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The *Pseudomonas aeruginosa* Global Regulator VqsR Directly Inhibits QscR To Control Quorum-Sensing and Virulence Gene Expression

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The opportunistic pathogen *Pseudomonas aeruginosa* has at least three quorum-sensing (QS) systems, including the acyl-homoserine lactone (acyl-HSL)-mediated *las* and *rhl* systems, as well as the 2-alkyl-4(1H)-quinolone (AHQ) signal-based system. A group of key regulators of these QS systems have been identified, such as *qteE*, *vqsM*, *vqsR*, and *vfr*. However, the underlying regulatory mechanisms of these QS systems are not yet fully understood. Here, using electrophoretic mobility shift assays, we demonstrated that VqsR indirectly regulates acyl-HSL systems but specifically binds to the *qscR* promoter region, which indicates that VqsR influences QS-controlled pathways through QscR. Through a dye-based DNase I footprint assay, we showed that VqsR interacts with an inverted repeat (IR) motif (TCGCCN$_8$GGCGA, where N is any nucleotide) in the promoter region of *qscR*. A genome-wide search identified 50 other promoter regions carrying the same putative IR motif. The recombinant VqsR protein exists as a homodimer in solution. In addition, using a *qscR-lux* reporter assay and Northern blot hybridization, we found that the transcription level of *qscR* increased 4-fold in the *vqsR* deletion strain compared to the wild-type PAO1 strain, indicating *vqsR* as a negative regulator of *qscR*. Taken together, these findings provide new insights into the complex regulation network of QS systems in *P. aeruginosa*.

Pseudomonas aeruginosa is a major opportunistic pathogen capable of causing a variety of soft tissue infections in susceptible hosts. *P. aeruginosa*-associated chronic infection and pulmonary inflammation are mostly responsible for the morbidity and mortality of patients with cystic fibrosis (8). Many virulence factors, including toxins (exotoxin A and exoenzyme S), proteases (elastase, LasA protease, and alkaline protease), hemolysins, and phenazines, have been demonstrated to contribute to the pathogenicity of *P. aeruginosa* in multiple animal models (19, 55).

P. aeruginosa possesses at least two well-defined quorum-sensing (QS) systems, the *las* and *rhl* systems, which control the production of numerous virulence factors, including LasA, LasB, and RhlA (37, 42). Each system consists of a transcriptional activator (LasR or RhlR) and its cognate autoinducer synthase (LasI or RhlI). In the *las* system, the transcriptional activator LasR positively regulates the expression of the quorum signal synthase LasI, which is responsible for the biosynthesis of *N*-3-oxo-dodecanoyl-homoserine lactone (3OC$_{12}$-HSL). In the *rhl* system, the transcriptional activator RhlR activates the expression of RhlI, which synthesizes the second quorum signal, *N*-butanoyl-homoserine lactone (C$_4$-HSL). 3OC$_{12}$-HSL and C$_4$-HSL specifically bind and activate LasR and RhlR, respectively (25). Upon activation by 3OC$_{12}$-HSL, LasR turns on the production of a variety of virulence factors, such as LasA protease, LasB elastase, exotoxin A, and alkaline protease (45, 52). Activation of RhlR by C$_4$-HSL promotes the production of rhamnolipid, hydrogen cyanide, pyocyanin (34, 38), and effectors of the type III secretion system (3). Microarray analyses revealed that both the *las* and *rhl* systems also control the expression of over 600 genes in *P. aeruginosa* (47, 58). Furthermore, the autoinducers alone appear to negatively modulate the host immune responses (41). LasR directly binds to the promoter region of *vqsR*, another positive QS regulator (28). QscR, a LasR-RhlR homologue without a cognate acyl-HSL synthase gene, has been demonstrated to be a repressor of both *las* and *rhl* systems. QscR binds to promoters that have elements similar in sequence to the *las* box but does not bind to the *las* box (5). In addition, the binding of QscR to promoter DNA is dependent on 3OC$_{12}$-HSL (27).

Besides 3OC$_{12}$-HSL and C$_4$-HSL, *P. aeruginosa* produces diverse 2-alkyl-4-quinolones (AHQs) as the third group of QS signal molecules (39). The major AHQ signals include 2-heptyl-3-hydroxy-4-quinolone (the *Pseudomonas* quinolone signal [PQS]) and 2-heptyl-4-quinolone (HHQ) (9, 10, 39). PQS synthesis is catalyzed by enzymes encoded by the *pqsABCDE* and *phnAB* operons as well as *pqsH* (9), which are regulated by a transcriptional regulator in the PQS system, PqsR (also known as MvfR) (9). Mutations in *pqsR* interfere with PQS synthesis and compromise the production of pyocyanin as well as other QS-controlled virulence factors (9, 16). Our previous work showed that PmpR directly binds to the *pqsR* promoter region (30).

Including the aforementioned regulators, a complex regulatory network comprising at least 16 regulators controls the QS systems at both the transcriptional level and the posttranscriptional level in *P. aeruginosa*. However, only seven of them have so far been linked to their direct targets in the QS network (56, 60). Among these mysterious factors, VqsM regulates QS-controlled genes by tuning the transcription of another regulator, VqsR (12). Recently, Siehnel and colleagues showed that *qteE* serves as a unique negative regulator to blocks QS gene expression and decreases the half-life of the LasR protein without affecting its transcription or translation (51). On the other hand, we have shown

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype	Source or reference
E. coli strains		
DH5α	*endA hsdR17 supE44 thi-1 recA1 gyrA relA1* D (*lacZYA-argF*)*U169 deoR* (ϕ80d*lacD* (*lacZ*) M15)	Lab stock
BL21	F^- *ompT hsd*S_B (r_B^- m_B^-) *gal dcm met* (DE3)	Lab stock
P. aeruginosa strains		
PAO1	Wild type	Lab stock
PAO1(Δ*vqsR*)	*vqsR* replacement mutant of PAO1; *vqsR*::Gm^r	This study
PAO1(Δ*vqsR*)C	PAO1(Δ*vqsR*) complemented strain	This study
Plasmids		
pMS402	Expression reporter plasmid carrying the promoterless *luxCDABE*; Kn^r Tmp^r	13
pMCSG7	Protein expression vector	53
pEX18AP	$oriT^+$ $sacB^+$ gene replacement vector with multiple-cloning site from pUC18; Ap^r	18
pPS858	pBR322 derivative carrying a FRT-Gm cassette, Ap^r	18
pLH-*lasI*	pMS402 containing *lasI* promoter region	This study
pLH-*qscR*	pMS402 containing *qscR* promoter region	This study
pMCSG7-*lasR*	Protein expression construct, *lasR* cloned in pMCSG7 vector	This study
pMCSG7-*VqsR*	Protein expression construct, *vqsR* cloned in pMCSG7 vector	This study
PAK1900	*E. coli-P. aeruginosa* shuttle cloning vector, Ap^r	20
PAK1900-*vqsR*	PAK1900 derivative carrying the whole operon of *vqsR*	This study
PAK1900-*qscR*	PAK1900 derivative carrying the whole operon of *qscR*	This study
pEX18Ap-*vqsR*	pEX18Ap carrying the up and down fragment of *vqsR*	This study
pEX18Ap-*vqsR*Gm	pEX18Ap-*vqsR* derivative, for replacing *vqsR* locus with a gentamicin resistance cassette	This study

that overexpression of QteE represses the expression of the PQS system in *P. aeruginosa* (29).

