African Journal of Microbiology Research Vol. 6(34), pp. 6333-6339, 6 September, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR12.051
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Cloning and preliminary identification of SptR, a LuxR-like regulator from *Serratia plymuthica*

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Accepted 21 August, 2012

***N*-acylhomoserine lactone (AHL)-mediated Quorum sensing (QS) allows bacterial populations to communicate via the exchange of diffusible signals to coordinate gene expression and control adaptive behaviors in response to cell density in Gram-negative bacteria. Two *luxIR*-like QS systems *splIR* and *spsIR* have been characterized in an endophytic strain G3 of *Serratia plymuthica*. Here we reported a third LuxR homolog, SptR for better understanding the QS network by strain G3. Firstly, cloning and sequencing of a *sptR* gene was performed using PCR; phylogenetic analysis revealed that SptR was not closely correlated to other members of LuxR-family protein in *Serratia*, but clustered into one clade with LuxR-family members from *Enterobacter cloacae* SCF1 and *Erwinia tasmaniensis* Et1/99 sharing a more common ancestor. Further mutational analysis of biocontrol-related phenotypes demonstrated that SptR served as a positive regulator to control production of exenzymes, as well as swimming motility and biofilm formation contrary to most LuxR homologs from *Serratia* as repressor. However, no significant impact on production of auxin indole-3-acetic acid (IAA) and antagonistic ability was observed. The results paved the way for further studies of the complex QS network and regulatory mechanisms involved in fine tuning of beneficial bacteria-plant interactions by *S. plymuthica*.**

Key words: *Serratia plymuthica*, a LuxR-like SptR regulator, quorum sensing, exoenzymes, swimming motility, biofilm formation.

INTRODUCTION

N-acylhomoserine lactone (AHL)-based quorum sensing (QS) systems have been described in many plant-associated Gram-negative bacterial species to regulate gene expression in response to cell density. These signal molecules are produced by LuxI family of AHL synthase and sensed by the LuxR family of response regulator. QS controls diverse biological functions including bioluminescence, conjugation, pathogenicity or virulence, symbiosis, production of exoenzymes and secondary metabolites, and biofilm formation (Pierson et al., 1998; Miller and Bassler, 2001; Whitehead et al., 2001). In addition, QS also controls interactions between bacteria and higher organisms, thus has major impacts on agriculture, ecology, industry and medicine where QS systems control the adaptive behaviour of microbes (Williams and Cámara, 2009).

Serratia plymuthica G3 is an endophytic strain isolated from the stems of *Triticum aestivum* L. with a broad spectrum of antifungal activities and can produce an array of antimicrobial exoproducts such as chitinase, protease, antibiotic pyrrolnitrin, and siderophores. In addition it also produced the plant hormone indole-3-acetic acid (IAA) (Liu et al., 2010). Previous studies have characterized two QS systems (*splIR* and *spsIR*) in biocontol strains of *S. plymuthica* and shown to control biocontrol-related traits (Liu et al., 2007; Liu et al., 2011). Recently we obtained a draft genome of *S. plymuthica* G3 (Liu et al., unpublished data), and preliminary sequence analysis identified the presence of a third LuxR-like protein SptR, although its cognate AHL synthase gene *sptI* seems no complete open reading

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Table 1. Bacterial strains and plasmids used in this study.

