Delays in *Pseudomonas aeruginosa* quorum-controlled gene expression are conditional

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Acyl-homoserine lactone (acyl-HSL) signaling is thought to mediate quorum sensing in many species of Proteobacteria. The opportunistic human pathogen *Pseudomonas aeruginosa* uses acyl-HSLs to regulate hundreds of genes, including many that code for extracellular virulence factors. The idea that the *P. aeruginosa* acyl-HSLs serve as quorum-sensing signals has been questioned recently because microarray experiments show that the addition of signals to cultures of *P. aeruginosa* does not advance the onset of transcription for most acyl-HSL-dependent genes. We show that, under specific conditions, the expression of many acyl-HSL-dependent genes can be triggered at low culture density by signal addition. If complex medium is conditioned by growth of a non-acyl-HSL-producing *P. aeruginosa*, signals can eliminate the delay in expression of a battery of acyl-HSL-dependent genes. Furthermore, for one representative gene, lasB, there is no delay when signals are added to *P. aeruginosa* growing in conditioned complex medium or in minimal medium. We conclude that complex medium contains an inhibitor or inhibitors that can prevent induction of many, but not all, acyl-HSL-regulated genes and that the inhibitor is consumed by *P. aeruginosa*. Our results show that acyl-HSL signals can trigger expression of a large number of acyl-HSL-dependent genes regardless of growth phase. In this way, signaling in *P. aeruginosa* appears similar to quorum sensing in other Proteobacteria.

The opportunistic pathogen *Pseudomonas aeruginosa* requires two self-produced extracellular acyl-homoserine lactone (acyl-HSL) signals to activate the expression of hundreds of its ∼5,500 genes (1–4). Many other Proteobacteria use acyl-HSLs as signals to regulate gene expression in a cell-density-dependent fashion. Thus, acyl-HSL signaling is generally considered to be involved in quorum sensing (reviewed in refs. 5–7). The view of acyl-HSLs as quorum-sensing signals in *P. aeruginosa* has recently been questioned (8–10). The idea that acyl-HSLs might be signals that do not play a role in quorum sensing comes from experiments showing that the expression of many acyl-HSL-controlled *P. aeruginosa* genes is delayed until the transition from logarithmic growth to stationary phase, despite adding high concentrations of these signals during early log-phase growth (1, 2, 9). The ability to show that signal concentration defines the density at which quorum-controlled genes are induced has served as a critical proof for the role of acyl-HSLs in quorum sensing by other bacteria (11–14).

The two acyl-HSL signaling systems in *P. aeruginosa* are LasR-RhlR (15). LasI codes for an enzyme responsible for the production of N-3-oxododecanoyl-HSL (3OC12-HSL), and LasI is a transcription factor that responds to 3OC12-HSL. RhlI is responsible for the synthesis of N-butanoyl-HSL (C4-HSL), and RhlI is a transcription factor that responds to C4-HSL. The LasI-RhlI-R systems control overlapping, but not identical, sets of genes constituting ∼6% of the *P. aeruginosa* genome (1, 2). Because they are self-produced diffusible molecules, both signals accumulate in the growth medium as a *P. aeruginosa* culture increases in density. These signals are required to activate or repress the genes that they control (2–4, 16, 17). A classical proof that acyl-HSLs serve as quorum-sensing signals that allow cells to monitor their species’ population density is to show that addition of an acyl-HSL signal to a culture early in growth elicits the premature expression of signal-dependent genes (11–14). However, recent transcription-profiling studies revealed that the timing of expression for most *P. aeruginosa* acyl-HSL-dependent genes was not influenced by the addition of 3OC12-HSL and C4-HSL to the culture medium (2). For most of these 300 genes studied by Schuster et al. (2), induction occurred at the transition between logarithmic growth and the stationary phase, independent of the presence or absence of exogenously added acyl-HSLs. An explanation for a subset of the genes that showed a delayed response to signals might lie in the fact that they require the stationary-phase σ factor RpoS for activation (18). This subset consists of 84 genes, and, even for this subset, there is no evidence that high levels of RpoS and acyl-HSLs early in growth can eliminate the delay in expression.

