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Roles of a Solo LuxR in the Biological Control Agent *Lysobacter enzymogenes* Strain OH11

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ABSTRACT

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Lysobacter enzymogenes is a ubiquitous plant-associated and environmentally friendly bacterium emerging as a novel biological control agent of plant disease. This bacterium produces diverse antifungal factors, such as lytic enzymes and a secondary metabolite (heat-stable antifungal factor [HSAF]) having antifungal activity with a novel structure and mode of action. The regulatory mechanisms for biosynthesis of antifungal factors is largely unknown in *L. enzymogenes*. The solo LuxR proteins have been shown to be widespread, playing important roles in plant-associated bacteria. Here, we cloned and studied a solo LuxR protein, LesR, from *L. enzymogenes* strain OH11. Overexpression but not deletion of *lesR* significantly impaired HSAF biosynthesis levels and antimicrobial activities but did not show visible effect on production of major lytic enzymes. Overexpression of *lesR* also led to remarkably accelerated cell aggregation and induced production of a melanin-like pigment in *L. enzymogenes*; these two phenotypes are mediated by the diffusible factor cell-to-cell signaling system of *L. enzymogenes*. The C-terminus helix-turn-helix domain was shown to be critical for several *lesR*-controlled functions. Overall, our study provides the first example of the roles and mechanisms of a solo LuxR protein in a plant-associated *L. enzymogenes*.

Additional keywords: quorum sensing.

Lysobacter, a genus belonging to the Xanthomodaceae family, comprises a number of biological control agents (8). This genus displays unique characteristics, such as high genomic G+C content (≈70%) and production of abundant and antimicrobial secondary metabolites, distinguished from other taxonomically and ecologically related bacterial microbes (8,28,44). Of these, *Lysobacter enzymogenes*, first proposed in 1978, is a biocontrol agent for suppressing fungal and oomyceteous diseases (8,10). Previous evidence demonstrated that lytic enzymes produced by this bacterium, such as chitinase, protease, and glucanase, are important biocontrol factors (24). Importantly, a bioactive secondary metabolite called heat-stable antifungal factor (HSAF) was recently identified as an antifungal factor with a novel structure and mode of action (26–28,42). In previous studies, we cloned the gene cluster responsible for HSAF biosynthesis and presented its unique biosynthetic mechanism (28). However, the regulatory mechanisms for biosynthesis of HSAF and lytic enzymes remain unclear.

Bacterial quorum sensing (QS) is a form of gene regulation mediated by the accumulation and recognition of self-produced signals in local environments; bacteria use this system to regulate gene expression in a cell-density manner. Thus far, the physiological functions of QS are linked to virulence, symbiosis, biofilm formation, motility, and biosynthesis of secondary metabolites in diverse bacteria with different ecological niches (30,41). *N*-acyl-homoserine lactones (AHLs) are a major class of signal molecules produced by a variety of animal- and plant-associated bacteria (30,41). A typical AHL-responsive QS system is mediated by two proteins belonging to the LuxI and LuxR protein families, as first observed in *Vibrio fischeri* (15,32). The QS-type LuxR-family proteins ($LuxR_{QS}$) are response regulators, composed of two functional domains: an *N*-terminal AHL-binding domain and a C-terminal domain containing a helix-turn-helix (HTH) DNA-binding motif. The QS-type LuxI-family synthase ($LuxI_{QS}$) is responsible for synthesizing the AHL, which then interacts directly with the cognate $LuxR_{QS}$ protein at a high cell density or quorum concentration (13,31). Consequently, the stable protein–AHL complex binds at specific gene promoter sequences called *lux* boxes to activate or repress the transcription of target genes (14). In AHL-responsive QS, the $LuxR_{QS}$ protein is most often genetically paired with the $LuxI_{QS}$ protein in diverse AHL-producing bacteria.

Recent studies demonstrated that a number of bacterial genomes contain another subgroup of *luxR*-family proteins, which also contains the classical AHL-binding domain at the N-terminus and an HTH DNA-binding domain at the C terminus but is not genetically linked with a cognate *luxI* gene. These LuxR-type homologues have been called orphans and, more recently, solo LuxR proteins (12,16,33,40). Experimental evidence shows that several bacterial solo LuxR proteins evolved to acquire the ability to sense exogenous AHL signals produced by neighboring bacteria, leading to regulation of transcription of target genes. For example, SdiA, the solo LuxR protein of *Salmonella enterica* and *Escherichia coli*, responds to exogenous AHLs produced by neighboring bacteria (1,22). Another important subfamily of solo LuxR proteins is found in plant-associated bacteria and called $LuxR_{PA}$ (16). They sense as-yet-unidentified plant signals being involved in inter-kingdom signaling (7,9,16). Well-studied ex-

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*The ***e*-Xtra** logo stands for "electronic extra" and indicates that the online version contains one supplemental file, six supplemental figures, and two supplemental tables. Figures 1 to 7 appear in color online.

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amples of $LuxR_{PA}$ include XccR of *Xanthomonas campestris* binding a signal present in cabbage; OryR of *X. oryzae* binding a rice-produced signal compound; XagR of *X. axonopodis* binding soybean-, rice-, and cabbage-produced molecules; PsoR of *Pseudomonas fluorescens* binding rice and wheat molecules; and NesR in *Sinorhizobium meliloti* (the plant response has not been determined) (7,9,33,39,43). All of the above-mentioned $LuxR_{PA}$ proteins in the corresponding bacterium play important biological roles related to plant–bacteria interactions. For examples, XccR, OryR, and XagR are involved in the virulence of *X. campestris*, *X. oryzae*, and *X. axonopodis*, respectively; NesR is associated with stress response and symbiosis in *S. meliloti*; and PsoR is linked to antibiotic production and biological control in *P. fluorescens*.

Diffusible signal factor (DSF) and diffusible factor (DF), originally identified from *X. campestris* pv. *campestris*, are two chemically distinct cell-to-cell signals. DSF was initially characterized in *X. campestris* pv. *campestris* as *cis*-11-methyl-2-dodecenoic acid. Currently, DSF family molecules were identified from diverse bacteria, including *cis*-2-dodecenoic acid produced by *Burkholderia cenocepacia* (6), *cis*,*cis*-11-methyldodeca-2,5-dienoci acid produced by *X. oryzae* pv. *oryzae* (19), and 2 (*Z*)-tetradecenoic acid produced by *Xylella fastidiosa* (5). The biosynthesis of DSF signals needs a regulation of pathogenicity factor F (*rpfF*) gene, which encodes a putative enoyl-CoA hydratase in *Xanthomonas* spp. (4,17,20). Disruption of *rpfF* impaired DSF production ability as well as virulence in diverse *Xanthomonas* spp. (17,20). DF was recently characterized as the mixture of 3-hydroxybenzoic acid (3-HBA) and 4-HBA in *Xanthomonas campestris* pv. *campestris*, and further found to be widespread in variety of bacterial species with diverse ecological niches (18). The biosynthesis of DF needs a *xanB2* gene in *X. campestris* pv. *campestris*. The product of this gene is a pteridine-dependent dioxygenase-like protein, which hydrolyses chorismate, the end-product of the shikimate pathway, to produce 3-HBA and 4-HBA (45). Disruption of *xanB2* in *X. campestris* pv. *campestris* impaired the biosynthesis of DF, polyene yellow pigments (also called xanthomonadins), virulence, and antioxidant ability (45). Recently, we identified these two distinct cell-to-cell signaling factors (DSF and DF) and elucidated their differentially regulatory roles on HSAF biosynthesis in *L. enzymogenes* (35).

We recently determined the genome sequence of strain OH11, a Chinese *L. enzymogenes* isolate. Genome survey and sequence analysis revealed a solo LuxR protein in *L. enzymogenes* (Fig. 1), which was named LesR. Here, we demonstrated the critical roles that *lesR* played in HSAF biosynthesis, antimicrobial activities, cell aggregation, and induction of melanin-like pigment. We also showed that the DF cell-to-cell signaling system of *L. enzymogenes* was involved in partial *lesR*-controlled functions. The findings add to our current knowledge of roles of a new member of solo LuxR proteins from a plant-associated *L. enzymogenes*. Our results also provided evidence, for the first, time to show the close relationship between solo LuxR and DF-mediating cell-to-cell signaling in bacteria.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Supplemental Table 1. *E. coli* strain Top10 was used for plasmid constructions, which were grown in Luria-Bertani (LB) broth at 37°C. Unless otherwise stated, *L. enzymogenes* strains were grown in 1/10 tryptic soy broth (TSB) (Sigma Chemical, St. Louis) with shaking at 200 rpm. *Pythium ultimum* and *Rhizoctonia solani* were grown in 1/10 TSB plus agar (TSA) at 25°C. When required, antibiotics were added to medium at the following concentrations: kanamycin (Km) at 100 μg/ml for *L. enzymogenes* strains, gentamicin (Gm) at 50 μg/ml for gene over-expression strains of *L. enzymogenes*, and ampicillin (Amp) at 100 μg/ml or Gm 25 μg/ml for *E. coli* strainTop10.

