JOURNAL OF BACTERIOLOGY, Feb. 1995, p. 654–659
0021-9193/95/$04.00+0

Vol. 177, No. 3

Activation of the *Pseudomonas aeruginosa lasI* Gene by LasR and the *Pseudomonas* Autoinducer PAI: an Autoinduction Regulatory Hierarchy

PATRICK C. SEED, LUCIANO PASSADOR, AND BARBARA H. IGLEWSKI*

Department of Microbiology and Immunology, University of Rochester, Rochester, New York 14642

Received 29 June 1994/Accepted 21 November 1994

In *Pseudomonas aeruginosa*, the transcriptional activator LasR and the *Pseudomonas* autoinducer PAI, are necessary for efficient transcriptional activation of the *lasB* gene, encoding elastase (L. Passador, J. M. Cook, M. J. Gambello, L. Rust, and B. H. Iglewski, Science 260:1127–1130, 1993). The transcriptional start points of *lasI* in *Escherichia coli* and *P. aeruginosa* were determined by S1 nuclease mapping. In the presence of both LasR and PAI, the start site, T1, is located at position −25 relative to the ATG translational start codon. A minor transcriptional start, T2, is found at position −13 when *lasI* is transcribed in the absence of either LasR or PAI in *P. aeruginosa* and *E. coli*, respectively. To begin to closely examine the regulation of *lasI*, whose product is involved in the synthesis of PAI, a *lasI-lacZ* fusion on a lambda phage was constructed to form monolysogens of *E. coli* MG4. Lysogens supplied only with either *lasI* or *lasR* via multicopy plasmids demonstrated no significant increase in β-galactosidase expression compared with control levels. Lysogens in which both *lasR* and *lasI* were supplied in multicopy exhibited a 62-fold increase in expression, and a lysogen in which *lasR* was supplied in *trans* and which was grown in the presence of exogenous PAI exhibited a 60-fold increase. Thus, LasR and PAI are necessary for the full expression of *lasI* in *E. coli*. The interchangeability of the *P. aeruginosa* and *Vibrio fischeri* homologs LasR and LuxR and their respective autoinducers, PAI and VAI, as activators of *lasI-lacZ* was examined. Only the combination of LasR and PAI significantly increased the expression of *lasI*. The comparison of *lasI-lacZ* and *lasB-lacZ* expression in lysogens grown in the presence of *lasR* and PAI revealed that half-maximal expression of *lasI* required 0.1 nM PAI, in contrast to the 1.0 nM PAI necessary for *lasB* half-maximal expression. These results suggest an autoinduction regulatory hierarchy in which LasR and low PAI concentrations primarily activate *lasI* expression in a regulatory loop. With the accumulation of PAI, secondary activation of virulence product genes such as *lasB* occurs.

Multiple homologous regulatory systems which entail the interaction of a diffusible effector molecule, termed an autoinducer, with a transcriptional activating protein to induce expression of different target genes have been described for gram-negative bacteria. The transcriptional activators and autoinducers in these processes are closely related, and the protein activators display significant peptide sequence homology, particularly in their putative DNA-binding domains. *Vibrio fischeri* (6, 7, 20), *Agrobacterium tumefaciens* (24, 33), *Erwinia carotovora* (2, 17), and *Pseudomonas aeruginosa* (22) are among the prokaryotic organisms which utilize this type of regulation, to control bioluminescence (*V. fischeri*), conjugation (*A. tumefaciens*), and virulence factor (*E. carotovora* and *P. aeruginosa*) regulation.

P. aeruginosa, a gram-negative opportunistic human pathogen, uses the transcriptional activator protein, LasR, to control the expression of a number of extracellular factors believed to be important in pathogenesis, such as elastase (LasB) (12), LasA protease (31), alkaline protease (13), and toxin A (13). A *Pseudomonas* autoinducer, PAI, is necessary in conjunction with the transcriptional activator LasR for the maximal activation of the *lasB* gene (22). Together LasR and PAI may form a critical global regulatory system for the expression of important *Pseudomonas* virulence factors.

The production of the *Pseudomonas* autoinducer involves a putative autoinducer synthase (LasI) coded for by the *lasI* gene (22, 23). Since both LasR and PAI are required for the high-level expression of the *lasB* gene, *lasI* expression must be coordinated to provide the putative synthase for the production of PAI when the expression of *lasB* and other virulence factors is required. A better understanding of *lasI* regulation is central to describing the capability of LasR and PAI as a global autoinduction regulatory system. This report details the mapping of the *lasI* transcriptional start site in *Escherichia coli* and *P. aeruginosa*, initial studies of *lasI* regulation by LasR and PAI, the autoinduction hierarchy of *lasI* and *lasB* expression, and the limited interchangeability between the Las elements of *P. aeruginosa* and the Lux elements of *V. fischeri*.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains, plasmids, and bacteriophages used in this study are listed in Table 1. Monolysogens were formed in *E. coli* MG4, a *recA* derivative of *E. coli* MG1655 which also carries a Δ(*argF-lacIPOZYA*)*205* mutation (25). *E. coli* C600 was used for plaque purification and phage lysates (1). Recombinant plasmids were recovered in *E. coli* TB1 (3).

