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Biofilm Formation, Host-Cell Adherence, and Virulence Genes Regulation of *Streptococcus suis* in Response to Autoinducer-2 Signaling

Yang Wang · Li Yi · Zhicheng Zhang · Hongjie Fan · Xiangchao Cheng · Chengping Lu

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Abstract Autoinducer-2 (AI-2) is a universal signal molecule mediating intra- and interspecies communication among bacteria. AI-2 is a byproduct of the LuxS enzyme during the catabolism of S-adenosylhomocysteine and plays critical roles in regulating various behaviors of bacteria. In our previous study, the function of LuxS in AI-2 production was verified in *Streptococcus suis* (SS). Decreased levels of SS biofilm formation and host-cell adherence as well as the inability to produce AI-2 were observed in SS having a *luxS* mutant gene. In this study, exogenous addition of a low concentration of AI-2 synthesized in vitro was found to promote biofilm formation and host-cell adherence. However, higher concentrations of AI-2 inhibited SS biofilm formation and host-cell adherence. Real-time PCR results showed that the mRNA level of virulence factors of SS biofilm, *gdh*, *cps2*, *sly*, and *mrp* increased and *ef*, *fbps*, *and gapdh* decreased with increasing AI-2 concentrations. These findings demonstrated that AI-2 supplemented exogenously acted as a concentration-dependent signaling molecule to regulate SS biofilm formation, host-cell adherence, and transcription levels of many virulence genes.

Yang Wang and Li Yi have contributed equally to this study.

Y. Wang · X. Cheng (✉)
College of Animal Science and Technology, Henan University of Science and Technology, Luoyang 471003, China
e-mail: chengxch@126.com

Y. Wang
e-mail: wangyocean@163.com

Y. Wang · L. Yi · Z. Zhang · H. Fan · C. Lu
OIE Reference Laboratory for Swine Streptococcosis, Nanjing 210095, China

Y. Wang · L. Yi · Z. Zhang · H. Fan · C. Lu (✉)
Key Lab of Animal Bacteriology, Ministry of Agriculture, Nanjing Agricultural University, Nanjing 210095, China
e-mail: lucp@njau.edu.cn

L. Yi
Department of Life Science, Luoyang Normal University, Luoyang 471022, China

Z. Zhang
Department of Animal Science and Technology, Jinling Institution of Technology, Nanjing 210038, China

Introduction

Quorum sensing is an intercellular communication system bacteria use to indirectly monitor their own population density through signaling compounds that diffuse through the environment [13]. One of the regulatory systems is involved in the production of cell signaling molecules via *luxS*-based autoinducer-2 (AI-2). When the concentration of signaling molecules accumulates and reaches a threshold level, bacteria can alter their genes expression in unison and participate in diverse behaviors such as bioluminescence, biofilm formation, adherence, and virulence [26].

AI-2 synthesis is linked to the metabolism of S-adenosylmethionine. Methylation reactions frequently use S-adenosylmethionine as the methyl donor to generate S-adenosylhomocysteine (SAH) [19]. SAH is hydrolyzed to adenine and 4,5-dihydroxy-2,3-pentanedione (DPD) by the nucleosidase Pfs and the LuxS enzyme [14]. Upon formation, DPD spontaneously cyclizes to form at least two different interspecies communication molecules described as AI-2 [10]. A broad range of Gram-positive and Gram-negative bacteria has been suggested to harbor *lux*S orthologs, most of which can produce AI-2 [9, 19]. AI-2 has been shown to be involved in biofilm formation and host-cell adherence in many bacterial species.

Streptococcus suis (SS) is a zoonotic pathogen associated with a wide range of diseases in pigs, including meningitis, septicemia, pneumonia, endocarditis, and arthritis [15]. It is also a problematic zoonotic agent for humans exposed to diseased pigs or their products [15]. It has been suggested that SS infection begins with its colonization on the nasopharyngeal tissue and that the interaction of SS with respiratory tract epithelial cells is central to the initiation of the infection [22]. Different forms of interaction between SS and HEp-2, such as adhesion, invasion, and toxic effects, have been studied [7, 22, 30, 31]. SS is considered to be a normal inhabitant of a variety of ruminants, and they have the ability to form biofilms in vitro that are highly resistant to cleaning procedures [5, 16, 30]. In our previous study, we found that *luxS* gene is important for AI-2 production. SS mutant with Δ*luxS* has decreased ability in biofilm formation. It also shows lower adherence and reduced virulence [7, 31]. However, the role of AI-2, especially in SS biofilm formation, host-cell adherence, and virulence genes expression, has not been completely elucidated.

