Signal-Amplifying Genetic Circuit Enables In Vivo Observation of Weak Promoter Activation in the Rhl Quorum Sensing System

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Abstract: Small changes in transcriptional activity often significantly affect phenotype but are not detectable in vivo by conventional means. To address this problem, we present a technique for detecting weak transcriptional responses using *signal-amplifying* genetic circuits. We apply this technique to reveal previously undetectable log phase responses of several Rhl quorum sensing controlled (qsc) promoters from *Pseudomonas aeruginosa*. Genetic circuits with Rhl promoters and transcriptional amplification components were built and tested in *Escherichia coli*. This enabled us to isolate the behavior of the promoters under study from Las and quinolone interactions. To amplify qsc promoter responses to acyl-homoserine lactones (AHL), the highly efficient λ repressor gene was placed downstream of several Rhl promoters and coupled to a fluorescent reporter under the control of the λ $P_{(R)}$ promoter. With amplification, up to ~ 100-fold differences in fluorescence levels between AHL induced and noninduced cultures were observed for promoters whose responses were otherwise not detectable. In addition, the combination of using signal amplification and performing experiments in *E. coli* simplified the analysis of AHL signal crosstalk. For example, we discovered that while a C4HSL/RhlR complex activates both qsc*rhlA* and qsc*phzA1*, a 3OC12HSL/RhlR complex activates qsc*phzA1* but not qsc*rhlA* in our system. This crosstalk information is particularly important since one of the potential uses of amplification constructs is for the detection of specific quorum sensing signals in environmental and clinical isolates. Furthermore, the process of decomposing networks into basic parts, isolating these components in a well-defined background, and using amplification to characterize both crosstalk and cognate signal responses embodies an important approach to understanding complex genetic networks. © 2004 Wiley Periodicals, Inc.

INTRODUCTION

Weak transcriptional responses often play important regulatory roles in complex genetic networks (Freeman, 2000). They can control the expression of transcription factors such as AtZFP1 in *Arabidopsis thaliana* (Mantis and Tague, 2000), tissue- and tumor-specific genes (Nettelbeck et al., 2000), and gene products that trigger positive feedback loops (Schwartz et al., 1999; Laratta et al., 2002). Also, many weak promoters are useful in biomedical and biosensing applications due to their ability to report the presence of a specific chemical, event, or environmental condition (Billinton et al., 1998). However, responses of such promoters are often difficult to detect in vivo. Here, we examine weak transcriptional responses and the specific roles of different quorum sensing signals in the opportunistic human pathogen *Pseudomonas aeruginosa*.

Quorum sensing enables many bacteria to measure population density and coordinate various cellular behaviors in a population (Miller and Bassler, 2001; Taga and Bassler, 2003). In *P. aeruginosa*, the Las and Rhl quorum sensing systems control expression of hundreds of genes (Whiteley et al., 1999; Schuster et al., 2003; Wagner et al., 2003; Hentzer et al., 2003; Arevalo-Ferro et al., 2003). Specifically, LasI catalyses synthesis of the diffusible acyl-homoserine lactone (AHL) molecule 3OC12HSL, which forms a complex with LasR (Pearson et al., 1994). The LasR/3OC12HSL complex then regulates a set of genes including *lasI* and *rhlR* (Pesci et al., 1997). In the Rhl system, RhlI catalyzes synthesis of another diffusible molecule, C4HSL. This AHL molecule binds RhlR and regulates another set of genes including *rhlI* (Winson et al., 1995). The expression of many genes has been found to be con-

trolled by these systems as well as the coordination of processes including virulence factor production (Brint and Ohman, 1995; Winzer et al., 2000), biofilm formation (Davies et al., 1998), and antimicrobial resistance (Hassett et al., 1999). However, the task of determining which promoters are regulated by quorum sensing is complicated by the fact that the strength of transcriptional response to AHL depends on various conditions such as growth phase and media (Vasil, 2003; Wagner et al., 2003; Medina et al., 2003a). Interactions between the Las and Rhl systems (Van Delden and Iglewski, 1998) and the quinolone signaling system (McGrath et al., 2004) add further complexity to the challenge of identifying the specific roles of each signal. In this article, we introduce a method of transcriptional amplification for the purpose of detecting weak responses to signals that cannot be observed directly in vivo and use this method to study several Rhl system promoters in isolation from Las and quinolone interactions.

To establish baseline detection levels, we first constructed a set of "direct detection" circuits that express *rhlR* constitutively and express enhanced yellow fluorescent protein (*eyfp*) under control of the qsc promoters shown in Table I. The fact that only one of these promoters exhibited a significant log phase response to C4HSL motivated the construction of the signal amplification circuits. Rather than placing the *eyfp* reporter gene directly under control of the qsc promoter, our signal amplification technique consists of inserting a destabilized version (Andersen et al., 1998) of the λ repressor gene *cI(LVA)* (Ptashne, 1986) downstream of the qsc promoter and coupling it to a λ P(R-O12) promoter (Weiss, 2001) that regulates *eyfp* expression (Fig. 1). Since CI is a highly efficient repressor, even a low concentration of CI can completely repress λ P(R-O12) and dramatically change the reporter concentration. We developed a computational model to explore the fundamental issues in engineering transcriptional amplifiers that work well over a wide range of promoter strengths. The model guided the development of different constructs (through mutations in λ P(R-O12)) that successfully amplified the different qsc promoters in the experiments.

Based on these experimental results, we classify qsc promoter responses to a particular AHL for a given growth phase as directly observable, observable through amplification, or nonresponsive (Table I). Without amplification, several qsc promoters appear to be nonresponsive to various AHLs in *E. coli* during log phase. Here we demonstrate that the response of three of these promoters (qsc*rhlA*, qsc*phzA1*, and qsc*lasB*) to C4HSL should rather be classified as observable through amplification. Since transcriptional amplification can be useful for detecting specific quorum sensing signals in environmental and clinical isolates, we characterized qsc promoter responses to different AHLs using our amplification constructs. These crosstalk properties, which should ultimately be investigated in *P. aeruginosa*, also have implications for understanding the intricate regulation of qsc genes and for interspecies communication.