Recombinant DNA techniques. Restriction enzymes, DNA-modifying enzymes, and T4 DNA ligase were obtained from Gibco-Bethesda Research Laboratories (Gaithersburg, Md.), U.S. Biochemicals (Bethesda, Md.), or Boehringer Mannheim (Indianapolis, Ind.). Standard recombinant techniques were used as described by Sambrook et al. (28). λ DNA was prepared for cloning and screening by the methods of Miller (21) and Sambrook et al. (29). Plasmid DNA was prepared by the Magic Miniprep protocol (Promega, Inc., Madison, Wis.). Plasmid and λ DNA fragments were generated by restriction digestion and resolved by agarose gel electrophoresis. Plasmid DNA bands were excised and purified by the GeneClean protocol (Bio101 Inc., La Jolla, Calif.). λ arms were excised from agarose gels and purified by adding 2 volumes of phenol to the agarose containing the λ DNA and frozen at −70°C overnight. The phenol-

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Rochester, 601 Elmwood Ave., Rochester, NY 14642. Phone: (716) 275-3402. Fax: (716) 473-9573.

TABLE 1. Bacterial strains, plasmids, and bacteriophages

Strain, plasmid, or bacteriophage	Relevant genotype or characteristics	Source or reference
Strains		
MG4	Δ(*argF-lac*)*U169 zah-735*::Tn*10* *recA56 srl*::Tn*10*	25
C600	*thi-1 thr-1 leuB6 lacY1 tonA21 supE44*	1
TB1	JM83 *hsdR* (r_K^- m_K^+)	3
Plasmids		
pBluescriptII SK$^+$	Amp^r	Stratagene
pGS560	Cm^r *luxR*	30
pJMC30	Amp^r *lasR′ lasI*	22
pKSBPro	Amp^r *lasBp* (200-bp *Eco*RI-*Hin*dIII fragment)	27
pLPL101	Amp^r *lasI* (1.34-kb *Ssp*I-*Sal*I fragment)	This study
pLPL104	Amp^r *lasR lasI*	22
pLPL105	Amp^r *lasR lasI* (2.4-kb *Bam*HI-*Xho*I fragment)	This study
pMJG1.7	Amp^r *lasR*	12
pPCS1	Amp^r *lasR* (1.1-kb *Bam*HI fragment)	This study
pSW200	Amp^r 1.8-kb stabilizing fragment	12
pUC18	Amp^r	32
pUCP18	Amp^r 1.8-kb stabilizing fragment	29
Bacteriophages		
λTL61	*lacZYA*	19
λB$_2$1P1	*lasB-lacZ*	This study
λI$_1$4	*lasI-lacZ*	This study

agarose mixture was spun at high speed in a microcentrifuge at 4°C for 15 min. The aqueous phase was removed and further extracted with phenol-chloroform (1:1) and then with chloroform. DNA was precipitated with 0.1 volume of 3 M sodium acetate (pH 5.0) and 1 volume of isopropanol at −20°C (28).

Fragments were ligated in vitro with T4 DNA ligase. Plasmid recombinants were recovered in *E. coli* TB1 by $CaCl_2$ transformation (28). Transformed bacteria were selected on LB agar plates (21) containing 100 μg of ampicillin per ml. λ recombinants were recovered by in vitro packaging with the Gigapack Gold protocol (Stratagene, La Jolla, Calif.). Recombinant phage particles were plaque purified by infecting *E. coli* C600 (1) at a multiplicity of infection of approximately 2 and plating dilutions in agar (Difco, Detroit, Mich.). Single plaques picked by drawing an agar plug into a Pasteur pipette were resuspended in 1 ml of SM buffer (28) at 4°C overnight. Five microliters of the resuspension was dotted on a lawn of *E. coli* MG4 over MacConkey agar and grown overnight at 37°C. Recombinant phage suspensions producing pink or red plaques on the lawn, indicating Lac$^+$ phenotypes, were further examined. Stock lysates were made by plate lysates or small liquid lysates essentially as described by Linn and St. Pierre (19) and Sambrook et al. (28). Phage and plasmid constructs were verified by using standard restriction analysis methods (28).

Phage and plasmid constructions. For the construction of λI$_1$4, a *lasI-lacZ* transcriptional fusion in λTL61, pLPL101 was digested with *Eco*RI to obtain the 658-bp fragment containing the last 73 bp of the *lasR* open reading frame, the 365-bp intergenic region between *lasR* and *lasI*, and the first 209 bp of *lasI*. The fragment was ligated into the *Eco*RI site of λTL61 (a kind gift from T. Linn [19]). The lambda phage carrying *lasB-lacZ*, λB$_2$1P1, was constructed by isolating the 200-bp *Eco*RI-*Hin*dIII *lasB* promoter fragment (positions −193 to +2) from pBluescriptII KS$^+$ (27) and ligating it into the respective sites of λTL61. Ligated DNA was packaged, and recombinant particles were examined for β-galactosidase activity as described above. Positive plaque-purified phage DNA was examined for the appropriate fusion and orientation of the insert.

