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The Distinct Quorum Sensing Hierarchy of *las* and *rhl* in *Pseudomonas* sp. M18

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Abstract *Pseudomonas* sp. M18 is a rhizosphere isolate capable of producing two kinds of antifungal agents: phenazine-1-carboxylic acid (PCA) and pyoluteorin. Recently, the two well-studied quorum sensing (QS) systems of *Pseudomonas aeruginosa*, LasR/LasI and RhlR/RhlI, have also been identified in this strain. However, in this study, through the use of *lacZ* translational fusion expression analysis and acyl-homoserine lactone thin-layer chromatography (TLC) bioassays, we clearly display a more complex and distinctive hierarchy of the *las* and *rhl* QS systems in strain M18. In this QS cascade, expression of *rhlI* was negatively controlled by the LasR/LasI QS system. In contrast with *lasI*, which negatively regulated the *rhlR* induction, *lasR* exerted a positive influence on *rhlR* expression during the log-phase. This interrelationship indicated that the response regulators (LasR and RhlR) of the QS system are expressed independently of their cognate synthases (LasI and RhlI). Furthermore, the *las* system also modulated the timing and magnitude of the *rhlI* and *rhlR* maximal expression. In addition, our data imply that the *lasR* gene exerts its negative control on PCA production through modulation of *rhlI* expression. Thus, interactions between the two QS systems are strain specific.

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Introduction

Quorum sensing (QS) is a process employed by many bacteria for communication and for synchronization of group behavior. It is affected *via* the secretion of specific signal molecules (autoinducers) and occurs in a cell density-dependent manner. The QS system is typically comprised of the synthase gene, whose product catalyzes the biosynthesis of signals, and the receptor (regulator) gene, whose product responds to the signal and subsequently regulates the target genes.

The QS systems are widespread in pseudomonads. The most extensively studied of these are the *las* and *rhl* systems in strain *Pseudomonas aeruginosa*. The *las* QS system is composed of LasI, which directs the synthesis of the signal molecule *N*-3-oxododecanoyl-homoserine lactone (OdDHL, also known as 3-oxo-C_{12}-HSL) and of its receptor LasR. Similarly, the *rhl* QS system consists of RhlI, which is responsible for the synthesis of the signals *N*-butanoyl-homoserine lactone (BHL, major product, also known as C_4-HSL) and *N*-hexanoyl-homoserine lactone (HHL) and of its receptor RhlR. The two systems themselves are subject to a positive feedback and regulate overlapping sets of genes concerning the virulence factor production, biofilm formation, and many other genes [1]. Some of these, such as *rhlAB* (rhamnolipid) and the *phzABCDEFG* operon (phenazine biosynthesis), have been identified as being specifically and directly activated by the *rhl* regulon. For other numerous *las*-controlled genes, the situation is not as clear [2–4]. Previously published studies have shown that the two systems are organized hierarchically and are interdependent, so that the *las* QS system has a positive effect on *rhlI* and *rhlR* expression [5, 6].

Pseudomonas sp. M18 is a fluorescent *Pseudomonas* strain that can suppress diseases caused by pathogenic

fungi in crop plants. This biocontrol capacity largely depends on the production of its two kinds of antibiotics: phenazine-1-carboxylic acid (PCA) and pyoluteorin (Plt). Strain M18 has a similar genetic background to that of *P. aeruginosa* PAO1, shown by the 99% identity between the *16S rRNA* gene sequences of M18 (AY696302) and PAO1. Although the production of antifungal compounds (e.g., phenazine derivatives) is common in the strains of *P. aeruginosa*, some marked characteristics of strain M18 clearly reflect that it is distinct from *P. aeruginosa* strain reported before, such as Plt production and its biosynthetic structural, regulatory, and ABC export (or resistance) gene cluster (GenBank accession number AY394844), the high production of PCA, and remarkably different regulatory mechanisms of secondary metabolisms including Plt and PCA biosynthesis [7–10].