Table I. Quorum sensing controlled promoters studied.

| Promoter	Promoter region from PAO-1 used in plasmids	Downstream gene functions	Classification
qsc*rhll* (PA3476)	GGTCGGCGGCGCCTACCAGATCTGGCAGGTTGCCTGCCGTTCATCCTCCTTTAGTCTTCCCCCTCATGTGTG	C4HSL synthesis	direct
qsc*rhlA* (PA3479)	CGGCCGCCCGTCTGTGAAATCTGGCAGTTACCGTTAGCTTTCATCGGCTAC	biofilm development and maintenance	amplified
qsc*nrps* (PA3327)	GTTTCCCGGAACCTAACAGATTTGTAAGTTGCCTCGTAAATACCTACCAGAATTAACAGTTAACAACGCGAT	adaptation, protection, microbial competitiveness	not responsive
qsc*hcnA* (PA2193)	CCTGCACCCCACCTACCAGAATTGGCAGGGGAAGATACCCACCTGTCATGGATCAATGTCCGCGCCGGCCC	microbial competitiveness	not responsive
qsc*phzA1* (PA4210)	ACCCTGTCTGGCACCTACCAGATCTTGTAGTTGAGCCGGTACGAGCGTTCTGTGTTTATGCAATCCACATCA	virulence/microbial competitiveness	amplified
qsc*phzS* (PA4217)	CGGTTATTCGCCCTACGCGGCCTTGGAGCCCATCTAACCGCACGCGGTTCTTTTGGTACACGAAAGCACCGTCGAAA	virulence/microbial competitiveness	not responsive
qsc*lasB* (PA3724)	AATCAAGGCTACCTGCCAGTTCTGGCAGGTTTGGCCGGCGGGTTCTTTTCCTGTTGCTGTGCTTTGCTAACAGGGAAGGATCGC	virulence	amplified
qsc*palL* (PA2570)	CCTCTTTTCTCTGCATGAATTGGTAGGCGGTACTTCCTCGTTGCTGTGCTTTGCTAACAGGGAAGGATCGC	virulence	not responsive

The promoter name is shown in the first column. These names are based on the first gene found upstream of the promoter, and the corresponding numbers assigned by the *Pseudomonas* genome project (Stover et al., 2000) are shown in parentheses. Rhl box sequence regions are underlined, and conserved sites in these regions are shown in bold. The Rhl box consensus sequence is $A_4C_5C_5A_6G_7A_6T_5C_5T_8G_6G_7C_4A_8G_8G_3T_5$. Promoters are classified as "direct" if they exhibit a distinct log phase response to C4HSL, as "amplified" if they respond only when amplified, or as "not responsive".
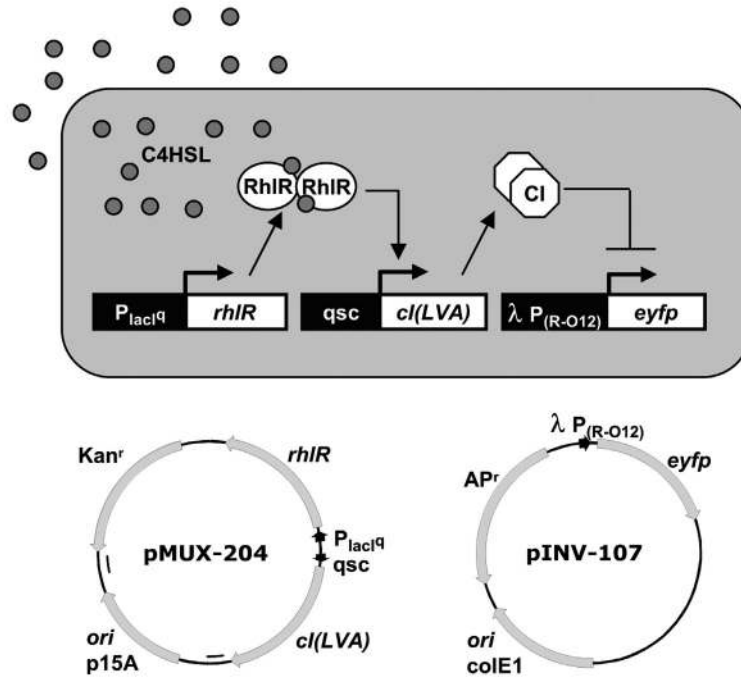
MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Escherichia coli JM2.300 (F⁻ *lacI22* λ⁻ e14⁻ *rpsL135*(StrR) *thi-1*) from the *E. coli* Genetic Stock Center (strain 5002) was used in all phenotype experiments; *E. coli* DH5α (F⁻ Θ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR*17(r$_k^-$, m$_k^+$) *phoA supE44 thi-1 gyrA96 relA1* λ⁻) was used for plasmid building and propagation. LB broth (Difco, Detroit, MI) with the appropriate antibiotic(s) was used as a growth medium in all experiments. For direct detection experiments, 50 μg/mL kanamycin (Shelton Scientific, Shelton, CT) was used. In amplifier experiments, 50 μg/mL kanamycin (Shelton Scientific) and 100 μg/mL ampicillin (Sigma, St. Louis, MO) were used. AHL was added at the specified concentration. The AHLs butanoyl-homoserine lactone (C4HSL), hexanoyl-homoserine lactone (C6HSL), and 3-oxohexanoyl-homoserine lactone (3OC6HSL) were acquired from Sigma-Aldrich, while 3-oxo-dodecanoyl-homoserine lactone (3OC12HSL) was the gift of L. Passador (Passador et al., 1996; Pearson et al.,
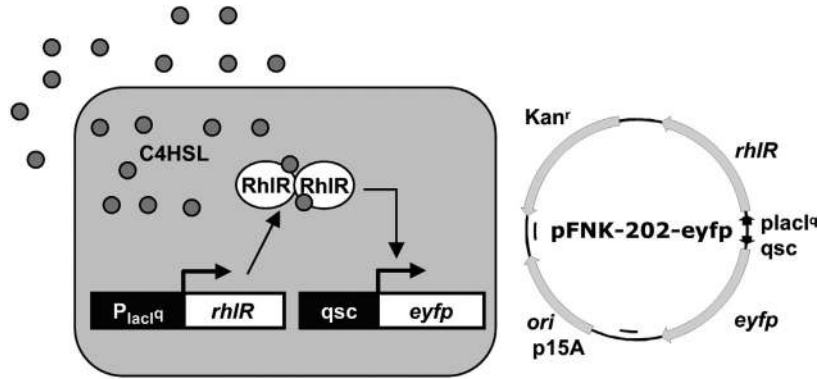
Figure 1. Amplifier genetic circuit and plasmid maps for (**a**) direct detection and (**b**) amplification. Exogenous C4HSL diffuses into the cell and binds the RhlR receptor protein. The C4HSL/RhlR complex activates the qsc promoter, resulting in EYFP production with the direct detection constructs and CI production with the amplification constructs. In the amplification constructs, CI then represses λ P$_{(R-O12)}$ and lowers the level of the fluorescent reporter EYFP.

