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Characterization of LuxI/LuxR and their regulation involved in biofilm formation and stress resistance in fish spoilers *Pseudomonas fluorescens*

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ABSTRACT

Quorum sensing (QS) is crucial for adaption and development of foodborne bacteria in diverse environments. *Pseudomonas fluorescens* PF07 with QS mediated acylated homoserine lactones (AHLs) activity was isolated from spoiled large yellow croaker (*Pseudosciaena crocea*). In this study AHL-mediated QS system was characterized and their roles in biofilm formation, motility, stress response and spoilage of *P. fluorescens* were evaluated. A LuxI/LuxR homolog consisting of a conserved AHL synthase gene (*luxI*) and a transcriptional regulator gene (*luxR*) was identified in the strain. Two in-frame deletion mutants of *luxI* and *luxR*, Δ*luxI* and Δ*luxR*, were constructed to explore their QS signaling function in *P. fluorescens*. Three types of AHLs were detected in PF07 culture by LC-MS/MS, and *N*-butanoyl-L-homoserine lactone (C_4-HSL) was a major signal molecule. The C_4-HSL activities was almost abolished in Δ*luxI,* and decreased greatly in Δ*luxR*. Compared with wild type (WT) strain, both Δ*luxI* and Δ*luxR* showed the significant decrease of biofilm biomass and expolysaccharide production, resulting in thinner and incompact biofilm structure, but promoted swimming motility. The resistance of *P. fluorescens* to H_2O_2, heat, NaCl and crystal violet apparently declined in two mutants compared to WT. Spoilage factors, siderophore and protease, apparently attenuated due to deletion of *luxI* or *luxR* gene, while the growth and TVB-N production did not differ. Furthermore, the changes of the biofilm formation, motility and protease in Δ*luxI* strain were partially restored by the exogenous C_4-HSL. In agreement with the effect of two mutants on various phenotypes, the transcriptions of *alg*, *lapA*, *flgA*, *rpoS*, and *aprX* were significantly down-regulated, and *flgA* was up-regulated in Δ*luxI* and Δ*luxR*. Therefore, the present study highlighted that the co-operation of LuxI/LuxR homolog was an important QS regulator in biofilm formation, motility and spoilage potential, and hinted its positive regulation of stress resistance with RpoS in *P. fluorescens*.

1. Introduction

Quorum sensing (QS) is thought to play a role in microbial related food spoilage and food safety. Microbes use this cell-to-cell communication to monitor their population density and initiate a number of physiological behaviors by releasing and receiving of signal molecules (Miller and Bassler, 2001). In Gram-negative proteobacteria, QS is mainly mediated by *N*-acylhomoserine lactones (AHLs) signal molecules (Kai and Bassler, 2016), which have been found in several bacteria related to food spoilage, such as the members of the genus *Pseudomonas*, *Serratia*, *Aeromonas*, and *Hafnia*. Various AHLs signal molecules have been reported to be either present or to increase their concentration in spoiled seafood product, milk, meat and vegetables (Skandamis and Nychas, 2012). AHLs dependent QS are typically synthesized by a LuxI-type enzymes and detected by LuxR-type transcriptional regulators. LuxI enzymes produce AHLs by deriving the lactone moiety from *S*-adenosyl methionine (SAM), and, in most cases, the particular acyl chain is obtained from intermediates of fatty acid biosynthesis. AHLs have a core *N*-acylated homoserine-lactone ring and a 4–18 carbon cayl chain that can contain modifications (Watson et al., 2002). LuxR homolog consists of two domains, including an amino-terminal AHL-binding domain and a carboxy-terminal helix-turn-helix (HTH) DNA-binding domain (Zhang et al., 2002). Most LuxR homolog receptor binds the cognate AHLs, and LuxR/AHLs complexes regulate multiple physiological processes such as bioluminescence, production of virulence factors, biofilm formation, secretion of extracellular enzymes in Gram-negative bacteria isolated from food matric (Skandamis and Nychas, 2012; Watson et al., 2002; Zhang et al., 2002).

More than 70 different Gram-negative bacteria have been reported to possess a LuxI/LuxR-type QS system. The LuxI/R system was first

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discovered in *Vibrio fisheri* during the investigation of the phenomenon of bioluminescence (Schaefer et al., 1996). SplI/R in *Serratia plymuthica* RVH1, involving in the deterioration of carrot slices, regulated the production of nuclease, chitinase, protease and butanediol fermentation (Houdt et al., 2007). The spoilage potential in *Aeromonas salmonicida* isolated from spoiled marine fish was associated with the negative control of AsaI/R-type system (L. Liu et al., 2018). There are currently well-known AHLs mediated QS pathways in *Pseudomonas aeruginosa*: two LuxR and LuxI-type systems called LasI/R and RhlI/R. LasR, in complex with 3-oxo-C_{12}-HSL, activates a large regulon of downstream genes that includes the *lasI* synthase gene (Chugani and Greenberg, 2010). The LasR-autoinducer complex also activates *rhlR* and *rhlI* expression. RhlR bounding to C_4-HSL activates its own regulon that includes *rhlI*, and thereby establishes the second autoinduction feed-forward loop (Jimenez et al., 2012). However, until now, few studies have been investigated the LuxI/LuxR homologs in *Pseudomonas fluorescens* as a food spoilage isolate.

Pseudomonas have high proteolytic, lipolytic and psychrotrophic abilities as important gram-negative spoilage organisms in raw fish, meat and dairy products (Caldera et al., 2016). Moreover, bacteria from *Pseudomonas* genera, especially *Pseudomonas fluorescens*, are known to be good biofilm producers, which is of particular concern in the food industry. Biofilm formation not only cause processing equipment corrosion and increase of food spoilage, but also provide appropriate substratum for the growth of other bacteria including pathogens (Coughlan et al., 2016). *P. fluorescens* biofilm is difficult to remove from abiotic surface due to an increased accumulation of extracellular polymeric substances (EPS) when the biofilm matures. In *Pseudomonads*, QS regulates the production of extracellular DNA, lectins, and biosurfactants, which all play a role in biofilm formation (Fazli et al., 2014). AHLs system in *P. aeruginosa* can positively regulate the biofilm maturation and promote dispersal (De Kievit, 2009), while QS system PpuI/R in *Pseudomonas putida* negatively affected its biofilms (Steidle et al., 2002).

In the processing of seafood, microorganisms in the surface of fish encounter a series of stresses, such heat, cold, salt, and preservatives. The adaptation to extracellular unfavorable environment is critical for the survival and growth of spoilage related bacteria. Several bacterial species respond to environmental stresses by modulation of QS components (Joelsson et al., 2007). In *P. aeruginosa* QS system, nutrient starvation preferentially induces the *rhl* system, with the *las* system appearing to respond predominantly to AHL signal accumulation (Mellbye, and Schuste,R, 2014). QS in *P. aeruginosa* has been linked to the expression of catalase and superoxide dismutase genes for protection against oxidative stress of hydrogen peroxide (Hassett et al., 2010). Although LuxI/LuxR mediated QS co-exist in bacterial community widely modulate virulence factors and adaption in *P. aeruginosa*, its roles in biofilm formation and response to food environmental stresses are far from fully understood in *P. fluorescens*.

Here, we explored the role of *luxI/luxR* mediated QS in biofilm formation and environmental stress in *P. fluorescens* PF07, isolated from *Pseudosciaena crocea*, a commercially popular seafood in China. One AHL-dependent QS system, LuxI-type synthase and LuxR-type regulatory proteins, was identified in *P. fluorescens*. To determine whether *luxI* and *luxR* contributes to QS, *luxI* and *luxR* were each mutated, and AHLs profiles, biofilm formation, moility, stress and spoilage potential between wild type (WT) strain and mutants were comparatively evaluated. The aim of this study was to contribute better understand the potential role of LuxI/LuxR type QS for environmental adaption and bacterial physiology in food spoilage bacteria.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The *P. fluorescens* PF07 strain, originating from spoiled large yellow croaker (*P. crocea*) stored at 4 °C was previously identified as a strong spoiler in our laboratory (Zhao et al., 2016). The 16S rRNA gene sequences of strain PF07 was deposited under the accession number KT716389. *P. fluorescens* PF07 and its mutants were grown in Luriae-Bertani (LB) or Tryptic Soy (TSB) broth at 30 °C. *Chromobacterium violaceum* CV026 obtained from Dr. Yang, Zhejiang A&F University, was cultured in LB broth supplemented with kanamycin (20 μL/mL) at 30 °C. The suicide plasmid pLP12 used for the construction of mutants was induced genes expression under the control of PBAD promoter. Genes expression of the plasmid was achieved by the addition of the 0.2% L-arabinose to the growth media, and repressed by the additon of 0.3% D-glucose.

2.2. Identification and sequence analysis of luxI and luxR

Bacterial genomic DNA was extracted with according to the instruction of Biospin Bacteria Genomic DNA Extraction Kit (BioFlux, Alameda, CA, USA). Whole-genome sequencing was performed on the Illumina HiSeq PE150 platform (Illumina, USA) to get the bacteria frame diagram at the Beijing Novogene Bioinformatics Technology Co., Ltd. Sequences of LuxI/LuxR were found in scaffold22 based on the database of QS related LuxI/LuxR homologs in *P. fluorescens* UK4 (GenBank Accession No. CP008896.1) by using Gapped BLAST and PSI-BLAST. The *luxI* and *luxR* genes with an expected product size of 1600 bp and 1281 bp were amplified by using two primer pairs of luxI-TF/luxI-TR and luxR-TF/luxR-TR, respectively (Table 1). The PCR mixture contained, 50–100 ng DNA extract, 0.5 μL of reverse and forward primer (2 μM), respectively, 2 μL of deoxynucleoside triphosphate (2.5 mM), 2.5 μL of 10 × Tag PCR buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$), 0.25 μL Taq DNA polymerase (5 U/μL, Takara Biotech, Dalian, China) and sterile pure PCR water to a final volume of 25 μL. The conditions for the PCR amplification consisted of 94 °C for 5 min, followed by 30 cycles of 94 °C for 40 s, 49 °C for 30 s, 72 °C for 40 s, followed by 72 °C for 10 min. The amplified product was analyzed on 1.0% (m/v) agarose gels, and a ladder marker DL 2000 (Takara Biotech) was used to estimate the size of the product. Two PCR products were purified using a PCR purification kit (Takara Biotech) and were sequenced.