We hypothesize that, for many, if not all, of the acyl-HSL-dependent genes that show a population-density-dependent delay in gene expression regardless of acyl-HSL concentration, the medium contains an inhibitor of their expression that must be removed by cellular metabolism before the signals can function. This hypothesis draws on early work with *Vibrio fischeri* (13, 19). Acyl-HSL-mediated quorum sensing was first discovered in this marine bacterium (11, 12). Notably, there was a delay in the transcription of the genes controlled by acyl-HSL signaling in *V. fischeri*, even when the signal was added at high concentrations early in growth. Conditioning the culture medium by growth of a non-signal-producing bacterium removed the inhibitor, and addition of the signal to cells in conditioned medium led to an immediate induction of the quorum-controlled genes (13). Here, we show that, for many, but not all, of the acyl-HSL-dependent *P. aeruginosa* genes, signal addition can eliminate the lag in expression during early logarithmic growth in properly conditioned medium.

**Materials and Methods**

**Bacterial Strains, Media, and Growth Conditions.** The bacterial species and strains used were *P. aeruginosa* PA01 (provided by B. Iglewski, University of Rochester, Rochester, NY), MW1 a tetracycline- and mercury-resistant lasI, rhlI mutant of PA01 (1), JY500 (see below), JY501 (see below), and PA14 (20). We also used *Escherichia coli* SM10 (21), *Pseudomonas putida* PRS2000 (22), *Burkholderia cepacia* K56–2 (23), *Staphylococcus aureus* MN8 (24), and *Vibrio harveyi* MAV (25). A PA01 pgsR mutant was provided by the University of Washington Genome Center (www.genome.washington.edu/uwgc/pseudomonas/index.cfm). The media we used were LB broth (1% tryptone/0.5% yeast extract/0.5% NaCl) buffered with 50 mM MOPS, pH 7.0.

**Abbreviations:** acyl-HSL, acyl-homoserine lactone; BLB, buffered LB broth.

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7.0 (BLB broth), conditioned BLB broth, or minimal medium (26) containing 0.2% glycerol. Unless otherwise indicated, 10 μM 3OC12-HSL and 2 μM C4-HSL were added to the medium just before inoculation with P. aeruginosa. Conditioned BLB broth was prepared by growth of P. aeruginosa MW1 with shaking at 37°C to an optical density of 0.8 (at 600 nm), unless otherwise specified. Inoculation was with an overnight culture (1%). The media were clarified by centrifugation at 2,500 x g for 10 min at 4°C, and the remaining cells were removed by filtration through a 0.2-μm filter. Where indicated, other bacteria were used to generate conditioned medium. With the exception of P. putida, which was grown at 30°C, the procedure for conditioning the medium with these other bacteria was identical to the procedure described above for conditioning with P. aeruginosa MW1. Where indicated, conditioned medium was supplemented with tryptone [1% (wt/vol)], yeast extract [0.5% (wt/vol)], or both. For experiments in which lasB-lacZ transcription was monitored, the inoculum was from a logarithmic-phase culture, and the initial density was 0.001 at 600 nm.

Plasmid and Strain Construction. Plasmids were constructed by standard procedures. For construction of pJY301, regions flanking lasI were synthesized by PCR [primers 5'-GGGAGGCTTCA-CAGACGTCGCGCC-3' and 5'-CGGGATCCCGGA-ATTTGATCATATCTACGCTAC3'- 5'-GGGGGATCCCGGA-CAGACGTCGCGCC-3' and 5'-GGGGGATCCCGGA-ATTTGATCATATCTACGCTAC3'- (restriction sites are italicized)] and cloned into pEX18Tc (27). To construct pJY302, we cloned the gentamicin (Gm)-resistance cassette from pGm (28) into BamHI-digested pJY301. To construct pJY310, the lasB promoter region from P. aeruginosa PA01 was synthesized by PCR [primers 5'-GGGGGATCCCGGA-CAGACGTCGCGCC-3' and 5'-GGGGGATCCCGGA-CAGACGTCGCGCC-3'- (restriction sites are italicized)] and cloned upstream of the promoterless lasZ gene in the miniCTX lasZ plasmid (29).