1994). For all growth experiments, cultures were incubated at 37°C in a shaker at 250 rpm.

Plasmids

Table II and Figure 1 describe the plasmids used in this study. Constructs designed for direct detection contain *eyfp* under control of the specified qsc promoter, kanamycin resistance, and *rhlR* constitutively expressed from the P_{lacIq} promoter. The P_{lacIq} promoter is the *lacI* promoter with a 1 bp mutation in the -35 region to enhance transcriptional efficiency (Calos, 1978). The direct detection plasmids by name are pFNK-202-eyfp-qsc118, -qsc119, -qsc126, -qsc128, -qsc131, -qsc132, -qsclasB, and -qsclecA. The signal amplifiers are two-plasmid systems. The first plasmid is one of the direct detection plasmids with *eyfp* replaced by *cI(LVA)*. These are pMUX-204-qsc119, -qsc126, -qsc128, -qsc131, -qsc132, -qsclasB, and -qsclecA. The second plasmid (either pINV-107-mut0, -mut5, or -mut6) contains ampicillin resistance along with *eyfp* regulated by a variant of the Bacteriophage λ promoter. Specifically, the O_R1 sequence of the mut0 plasmid is TACCTCTGGCGGTGATA, while the mut5 and mut6 O_R1 sequences are TAC**A**T**A**TGGCGGTGATA and TAC**AGA**TGGCGGTGATA, respectively (Weiss, 2001). The qsc promoter sequences for the pMUX and pFNK plasmids are shown in Table I. Although the *lasB* promoter has two R-protein binding boxes (OP1 and OP2) in *P. aeruginosa* (Rust et al., 1996; Anderson et al., 1999), only OP1 was included in the qsc*lasB* constructs. A strong synthetic ribosome binding site, RBSII, was placed upstream of all *eyfp* genes (Weiss and Basu, 2002).

The plasmid pTKU-105 is similar to pINV-110 (Yokobayashi et al., 2002) with the P_{lac} promoter replaced by qsc*phzA1*. This plasmid was created by ligating annealed oligos containing the qsc*phzA1* promoter to a PCR-amplified pINV-110 fragment. The *lacI* gene was then replaced with *rhlR* from pECP61.5 (Pearson et al., 1997) to form pTKU-106. Next, pFNK-202 was created by replacing the region of pTKU-106 containing *cI* and *ecfp* with *gfp(LVA)* from pRCV-3 (Weiss, 2001). The *eyfp* gene from pINV-107 was added in place of the *gfp(LVA)* in pFNK-202 to form pFNK-202-eyfp-qsc131. Annealed oligos containing the seven other qsc promoters shown in Table I were then inserted into pFNK-202-eyfp-qsc131 in place of qsc*phzA1* to create the remaining pFNK-202-eyfp variants. The pTKU-106 plasmid also served as a parent for pMUX-204-qsc131, which was formed by removing the *ecfp* gene from pTKU-106 and adding an LVA tail to *cI*. Finally, the same annealed oligos used to create the pFNK-202-eyfp variants were used to make pMUX-204-qsc119, -qsc126, -qsc128-, qsc-132, -qsclasB, and -qsclecA from pMUX-204-qsc131.

PCR reactions were carried out using Invitrogen (La Jolla, CA) Hi Fidelity PCR supermix. Qiagen's (Chatsworth, CA) QIAquick Gel Extraction Kit was used for gel extraction of PCR products. Digestions were performed as instructed by the manufacturer (NEB and Fermentas). PCR purification was done with Qiagen's PCR purification kit. T4 DNA ligase from NEB was used for ligations, and either Bio-Rad (Hercules, CA) or Eppendorf (Hamburg, Germany) miniprep kits were used. The primer 5′ AAGGGAATCG TGTGGCGCACGC 3′, which binds the N-terminal region

Table II. Plasmids used in this work.