Generation of deletion mutant, complemented, or over-expression strains of *lesR* in *L. enzymogenes*. The homologous recombination-based in-frame deletion of *lesR* was performed as described previously in our laboratory (36). In brief, the wild-type strain OH11 of *L. enzymogenes* was used as a parental strain to generate the in-frame deletion mutant. Two flanking regions of *lesR* genes were generated by the polymerase chain reaction (PCR) method using primer pairs and cloned into the corresponding sites of suicide-vector pEX18GM (21) digested with appreciated enzymes. The final constructs were transformed into the wild-type strain OH11 by electroporation. *Lysobacter* transconjugants were selected on LB plates without sucrose and with Km (100 μg/ml) and Gm (150 μg/ml). The positive colonies were plated on LB plates containing 10% (wt/vol) sucrose and Km (20 μg/ml) to select for resolution of the construct by a second crossover event. The resultant mutants were confirmed by PCR and Southern blotting (data not shown).

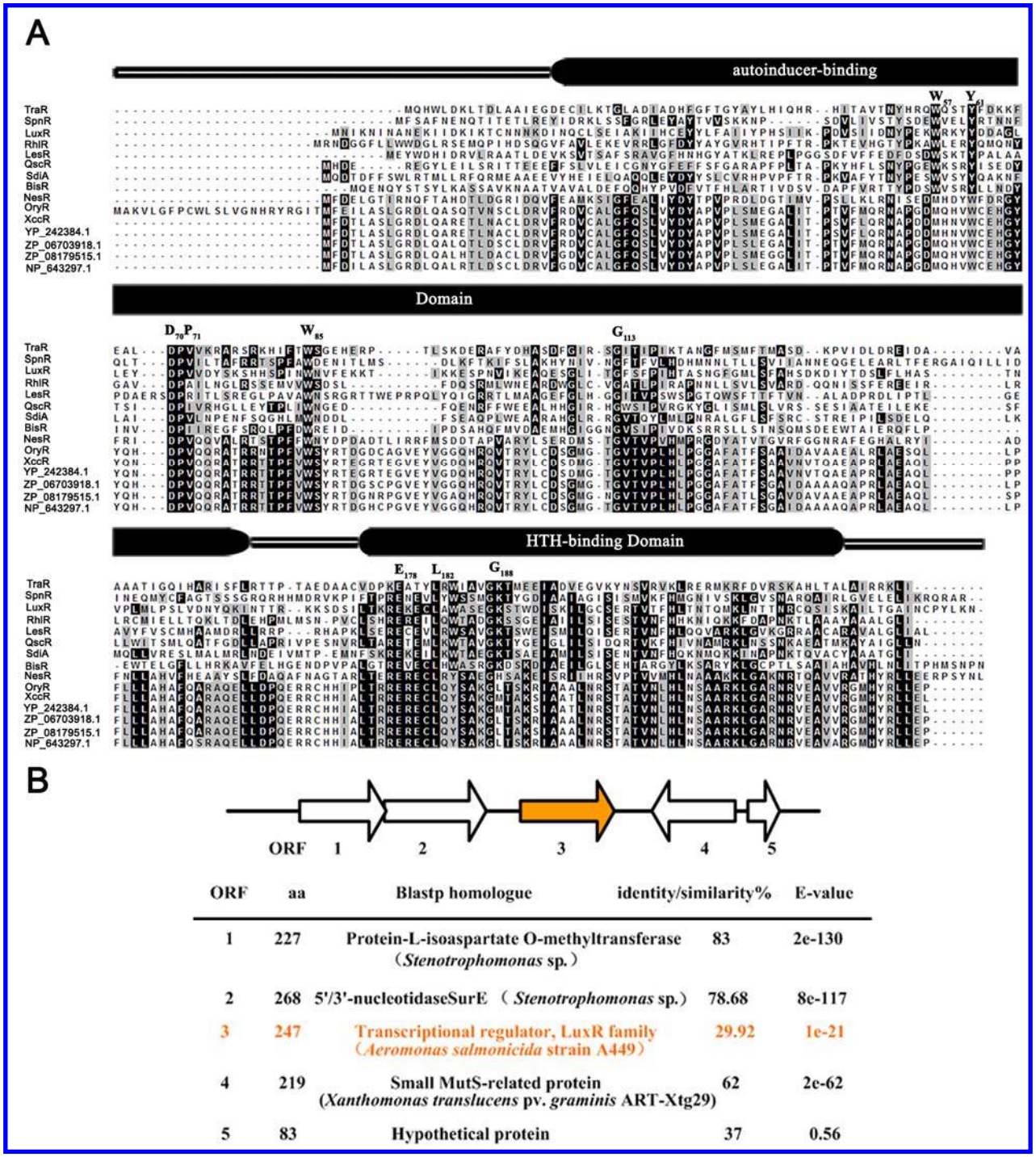
For generation of complemented or overexpression strains, *lesR*, together with its predicted promoter, was amplified from strain OH11 and cloned into the broad-host vector pBBR1MCS-5 (25), creating final construct. This construct was transformed into the *lesR*-deletion mutant for generation of corresponding complemented strains or into wild-type OH11 for constructing the overexpression strain. The expression of *lesR* in the wild-type, deletion mutant, complemented, or overexpression strains was determined by quantitative reverse-transcription (qRT)-PCR and RT-PCR, which was described below and in the Supplemental file, respectively. This final vector construct was also ransformed into target mutants of *L. enzymogenes*, including *rpfF* and *lenB2* deletion mutants (named Δ*rpfF* and Δ*lenB2*, respectively). Mutation of *rpfF* and *lenB2* impaired the ability of *L. enzymogenes* in producing the cell-to-cell signals DSF and DF, respectively (35).

Domain analysis and expression. The domain analysis of LesR was performed on the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and Pfam (http://pfam.sanger.ac.uk/) databases. To express the domain, each of two domains of LesR was amplified by PCR amplification and spliced with the promoter of *lesR* by overlapping PCR method. The final DNA fragment (promoter plus domain) was cloned into the broad-host vector pBBR1MCS-5, creating final constructs. These constructs were transformed into wild-type strain OH11, and the positive colonies were selected on LB plates with Km (100 μg/ml) and Gm (150 μg/ml).

HSAF extraction and detection. Extraction of HSAF from *L. enzymogenes* was performed as described previously (28,42). We cultured the OH11 and its derivative strains in 20 ml of 1/10 TSB medium, supplement with Km at 100 μg/ml at 28°C in a rotary shaker at 200 rpm for 3 days. Then, 3 ml of culture from 50-ml bacterial suspensions was selected for HSAF extraction. For details, after centrifugation (10,000 × *g* at 4°C for 30 min), the supernatant was collected and an equal volume of ethyl acetate was added. After being fully shaken three times in a separating funnel, the ethyl acetate phase was collected and fully evaporated. Finally, the collection (HSAF) was dissolved in 200 μl of methanol, and 20 μl was used for high-performance liquid chromatography (HPLC) analysis. The HPLC program was described as follows: 5 to 40% B (acetonitrile/0.025% TFA) in A (water/0.025% TFA) in the first 0 to 10 min, 40 to 60% mobile phase B in mobile phase A from 10 to 15 min, 60% mobile phase B in mobile phase A from 15 to 20 min, 60 to 100% mobile phase B in mobile phase A from 20 to 22 min, 100% mobile phase B in mobile phase A from 22 to 24 min, and 100 to 5% mobile phase B in mobile phase A from 24 to 26 min. HSAF were detected at 318 nm on a UV detector (Agilent 1200 Diode array G1315D; Agilent, USA). Three replicates for each treatment were used, and the experiment was repeated three times.

Antimicrobial activity assays. The antimicrobial activity assays were performed as described previously (36). In brief, a plate inhibition assay was used to determine antimicrobial activity of strain OH11 and its derivative strains. In tests with filamentous fungi and oomycetes, *L. enzymogenes* strains were co-cultured with the oomycete *P. ultimum*, and the fungi *R. solani*. The *L. enzymogenes* strains were grown in 1/10 TSB broth for 48 h, then adjusted to similar concentrations (≈1 × 10^7 CFU/ml). Then, 5 µl of the culture of each *L. enzymogens* strain was spot inoculated on the periphery of 1/10 TSA plates. The center of each plate was inoculated with a mycelial plug of tested oomycete or fungal pathogens (three plates per pathogen). After the plates were incubated for 2 to 5 days at 25°C, the radii of the growth inhibition zones around the colonies were measured.

