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Regulation of *las* and *rhl* Quorum Sensing in *Pseudomonas aeruginosa*

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The production of several virulence factors by *Pseudomonas aeruginosa* is controlled according to cell density through two quorum-sensing systems, *las* and *rhl*. The *las* system is comprised of the transcriptional activator protein LasR and of LasI, which directs the synthesis of the autoinducer PAI-1. Similarly, the *rhl* system consists of the transcriptional activator protein RhlR and of RhlI, which directs synthesis of the autoinducer PAI-2 (formerly referred to as factor 2). To study the interrelation between the two *P. aeruginosa* quorum-sensing systems, we fused a *lacZ* reporter gene to *lasR*, *rhlR*, and *rhlA* and monitored expression of these three genes under various conditions. Our data indicate that *lasR* and *rhlR* are expressed in a growth-dependent manner, with activation of each gene occurring during the last half of log-phase growth. We also show that the *las* quorum-sensing system controls the *rhl* quorum-sensing system in two ways. First, we found that LasR and PAI-1 activated *rhlR* transcription. Second, we showed that PAI-1 blocked PAI-2 from binding to RhlR, thereby inhibiting the expression of *rhlA*. Our data thus indicate that the *las* system exerts two levels of control on RhlR, transcriptional and posttranslational.

At least two complete quorum-sensing systems, *las* and *rhl*, are present in the opportunistic human pathogen *Pseudomonas aeruginosa*. These systems are known to control the expression of a number of virulence genes in response to bacterial cell density (5), but their specific effect on each other has not been studied. The *las* and *rhl* systems each contain homologs of the LuxR and LuxI proteins of the prototypic *lux* quorum-sensing system from *Vibrio fischeri* (see reference 5 for a review). The *las* system consists of the transcriptional activator protein LasR and of LasI, which directs the synthesis of the autoinducer PAI-1 [*N*-(3-oxododecanoyl)-L-homoserine lactone] (6, 20, 22). This system has been shown to activate the expression of *lasI*, *lasB*, *lasA*, *apr*, and *toxA* (7, 20, 27, 30). Similarly, the *rhl* system consists of the transcriptional activator protein RhlR and RhlI, which directs the synthesis of the autoinducer PAI-2 (*N*-butyryl-L-homoserine lactone; formerly known as factor 2) (16, 17, 23). This system controls the expression of *rhlI* and *rhlAB*, which codes for a rhamnosyltransferase required for rhamnolipid (heat-stabile hemolysin) production (11, 15–17). It has also been reported that *rhl* quorum sensing activates the expression of *rpoS*, a stationary-phase sigma factor that controls numerous genes (11).

The general model for quorum sensing (5) begins with the autoinducer, which is a diffusible molecule, being produced at a basal level at low cell densities. The autoinducer concentration then increases with cell density until a threshold concentration is reached. At this concentration, the autoinducer binds to its specific target protein (i.e., LasR or RhlR), and the autoinducer-protein complex activates genes that it controls.

Experiments on the interchangeability of the *las* and *rhl* system components showed that they were not compatible, in that PAI-2 does not activate LasR nor does PAI-1 activate RhlR in *Escherichia coli* (23, 24). However, it was apparent that these two systems were not completely independent of one another. It was first indicated that the *las* and *rhl* systems may be linked when Pearson et al. (23) showed that PAI-2 was poorly expressed in a *P. aeruginosa lasR* strain, which led to the conclusion that LasR may control PAI-2 production. After this, it was reported that the *rhl* system was working in tandem with the *las* system to control the production of elastase in *P. aeruginosa* (3, 17). While this paper was being written, an article was published that showed, as we do below, that *rhlR* transcription was controlled by LasR-PAI-1 (11). For our study, we sought to determine the effect, if any, of the *las* and *rhl* systems on each other. We report here that the *las* quorum-sensing system controls RhlR, the transcriptional activator of the *rhl* system, at both the transcriptional and posttranslational levels. In addition, we show that *lasR* and *rhlR* are expressed in a growth-dependent manner, with each gene being activated during the second half of log-phase growth.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. Plasmid pECP60 was constructed by ligating the 623-bp, *rhlA* promoter-containing (24) *Bam*HI/*Bgl*II fragment of pUO101 into the *Bam*HI site of pSW205 to create a *rhlA′-lacZ* translational fusion. Plasmid pUO101 was kindly provided by U. Ochsner and carries a 5.8-kb *Hin*dIII/*Eco*RI fragment of *P. aeruginosa* PG201 DNA which contains the entire *rhl* regulon (*rhlABRI*). Transcriptional fusions of *lasR′-lacZ* and *rhlR′-lacZ* were constructed in the vector pLP170. A *lasR* promoter-containing fragment was generated through PCR, using primers that contained an *Eco*RI or *Bam*HI site. The 3′ end of the primer containing the *Eco*RI site (5′-GCGTGGGGAATTCCGCGTGC GCCGCGC-3′) corresponded to the nucleotide 395 bp upstream from the *lasR* start codon. The 3′ end of the primer containing the *Bam*HI site (5′-CCGTCG GATCCACCCGCGCGTAGCC-3′) corresponded to the nucleotide 197 bp downstream from the *lasR* start codon. The transcriptional start sites of *lasR* have been mapped to nucleotides 201 and 231 bp upstream from the *lasR* start codon (1), which ensured that the *lasR* regulatory region is contained on the sequence used to make the *lasR′-lacZ* fusion. The PCR product obtained by the use of a *lasR* template was digested with *Eco*RI and *Bam*HI and ligated into the transcriptional fusion vector pTL61T, which had been digested with the same enzymes. The resulting *lasR′-lacZ* transcriptional fusion was released from this vector by digestion with *Eco*RI and *Eco*RV and ligated into pLP170 that had been digested with the same enzymes to yield pPCS1001. The PCR-generated fragment was sequenced to ensure that mutations had not been incorporated. The *rhlR* promoter-containing fragment was released from pUO101 by using the enzymes *Pst*I and *Bam*HI and was ligated into *Pst*I/*Bam*HI-digested pTL61T. The recognition sites for the enzymes *Pst*I and *Bam*HI are located at nucleotides −500 and +242 relative the *rhlR* start codon. Through deletion analysis of the DNA upstream from *rhlR*, Ochsner et al. (16) mapped the promoter region of *rhlR* to the region within 80 nucleotides upstream from the *rhlR* start codon. This