Strain and plasmid	Description	Reference
S. plymuthica G3	Endophytic strain, wild type, Rif ^{R}	Liu et al. (2010)
Δ*sptR*	A sptR::Gm mutant, Gm ^{R}	This study
Δ*sptR*/*sptR*$^{+}$	Δ*sptR* carrying pUCP26-*sptR* for *trans*-complement	This study
Δ*sptR*/pUCP26	Δ*sptR* carrying pUCP26 as control	This study
S. plymuthica HRO-C48	Rhizospheric strain, wild type, Rif ^{R}	Liu et al. (2007)
AHL-4	A mini-Tn5 insertion mutant in *splI* gene of HRO-C48, Km ^{R}	Liu et al. (2007)
E. coil DH5α	A host strain for cloning	Ausubel et al. (1994)
E. coil S17-1	thi pro hsdR recA; chromosomal RP4; Tra^{+}; Sm/Sp^{R}	Ausubel et al. (1994)
pMD19-T	Cloning vector, Amp ^{R}	Takara
pMD19-T-*sptR*	Plasmid pMD19-T harboring the G3 *sptR* gene	This study
p34S-Gm	Source of Gm ^{R} cassette	Dennis et al.(1998)
pDM4	Gene replacement vector, Cm ^{R}, *SacB*	O'Toole et al. (1996)
pUCP26	Broad-host-range cloning vector; Tc ^{R}	West et al. (1994)
pBluelux	A promoter-less *luxCDABE* cassette in pBluescript II, Amp ^{R},	Atkinson et al. (2008)

frame (ORF) and remain to be further confirmed through experiments. The findings indicated the complexity of QS network in strain G3. To lay a foundation for better investigation of QS networks and their interactions in *S. plymuthica,* here we firstly performed the cloning, sequencing and phylogenetic analysis of the *sptR* gene encoding a third LuxR homolog in strain G3, followed by mutational analysis to characterize several *sptR*-regulated biocontro-related phenotypes using a mutant deficient in *sptR* preliminarily.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *S. plymuthica* strains G3, HRO-C48 and their derivatives were cultured at 28°C and *Escherichia coli* strains were cultured at 37°C in Luria-Bertani broth (LB) or on agar medium (LA). Antibiotics, when required, were added to the growth media at the following concentrations: rifampicin, 40 μg/ml for strain G3, a natural mutant resistant to rifampicin; ampicillin, 100 μg/ml; gentamycin, 20 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 25 μg/ml.

DNA preparation and manipulations

Standard methods were used for plasmid and genomic DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, ligation, and transformation (Ausubel et al., 1994) or follow the manufacturer's directions.

Cloning of *sptR* gene encoding a third LuxR regulator from strain G3

We have cloned *splIR* and *spsIR* encoding two LuxIR-like QS systems in strains G3 and HRO-C48 of *S. plymuthica*. Very recently, the draft genome of strain G3 (Liu et al., unpublished data) allowed us *in silicon* analysis and identification of a third *luxR* homologous gene, designated as *sptR*. For cloning and sequencing, a primer pair sptR-F and sptR-R in Table 2 was designed to amplify *sptR* by PCR using strain G3 genomic DNA as template and LA-Taq polymerase (Takara, Dalian, China). The following program was used for thermal cycling: 94°C for 5 min, then 30 cycles at 94°C for 1 min, 50°C for 45 s, and 72°C for 1 min, and a final extension at 72°C for 10 min. After purification, 1056-bp PCR products were cloned into pMD19-T (Takara, Dalian, China) followed by transformed into *E. coli* DH5α. The positive clones were selected through PCR and sequencing (Sangon Co. Ltd., Shanghai, China).

Further analysis of the phylogeny of the 738-bp *sptR* homologs searching related amino acid sequences currently available in the GenBank database was carried out using the neighbor-joining method of MEGA 4. Bootstrap test (1000 repetitions) was performed as previously described (Liu et al., 2011).

Construction of a Δ*sptR* mutant and complement strain

To investigate the role of a third LuxR homolog SptR in strain G3, a *sptR* mutant Δ*sptR*::Gm was constructed and isolated using the homologous gene replacement strategy as previously described (Atkinson et al., 2008). Briefly, using primer pairs sptR1-F/R and sptR2-F/R in Table 2, 331-bp upstream fragment and 324-bp downstream region of the *sptR* gene were amplified, respectively. Two PCR products were ligated by stepwise cloning into pMD19-T with 110-bp deletion of *sptR* coding region. Then, a Gm cassette from p34S-Gm (Dennis and Zylstra, 1998) excised by *Sma*I was inserted into the *Sal*I site after blunting end between two PCR fragments. Next, the *Xba*I–*Pst*I sptR1-Gm-sptR2 fragment was excised from pMD19-T and subcloned into the pDM4 suiside plasmid (O'Toole et al., 1996), and then transformed into *E. coli* S17-1(λ-pir). The protocol for obtaining a mutant through the stable integration of deletion/insertion constructs of *sptR* into the parent chromosome was carried out as previously described (Atkinson et al., 1999). A Δ*sptR* mutant with resistance to gentamycin and sensitive to chloramphenicol was selected and verified by PCR and sequencing. Additionally, the complement strain Δ*sptR*/pUCP26-*sptR* was constructed through subcloning of 1525-bp PCR product using primers sptR-F and sptR-pR in Table 2 including the full length *sptR* ORF with its 469-bp native promoter region into the