For the formation of a lysogen, a verified *lasI-lacZ* or *lasB-lacZ* phage clone was chosen to infect *E. coli* MG4 at an approximate multiplicity of infection of 0.1 as follows. MG4 was grown overnight in YT medium (21) with 2% maltose, pelleted, and resuspended in an equal volume of SM buffer (28) with 10 mM $MgSO_4$. MG4-phage suspensions (90 μl:10 μl) were mixed and incubated at 23°C for 10 min and at 37°C for an additional 15 min. The phage-bacterium mixture was plated on MacConkey agar (10^{-6} to 10^{-8} dilutions). Lysogens were selected as pink or red colonies after overnight growth at 37°C. Monolysogens were confirmed with the Ter test as described previously (19).

Plasmids were constructed to study the effects of *lasI* and *lasR* on *lasI-lacZ* expression. pLPL101, containing the *lasI* gene, was constructed by ligating the 1.34-kb *Ssp*I-*Sal*I fragment of pJMC30 (22) into *Eco*RV-*Sal*I-digested pBluescriptII SK$^+$ (Stratagene). To construct pPCS1, the *lasR*-containing 1.2-kb *Bam*HI-*Cla*I fragment from pMJG1.7 (12) was ligated into *Bam*HI-*Acc*I-digested pUC18 (32). A 1.1-kb *Bam*HI fragment from this intermediate was subcloned into *Bam*HI-digested pUCP18 (29). pLPL105, containing the *lasR* and *lasI* genes, was constructed by ligating the 2.4-kb *Bam*HI-*Xho*I fragment of pLPL104 (22) into pSW200 (13) digested with *Bam*HI-*Sal*I.

Assay of β-galactosidase activity. Strains were routinely grown at 37°C with shaking at 250 rpm in modified A medium (A medium [28] supplemented with 0.4% glucose, 0.05% yeast extract, and 1 mM $MgSO_4$) and the appropriate antibiotic. Overnight cultures in modified A medium were diluted 1:1,000 in the same medium. Synthetic *Pseudomonas* autoinducer (23) in ethyl acetate was added to each culture tube prior to the addition of culture medium. Ethyl acetate was removed with a stream of nitrogen gas. Except as noted, the final concentration of PAI in experimental cultures was 100 nM. Cultures were grown to an optical density at 600 nm of 0.4 to 0.6. β-Galactosidase activity was measured by the method of Miller (21). The K_{ind} value is defined as the concentration of PAI (nanomolar) required for the half-maximal expression of a *lacZ* fusion.

S1 mapping. Overnight cultures of the desired strains were grown LB medium at 37°C with shaking. Subcultures (0.1% inoculum) in the same medium were grown to early logarithamic growth phase, and RNA from cultures of *P. aeruginosa* PAO1 and PAOR1 and *E. coli* λI$_1$4(pPCS1) grown in the presence and absence of 100 nM PAI was prepared as described by Deretic et al. (4). S1 nuclease mapping was performed as described by Gerhardt et al. (15). The antisense strand of a 621-bp *Eco*RV-*Cla*I fragment from pLPL101 containing the intergenic region of *lasR-lasI* was end labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase as a probe. The radiolabelled probe (3.6×10^4 cpm) was hybridized with 50 μg of total RNA for each bacterial sample. S1 nuclease was added to each reaction mixture in 50- or 400-U amounts. DNA sequencing reactions for the *lasR-lasI* intergenic region were accomplished by using a primer whose end point was identical to that of the labelled probe strand and the Circumvent Thermal Cycling Kit (New England Biolabs, Beverly, Mass.). Sequencing and S1 nuclease reactions were run on a denaturing 8% Tris-borate-EDTA–polyacrylamide gel electrophoresis sequencing gel (28).

RESULTS

Maximal expression of *lasI* requires both LasR and the *Pseudomonas* autoinducer, PAI. Northern (RNA) analysis of the *P. aeruginosa* LasR$^-$ mutant PAOR1 has previously shown this mutant to have a reduced amount of *lasI* mRNA (22). These results suggested the possible requirement for LasR for the expression of *lasI*. The expression of the *lasI* gene, however, had not been measured quantitatively. To elucidate the role of LasR in the regulation of *lasI*, a *lasI-lacZ* transcriptional fusion, λI$_1$4, was constructed on λTL61 (19). This λ-based reporter system contains an RNase III site between the multicloning site and the Shine-Dalgarno sequence upstream of *lacZ*. Cleavage of the resultant fusion mRNA at the RNase III site ensures the independent translation of *lacZ*. In the construction of the *lasI-lacZ* transcriptional fusion, the entire intergenic region between *lasR* and *lasI* was provided to attempt to include the *lasI* promoter and its potential regulatory sequences. The creation of the fusion on a λ phage had three primary advantages to study the regulation of *lasI*. First, *E. coli* was a useful non-*Pseudomonas* gram-negative background to focus on the individual effects of *Pseudomonas* regulatory factors. This model system is particularly important since a defined LasI$^-$ *P. aeruginosa* mutant in which to study the effects of the *Pseudomonas* autoinducer, PAI, has not been successfully obtained. Second, monolysogens can be obtained to eliminate the effects of multicopy vectors. Third, the chromosomal location of the fusion facilitated testing of the effects of regulatory factors whose genes were located on plasmid vectors.