Plasmid	Relevant properties	Reference or source
pINV-107	*ori* colE1 AP^r; λP$_{(R-O12)}$-RBSII-*eyfp*	Weiss, 2001
pINV-107-mut5	*ori* colE1 AP^r; λP$_{(R-O12mut5)}$-RBSII-*eyfp*	Weiss, 2001
pINV-107-mut6	*ori* colE1 AP^r; λP$_{(R-O12mut6)}$-RBSII-*eyfp*	Weiss, 2001
pINV-110	*ori* p15A Km^r; P$_{lac}$-RBSII-*cI*-RBSII-*ecfp* P$_{lacIq}$-*lacI*	Weiss, 2001
pRCV-3	*ori* colE1 AP^r; luxP(L)-*luxR* luxP(R)-RBSII-*gfp(LVA)*	Weiss, 2001
pECP61.5	*ori* colE1 AP^r; *rhlA'*-*lacZ* tacp-*rhlR*	Pearson et al., 1997
pTKU-105	*ori* p15A Km^r; qsc*phzA1*-RBSII-*cI*-RBSII-*ecfp* P$_{lacIq}$-*lacI*	This Study
pTKU-106	*ori* p15A Km^r; qsc*phzA1*-RBSII-*cI*-RBSII-*ecfp* P$_{lacIq}$-*rhlR*	This Study
pFNK-202-qsc131	*ori* p15A Km^r; qsc*phzA1*-RBSII-*gfp(lva)* P$_{lacIq}$-*rhlR*	This Study
pFNK-202-eyfp-qsc118	*ori* p15A Km^r; qsc*rhlI*-RBSII-*eyfp* P$_{lacIq}$-*rhlR*	This Study
pFNK-202-eyfp-qsc119	*ori* p15A Km^r; qsc*rhlA*-RBSII-*eyfp* P$_{lacIq}$-*rhlR*	This Study
pFNK-202-eyfp-qsc126	*ori* p15A Km^r; qsc*nrps*-RBSII-*eyfp* P$_{lacIq}$-*rhlR*	This Study
pFNK-202-eyfp-qsc128	*ori* p15A Km^r; qsc*hcnA*-RBSII-*eyfp* P$_{lacIq}$-*rhlR*	This Study
pFNK-202-eyfp-qsc131	*ori* p15A Km^r; qsc*phzA1*-RBSII-*eyfp* P$_{lacIq}$-*rhlR*	This Study
pFNK-202-eyfp-qsc132	*ori* p15A Km^r; qsc*phzS*-RBSII-*eyfp* P$_{lacIq}$-*rhlR*	This Study
pFNK-202-eyfp-qsclasB	*ori* p15A Km^r; qsc*lasB*-RBSII-*eyfp* P$_{lacIq}$-*rhlR*	This Study
pFNK-202-eyfp-qsclecA	*ori* p15A Km^r; qsc*pa1L*-RBSII-*eyfp* P$_{lacIq}$-*rhlR*	This Study
pMUX-204-qsc119	*ori* p15A Km^r; qsc*rhlA*-RBSII-*cI(LVA)* P$_{lacIq}$-*rhlR*	This Study
pMUX-204-qsc126	*ori* p15A Km^r; qsc*nrps*-RBSII-*cI(LVA)* P$_{lacIq}$-*rhlR*	This Study
pMUX-204-qsc128	*ori* p15A Km^r; qsc*hcnA*-RBSII-*cI(LVA)* P$_{lacIq}$-*rhlR*	This Study
pMUX-204-qsc131	*ori* p15A Km^r; qsc*phzA1*-RBSII-*cI(LVA)* P$_{lacIq}$-*rhlR*	This Study
pMUX-204-qsc132	*ori* p15A Km^r; qsc*phzS*-RBSII-*cI(LVA)* P$_{lacIq}$-*rhlR*	This Study
pMUX-204-qsclasB	*ori* p15A Km^r; qsc*lasB*-RBSII-*cI(LVA)* P$_{lacIq}$-*rhlR*	This Study
pMUX-204-qsclecA	*ori* p15A Km^r; qsc*pa1L*-RBSII-*cI(LVA)* P$_{lacIq}$-*rhlR*	This Study

of *rhlR* was used to sequence the qsc promoter regions of all pFNK and pMUX plasmids. The sequencing primer 5′ TGCAGGGCTTCCCAACCTTACC 3′ was used to verify the C-terminal region of *rhlR* in pFNK-202-eyfp-qsc131 and pTKU-106. In addition, the primer 5′ TACAACCAC TAAACCCACAG 3′ was used to verify the LVA region of *cI(LVA)*. Sequencing was performed with an Applied Biosystems (Foster City, CA) 3100 PRISM 16-array capillary automated fluorescent DNA sequencer. Stratagene's (La Jolla, CA) site-directed mutagenesis kit was used to fix a single basepair mutation in the -35 region of pFNK-202-qsc131 and pMUX-204-qsc131.

Data Acquisition and Analysis

For all experiments, overnight cultures were diluted to an effective OD_{600} of $\sim 10^{-5}$ in 14-mL culture tubes containing 2 mL of the appropriate media. Cells were then grown to an OD_{600} of ~ 0.3 for log phase measurements. For stationary phase experiments, 1 mL of the appropriate fresh media was added to 1 mL of culture at OD_{600} 0.3, and the culture was grown to stationary phase by additional incubation for 14 ± 2 h. All experiments were performed in triplicate. Prior to flow cytometry (FACS) measurements, cells were washed twice in 0.2 μm filtered phosphate-buffered saline to minimize media background fluorescence. All FACS measurements were performed on a Beckman Coulter (Palo Alto, CA) Altra flow cytometer equipped with a 488 nm argon excitation laser and a 515–545 nm emission filter. Median fluorescence values were converted to equivalent fluorescein molecule counts using SPHERO Rainbow Calibration Particles (Spherotech, Libertyville, IL, RCP-30-5A) that were measured before each session.

Model and Simulations

The direct detection circuits and signal amplifier circuits were modeled and simulated. The supporting information (available via the Internet; see footnote) describes the specific chemical reactions modeled and kinetic parameters used. Biochemical reactions for transcription, translation, repression through cooperative binding, AHL complex formation, and degradation were simulated with deterministic ordinary differential equations (Kierzek et al., 2001; Weiss et al., 2003). Matlab's ode15s, a variable order solver of the numerical differential formulas, was used to find the steady-state solutions to these differential equations (Shampine and Reichelt, 1997).

