm using a spectrophotometer (Multiskan FC, Thermo, China). All assays were performed in triplicate and repeated three times.

2.5. Time-kill study

The time-kill kinetics of *S. suis* WT and Δ*luxS* strains in the presence of norfloxacin or enrofloxacin were determined. Cells from 24-h cultures were harvested by centrifugation and suspended in phosphate-buffered saline (PBS, pH 7.2) to a final concentration of 1×10^6 cfu/mL in the presence of norfloxacin at 20, 10, 5, and 2.5 mg/L or enrofloxacin at 5, 2.5, 1.25, and 0.625 mg/L. The control sample was cultured without antibiotics. Time-kill assays in the presence of exogenous AI-2 were also performed. At specific time points (0, 1, 2, 3, 4, 6, 8, and 12 h), 10-μL samples were taken aseptically for determination of bacterial counts on TSB culture plates. The colonies were counted only on plates that had between 30 and 300 colonies. Three independent experiments were performed to ensure reproducibility.

2.6. Detection of intracellular enrofloxacin concentrations by HPLC

Accumulation of enrofloxacin into cells of *S. suis* WT and Δ*luxS* strains, in absence and presence of AI-2, was determined by HPLC. Bacterial strains were cultured in TSB medium to an OD_{600} of 0.8 and then incubated with enrofloxacin (200 μg/L) at 37 °C. Aliquots of 1.5 mL were taken out at 0, 5, 10, 20, 40, 60, 120, and 180 min. Cells were harvested by centrifugation (8,000 g for 5 min), and washed three times with 1 mL of PBS (pH 7.0). The wet weight of bacteria was adjusted to 40 mg, and cells were lysed by repeated freezing-thawing cycles and with sonication. The cell lysate was subjected to centrifugation at 12,000 g for 10 min. The supernatant was recovered and 1 mL of ethyl acetate was added. The mixture was then shaken for 20 min and after standing for 10 min, the ethyl acetate layer was harvested. The extraction procedure was repeated and the two extracts were combined and evaporated to dryness in a water bath (45 °C) under a gentle stream of nitrogen gas. One mL of mobile phase standard working solution was added; after shaking for 2 min to fully dissolve the extracted material, the solution was filtered. The concentration of enrofloxacin was determined by HPLC using an X Bridge™ C185 column (150 mm × 4.6 mm, 5 μm; the mobile phase was a mixture of acetonitrile: 0.05 mol/L H_3PO_4 (pH = 3) with a ratio of 80:13, v/v, a flow rate of 1.0 mL/min, and UV detection at a wavelength of 280 nm. The concentration of enrofloxacin was calculated by comparison with a standard curve of enrofloxacin (0.025–2.0 mg/L). Determination of intracellular enrofloxacin was performed on at least three separate experiments.

2.7. Detection of mRNA expression of related efflux pumps

RT-PCR was used to analyze the relative expression of relevant efflux pump genes, including *satA*, *satB* and *smrA*. The primers used were designed using Primer premier 5 software and are listed in Table 1. Overnight cultures of *S. suis* WT and Δ*luxS* strains, in the presence and absence of exogenous AI-2 were diluted 1:100 in fresh TSB and grown

Table 1
Primers used for the quantitative RT-PCR analysis.

Genes	Primer sequence
SatA	GCTCA TTCGA CCAAC CGTAT
	CACCA CTTGG GTCAA GGACT
SatB	TCGGC TCCGT ATCGT GT
	TCGGT GGCTT TACTT CC
SmrA	AAGCA GAATT TGAAG GTG
	AAGGG CATTA ACAGA TACCG