The independent effects of the *lasR* and *lasI* genes were tested in *trans* to the *lasI-lacZ* transcriptional fusion. Neither the presence of *lasI* (pLPL101) nor that of *lasR* (pPCS1) alone on a multicopy plasmid had a significant effect on the expression of *lasI-lacZ* (Fig. 1). Growth of the lysogens in PAI-containing medium also had no significant effect on the expression of *lasI-lacZ* (Fig. 1, pUCP18+PAI). The presence of both *lasR* and *lasI* in multicopy (pLPL105) yielded a sizable increase (62-fold) in expression compared with the control level (Fig.

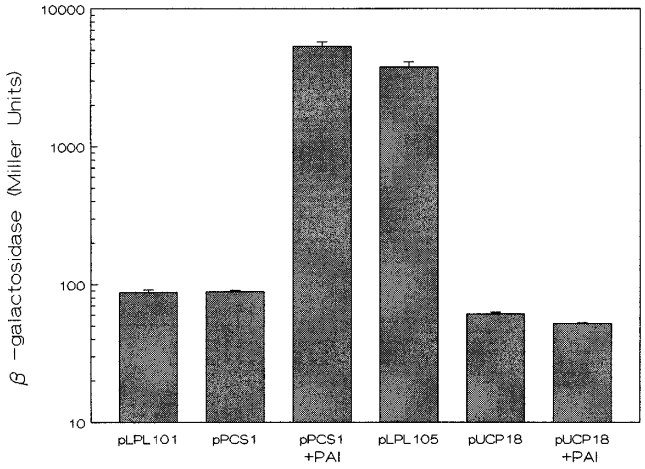

FIG. 1. Effects of LasI and LasR on expression of *lasI-lacZ*. In the monolysogen $\lambda I_1 4$, *lasI* was supplied in *trans* on pLPL101 or *lasR* was supplied in *trans* on pPCS1. pLPL105 carries *lasI* and *lasR*. Control levels of expression were measured with pUCP18 in *trans* (29). Monolysogens of λTL61 were previously shown to produce 71 U of β-galactosidase (19). Where indicated, PAI was present at a concentration of 100 nM. Cultures were grown in modified A medium at 37°C and assayed as described in the text. Values represent data obtained from three independent cultures, each assayed in duplicate. Error bars indicate standard deviations.

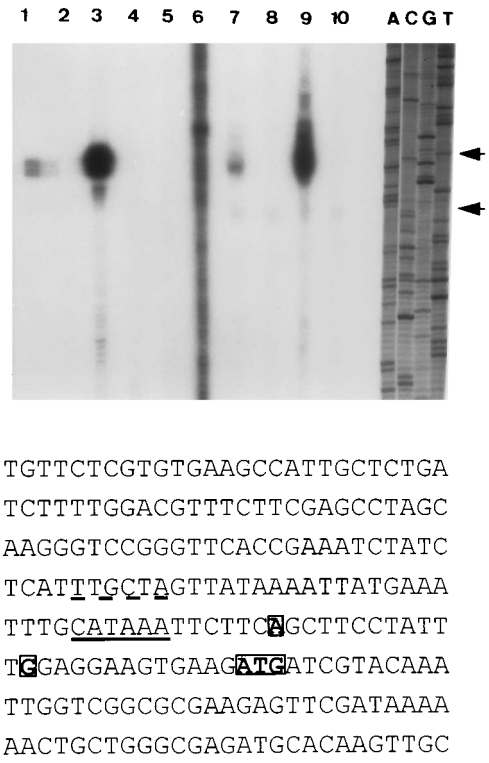

FIG. 2. S1 nuclease mapping of the *lasI* transcriptional start in *P. aeruginosa* and *E. coli*. Fifty micrograms of total RNA was hybridized with 3.6×10^4 cpm of a 621-bp *Eco*RV-*Cla*I probe. S1 nuclease (400 U [lanes 1 to 4] or 50 U [lanes 7 to 10]) was added to the total RNA-probe mixture. Lanes 1 to 4, PAO1, PAOR1, and $\lambda I_1 4$(pPCS1) grown in 100 nM PAI and $\lambda I_1 4$(pPCS1) total RNA, respectively; lane 5, 400 U of S1 nuclease and 3.6×10^4 cpm of probe; lane 6, 3.6×10^4 cpm of probe; lanes 7 to 10, same as lanes 1 to 4, respectively. The upper and lower arrows indicate the primary and secondary transcriptional start sites, respectively. The nucleotide sequence of the region upstream of the *lasI* translational start is also illustrated. The ATG translational start of *lasI* is indicated with a box. The −35 and −10 regions defining the proposed promoter are indicated by dashed and solid underlining, respectively. The dominant start site is indicated by the boxed A nucleotide. The boxed G nucleotide indicates the position of the second identified transcriptional start site.