VqsR is another important QS regulator whose direct targets remain unknown. It has been shown that a *vqsR* mutant displays reduced production of quorum signals and virulence factors relative to the wild type (21). Microarray analysis revealed that VqsR controls the expression of approximately 200 genes (21, 22). Comparison of the microarray data of VqsR with those of the QS regulon revealed that 101 genes are shared in the two pathways (22, 47), which suggests that VqsR may directly control other known QS regulators. In this study, we reveal that purified VqsR indirectly controls *las* or *rhl* systems but directly regulates the LasR-RhlR homolog QscR. A DNase I footprint assay showed that unlike other QS regulators, such as LasR and QscR, that bind to the *las* box sequence ($NNCTN_{12}AGNN$), VqsR binds to an inverted repeat sequence ($TCGCCN_8GGCGA$). Furthermore, the association between VqsR and the *qscR* promoter region is independent of $3OC_{12}$-HSL. Taken together, these results extend our understanding of complicated regulatory networks of QS systems in *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial stains and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* PAO1 and derivatives were grown at 37°C on LB agar plates or in broth with shaking at 200 rpm. Antibiotics were used at the following concentrations: for *Escherichia coli*, gentamicin (Gm) at 10 μg/ml and ampicillin (Ap) at 100 μg/ml; for *P. aeruginosa*, gentamicin (Gm) at 50 μg/ml in LB.

Expression and purification of VqsR protein. The gene encoding VqsR was PCR amplified from *P. aeruginosa* chromosomal DNA by using the primers *vqsR*-For and *vqsR*-Rev (Table 2). The PCR product was introduced into pMCSG7 (53) by ligation-independent cloning to generate pMCSG7-VqsR. The resulting plasmid was transformed into BL21star(DE3). Ten milliliters of overnight precultures grown from a single colony was inoculated into 1 liter of autoclaved LB medium containing 100 μg/ml ampicillin. The cells were grown at 37°C and 250 rpm to an optical density at 600 nm (OD_{600}) of ~0.6, and then the temperature was reduced to 16°C. Protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The overnight culture was harvested at 4°C by centrifugation at 6,000 rpm for 8 min. All subsequent steps were performed at 4°C. The pellet was suspended in 20 ml buffer A (10 mM Tris-HCl [pH 7.4], 500 mM NaCl, 1 mM dithiothreitol [DTT]) and 10 mM phenylmethylsulfonyl fluoride (PMSF). The cells were lysed by sonication and centrifuged at 12,000 rpm for 25 min. The supernatant was filtered through a 0.45-μm filter and applied to a nickel-nitrilotriacetic acid (Ni-NTA) column. The column was washed with 5% buffer B (10 mM Tris-HCl [pH 7.4], 500 mM imidazole, 300 mM NaCl, 1 mM DTT) and eluted with a linear gradient from 5% to 100% buffer B over 40 ml. Peak fractions were pooled and kept at 4°C. The purity was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (see Fig. 1A).

Construction of a *P. aeruginosa vqsR* deletion mutant. For *vqsR* gene replacement, a *sacB*-based strategy was employed (49). To construct the *vqsR*-null mutant (Δ*vqsR*), PCRs were performed to amplify sequences upstream (706 bp) and downstream (764 bp) of the intended deletion. The upstream fragment was amplified from PAO1 genomic DNA using the primers *vqsR*kf1 (with an EcoRI site) and *vqsR*kr1 (with an XbaI site), while the downstream fragment was amplified with the primers *vqsR*kf2 (with an XbaI site) and *vqsR*kr2 (with a PstI site). These primers are listed in Table 2. The two PCR products were digested with EcoRI-XbaI or XbaI-PstI, respectively, and then cloned into the EcoRI-PstI-digested gene replacement vector pEX18Ap via a three-piece ligation, which yielded pEX18Ap-*vqsR*. A gentamicin resistance cassette was digested from pPS858 (18) with XbaI. The fragment was cloned into XbaI-digested pEX18Ap-*vqsR*. The resulting plasmid, pEX18Ap-*vqsR*Gm, was electroporated into wild-type PAO1 with selection for gentamicin resistance. Colonies were screened for gentamicin resistance, carbenicillin sensitivity, and loss of sucrose (5%) sensitivity, which typically indicates a double crossover event and thus the occurrence of gene replacement. The Δ*vqsR* strain was further confirmed by PCR and Southern blot analysis.

Construction of the *qscR* reporter plasmid. Carrying a promoterless *luxCDABE* reporter gene cluster, plasmid pMS402 was used to construct

TABLE 2 Primers used in this study

Primer pair	Purpose	Primer name	Sequence (5′–3′)[a]
HL1	Cloning VqsR into pMCSG7	VqsRf	TACTTCCAATCCAATGCCATGCCGTGGATATCGCATTGCACG
		VqsRr	TTATCCACTTCCAATGTTAGCGTGCGTCGGCGAGCCGCG
HL2	Construction of *vqsR* deletion mutant	*vqsR*kf1	TCGGAATTCAGCGCATCACACTGAATCC
		*vqsR*kr1	TGCTCTAGACTGCACCCCTCTTCACAGC
HL3	Construction of *vqsR* deletion mutant	*vqsR*kf2	TCGTCTAGACATGTCGGCGATTTCCAC
		*vqsR*kr2	GGTCTGCAGCGATGAAGCCGAGGAGTG
HL4	Complementation with *vqsR* mutant	*vqsR*cf	GCGAAGCTTCATGACATAACGCCGAGTG
		*vqsR*cr	GCTTCTAGAGTCGGGCAATATGAATTTCG
HL5	*lasI* gel shift assay	*lasI*gf	CAGAAAGTTTCCTGGCTTTCC
		*lasI*gr	CACTTGAGCACGCAACTTGT
HL6	*lasR* gel shift assay	*lasR*gf	GATGGGCCGACAGTGAAC
		*lasR*gr	AATCAGCCAAATATGGATTCG
HL7	*rhlI* gel shift assay	*rhlI*gf	GAACATCCAGAAGAAGTTCGAC
		*rhlI*gr	AAAAGGCGGCATCCCTAC
HL8	*rhlR* gel shift assay	*rhlR*gf	GCGTTTCATGGAATTGTCAC
		*rhlR*gr	AAAAAGCCTCCGTCATTCCT
HL9	*vfr* gel shift assay	*vfr*gf	CCGAGTCCCGAAAGAATAAA
		*vfr*gr	CAAAGGGCGCCAGCTTAG
HL10	*mvfR* gel shift assay	*mvfR*gf	GTTGCCGCTACTGTGGAAGA
		*mvfR*gr	TTTCGTCGAATTTACGAGCA
HL11	*pqsABCDE* operon gel shift assay	*pqsA*gf	GTTTGCCATCTCATGGGTTC
		*pqsA*gr	CAGAACGTTCCCTCTTCAGC
HL12	*pqsR* gel shift assay	*pqsR*gf	GTTGCCGCTACTGTGGAAGA
		*pqsR*gr	TTTCGTCGAATTTACGAGCA
HL13	*pqsH* gel shift assay	*pqsH*gf	TCCGTTGCTCCTTAGCAGCG
		*pqsH*gr	TGCGCCTGGATGATCGTCGTG
HL14	*ptxR* gel shift assay	*ptxR*gf	ACGGGTCTTGTCGAAAAAT
		*ptxR*gr	GGACCTGGTACCTCTTGGTG
HL15	*rpoS* gel shift assay	*rpoS*gf	CGCAACCTGCCAGAAAAC
		*rpoS*gr	CGGAAAACCTTAGACCCACT
HL16	*qscR* gel shift assay	*qscR*gf	CGTGCGAGAAGAACAATGAG
		*qscR*gr	AGCTTCACCAGTCAACAA
HL17	*qscR* gel shift assay	*qscRgf1*	GGGCTCGACCTCGTCCGGAA
		qscRgfr	AGCTTCACCAGTCAACAA
HL18	*dksA* gel shift assay	*dksA*gf	ACATGGAAAGGCTCGATG
		*dksA*gr	CGCCTCTCACTTTCGCTAAT
HL19	*rhlA* gel shift assay	*rhlA*gf	TTCGAACAGGCAAACAGCTA
		*rhlA*gr	GTTTCGACACCGGAAACC
HL20	*phzA1* gel shift	*phzA1*gf	TTCCTGTGGCTCTTCCTGTT
		*phzA1*gr	CGATGTTGGTGTTGAGGATG
HL21	*lasB* gel shift assay	*lasB*gf	TCTTGTTCAGTTCTCCTGG
		*lasB*gr	CGAGCAGTGGCCCAACCCTA