Recently, two complete *las* and *rhl* QS systems identified in strain M18 have been shown to play critical roles in the regulation of secondary metabolites [7, 10]. However, the *las* QS system in strain M18 has been reported to negatively control *phzABCDEFG* expression. In contrast, the *las* system in *P. aeruginosa* mutants exhibits a significant delay of *phzABCDEFG* expression and pyocyanin production, through modulation of the *rhl* regulon [7, 11]. The aim of this study was therefore to further investigate this apparent discrepancy, explore the role of the *rhl* system on PCA production, and the interaction between the *las* and *rhl* systems in *Pseudomonas* sp. M18.

Materials and Methods

Bacterial Strains and Growth Conditions

Pseudomonas sp. M18 is a fluorescent *Pseudomonas* strain isolated from the watermelon rhizosphere. It is an unusual strain in that it shares some distinct features with both *P. aeruginosa* and *Pseudomonas fluorescens* [7–10]. The other bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was routinely grown at 37°C in Luria-Bertani (LB) medium. *Pseudomonas* sp. M18 and its derivatives were incubated at 28°C in King's medium B (KMB) [12] or pigment-producing medium (PPM) [13]. The antibiotics added to media were ampicillin (Ap) (100 μg ml^{-1}), spectomycin (Sp) (100 μg ml^{-1}), kanamycin (Km) (50 μg ml^{-1}), tetracycline (Tc) (120 μg ml^{-1}), and gentamicin (Gm) (40 μg ml^{-1}) for

Table 1 Strains and plasmids used in this study

Materials	Characteristics	Source
Strains		
E. coli		
SM10	*thi-1 thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km^r	[27]
DH5α	*recA1 endA1 gyrA96 thi1 hsdR*17 (rk^- mk^+) *supE*44 *re1A*1	[27]
Pseudomonas sp.		
M18	PCA, Plt producer, Sp^r	[9]
M18LIG	M18 *lasI*::Gm^r, Sp^r Gm^r	[7]
M18LRK	M18 *lasR*::Km^r, Sp^r Km^r	[7]
M18IG	M18 *rhlI*::Gm^r, Sp^r Gm^r	[10]
M18RK	M18 *rhlR*::Km^r, Sp^r Km^r	[10]
M18IR	M18 *rhlI*::Gm^r, *lasR*::Km^r Sp^r, Gm^r Km^r	This study
C. violaceum CV026	Signal molecule reporter, Km^r	[14]
Plasmids		
pME6032	pVS1–p15A *E. coli—Pseudomonas* shuttle vector, $lacI^q$-P_{tac} expression vector, Tet^r	[28]
pME6032lasI	pME6032 with *Bgl*II-*Eco*RI insert of 1.2 kb, including gene *lasI* and partial flanking sequence, Tc^r	[7]
pME6032lasR	pME6032 with *Xho*I-*Nco*I insert of 0.9 kb, including gene *lasR* and partial flanking sequence, Tc^r	[7]
pMETcLRK	*lasR*::ΩKm in pEXTcLASR, Tc^r Km^r	[7]
pME6015	pVS1–p15A *E. coli—Pseudomonas* shuttle vector for translational *lacZ* fusions and promoter probing, Tc^r	Dieter Haas
pRRL	357-bp *Eco*RI-*Pst*I PCR amplified fragment containing *rhlR* upstream region cloned into pME6015	This study
pMEIZ	898-bp *Eco*RI-*Pst*I PCR amplified fragment containing *rhlI* upstream region cloned into pME6015	[29]

Plt pyoluteorin, *PCA* phenazine-1-carboxyl acid, *r* antibiotics-resistant, *Km* kanamycin, *Tc* tetracycline, *Gm* gentamicin, *Amp* ampicillin, *Sp* spectinomycin

pseudomonads, and Ap (100 μg ml^{-1}), Km (50 μg ml^{-1}), Tc (15 μg ml^{-1}), and Gm (10 μg ml^{-1}) for *E. coli*.

