Reversible Signal Binding by the *Pseudomonas aeruginosa* Quorum-Sensing Signal Receptor LasR

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**ABSTRACT** Many members of the LuxR family of acyl-homoserine lactone (acyl-HSL)-dependent quorum-sensing transcriptional activators are thought to have the unusual characteristics of requiring the signal ligand during polypeptide synthesis to fold into an active conformation and of binding signal extraordinarily tightly. This is the case for the N-3-oxo-dodecanoyl-HSL-dependent *Pseudomonas aeruginosa* virulence regulator LasR. We present evidence that LasR can fold into an active conformation *in vivo* in the absence of the acyl-HSL ligand. We also present evidence indicating that in the cellular environment, LasR and N-3-oxo-dodecanoyl-HSL readily dissociate. After dissociation, LasR can remain in a properly folded conformation capable of reassociating with signal. We present a new model for the folding and signal binding of LasR and other members of the family of transcription factors to which LasR belongs. Our findings have important implications concerning the cellular responses to decreased environmental concentrations of signals and have implications about potential quorum-sensing inhibition strategies.

**IMPORTANCE** The opportunistic pathogen *Pseudomonas aeruginosa* causes difficult to treat and incurable diseases. Quorum sensing controls expression of many virulence factors in this bacterium, and the quorum-sensing signal receptor LasR has been targeted for anti-quorum-sensing therapeutic development. The work described here changes the current view about how LasR interacts with the quorum-sensing signal to which it responds. This is of importance for therapeutic development. The experiments also address a poorly studied aspect of quorum sensing: the response to decreases in quorum-sensing signals. This has importance with respect to understanding the ecology and evolution of quorum sensing.
target DNA in the absence of signal and serves as a repressor. Signal binding results in derepression (19).

If this belief that LasR and certain other LasR homologs require their signal as a folding scaffold during their synthesis is correct, it has important implications about cellular responses to decreasing environmental acyl-HSL levels and about designing inhibitors of quorum sensing that might have utility as antivirulence therapeutics. For example, if signal binding is essentially irreversible, then one would predict that gene activation by 3OC12-HSL–LasR would persist when a cell moves from a high-population-density (high-3OC12-HSL concentration) environment to a low-population-density (low-3OC12-HSL) environment, at least until active LasR is decreased by proteolysis or cell division. One might also predict that competitive inhibitors of 3OC12-HSL might work via binding to the nascent LasR polypeptide chain prior to 3OC12-HSL binding but would not affect preexisting LasR–3OC12-HSL complexes.

There has been an enormous amount of effort aimed at understanding the influence of increased acyl-HSL levels on the activity of LuxR homologs, but almost no attention has been paid to the immediate cellular response to decreases in acyl-HSL concentrations. We are aware of only three reports on the influence of a drastic decrease in signal levels on LuxR homolog-dependent gene transcription (20–22). When Vibrio fischeri, the bacterium in which acyl-HSL quorum sensing was first discovered, is rapidly transferred from a signal-replete medium to a signal-depleted medium but kept at the same population density, transcription of quorum-sensing-controlled genes returns to the basal level within minutes. This suggests that LuxR binds to its cognate signal, 3OC6-HSL, reversibly. This is consistent with the fact that, unlike LasR and TraR, purified LuxR does not appear to bind signal irreversibly (13). LuxR is considered an exception rather than the rule. Somewhat similar experiments on the A. tumefaciens LuxR homolog TraR showed a precipitous drop in the environmental signal concentration that resulted in a rather rapid decrease in TraR-dependent reporter transcription, but a low level of transcription appeared to persist for several cell divisions. The investigators came to the conclusion that the low level of transcription indicated that some TraR retained signal and continued to function (22), but they did not discriminate this possibility from the possibility that the low level of transcription represented basal-level TraR-independent transcription of the reporter. None of these studies (20–22) addressed the issue of whether functional acyl-HSL receptors capable of signal binding and DNA binding remained after signal removal from the medium.

