LuxR homolog-independent gene regulation by acyl-homoserine lactones in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa **quorum control of gene expression involves three LuxR-type signal receptors LasR, RhlR, and QscR that respond to the LasI- and RhlI-generated acyl-homoserine lactone (acyl-HSL) signals 3OC12-HSL and C4-HSL. We found that a LasR-RhlR-QscR triple mutant responds to acyl-HSLs by regulating at least 37 genes. LuxR homolog-independent activation of the representative genes *antA* and *catB* also occurs in the wild type. Expression of *antA* was influenced the most by C10-HSL and to a lesser extent by other acyl-HSLs, including the *P. aeruginosa* 3OC12-HSL and C4-HSL signals. The *ant* and *cat* operons encode enzymes for the degradation of anthranilate to tricarboxylic acid cycle intermediates. Our results indicate that LuxR homolog-independent acyl-HSL control of the *ant* and *cat* operons occurs via regulation of *antR*, which codes for the transcriptional activator of the *ant* operon. Although *P. aeruginosa* has multiple pathways for anthranilate synthesis, one pathway—the kynurenine pathway for tryptophan degradation—is required for acyl-HSL activation of the *ant* operon. The kynurenine pathway is also the critical source of anthranilate for energy metabolism via the *antABC* gene products, as well as the source of anthranilate for synthesis of the *P. aeruginosa* quinolone signal. Our discovery of LuxR homolog-independent responses to acyl-HSLs provides insight into acyl-HSL signaling.**

anthranilate | gene regulation | quorum sensing

The metabolically versatile bacterium *Pseudomonas aeruginosa* is ubiquitous and can adapt to diverse habitats. For example, it can be found in soil and water or as an opportunistic pathogen in a wide range of hosts, including plants and humans. The expression of a large number of *P. aeruginosa* genes, including virulence genes, is controlled by a signaling mechanism called quorum sensing. Quorum sensing involves production and sensing of chemical signals that allow bacteria to regulate gene expression in response to alterations in cell density (1–4). The archetypical LuxI-R quorum-sensing system that controls luminescence in *Vibrio fischeri* uses the enzyme LuxI to generate a diffusible acyl-homoserine lactone (acyl-HSL) signal, 3-oxo-hexanoyl-HSL, which binds to the acyl-HSL receptor and transcription factor LuxR (5, 6). Similar quorum-sensing systems have been found in about 100 species of *Proteobacteria*, where they function as regulators of a range of functions. Acyl-HSL quorum sensing commonly controls extracellular products, such as exoproteases, exopolysaccharides, antibiotics, and aggregation factors (7–10).

P. aeruginosa has two acyl-HSL synthases and three receptors (11–14). The LasI synthase produces 3-oxo-C12-HSL, for which there are two receptors, LasR and QscR. The RhlI synthase produces C4-HSL, for which the receptor is RhlR. Together these quorum-sensing systems regulate hundreds of *P. aeruginosa* genes. Different elements of the *P. aeruginosa* quorum sensing circuit also influence each other at multiple levels; for example, LasR-3OC12-HSL activates *rhlR* and *rhlI* transcription, and QscR influences expression of a subset of *las*- and *rhl*- controlled genes (15, 16). In fact, the regulons of LasR, RhlR, and QscR are partially overlapping. From previous work, it is evident that many factors, both known and unknown, influence the control of gene expression by acyl-HSLs in *P. aeruginosa* (17, 18).

Integrated into the acyl-HSL quorum-sensing circuits is a third signal, 2-heptyl-3-hydroxy-4-quinolone, known as the *Pseudomonas* quinolone signal (PQS) (19). Transcriptome analyses have shown that quinolone signaling directly or indirectly controls the expression of at least 90 genes (20, 21). The acyl-HSL and PQS signaling systems influence each other; the *las* system activates synthesis of PQS, which in turn activates *rhlI* expression (22, 23). In addition, LasR, RhlR, and QscR influence expression of genes that can potentially alter intracellular levels of the PQS biosynthesis precursor anthranilate (18).