To construct the lasI, rhlI mutant strain JY500, we transformed P. aeruginosa MW1 with pJY302 by mating and screened for a tetracycline-sensitive, gentamicin-resistant exconjugant (JY500). Disruption of lasI was confirmed by DNA sequencing. The lasB-lacZ reporter strain in strain JY501 was constructed by introducing pJY310 into JY500 by mating. The miniCTX-based reporter fusion in the resulting strain JY501 was verified by lacZ monitor transcription from the lasI promoter region. lasI was confirmed by DNA sequencing. For experiments in which lasB-lacZ transcription was monitored, the inoculum was from a logarithmic-phase culture, and the initial density was 0.001 at 600 nm.

Transcriptome Analysis. Cultures of P. aeruginosa MW1 were harvested at optical densities (600 nm) of 0.05 and 0.5, and RNA was isolated as described in ref. 2. Chromosomal DNA contamination was monitored by PCR with primers specific to the ribosomal protein gene rplU and subsequent agarose gel electrophoresis. Amplification products were not detected. The integrity of total RNA was assessed by agarose gel electrophoresis of glyoxylated samples, QDNA synthesis, fragmentation, labeling, and hybridization as well as P. aeruginosa GeneChip (Affymetrix) genome-array processing were performed as described in ref. 2. The Affymetrix program GCOS 1.1 was used for data processing. Those genes that were given a significant “call” by the GeneChip software in each of two independent experiments were selected for further analysis. Transcript data were further analyzed with the web-based program CYBER-T (http:// visitor.ics.uci.edu/genex/cybert) (2) to identify those genes whose expression was significantly different between conditions. The P-value threshold was 0.001, and the posterior probability of differential gene expression for these genes was >0.95.

β-Galactosidase Activity in the lasB-lacZ Reporter Strain JY501. To monitor transcription from the lasB promoter, we measured β-galactosidase activity in P. aeruginosa JY501, which contains a lasB-lacZ chromosomal fusion. Activity of β-galactosidase was measured in 10–20 μl of chloroform-treated culture by using a chemiluminescent microtiter dish assay (Tropix Galacto-Light Plus, Applied Biosystems). Background, as measured with a reporterless strain of P. aeruginosa, was subtracted, and relative units were luminescence divided by the optical density (600 nm) of the culture from which the sample was taken.

Results

Conditional Elimination of the Lag in Acyl-HSL-Dependent Gene Expression: A Transcriptome Analysis. Previous studies have shown that a majority of acyl-HSL-dependent genes in P. aeruginosa are not expressed early in response to exogenous addition of acyl-HSL signals. For example, the transcriptome analysis of Schuster et al. (2) identified 315 acyl-HSL-activated genes, but only 30 were activated by acyl-HSLs in early to mid-log phase, with the remaining signal-responsive genes showing a delay until the culture density exceeded 0.4 at 600 nm. Data such as these have led to concerns that the acyl-HSL response in P. aeruginosa is, in most cases, not a quorum-sensing response, and a dichotomy is drawn with the paradigm of acyl-HSL-mediated quorum-sensing luminescence in V. fischeri (8–10). However, initial investigations of quorum sensing in V. fischeri were published >30 years ago, and some important work (13, 19) is often overlooked. In fact, when V. fischeri was grown in a complex medium, there was a delay in expression of luminescence genes that could not be overcome by exogenous signal addition. Conditioning the medium by prior growth of a non-signal-producing bacterium could eliminate the delay, presumably by elimination of an inhibitor (13). We have used transcript-profiling technology to investigate whether conditioning the P. aeruginosa medium by prior growth with a non-acyl-HSL-producing strain might allow early acyl-HSL-dependent activation of a larger subset of genes than the subset identified by Schuster et al. (2). We identified 48 genes in the P. aeruginosa signal-generation mutant strain MW1 that were activated early in unconditioned complex medium containing 3OC12- and C4-HSL (Fig. 1, and see Table 1, which is published as supporting information on the PNAS web site). Of these genes, 33 had been previously described as acyl-HSL-dependent (2). Two of the other 15 genes are linked in putative operons to previously identified acyl-HSL-dependent genes. Growth of P. aeruginosa MW1 in conditioned medium was indistinguishable from growth in fresh medium (data not shown). In conditioned medium, an additional 103 genes were activated early by signal addition. Of these, 93 were previously described as acyl-HSL-dependent (2), and 5 of the remaining 10 are linked to putative acyl-HSL-dependent operons. Thus, given the proper conditions, nearly half of the acyl-HSL-dependent genes identified by Schuster et al. (2) can be induced in early logarithmic growth by the addition of the acyl-HSL signals. The expression of several hundred non-acyl-HSL-dependent genes was also affected by conditioning. However, for the purpose of understanding delays in acyl-HSL-dependent gene expression, we have focused on the acyl-HSL-responsive genes.

