Regulation of the *Pseudomonas aeruginosa* Quorum-Sensing Regulator VqsR[∇]

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Bacteria communicate with each other to regulate cell density-dependent gene expression via a quorum-sensing (QS) cascade. In *Pseudomonas aeruginosa*, two known QS systems, *las* and *rhl*, control the expression of many factors that relate to virulence, pathogenicity, and biofilm development. Microarray studies of the *las* and *rhl* regulons led to our hypothesis that a complicated hierarchy in the QS regulon is composed of multiple transcriptional regulators. Here, we examined a QS-regulated gene, *vqsR*, which encodes a probable transcriptional regulator with a putative 20-bp operator sequence (*las* box) upstream. The transcriptional start site for *vqsR* was determined. The *vqsR* promoter was identified by examining a series of *vqsR* promoter-*lacZ* fusions. In addition, an *Escherichia coli* system where either LasR or RhlR protein was expressed from a plasmid indicated that the *las* system was the dominant regulator for *vqsR*. Electrophoretic mobility shift assays (EMSA) demonstrate that purified LasR protein binds directly to the *vqsR* promoter in the presence of 3O-C₁₂-HSL. Point mutational analysis of the *vqsR las* box suggests that positions 3 and 18 in the *las* box are important for *vqsR* transcription, as assayed with a series of *vqsRp-lacZ* fusions. EMSA also shows that positions 3 and 18 are important for binding between the *vqsR* promoter and LasR. Our results demonstrate that the *las* system directly regulates *vqsR*, and certain nucleotides in the *las* box are crucial for LasR binding and activation of the *vqsR* promoter.

Pseudomonas aeruginosa is an opportunistic human pathogen and a major cause of nosocomial infections. It infects immunocompromised individuals and patients with the pulmonary disorder cystic fibrosis (10). The expression of many *P. aeruginosa* virulence factors is controlled by a regulatory mechanism known as quorum sensing (QS) (5, 23). QS is a form of intercellular communication that allows bacteria to coordinate gene expression in response to cell density. In many gram-negative bacteria, QS is accomplished by the production of diffusible signal molecules in the form of acyl homoserine lactones, or autoinducer, and a transcriptional regulatory protein (R protein) that serves as a signal receptor and transcriptional regulator. Both autoinducer and R protein are produced at basal levels when the population density is low. As cell density increases, autoinducer molecules accumulate. As a threshold concentration of autoinducer is reached, it forms a complex with the R protein. The R protein-autoinducer complex is able to regulate its target genes (9).

In *P. aeruginosa*, two primary QS systems, *las* and *rhl*, have been identified. The *las* system includes LasI and LasR, and the *rhl* system includes RhlI and RhlR. LasI catalyzes the synthesis of the *N*-(3-oxododecanoyl)-L-homoserine lactone (3O-C₁₂-HSL) signal molecule, which binds to the transcriptional regulator protein LasR. RhlI catalyzes the synthesis of *N*-butanoyl-L-homoserine lactone (C₄-HSL), which binds to the RhlR protein. These two systems form a hierarchical cascade that regulates the transcription of multiple structural and

regulatory genes (17, 26). A conserved dyad symmetry DNA sequence, termed the *las* box, has been found upstream of some genes known to be directly regulated by the *las* or the *rhl* system (7, 37). Many studies suggest that the *las* and *rhl* systems regulate their target genes by recognizing the *las* box (2, 6, 20, 29).

In addition to the *las* and *rhl* systems, other QS signal molecules and regulators have been identified. Yet, their roles in QS are not fully understood. For example, the *P. aeruginosa* quinolone signal is regulated by the *las* system (25) and also regulates the *rhl* system (19). Also, QscR, a LuxR homolog, uses 3O-C₁₂-HSL to control a specific regulon that partially overlaps the *las* and *rhl* regulon (3, 18). These add a further level of complexity to the QS circuit.

Over the past few years, global gene expression modulated by the *las* and *rhl* QS regulons in *P. aeruginosa* have been analyzed (11, 31, 36). Transcription profiling suggests that the *las* and/or *rhl* regulon influences the transcription of 3 to 11% of the *P. aeruginosa* genome. However, many of the genes in this regulon appear to lack a *las* box in their promoter region (36). This implies that the expression of many QS genes may be indirectly controlled by the *las* and/or *rhl* system. For instance, *las* and/or *rhl* may regulate additional transcriptional regulator(s). Further analysis of the hierarchical cascade in the QS regulon will be required to test this hypothesis and may elucidate one or more currently unknown critical regulatory factors other than the LasR:3O-C₁₂-HSL and RhlR:C₄-HSL complexes. These regulatory factors, by themselves or together with the *las* and *rhl* systems, could be potential targets for disruption for altering virulence.

In a *P. aeruginosa* strain isolated from a cystic fibrosis patient, inactivation of *vqsR* abrogated the production of acyl

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[∇] Published ahead of print on 20 April 2007.