Here we report acyl-HSL regulation of gene expression in *P. aeruginosa* that does not follow the classical quorum-sensing tenet, in that it is not mediated by a LuxR-type transcription factor. The LuxR homolog-independent acyl-HSL regulon includes the *antABC* and *catBCA* genes, which encode enzymes for the degradation of anthranilate. We show that acyl-HSL regulation of the *ant* and *cat* operons correlates with altered expression of the transcriptional regulator AntR. In *P. aeruginosa*, anthranilate is a pivotal branch-point metabolite; it can be directed either via the TCA cycle for energy metabolism or into the PQS synthesis pathway (18). The flux of anthranilate into the diverging pathways is tightly controlled by transcriptional and posttranscriptional regulatory mechanisms that are dictated variously by iron and anthranilate availability, as well as quorum sensing (18, 24). Our study reveals another layer in the extensive control that *P. aeruginosa* places on the metabolism of anthranilate.

Results

Signal Receptor-Independent Response to Acyl-HSLs. As a control for experiments designed to determine the transcriptome response to QscR and C10-HSL, we performed microarray analyses on a *P. aeruginosa* PAO LasR-RhlR-QscR triple mutant (DA15) with or without added C10-HSL. To our surprise, we identified 37 genes that showed a significant C10-HSL response (Table S1). Of these, 13 genes were induced and 24 genes were repressed. Many, but not all, of the genes that we identified are not predicted to be parts of operons. Examination of cases in which only part of an operon showed a C10-HSL response invariably revealed that the remaining genes also showed an appropriate response, but not of a magnitude to provide statistical significance. We were particularly interested in cases where all genes in an operon showed similar statistically significant C10-HSL responses. Most likely the responses of such genes are biologically important; thus, for our subsequent experiments, we concentrated on the *ant* and *cat* operons. All of the genes in these operons were activated by

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C10-HSL, and the responses were somewhat greater than the responses of other genes (Table S1).

The *P. aeruginosa* PAO1 genome contains an ORF (PA1136) coding for a protein with sequence similarity to LasR, RhlR, QscR, and other LuxR homologs (25). There is a notable similarity in the acyl-HSL–binding regions (Fig. S1), and PA1136 shows the same extent of identity to each of the other LuxR homologs shown in Fig. S1 as they do to one another other; however, PA1136 has only five of the seven residues conserved among all characterized LuxR homologs (3). Nevertheless, PA1136 might code for a C10-HSL receptor. Therefore, we constructed a signal receptor null strain lacking *lasR*, *rhlR*, *qscR*, and PA1136 (SC20) and measured transcript levels of the first genes in the *cat* and *ant* operons, *catB* and *antA*, in the null mutant. The null mutant and the LasR-RhlR-QscR triple mutant showed the same response to C10-HSL (Table 1). Thus, PA1136 does not encode a LasR homolog responsible for mediating the C10-HSL–dependent expression of *antA* and *catB*.

Do other acyl-HSLs substitute for C10-HSL as LuxR homolog-independent activators of gene expression? To answer this question, we measured transcript levels of *catB* in the LasR-RhlR-QscR triple mutant grown in the presence of a number of different acyl-HSLs (Fig. 1). Various acyl-HSLs activated *catB*, and the response was most sensitive to C10-HSL. Although not as sensitive to the *P. aeruginosa* LasR and RhlR signals 3OC12-HSL and C4-HSL, a response to physiological levels of C4-HSL (~10 μM) and a small response to physiological levels of 3OC12-HSL (~5 μM) were noted (26).

