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RsaL, a Novel Repressor of Virulence Gene Expression in *Pseudomonas aeruginosa*

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As components of a *Pseudomonas aeruginosa* quorum-sensing system, LasR and PAI-1 globally regulate expression of multiple virulence determinants, as well as the second *P. aeruginosa* quorum-sensing system. To date, no information exists on negative regulation of the quorum-sensing cascade in *P. aeruginosa*. Here we describe a novel gene, *rsaL*, which is located downstream from *lasR* and transcribed antisense relative to *lasR*. In *P. aeruginosa*, overexpression of *rsaL* results in reduced *lasB* expression and decreased elastase activity. With the use of a six-His protein fusion system, we demonstrate that *rsaL* encodes an 11-kDa protein. Direct quantitation of PAI-1 levels in cultures and studies utilizing *Escherichia coli* lambda lysogens carrying *lacZ* transcriptional fusions reveal that RsaL specifically represses transcription of the PAI-1 autoinducer synthase gene, *lasI*. RsaL's repressive effect on *lasI* and the associated decrease in elastase activity have important implications for the expression of all LasR–PAI-1-dependent virulence genes and the overall pathogenicity of *P. aeruginosa*.

The expression of many virulence factors produced by the gram-negative opportunistic pathogen *Pseudomonas aeruginosa* is regulated by a cell density-dependent mechanism known as quorum sensing (9, 10, 38). Quorum sensing utilizes a transcriptional activator protein (R protein) which acts in concert with a small, signaling molecule, known as the autoinducer (AI), to stimulate the expression of target genes. At low cell density, AI is produced at a basal level. As the cell population increases, so does the concentration of AI, providing a chemical signal with which to monitor cell density. Once a threshold level of AI is reached, it binds to and activates the R protein, resulting in expression of quorum-sensing-controlled genes. A number of AI molecules from various organisms have been identified and characterized (see reference 8 for a review). Most of these molecules are N-acylated homoserine lactones and differ only in the length and substitution of their acyl side chain. The phenomenon of quorum sensing appears to be nearly ubiquitous among gram-negative bacteria (8), and a regulatory system employing a γ-butyrolactone signaling molecule has been identified in gram-positive *Streptomyces* spp. (14).

P. aeruginosa contains two known quorum-sensing systems, each with its own R protein-AI pair. The better understood of these is the *las* system, which consists of the transcriptional activator protein, LasR, and an AI called PAI-1. The synthesis of PAI-1 (*N*-3-oxo-dodecanoyl homoserine lactone) is directed by the autoinducer synthase LasI (23, 25). LasR and PAI-1 work in concert to increase the expression of a number of virulence genes, including those for several proteases (*lasA, lasB*, and *aprA*) and exotoxin A (*toxA*) (9, 10, 23, 33, 38). LasR–PAI-1 is also required for expression of the AI synthase gene, *lasI*, creating a positive feedback loop (35). Studies done with *Escherichia coli* demonstrated that 10-fold-less PAI-1 was required to activate *lasI*, as compared to *lasB* (35). Therefore, it appears that a hierarchy of gene regulation exists, with particular genes activated or turned on earlier than others. In this hierarchy, low levels of PAI-1 apparently bind to LasR, which then induces expression of *lasI* and increases PAI-1 production. After sufficient PAI-1 has accumulated, secondary activation of virulence genes such as *lasB* occurs. Maintaining tight control of *lasI* expression is therefore a key feature of the *P. aeruginosa* quorum-sensing process.

The second *P. aeruginosa* quorum-sensing system consists of the regulatory protein RhlR and PAI-2 (*N*-butyryl homoserine lactone) and has been shown to regulate the biosynthesis of rhamnolipid, RpoS, and, to some extent, the *lasB*-encoded elastase (4, 16, 21, 22, 26, 27, 39). Interestingly, LasR and PAI-1 have been found to regulate the expression of RhlR, making LasR and PAI-1 the dominant regulators in *P. aeruginosa* (16, 28).

It is apparent that quorum sensing plays a key role in the regulation of virulence gene expression in *P. aeruginosa*, and recently quorum sensing has been shown to be involved in the differentiation of *P. aeruginosa* biofilms (6). Despite the fact that this cellular signaling mechanism plays a critical role in *P. aeruginosa* virulence, as well as facilitating its survival in hostile environments, very little is known about how the regulators of these systems are themselves regulated. All experiments reported to date have focused on positive gene regulation, and specific repressor molecules have not been reported for the *P. aeruginosa* quorum-sensing systems. Here we report the identification of a novel gene, *rsaL*, which encodes a negative regulatory protein. We show that RsaL completely represses *lasI* transcription, thereby blocking activation of the *P. aeruginosa* quorum-sensing cascade.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains are listed in Table 1. Plasmids are shown in both Table 1 and Fig. 1B. Oligonucleotide primers were synthesized by the University of Rochester Medical Center Nucleic Acid Core Facility. In all cases in which PCR was used to generate plasmid constructs, DNA sequencing

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant phenotype of characteristic(s)	Source or reference
Strains		
P. aeruginosa		
PAO1	Prototrophic	13
PAO-JP1	Δ*lasI*::*tet*, LasI null mutant	27
PAO-R1	Sm^r Δ*lasR*::*tet*, LasR null mutant	9
PDO111	*rhlR*::Tn*501* derivative of PAO1, Hg^r, RhlR null mutant	4
E. coli		
MG4 $\lambda I_1 4$	Δ(*argF-lac*)*U169 zah-735*::Tn*10 recA56 srl*::Tn*10 lasI*::*lacZ* lysogen	35
MG4 λB_2	Δ(*argF-lac*)*U169 zah-735*::Tn*10 recA56 srl*::Tn*10 lasB*::*lacZ* lysogen	35
Plasmids		
pEX1.8	Amp^r, stabilizing fragment (1.8-kb *Pst*I fragment)	27
pEXR	Amp^r $lasR_{pwt}$	This study
pEXRL	Amp^r $rsaL_{ptac}$	This study
pEXRR	Amp^r $rsaL_{ptac}$ (800-bp *Sal*I-*Hph*I fragment) $lasR_{pwt}$	This study
pEXRR2	Amp^r $rsaL_{ptac}$ (307-bp *Sfi*I-*Hph*I fragment) $lasR_{pwt}$	This study
pEXRR/NS	Amp^r $rsaL_{ptac}$ (start methionine ATG to CTG) $lasR_{pwt}$	This study
pPCS2001	Amp^r *lasI-lacZ*	This study
pPCS2002	Amp^r *rsaL-lacZ*	This study
pKDT17	Amp^r *lasB-lacZ* $lasR_{plac}$	25
pPCS15	Amp^r *lasB-lacZ* $lasR_{plac}$ $rsaL_{pwt}$	This study
pTS4001.7	Amp^r *lasB-lacZ* $lasR_{pwt}$ $rsaL_{pwt}$	23
pPCS16	Amp^r *lasB-lacZ* $rsaL_{plac}$ $lasR_{pwt}$	This study
pPCS15/*rsaL-lacZ*	Amp^r $lasR_{plac}$ $rsaL_{pwt}$-*lacZ*	This study
pTS4001.7/*rsaL-lacZ*	Amp^r $lasR_{pwt}$ $rsaL_{pwt}$-*lacZ*	This study
pPCS16/*rsaL-lacZ*	Amp^r $lasR_{pwt}$ $rsaL_{plac}$-*lacZ*	This study
pPCS16/ES	Amp^r *lasB-lacZ* $rsaL_{plac}$ (codon 3 TCA to TAA) $lasR_{pwt}$	This study
pPCS16/NS	Amp^r *lasB-lacZ* $rsaL_{plac}$ (start methionine ATG to CTG) $lasR_{pwt}$	This study
pTS400	Amp^r, *lasB-lacZ* translational fusion on pSW205	23
pSW205	Amp^r *lacZ′*, stabilizing fragment (1.8-kb *Pst*I fragment)	10
pSWRL	Amp^r *rsaL-lacZ* (out-of-frame translational fusion)	This study
pSWRL2	Amp^r *rsaL-lacZ* (in-frame translational fusion)	This study
pTrcHis	Amp^r, A, B and C vectors for cloning in all three frames	Invitrogen

was performed to verify the sequences of the PCR products. Transformants were selected on agar medium containing the appropriate antibiotic(s).