In assays involving yeast (*Saccharomyces cerevisiae*), the plates were prepared by adding 200 µl of yeast cell suspension (optical density at 600 nm [OD_{600}] = 0.1) into the 100-ml 1/10 TSA medium. Then, the surfaces of tested plates containing the



ORF	aa	Blastp homologue	identity/similarity%	E-value
1	227	Protein-L-isoaspartate O-methyltransferase (*Stenotrophomonas* sp.)	83	2e-130
2	268	5'/3'-nucleotidaseSurE (*Stenotrophomonas* sp.)	78.68	8e-117
3	247	Transcriptional regulator, LuxR family (*Aeromonas salmonicida* strain A449)	29.92	1e-21
4	219	Small MutS-related protein (*Xanthomonas translucens* pv. *graminis* ART-Xtg29)	62	2e-62
5	83	Hypothetical protein	37	0.56

Fig. 1. Sequence characterization of LesR in *Lysobacter enzymogenes* strain OH11. **A,** Alignment of LesR with other LuxR-type transcriptional regulators. Solo LuxRs: QscR, *Pseudomonas aeruginosa* PAO1; SdiA, *Escherichia coli* LF82; BisR, *Rhizobium leguminosarum* pv. *viciae*; NesR, *Sinorhizobium meliloti* 2011; OryR, *Xanthomonas oryzae* pv. *oryzae* KACC10331; XccR, *X. campestris* pv. *campestris* 8004; YP_242384.1, *X. gardneri* ATCC 19865; ZP_06703918.1, *X. fuscans* subsp. *aurantifolii* ICPB 11122; ZP_08179515.1, *X. vesicatoria* ATCC 35937; and NP_643297.1, *X. axonopodis* pv. *citri* 306. Quorum-sensing LuxR: TraR of *Agrobacterium tumefaciens*; SpnR, *Serratia marcescens*; LuxR of *Vibrio fischeri*; and RhlR of *Pseudomonas aeruginosa*. The nine highly conserved amino acids are highlighted in black and their positions with respect to TraR are indicated above. **B,** *lesR* (highlighted in orange) organization in the genome of *L. enzymogenes* strain OH11. Homologues and predicated functions of *lesR* flanking genes were presented. ORF = open reading frame and aa = amino acids.

yeast were inoculated with 5 µl of culture fluid of *L. enzymogenes* strains. These cultures were incubated at 28°C for 1 to 4 days, depending upon the organism, prior to the size of growth inhibition zones surrounding the bacterial colonies and disks being measured and photographed.

For determination of HSAF-based antimicrobial activity, 2 µl of HSAF extraction used for the HPLC assay was added into the center of tested plates (1/10 TSA). A mycelial plug of the pathogen *P. ultimum* was inoculated on the periphery of 1/10 TSA plates. After the plates were incubated for 2 days at 25°C, the radii of the growth inhibition zones around HSAF spots were measured and photographed.

Cell aggregation and melanin-like pigment observation assays. The wild-type strain OH11 and its derivative strains were grown in 3 ml of 1/10 TSB at 28°C in a rotary shaker at 200 rpm for 3 days. Then, these bacterial suspensions were selected for melanin-like pigment observation. For a cell aggregation assay, the strains were grown in 3 ml of 1/10 TSB at 28°C in a rotary shaker at 200 rpm until an OD_{600} of 3.0. Then, these cultures were vertically placed without movement in a test-tube support. After 12 h, the cell aggregation was observed and photographed. Next, the cells in these cultures were selected for pigmentation of crystal violet. In brief, the culture of each strain was shaken up again, and 2 µl of culture was spotted on the glass slide and dried at 28°C for 5 h. Next, 5 µl of 0.2% crystal violet solution was added into the spots for 1 min, following by washing gently by double-distilled H_2O. After removing the residual water, the cells were observed with an Olympus microscope (Tokyo, Germany).

Real-time PCR. Real-time qRT-PCR was carried out by using the SYBR Premix EX Tag II kit (TaKaRa, Japan) in an ABI PRISM 7500 Real-Time PCR System (Applied Biosystems, USA). Primer sequences used in this assay are listed in Supplemental Table 2. RNA was extracted from various *L. enzymogenes* strains at different growth stages and using the RNAiso plus reagent (TaKaRa) following the manufacturer's instructions. To remove genomic DNA, the eluted RNA samples were treated with RNase inhibitors and DNaseI (TaKaRa). RNA integrity was confirmed by electrophoresis using 1.2% agarose gels. Then, 2 µg of each RNA sample was used to synthesize cDNA with a cDNA synthesis kit (TaKaRa). The same experiment was repeated three times, and each treatment involved three replicates. In this study, the transcriptional level of *lesR* in the wild-type OH11, *lesR*-deletion mutant, and *lesR* overexpression strain was determined. The transcriptional levels of five characterized HSAF biosynthesis-related genes (28) were assessed and compared in target strains. In addition, based on the references to a cell-to-cell regulatory mechanism, the 16S rRNA gene was a preferred housekeeping gene (internal control) in a *Xanthomonas* sp. (a species related to *L. enzymogenes*) when qRT-PCR experiments were used (2,37). Thus, we also selected this gene as a housekeeping gene in a qRT-PCR assay in *L. enzymogenes*.

Data analysis. All analyses were conducted using SPSS 14.0 (SPSS Inc., Chicago). The hypothesis test of percentages (*t* test, $P = 0.05$) was used to determine significant differences in the gene expression and antimicrobial activity.

RESULTS AND DISCUSSION

LesR is a solo LuxR protein. The primary structure of LesR consists of 246 amino acids; it possesses a typical AHL-binding domain (position 15 to 170) at the N terminus and an HTH DNA-binding domain (position 183 to 236) at the C terminus but its gene is not directly associated in the genome with a cognate *luxI* and, hence, it is a solo (Fig. 1B). Furthermore, LesR possesses all six highly conserved amino-acid substitutions (W57, Y61, D70, P71, W85, and G113) found in the AHL-binding motif in QS LuxR proteins (Fig. 1A) (16) Therefore, it does not belong to the subfamily of LuxR solos present in some plant-associated bacteria which respond to plant compounds (16) and is probably a canonical LuxR family protein capable of binding AHLs. Our recent studies show that no AHL-like molecules are produced by this bacterium, indicating that this solo might be involved in the response to AHLs produced by neighboring bacteria. Further genome survey demonstrates that no autoinducer synthase LuxI homologue was found in strain OH11.

Two protein-encoding genes are located adjacent to *lesR* in the genome of strain OH11. One is the homologue of SurE, a stationary-phase survival protein first discovered in *E. coli* (34). The other is homologue of a small MutS-related (Smr) domain-containing protein. MutS family proteins are shown to be widely

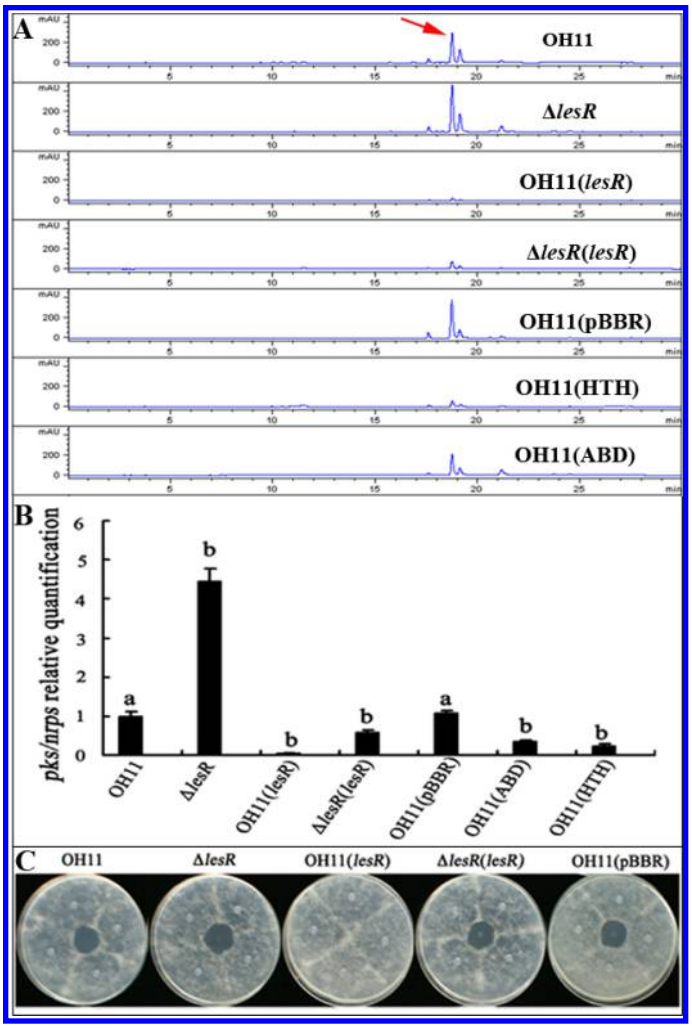


Fig. 2. Overexpression of completed *lesR* or its helix-turn-helix (HTH) DNA-binding domain impaired heat-stable antifungal factor (HSAF) production in *Lysobacter enzymogenes*. **A,** High-performance liquid chromatography determination of HSAF production level. The arrow indicates the peak of standard HSAF (42). **B,** Impact of *lesR* overexpression on the transcriptional level of a characterized HSAF biosynthesis gene *pks/nrps*, which encodes a hybrid polyketide synthase/nonribosomal peptide synthetase in *L. enzymogenes* (28,42). **C,** Inhibitory activities of HSAF extracts against *Pythium ultimum* (a ubiquitous soilborne pathogen which causes damping-off and root rot on plants). OH11, the wild-type *L. enzymogenes* strain; Δ*lesR*, the *lesR* deletion mutant of strain OH11; Δ*lesR*(*lesR*), the complemented strain of Δ*lesR*; OH11(*lesR*), overexpression strain of *lesR*; OH11(pBBR), the wild-type strain haboring the empty vector; OH11(ABD), overexpressing *N*-acyl-homoserine lactone-binding domain of *lesR*; OH11(HTH), overexpressing HTH DNA-binding domain of *lesR*. Three replicates for each treatment were used, and the experiment was repeated three times. Vertical bars represent standard errors. Different letters above bars indicate a significant difference between the wild-type strain OH11 and the tested mutants ($P < 0.05$; *t* test). Identical letters above the data bars indicate that there is no significant difference between the wild-type strain and the tested strains ($P > 0.05$; *t* test).

distributed in bacteria and play important roles in diverse DNA transactions, including DNA mismatch repair and recombinational events (11). Because LuxR solos often regulate adjacently located genes, it could be important to determine whether *surE* and *smr* are regulated by LesR; however, no evident *lux*-box-like sequences were detected in the promoters of these two genes in *L. enzymogenes*.