Gene Regulation by C10-HSL Occurs in WT *P. aeruginosa*. In *P. aeruginosa*, the acyl-HSL quorum-sensing circuits are interconnected with LasR-, RhlR-, and QscR-controlling overlapping regulons. C10-HSL as well as 3OC12-HSL, can serve as a signal for gene activation by QscR. To test whether LuxR homolog-independent C10-HSL gene regulation is relevant in the context of an intact quorum-sensing circuit, we measured the abundance of the *catB* and *antA* transcripts in WT *P. aeruginosa* PAO1 (Table 1). Real-time RT-PCR analysis showed that transcript levels of both *catB* and *antA* were stimulated when the WT was grown with added C10-HSL. Controls demonstrated that the QscR-dependent PA1897 did not respond to C10-HSL in the LasR-RhlR-QscR triple mutant. As expected, given its functional QscR and production of 3OC12-HSL, the WT had a much higher PA1897 transcript level than the triple mutant.

C10-HSL–Dependent Induction of *catB* Relies on Anthranilate Provided via the Kynurenine Pathway for Tryptophan Degradation. Although a number of factors affect *antABC* expression, the regulation of this operon cannot be fully explained based solely on the influence of these factors, suggesting the presence of additional factors that contribute to this regulation (18, 24). Previous work has shown that the transcriptional activator AntR activates the expression of *antABC* in response to the inducer anthranilate (18). *P. aeruginosa* can acquire anthranilate from the environment or synthesize it via one of three pathways: the kynurenine pathway, the tryptophan synthesis pathway, or the PQS pathway (Fig. S2) (27–30). We hypothesized that acyl-HSL activation of the *ant* and *cat* operons proceeds through AntR, which requires anthranilate as a coactivator. To begin to test this hypothesis, we generated strains with mutations in each of the three anthranilate synthesis pathways in *P. aeruginosa* DA15, into which we engineered a chromosomally integrated *catB-lacZ* transcriptional fusion. Expression of *catB–lacZ* fusion required a functional *kynA,* but a *pqsR* mutation and a *trpE* mutation did not eliminate C10-HSL induction of *catB–lacZ* (Fig. 2*A*). C10-HSL induction in the *kynA* mutant was recovered by exogenous addition of anthranilate (Fig. 2*B*). These data support the view that anthranilate and AntR mediate C10-HSL induction of *cat* and *ant* genes. Under our experimental conditions, the source of the anthranilate is the kynurenine pathway. A previous study showed that the kynurenine pathway is the primary source of anthranilate for PQS biosynthesis in rich media (27) similar to the medium that we used in the present study.

C10-HSL Advances *antR* Expression. Our findings with anthranilate synthase mutants demonstrate that anthranilate is required for the C10-HSL induction of *catB* and presumably other genes affected by C10-HSL in the LasR-RhlR-QscR triple-mutant background. This effect could result from the concerted action of anthranilate and AntR, the anthranilate-dependent activator of *antABC*. In support of this idea, we found that transcription of *catB* in an AntR mutant is low and not influenced by C10-HSL.

If C10-HSL affects *ant* and *cat* gene expression through AntR, then it also might be expected to influence *antR* expression. To explore this possibility, we measured β-galactosidase activity in the *lasR-rhlR-qscR* mutant strain DA15 with a WT *antR* plus either an engineered chromosomal *antR-lacZ* transcriptional or translational fusion. When grown in the presence of added C10-HSL, both fusion strains showed earlier induction of the reporter (Fig. 3). This pattern of expression also was observed when the fusions were in the WT. Control strains with a chromosomally integrated promoterless *lacZ* gene exhibited low basal β-galactosidase activity that was not influenced by C10-HSL.

To track expression of AntR, we constructed strain SC30 by generating a chromosomal fusion of a sequence encoding the VSV-G epitope to *antR* in strain DA15. We found that adding the tag did not interfere with the function of AntR by monitoring expression of the chromosomal *catB-lacZ* reporter in strain SC31, a strain containing the epitope-tagged AntR and a *catB-lacZ* reporter in the chromosomal *attB* site. Consistent with results of the *antR* fusion studies, immunoblot analysis of strain SC30 showed that AntR accumulated at higher levels earlier during growth with C10-HSL (Fig. 4). Thus, our data are consistent with the idea that AntR mediates C10-HSL activation of the *ant* and *cat* operons.