In light of the above-described considerations, we have examined the activity of LasR in recombinant Escherichia coli and in P. aeruginosa after a rapid decrease in 3OC12-HSL from above a threshold level for LasR activity to below that level. The experiments we present are consistent with the view that LasR folds into an active conformation in the absence of any acyl-HSL signal and can remain in a properly folded signal-free state. Our experiments are also consistent with the view that, in the context of the cellular environment, signal binding is reversible.

RESULTS
In vivo LasR activity after removal of extracellular 3OC12-HSL.

Purified LasR binds to the cognate signal 3OC12-HSL so avidly that even after extensive dialysis against signal-free buffer, this 3OC12-HSL-dependent transcription factor retains signal and a capacity to bind target DNA in the absence of added signal (12).

These in vitro findings suggest that when cells growing in the presence of 3OC12-HSL are challenged with a steep decrease in external 3OC12-HSL concentration, LasR activation of gene expression should persist until cellular LasR is degraded or diluted by cell division. Removal of extracellular 3OC12-HSL should not affect ongoing transcriptional activation by intact LasR-signal complexes. We first tested this prediction in recombinant E. coli containing an arabinose-inducible lasR and a last-lacZ reporter of LasR activity. Cells were grown in the presence of arabinose and 200 nM 3OC12-HSL. This level of 3OC12-HSL is about 10 to 20 times the concentration necessary to saturate the signal response. During the active last-lacZ induction phase, the cells were pelleted and then suspended in a volume of fresh medium equal to that from which they were pelleted with or without added 3OC12-HSL. The measured 3OC12-HSL concentration after suspension in fresh medium without added 3OC12-HSL was about 2 nM. When centrifuged cells were suspended in fresh medium containing 200 nM 3OC12-HSL, induction of the last-lacZ fusion appeared similar to induction in continuously grown uncentrifuged cells. However, when the cells were suspended in signal-free medium, there was no measurable increase of β-galactosidase (Fig. 1). In fact, β-galactosidase normalized to cell mass decreased with time after removal of external signal as we would expect if transcription of the last-lacZ reporter were to drop to basal levels. The most plausible explanation for this result is that when the concentration of free 3OC12-HSL drops below a threshold, there is a dissociation of LasR and 3OC12-HSL.

We chose to first examine LasR in recombinant E. coli. It is important to determine whether the LasR response to decreased 3OC12-HSL in P. aeruginosa is similar to the response in E. coli. Experiments with P. aeruginosa require a somewhat more complicated design because wild-type P. aeruginosa produces 3OC12-HSL and because factors present in the growth medium other than 3OC12-HSL can influence the timing of quorum-sensing-regulated gene expression (23). To circumvent these issues, we introduced the last-lacZ reporter plasmid into a P. aeruginosa lasR rhl double mutant that does not produce acyl-HSLs, and after centrifugation, we suspended cells in sterile medium that had been conditioned by pregrowth of the signal...
reached a plateau. We presume that this is because without evidence in support of the view that removal of arabinose effectively de novo believe that the response involved a leaky arabinose promoter and in arabinose-containing medium, and the response was not as large as the response to 3OC12-HSL for at least 20 min after arabinose deple-
tion. The response was not as large as the response to 3OC12-HSL in arabinose-containing medium, and 3OC12-HSL expression rapidly reached a plateau. We presume that this is because without de novo synthesis, the diminishing concentration of LasR becomes limiting. The response to signal addition was rapid. Thus, we do not believe that the response involved a leaky arabinose promoter and de novo synthesis of LasR after signal addition.

We performed two additional experiments to gain additional evidence in support of the view that removal of arabinose effectively stopped lasR transcription and that the response to signal was a response of preexisting LasR. We used reverse transcription-PCR (RT-PCR) to measure lasR transcripts before and 20 min after arabinose removal (Fig. 2). After 20 min, cells suspended in medium without arabinose had very little lasR transcript. We obtained quantitative information by using real-time RT-PCR and found about 3,000 times more lasR transcript in cells incubated with arabinose than in cells incubated without arabinose for 20 min. We also grew E. coli containing an arabinose-inducible lasR and the last-lacZ fusion in the presence of 3OC12-HSL but without arabinose to determine the amount of β-galactosidase activity that might be attributed to a leaky arabinose promoter and found a negligible level of last-lacZ expression (Fig. 1, left panel, inset).

