Acyl-homoserine-lactones receptor LuxR of *Shewanella baltica* involved in the development of microbiota and spoilage of refrigerated shrimp

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Abstract Numerous bacterial species utilize quorum sensing molecules acyl-homoserine-lactones (AHLs) to communicate, however, crosstalk often complicates the dynamics and behaviors of mixed populations. In this study, we developed a *luxR* mutant of wild type *Shewanella baltica* SA03 (WT SA03), and aimed to investigate the role of *S. baltica* LuxR (AHLs receptor) involved in the spoilage of refrigerated shrimp (*Litopenaeus vannamei*) by inoculating WT SA03 and *luxR* mutant of *S. baltica* SA03 ($\Delta luxR$ SA03), respectively. The results indicated the maximum growth rate of total viable bacteria in shrimp inoculated with $\Delta luxR$ SA03 was 73.34% lower than that of WT SA03. The lag time of total bacteria in shrimp treated with $\Delta luxR$ SA03 were 87.6 h, significantly longer than that of WT SA03. Meanwhile, the total volatile basic nitrogen concentrations of shrimp treated with WT SA03 were significantly higher than that of $\Delta luxR$ SA03 after 2 days of storage, which were in agreement with the decrease of the content of AHLs of the shrimp. The results indicated *S. baltica* might utilize AHLs produced by other bacteria and accelerate the shrimp spoilage process through LuxR receptor system.

Keywords Acyl-homoserine-lactones · Quorum sensing · Eavesdropping · Spoilage · Shrimp

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Introduction

Quorum sensing (QS) is a cell to cell communication mechanism through which bacteria employ signal molecules to regulate their group behaviors responding to their local environment changes (Chhabra et al. 2005). Acyl-homoserine-lactones (AHLs) are the most studied signal molecule in gram-negative bacteria (Yang et al. 2016; Li et al. 2018). Generally, AHL-QS circuits contain a LuxR and LuxI homolog. LuxI is responsible for the production of AHLs, and LuxR can response to the AHLs to coordinate the bacteria behavior. However, LuxR solos have been shown to respond to exogenous AHLs produced by neighboring cells as well endogenously produced AHLs and some LuxR proteins have evolved from the ability to binding AHLs and respond to other molecules/signals (Patel et al. 2013). In order to compete bacteria and modulate the interaction with its competitor, some bacteria do exist eavesdropping on quorum sensing autoinducers (Case et al. 2008), such as *Escherichia coli* and *Salmonella Enteritidis* (Kim et al. 2014; Almeida et al. 2016).

The recent studies showed that *Shewanella baltica*, the specific spoilage organism in shrimp and fish, were capable of producing AI-2 and cyclic dipeptides as the signal molecules (Zhu et al. 2015, 2016; Gu et al. 2013). Though *S. baltica* possesses AHLs receptor LuxR, AHLs signal molecule was seldom detected in *S. baltica*. QS signal molecular and responses can be cooperative in vitro, but it remains unclear whether QS is cooperative in nature. Little is known about the relationship between *S. baltica* and other bacteria produced AHLs in the spoilage of shrimp, and *S. baltica* may do poorly in artificial petri dishes conditions because of the importance of metabolism networks in spoiled shrimp. Here, we constructed a *luxR* mutant of *S. baltica*, and the purpose of the study was to

elucidate: (1) Can *luxR* mutant of *S. baltica* use exogenous AHLs to keep the quantity advantage in the microbiota development? (2) Whether the LuxR receptor system of *S. baltica* play an important role in the spoilage of shrimp or not.

Materials and methods

Materials and bacterial strains

Shrimp (*Litopenaeus vannamei*) with average length of 13.59 ± 0.52 cm and weight of 13.83 ± 0.32 g was purchased from a local market. The shrimp was transported in oxygenated package to laboratory within 1 h and was approved by the local ethical committee, Ocean university of China. Plate count agar (PCA) and iron agar (IA) mediums were purchased from Haibo Biotechnology Co., Ltd. (Qingdao, China). The synthetic *N*-hexanoyl-L-homoserine lactone (C6-HSL) and DNA kits were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals used in this study were of analytical grade and are commercially available.

The *Shewanella baltica* SA03 was isolated from spoiled refrigerated *Litopenaeus vannamei* using agar plate method and kept in our laboratory (*Shewanella baltica* SA03 has identified using 16 s rDNA method, data not shown). *S. baltica* SA03 was routinely grown at 30 °C in Luria–Bertani (LB) broth under aerobic condition. *E. coli* WM3064 was grown at 37 °C in LB medium under aerobic condition. Appropriate concentration of antibiotics was added in *E. coli* WM3064.

