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Vfr Controls Quorum Sensing in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa **controls several genes in a cell density-dependent manner through a phenomenon termed quorum sensing. The transcriptional activator protein of the *las* quorum-sensing system is encoded for by the *lasR* gene, which is at the top of a quorum-sensing hierarchy. The activation of LasR as a transcriptional activator induces the expression of multiple genes that code for factors important for virulence, and *rhlR*, which encodes the transcriptional activator protein of the *P. aeruginosa rhl* quorum-sensing system. Elucidating the method of *lasR* regulation is crucial to understanding *P. aeruginosa* quorum sensing. In this report, we present studies on the transcriptional control of *lasR*. We identified two distinct transcriptional start sites for *lasR* that were located 201 bp (transcript T1) and 231 bp (transcript T2) upstream from the *lasR* start of translation. With the use of transcriptional *lasRp-lacZ* fusions, we showed that in *P. aeruginosa*, *lasR* expression is cell density dependent. This gene was expressed at a basal level until it was induced during the second half of log-phase growth, with expression becoming maximal during stationary-phase growth. We also showed that *lasR* expression was regulated through the cyclic AMP receptor protein (CRP)-binding consensus sequence in its promoter region. Our results from *P. aeruginosa* mutant studies and gel retardation assays indicated that this regulation was mediated by Vfr, a homolog of the *Escherichia coli* CRP.**

The opportunistic pathogen *Pseudomonas aeruginosa* uses quorum sensing to regulate specific genes in a cell density-dependent manner. Quorum-sensing systems are found in many gram-negative bacteria, the first of which was the marine symbiont *Vibrio fischeri*, and consist of a transcriptional activator protein and a small diffusible chemical molecule termed autoinducer (see reference 14 for a review). *P. aeruginosa* has two distinct quorum-sensing systems, *las* and *rhl*. The *las* quorum-sensing system consists of the transcriptional activator protein LasR and the *Pseudomonas* autoinducer PAI-1 [*N*-(3-oxododecanoyl)-L-homoserine lactone] (16, 32, 33). At high cell densities, PAI-1 reaches a threshold concentration and forms a complex with LasR, thereby converting the protein into a transcriptional activator that has been shown to be important for transcription of *lasI*, *lasB*, *lasA*, *apr*, *toxA*, and *rhlR* (17, 23, 32, 36, 42, 48). The *rhl* quorum-sensing system consists of the transcriptional activator protein RhlR and the autoinducer PAI-2 (*N*-butyryl-L-homoserine lactone; formerly named factor 2 [14]) (30, 31, 34). So far, *rhl* quorum sensing has been shown to control the transcription of *rhlA*, *rhlI*, *lasB*, and *rpoS* (4, 23, 29, 35).

In *P. aeruginosa*, a quorum-sensing hierarchy exists in which *las* quorum sensing is dominant. The *las* quorum-sensing system regulates *rhlR* at both the transcriptional and translational levels, which means that the quorum-sensing chain of events begins with *lasR* transcription (23, 36). Therefore, determining the manner in which *lasR* is regulated has become of utmost importance.

Studies of the *V. fischeri luxR* gene, which encodes a LasR homolog, showed that this gene is positively and negatively regulated by LuxR protein and is transcribed in a cell density-dependent manner (8, 44). We have shown that *lasR* is expressed in a cell density-dependent manner and not constitutively as reported by others (23), with expression at a basal level until it is induced in the second half of log-phase growth and then becomes maximal during stationary phase (36). It is also well documented that *luxR* transcription is controlled by the cyclic AMP (cAMP) receptor protein (CRP), which binds to a CRP-binding consensus sequence (CCS) located in the *lux* operon promoter region (9, 43, 47). The report of a perfect CCS in the DNA directly upstream from *lasR* (17), and the identification of the *P. aeruginosa* CRP homolog, Vfr, which was shown to be involved in the control of protease and exotoxin A production (51), hinted that *lasR* may be a CRP-controlled gene.

We present here a study on the transcriptional control of *lasR*. We have determined that *lasR* is transcribed from two distinct transcriptional start sites and confirm that it is expressed in a cell density-dependent manner. Our data also indicate that the CCS upstream from *lasR* is a *lasR* regulatory element. Finally, we show that in *P. aeruginosa*, *lasR* expression requires the *vfr* gene and that Vfr binds specifically to the *lasR* CCS in vitro.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The strains and plasmids referred to in this paper are listed in Table 1. *Escherichia coli* strains were routinely grown in Luria-Bertani (LB [26]) medium or, for single-stranded phage rescue, in 2xYT medium (26) at 37°C. *P. aeruginosa* strains were grown on LB medium or Vogel-Bonner minimal medium (VBMM [50]) agar plates at 37°C. For β-galactosidase (β-Gal) activity assays, *P. aeruginosa* strains were grown at 32°C in TSBD medium, which consists of a deferrated dialysate of trypticase soy broth (Difco Laboratories, Detroit, Mich.) supplemented with 1% glycerol and 50 mM monosodium glutamate (28). The following concentrations of antibiotics

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TABLE 1. Strains and plasmids used