Nucleic acid manipulation. Purification, cloning, electrophoresis, and other manipulations of nucleic acid fragments and constructs were performed using standard techniques (12, 34).

PCR. PCR was performed under standard conditions as presented by Gibco Life Technologies (Gaithersburg, Md.) on data sheets supplied with their *Taq* polymerase. $MgCl_2$ was used at a concentration of 3 mM. In a 100-μl reaction mixture, 100 pmol of each primer was used. DNA fragments were isolated from agarose gels by using the GeneClean system (Bio101, La Jolla, Calif.).

β-Galactosidase assays. *E. coli* strains were grown at 37°C in supplemented A medium (28) with ampicillin (100 μg/ml). In addition, synthetic PAI-1 (final concentration, 100 nM) was added to the flasks before addition of culture medium. *P. aeruginosa* strains were grown at 37°C in peptone Trypticase soy broth (PTSB) medium supplemented with carbenicillin (200 μg/ml), and where stated, synthetic PAI-1 (generated as previously described [24]) was added to the flasks to a final concentration of 1 μM before addition of culture medium. For both *E. coli* and *P. aeruginosa*, overnight cultures were diluted 1 to 100 and allowed to grow until they had reached an optical density of 0.5 to 0.8. Optical density was measured at 540 nm for *P. aeruginosa* and 600 nm for *E. coli*. β-Galactosidase activity was assayed in triplicate as described by Miller (19).

Protein expression. To purify RsaL protein, *rsaL* was ligated into a commercially available expression vector that generates a protein bearing six tandem histidine residues at its amino terminus. *rsaL* was ligated into pTrcHisA, -B, and -C (Promega Corp., Madison, Wis.) to ensure that all three translational reading frames were examined. DNA sequencing verified that *rsaL* is in the correct frame for translation in pTrcHisB/*rsaL*. Overnight cultures of *E. coli* JM109 harboring these plasmids were subcultured 1 to 20 in Luria-Bertani medium supplemented with ampicillin (100 μg/ml) and allowed to grow for 30 min before addition of 2 mM IPTG (5-bromo-4-indol-3-chloro-isopropyl β-D-galactopyranoside). Following 4 h of growth, 1-ml aliquots of the cultures were sedimented (12,000 × *g*) and the cell pellets were lysed in buffer B (8 M urea, 0.1 M Na-phosphate, 0.01 M Tris-HCl [pH 8.0]). A 50-μl aliquot of Ni-nitrilotriacetic acid (NTA) resin (Qiagen, Valencia, Calif.) was added to the cell lysates, which were incubated for 30 min at room temperature with frequent inversion. The resin was washed three times with buffer C (8 M urea, 0.1 M Na-phosphate, 0.01 M Tris-HCL [pH 6.3]) and then resuspended in 20 ml of buffer C containing 100 mM EDTA to elute any proteins which had bound to the resin.

Lysates from *E. coli* harboring pTrcHisA/*rsaL*, pTrcHisB/*rsaL*, and pTrcHisC/*rsaL* were analyzed on 15% acrylamide gels according to established methods (34). Twenty-microliter aliquots of lysates taken both prior to and after incubation with Ni-NTA resin were boiled with sample buffer containing 2-mercaptoethanol and loaded alongside commercial molecular weight markers (Bio-Rad, Hercules, Calif.). Gels were stained for 3 h in a solution of 30% methanol, 10% acetic acid, and 0.05% Coomassie brilliant blue and then destained in 30% methanol–10% acetic acid.

Elastolytic activity determination. Cultures were streaked onto elastin agar containing carbenicillin (200 μg/ml) and incubated at 30°C for 48 h as previously described (32). The zones of clearing (elastin solubilization) were measured, and the average of four independent test streaks was reported for each bacterial strain.

Extraction of PAI-1 from *P. aeruginosa* culture supernatants. Cultures were grown to late log phase (optical density at 540 nm of 0.8) in PTSB medium. Extraction of PAI-1 was done as described previously (25). As a control, synthetic PAI-1 was added to PTSB medium at a final concentration of 1 μM. This sample was treated identically to the supernatants. A 1-ml aliquot of an *E. coli* MG4(pKDT17) (23, 25) overnight culture diluted 1:100 in modified A medium containing 100 μg of ampicillin per ml was added to each tube, which contained either experimental extract or control PAI-1. The strains were grown for 5 h at 32°C and assayed for β-galactosidase. Comparison of β-galactosidase values obtained against those of a standard curve plotted with the synthetic PAI-1 allowed estimation of the PAI-1 content of each sample.

Western blot analysis of LasR. Cell pellets from cultures to be tested were obtained by centrifugation (6,000 × *g*, 5 min) of 1 to 2 ml of the culture. Each pellet was solubilized in lysis buffer (6 M urea, 1% sodium dodecyl sulfate [SDS], 10% glycerol, and 1% β-mercaptoethanol in 50 mM Tris-HCl [pH 7.5]) by incubation for 30 min or until samples were clear. The protein content of each sample was determined using the commercially available bicinchoninic acid method (Pierce, Rockford, Ill.). Equal amounts of protein (usually 25 μg) were prepared in a total volume of 25 μl. The samples were separated on an SDS–10 to 12% polyacrylamide gel and transferred to a nitrocellulose membrane according to standard techniques (34).

Chemiluminescent detection of the protein of interest was carried out using a commercially prepared (CoCalico Biologicals, Reamstown, Pa.) anti-LasR antibody and the protocol supplied with the ECL chemiluminescence Western blotting kit (Amersham Life Science Inc., Arlington Heights, Ill.).

Computer analysis of nucleic acids and proteins. Computer analysis of both nucleic acids and proteins was carried out using the Genetics Computer Group (GCG) Wisconsin Package software version 8 (Genetics Computer Group, Madison, Wis.).

RESULTS

A novel gene is contained in the intergenic region between *lasR* and *lasI*. In *P. aeruginosa*, *lasR* and *lasI* are separated by a 367-bp intergenic region and are transcribed in the same direction from independent promoters (Fig. 1A and C) (9, 23, 35). Preliminary studies indicated that regulatory elements controlling *lasI* expression exist within the *lasR-lasI* intergenic region, outside the *lasI* promoter. While studying this region, we discovered that a novel gene was located on the strand opposite to *lasR*. Two plasmids were constructed which contained a 658-bp *Eco*RI fragment (Fig. 1A) encompassing the *lasRI* intergenic region fused in both the forward (pPCS2001) and reverse (pPCS2002) orientations relative to *lacZ*. Both the *plasI-lacZ* fusion (pPCS2001) and the reverse *lacZ* fusion (pPCS2002) were expressed in the wild-type *P. aeruginosa* strain PAO1 (data not shown), suggesting the presence of a novel gene, which we have termed *rsaL*.

