QscR, a modulator of quorum-sensing signal synthesis and virulence in *Pseudomonas aeruginosa*

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The opportunistic pathogenic bacterium *Pseudomonas aeruginosa* uses quorum-sensing signaling systems as global regulators of virulence genes. There are two quorum-sensing signal receptor and signal generator pairs, LasR–LasI and RhlR–RhlI. The recently completed *P. aeruginosa* genome-sequencing project revealed a gene coding for a homolog of the signal receptors, LasR and RhlR. Here we describe a role for this gene, which we call *qscR*. The *qscR* gene product governs the timing of quorum-sensing-controlled gene expression and it dampens virulence in an insect model. We present evidence that suggests the primary role of QscR is repression of *lasI*. A *qscR* mutant produces the LasI-generated signal prematurely, and this results in premature transcription of a number of quorum-sensing-regulated genes. When fed to *Drosophila melanogaster*, the *qscR* mutant kills the animals more rapidly than the parental *P. aeruginosa*. The repression of *lasI* by QscR could serve to ensure that quorum-sensing-controlled genes are not activated in environments where they are not useful.

Pseudomonas aeruginosa is a versatile bacterium that can be found in many different environments. For example, it can be found in lakes, soils, and on plants (1). It is also an emerging opportunistic pathogen of humans (2, 3). The genome sequence of this bacterium was completed recently. The sequence analysis revealed a great diversity of genes, including a high number of putative transcription regulators. Nearly 10% of the 5,570 predicted *P. aeruginosa* genes appeared to be transcription factors (4).

A large number of genes (perhaps as many as 200–300), including virulence factor genes and genes involved in biofilm development, are activated by two homologous acyl-homoserine lactone (AHSL) quorum-sensing systems. These two systems are the LasR–LasI and RhlR–RhlI systems. LasR is a transcriptional activator that responds to the product of the LasI protein, *N*-3-(oxododecanoyl)homoserine lactone ($3OC_{12}$-HSL). At sufficient environmental concentrations of this AHSL signal, a number of genes are activated, including *rhlR*, which codes for the *N*-butyrylhomoserine lactone (C_4-HSL) receptor, and *rhlI*, which codes for the C_4-HSL signal generator. RhlR and C_4-HSL activate many other genes. As might be expected in a bacterium with so many lifestyles, and so many regulatory genes, the elements of the two quorum-sensing systems are controlled by other factors (5–8).

The analysis of the *Pseudomonas* genome revealed a gene coding for a homolog of LasR and RhlR but no additional genes coding for LasI and RhlI homologs. The predicted ORF for the LasR homolog, PA1898, begins at bsae pair 2,069,490 and terminates at base pair 2,070,203 (see http://www.pseudomonas.com). The gene is linked to a group of genes with similarity to the *phz* operon, which is required for production of the phenazine pigment, pyocyanin, itself a virulence factor (8, 9). However, this operon cannot substitute for the *phz* operon in the synthesis of pyocyanin (8). Here we investigate the role of the LasR, RhlR homolog in *P. aeruginosa* quorum sensing and virulence. Our evidence indicates that the homolog is a negative regulator of quorum-sensing-controlled genes and that it likely exerts its affect by repressing *lasI*. Thus we have termed the gene *qscR* for quorum-sensing-control repressor.

Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions. The strains and plasmids we used are shown in Table 1. *Escherichia coli* and *P. aeruginosa* were routinely grown at 37°C in Luria–Bertani broth or Luria–Bertani agar. For virulence experiments, bacteria were grown in Brain Heart Infusion broth (Difco) for 8 h. Pyocyanin assays were performed with *P. aeruginosa* grown in pyocyanin production medium (PPM) (10). Media were supplemented with antibiotics for selection as follows: for *E. coli*, ampicillin (Ap) at 100 μg/ml, gentamicin (Gm) at 15 μg/ml, chloramphenicol (Cm) at 30 μg/ml, and spectinomycin (Sp) at 100 μg/ml; for *P. aeruginosa*, carbenicillin (Cb) at 300 μg/ml, Gm at 100 μg/ml, nalidixic acid at 20 μg/ml, and streptomycin (Sm) at 500 μg/ml. For promoter activity assays, pyocyanin production measurements, and AHSL production studies, the OD_{600} at the start of an experiment was 0.005–0.05. Starter cultures were in the mid-log phase. Analyses were performed on samples taken at the indicated points during culture growth. For experiments with *tac* promoter-controlled genes, we included isopropyl-β-D-thiogalactopyranoside (1 mM) in the culture medium.

