A unique regulator controls the activation threshold of quorum-regulated genes in *Pseudomonas aeruginosa*

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Quorum-sensing (QS) systems allow organisms, such as the pathogen *Pseudomonas aeruginosa*, to link gene expression with their population density and the diffusion and flow characteristics of their environment. The leading hypotheses about QS systems' biological functions necessitate that QS-controlled gene expression be suppressed until a threshold culture density (the quorum) is reached. Despite a detailed understanding of QS in *P. aeruginosa*, known regulatory elements do not fully explain how the quorum threshold for gene activation is produced. Here we investigated the mechanism with a screening approach that used random gene activation. These experiments uncovered a regulator without close homologs in other species that produces the quorum expression threshold. Expression of this regulator (named QteE) reduces LasR protein stability without affecting LasR transcription or translation. QteE also independently reduces RhlR levels. Because QteE can block QS when signal levels are high, it could provide a mechanism for individual cells to exert autonomous control over their QS regulons. This unique regulator governs two central QS control points in *P. aeruginosa* and shapes the expression pattern thought fundamental to the biological functions of QS.

cell to cell signaling | quorum sensing | LasR | gene regulation

Much understanding of quorum sensing (QS) has come from studies with the bacterium *Pseudomonas aeruginosa*, which uses QS to regulate exoproducts and virulence factors that mediate some invasive infections (1–3). In QS, bacteria produce extracellular signals and the receptor proteins that bind them, and the signal–receptor complex regulates QS-controlled genes (3). In the best-characterized *P. aeruginosa* systems, the extracellular signals are the acylated homoserine lactones (HSLs), 3-oxo-dodecanoyl-HSL (C12) and butanoyl-HSL (C4), which are synthesized by the products of the *lasI* and *rhlI* genes. The signal receptors are encoded by *lasR* and *rhlR*. Controlling genes by QS is thought to provide key advantages to bacteria (see below).

Although patterns differ, many QS-controlled genes in *P. aeruginosa* show a characteristic expression curve when studied in batch cultures. Expression of these genes exhibit a sigmoidal (or "S-shaped") pattern, with negligible expression when the cell number is low and a rapid upswing when population density reaches a critical threshold (4). The threshold population density at which gene expression is triggered is called the "quorum" (4, 5); the phase before expression, the "prequorum" period; and the overall pattern has been called quorum-dependent expression by some investigators (5).

The mechanism that generates this pattern is of great interest because restrained gene expression in the prequorum period is thought to be central to QS's biological functions in *P. aeruginosa* and other organisms (6–8). One hypothesis is that QS enables bacteria to coordinate activities so they can operate in groups. Inhibiting gene expression when population density is low could serve this purpose, for example, by delaying virulence factor production until enough cells amass to produce effective levels (9, 10). Restrained prequorum gene expression may also benefit groups by enabling coordinated "sneak attacks" during infection (7, 11). This may be an advantage because QS-controlled factors would be hidden until a large force assembles.

Another view is that the benefits of QS gene regulation do not require that bacteria engage in group or social activities (12, 13). According to this idea, QS signals are used to gauge the rate at which secreted products would be lost by diffusion and flow, rather than to measure population density (12). The expression threshold of QS could also serve this function by enabling bacteria to conserve energy for exoproduct synthesis until conditions permit signal (and hence exoproduct) accumulation. Thus, restrained gene expression in prequorum conditions is critical to the postulated benefits of QS for bacterial groups and individual cells.

How is the quorum threshold pattern of gene expression produced? Clearly, the accumulation of signals caused by increasing population density, limited signal loss, and the positive feedback regulation of signal synthesis is important (14, 15). However, experiments by Whiteley et al. (16) showed that signal accumulation alone does not account for the quorum-dependent expression pattern of many genes. Whiteley et al. (16) exogenously added saturating levels of acyl–HSL signals to *P. aeruginosa* cultures and found that many QS-controlled genes continued to exhibit restrained expression at low culture densities. This result has been confirmed by other investigators (14, 17, 18) and indicates that additional control mechanisms are required.

Here we explored the possibility that previously undiscovered negative regulators might inhibit prequorum transcription in *P. aeruginosa* and produce the quorum threshold. Using a random gene activation strategy, we found a unique regulator (named QteE) that blocks QS gene expression and decreases the half-life of the LasR protein without affecting *lasR* transcription or translation. Our data also show that QteE independently blocks RhlR protein accumulation and signaling by the *rhl* system. Furthermore, we found that all QS-controlled genes that we tested lose their characteristic quorum expression threshold when *qteE* is inactivated.