***rsaL* encodes a unique protein.** To determine whether the *rsaL* open reading frame (ORF) encodes a protein, an in-frame translational fusion to *lacZ* (pSWRL2) was constructed and introduced into *P. aeruginosa* PAO1. Significant β-galactosidase expression was observed in strain PAO1 containing the translational fusion (9,246 ± 322 Miller units). As a control, an out-of-frame *rsaL-lacZ* translational fusion (pSWRL) was also examined. The strain carrying the out-of-frame fusion expressed only slightly more β-galactosidase (41 ± 3 Miller units) than strain PAO1 carrying the vector control (3 ± 1 Miller units). These findings suggested that a protein is produced from the *rsaL* transcript.

To visualize the RsaL protein, *rsaL* was cloned into pTrcHisA, -B, and -C. These vectors generate proteins with six tandem histidine residues at their amino terminus. Cell lysates of *E. coli* JM109 harboring these plasmids were incubated with Ni-NTA resin to allow binding of proteins containing the six-His tag. SDS-polyacrylamide gel electrophoresis (PAGE) analysis revealed that a major protein of approximately 17 kDa was present in the *E. coli*(pTrcHisB/*rsaL*)-eluted sample (Fig. 2, lane 3). RsaL is predicted to have a molecular mass of 10.6 kDa. The discrepancy in size can be accounted for by the presence of the six-His tag and an additional 30 linking amino acids contributed by the pTrcHisB vector. When RsaL was expressed under the control of the *tac* promoter and did not contain the six-His linker, a protein of approximately 11 kDa was observed (data not shown). DNA sequencing verified that in pTrcHisB/*rsaL*, the *rsaL* ORF is in the correct frame for translation. No observable protein of the correct size was produced from the out-of-frame construct (Fig. 2, lane 4).

The *rsaL* translated sequence was compared (TFASTA program) against known sequences by using the GCG computer software. The deduced sequence of the RsaL protein bears no significant similarity to any protein contained in the sequence data bank, including ORFs encoded on the antisense strand of known transcriptional activator and AI synthase genes (*luxR, luxI*, etc.; see reference 8 for a review).

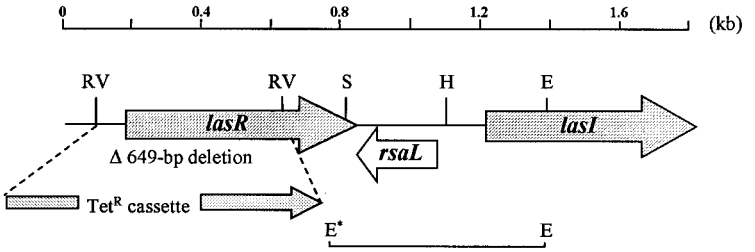
Expression of *rsaL* requires LasR and not RhlR for expression. To assess which, if any, of the quorum-sensing systems regulate *rsaL* expression, cultures of *P. aeruginosa* wild-type strain PAO1 or strains that contain either a LasR (PAO-R1 [9]), RhlR (PDO111 [4]), or LasI (PAO-JP1 [26]) null phenotype and that carry an *rsaL-lacZ* translational fusion (pSWRL2) were assayed for expression of the fusion. Those strains that were LasR or LasI null mutants demonstrated a lack of *rsaL* expression (Fig. 3). Conversely, an RhlR null mutant exhibited wild-type levels of *rsaL* expression. Addition of exogenous PAI-1 to a LasI null mutant carrying the *rsaL-lacZ* fusion resulted in the restoration of *rsaL* expression to approximately wild-type levels. Taken together, the data indicate that *rsaL* expression requires both LasR and PAI-1. Furthermore, RhlR does not significantly affect *rsaL* expression.

Effects associated with *rsaL* overexpression. To assess the function of *rsaL*, multiple plasmids containing *lasR* alone or in combination with *rsaL* were examined in *P. aeruginosa* PAO-R1. Expression of the *lasR* and *rsaL* genes on these constructs is driven by either their native promoters or the *lac* promoter. In addition, the plasmids carry a *lasB-lacZ* translational fusion that functions as a reporter system for LasR–PAI-1-dependent activation. Strains PAO-R1(pLasR) and PAO-R1(pKDT17), which contain *lasR* under control of the wild-type promoter and the *lac* promoter, respectively, but no *rsaL*, showed high levels of *lasB* expression (Fig. 4). The increased *lasB* expression in pLasR as compared to that in pKDT17 is most likely because *lasR* expression is positively regulated in *P. aeruginosa*, albeit modestly, by LasR–PAI-1 (28). Plasmid pPCS15 is a derivative of pKDT17 that contains both $lasR_{plac}$ and *rsaL* under control of its own promoter. In strain PAO-R1(pPCS15 $lasR_{plac}$ $rsaL_{pwt}$ *lasB-lacZ*), *lasB* expression was markedly lower than in strain PAO-R1(pKDT17 $lasR_{plac}$ *lasB-lacZ*), suggesting that the presence of *rsaL* decreased *lasR* expression or activity (Fig. 4). The decrease in *lasB* expression is even more pronounced when *lasR* is expressed from its native promoter in the presence of *rsaL*, as seen in strains PAO-R1(pTS4001.7 $lasR_{pwt}$ $rsaL_{pwt}$ *lasB-lacZ*) and PAO-R1(pPCS16 $lasR_{pwt}$ $rsaL_{plac}$ *lasB-lacZ*), particularly when *rsaL* is constitutively expressed from the *lac* promoter [strain PAO-R1(pPCS16 $lasR_{pwt}$ $rsaL_{plac}$ *lasB-lacZ*)] (Fig. 4). These findings indicated that in *P. aeruginosa*, *rsaL* caused a strong repressive effect on *lasB* expression.

To confirm that the relative decrease in *lasB* expression in PAO-R1 containing plasmid pPCS15, pTS4001.7, or pPCS16 is due to an increased level of *rsaL* expression, we constructed plasmids pPCS15/*rsaL-lacZ*, pTS4001.7/*rsaL-lacZ*, and pPCS16/*rsaL-lacZ* (Fig. 1B). These plasmids have an *rsaL-lacZ* translational fusion in place of the *rsaL* gene, which enabled us to measure the level of *rsaL* translation from these fusions and, thus, to indirectly gauge RsaL production. Although these new plasmids no longer have *lasB-lacZ* fusions, the promoters controlling expression of *lasR* and *rsaL-lacZ* are the same as those found on pPCS15 ($lasR_{plac}$ $rsaL_{pwt}$ *lasB-lacZ*), pTS4001.7 ($lasR_{pwt}$ $rsaL_{pwt}$ *lasB-lacZ*), and pPCS16 ($lasR_{pwt}$ $rsaL_{plac}$ *lasB-lacZ*). As seen in Fig. 4, increasing levels of *rsaL* expression are observed in plasmids pPCS15/*rsaL-lacZ*, pTS4001.7/*rsaL-lacZ*, and pPCS16/*rsaL-lac*, respectively. From this data it appears that a direct correlation exists between the amount of RsaL protein produced and the level of *lasB-lacZ* activity observed in strains PAO-R1(pPCS15 $lasR_{plac}$ $rsaL_{pwt}$ *lasB-lacZ*), PAO-R1 (pTS4001.7 $lasR_{pwt}$ $rsaL_{pwt}$ *lasB-lacZ*), and PAO-R1(pPCS16 $lasR_{pwt}$ $rsaL_{plac}$ *lasB-lacZ*).