Construction and Complementation of a *P. aeruginosa qscR* Mutant. A *qscR* mutant was constructed as follows. A 3.1-kb *P. aeruginosa* PAO1 chromosomal DNA fragment containing *qscR* (from base pair 2,067,814 to base pair 2,070,999 on the *P. aeruginosa* chromosome) was amplified by Expand long-template PCR (Boehringer Mannheim), and cloned into *Bam*HI-digested pMP7. The resulting plasmid was pKL13. A 1.6-kb *Sma*I fragment of pGmΩ1 that contained the *aacC1* gene (encoding Gm acetyltransferase) was ligated with *Eco*RV-digested pKL13 to create pKL14. This construct has the *aacC1* gene inserted 19 bp downstream of the translation start site of *qscR,* and *aacC1* is flanked by 1.7 kb of upstream and 1.5 kb of downstream *P. aeruginosa* DNA. A 1.6-kb *Kpn*I fragment carrying a mobilizing (mob) cassette from pKV69 was cloned into *Kpn*I-digested pKL14 to create pMW16, a suicide plasmid with a ColE1 *ori.* pMW16 was mobilized from *E. coli* S17–1 into *P. aeruginosa* PAO1 by conjugation. Selection for Gm^R colonies followed by screening for Cb^S yielded a *qscR* insertion mutant, *P. aeruginosa* PAOR3. We confirmed that *P. aeruginosa* PAOR3 contained an insertion of the Gm cassette in *qscR* at the expected location by Southern blotting (Genius, Boehringer Mannheim) with *aacCI* and *qscR* gene probes.

The *qscR* mutation in strain PAOR3 was complemented by introduction of pKL9, which contains *qscR* under the control of the *tac* promoter. To construct pKL9, a 714-bp DNA fragment carrying *qscR* was amplified from pKL13 with Expand long-template PCR and T/A cloned using the Original TA cloning kit (Invitrogen) to form pKL8, which was digested with *Mfe*I and

Abbreviations: AHSL, acyl-homoserine lactone; $3OC_{12}$-HSL, *N*-(3-oxododecanoyl)homoserine lactone; C_4-HSL, *N*-butyrylhomoserine lactone; Ap, ampicillin; Gm, gentamicin; Cm, chloramphenicol; Sp, spectinomycin; Cb, carbenicillin; Sm, streptomycin.

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Table 1. Bacterial strains and plasmids used

Strain or plasmid	Genotype	Source/ref.
P. aeruginosa		
PAO1	Wild-type prototroph	30
PAOR3	*qscR* mutant of PAO1, Gm^R	This study
E. coli		
DH5α	F^- ϕ80d*lacZ*Δ*M15*Δ(*lacZYA-argF*)U169*endA1 recA1 hsdR17 deoR gyrA96 thi-1 relA1 supE44*	31
S17-1	*thi pro hsdR recA RP4-2* (Tet::Mu) (Km::Tn7)	32
CM830	*E. coli* K-12 derivative	
Plasmids		
pGmΩ1	Broad-host-range vector containing the *aacC1* gene, Gm^R	33
pCR2.1	TA cloning vector, Ap^R	Invitrogen
pEX1.8	Broad-host-range expression vector, Ap^R	34
pQF50	Broad-host-range *lacZ* transcriptional fusion vector, Ap^R	35
pMP7	*E. coli* cloning vector, (ColE1) *ori*, Ap^R	36
pKL13	pMP7 containing 3.1 kb *qscR* fragment, Ap^R	This study
pKL14	pKL13 with 1.6 kb *aacC1* interrupting *qscR* Ap^RGm^R	This study
pKV69	Vector carrying mobRP4 Cm^RTc^R	K. Visick, Loyola Univ., Chicago
pMW16	pKL14 containing 1.6 kb mobRP4 from pKV69, $Ap^RGm^RCm^R$	This study
pKL8	pCR2.1 containing 714 bp *qscR* fragment, Ap^R	This study
pKL9	pEX1.8 containing p*tac-qscR*, Ap^R	This study
pMW303	Broad-host-range *phzABC-lacZ* reporter, Ap^R	15
pMW301	Broad-host-range *hcnAB-lacZ* reporter, Ap^R	15
pSC11	Broad-host-range *lasI-lacZ* reporter, Ap^R	This study
pSC10	Broad-host-range *lasR-lacZ* reporter, Ap^R	This study
pMW305	Broad-host-range *rhlI-lacZ* reporter, Gm^R, Sp^R, Sm^R	15
pMW304	Broad-host-range *rhlR-lacZ* reporter, Gm^R, Sp^R, Sm^R	15
pMW105B	Broad-host-range qsc105-*lacZ* reporter, Ap^R	This study