at 37 °C with shaking for 5 h. Bacteria were collected by centrifugation (10,000 × g for 5 min). Total RNA was extracted using the TRIzon (ComWin Biotech Co.,Ltd, Biejing, China) method. The RNA was subjected to a DNase I (Promega, Madison, USA) treatment to remove DNA contamination. The cDNA synthesis was performed using the PrimeScript™ reagent kit (ComWin Biotech Co.,Ltd, Biejing, China) following the manufacturer's directions. Relative expression was normalized using the 16S rRNA gene as the endogenous control (Wang et al., 2011a). Real-time PCR was performed as follows: initial denaturation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 15 s, annealing and extending at 60 °C for 1 min in each cycle. Melting curve analysis were performed at 95 °C for 15 s 60 °C for 1 min, 95 °C for 15 s, and 60 °C for 15 s. To assess amplification specificity, a melting curve analysis was drawn at the end of each PCR run. The $2^{-\Delta\Delta CT}$ method was calculated and analyzed the threshold cycle values (CT) obtained from the melting curve (Wang et al., 2011b). Each sample was analyzed in duplicate, and expression data was collected from three biological replicates.

2.8. Statistical analysis

Statistical analyses were carried out using the Graphpad Software package (GraphPad Software, La Jolla, CA). One-way analysis of variance (ANOVA) followed by the Dunnett's multiple comparison test was used for biofilm formation and CFU counts. The mean values are shown in the figures. Where appropriate, the data were analyzed using the Student's t test, and a value of $P \leq 0.05$ was considered significant. Pairwise comparisons with $P \leq 0.05$ were considered statistically significant; $P \leq 0.01$ was considered extremely significant.

3. Result

3.1. Antibiotic susceptibility

The MIC and MBC values of enrofloxacin and norfloxacin for *S. suis* are presented in Table 2. The Δ*luxS* strain showed an increased susceptibility to both antibiotics compared to the wild type strain. The MIC values of enrofloxacin and norfloxacin were 1.25 mg/L and 5 mg/L, respectively for the WT strain and 0.625 and 2.5 mg/L, respectively for the Δ*luxS* strain. When AI-2 was added to culture medium, the mutant Δ*luxS* showed a decreased susceptibility to enrofloxacin (1.25 vs 0.625 mg/L) and norfloxacin (10 vs 2.5 mg/L). The WT strain was also less susceptible to both antibiotics in the presence of AI-2.

Table 2
MIC and MBC of enrofloxacin and norfloxacin for *S. suis*.

Strain	MIC(mg/L)		MBC(mg/L)	
	norfloxacin	enrofloxacin	norfloxacin	enrofloxacin
WT[a]	5	1.25	20	1.25
Δ*luxS*[b]	2.5	0.625	5	0.625
WT + AI-2[c]	20	2.5	40	10
Δ*luxS* + AI-2[d]	10	1.25	20	2.5

[a] WT = wild-type strain.
[b] Δ*luxS* = *luxS* mutant.
[c] WT + AI-2 = wild-type strain with exogenous 3.9 μM AI-2.
[d] Δ*luxS* + AI-2 = *luxS* mutant with exogenous 3.9 μM AI-2.

3.2. Bacterial growth curve

Growth curves of *S. suis* WT and Δ*luxS* strains in the presence of enrofloxacin and norfloxacin are presented in Figures S1 and S2 (Supplementary material). The growth of the WT strain was attenuated by 1.25 mg/L of norfloxacin, and 0.625 mg/L of enrofloxacin, while Δ*luxS* strain was attenuated by 0.625 mg/L of norfloxacin (Supplementary material, Figure S1), and 0.316 mg/L of enrofloxacin (Supplementary material, Figure S2). The difference in the growth curves between WT and Δ*luxS* strains with exogenous AI-2 in the presence of antibiotics was already evident at early exponential growth phase and continued throughout stationary phase.

3.3. Biofilm formation in the presence of antibiotics

Antibiotics at sub-MICs increased biofilm formation by *S. suis* WT strain. In the absence of antibiotics, the Δ*luxS* strain displayed an approximately 40% reduction in the level of biofilm formation compared to that of the WT strain (Fig. 1). The level of biofilm formation by the WT significantly increased by 20–30% in the presence of 0.50–3.5 mg/L of norfloxacin or 0.05 to 0.30 mg/L of enrofloxacin (Fig. 1a and b). At a corresponding range of sub-MIC of norfloxacin, biofilm formation by the Δ*luxS* strain was either unaffected or slightly reduced (Fig. 1a). However, when exogenous AI-2 was added, both WT and Δ*luxS* strains showed an increased ability to form biofilm.