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TABLE 1. Strains and plasmids

Strain or plasmid	Relevant genotype or phenotype	Reference
E. coli		
DH5α	F′/*endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*) U169 *deoR* [φ*80 dlacZ*ΔM15 *recA1*]	31
JM109	F′ *traD36 lacI*^{q} Δ(*lacZ*)M15 *proA*$^+$*B*$^+$/*el4*$^-$ Δ(*lac-proAB*) *thi gyrA96 endA1 hsdR17 relA1 supE44 recA1*	32
P. aeruginosa		
PAO1	Wild type	9
PDO100	Δ*rhlI*::Tn*501-2* strain PAO1 derivative	3
PAO-JP2	Δ*lasI*::Tet, Δ*rhlI*::Tn*501-2* strain PAO1 derivative	24
Plasmids		
pSW205	Translational *lacZ* fusion vector that contains an origin of replication for both *E. coli* and *P. aeruginosa* and *bla* (Amp)	7
pECP60	pSW205 containing an *rhlA′-lacZ* translational fusion	This study
pEX1	Expression vector for constructing *tacp* fusions; carries *lacI*^{q} and *bla*	19
pEX1.8	pEX1 containing a *P. aeruginosa* origin of replication	24
pJPP8	pEX1.8 containing *tacp-rhlR*	24
pECP61.5	pJPP8 containing the *rhlA′-lacZ* fusion from pECP60	24
pACYC184	General-purpose cloning vector, Tet Chlor	4
pPCS11	*tacp-lasR* on pACYC184	26
pLP170	*lacZ* transcriptional fusion vector that contains an origin of replication for both *P. aeruginosa* and *E. coli* and *bla*	25
pPCS1001	pLP170 containing a *lasR′-lacZ* transcriptional fusion	This study
pPCS1002	pLP170 containing a *rhlR′-lacZ* transcriptional fusion	This study
pGroESL	pEX1.8 containing *lacp-groESL*	24
pTL61T	*lacZ* transcriptional fusion vector	12

confirmed that the sequence used to construct the *rhlR′-lacZ* fusion contains the *rhlR* promoter. The *rhlR′-lacZ* transcriptional fusion was released by digestion with *Eco*RI and *Eco*RV and ligated into pLP170 digested with the same enzymes to yield pPCS1002. Nucleotide fusions were specifically verified by sequencing to ensure integrity of the fusion junction.

DNA techniques. Standard techniques were used for DNA manipulation (13). PCR was performed using *Taq* polymerase (Gibco/BRL, Gaithersburg, Md.). Oligonucleotides used for PCR were synthesized by G. Kampo and J. Maniloff at the Core Nucleic Acid Laboratory at the University of Rochester Medical Center. Restriction endonucleases were purchased from Gibco/BRL or New England Biolabs (Beverly, Mass.). Plasmids were introduced into *E. coli* and *P. aeruginosa* by transformation (13) or electroporation (28), respectively. *E. coli* containing plasmid DNA was selected on L-agar (13) plates containing ampicillin (100 μg/ml) and/or chloramphenicol (30 μg/ml) when appropriate. *P. aeruginosa* containing plasmid DNA was selected on peptone tryptic soy broth (PTSB) (18) agar plates containing carbenicillin (200 μg/ml). Nucleotide sequencing was accomplished by using the Sequenase kit (U.S. Biochemical Corp., Cleveland, Ohio) and [α-^{35}S]dATP (NEN Research Products, Boston, Mass.).