*Hin*dIII, and cloned into *Eco*RI–*Hin*dIII-digested pEX1.8 to generate pKL9.

Construction of Reporter Gene Fusions. To create the *lasR–lacZ* reporter plasmid, pSC10, a 623-bp PCR fragment with engineered *Sal*I and *Bam*HI sites flanking bp −410 to +213 relative to the *lasR* translational start was ligated to *Sal*I–*Bam*HI-digested pQF50. To construct the *lasI–lacZ* reporter plasmid, pSC11, a 505-bp PCR fragment with engineered *Sal*I and *Bam*HI sites flanking base pairs −282 to +223 relative to the *lasI* translational start site was cloned into *Sal*I–*Bam*HI-digested pQF50. A 289-bp PCR-generated fragment flanking base pairs −273 to +16 relative to the translational start site of the ORF PA2587 (http://www.pseudomonas.com) was polished with T4 polymerase and cloned into *Sma*I-digested pQF50 to construct the qsc105-*lacZ* reporter plasmid, pMW105B. All constructs were verified by DNA sequencing.

AHSL, Pyocyanin, and β-Galactosidase Measurements. Concentrations of $3OC_{12}$-HSL and C_4-HSL were measured with bioassays as described (11, 12). Synthetic $3OC_{12}$-HSL and C_4-HSL (Quorum Sciences, Coralville, IA) were used to generate standard curves. Pyocyanin was extracted from culture fluid with chloroform and then extracted from the chloroform with 0.2 M hydrochloric acid in water. The absorbance at 520 nm was a measure of the amount of extracted pyocyanin as described elsewhere (10). β-Galactosidase activity was measured as described (8). Results are given in units of β-galactosidase, activity per OD_{600}.

Fruit Fly Virulence Studies. Approximately 8×10^9 viable bacterial cells were pelleted from cultures by centrifugation. These cells were suspended in 170 μl of 5% sucrose solution (13) and added to a filter disk (2.3-cm diameter Whatman paper) that completely covered the agar surface (5 ml of 2.4% agar with 5% sucrose) at the bottom of a standard glass fly culture vial. Adult male 3- to 5-day-old fruit flies (*Drosophila melanogaster* Canton S) that had been starved for food and water for 5 h were added to each vial (9–12 flies per vial). The vials were capped with cotton, inverted over a tray with water for humidity, and incubated at 25°C. To determine the number of viable bacterial cells associated with individual flies, single flies were ground with a Teflon pestle in an Eppendorf tube with 100 μl of 10 mM $MgSO_4$, and serial dilutions of the homogenate were spread on Luria–Bertani agar.

Results

The Relationship of QscR to AHSL Signal Receptors. The *qscR* ORF consists of 714 nucleotides, and it codes for a polypeptide of 27,236 Da. A sequence comparison shows that QscR is most similar to *Ralstonia solanacearum* SolR (33% identity, 49% similarity), *P. aeruginosa* RhlR (32% identity, 53% similarity), *P. aeruginosa* LasR (29% identity, 46% similarity), and *Vibrio fischeri* LuxR (27% identity, 45% similarity). An alignment of these polypeptides is shown in Fig. 1. QscR has the seven residues conserved among all of the members of this family of transcription factors (14). The *qscR* gene is linked to a cluster of genes that is nearly identical to the *phz* operon (Fig. 1). The DNA immediately upstream of this gene cluster bears no relation to the *phz* promoter region. Furthermore, *P. aeruginosa* strain PAO1 with a mutation in the *phz* operon does not produce pyocyanin (10). Thus, the genes linked to *qscR* do not direct the synthesis of pyocyanin in *P. aeruginosa* strain PAO1.