Table 1. Transcript abundance of selected C10-HSL–regulated genes

	Transcript level*		
Strain	*catB*	*antA*	PA1897
DA15 (Δ*lasR*, Δ*rhlR*, Δ*qscR*)	0.02	0.74	0.06
DA15 (Δ*lasR*, Δ*rhlR*, Δ*qscR*) plus 2 μM C10-HSL	0.92 (55)	3.2 (4.3)	0.06 (0.9)
SC20 (Δ*lasR*, Δ*rhlR*, Δ*qscR*, ΔPA1136)	0.01	0.43	0.06
SC20 (Δ*lasR*, Δ*rhlR*, Δ*qscR*, ΔPA1136) plus 2 μM C10-HSL	1.6 (93)	4.8 (6.5)	0.06 (1)
PAO1 (WT)	0.08	1.5	1.2
PAO1 (WT) plus 2 μM C10-HSL	1.7 (20)	7.2 (5)	2.7 (2.4)

*Numbers in parentheses indicate fold changes in transcript abundance on growth in the presence of C10-HSL. Transcript abundance was measured by real-time RT-PCR.

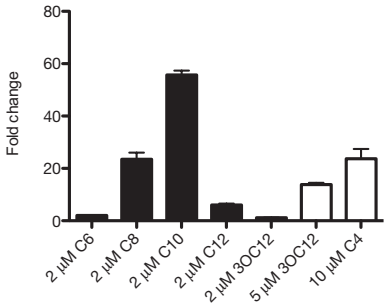

Fig. 1. The influence of various acyl-HSLs on *catB* expression in the LasR-RhlR-QscR triple-mutant DA15. Black bars indicate signals tested at 2 μM; white bars, signals tested at higher concentrations. Transcript abundance was measured by real-time RT-PCR.

Discussion

Acyl-HSLs have received considerable attention as signaling molecules that control community behavior in many *Proteobacteria* (2, 31, 32). Typically, acyl-HSLs regulate gene expression by binding to LuxR-type receptors and promoting their transcriptional regulatory activity. Here we have described acyl-HSL control of gene expression in *P. aeruginosa* that does not use a LuxR-type signal receptor and thus operates beyond the classical LuxI-R quorum regulation. To the best of our knowledge, this aspect of acyl-HSL control of gene expression has not been described for other bacteria. We have identified 37 genes that respond to C10-HSL in a *P. aeruginosa* signal receptor mutant strain, and provided evidence that this C10-HSL response is relevant in the WT strain as well. Although C10-HSL was the most effective of the signals that we tested, all signals were active to varying extents.

Our findings point to the need to reconsider the *P. aeruginosa* quorum-sensing paradigm on multiple levels. We have presented evidence suggesting the presence of at least one mechanism other than that involving a LuxR homolog through which both *P. aer-*

