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The Quorum-Sensing Negative Regulator RsaL of *Pseudomonas aeruginosa* Binds to the *lasI* Promoter

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A mutation in the *rsaL* gene of *Pseudomonas aeruginosa* produces dramatically higher amounts of *N*-acyl homoserine lactone with respect to the wild type, highlighting the key role of this negative regulator in controlling quorum sensing (QS) in this opportunistic pathogen. The DNA binding site of the RsaL protein on the *rsaL-lasI* bidirectional promoter partially overlaps the binding site of the LasR protein, consistent with the hypothesis that RsaL and LasR could be in binding competition on this promoter. This is the first direct demonstration that RsaL acts as a QS negative regulator by binding to the *lasI* promoter.

Quorum sensing (QS) is a widespread bacterial intercellular communication system based on the production of signal molecules of which the extra cellular concentration is related to cell density. Individual cells sense the presence of the signal molecule, which allows the whole bacterial population to control gene expression in response to cell density. This form of regulation controls a diverse range of phenotypes, including various pathogenicity determinants, conjugation, biofilm formation, and production of antibiotics and secondary metabolites (16, 27, 31).

Several gram-negative bacteria use acylated homoserine lactones (acyl-HSL) as signal molecules in quorum sensing. The *Pseudomonas aeruginosa* QS system is one of the most extensively studied within this group of bacteria, reflecting the importance of this microorganism as an opportunistic pathogen of humans, other animals, and plants (10, 15). *P. aeruginosa* possesses two homologous QS systems encoded by the *lasR*/*lasI* and *rhlR*/*rhlI* gene pairs. The *lasI* and *rhlI* genes encode acyl-HSL synthase enzymes (LasI and RhlI) responsible for the synthesis of the *N*-3-oxo-dodecanoyl homoserine lactone ($3OC_{12}$-HSL) and *N*-butyryl homoserine lactone (C_4-HSL) signal molecules, respectively. The *lasR* and *rhlR* genes encode the regulators (i.e., LasR and RhlR) that respond to their cognate signals (i.e., $3OC_{12}$-HSL and C_4-HSL) and activate transcription of *lasI* and *rhlI*, respectively, thus creating a positive feedback loop. Moreover, the two *P. aeruginosa* quorum-sensing systems are organized in a hierarchical manner, where the RhlR/RhlI system is subordinate to the LasR/LasI system, since expression of *rhlR* and *rhlI* is dependent upon LasR (18, 24).

In *P. aeruginosa*, QS is a major global regulatory system that has been estimated to control approximately 5% of the genes, including the most important virulence genes (21, 26). Indeed, *P. aeruginosa* quorum-sensing null mutants are severely impaired in virulence in all the infection model systems examined (reviewed in reference 24).

The *P. aeruginosa* QS system is intricately connected with other cellular global regulatory networks, since it is regulated by a number of regulatory factors, including Vfr (1), GacA (20), the LuxR homologues QscR and VqsR (3, 11), MvaT (5), the alternative sigma factors RpoS and RpoN (8, 29), PprB (6), RsmA (19), DksA (9), and RsaL (4). The additional regulation of QS by the above-mentioned factors most likely affects the timing of the response and increases the range of environmental and metabolic signals, in addition to cell-density, to which QS responds. The mode of action of these regulators in most cases is unknown, and with the exception of LasR (22), none have been shown to directly regulate *lasI* gene expression.

This study is focused on the RsaL protein encoded by the *rsaL* gene located between the *lasR* and *lasI* genes (Fig. 1). Overexpression of *rsaL* in *P. aeruginosa* PAO1 resulted in a 20-fold reduction of $3OC_{12}$-HSL production (4). However, the effect of an *rsaL* mutation on $3OC_{12}$-HSL synthesis in *P. aeruginosa* has never been investigated. Therefore, we compared $3OC_{12}$-HSL production in *P. aeruginosa* PAO1 and its *rsaL*-derivative mutant. A *P. aeruginosa rsaL* transposon insertion mutant was supplied by the University of Washington Genome Center (www.genome.washington.edu/UWGC/pseudomonas) (Tables 1 and 2). The lactones produced by both strains were extracted and separated by thin-layer chromatography (TLC) and revealed by overlaying the TLC plate with a thin layer of Luria Bertani top agar seeded with *Escherichia coli*(pSB1075), a sensor strain for the detection of $3OC_{12}$-HSL and $3OC_{10}$-HSL (30). Results showed that $3OC_{12}$-HSL production is dramatically enhanced in the *rsaL* mutant (Fig. 2). Moreover, wild-type $3OC_{12}$-HSL levels were restored upon complementation of the *rsaL* mutant with the plasmid $pPSRsaL_{PAO}$ (Tables 1 and 2), expressing the *rsaL* gene, confirming that the mutated phenotype was due to *rsaL* inactivation and ruling out the occurrence of polar effects due to transposon insertion (Fig. 2). This result is consistent with what was recently observed in the rhizosphere-colonizing plant growth-promoting organism *Pseudomonas putida* WCS358, which has a QS genetic locus highly homologous to the *las* system of *P. aeruginosa*