β-Gal activity assays. β-Galactosidase (β-Gal) activity was assayed according to Miller (14), and the mean ± 1 standard deviation (SD) was reported.

Media and culture conditions for β-Gal activity assays. (i) *P. aeruginosa*. *P. aeruginosa* cultures were grown for 18 h at 37°C with shaking in PTSB (supplemented with 200 μg of carbenicillin per ml when appropriate) and subcultured into the same medium to a starting A_{660} of 0.05. When indicated, PAI-1 and/or PAI-2 was added at the start of subculturing to achieve a final concentration of 1 μM. For Fig. 2, cultures were assayed for β-Gal activity throughout the growth cycle and growth was monitored by measuring cell density (absorbance at 660 nM). For Fig. 1 and Fig. 3, cultures were assayed for β-Gal activity after 7.5 h of growth. This time point was chosen because it was the time point when all cultures were always at approximately the same cell density (see Fig. 2A) and therefore the assays would be comparable.

(ii) *E. coli*. *E. coli* cultures were grown in A medium (13) supplemented with 0.4% glucose, 1 mM $MgSO_4$, and 0.05% yeast extract. When appropriate, 100 μg of ampicillin per ml and/or 30 μg of chloramphenicol per ml was added to the medium. Cultures were shaken at 37°C for 18 h and subcultured to a starting A_{600} of 0.08. At an A_{600} of 0.3, autoinducers were added where indicated. IPTG (5-bromo-4-indole-3-chloro-isopropyl-β-D-galactopyranoside) was also added at this time to a final concentration of 1 mM for Fig. 4. For Fig. 6., IPTG was omitted because the *tac* promoter controlling *rhlR* allows enough RhlR to be produced to activate *rhlA* in the presence of PAI-2. When IPTG was added, the massive overexpression of RhlR lessened the effect shown here (data not shown). After additions were made to *E. coli* cultures, growth was continued for 1.5 h, at which time assays for β-Gal activity were completed.

PAI-2 binding assays. Binding assays followed the method of Hanzelka and Greenberg (8) with the following modifications. *E. coli* DH5α cultures containing two plasmids, pJPP8 and pGroESL, were grown (starting A_{600} of 0.5) for 2 h in Luria-Bertani (LB) medium (containing 100 μg of ampicillin per ml and 30 μg of chloramphenicol per ml) at 37°C with shaking in the presence of 1 mM IPTG. Plasmid pGroESL was included because GroES and GroEL were shown to enhance binding of the *Vibrio* autoinducer VAI-1 to LuxR (8). Culture aliquots were preincubated (25°C, 30 min) in the presence or absence of PAI-2 or PAI-1 (1 or 10 μM) followed by a 30-min incubation in the presence of 250 nM [^{3}H]PAI-2 (24). Radioactivity remaining with the cells was determined as described previously (8). All binding experiments were completed twice in duplicate. We have shown elsewhere that *E. coli* cells which contained pJPP8 and pGroESL bound approximately 20-fold-more [^{3}H]PAI-2 than cells which contained pEX1.8 (control vector) and pGroESL (24).

RESULTS AND DISCUSSION

The *las* quorum-sensing system affects the *rhl* quorum-sensing system. It became clear that the two quorum-sensing systems of *P. aeruginosa* were communicating when we studied the effects of exogenous autoinducers on *rhlA* expression in cells grown in PTSB medium. It has been shown that PAI-2 and RhlR are required for the activation of *rhlA* in *E. coli* (24) and that *rhlR* is required for rhamnolipid production in *P. aeruginosa* (16). To study *rhlA* expression in *P. aeruginosa*, β-Gal activity was assayed to monitor activation of an *rhlA′-lacZ* fusion in early-stationary-phase cultures of *P. aeruginosa* PDO100 *rhlI*(pECP60) and PAO-JP2 *rhlI lasI*(pECP60) (Fig. 1). As expected, strain PDO100(pECP60) exhibited *rhlA* activation only when exogenous PAI-2 was provided. However, in strain PAO-JP2(pECP60), maximal *rhlA* activation required both PAI-2 and PAI-1. The addition of PAI-2 alone allowed only 8% (100 Miller units) of the *rhlA* activation seen when both PAI-1 and PAI-2 were added (1,212 Miller units) (Fig. 1). We have also qualitatively shown that a *P. aeruginosa lasI* mutant strain does not make rhamnolipid (24). It was obvious