1). Similarly, high-level expression (60-fold increase) was achieved when *lasR* was present in *trans* (pPCS1) and 100 nM exogenous PAI was added (Fig. 1). LasR appears to be necessary but not sufficient for *lasI* expression. Maximal expression of *lasI* requires both LasR and PAI derived from either the presence of the *lasI* gene or exogenous supplementation.

***E. coli* and *P. aeruginosa* RNA polymerases transcribe *lasI* from the same start point.** S1 mapping demonstrated that *lasI* is expressed from its own promoter contained within the intergenic *lasR-lasI* region (Fig. 2). In the presence of LasR and PAI, both *E. coli* $\lambda I_1 4$(pPCS1) plus PAI and *P. aeruginosa* PAO1 demonstrated the same start of transcription for *lasI* (Fig. 2). This site is located at nucleotide −25 relative to the ATG translational start codon (Fig. 2). Putative σ^{70} −10 (CATAAA) and −35 (TTGCTA) consensus sequences, centered at nucleotides −35 nt and −62, respectively, relative to the *lasI* translational start, have been identified (Fig. 2). In the absence of LasR (PAOR1) or PAI [$\lambda I_1 4$(pPCS1)], a secondary transcriptional start site was consistently observed at nucleotide −13 (Fig. 2). This secondary site suggests that *lasI* is expressed at low levels in the absence of autoinductory regulation.

***lasI* transcriptional activation has a lower K_{ind} and higher maximal expression than *lasB* transcriptional activation.** The levels of expression of *lasI-lacZ* ($\lambda I_1 4$) and *lasB-lacZ* ($\lambda B_2 1P1$) were compared to quantify differences in the strengths of activation by LasR and PAI. The fusions were monitored in the presence of LasR (pPCS1) and a range of PAI concentrations. In the presence of LasR and saturating PAI concentrations, the maximal expression of *lasI-lacZ* was 3.6-fold higher than *lasB-lacZ* expression (Fig. 3). Through graphical analysis (Fig. 3), K_{ind} values were determined as the concentrations of autoinducer required for half-maximal expression. K_{ind} values for *lasI-lacZ* and *lasB-lacZ* were determined to be 0.1 and 1.0 nM, respectively (Fig. 3). The 10-fold difference in K_{ind} values suggests that *lasI* is preferentially activated by LasR and PAI.

LuxR and the *V. fischeri* autoinducer, VAI, do not activate *lasI* expression. LasR and PAI of *P. aeruginosa* and LuxR and VAI of *V. fischeri* were interchanged within the *lasI-lacZ* lysogen to assess whether *lasI* promoter elements are recognized by the *lux* components. *lasR* carried on pPCS1 or *luxR* on pGS560 (a kind gift from T. Baldwin [30]) was put in *trans* in the *lasI-lacZ* lysogen. PAI or VAI (synthesized by Kaplan et al. [18]) was supplied exogenously to the culture medium at a concentration of 100 nM.

As demonstrated in Fig. 4, *lasI* expression increased 49-fold in the presence of LasR and PAI. The *lux* plasmid, pGS560, carrying LuxR, plus 100 nM VAI was insufficient for *lasI* positive regulation, effecting no change in expression over the control level. The combination of LasR and VAI or of LuxR and PAI also caused insignificant changes in *lasI* expression, i.e., β-galactosidase activity remained at control levels.

DISCUSSION

Since the major gene products involved in *P. aeruginosa* autoinduction, LasR and LasI, have close homology to LuxR and LuxI, respectively, of *V. fischeri* (12, 22), common regulatory features of the autoinduction processes might also be found. Among those organisms for which the process of autoinduction has been described, *V. fischeri* has been best studied, and the regulation of bioluminescence is well understood (6, 7,