(Continued on following page)

TABLE 2 (Continued)

Primer pair	Purpose	Primer name	Sequence (5′–3′)[a]
HL22	*qscR* expression assay	*qscRpf*	GCGCTCGAGCGTGCGAGAAGAACAATGAG
		*qscR*pr	CGCGGATCCAGCTTCACCAGTCAACAA
HL23	Cloning *lasR* into pMCSG7	*lasRpf*	TACTTCCAATCCAATGCCATGGCCTTGGTTGACGGTTT
		lasRr	TTATCCACTTCCAATGTTAGAGAGTAATAAGACCCAAAT

[a] Restriction sites are underlined.

promoter-*luxCDABE* reporter fusions with the *qscR* promoter region as reported previously (13, 30). The *qscR* promoter region was amplified by PCR using the primers *qscR*pf and *qscR*pr in Table 2 (54). The promoter region was cloned into the BamHI-XhoI site upstream of the *lux* genes in pMS402. The construct was transformed into PAO1 strains by electroporation. Cloned promoter sequences were confirmed by DNA sequencing.

Electrophoretic mobility shift assays (EMSA). Various amounts of VqsR protein were incubated with different radioactive DNA probes (Table 3) in 25 μl of gel shift loading buffer (10 mM Tris-HCl [pH 7.4], 50 mM KCl, 5 mM $MgCl_2$, 10% glycerol, and 3 μg/ml sheared salmon sperm DNA). After incubation at room temperature for 20 min, the samples were analyzed by 6% polyacrylamide gel electrophoresis in 0.5× Tris-borate-EDTA (TBE) buffer at 90 V for 90 min. The gels were dried and subjected to autoradiography on a phosphor screen (BAS-IP; Fuji).

Luminescence screening assays. Expression of *lux*-based reporters from cells grown in liquid culture was measured as counts per second (cps) of light production in a Victor3 multilabel plate reader (Perkin-Elmer) or Synergy 2 (Biotek) as previously described by our group (30). Overnight cultures of the reporter strains were diluted to an A_{600} of 0.2 and cultivated for an additional 2 h before use. The cultures were inoculated into parallel wells of a black 96-well plate with a transparent bottom. A 5-μl volume of the fresh cultures was inoculated into the wells containing a total volume of 95 μl medium plus other components, and the A_{600} value in the wells was adjusted to around 0.07. A 60-μl volume of filter-sterilized mineral oil was added to prevent evaporation during the assay. Promoter activities were measured every 30 min for 24 h. Bacterial growth was monitored at the same time by measuring the OD at 595 nm in a Victor3 multilabel plate reader. Expression on solid medium was carried out by plating the reporter strains onto soft-top LB agar and imaging in a LAS300 imaging system (Fuji Corp.).

RNA extraction and Northern blot. Procedures were modified from the protocol previously reported (24). Briefly, overnight *P. aeruginosa* cultures were diluted 100-fold and continually grown at the same medium for about 3 h to an OD_{600} of ~0.6. Total RNA was extracted by using a Qiagen RNeasy kit following the manufacturer's instructions. For Northern blot assays, 5 μg RNA was loaded onto a formaldehyde agarose gel. RNA samples were separated by electrophoresis and blotted onto a nylon membrane (Hybond N; Amersham). Northern blots were hybridized with a *qscR* probe, which was labeled with [α-^{32}P]dCTP by using a randomly primed DNA labeling kit (Ambion). After hybridization and washing, the blots were subjected to autoradiography on a phosphor screen (BAS-IP; Fuji).

TABLE 3 Promoter regions used in this study

Gene name	Probe size (bp)[a]	Transcriptional start site (bp)[a,b]	Reference
lasR	345	−231 (A), −201 (A)	2
lasI	223	−25 (A), −13 (G)	50
rhlR	400	−225 (A), −160 (G), −31 (T), −24 (T)	6
rhlI	257	−100 (C)	7
pqsA	363	−71 (G)	31
pqsR	360	−190 (T), −278 (T)	57
pqsH	324	ND	
rhlA	361	−228 (C), −183 (A)	38
lasB	286	−141 (A)	15
qscR	330	ND	
vfr	273	−146 (C)	46

[a] Probe sizes and transcriptional start sites were measured from the translational start site. Multiple transcriptional start sites have been determined by previous studies.
[b] ND, not determined.

Dye primer-based DNase I footprint assay. The DNA footprint assay was carried out as previously described (62). Briefly, a 330-bp promoter fragment of the *qscR* promoter region that encompasses bases from −231 to +95 was generated by PCR with the primers *qscR*f1 (carrying 6-carboxyfluorescein [6-FAM] at the 5′ end) and *qscR*r1. One 6-FAM-labeled *qscR* promoter probe (40 nM) was incubated with various amounts of His6-VqsR protein ranging from 0 to 4 μM in gel shift loading buffer (10 mM Tris-HCl [pH 7.4], 50 mM KCl, 5 mM $MgCl_2$, 10% glycerol, and 3 μg/ml sheared salmon sperm DNA). After several optimization experiments, the nuclease digestion was found to work best with 0.05 U of DNase I (New England BioLabs) per 20-μl reaction mixture for 5 min at 25°C. The reaction was stopped with 0.25 M EDTA, and DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1). Control digestions with the *qscR* promoter probe were done with 20 μg of bovine serum albumin (BSA) instead of His-VqsR. The DNA fragments were purified with a QIAquick PCR purification kit (Qiagen) and eluted in 15 μl distilled water. About 5 μl of digested DNA was added to 4.9 μl HiDi formamide (Applied Biosystems) and 0.1 μl GeneScan-500 LIZ size standards (Applied Biosystems). The samples were analyzed with a 3730 DNA analyzer, with a G5 dye set, running an altered default genotyping module that increased the injection time to 30 s and the injection voltage to 3 kV, in the sequencing facility at the University of Chicago. Results were analyzed with Peak Scanner software (Applied Biosystems).

Measurement of pyocyanin production. Pyocyanin was extracted from culture supernatants and measured using previously reported methods (14, 23). Briefly, 3 ml of chloroform was added to 5 ml of culture supernatant. After extraction, the chloroform layer was transferred to a fresh tube and mixed with 1 ml of 0.2 N HCl. After centrifugation, the top layer (0.2 M HCl) was removed and its absorption measured at 520 nm. Concentrations, expressed as micrograms of pyocyanin produced per ml of culture supernatant, were determined by multiplying the optical density at 520 nm (OD_{520}) by 17.072 (23).