RESULTS

Direct Detection of Rhl qsc Promoters

Figure 2a shows that the responses of the qsc*rhlI*, qsc*rhlA*, qsc*phzA1*, qsc*lasB*, and qsc*pa1L* promoters to C4HSL are directly detectable in stationary phase *E. coli* cells, confirming that these constructs are operational. However, of these promoters only qsc*rhlI* exhibits a reasonably strong, directly detectable response to C4HSL in log phase (Fig. 2b). In addition to the above promoters, the responses of qsc*nrps*, qsc*hcnA*, and qsc*phzS* were measured, but these promoters exhibited no response in either stationary or log phase (data not shown). Our qsc*rhlA* results agree with previous findings that this promoter responds in stationary but not log phase *E. coli* cells grown in LB media (Medina et al., 2003a). Also, the qsc*pa1L* promoter has been shown to respond to C4HSL in *E. coli* and has a −10 sequence
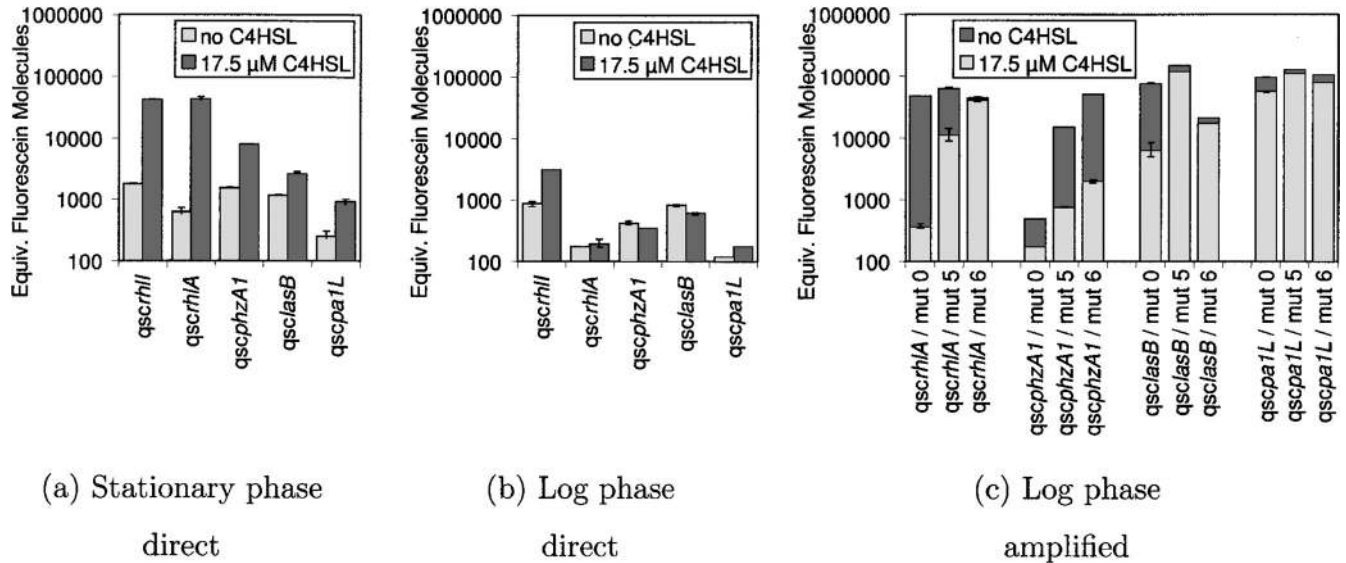

Figure 2. **a:** Directly detected response of qsc promoters in stationary phase. **b:** Directly detected response of qsc promoters in log phase. **c:** Amplified response in log phase with and without 17.5 μM C4HSL. Bar heights represent the average of fluorescence intensities from triplicate experiments, while error bars show the lowest and highest fluorescence levels.

Figure 3. C4HSL dosage response of signal amplifier constructs qsc*rhlA*/mut0 (squares), qsc*rhlA*/mut5 (triangles), qsc*phzA1*/mut5 (diamonds), and qsc*phzA1*/mut6 (circles) in log phase. Symbol markers represent the average of fluorescence intensities from triplicate experiments, while error bars show the lowest and highest fluorescence levels.
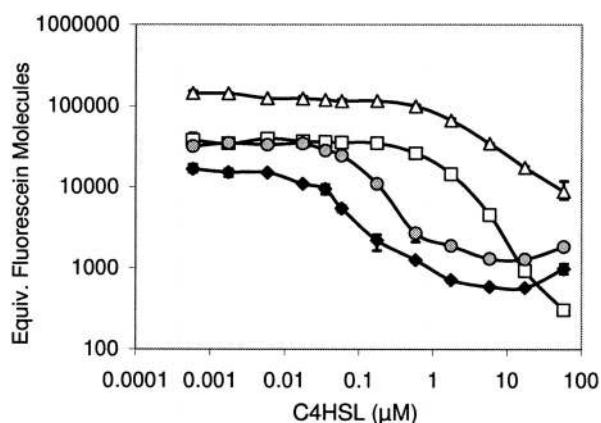
homologous to the σ^S consensus (Winzer et al., 2000; Espinosa-Urgel et al., 1996).

Weak Responses Are Detectable Using Transcriptional Amplification Circuits

The inability to directly detect log phase activation of seven of the eight promoters in this study motivated the use of transcriptional amplification, whereby the *cI* gene is inserted downstream of the qsc promoter and is coupled to a construct expressing *eyfp* under control of the λ P$_{(R-O12)}$ promoter (Fig. 1). The results shown in Figure 2c indicate that weak log phase activation of qsc promoters can be detected using amplification. For all amplifiers shown, high C4HSL concentrations result in elevated CI levels, and thus lower EYFP fluorescence. Figure 2c also demonstrates the effect of using the three different variations of λ P$_{(R-O12)}$ (mut0, mut5, mut6) for each qsc promoter. The

mut0 version has the original O$_R$1 and O$_R$2 sequences of λ P$_{(R-O12)}$, while mut5 and mut6 contain mutations of O$_R$1 that decrease the binding efficiency of CI (Weiss and Basu, 2002). As shown by Figure 2c, for a given qsc promoter the mut5 and mut6 versions of the amplification detection circuit result in higher fluorescence values than the original mut0 construct. To ascertain that decreases in fluorescence in the amplifier cells are due to repression rather than a toxic effect of C4HSL, cells containing pINV-107 were grown in similar C4HSL concentrations and showed no noticeable change in fluorescence (data not shown).