It is logical to postulate that any mutation that abolishes the production of RsaL should result in a loss of *lasB-lacZ* repression. In order to examine this possibility, mutations which removed the start codon (ATG to CTG) or which created a premature translational stop codon (TCA to TAA at codon 3) were introduced into the RsaL coding region by using the PCR and mutagenic primers. These mutated versions of *rsaL* were used to replace the wild-type *rsaL* on pPCS16 ($lasR_{pwt}$ $rsaL_{plac}$ *lasB-lacZ*). As expected, strain PAO-R1 carrying the mutated *rsaL* (on both pPCS16/NS and pPCS16/ES) and an intact *lasR* demonstrated high levels of *lasB* expression (Fig. 4), indicating

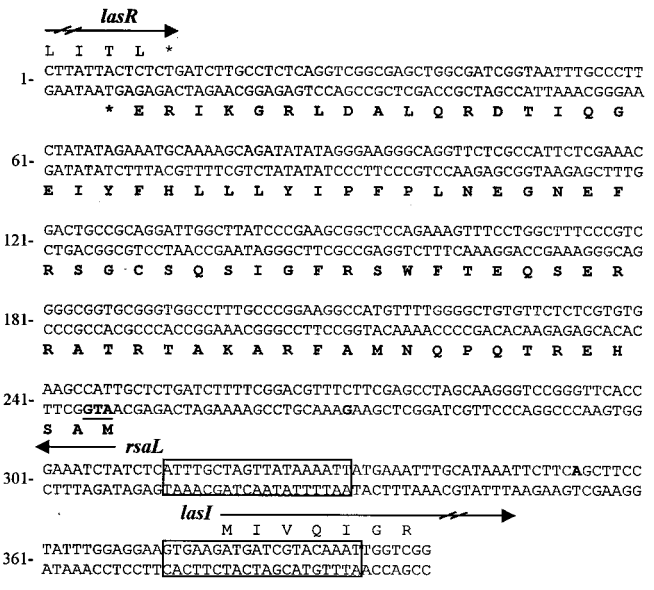
A



B

Plasmid		Genes Expressed
pLasR		*lasR*$_{pwt}$; *lasB-lacZ*
pKDT17		*lasR*$_{plac}$; *lasB-lacZ*
pPCS15[a]		*lasR*$_{plac}$; *rsaL*$_{pwt}$; *lasB-lacZ*
pTS4001.7[a]		*lasR*$_{pwt}$; *rsaL*$_{pwt}$; *lasB-lacZ*
pPCS16[a]		*lasR*$_{pwt}$; *rsaL*$_{plac}$; *lasB-lacZ*
pPCS16/NS[a]		*lasR*$_{pwt}$; *rsaL*$_{plac}$-no start; *lasB-lacZ*
pPCS16/ES[a]		*lasR*$_{pwt}$; *rsaL*$_{plac}$-early stop; *lasB-lacZ*
pPCS15/*rsaL-lacZ*[b]		*lasR* $_{plac}$; *rsaL-lacZ*$_{pwt}$
pTS4001.7/*rsaL-lacZ*[b]		*lasR*$_{pwt}$; *rsaL-lacZ*$_{pwt}$
pPCS16/*rsaL-lacZ*[b]		*lasR*$_{pwt}$; *rsaL-lacZ*$_{plac}$
pTS400	vector	*lasB-lacZ*
pEXR		*lasR*$_{pwt}$
pEXRL		*rsaL*$_{ptac}$
pEXRR[b]		*lasR*$_{pwt}$*rsaL*$_{ptac}$
pEXRR2[b]		*lasR*$_{pwt}$*rsaL*$_{ptac}$
pEXRR/NS[b]		*lasR*$_{pwt}$*rsaL*$_{ptac}$-no start codon
pEX1.8	vector	

C



a loss of repression. These findings suggested that the protein identified as RsaL was responsible for the repression of *lasB*.

The repressive effect of RsaL on *lasB* expression was also evident when observing the elastolytic activity of the strains carrying the plasmids mentioned above. Strain PAO-R1 containing pLasR ($lasR_{pwt}$ *lasB-lacZ*), pKDT17 ($lasR_{plac}$ *lasB-lacZ*), or pPCS15 ($lasR_{plac}$ $rsaL_{pwt}$ *lasB-lacZ*) exhibited marked zones of clearing (elastin solubilization) measuring 2.5, 2.13, and 1.58 mm, respectively, after 48 h at 30°C on elastin agar. These zones of clearing are produced primarily by the activity of LasB elastase (33). Strain PAO-R1(pTS4001.7 $lasR_{pwt}$ $rsaL_{pwt}$ *lasB-lacZ*) produced a smaller zone of clearing (0.88 mm), while strains PAO-R1(pPCS16 $lasR_{pwt}$ $rsaL_{plac}$ *lasB-lacZ*) and PAO-R1(pTS400 *lasB-lacZ*) produced no observable zones of elastin solubilization (0.0 mm), remaining elastolytically negative even after 48 h at 30°C. These findings correlated well with the expression of *lasB-lacZ* in each strain, confirming that *rsaL* expression had a significant negative effect on the ability of LasR to activate *lasB* expression.

RsaL directly affects *lasI* expression. The finding that *lasB*, which is dependent on LasR for expression, is downregulated by RsaL led to the question of how RsaL is able to mediate this regulation. At least two possibilities exist. First, RsaL may bind to sequence upstream of, or within, *lasB*, thereby directly blocking transcription or translation. Second, RsaL may affect a common mediator required for *lasB* activation, the two most obvious candidates being LasR and PAI-1. To address each of these possibilities, we made use of *E. coli* single-copy λ lysogens carrying either a *lasI-lacZ* (*E. coli* MG4 $\lambda I_1 4$) or *lasB-lacZ* (*E. coli* MG4 λB_2) transcriptional fusion on the prophage (35). Plasmids containing either *lasR* under its own promoter (pEXR), *lasR* under its own promoter and *rsaL* under the *tac* promoter (pEXRR), *rsaL* under the *tac* promoter (pEXRL), or a vector control (pEX1.8) were mobilized into the two *E. coli* λ lysogens. Plasmid pEXRR/NS, which contains *rsaL* with its start codon removed and a functional copy of *lasR*, was also examined. In all cases, the *E. coli* cultures were grown in the presence of 100 nM exogenous PAI-1, which was required for LasR activation. In the *E. coli* MG4 λB_2 cells expressing both *rsaL* and *lasR* (pEXRR $lasR_{pwt}$ $rsaL_{ptac}$), β-galactosidase activity was similar to that in cells expressing only *lasR* (pEXR $lasR_{pwt}$), indicating that *rsaL* does not directly affect *lasB* expression (Fig. 5). However, *rsaL* did have a significant effect on *lasI* expression. In the *E. coli* MG4 $\lambda I_1 4$ cells containing both *rsaL* and *lasR* (pEXRR $lasR_{pwt}$ $rsaL_{ptac}$), very low β-galactosidase levels were observed compared to those in cells containing *lasR* alone (pEXR $lasR_{pwt}$) (Fig. 5). These findings indicate that RsaL is able to act directly on *lasI* to downregulate its expression. In the *lasI* λ lysogen containing pEXRR/NS ($lasR_{pwt}$ $rsaL_{ptac}$ [no start codon]), *lasI* expression was significantly increased compared to that in the same lysogen containing pEXRR ($lasR_{pwt}$ $rsaL_{ptac}$). However, the repressive activity associated with RsaL does not appear to be totally abolished by removal of the RsaL start methionine. Inspection of the nucleotide sequence of the pEXRR/NS ($lasR_{pwt}$ $rsaL_{plac}$ [no start codon]) construct revealed the presence of an in-frame GTG codon 19 amino acids upstream of the native RsaL start methionine. Data from previous studies have shown that a GTG codon can be used as a translational start codon approximately 8% of the time (11). Therefore, the partial repression seen from pEXRR/NS ($lasR_{pwt}$ $rsaL_{ptac}$ [no start codon]) may be due to the synthesis of a modified RsaL protein using this GTG start codon. To verify that the 81-amino-acid ORF believed to encode RsaL is sufficient to produce a functional protein, plasmid pEXRR2 was created. Plasmid pEXRR2 is identical to pEXRR ($lasR_{pwt}$ $rsaL_{ptac}$) except that it contains the *rsaL* gene on a 307-bp DNA fragment (Fig. 1A) instead of an 800-bp fragment (pEXRR). Similar to pEXRR ($lasR_{pwt}$ $rsaL_{ptac}$), pEXRR2 inhibited *lasI* expression in the *lasI* λ lysogen strain (data not shown), confirming that this smaller fragment encodes a functional RsaL protein.