The deduced amino acid sequence identity of LuxI and LuxR obtained from *P. fluorescens* PF07 was determined using Needleman-Wunsch global sequence alignment tool of BLAST. The Protein Data Bank (PDB) profile of LuxI and LuxR protein in *P. fluorescens* were gained in the web of Collaboratory for Structural Bioinformatics. Multiple sequence alignments of LuxI and LuxR in the isolate and other LuxI/LuxR-homologs were conducted using MEGA7.0 and presented using ESPript 3.0 (http://espript.ibcp.fr/ESPript/). The sequence of LuxI-homologs, including LasI (*P. aeruginosa*), LuxI (*V. fischeri*), RhlI (*P. aeruginosa*), AsaI (*A. salmonicida*), TraI (*A. tumefaciens*), and the LuxR-homologs sequence, including LuxR (*P. fluorescens* UK4), LuxR (*V. fischeri*), RhlR (*P. aeruginosa*), LasR (*P. aeruginosa*), AsaR (*A. salmonicida*), TraR (*A. tumefaciens*), were obtained from NCBI protein database. In order to generate 3-D structure models, the LuxI and LuxR proteins of *P. fluorescens* PF07 was constructed by SWISS-MODEL and was further evaluated by the program PyMOL.

2.3. In-frame delection of luxI and luxR

Two *luxI* and *luxR* in-frame delection mutants of *P. fluorescens* PF07 was constructed by allelic replacement (L. Liu et al., 2018; Luo et al., 2015). All primers used in mutant construction were shown in Table 1. Briefly, two fragments flanking the *luxI* gene were amplified by PCR with primers luxI-*Eco*RI-MF1/luxI-MR1 and luxI-MF2/luxI-*Spe*I-MR2. Similarly, two fragments franking the *luxR* gene were obtained by PCR with primers luxR-MF1/luxR-MR1 and luxR-MF2/luxRI-MR2. The fragments were purified and fused in subsequent PCR reaction using primers luxI-EcoRI-MF1/luxI-SpeI-MR2 and luxR-MF1/luxRI-MR2,

Table 1
Primers used in *luxI/R* mutants and qRT-PCR.

Primers	Sequence(5′-3′) description	Product size (bp)
luxI mutant		
luxI-EcoRI-MF1	TGACGATGAATTCCGATCCTCAGTGGCTTGCGTTC	542
luxI-MR1	GAATCAGCGTGCAGCGCGATCAGTTCATGGTGATGGCGGGTG	
luxI-MF2	CACCCGCCATCACCATGAACTGATCGCGCTGCACGCTGATTC	511
luxI-SpeI-MR2	TAGTCGTACTAGTCAAGGCGCAGAACCTGTCGATC	
luxI-TF	TGTTTCTGGCGGTGTACCTGG	2100 (WT)
luxI-TR	CATGGAGAAGGAAAACGACCTCA	1600 (△*luxI*)
luxR mutant		
luxR-MF1	GGAATCTAGACCTTGAGTCGTGCCAGGCCGGAAAACTCAG	608
luxR-MR1	GGTGGAATCGCTTGCGGATAAACAAGACACTGGCGGCTGC	
luxR-MF2	GCAGCCGCCAGTGTCTTGTTTATCCGCAAGCGATTCCACC	530
luxR-MR2	ACAGCTAGCGACGATATGTCTTGCCGATGAGTTCGCTGCT	
luxR-TF	GACCTCGGCGTTACTCATATCTC	1911 (WT)
luxR-TR	GCGGTATTTTTCTGACCAGTGAT	1281 (△*luxR*)
qRT-PCR		
alg-F	ATGCCTATGTATTCAGCCAAC	201
alg-R	ATTCCTCGCCGTCTTCTTC	
lapA-F	GATGGTGCCGTCGGTTTC	127
lapA-R	GGGTGCCGATACCTTTGTC	
flgA-F	GTGTTGAGCCTGGTTGGGA	173
flgA-R	ATCGCCCGCTTGAATGAG	
rpos-F	CGCTTGATGCGACCCAGTT	189
rpos-R	ACAGGGACAGCCCACGAT	
aprX-F	TAACGAGCCGCAGACCCT	83
aprX-R	AGCCATCAACCGCTACGC	
16 s rRNA-F	GCCCCCTGGACAAAGACTGAC	88
16 s rRNA-R	CATCGTTTACGGCGTGGACTACC	

respectively. The fused segments were sequenced and ligated into suicide vector pLP12. Recombinant plamids pLP12-luxI or pLP12-luxR were transformed into *E. coli* β2163 by electroporation, and conjugated with PF07. To screen stable insertional mutants, the cell of conjugation was spread on LB plates supplemented with chloramphenicol (20 μg/ml) and 0.3% D-glucose. These mutants were verified by PCR and sequencing using primer pairs luxI-TF/luxI-TR and luxR-TF/luxR-TR.

2.4. AHLs analyses by biosensor and LC-MS/MS

AHLs activities in *P. fluorescens* WT strain and two mutants, Δ*luxI* and Δ*luxR*, incubated at 30 °C for 24 h were primilary detected using *C. violaceum* CV026 biosensor strain by a parallel streak method (X. Liu et al., 2018). The biosensor strain produces a purple pigment when induced by C_4-HSL as positive control. Furthermore, the AHL profiles produced by WT and two mutants were further determined by LC-MS/MS as described by Zhao et al. (2016). The cultures in LB broth for 24 h at 30 °C were centrifuged at 10,000 × *g* for 10 min, then extracted with an equivalent volume of ethyl acetate with 0.1% (v/v) glacial acetic acid. The mixture was shaken adequately for 30 s and stayed for layer, which was repeated three times. The extract resuspended in 99.9% HPLC-grade methanol (Thermo Fisher, USA) and was injected for LC-MS/MS analysis (1290 infinityII-6460 triple quad, Agilent Technologies Inc., Santa Clara, CA) using a RRHD Eclipse Plus C18 column (50 mm × 2.1 mm × 1.8 μm) (Agilent Technologies Inc., USA). Ten synthetic AHLs and oxo-derivatives of known carbon chain lengths were used as standards for comparison. These synthetic AHLs, including *N*-butanoyl-L-homoserinelactone (C_4-HSL), *N*-hexanoyl-L-homoserinelactone (C_6-HSL), *N*-(3-oxohexanoyl)-L-homoserine lactone (O-C_8-HSL), *N*-decanoyl-L-homoserine lactone (C_{10}-HSL), *N*-dodecanoyl-L-homoserine lactone (C_{12}-HSL) and *N*-tetradecanoyl-L-homoserinel actone (C_{14}-HSL), were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.5. Biofilm and EPS assay

Overnight cultures of WT strain, Δ*luxR* and Δ*luxI* were diluted at 1:1000 ratio in a fresh sterile TSB medium. The dilutions were transferred into the wells of 96-well flat-bottomed polystyrene culture microtiter plate. After the plates were statically incubated at 30 °C for 6 h, 9 h, 12 h, 18 h, 24 h and at 4 °C for 72 h, 96 h, 120 h, 144 h, biofilm biomass were measured quantitatively by crystal violet assay (Djordjevic et al., 2002). Finally, the absorbance was measured at 590 nm using a microplate reader (Infinite 200, Tecan, Switzerland). Similarly, the above dilutions of WT strain, Δ*luxR* and Δ*luxI* without or with 40 μM C_4-HSL were transferred into the wells of 6-well polystyrene culture microtiter plate. After cultured at 30 °C for 6 h, 9 h, 12 h, 18 h, 24 h, and at 4 °C for 72 h, 96 h, 120 h, 144 h. The extraction of extracellular polysaccharides was slightly modified according to the method of Pang et al. (2017). The cultures in the wells were then carefully aspirated, and plate well were wash thrice with sterile PBS to remove loosly adherent cells. The biofilm in each wells was re-dissolved with PBS and treated by sonicator with 50 kHz for 5 min for polysaccharides extraction. After centrifugation at 10,000 × *g* for 30 min, the supernatant was filtered through 0.2-μm pore size filter, and the filtrate was subjected to the polysaccharides analysis. Total polysaccharides (expressed as μg per mL) of filtrate were quantified by the phenol-sulphuric acid with glucose as standard.