Results

An Induced Expression Screen for Negative QS Regulators. Regulators that restrain prequorum gene expression may have their greatest effects at low cell densities. Thus, identifying these regulators by mutagenesis could be difficult as the mutants and wild type may exhibit similar phenotypes once colonies are large enough to be screened. To avoid this problem, we used an

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approach in which potential regulators could be inducibly expressed. We accomplished this by engineering the arabinose-inducible *araC*-P_{BAD} promoter onto one end of the mini-Tn*5* transposon (19), with the promoter and transcriptional start site facing outward (Fig. 1). We reasoned that induced expression would likely produce observable phenotypes even if the physiological function of the regulator was transient, conditional, or redundant.

To maximize the chances of finding negative regulators with robust activity, we used a *P. aeruginosa* PAO1 variant (20) that overexpressed rhamnolipid biosynthetic genes (*rhlAB*), which are QS controlled, as the reporter strain. The *rhlA* promoter linked to a *gfp* reporter (P_{rhlA}*::GFP*) was inserted on the chromosome of this strain and, as expected, colonies were brightly fluorescent (Fig. S1*A*). Thus, if our transposon inserted upstream of a potent negative QS regulator, colonies should show arabinose-induced reduction of GFP fluorescence.

Expression of PA2593 Represses Multiple QS-Controlled Phenotypes. One transposon insertion mutant (of ~20,000 screened) exhibited reversible arabinose-induced repression of *rhlA* (Fig. S1*A*). Sequence analysis showed that the transposon inserted 343 bp upstream of gene PA2593, which encodes for a hypothetical protein of 190 amino acids (21). We termed this gene *qteE* (*q*uorum *t*hreshold *e*xpression *e*lement) because of its effects on QS-controlled genes (see below).

We verified that *qteE* was responsible for inhibiting P_{rhlA}*::GFP* activity by expressing a copy in an unmanipulated wild-type PAO1 clone (Fig. S1*B*). Induced expression of *qteE* also reduced rhamnolipid, protease, elastase, and pyocyanin levels to those seen in a *P. aeruginosa* mutant lacking QS (Fig. 2 *A–D*). Although *qteE* is present in all sequenced *P. aeruginosa* strains, we found only moderate sequence homologs (to proteins of unknown function) in other species and no conserved domains (Fig. S2). This suggests that *qteE* may have a unique mechanism of action or that its homologs evolve rapidly, making them undetectable by conventional homology searches.

QteE Does Not Act by Blocking QS Signal Activity. The fact that induction of *qteE* repressed many QS-controlled phenotypes suggested that it could act at a central point in the system, perhaps by interfering with the acyl–HSL signals or the receptor proteins. We began by examining the effect on signals and found that induced expression of *qteE* eliminated the activity of both acyl–HSL signals in culture supernatants (Fig. S3*A*). This result raised the possibility that QteE acts by interfering with signal synthesis. However, because the signal synthases are themselves QS controlled (14, 22, 23), QteE could produce low signal levels by acting at some other central point in the regulatory network.

To distinguish between these possibilities, we added inducing levels of both signals to cultures expressing *qteE*. We reasoned that this intervention should reverse *qteE*'s effects if it functioned by reducing signal activity. As shown in Fig. 3*A*, *qteE* repressed *rhlA* transcription even when saturating levels of both signals were exogenously supplied. We confirmed that the added signals were active in *qteE*-expressing cultures using a bioassay that measured acyl–HSLs. These data indicate that *qteE*'s repression of QS was not likely due to inhibited signal production or signal degradation.

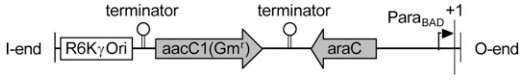

Fig. 1. The mini-Tn*5*-Pro transposon. The transposon contains the *araBAD* promoter and its *araC* regulator, a R6Kγ origin of replication, and the *accC1* gentamycin resistance gene. The promoter transcriptional start site is 42 bp from the Tn::Chromosome junction. Not to scale.