A *qscR* Mutant Overexpresses Quorum-Sensing-Controlled Virulence Factors. To study the role of *qscR* in *P. aeruginosa*, we constructed a null mutant, PAOR3. Cultures of this *qscR* mutant grew at the same rate as the parent strain PAO1 (data not shown); however, they were noticeably different from the parent in that they were

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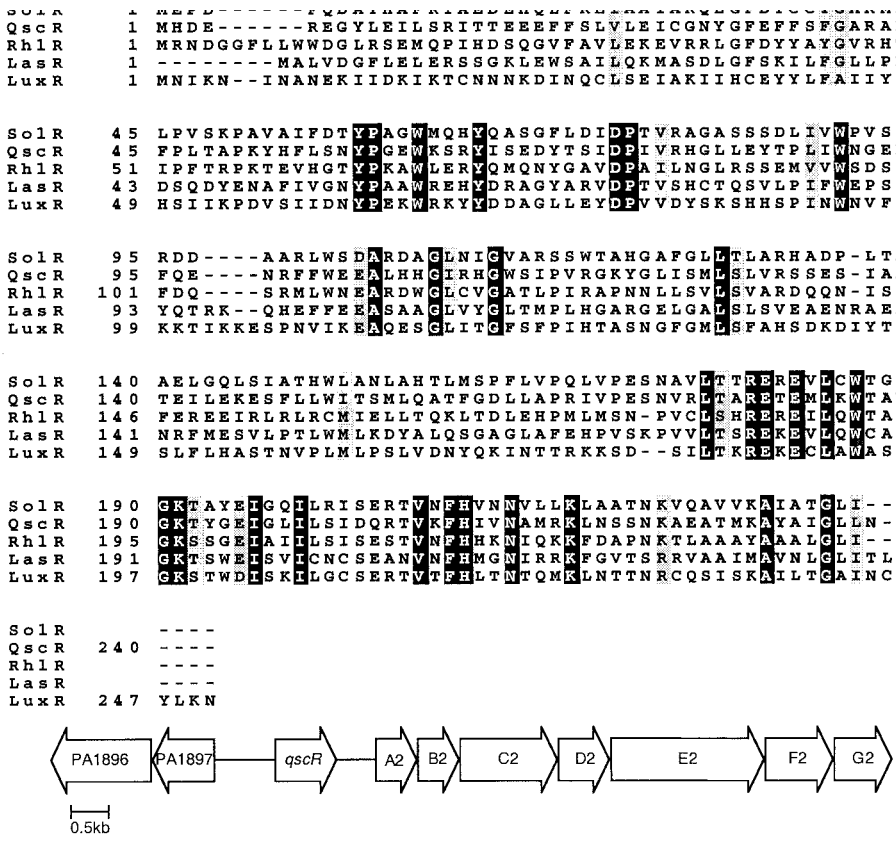

Fig. 1. Relationship of the *qscR* gene and gene product to other genes and their products. (*Upper*) Alignment of the QscR sequence with SolR, RhlR, LasR, and LuxR. Conserved amino acids are shaded in black. Gray shading indicates that 100% of the residues are similar at that position. The alignment was constructed by using the CLUSTAL W multiple alignment program and the degree of residue shading was determined by using Boxshade at 1.0 setting (37). The sequences used in the alignment are *R. solanacearum* SolR (GenBank accession no. AF021840), *P. aeruginosa* RhlR (L08962), *P. aeruginosa* LasR (M59425), and *V. fischeri* LuxR (M96844). (*Lower*) A map of *qscR* and the genes flanking *qscR* in the *P. aeruginosa* genome. In the *P. aeruginosa* genome annotation, genes designated *A2–G2* are called *phzA2-G2* based on similarity to genes in the *phz* operon (http://www.pseudomonas.com). The genes in the *phzA2-G2* operon are each over 98% identical at the DNA sequence level to the bona fide *phzA-G* genes. Other phenazine-producing species of *Pseudomonas* have operons with related genes. The *qscR* gene is called *phzR* in the *P. aeruginosa* genome annotation.