2.6. Confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM) observation

The biofilm formed by WT strain and mutants were further observed by CLSM (L. Liu et al., 2018). Three milliliters of the above diluted cultures of WT strain, Δ*luxR* and Δ*luxI* with or without 40 μM C_4-HSL were deposited in the FluroDish. After incubation for 120 h at 4 °C, bacterial suspensions were removed and biofilm were stained with the SYTO-9 in the dark at 30 °C for 15 min, according to the manufacturer's instructions. The maximum excitation/emission used for these stains was approximately 480/500 nm for SYTO-9. The CLSM images were obtained with a Zeiss LSM 710 (Carl Zeiss, Jena, Germany) and an oil immersion objective lens 43 ×. The Zeiss confocal software was used to analyze the biofilm images, allowing for collection of z-stacks. Additionally, the WT strain, Δ*luxR* and Δ*luxI* without or with 40 μM C_4-HSL inoculated in a 24-well plate containing sterile glass slip (8 mm × 8 mm) in each well at 4 °C for 120 h was also analyzed

qualitatively using SEM (Remuzgo-Martínez et al., 2015). After the removal of culture media and washing, the glass slips were collected and fixed with ice-cold 2.5% glutaraldehyde overnight at 4 °C. Glass slips were postfixed with 1% osmium tetroxide for 1 h after washing. The samples were washed in PBS (pH 7.0) and dehydrated by a graded series of ethanol from 30% to 100% for about 15 to 20 min at each step. The dehydrated samples were coated with gold-palladium and were observed by SEM (Hitachi SU8010, Japan).

2.7. Motility assay

Swimming motility was performed by following a method described previously (L. Liu et al., 2018). About 5 μl of overnight cultures of WT strain, Δ*luxR* and Δ*luxI* without or with 40 μM C_4-HSL (C_4-HSL was added in the medium) were inoculated at the center of a swimming agar plate consisting of 1% tryptone, 0.5% NaCl, and 0.3% agarose. Plates were incubated at 30 °C for 48 h, and images of the plates were captured every 12 h.

2.8. Stress resistance assays

The WT strain, Δ*luxR* and Δ*luxI* of *P. fluorescens* were incubated in LB broth at 30 °C for 24 h to reach the stationary phase (OD600 nm ≈ 1.2). Bacterial cells were collected by centrifugation (8000 × *g*, 10 min), washed thrice with 0.2 M phosphate buffer (pH 7.0), and were suspended and diluted with the same buffer. Each culture received one of the following treatments at an initial population of about 7 log cfu/mL: (a) 20 mM H_2O_2; (b) 30% (m/v) NaCl; (c) 50 °C; (d) 150 μg/mL crystal violet. After 0 min, 10 min, 20 min, 30 min and 40 min of treatments, survival bacterial population was checked by plating with appropriate dilutions. Viable counts were performed after 48 h of incubation at 30 °C.

2.9. Spoilage potential in sterile fish juice media

The preparation and inoculation in sterile muscle juice media of *P. crocea* was performed according to the method reported by Zhao et al. (2016). Overnight cultures were diluted and inoculated into juice media to reach an inoculated level of 4–5 log cfu/mL, and exogenous C_4-HSL at 40 μM was added to fish juice inoculated by Δ*luxI*. The sterile juice supplemented with sterile water was used as a control. All batches of inoculated fish juice were stored at 4 °C, and the samples taken at 3 d, 5 d, 7 d, and 9 d were subjected to determine bacterial growth, total volatile basic nitrogen (TVB-N), siderophore and extracellular protease activity. Ten-fold dilutions of samples were made with saline peptone and plated. The agar plates were incubated at 30 °C for 48 h. TVB-N (mg N/100 ml juice) was measured using steam distillation with FOSS Kjeltec 8400 Automatic Nitrogen Determination apparatus (Foss, Denmark). The juice samples were centrifuged at 10,000 × *g* for 1 min, and the supernatants were used for analysis of siderophore and protease activity. Siderophore activity was measured according to the methods of Wongtrakoongate et al. (2012) with some modifications. Briefly, the supernatants were mixed with an equal volume of chrome azurol sulphonate (CAS) solution containing 0.6 mM hexadecyltrimethylammonium, 1.5×10^{-2} mM $FeCl_3.6H_2O$, 0.15 mM CAS, 50 mM anhydrous piperazine, 0.75 M HCl. After the mixture was incubated for 60 min at room temperature in the dark, an increase absorption in orange color was then measured using a spectrophotometer at OD_{630} nm. The relative quantity of siderophore activity (%) was calculated by $(A_{control} - A_{sample}) / A_{control} \times 100\%$. Extracellular protease activity in the supernatants as crude enzyme was performed according to Liu et al. (2007).

2.10. RNA isolation and RT-PCR

These genes of alginate (*alg*), adhesins (*lapA*), flagellar biosynthetic protein (*flgA*), RNA polymerase sigma factor (*rpos*), and alkaline protease (*aprX*) were found in *P. fluorescens* by searching in the GenBank sequence database. Five genes of *alg*, *lapA*, *flgA*, *rpoS* and *aprX* in WT strain and two mutants were amplified by specific primers (Table 1) designed according to the known sequences. The WT strain, Δ*luxR* or Δ*luxI* supplemented with or without 40 μM C_4-HSL were cultured in LB medium at 30 °C for 12 h and 24 h. The cultures were collected, and total RNA was extracted by using Trizol method (ThermoFisher Scientific, USA). cDNA synthesis was performed with GoScriptTM Reverse System (Promega, USA) from four independent samples. qRT-PCR reaction was carried out by using the PowerUp™ SYBR™ Green Master Mix (ThermoFisher Scientific,USA). In bacterial mRNA transcriptional analysis 16S rRNA was used as internal control due to its stability for *P. fluorescens*. Specificity of RT-PCR was confirmed by agarose gel and melting curve analysis. Then the $\triangle\triangle$Ct was calculated by the different value between $\triangle$Ct for each sample and the $\triangle$Ct for the calibrator. Finally, the relative mRNA expression was showed as $2^{-\triangle\triangle Ct}$ for bacterial spoilage related genes and internal control.

2.11. Statistical analysis

Three replicate trials were done for each sample, and all the experiments were repeated for three times. The results concerning this study were expressed as means ± standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA) using SPSS software (version 18.0) (SPSS Inc., Chicago, IL, USA). Differences between experimental groups were considered to be significant at a p value of < 0.05.

3. Results

3.1. Identification and sequence analysis of LuxI/LuxR homologs in P. fluorescens

To mine QS systems consisting of LuxI-type synthase and LuxR-type regulator from the genome, homologs of one *luxI* and three *luxR* genes of PF07 isolate were found (Fig. 1) by the alignment of *P. fluorescens* UK4 database. LuxI encodes a protein of predicted 192 amino acids (Fig. 1A). The deduced amino acid sequence of LuxI in PF07 shared 99.0% identity of that in *P. fluorescens* UK4, 65.0% of RhlI in *P. aeruginosa*, and 59.0% of LuxI in *V. fischeri* (Fig. 1B). However, lower than 30.0% identity of amino acid sequence was showed between LuxI in PF07 and LasI in *P. aeruginosa* (30%), AsaI in *A. salmonicida* (15%), or TraI in *A.tumefaciens* (14%). Additionally, LuxI protein of PF07 had 21 conserved residues with other six LuxI-type AHL synthases, which contained 8 invariant residues, including R24, F28, W34, D48, D51, R72, E102 and R105.

Total of 3 LuxR homologs, including LuxR1 (GM004425), LuxR2 (GM001489), LuxR3 (GM002298), have been distributed in the *P. fluorescens* PF07. The predicted protein motif analysis revealed that N- and C-terminal domain of LuxR1, LuxR2, and LuxR3 possessed key conserved residues that match the consensus for LuxR homolog. The *luxR*1 gene was genetically linked to a *luxI* gene, and located to 28 bp upstream of *luxI* gene, while the *luxR*2 and *luxR*3 genes were distantly located > 68 kb and 99 kb upstream of the *luxI*/*luxR* locus respectively. So, *luxR*1 (*luxR*) gene was further amplified and shown to encode 261 deduced amino acid in the isolate by sequencing. LuxR protein in PF07 shared 97.0% identity with LuxR from *P. fluorescens* UK4, 75.0% identity with RhlR from *P. aeruginosa,* and 50.0% of LuxR from *V. fischeri* (Fig. 1C). However, it was shown lower than 40% identity between LuxR in PF07, and LasR in *P. aeruginosa* (39.0%), TraR from *A. tumefaciens* (35.0%) or AsaR in *A. salmonicida* (21.0%). There were same 19 residues among six LuxR-type AHL receptors, in which nine conserved residues, including W68, Y72, D81, P82, W96, G124, E189, L193 and G199, were observed (Fig. 1C). The 3D models of the LuxI and LuxR in *P. fluorescens* PF07 was shown that LuxI protein consists of