Table 2. PCR primers used in this study.

Primer	Sequence (the introduced restriction sites were underlined)	Reference
sptR-F	5'- ATGCTAAGAAAGAACCCA- 3'	This study
sptR-R	5'- GATGGAATATGAAGACAATATC -3'	This study
sptR-pR	5'- AATATGTGGAGCAAGGTG-3'	This study
sptR1-F	5'- CTGCAGAGCCGGCAGTTAATGGAA- 3' (*Pst*I)	This study
sptR1-R	5'- GTCGACAAACTCGCGTGCCTGAT- 3' (*Sal*I)	This study
sptR2-F	5'- CGAGAATAAAGGCGACAT- 3'	This study
sptR2-R	5'- CAGAGCCGGCAATCTAAT- 3'	This study
sptRlux-F	5'-AATATGTGGAGCAAGGTG-3'	This study
sptRlux-R	5'- ACCGCTCAGCAGAATATA -3'	This study

broad-host-range vector pUCP26 (West et al., 1994), followed by transformation into the Δ*sptR* mutant.

Assays for proteolytic activity and IAA production

To determine the impact of SptR on production of exoenzymes and secondary metabolite, firstly proteolytic activities were assayed on skimmed milk agar plates by seeding 2 µl of bacterial overnight cultures onto the plate and monitoring the appearance of haloes around the developing bacterial colonies after 48 h at 28°C as previously described (Liu et al., 2010). Furthermore, overnight cultures of bacteria in LB broth with appropriate antibiotics were diluted (OD_{600} 0.02) into 2 ml of LB with 200 µg/ml of tryptophan. After further incubation for 42 h, Salkowski's method was used to determine IAA production (Gordon and Weber, 1951). A standard curve generated from known concentrations of IAA was used to calculate the concentration of IAA.

Swimming motility assays

Flagellar function has been demonstrated to be necessary for initial bacterial attachment and subsequent biofilm formation (O'Toole and Kolter, 1998). For detection of flagellar swimming motility, minimal swim motility agar plates contained 10 g/liter tryptone, 5 g/liter NaCl and 0.3% (wt/vol) Bacto agar were prepared. A 1 µl volume of overnight seed cultures grown at 28°C was stabbed into swim agar plates and incubated at root temperature for 16 h (Liu et al., 2011).

Abiotic surface adhesion assays

Adhesion is considered to be the first step in the development of bacterial biofilm. Bacterial adhesion on an abiotic surface was measured using polystyrene microtitre plates in triplicate as described by O'Toole and Kolter (1998) with a few modifications. Overnight bacterial cultures were inoculated into the wells of microtiter in 100 µ l of LB (adjusted to OD_{600} 0.02) without shaking and incubated at 28°C for 48 h. The cell densities were determined at 600 nm, followed by quantification of adhesion through staining with 0.1% solution of crystal violet (CV). The absorbance was measured at 570 nm (Müller et al., 2009).

Bioassay of the antifungal activity *in vitro*

To evaluate the effects of SptR in the suppression of pathogenic fungi, strain G3 and the Δ*sptR* mutant were tested for *in vitro* antagonistic activity against gray mould *Botrytis cinerea* as previously described (Liu et al., 2010). Briefly, overnight culture of strain G3 and its derivatives in LB at 28°C were spotted (2 µl) in a line 3 cm away from the centre of the PDA plate, respectively; 5 mm diameter agar disks from an actively growing fungal culture were seeded at the centre of PDA plates (60 mm diameter). After incubating at 25°C for 4 days, the diameter of the inhibition zone was measured.