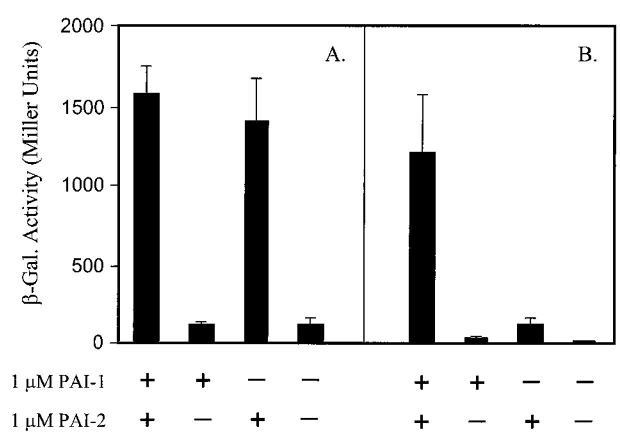

FIG. 1. Autoinducer requirements for *rhlA* activation in *P. aeruginosa*. *P. aeruginosa* PAO1 derivatives PDO100 (*rhlI*) and PAO-JP2 (*lasI rhlI*) containing pECP60 were grown as described in Materials and Methods in the presence of the indicated autoinducer. During the early stationary phase of growth, β-Gal activity was assayed. Data represent the means of duplicate β-Gal assays from four separate experiments, and activity is expressed as Miller units ± SD^{n-1}. (A) Strain PDO100(pECP60); (B) strain PAO-JP2(pECP60).

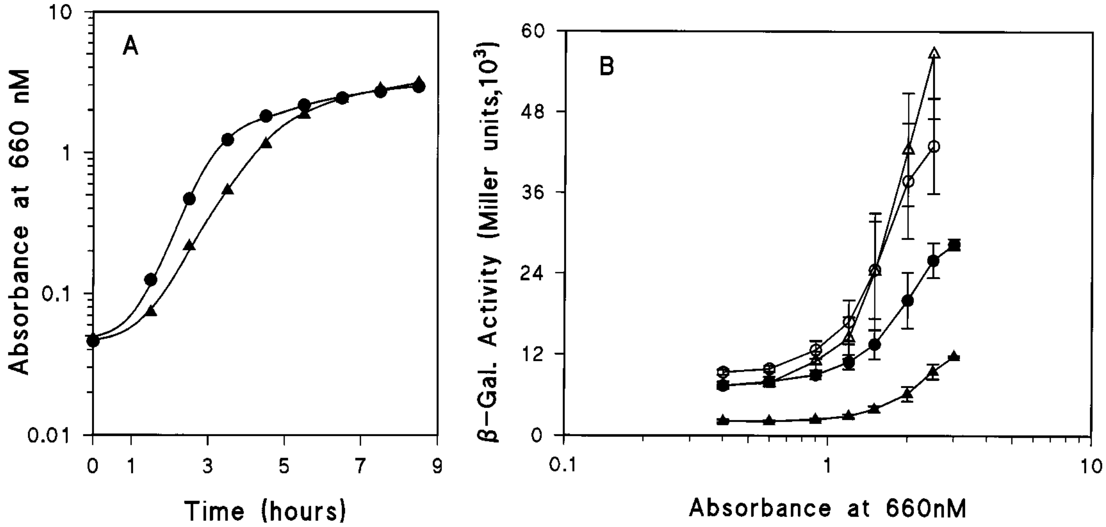

FIG. 2. Time course of *lasR* and *rhlR* expression in strains PAO1 (wild type) and PAO-JP2 (*lasI rhlI*). Cultures containing plasmids with *lasR'-lacZ* and *rhlR'-lacZ* transcriptional fusions were grown as described in Materials and Methods, and aliquots were taken throughout growth and assayed for cell density (A, absorbance at 660 nM; B, β-Gal activity). (A) Curves represent the averages of at least three separate experiments. Closed circles indicate strain PAO1 containing either pPCS1001 or pPCS1002. Closed triangles indicate strain PAO-JP2 containing either pPCS1001 or pPCS1002. The A_{660} measurements for a strain carrying either pPCS1001 or pPCS1002 were averaged because growth curves for an individual strain were superimposable regardless of which plasmid was present. (B) Each curve is the result of at least three separate growth curve experiments with duplicate β-Gal assays performed on each aliquot. Activity is expressed as Miller units ± SD^{n-1}. ○, strain PAO1(pPCS1001); △, strain PAO1(pPCS1002); ●, strain PAO-JP2(pPCS1001); ▲, strain PAO-JP2(pPCS1002).

from these data that *rhlA* expression was dependent on components of both the *rhl* and *las* quorum-sensing systems. This result was a surprise because we had expected that activation of *rhlA* in *P. aeruginosa* would occur in the presence of PAI-2 and RhlR as it had in *E. coli* (24). However, in our *E. coli* experiments, *rhlR* expression was controlled by the inducible *tac* promoter instead of its own promoter, thus eliminating the wild-type regulatory sequences upstream from *rhlR*. This led us to speculate that perhaps RhlR was not expressed in strain PAO-JP2 and that *rhlR* expression may be regulated through quorum sensing. To test this theory, we examined the activation of our *rhlR'-lacZ* and *lasR'-lacZ* transcriptional fusions in *P. aeruginosa* strains PAO1 and PAO-JP2 grown in PTSB medium (Fig. 2). We feel that it is very important to study quorum sensing in a well-defined *P. aeruginosa* mutant strain in which a critical portion of each quorum-sensing system is absent. The use of strain PAO-JP2 *lasI rhlI* allowed us to test our fusions in the absence of the two known *P. aeruginosa* autoinducers or in the presence of exogenously added autoinducers (see below), thus ensuring that our data did not result from partial effects caused by interference between the *las* and *rhl* systems. This is in contrast to a similar study by Latifi et al. (11) in which they used strain PAO1 with a *lasR* mutation and the undefined mutant strain PAN067 to test the effects of quorum sensing on *lasR* and *rhlR* expression.