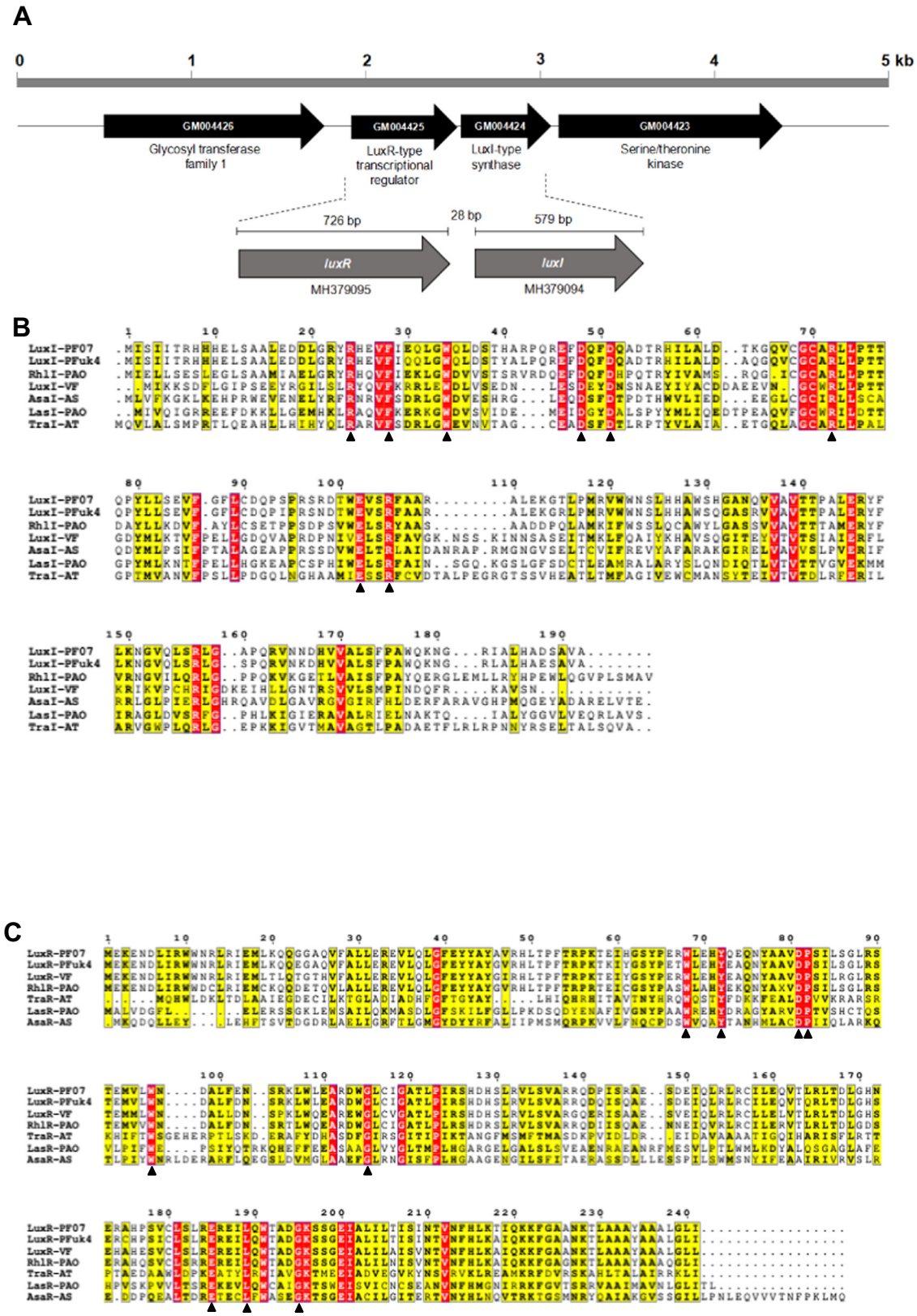


Fig. 1. Physical map and sequence of analysis of LuxI and LuxR obtained for *P. fluorescens* PF07. (A) The arrows indicate the coding region of *luxI* and *luxR*, and partial near genes obtained by PCR verification based on whole-genome sequencing. Sequencing number of serine/theronine, LuxI, LuxR and glycosyl transferase family were GM004423, GM004424, GM004425, GM004426, respectively. GenBank accessions of *luxI* and *luxR* genes were MH379094 and MH379095, respectively. (B) Alignment of LuxI (PF07, GM00424) and LuxI (PFuk4, AIG00862.1), RhlI (*P. aeruginosa*, NP_252166.1), LuxI (*V. fischeri*,YP_206882.1), AsaI (*A. salmonicida*, ADE09299.1), LasI (*P. aeruginosa*, NP_250123.1), TraI (*A. tumefaciens*, AFX65742.1). (C) Alignment of LuxR (PF07, GM00425) and LuxR (PFuk4, AIG00863.1), LuxR (*V. fischeri*, YP_206883.1), RhlR (*P. aeruginosa*, NP_252167.1), LasR (*P. aeruginosa*, NP_250121.1), AasR (*A. salmonicida*, ADE09301.2), TraR (*A. tumefaciens*, AAD31606.2). The conserved residues of LuxI and LuxR were highlighted with filled triangles under every row.

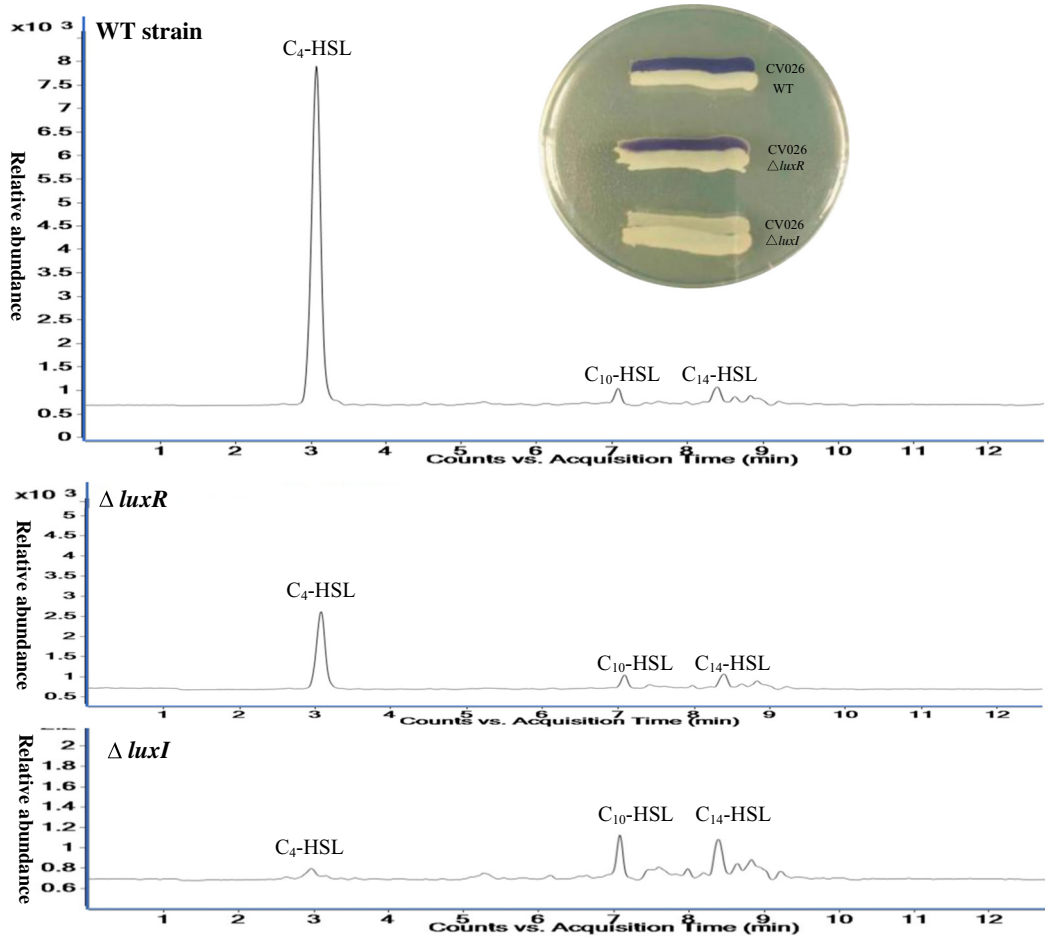


Fig. 2. Mass spectra of N-acylhomoserine lactones (AHLs) profiles in *P. fluorescens* WT strain, Δ*luxR* and Δ*luxI* at 30 °C for 24 h. Inset, streak assay to of screen AHLs activities in wild type strain and mutants using test strain *C. violaceum* CV026 for incubation of 24 h.

seven α-helices and eight β-sheet, and LuxR protein consists of fifteen α-helices and eight β-sheet (Fig. S1).

3.2. Construction of luxI/luxR in-frame deletion mutant

To evaluate the roles of LuxI as a synthase and LuxR as a receptor of AHLs in *P. fluoresces*, two in-frame deletion mutants, Δ*luxI* and Δ*luxR*, were constructed by allelic exchange. Genes of *luxI* and *luxR* were successfully deleted by suicide plasmid pLP21. The PCR in the Δ*luxI* and Δ*luxR* generated a short 1600-bp and 1281-bp fragment as expected, while a normal 2100-bp and 1911-bp DNA fragment was amplified in wild type (WT) strain, respectively (Fig. S2). The sequencing of PCR products confirmed that Δ*luxI* and Δ*luxR* were successfully achieved.

3.3. LuxI/LuxR deletion decreased AHLs activity

The AHLs assay with reporter strain *C. violaceum* CV06 and LC-MS/MS was performed in WT, Δ*luxI* and Δ*luxR*. As shown in Fig. 2, the WT PF07 induced purple pigment violacein production by *C. violaceum* for 24 h of incubation at 30 °C, indicating that the isolate had a strong short chain AHLs activities. Compared with WT strain, Δ*luxR* only produced the slight violacein and Δ*luxI* failed to trigger violacein production by preliminarily screening. Moreover, three types of AHLs including C_4-HSL (*m/z* 172), C_{10}-HSL (*m/z* 256) and C_{14}-HSL (*m/z* 312) were identified in the supernatant extracts of WT strain, in which C_4-HSL was the predominant AHLs signal (Fig. 3 and Table 2). The deletion of *luxR* and *luxI* significantly decreased the AHLs activities, especially C_4-HSL. After inoculation for 24 h at 30 °C, the concentration of C_4-HSL was 276.88 ng/mL, 106.48 ng/mL and 0.31 ng/mL in WT, Δ*luxI and* Δ*luxR*, respectively. Additionally, low activities of C_{10}-HSL and C_{14}-HSL did not significantly differed among three strains.