Swarming and swimming motility assays. The motility assay was carried out as described previously (43). Swarming medium was based on M8 minimal medium, supplemented with $MgSO_4$ (1 mM), glucose (0.2%), and Casamino Acids (CAA) (0.5%) and solidified with agar (0.5%). Bacteria were spot inoculated on swarm agar plates as 2.5-μl aliquots taken directly from overnight LB cultures. Swarming agar plates were incubated for 24 h at 37°C and then incubated an additional 24 h at room temperature for a total of 48 h. Swimming motility medium was 10 g/liter tryptone, 5 g/liter NaCl, and 0.3% agar (43), and 2 μl of overnight cultures was spotted carefully on the plates and incubated at room temperature for 16 h.

Biofilm formation assay. Biofilm formation was measured in a static system as previously described (36) with minor modifications. Cells from overnight cultures were inoculated at 1:100 dilutions into LB medium in

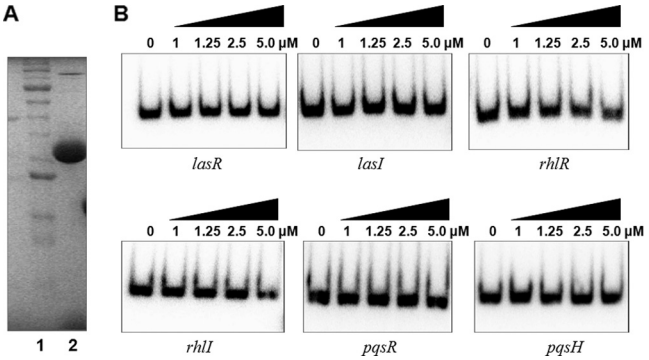

FIG 1 Purified VqsR protein does not bind to the promoter regions of many quorum-sensing genes. (A) SDS-PAGE gel of VqsR after Ni-NTA column affinity chromatography purification. Lane 1, standard protein markers; lane 2, Purified VqsR protein. (B) EMSA showed that VqsR did not bind to the promoter regions of many quorum-sensing genes. PCR products containing the *pqsR*, *pqsA*, *lasR*, *lasI*, *rhlR*, and *rhlI* promoter regions were added to the reaction mixtures at 0.1 μM each. VqsR protein was added to reaction mixtures in lanes 2 to 5 at 0.5, 1.25, 2.5 and 5.0 μM, respectively. No protein was added in lane 1.

96-well polystyrene microtiter plates (Costar) and grown at 30°C for 10 h. A 25-μl volume of 1% crystal violet was added to each well and allowed to stain for 15 min prior to removal by aspiration. Wells were rinsed three times by submerging the plates in distilled water, and the remaining crystal violet was dissolved in 200 μl of 95% ethanol. A 125-μl portion of this solution was transferred to a new polystyrene microtiter plate, and the absorbance was measured at 595 nm.

Gel filtration analysis of purified VqsR. Gel filtration was performed by following previously reported procedures (40). Briefly, purified VqsR protein (0.5 mg/ml) was eluted on a Superdex 75 column (Amersham Pharmacia) at 4°C using a fast protein liquid chromatography (FPLC) apparatus with gel filtration buffer (10 mM Tris-HCl [pH 7.4], 100 mM NaCl, and 1 mM DTT) at a flow rate of 1 ml/min. The protein fractions were collected and kept at −80°C for future experiments.

Measurement of $3OC_{12}$-HSL content of VqsR. Procedures were modified from the protocol described previously (35). Briefly, 0.5 μM VqsR was digested in 500 μl of PBS buffer with 5 μg of proteinase K for 1 h at room temperature and then extracted by $3OC_{12}$-HSL with three volumes of acidified ethyl acetate containing 0.01% glacial acetic acid. The ethyl acetate extracts were evaporated to dryness, and the 3-oxo-C_{12}-HSL was dissolved in 500 μl of acidified ethyl acetate. To measure $3OC_{12}$-HSL, we used a bioassay with *E. coli* DH5α carrying pKD201 (pMC402-*lasI-lux*) and pMCSG7-LasR. Synthetic $3OC_{12}$-HSL was used to prepare a standard curve. The reporter was grown in LB medium plus 50 μg/ml kanamycin and 100 μg/ml ampicillin overnight at 37°C and diluted to an OD_{600} of 0.05 in fresh LB plus kanamycin and ampicillin. Ethyl acetate extracts were added into 2-ml plastic tube and was evaporated spontaneously. One hundred microliters of diluted reporter culture was added to each tube, and luminescence was measured after 4 h at 37°C with shaking by using an AD Analyst 96 384 microplate reader (LJL Biosystems). A standard curve with synthetic $3OC_{12}$-HSL was used to determine the amount of $3OC_{12}$-HSL extracted from VqsR. LasR (0.5 μM) was used as a positive control.

RESULTS

VqsR indirectly regulates three quorum-sensing systems. As a first step in identifying the molecular mechanism utilized by VqsR, we expressed and purified 6×His-tagged VqsR protein in *E. coli*. After Ni-NTA column purification, we obtained a highly purified fraction as shown in an SDS-PAGE gel (Fig. 1A). Unlike LasR and QscR, which need $3OC_{12}$-HSL to be highly soluble and functional, soluble VqsR was obtained in the absence of any acyl-HSL in the growth medium, suggesting that VqsR may function without any QS signal. Given that the *vqsR* regulon contains over 100 QS-controlled genes, we speculated that VqsR protein might directly control some known QS regulators via direct interaction with their promoter regions. To test this hypothesis, we first performed the EMSA experiments using the VqsR protein and the promoter regions of *lasRI* or *rhlRI*. The promoters were amplified by PCR to cover the entire intergenic regions in front of these genes (primers are listed in Table 2; promoters are listed in Table 3). It turned out that VqsR did not physically associate with the *lasRI* or *rhlRI* promoter regions (Fig. 1B), indicating that VqsR indirectly regulates the *las* and *rhl* systems.

It has been shown that the *rhl* system negatively regulates the PQS system (11), while the PQS system positively regulates the *rhl* system (26). Previous microarray analyses demonstrated that VqsR positively controls the expression of *pqsH* (21), which led us to test whether VqsR directly controls the PQS system. Subsequently, EMSA was performed using VqsR protein and three candidate DNA probes containing the *pqsH*, *pqsR*, or *pqsA* promoter region in the PQS system (primers are listed in Table 2; promoters are listed in Table 3). As shown in Fig. 1B, VqsR did not bind to these probes, suggesting that VqsR also indirectly regulates the PQS system.

Previous studies demonstrated that a VqsR mutant displayed reduced production of pyocyanin, rhamnolipid, and elastase (21), which led us to further test whether VqsR can bind to the promoters of the corresponding genes. Again, EMSA results showed no interaction between VqsR protein and DNA probes containing these three promoters (data not shown). Taking these negative results together, we concluded that VqsR indirectly regulates *las*, *rhl*, and PQS systems.

VqsR directly regulates the orphan regulator QscR. Since there was no direct link between VqsR and the promoters that we have tested, we speculated that it may bind to promoter regions of other important QS regulators. In the *vqsR* regulon, there are several QS regulators that have been recently identified. These novel regulators include the novel AraC-type global regulator VqsM (12), Vfr, which is a homolog of the *E. coli* cyclic AMP (cAMP) receptor protein (CRP) (2), and an orphan QS transcriptional factor, QscR, that appears to repress *lasI* (5, 27). Given that VqsR regulates the expression of these regulators, we speculate that VqsR protein might interact with their promoters (primers shown in Table 2; promoters shown in Table 3). VqsR did not bind to DNA fragments carrying the *vfr* or *vqsM* promoter but specifically bound to the *qscR* promoter probe (Fig. 2), indicating that VqsR regulates QS systems by directly controlling the expression of QscR.