blue. This is a characteristic of strains that overproduce the phenazine pigment pyocyanin (15). Direct measurements confirmed that the *qscR* mutant produced more pyocyanin than the parent. It also produced pyocyanin at a lower culture density than the wild type. Complementation of the *qscR* mutation in PAOR3 restored the parental pyocyanin levels and timing of pyocyanin synthesis (Fig. 2*A*).

The influence of QscR on pyocyanin production could be at the level of *phz* operon transcription or at a posttranscriptional level. It could be direct or it could be a general repression of quorum-sensing-controlled genes. To address these questions, we tested the influence of QscR on the expression of β-galactosidase in strains with the *lacZ* gene fused to promoters of quorum-controlled genes (including a *phzABC–lacZ* fusion). Besides the *phz* operon, we examined transcription of the *hcn* operon. This operon appears to be required for production of hydrogen cyanide, which has been established as a virulence factor for *P. aeruginosa* (16), and like the *phz* operon, we believe it is controlled by C_4-HSL and RhlR, directly (8). We also examined a *lacZ* fusion to an ORF controlled by $3OC_{12}$-HSL and LasR, qsc105 (8). The *phzC–lacZ* fusion was expressed earlier in the *qscR* mutant than the wild-type (Fig. 2*B*). This indicates that the early induction of pyocyanin synthesis in the *qscR* mutant can be attributed to derepression of *phz* operon transcription. Early expression of the *phz* operon could also account for increased levels of pyocyanin in cultures of the *qscR* mutant. The patterns of β-galactosidase expression in the parent and the *qscR* mutant carrying an *hcnAB-lacZ* fusion were similar to the patterns with the *phzC-lacZ* fusion (Fig. 2*C*). Thus, we conclude that both the *hcn* and *phz* promoters are affected similarly by QscR. Our analysis of expression of the LasR-$3OC_{12}$-HSL-controlled qsc105–*lacZ* fusion (Fig. 2*D*) showed that a mutation in *qscR* resulted in premature transcription from the qsc105 promoter. The expression of qsc105–*lacZ* was even earlier than expression from the *phz* or *hcn* promoters (Fig. 2).

Regulation of Quorum-Sensing Signal Generator and Signal Receptor Gene Expression by QscR. One hypothesis for the finding that transcription from each of the quorum-sensing-controlled promoters we examined was influenced by QscR is that QscR functions to regulate components of the LasR–LasI or RhlR–RhlI quorum-sensing signal generator and signal receptor pairs. To test this hypothesis, we examined expression of *lacZ* fusions to the genes coding for each of these four polypeptides in the *P. aeruginosa qscR* mutant and the parental strain. Expression of the *lasR–lacZ* fusion in the parent and mutant was indistinguishable (Fig. 3*A*). A *rhlR-lacZ* fusion showed slightly elevated expression in early logarithmic phase (Fig. 3*B*). Expression of both the *lasI–lacZ* fusion and the *rhlI–lacZ* fusion occurred early in the *qscR* mutant as compared with the parent (Fig. 3 *C* and *D*).

The results described above lead to the hypothesis that a mutation in *qscR* leads to early production of the acyl-HSL signals, which attain a critical concentration for activation of quorum-sensing-controlled genes at a lower culture density in the mutant compared with the parent. In one test of this