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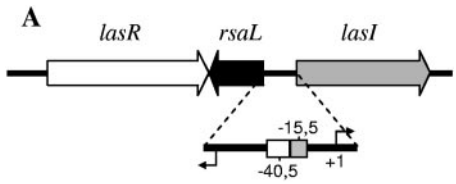

FIG. 1. Genetic organization of the *P. aeruginosa las* quorum sensing locus and detail of the *rsaL-lasI* bidirectional promoter (below). The *lasR*, *rsaL*, and *lasI* genes are depicted as arrows. Bent arrows indicate the *rsaL*- and *lasI*- divergent transcripts. The open box and the gray box indicate the LasR and RsaL binding sites, respectively. Numbers indicate the center of the LasR and RsaL binding sites with respect to the *lasI* transcription starting site (22).

and organized in the same way (2). A *P. putida* WCS358 *rsaL* mutant discloses strong enhancement of the transcriptional activity of the *ppuI* gene (the *lasI* orthologue), producing dramatically higher levels of acyl-HSL with respect to the wild type (2).

Therefore, RsaL plays a pivotal role in the hierarchy of regulators controlling quorum sensing, both in *P. aeruginosa* PAO1 and *P. putida* WCS358. This finding is also in agreement with a study in which the key role of RsaL in the repression of *P. aeruginosa* QS was inferred by mathematical modeling (7).

Genetic studies have shown that in *E. coli* the *lasI* promoter is not activated by the $3OC_{12}$-HSL/LasR complex when RsaL is also present (4). It was therefore suggested that RsaL could reduce $3OC_{12}$-HSL production by binding to the *lasI* promoter and inhibiting transcription. Since the repressive effect of RsaL was reduced upon the addition of exogenous $3OC_{12}$-HSL, it has also been speculated that RsaL and LasR could be in binding competition to the *lasI* promoter (4). However, another plausible explanation of the above genetic data could be that RsaL impairs binding of LasR to the *lasI* promoter by directly interacting with LasR and not with DNA. Therefore, in order to investigate the DNA binding properties of RsaL, we purified the recombinant protein $RsaL_{PAO}6H$, which differs from the wild type for the presence of a six-histidine tag extension at the C terminus. The functionality of $RsaL_{PAO}6H$ with respect to the wild-type counterpart has been controlled by an in vivo assay system consisting of an *E. coli* strain expressing the *lasR* gene in *trans* and carrying a transcriptional fusion between the *lacZ* reporter gene and the *lasI* promoter. In this strain, the activity of the *lasI* promoter is activated upon the addition of exogenous $3OC_{12}$-HSL and can be measured quantitatively. In this assay system, introduction of plasmids $pRsaL_{PAO}WT$ and $pRsaL_{PAO}6H$ (Table 1) showed that the recombinant $RsaL_{PAO}6H$ protein can repress the activity of the *lasI* promoter to the same extent as the wild-type RsaL protein (data not shown).

$RsaL_{PAO}6H$ was overexpressed in *E. coli* BL21(pLysS, $pRsaL_{PAO}6H$) (Tables 1 and 2) and purified from the soluble