Construction of *lasR rhlI* Double Genes Mutant from *rhlI* Mutant Strain M18IG

The plasmid pMETcLRK was first transformed into the *E. coli* SM10. Then it was mobilized from the *E. coli* SM10 donor into strain M18IG by biparental mating. Transconjugants were selected on LB plates containing Sp to counter-select *E. coli* SM10 and Km. After a second crossing-over, Km-resistant, Tc-sensitive, and sucrose-resistant recombinants with the chromosomally inactivated *lasR* gene in strain M18IG were obtained. The resultant *lasR rhlI* double mutant, designated M18IR, was confirmed by PCR and sequencing with the primers LasR-UP and LasR-Down [7].

Construction of Translational *rhlR′–′lacZ* Fusions

A 357-bp PCR fragment carrying the promoter region and part of the *rhlR* gene was amplified from the strain M18 genomic DNA using primers PrhlR-UP (5′-CGT CGA ATT CTG TCA CAA CCG CAC AGT ATC-3′) and PrhlR-DOWN (5′-ACT GCT GCA GAT AGG CGT AGT AAT CGA AGC-3′) (*Eco*RI and *Pst*I sites are underlined). The 357 bp PCR fragment shared 99.5% identity with that from *P. aeruginosa* PAO1. The fragment was cloned into *Eco*RI-*Pst*I-digested pME6015 to generate pRRL, a translational *rhlR′–′lacZ* fusion.

Quantitative Assay of PCA by HPLC

Pseudomonas sp. M18 and its derivative strains were grown in PPM media as indicated in the caption of Fig. 1. Extraction and quantification of PCA were carried out as previously described [7].

β-Galactosidase Assay

Pseudomonas sp. M18 and its derivatives containing the *lacZ* reporter plasmids were cultivated in KMB media, as indicated in the caption of Fig. 3. The β-galactosidase activities were assayed as previously described [7].

TLC Analysis of Acyl-Homoserine Lactone (AHL) Signals

The AHL signals, BHL and HHL, were detected using a TLC bioassay, as described by McClean et al. [14] and Shaw et al. [15]. M18 and its derivative strains were grown to an OD_{600} of 7.5 to 8.5 in KMB media. The AHL extraction and analysis were performed as described previously [10]. Synthetic AHL standards of BHL and HHL (catalogue no. 09945 and 09926; Fluka, Shanghai, China) served as controls. *Chromobacterium violaceum* CV026 was grown overnight at 28°C in LB broth and used as the indicator strain [14, 15].

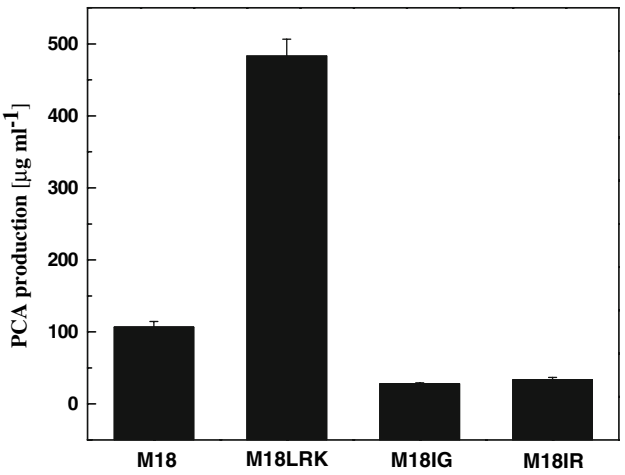

Fig. 1 Effect of LasR and RhlI on PCA production. PCA production was assayed in strain M18, the *lasR* mutant M18LRK, the *rhlI* mutant M18IG, and the *lasR rhlI* double mutant M18IR, in PPM broth, after 72 h incubation of the cultures

Results

The Effect of QS System on PCA Production

The *las* and *rhl* systems in strain M18 appeared to be organized in a different manner to that seen in *P. aeruginosa*, in terms of the production of PCA. As shown in Fig. 1, *lasR* disruption led to a substantial enhancement of PCA production, in agreement with our previous report that the *las* system negatively controls PCA biosynthesis in strain M18 [7]. A similar reduction in PCA production was caused by the *rhlI* mutation and the *lasR rhlI* double mutation (Fig. 1). This result not only indicates that *rhlI* positively controls PCA biosynthesis, but also implies that *lasR* may exert some effect through *rhlI*.