These experiments support the view that a pool of signal-free LasR exists in cells and that the LasR in this pool is capable of binding 3OC12-HSL and activating transcription of a LasR-dependent promoter. We also grew cultures in arabinose-containing medium without 3OC12-HSL, centrifuged the cells, and suspended them in arabinose-free medium. We then examined whether we could measure induction of a pascal-lacZ reporter upon the addition of 3OC12-HSL to these cells that had not seen any previous exposure to 3OC12-HSL (Fig. 3). As was the case for the experiment in Fig. 2, there was a response that persisted for over 20 min in the absence of arabinose.

Extracts of cells grown without acyl-HSLs have detectable LasR activity. If LasR can fold into an active conformation in the absence of acyl-HSLs, it should be possible to demonstrate the existence of functional protein in cell-free extracts of LasR-expressing E. coli grown without signal. We hypothesized that active LasR has not been found in lysates of cells grown without signal previously, because signal-free protein is very unstable in the lysis buffers that have been used. This hypothesis is in opposition to the hypoth-
thesis that nascent LasR polypeptides must fold around the signal to attain an active conformation (12). To test our new hypoth-
esis, we grew E. coli strain BL21 (DE3) carrying plasmids pLYS and pET17b.lasR with isopropyl-β-D-thiogalactopyranoside (IPTG) overnight at 17°C (12). The cells were broken on ice in a buffer containing 3OC12-HSL, which we hoped would bind

FIG 2  Addition of signal to signal-free cells restores LasR activity. (Left) E. coli containing an arabinose-inducible LasR expression vector and a last-lacZ reporter construct was grown to OD_{600} of 0.2 in the presence of arabinose and 100 nM 3OC12-HSL. Cells were pelleted by centrifugation (arrow pointing down) and then suspended in LB MOPS medium without arabinose (arrow pointing up). One hundred nanomolar 3OC12-HSL was added back at various time points, and β-galactosidase activity (×10^4) was monitored. Symbols: solid squares, no centrifugation; solid triangles, signal added back immediately upon suspension in fresh medium; solid diamonds, 20 min after centrifuga-
tion; open squares, 40 min after centrifugation; open triangles pointing down, no signal added after centrifugation. (Right) RT-PCR of lasR mRNA and 16S RNA transcript levels. Lanes 1, E. coli at an OD_{600} of 0.5 with 0.1% arabinose present; lanes 2, 20 min after centrifugation and removal of arabinose.

FIG 3 LasR produced in the absence of signal can be activated by subsequent signal addition. E. coli containing an arabinose-inducible LasR expression vector and a last-lacZ reporter construct was grown in the presence of 0.1% arabinose without 3OC12-HSL. After centrifugation, we suspended the cells in arabinose-free medium, added 100 nM 3OC12-HSL at various times and measured β-galactosidase activity over time. Symbols: solid squares, signal added without centrifugation; solid triangles pointing up, signal added immediately after centrifugation; solid triangle pointing down, signal added 10 min after centrifugation; solid diamonds, 20 min after centrifugation; open squares, 30 min after centrifugation; open triangles pointing down, no signal added after centrifugation. Note that β-galactosidase activity levels are somewhat lower than those shown in Fig. 2, presumably because 3OC12-HSL was not present in this experiment prior to cen-
trifugation.