3.4. LuxI/LuxR deletion weakened biofilm formation

Biofilm formation among WT strain, Δ*luxR* and Δ*luxI* incubated in TSB was shown in Fig. 3. It was observed that the biofilm biomass of *P. fluorescens* PF07 increased with the length of incubation, and arrived to the highest biomass for 12 h at 30 °C and 4 °C for 5 d, then slowly decreased. During the whole biofilm phase, the biomass of Δ*luxR* and Δ*luxI* was significantly lower than that of WT ($p < 0.05$) (Fig. 4AB). Compared with WT strain, Δ*luxR* and Δ*luxI* reduced biomass by 15.97% and 19.26% at 30 °C for 12 h, and decreased by 20.09% and 30.43% at 4 °C for 5 d, respectively. Additionally, the biofilm biomass greatly increased in Δ*luxI* supplemented with 40 μM C_4-HSL, indicating that complementation of exogenous C_4-HSL partially recovered biofilm formation in Δ*luxI*.

EPS production is essential for the development of biofilm structure and maturation (Billings et al., 2013). As shown in Fig. 3 CD, the production of EPS exhibited the similar tendency with biofilm biomass, and the more EPS was secreted during the biofilm formation in *P. fluorescens* at 4 °C compared to 30 °C. In contrast to WT strain, Δ*luxR* and Δ*luxI* greatly decreased the EPS production by 29.42% and 35.94% for 12 h incubation at 30 °C, and reduced by 25.21% and 22.36% for 5 days at 4 °C respectively. Two mutants were observed to decrease significantly EPS production during the entire biofilm growth period ($p < 0.05$), however, the supplement of C_4-HSL in Δ*luxI* caused to

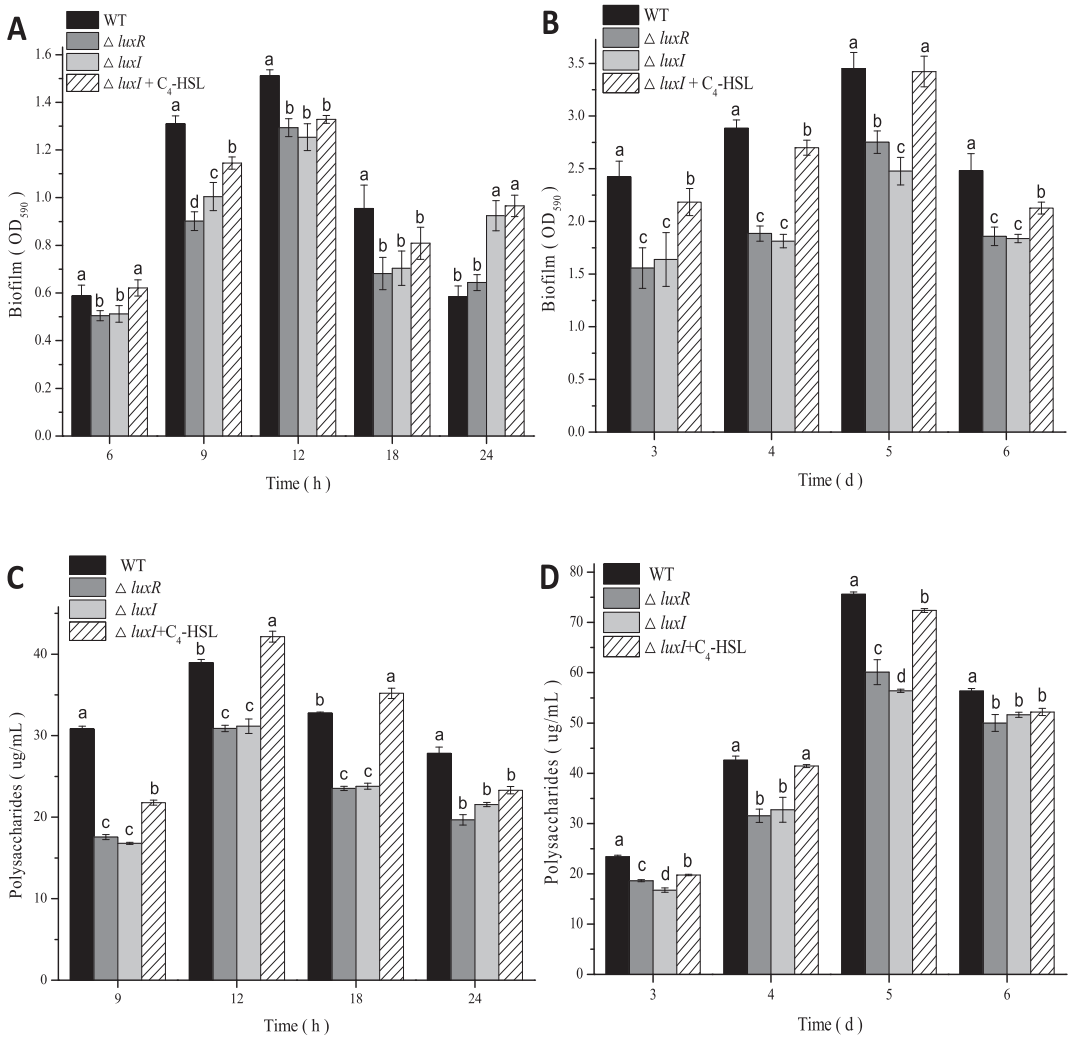


Fig. 3. Biofilm biomass and polysaccharides production of *P. fluorescens* WT, Δ*luxR* and Δ*luxI* without or with C_4-HSL incubated 30 °C (A, C) and 4 °C (B, D). Data was mean ± SD of three independent experiments. Different letters at each column indicated significant differences at $p < 0.05$ among WT and mutants.

restore EPS production.

We further observed the attachment of WT strain and two mutants by CLSM and SEM to assess the changes of biofilm structure, as presented in Fig. 4. The WT strain of *P. fluorescens* aggregated together to form rather thick and compact biofilm, while two mutants formed the thin biofilm, especially Δ*luxI*. The thickness of biofilm formed by WT, Δ*luxR* and Δ*luxI* reached 50.0 μm, 25.0 μm, and 22.0 μm at 4 °C for 5 d respectively (Fig. 4A). SEM images showed WT strain developed well growing biofilm connected by EPS matrix, while, biofilm of Δ*luxR* and Δ*luxI* showed apparent reduction of EPS (Fig. 4B). Moreover, the Δ*luxI* supplemented with exogenous C_4-HSL not only resulted in great increase of biofilm thickness (35.0 μm at 4 °C), but also stimulated the production of EPS compared to Δ*luxI*.

3.5. LuxI/LuxR deletion effect on motility

Flagellar-dependent swimming motility is related to biofilm formation, which are coordinately regulated by AHLs-mediated QS (O'Toole and Kolter, 1998). Swimming motility among the WT, Δ*luxR* and Δ*luxI* strains was presented in Fig. 5. In the initial 12 h at 30 °C WT strain and two mutants showed the slow motility, and Δ*luxR* and Δ*luxI* exhibited gradually stronger swimming than WT strain ($p < 0.05$) for 24 h incubation. Compared to the WT strain, swimming increased by 20.32%, 25.76%, 8.01% in Δ*luxR,* and promoted by 12.92%, 16.23%, 8.12% in Δ*luxI* for 24 h, 36 h and 48 h, respectively. Furthermore, supplement of C_4-HSL in Δ*luxI* strain resulted in a significant decrease of swimming, even was weaker than the WT strain.

Table 2
AHLs activity of in *P. fluorescens* PF07 WT strain, Δ*luxR* and Δ*luxI*.

AHLs	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	Concentration (ng/mL)		
				WT	△*luxR*	△*luxI*
C_4-HSL	3.06	172.0	102.2	276.88 ± 1.32[a]	106.48 ± 2.81[b]	0.31 ± 0.02[b]
C_{10}-HSL	7.39	256.0	102.2	6.52 ± 0.16[a]	6.76 ± 1.12[a]	5.98 ± 0.32[a]
C_{14}-HSL	8.22	312.0	102.2	7.01 ± 0.67[a]	6.87 ± 0.29[a]	6.53 ± 0.66[a]

Data was mean ± SD of three independent experiments. Different letters at each column indicated significant differences at $p < 0.05$ among WT and mutants.