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics and plasmid construction	Reference or source
Strain		
P. aeruginosa PAO1	Wild type	American Type Culture Collection
P. aeruginosa PAO1 *rsaL*	*rsaL*::IS*lacZ/hah;* Tet^r	University of Washington Genome Center
E. coli DH5α	*endA1 hsdR17 supE44 thi-1 recA1 gyrA96* (Nal^r) *relA1* Δ(*lacIZYA-argF*)*U169 deoR*(ϕ80*dlacZ*Δ*M15*)	Bethesda Research Laboratories
E. coli BL21(DE3)pLysS	High-stringency expression host; Chl^r	Novagen
E. coli(pBS1075)	Acyl-HSL sensor strain; Amp^r	30
Plasmid		
pBluescript II KS^+	Cloning vector; Amp^r	Stratagene
pDrive	TA-based cloning vector; Kan^r, Amp^r	QIAGEN
pET28b	Expression vector, Kan^r	Novagen
pBBR1MCS-5	Broad-host-range vector; Gen^r	12
$pRsaL_{PAO}6H$	pET28b derivative in which a 255-bp PCR fragment, originated with primers P133FW and P134RV (Table 2), was cloned into the NcoI-HindIII sites of the vector; allows the overexpression in *E. coli* of a recombinant RsaL protein carrying a six-histidine tag at the C terminus	This study
$pRsaL_{PAO}WT$	pET28b derivative in which a 258-bp PCR fragment originated with primers P133FW and P136RV (Table 2), was cloned into the NcoI-HindIII sites of the vector; allows the overexpression in *E. coli* of the wild-type RsaL protein	This study
$pPSRsaL_{PAO}$	PCR fragment encompassing the wild-type *rsaL* gene was originated with primers P0FW and P0RV and plasmid $pRsaL_{PAO}WT$ as the template (Table 2) and was cloned into the KpnI-BamHI sites of pBBR1MCS-5	This study
p*PlasIS*	pDrive derivative containing a 103-bp PCR fragment, originated with primers P193FW and P194RV (Table 2)	This study
p*PlasI5′*	pBluescript II KS^+ derivative in which a 313-bp PCR fragment, originated with primers P137FW and P138RV (Table 2), was cloned into the EcoRI-BamHI sites of the vector	This study
p*PlasI3′*	pBluescript II KS^+ derivative in which a 313-bp PCR fragment, originated with primers P139FW and P138RV (Table 2), was cloned into the XbaI-BamHI sites of the vector	This study

TABLE 2. Oligonucleotides used in this study

Name[a]	Sequence (5′-3′)[b]	Position[c]	Site[d]
P133FW	5′-CATGCCATGGCTTCACACGAGAGAA-3′	+1	NcoI
P134RV	5′-CCCAAGCTTCTCTCTGATCTTGCCTCTC-3′	+240	HindIII
P136RV	5′-CCCAAGCTTTTACTCTCTGATCTTGCCTC-3′	+243	HindIII
P137FW	5′-CGGAATTCGGTGGCCTTTGCCCGGA-3′	+57	EcoRI
P138RV	5′-CGGGATCCCACTAACGTCCCAGCCTTT-3′	−240	BamHI
P139FW	5′-GCTCTAGAGGTGGCCTTTGCCCGGA-3′	+57	XbaI
P193FW	5′-CTTCGAGCCTAGCAAGGG-3′	−24	
P194RV	5′-CTTCCTCCAAATAGGAAGCT-3′	−126	
P0FW	5′-GGGTACCAATAATTTTGTTTAACTTTA-3′	329	KpnI
P0RV	5′-GGGATCCATTGCTCAGCGGTGGCAGC-3′	71	BamHI

[a] All PCR was performed using *Pfu* polymerase (Stratagene) and *P. aeruginosa* genomic DNA as templates except PCR performed with P0FW and P0RV, for which the DNA template was plasmid pRsaL$_{PAO}$WT (Table 1).
[b] Introduced restriction sites are underlined.
[c] Distance in base pairs from the *rsaL* ATG (for P0FW and P0RV, the position with respect to the pET28b sequence is given).
[d] Restriction recognition sites.

cellular fraction by Ni^{2+} affinity under nondenaturing conditions according to the standard procedure suggested by the column manufacturer (Sigma-Aldrich, St. Louis, Mo.). The fractions containing highly purified RsaL$_{PAO}$6H (>90% pure) were pooled, dialyzed against wash buffer (500 mM NaCl, 50% [vol/vol] glycerol, and 50 mM NaH_2PO_4 [pH 8.0]), and stored at −20°C. Key steps of the purification procedure are shown in Fig. 3.

The DNA binding properties of RsaL$_{PAO}$6H were investigated by performing an electrophoretic mobility shift assay (EMSA), using a DNA probe encompassing the *lasI* promoter. The probe was obtained by 5′-end labeling of an EcoRI fragment derived from plasmid p*PlasIS* (Tables 1 and 2). The procedures for 5′-end labeling as well as for the EMSA have been previously described (13).