Confirmation of the Transcriptome Analysis: Early Induction of a lasB-lacZ Fusion in Conditioned Medium. The P. aeruginosa elastase gene lasB represents a particularly well-studied acyl-HSL-dependent gene. This gene was not only shown to be a late gene in transcriptome experiments (2), but gene-reporter studies also

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show that \textit{lasB} is not transcribed until the transition between logarithmic and stationary phase (1). Our transcriptome analysis indicated that \textit{lasB} was induced early in growth by addition of 3OC12- and C4-HSL to \textit{P. aeruginosa} MW1 in conditioned, but not unconditioned, medium (Fig. 2). To confirm the transcriptome results, we monitored \textit{lacZ}-galactosidase activity in \textit{P. aeruginosa} JY501, an acyl-HSL-synthesis mutant that carries a chromosomal \textit{lasB}-\textit{lacZ} reporter. Acyl-HSL-dependent induction of the \textit{lasB}-\textit{lacZ} reporter commenced immediately after inoculation of conditioned medium, but, as expected, there was a delay in \textit{lasB-lacZ} induction in fresh medium (Fig. 3). These results extend the microarray analysis by showing that there was little or no lag in acyl-HSL-dependent \textit{lasB} induction, even at very low cell densities, and confirm that early expression of \textit{lasB} was quorum-signal- and conditioning-dependent.

The Delay in \textit{lasB} Expression Is Caused by an Inhibitor Present in Fresh Medium. We questioned whether the growth of bacteria other than \textit{P. aeruginosa} could condition the medium. Thus, we conditioned the medium by growth of \textit{E. coli}, \textit{S. aureus}, \textit{V. harveyi}, the more closely related \textit{B. cepacia}, the very closely related \textit{P. putida}, and a different \textit{P. aeruginosa} strain, PA14. Other than \textit{P. aeruginosa}, none of the species tested greatly influenced the timing of \textit{lasB-lacZ} induction (data for \textit{P. putida} are shown in Fig. 4; data for other species are not shown). This finding indicates that either \textit{P. aeruginosa} produces an as yet unidentified additional signal required for \textit{lasB} induction or that there is an inhibitor of \textit{lasB} induction in the growth medium that is specifically removed by the growth of \textit{P. aeruginosa} but not the several other bacterial species we tested.

We performed several experiments to discriminate between the ideas that conditioning with \textit{P. aeruginosa} was removing an inhibitor or was adding a non-acyl-HSL signal. There is one other known non-acyl-HSL signal produced by \textit{P. aeruginosa}, the \textit{Pseudomonas} quinolone signal (PQS) (31). Might the accumulation of this signal account for elimination of the lag in \textit{lasB} expression in conditioned medium? To address this question, we conditioned the medium by using a \textit{pqsR} mutant. \textit{pqsR} encodes a transcriptional regulator required for PQS signal synthesis (32). Conditioning with the \textit{pqsR} mutant led to a \textit{lasB-lacZ} induction pattern that was identical to preconditioning with the
The activity of means squares) or without (open squares) added acyl-HSLs. The values given are a control (added as tryptone (T) and nm. (pursue this idea further, we added dry-powder medium or the conditioned medium contains an unidentified activator. To with the idea that fresh medium contains an inhibitor rather than result in a loss of premature expression in the complex medium used, we first mixed fresh media with conditioned medium (Fig. 5). Thus, PQS does not appear to be responsible for early lasB-lacZ expression in conditioned medium.