Overexpression of *lesR* remarkably impaired the HSAF biosynthesis level. To address the role of *lesR* in *L. enzymogenes*, an *lesR* deletion mutant and overexpression strain were generated and studied. We clearly observed that overexpression of *lesR* almost completely impaired HSAF production in *L. enzymogenes* as determined by HPLC (Fig. 2A). To further confirm this result, the HSAF extracts were then used to test for antifungal activities. As expected, the HSAF extracts from wild-type OH11 or control (wild type containing the empty plasmid vector) displayed similar and strong antifungal activity against *P. ultimum*, the pathogen causing damping-off and root rot on plants. No antifungal activity was observed, however, in extracts from the *lesR* overexpression strain (Fig. 2C). Subsequently, the transcriptional activity of the hybrid polyketide synthase/nonribosomal peptide synthetase encoding gene (*pks/nrps*) responsible for HSAF biosynthesis was tested to determine whether it was influenced by overexpressing *lesR*. As expected, the expression level of this gene was significantly reduced at a transcriptional level when *lesR* was overexpressed in wild-type background (Fig. 2B). On the other hand, deletion of *lesR* did not show visible effects on these phenotypes (Fig. 2). Taken together, these results indicated that LesR, at some level, is involved in the modulation of HSAF biosynthesis in *L. enzymogenes* because its overexpression has dramatic effects on HSAF production and the transcription of its biosynthetic gene. It is interesting to note that no putative *lux*-box-like element was found in the promoter of *pks/nrps*, indicating that LesR might use an indirect mechanism to modulate HSAF biosynthesis. We have looked for a *lux* box in the genome of strain OH11 and could not find a clear *lux*-box site.

Overexpression of *lesR* impaired the antimicrobial activities against yeast and plant pathogens. As described above, HSAF production was significantly reduced in an *lesR* overexpression strain; thus, we hypothesized that overexpression of *lesR* might reduce inhibitory activity against plant pathogens. In order to test this, we performed experiments along this line. It was determined that the antagonistic activities against both *R. solani* and *P. ultimum* were significantly decreased in the *lesR* overexpression strain compared with the wild type harboring the empty vector. However, the *lesR* deletion mutant as well as wild-type OH11 displayed similar antagonistic activities against these two pathogens (Fig. 3A and B). The antimicrobial activity of *lesR* overexpression strains against these two pathogens was significantly reduced but not abolished. This result indicated that other HSAF-independent antifungal factors possibly might be present in the antimicrobial activities; this was subsequently confirmed by the antagonistic assay with the model fungus yeast (*S. cerevisiae*), as described here below.

We observed that overexpression of *lesR* (and not the *lesR* knock-out mutant) remarkably reduced the antifungal activity against *S. cerevisiae* (Fig. 3C). It is known that HSAF plays no inhibitory activity against *S. cerevisiae* (27); therefore, our results indicated that the involvement of *lesR* in inhibiting yeast growth occurs most likely via the regulation of unknown and HSAF-independent antifungal factors in *L. enzymogenes*. Further evidence showed that overexpression of *lesR* did not impair the production of four major lytic enzymes (Supplemental Figure 3), indicating that this HSAF-independent antifungal factor was not associated with lytic enzymes. Nevertheless, our results demonstrated that *lesR* played a critical role in inhibiting fungal pathogens.

Overexpression of *lesR* accelerated cell aggregation and induction of a melanin-like pigment production. It was of interest to test whether other phenotypes were affected by *lesR* overexpression. We observed that overexpression of *lesR* accelerated cell aggregation compared with the wild-type OH11 with or without the empty vector (Fig. 4). After 12 h of vertical stewing, the bacterial culture became clear, and most cells of the *lesR* overexpression strain were found to locate in the bottom of the test tube. Crystal-violet pigmentation showed that the cells of the *lesR* overexpression strain assembled together. Under the same conditions, the bacterial culture of the *lesR* deletion mutant as well as the wild-type OH11 were found to be turbid, and only a very few cells sank to the bottom of the tube. It must be noted that the complementation strain of the *lesR* deletion mutant also showed cell aggregation in both tube and crystal-violet pigmentation assays compared with the wild-type strain and *lesR* mutant. This is probably due to the expression of *lesR* being higher than the wild type, as was also determined by qRT-PCR and RT-PCR (Supplemental Figure 2). The phenotype of cell aggregation implied that surface components were likely to be regulated in an *lesR*-dependent manner. LuxR-mediating cell aggregation has been demonstrated in AHL-QS bacterium (23); however, the role of solo LuxRs in this phenotype has not yet been reported. In the animal-pathogenic bacterium *Yersinia pseudotuberculosis*, mutation of *ypsR* (a part of the canonical complete QS system) induces a cell aggregation phenotype, which was shown to be correlated to the flagellin structural protein (a flagella biosynthesis-related protein), with similarities to FleA, FleB, and FleC of *Y. enterocolitica* (23). Furthermore, extracellularly secreted polysaccharides (i.e., cellulose and an arabinose-rich exopolysaccharide) were also shown to be involved in autoaggregation in the soil-

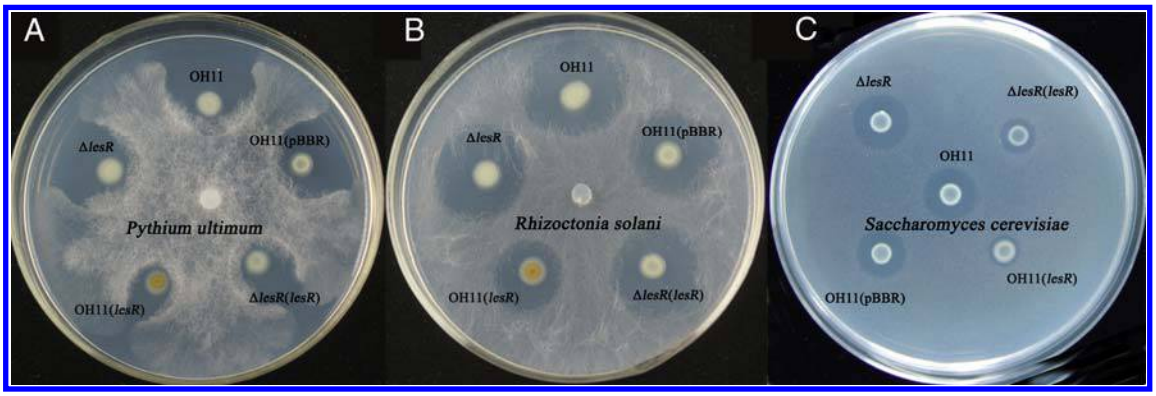


Fig. 3. Inhibitory activity of *lesR* deletion mutant against **A,** oomyceteous and **B and C,** fungal strains. OH11, the wild-type strain *Lysobacter enzymogenes*; Δ*lesR*, the *lesR* deletion mutant of strain OH11; Δ*lesR*(*lesR*), the complemented strain of Δ*lesR*; OH11(*lesR*), overexpression strain of *lesR*; OH11(pBBR), the wild-type strain haboring the empty vector. *Pythium ultimum* is a ubiquitous soilborne pathogen which causes damping-off and root rot on plants. *Rhizoctonia solani* is the rice sheath-blight fungal pathogen and *Saccharomyces cerevisiae* is a model yeast strain.

borne bacteria *Agrobacterium tumefacines* and *Azospirillum brasilense*, as well as the symbiotic gram-negative rhizobacterium *S. meliloti* (3,29,38). It is important to note that *L. enzymogenes* lacks flagella and displays flagellum-independent gliding motility and abundance of extracellular polysaccharide (8). Therefore, whether extracellular polysaccharide plays critical contributions to the process of *lesR*-mediating cell aggregation needs further investigation.

Another interesting finding was that a melanin-like pigment was produced in the *lesR* overexpression strain after growth of 3 days in 1/10 TSB broth (Fig. 5). Other tested *Lysobacter* strains, including the *lesR* deletion mutant, did not exhibit such a trait. Currently, we do not know the chemical structure and potential roles of this melanin-like pigment, which deserves further investigation. Nevertheless, our results indicated that *lesR* was involved in cell aggregation and induction of a melanin-like pigment in *L. enzymogenes*.

C-terminal HTH-binding domain was responsible for several *lesR*-controlled functions. LesR contained an AHL-binding domain at the N terminus and an HTH DNA-binding domain at the C terminus. To address the contribution of each domain to *lesR*-controlled functions, each of them was overexpressed in *L. enzymogenes* (Supplemental Table 1). It was evident that overexpression of the single C-terminal HTH-binding domain impaired the HSAF production, accelerated cell aggregation, and induced the production of a melanin-like pigment (Figs. 2A, 4, and 5), which was consistent with the phenotypes of completed *lesR* overexpression strain. However, overexpression of the single N-terminal AHL-binding domain did not show visible effects on these concerned functions in *L. enzymogenes*. These results indicated that overexpressing the C-terminal HTH DNA-binding domain alone has a drastic effect on phenotypes, just like expressing the complete *lesR*. This result validated the roles of full-length *lesR* in *L. enzymogenes* presented in this study.