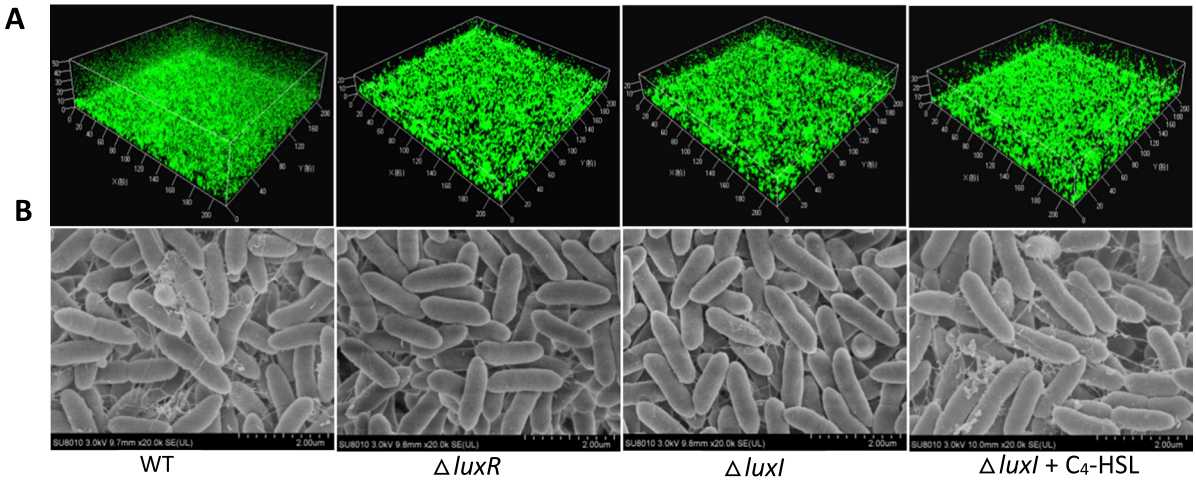


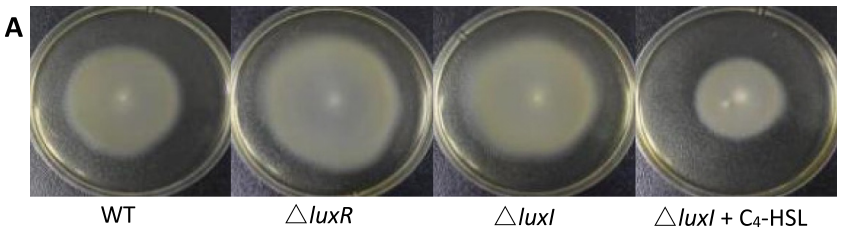
Fig. 4. Attachment and biofilm structure of *P. fluorescens* WT, Δ*luxR* and Δ*luxI* without or with C_4-HSL on the biotic surface. (A) Confocal laser scanning microscopy (CLSM) images (43 × oil) of WT strain and mutants at 4 °C for 120 h. (B) Scanning electron microscopy image of WT strain and two mutants at 4 °C for 120 h.

3.6. LuxI/LuxR deletion reduce on stress resistance

The survival rates of WT strain, Δ*luxR,* and Δ*luxI* in stationary-phase were determined after exposure to diverse stress conditions, including H_2O_2, thermal, NaCl and crystal violet treatments. As shown in Fig. 6, two mutants Δ*luxR* and Δ*luxI* exhibited significantly lower survival rates than WT cells after four treatments of stress ($p < 0.05$). With the length of treated time, the different viability between WT and two mutants cells increased. After 40 min of exposure for 20 mM H_2O_2, 50 °C thermal, 30% NaCl and 150 μg/mL crystal violet, the survival rates of WT were 15.31, 9.71, 1.79 and 1.51 fold higher than those of Δ*lux*R, and 18.71, 10.88, 3.01,1.35 fold higher than Δ*luxI,* respectively. Both Δ*luxI and* Δ*luxR* mutants exhibited the similar decrease of survival cell in four stress conditions, and Δ*luxI* was slightly sensitive compared with Δ*luxR* ($p < 0.05$).

3.7. LuxR/LuxI affect spoilage potential

Spoilage potential of WT strain, Δ*luxR* and Δ*luxI* with or without C4-HSL was investigated in the fish juice media stored at 4 °C (Fig. 7). The strains in all groups grew slowly and reach a early stationary phase after 5 days at 4 °C (Fig. 7A). *P. fluorescens* PF07 had weak production of TVB-N, and no significant difference of TVB-N were accumulated in four groups during the storage ($p > 0.05$) (Fig. 7B). The siderophore



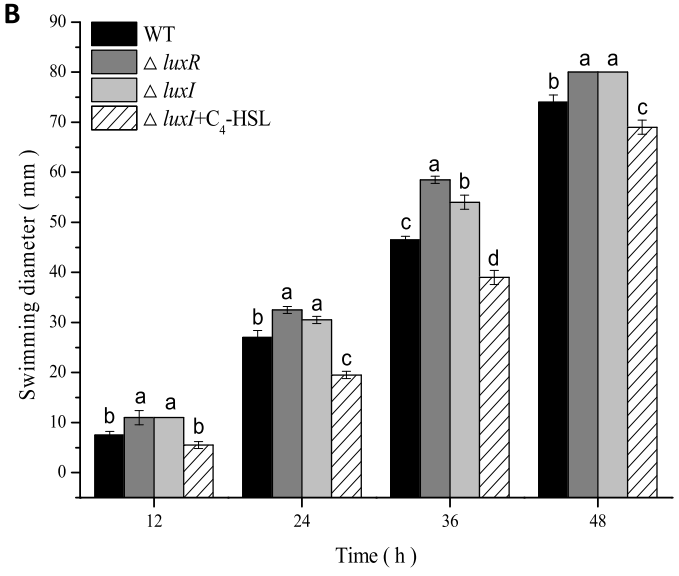


Fig. 5. Swimming motility change of *P. fluorescens* WT, Δ*luxR* and Δ*luxI*. (A) Swimming of WT, Δ*luxR* and Δ*luxI* inoculated onto the surface of 0.3% of LB agar at 30 °C for 36 h. (B) Swimming diameter of WT, Δ*luxR* and Δ*luxI* at 30 °C for the different incubation time. Data was mean ± SD of three independent experiments. Different letters at each column indicated significant differences at $p < 0.05$ among WT and mutants.

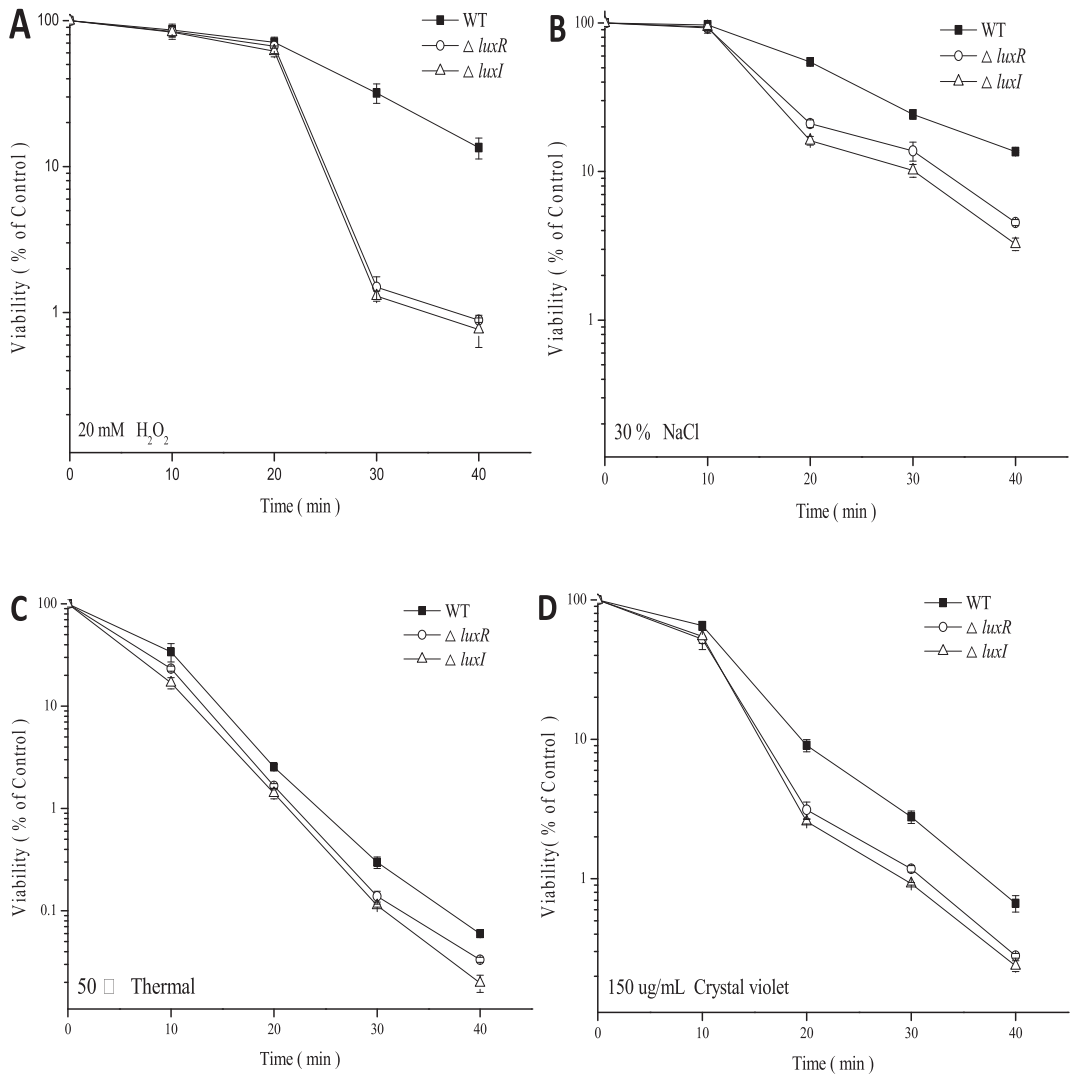


Fig. 6. Stress resistance assays of *P. fluorescens* WT, △*luxR* and △*luxI* exposing to 20 mM H_2O_2 (A), 30% NaCl (B), 50 °C (C) and 150 μg/mL crystal violet (D). The initial population of *P. fluorescens* was 6–7 log cfu/mL. Survival percentage was obtained by dividing the surviving population by the initial population, which corresponds to 100%. Data was mean ± SD of three independent experiments. Different letters at a given time indicated significant differences at $p < 0.05$ among WT and mutants. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

production between WT and Δ*luxR* or Δ*luxI* did not differ in initial 5 days, and exhibited significant decrease in Δ*luxR* or Δ*luxI* after 7 days compared to WT strain ($p < 0.05$) (Fig.7C). Interestingly, extracellular protease activity in two mutants was remarkably lower than WT strain during the whole storage time ($p < 0.05$). Compared to WT strain, protease activity in Δ*luxR* or Δ*luxI* dropped by 40.23% and 37.54% after incubation for 9 day at 4 °C. Moreover, complementation of C4-HSL in Δ*luxI* slightly increased two spoilage phenotypes to WT strain ($p < 0.05$).