As shown in Fig. 4, incubation of the probe with increasing amounts of purified RsaL$_{PAO}$6H leads to the formation of a complex endowed with lower electrophoretic mobility with respect to the probe alone. The minimum molar concentration of RsaL$_{PAO}$6H able to shift almost all the probe is 8 nM, and since the protein preparation is >90% pure, it would contain a fairly low concentration of contaminants (≤0.8 nM), at least twofold lower than that of the DNA probe (2 nM). Therefore, the possibility that the observed complex may be due to coeluted proteins different from RsaL$_{PAO}$6H can be ruled out. Moreover, the addition of an excess of unlabeled probe inhibited the formation of the complex, while the nonspecific competitor had no effect. The above results show that RsaL$_{PAO}$6H is able to specifically bind the *lasI* promoter, demonstrating for the first time that RsaL is a DNA binding factor.

In order to characterize the RsaL binding site, DNase I protection assays were performed on both strands of a DNA fragment encompassing the entire *rsaL-lasI* intergenic region (4, 23, 28) (Fig. 5A). Plasmids p*PlasI3′* and p*PlasI5′* were used to obtain the DNA probes, spanning from the first 19 codons of *rsaL* to the first 36 codons of *lasI* (Tables 1 and 2). The procedures for the DNase I protection assay have been described previously (14).

The DNA region involved in RsaL binding spans at least

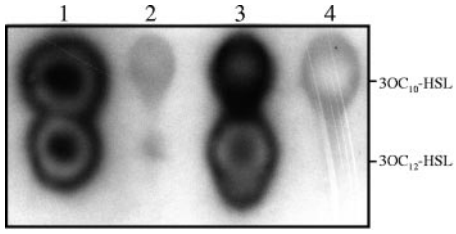

FIG. 2. TLC analysis of acyl-HSL produced by parent strain *P. aeruginosa* PAO1 and its *rsaL*-negative mutant derivative visualized by overlaying with acyl-HSL sensor strain *E. coli*(pSB1075) (30). Lane 1, synthetic 3OC$_{12}$-HSL and 3OC$_{10}$-HSL (0.8 μg); lane 2, PAO1; lane 3, PAO1 *rsaL*; lane 4, PAO1 *rsaL* carrying plasmid pPSRsaL$_{PAO}$. Acyl-HSLs were extracted from spent supernatants, and a volume corresponding to 8×10^8 CFU was loaded for the TLC assay.

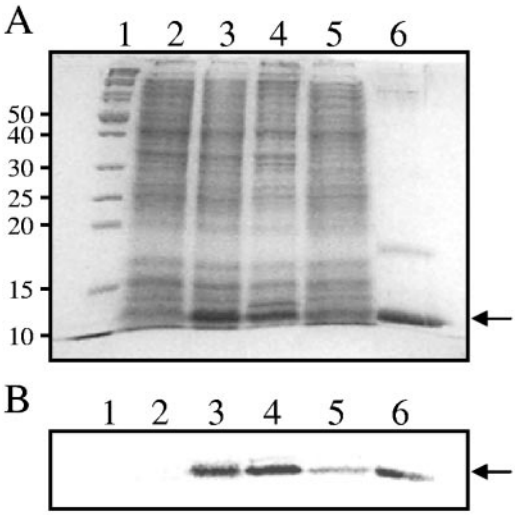

FIG. 3. Results of overexpression and purification of recombinant RsaL$_{PAO}$6H are shown. (A) Analysis of protein samples from key steps of purification by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lane 1, PageRuler protein ladder (Fermentas Inc.) (only molecular mass markers from 50 kDa to 10 kDa are indicated); lane 2, uninduced whole-cell extract; lane 3, induced whole-cell extract; lane 4, insoluble cell extract; lane 5, soluble cell extract; lane 6, pooled fractions eluted at 250 mM imidazole. The arrow indicates the purified RsaL$_{PAO}$6H protein (10.8 kDa). (B) Western blot with anti-six-histidine antibodies of a sodium dodecyl sulfide-polyacrylamide gel identical to that shown in panel A.