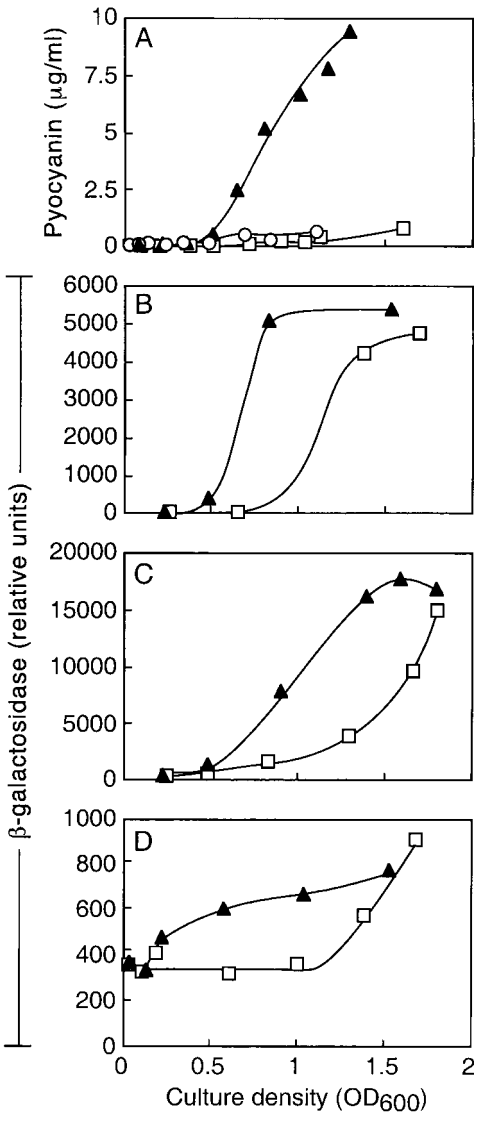

Fig. 2. The influence of *qscR* on quorum-controlled factors in *P. aeruginosa*. (*A*) Pyocyanin levels in *P. aeruginosa* PAO1 (□), PAOR3 (▲), and PAOR3 with the *qscR* plasmid pKL9 (○). β-Galactosidase in *P. aeruginosa* PAO1 (□), and PAOR3 (▲) containing the *phzABC-lacZ* plasmid, pMW303 (*B*), the *hcnAB-lacZ* plasmid, pMW301 (*C*), and the qsc105-*lacZ* plasmid, pMW105B (*D*).

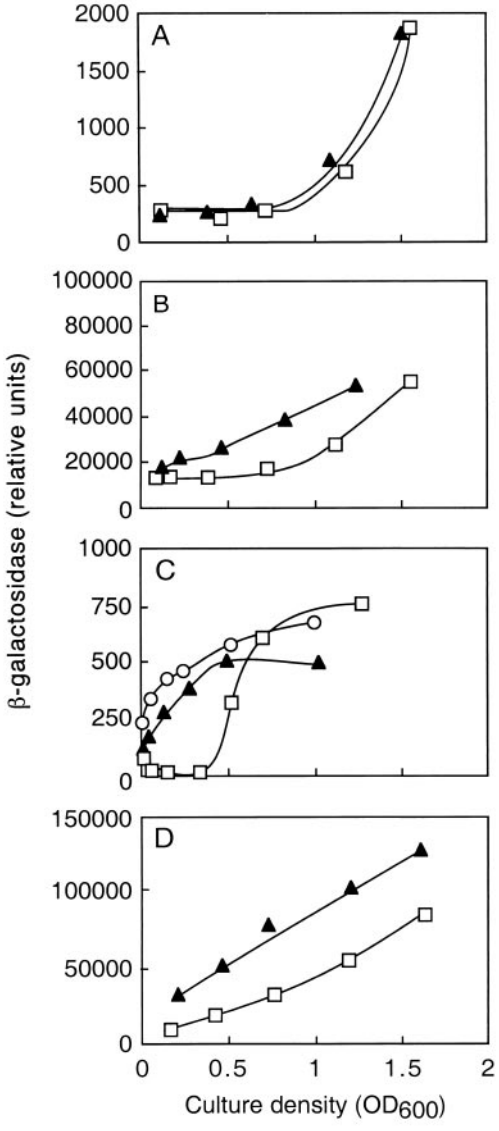

Fig. 3. Expression of the *lasR–lacZ* (pSC10) (*A*), *rhlR–lacZ* (pMW304) (*B*), *lasI–lacZ* (pSC11) (*C*), and the *rhlI–lacZ* (pMW305) (*D*) transcriptional fusions in *P. aeruginosa* PAO1 (□), PAO1 with exogenously added 2 μM $3OC_{12}$-HSL (○), and PAOR3 (▲).