Construction of the *luxR* mutant of *S. baltica* SA03

The *S. baltica* SA03 *luxR* mutant was constructed using the *att*-based fusion PCR method as described by Jin et al. (2013). Briefly, the target flanking gene was amplified with the primer containing *attB* and the specific sequence, then followed by a second round of PCR. The fusion PCR fragments were introduced into plasmid pHG1.0 by site-specific recombination using BP Clonase (Invitrogen, USA). The verified recombinant plasmid was transformed into and maintained in *E. coli* WM3064 and then transferred into *S. baltica* SA03 by conjugation with the ratio of the donor to the recipient being 2:1. Gentamycin-sensitive and sucrose-resistant colonies were screened by PCR for deletion of the target gene. Mutants were verified by sequencing the mutated regions. The primer used in this study was as follows: LF: TCACGGACTTGATAGACAA; LR: GTACTGGGATGCTGGTTT; SF: AATTGAGCCTGACACAGAC; SR: GTAGTTACGGTATCTTGAATGG.

Bacterial growth assay

The wild-type strain WT SA03 and $\Delta luxR$ SA03 mutant were incubated in AB medium in the presence of C6-HSL (10 μ M) with shaking (biochemical incubator, 200 rpm) for 24 h at 30 °C, respectively. The control is added equivalent dimethyl sulfoxide. Bacterial population was estimated by the measure of OD₆₀₀ using a spectrophotometer in 4 h intervals.

Shrimp inoculation and spoilage assays

Live shrimp were painless killed using – 20 °C sterile ice. The peeled and sterilized samples were randomly assigned into three groups (each group 9.0 ± 0.2 kg) and each subjected to processing in triplicate. The shrimp samples was combined inoculated with 1.0×10^4 colony-forming units (cfu)/mL initial microbiota (H_2S bacteria (black colony) together with medium were removed using the small needle, then rinse the culture plate and suspension) of shrimp and 1.0×10^4 cfu/mL wild type *S. baltica* SA03 (WT SA03), or 1.0×10^4 cfu/mL initial microbiota of shrimp and 1.0×10^4 cfu/mL *luxR* mutant of *S. baltica* SA03 ($\Delta luxR$ SA03). The sterilized sample without inoculation was appointed as the control. Treated shrimp samples were air packaged and stored at 4 ± 1 °C inside the refrigerator for 6 days. Average 600 g samples were taken from each group for analysis at each sampling time. The experiments were performed in triplicates.

Total aerobic plate counts (TPC) were enumerated using nutrient agar, and incubated at 37 °C for 24 h. The results were expressed as the colony-forming units (cfu) per g of the sample. H_2S -producing bacteria were determined in iron agar as described by Gu et al. (2013). The growth dynamics of total viable bacteria and H_2S -producing bacteria were determined using the modified Gompertz model, which uses a static equation to describe bacterial growth N [cfu/g] as a function of time t [h] (Zwietering et al. 1990).

$$\log N(t) = \log N_0 + \log(N_{\max}/N_0) \times \exp\{-\exp[(2.718\mu_{\max}(\text{Lag}-t)/(\log(N_{\max}/N_0)) + 1])\} \quad (1)$$

N_0 is the initial population level of the bacteria at time $t = 0$ h (cfu/g); $N_{\max}$ is the maximum microbial cell density (cfu/g); Lag is the lag time (h); and $\mu_{\max}$ is the maximum growth rate.

Total volatile basic nitrogen (TVB-N) of the shrimp was measured using steam distillation with a Kjeldahl distillation apparatus and titration (Kaewprachu et al. 2017). The distillate was collected in boric acid (0.3% w/v) and titrated with 0.05 mol/L standard hydrochloric acid using phenolphthalein as an indicator.