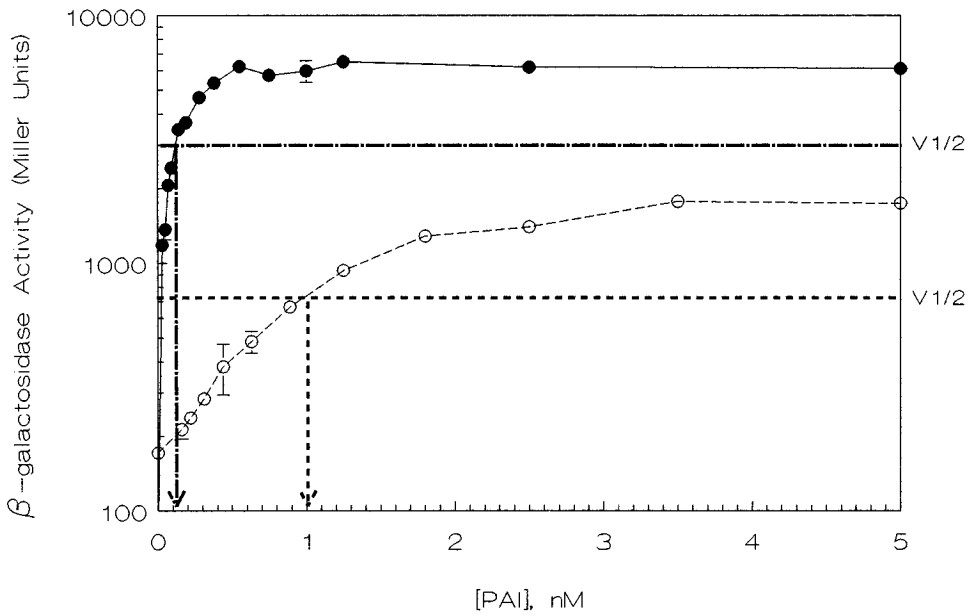

FIG. 3. Comparison of the expression of *lasI-lacZ* and *lasB-lacZ* in the presence of LasR and PAI. Monolysogens of $\lambda I_1 4$(pCS1) (●) and $\lambda B_2 1$P1(pPCS1) (○) were grown for 4 h in modified A medium containing PAI as indicated. Half-maximal expression (V1/2) is shown for *lasI-lacZ* (–·–·–) and *lasB-lacZ* (– – –), and vertical arrows indicate K_{ind} values. Values are means (± standard deviations) for three independent trials, each assayed in duplicate.

11). Studies of the *lux* operon have shown that LuxR and VAI are responsible for the positive transcriptional regulation of *luxI* (8, 9). Similar findings on TraR-AAI regulation of *traI* have been published (10, 16). This report demonstrates that *lasI* is also positively regulated by LasR and PAI at the level of transcription. In addition, S1 mapping of the primary *lasI* transcriptional start point in both *E. coli* and *P. aeruginosa* has produced identical findings, suggesting that the RNA polymerases from the two genera have similar promoter recognitions and interactions with LasR. The *lasB* transcriptional start points in *P. aeruginosa* and *E. coli* have also been S1 nuclease mapped to within 3 bases of each other (26). These findings suggest that *E. coli* can serve as a functionally valid heterologous system in which to better examine and compare the transcriptional activation of genes involved in the *P. aeruginosa* autoinduction process.

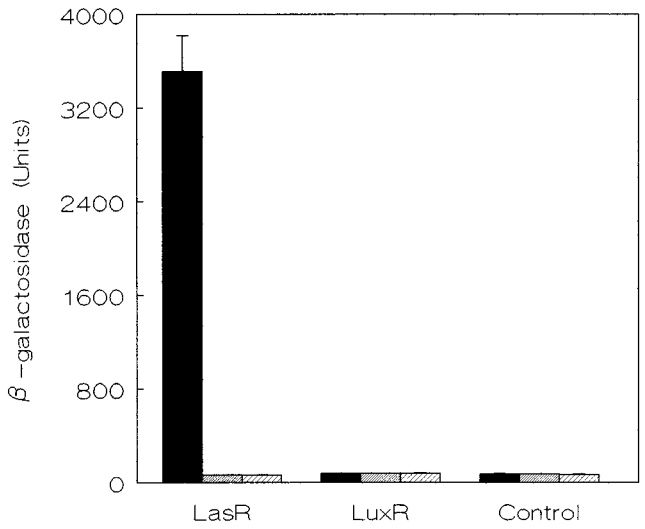

FIG. 4. *lasI-lacZ* expression in the presence of LasR or LuxR and the autologous or homologous autoinducer, PAI or VAI. PAI and VAI were added to culture medium at a concentration of 100 nM. Monolysogens containing *lasR* on pPCS1, *luxR* on pGS560 (30), and the pUCP18 control (29) were grown in modified A medium at 37°C and assayed as described in the text. ■, PAI added; ▨, VAI added; ▨, no addition. Values represent data obtained from three independent cultures, each assayed in duplicate. Error bars indicate standard deviations.

The experiments described herein also show that *lasI* is regulated positively by both LasR and endogenous (by supplying the *lasI* gene) or exogenous PAI. When expression was dependent on endogenous PAI production by LasI (Fig. 2, pLPL105 in *trans*), lower-level expression was detected than when saturating PAI concentrations were added in the presence of LasR (Fig. 2, pPCS1). Additional experiments have shown that expression correlates with the amount of exogenous synthetic autoinducer until a saturating concentration is reached (Fig. 3). The dependence of *lasB-lacZ* expression on autoinducer concentration was previously shown by Pearson et al. (23). The regulation is therefore sensitive to autoinducer concentration, and apparently, enough LasR is present to accommodate even high levels of autoinducer to stimulate increasing levels of expression of *lasI*. High levels of LasR may, however, be from the use of multicopy plasmids.

Despite the close similarities of the *V. fischeri* and *P. aeruginosa* factors for autoinduction, *lasI* cannot be activated by *V. fischeri* LuxR and LuxI. Neither LasR nor LuxR in concert with VAI was able to significantly elevate *lasI* expression. This experiment confirms previous findings that LasR function is specifically activated by PAI. Since the primary structures of the *P. aeruginosa* and *V. fischeri* autoinducers differ only in the length of the fatty acyl side chain (12 and 6 carbons, respectively [5, 23]), this side group must confer the specificity for activation. The specificities of the molecules could be due to their conformational differences or the hydrophobicity-hydrophilicity profile of each molecule. While LuxR and VAI do not affect *lasI* expression, *lasB* expression was previously shown to be increased fourfold by LuxR and VAI as demonstrated with a

lasB-lacZ fusion (14). DNA sequence comparisons demonstrate a "*lux* box-like" 20-bp palindrome in the *lasB* operator region. The *A. tumefaciens traA* and *traI* genes, which are controlled by autoinduction, also have a similar consensus sequence in their operator regions (10, 16). This consensus sequence is not present in the *lasI* upstream region (see reference 12 for sequence information). A unique *las* box could exist as the *cis*-acting element for *lasI*, although its nature remains to be determined.