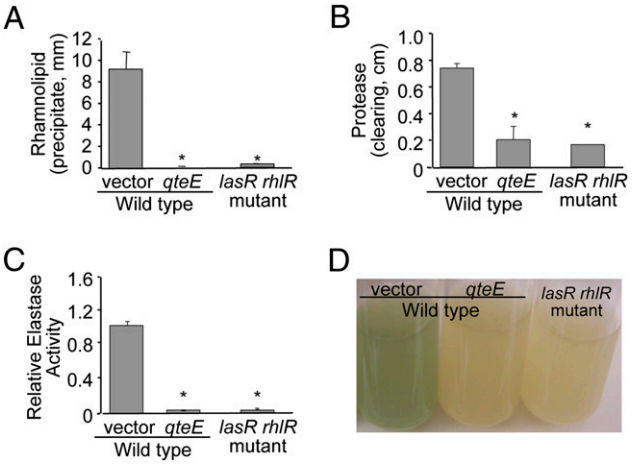

Fig. 2. Expression of *qteE* suppresses multiple QS-controlled phenotypes. (*A*) Rhamnolipid production by wild-type *P. aeruginosa* containing control and *qteE*-expressing vectors and a *lasR*/*rhlR* mutant as measured by the zone of precipitate produced on indicator plates. Data are the mean of six replicates and are representative of two experiments; error bars show SEM; **P* < 0.00001 versus *P. aeruginosa* containing the control vector. (*B*) Protease activity of the strains described in *A* measured by zones of clearing on plates containing casein. Data are the mean of three replicates and are representative of three experiments; error bars show SEM; **P* < 0.0002 versus *P. aeruginosa* containing the control vector. (*C*) Elastase activity of the strains described in *A* measured by the release of Congo Red from elastin–Congo Red complexes. Data are the mean of three replicates and are representative of three experiments; error bars show SEM; **P* < 0.00001 versus the control vector. (*D*) Pyocyanin production of the strains described in *A* as observed by the green color of cultures grown in liquid medium. Data are representative of three experiments.

QteE Inhibits QS by Reducing LasR Protein Stability. Another potential central control point is the LasR protein because the LasR-C12 complex regulates the expression of both signal syn-

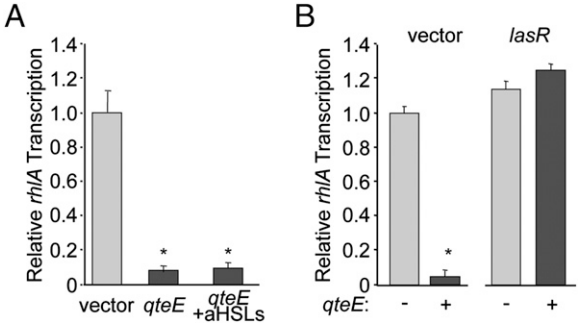

Fig. 3. QteE does not act by inhibiting signal activity but by inhibiting LasR function. (*A*) QteE represses QS-controlled gene expression even when inducing levels of acyl–HSL signal are exogenously added. *rhlA* transcription was measured in *P. aeruginosa* containing control and *qteE*-expressing vectors with and without added acyl–HSL signals (1 μM of C12, 5 μM of C4). Data are the mean of three replicates and are representative of two experiments; error bars show SEM; **P* < 0.01 versus the vector control. (*B*) Excess LasR overcomes *qteE*'s inhibitory effects. Induced expression of *qteE* from a single copy on the chromosome reduces expression of a *rhlA*::*gfp* reporter. When *lasR* is expressed in multicopy, *qteE*'s inhibitory action is lost. Data are the mean of three replicates and are representative of two experiments; error bars show SEM; **P* < 0.0001 versus the uninduced culture. Changes in the presence of excess LasR are nonsignificant.

thases and other QS-controlled genes. To investigate whether QteE blocks QS by inhibiting LasR function, we expressed *lasR* on a multicopy plasmid whereas *qteE* was expressed in single copy. Overexpressing *lasR* eliminated *qteE*'s ability to repress *rhlA* transcription (Fig. 3*B*) and elastase production (Fig. S3*B*). The fact that *qteE*'s QS inhibitory activity was overcome by increased *lasR* expression suggests that QteE may work by inhibiting LasR function. These data also raise the possibility that the LasR:QteE stoichiometry might be a key factor determining whether QS is activated or repressed.

To investigate how *qteE* might inhibit LasR, we measured *lasR* transcription and translation in wild-type and *qteE*-expressing cells and found no difference (Fig. S4). In contrast, Western blots showed that expressing *qteE* from the beginning of culture growth markedly reduced LasR protein accumulation (Fig. 4*A*). Induction of *qteE* also reduced LasR that was expressed from an isopropyl-β-D-thiogalactopyranoside (IPTG)-controlled promoter, providing additional evidence that QteE's action is not due to an inhibitory effect on *lasR* promoter activity (Fig. S5).

Because previous work suggests that LasR may be unstable in the absence of its acyl–HSL signal (24) and because *qteE* expression results in very low signal levels (Fig. S3*A*), we explored the possibility that QteE may lower LasR through its inhibitory effect on signal production. As shown in Fig. 4*B*, LasR accumulates in a *lasI*/*rhlI* mutant (that does not produce signals) and *qteE* expression reduced LasR in this strain as it did in wild-type *P. aeruginosa*. Furthermore, *qteE* reduced LasR when excess signal was exogenously added to the cultures in which *lasR* was IPTG-controlled (Fig. S5). These data indicate that *qteE* reduces LasR accumulation independently of its effect on acyl–HSL production.