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant genotype and/or phenotype	Source or reference
Strains		
<i>P. aeruginosa</i>		
PAO1	Wild type	13
JP1	$\Delta lasI$ derivative of PAO1; Tc ^r	24
JM100	$\Delta rhII$ derivative of PAO1; unmarked	This study
JM2	$\Delta rhII$ derivative of JP1; Tc ^r	This study
<i>E. coli</i>		
DH5 α	F ⁻ $\phi 80 lacZ \Delta M15 \Delta(lacZYA-argF)U169 endA1 hsdR17 deoR gyrA96 thi-1 relA1 supE44$	Gibco
BL21	<i>E. coli</i> B F ⁻ <i>dcm ompT hsdS</i> (r _B m _B ⁻) <i>gal</i>	Stratagene
Plasmids		
For gene replacement		
pEX18Ap	<i>oriT</i> ⁺ <i>sacB</i> ⁺ ; gene replacement vector with MCS from pUC18; Ap ^r	12
pPS858	Source of Gm ^r <i>Gfp FRT</i> cassette; Ap ^r ; Gm ^r	12
pEX18ApEK	An 8-bp EcoRI-KpnI fragment was removed from pEX18Ap, followed by Klenow fragment treatment and ligation	This study
pEX18Ap- <i>rhII</i>	3-kb HindIII fragment of the <i>rhII</i> ORF and flanking regions from strain PAO1 on pEX18ApEK	This study
pJEM- <i>rhII</i> ⁻	pEX18Ap- <i>rhII</i> with 251 bp EcoRI-KpnI fragment removed	This study
pFLP2	Broad-host-range Flp- <i>FRT</i> site-specific excision vector; Ap ^r	12
For gene expression analysis		
pLP170	Promoterless <i>lacZ</i> transcriptional fusion vector	27
pECP8	<i>tacp-lasR</i> on broad-host-range vector pEX1.8	35
pJPP8	<i>tacp-rhlR</i> on broad-host-range vector pEX1.8	24
pLL3	418-bp <i>vqsR</i> promoter (nt -248 to +170) ^a fragment inserted into HindIII-XbaI-digested pLP170 to create a <i>vqsRp-lacZ</i> transcriptional fusion	This study
pLL3A	Derived from pLL3; <i>vqsRp'</i> (nt -112, C→T) ^a - <i>lacZ</i>	This study
pLL3B	Derived from pLL3; <i>vqsRp'</i> (nt -97, G→A) ^a - <i>lacZ</i>	This study
pLL3C	Derived from pLL3A; <i>vqsRp'</i> (nt -112, C→T; nt -97, G→A) ^a - <i>lacZ</i>	This study
pLL3D	Derived from pLL3; <i>vqsRp'</i> (nt -114, A→G) ^a - <i>lacZ</i>	This study
pLL3E	Derived from pLL3; <i>vqsRp'</i> (nt -95, T→C) ^a - <i>lacZ</i>	This study
pLL3F	Derived from pLL3D; <i>vqsRp'</i> (nt -114, A→G; nt -95, T→C) ^a - <i>lacZ</i>	This study
pLL4	Same as pLL3 but <i>vqsRp-lacZ</i> has <i>vqsR</i> promoter region from nt -166 to +170 ^a	This study
pLL5	Same as pLL3 but <i>vqsRp-lacZ</i> has <i>vqsR</i> promoter region from nt -65 to +170 ^a	This study
pLL6	pECP8 carrying 4.6-kb XmnI fragment of pLP170 containing promoterless <i>lacZ</i> in BamHI site	This study
pLL7	pJPP8 carrying 4.6-kb XmnI fragment of pLP170 containing promoterless <i>lacZ</i> in NdeI site	This study
pLL8	pECP8 carrying 5-kb XmnI fragment of pLL3 containing <i>vqsRp-lacZ</i> in BamHI site	This study
pLL8A	pECP8 carrying 5-kb XmnI fragment of pLL3A containing <i>vqsRp'-lacZ</i> in BamHI site	This study
pLL8B	pECP8 carrying 5-kb XmnI fragment of pLL3B containing <i>vqsRp'-lacZ</i> in BamHI site	This study
pLL8C	pECP8 carrying 5-kb XmnI fragment of pLL3C containing <i>vqsRp'-lacZ</i> in BamHI site	This study
pLL8D	pECP8 carrying 5-kb XmnI fragment of pLL3D containing <i>vqsRp'-lacZ</i> in BamHI site	This study
pLL8E	pECP8 carrying 5-kb XmnI fragment of pLL3E containing <i>vqsRp'-lacZ</i> in BamHI site	This study
pLL8F	pECP8 carrying 5-kb XmnI fragment of pLL3F containing <i>vqsRp'-lacZ</i> in BamHI site	This study
pLL9	pJPP8 carrying 5-kb XmnI fragment of pLL3 containing <i>vqsRp-lacZ</i> in NdeI site	This study

^a Location of the corresponding sequence within the *vqsR* promoter region relative to the translational start codon.