The Regulation of *las* QS System on the *rhlI′–′lacZ* Translational Fusion Expression

To confirm the latter hypothesis and to determine the influence of *las* QS system on the *rhl* QS system, we performed *lacZ*-based fusion analysis in strain M18 and its *lasI* or *lasR* inactivated mutant. The regulation of *las* and *rhl* QS systems may occur at transcriptional and/or post-transcriptional levels in strain M18. Since the *rhlI′–′lacZ* and *rhlR′–′lacZ* transcriptional fusions cannot reflect post-

transcriptional regulation of *rhlI* and *rhlR*, we constructed two translational fusions pMEIZ (*rhlI′–′lacZ*) and pRRL (*rhlR′–′lacZ*) to, respectively, assay the *rhlI* and *rhlR* expressions. These two translational fusions contain the flanking region of the transcriptional start site (TSS) and the first several codons of *rhlI* and *rhlR*, they can, respectively, reflect the combined transcriptional and translational expression levels of *rhlI* and *rhlR* (Fig. 2c). The *rhlI* and *rhlR* genes expression in corresponding strains were monitored in KMB media throughout cell growth, as described in Fig. 2. Both *rhlI* and *rhlR* genes were

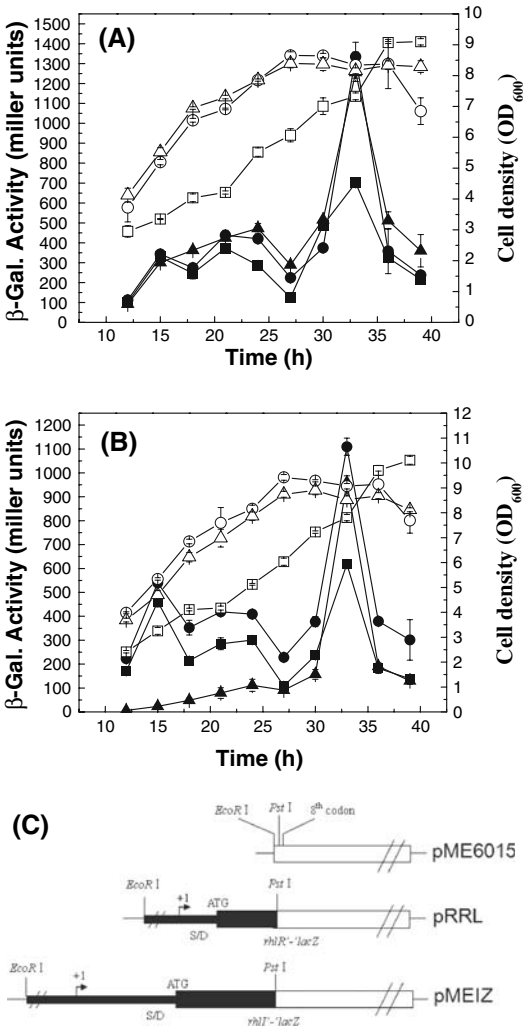

Fig. 2 Effect of LasI and LasR on *rhlI* and *rhlR* gene expression levels and cell growth. OD_{600} (*open symbols*) value and *β*-galactosidase expression (*solid symbols*) of the *rhlI′–′lacZ* translational fusion expression plasmid pMEIZ (**a**), the *rhlR′–′lacZ* translational fusion expression plasmid pRRL (**b**) were followed over time in cultures of wild type M18 (*square*), *lasI* mutant M18LIG (*circle*), and *lasR* mutant M18LRK (*triangle*) in KMB media. Data represent the means (±SD) of triplicate cultures. (**c**) Maps of plasmid pRRL and pMEIZ. S/D, putative Shine-Dalgarno sequence. +1, putative TSS. The putative promoter region and its flanking sequence are shown as a *thick black line*