3.8. Related gene transcription

The transcription of *alg*, *lapA*, *flgA*, *rpoS* and *aprX*, were further determined using qPCR with RNA isolated from WT strain, Δ*luxR* and Δ*luxI* with or without exogenous C4-HSL as shown in Fig. 8. The transcriptions of four genes related biofilm, protease and stress in Δ*luxR* and Δ*luxI* were found to significantly decrease except *flgA* gene. Compared to WT strain, the transcript levels of *alg*, *lapA*, *rpoS* and *aprX* genes in Δ*luxR* were 0.79, 0.76, 0.83, 0.80 fold, and were 0.29, 0.38, 0.25, 0.24 fold in Δ*luxI* at exponential phase (12*h*), respectively. During the stationary phase (24 h), similar transcript changes of four genes in Δ*luxR* and Δ*luxI* were observed, except the less transcript levels in Δ*luxR* (about from 0.33 to 0.37 fold). Conversely, the transcription of *flgA* gene in Δ*luxR* or Δ*luxI* was significantly higher than that in WT strain ($p > 0.05$) for 24 h of the culture, and up-regulated by 1.31 and 1.36 fold in Δ*luxR* and Δ*luxI,* respectively. Meanwhile, the transcriptions of *alg*, *lapA*, *rpoS* and *aprX* genes partially restored the similar levels of WT strain, and the transcript levels of *flgA* gene greatly decreased in Δ*luxI* supplemented with 40 μM C4-HSL at exponential and stationary phase respectively, which revealed that LuxI/LuxR homolog in *P. fluorescens* affected markedly expression of genes related biofilm, motility, protease and stress.

4. Discussion

Various Gram-negative bacteria use LuxI/LuxR-type QS system to orchestrate target genes expression related the virulence and biofilm formation (Kai and Bassler, 2016). The identification of key genes related to AHLs mediated QS will benefit to the understanding of adaption, biofilm formation and bacterial spoilage mechanism. In the present study, one AHL synthase gene (*luxI*), and three transcriptional regulators (*luxR*) homologs were found in *P. fluorescens* PF07, as most important spoilage microorganism in refrigerated seafood product. Members of *luxR* family of transcriptional regulators are often, but not

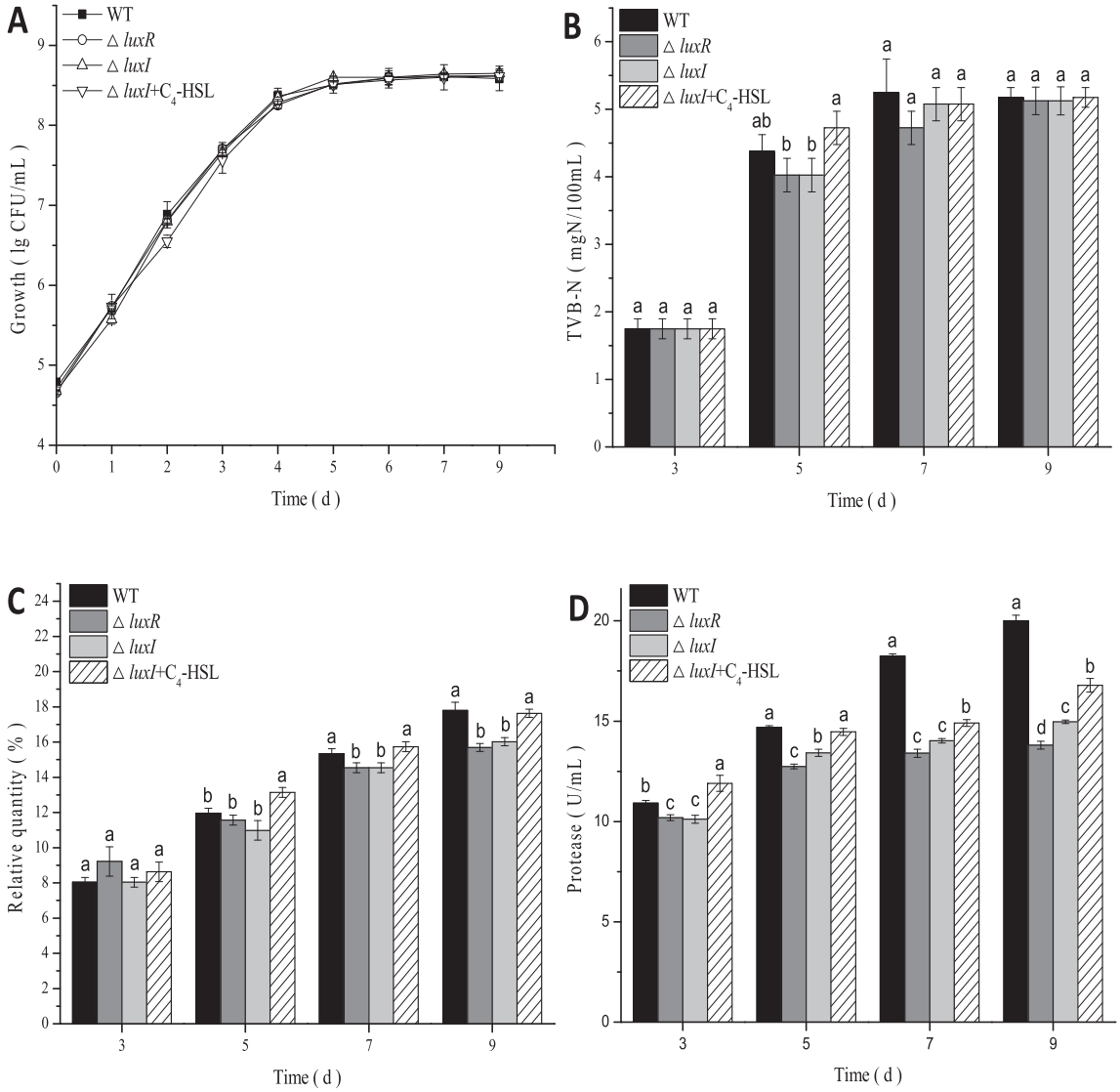


Fig. 7. Growth curve (A), TVB-N production (B), siderophore (C) and proteolytic activity (D) of *P. fluorescens* WT, Δ*luxR* and Δ*luxI* with or without C_4-HSL in fish juice media stored at 4 °C. Data was mean ± SD of three independent experiments. Different letters at each column indicated significant differences at $p < 0.05$ among WT and mutants.

always, linked to a *luxI*. Two AHL synthase genes and three LuxR-like transcription factor genes are observed in *P. aeruginosa* (Jimenez et al., 2012) and *P. fluorescens* UK4 (X. Liu et al., 2018). In *P. fluorescens* PF07, only one *luxR* (*luxR1*) is located upstream of *luxI*, and the other *luxR* might be as LuxR "solos" (term used to define LuxR proteins without a cognate LuxI). The possible function of these LuxR "solos" proteins could be to sense and respond to foreign and intracellular molecules and to extend the AHL QS regulon to other gene targets, such as *P. aeruginosa* QscR (Chugani and Greenberg, 2010). In the present study, we characterized the structure of one LuxI/LuxR homolog and evaluated its role in *P. fluorescens* PF07. According to bioinformatics analysis, two deduced complete amino acid sequence of *luxI* and *luxR* genes in *P. fluorescens* PF07 shared high identity with LuxI/LuxR homologs in *Pseudomonas*, especially *P. fluorescens* UK4. The LuxI protein of PF07 shared 21 conserved residues with other six LuxI-type AHL synthases, which are crucial for enzymatic activity (Watson et al., 2002). The high degree of LuxI sequence conservation in the N-terminal region suggested a role in catalysis and binding of the common substrate SAM. The LuxR protein in PF07 with nine conserved residues was a close homolog of other LuxR-type receptors and displays similar properties, including N-terminal domain as a AHL-binding site, and C-terminal domain having a helix-turn-helix DNA binding motif (Zhang et al., 2002). Additionally, a considerable heterogeneity of sequences between LuxI and LuxR in *P. fluorescens* and homologs in *A. salmonicida* or *A. tumefaciens* might be associated with the different genus/species and different AHLs activities. The 3D structures of LuxI- and LuxR-type proteins were a mixed α-β-α sandwich just like other homology proteins, and the residues in the active site were strictly conserved. It was revealed that LuxI/LuxR in *P. fluorescens* PF07 had a characteristic protein structure of AHL-QS homologs found in many *Proteobacteria* (Churchill and Chen, 2011).