is there a pool of ligand-free LasR that retains a capacity for 3OC12-HSL binding and gene activation? The signal depletion experiments described above indicate that 3OC12-HSL can dissociate from LasR in vivo, but is the putative signal-free LasR functional? Can it respond to the addition of 3OC12-HSL? For simplicity, we used recombinant E. coli to address this question. The experimental protocol was similar to that used in Fig. 1 except that when the cells were suspended in fresh medium, we omitted both 3OC12-HSL and L-arabinose, the inducer of lasR transcription. At increasing periods of time after arabinose removal, we added 3OC12-HSL and asked whether the signal addition stimulated lasI-lacZ transcription (Fig. 2). In fact, there was a measurable response to 3OC12-HSL for at least 20 min after arabinose deple-
tion. The response was not as large as the response to 3OC12-HSL in arabinose-containing medium, and lasI-lacZ expression reached a plateau. We presume that this is because without de novo synthesis, the diminishing concentration of LasR becomes limiting. The response to signal addition was rapid. Thus, we do not believe that the response involved a leaky arabinose promoter and de novo synthesis of LasR after signal addition.

We obtained quantitative information by using real-time RT-PCR and found about 3,000 times more lasR transcript in cells incubated with arabinose than in cells incubated without arabinose for 20 min. We also grew E. coli containing an arabinose-inducible lasR and the last-lacZ fusion in the presence of 3OC12-HSL but without arabinose to determine the amount of β-galactosidase activity that might be attributed to a leaky arabinose promoter and found a negligible level of last-lacZ expression (Fig. 1, left panel, inset).
to LasR and stabilize it. If correct, our hypothesis predicts that we should be able to detect active protein in the lysate.

We used lysates of cells grown with or without 3OC12-HSL as a source of LasR in DNA electrophoretic mobility shift assays (EMSAs) with a LasR-specific DNA fragment as a target (Fig. 4). Although extracts from cells grown with 3OC12-HSL appeared to have about 10 times more active LasR than cells grown without 3OC12-HSL, we detected active LasR in extracts of cells grown without 3OC12-HSL. For a control, we prepared a lysate of cells grown without added signal, but we did not supply 3OC12-HSL in the lysis buffer. Although we included 3OC12-HSL in the EMSA reaction buffer, we could not detect LasR binding to target DNA when using this lysate. There was also no specific DNA binding when we used extracts from cells that did not contain a functional lasR gene (Fig. 4).

The difference in ability to bind LasR-specific DNA may reflect an increased amount of stable LasR in the soluble fraction or it could represent a small percentage of soluble LasR with the remainder being inactive. To address this issue, we performed an anti-LasR Western immunoblot analysis of the cell lysates analyzed by an EMSA (Fig. 4D). There is about 10 times more soluble LasR in the extracts of cells grown with 3OC12-HSL than in the extracts of cells grown without 3OC12-HSL. This leads us to conclude that when cells grown without 3OC12-HSL are broken in ice-cold buffer containing 3OC12-HSL, the majority of the soluble LasR is active as judged by EMSAs. When cells are broken in buffer without 3OC12-HSL, the amount of soluble LasR is roughly equivalent to the level when cells are broken in buffer with 3OC12-HSL, but the soluble protein is inactive as assessed by an EMSA.

These in vitro studies support the view that LasR can fold into a functional conformation in the absence of an acyl-HSL signal. Consistent with previous reports on TraR and QscR (15, 24), we believe that signal-free LasR is relatively unstable in vivo, and thus, we find relatively low levels of active protein in cells grown without 3OC12-HSL.

**DISCUSSION**

Acyl-HSL-dependent transcriptional activators such as TraR and LasR form insoluble aggregates when expressed in recombinant bacteria grown without the cognate signal but show increased solubility when cells are grown in the presence of signal (10, 12, 13, 15). What little soluble protein there might be in extracts of cells grown without signal does not appear to be active as assessed by EMSA in the absence or presence of the cognate signal (12). Active TraR or LasR can be obtained from cells grown with the cognate signal. When these proteins are purified, they retain signal even after purification steps involving dialysis and column chromatography (12, 15). Moreover, LasR retained signal even after extensive dialysis against signal-free buffer. Furthermore, the crystal structure of TraR in its DNA-bound state shows that the acyl-HSL signal is completely buried in the protein (14). These sorts of data have led to the view that during polypeptide synthesis many LuxR homologs require their cognate signal to fold properly and that once the active protein has formed, it retains signal (17). If this were the case, these would represent very unusual properties. This sort of behavior would have important implications related to the ability of cells to respond rapidly to decreases in environmental signal levels and the ability of small molecules to competitively inhibit activity of functional LasR or TraR proteins.