homoserine lactone signal molecules, decreased the production of virulence factors, and reduced the pathogenicity in a nematode infection model system (15). Preliminary transcriptome analysis also suggested that *vqsR* may regulate QS-controlled genes (15, 16). Expression of *vqsR* itself appears to be regulated by *las* and/or *rhl* in *P. aeruginosa* PAO1 (11, 31, 36). The goal of the present study was to determine if *vqsR* is directly regulated by the *las* and/or *rhl* system. The regulation of *vqsR* was studied in vivo with *vqsRp-lacZ* transcriptional fusions. A direct interaction between the *vqsR* promoter and purified LasR protein in vitro was also demonstrated. This work establishes the importance of the *las* box found in the *vqsR* promoter region.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are shown in Table 1. *P. aeruginosa* strains were cultured at 37°C in peptone-tryptic soy broth (PTSB) (22) or modified FAB [0.1 mM CaCl₂, 0.1 mM FeSO₄, 0.15 mM (NH₄)₂SO₄, 0.33 mM Na₂HPO₄, 0.2 mM KH₂PO₄, 0.5 mM NaCl, 40 mM KNO₃, 10 mM glucose, 0.17% yeast extract (pH 7.0)] (4). When appropriate, carbenicillin (200 µg/ml), exogenous 3O-C₁₂-HSL, or C₄-HSL was added where indicated. *Escherichia coli* strains containing plasmids were grown at 37°C in Luria-Bertani (LB) broth with 100 µg/ml ampicillin (for cloning) or modified A medium (26) with 100 µg/ml ampicillin (for assaying β -galactosidase [β -Gal] activity).

DNA manipulations. Standard techniques were used for purification and manipulation of DNA (30). Restriction endonucleases, DNA ligase, and terminal deoxynucleotidyl transferase (TdT) were purchased from Invitrogen (Carlsbad, CA) or New England Biolabs (Beverly, MA). Platinum *Pfx* DNA polymerase and

TABLE 2. Oligonucleotides used for construction of *vqsR-lacZ* fusions

Plasmid ^a	Strand ^b	Oligonucleotide sequence ^c (5'→3')	Location ^d
pLL3	+	TTAAGCTTAAGATGGACGCTGGTTTGC	−248 to −230
	−	TTTCTAGAGGAAAGAATCAAGGGACTGG	+179 to +151
pLL3A	+	CGAAACAAGTTGGATTTTGCACITACCAGAACTGG	−134 to −100
	−	CCAGTTCTGGTAAAGTGCAAAATCCAACCTGTTTCG	−100 to −134
pLL3B	+	CCAGAACTGGTAATTCTGACCTGTGGCTATCTTCG	−109 to −75
	−	CGAAGATAGCCACAGGTCAGAAITACCAGTTCTCG	−75 to −109
pLL3D	+	CGAAACAAGTTGGATTTTGCCTACCAGAACTGG	−134 to −100
	−	CCAGTTCTGGTAGGCGCAAAATCCAACCTGTTTCG	−100 to −134
pLL3E	+	CCAGAACTGGTAGTCTCTGACCTGTGGCTATCTTCG	−109 to −75
	−	CGAAGATAGCCACAGGTCAGGACTACCAGTTCTCG	−75 to −109

^a Plasmid that was constructed by using the respective oligonucleotide.

^b DNA strand containing the respective oligonucleotide. +, *vqsR* coding strand; −, *vqsR* noncoding strand.

^c Relevant restriction endonuclease sites are underlined, and nucleotide changes are in boldface and underlined.

^d Location of the corresponding sequence within the *vqsR* promoter region relative to the *vqsR* GTG start codon.

large (Klenow) fragment DNA polymerase I were purchased from Invitrogen (Carlsbad, CA). The oligonucleotides used for PCR were synthesized by Invitrogen. Calf intestine alkaline phosphatase was purchased from Roche (Indianapolis, IN). Plasmids were isolated using QIAprep spin miniprep kits (QIAGEN, Chatsworth, CA), and DNA fragments were purified using QIAquick PCR purification kits or QIAquick gel extraction kits (QIAGEN). DNA was sequenced at the DNA core facility, Functional Genomics Center, at the University of Rochester (Rochester, NY).

Construction of *P. aeruginosa* $\Delta rhlI$ and $\Delta lasI$ $\Delta rhlI$ mutant strains. A 3-kb, HindIII-digested PCR fragment carrying the *rhlI* open reading frame (ORF) and flanking regions (amplified by primers 5'-AAAAAGCTTGACCAGGCACCA GGATGGG-3' and 5'-AAAAAGCTTCGGGCCAATTCTGCTGTGATG C-3' from PAO1 chromosomal DNA) was ligated into the vector pEX18ApEK. The resulting plasmid, pEX18Ap-*rhlI*, was digested with EcoRI and KpnI to make a 251-bp deletion in the *rhlI* ORF, treated with Klenow fragment, and then ligated with a SmaI-digested Gm^r *Gfp FRT* cassette to generate pJEM- $\Delta rhlI$. Plasmid pJEM- $\Delta rhlI$ was transferred into *P. aeruginosa* PAO1 and JP1 (24) to generate an isogenic $\Delta rhlI$ mutant (JM100) and a $\Delta lasI$ $\Delta rhlI$ mutant (JM2) by using allelic exchange and the Flp-*FRT* recombination protocol as previously described (12). The *rhlI* deletion in both JM100 and JM2 was confirmed by DNA sequencing.

Mapping the transcriptional start site. For RNA isolation, *P. aeruginosa* PAO1 cultures were collected at the mid-logarithmic growth phase (optical density at 660 nm [OD₆₆₀], 0.5) and early stationary-growth phase (OD₆₆₀, 1.0). RNA was isolated using RNawiz and a DNA-Free kit (Ambion, Austin, TX) as previously described (36). Rapid amplification of cDNA ends (5'RACE) was performed to identify the transcriptional start site of *vqsR* by using the 5'RACE system (Invitrogen) according to the manufacturer's instructions. Briefly, gene specific primer 5'-TCCGTCACACATAATGC-3', RNA from *P. aeruginosa* PAO1, and reverse transcriptase were used for first-strand cDNA synthesis. A homopolymeric tail was then added to the 3' end of the cDNA by using TdT and dCTP/dATP. PCR amplification was accomplished using a nested gene-specific primer (5'-CGACAGTTCTCTGGATACC-3'), a novel deoxinosine-containing anchor primer, and the poly(C) tail cDNA as a template. PCR products were sequenced, and transcriptional start sites were identified.