Fig. 1 Construction and agarose gel result of *Shewanella baltica* SA03 *luxR* mutant. M: D2000 marker, 1. Fusion fragments of the target gene. 2. Gene products of primer LF/SR. 3. Gene products of

primer SF/LR. 4. *luxR* gene fragment of wild type *Shewanella baltica* SA03. 5. *luxR* gene fragment of mutant *luxR* *Shewanella baltica* SA03

Extraction and detection of AHLs

Shrimp meat samples (100 g) were aseptically removed and subsequently homogenized with 200 mL ethyl acetate containing 0.1% (v/v) formic acid for 3 min in a stomacher and then centrifugation at 8000 rpm for 10 min at 4 °C. Ethyl acetate fractions were evaporated to dryness at 30 °C. The residues were re-dissolved in dimethyl sulfoxide (about 1.0 mL) to obtain the AHLs extracts. The AHLs was measured using the bioreporter strains *A. tumefaciens* A136 according to the method of Kawaguchi et al. (2008). A 200 µL mixture containing AHLs extracts, *A. tumefaciens* A136 cell-free lysate and 20 mM KH₂PO₄ buffer (pH 7.0) (v:v:v = 1:1:2) added into a 96-well plate, and then X-gal (500 µg/mL) was added to each solution and mixed. The plate was incubated at 30 °C for 3 h, and the absorbance at 635 nm was measured. The AHLs activity was expressed as OD₆₃₅ value.

Statistical analysis

Analysis of variance (ANOVA) was performed and the mean comparisons were done by Duncan's multiple range tests. The microbial growth dynamics were fitted using the modified Gompertz model by Sigmaplot 10.0 (Jandel

Scientific, San Francisco, CA). All experiments were performed in triplicates (n = 3).

Results and discussion

Construction of *ΔluxR* SA03 mutant

In order to confirm the function of LuxR as a AHLs receptor, a knockout mutant of *luxR* was constructed. The strategy and results of *ΔluxR* SA03 mutant construction was shown in Fig. 1. *ΔluxR* SA03 was confirmed by PCR amplification and sequencing of *luxR* gene from chromosomal DNA of *S. baltica*. In the WT SA03, the intergenic region of *luxR* consists of 700 bp, whereas, in *ΔluxR* SA03, it consists of 200 base pairs, meaning that 500 bp upstream were lost during the recombination process. The results indicated that the *luxR* gene in *S. baltica* SA03 was knocked out successfully.

Growth curve of WT SA03 and *ΔluxR* SA03

The growth curves of WT SA03 and *ΔluxR* SA03 were similar (Fig. 2). However, exogenous signal molecule C6-HSL significantly improved the growth of *S. baltica* SA03, and showed no effect on *ΔluxR* SA03. It is widely believed

Fig. 2 The growth changes of wild type *Shewanella baltica* SA03 (WT SA03) and *luxR* mutant *Shewanella baltica* SA03 ($\Delta luxR$ SA03) during incubation, and the total volatile basic nitrogen (TVB-N), acyl-

homoserine-lactones (AHLs) activity changes of shrimp inoculated with WT SA03 and $\Delta luxR$ SA03 during cold storage

Table 1 Total viable bacteria growth changes in shrimp inoculation with WT SA03 or $\Delta luxR$ SA03

Time of storage (days)	Total viable bacteria ($\times 10^5$ CFU/g)		
	Control	WT SA03	$\Delta luxR$ SA03
0	0.1 $\pm$ 0.0 ^a	0.5 $\pm$ 0.1 ^c	0.4 $\pm$ 0.2 ^b
1	0.1 $\pm$ 0.0 ^a	1.1 $\pm$ 0.1 ^c	0.1 $\pm$ 0.0 ^a
2	0.1 $\pm$ 0.0 ^a	9.5 $\pm$ 1.1 ^c	0.3 $\pm$ 0.0 ^b
3	0.1 $\pm$ 0.0 ^a	5.3 $\pm$ 0.7 ^c	0.8 $\pm$ 0.1 ^b
4	0.2 $\pm$ 0.0 ^a	13.3 $\pm$ 0.7 ^c	2.2 $\pm$ 0.2 ^b
5	1.3 $\pm$ 0.1 ^a	42.0 $\pm$ 0.7 ^c	3.7 $\pm$ 0.7 ^b
6	2.1 $\pm$ 0.2 ^a	165.0 $\pm$ 0.2 ^c	81.0 $\pm$ 7.1 ^b
$\mu_{\max}$ /h	0.043 $\pm$ 0.006 ^a	0.236 $\pm$ 0.001 ^b	0.063 $\pm$ 0.006 ^c
Lag (h)	124.3 $\pm$ 0.6 ^c	37.2 $\pm$ 0.7 ^a	87.6 $\pm$ 4.3 ^b
R ²	0.9936	0.9838	0.9810