Because there is little known about cellular responses to precipitous decreases in external acyl-HSL signals, we sought to investigate such a response. We chose to study LasR because production of significant levels of soluble LasR in recombinant bacteria requires its cognate acyl-HSL, it retains the acyl-HSL even after extensive dialysis against signal-free buffer, and it has been a target for development of quorum-sensing inhibitors that might have value as anti-*Pseudomonas* virulence therapeutics (12, 17).

Our in vivo experiments with either recombinant *E. coli* or
**P. aeruginosa** show that there is a rapid cessation in transcription of a LasR-dependent gene after 3OC12-HSL is decreased from a saturating level to a level below the threshold for gene activation (Fig. 1). These experiments indicate that in contrast to *in vitro* results, 3OC12-HSL can rapidly dissociate with LasR in *vivo*. These experiments also show that LasR-dependent quorum control of gene expression can respond rapidly to decreased signal concentration just as it can respond rapidly to increased signal concentration.

If it is true that LasR and 3OC12-HSL can dissociate rapidly *in vivo*, it becomes an open question as to whether signal-free LasR can bind added 3OC12-HSL and regain its ability to activate quorum-controlled genes. We addressed this question in two ways. First, we used recombinant *E. coli* containing an arabinose-inducible lasR and a LasR-responsive reporter in the presence of arabinose and 3OC12-HSL. During the induction phase of the reporter, we removed arabinose and 3OC12-HSL, and we asked whether the existing pool of LasR maintained an ability to activate the reporter in response to 3OC12-HSL added over increasing periods of time (Fig. 2). Our evidence indicates that the bacteria retained a pool of functional, 3OC12-HSL-responsive LasR for at least 20 min (Fig. 2 and 3). Second, we were able to demonstrate the existence of active LasR in extracts of bacteria that were grown without any added acyl-HSL (Fig. 4). We believe that this has been problematic for LasR and other LasR homologs in the past because unless they are bound to their signal, these proteins are very unstable in cell-free extracts. We overcame this obstacle by breaking the cells in ice-cold buffer with 3OC12-HSL.