Plasmid construction and mutagenesis. (i) **Construction of deletion mutations in the *vqsR* promoter region.** To construct pLL3, a HindIII-XbaI *vqsR* promoter fragment from nucleotide (nt) −248 to +170 (relative to the translational start codon) was generated from a *P. aeruginosa* genomic DNA template by using PCR and inserted into HindIII- and XbaI-digested vector pLP170 (27). To construct pLL4, pLL3 was digested with ApaI and SmaI, filled in with the Klenow fragment, and religated to create a promoter-*lacZ* fusion from nt −166 to +170. For the construction of pLL5, the same PCR fragment containing the *vqsR* promoter, which was used to construct pLL3, was digested with ClaI and gel purified. A 235-bp fragment recovered from the gel was treated with Klenow fragment, followed by XbaI digestion, and then inserted into a SmaI- and XbaI-digested pLP170 plasmid to create a promoter-*lacZ* fusion from nt −65 to +170.

(ii) **Site-directed mutagenesis of the *vqsR* las box.** Plasmids pLL3A through pLL3F, containing point mutations in the *vqsR* las box, were constructed by using

pLL3 as a template and a QuikChangeXL site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The oligonucleotides used in the construction of *vqsR-lacZ* fusion plasmids are listed in Table 2. The promoter sequences of *vqsR* in pLL3 and pLL3A-pLL3F were verified by DNA sequencing.

(iii) **Construction of *vqsR-lacZ* plasmids for expression in *E. coli*.** XmnI DNA restriction fragments containing *vqsR-lacZ* fusions from pLP170, pLL3, and pLL3A through pLL3F were inserted into pECP8 (treated with BamHI, Klenow fragment, and alkaline phosphatase according to the manufacturers' protocols) and pJPP8 (treated with NdeI, Klenow fragment, and alkaline phosphatase according to the manufacturers' protocols) separately to construct plasmids pLL6, pLL7, pLL8, pLL8A through pLL8F, and pLL9.

Monitoring promoter activity in *P. aeruginosa*. Promoter activity in *P. aeruginosa* was analyzed according to previously described methods (26). Briefly, cultures were grown in PTSB (supplemented with 200 µg/ml carbenicillin) at 37°C with shaking for 16 h and subcultured into the same medium to a starting OD₆₆₀ of 0.05. When indicated, 3O-C₁₂-HSL or C₄-HSL was added at the start of subculturing. When cultures were grown to an OD₆₆₀ of 1, cells were collected, resuspended in phosphate-buffered saline, and assayed for β-Gal activity as described previously (21).

Autoinducer sensitivity assays. (i) ***P. aeruginosa*.** The autoinducer sensitivity of the *vqsR* promoter in *P. aeruginosa* was assayed as described previously (24). Briefly, cells from overnight PTSB-carbenicillin (200 µg/ml)-grown cultures were subcultured into the same medium to an OD₆₆₀ of 0.05 and then grown at 37°C with shaking to an OD₆₆₀ of 0.3. At this time, 1-ml aliquots of culture were transferred to tubes containing various concentrations of autoinducer. Growth was continued for 1 h, and the cultures were assayed for β-Gal activity (21).

(ii) ***E. coli*.** The autoinducer sensitivity of the *vqsR* promoter in *E. coli* was assayed as described previously (24). Briefly, cultures were grown in medium A supplemented with 100 µg/ml ampicillin at 37°C with shaking overnight and subcultured into the same medium to a starting OD₆₆₀ of 0.08. After growth at 37°C with shaking to an OD₆₆₀ of 0.3, IPTG (isopropyl-β-D-thiogalactoside) was added to a final concentration of 0.2 mM, and then 1-ml aliquots of culture were transferred to tubes containing various concentrations of autoinducer. Growth was continued for 90 min, and the cultures were assayed for β-Gal activity (21).

Overproduction and purification of the LasR protein. For LasR expression, the culture was grown as previously described (32), with some modifications. Briefly, overnight colonies of *E. coli* BL21 carrying pECP8 on LB-ampicillin plates were used to inoculate 500 ml LB containing 100 µg/ml ampicillin and 10 µM 3O-C₁₂-HSL. The culture was incubated at 37°C until its OD₆₆₀ reached 0.5. Then, the culture was rapidly chilled to 20°C, induced with 0.2 mM IPTG (final concentration), and incubated at 17°C overnight. Cells were harvested from the overnight culture and frozen at −80°C. All subsequent steps for purification of LasR were performed as previously described (32). The resulting LasR preparation was verified by Western blot analysis with anti-LasR antibody and was 96% pure as judged by silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis and a densitometric scan. The Bradford assay (Bio-Rad) was used to determine protein concentrations.