In this study, in order to find out how SS responded to AI-2, we added different concentrations of synthetic AI-2 to the media of different SS strains and evaluated biofilm formation, host-cell adherence, and virulence genes expression.

Materials and Methods

Bacterial Strains and Culture Conditions

Bacterial strains, plasmids, and growth conditions used are listed in Table 1. The HA9801 strain was isolated from SS infected pigs in the Jiangsu Province in 1998 and was confirmed as a virulent strain [34]. The *luxS* mutant of HA9801 (Δ*luxS*) was constructed in a previous study [31]. SS strains were grown in Todd-Hewitt broth (THB) (Difco Laboratories, Detroit, MI) medium or plated on THB agar with 5 % (v/v) sheep blood. THB medium supplemented with 1 % fibrinogen was used in the biofilm assay.

In Vitro Production of AI-2

In vitro AI-2 synthesis reactions were carried out at 37 °C, according to the method previously described [18, 31]. Briefly, AI-2 was produced by incubation with 1 mM SAH (Sigma, USA) and 1 mg/ml of purified Pfs and LuxS for 1 h at 37 °C in 10 mM sodium phosphate buffer at pH 8.0. The AI-2 concentration was estimated using Ellman's assay to quantify homocysteine concentration and measuring the absorbance at 412 nm.

Biofilm Plate Assay

SS were tested for production of biofilm using the protocol described in our previous report [31]. Briefly, an overnight culture of SS was diluted to obtain an OD600 of 0.2 into fresh medium and incubated at 37 °C for 24 or 48 h before being stained with crystal violet. After fixing of methanol and then staining was measured at 595 nm. All assays were performed in triplicate and repeated three times.

Effect of AI-2 on Biofilm Formation

Furthermore, AI-2 (1, 2, 4, 6, 8, 10, 15 μM) was added to cultures of the *luxS* mutant and the wild-type strain. Plates were incubated at 37 °C for 24 h without agitation. To assess the time course of biofilm formation in the presence of AI-2, the plates supplemented with 2 μM AI-2 were incubated at 37 °C for 24 or 48 h without agitation. The biofilm density of different conditions was tested as described above. The experiments were done in triplicate.

The biofilm counts were evaluated according to the method described [1]. Briefly, the biofilms were formed in 12-well microtiter plates and scraped with a disposable cell scraper (BD Falcon) into fresh THB medium, and total colony forming unit (CFU) was determined by appropriate dilution and plating on THB agar.

Table 1 Characteristics of bacterial strains, plasmids, and primers used in this study

Strain and plasmid	Relevant characteristics	Source of references
Strains		
HA9801	Virulent strain of SS2 isolated from dead pig	Collected in our laboratory
Δ*luxS*	Mutation in *luxS* gene of HA9801; Cm^r	[31]
E. coli BL21	*DE3*	Invitrogen, Shanghai
V. harveyi BB170	BB120 luxN::Tn5 (sensor 1^-, sensor 2^+) *V. harveyi*	[4]
V. harveyi BB120	Wild type *V. harveyi*	[4]
Plasmids		
pET28a-*luxS*	Containing the *luxS* gene of SS2	[31]
pET28a-*pfs*	Containing the *pfs* gene of SS2	[31]

Cm^r chloramphenicol resistant

Adherence Assay

The adherence assay was performed on HEp-2 cells (ATCC CCL23) according to our previous report [31]. Briefly, semi-confluent monolayers were washed and incubated with experimental medium (without fetal bovine serum) containing bacteria with a multiplicity of infection (MOI) of 100 for 3 h at 37 °C with 5 % CO_2. To determine the effect of AI-2 on the adhesion of SS to HEp-2 cells, various concentrations of AI-2 (0, 2, 4, 6, 8, 10, 15 μM) were added to the media of wild-type strain and *luxS* mutant during the bacteria-cell contact. All plates were washed three times with PBS. Adherent cells were detached using 0.25 % trypsin, serially diluted tenfold in sterile PBS and plated onto THB agar plates. Results are expressed as the average number of bacteria adhering to HEp-2 cells [31]. The uninfected cells were used as the negative control in all experiments. The assay was performed at least three times.

The Influence of AI-2 on the Transcription of the Virulence Genes of SS

To test the effects of AI-2 on the transcription of the virulence genes of SS, different concentrations of AI-2 were added to SS and cultured 24 h till SS biofilm formed. Total RNA was isolated from SS grown as biofilms cells for 24 h, and qRT-PCR was carried out according the method previously described [29]. The related genes of adhesion primers used for the various RT-PCR assays are listed in Table 2.