P. aeruginosa and *V. fischeri* differ extensively in the organization of their autoinducible genes. In *P. aeruginosa*, these differences may necessitate the presence of a unique *las cis* element. In *V. fischeri*, the bioluminescence genes are joined in an operon in which *luxI* expression is coupled with the downstream genes. Transcription of the distal genes is dependent on the *luxI* operator and its regulation. The *P. aeruginosa lasR*, *lasI*, and virulence factor genes such as *lasB*, however, are part of a larger regulon in which each of the virulence factor genes has individual operators with separate LasR-PAI regulatory elements distinct from the *lasI* operator. Some hierarchy in the regulation must therefore exist to ensure that sufficient LasI is produced to provide the PAI required for activation, with LasR, of the virulence factor genes. In *P. aeruginosa*, the correct stoichiometry of *lasI* transcription to the transcription of the virulence factor genes is not ensured simply by an operon structure. The functional differences between the LasR-PAI regulatory elements could, however, provide the balanced expression of *lasI* and the virulence factor genes.

The experiments described in this report demonstrate that *lasI* expression in *E. coli* requires significantly lower concentrations of PAI for half-maximal expression than are required by *lasB*. The differences in activation by LasR and PAI suggest an autoinduction regulatory hierarchy. Although LasR has not yet been shown to directly interact with DNA, it may have a stronger affinity for a unique *las* element possibly involved with *lasI* expression than for the *lux* box-like element in the *lasB* operator. With low levels of PAI, LasR could interact with the higher-affinity element in *lasI*. Larger amounts of the LasI protein would result in additional PAI which would accumulate to a level where enough activated LasR would be present to associate with the lower-affinity elements such as the *lasB lux* box-like element. These primary and secondary tiers may be significant for the temporal aspects of virulence factor production. Even genes in the secondary tier could be differentially regulated by LasR and PAI on the basis of modifications of their *cis*-acting elements. The regulon organization also allows the virulence genes to have operators independent from *lasI* such that other regulatory factors can influence the expression of the *P. aeruginosa* virulence genes independently from autoinduction, as occurs with *toxA* regulation (13).

ACKNOWLEDGMENTS

The *V. fischeri* autoinducer (VAI) used in these studies was furnished by E. P. Greenberg. K. Guertin and A. S. Kende generously synthesized the *Pseudomonas* autoinducer (PAI). We thank K. D. Tucker for his helpful insights.

This research was supported by grant AI 33713 from the National Institute of Allergy and Infectious Disease, and P. C. Seed is supported by NIH grant T32AT07362.