To test the hypothesis that there is an inhibitor of lasB transcription in the complex medium used, we first mixed fresh medium with conditioned medium (Fig. 5A). Dilution of conditioned medium by even a small amount of fresh medium resulted in a loss of premature lasB-lacZ induction, consistent with the idea that fresh medium contains an inhibitor rather than that conditioned medium contains an unidentified activator. To pursue this idea further, we added dry-powder medium or the components of the medium (yeast extract and tryptone) to conditioned medium and measured lasB-lacZ induction (Fig. 5B). The delay in induction was recovered by addition of dry powder to conditioned medium. Both of the components of the complex medium exerted some inhibitory effect. If there is an inhibitor present in complex medium, one might predict that the lag in acyl-HSL-dependent lasB expression might not occur in a minimal medium. In fact, when grown in minimal medium containing the acyl-HSL signals, the lasB reporter strain P. aeruginosa JY501 showed an early induction of the reporter (Fig. 5C).

Discussion

Considerable attention has surrounded the finding that the addition of acyl-HSLs to the growth medium does not influence the timing of expression for many, if not most, acyl-HSL-dependent P. aeruginosa genes. Although the activation of such genes depends on acyl-HSLs, their transcription is not triggered early in growth by the inclusion of acyl-HSLs in the medium (1, 2, 9). A number of investigators have searched for other factors that might be responsible for the delay in acyl-HSL-dependent gene transcription, and several factors have been implicated in the delayed responses for some genes. These factors include RpoS, QscR, MvaT, RsmA, and PqsR (reviewed in ref. 33). Some of these factors shorten, but do not eliminate, the lag in transcription for the genes they control. For example, when an mvaT mutant is grown in the presence of acyl-HSLs, induction of lecA expression is advanced but by only one cell division (9). For other factors, it is difficult to assess from published data whether mutations eliminated or just reduced the lag in acyl-HSL-dependent gene expression.

This delay in the activation of P. aeruginosa quorum-responsive genes is reminiscent of the quorum response in V. fischeri, the marine bacterium in which acyl-HSL-mediated quorum sensing was first described (11–13, 19). For V. fischeri, there was a delay of luminescence induction in complex medium, and the delay was shown to result from the presence of an inhibitor in the medium. This inhibitor could be removed by conditioning the medium with pregrowth of a non-signal-producing marine bacterium (13). We drew upon these findings with V. fischeri and performed experiments with P. aeruginosa in conditioned medium. Conditioning the medium by pregrowth with a P. aeruginosa mutant incapable of acyl-HSL production did not influence the subsequent growth of P. aeruginosa. However, as shown by using microarray technology, 136 previously identified quorum-sensing genes (2) were activated early when P. aeruginosa MW1 was grown in conditioned medium with acyl-HSL signals added vs. 33 genes when the medium was not conditioned (Fig. 1). Thus, a significant percentage of the 315 genes we previously identified as acyl-HSL-responsive could be activated prematurely by the addition of signals in conditioned medium.