3.4. Time-kill curve of norfloxacin and enrofloxacin

Time-kill curve analyses of norfloxacin and enrofloxacin were performed against *S. suis* WT and Δ*luxS* strains. The Δ*luxS* strain was found to be more susceptible to both antibiotics than the WT strain (Supplementary material, Figure S3 and S4). More specifically, for the Δ*luxS* strain, norfloxacin exerted bacteriostatic and bactericidal effects at concentrations of 2.5 mg/L and 5 mg/L, respectively (Supplementary material, Figure S3), while for the WT strain, bacteriostatic and bactericidal effects were observed at concentrations of 10 mg/L and 20 mg/L, respectively (Supplementary material, Figure S3). When exogenous AI-2 added to AI-2, the antibacterial and bactericidal effects of norfloxacin against WT and Δ*luxS* strains were significantly decreased. With regard to enrofloxacin, similar tendencies were observed for WT and Δ*luxS* strain (Supplementary material, Figure S4).

3.5. Intracellular enrofloxacin accumulation

Intracellular accumulation of enrofloxacin in *S. suis* WT and Δ*luxS* strains were monitored by HPLC. Enrofloxacin accumulation was found to be drastically lower in the WT strain compared to the Δ*luxS* strain. More specifically, the WT strain accumulated about 2-fold less enrofloxacin than the Δ*luxS* strain after 10, 20, 40, 60 and 120 min exposures to the antibiotic (Fig. 2). Intracellular enrofloxacin concentrations reached a peak after 5 min in the WT strain (exogenous AI-2) while it reached a peak after 10 min in the Δ*luxS* (exogenous AI-2) (Fig. 2). When exogenous AI-2 was added, the accumulation of enrofloxacin WT and Δ*luxS* strains decreased more particularly following a 1-h exposure; this suggests that AI-2 may increase the efflux pump activity.

3.6. Expression of quinolone efflux pump genes

To determine whether the LuxS/AI-2 system could influence the susceptibility of *S. suis* to quinolones by regulating the efflux pump, we analyzed by real-time RT-PCR the mRNA expression of genes

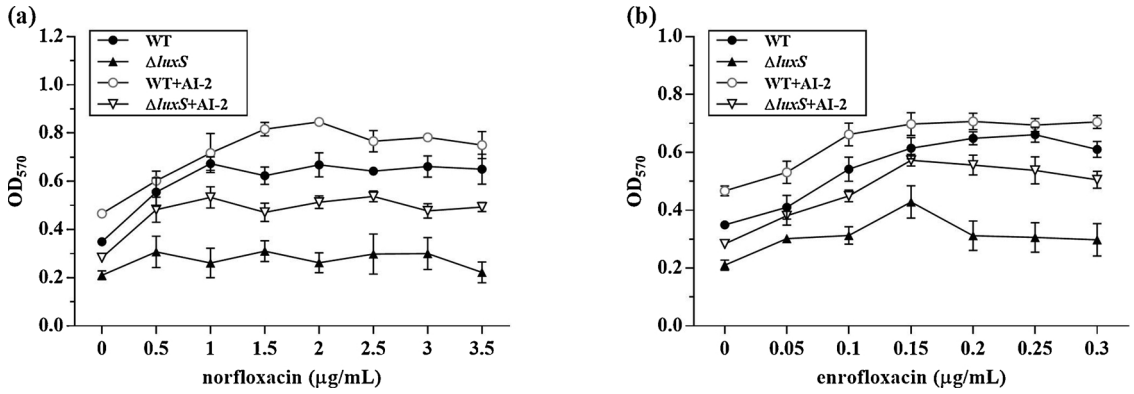

Fig. 1. Biofilm formation in the presence of norfloxacin (a) and enrofloxacin (b). The wild-type (WT) strain, the *luxS* mutant (Δ*luxS*), the wild-type strain with exogenous 3.9 μM AI-2 (WT + AI-2), and the *luxS* mutant with exogenous 3.9 μM AI-2 (Δ*luxS* + AI-2) were grown in the presence of different concentrations of norfloxacin and enrofloxacin for 24 h at 37°C prior to determine biofilm formation by crystal violet staining. The control sample was cultured without antibiotics. The data points represent mean values (n = 3) optical density at 570 nm.