Strain or plasmid	Relevant genotype or description[a]	Reference
P. aeruginosa		
PAO1	Wild type	21
PAO-R1	Δ*lasR*::Tet^r derivative of strain PAO1	16
PAO9001	Strain PAO1 derivative with a 1.2-kb deletion of the chromosomal region containing *vfr*, the 5′ end of *orfX*, and the 3′ end of *trpC*; inserted into the deleted region is a 0.85-kb *Sal*I fragment carrying the *accC1* gene encoding gentamicin resistance	38
PA103	Hypertoxigenic clinical isolate	25
E. coli		
HB101	F$^-$ *mcrB mrr hsdS20 recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20* (Sm^r) *supE44*	3
JM109	F′ *traD36 lacI*^{q} Δ (*lacZ*) M15 proA$^+$B$^+$/e14$^-$ Δ (*lac-proAB*) *thi gyrA endA1 hsdR17 relA1 supE44 recA1*	53
MM294	*endA1 hdR17 thi supE44*	1
TB1	*ara* Δ (*lac-proAB*) *rspL lacZΔM15 hsdR*	2
XL1-blue	*recA1 endA1 thi-1 hsdR17 supE44 lac* [F′ *proAB lacI*^{q} Δ (*lacZ* M15 Tn*10* (*Tc*^{r})]	Stratagene
pBluescript II/KS$^+$	General purpose cloning vector; Ap^r	Stratagene
pKS$^+$1.7	pBluescript II/KS$^+$ with 1.7-kb *Sac*II-*Eco*RI fragment containing the strain PAO1 *lasR* gene; Ap^r	16
pKS$^+$0.4	pKS$^+$1.7 deletion derivative; contains *lasR* promoter region from nt −428 to −4	This study
pQF50	Multicopy, broad-host-range, transcriptional *lacZ* fusion vector; Ap^r	10
pAMA301	pQF50 containing *lasR* promoter region from nt −324 to −4 transcriptionally fused to *lacZ*	This study
pCP13	Low-copy-number broad-host-range cloning vector; Tc^r Km^r	12
pAMA304	*Pst*I fragment that contains *lasRp-lacZ* from pAMA301 in *Pst*I site of pCP13	This study
pUC18	General purpose cloning vector; Ap^r	53
pKDT3	pUC18 containing *lasR* region from nt −428 to +1212	48
pTL61T	*lacZ* transcriptional fusion vector; Ap^r	24
pTL61T0.7	pTL61T containing *lasR* region from nt −428 to +214	This study
pAMA700	pQF50 containing *lasR* region from nt −428 to +214 transcriptionally fused to *lacZ*	This study
pMJG3.9	pBluescript II/KS$^+$ containing *lasR* on a 3,900-bp *Kpn*I-*Eco*RI fragment	16
pCP/Z	pCP13 containing a promoterless *lacZ* on a *Hin*dIII fragment	39
pKS$^+$/R-Z	pBluescript II/KS$^+$ containing *lasR* promoter region from nt −2800 (approximately) to nt −4 fused to *lacZ*	This study
pAMA310	pCP13 containing the *lasRp-lacZ* transcriptional fusion from pKS$^+$/R-Z	This study
pAMA301D	pAMA301 deletion derivative with *lasRp* (nt −280 to −4) fused to *lacZ*	This study
pAMA301A8	pAMA301 insertion derivative with *lasRp*::(8-bp *Kpn*I linker at nt −267) fused to *lacZ*	This study
pAMA301A4	pAMA301A8 deletion derivative with 4 bp removed from center of *Kpn*I site of linker insert	This study
pKS$^+$0.4M1	pKS$^+$0.4 derivative; *lasRp* (nt −285:G→C; nt −272:G→C)	This study
pKS$^+$0.4M2	pKS$^+$0.4 derivative; *lasRp* (nt −287:G→C; nt −274:C→G)	This study
pKS$^+$0.4M3	pKS$^+$0.4 derivative; *lasRp* (nt −286:T→C; nt −271:A→G)	This study
pAMA301M1	pQF50 containing mutated *lasRp* from pKS$^+$0.4M1 transcriptionally fused to *lacZ*	This study
pAMA301M2	pQF50 containing mutated *lasRp* from pKS$^+$0.4M2 transcriptionally fused to *lacZ*	This study
pAMA301M3	pQF50 containing mutated *lasRp* from pKS$^+$0.4M3 transcriptionally fused to *lacZ*	This study
pMMB66HE	broad-host-range *tacp* expression vector; *lacI*^{q} Ap^r	15
ptac917Δp	pMMB66EH containing *tacp-vfr*	52
pUCP18	pUC18 containing a *P. aeruginosa* origin of replication	41
pKF812	pUCP18 containing the *P. aeruginosa vfr* gene	51
pRK2013	conjugation helper plasmid, Tra, ColE1 replicon; Km^r	11

[a] Nucleotides are numbered relative to the start codon of *lasR*. Ap^r, ampicillin-carbenicillin resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance.

were used as needed in the appropriate medium: for *E. coli*, 100-μg/ml ampicillin, 50-μg/ml kanamycin, or 20-μg/ml tetracycline; and for *P. aeruginosa*, 100-μg/ml carbenicillin or tetracycline.

Enzymes and reagents. A Qiagen Mini Plasmid kit (Qiagen Inc., Chatsworth, Calif.) was used for plasmid DNA purification. Restriction-digested or modified DNA was purified from agarose gels with the use of the GeneClean kit (Bio 101, La Jolla, Calif.). Enzymes were purchased from Gibco-BRL (Gaithersburg, Md.), New England Biolabs (Beverly, Mass.), or Boehringer Mannheim (Indianapolis, Ind.) and used as recommended by the manufacturers. DNA sequencing was completed with the use of a Sequenase kit (U.S. Biochemical Corp., Cleveland, Ohio) or the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Perkin-Elmer Corporation, Norwalk, Conn.). Radioisotopes were obtained from Amersham (Arlington Heights, Ill.).

Genetic techniques. Plasmid DNA was introduced into *E. coli* by standard protocols (26). Transformation of plasmids into *P. aeruginosa* was achieved by electroporation (46). For triparental matings in which *P. aeruginosa* received pCP13 or its derivatives, *E. coli* HB101 and MM294(pRK2013) were used as a donor strain and a helper strain, respectively. For experiments in which *P. aeruginosa* contained two compatible plasmids, the second plasmid was always introduced immediately prior to analysis due to plasmid instability. Oligonucleotides were synthesized by George Kampo and Jack Maniloff at the University of Rochester Nucleic Acid Core Facility. Oligonucleotides AA3 to AA10 were synthesized with 5′ end phosphorylation in order to minimize manipulations after PCR.