The dosage responses of the strongly amplified promoters qsc*rhlA* and qsc*phzA1* (Fig. 3) provide important signal sensitivity information. The results further demonstrate the effect of different λ P$_{(R-O12)}$ promoters on amplification of different qsc promoters. For example, cells with the qsc*rhlA*/mut5 amplifier exhibit higher fluorescence than cells with the qsc*rhlA*/mut0 amplifier due to the weaker CI-O$_R$1 binding efficiency of the mutant promoter. While mut0 provides the best amplification for qsc*rhlA*, we did not use mut0 for qsc*phzA1* dosage response experiments, since Figure 2c indicates that mut5 and mut6 work better for this promoter. Inspection of Figure 2c and Figure 3 reveals that amplifiers qsc*rhlA*/mut0, qsc*phzA1*/mut5, qsc*phzA1*/mut6, and qsc*lasB*/mut0 exhibit a greater difference in fluorescence levels between induced and noninduced conditions than even the stationary phase direct detection measurements of the corresponding qsc promoters shown in Figure 2a. This enhanced sensitivity provides a means of detecting crosstalk responses with greater resolution than with direct detection circuits, and we utilize this advantage in the following section.

Crosstalk

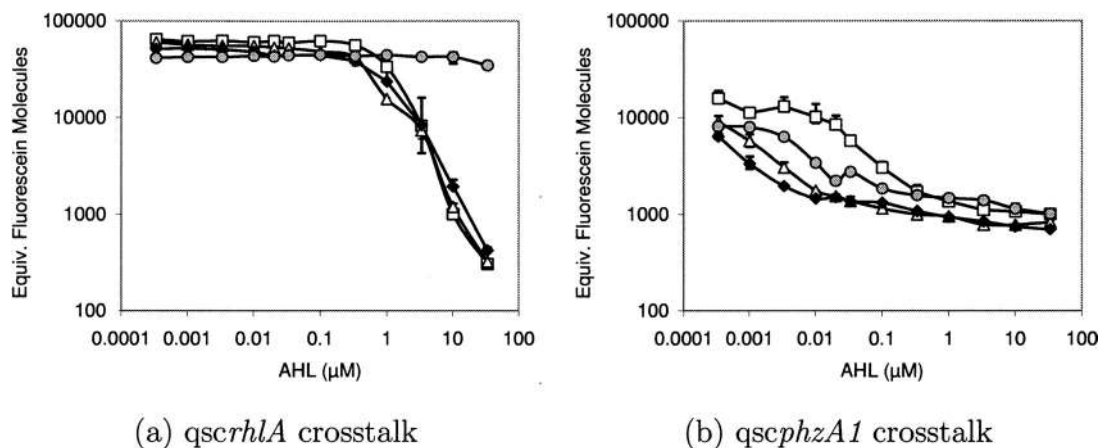Using signal amplification, we examine the ability of AHL molecules other than C4HSL to bind RhlR and activate

(a) qsc*rhlA* crosstalk
(b) qsc*phzA1* crosstalk

Figure 4. Crosstalk results of signal amplifiers (**a**) qsc*rhlA*/mut0 and (**b**) qsc*phzA1*/mut5 grown to log phase in a range of C4HSL (squares), C6HSL (triangles), 3OC12HSL (circles), and 3OC6HSL (diamonds) concentrations. Symbol markers represent the average of fluorescence intensities from triplicate experiments, and error bars show the lowest and highest fluorescence levels.

qsc*rhlA* and qsc*phzA1*. Figure 4 shows the dosage response of the qsc*rhlA*/mut0 and qsc*phzA1*/mut5 signal amplifiers to C6HSL, 3OC12HSL, 3OC6HSL, and C4HSL. Small amounts of C6HSL are produced by RhlI (Winson et al., 1995), and very small amounts of 3OC6HSL are generated by LasI (Pearson et al., 1994). In addition, several organisms found in the same environments as *P. aeruginosa* produce significant amounts of 3OC6HSL and C6HSL, and we discuss below the possibility of interspecies communication. In accord with previous findings (Ochsner and Reiser, 1995), qsc*rhlA*/mut0 responds almost as well to C6HSL and 3OC6HSL as it does to C4HSL, but it does not respond to 3OC12HSL. On the other hand, qsc*phzA1*/mut5 exhibits distinct responses to all of the AHLs shown. Since these experiments were carried out in *E. coli* in isolation from Las and quinolone interactions, the different responses are due only to specific promoter activation by the RhlR-AHL complex.