On the basis of all of the available results on LasR and related transcription factors, we propose a new model for the interactions of LasR with its cognate signal ligand 3OC12-HSL. We believe this model might hold for TraR, but we have no experimental data to bring to bear on this possibility. Our model is perhaps more congruent with our general understanding of protein-ligand interactions than the previous model. We believe that LasR can fold into a functional conformation in the absence of an acyl-HSL ligand. Consistent with previous reports on TraR and QscR (15, 24), we believe that signal-free LasR is relatively unstable in comparison to signal-bound LasR. However, it is sufficiently stable for a pool of functional cellular signal-free LasR to exist. We believe that other transcription factors, LasR binds to its coinducig ligand (3OC12-HSL) reversibly. It is generally assumed that members of the LuxR family fall into one of three general categories based on interactions with their signals (17). One category is represented by LasR and TraR, both of which were thought to require signal as a folding scaffold during synthesis and to bind signal in a virtually irreversible manner. Those like LuxR from *V. fischeri* were thought to require signal as a scaffold but once folded exhibit reversible signal binding. Finally, members of a class represented by *M. tianshanense* MrtR and *P. stewartii* EsaR do not require an acyl-HSL as a folding scaffold. Our new evidence leads us to speculate that all LuxR homologs might interact with their signals in a fashion analogous to MrtR and EsaR in that they can fold into active forms in the absence of an acyl-HSL. Our recent biochemical analysis of QscR (24) is consistent with this new view, and it suggests that the differences in behavior of different LuxR homologs can be accounted for by signal and DNA binding affinities as well as stability of signal-free forms *in vivo*. Considerable attention has been given to cellular responses to increasing acyl-HSL levels, either natural accumulations or artificial additions. Unfortunately, little attention has been given to the responses resulting from rapid decreases in signal levels. This report shows there is information to be gained by studying the effects of decreasing environmental signal concentrations on quorum-sensing regulation of gene expression.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** We used *E. coli* DH5α (25) and *P. aeruginosa* MW1, a ∆lasR mutant derived from strain PA01 (26) for all *in vivo* experiments. We used *E. coli* BL21(DE3) carrying plasmids pLysS and pET17b.lasR, which contains an IPTG-inducible T7 RNA polymerase and lasR under the control of a T7 promoter (12) as a source of cell extracts for *in vitro* LasR activity experiments. pLysS contains a gene encoding T7 lysozyme and a chloramphenicol resistance marker. The following additional plasmids were used: pN105L, which contains an arabinose-inducible lasR and a gentamicin resistance marker (10), and pSC11, which contains a plasmid-lasR and an ampicillin resistance marker (9). Unless indicated otherwise, the bacteria were grown in Luria-Bertani (LB) broth containing 50 mM morpholinopropanesulfonic acid (MOPS) at pH 7.0 and 37°C with shaking. The optical density at 600 nm (OD600) was used to monitor growth. The following antibiotics were added at the following concentrations as appropriate: 100 μg of ampicillin per ml, 10 μg of gentamicin per ml, 150 μg of carbenicillin per ml, and 34 μg of chloramphenicol per ml. Where indicated, 0.1% arabinose or 0.4 mM IPTG was added to induce LasR production. Plasmids were introduced into *E. coli* or *P. aeruginosa* by transfection (25). The additions of 3OC12-HSL were as indicated.

**Signal removal and signal add-back experiments.** The inocula for *E. coli* experiments were from overnight cultures. The starting culture density was 0.01 at 600 nm. For all experiments, bacteria were grown in 50 ml LB supplemented with MOPS in 250-ml flasks until the culture density reached 0.2. At an OD600 of 0.2, cells were pelleted by centrifugation (2,000 × g for 5 min at room temperature) and cells from 6 ml of culture medium were suspended in 6 ml of medium as indicated. For *E. coli*, cells were suspended in fresh LB supplemented with MOPS. For *P. aeruginosa*, we used a medium that had been conditioned by growth of *P. aeruginosa* MW1 to an optical density of 0.2. The *P. aeruginosa* MW1 cells were removed from this medium by centrifugation as described above. We manipulated the signal concentration in the growth medium after centrifugation or the expression of LasR by 1-arabinose as indicated.