Construction of *lux*-based transcriptional fusion to *sptR* promoter

It has been reported that the expression of *splR* is AHL-dependent in *S. plymuthica* RVH1 (Van Houdt et al., 2007a). To examine whether the expression of *sptR* from G3 is the same case, the 440-bp *sptR* gene promoter region including its upstream putative *lux*-box like sequence by strain G3 were amplified with the primers sptRlux-F and sptRlux-R in Table 2. The PCR fragment was digested with *Xba*I and *Sal*I followed by Klenow blunting, then ligated into the *Sma*I site of pBluelux (Atkinson et al., 2008). The positive clones were selected according to bioluminescence, then excised with *Pst*I and ligated into the same site of the broad-host-range vector pUCP26, resulting in pUCP26/*sptR*::*lux* was introduced into the closely related strain HRO-C48 and its *splI* mutant AHL-4 (Liu et al., 2007), respectively. Bioluminescence was determined with a combined luminometer/spectrophotometer (Bio-Tek, Massachusetts, USA).

Statistical analysis

All data were subjected to One-Way ANOVA statistical analysis using SPSS11.5. Each assay was performed at least in triplicate, and the average results are shown.

Nucleotide sequence accession numbers

The GenBank accession numbers for the *sptR* gene from strain G3 is JF274255.

RESULTS

Cloning and phylogenetic analysis of a LuxR-like SptR from *S. plymuthica* G3

Whole-genome sequencing searches predicted the

Table 3. Effects of sptR on swimming motility and biofilm formation in G3.

Phenotype	WT-G3	ΔsptR	ΔsptR/sptR+	ΔsptR/pUCP26
Swimming motility (mm)	42.5 ±2.1 (A)	24.0 ± 1.4 (B)	37.8 ±2.5 (A)	23.8 ± 1.1 (B)*
Biofilm (OD570)	0.47 ± 0.01 (A)	0.37 ±0.02 (B)	0.45 ± 0.02 (A)	0.37 ± 0.02 (B)

* Different letters indicate significant differences at $p<0.01$.

presence of a third LuxR-like protein SptR in strain G3. The *sptR* gene was amplified using primers sptR-F and sptR-R. Sequence analysis of the 1056-bp PCR product included a putative ORF of the 738-bp *luxR*-like gene from strain G3 (tentatively designated as *sptR* for *S. plymuthica* third *luxR*), and encodes a protein with 245 amino acids. A database search in GenBank revealed 74 and 67% identity between SptR and LuxR family transcriptional regulator from *Erwinia tasmaniensis* strain ET1/99 and *Enterobacter cloacae* SCF1, respectively. The N-terminal autoinducer (AHL) binding domain and the C-terminal DNA-binding domain of LuxR-like proteins including the helix-turn-helix motif (HTH_LUXR) described in similar LuxR homologs of other gram-negative bacteria, appear to be conserved.

Further phylogenetic analysis revealed that SptR from G3 was clustered into the same clade with the LuxR family regulators from *E. tasmaniensis* strain ET1/99 and *E. cloacae* SCF1, but has no close correlation with the other two known LuxR homologs SplR and SpsR from G3, as well as CarR form *Serratia odorifera* DSM 4582 (Figure 1).

Isolation and verification of a *sptR* deletion/insertion mutant

To investigate the functions of the SptR, a deletion/insertion mutant Δ*sptR* was constructed by homologous recombination with the pDM4 suicide vector carrying the *sptR* gene disrupted by deletion of 110-bp coding region and insertion of the gentamicin resistance cassette. Double recombinants with resistance to gentamycin and rifampicin, and sensitive to chloramphenical were selected and verified by PCR (Figure 2) and sequencing. Additionally, a complement strain Δ*sptR*/pUCP26-*sptR* was obtained through providing *sptR in trans*.