We next sought to determine the specific DNA sequence that VqsR recognizes in the *qscR* promoter region by performing a dye-primer-based DNase I footprint assay (primers are shown in Table 2). PCR was performed with a 6-FAM-labeled primer (*qscR*f1 carrying 6-FAM at the 5′ end) and *qscR*r1 to amplify a 330-bp *qscR* promoter region. The 6-FAM-labeled *qscR* promoter probe was incubated with 0.5 μM VqsR protein and then treated with 0.05 U of DNase I. After a 5-min incubation, the reaction was terminated and the product was analyzed on a 3730 DNA analyzer with the default genotyping module and the G5 dye set. By comparing electropherograms with and without VqsR (Fig. 3A) by using Peak Scanner software (Applied Biosystems), we were able

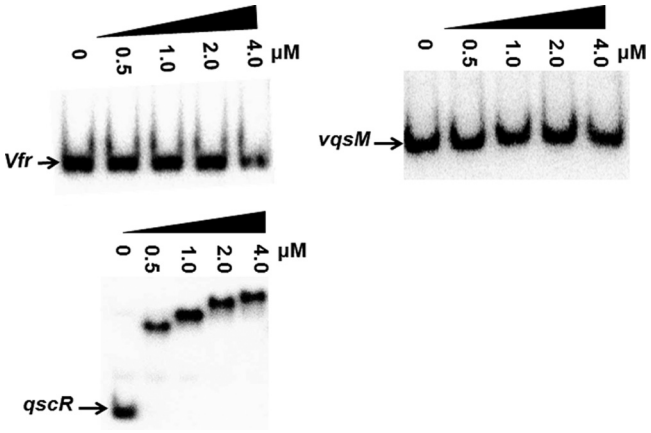

FIG 2 VqsR directly binds to the *qscR* promoter region. PCR products containing the *qscR*, *vfr*, and *vqsM* promoter regions were added to the reaction mixtures at 0.1 μm each. VqsR protein was added to reaction mixtures in lanes 2 to 5 at 0.5, 1.0, 2.0 and 4.0 μM, respectively. No protein was added in lane 1.

to uncover a specific VqsR-protected region containing a putative18-bp inverted repeat (IR) motif ($TCGCCN_8GGCGA$, where N is any nucleotide). Subsequently, we used PCR (with the primers *qscRgf1* and *qscRgfr* [Table 2]) to amplify a truncated *qscR* promoter region without the IR motif. We repeated the EMSA using the truncated *qscR* promoter probe. As expected, VqsR no longer bound to DNA once the IR motif was removed (Fig. 3B), which confirms that the motif is crucial to the DNA-binding ability of VqsR.

To further confirm that the sequence of the IR motif is exclusively responsible for the interaction with VqsR, additional EMSAs were performed using the *qscR* promoter DNA containing single-point mutations at each nucleotide of the IR modules (−188T to C, −187C to G, −186G to C, −185C to T, and −184C to A). The results showed that the substitution −186G to C, −185C to T, or −184C to A completely abolished binding with VqsR, which demonstrates that these three sites are required for VqsR binding (Fig. 3B). Meanwhile, VqsR still

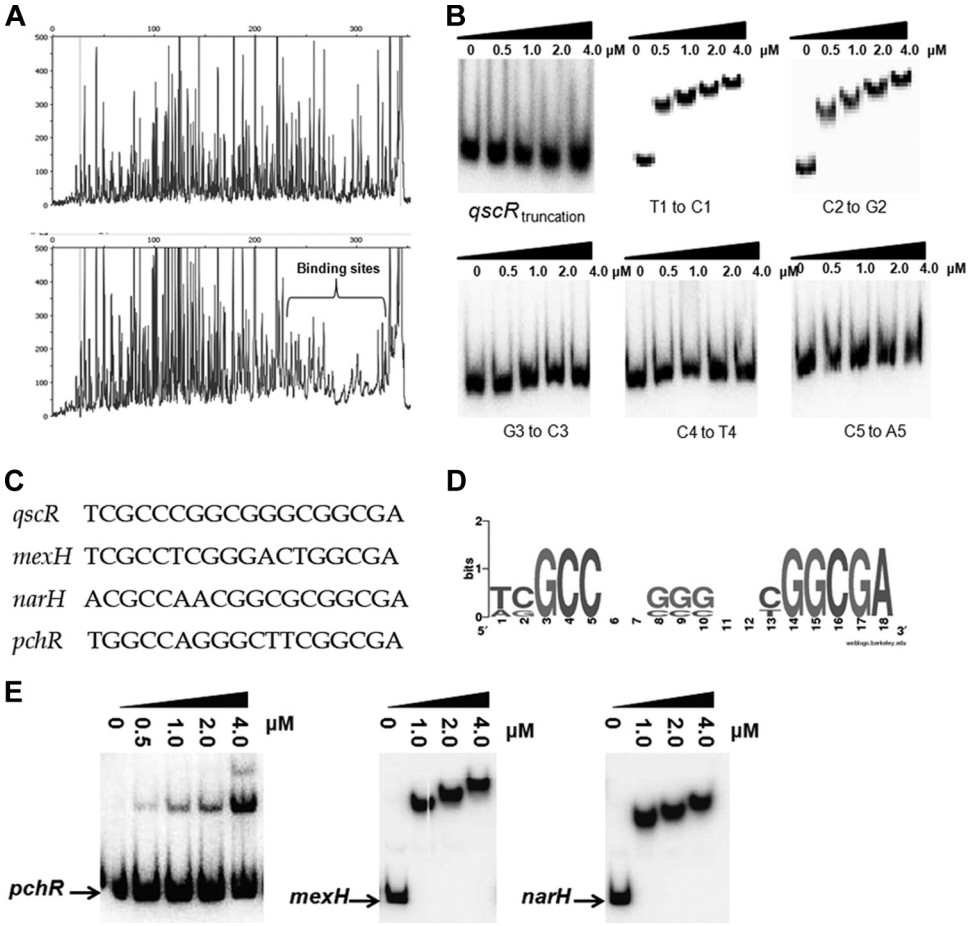

FIG 3 VqsR directly binds to an inverted repeat motif in the *qscR* promoter. (A) Electropherograms showing the protection pattern of the *qscR* promoter after digestion with DNase I following incubation in the absence or presence of 500 nM VqsR. The region of interest identifies the area that shows significant reduction in the peak pattern compared with the control. (B) Mutations in the inverted repeat affect the DNA-binding affinity of VqsR. VqsR does not bind to the DNA probe containing the *qscR* promoter region with the inverted repeat motif removed ($qscR_{truncation}$). Single-point mutations in the first half of the inverted repeat motif showed varied influence on the DNA-binding affinity of VqsR. (C) Besides *qscR*, three other genes carry the conserved inverted repeat sequences in their promoter regions, as determined by a bioinformatic search. (D) VqsR binding sequence. The size of each letter indicates the relative abundance at the respective position in a matrix generated with CONSENSUS software. (E) VqsR binds to the promoters of *pchR*, *mexH*, and *narH*. PCR products containing the *pchR*, *mexH*, and *narH* promoter regions were added to the reaction mixtures at 0.1 μM each. VqsR protein was added to reaction mixtures in lanes 2 to 5, respectively. No protein was added in lane 1.

bound to the DNA probe with the mutation from −188T to C, or from −187C to G, indicating that these two sites are dispensable (Fig. 3B).