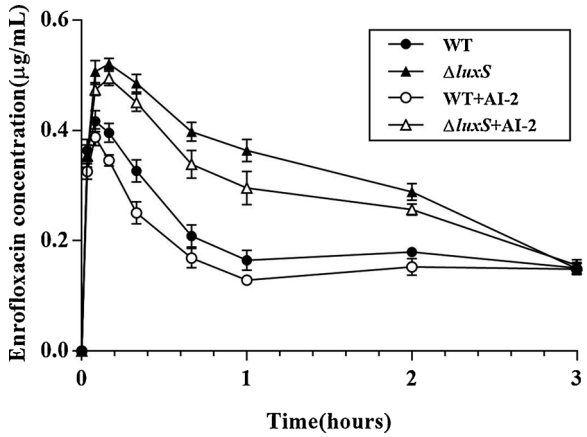

Fig. 2. Intracellular accumulation of enrofloxacin. The wild-type (WT) strain, the *luxS* mutant (Δ*luxS*), the wild-type strain with exogenous 3.9 μM AI-2 (WT + AI-2), and the *luxS* mutant with 3.9 μM AI-2 (Δ*luxS* + AI-2) were incubated in the presence of enrofloxacin (200 mg/L) and intracellular enrofloxacin was determined by HPLC after 0, 2, 5, 10, 20, 40, 60, 120, and 180 min. Each value represents the mean ± SD from three experiments.

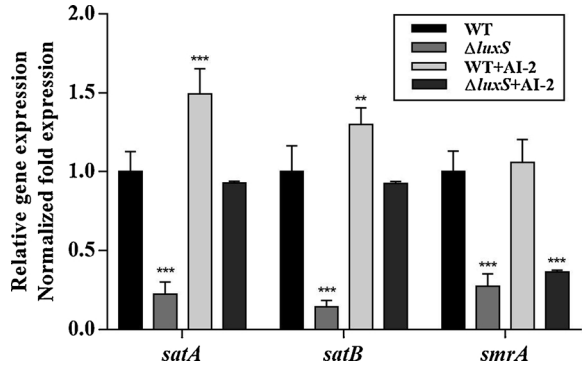

Fig. 3. Expression levels of *satA*, *satB* and *smrA* in *S. suis*. The wild-type (WT) strain, the *luxS* mutant (Δ*luxS*), the wild-type strain with 3.9 μM AI-2 (WT + AI-2) and the *luxS* mutant with 3.9 μM AI-2 (Δ*luxS* + AI-2) were analyzed by real-time RT-PCR. Data are expressed as means ± standard deviations. The expression was normalized to 16S rRNA. Statistical analysis was done using the two-tailed Student's *t*-test to compare the expression levels of *satA, satB,* and *smrA*. Significantly different (**$p < 0.01$; ***$p < 0.001$) compared to untreated control bacteria.

corresponding to the efflux pump proteins *satA, satB* and *smrA*. The mRNA expression levels of *satA, satB* and *smrA* were significantly decreased in the Δ*luxS* strain, respectively in comparison to the WT strain. The mRNA expression levels of *satA* and *satB* significantly increased by in the WT strain exposed to exogenous AI-2 compared to the control (absence of AI-2) (Fig. 3). The mRNA expression levels of *satA, satB* and *smrA* were not significantly different between the WT strain and the Δ*luxS* strain exposed to AI-2. This suggests that the LuxS/AI-2 signalling might be associated with the quinolone antibiotic susceptibility by regulating the efflux pump in *S. suis*.