Since *lasI* directs the synthesis of PAI-1, culture supernatants of parent strain PAO1 and strain PAO-R1 carrying pKDT17, pPCS15, pTS4001.7, pPCS16, or pTS400 were assayed for the presence of PAI-1. These assays were performed

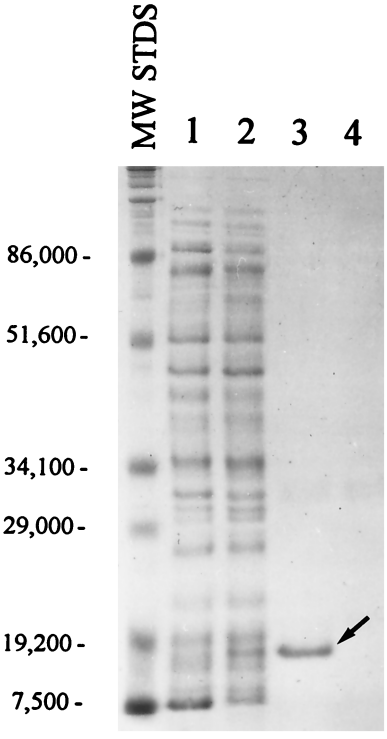


FIG. 2. Expression of RsaL as a fusion protein. Lysates from *E. coli* JM109 carrying either the control vector (pTrcHisB) (lane 1) or the vector with an in-frame fusion of *rsaL* (pTrcHisB/*rsaL*) (lane 2) were separated by SDS-PAGE. Ni-NTA resin was used to purify the fusion protein from *E. coli* JM109 (pTrcHisB/*rsaL*) (lane 3) and from *E. coli* JM109 carrying *rsaL* cloned onto pTrcHisA (lane 4) to serve as an out-of-frame control. Molecular mass standards are shown in daltons. The arrow indicates the protein observed in lane 3, which is believed to be RsaL.

FIG. 1. Plasmid inserts and DNA sequence of the *rsaL* gene. (A) The region including and surrounding the *lasR, rsaL*, and *lasI* genes is depicted. Restriction sites include *Eco*RV (RV), *Eco*RI (E; E* indicates that this restriction site was located in the multiple cloning site of vector pLP170 [29] and not in *P. aeruginosa* DNA), *Sfi*I (S), and *Hph*I (H). The 658-bp *Eco*RI DNA insert found on pPCS2001 and pPCS2002 is indicated. The 649-bp *Eco*RV fragment that was deleted and replaced with a tetracycline resistance (Tet^r) cassette during the generation of strain PAO-R1 (Δ*lasR*) (9) is also shown. The 307-bp *Sfi*I-*Hph*I fragment encodes a functional RsaL protein; thus, the Tet^r cassette insertion in strain PAO-R1 does not interrupt the *rsaL* gene. (B) DNA inserts of the plasmids used in this study are indicated. [a], plasmids containing both *lasR* and *rsaL* in their native conformation on a single DNA fragment; [b], plasmids that contain the *lasR* and *rsaL* genes on separate DNA fragments. (C) *lasR-lasI* intergenic region. The deduced amino acid sequence of RsaL is indicated in bold. The *rsaL* translational start codon (ATG) is underlined, and potential *lux*-box-like elements are enclosed by boxes.

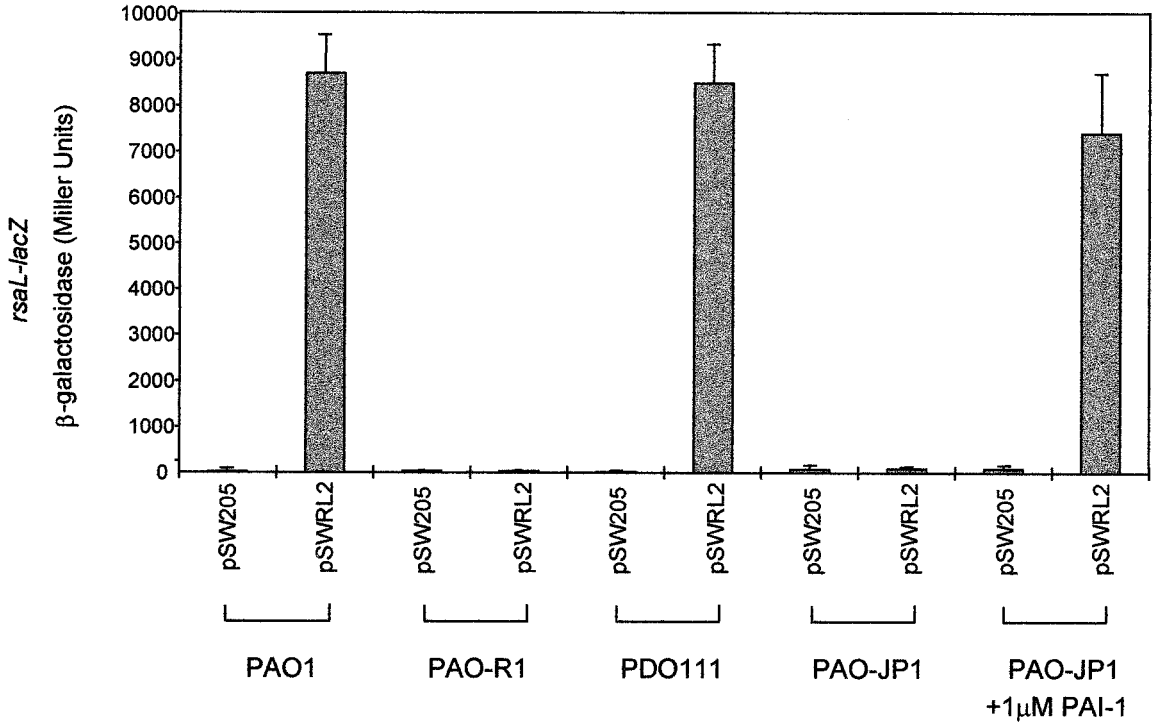


FIG. 3. Effect of the *P. aeruginosa* quorum-sensing regulators on expression of *rsaL*. Strains carrying a plasmid-borne *rsaL-lacZ* translational fusion (pSWRL2) or the control plasmid (pSW205) were monitored for expression of β-galactosidase. Expression of the fusion was analyzed in the wild-type strain PAO1, the LasR null mutant PAO-R1, the RhlR null mutant PDO111, and the LasI null mutant PAO-JP1. PAO-JP1 grown in the presence of 1 μM exogenous PAI-1 was also examined.