Genome-wide search of the genes regulated by the putative IR elements. The identification of a VqsR-regulated IR element enabled us to search for putative *vqsR*-regulated promoters in *P. aeruginosa*. The *P. aeruginosa* genome was searched for the IR sequence (perfect or with one mismatch) by using the program Regulatory Sequence Analysis Tools (http://rsat.ulb.ac.be/rsat/), which uncovered 51 promoters with perfect IR (Table 4) and 700 others with one mismatch (data not shown). Interestingly, among the genes flanked by the putative IR motif, some belong to the *vqsR* regulon, such as *mexH*, which encodes a component in the efflux pump MexGHI-OpmD (1), the nitrate reductase gene *narH* (33), and *pchR*, encoding an AraC-type regulator (32) (Fig. 3C and D). We next sought to determine if VqsR directly binds to these promoters by EMSA. As shown in Fig. 3E, VqsR indeed binds to the promoter regions of *mexH*, *narH*, and *pchR*, indicating the direction regulation of these genes by VqsR.

VqsR exists as a homodimer in solution. Previous studies demonstrated that most QS regulators such as LasR (48), TraR (61), and CarR (59) exist as homodimers, while a few, such as QscR of *P. aeruginosa*, are monomers in solution (27). The binding of VqsR to promoters containing an IR motif strongly suggests that VqsR may also exist as a dimer. This was further supported by a gel filtration chromatography analysis of purified VqsR. On a Superdex 75 column (Amersham Pharmacia), purified VqsR eluted with a molecular mass of about 62 kDa, which is exactly twice its predicted molecular mass (Fig. 4A and B). In addition, EMSA confirmed that the 62-kDa fraction efficiently bound to the *qscR* promoter region (Fig. 4C).

3OC$_{12}$-HSL does not affect DNA binding of VqsR. Since VqsR is an important QS regulator, we next sought to investigate if VqsR binds to any acyl-HSL signals, which is a characteristic of LasR (48). To examine if VqsR binds to 3CO$_{12}$-HSL, we incubated purified VqsR with 20 μM 3OC$_{12}$-HSL at 4°C overnight. Gel filtration chromatography was then performed, and each fraction was collected. After digestion with proteinase K, the samples were extracted twice with ethyl acetate, evaporated to dryness, and resuspended in ethyl acetate. The amount of 3OC$_{12}$-HSL in the ethyl acetate extract was determined using a 3OC$_{12}$-HSL bioassay in *E. coli* (48). As shown in Fig. 5A, the expression of *lasI-lux* in the *E. coli* strain treated with ethyl acetate from digested VqsR was at the same level as that of the negative control. As a positive control, the activity of *lasI-lux* was strongly induced by proteinase K-treated LasR, which carries 3OC$_{12}$-HSL. These results clearly indicate that VqsR does not bind to 3OC$_{12}$-HSL. In addition, we performed EMSA using VqsR and the *qscR* promoter DNA in the absence and presence of 3OC$_{12}$-HSL. As shown in Fig. 5B, the binding affinity of VqsR to the *qscR* promoter remained the same with and without 3OC$_{12}$-HSL, suggesting that 3OC$_{12}$-HSL is not involved in DNA binding of VqsR.

VqsR affects the expression of *qscR* and other phenotypes *in vivo*. Since we have shown that VqsR directly binds to the *qscR* promoter *in vitro*, we subsequently sought to test this regulation *in vivo* by a *qscR-lux* reporter assay and a Northern hybridization. We constructed a *qscR* promoter-*lux* fusion and then measured its activity in wild-type PAO1, a *vqsR* mutant, and a *vqsR* mutant complemented with PAK1900-*vqsR* as described in Materials and Methods. As shown in Fig. 6A, the activity of *qscR-lux* in the *vqsR* mutant was about 4-fold higher than that in the wild-type PAO1 strain. The result was further verified by the Northern blot assay probed by the *qscR* (Fig. 6B), demonstrating that VqsR negatively regulates *qscR* expression.

TABLE 4 Genes containing inverted repeat sequences

Gene name or PA no.	Start	End	Product
PA0269	−227	−210	Conserved hypothetical protein
PA0663	−147	−130	Hypothetical protein
PA0810	−224	−207	Probable haloacid dehalogenase
PA0961	−164	−147	Probable cold-shock protein
PA1263	−237	−220	Hypothetical protein
ggt	−373	−356	Gamma-glutamyltranspeptidase precursor
PA1364	−244	−227	Probable transmembrane sensor
fliM	−357	−340	Flagellar motor switch protein FliM
alc	−307	−290	Allantoicase
modB	−341	−324	Molybdenum transport protein ModB
PA1955	−157	−140	Hypothetical protein
PA2174	−310	−293	Hypothetical protein
PA2307	−387	−370	Probable permease of ABC transporter
xylX	−168	−151	Toluate 1,2-dioxygenase alpha subunit
greB	−374	−357	Transcription elongation factor GreB
asd	−278	−261	Aspartate semialdehyde dehydrogenase
PA3314	−130	−113	Probable ATP-binding component of ABC transporter
PA3472	−342	−325	Hypothetical protein
PA3973	−314	−297	Probable transcriptional regulator
PA4191	−313	−296	Probable iron/ascorbate oxidoreductase
PA4312	−345	−328	Conserved hypothetical protein
PA4357	−199	−182	Conserved hypothetical protein
rpsO	−388	−371	30S ribosomal protein S15
PA4841	−215	−198	Conserved hypothetical protein
ureG	−297	−280	Urease accessory protein UreG
qscR	−188	−171	Quorum-sensing control repressor
PA0484	−206	−189	Conserved hypothetical protein
PA0678	−207	−190	HxcU putative pseudopilin
PA0839	−315	−298	Probable transcriptional regulator
PA1255	−309	−292	Hypothetical protein
PA1309	−390	−373	Probable transcriptional regulator
PA1356	−95	−78	Hypothetical protein
PA1407	−326	−309	Hypothetical protein
PA1478	−322	−305	Hypothetical protein
PA1527	−117	−100	Conserved hypothetical protein
PA1884	−120	−103	Probable transcriptional regulator
PA1993	−99	−82	Probable major facilitator superfamily (MFS) transporter
hcnC	−352	−335	Hydrogen cyanide synthase HcnC
PA2477	−34	−17	Probable thiol-disulfide interchange protein
PA2533	−183	−166	Probable sodium-alanine symporter
cif	−329	−312	CFTR inhibitory factor, Cif
PA3133	−378	−361	Probable transcriptional regulator
PA3471	−132	−115	Probable malic enzyme
ygbP	−239	−222	4-Diphosphocytidyl-2-*C*-methylerythritol synthase
PA4090	−301	−284	Hypothetical protein
mexH	−375	−358	Probable resistance-nodulation-cell division (RND) efflux membrane fusion protein precursor
PA4320	−209	−192	Hypothetical protein
PA4455	−352	−335	Probable permease of ABC transporter
PA4773	−154	−137	Hypothetical protein
ureC	−304	−287	Urease alpha subunit
argE	−338	−321	Acetylornithine deacetylase