Table 2 Primers used for qRT-PCR

Name	Oligonucleotide sequence (5′–3′)	Target gene
GDH-S	CACCTTTACCACCGCCGATTG	*gdh*
GDH-A	GGAAATGTTCAAGTCAACCGTGG	*gdh*
CPS2-S	ATTGGTAGGCACTGTCGTTGGTC	*cps2*
CPS2-A	AGAACTTAGCATTGTTGCGGTGG	*cps2*
SLY-S	TCATTCAGGTGCTTATGTTGCG	*sly*
SLY-A	GAAGATTGCGAGCATTTCCTGG	*sly*
EF-S	TCCAATCACAGATCCAGATAGCG	*ef*
EF-A	CTGACCCATTTGGACCATCTAAG	*ef*
MRP-S	CAAGGAAAGTGAACAGAACGAGC	*mrp*
MRP-A	TAGTCGTCCAAACCTGAGTAGCG	*mrp*
FBPS-S	AACCATCTTGCCAGGCTCCAC	*fbps*
FBPS-A	CAGTTCAGAAGCCGTATCCCGAC	*fbps*
GAPDH-S	CTTGGTAATCCCAGAATTGAACGG	*gapdh*
GAPDH-A	TCATAGCAGCGTTTACTTCTTCAGC	*gapdh*
16S RNA-S	GTTGCGAACGGGTGAGTAA	*16sRNA*
16S RNA-A	TCTCAGGTCGGCTATGTATCG	*16sRNA*

Statistical Analyses

Statistical analyses were carried out using the Graphpad Software package (GraphPad Software, La Jolla, CA). One way ANOVA was used in analysis of the biofilm formation and biofilm 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 $P < 0.05$ was considered significant.

Results and Discussion

Effect of AI-2 on Biofilm Formation

Microorganisms in the environment live predominantly in biofilms, and environmental signals and bacterial interactions have been shown to be very important for biofilm formation [8]. Therefore, we studied the influence of the universal quorum-sensing signaling molecule AI-2 on SS biofilm formation and biofilm CFU counts. Various concentrations of synthetic AI-2 were added to the biofilm plate to test whether the AI-2 signal regulates SS biofilm formation. After incubating HA9801 for 24 h, we added various concentrations of AI-2 in the medium (0–2 μM). We observed an increase in the biofilm density and biofilm CFU counts. The biofilm formation and biofilm CFU counts significantly decreased when the cells were incubated with AI-2 from 2 to 15 μM (Fig. 1a, b). Similar results were found in biofilm formation associated with the Δ*luxS* strain, that is, low doses of AI-2 (0–4 μM) promoted biofilm formation and biofilm CFU counts in the Δ*luxS* strain, while high doses of AI-2 inhibited the biofilm formation and biofilm CFU counts. Adding low concentrations of AI-2 leaded to the increase of SS biofilm formation, indicating that SS was able to sense the molecule. Furthermore, the concentration of AI-2 added to the culture medium was important, given that biofilm formation occurred at an optimal AI-2 concentration and declined at higher AI-2 concentrations. This finding indicates that when the amount of AI-2 signaling molecules reaches a threshold level, the bacteria alter some of their biological properties, especially those related to biofilm formation. The concentration-dependent effect of AI-2 on biofilm formation has been reported for *Bacillus cereus* [2], *Streptococcus oralis* [24], and *Mycobacterium avium* [11], but higher concentration of AI-2 had different effects on SS biofilm formation, which was different from the other bacterial species. One possible explanation for this is that AI-2 might not act as the only AI in SS, because many peptides (e.g., the *staphylococcal* autoinducing peptides, the 2-alkyl-4-quinolones, the Phr peptides of *Bacillus subtilis*, and the mating pheromones of *Enterococcus*