**Monitoring LasR activity.** We monitored LasR activity *in vivo* by following expression of a lasR promoter-driven lasZ with a Galacto-Light Plus kit (Tropix) as described elsewhere (10). The activity is given as relative luminescence units per OD600 of the cell cultures. We monitored LasR activity *in vitro* by using an EMSA as described elsewhere (12). Cell extracts for EMSAs were prepared as follows. We grew *E. coli* BL21(DE3) carrying pLysS and pET17b.lasR with or without 2 mM 3OC12-HSL. For a control, we used *E. coli* BL21(DE3) carrying pLysS with the empty vector pET17b. The cultures were grown to an optical density at 600 nm of 0.5 and rapidly cooled to 17°C. We then added IPTG and continued incubation at 17°C overnight with shaking. Cells were harvested by centrifugation, and the cell pellets were quickly frozen at −80°C for 90 min. The pellets were thawed on ice and suspended in cold LasR purification buffer (25 mM Tris-HCl [pH 7.8], 150 mM NaCl, 1 mM diithiothretol [DTT], 1 mM EDTA, 10% glycerol, 0.05% Tween 20) in the presence or absence of 2 μM 3OC12-HSL and immediately sonicated (Branson) three times for 10 s each. Insoluble material was removed by ultracentrifugation (100,000 × g for 30 min), and soluble cell-free extracts were used in EMSAs. The EMSA reaction mixtures contained a 290-hp LasR binding site containing the rsaL-lasI intergenic region and a nonspecific 176-bp fragment of the rsaL coding region. Both DNAs were generated by PCR amplification as previously described (12). PCR products were end labeled by using [γ-32P]ATP (Perkin Elmer) and T4 polynucleotide kinase (NEB). Binding reaction mixtures contained a 0.5 μM concentration of each.
probe in a final volume of 19 μl of DNA binding buffer (20 mM Tris-HCl [pH 7.5], 50 mM KCl, 1 mM EDTA, 1 mM DTT, 100 μM bovine serum albumin [BSA], 10% glycerol) and 1 μl of a dilution of cell-free extract as indicated. Reaction mixtures were incubated for 20 min and then separated at room temperature on a 5% Tris-glycine-EDTA polyacrylamide gel at 50 V using a mini-Protean tetra cell (Bio-Rad) for 120 min. The gels were dried and used to expose a storage phosphor screen, and exposure was visualized by using a Storm PhosphorImager and ImageQuant software (GE Healthcare).

**Quantitative 3OC12-HSL bioassay.** We measured the amount of 3OC12-HSL in culture fluid by using the bioassay strain E. coli DH5α(pN105L, pSC11) as previously described (10). A standard curve was generated by using synthetic 3OC12-HSL.

**Western immunoblot analysis of LasR.** Cell extracts were prepared as described above for EMSAs. Soluble and insoluble proteins were separated by centrifugation as described above. The analysis involved previously described procedures (27). Polypeptides were separated by SDS-polyacrylamide gel electrophoresis, and for immunoblotting, we used rabbit anti-LasR IgG and horseradish peroxidase-conjugated goat anti-rabbit IgG as the primary and secondary antibodies, respectively. Chemiluminescence detection was with a SuperSignal West Pico kit (Thermo Scientific).

**RT-PCRs.** Ten-milliliter cultures of E. coli DH5α(pN105L, pSC11) were grown to a density of 0.8 in the presence of t-arabinose. Cells from 4 ml of each culture were pelleted by centrifugation and suspended in fresh medium without arabinose. After 20 min without arabinose, the cells were pelleted by centrifugation, and RNA was extracted from the pelleted cells by using a Qiagen RNeasy kit. We also isolated RNA from cells that had been incubated with arabinose. After DNase (Promega) treatment as described elsewhere (27), we incubated 1 μg of RNA with random primers and Superscript II reverse transcriptase (Invitrogen) at 35°C for 10 min and then at 42°C for 30 min. The reverse transcriptase was then deactivated by heat treatment (70°C for 10 min). The cDNAs prepared in this manner were analyzed by real-time PCR. We used the SYBR green reagent (Applied Biosystems) and the following primers: primers LasR258F (TTTCTGGGAACCGTCCATCT) and LasR320R (GCCGAGGGCCGCTCTCGGA) for measurement of LasR mRNA (at the end of the primer designation, F stands for forward and R stands for reverse) and primers 16SNA312F (CACACTGGAACTGAGCACAGC) and 16SNA437R (AGTACTTTACAACCCGAAGC) for measurement of 16S RNA. *Pseudomonas aeruginosa* (strain PAO1) genomic DNA was used as a standard.

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**REFERENCES**

1. Fuqua, C., and E. P. Greenberg. 2002. Listening in on bacteria: acyl-homoserine lactone signalling. Nat. Rev. Mol. Cell Biol. 3:685–695.

2. Waters, C. M., and B. L. Bassler. 2005. Quorum sensing: cell-to-cell communication in bacteria. Annu. Rev. Cell Dev. Biol. 21:319–346.