***lesR*-controlled cell aggregation and melanin-like pigment induction was mediated by the DF cell-to-cell signaling pathway in *L. enzymogenes*.** LesR is a solo LuxR-family protein, and is hypothesized to be involved in responding to AHL signals from neighboring bacteria. Because *L. enzymogenes* itself is able to produce two cell-to-cell signals (DSF and DF), it was of interest to investigate the relationship between *lesR*-controlled functions and a DSF or DF cell-to-cell signaling system. Therefore, we determined the contribution of DSF and DF signaling system to *lesR*-controlled functions. It was surprising to find that overexpression of *lesR* in an *lenB2* mutant (DF-disruption mutant) did not show the phenotype of cell aggregation and pigment induction (Fig. 6). However, overexpression of *lesR* in the background of the *rpfF* mutation (DSF-disruption mutant) also accelerated cell aggregation and induction of pigment production. Taken together, these results indicated that *lesR*-controlled above two phenotypes (cell aggregatin and pigment induction) was mediated by DF cell-to-cell signaling but not by DSF in *L. enzymo-*

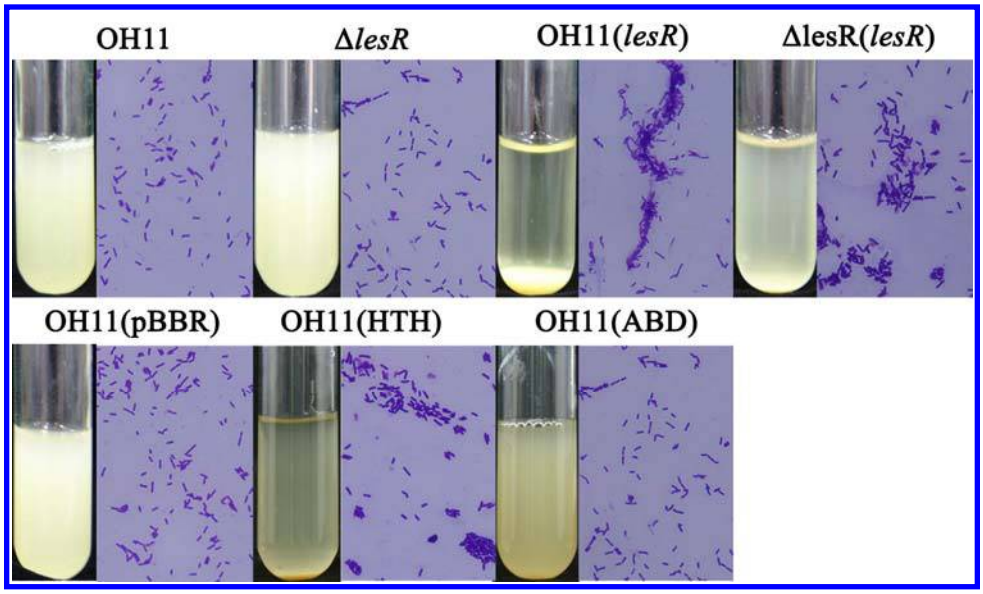


Fig. 4. Overexpression of completed *lesR* or its helix-turn-helix (HTH) DNA-binding domain accelerated cell aggregation in *Lysobacter enzymogenes*. The cell aggregation in the tube was observed after the strain was grown in liquid 1/10 tryptic soy broth until an optical density at 600 nm of 3.0. The culture of each strain was shaken up and the cells were used for pigmentation of crystal violet. Details are provided in Materials and Methods. OH11, the wild-type *L. enzymogenes* strain; Δ*lesR*, the *lesR* deletion mutant of strain OH11; Δ*lesR*(*lesR*), the complemented strain of Δ*lesR*; OH11(*lesR*), overexpression strain of *lesR*; OH11(pBBR), the wild-type strain haboring the empty vector; OH11(ABD), overexpressing *N*-acyl-homoserine lactone-binding domain of *lesR*; OH11(HTH), overexpressing HTH DNA-binding domain of *lesR*.

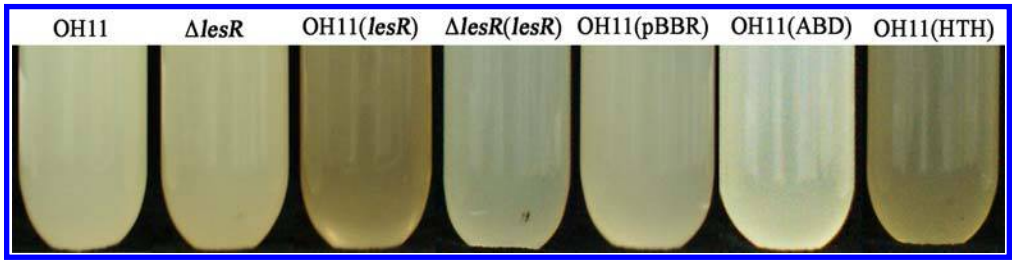


Fig. 5. Overexpression of completed *lesR* or its helix-turn-helix (HTH) DNA-binding domain induced production of a melanin-like pigment production in *Lysobacter enzymogenes*. Representative images of melanin-like pigment production in liquid Luria-Bertani broth. Strains OH11 (*lesR*) and OH11 (HTH) produced a melanin-like pigment after 3 days of growth in liquid 1/10 tryptic soy broth. OH11, the wild-type *L. enzymogenes* strain; Δ*lesR*, the *lesR* deletion mutant of strain OH11; Δ*lesR*(*lesR*), the complemented strain of Δ*lesR*; OH11(*lesR*), overexpression strain of *lesR*; OH11(pBBR), the wild-type strain haboring the empty vector; OH11(ABD), overexpressing *N*-acyl-homoserine lactone-binding domain of *lesR*; OH11(HTH), overexpressing HTH DNA-binding domain of *lesR*.

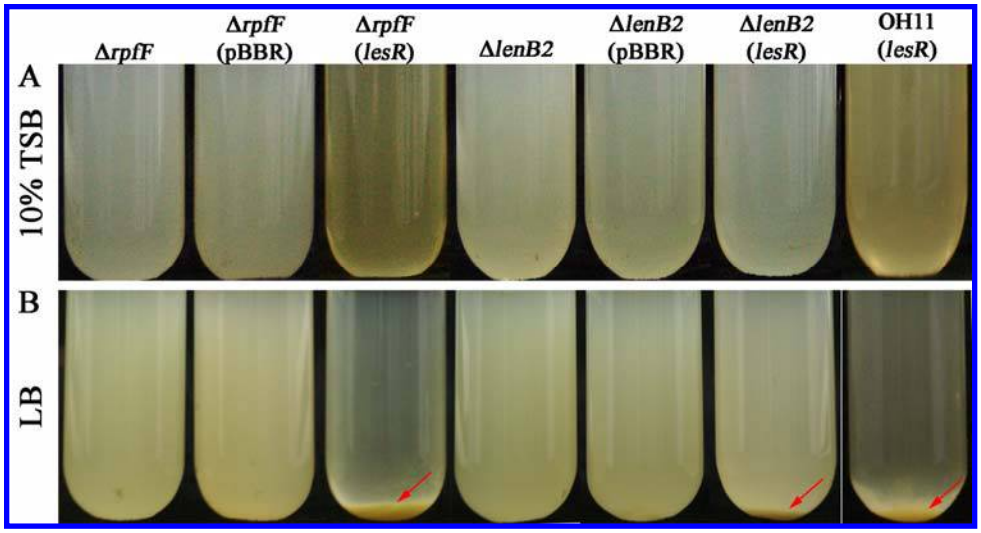


Fig. 6. Contribution of diffusible signal factor (DSF) or diffusible factor (DF) cell-to-cell signaling system to **A,** melanin-like pigment production and **B,** *lesR*-controlled cell aggregation in *Lysobacter enzymogenes*. Compared with overexpression of *lesR* in the wild-type background, overexpression of *lesR* in the DF-disrupted mutant (Δ*lenB2*) showed **A,** no production of melanin-like pigment in 1/10 tryptic soy broth and **B,** decreased ability in cell aggregation in Luria-Bertani (LB) broth. Arrows indicated the phenotype of cell aggregation from overexpression strain of *lesR* in the background of DSF-disrupted mutant (Δ*rpfF*), DF-disrupted mutant (Δ*lenB2*). and wild-type OH11, respectively. The source of Δ*rpfF* and Δ*lenB2* was provided by Qian et al. (35).