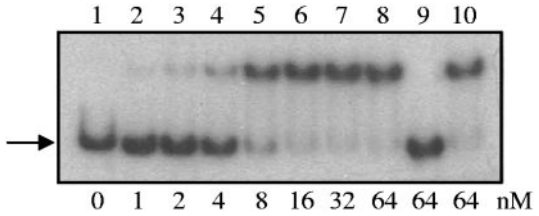

FIG. 4. EMSA of $RsaL_{PAO}6H$ binding to the *lasI* promoter. The ^{32}P-labeled DNA fragment contained nucleotides −83 to +20 with respect to the *lasI* transcriptional starting site. $RsaL_{PAO}6H$ concentrations (nM) are indicated below the lanes. The probe concentration for each sample was 2 nM. As specific and unspecific competitors, unlabeled probe (100 nM) and calf thymus DNA (1 μg) were added to lane 9 and 10, respectively. The arrow indicates the unshifted DNA probe.

from nucleotides −22 to −9 with respect to the *lasI* transcription starting point and is located approximately at the center of a palindromic region consisting of two 5′-AAnTTATGnAA-3′ inverted sequences interrupted by a single nucleotide (Fig. 5B). This dyad symmetry suggests that RsaL could bind DNA as a dimer. Even if the boundaries of the protection cannot be defined precisely due to the high AT content of this DNA region that makes it partly resistant to DNase I attack, the real RsaL-mediated protection could extend up to the extremes of the palindrome, as indicated by the presence of hypersensitive sites at the palindrome boundaries (Fig. 5A and B).

The RsaL binding site partially overlaps the −10 consensus for σ^{70} recognition (Fig. 5B), consistent with the proposed mechanism of action of RsaL as a transcriptional repressor (2, 4). Previous studies have shown that binding of LasR to a unique site located in the *rsaL-lasI* intergenic region activates the transcription of both *rsaL*- and *lasI*-divergent genes (22, 28). RsaL is implicated as well in the negative regulation of both *rsaL* and *lasI* transcription (4). Interestingly, the DNA region protected by LasR (22) is adjacent to the minimum RsaL-protected region and partially overlaps the palindromic sequence involved in RsaL binding (Fig. 5B). The close proximity of the LasR- and RsaL-protected sites supports the hypothesis that LasR and RsaL compete for binding to the *rsaL-lasI* bidirectional promoter. Therefore, it is likely that binding of RsaL to the *rsaL-lasI* promoter simultaneously inhibits *lasI* and *rsaL* transcription by impairing LasR binding. However, the possibility that LasR and RsaL could bind simultaneously to the *lasI* promoter cannot be ruled out, in which case, the RsaL-mediated repression of *lasI* transcription would lead to reduced synthesis of $3OC_{12}$-HSL and consequently to reduced expression of all the genes dependent on the LasR/$3OC_{12}$-HSL complex for their transcription, including the *rsaL* gene itself.

Conclusions. The *P. aeruginosa* QS system is finely regulated and integrated within the global cell regulatory network. Thanks to genetic and microarray studies, knowledge of the number of regulatory factors involved in *P. aeruginosa* QS regulation is increasing. However, the real impact of these regulators in determining the amount of acyl-HSL produced and the molecular mechanisms underlying their interaction with *las* and *rhl* genes in most cases remains unknown (10, 17).

This study shows the dramatic effect of the *rsaL* mutation in $3O\text{-}C_{12}$-HSL production, highlighting the importance of RsaL as a major negative regulator of QS in *P. aeruginosa*. Moreover,