Simulation Results

We created a model to examine the fundamental aspects of transcriptional amplification and to assist in the forward engineering of amplifier circuits that work well with the different qsc promoters. In this model, ordinary differential equations were used to simulate transcription, translation, repression through cooperative binding, AHL complex formation, and degradation. Each qsc promoter exhibits a particular range of transcriptional activity, from basal expression when no AHL is present to fully induced expression under saturating AHL concentrations. These ranges differ for the various promoters, and Figure 5a depicts the simulated direct responses of two hypothetical qsc promoters. One obstacle to the actual detection of such responses is that the background fluorescence of the cell and the detection capabilities of the instrumentation impose a detection threshold that EYFP expression levels must cross.
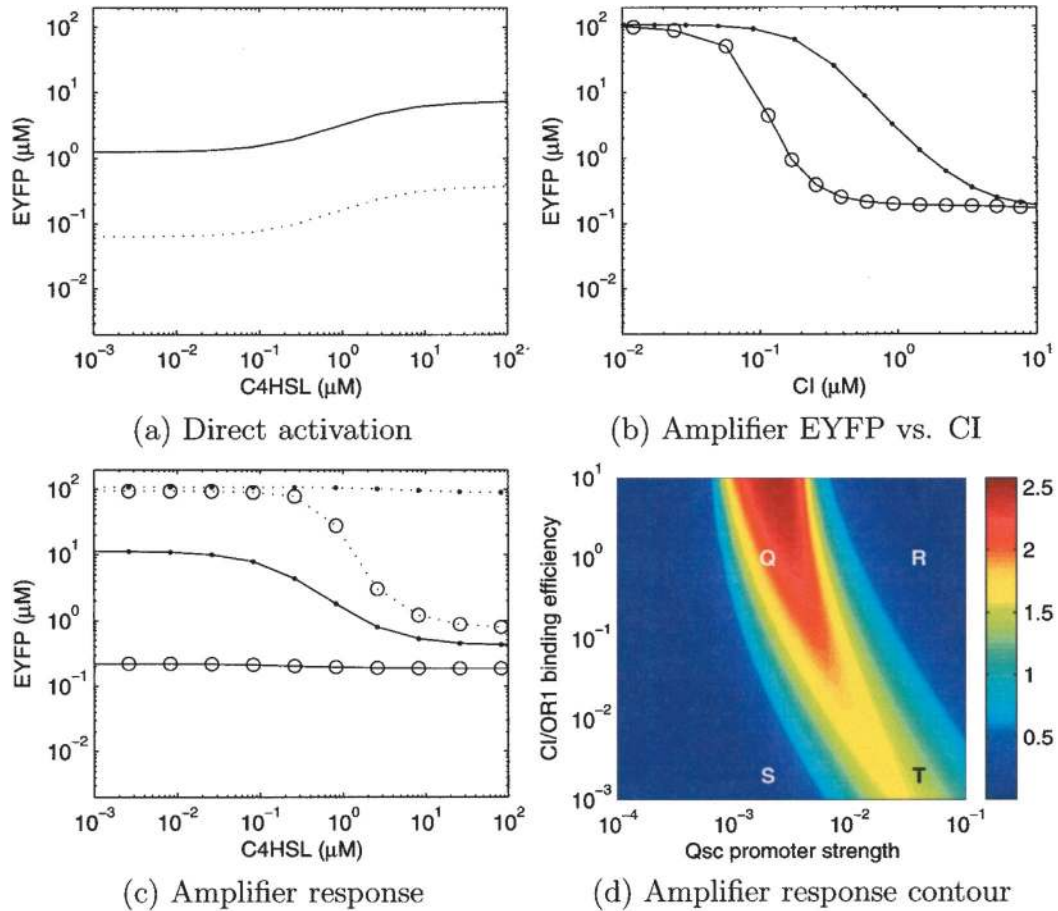

(a) Direct activation

(b) Amplifier EYFP vs. CI

(c) Amplifier response

(d) Amplifier response contour

Figure 5. **a:** Simulated direct detection response of two hypothetical qsc promoters, qsc1 (dotted) and qsc2 (solid). **b:** Relationship between CI and EYFP for AmpA (open circles) and AmpB (closed circles). AmpA has the original O_R1 and a AmpB has a variant with a lower CI-O_R1 binding efficiency. **c:** Simulated response of these two amplifiers to the two hypothetical qsc promoters. The curves shown are AmpA/qsc1 (dotted, open circles), AmpA/qsc2 (solid, open circles), AmpB/qsc1 (dotted, closed circles), AmpB/qsc2 (solid, closed circles). **d:** Contour representing the log of the maximum EYFP concentration minus the log of the minimum EYFP concentration of the amplified response as a function of qsc promoter strength and CI/O_R1 binding efficiency. The qsc values shown represent the induced transcription rate of the qsc promoter as a factor of the transcription rate of a typical strong promoter. The rate of basal transcription of the qsc was set to one-sixth of the induced rate. The CI/O_R1 binding efficiency values represent binding efficiency relative to the original O_R1. Thus, 10^0 represents the original O_R1 sequence. Points Q, R, S, and T correspond, respectively, to the qsc strengths and binding efficiencies for the AmpA/qsc1, AmpA/qsc2, AmpB/qsc1, and AmpB/qsc2 curves in (**c**).

Even if this threshold is crossed, small differences between the fully induced and noninduced EYFP concentrations may be difficult to observe. Figure 5b, which shows the relationship between CI and EYFP for two variations of λ $P_{(R-O12)}$, delineates the mechanism of amplification. AmpA models the original λ $P_{(R-O12)}$, while AmpB represents a variation of λ $P_{(R-O12)}$ with a weaker CI-O_R1 binding efficiency. Notice that low levels of CI correspond to high levels of EYFP that are likely above the previously discussed EYFP detection limit. Furthermore, each amplifier has a particular range of CI values for which small increases in CI concentration result in dramatic decreases in EYFP concentration. This is considered the *dynamic operating range* of the amplifier.

Since ranges of CI expression vary among the different qsc promoters, it is imperative to match these promoters with corresponding λ $P_{(R-O12)}$ variations so that any particular range of CI levels will map correctly to the operating range of at least one λ $P_{(R-O12)}$ variation. Figure 5c, which depicts the responses of AmpA and AmpB to qsc1 and qsc2, demonstrates this need for different variations of λ $P_{(R-O12)}$. Specifically, AmpA strongly amplifies qsc1, but exhibits a weak, low level response to qsc2. On the other hand, AmpB properly amplifies qsc2 but responds weakly to qsc1. Also note that when the qsc promoters are matched with the appropriate amplifiers, the resulting difference between the fully induced and noninduced EYFP concentrations is improved. For example, there is less than a 10-fold difference between the high and low EYFP concentrations for qsc1 in Figure 5a. However, matching qsc1 with AmpA results in 100-fold difference between the high and low EYFP concentrations in Figure 5c.

Figure 5d depicts the difference between the log of the maximum and the log of the minimum EYFP concentrations as a function of qsc promoter strength and CI-O_R1 binding efficiency. Points Q, R, S, and T in the contour correspond to curves Q, R, S, and T in Figure 5c. One important trend is that lower CI-O_R1 binding efficiencies accommodate stronger promoters. However, as binding efficiencies are lowered, the maximum possible difference between high and low EYFP values is reduced. This is due to the fact that lowering the CI-O_R1 binding efficiency decreases the maximum slope in the EYFP vs. CI characteristic (Fig. 5b). By modifying CI-O_R1 binding efficiency, a library of amplifiers can be made to accommodate a wide range of qsc promoter strengths. This correlates well with our experimental results, since the mut0 amplifier works best for qsc*rhlA*, while mut5 and mut6 work best for qsc*phzA1*.