sptR is required for protease production in *S. plymuthica* G3

Extracellular enzymes and secondary metabolites are important biocontrol determinants of strain G3 (Liu et al., 2010). To investigate whether *sptR* plays a role in production of exoenzymes and secondary metabolites, we compared the proteolytic activity and IAA levels of the Δ*sptR* mutant with the wild type G3. Examination of growth rates over a 24 h time period revealed that the mutation in *sptR* has no significant influence on bacterial growth, as well as IAA production using Salkowski's reagent (data not shown). However proteolytic activity was abolished in the Δ*sptR* mutant compared with the wild type G3, and the complement strain restored the proteolytic activity to the wild type level (Figure 3), suggesting that *sptR* is required for production of extracellular protease by G3.

sptR positively controls swimming motility and biofilm formation of G3

Swimming motility of strain G3 and the Δ*sptR* mutant using flagellar swim plates was examined, and biofilm formation on an abiotic surface under static conditions was tested in a microtiter-plate assay. The results showed that both swimming motility and biofilm formation capacity (Table 3) were impaired in the Δ*sptR* mutant compared with the wild type G3 ($P<0.01$). Mutation in *sptR* gene resulted in significant decrease in swimming zone and adhesion ability compared with G3. However, the *trans*-complement strain almost restored the ability to swim and adhere on an abiotic surface to the wild type level, suggesting that the SptR is involved in swimming motility and biofilm formation.

SptR has no impact on the antagonistic activity

In vitro antagonistic ability to suppress gray mould *B. cinerea* was tested. Figure 4 illustrated that no significant difference in antifungal activity against *B. cinerea* was observed among G3 and its derivatives, indicating that the SptR is not required for production of antimicrobial metabolites.

Heterologous expression of G3 *sptR* promoter is AHL-dependent

Sequence analysis predicted that the *sptI* gene of G3 has imperfect ORF. To obtain preliminary information on whether other AHL synthase has influence on the expression of *sptR*, we introduced the plasmid-based *sptR*::*lux* reporter fusion into the highly closely related strain HRO-C48 and its *splI* mutant AHL-4 deficient in