Given LasR's central position in QS regulation and previous work suggesting that LasR-signal complexes are stable and could have lasting effects (24), we performed additional experiments to determine if *qteE* could reduce LasR after LasR had already accumulated. Using a *P. aeruginosa* strain in which the native copies of *lasR*, *qteE*, *lasI*, and *rhlI* had been inactivated, we expressed *lasR* from the beginning of culture growth and waited until midlog phase to express *qteE*. Delayed induction of *qteE* reduced LasR levels, whether excess signals were absent or present (Fig. 4*C*). We also induced *qteE* after LasR had accumulated by expression from its native promoter in a *lasI*/*rhlI* mutant. Again, delayed *qteE* induction reduced LasR levels whether signals were absent or present (Fig. 4 *D* and *E*).

The fact that *qteE* expression reduces LasR protein levels without affecting *lasR* transcription or translation suggested that it may work by a post-translational mechanism. To test this, we performed pulse-chase experiments. Inducing *qteE* from the beginning of culture growth produced no appreciable change in the abundance of labeled LasR present immediately after a [^{35}S] methionine pulse (Fig. 4*F*: compare *Upper* and *Lower*; $T = 0$ min). However, during the chase period, cultures expressing *qteE* exhibited a pronounced reduction in LasR protein levels (Fig. 4*F*, *Lower*). Thus, although *qteE* expression does not appear to affect LasR synthesis, it does reduce LasR protein stability.

QteE Blocks QS When Heterologously Expressed. The fact that multiple regulatory inputs converge on QS control points led us