S1 nuclease analysis and sequencing. Purification of RNA from *P. aeruginosa* grown in LB medium at 37°C was achieved by centrifugation through a CsCl cushion as described by Deretic et al. (5). The transcriptional start site of *lasR* was determined by S1 nuclease analysis as described by Deretic et al. (6). Single-stranded DNA template for probe generation was produced from pKS$^+$1.7. This template included 213 nucleotides (nt) of the *lasR* coding sequence and 324 nt of the *lasR* upstream region. Two different oligonucleotides, termed AA1 (5′ AACCGTCAACCAAGGC 3′) and AA2 (5′ ATCCAAGGTC GCTCGC 3′), which are complementary to nt +19 to +4 and nt +93 to +78, respectively, relative to the *lasR* translational start codon, were hybridized to the template in duplicate S1 nuclease analysis experiments. Hybridized oligonucleotides were extended with Klenow fragment in the presence of [α-^{32}P]dCTP to create uniformly labeled probes. Before treatment with S1 nuclease, purified probes were hybridized with 75 μg of total RNA from strain PAO1 or PAO-R1. Sequencing reactions were performed with the same single-stranded DNA and oligonucleotide primers that were used for probe preparation. The sequencing reactions were subjected to electrophoresis on a 6% acrylamide–8 M urea gel in lanes adjacent to the S1 nuclease reaction mixtures.

Construction of *lasR-lacZ* transcriptional fusions. To begin the construction of a *lasRp-lacZ* transcriptional fusion vector, the *Eco*RI-*Eco*47III fragment contain-

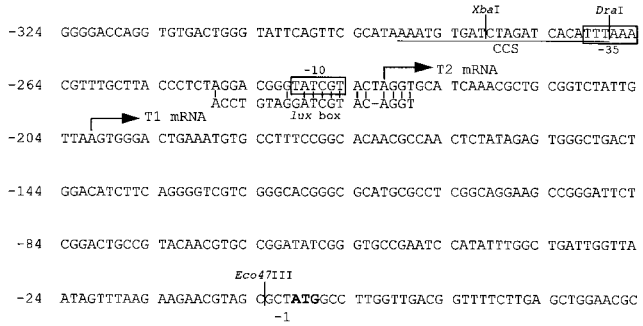

FIG. 1. Nucleotide sequence of the *lasR* promoter region. Numbering is relative to the *lasR* ATG start codon, which is indicated by boldface type. The T1 and T2 *lasR* mRNA start sites mapped by S1 nuclease analysis (see Fig. 2) are indicated by arrows pointing in the direction of transcription. The CCS is underlined, and the potential *lux* box is indicated by alignment with the *V. fischeri lux* box. Potential −35 and −10 regions for a promoter for the T2 start site are boxed. The identified restriction sites were used in plasmid constructions (see Materials and Methods).

ing the *lasR* structural gene was removed from plasmid pKS$^+$1.7. This deletion created plasmid pKS$^+$0.4 which contains a 424-bp fragment of the *lasR* upstream region (−428 to −4 with respect to the translational start codon). A 320-bp *Sma*I-*Hin*dIII fragment was released from pKS$^+$0.4 and ligated into pQF50 digested with the same enzymes to create pAMA301. This plasmid contains the DNA region spanning from −324 to −4 relative to the *lasR* start codon fused transcriptionally to the *lacZ* reporter gene.

To obtain a low-copy-number expression plasmid, an approximately 4-kb *Pst*I fragment that contains the *lasRp-lacZ* fusion was released from pAMA301 and ligated into *Pst*I-digested pCP13 to create pAMA304. To determine if the *lasR* region used to make the *lasRp-lacZ* fusion described above contains all of the sequence necessary for optimal *lasR* transcription, two more plasmids were created. First, a 700-bp *Eco*RI-*Sal*I fragment encompassing the above described 320-bp region plus 214 bp of the *lasR* open reading frame was transferred from pKDT3 into pTL61T, resulting in pTL61T-0.7. The fragment was released from this plasmid via *Bam*HI digestion and ligated into *Bam*HI-digested pQF50. A construct with the proper orientation to create a *lasR′-lacZ* transcriptional fusion was named pAMA700. The second construct was created by transferring an approximately 2,800-bp *Bam*HI-*Eco*47III fragment from pMJG3.9 to pBluescript II KS$^+$. The *Bam*HI-*Eco*47III fragment includes approximately 2.5 kb of DNA upstream from the published *lasR* sequence (16) and spans nt −4 to approximately nt −2800 relative to the *lasR* start codon. The *lacZ* gene contained on a *Hin*dIII fragment released from pCP/Z was added to the unique *Hin*dIII site on this plasmid to create a transcriptional *lasRp-lacZ* fusion. The resulting plasmid, pKS$^+$/R-Z, was digested with *Bam*HI and partially digested with *Hin*dIII to release an approximate 6,500-bp fragment containing the *lasRp-lacZ* fusion which was ligated into *Bam*HI/*Hin*dIII-digested pCP13, resulting in plasmid pAMA310. All *lasR-lacZ* fusion junctions were sequenced to ensure DNA integrity.

Constructing mutations within the *lasR* promoter region. To create pAMA301D, a 50-bp *Xba*I fragment was released from pAMA301 and the plasmid was religated. This resulted in a deletion of the *lasR* upstream region from −324 to −281 relative to the *lasR* start codon, which removes half of the CCS upstream from *lasR* (Fig. 1). Plasmid pAMA301A8 was constructed by inserting an 8-bp *Kpn*I linker (purchased from Promega [Madison, Wis.]) into the unique *Dra*I site (Fig. 1) immediately downstream from the CCS. This 8-bp insertion increases the spacing between the center of the CCS and *lasR* T1 and T2 transcriptional start sites to 85.5 and 55.5 bp, respectively. The spacing in pAMA301A8 was decreased 4 bp by digesting the plasmid with *Kpn*I, removing the overhanging 3′ ends with T4 DNA polymerase, and religating the plasmid to create pAMA301A4.