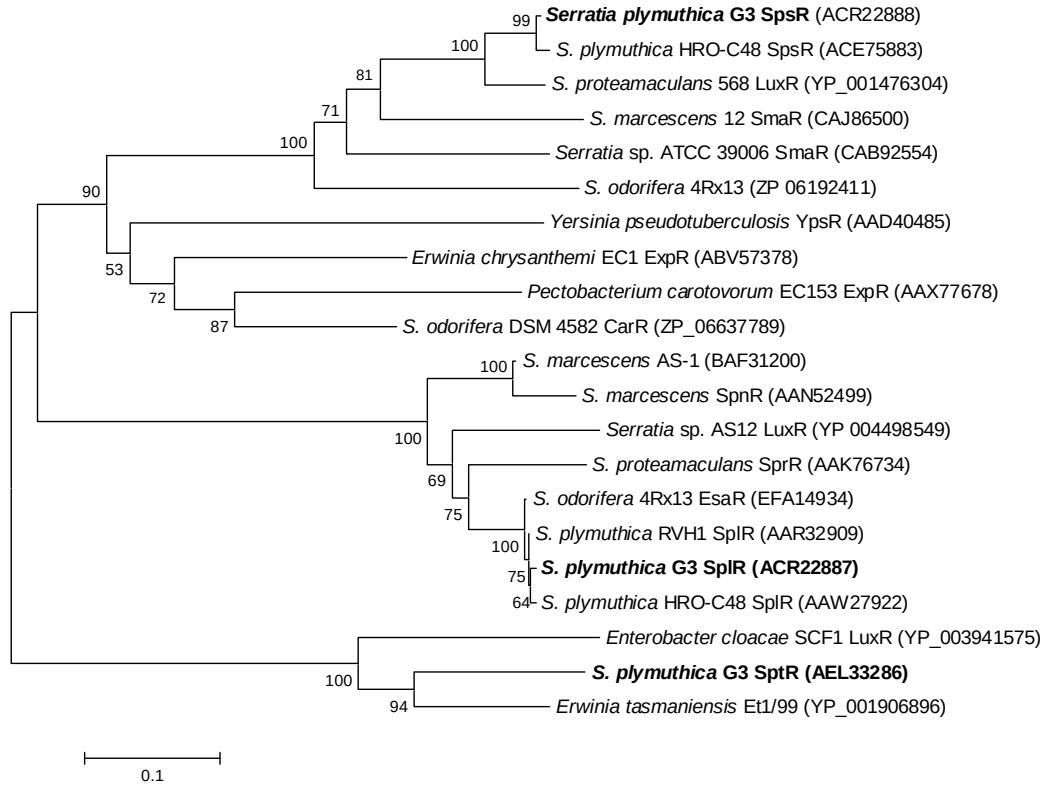

Figure 1. Phylogenetic analysis of the SptR and other LuxR homologs by amino acid. Neighbor-joining tree was constructed using MEGA 4. Multiple sequence alignment of SptR and related LuxR-family proteins in the GenBank database was performed by ClustalW. The significance of each branch is bootstrap value calculated for 1000 subsets. Scale bar indicates the mean number of substitutions per site.

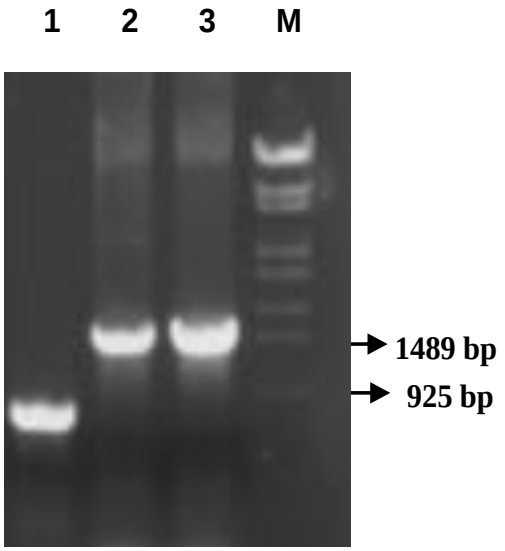

Figure 2. PCR verification of the ΔsptR mutant of strain G3 using primers sptR1-F and sptR2-R. Lane 1 Genomic DNA of G3 WT as template (767-bp); Lane 2 & 3 Genomic DNA of two clones of the ΔsptR mutants as template (1457-bp); M λEcoT14 I digest-Marker.

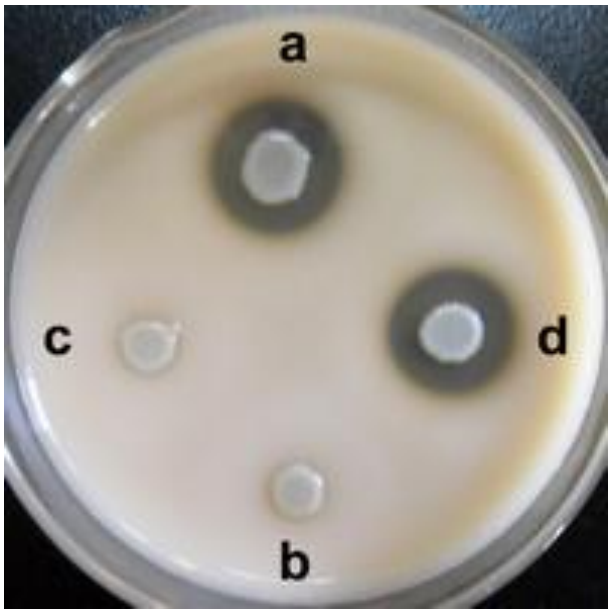

Figure 3. Proteolytic activity of strain G3 and its derivatives after incubation for 48 h on skim milk plates. a G3 WT; b The ΔsptR mutant; c The ΔsptR/pUCP26 as control; d The complement strain ΔsptR/pUCP26-sptR.