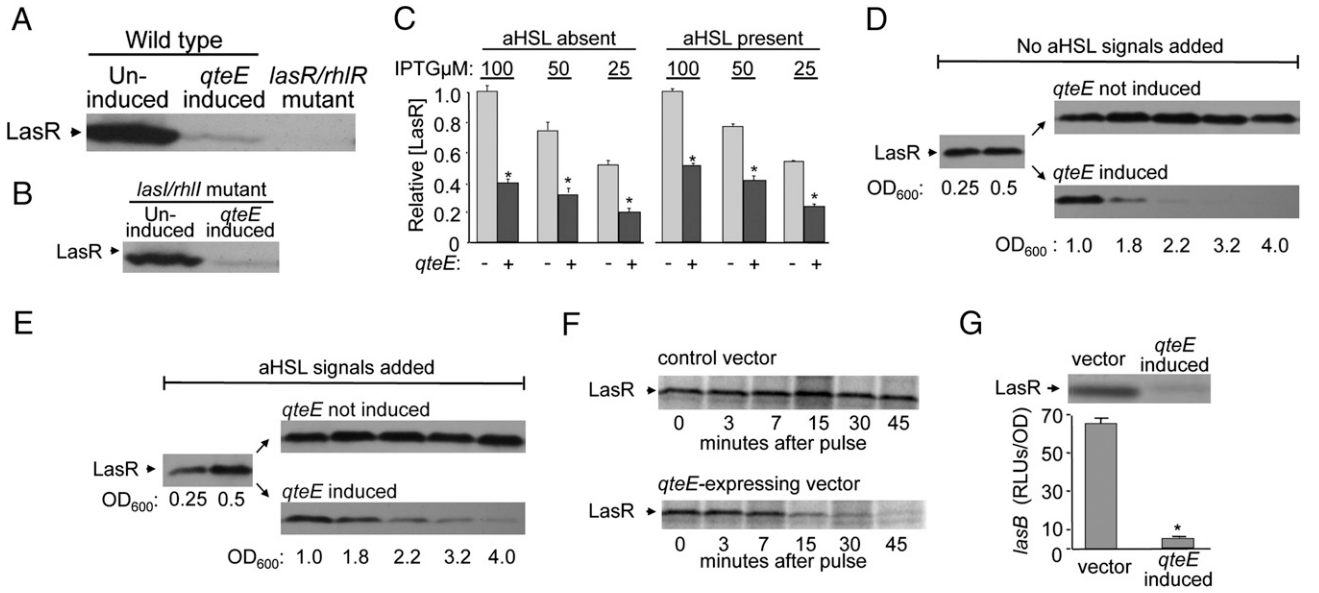

Fig. 4. Expression of *qteE* inhibits LasR protein stability. (*A*) Expressing QteE from the beginning of culture growth reduces LasR accumulation. LasR immunoblots from wild-type *P. aeruginosa* containing control and *qteE*-expressing vectors and a *lasR*/*rhlR* mutant. Data are representative of three experiments. (*B*) QteE reduces LasR accumulation in a *lasI*/*rhlI* mutant. Data are representative of three experiments. (*C*) QteE reduces LasR levels even if it is induced after LasR. *LasR* controlled by an IPTG-inducible promoter was expressed from the beginning of culture growth (using the indicated IPTG concentrations) in a *P. aeruginosa lasI*/*rhlI*/*lasR*/*qteE* mutant in the absence of signal (*Left*) or in the presence of excess C4- and C12-acyl–HSL (*Right*). At a culture OD_{600} of 0.4, *qteE* was expressed under arabinose control or was not induced. Data show relative LasR levels present at a culture OD_{600} of 3.0, normalized to the LasR present after 100 μM IPTG induction and no arabinose addition. Data are the mean of three measurements and are representative of two experiments; error bars show SEM; $^*P < 0.002$ versus the *qteE*-uninduced condition. (*D* and *E*) QteE reduces LasR levels if induced after LasR has accumulated from its native promoter. A *P. aeruginosa lasI*/*rhlI* mutant containing inducible *qteE* was grown in the absence (*D*) and the presence (*E*) of excess C12- and C4-acyl–HSL signals. Cultures were initially grown without inducing *qteE*. At an OD_{600} of 1.0, the culture was split. *QteE* remained uninduced in cells represented in the *Upper* immunoblots in *D* and *E*. *QteE* was induced in cells shown in the *Lower* panels in *D* and *E*. Immunoblots are representative of two experiments. (*F*) QteE decreases the stability of LasR in *P. aeruginosa*. Autoradiographs of immunoprecipitated ^{35}S-labeled-LasR from cells containing control and *qteE*-expressing vectors immediately after a 60-s pulse with [^{35}S]methionine (time 0) or at the indicated times following chase with cold methionine. Data are representative of two experiments. (*G*) QteE inhibits LasR in *E. coli*. *E. coli*-expressing *lasR*, a *lasB* reporter, and either control or *qteE*-expressing vectors were grown with inducing levels of C12-HSL. (*Upper*) LasR immunoblot; (*Lower*) bar graph shows *lasB* transcription. Data in graph are the mean of three replicates and are representative of two experiments; error bars show SEM; $^*P < 0.0001$ versus the vector control. The immunoblot is representative of two experiments.

to investigate whether QteE independently reduces LasR accumulation or if it requires other elements of the *P. aeruginosa* QS system. To accomplish this, we moved *qteE*, *lasR*, and a LasR-responsive transcriptional reporter (P_{lasB}::*lacZ*) into *Escherichia coli*. Induction of *qteE* in *E. coli* lowered LasR levels (Fig. 4*G*, *Upper*), blocked *lasB* expression (Fig. 4*G*, *Lower*), and reduced LasR protein stability (Fig. S6) in *E. coli* as it did in *P. aeruginosa*.

QteE Decreases RhlR Accumulation and Blocks Rhl-Mediated Signaling. The strong inhibitory effect of *qteE* on LasR activity led us to hypothesize that it might have similar effects on RhlR, and we tested this in two ways. Because the Las system controls both *rhlR* and *rhlI* transcription (14, 22, 23), studies of Rhl signaling in *P. aeruginosa* could be confounded by *qteE*'s inhibitory effect on LasR. To generate a system in which Rhl signaling could be independently studied, we used a *lasR*/*rhlR* mutant in which *rhlR* was inducibly expressed, and exogenously added the C4 signal to cultures. Expression of *qteE* blocked activation of *rhlA* in this system, indicating that *qteE* independently inhibits Rhl signaling (Fig. 5*A*). As an additional test, we expressed *qteE* in an *E. coli* strain to which *rhlR*, a transcriptional reporter of *rhlA*, and C4 signal was added. Again, *qteE* was found to repress the expression of a *rhl*-controlled gene (Fig. 5*B*, *Upper*). Furthermore, Western blots showed that *qteE* expression also reduced accumulation of the RhlR protein in this *E. coli* system (Fig. 5*B*, *Lower*) as was observed for LasR (Fig. 4*G*).

Inactivation of *qteE* Increases LasR Levels and Eliminates the Characteristic Quorum Threshold. Artificial overexpression of genes can produce aberrant effects due to high transcript levels or because expression is dissociated from normal cell physiology. Thus, we used a *P. aeruginosa* mutant with *qteE* inactivated to investigate its physiologic functions. As shown in Fig. 6, inactivation of *qteE* increased LasR levels, and the effect was most pronounced at low culture densities. For example, at a culture OD_{600} of 0.17, the mutant contained approximately threefold more LasR than the wild type, whereas little difference was seen at OD_{600} 2.3 (Fig. 6).

We also examined the expression patterns of several QS-controlled genes in the *qteE* mutant. To ensure that QS genes were not already induced in the inocula used for these experiments, we grew cultures to an OD_{600} of 0.2, washed the cells, diluted them 1:100, and repeated this sequence twice before the measurements. Inactivation of *qteE* had two effects on gene expression. First, inactivation of *qteE* raised the maximum expression level of some of the genes. For example, *lasB* expression was increased by >10-fold (Fig. S7*A*).