To explore the role of LuxI/LuxR, two mutants Δ*luxI* and Δ*luxR* had been successfully constructed by deleting *luxI* and *luxR* genes in PF07. It was confirmed high short-chain AHLs secreted by *P. fluorescens* PF07, particularly C_4-HSL. In several *Pseudomonoas* isolates from food, the production of a diverse range of AHLs has been widely reported. *P. fluorscens* 395 from raw milk was capable of producing C_4-HSL and 3-oxo-C_8-HSL (Liu et al., 2007). *P. fluorscens* PF01 as a spoilage isolate secreted seven types of AHLs, 3-oxo-C_8-HSL as a major signaling in our previous work (Zhao et al., 2016). Furthermore, the deficiency of *luxR* gene weakened AHLs activities, and C_4-HSL production was mostly abolished in Δ*luxI*, indicating that LuxI was indeed essential for the

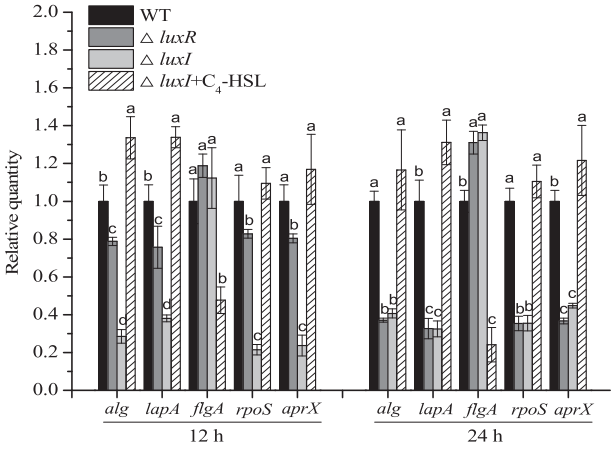


Fig. 8. Comparison of transcription of *alg*, *lapA*, *flgA*, *rpoS* and *aprX* in *P. fluorescens* WT strain, Δ*luxR* and Δ*luxI* with or without C_4-HSL at 30 °C. Relative *alg*, *lapA*, *flgA*, *aprX*, and *rpoS*, transcript levels in WT strain Δ*luxR* and Δ*luxI* by qRT-PCR, and 16S rRNA was used as reference gene. Data was presented as the mean ± standard deviation ($n = 5$). Different letters at each column indicated significant differences at $p < 0.05$ among WT and mutants at the same gene and time.

production of C_4-HSL-mediated autoinduction, and LuxR in upstream of *luxI* gene may be partially involved in the modulation of AHLs synthesis. LuxR seems both to activate and to inhibit the *luxI* gene in a number of cases (Churchill and Chen, 2011). Similar results were found by Milton et al. (1997), who reported that the deletion of *vanI* and *vanR*, belonging to LuxI/LuxR family in *Vibrio anguillarum,* abolished *N*-(3-oxodecanooyl)-L-homoserine lactone. Additionally, two mutants still remained the low activities of C_{10}-HSL and C_{14}-HSL, hinting that there may be other LuxI homolog responsible for the synthesis and production of these AHLs.

Pseudomonas with strong biofilm-forming ability can produce several biofilm matrix molecules, including polysaccharides, nucleic acids and proteins. Our results showed that *P. fuorescens* isolate was a good biofilm producer with high biomass. The *luxI* or *luxR* deficiency of *P. fuorescens* PF07 induced the decline of biofilm biomass and EPS production during the whole development phase compared to WT strain. This phenomenon was in agreement with observations obtained by Mukherjee et al. (2017), who found that the deletion of RhlI in *P. aeruginosa* may have caused the less biofilm formation, lower ability of EPS production and attachment. The observations by CLSM clearly revealed that biofilm of Δ*luxR* or Δ*luxI* exhibited less thickness than that of WT strain, and exogenous C_4-HSL in Δ*luxI* partially restore to the biofilm structures of WT. Additionally, the more EPS produced from WT and Δ*luxI* with exogenous C_4-HSL were found by SEM observation in spite of the similar bacterial morphology between WT and two mutants. Polysaccharides, such as alginate, Pel and Psl, play critical roles in the biofilm development of by providing structural support in *P. aeruginosa*. Consistently, the expression of alginate (*alg*) as an important compound of EPS and adhesins (*lapA*) was significantly down-regulated in two mutants. Those results suggested that LuxR/LuxI could positively modulated the biofilm development and maturation in *P. fluorescens.*

P. fluorescens has multiple flagella for motion. Swimming motility of bacterial strains plays an important role in the early stage of biofilm formation to allow bacterial spread on surfaces (O'Toole and Kolter, 1998). In contrast to the decrease of biofilm formation, Δ*luxR* and Δ*luxI* greatly promoted the swimming motility, and up-regulated the expression of flagellar biosynthetic protein (*flgA*) in *P. fluorescens*. The inverse influence of LuxR and LuxI on biofilm formation and flagella-driven motility in *P. fluorescens* was also found *in P. fluorescens* F113 (Barahona et al., 2010) and *A. salmonicida* AE03 (L. Liu et al., 2018). In *P. aeruginosa*, growth as a biofilm was inversely regulated with a cooperative form of multicellular surface motility (Kuchma et al., 2007), and the transcription factor FleQ inversely control flagellar motility and EPS production in the response to cellular cyclic-di-GMP (Baraquet et al., 2012). These findings suggested that the deficiency of *luxR* or *luxI* gene could inhibit expression of EPS matrix and induce flagellar-driven motility by affecting the concentration of intracellular second messenger *c*-di-GMP content, which led to repress the conversion of biofilm cell status from planktonic cell.

Previous findings implied that QS may play an important role in the response of Gram-negative bacteria to environmental stresses. Our studies indicated that *luxR* or *luxI* might be involved in the positive regulation of resistance of *P. fluorescens* aganist H_2O_2, NaCl, heat, and crystal violet. Compared to WT strain, Δ*luxR* and Δ*luxI* exhibited the lowest survival rate to hydrogen peroxide in four environmental stresses. Similarly, QS system of *P. aeruginosa* and *Salmonella typhi* regulates superoxide dismutase and catalase during oxidative stress and aids in oxidative stress (Hassett et al., 2010; Walawalkar et al., 2016). QS was also linked to the *Vibrio harveyi* osmotic stress response (Van Kessel et al., 2015). Our observations imply that LuxI/LuxR mediated QS may play important roles in the response of Gram-negative spoilage bacteria to oxidative, osmotic, thermal and preservative stresses in food processing. Additionally, as a master regulator of the general stress response in Gram-negative bacteria, RpoS, regulates the expression of various genes related with stress protective functions (Dodd and Aldsworth, 2002). In the present study, the deletion of *luxI* and *luxR* significantly inhibited the transcript levels of *rpoS* in *P. fluorescens*, as similarly observed by Latifi et al. (1996), who reported that the expression of *rpoS* was abolished in a *P. aeruginosa lasR* mutant. Our recent studies revealed that RpoS-deleted *P. fluorescens* UK4 attenuated AHLs activities and transcription of AHLs synthase genes (X. Liu et al., 2018). It was hinted that AHLs mediated QS interlinked via RpoS to integrate the regulation of stress response in *P. fluorescens.*

P. fluorescens is widely known to be responsible for the specific spoilage in iced fish (Zhao et al., 2016). In refrigerated fish juice media, *P. fluorescens* PF07 with high amount of siderophores and protease activity promoted the microbial spoilage in spite of low TVB-N production. Siderophore has high affinity to iron from food environment to get competitive advantage for some *Pseudomonas*. Our results indicated that Δ*luxR* and Δ*luxI* significantly repressed the siderophore activity. Indeed, the biosynthesis of siderophore in *P. putida* and *P. aeruginosa* were controlled by AHL-based QS system (Ren et al., 2004). Protease as a major factor in spoilage related bacteria degrades proteins into volatile nitrogen- and sulfur-containing compounds with offensive odours. The proteolytic activity decreased greatly in Δ*luxR* and Δ*luxI*, which seemed to be a AHL regulated phenotype in PF07. Similarly, QS regulation had influence on the exoenzyme (lipases and proteases) synthesis in *P. fluorescens* 395 (Liu et al., 2007) and *Pseudomonas psychrophila* PSPF19 (Jamuna and Vittal, 2014). The tanscription of *aprX* gene coding alkaline metalloprotease was down-regulated in Δ*luxR* and Δ*luxI* of PF07, which was in line with previous results in *P. fluorescens* 395 (Liu et al., 2007). Our data was further indicated that complementation of Δ*luxI* with exogenous C_4-HSL partially restored two spoilage phenotypes, which was in agreement with observations obtained by Wevers et al. (2009). Additionally, it was indicated that Δ*luxR* or Δ*luxI* had no influence on the growth in juice media, as well as TVB-N production, during the refrigerated storage. These above results highlighted the import role of LuxI/LuxR homolog as a QS factor in the regulation of spoilage ability, and suggested that other unknown signaling might be also involved in the modulation of production of spoilage metabolite in the psychrotrophic *P. fuorescens*.

Our results apparently revealed that the absence of *luxI* or *luxR* in *P. fluorescens* PF07 decreased transcription of of alginate, adhesins, RpoS and protease, however, largely promoted flagellar biosynthesis, which was consistent with the phenotypes changes of biofilm, stress and spoilage between the WT strain and Δ*luxR* or Δ*luxI*. To our knowledge, this is the first report identifying the roles of LuxI/LuxR in *P. fluorescens*

isolated from food as a psychrotrophic spoilage strain.

Overall, the data presented here revealed that the LuxI/LuxR homolog plays a crucial role in the regulation of biofilm formation, motility and stress response for adaption in food processing environment in *P. fluorescens* PF07. The LuxI was responsible for the production of C_4-HSL as a major AHLs signal molecule, while LuxR was harbored by *P. fluorescens* as a transcription factor to coordinated AHLs mediated QS system. The biofilm formation, exopolysaccharides production, and protease were positively regulated by LuxI/LuxR as co-operation. Moreover, this is the interesting report hinting that LuxI/LuxR mediated QS could modulated the resistance to various stress environment by influencing the global regulator RpoS. Future work will focus on unraveling the precise regulatory network of LuxI/LuxR complex in transcriptional regulation of target genes and identifying the new cognate LuxI/LuxR homologs in *P. fluorescens*.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2018.12.011.

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