To construct point mutations within the CCS, we used the inverse PCR technique described by Hemsley et al. (20). Briefly, sets of 5′ phosphorylated primers were designed so that each primer in a set contained a point mutation that would change the CCS upstream from *lasR*. The 5′ ends of the primers in a set annealed to adjacent nucleotides on opposite DNA strands so that no overlap between primers occurred. In this manner, the primers are designed back to back so that they will produce a PCR product that can be ligated to form a plasmid with the directed mutations. The plasmid pKS$^+$0.4 was used as a template for PCR (with *Taq* polymerase [Gibco-BRL]) with the following parameters: 25 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 12 min. Ligated products were transformed into *E. coli* XL1-Blue. Three sets of mutant primers (AA3 and AA4, AA5 and AA6, and AA7 and AA8) were designed to create changes in the CCS while preserving its palindromic nature. Primers AA3 and AA4 resulted in a product in which the G at position 5 and the C at position 18 of the CCS were changed to a C and a G, respectively. The ligated product of this reaction was named pKS$^+$0.4M1. Primers AA5 and AA6 resulted in pKS$^+$0.4M2, which has the G at position 7 and the C at position 16 of the CCS changed to a C and a G, respectively. Primers AA7 and AA8 resulted in pKS$^+$0.4M3, which has the T at position 4 and the corresponding A at position 19 of the CCS changed to C and G, respectively. The *Sma*I-*Hin*dIII, mutation-containing fragments from pKS$^+$0.4M1, pKS$^+$0.4M2, and pKS$^+$0.4M3 were ligated into *Sma*I-*Hin*dIII digested pQF50, resulting in pAMA301M1, pAMA301M2, and pAMA301M3, respectively. The *lasR* promoter region of all mutated plasmids was sequenced to ensure DNA integrity.

Purification of Vfr. Vfr was purified from a crude cell lysate of *P. aeruginosa* PA103(ptac917Δp) by affinity chromatography on cAMP-agarose as described by Ghosaini et al. (18), with the following modifications. To overexpress Vfr, isopropyl-β-D-thiogalactopyranoside (IPTG) was added at mid-log phase to a final concentration of 1 mM. After an additional 6 h of growth, the cells were harvested by centrifugation for 15 min at 5,000 × *g*. To prepare the crude lysate, the cell pellet was resuspended in 100 mM Tris-HCl (pH 8.0)–50 mM KCl–2 mM EDTA–5 mM β-mercaptoethanol–1 mM sodium azide–200-μg/ml lysozyme, and the suspension was incubated at 25°C for 20 min. The lysate was adjusted to 25 mM Tris-HCl (pH 8.0), 25 mM $MgSO_4$, and 1% Brij 35 and incubated at 4°C for 10 min. To decrease the viscosity due to nucleic acids, the lysate was sonicated at approximately 100 W for three 10-s intervals on ice with a Vibra Cell sonicator (Sonics and Materials, Inc., Danbury, Conn.). The lysate was centrifuged at 16,000 × *g* for 30 min at 4°C to remove cellular debris and was applied to a 5-ml cAMP-agarose column (Sigma Chemical Co., St. Louis, Mo.) as described by Ghosaini et al. (18). Vfr was determined to be greater than 90% pure as measured by Commasie brilliant blue staining of a polyacrylamide gel. The amino terminus of Vfr was sequenced at the Howard Hughes Medical Institute, Columbia University Protein Chemistry Core Facility (New York, N.Y.). The sequence obtained (VAITHTPKLK) matched the deduced amino acid sequence of the cloned *vfr* gene (51).

Gel retardation assays. The *lasR* promoter region was isolated from either pAMA301, pAMA301D, pAMA301M1, pAMA301M2, or pAMA301M3 as a 350-bp *Pst*I-*Hin*dIII fragment. The fragments were end labeled with the use of α-^{32}P-labeled deoxynucleoside triphosphates (NEN Research Products, Boston, Mass.) and the Klenow fragment (U.S. Biochemical Corp.). The binding reactions were carried out as described by DeVault et al. (7), with the following modifications. The radiolabeled DNA fragments (at concentrations of approximately 15 to 40 pM) and various concentrations of purified Vfr were incubated in 10 mM Tris-HCl (pH 7.4)–100 mM KCl–1 mM EDTA–5% glycerol–1 mM dithiothreitol–50-μg/ml bovine serum albumin–20 μM cAMP for 20 min at room temperature. To detect binding, the samples were electrophoresed on a 6% native polyacrylamide gel in 10 mM Tris-HCl (pH 7.4)–1 mM EDTA–20 μM cAMP for 1.5 h at 10 V/cm at 4°C. The gel was dried and exposed to Kodak X-Omat AR5 film at −70°C overnight with an intensifying screen. Control experiments showed that Vfr did not bind to the *lasR* promoter when cAMP was omitted from the binding assays (data not shown).

β-Gal activity assays. For assays involving dual-plasmid systems, freshly created transformants were used to avoid plasmid rearrangements. Single-colony isolates of *P. aeruginosa* strains were inoculated into TSBD medium (with appropriate antibiotics) and grown overnight at 32°C with shaking. Overnight cultures were subcultured into fresh medium at a ratio of 1:200 and were grown under the same conditions. At the indicated time points, aliquots were removed and centrifuged at 14,000 rpm in an Eppendorf Microfuge for 5 min. Cell pellets were resuspended in 0.1 M phosphate buffer (pH 7.0) at ratios of 1:1 to 1:20 to obtain a final optical density at 540 nm (OD_{540}) of 0.2 to 0.5 and were stored at −20°C. Samples were assayed in triplicate, from at least two independent cultures, for β-Gal activity as described by Miller (27). The *t* distribution was used to establish 95% confidence limits.