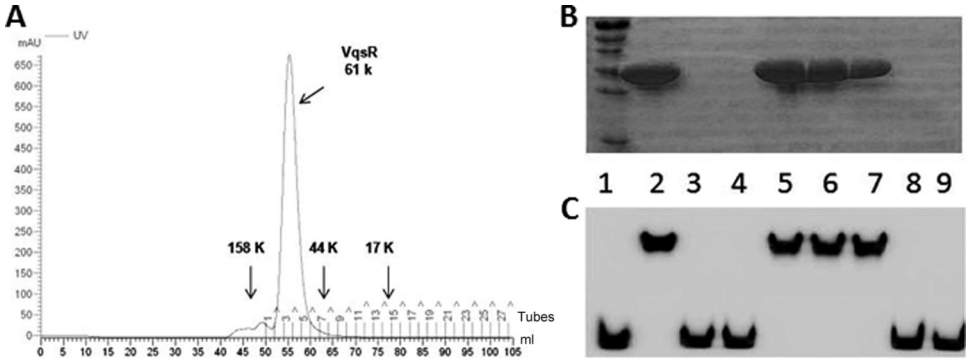

FIG 4 Purified VqsR exists as a homodimer in solution. (A) The molecular mass of His-tagged VqsR was estimated by gel filtration column chromatography. Arrows indicate the standard molecular masses according to the volumes of elution. The standards for the Superdex 75 16/60 column, presented as elution volume (ml)/molecular mass (kDa), are 76/17, 60/43, 54/67, and 42/158. (B) The elution samples were subjected to SDS-PAGE. The samples were collected from tubes 1 to 7 (corresponding to tubes 1 to 7 in panel A). Lane 1 was the positive control, containing purified VqsR before gel filtration. (C) Elution products from tube 1 to 7 were reacted with the *qscR* promoter DNA and then assayed by EMSA. Lane 1 contained purified VqsR as a positive control.

Previous microarray analysis showed that a *vqsR* mutant exhibited decreased production of pyocyanin and acyl-HSL signals (21). To investigate the regulation *in vivo*, we tested phenotypes such as pyocyanin production, biofilm formation, motility, and colony morphology in the wild-type, the *vqsR* mutant, the *vqsR* mutant complemented with *vqsR*, and the *vqsR* mutant complemented with *qscR*. The production of pyocyanin and the formation of biofilms in the *vqsR* mutant was significantly lower than those in the wild-type strain, and this could be complemented by overexpression of either *vqsR* or *qscR* in the *vqsR* mutant (Fig. 6C and D). On the other hand, the loss of *vqsR* also compromised the motility and altered colony morphology, which could also be complemented by overexpression of *qscR* (Fig. 6E, F, and G). Taken together, these results clearly indicate that VqsR regulates these phenotypes by controlling QscR expression *in vivo*.

DISCUSSION

LuxR-family regulators are key players in bacterial quorum-sensing systems among a wide range of organisms. QS coordinates the expression of a variety of genes, which are involved in virulence regulation, antibiotics biosynthesis, motility, and biofilm formation (4). Previous studies have shown that VqsR is a member of the LuxR family and possesses a *las* box in its promoter region. Sequence analysis showed that VqsR contains a carboxy-terminal helix-turn-helix (HTH) DNA binding domain. A *vqsR* transposon insertion mutant showed reduced acyl-HSL production and compromised expression of some QS-controlled virulence factors (21, 22). In addition, Dong et al. has reported that another global regulator VqsM positively influences the *las*- and *rhl*-mediated QS signaling pathways via modulating VqsR expression (12). Although the global regulator VqsR plays important roles in *P. aeruginosa*, its underlying regulatory mechanism has yet to be elucidated.

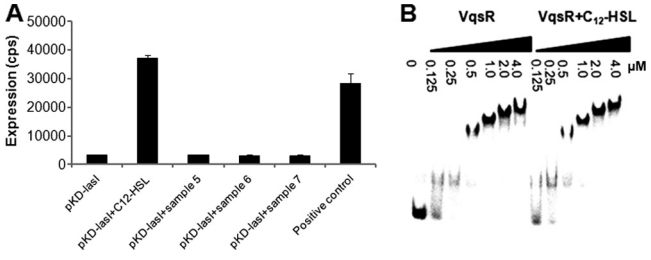

FIG 5 Purified VqsR protein does not contain $3OC_{12}$-HSL. (A) An AHL bioassay determined the $3OC_{12}$-AHL content of purified VqsR and LasR. Samples 5, 6, and 7 are gel filtration-purified VqsR protein used for Fig. 4. A 20-μl sample of the gel filtration fraction was digested with 0.2 mg/ml proteinase K in 200 μl gel filtration buffer for 1 h at 37°C, extracted twice with ethyl acetate, evaporated to dryness, and resuspended in ethyl acetate. The amount of extracted $3OC_{12}$-HSL from digested VqsR or LasR was determined through the use of a $3OC_{12}$-HSL bioassay (*E. coli* carries both pKD-*lasI-lux* and pMCSG7-*lasR*). (B) An EMSA was performed in the absence and presence of $3OC_{12}$-HSL. Lane 1, *E. coli* containing *lasI-lux* only.

Comparison of the microarray data for the *vqsR* mutant (22) with those for the QS regulons (47) revealed that 101 genes were shared by these transcriptomes, although these data were obtained under different experimental conditions. This strongly indicates that VqsR is part of the QS circuit in *P. aeruginosa*. We hypothesize that a complicated hierarchy exists in the QS regulon, consisting of multiple transcriptional regulators, in which VqsR may serve as a regulator of *las*, *rhl*, and AHQ systems. In order to test this possibility, we carried out EMSA experiments which showed that the purified VqsR does not bind to the promoter regions of major regulators of three QS systems (*las*, *rhl*, and AHQ). Interestingly, it specifically bound to the *qscR* promoter region. Subsequent genetic experiments demonstrated that VqsR negatively regulates *qscR*. In addition, we attempted to construct a *vqsR qscR* double deletion to strengthen this conclusion. However, we were unable to obtain the double deletion for unknown reasons. This result strongly suggests that VqsR regulates gene expression that is mainly dependent on the *qscR* regulator in *P. aeruginosa*.

Previous studies demonstrated that many QS regulators can bind to the conserved *las* box sequence ($NNCTN_{12}AGNN$). In addition, palindromic *las* box-like sequences are found in the promoter regions of about 7% of the QS-regulated genes. The *las* box is important for the transcription of a number of QS-regulated genes (17). DNase I footprinting results showed that VqsR protected an inverted repeat sequence ($TCGCCN_8GGCGA$) in the *qscR* promoter. Interestingly, the VqsR binding sequences are different from those of other reported QS regulators, indicating the specificity of VqsR regulation in the QS networks. By searching the *P. aeruginosa* genome with the program Regulatory Sequence Analysis Tools for additional genes whose promoters carry the