EMSA. A 309-bp DNA probe of the *rsaL* promoter region (nt -278 to ~+31) and a 292-bp DNA probe of the *vqsR* promoter region (nt -247 to ~+45) were generated by PCR from PAO1 genomic DNA. A 201-bp nonspecific DNA probe was also generated by PCR amplification of DNA encompassing the *rsaL* coding region and the intergenic region between *rsaL* and *lasR* (nt +601 of *lasR* to +144 of *rsaL*). All probes were labeled at the 3' end by using [α - 32 P]CTP and TdT according to the manufacturers' protocols. Electrophoretic mobility shift assay (EMSA) experiments were based on a protocol by Urbanowski et al. (34), and the reaction condition for LasR-DNA interaction was according to that previously described (32).

RESULTS

Determination of the *vqsR* transcriptional start site. The transcriptional start site of *vqsR* was determined by 5'RACE as described in Materials and Methods. With the poly(C) tail cDNA used as a template, PCR amplification with the nested gene-specific primer and the novel deoxinosine-containing anchor primer resulted in a single-band PCR product. This PCR product was analyzed with DNA sequencing, and the result revealed that the *vqsR* transcriptional start site was at 51 bp upstream of the translation start site (Fig. 1A). A putative *las* box is located from 115 bp to 96 bp upstream of the translation start site (as indicated in Fig. 1A) and exhibits substantial homology with other reported *las* box sequences (7, 37).

Identification of the probable promoter region of *vqsR*. To understand how *vqsR* is regulated, we constructed three *vqsR*-*lacZ* fusions: pLL3 (which contains *vqsR* upstream, from nt -248 to +170), pLL4 (which contains *vqsR* upstream, from nt -166 to +170), and pLL5 (which contains *vqsR* upstream, from nt -65 to +170) (the numbering is relative to the translational start site) (Fig. 1B). These three fusions were introduced into the wild-type strain PAO1 and JM2 (Δ *lasI* Δ *rhlI*), and β -Gal activity was measured to assay promoter activity. Compared to that in the control vector pLP170, β -Gal activity increased 14-fold in PAO1/pLL3, 10-fold in PAO1/pLL4, and minimally (control levels) in PAO1/pLL5 (Fig. 1C). A similar trend of promoter activity was observed in JM2 with both exogenous 3O-C₁₂-HSL and C₄-HSL. However, in the JM2 strain without added autoinducers, only background levels of β -Gal activity were detected with all three plasmid constructs. These findings suggested that *vqsR* is regulated by the *las* and/or *rhl* QS system, and the *vqsR* upstream region from nt -248 to +170 contains the promoter and elements for maximum transcription of *vqsR*. Thus, the fusion construct pLL3 was used for further transcriptional studies of *vqsR*.

QS control of *vqsR*. To identify which QS system of *P. aeruginosa* regulates *vqsR*, plasmid pLL3 (*vqsR*-*lacZ*) was introduced into JP1 (Δ *lasI*) and JM100 (Δ *rhlI*), and β -Gal activity was assayed. Compared with the β -Gal activity in PAO1/pLL3, only 15% β -Gal activity can be detected in JP1/pLL3, but 69% β -Gal activity can be detected in JM100/pLL3 (Fig. 2A). To investigate the response of the *vqsR* promoter to different doses of autoinducers in these two strains, we added exogenous 3O-C₁₂-HSL into JP1/pLL3 culture or C₄-HSL into JM100/pLL3 cultures to a final concentration from 1 nM to 10 μ M. As shown in Fig. 2B, in both JP1/pLL3 and JM100/pLL3 cultures, with increasing amounts of exogenous autoinducer in the cultures, higher β -Gal activities were detected. For strain JP1/pLL3, with exogenous 3O-C₁₂-HSL from 0 to 10 μ M, the β -Gal activity increased 5-fold; for strain JM100/pLL3, with

FIG. 1. Identification of the probable promoter region of *vqsR*. (A) Nucleotide sequence of the *vqsR* promoter region. Numbering is relative to the *vqsR* GTG start codon, which is indicated with a gray box. The *vqsR* mRNA start site, mapped by 5'RACE, is at position -51 (G), indicated by a black box with white type. The potential *las* box is as indicated. (B) Three *vqsR*-*lacZ* fusions, pLL3, pLL4, and pLL5, were constructed as indicated. Numbering is relative to the translational start site. (C) β -Gal expression of pLL3, pLL4, pLL5, and the control vector in the wild-type strain PAO1 (shaded bar), JM2 (black bar), and JM2 with exogenous 3O-C₁₂-HSL and C₄-HSL (white bar) is shown. Data represent the means for three independent experiments, each done in triplicate. PAI, *Pseudomonas aeruginosa* autoinducer.

exogenous C₄-HSL from 0 to 10 μ M, the β -Gal activity increased only 1.5-fold.