RESULTS

Determination of the *lasR* transcriptional start site. The transcriptional start site of *lasR* was mapped by S1 nuclease analysis. Two different oligonucleotide primers, AA1 and AA2 (see Materials and Methods), were used to make probes for analysis of total RNAs from strains PAO1 and PAO-R1. The results of S1 nuclease analysis with both primers are presented in Fig. 2. With both primers, the *lasR* transcriptional start sites were mapped to the same bases, showing that two distinct *lasR* transcriptional start sites existed. The lower and upper arrows in Fig. 2 point to the protected probes that correspond to nt −201 (T1 transcript) and −231 (T2 transcript), relative to the *lasR* start codon. The band for the larger protected probe in Fig. 2B is light; however, it was clearly visible on our original autoradiograph and is located exactly next to the same sequence band to which the larger protected probe band is adjacent in Fig. 2A. The S1 nuclease analysis also shows that the

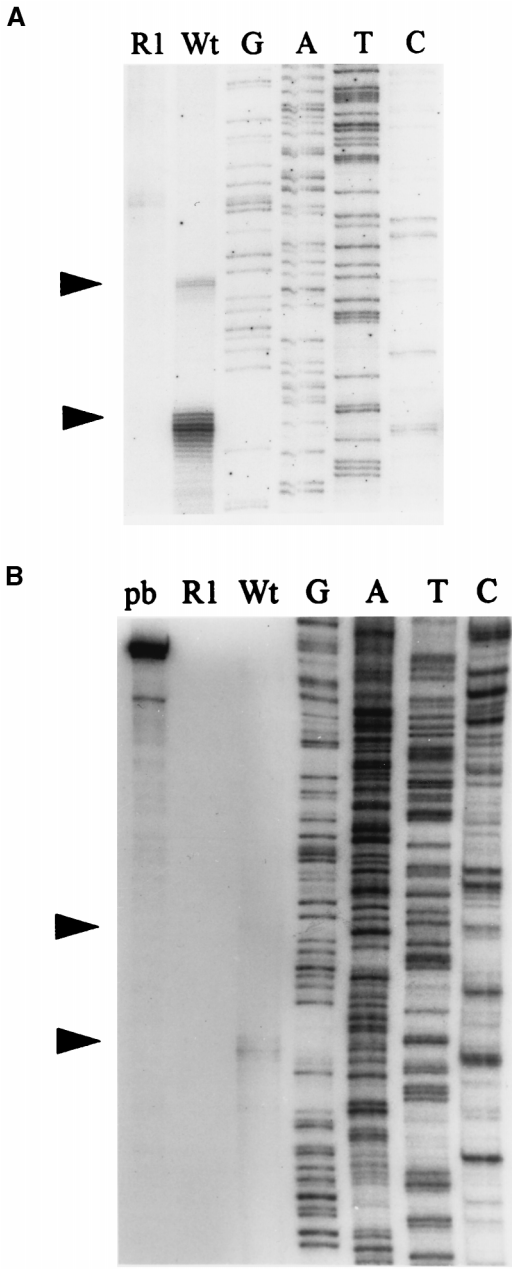

FIG. 2. S1 nuclease analysis of the 5′ end of the *lasR* transcript. Sequencing reaction lanes are labeled according to nucleotides (G, A, T, and C). Sequence was obtained by using the same DNA and oligonucleotide that were used to generate the probe for each experiment. The arrows point to the probe fragments in lanes Wt that remained after digestion with S1 nuclease. Lanes Wt and R1, total RNA purified from *P. aeruginosa* PAO1 and PAO-R1, respectively. Probes (lane pb) were generated with the use of oligonucleotide primers AA1 (A) and AA2 (B) as described in Materials and Methods.

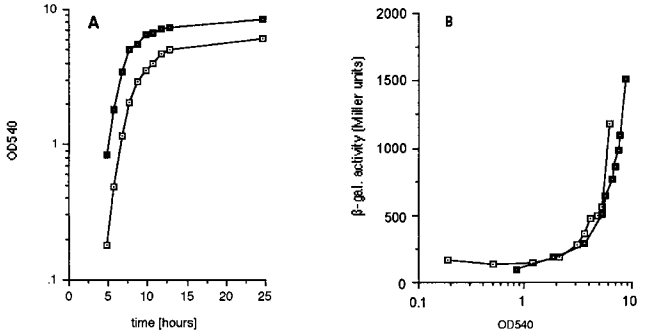

FIG. 3. Time course of *lasR* expression in *P. aeruginosa*. Expression from distinct *lasRp-lacZ* fusions was monitored at different time points during the growth cycle of *P. aeruginosa* PAO1 containing either pAMA304 (low copy number [□]) or pAMA301 (high copy number [■]). The data presented are from the averages of triplicate β-Gal assays from one experiment with one culture for each strain but are representative of multiple, independently performed time course experiments. (A) Growth curve showing culture absorbance at 540 nM plotted against time; (B) β-Gal activities at various culture absorbance measurements.

T1 transcript is more abundant than the T2 transcript (Fig. 2), suggesting that T1 may be the major start site for *lasR* transcription. As expected, neither of these transcriptional start sites was present when total RNA from strain PAO-R1, a *lasR* deletion mutant, was analyzed as a negative control (Fig. 2).

The DNA region directly upstream from the T1 site contains no sequence that is highly similar to a known sigma factor consensus sequence. However, the region directly upstream from the T2 site contains a sequence that is similar to a σ^{70}-type promoter consensus sequence. This potential promoter has −35 and −10 regions that begin 39 and 9 bp upstream from the T2 site, respectively, and these regions matched 4 of 6 bp with both the consensus −35 and the consensus −10 regions for a σ^{70}-type promoter (Fig. 1). More interestingly, the T2 and T1 sites are located 47.5 and 77.5 bp, respectively, downstream from the center of the CCS that was identified upstream from *lasR* (Fig. 1) (17). This sequence is a 22-bp palindrome that is 100% identical to the consensus sequence for *E. coli* CRP-binding sites (19), which caused us to hypothesize that the CCS is a *lasR* regulatory element (see below). We also point out that there is a sequence beginning 17 bp upstream from the T2 transcriptional start site that is reasonably similar to that of the *V. fischeri lux* box (Fig. 1). This sequence matches 13 of 20 bp with the *lux* box and is positioned so that it overlaps with the T2 transcriptional start site and is centered 37 bp upstream from the T1 transcriptional start site. The *lux* box of *V. fischeri* has been shown to be the binding site for LuxR, and similar sequences upstream from the *P. aeruginosa lasB* gene were shown to be transcriptional regulatory elements (40, 47). The location of a potential *lux* box upstream from *lasR* may be an indication of another level of *lasR* regulation which will be discussed below.