REFERENCES

1. **Appleyard, R. K.** 1954. Segregation of new lysogenic types during growth of a doubly lysogenic strain derived from *Escherichia coli* K12. Genetics **39:** 440–452.
2. **Bainton, N. J., B. W. Bycroft, S. R. Chhabra, P. Stead, L. Gledhill, P. J. Hill, C. E. D. Rees, M. K. Winson, G. P. C. Salmond, G. S. A. B. Stewart, and P. Williams.** 1992. A general role for the *lux* autoinducer in bacterial cell signalling: control of antibiotic biosynthesis in *Erwinia*. Gene **116:**87–91.
3. **Baker, T. A., A. D. Grossman, and C. A. Gross.** 1984. A gene regulating the heat shock response in *Escherichia coli* also affects proteolysis. Proc. Natl. Acad. Sci. USA **81:**6779–6783.
4. **Deretic, V., J. F. Gill, and A. M. Chakrabarty.** 1987. Gene *algD* coding for GDPmannose dehydrogenase is transcriptionally activated in mucoid *Pseudomonas aeruginosa*. J. Bacteriol. **169:**351–358.
5. **Eberhard, A., A. L. Burlingame, C. Eberhard, G. L. Kenyon, K. H. Nealson, and N. J. Oppenheimer.** 1981. Structural identification of autoinducer of *Photobacterium fischeri* luciferase. Biochemistry **20:**2444–2449.
6. **Engebrecht, J., K. Nealson, and M. Silverman.** 1983. Bacterial bioluminescence: isolation and genetic analysis of functions from *Vibrio fischeri*. Cell **32:**773–781.
7. **Engebrecht, J., and M. Silverman.** 1984. Identification of genes and gene products necessary for bacterial bioluminescence. Proc. Natl. Acad. Sci. USA **81:**4154–4158.
8. **Engebrecht, J., and M. Silverman.** 1986. Regulation of expression of bacterial genes for bioluminescence, p. 31–44. *In* J. K. Setlow and A. Hollaender (ed.), Genetic engineering, vol. 8. Plenum Publishing Corp., New York.
9. **Engebrecht, J., and M. Silverman.** 1987. Nucleotide sequence of the regulatory locus controlling expression of bacterial genes for bioluminescence. Nucleic Acids Res. **15:**10455–10467.
10. **Fuqua, W. C., and S. C. Winans.** 1994. A LuxR-LuxI-type regulatory system activates *Agrobacterium* Ti conjugal transfer in the presence of a plant tumor metabolite. J. Bacteriol. **176:**2796–2806.
11. **Fuqua, W. C., S. C. Winans, and E. P. Greenberg.** 1994. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. J. Bacteriol. **176:**269–275.
12. **Gambello, M. J., and B. H. Iglewski.** 1991. Cloning and characterization of the *Pseudomonas aeruginosa lasR* gene, a transcriptional activator of elastase expression. J. Bacteriol. **173:**3000–3009.
13. **Gambello, M. J., S. Kaye, and B. H. Iglewski.** 1993. LasR of *Pseudomonas aeruginosa* is a transcriptional activator of the alkaline protease gene (*apr*) and an enhancer of exotoxin A expression. Infect. Immun. **61:**1180–1184.
14. **Gray, K. M., L. Passador, B. H. Iglewski, and E. P. Greenberg.** 1994. Interchangeability and specificity of components from the quorum-sensing regulatory systems of *Vibrio fischeri* and *Pseudomonas aeruginosa*. J. Bacteriol. **176:**3076–3080.
15. **Hendrickson, W. G., and T. K. Misra.** 1994. Nucleic acid analysis, p. 437–459. *In* P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg (ed.), Methods for general and molecular bacteriology. American Society for Microbiology, Washington, D.C.
16. **Hwang, I., P. L. Li, L. Zhang, K. R. Piper, D. M. Cook, M. E. Tate, and S. K. Farrand.** 1994. TraI, a LuxI homologue, is responsible for the production of conjugation factor, the Ti plasmid N-acylhomoserine lactone autoinducer. Proc. Natl. Acad. Sci. USA **91:**4639–4643.
17. **Jones, S., B. Yu, N. J. Bainton, M. Birdsall, B. W. Bycroft, S. R. Chhabra, A. J. R. Cox, P. Golby, P. J. Reeves, S. Stephens, M. K. Winson, G. P. C. Salmond, G. S. A. B. Steward, and P. Williams.** 1993. The *lux* autoinducer regulates the production of exoenzyme virulence determinants in *Erwinia carotovora* and *Pseudomonas aeruginosa*. EMBO J. **12:**2477–2482.
18. **Kaplan, H., A. Eberhard, C. Widrig, and E. P. Greenberg.** 1984. Synthesis of 3-oxo-(4,5-tritium)-hexanoyl homoserine lactone: biologically active tritium-labelled *Vibrio fischeri* autoinducer. J. Labelled Compounds Radiopharm. **26**(4)**:**387–395.
19. **Linn, T., and R. St. Pierre.** 1990. Improved vector systems for constructing transcriptional fusions that ensures independent translation for *lacZ*. J. Bacteriol. **172:**1077–1184.
20. **Meighen, E. A.** 1991. Molecular biology of bacterial bioluminescence. Microbiol. Rev. **55:**123–142.
21. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Passador, L., J. M. Cook, M. J. Gambello, L. Rust, and B. H. Iglewski.** 1993. Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. Science **260:**1127–1130.
23. **Pearson, J. P., K. M. Gray, L. Passador, K. D. Tucker, A. Eberhard, B. H. Iglewski, and E. P. Greenberg.** 1994. Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. Proc. Natl. Acad. Sci. USA **91:**197–201.
24. **Piper, K. R., S. B. von Bodman, and S. K. Farrand.** 1993. Conjugation factor of *Agrobacterium tumefaciens* regulates Ti plasmid transfer by autoinduction. Nature (London) **362:**448–450.
25. **Ralling, G., S. Bodrug, and T. Linn.** 1985. Growth rate-dependent regulation of RNA polymerase synthesis in *Escherichia coli*. Mol. Gen. Genet. **201:**379–386.
26. **Rust, L.** 1994. Doctoral thesis. University of Rochester, Rochester, N.Y.
27. **Rust, L., and B. H. Iglewski.** 1994. Unpublished data.
28. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Schweizer, H. P.** 1991. *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19. Gene **97:**109–112.
30. **Shadel, G. S., and T. O. Baldwin.** 1991. Positive autoregulation of *Vibrio*

fischeri luxR gene: LuxR and autoinducer activate cyclic AMP-catabolite gene activation protein complex-independent and -dependent *luxR* transcription. J. Bacteriol. **173:**568–575.

31. **Toder, D. S., M. J. Gambello, and B. H. Iglewski.** 1991. *Pseudomonas aeruginosa* LasA: a second elastase under the transcriptional control of *lasR*. Mol. Microbiol. **5:**2003–2010.
32. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
33. **Zhang, L., P. J. Murphy, A. Kerr, and M. E. Tate.** 1993. *Agrobacterium* conjugation and gene regulation by *N*-acyl-L-homoserine lactone. Nature (London) **362:**446–448.