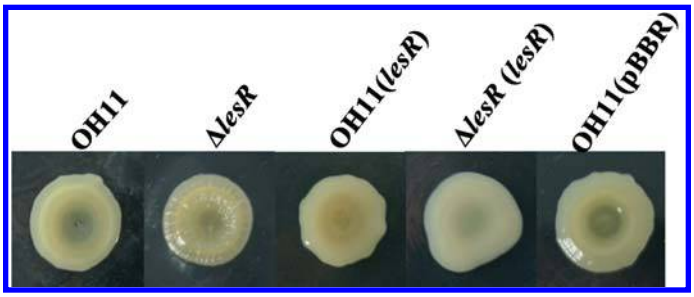


Fig. 7. Deletion of *lesR* affected the colony morphology of *Lysobacter enzymogenes*. The *lesR* deletion mutant displayed dry and crimpled colony morphologies when grown in Luria-Bertani medium after 3 days. OH11, the wild-type *L. enzymogenes* strain; Δ*lesR*, the *lesR* deletion mutant of strain OH11; OH11(*lesR*), overexpression strain of *lesR*; Δ*lesR*(*lesR*), the complemented strain of Δ*lesR*; OH11(pBBR), the wild-type strain haboring the empty vector.

genes. How DF affects these *lesR*-controlled phenotypes in *L. enzymogenes* should be further addressed. It is important to note that we were unable to determine the effect of DSF or DF on *lesR*-mediating HSAF production, because disruption of DSF or DF already impaired the HSAF production level (35).

***lesR* was not a pseudogene in *L. enzymogenes*.** The results above showed that overexpression of *lesR* but not deletion affected diverse biological functions in *L. enzymogenes*, indicating that *lesR* could possibly be a pseudogene. However, we were surprised to find that the *lesR* deletion mutant displayed dry and crimple colony morphologies when grown in LB medium compared with wild-type, *lesR*-complemented, or overexpressing strains (Fig. 7), providing evidence to show that *lesR* was a functional gene. Furthermore, we also compared the global protein expression levels between the wild-type strain and *lesR* deletion mutant by proteomics. We observed that mutation of *lesR* resulted in 33 protein spots with a significantly expressed level (>1.5-fold) compared with the wild-type strain (Supplemental Figure 4). Of these, 17 and 16 protein spots were down- and up-expressed, respectively, in the *lesR* mutant (Supplemental Figures 5 and 6). This result indicated that *lesR* was a functional gene in regulating protein expression. However, systematic proteomic analysis between wild-type OH11 and the *lesR* deletion mutant, as well as between wild-type OH11 containing the empty vector and the *lesR* overexpression strain, is needed in order to obtain the *lesR*-controlled proteins for further elucidation of the roles and regulatory mechanisms of *lesR* in *L. enzymogenes*. Moreover, why deletion of *lesR* did not show visible effects on tested phenotypes in this study is currently unknown.

Concluding remarks. This study reports, for the first time, the presence of an LuxR solo designated as LesR in a plant-associated and novel emerging biological control agent, *L. enzymogenes*. LesR has a typical modular structure of QS LuxR proteins consisting of an AHL-binding and an HTH DNA-binding motif. This indicates that LesR could be involved in binding AHL signals produced by neighboring bacteria. By using *L. enzymogenes* overexpressing *lesR* which could overcome, to some extent, the necessity of a neighboring AHL inducer, we described diverse functions of *lesR* in *L. enzymogenes*. Phenotypic studies determined that LesR was involved in the regulation of (i) biosynthesis of a novel antifungal factor (HSAF), (ii) cell aggregation, and (iii) a melanin-like pigment. We also provided experimental evidence to link a DF cell-to-cell signaling system to this solo LuxR in *L. enzymogenes*. In summary, *lesR* plays unique roles in the plant-associated and bacterial biocontrol agent *L. enzymogenes*. In our future studies, we aim to (i) identify the signal that LesR responds to, (ii) understand the indirect regulatory mechanisms of *lesR* on HSAF biosynthesis, and (iii) investigate the chemical structure of the melanin-like pigment induced by *lesR*.

ACKNOWLEDGMENTS

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Supplementary file

Roles of a solo LuxR in the biological control agent *Lysobacter enzymogenes* strain OH11

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1. Materials and Methods

1.1 Detection of lytic enzyme production in *Lysobacter enzymogenes*

Chitin hydrolysis was detected using 1% colloidal chitin as described previously (5). Hydrolysis of 1% carboxymethyl cellulose (CMC) was detected as described previously (5). Proteolytic activity was detected as diffusion-clearing zones on milk agar (3). β-1, 3-glucanase activity was detected by the appearance of zones of color differentiation surrounding colonies grown on medium 813 agar (6) containing 0.5% laminarin (soluble β-1, 3-glucans) after staining with 1% Congo red.

1.2 Protein extraction and purification

Total protein was extracted from bacterial cells according to description previously (7). Cells were harvested from the culture (cells were grown in 1/10 TSB broth) with an OD_{600} of 1.0 by centrifugation (10, 000×*g* at 4 ºC for 10 min). The cell pellets were re-suspended in 20-mL washing buffer (50 mmol/liter Tris-HCI, pH 7.2), and this step (centrifugation and washing) was repeated for two times. The finally washed cells were re-suspended in 400-μl Alklysis buffer (added 1×PMSF), and fragmentized by an ultrasonic machine. Then, the lysate was centrifuged at 10, 000×*g* at 4 ºC for 20 min. The supernatant was transferred into a fresh 2-mL reaction tube. Total protein of each strain was purified by CKEAN UP kit (GE, USA). Protein samples were stored at -20 ºC for 2-DE assay described below.

1.3 2-DE

Isoelectric focusing (IEF) was performed according to description previously (2). The protein concentration was adjusted to 200 μg in 450 μl RH buffer (proteins, 1% DTT, 1% IPG Buffer, 1×BPB, RB). The samples were applied to a 24-cm, linear pH 4-7 IEF strip (GE, USA). IEF was carried out on an IPGPHOR3 (GE, USA) system using the following program: 300 V×20 min; 700 V×0.5 h; and 1500 V×1.5 h. The gradient was raised from 1500 V to 9000 V within 3 hours, and subsequently 9000 V×6.5 h. At last, IEF was terminated at 9000 V. Before separating on SDS-PAGE, the IEP strips were equilibrated as described previously (2). The strip equilibrated was mounted on a 12.5% T acrylamide: bis-acrylamide (37.5:1) SDS-PAGE gel for second dimension electrophoresis using the following program: 2 W/gel, 45 min; and 17 W/gel, 4 hours.

At least three repetitions were performed for each condition. Silver staining was carried out according to the method described previously (1). The comparative analysis of the resulting images was performed by PDQuest v7.2 software (Biorad, Hercules, CA, USA).

1.4 RT-PCR

The expression of lesR was assayed by semi-quantitative RT-PCR (4) with corresponding primer pairs (lesR-1/lesR-2) (Supplemental Table 2). Single colony of *Lysobacter* was pre-incubated in 10-ml LB medium for 16–20 h, until the OD_{600} value reached 1.0, and 2% of this culture was sub-cultured into 10-ml of the fresh 1/10 TSB broth for 12-14 h incubation. When the OD_{600} value reached 1.0, the bacterial cells were collected. As a template, total RNAs were extracted using the Trizol reagent (Takara, Dalian, China) according to the manufacturer's protocol. To remove genomic DNA, the eluted RNA samples were treated with RNase inhibitors and DNaseI (TaKaRa). RNA integrity was confirmed by electrophoresis using 1.2% agarose gels. Then 2 μg of each RNA sample was used to synthesise cDNA with a cDNA synthesis kit (TaKaRa). Semi-quantitative RT-PCR was performed on the ordinary PCR apparatus and the PCR program was as follows: step 1, 95 °C for 5 min; step 2, 95 °C for 30 s; step 3, 65 °C for 30 s; step 4, 72 °C for 30 s; and step 5, 72 °C for 8 min, 34 cycles from steps 2 to 4 to amplify 16S rDNA and *lesR*.