hypothesis, we added $3OC_{12}$-HSL to the parent with the *lasI–lacZ* reporter. Consistent with the hypothesis, the exogenous addition of the signal compound resulted in a premature induction of β-galactosidase that was similar to the induction in the *qscR* mutant (Fig. 3*C*). In another test of the hypothesis we measured $3OC_{12}$-HSL and C_4-HSL in cultures of the mutant and parent (Fig. 4). Both of the AHSLs were synthesized earlier in the *qscR* mutant than the parent. The LasI-generated signal, $3OC_{12}$-HSL was synthesized earlier than the RhlI-generated signal, C_4-HSL (Fig. 4).

Virulence of a *qscR* Mutant. Unlike its homologs, LasR and RhlR, which are required for virulence gene activation (17–20), QscR serves to repress the synthesis of at least two virulence factors, pyocyanin and hydrogen cyanide. Thus, we would predict that QscR is not required for virulence. To test this, we used an insect model for *P. aeruginosa* pathogenesis. We tested the role of QscR in virulence by feeding the mutant and parent to the fruit fly *D. melanogaster*; a particularly convenient model host among the various insects in which *P. aeruginosa* causes disease (21–23). We fed fruit flies with *P. aeruginosa* cells suspended in a sucrose solution (see *Materials and Methods*) and monitored the flies for 2 weeks. *E. coli* was nearly completely harmless and the parent, *P. aeruginosa* PAO1 was relatively benign, slowly killing a minority of the flies (Fig. 5). The *qscR* mutant, however, was considerably more virulent than the parent, killing 100% of the flies (114 in total) by 12.5 days (Fig. 5). At least in this model, QscR functions as a governor that reduces the virulence of *P. aeruginosa.*

Discussion

Quorum sensing controls virulence and biofilm formation in *P. aeruginosa.* There are two well-studied quorum-sensing signal generator-receptor pairs in this bacterium, LasI–LasR, and RhlI–RhlR (for reviews, see refs. 24–26). As a result of the *P. aeruginosa* genome-sequencing project, a gene coding for a homolog of LasR and RhlR was discovered (http://www.pseudomonas.com). There is no apparent cognate signal gener-

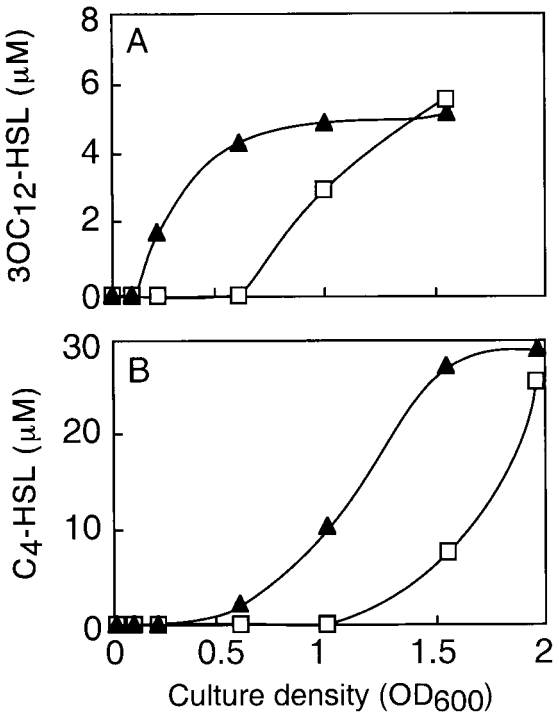

Fig. 4. The concentrations of $3OC_{12}$-HSL (*A*) and C_4-HSL (*B*) in cultures of *P. aeruginosa* PAO1 (□) and PAOR3 (▲).