expressed throughout the growth cycle in the wild-type strain M18. During the early exponential phase of growth both *lacZ* fusions were expressed at a relatively low level, but they were induced rapidly and reached maximal expression during the late exponential phase. This observation was in accordance with that seen in *P. aeruginosa* [5, 16]. In contrast, as reflected in Fig. 2a, both *lasI* and *lasR* mutants followed a similar pattern of *rhlI′–′lacZ* expression as that seen in the parent strain M18. However, the *rhlI′–′lacZ* expression in the strains showing disrupted *las* QS systems was significantly enhanced during some growth phases as compared with that seen in the wild-type strain. This increase could be readily observed at the maximal expression level, where the *rhlI′–′lacZ* expression was about twofold higher in the *lasI* or *lasR* mutants than it was in strain M18. Taken together, the *las* QS system appeared to be negatively controlling the *rhlI* expression in strain M18 (Fig. 2a). Furthermore, the maximal expression of *rhlI′–′lacZ* fusion in the *las* QS mutants was restricted to the early stationary phase, while it occurred at the late log-phase growth in the wild-type strain.

The Influences of the *las* QS System on *rhlR′–′lacZ* Expression

As shown in Fig. 2b, the delayed maximal expression of the *rhlR′–′lacZ* reporter was also observed in both *lasI* and *lasR* mutant strains. However, the *rhlR′–′lacZ* fusion exhibited significantly different patterns of expression in the *lasI* compared to the *lasR* mutants. Both basal and maximal levels of the *rhlR* gene expression in the *lasI* mutant were increased by approximately twofold, compared with those of the parental strain, although both strains had a similar expression profile (Fig. 2b). In contrast, the activity of *rhlR′–′lacZ* fusion in the *lasR* mutant was greatly reduced overall during the exponential phase, compared with the parental M18 strain, although it still showed a linear increase. Upon entry into the early stationary phase, the *rhlR* expression in the *lasR* mutant was substantially activated and showed maximal *rhlR* expression about twofold higher than that in the parental M18 strain (Fig. 2b). Overall, the *lasI* gene appeared to exert a negative control over *rhlR* expression in strain M18. However, the basal level of *rhlR* expression was positively regulated by *lasR* during the log-phase and the higher maximal activities of *rhlR* expression were delayed until the early stationary phase in *lasR* mutant.

The Modulation of QS Systems on BHL and HHL Production

TLC bioassay has been reported to detect and semi-quantify the AHLs signal molecules. The intensity of the

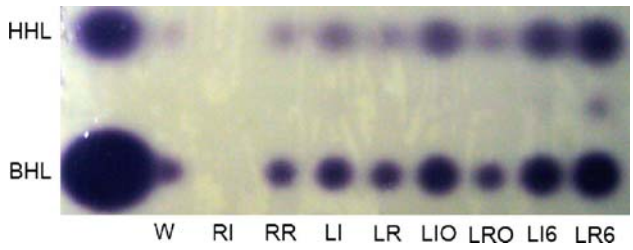

Fig. 3 TLC analysis of AHLs secreted by the wild-type M18 strain and its derivatives grown in KMB media. AHL samples were visualized with the *C. violaceum* CV026 reporter strain. *M* BHL and HHL markers, *W* the wild-type M18 strain, *RI* the *rhlI* mutant M18IG strain, *RR* the *rhlR* mutant M18RK strain, *LI* the *lasI* mutant M18LIG strain, *LR* the *lasR* mutant M18LRK strain, *LI6* the M18LIG strain harboring the empty vector pME6032, *LR6* the M18LRK strain harboring the empty vector pME6032, *LIO* the M18LIG strain harboring pME6032lasI in which the *lasI* gene was overexpressed, *LRO* the M18LRK strain harboring pME6032lasR in which the *lasR* gene was overexpressed