Second, and most notably, inactivation of *qteE* eliminated the characteristic quorum threshold expression pattern of *rsaL*, *lasB*, *pa1656*, and *lasI* (Fig. 7). Instead of being triggered at the threshold population density apparent in control cultures, gene expression was activated at the lowest culture densities we examined (OD = 0.02–0.05). Inactivation of *qteE* also advanced *rhlA* transcription (Fig. S7*B*). However, signal addition was required to achieve rhlA expression at very low culture densities (Fig. S7 *C* and *D*).

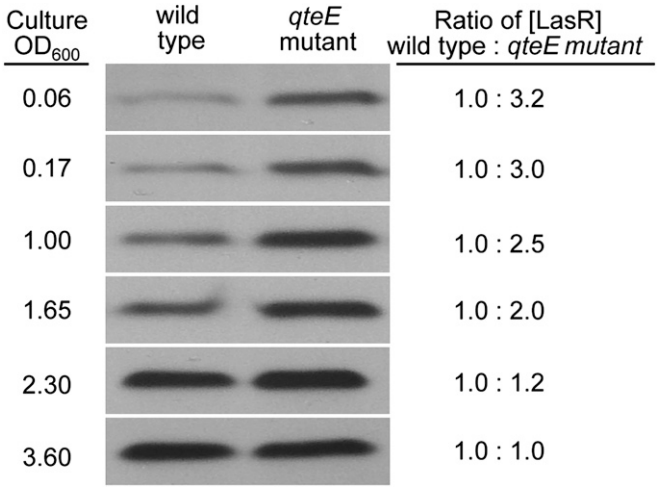

Fig. 6. Inactivation of *qteE* increases LasR levels at low culture densities. LasR immunoblots from wild-type and *qteE* mutant *P. aeruginosa* were grown to the indicated culture densities. Relative concentration of LasR in wild-type and *qteE* mutant *P. aeruginosa* was determined by densitometry measurements of immunoblots. Immunoblots are representative of three experiments. Relative LasR concentrations are the mean of three measurements.

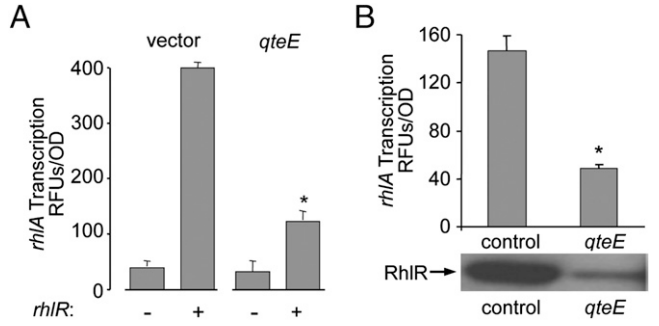

Fig. 5. Expression of *qteE* inhibits RhlR activity. (*A*) QteE decreased *rhlR*-induced *rhlA* expression in a *P. aeruginosa lasR/rhlR/qscR* mutant. Data are the mean of three replicates and are representative of three experiments; error bars show SEM; **P* < 0.003 versus the ara-induced culture containing the control vector. (*B*) QteE inhibits RhlR activity in *E. coli.* The graph shows that *qteE* inhibits *rhlA* transcription. The immunoblot shows that *qteE* inhibits accumulation of expressed RhlR. Data in graph are the mean of three replicates and are representative of two experiments; error bars show SEM; **P* < 0.013 versus the culture-containing control vector. The immunoblot is representative of two experiments.

Discussion

Here we identify a previously undescribed regulator (QteE) that blocks both major QS systems of *P. aeruginosa.* Induction of *qteE* prevents accumulation of LasR by reducing LasR protein stability; *qteE* also blocks RhlR accumulation, and this effect is independent of *qteE*'s action on LasR. QteE lowers the levels of both receptor proteins (R proteins) in *E. coli*. This suggests that QteE either acts on its own or in concert with general cellular machinery, rather than through a co-operating QS regulator.

Our data show that *qteE* is required to produce the quorum expression threshold characteristic of some QS-regulated genes including *rsaL*, *lasB*, *pa1656*, and *lasI*. In wild-type *P. aeruginosa*, these genes exhibit low expression levels until the quorum is reached. In bacteria lacking *qteE*, rapidly rising expression was seen at the lowest cell densities studied (OD_{600} of 0.02–0.05), and no quorum threshold was apparent. Although it is possible that some expression threshold exists for these genes in the *qteE* mutant, our data indicate that it would occur at culture densities ~10-fold lower than in the wild type. A threshold occurring at such low cell densities would seem unlikely to serve QS's postulated physiological functions. QteE also regulates the maximum expression level of some QS-controlled genes. Although the earlier onset of gene expression could increase reporter activity in stationary phase, the magnitude of some of the increases raises the possibility that *qteE* may also control the stationary phase expression of some genes.