Expression of *lasR* during *P. aeruginosa* growth. To determine the effect of copy number on the expression of *lasRp-lacZ* fusions, we monitored β-Gal expression in *P. aeruginosa* PAO1(pAMA301) and PAO1(pAMA304). Cultures grown at 32°C in TSBD medium supplemented with carbenicillin (100 μg/ml) were monitored throughout the growth cycle, and the results are presented in Fig. 3. Minor growth differences were seen between strains PAO1(pAMA301) and PAO1 (pAMA304) (Fig. 3A); however, these differences did not affect the temporal expression of *lasR*. When plotted against cell density as measured by OD_{540} values, *lasR* induction kinetics from strains PAO1(pAMA301) and PAO1(pAMA304) appeared identical (Fig. 3B). These results indicated that transcription of *lasR* was at a basal level until it was induced in the last half of log-phase growth and then reached a maximal level during the stationary phase. This is in exact agreement with the results obtained with cultures, which contained a different *lasR-lacZ* fusion plasmid, that were grown in PTSB medium at 37°C (36). This is another indication that *lasR* expression in-

TABLE 2. Comparison of expression levels from different *lasR-lacZ* fusion plasmids in strain PAO1

Plasmid	β-Gal activity[a]
pAMA304	1,502 (1,472/1,532)
pAMA310	1,449 (1,324/1,573)
pAMA301	1,760 (1,657/1,863)
pAMA700	1,124 (1,084/1,163)
pAMA301A8	1,247 (1,138/1,356)
pAMA301A4	390 (308/472)

[a] β-Gal activity was assayed after 24 h of growth from duplicate cultures of strain PAO1 containing the indicated plasmids. Data are the means of triplicate β-Gal activity assays from at least three separate experiments, and activity was expressed as Miller units (numbers in parentheses are 95% confidence intervals).

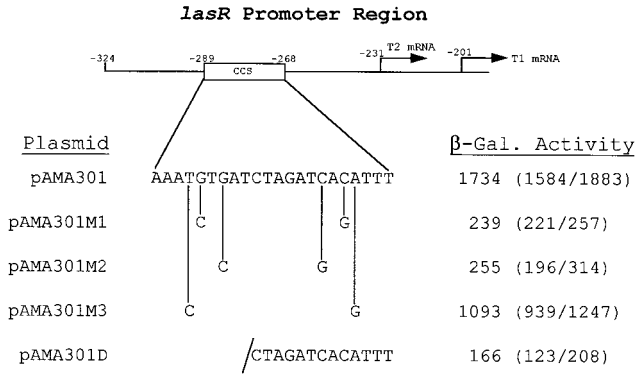

FIG. 4. Effect of the CCS on *lasR* expression in *P. aeruginosa*. Expression from wild-type and mutated *lasRp-lacZ* fusions was monitored by measuring β-Gal activity to determine the influence of the CCS on *lasR* transcription. β-Gal activity was measured from cultures of strain PAO1 containing the indicated plasmids after 24 h of growth. Data are β-Gal activity measurements (in Miller units) and are the means of at least three independent experiments with duplicate cultures. Values in parentheses are 95% confidence intervals. Nucleotide changes (or deletion) in the CCS of the *lasRp-lacZ* fusion on each plasmid are indicated. Plasmid pAMA301 contains a *lasRp-lacZ* fusion with the wild-type CCS. Numbering of the *lasR* promoter region is relative to the *lasR* start codon.

creases at higher cell densities, which conflicts with a report claiming that *lasR* is expressed constitutively (23).

To ensure that pAMA301 and pAMA304 contained all sequences upstream from *lasR* required for maximal *lasR* expression, pAMA310 was constructed. This plasmid contains the approximately 2,800 bp of DNA directly upstream from the *lasR* start codon transcriptionally fused to *lacZ*. Comparison of β-Gal activities from strain PAO1 harboring either pAMA310 or pAMA304 (both contain the same base vector) showed no significant difference in *lasR* expression from these plasmids (Table 2). This indicated that the DNA upstream from *lasR* that is required for maximal expression is contained in the region from nt −324 to −4 relative to the *lasR* start codon.

To evaluate whether portions of the *lasR* sequence downstream from the translational start site might also be involved in *lasR* transcriptional regulation, pAMA700, which contains nt −428 to +214 relative to the *lasR* start codon, was constructed. A comparison of β-Gal activities from strain PAO1 harboring pAMA700 or pAMA301 (both contain the same base vector) showed that *lasR* transcription in strain PAO1(pAMA700) was 36% less than that in strain PAO1(pAMA301) (Table 2). This suggested that the DNA of the *lasR* open reading frame had a negative effect on *lasR* transcription. The reason for this effect is not apparent. The *lasR* open reading frame may contain regulatory elements such as a DNA-binding site for a repressor or a transcription attenuation region that cause this effect. Alternatively, including part of the *lasR* open reading frame in the fusion might have resulted in a hindrance of *lacZ* transcription by *lasR* translational machinery.

Involvement of the CCS in *lasR* transcriptional regulation. To determine if the CCS in the *lasR* promoter region was involved in the regulation of *lasR*, we constructed a series of plasmids containing *lasRp-lacZ* fusions with mutations in the putative CCS (see Fig. 4 for mutated sequences). Deletion of nt −324 to −281 (relative to the *lasR* start codon) from pAMA301 removed the first 9 bp of the CCS and resulted in plasmid pAMA301D. Expression of *lasR* in strain PAO1(pAMA301D) was reduced by 90% compared to that seen in strain PAO1(pAMA301) (Fig. 4). This indicated that the 43-bp region (which contains part of the CCS) deleted from pAMA301 was extremely important for *lasR* transcription. To determine if specific base pairs within the CCS were involved in *lasR* regulation, site-directed mutations that preserved the CCS palindromic nature were made. Compared to the wild-type promoter (pAMA301), mutations at positions 5 and 18 (pAMA301M1) and at positions 7 and 16 (pAMA301M2) of the CCS caused *lasR* expression to decrease by 86 and 85%, respectively (Fig. 4). This not only showed that these bases of the *lasR* promoter region were important for *lasR* expression but also suggested that the CCS in which they were contained was also involved in *lasR* regulation. Mutations at positions 4 and 19 (pAMA301M3) of the CCS caused *lasR* expression to decrease by 37% compared to that of the wild-type promoter (pAMA301) (Fig. 4). This indicated again that these bases were important for *lasR* regulation and provided more evidence to support CCS involvement in *lasR* control. However, these bases were obviously not as critical as those mutated at positions 5 and 18 or at positions 7 and 16 of the CCS. The conclusions from our site-directed mutation studies are in agreement with those made from similar mutation studies regarding the affinity of CRP for the CCS of *E. coli* in vitro (18, 22).