R² is the coefficient of determination. Control: no inoculation

$\mu_{\max}$ is the maximum growth rate (per hour); Lag is the lag time (h)

Different letters in a row indicate significant differences ($P < 0.05$)

that QS can regulate bacteria growth, and microbial growth and metabolism is the main major cause resulting in food spoilage or deterioration (Gram and Dalgaard 2002). Blana et al. (2017) found the growth of *Salmonella* may be affected by the presence of quorum sensing signaling compounds. Zhang et al. (2016) also found exogenous *N*-

butanoyl-L-homoserine lactone (C4-HSL) and C6-HSL promoted the growth of *Shewanella* sp. Our results showed WT SA03 and $\Delta luxR$ SA03 have different response to exogenous C6-HSL, indicating LuxR system was involved in *S. baltica* QS system.

AHLs receptor LuxR of *S. baltica* and shrimp spoilage

In our previous study we have found *Shewanella* spp. was the only H₂S-producing bacteria in spoiled shrimp, and exogenous C6-HSL can affect the microbiota change in refrigerated shrimp (Zhu et al. 2015). In this study, the effects of AHLs receptor LuxR on the microbiota changes in refrigerated shrimp were analyzed. The results showed total viable bacteria of *ΔluxR* SA03 treated samples exhibited a longer lag time ca. 87.6 h, and the maximum growth rate was significantly lower than that of WT SA03 ($P < 0.05$) (Table 1). Microorganisms are the major cause of spoilage of most seafood products, and extending lag phase durations of microorganism may extend the shelf-life of seafood products (Remya et al. 2017). Thus, we suggested that the LuxR-type QS may accelerate the development of microbiota in refrigerated shrimp.

Level of TVB-N production is important quality parameter for the assessment of shrimp spoilage. As shown in Fig. 2, the TVB-N values of all treatments increased with storage time. However, the TVB-N concentrations of the samples treated with WT SA03 were significantly higher than those of *ΔluxR* SA03 and control after 2 days of storage ($P < 0.05$). These trends were in agreement with the changes of total viable bacteria. Meanwhile, the AHLs contents changes of three treatments were very different. The peak of AHLs content of WT SA03 treated samples appeared at the first day, and the *ΔluxR* SA03 and control appeared at the third and fourth day, respectively. The AHLs of WT SA03 treated samples kept lower level after 2 days of storage. We deduce WT SA03 might utilize AHLs produced by other bacteria through LuxR receptor system and avoid the accumulation of AHLs during storage, which accelerate the increase of total viable bacteria and the spoilage process of shrimp. However, the AHLs activity of control samples showed an obvious decrease at the storage day 3, which may be attributed to the errors of limited samples, or the utilization of AHLs as autoinducers, or the degradation of AHLs by quorum quenching enzymes (Chen et al. 2013).

Bacteria are now known to be highly social organisms. Their behaviors range from cooperatively forming complex multispecies communities to fiercely competing for resources (Michie et al. 2016). Since AHLs-based QS regulate the expression hydrolytic enzymes and other metabolic process, we hypothesized that AHL molecules may promote the competitiveness of *S. baltica* in refrigerated shrimp. If QS-regulated spoilage factors are cooperative, the benefits of signal response should be shared between both producers and nonproducers within the host (Zhou et al. 2014). We found WT SA03 tended to reach higher population densities, and *ΔluxR* SA03 did not

benefit from the presence of AHLs. We inferred that WT SA03 gain a growth advantage in spoiled shrimp by avoiding the metabolic costs of QS-regulated traits (Zhou et al. 2014; Srimani and You 2014), and this reason may be related to the lower TVB-N level of *ΔluxR* SA03 treated samples during cold storage.

Conclusion

In this study, the *luxR* mutant of *S. baltica* *ΔluxR* SA03 was successfully developed. The strain of *ΔluxR* SA03 lost its sensitivity to AHLs in vitro and in vivo. *S. baltica* do not produce AHLs, however, *S. baltica* might utilize environmental AHLs produced by other bacteria through LuxR receptor system and accelerate the shrimp spoilage process, and *ΔluxR* SA03 did not benefit from the presence of AHLs. Our results suggest AHLs receptor LuxR of *S. baltica* was involved in the development of microbiota and spoilage of refrigerated shrimp, and the reason may be related to the AHLs eavesdropping of *S. baltica* through LuxR system.

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Compliance with ethical standards

Conflict of interest All authors have no conflicts of interest.

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