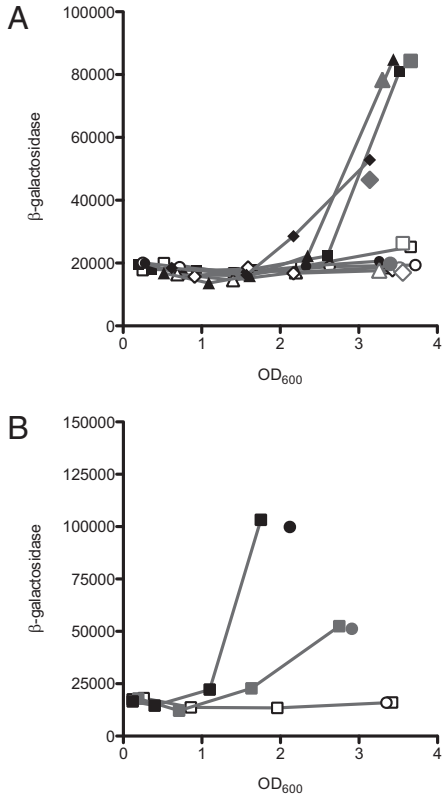

Fig. 2. Transcriptional activation of a *catB-lacZ* chromosomal fusion. β-galactosidase activity is given as relative light units normalized to OD_{600}. For one of the replicate experiments, only the final values are given for clarity. (*A*) β-galactosidase activity with (closed symbols) or without (open symbols) 2 μM C10-HSL in the following strains: LasR-RhlR-QscR triple-mutant SC24 (squares), SC24Δ*pqsR* (triangles), SC24Δ*trpE* (diamonds), and SC24Δ*kynA* (circles). The gray symbols in large type represent final values from an independent replicate. (*B*) β-galactosidase activity of the SC24Δ*kynA* strain (open symbols), with 0.2 mM anthranilate (gray symbols), and with 0.2 mM anthranilate and 2 μM C10-HSL (black symbols). Circles indicate the final values of an independent replicate.

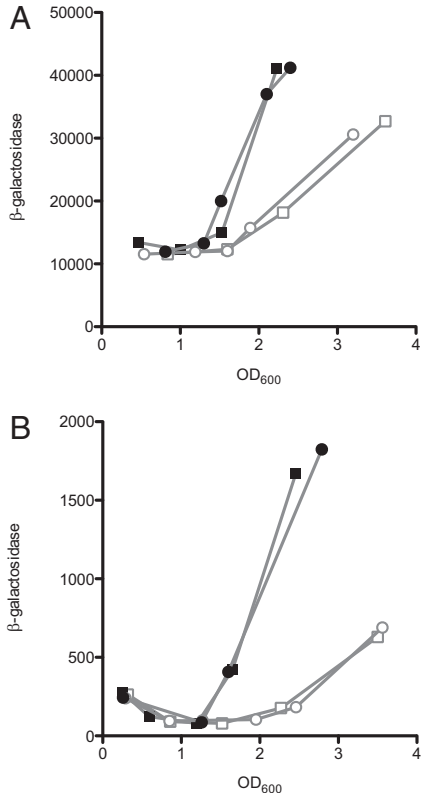

Fig. 3. Expression of chromosomal *antR-lacZ* transcriptional (*A*) and translational fusion (*B*) in the LasR-RhlR-QscR triple-mutant strain DA15. The results of two independent experiments are shown. Open symbols indicate activity in the absence of added acyl-HSL, and closed symbols indicate activity in the presence of 2 μM C10-HSL. β-galactosidase activity is given as relative light units normalized to OD_{600}.

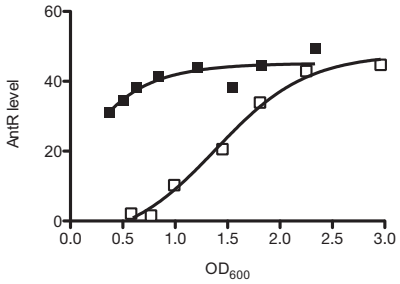

Fig. 4. A representative experiment showing AntR protein levels in the LasR-RhlR-QscR triple-mutant strain DA15 expressing AntR with an amino terminal VSV-G tag. Cells were grown with (closed squares) or without (open squares) 2 μM C10-HSL. Levels were monitored by Western blotting with VSV-G antiserum, and the intensity of the bands was quantitated as described in *Materials and Methods*. AntR levels are the band intensities in the Western blot.

uginosa quorum-sensing signals 3OC12-HSL and C4-HSL can regulate gene expression. Our evidence indicates that this type of gene regulation also can be mediated by acyl-HSLs produced by other bacteria, thus allowing *P. aeruginosa* to potentially respond to certain cohabiting microbes. We do not yet know whether this type of activity might extend beyond *P. aeruginosa*.