In our experiments, we found that activation of *rhlR* and *lasR* in strain PAO1 was very similar (Fig. 2B), with both genes exhibiting a basal level of transcription until they became activated in the last half of log-phase growth (culture A_{660}, >1.0) (Fig. 2A). This result differs significantly from that of Latifi et al. (11), who report that *lasR* is expressed constitutively in *P. aeruginosa*. We found that *rhlR* was also expressed in a growth-dependent manner (Fig. 2B), while they (11) report that expression is constant until stationary phase. The reason for this conflict is not apparent. It could result from the use of different media (PTSB for this work and LB medium by Latifi et al. [11]), from differences between the specific *P. aeruginosa* PAO1 strains used by the two laboratories, or from an unknown factor.

In the *lasI rhlI* mutant (strain PAO-JP2), basal *lasR* transcription was unaffected and maximal expression was decreased by approximately 50% compared to that seen in the parental strain, PAO1 (Fig. 2B). However, *rhlR* transcription was critically affected in the absence of both PAI-1 and PAI-2. In strain PAO1, both basal and maximum levels of *rhlR* transcription were approximately fivefold higher than the levels seen in strain PAO-JP2 (Fig. 2B). These results indicate that even the basal levels of *rhlR*, but not those of *lasR*, are dependent on the presence of PAI-1 and/or PAI-2. These data also show that both *lasR* and *rhlR* are expressed to at least some extent in the absence of autoinducers, indicating that these genes are controlled by more than just quorum sensing. Most importantly, these data showed that during all phases of *P. aeruginosa* growth, maximum *rhlR* expression was dependent on PAI-1 and/or PAI-2, indicating that *rhlR* was controlled by one or both quorum-sensing systems.

Transcription of *rhlR* is positively regulated by LasR-PAI. To determine which quorum-sensing system controlled *rhlR*, PAI-1 and/or PAI-2 was added exogenously to cultures of strain PAO-JP2 containing pPCS1001 (*lasR'-lacZ*) or pPCS1002 (*rhlR'-lacZ*) (Fig. 3). Interestingly, these data showed that PAI-2 had no effect on *rhlR* or *lasR* transcription, but PAI-1 alone was capable of restoring expression of both gene fusions to normal levels (Fig. 3). The ability of PAI-1 to restore full *rhlR* expression to strain PAO-JP2(pPCS1002) suggests that the *las* quorum-sensing system controls *rhlR* transcription. In this scenario, the *rhl* system indirectly responds to cell density through the *las* system and only the *las* system directly "senses a quorum."

To ensure that the affect of PAI-1 on *rhlR* required LasR, we determined the effects of LasR and PAI-1 on *rhlR'-lacZ* expression in *E. coli* (Fig. 4). In the absence of LasR or PAI-1, *rhlR* expression remained at background level (Fig. 4). In the presence of both LasR and PAI-1, *rhlR* expression increased

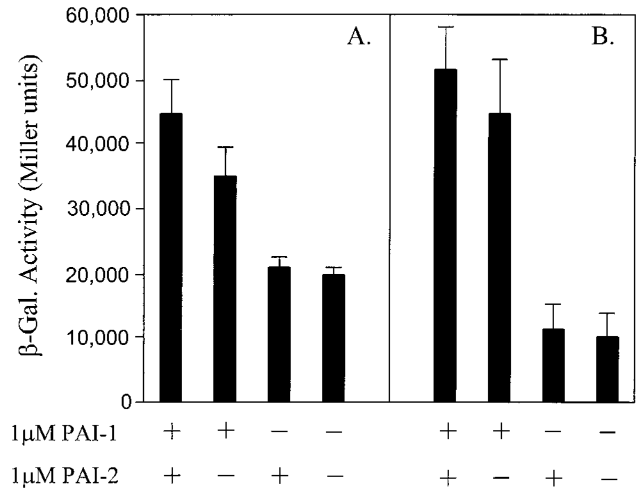

FIG. 3. Autoinducer effects on *lasR* and *rhlR* expression. *P. aeruginosa* PAO-JP2 (*lasI rhlI*) containing a plasmid with either a *lasR′-lacZ* or *rhlR′-lacZ* transcriptional fusion was grown in the presence of the indicated autoinducer as described in Materials and Methods. Aliquots were taken during early-stationary-phase growth and assayed for β-Gal activity. The control plasmid, pLP170, containing a promoterless *lacZ* gene always yielded less than 2,500 units of β-Gal activity when carried in strain PAO-JP2 (data not shown). Data represent the means of duplicate β-Gal activity assays from three separate experiments, and activity is expressed as Miller units ± SD^{n-1}. (A) Strain PAO-JP2(pPCS1001); (B) strain PAO-JP2(pPCS1002).