To further investigate the involvement of the CCS in the activation of *lasR* transcription, the spacing between the CCS and the transcriptional start sites was altered. It has been shown that insertions or deletions of nonintegral helical turns between a CCS and a transcriptional start site eliminated activation of transcription by CRP in *E. coli* (49). In the wild-type *lasR* promoter, spacings between the center of the CCS and the T1 and T2 starts of transcription are 77.5 and 47.5 bp, respectively. Increasing this spacing by 4 bp (pAMA301A4) or 8 bp (pAMA301A8) caused a decrease in *lasR* transcription of 78 or 29%, respectively, compared to that with the wild-type promoter (pAMA301) (Table 2). This result indicated that the spacing between the transcriptional start sites and CCS of *lasR* is important but not crucial for *lasR* transcription.

Effect of *vfr* on *lasR* transcription. The *P. aeruginosa* Vfr protein is a homolog (67% identical and 91% similar) of the *E. coli* CRP (51). Our data presented above showed that *lasR* is regulated by a CCS; thus, it follows that Vfr should be involved in *lasR* expression. To determine whether Vfr is required for *lasR* transcription, we monitored β-Gal activity in the defined, *P. aeruginosa vfr* deletion mutant strain PAO9001, which contained pAMA304. The expression of *lasR* in strain PAO9001 (pAMA304) was decreased by 90% compared to that seen in the wild-type strain PAO1(pAMA304) (Table 3), indicating that Vfr was required for *lasR* transcription. To demonstrate that the cloned *vfr* gene could restore *lasR* transcription to a *vfr* mutant, strains PAO1 and PAO9001 containing pAMA304 were transformed with pKF812 and were monitored for β-Gal

TABLE 3. Effect of Vfr on *lasR* expression in *P. aeruginosa*

Strain (containing pAMA304)	β-Gal activity from strain containing the following second plasmids[a]		
	None	pUCP18	pKF812
PAO1	1,502 ± 45	1,977 ± 61	2,338 ± 73
PAO9001	153 ± 7	430 ± 46	1,444 ± 173

[a] β-Gal activity was assayed from 24-h cultures of *P. aeruginosa* containing pAMA304 and the indicated second plasmids. Data are the means of triplicate β-Gal activity assays from at least two separate experiments, and activity was expressed as Miller units ± standard deviations.

expression. Strain PAO1(pAMA304, pKF812) showed a slight increase in *lasR* expression compared to that for the control, strain PAO1(pAMA304, pUCP18) (Table 3). More interestingly, strain PAO9001(pAMA304, pKF812) had 3.4-fold more *lasR* expression than the control, strain PAO9001(pAMA304, pUCP18) (Table 3). This increase restored *lasR* expression to 62% of that measured in strain PAO1(pAMA304, pKF812) and restored expression to that measured in strain PAO1(pAMA304) (Table 3). The modest effect seen on *lasR* expression by the addition of pUCP18 as a control vector to either strain PAO1(pAMA304) or strain PAO9001 (pAMA304) was unexpected and does not have an apparent explanation (Table 3). Nevertheless, these results showed that as our studies of the *lasR* CCS predicted, *vfr* was important for *lasR* expression in *P. aeruginosa.*

Binding of Vfr to the *lasR* promoter. To determine whether the *vfr*-dependent activation of *lasR* transcription presented above occurred via an interaction between Vfr and the CCS in the *lasR* promoter, gel retardation assays were performed. Purified Vfr was tested for the ability to bind a 350-bp *Pst*I/*Hin*dIII DNA fragment which contained the wild-type *lasR* promoter from pAMA301. As shown in Fig. 5, Vfr altered the mobility of the wild-type *lasR* promoter fragment in a concentration-dependent manner, indicating that Vfr bound to the DNA upstream from *lasR*. We also found that addition of excess, unlabeled *lasR* promoter DNA prevented Vfr from binding the labeled *lasR* promoter fragment and that the mobility of the *lasR* promoter fragment from pAMA301D was not altered in a gel retardation assay (data not shown). This indicated that Vfr binding to *lasR* promoter DNA was specific and that the region from bp −324 to −281 (which contains part of the CCS) relative to the *lasR* start codon was required for this interaction. To determine if the CCS was important for Vfr to bind upstream from *lasR*, we performed gel retardation assays using the *Pst*I/*Hin*dIII DNA fragment from the site-directed,

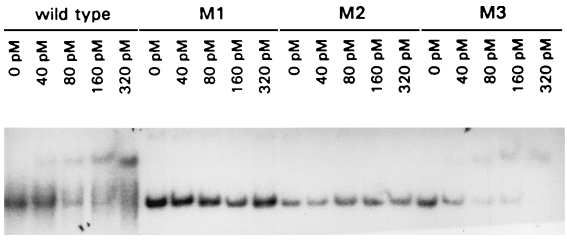

FIG. 5. Vfr binds to the *lasR* promoter region. A gel retardation experiment was performed to show that Vfr interacts with the *lasR* promoter region. Radiolabeled DNA fragments containing either the wild-type or the mutated *lasR* promoter were incubated with purified Vfr as described in Materials and Methods. Headings indicate the sources of *lasR* promoter-containing fragment as follows: wild type, pAMA301; and M1, M2, and M3, pAMA301M1, pAMA301M2, and pAMA301M3, respectively (see Fig. 4 for sequence changes). Protein concentrations are indicated above each gel lane.