3. Gambelli, M. J., and B. H. Iglewski. 1991. Cloning and characterization of the *Pseudomonas aeruginosa* lasR gene, a transcriptional activator of elastase expression. J. Bacteriol. 173:3231–3238.

4. Ochsner, U. A., A. K. Koch, A. Fiechter, and J. Reiser. 1994. Isolation and characterization of a regulatory gene affecting thiaminoid biosurfactant synthesis in *Pseudomonas aeruginosa*. J. Bacteriol. 176:2044–2054.

5. Passador, L., J. M. Cook, M. J. Gambelli, L. Rust, and B. H. Iglewski. 1993. Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. Science 260:1127–1130.

6. Pearson, J. P., K. M. Gray, L. Passador, K. D. Tucker, A. Eberhard, B. H. Iglewski, and E. P. Greenberg. 1994. Structure of the autoinducer signal required for expression of *Pseudomonas aeruginosa* virulence genes. Proc. Natl. Acad. Sci. U. S. A. 91:197–201.

7. Pearson, J. P., L. Passador, B. H. Iglewski, and E. P. Greenberg. 1995. A second N-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. U. S. A. 92:1490–1494.

8. Winson, M. K., M. Camara, A. Latifi, M. Foglino, S. R. Chhabra, M. Daykin, M. Bally, V. Chapon, G. P. Salmond, B. W. Bycroft, A. Lazdunski, G. S. Stewart, and P. Williams. 1995. Multiple N-acyl-l-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. U. S. A. 92:9427–9431.

9. Chuang, A. S., M. Whiteley, K. M. Lee, D. D’Argenio, C. Manoil, and E. P. Greenberg. 2001. QscR, a modulator of quorum-sensing signal synthesis and virulence in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. U. S. A. 98:2752–2757.

10. Lee, J. H., Y. Lequette, and E. P. Greenberg. 2006. Activity of purified QscR, a *Pseudomonas aeruginosa* orphan quorum-sensing transcription factor. Mol. Microbiol. 59:1342–1352.

11. Pearson, J. P., C. Van Delden, and B. H. Iglewski. 1999. Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals. J. Bacteriol. 181:1203–1210.

12. Schuster, M., M. L. Urbanowski, and E. P. Greenberg. 2004. Promoter specificity in *Pseudomonas aeruginosa* quorum sensing revealed by DNA binding of purified LasR. Proc. Natl. Acad. Sci. U. S. A. 101:15833–15839.

13. Urbanowski, M. L., C. P. Lostroh, and E. P. Greenberg. 2004. Reversible acyl-homoserine lactone binding to purified *Vibrio fischeri* LuxR protein. J. Bacteriol. 186:631–637.

14. Zhang, R. G., T. Pappas, J. L. Brace, P. C. Miller, T. Oulmassov, J. M. Molyneaux, J. C. Anderson, J. K. Bashkin, S. C. Winans, and A. Joachimiak. 2002. Structure of a bacterial quorum-sensing transcription factor complexed with pheromone and DNA. Nature 417:971–974.

15. Zhu, J., and S. C. Winans. 1999. Autoinducer binding by the quorum-sensing transcriptional regulator TraR increases affinity for target promoters in vitro and decreases TraR turnover rates in whole cells. Proc. Natl. Acad. Sci. U. S. A. 96:4832–4837.

16. Zhu, J., and S. C. Winans. 2001. The quorum-sensing transcriptional regulator TraR requires its cognate signaling ligand for protein folding, protease resistance, and dimerization. Proc. Natl. Acad. Sci. U. S. A. 98:1507–1512.

17. Schuster, M., and E. P. Greenberg. 2008. LuxR-type proteins in *Pseudomonas aeruginosa* quorum sensing: distinct mechanisms with global implications. J. Bacteriol. 190:343–348.

18. von Bodman, S. B., J. K. Ball, M. A. Faini, C. M. Herrera, T. D. Minogue, M. L. Urbanowski, and A. M. Stevens. 2003. The quorum sensing negative regulators EsaR and ExpR (strain PAO1) genomic DNA was used as a standard.