approximately threefold (to the same level as that seen for our *lasR′-lacZ* fusion in *E. coli*) (Fig. 4), indicating that *rhlR* was directly controlled by LasR and PAI-1. (Experiments in which we tested the ability of RhlR to activate *lasR* and *rhlR* in the presence of 50 nM, 1 μM, and 10 μM PAI-1 or PAI-2 showed no activation of either gene in *E. coli* [data not shown].) Furthermore, the expression of *lasR* was unaffected by the presence of LasR and PAI-1 in *E. coli* (Fig. 4). However, it is apparent that in *P. aeruginosa* PAO-JP2, PAI-1 caused a slight increase (approximately twofold) in expression from the *lasR′-lacZ* fusion (Fig. 2B and 3). This distinct difference in *lasR* expression between *P. aeruginosa* and *E. coli* makes it difficult to determine the role of autoregulation in *lasR* expression. It has also been reported that *lasR* transcription was unaffected in a strain PAO1 *lasR* mutant but negatively affected by the presence of LasR in *E. coli* (11), phenomena which we have also observed (2). Additionally, we have shown elsewhere that *lasR* transcription requires *vfr* (1), which encodes a homolog of the *E. coli* Cap protein. Taken together, these data do not lead to a conclusion concerning the effect of quorum sensing on LasR but suggest that *lasR* expression is complex and controlled by multiple factors.

The data in Fig. 2, 3, and 4 show that the *las* quorum-sensing system controls *rhlR* at the transcriptional level, providing direct evidence of communication between two quorum-sensing systems of the same organism. This role for LasR–PAI-1 adds to the importance of the complex as a global regulator of *P. aeruginosa* virulence. In addition, the control of *rhlR* by the *las* system implies that the *las* system is activated before the *rhl* system, indicating that, as suggested by Latifi et al. (11), a hierarchy exists between the two systems where *las* quorum sensing is dominant. This conclusion appears to be in direct conflict with earlier reports from our laboratory that indicated *lasR* could require a factor other than PAI-1 (possibly PAI-2) to function properly (20, 23). Subsequent investigation has shown that these conclusions were invalid because two *P. aeruginosa* strains, PAO-R1(pTS400-1.7) and PAO-R1(pTS400), used to produce data for those papers contained improper plasmids.

Often, gene control through quorum sensing is associated with the presence of a "*lux* box" consensus sequence which is usually found very close to, or overlapping with, the promoter of a quorum-sensing-controlled gene (5). Upon inspection, we discovered that the DNA sequence upstream from *rhlR* contains a potential *lux* box consensus sequence centered 81 nucleotides upstream from the *rhlR* translational start site. This sequence (5′-TTTTGCCGTATCGGCAAGGC-3′) matched 10 of 20 nucleotides with the *V. fischeri lux* box and 11 of 20 nucleotides with OP1 of *lasB* from *P. aeruginosa* (26). The importance of these sequences in quorum sensing was emphasized when Stevens et al. (29) showed that the *lux* box of *V. fischeri* specifically associates with the DNA-binding domain of LuxR and Rust et al. (26) demonstrated that OP1 of *lasB* is important for LasR–PAI-1-mediated activation of *lasB*. Additionally, Ochsner et al. (16) mapped the *rhlR* promoter region by deletion analysis of the DNA upstream from *rhlR* and identified a potential *rhlR* promoter that begins only 7 nucleotides from the 3′ end of the possible *rhlR lux* box. The discovery and location of this sequence upstream from *rhlR* lead us to speculate that it may be involved in the control of *rhlR* by LasR–PAI-1.

RhlR activity is also controlled at the posttranslational level by the *las* quorum-sensing system. To further explore the control of *rhl* by *las*, we tested the specificity of RhlR-autoinducer interactions. Tritium-labeled PAI-2 ([^{3}H]PAI-2) was used to develop a binding assay (see Materials and Methods) where *E. coli* cells overexpressing RhlR and GroES and GroEL were pretreated with unlabeled PAI-2 or PAI-1 in an amount either 4- or 40-fold in excess of the [^{3}H]PAI-2 (250 nM). After pretreatment with 4- or 40-fold-excess PAI-2, [^{3}H]PAI-2 binding decreased 64 and 89%, respectively (Fig. 5), indicating that unlabeled PAI-2 specifically competed (as expected) with [^{3}H]PAI-2 for binding to cells expressing RhlR. Interestingly, pretreatment with 4- or 40-fold-excess unlabeled PAI-1 decreased [^{3}H]PAI-2 binding to cells expressing RhlR by 86 and