CCS mutant plasmids pAMA301M1, pAMA301M2, and pAMA301M3 (see Fig. 4 for CCS mutations). As shown in Fig. 5, purified Vfr protein did not bind to fragments containing mutations at either positions 5 and 18 (pAMA301M1) or at positions 7 and 16 (pAMA301M2) of the CCS. This showed that DNA within the CCS was required for Vfr binding, suggesting that the CCS upstream from *lasR* is a Vfr-binding site. The *lasR* promoter fragment containing mutations at positions 4 and 19 of the CCS was bound by Vfr (Fig. 5), indicating that these base pairs are not critical for Vfr binding to the CCS. These results are consistent with those shown in Fig. 4, where we found that changes in positions 4 and 19 of the CCS are not as important for CCS control of *lasR* expression. The overall conclusion from our studies of the CCS upstream from *lasR* is that this sequence is a *lasR* regulatory element that is a binding site for Vfr.

DISCUSSION

With the use of two distinct probes, our S1 nuclease analysis of the *lasR* promoter region showed that two *lasR* transcriptional start sites exist. A major start site (T1) and a minor start site (T2) were located at nt −201 and −231, respectively, relative to the *lasR* start codon. Subsequent inspection of the DNA sequence upstream from these start sites revealed two very interesting regions. Gambello et al. (17) reported that a 22-bp palindromic sequence identical to an *E. coli* CCS was present upstream from *lasR*. We determined that this CCS was centered at bp −77.5 and −47.5 upstream from the T1 and T2 start sites, respectively. This spacing placed the CCS in a location where it could control transcription from either *lasR* mRNA start site. The second interesting sequence found upstream from *lasR* is a region that matches 13 of 20 bp with the *lux* box of *V. fischeri*. This potential *lux* box is centered 37 bp upstream from the T1 transcriptional start site and only 7 bp from the T2 transcriptional start site. Genes that are controlled by quorum sensing often have a *lux* box-like sequence centered approximately 40 bp upstream from their transcriptional start site (14), which adds evidence to our argument that *lasR* is controlled in a cell density-dependent manner. The fact that the potential *lux* box upstream from *lasR* also covers the T2 transcriptional start site allows for numerous potential avenues of control. Obviously, the DNA in and around the potential *lux* box of the *lasR* promoter region will require further study to determine if it is involved in *lasR* regulation.

Our experiments on the temporal expression of *lasR* showed that in *P. aeruginosa* PAO1 grown in TSBD medium at 32°C, *lasR* expression from low- or high-copy-number *lasRp-lacZ* fusions is induced during the second half of log-phase growth and steadily increases to maximal levels at stationary phase. We have also shown elsewhere that *lasR* is expressed in the same manner when grown in PTSB medium at 37°C (36). These two studies show independently that *lasR* is expressed in a cell density-dependent manner rather than constitutively as reported by Latifi et al. (23). It was also shown by Pesci et al. (36) that *lasR* expression does not reach a maximal level in the absence of PAI-1 and PAI-2. Evidence for autoregulation of *lasR* was not surprising in light of reports that show autoregulation of *V. fischeri luxR* and *Agrobacterium tumefaciens traR* (13, 45). This may be an indication that the potential *lux* box in the *lasR* promoter region is acting as a regulatory element through which LasR could bind and exert another level of control on *lasR* induction.

West et al. (51) showed that Vfr is required for exotoxin A and protease production by *P. aeruginosa*. LasR is also known to be required for protease production and maximal exotoxin

A production (16, 48). These facts, accompanied by the report of Gambello et al. (17) of a perfect CCS located upstream from *lasR*, led us to hypothesize that Vfr controlled *lasR* expression. To determine if this was true, we began by studying the CCS upstream from *lasR*. These studies showed that this sequence is a significant *lasR* regulatory element. Deletion of an upstream portion of the *lasR* promoter which removed the first 9 bp of the CCS caused *lasR* expression to be at a basal level in *P. aeruginosa*. We then showed that specific base pairs within the CCS were crucial for *lasR* transcription. Double point mutations that preserved the palindromic nature of the CCS resulted in significant decreases in *lasR* expression, indicating that the CCS functions as a *lasR* regulatory element. This conclusion was also supported by our finding that changes in spacing between the CCS and the *lasR* transcriptional start sites affect the level of *lasR* transcription. *E. coli* promoters which are positively regulated by the transcriptional activator CRP usually have a CCS centered at bp −41, −61, or −72 upstream from the start of transcription (22, 37). In the wild-type *lasR* promoter, the CCS is centered at nt −77.5 and −47.5 from the T1 and T2 start sites, respectively. Our experiments suggest that spacing is important for the CCS to regulate *lasR*, but that the optimal spacing between a CCS and transcriptional start site in *P. aeruginosa* may be different from that of *E. coli.*

Our in vivo and in vitro studies of Vfr's effect on *lasR* prove that Vfr is the major regulator of *P. aeruginosa* quorum sensing. We demonstrate that a defined *P. aeruginosa vfr* mutant strain has greatly reduced levels of *lasR* expression which can be restored to a wild-type level by complementing with the *vfr* gene in *trans*. We followed this with an in vitro analysis that showed that Vfr binds specifically to the *lasR* promoter region. Mutations within the CCS stopped this binding, indicating that the binding affinity of Vfr for the *lasR* promoter region is dependent on the CCS. Thus, it appears that activation of *lasR* transcription is dependent on the interaction of the Vfr protein with the CCS present in the *lasR* promoter region. This means that the *P. aeruginosa* quorum-sensing cascade begins with the activation of *lasR* transcription by Vfr. Its place at the top of this cascade, which includes regulation of *rhl* quorum sensing by *las* quorum sensing and the activation of multiple virulence genes by both systems, leads us to summarize that Vfr is a crucial component of *P. aeruginosa* virulence.

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