Additional work will be required to determine how QteE produces the quorum threshold. However, the experiments

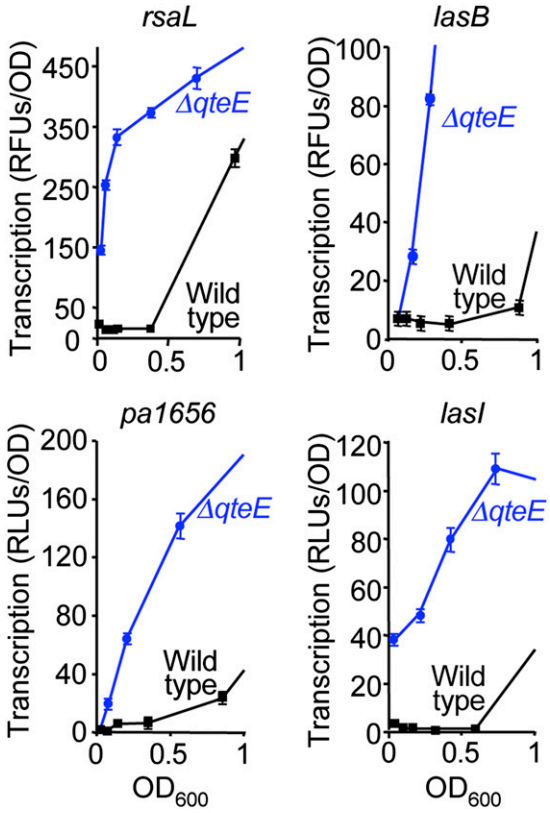

Fig. 7. Inactivation of *qteE* eliminates the quorum-dependent expression pattern of several QS-controlled genes. Reporter activity was measured in wild-type or *qteE* mutant *P. aeruginosa* carrying transcriptional reporters of *rsaL*, *lasB*, *pa1656*, and *lasI*. Panels show a detailed view of gene expression from OD_{600} of 0–1.0 (Fig. S7*A* shows OD_{600} of 0–4.0). Data are the mean of three replicates and are representative of at least two experiments; error bars show SEM.

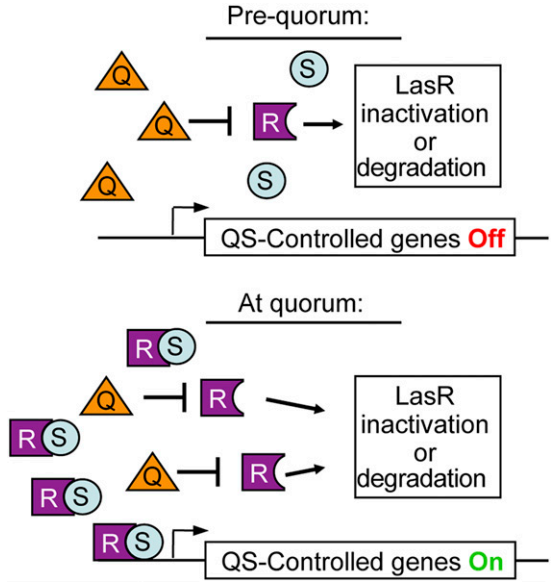

Fig. 8. Model of how QteE produces the quorum-threshold expression pattern. At low (prequorum) culture densities, QteE activity dominates, producing inactivation or degradation of LasR. At a threshold population density (the quorum), the balance between QteE and LasR shifts so that QteE's inhibitory activity is overcome. This could occur because *lasR* expression increases, because the activity of another inhibitor of LasR wanes, or by activation of a QteE inhibitor. However it occurs, an increase in the relative activity of LasR to QteE could induce the expression of quorum-responsive genes, including the signal synthases, and initiate the positive feedback of the QS circuit. The fact that QteE reduces LasR stability even when LasR has accumulated in the presence of high signal levels raises the possibility that QteE may act on both signal-bound and unbound LasR.

showing that overexpression of LasR overcomes *qteE*'s effects (Fig. 3*B* and Fig. S3*B*), coupled with previous work indicating that *lasR* expression increases in exponential phase (when many QS genes are activated) (14, 25), suggests a provisional model in which the stoichiometry between LasR and QteE is a key factor. This model (Fig. 8) postulates that at the low cell densities of the prequorum state the relative activity of QteE may exceed that of LasR. This would repress QS-controlled gene expression because of QteE's inhibitory effect on LasR activity. At the quorum, the relative activity of LasR and QteE may shift, favoring LasR. This change could be a critical tipping point that permits LasR to become active, provided that sufficient acyl–HSL signal is present. After this, the positive feedback inherent to QS could be triggered by the induction of the signal synthases and the increased *lasR* expression that occurs with rising population density. Together, these could produce the characteristic upswing in QS gene expression.