DISCUSSION

We present a method to detect small transcriptional changes that enabled us to characterize the log phase behavior of several *P. aeruginosa* qsc promoters in *E. coli*. Our amplification system is based on strong repression. Because EYFP is a stable protein with a half-life of around 24 h (CLONTECHniques XII, Oct. 1997, 4–15; CLONTECHniques XIV, July 1999, 1–2), its effective rate of decay is determined by dilution through cell growth. Thus, with the signal amplifiers, expression of CI that sufficiently represses λ $P_{(R-O12)}$ will cause exponential decay of EYFP as long as the cultures are growing (i.e., in log phase). Hence, activation of the weak qsc promoter can decrease reporter concentration significantly, resulting in inverted amplification. Previously, noninverted methods have been used to amplify weak promoters. For example, the strong activator GAL4-VP16 has been used to amplify tissue-specific promoters for imaging and gene therapy applications (Iyer et al., 2001; Segawa et al., 1998; Nettelbeck et al., 1998, 2000). However, reporter expression from noninverted amplifiers may still be below the detection limit, since activation of an amplified weak promoter raises reporter levels from initially low levels. This detection problem will not occur with an inverted amplifier, since similar activation of the amplified promoter instead results in a decrease in reporter expression from an initially high concentration that is well above background levels.

Since a log phase response to C4HSL was directly detected for qsc*rhlI* alone, we applied our amplification technique to the seven other promoters in our study. Amplification successfully revealed the responses of qsc*rhlA*, qsc*phzA1*, and qsc*lasB*. Note the high concentration of C4HSL required for half maximal induction of qsc*rhlA* (Fig. 3), a promoter that controls rhamnolipid synthesis (Ochsner and Reiser, 1995; Pearson et al., 1997). Rhamnolipids are synthesized during later stages of biofilm development and are required to maintain biofilm architecture (Davey et al., 2003). The highly populated, diffusion-limited environment of the biofilm allows AHLs to readily accumulate within cells. Thus, the requirement of high C4HSL concentrations for qsc*rhlA* activation may prevent premature rhamnolipid synthesis during early stages of biofilm formation. The fact that RhlR acts as a transcriptional repressor of qsc*rhlA* when not bound to C4HSL (Medina et al., 2003b) likely contributes to the high concentration of C4HSL needed for activation. However, it should be considered that our constructs constitutively express *rhlR* with a strong ribosome binding site, potentially resulting in higher levels of free RhlR than found naturally in *P. aeruginosa*. In agreement with the simulation results, the amplifier responses shown in Figures 2 and 3 support the need for a library of amplifiers with different CI-O_R1 binding efficiencies.

Although findings should ultimately be verified in *P. aeruginosa*, the use of an *E. coli* host provides the benefit of isolating the qsc promoters from the Las and quinolone signaling systems, as well as other relevant regulatory mechanisms present in *P. aeruginosa*. Thus, observed responses to C4HSL in Figures 1, 2, and 3 can be attributed to direct activation of the qsc promoters by the C4HSL/RhlR complex. However, the promoters in this study that do not exhibit distinct responses to C4HSL in *E. coli* may

respond to C4HSL in *P. aeruginosa* either through distal promoter regulation, additional regulatory mechanisms, or other indirect means. Specifically, in contrast to previous findings in *P. aeruginosa* (Whiteley et al., 1999; Schuster et al., 2003; Wagner et al., 2003; Hentzer et al., 2003), qsc*nrps*, qsc*hcnA*, and qsc*phzS* do not respond to C4HSL in *E. coli*. Care must be taken when comparing growth phase regulation in *E. coli* to growth phase regulation in *P. aeruginosa*, since mechanisms of regulation, e.g., RpoS (Venturi, 2003), may differ.

The crosstalk results shown in Figure 4 have a number of potential implications. We see that qsc*phzA1* but not qsc*rhlA* responds to 3OC12HSL-RhlR. One important conclusion is that the AHL/R-protein binding domains of qsc promoters are important in determining specificity. This is supported by the fact that certain point mutations in the LasR-specific PA1896 promoter (qsc102) enable this promoter to additionally respond to RhlR (Whiteley and Greenberg, 2001). Furthermore, crosstalk can enable interspecies communication, allowing for more advantageous regulation of processes such as biofilm formation, antibiotic secretion, and virulence. For example, *Burkholderia cepacia*, which forms biofilms with *P. aeruginosa*, responds to 3OC12HSL (Riedel et al., 2001). Also, in addition to its presence in animals, *P. aeruginosa* is found in soil and water and is known to infect plants. Many other soil bacteria and plant pathogens are known to produce 3OC6HSL and/or C6HSL, including *Chromobacterium violaceum* (Chernin et al., 1998), *Erwinia carotovora, Pantoea stewarii*, and various other pseudomonads (von Bodman et al., 2003). Furthermore, a number of bacterial strains that degrade specific types of AHL molecules have been isolated (Uroz et al., 2003). Thus, the ability of a promoter to respond to multiple different AHLs would enhance robustness of quorum sensing responses in the presence of such organisms. This said, it must again be considered that in *P. aeruginosa*, crosstalk responses to 3OC6HSL and C6HSL may be less significant due to lower RhlR levels.

The signal-amplifying genetic circuits described here have a number of potential applications. Amplification may resolve debates such as whether or not quorum sensing regulates RpoS (Latifi et al., 1996; Whiteley et al., 2000; van Delden et al., 2001) and which genes are truly quorum-sensing repressed in *P. aeruginosa* (Schuster et al., 2003; Wagner et al., 2003; Vasil, 2003). Also, since transcriptional responses can be detected with greater sensitivity through amplification, this technique has a number of biomedical and biosensing applications. For example, amplification enables the engineering of highly sensitive bioassays that can detect trace amounts of toxins, pollutants, or molecules indicating the presence of a particular pathogen.

In the future, more sophisticated synthetic genetic circuits (Ferber, 2004) could be developed to improve our understanding of weak, transient, or multielement responses in complex genetic networks.

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