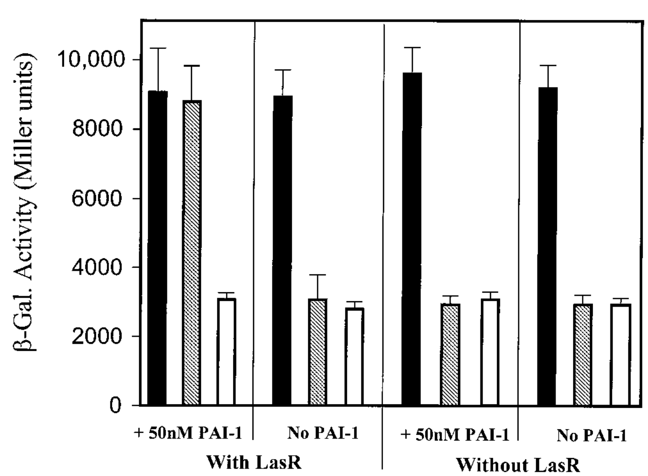

FIG. 4. Effects of LasR and PAI-1 on *lasR* and *rhlR* expression in *E. coli*. Cultures of *E. coli* JM109 containing two plasmids were grown in the presence or absence of PAI-1 and assayed for β-Gal activity as described in Materials and Methods. Data represent the means of duplicate β-Gal activity assays from three separate experiments, and activity is expressed as Miller units ± SD^{n-1}. "With LasR," containing plasmid pPCS11; "Without LasR," containing plasmid pACYC184. The second plasmid contained by each culture is indicated as follows: solid bars, pPCS1001; cross-hatched bars, pPCS1002; open bars, pLP170.

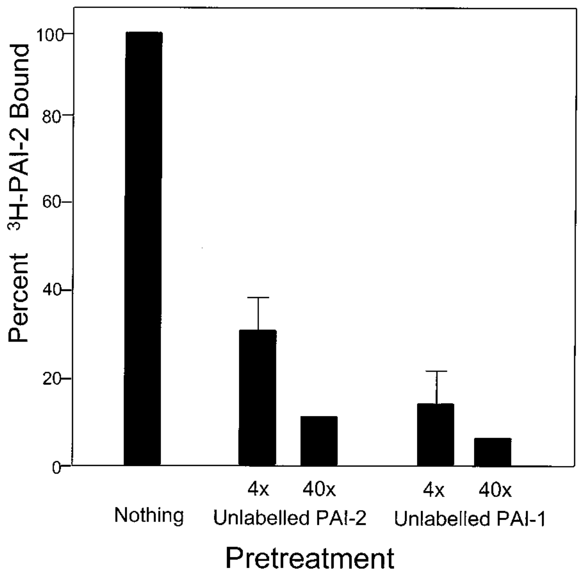

FIG. 5. PAI-1 blocks the interaction of PAI-2 and RhlR. Cells overexpressing RhlR were pretreated with the indicated unlabelled autoinducer in an amount either fourfold (4×) or 40-fold (40×) in excess of the amount of [^{3}H]PAI-2 added. Results are expressed as the percentage's of cell-bound radioactivity relative to that bound to cells not pretreated before the addition of [^{3}H]PAI-2.

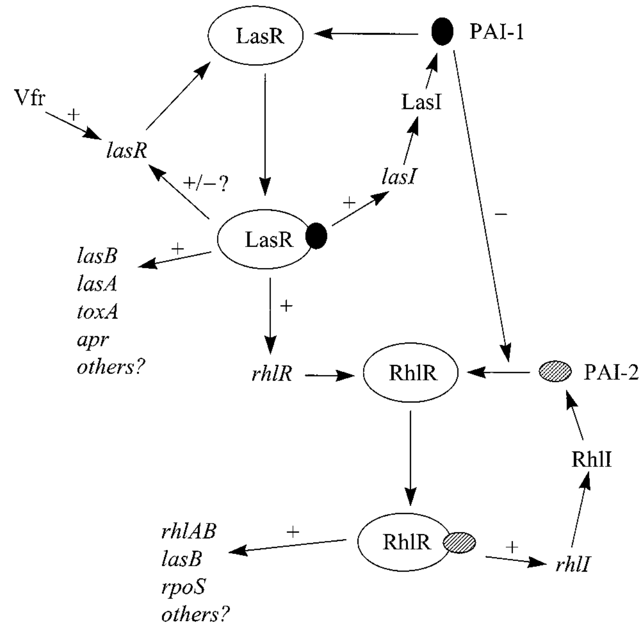

FIG. 7. Model of the *P. aeruginosa* quorum-sensing circuitry. LasR, RhlR, PAI-1, and PAI-2 are symbolized by circles. Plus symbols indicate transcriptional activation of the gene(s) at the end of an arrow. The effect of the LasR–PAI-2 complex on *lasR* is unclear, so this was indicated by "+/−?". The minus symbol by the arrow extending from PAI-1 to the arrow between PAI-2 and RhlR indicates blocking of the association between PAI-2 and RhlR. To begin the quorum-sensing cascade, LasR and PAI-1 are both produced at a basal level. As culture density increases, *lasR* is activated by Vfr (1), and PAI-1 reaches a threshold concentration and binds to LasR. We speculate that at low culture densities, the PAI-1 concentration is in excess of the PAI-2 concentration, allowing PAI-1 to block the interaction of RhlR and PAI-2. The autoinduction of *lasI* by LasR–PAI-1 could keep the PAI-1 concentration well above that of PAI-2 until enough RhlR and/or PAI-2 is produced to overcome the blocking effect of PAI-1. Once RhlR associates with PAI-2, autoinduction of *rhlI* occurs and the remainder of the RhlR–PAI-2-controlled genes are activated.