For further analysis, we chose one gene, lasB, that shows early induction by acyl-HSL signaling in conditioned medium. Although lasB induction could be triggered early in growth when acyl-HSLs were added to P. aeruginosa MW1-conditioned medium, conditioning with several other bacterial species did not affect the delay in signal-dependent induction. Several lines of evidence (Fig. 5) indicate that the early induction of lasB results from the removal of an inhibitor or inhibitors of quorum-controlled gene expression by metabolism of P. aeruginosa, specifically. The inhibitory substance(s) appear to be present in both the yeast extract and the tryptone components of the complex medium. One possibility is that the inhibitor is iron. We believe this is unlikely, because we would expect iron to be consumed by the growth of bacteria other than P. aeruginosa, and, as discussed above, conditioning with several other species did not eliminate the lag in response to acyl-HSLs. However, P.
*P. aeruginosa* has a particularly high-affinity iron-acquisition system (34), and medium conditioned by pregrowth with this species might have lower levels of iron than media conditioned by other bacteria. Iron is known to be a repressor of a battery of *P. aeruginosa* genes, including some quorum-controlled genes (35). We observed early production of pyocyanin, a quorum-controlled pigment involved in iron metabolism (36), when cultures were grown in conditioned medium with acyl-HSL signals. In fact, when iron is added to conditioned medium containing signals, there is a delay in pyocyanin synthesis (data not shown); however, this is not the case with lasB expression. Iron does not reconstitute the delay in lasB expression when *P. aeruginosa* is grown in conditioned medium (data not shown). Thus, we conclude that iron can delay the expression of some quorum-sensing genes but is not the inhibitor of lasB expression removed by conditioning complex medium.

We have not identified the inhibitory substance (or substances), but their identity could be important. There is considerable interest and effort directed toward the development of small-molecule inhibitors of *P. aeruginosa* quorum sensing as potential antivirus agents (4, 10, 37). It might be possible to develop a nonmetabolizable analog of an inhibitor present in the standard complex medium we have used. However, we must point out that the inhibitor is not a general *P. aeruginosa* quorum-sensing inhibitor and does not prevent expression of a considerable portion of acyl-HSL-dependent genes (Fig. 1). Whether the subset of genes affected by the inhibitor constitutes a therapeutic target remains an open question.

In *P. aeruginosa*, many quorum-activated genes are coregulated by the stationary-phase σ factor RpoS (18). Even though growth of *P. aeruginosa* in conditioned medium was indistinguishable from growth in fresh medium, one might argue that cellular RpoS levels were higher in conditioned medium than they were in fresh medium, and that this resulted in early acyl-HSL induction of RpoS coregulated quorum-controlled genes. However, we do know that some genes that show conditionally early acyl-HSL-dependent transcription, including lasB, are not coregulated by RpoS (18). A further comparison of our transcriptome data with the 80 genes coactivated by RpoS and not activated by RpoS, and expressed late in conditioned medium, an additional 103 are activated early in conditioned medium, and 74 of the genes that are not activated by acyl-HSLs in conditioned medium depend on RpoS for activation. In all, there are 210 acyl-HSL-dependent genes either expressed early in conditioned medium or RpoS activated and 105 genes that are acyl-HSL dependent, not activated by RpoS, and expressed late in conditioned medium. We have not accounted for these 105 genes. We suppose that some of these genes might be in multi-input dense-overlapping regulons other than the RpoS regulon. Others might be controlled by feed-forward loops (39), regulatory networks in which they are activated by acyl-HSLs indirectly because quorum sensing directly controls the regulators that affect them.

Our results add to mounting evidence that the acyl-HSL response in *P. aeruginosa* is a fluid phenomenon continually being modified by environmental conditions. We know that the identity of acyl-HSL-responsive genes can vary depending on environmental conditions [we chose to use conditions similar to those used in our previous transcriptome analyses of *P. aeruginosa* quorum sensing and RpoS control of gene expression (2, 18)], and there are, likely, other genes controlled directly or indirectly by quorum sensing under conditions different from those we have used (3, 4). *P. aeruginosa* inhabits diverse environments in which the expression of some quorum-controlled genes would be appropriate, whereas expression of others may not be appropriate. The lasB gene, which codes for an extracellular protease, might be a good example of a gene requiring multi-inputs for activation. In media replete with amino acids, expression of a protease might not be beneficial. Thus, expression of lasB might require two conditions: amino-acid limitation and a sufficient population density. In fact, with different environmental conditions, genes that show a delayed response to acyl-HSLs even in our conditioned medium might be expressed early in growth. We conclude that, at least for a considerable number of the acyl-HSL genes identified in our experiments, the acyl-HSLs can function as quorum-sensing signals.

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