In strain JM100, only the *las* system is functional because of the mutation in *rhlI*. In strain JP1, however, the lack of a functional *las* system causes a failure to activate the *rhl* system. Thus, both the *las* and the *rhl* systems are absent (6, 25). Therefore, in order to test the direct effect of the *las* or the *rhl* system on *vqsR* regulation, it is necessary to examine the *vqsR* promoter activity in an environment that can separately provide either the *las* or the *rhl* system. *P. aeruginosa* homoserine lactone autoinducers as well as *lasR* and/or *rhlR* are absent in *E. coli* DH5 α (33). Using *E. coli* DH5 α as a host, we analyzed the direct effect of the *las* and *rhl* QS systems on *vqsR* expression. To supply the *las* system in *trans*, a fragment containing

FIG. 2. Regulation of *vqsR* by the *las* and/or *rhl* QS system in either *P. aeruginosa* or *E. coli*. (A) β -Gal activities demonstrated the response of the *vqsR* promoter in *P. aeruginosa* PAO1 (white bar), JP1 (dotted bar), and JM100 (black bar) without exogenous autoinducer. (B) The response of the *vqsR* promoter in JP1 with increasing doses of 3O-C₁₂-HSL (dashed line) or in JM100 with increasing doses of C₄-HSL (solid line). (C) Response of *vqsR* promoter in an *E. coli* background. Symbols: $\square$, LasR plus 3O-C₁₂-HSL; $\blacksquare$, LasR plus C₄-HSL; $\triangle$, RhlR plus C₄-HSL; $\blacktriangle$, RhlR plus 3O-C₁₂-HSL. The β -Gal activity values shown are normalized by subtracting the background levels (β -Gal values of pLL6 and pLL7 in *E. coli* with increasing doses of 3O-C₁₂-HSL or C₄-HSL). Data represent the means for three independent experiments, each done in triplicate. (D) EMSA for the *vqsR* promoter and purified LasR. All lanes contained ≈ 10 fmol of an equimolar mixture of the two probes (negative control and positive control or the *vqsR* promoter) and the indicated concentration of LasR in the presence of 10 μ M 3O-C₁₂-HSL. (Left) Positive control (the *rsal* promoter); (right) the *vqsR* promoter. The film was exposed for 24 h. PAI, *Pseudomonas aeruginosa* autoinducer.

the *vqsR*-*lacZ* fusion (from pLL3) was inserted into pECP8, which contains an IPTG-inducible *tacp-lasR* fusion, to construct plasmid pLL8. Similarly, plasmid pLL9 was constructed with insertion of the *vqsR*-*lacZ* fusion into pJPP8, which contains the *tacp-rhlR* fusion. A promoterless *lacZ* fragment was also inserted into plasmids pECP8 and pJPP8 to construct pLL6 and pLL7 for use as controls. The autoinducer sensitivity of the *vqsR* promoter was assayed by pairing pLL8 or pLL9 with various concentrations of either 3O-C₁₂-HSL or C₄-HSL. As shown in Fig. 2C, without their cognate autoinducer, neither LasR nor RhlR could activate the *vqsR* promoter in *E. coli* DH5 α . In the presence of a relatively low concentration of 3O-C₁₂-HSL (1 nM), the *vqsR* promoter was activated by the *las* system more than twofold compared to the control. However, the *rhl* system did not activate the *vqsR* promoter in the presence of 1 nM C₄-HSL. With a higher concentration of C₄-HSL (100 nM), the *vqsR* promoter showed modest activation by the *rhl* system. This modest activation by the *rhl* system was threefold less than the activation by the *las* system at the same concentration of autoinducer. These results suggest that the *vqsR* promoter is more sensitive to activation by the *las* system than the *rhl* system.

Interaction between the *vqsR* promoter and the *las* system.

To investigate whether the LasR:3O-C₁₂-HSL complex binds to the *vqsR* promoter directly, EMSAs were performed. Based on protocols by Schuster et al. (32), the LasR protein was purified to 96% homogeneity as judged by a silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis and a densitometric scan (data not shown), and the *rsaL* promoter region was used as a positive-control DNA for the EMSA. A DNA fragment encompassing the *rsaL* coding region and the intergenic region between *rsaL* and *lasR*, which does not bind to LasR (as shown by Schuster et al. [32]), was also included in each reaction as a negative control (see Materials and Methods for details of sequences). As shown in Fig. 2D, in the presence of 3O-C₁₂-HSL, LasR caused a shift in the migration of the *vqsR* promoter DNA fragment similar to that for the positive-control fragment (a DNA that contains a known *las* box and binds to LasR, as shown by Schuster et al. [32]).

The importance of the *las* box for *vqsR* transcription. (i) **In a *P. aeruginosa* background.** Conserved nucleotides of the *las* box are critical for QS control of *lasB* (29). To examine whether the apparent *las* box sequence/motif in the *vqsR* promoter is critical for QS regulation, a series of *vqsR*-*lacZ* fusions containing point mutations in the apparent *las* box were constructed. These plasmids were introduced into *P. aeruginosa* PAO1, and β -Gal activity was measured. According to the study of Rust et al. (29), the expression of *lasB*-*lacZ* decreased dramatically when point mutations at positions 3, 18, and both 3 and 18 were introduced in the OP1 *las* box of *lasB*. Here, we report that in *P. aeruginosa* PAO1, a similar effect was also observed in the same point mutations in the *vqsR* *las* box. The levels of expression were reduced to 7% of that for the wild-type *vqsR* *las* box when the mutation was at position 3 (nt -113; C to T; pLL3A), 18% when the mutation was at position 18 (nt -98; G to A; pLL3B), and 12% when mutations were introduced at both position 3 and position 18 (pLL3C) (Table 3). These data suggest that both position 3 and position 18 of the *vqsR* *las* box are critical for *vqsR* transcription. We also examined the expression levels of *vqsR* with point muta-