We focused most of our attention on two particular operons induced by C10-HSL in the LasR-RhlR-QscR mutant, the *antABC* and *catBCA* operons. It is probably not a coincidence that the *ant* operon has been previously reported to show a curious pattern of quorum control (18, 33). There is considerable evidence linking its regulation to each of the key elements of the quorum-sensing circuit; however, a coherent picture of how the different connections are integrated is not clear from the available data. Before the present study, its acyl-HSL regulation had been examined only in the context of LasR, RhlR, and QscR dependence. Although we describe an additional layer of control, our understanding of this layer is limited at this time.

Classical quorum-sensing regulation of gene expression is a dynamic process. Different genes are activated at different times in the mid-logarithmic to late stationary phase of growth (33). This sort of continuum of responses could apply to LuxR-independent acyl-HSL regulation as well, with different genes responding at different times during growth. Our transcriptome analyses were performed at OD_{600} 2; thus, it is possible that our findings provide only a snapshot of the C10-HSL response, and the genes identified in this study are a subset of the genes regulated by C10-HSL in the LasR, RhlR, QscR mutant. A case in point is *antR,* which codes for the transcriptional activator of the *ant* operon. Unlike the *ant* operon, *antR* was not identified in the initial set of C10-HSL–regulated genes; however, we provide evidence that *antR* expression is activated by C10-HSL. Our experiments indicate that the *antR* response is proximal to the C10-HSL induction of the *ant* and *cat* operons. How C10-HSL affects activation of *antR* remains unclear, however.

A key question is whether LuxR homolog-independent acyl-HSL control of *P. aeruginosa* metabolism is relevant in human infections. *P. aeruginosa* metabolism in infected patients is certain to be complex, and the effects of nutritional substrates on bacterial physiology in vivo are poorly understood. Thus, any assessment of the relevance of in vitro findings on bacterial physiology during an infection should be made with caution. Nonetheless, there is considerable evidence in the literature supporting our view that acyl-HSL control of *P. aeruginosa* metabolism is relevant in conditions extant in the pathogenic environment of the cystic fibrosis (CF) lung. Previous studies documenting the presence of PQS in CF sputum imply the availability of anthranilate, a PQS precursor (34, 35). Together with previously reported findings, our results demonstrate that the PQS biosynthesis pathway and the anthranilate degradation pathway for energy metabolism draw from the same pool of intracellular anthranilate (27). This pool derives from the degradation of the aromatic amino acid tryptophan via the kynurenine pathway. The high levels of aromatic amino acids in CF sputum and the availability of anthranilate as a growth substrate, coupled with the fact that *P. aeruginosa* accumulates at high densities in CF airways, makes acyl-HSL control of *P. aeruginosa* metabolism during chronic CF lung infections a distinct possibility (36–40). It is interesting to note that a number of the repressed genes in the C10-HSL regulon have known or putative tricarboxylic acid (TCA) transport functions (25). The regulation of these genes could be a consequence of activation of the anthranilate degradation pathway, which eventually feeds into the TCA cycle. The pattern of C10-HSL–regulated gene expression can be interpreted as reflecting a concerted shift in carbon source preference. If this is so, then our finding that acyl-HSLs can induce or facilitate this shift is of some physiological significance. The impact of nutritional cues on diverse phenotypes, such as biofilm development, surface motility, and cell-cell signaling, has been well documented, and metabolic pathways have been discussed as possible targets for drug development (40–42).