on supernatants from late-logarithmic-phase cultures (optical density at 540 nm of 0.8), which is coincident with RsaL's repressive effect. As expected, the supernatant from the wild-type strain, PAO1, contained high levels of PAI-1 (1.2 μM). Strain PAO-R1(pKDT17 *lasR*$_{plac}$ *lasB-lacZ*) produced substantial PAI-1 (370 nM), while each of the other strains yielded much lower concentrations. Supernatants from strains PAO-R1(pPCS15 *lasR*$_{plac}$ *rsaL*$_{pwt}$ *lasB-lacZ*) and PAO-R1 (pTS4001.7 *lasR*$_{pwt}$ *rsaL*$_{pwt}$ *lasB-lacZ*) were estimated to contain 50 and 45 nM PAI-1, respectively. Strains PAO-R1 (pPCS16 *lasR*$_{pwt}$ *rsaL*$_{plac}$ *lasB-lacZ*) and PAO-R1(pTS400 *lasB-lacZ*) each produced less than 10 nM PAI-1. The finding that the PAO1 culture supernatant contained a much higher PAI-1 concentration than that of strain PAO-R1(pKDT17 *lasR*$_{plac}$ *lasB-lacZ*) is interesting and may result because this plasmid contains the promoters of two genes that are potentially controlled by LasR–PAI-1 (*lasR* and *lasB*). Therefore, PAI-1 is possibly being titrated out in strain PAO-R1(pKDT17) by the multiple copies of these promoters. Nonetheless, the decreased levels of PAI-1 observed in the cells expressing RsaL support the conclusion that RsaL mediates its repressive effect by targeting *lasI* expression.

To examine whether exogenously supplied PAI-1 could circumvent RsaL's repressive effect, cultures of strain PAO-R1 containing pLasR, pKDT17, pPCS15, pTS4001.7, pPCS16, or pTS400 were monitored for *lasB* expression after growth in either the presence or absence of 1 μM PAI-1 (Fig. 6). A marked increase in *lasB* expression was observed in strains PAO-R1(pPCS15 *lasR*$_{plac}$ *rsaL*$_{pwt}$ *lasB-lacZ*), PAO-R1 (pTS4001.7 *lasR*$_{pwt}$ *rsaL*$_{pwt}$ *lasB-lacZ*), and PAO-R1(pPCS16 *lasR*$_{pwt}$ *rsaL*$_{plac}$ *lasB-lacZ*) grown in the presence of 1 μM PAI-1 as compared to that in the same strains grown in the absence of PAI-1. We speculate that the addition of PAI-1 abrogated RsaL's repressive effect in two ways. First, PAI-1 activated LasR, enabling it to directly induce *lasB* expression. Second, the increased concentration of PAI-1 enabled LasR–PAI-1 to compete with RsaL for binding to the *lasI* promoter, thereby increasing *lasI* transcription. The resultant increase in PAI-1 levels then enabled further activation of *lasB*. In the case of strain PAO-R1(pPCS16 *lasR*$_{pwt}$ *rsaL*$_{plac}$ *lasB-lacZ*), addition of 1 μM PAI-1 did not completely relieve RsaL's repression. It is possible that the high concentration of RsaL present in these cells hinders the ability of LasR–PAI-1 to compete for binding to the *lasI* promoter. Thus, no additional PAI-1 is produced and *lasB* expression is lower than that observed when higher levels of PAI-1 are available for LasR activation. Interestingly, an increase in *lasB* expression was also observed when 1 μM PAI-1 was added to strains PAO-R1(pLasR *lasR*$_{pwt}$ *lasB-lacZ*) and PAO-R1(pKDT17 *lasR*$_{plac}$ *lasB-lacZ*), which do not express *rsaL*. This enhanced expression is probably caused by the more efficient activation of the LasR molecules present due to the higher concentration of PAI-1.

RsaL does not inhibit LasR expression. To determine whether RsaL has any effect on LasR expression, Western blot analysis of total protein obtained from cells collected in the early stationary phase of growth was performed. Representative results of three separate blots are shown in Fig. 7. In each assay, strains PAO-R1(pKDT17 *lasR*$_{plac}$ *lasB-lacZ*), PAO-R1(pPCS15 *lasR*$_{plac}$ *rsaL*$_{pwt}$ *lasB-lacZ*), PAO-R1(pTS4001.7 *lasR*$_{pwt}$ *rsaL*$_{pwt}$ *lasB-lacZ*), and PAO-R1(pPCS16 *lasR*$_{pwt}$ *rsaL*$_{plac}$ *lasB-lacZ*) demonstrated similar signals for LasR antigen, indicating that the presence of RsaL does not inhibit LasR production. Strain PAO-R1(pLasR *lasR*$_{pwt}$ *lasB-lacZ*) exhibited slightly elevated levels of LasR, as noted by the broader, more intense signal, and PAO-R1(pTS400 *lasB-lacZ*), which does not produce LasR, did not exhibit any signal in the assays.