4. Discussion

Bacteria use QS systems to coordinate gene expression in response to cell density. The LuxS/ AI-2 quorum-sensing system which consists of the LuxS enzyme responsible for AI-2 production has been recognized as a universal signaling molecule for bacterial communication (Vendeville et al., 2005). In a previous study, we brought evidence for a role of *luxS* gene in biofilm formation in *S. suis* (Wang et al., 2011b). We further showed that exogenous AI-2 acted as a concentration-dependent signaling molecule to regulate *S. suis* biofilm formation, host-cell adherence, and transcription levels of many virulence genes (Wang et al., 2014). These findings confirmed that LuxS/AI-2 is a complex network that regulate several functions in *S. suis*. However, the relationship between the LuxS/AI-2 system and antibiotic resistance in *S. suis* has not been investigated. Our present work demonstrated that *luxS* inactivation results in a mutant with an increased susceptibility to both norfloxacin and enrofloxacin compared to the wild type strain. Moreover, the *luxS* mutant had decreased growth rate at sub-MICs of antibiotics. In addition, it was found that deletion of *luxS* resulted in stronger bactericidal effects of norfloxacin and enrofloxacin. Of importance, all the above effects could be complemented by the presence of exogenous AI-2. Although the Δ*luxS* strain grew normally in low concentrations of quinolone antibiotics, there was a major growth defect in higher concentration, that could be complemented with exogenous AI-2. These results suggest that the susceptibility of Δ*luxS* to antibiotic could be attributed to the loss of a signaling mechanism associated with the AI-2 molecule.

In our study, the LuxS/AI-2 system appears to influence the quinolone susceptibility of *S. suis*. Xue et al. previously reported that the LuxS/AI-2 system is involved in vancomycin, teicoplanin, penicillin G, and oxacillin susceptibility in *Staphylococcus aureus* (Xue et al., 2013). Since it has been shown that LuxS/AI-2 system affect antibiotic susceptibility, this then leads to the question of what are the specific regulatory mechanism. In Gram-negative bacteria such as *Escherichia coli* and *Salmonella* spp., AI-2 is imported by the Lsr transport system, which is repressed in the absence of an AI-2 signal by LsrR (Pereira et al., 2012). It has been suggested that the sensitivity of bacteria to

antibiotics is due to the up-regulation of their LsrR, however, Gram-positive bacteria lack any homologues of either the Lsr permease or the LuxP/LuxQ phosphorelay system. Gram-positive species have not been confirmed that specific mechanisms for regulating bacterial susceptibility. Few studies have clearly addressed the relationship between QS systems and antimicrobial susceptibility mechanisms. We found that the LuxS/AI-2 signalling might be associated with the quinolone efflux pump in *S. suis*.

HPLC analyses showed significant differences in the intracellular accumulation of enrofloxacin in the WT and Δ*luxS* strains. When exogenous AI-2 was added, the concentration of enrofloxacin in cells was reduced, which suggests the strengthening of the efflux pump activity. Moreover, the gene expression of *satA* and *satB* was increased in the presence of AI-2, while *smrA* was not modified in both WT and Δ*luxS* strains. This is in agreement with Jose et al. who showed that fluoroquinolone efflux in *S. suis* is mediated by SatAB and not by SmrA.

5. Conclusions

Our findings provide strong evidence that the LuxS/AI-2 system in *S. suis* is involved in fluoroquinolones susceptibility by regulating the efflux pump SatAB. Given that LuxS is highly conserved in the bacteria, it may represent a target for novel antimicrobials that would attenuate colonization and interfere with disease progression.

Conflict of interest statement

The authors have not declared any conflict of interest.

Author contributions

Conceived and designed the experiments: YW and LY. Performed the experiments: BBL, XD and JPL. Analyzed the data: YW, BBL and SLG. Contributed reagents/materials/analysis tools: CLM and JPL. Wrote the paper: YW and BBL.

Ethical approval

Not required

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.05.006.

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