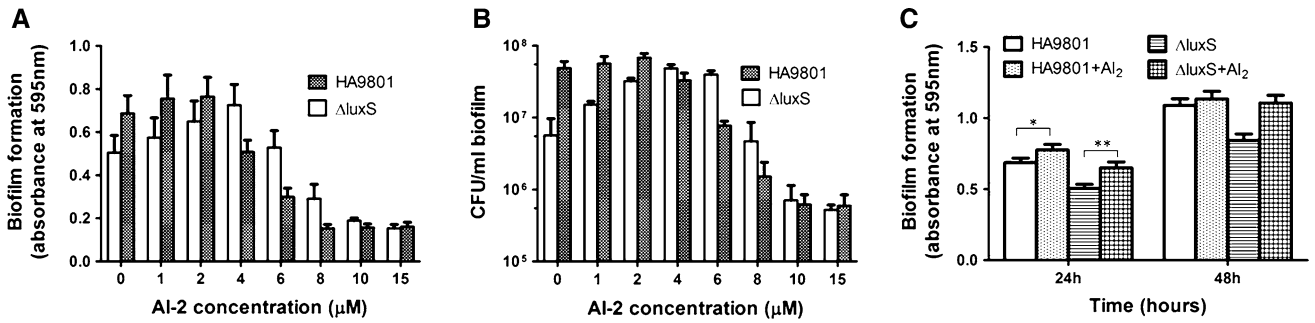

Fig. 1 Effect of AI-2 on biofilm formation in *S. suis* HA9801 and Δ*luxS*. Different concentrations of in vitro synthesized AI-2 were added to microtiter wells inoculated with strain HA9801 and Δ*luxS* in THB medium supplemented with 1 % fibrinogen. **a** After 24 h of incubation, the biofilm density was measured. **b** Change in the biofilm CFU counts of SS as the concentration of AI-2 increases. The columns represent the means and standard deviations of four or more experiments. Statistic analysis showed that $P < 0.01$ at various concentrations of synthetic AI-2, indicating that biofilm formation and biofilm CFU counts of strain HA9801 and Δ*luxS* were affected by AI-2. **c** Time course of biofilm formation of strain HA9801 and Δ*luxS* in the presence or absence of 2 μM AI-2. Experiments were run in triplicate. The *asterisk* showed significant difference ($P < 0.05$)

faecalis) was used as the autoinduce signal molecules in the most Gram-positive bacteria which play a vital role in regulating bacterial biofilm formation and virulence [32]. Instead, AI-2 produced by a different bacterial species might act as a cross-species signaling molecule or a parainducer. AI-2 has been shown to be involved in biofilm formation in many bacterial species, but the role of AI-2 has not yet been completely elucidated. Auger reported that the exogenous addition of AI-2 synthesized in vitro at concentrations from 0 to 6.8 μM had an inhibitory effect on *B. cereus* biofilm formation [2]. In *Vibrio cholerae* [17] and *Eikenella corrodens* [3], AI-2 was found to inhibit biofilm formation, while it was found to promote biofilm formation in *Escherichia coli* [12, 20], *S.mutans* [35], *S.pneumoniae* [27], *S.intermedius* [1], and *Actinobacillus actinomycetemcomitans* [25]. Furthermore, AI-2 seems to play an important ecological role in the formation of multispecies biofilms [24, 35].

Finally, we assessed the time course of biofilm formation in the presence of 2 μM AI-2 (Fig. 1c). During 24 h of growth, HA9801 showed a significant increase in biofilm formation when AI-2 was present ($P < 0.05$). However, when the culture time was prolonged to 48 h, AI-2 was not able to increase biofilm formation of HA9801 ($P > 0.05$). Conversely, we observed a significant increase in the biofilm when the medium was supplemented with 2 μM AI-2 in Δ*luxS* strain after 24 and 48 h of incubation ($P < 0.05$). When Δ*luxS* was incubated with 2 μM AI-2 for 48 h, the biofilm formation ability reached the level of HA9801 strain (Fig. 1c). According to these results, the decrease in biofilm formation observed in the Δ*luxS* SS strain (no AI-2 supplementation) was somehow related to AI-2, because supplementing the medium with low concentrations of AI-2 increased the amount of biofilm.

Effect of AI-2 on the Ability of SS to Adhere to Host Cells

In many bacteria, adhesion to host cells is reduced when a *luxS* mutation is introduced in *Lactobacillus acidophilus* [6], *Campylobacter jejuni* [23], and SS [31]. However, whether the reduction was related to AI-2 has not been investigated. To determine the effect of AI-2 on the adhesion of SS to HEp-2 cells, various concentrations of synthetic AI-2 were added to the media of HA9801 (Fig. 2a) and Δ*luxS* (Fig. 2b) when the bacteria contacted with the cells. We found that AI-2 significantly promoted the adherence of both HA9801 and Δ*luxS* in the presence of a low concentration of AI-2. The maximal adherence of SS was 145 % at a concentration of 4 μM AI-2 in the HA9801 culture medium. However, as the concentration of AI-2 increased, the adherence gradually decreased; at a concentration of 15 μM AI-2, the adherence of HA9801 dropped by 51 % from its maximum value. This phenomenon was similar in the Δ*luxS* culture, which had a maximal SS adherence of 151 % when the concentration of AI-2 was 6 μM. At a concentration of 15 μM AI-2, the SS adherence dropped by 42 %. In general, AI-2 has been shown to control a variety of cellular processes. Bacterium can utilize its own and the other bacteria AI-2 in vivo. The changes of AI-2 populations in vivo provide a mechanism for the regulation of virulence gene expression and biofilm formation during the infection process [33], and the concrete mechanism is still to be further studied.