This model is consistent with and was influenced by work in *Agrobacterium tumefaciens* showing that an R-protein anti-activator (TraM) inhibits QS in the prequorum period (5, 26, 27) and by studies in *P. aeruginosa* showing that some QS genes respond to ectopic R-protein expression (28). Furthermore, the model does not exclude the contribution of regulators like *mvaT* (17), *qscR* (29), and others such as *rsmA*, *rsaL*, *algQ*, *vfr*, *vqsR*, *rpoS*, *rpoN*, and *dksA* (reviewed in ref. 10); the sequestration of R proteins by promoters (14); or the binding dynamics of signal–R protein complexes (28). However, the fact that *qteE* inactivation causes the expression of several QS genes to increase early in culture growth, without an apparent threshold, indicates that *qteE* has a central role in producing the quorum-dependent expression pattern.

How does QteE inhibit R-protein activity? One possibility is that QteE functions analogously to TraM of *A. tumefaciens.* TraM binds the R protein of *A. tumefaciens* (TraR). When the stoichiometry between the two proteins favors TraM, it can prevent and disrupt TraR's interaction with target promoters (30–32). When TraR levels rise, it likely becomes dominant and QS can be activated (5, 26, 27). However, we have not yet determined if QteE disrupts LasR's interaction with promoter sequences, and QteE's pronounced effect on LasR stability raises the possibility that QteE could act primarily by lowering LasR protein levels. Furthermore, *traM* is only about half the size of *qteE* and no sequence homology exists. Nevertheless, it remains possible that QteE and TraM function similarly because anti-activator proteins may share little sequence homology (33). It is also important to note that we do not yet know if reduced LasR protein levels are required for QteE's function or if LasR instability is secondary to some other QteE–LasR inactivating interaction.

Regardless of its precise mechanism, our data suggest that QteE could be a particularly powerful QS regulator. For example, mechanisms that act by blocking R protein or signal synthesis could have limited efficacy because existing pools of LasR and signal could continue to activate QS genes even after production stops (24). Furthermore, signal concentrations depend upon environmental conditions and the actions of neighboring cells. In contrast, QteE can repress QS when signal levels are high (Fig. 3*A*). QteE can also reduce LasR after LasR has accumulated and excess signal is added to increase the relative proportion of LasR that is signal-bound (Fig. 4 *C* and *E*). These attributes could help reset cells to the prequorum state when

bacteria leave high-density communities or if diffusion or flow conditions suddenly change. Such regulation could also enable cells to fine-tune the threshold density at which their own QS regulons are activated. This capability could be particularly important for cells in biofilms because nutrient gradients produced by high-density growth could make the expression of multigene QS regulons excessively costly for some cells.

The regulatory actions described above would require that cells modulate QteE activity, and additional work will be required to determine if this occurs. However, if QteE or other regulators provide a mechanism for cells to exert autonomous control over their QS regulons, this could have implications for the competing evolutionary hypotheses on QS's function. Evolutionary biologists have pointed out that the postulated group fitness benefits of QS may be diminished by circumstances that bacteria commonly encounter (12, 13). For example, biofilm growth could limit the reliability of population density measurements, competing species could cause interference by signal production or degradation, and the evolution of receptor mutants could force wild-type cells to produce an unfair share of public goods. Autonomous control of QS using QteE or other regulators could mitigate these problems because individual cells could regulate QS-controlled genes independently of the actions of neighboring cells.

Materials and Methods

Bacterial Strains, Growth Conditions, and Screening Methods. Bacterial strains and plasmids are described in Table S1. Transposon, plasmid, and strain construction are described in *SI Materials and Methods*. *P. aeruginosa* was grown at 37 °C in Luria–Bertani (LB) or Vogel-Bonner Minimal Media (VBMM), and *E. coli* was grown in LB or M63 medium (with antibiotics as needed to maintain plasmids) as described in *SI Materials and Methods*.

Exoproduct, Gene Expression, and Acyl–HSL Assays. GFP fluorescence in reporter assays was measured with a microplate reader using $\lambda_{ex}/\lambda_{em}$ of 435/535 nm and normalized to OD_{595} values. β-Galactosidase assays used the Tropix Galacto-Light Plus kit (Applied Biosystems) and a microplate reader for luminescence measurements. Acyl–HSLs were measured using pSC11/*lasI-lacZ*, pJN105P*araBAD-lasR*, and pECP61.5. Additional information on acyl–HSL, rhamnolipid, protease, elastase, and pyocyanin measurements is provided in *SI Materials and Methods*.

Immunoblots and Pulse-Chase Experiments. Immunoblots were performed using whole-cell lysates with equal amounts of total protein in each lane. Pulse-chase labeling used 80 μCi/mL of [^{35}S]methionine for 60 s at OD_{600} = 0.5 for *E. coli* and 0.8 for *P. aeruginosa*, followed by chase with excess cold methionine. Antibodies and detection methods are described in *SI Materials and Methods*.

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