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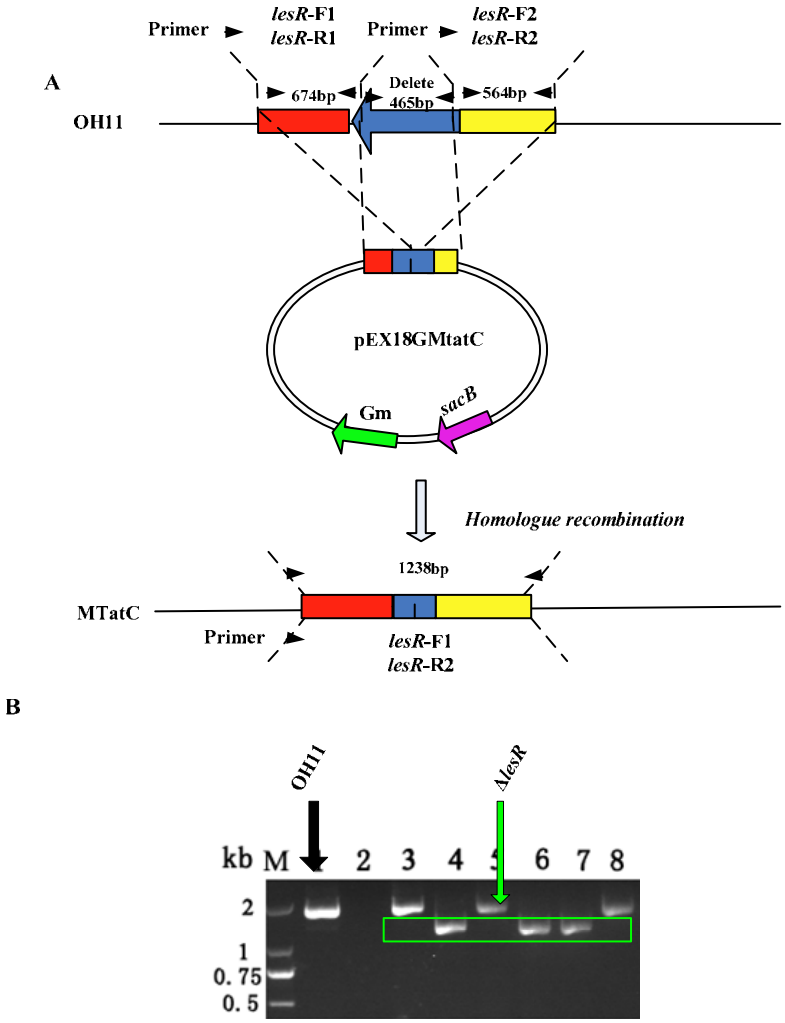
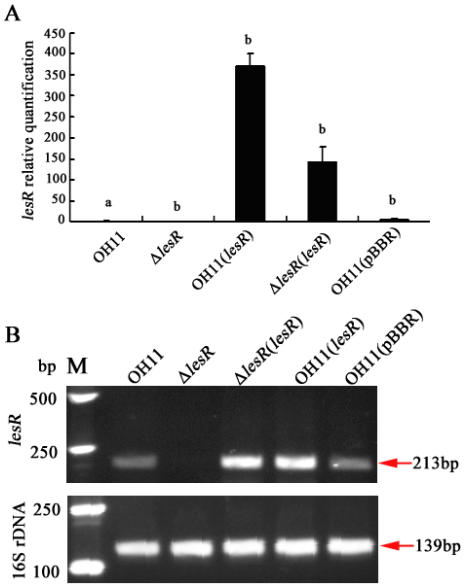
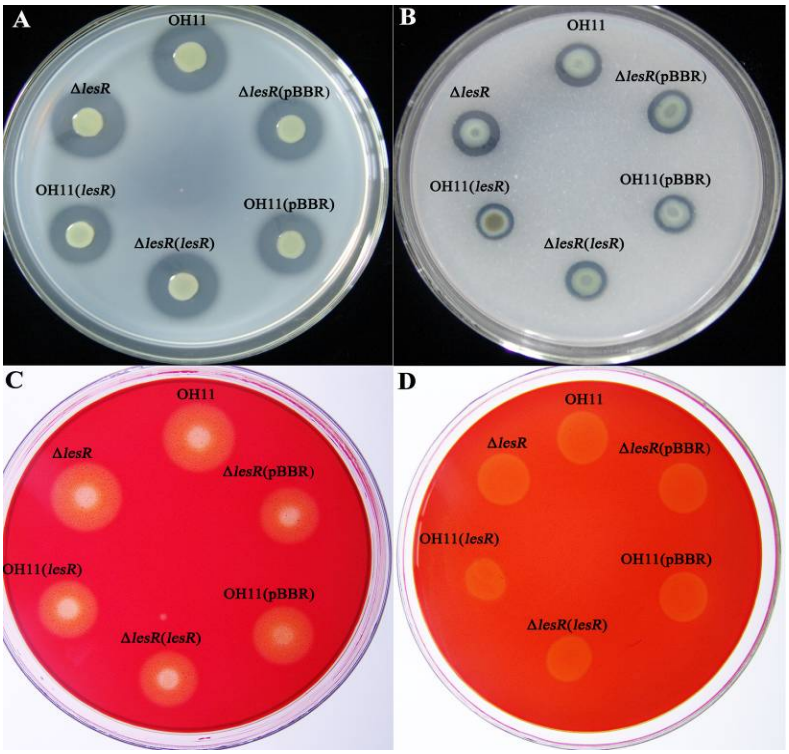


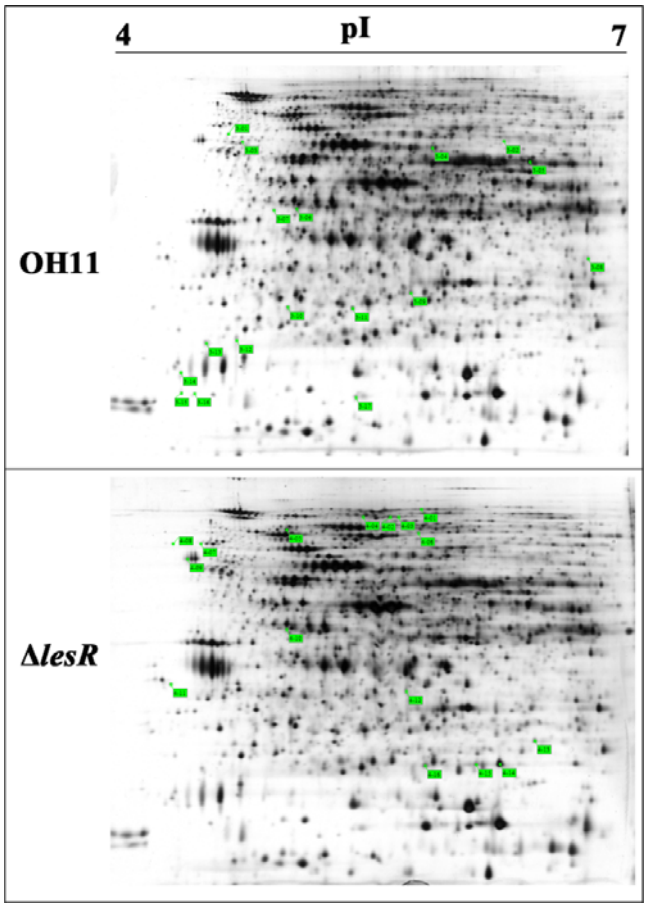
Supplemental Figure 1. The scheme of *lesR* mutant construction and molecular confirmation in *Lysobacter enzymogenes*. (A) The scheme of gene deletion was shown. The 674-bp (amplified by *lesR* -F1/ *lesR* -R1) (Supplemental Table 2) and 564-bp (amplified by *lesR* -F2 *lesR* -R2) (Supplemental Table 2) DNA fragment was used as 5' and 3' fragment for homologue recombination, respectively. The internal 465-bp DNA fragment would be deleted in the *lesR* mutant. The primers *lesR* -F1/ *lesR* -R2 (Supplemental Table 2) was used for molecular confirmation of *lesR* mutant. If the 465-bp internal fragment of *lesR* was successfully deleted, a 1238-bp DNA fragment would be amplified from *lesR* mutant. (B) PCR confirmation of *lesR* mutant was shown. Due to deletion of 465-bp internal fragment of *lesR*, only ≈1.3-kb DNA fragment was amplified from *lesR* -deletion mutant.



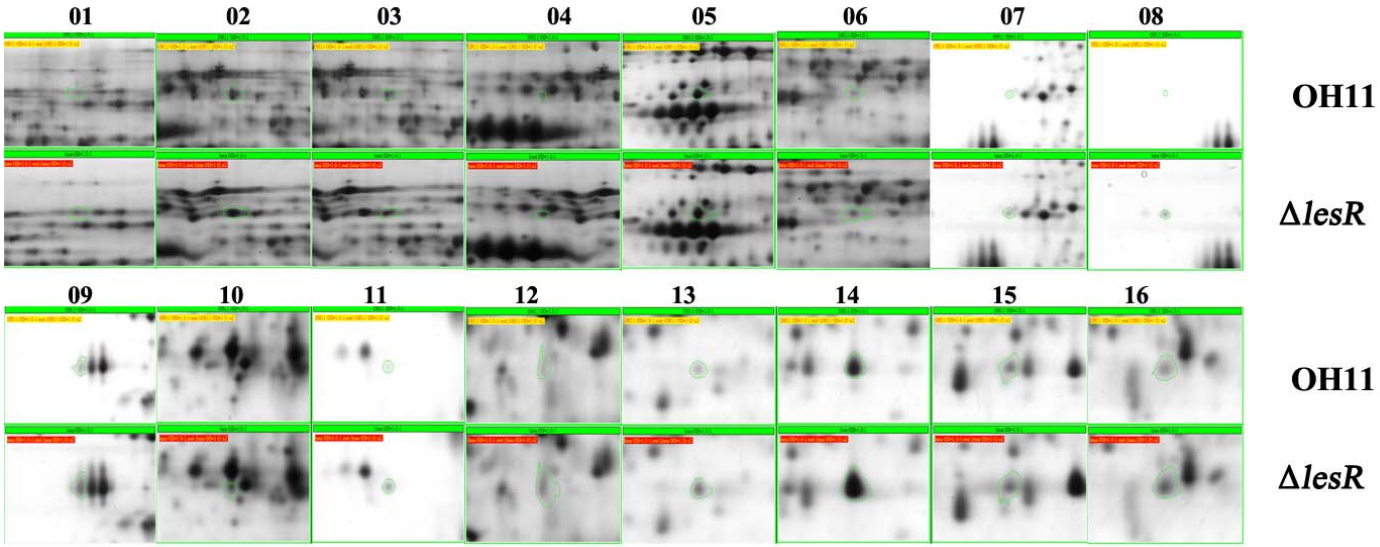
Supplemental Figure 2. Determination of transcriptional expression of *lesR* in wild-type OH11 and its derivative strains by qRT-PCR (A) and RT-PCR (B). OH11, the wild-type strain *L. enzymogenes*; Δ*lesR*, the *lesR* deletion mutant of strain OH11, Δ*lesR*(*lesR*), the complemented strain of Δ*lesR*$_1$; OH11(*lesR*), over-expression strain of *lesR*; OH11(pBBR), the wild-type strain haboring the empty vector. Different letters above bars indicate a significant difference between the wild-type strain OH11 and the tested mutants (P<0.05; t-test). Identical letters above the data bars indicate that there is no significant difference between the wild-type strain and the tested strains (P>0.05; t test).