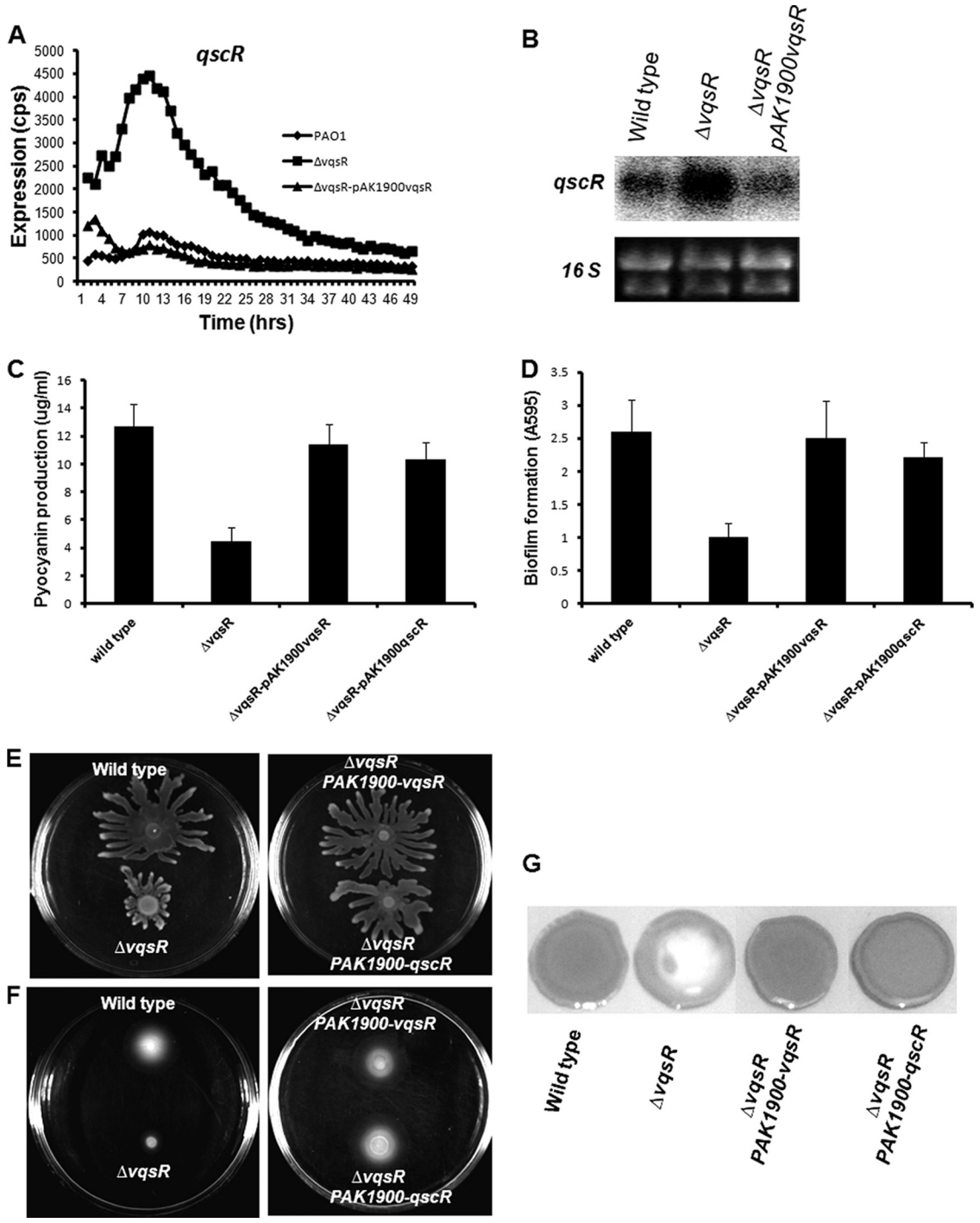

FIG 6 VqsR negatively regulates *qscR* expression and positively controls other phenotypes, such as pyocyanin, motility, and colony morphology, *in vivo*. (A) The expression of *qscR* was assayed in the wild type, the Δ*vqsR* mutant, and the Δ*vqsR* mutant complemented with PAK1900-*vqsR*. The assay was independently repeated at least three times, and the data are representative results. (B) Northern blotting showed the expression of *qscR* in different *P. aeruginosa* strains, including the wild type, the Δ*vqsR* mutant, and the Δ*vqsR* mutant complemented with PAK1900-*vqsR*, on the ethidium bromide-stained RNA gel. (C through G) Production of pyocyanin (C), biofilm formation (D), swarming (E), swimming (F), and colony morphology (G) in the wild-type and the indicated mutants. (C and D) Values are means from three independent experiments plus standard deviations.

same motif, we were able to identify 50 other such genes (Table 4), such as *mexH*, as well as over 700 genes (data not shown) containing the motif with one mismatch, such as *pchR* and *narH* (Fig. 3C). Strikingly, among the 51 genes identified as having a perfect flanking IR motif, only one (*hcnC*) belongs to the *vqsR* regulon, which does not even include *qscR* (21, 22). Given that the previous microarray analyses were performed under different conditions (such as under oxidative stress, using supplementation with hu-

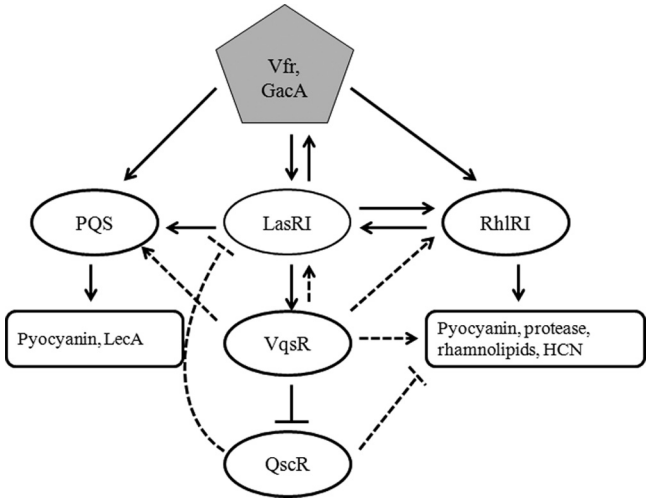

FIG 7 Schematic diagram of VqsR regulation in the complex QS systems of *P. aeruginosa*. The potential regulatory pathways and interplays are proposed according to our observations and previous studies. GacA and Vfr positively regulate Las and Rhl QS systems (2, 44). On the other hand, LasR directly controls VqsR expression (28). In the present study, we found that VqsR directly regulates QscR. However, VqsR indirectly regulates the Las, Rhl, and PQS systems. Solid arrows indicate positive regulation. T bars indicate negative regulation. Dotted lines indicate indirect regulation.

man serum, or in ABC medium), we speculate that the majority of the 51 genes may not be expressed differentially under these conditions. It is also possible that functional IR motifs with one or more mismatches are located in the promoter regions of the genes that show altered expression in *vqsR* mutants. It will be interesting to investigate the direct interaction between VqsR and the newly identified genes carrying an IR motif in their promoters.

As previous studies reported, many QS regulators bind to promoter DNA in a QS signal-dependent manner. For example, LasR (28, 48) and QscR (27) rely on the $3OC_{12}$-HSL signal to control downstream gene expression. However, unlike LasR, VqsR is soluble in solution in the absence of $3OC_{12}$-HSL (Fig. 1A). In agreement with this observation, a $3OC_{12}$-HSL bioassay also suggests no interaction between VqsR and $3OC_{12}$-HSL (Fig. 5). We postulate that VqsR may require an unknown signal to regulate gene expression.

In conclusion, we have demonstrated that the global regulator of VqsR indirectly regulates QS systems and QS-controlled virulence factors. However, it specially binds to the *qscR* promoter region. Taking our observations and those of previous studies together, we propose a working model to illustrate the pathways and interplays, in which VqsR modulates QS and the production of virulence factors mainly through negative regulation of *qscR* (Fig. 7). In this model, the global regulators GacA (44) and Vfr (2) positively regulate *las* and *rhl* systems. It has been shown that LasR can bind to the *vqsR* promoter region and positively controls VqsR expression (28). Finally, VqsR positively influences the expression of virulence factors by inhibiting the expression of QscR. The expression of VqsR and QscR could be subject to the positive (for VqsR) or negative (for QscR) feedback regulation of QS. These findings provide further information en route to establishing the connections in the complex QS regulatory networks in *P. aeruginosa*, which are important to a full understanding of how QS promotes virulence in the host.

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