TABLE 3. The importance of the *las* box in the *vqsR* promoter region

Plasmid	Mutation in <i>las</i> box (nt -115 to -96) ^a	Promoter activity (%) ^b for indicated series	
		<i>P. aeruginosa</i> PAO1 pLL3	<i>E. coli</i> DH5 α pLL8 (plus 3O-C ₁₂ -HSL)
pLL3/8	ACCTA CCAGA ACTGG TAGTT	100	100
pLL3A/8A	ACTTA CCAGA ACTGG TAGTT	7.4 $\pm$ 0.4 ^c	10.5 $\pm$ 6.7 ^c
pLL3B/8B	ACCTA CCAGA ACTGG TAATT	18.4 $\pm$ 4.0 ^c	23.7 $\pm$ 5.0 ^c
pLL3C/8C	ACTTA CCAGA ACTGG TAATT	11.8 $\pm$ 1.4 ^c	7.1 $\pm$ 3.1 ^c
pLL3D/8D	GCCTA CCAGA ACTGG TAGTT	39.9 $\pm$ 8.2 ^c	88.5 $\pm$ 12.3
pLL3E/8E	ACCTA CCAGA ACTGG TAGTC	123.4 $\pm$ 10.5	45.7 $\pm$ 8.7 ^c
pLL3F/8F	GCCTA CCAGA ACTGG TAGTC	46.3 $\pm$ 12.1 ^c	58.2 $\pm$ 19.4 ^c

^a Nucleotide changes are in boldface and underlined.

^b Promoter activity was determined by measuring the β -Gal activities of *vqsR*'-lacZ fusions grown in PAO1 or in *E. coli* supplemented with 100 nM 3O-C₁₂-HSL. Values are the means for four independent experiments, each done in duplicate.

^c $P < 0.05$.

tions in the *las* box at position 1 (nt -115; A to G; pLL3D) and position 20 (nt -96; T to C; pLL3E) and double-point mutations at both position 1 and position 20 (pLL3F). The levels of expression were reduced to 40% of that for the wild-type *vqsR* *las* box when the mutation was at position 1 and 46% when the mutations were introduced at both position 1 and position 20. The level of expression showed no significant change only when the mutation was at position 20. (The Student *t* test was used to identify mutations that showed significant changes of promoter activity [$P < 0.05$].)

(ii) **In an *E. coli* background.** To further examine the role of the apparent *las* box in regulation of the *vqsR* promoter by the *las* system, the expression levels of the *vqsR* promoters containing point mutations in this motif were examined using a reporter system in *E. coli* DH5 α as described above. Promoter-lacZ fusions containing point mutations in the *vqsR* *las* box from plasmids pLL3A-F were inserted into pECP8 (containing an IPTG-induced *lasR*) to create pLL8A-F. Plasmids were introduced into *E. coli* DH5 α , and β -Gal activities were measured. As shown in Table 3, when position 3 of the *vqsR* *las* box was mutated (pLL8A), the level of expression was reduced to 10% of that for the wild-type *vqsR* *las* box. When position 18 was mutated (pLL8B), the level of expression was reduced to 24%. When both position 3 and position 18 were mutated (pLL8C), the level of expression was reduced to 7%. However, the effect of mutating position 1 (pLL8D) or 20 (pLL8E) was less severe; the levels of expression were reduced to 88% and 46% separately. These data indicate that within the conserved *las* box sequence, these positions are crucial for *las* regulation of *vqsR* transcription.

The importance of the *vqsR* *las* box for LasR binding. The involvement of the *vqsR* *las* box for LasR binding was further tested by performing EMSA. DNA fragments containing mutations in the conserved *las* box of the *vqsR* promoter were generated by PCR amplification of plasmids pLL3A through pLL3F and were labeled with ³²P as described in Materials and Methods. As shown in Fig. 3, a mutation at *vqsR* *las* box position 3 appears to slightly decrease the binding of LasR to the *vqsR* promoter compared to the wild-type *vqsR* *las* box. A similar result was also observed when a point mutation was introduced at *las* box position 18. Single mutations at position 1 and position 20 and a double mutation at positions 1 and 20 slightly enhance the LasR binding. Interestingly, a *las* box

containing mutations at both position 3 and position 18 completely abolished binding by LasR. These results further support the idea that the *las* box of *vqsR* is important for binding of LasR to the *vqsR* promoter and show that conserved positions 3 and 18 in the *las* box are the most crucial for binding of LasR to the *vqsR* promoter.

DISCUSSION

Previous transcriptome analyses have suggested that the fourth LuxR homologue in *P. aeruginosa*, VqsR, is controlled by the *las* and/or *rhl* QS system (11, 31, 36). VqsR also appears to be involved in the regulation of virulence and QS-controlled genes (15, 16). Therefore, VqsR is part of the QS circuit in *P. aeruginosa*. We hypothesized previously that a complicated hierarchy in the QS regulon is composed of multiple transcriptional regulators. The *las* and/or *rhl* system may control additional transcriptional regulator(s), and VqsR might be among these regulators.

In this study, we determined the *vqsR* transcriptional start site. We also examined the *vqsR* upstream region to identify the promoter. A 20-bp *lux* box-like element that matches the minimal consensus sequence and position for the *P. aeruginosa* *las* box (37) was found upstream of the transcriptional start site (Fig. 1A). This region was confirmed to contain the probable promoter region of *vqsR* by β -Gal activity analysis of serially truncated upstream region-lacZ fusions (Fig. 1B).