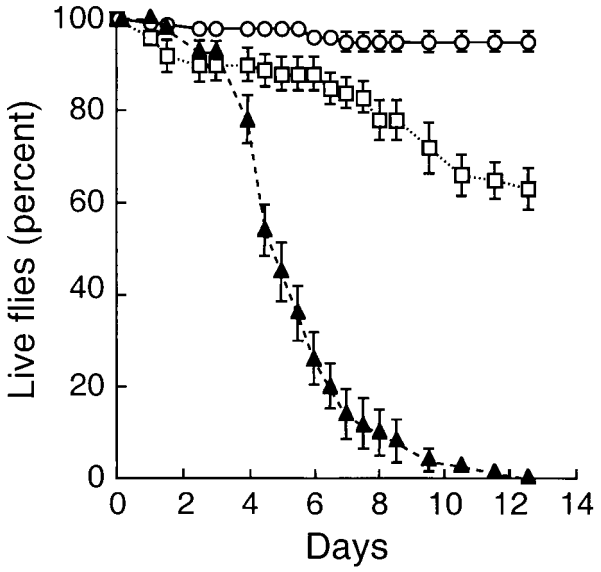

Fig. 5. Virulence of the *P. aeruginosa qscR* mutant in fruit flies. Death of flies over time when fed *P. aeruginosa* PAO1 (□), PAOR3 (▲), or *E. coli* CM830 (○). The values are the averages from 10 replicate experiments, each with 9–12 flies, and the SEM is shown for each value plotted. The parent and mutant bacteria survived equally well in the dry culture vials during the course of the experiments (data not shown). The number of bacteria recovered from flies just after death was 12 (± an SEM of 2) × 10^6 PAO1, and 8 (±3) × 10^6 PAOR3.

ator gene for this putative signal receptor gene. We show that in a wild-type strain of *P. aeruginosa*, the LasR–RhlR homolog represses transcription of three quorum-sensing-controlled genes, two of which are primarily activated by RhlR and one activated by LasR. This repression is in the logarithmic phase of growth (Fig. 2). The LasR-controlled genes are expressed earlier than the RhlR-controlled genes in a mutant in which the LasR–RhlR homolog has been inactivated (Fig. 2). Furthermore, we show that *lasI* and *rhlI* (both quorum-sensing-controlled themselves) are both prematurely transcribed in a mutant with an inactivated gene for this newly described transcription factor (Fig. 3). The mutant also produces the AHSL signals prematurely with the LasI-generated $3OC_{12}$-HSL produced earlier than the RhlI-produced C_4-HSL (Fig. 4). This leads to a model whereby this newly described transcription factor represses all quorum-sensing-controlled genes by repressing transcription of *lasI*, and thus retarding production of $3OC_{12}$-HSL in the early logarithmic phase of growth. Because $3OC_{12}$-HSL and LasR are activators of *rhlR* and *rhlI*, activation of *lasI* commences a cascade of activation of all quorum-sensing-controlled genes. We have called this transcription factor QscR, a quorum-sensing-control repressor.

We do not yet know whether QscR responds to a signal itself, and we do not know how it affects repression of *lasI* transcription. QscR could bind to the promoter region of *lasI* itself or it could form inactive heteromultimers with LasR. It could require binding of a signal, perhaps even $3OC_{12}$-HSL for its release from the *lasI* promoter or from LasR, or $3OC_{12}$-HSL binding to LasR could overcome the activity of QscR. There are examples of proteins in other bacteria that serve to govern the activity of transcription factors related to LasR. Perhaps the closest analogy comes from the plant pathogen *Agrobacterium tumefaciens*, which has a gene called *traS*. The *traS* gene codes for a truncated polypeptide that resembles the quorum-sensing activator TraR, and TraS blocks the function of TraR, presumably by forming inactive heterodimers with TraR (27).

It may be of relevance that AHSLs with long acyl tails do not diffuse through the cell membrane as readily as those with shorter acyl tails (28). In fact efflux pumps appear to be involved in export of $3OC_{12}$-HSL (28, 29). It is conceivable that the diffusion limitation could create a short circuit in the extracellular signaling process, and that transcription factors like QscR may circumvent this problem.

Our analysis of virulence (Fig. 5) shows that a mutation in QscR gives a hypervirulence phenotype. We hypothesize that there are disadvantages of this phenotype for *P. aeruginosa.* It is conceivable that in the initial stages of some infections, the display of quorum-sensing-controlled virulence factors allows the host the opportunity to mount a response. We also suspect that a *qscR* mutation could render *P. aeruginosa* less fit for survival in environments like soils or lakes where quorum-sensing-controlled virulence factors might be of no selective advantage.

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