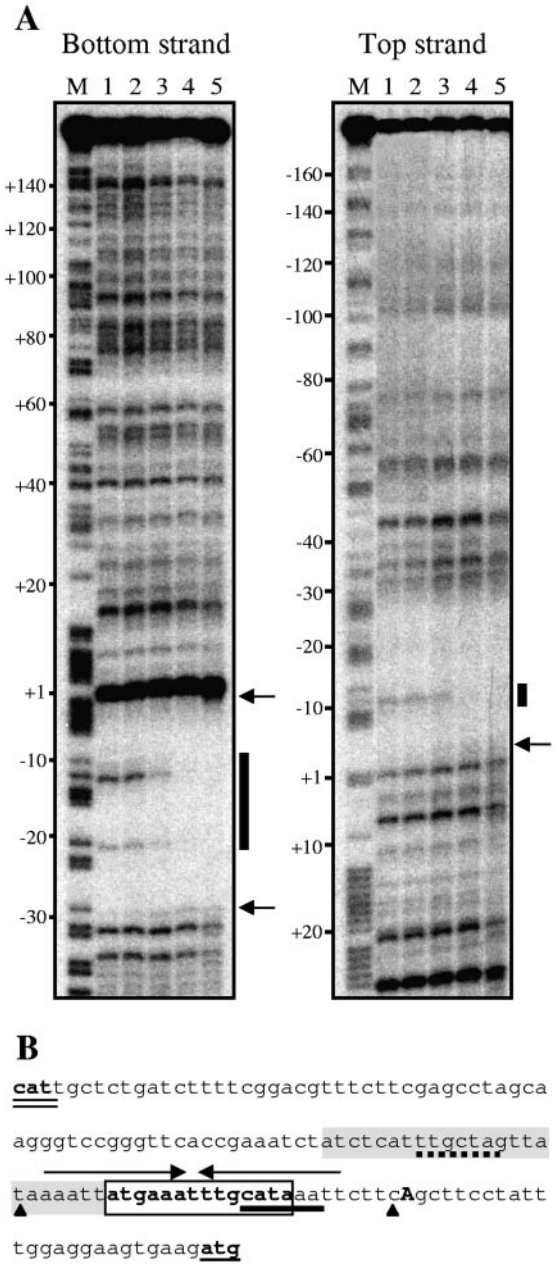

FIG. 5. (A) DNase I footprints of $RsaL_{PAO}6H$ on the *rsaL-lasI* intergenic region. The plasmids p*PlasI*5′ (for labeling of the bottom strand) and p*PlasI*3′ (for labeling of the top strand) were utilized to generate EcoRI/SacI fragments used as probes (Tables 1 and 2). The probes were mixed with different amounts of $RsaL_{PAO}6H$ protein prior to DNase I digestion. Thick lines indicate the regions showing specific protection by $RsaL_{PAO}6H$; arrows indicate hypersensitive sites. All numbering is in reference to the transcriptional starting site from the *lasI* promoter (23). M, Maxam and Gilbert sequencing reactions (A+G); lane 1, no $RsaL_{PAO}6H$ added; lanes 2 to 5, $RsaL_{PAO}6H$ added to a final concentration of 0.05, 0.5, 5, or 50 μM, respectively. (B) Sequence of the *rsaL-lasI* intergenic region. The *lasI* ATG starting codon is boldface and underlined, and the nucleotides complementary to the starting codon of *rsaL* (CAT) are boldface and double underlined. The *lasI* transcriptional starting site is boldface and capitalized (23). The sequence protected by RsaL in the DNase I protection assay is boldface and boxed, and hypersensitive sites are indicated by triangles. The sequence protected by LasR is gray shaded. The 5′-AAnTTATGnAA-3′ inverted repeats are indicated by arrows. The potential σ^{70}-dependent −35 and −10 consensus sequences are indicated by dashed and solid thick lines, respectively.

the demonstration that this protein is a DNA binding factor is of particular interest, considering that BLASTP and PSI-BLASTP (http://www.ncbi.nlm.nih.gov) analysis of the RsaL amino acid sequence did not show any significant homology with functionally characterized proteins, suggesting that RsaL could belong to a new class of transcriptional regulators. Interestingly, up to now, *rsaL* homologues have been reported only in the QS genetic loci of *P. aeruginosa* PAO1, *P. putida* WCS358, and *P. putida* IsoF (2, 4, 25).

To our knowledge, this is the first report of the direct binding of a QS regulator to the *lasI* promoter, with the exception of LasR (22).

The close proximity of the RsaL and LasR binding sites is in agreement with the previous hypothesis that these two proteins are in competition for binding to the *rsaL-lasI* bidirectional promoter (4). In this view, LasR would be able to trigger *lasI* transcription by outcompeting the transcriptional repressor RsaL at high cell densities, when the $3OC_{12}$-HSL levels reach a certain threshold, and/or if the levels of RsaL decrease in response to an unknown environmental/metabolic stimulus. In addition, an unknown signal molecule could bind RsaL and impair its binding to the DNA. The fact that *rsaL* expression itself is also dependent upon LasR adds further complexity to this regulatory mechanism. Thus, the interplay of RsaL and LasR on the *lasI* promoter, as well as the stimulus to which RsaL could respond, deserves further investigation.

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