We do not yet know the precise mechanism of signal receptor-independent control of gene regulation by acyl-HSLs. A better understanding of LuxR homolog-independent responses to acyl-HSLs could provide some insight. We do know that AntR is required for the response. The function of AntR is either parallel to or downstream of the acyl-HSL control of *antABC* and *catBCA*; regardless, it mediates the response. The fact that C10-HSL triggers increased AntR expression early in growth suggests that AntR mediates the C10-HSL response. But how does C10-HSL influence AntR expression? Several possibilities can be envisioned. There may be a non-LuxR type of transcription factor that interacts with acyl-HSLs. Perhaps there is an acyl-HSL–responsive small regulatory RNA that affects AntR expression either directly or indirectly. We hope that a more detailed understanding of the scope and mechanism of this type of acyl-HSL control might provide important insight into the evolution of acyl-HSL quorum sensing. It is clear that LuxI family-dependent signal generation and LuxR family signal receptor systems have coevolved. But which appeared first? Did signal synthesis evolve first, with signals functioning in some as yet unknown way? Was this followed by coevolution of receptors, or was it the other way around?

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions. The bacterial strains and plasmids used are listed in Table 2. For plasmid and strain constructions, bacteria were grown in LB broth, supplemented with antibiotics when appropriate to maintain plasmids and select for recombinants. For transcript profiling, real-time RT-PCR, β-galactosidase assays, and Western blot analyses, bacteria were grown in LB broth buffered with 50 mM 3-(N-morpholino) propanesulfonic acid (pH 7.0). The optical densities (OD_{600}) at inoculation were 0.01 for strain DA15 and its derivatives and 0.005 for all other strains.

To construct chromosomal deletions, alleles were generated by PCR amplification of DNA flanking the gene of interest and cloned into pEXG2 (43). The resulting plasmids were used to transform *Escherichia coli* S17-1, and then mobilized into *P. aeruginosa*. Transconjugants were selected on *Pseudomonas* isolation agar (PIA) containing gentamicin (100 μg/mL), followed by recovery of deletion mutants on PIA plates containing 5% sucrose. Candidate deletion mutants were screened by PCR; real-time RT-PCR was used to demonstrate a lack of detectable mRNA for the deleted gene.

The *catB-lacZ* transcriptional fusion was constructed by directional cloning of a 281-bp PCR product beginning 190 bp upstream of the *catB* translational start site into the mini-CTX-*lacZ* plasmid (44). A 278-bp PCR product beginning 252 bp upstream of the *antR* translational start site was directionally cloned into mini-CTX-*lacZ* and mini-CTX-*lacZ*-EB to generate *antR-lacZ* transcriptional and translational fusions, respectively. The resulting plasmids were verified by DNA sequencing and mobilized into *P. aeruginosa* to allow chromosomal integration. Transconjugants were selected on PIA-containing tetracycline (100 μg/mL). The plasmid backbone was excised using the pFLP2-encoded Flp recombinase, and the pFLP2 plasmid was cured via *sacB* counterselection (45). PCR analysis was performed to verify that the insertions occurred at the chromosomal *attB* site. Growth of the two fusion strains was slightly affected by C10-HSL (Fig. S3). To construct a N-terminal vesicular stomatitis virus glycoprotein (VSV-G) epitope–AntR fusion, a PCR-generated fragment with the sequence 5′-TATACAGATATTGAAATGAATAGATTAGGAAAA-3′ inserted in-frame after the initiation codon of *antR* was cloned into pEXG2. The resulting plasmid was verified by sequencing and mobilized into strains DA15 and SC24 to generate strains SC30 and SC31, respectively. Allelic replacement was confirmed by PCR and Western blot analysis.

Microarray Experiments. Cells were harvested at OD_{600} 2. RNA isolation and cDNA synthesis, labeling, and *P. aeruginosa* GeneChip array (Affymetrix) processing were performed as described previously (33). Where appropriate, synthetic C10-HSL was added to the medium at a final concentration of 2 μM before inoculation. Each experiment was done in duplicate. Affymetrix GeneChip Operating Software was used for initial data analysis. Transcripts assigned an "absent" call in both conditions (with or without C10-HSL) were excluded from further analyses. Cyber-T (http://cybert.microarray.ics.uci.edu) was used for statistical analyses. The *P* value threshold was set at 0.007, and the fold change cutoff was set at >2.5.