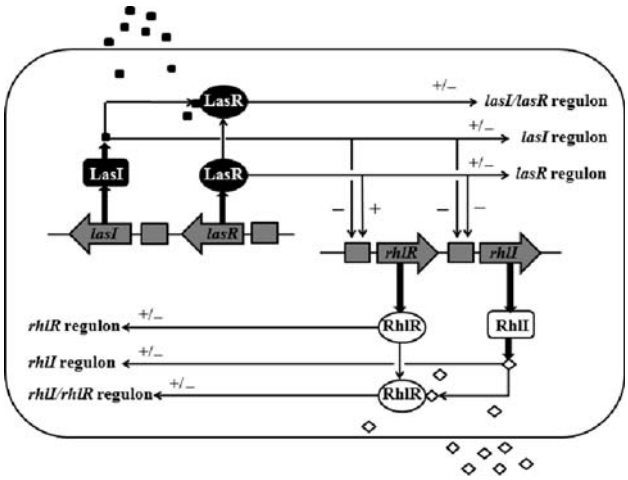

Fig. 4 Proposed model for the hierarchical QS systems in *Pseudomonas* sp. M18. +, activation; −, repression; ■, OdDHL; ◇, BHL; ➡, production

indicator strain's response to signal molecules can reflect the levels of AHLs amounts [14, 15]. To further corroborate the hypothesis that the *rhlI* gene was negatively modulated by the *las* QS system, TLC assay was performed to evaluate the levels of the signal molecules BHL and HHL in the wild-type strain M18 and its derivative strains when grown to an OD_{600} of 7.5 to 8.5. As shown in Fig. 3, neither BHL nor HHL was detected in the *rhlI* mutant strain, in agreement with the supposition that RhlI is responsible for the synthesis of these two effectors [10]. The chromosomal inactivation of *lasI* or *lasR* resulted in an increase in BHL and HHL production compared to that in the parental M18 strain. Conversely, the *lasI* or *lasR* overexpression (LIO and LRO in Fig. 3) led to a reduction in both BHL and HHL production in comparison with that observed in corresponding control strains (LI6 and LR6 in Fig. 3), which carry the empty vector pME6032 excluding the influence of plasmid on the BHL and HHL production (Fig. 3). The semi-quantified result of TLC bioassay is in consistent with the hypothesis above that the *las* QS system has a negative effect on the *rhlI* expression. In addition, the *rhlR* mutant strain produced similar amounts of BHL and HHL as did the wild-type strain (Fig. 3), indicating that the *rhlI* expression may be independent of the *rhlR* gene in strain M18.

Discussion

In this study, *rhlI* specifically upregulated PCA production, which is in accordance with similar observations in *P. aeruginosa*. In addition, *lasR* exerted a negative control over phenazine biosynthesis through the intermediate modulation of *rhlI*. Thus, in contrast to the situation in *P. aeruginosa*, *lasR* may have a negative effect on *rhlI* expression.

Pseudomonas sp. M18 appeared to employ a novel and complex hierarchical cascade of *las* and *rhl* systems that was complex and distinctly different from that seen in *P. aeruginosa* (Fig. 4). The *rhlI* gene expression was negatively controlled by the *las* system. This conclusion can be drawn from the *lacZ*-based fusion analysis (Fig. 2b) and TLC bioassay (Fig. 3). As shown in Fig. 2b, *lasI* negatively regulated the expression of *rhlR*, while the inactivation of *lasR* gene resulted in a significant delay and overexpression of *rhlR* activities. This might imply the LasR acts primarily as an activator of the *rhlR* expression that predominates during the exponential growth phase. A surrogate activator of *lasR* or another inducer of maximal activation may exist during the stationary phase. In previous studies of *P. aeruginosa*, control of *rhl* expression was apparently achieved by more than just the *las* QS system [5, 11].

The expression of the QS regulatory gene was not fully coupled with its cognate synthase gene. As indicated in Fig. 2, the *rhlR*-fusion expression was differentially modulated by *lasI* and *lasR*. Likewise, *rhlI* and *rhlR* were also regulated by *lasR* in distinctly different ways in strain M18. These findings were inconsistent with those reported for *P. aeruginosa* where both LasI and LasR have a positive effect on the expression of *rhlI* and *rhlR* [5, 6]. This is explainable since the QS regulatory genes (*lasR* and *rhlR*) and their cognate synthase genes (*lasI* and *rhlI*) have been shown to be transcriptionally coupled and they function synergistically in *P. aeruginosa* [1]. This significant difference between strains M18 and *P. aeruginosa* provides an obvious hint that the major regulators (*lasR* and *rhlR*) of QS system are expressed and may work independently of

their cognate synthase genes (*lasI* and *rhlI*) in strain M18. As shown in Fig. 3, the *rhlR* mutation had little effect on either BHL or HHL production, demonstrating that *rhlI* could be expressed independent of *rhlR*.