The Synthesis of AI-2 Effects on Transcriptional Level of Adhesion of SS

AI-2 molecule is located high in the hierarchy of regulation, as many transcriptional regulators are regulated,

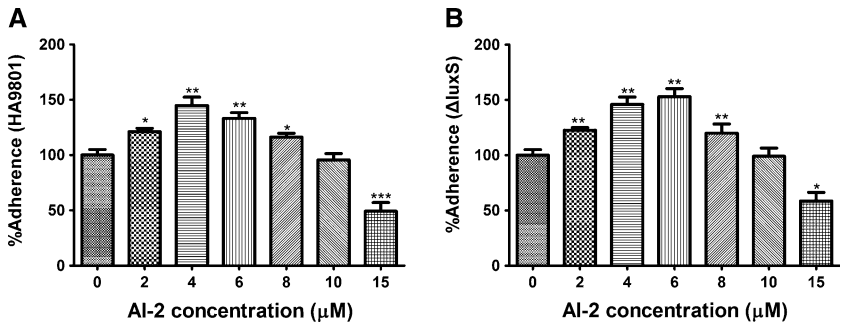

Fig. 2 Effect of AI-2 on HA9801 and Δ*luxS* adherence to HEp-2 cells. The adherence ability of the HA9801 **a** and Δ*luxS*. **b** were tested using HEp-2 cell line cultured in 24-well plates with MOI of 100:1 for 3 h. Different concentrations of in vitro synthesized AI-2 were added to microtiter wells when bacteria-cell contact. The *columns* represent the means and standard deviations of three or more experiments. The adhesion of HA9801 (**a**) or Δ*luxS* (**b**) to HEp-2 cells was considered 100 % in the absence of AI-2 in the media. The *asterisk* showed significant difference ($P < 0.05$)

Table 3 Virulence genes regulated by AI-2 of SS biofilm

Gene	Fold change in expression				Protein ID	Description
	0 μmol	4 μmol	8 μmol	15 μmol		
gdh	1	1.20 ± 0.13	1.34 ± 0.14	2.96 ± 0.23	ABP91406	Glutamate dehydrogenase
cps2	1	1.33 ± 0.18	2.17 ± 0.09	2.77 ± 0.21	ABP91457	Capsular polysaccharide
sly	1	1.19 ± 0.09	1.82 ± 0.15	2.72 ± 0.18	ABP92574	Suilysin
ef	1	1.59 ± 0.13	1.27 ± 0.12	0.86 ± 0.10	ABP91339	Extracellular protein factor
mrp	1	0.88 ± 0.11	1.61 ± 0.13	2.64 ± 0.25	ABP91914	Muramidase-released protein
fbps	1	1.56 ± 0.13	0.91 ± 0.08	0.86 ± 0.08	ABP92661	Fibronectin–fibrinogen-binding protein
gapdh	1	2.56 ± 0.13	1.57 ± 0.13	1.31 ± 0.11	ABP91318.1	Glyceraldehyde-3-phosphate dehydrogenase

especially for virulence genes [21]. In our previous studies, many virulence genes were found to be affected and were significantly decreased in the *luxS* deletion strain [31]. But most of the investigations did not perform the effects of the addition of AI-2 on virulence genes transcriptional level. Real-time PCR results showed that the mRNA level of virulence factors of SS biofilm, including *cps2*, *sly*, and *mrp*, increased with increasing AI-2 concentrations (from 0 to 15 μmol). The expression of *gdh* only increased when incubated with 15 μM of AI-2 but not with 4 and 8 μM of AI-2. However, three virulence genes (*ef*, *fbps*, *and gapdh*) were downregulated from addition of 4 μmol AI-2 to 15 μmol AI-2 (Table 3). In SS, fibrinogen-binding protein (FBPS) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were previously shown to mediate cell adhesion and play important roles in bacterial infection and invasion [28]. The decreased expression levels of these two genes related of adherence maybe resulted that the biofilm formation and adherence ability decreased as the concentration of AI-2 increased. These results showed that AI-2 could regulate the expression of virulence genes of SS.

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