Table 2. Bacterial strains used

Strain or plasmid	Relevant properties	Source or reference
E.coli		
DH5α	F⁻, φ80d*lacZ*ΔM15Δ(*lacZYA-argF*)U169 *deoR recA*1 *endA*1 *hsdR*17(rk⁻, mk⁺) *phoA supE*44 λ⁻ *thi*-1 *gyrA*96 *relA*1	Invitrogen
S17-1	*recA pro hsdR* RP4-2-Tc::Mu-*km*::Tn7	(46)
P. aeruginosa		
PAO1	WT	(42)
DA15	PAO1 derivative, Δ*lasR*, Δ*rhlR*, Δ*qscR*	(47)
SC20	DA15 ΔPA1136	This study
SC21	SC24 Δ*pqsR*	This study
SC22	SC24 Δ*trpE*	This study
SC23	SC24 Δ*kynA*	This study
SC24	DA15 with a *catB-lacZ* transcriptional fusion at the *attB* site	This study
SC26	DA15 with a *antR-lacZ* transcriptional fusion at the *attB* site	This study
SC27	DA15 with a *antR-lacZ* translational fusion at the *attB* site	This study
SC28	PAO1 with a *antR-lacZ* transcriptional fusion at the *attB* site	This study
SC29	PAO1 with a *antR-lacZ* translational fusion at the *attB* site	This study
SC30	DA15 with a VSV-G epitope sequence tagged to the N terminus of AntR	This study
SC31	SC24 with a VSV-G epitope sequence tagged to the N terminus of AntR	This study
Plasmids		
pEXG2	Gene-replacement vector; *sacB* Gm^r	(43)
mini-CTX-*lacZ*	Integrative reporter vector; Tc^r	(44)
mini-CTX-*lacZ*-EB	Integrative reporter vector; Tc^r	Brutinel and Yahr, University of Iowa
pFLP2	Source of Flp recombinase; Ap^r/Cb^r	(45)

km, kanamycin resistance; Ap^r, ampicillin resistance; Cb^r, carbenicillin resistance; Tc^r, tetracycline resistance; Gm^r, gentamicin resistance.

Real-Time RT-PCR. The procedures for RNA isolation and cDNA synthesis were similar to those used for the microarray experiments. Comparative RT-PCR was performed on an ABI Prism 7900 system using SYBR Green PCR Master Mix (Applied Biosystems). Each reaction used 5 ng of cDNA template in a 25-μL volume. Relative transcript levels were determined by the comparative standard curve method. Standard curves were generated using gene-specific primers with 10 ng to 0.1 pg of RNA-free genomic DNA purified from *P. aeruginosa* PAO1. A dissociation curve step was included at the end of each PCR to verify that a single specific product was amplified and confirm the absence of primer dimers. PA1897, a QscR-activated gene, was used as an internal control to verify the absence of significant nonspecific variation between samples.

β-Galactosidase Assays. Galacto-Light Plus (Tropix) was used to measure β-galactosidase activity in *lacZ* reporter fusion strains. Results are given in units of β-galactosidase activity per OD_{600}.

Western Blot Analysis. At each time point, ~3E+09 bacteria was harvested by centrifugation, and the cell pellet was suspended in 150 μL of 150 mM Tris-HCl (pH 7.5) and 150 μL of 2× Laemlli buffer. Samples were boiled for 5 min and sonicated with a Branson Microtip Sonifier (10 s at an output of 0.4). Protein concentration of the lysates was determined using a 660-nm protein assay (Pierce); 15 μg of protein for each sample was separated by SDS/PAGE, followed by polypeptide transfer to a nitrocellulose membrane. The membrane was probed with a 1:4,000 dilution of anti–VSV-G antibody (V4888; Sigma-Aldrich) and a 1:50,000 dilution of peroxidase conjugated goat anti-rabbit IgG, followed by detection of the conjugates with the Supersignal West Pico chemiluminescent substrate (Pierce). The intensity of the bands was determined using ImageQuant 5.1 (GE Healthcare).

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