94%, respectively (Fig. 5). This result was quite surprising and showed that PAI-1 also competed with PAI-2 for binding to cells expressing RhlR. We have shown elsewhere that PAI-1 and RhlR will not activate *rhlA* and that PAI-2 will not compete with PAI-1 for binding to LasR (21, 24). With this in mind, we speculated that perhaps PAI-1 blocked RhlR activity in vivo. To test this hypothesis, we assayed *rhlA* expression in the presence of RhlR, 1 μM PAI-2, and various concentrations of PAI-1 (Fig. 6). The ability of RhlR and PAI-2 to activate

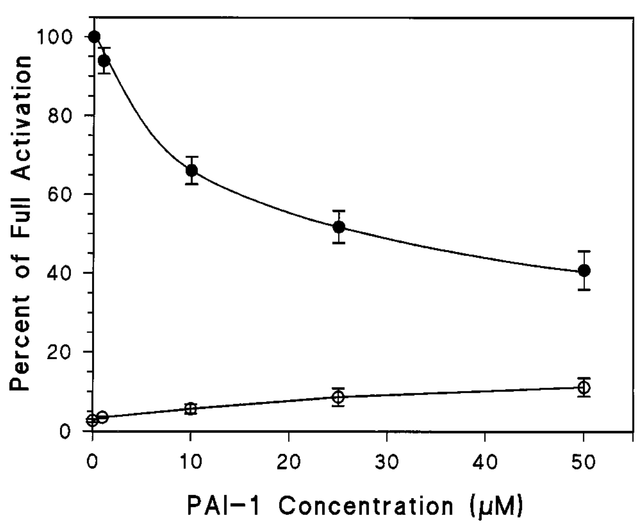

FIG. 6. PAI-1 blocks PAI-2 activity in *E. coli*. β-Gal activity expressed from an *rhlA'-lacZ* fusion was assayed from cultures of *E. coli* DH5α(pECP61.5) in the presence of 1 μM PAI-2 and various concentrations of PAI-1 as indicated. Cultures were grown as described in Materials and Methods. The data are the means of duplicate β-Gal assays from four separate experiments and are expressed as percentages of the activity from a culture that received only 1 μM PAI-2 (100% = 86 Miller units). ●, with PAI-2; ○, without PAI-2.

rhlA'-lacZ in *E. coli* decreased in a dose-dependent manner with increasing concentrations of PAI-1 (Fig. 6), suggesting that PAI-1 could block the PAI-2-binding site(s) of RhlR and cause the inhibition of a RhlR-controlled gene. This indicated that PAI-1 controls RhlR activity at a posttranslational level. Kuo et al. (10) have reported that a secondary autoinducer of *V. fischeri* inhibits activation of the *lux* operon, but it is not known whether this autoinducer is part of the *lux* system or primarily involved in a separate quorum-sensing system. The phenomenon shown here is the first report of an autoinducer from a defined quorum-sensing system within a bacterium that inhibits the activity of a second LuxR-type protein within the same bacterium.

To help clarify the circuitry involved in this complex regulation hierarchy, we present a model of *P. aeruginosa* quorum sensing (Fig. 7). We speculate that in *P. aeruginosa*, the posttranslational control of RhlR by PAI-1 occurs before *rhlI* is induced, when the concentration of PAI-1 is higher than the concentration of PAI-2. PAI-1 could block PAI-2 from associating with RhlR until enough RhlR and/or PAI-2 was present to overcome the PAI-1 blocking effect. At that point, RhlR–PAI-2 could autoinduce *rhlI*, which would allow the concentrations of PAI-2 and PAI-1 to become approximately equal, as we have seen in stationary-phase cultures (5 to 10 μM [22, 23]). This would allow *P. aeruginosa* to delay the induction of genes controlled by *rhl* quorum sensing and provide this organism with yet another mechanism to temporally control the activation of important factors.

The control of RhlR at both the transcriptional and posttranslational levels provides *P. aeruginosa* with an elegant mechanism through which it can use the *las* quorum-sensing system to control the *rhl* quorum-sensing system. The importance of this discovery becomes evident when one considers both the multiple virulence factors controlled by these two systems and the report (11) that RhlR–PAI-2 activates transcription of the sigma factor *rpoS*. The results presented here also lead us to speculate that it may be possible to develop a single therapeutic autoinducer analog of PAI-1, rather than two different analogs, which will inhibit expression of virulence genes controlled by both LasR and RhlR.

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