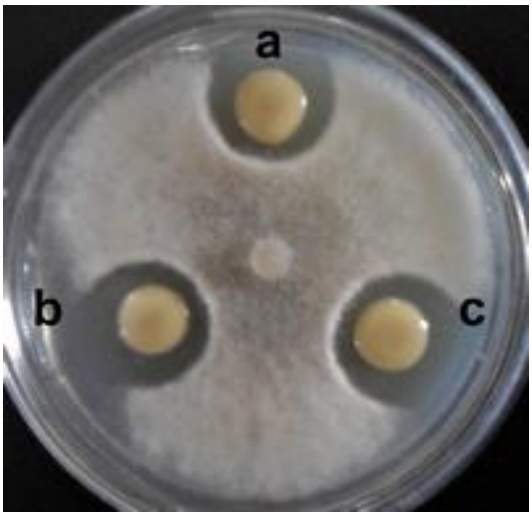

Figure 4. Antifungal activity against Botrytis cinerea by strain G3 and its derivatives after incubation for 4 d in a dual culture plate assay. a G3 WT; b The ΔsptR mutant; c The complement strain ΔsptR/pUCP26-sptR.

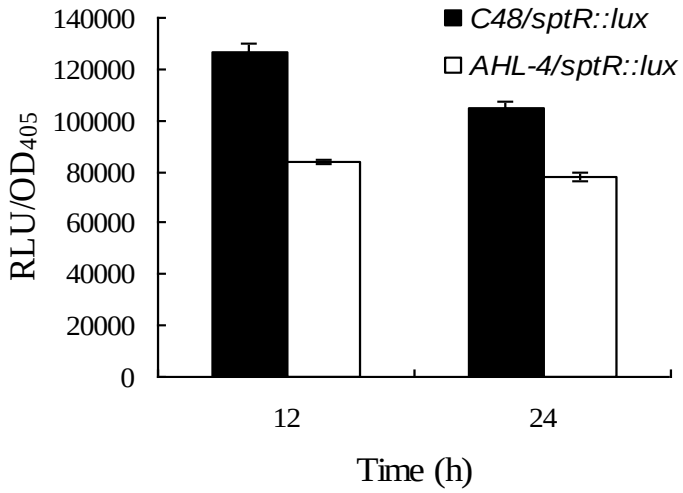

Figure 5. Analysis of *lux*-based reporter fusions to *sptR* promoter to reveal the impact of AHL production on the gene transcription of *sptR* with significant differences at *P* <0.01 between the wild type HRO-C48 and AHL-4, a *splI*- mutant deficient in AHL production after incubation for both 12 h and 24 h.

AHL production (Liu et al., 2007). Figure 5 showed that heterologous expression of *sptR* promoter in HRO-C48 is AHL-dependent. Bioluminescence was observed to be at significantly lower level in the AHL-4 mutant when compared with the wild type HRO-C48 after incubation for both 12 and 24 h (P<0.01). At present, the AHL mutants deficient in two AHL synthases *splI* and *spsI* of strain G3 is being constructed in our lab for reconfirming if it would be the same case as that in HRO-C48.

DISCUSSION

AHL-mediated QS systems have well been documented in *Serratia* spp., such as the important nosocomial pathogen *Serratia marcescens* (Wei et al., 2006) and plant beneficial biocontrol strains of *S. plymuthica* (Liu et al., 2007; Liu et al., 2010). AHL-regulated physiological functions in *Serratia* are remarkably diverse and of profound biological and ecological significance, including motility, biofilm formation and production of exoenzymes and secondary metabolites such as 2,3-butanediol and antibiotic pyrrolnitrin, as well as the interaction with the host plant (Van Houdt et al., 2007b; Müller et al., 2009). Recently, we have characterized two *luxIR*-like QS systems in the same isolate of the genus *Serratia* for the first time and unraveled that AHL signaling is involved in the global regulation of biocontrol-related traits in the endophytic strain G3 of *S. plymuthica* (Liu et al., 2011).