In *P. aeruginosa*, similar observations were reported, although it has been shown that the *las* and *rhl* systems are subject to a self-reinforcing positive feedback [1]. A recent report has suggested that the correlation between each synthase and its cognate transcriptional regulator can vary under different growth conditions [16]. More recently, a *lasR* (or *rhlR*) mutant was shown to retain its capacity to express *lasI* (or *rhlI*) and to produce its corresponding signal 3-oxo-C12-HSL (or C4-HSL) [7, 11, 17]. Furthermore, there is increasing evidence that implicates the QS signal molecule (3-oxo-C_{12}-HSL or C_4-HSL), or its receptor (LasR or RhlR) alone, in a function for avoiding the wasting of resources. Thus, the signal molecules have been shown to work independently of their corresponding regulators (LasR and RhlR) in many circumstances, including in vivo inflammatory responses and intra- and inter-species communication [18–22]. In addition, RhlR can regulate *rhlAB* expression, with or without the autoinducer [23]. Both of these molecules can bind to QscR and regulate the expression of other genes [24–26].

In strain M18, QS regulation does not appear to be a straightforward process. The expression of a receptor and its cognate synthase were not correlated with each other and this feature may allow fine-tuning of each regulator. Further insights into the nature of the interrelationship between the *las* and *rhl* systems in M18 will require detailed investigation into the regulatory mechanism of each of its major QS components.

The *las* QS system affected the timing and strength of *rhlI* and *rhlR* maximal expression. As shown in Fig. 2, the chromosomal inactivation of either the *lasI* or *lasR* gene caused a lag in the maximal expression of *rhlI* and *rhlR* fusion during the early stationary phase. A similar delayed effect resulting from *las* QS system disruption has also been reported in *P. aeruginosa* [11]. However, unlike *P. aeruginosa*, the *las* QS mutants of M18 expressed a much higher maximal activity of both the *rhlI* and *rhlR* genes than did their parental strain. This type of effect of the *lasR* mutation is conceivable since the *las* QS system primarily functions to activate gene expression in response to cell density.

Finally, it is also worth noting that the same experimental effects were observed with mutants grown in standard conditions in LB medium (data not shown). However, the *las* and *rhl* QS systems are known to be directly or indirectly regulated by a number of transcriptional regulators, and consequently, their expression is sensitive to environmental conditions. In *P. aeruginosa* PAO1, it has been reported that the relative timing and strength of expression of these two systems varied significantly under different conditions [16]. Given both the KMB and LB mediums utilized in this study are rich mediums, more systematical experiments under different conditions should be designed to further investigate the influence of environmental factors on *lasR/I* and *rhlR/I* genes expression and their regulatory interrelationship.

In conclusion, complicated interaction between the *las* and *rhl* QS systems occurs in *Pseudomonas* sp. M18 that is significantly different from the QS hierarchy reported in *P. aeruginosa*. These differences occur even though both organisms share many similar genetic features indicating the strain specificity of QS cascade. Genome sequencing of strain M18 is being undertaken and it will better clarify the genetic basis and classification of the strain. Additionally, since TLC bioassay is a semi-quantified method and influenced by many factors, to more accurately evaluate the amounts of AHLs and subtly study the regulation of AHLs productions, HPLC assay of AHLs is needed to be established. Our previous work has revealed that the *las* and *rhl* QS systems control expression of many genes in strain M18, more complete understanding of the *las* and *rhl* QS hierarchy will aid in the elucidation of complex connected regulatory network and the development of engineered strains with high production of PCA or Plt. At the same time, it will also help to decipher the evolution and regulatory mechanism of the QS system in *Pseudomonas* as compared with that in other pseudomonads.

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