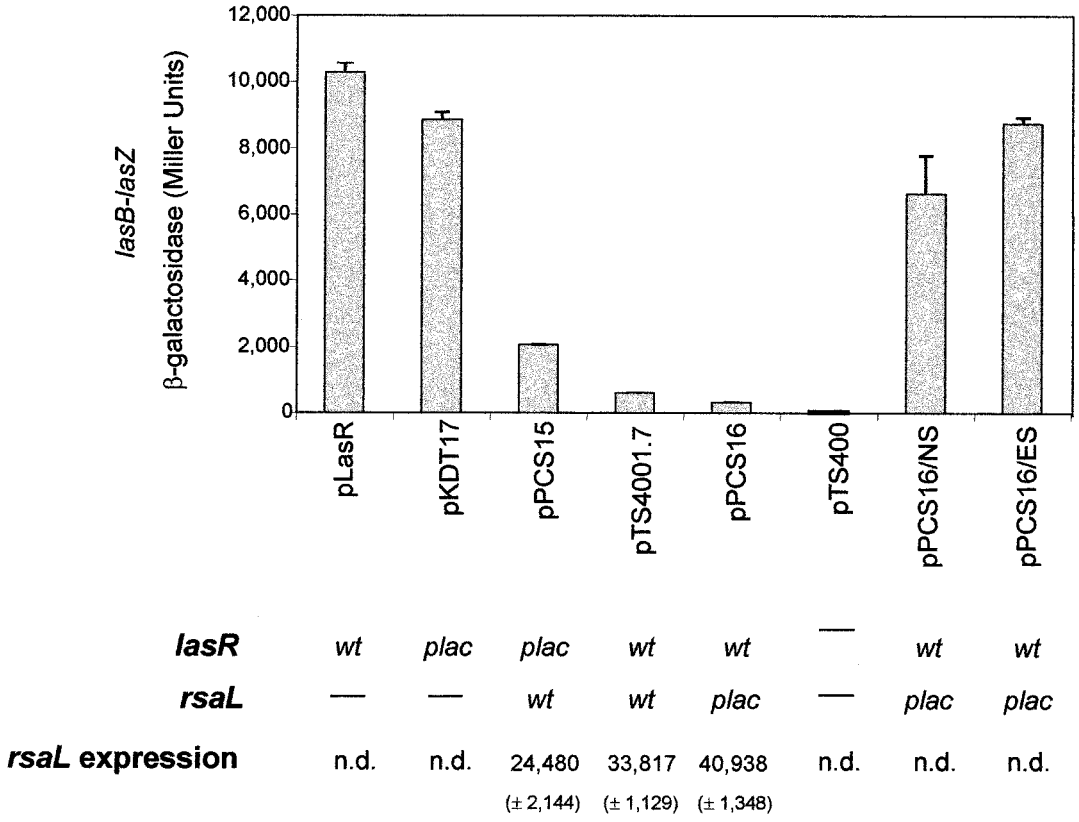


FIG. 4. Overexpression of *rsaL* inhibits expression of *lasB* in *P. aeruginosa*. β-Galactosidase activity from a plasmid-borne *lasB-lacZ* translational fusion was monitored in the presence of either *lasR* alone or both *lasR* and *rsaL* in *P. aeruginosa* PAO-R1. Expression of *lasR* and *rsaL* was directed by either their native promoters (*wt*) or the *lac* promoter (*plac*) as indicated under each plasmid. Plasmids in which the RsaL start methionine is removed (pPCS16/NS) or in which a stop codon is prematurely introduced (pPCS16/ES), but which otherwise are identical to pPCS16, were also examined for β-galactosidase production. To monitor the level of *rsaL* expression in plasmids pPCS15, pTS4001.7, and pPCS16, the *rsaL* gene located on these constructs was replaced by an *rsaL-lacZ* translational fusion. β-Galactosidase activity from the *rsaL-lacZ* fusions in *P. aeruginosa* PAO-R1 is indicated under each plasmid. n.d., not determined.

DISCUSSION

While details regarding the mechanism of quorum sensing in *P. aeruginosa* are continuously coming to light, very little is known about how the regulators themselves are regulated. Studies have revealed a variety of factors that affect the positive regulation of autoinduction (1, 30); however, no negative regulators of the *P. aeruginosa* quorum-sensing systems have been identified. This study describes a newly identified *P. aeruginosa* protein, RsaL, which negatively affects the *las* quorum-sensing system. Expression of *rsaL* resulted in decreased *lasB* expression, and mutations abolishing RsaL synthesis abrogated this effect, thereby confirming that RsaL was responsible for the repression.

The observation that *rsaL* decreased *lasB* expression led to the formulation of two hypotheses for its target of repression. First, RsaL could interact with a specific operator sequence upstream of the *lasB* gene. Evidence against this hypothesis was obtained from our data demonstrating that in the presence of LasR and PAI-1, *lasB* gene expression in an *E. coli* lysogen was not decreased when *rsaL* was expressed. Data from the *lasI* lysogen experiments supported the second hypothesis, in which RsaL directly affects one or more of the regulatory factors of the *las* quorum-sensing regulon. In *E. coli*, in the presence of LasR and added PAI-1, RsaL inhibited expression of *lasI* (Fig. 5). While *lasI* expression requires both LasR and PAI-1 (35), our Western blot analysis (Fig. 7) indicated that RsaL did not decrease LasR expression. Together these data indicated that RsaL specifically inhibits the expression of the *lasI* gene, preventing the production of PAI-1. In *P. aeruginosa*, in the absence of PAI-1, LasR would remain inactive and *lasB* would not be transcribed.

There have been only four reports to date of proposed negative regulators in quorum sensing. In *Vibrio harveyi*, LuxO was shown to repress autoinducible luminescence at the receptor portion of a two-component regulatory system (2). In *Pantoea stewartii*, the transcriptional activator EsaR acts as a repressor of quorum sensing at low cell density until sufficient AI becomes available for derepression to occur (3). TraM of *Agrobacterium tumefaciens* is proposed to repress autoinduction through protein-protein interactions with the transcriptional activator TraR (7, 15), and finally, in *Erwinia carotovora, rsmA* encodes a 6.8-kDa protein which has been shown to decrease synthesis of many extracellular enzymes (5, 20). The mode of action of the *Erwinia* protein, RsmA, is believed to be through repression of AI synthesis, a function similar to that predicted for RsaL. Cui and coworkers (5) demonstrated that multicopy *rsmA* suppressed the level of HSL [*N*-(3-oxohexanoyl)-L-homoserine lactone] in *Erwinia* culture supernatants and decreased the levels of the *hslI* transcript, which is required for HSL production. RsmA is a homolog of the *E. coli* CsrA protein, a negative regulator of carbon storage (31), and the predicted protein products of the two genes are 95% identical (5). CsrA is proposed to bind mRNA of its target gene, *glg*, to accelerate decay of the transcript (17, 18). The finding

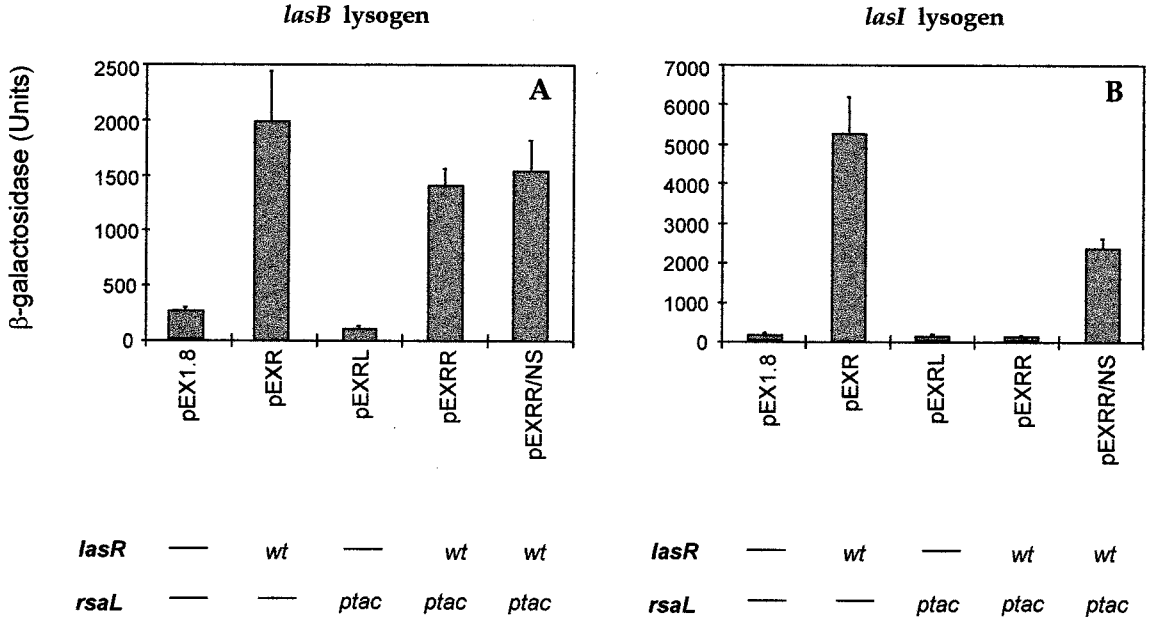


FIG. 5. Effect of RsaL on *lasI* expression. The expression of *lasB-lacZ* (A) and *lasI-lacZ* (B) fusions carried as prophages in *E. coli* MG4 was examined in the presence of *lasR* under control of its own promoter (pEXR), *rsaL* overexpressed from the *tac* promoter (pEXRL), or *lasR* expressed from its own promoter and *rsaL* overexpressed from the *tac* promoter (pEXRR). A construct identical to pEXRR but which has the start codon of RsaL removed through mutagenesis (pEXRR/NS) was also examined. Plasmid pEX1.8 was used as a control vector in these experiments. In all cases, 100 nM PAI-1 and 1 mM IPTG were added to the cultures at the initiation of growth.