In the present study, the presence of a third LuxR homolog, SptR from *S. plymuthica* G3 was verified through cloning and preliminary identification. Phylogenetic analysis of LuxR homologs available in GenBank from all *S. plymuthica* strains and representative strains of all other *Serratia* species implied that SptR is neither closely related to the known two LuxR homologs SplR and SpsR of G3, nor other LuxR proteins from *Serratia* spp. such as CarR from *S. odorifera* DSM 4582 (Figure 1). Unexpectedly it shared a more recent common ancestor with ExpR from *E. cloacae* SCF1 and *E. tasmaniensis* Et1/99. Gray and Garey (2001) have deduced that multiple LuxI and/or LuxR homologues present within single species have been usually acquired from independent sources.

It is worth noting that most LuxR proteins from *Serratia* have shown to be involved in negative control of QS-dependent phenotypes by LuxR-dependent repression of the target genes in the absence of AHL (Van Houdt et al., 2007b). For instance, SplR repressed protease production via *lipB*-secretion system in strain RVH1, since proteolytic activity was abolished in *splI*::*aacC1* mutant and could be restored in a double mutant of *splI* and *splR* (*splI*::Tn*10* *splR*::*aacC1*) (Van Houdt et al., 2007a). It is known that *lipB*-secretion system required for production of extracellular protease and lipase acts as direct target of AHL signaling in *Serratia* (Christensen et al., 2003). In contrast, mutational analysis revealed that SptR act as a positive regulator to regulate protease secretion, swimming motility and biofilm formation, although no difference in the antifungal activity against *B. cinrea* and production of IAA were observed between strain G3 and the Δ*sptR* mutant. Our previous quorum quenching analysis by expressing *aiiA*-lactonase in G3 showed no significant influence on swimming motility (Liu et al., 2011), but a *splI* mutant in HRO-C48 displayed increased swimming motility compared with the parent strain (Müller et al., 2009), confirming diversity and complexity of QS network in *Serratia*.

The prediction that the putative *sptI* ORF is incomplete

by sequence analysis suggested the possibility of SptR utilizing AHL synthases other than its cognate SptI in G3. Hence we constructed a plasmid-based *sptR::lux* reporter fusion, and bioluminescence analysis of the construct in a *spII* mutant AHL-4 and the wild type HRO-C48 verified that heterologous expression of G3 *sptR* promoter in HRO-C48 is AHL-dependent, in accordance with the previous report that expression of *splR* is AHL-dependent in *S. plymuthica* RVH1, since a plasmid-borne *splR*::*gfp* fusion was poorly expressed in a *spII* mutant when compared with the parent strain (Van Houdt et al. 2007a). Whether the expression of *sptR* gene in G3 is also AHL-dependent or auto-regulated is still to be explored, additional lines of evidence still need to verify whether the putative ORF of *sptI* is indeed incomplete. For example, liquid chromatography–mass spectrometry (LC-MS) identification of the AHL profiles of double mutant defective in two identified AHL synthase SpII and SpsI, as well as heterologous expression of the putative *sptI* gene region in *E. coli* to determine whether only SpII and SpsI are responsible for AHL production, and SptR as an orphan regulator with no cognate synthase.

The *splR* mutant in *S. plymuthica* has been reported to produce similar level of AHL signals to the parent strain RVH1 (Van Houdt et al. 2007a). Similarly, preliminary T-streak detection of AHL signals with *Chromobacterium violaceum* CV026 on LA plates showed that no difference was observed between the *sptR* mutant and the wild type G3 (data not shown). However, more sensitive quantitative techniques like LC-MS combined with assays of *lux*-based reporter fusions to *spII* and *spsI* promoters are necessary to confirm it.

In summary, the results described above highlight the diversity and complexity of QS networks in *Serratia*, and will help with further exploring and understanding the interplay among QS systems and how the QS network integrated into the complex regulatory networks to optimize the bacterial seed inoculants.

ACKNOWLEDGMENTS

This work was partially funded by the National Natural Science Foundation of China (NSFC) (no. 30670030), the NSFC-RS (Royal Society of UK) Joint Project (no. 30811130218); the funds from Agricultural Ministry of China (no. 201003004) The Talent Summit Project of Jiangsu Province and the Scientific Research Project from Shandong Province (no. 2010GNC10911).

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