Our next goal was to specifically address the involvement of the *las* and the *rhl* systems in regulation of the *vqsR* promoter. As shown in the result, the *vqsR* promoter appeared more sensitive to activation by the *las* system. When *lasR* was expressed, the *vqsR* promoter displayed relatively robust activation by 1 nM 3O-C₁₂-HSL. In comparison, when *rhlR* was expressed, only a modest induction was detectable, even when 100 nM C₄-HSL was added (Fig. 2B). These data suggest that the *las* system directly regulates *vqsR* and is the dominant regulator of *vqsR*. We further tested the idea that LasR directly regulates *vqsR* by performing EMSA with purified LasR and *vqsR* promoter DNA. Our results indicate that in the presence of 3O-C₁₂-HSL, purified LasR binds to the *vqsR* promoter. The shift was comparable to that for the positive control (the *rsaL* promoter DNA) (Fig. 2C).

According to a previous study from this laboratory (36),

FIG. 3. Importance of the *las* box for the binding of LasR to the *vqsR* promoter. Shown are EMSA results for the binding of LasR to the *vqsR* promoter with point mutations in the *las* box at the positions indicated. All lanes contained ≈ 10 fmol of an equimolar mixture of the two probes and purified LasR (the concentration is indicated on the top of each panel) in the presence of $10 \mu\text{M}$ 3O-C₁₂-HSL. The films were exposed for 48 h.

palindromic *las* box-like sequences can be found in the upstream regions of about 7% of the QS-regulated genes. Evidence from transcriptional fusion studies showed that the *las* box is important for the transcription of a number of QS-regulated genes. For example, the transcription of *rhlI* was dramatically decreased when half of the *las* box was deleted (6). In addition, point mutations at certain positions in the OP1 *las* box nearly abolished the transcription of *lasB* (29). In this study, we investigated the effects of point mutations in the *vqsR las* box. Individual mutations at positions 3 and 18 and a double

mutation at both position 3 and position 18 caused the promoter activity of *vqsR* to be reduced by 80% to 90% compared with that of the wild-type *vqsR las* box, while a double mutation at both position 1 and position 20 reduced promoter activity by 50% in both a *P. aeruginosa* PAO1 background and an *E. coli* DH5 α background (Table 3). These data agree with previous studies which showed that positions 3 and 18 in the *las* box upstream of *lasB* are crucial for promoter activity (29). We further studied the effects of *las* box mutations on the binding of LasR by using EMSA. Compared with the binding between LasR and the native *vqsR* promoter region, LasR binding was abolished by a double mutation at both position 3 and position 18 (Fig. 3). This is the first demonstration that mutations of certain nucleotides in the *las* box directly affect the binding of LasR to the *vqsR* promoter by using purified components.

Our results from transcriptional analysis and protein binding studies suggest that positions 3 and 18 in the *vqsR las* box are crucial for both LasR binding and activation of the *vqsR* promoter. Interestingly, our data show that mutations at positions 1 and 20 in the *vqsR las* box do not greatly interfere with LasR binding but still influence *vqsR* transcription. There are several possible reasons for this observation. First, the in vivo β -Gal reporter system may be more sensitive than the in vitro protein-DNA binding assay. Second, positions 3 and 18 in the *vqsR las* box may affect the on/off rate of LasR protein more severely in EMSA than positions 1 and 20. Third, the *vqsR las* box is located at nt -43 to -62 relative to the transcriptional start site and potentially overlaps the nt -35 promoter determinant. The point mutation at position 20 in the *las* box (nt -43) may affect the promoter interaction with the RNA polymerase. Fourth, the upstream sequence of the *vqsR las* box (nt -62 to -113) is very low in G+C content in contrast to the overall *P. aeruginosa* mol% G+C of 67 (2, 14). This AT-rich region may function as the UP element for RNA polymerase α CTD binding (1, 8, 28). Thus, the point mutation at position 1 (nt -62) in the *las* box may affect the UP element.

Previous studies have shown that *E. coli* DH5 α is a good host for in vivo reconstitution of the *P. aeruginosa* transcription regulation network (6, 26), because *P. aeruginosa* homoserine lactone autoinducers as well as *lasR* and/or *rhlR* are absent in *E. coli* DH5 α (33). By separately providing either the *las* or the *rhl* system in the *E. coli* host, we were able to examine the direct effect of the *las* and *rhl* QS systems on *vqsR* expression. Our results indicate that the minimal components of LasR together with 3-O-C₁₂-HSL are sufficient to reconstitute activation of the *vqsR* promoter in the *E. coli* "host" system.

In conclusion, we have demonstrated that the QS regulator VqsR is directly regulated by the *las* system. Our results also show that certain nucleotides in the *las* box are important for activation of the *vqsR* promoter. Therefore, in the QS cascade, we can place VqsR at the same level as RhlR and QscR, the other two LasR directly activated regulators. The results of this study provide information for a more complete understanding of the hierarchy in the QS regulon and hopefully identify a potential target for future treatment of *P. aeruginosa* infections.

ACKNOWLEDGMENTS

We thank M. Schuster for helpful information for LasR purification and H. Ngai, K. Picardo, G. Tomblin, and V. Wagner for insightful discussions and comments on the manuscript.

This work was supported by grant R37 AI033713 (to B. H. Iglewski) from the National Institutes of Health.

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