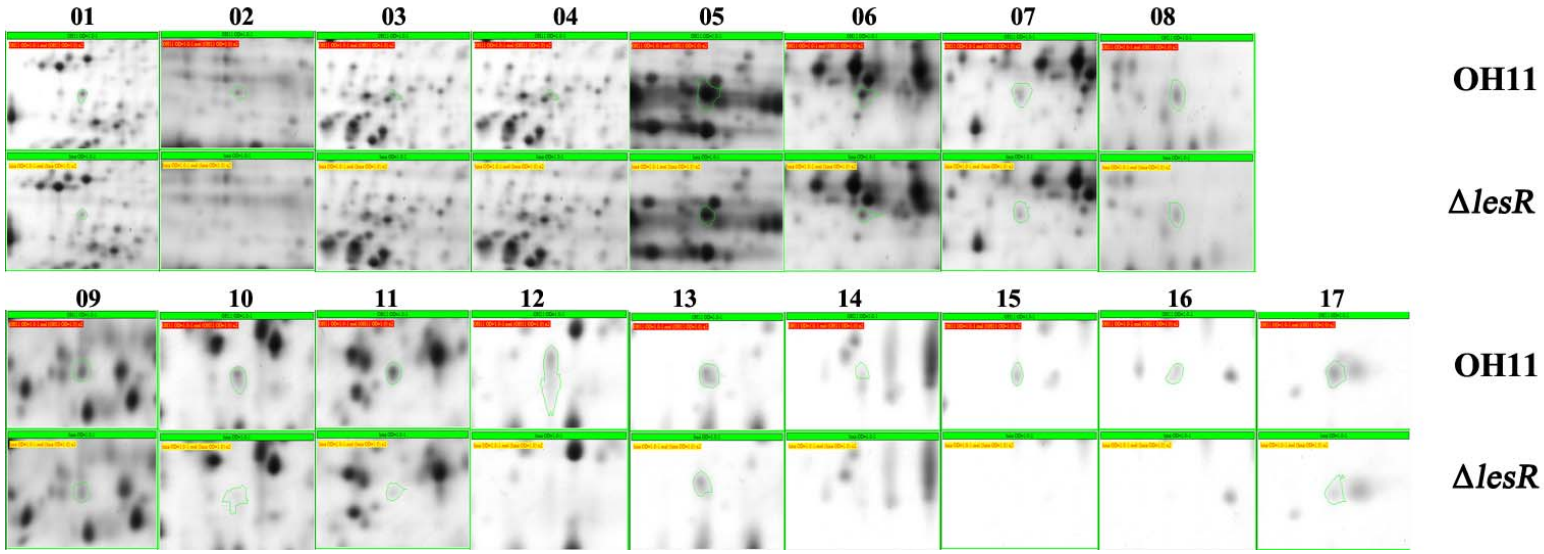
Supplemental Figure 3. Over expression of *lesR* did not impair the production of four-type lytic enzymes in *Lysobacter enzymogenes*. A: Protease activity detection; B: Chitinase activity; C: Cellulase activity; D: Glucanase activity. OH11, the wild-type strain *L. enzymogenes*; Δ*lesR*, the *lesR* deletion mutant of strain OH11, Δ*lesR*(*lesR*), the complemented strain of Δ$rpfF_{OH11}$; OH11(*lesR*), over-expression strain of *lesR*; OH11(pBBR), the wild-type strain haboring the empty vector.



Supplemental Figure 4. Deletion of *lesR* affected protein expression profile in *Lysobacter enzymogenes*. 2-DE gels determined the differentially-expressed protein spots between wild-type OH11 and *lesR*-deletion mutant (Δ*lesR*). The differentially expressed protein spots were numbered with green color with the threshold ratio of 1.5. Spots highlighted in green in strain OH11 and Δ*lesR* represent proteins positively or negatively regulated by *lesR*, which was provided in Figure S5 and S6 in Supplementary file 1 in details;



Supplemental Figure 5. Identification of 16 protein spots negatively regulated by *lesR* in *Lysobacter enzymogenes*. These 16 protein spots exhibited remarkably increased expression level (> 1.5 fold) in *lesR*-deletion mutant (Δ*lesR*) compared to wild type.



Supplemental Figure 6. Identification of 17 protein spots positively regulated by *lesR* in *Lysobacter enzymogenes*. These 17 protein spots exhibited remarkably increased expression level (> 1.5 fold) in *lesR*-deletion mutant (Δ*lesR*) compared to wild type.

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

Strains and plasmids	Characteristics[a]	Source
Lysobacter enzymogenes		
OH11	Wild type, Km^R	37
Δ*lesR*	*lesR* in-frame deletion mutant, Km^R	This study
Δ*lesR*(*lesR*)	Δ*lesR* harboring plasmid pBBR-*lesR*, Gm^R, Km^R	This study
Δ*lesR*(pBBR)	Δ *lesR* harboring plasmid pBBR1MCS-5, Gm^R, Km^R	This study
OH11(*lesR*)	OH11 harboring plasmid pBBR-*lesR*, Gm^R, Km^R	This study
OH11(HTH)	OH11 harboring plasmid pBBR-HTH, Gm^R, Km^R	This study
OH11(ABD)	OH11 harboring plasmid pBBR-ABD, Gm^R, Km^R	This study
Escherichia coli		
DH5α	*supE44lacU169* (Δ*lacZ*ΔM15) *hsdR17 recA lendA1gyrA96 thi-1 relA11*	Lab collection
Plasmids		
pEX18GM	Suicide vector with a *sacB* gene, Gm^R	21
pBBR1MCS-5	Broad-host-range vector with a P_{lac} promoter	25
pEX18-*lesR*	pEX18GM with two flanking fragments of *lesR*, Gm^R,	This study
pBBR-*lesR*	pBBR1MCS-5 cloned with a 1525-bp fragment containing intact *lesR* and its predicted promoter	This study
pBBR-HTH	pBBR1MCS-5 cloned with a 729-bp fragment containing intact HTH domain and the predicted promoter of *lesR*	This study
pBBR-ABD	pBBR1MCS-5 cloned with a 1029-bp fragment containing intact autoinducer binding domain and the predicted promoter of *lesR*	This study

[a] Km^R, Gm^R, Rif^R = Kanamycin-, Gentamicin-, Rifampicin-resistant, respectively.

Supplemental Table 2. Primers used for in-frame deletion, complementation, over-expression, real-time quantitative PCR and RT-PCR in this study

Primer	Sequence[a]	Purpose
lesR-F1	GGGGTACCATGTTGGCGGTGGTGTTGATG (KpnI)	To amplify a 674-bp upstream homologue arm of *lesR*
lesR-R1	CCCAAGCTT GCGTAGCCGTGGTTGTGGAA (*Hin*dIII)	
lesR-F2	CCCAAGCTT CTGGTCGGCGGTGGGCAAGA(*Hin*dIII)	To amplify a 564-bp downstream homologue arm of *lesR*
lesR-R1	GCTCTAGA GGCGATGGGGAAAAGAAAGC (*Xba*I)	
lesR-F	CCCAAGCTT ATGTTGGCGGTGGTGTTGATG (*Hin*dIII)	To amplify a 1525-bp fragment containing intact *lesR* and its predicted promoter
lesR-R	GCTCTAGA CGAGGGCGGTCCTGTCAGAAGT (*Xba*I)	
Prom-F	CCCAAGCTTATGTTGGCGGTGGTGTTGATG (*Hin*dIII)	To amplify a 561-bp fragment containing predicted promoter of *lesR*
Prom-R	GGGGTACC GCCGTGCCTGATCACCTGC (*Kpn*I)	
HTH-F	GGGGTACC ATGAAGCTCAGCGAGCGCGA (*Kpn*I)	To amplify a 168-bp DNA fragment containing intact HTH domain
HTH-R	CCCTCTAGACTACGCGCAGGCCGCGCGCC (*Xba*I)	
ABD-F	GGGGTACC ATGACGTTGGACGAGGTCA (*Kpn*I)	To amplify a 468-bp DNA fragment containing autoinducer binding domain
ABD-R	GCTCTAGA CTACGCCGCGTGCATGCACG (*Xba*I)	
16S rRNA-1	5'-ACGGTCGCAAGACTGAAACT-3'	The housekeeping gene for real-time quantitative PCR
16S rRNA-2	5'-AAGGCACCAATCCATCTCTG-3'	
PKS-NRPS-1	5'-CATCACATCATCTCCGATGC-3'	To determine the transcriptional level of *pks/nrps*
PKS-NRPS-2	5'-CAGTTCCACCTTCTCCTTGC-3'	
lesR-1	5'- AGTGGTCGTTCACCACCTTC-3'	To determine the transcriptional level of *lesR*
lesR-2	5'- TCATCGAGATCTCCCACGAG -3'	

[a]Restricted digestion enzyme site was underlined.