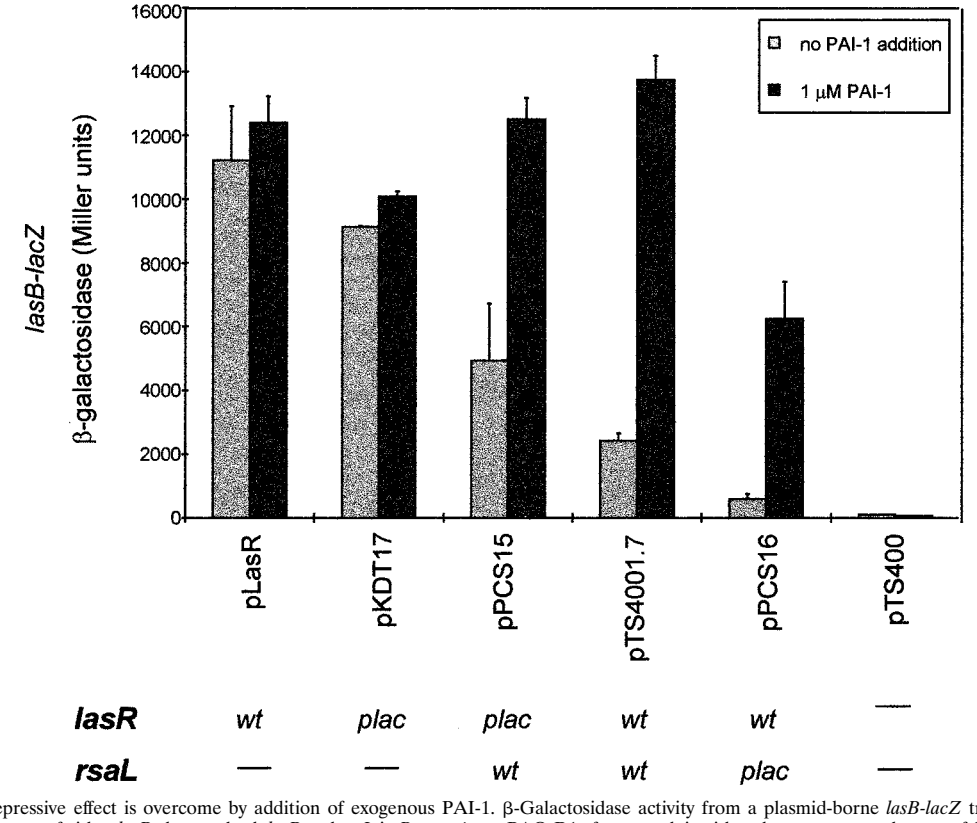


FIG. 6. RsaL's repressive effect is overcome by addition of exogenous PAI-1. β-Galactosidase activity from a plasmid-borne *lasB-lacZ* translational fusion was monitored in the presence of either *lasR* alone or both *lasR* and *rsaL* in *P. aeruginosa* PAO-R1 after growth in either the presence or absence of 1 μM exogenous PAI-1. Expression of *lasR* and *rsaL* was directed by either their native promoters (*wt*) or the *lac* promoter (*plac*) as indicated under each plasmid.

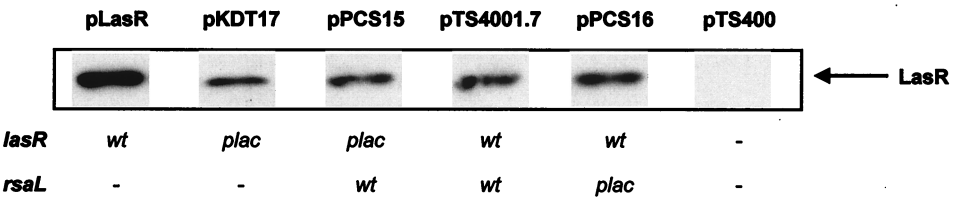


FIG. 7. Western blot analysis of LasR expression when *rsaL* is overexpressed in *P. aeruginosa* PAO-R1. Anti-LasR antibody (1:5,000) was used to visualize LasR in total cell lysates of strain PAO-R1 in the presence of *lasR* alone or both *lasR* and *rsaL*. Expression of *lasR* and *rsaL* was directed by either their native promoters (*wt*) or the *lac* promoter (*plac*) as indicated.

that the *Erwinia rsmA* gene was able to suppress glycogen synthesis in *E. coli*, together with the fact that, like CsrA, RsmA contains a putative RNA-binding domain, has led to the suggestion that RsmA may regulate gene expression by affecting mRNA stability (5). Analysis of the RsaL sequence revealed no similarities with either CsrA or RsmA. Furthermore, no RNA-binding motif equivalent to that described for RsmA or CsrA was identified in RsaL.

Whether RsaL binds directly to an operator element upstream of *lasI* and disrupts its expression or whether it affects the stability of the *lasI* mRNA has not been determined. However our λ lysogen experiments suggest that it is through the former mechanism. In the *E. coli* lysogen, the *lasI* transcriptional fusion contains an RNase III cleavage site between the *lasI* and *lacZ* genes; consequently, the mRNA is cleaved into two separate messages. Destabilization of the *lasI* message should therefore have no effect on stability of the *lacZ* mRNA. Thus, the absence of β-galactosidase activity in the *lasI* λ lysogen containing RsaL suggests that RsaL affects *lasI* transcription and not *lasI* message stability. An interaction between RsaL and an element upstream of *lasI* would be a novel finding in quorum-sensing systems. It is interesting to note the proximity of the *rsaL* promoter/operator region to that of *lasI*. The small region defining these two promoters suggests that overlapping operators, or perhaps even the same operator, may be used for expression of both the *rsaL* and *lasI* transcripts by LasR and PAI-1. Intriguingly, two putative *lux* boxes are located between the *rsaL* and *lasI* genes (Fig. 1C). One of the *lux* boxes is approximately centered in the *rsaL-lasI* intergenic region, and the second encompasses the *lasI* start of translation. Whether one or both of these regions is used for regulating the expression of *rsaL* and/or *lasI* has yet to be established. Recently, Fuqua and coworkers described the arrangement of the *traI* and *traC* genes of *A. tumefaciens* whereby the divergently transcribed genes share an operator targeted by TraR, a LasR homologue, and AAI, the *Agrobacterium* autoinducer (7). Previous studies have proposed that the LuxR homologues may function as activators by stabilizing and positioning the RNA polymerase complex at target gene promoters (36, 37). The commonly shared operator between two independent and divergent promoters implies that if LuxR-like proteins interact directly with RNA polymerase, they may dimerize and promote transcription in opposite directions.

In *P. aeruginosa*, LasR and PAI-1 globally regulate many products associated with virulence, as well as the second *P. aeruginosa* quorum-sensing system. We theorize that at low cell density, RsaL inhibits transcription of *lasI* by binding to the *lasI* operator region, thereby blocking activation by LasR–PAI-1. As the cell density increases, so does the basal level of PAI-1, which enables sufficient LasR–PAI-1 formation to outcompete RsaL for binding to the *lasI* operator. Thus, it appears that during the early stages of growth, RsaL blocks the quorum-sensing cascade by inhibiting the transcription of *lasI*. We speculate that in an RsaL mutant, *lasI* would be turned on much earlier in the growth cycle, resulting in premature activation of the *las* quorum-sensing-controlled genes. Studies are currently in progress to generate an RsaL null mutant and assess the phenotypic effects associated with this mutation. During the infection process, the repression of virulence factor expression may be critical for minimizing both the immunogenicity and host damage associated with these factors, allowing the organism to achieve a high population density prior to dissemination. Given the essential role of many of the quorum-sensing-controlled genes in *P. aeruginosa* virulence, RsaL appears as a pivotal regulator of *P. aeruginosa* pathogenicity. Further studies in progress should clarify the role of this unique regulator in *P. aeruginosa* virulence.

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T.D.K. and P.C.S. contributed equally to this work.

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