



# Evolution of the *Pseudomonas aeruginosa* quorum-sensing hierarchy

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The bacterial pathogen *Pseudomonas aeruginosa* activates expression of many virulence genes in a cell density-dependent manner by using an intricate quorum-sensing (QS) network. QS in *P. aeruginosa* involves two acyl-homoserine-lactone circuits, LasI-LasR and RhlI-RhlR. LasI-LasR is required to activate many genes including those coding for RhlI-RhlR. *P. aeruginosa* causes chronic infections in the lungs of people with cystic fibrosis (CF). In these infections, LasR mutants are common, but *rhlR-rhlI* expression has escaped LasR regulation in many CF isolates. To better understand the evolutionary trajectory of *P. aeruginosa* QS in chronic infections, we grew LasR mutants of the well-studied *P. aeruginosa* strain, PAO1, in conditions that recapitulate an environment where QS signal synthesis by other bacteria might still occur. When QS is required for growth, addition of the RhlI product butyryl-homoserine lactone (C4-HSL), or bacteria that produce C4-HSL, to LasR mutants results in the rapid emergence of a population with a LasR-independent RhlI-RhlR QS system. These evolved populations exhibit subsequent growth without added C4-HSL. The variants that emerge have mutations in *mexT*, which codes for a transcription factor that controls expression of multiple genes. LasR-MexT mutants have a competitive advantage over both the parent LasR mutant and a LasR-MexT-RhlR mutant. Our findings suggest a plausible evolutionary trajectory for QS in *P. aeruginosa* CF infections where LasR mutants arise during infection, but because these mutants are surrounded by C4-HSL-producing *P. aeruginosa*, variants rewired to have a LasR-independent RhlIR system quickly emerge.

acyl-homoserine lactone | cystic fibrosis | LasR | RhlR | sociomicrobiology

The opportunistic pathogen *Pseudomonas aeruginosa* is the cause of a variety of human infections, including chronic infections of the airways of people with cystic fibrosis (CF). Expression of many *P. aeruginosa* virulence factors and other genes is regulated by two acyl-homoserine lactone (AHL) quorum-sensing (QS) systems, LasI-LasR (LasIR) and RhlI-RhlR (RhlIR). LasI catalyzes production of the diffusible QS signal *N*-3-oxododecanoyl homoserine lactone (3OC12-HSL). 3OC12-HSL binds to its cognate receptor, the transcription factor LasR, which can then activate expression of multiple genes, including *lasI*, *rhlI*, and *rhlR*. In an analogous manner, RhlI catalyzes production of the diffusible signal butyryl-HSL (C4-HSL), which binds to the transcription factor RhlR. Signal-bound RhlR then activates expression of *rhlI* and other genes, some of which overlap with the LasR regulon (for reviews, see refs. 1–3). In commonly studied *P. aeruginosa* strains such as PAO1, the RhlIR QS system requires induction by LasR. As a result, deletion of either *lasR* or *lasI* in strain PAO1 diminishes expression of QS-activated genes. LasR-null mutants of strain PAO1 also show impaired virulence in acute infection animal models (for example, see refs. 4 and 5).

These two AHL QS systems interact with a third system, which is mediated by the signal 2-heptyl-3-hydroxy-4-quinolone [*Pseudomonas* quinolone signal (PQS)] and its biosynthetic precursor 2-heptyl-4-quinolone (HHQ) (6–9). The *pqs* operon *pqsABCDE*, together with *pqsH*, codes for HHQ and PQS production. PQS and HHQ bind to the transcriptional regulator PqsR (also called

MvfR), which then activates expression of the PQS synthesis genes. The PQS system activates multiple genes, many of which are also regulated by LasR or RhlR (8–10). LasR activates expression of the PQS genes, and in turn, a product of *pqsE* increases expression of RhlR-activated genes (9–11).

A majority of QS-activated genes code for secreted factors or the production of secreted factors. Such factors can serve as public goods if they benefit the entire population of cells. Public goods production carries a metabolic cost to individual cells, and this in turn provides an incentive for the emergence of cheating cells that do not produce those goods. When *P. aeruginosa* PAO1 growth requires QS-regulated protease production, LasR mutant cheaters emerge, and these mutants have a fitness advantage (12–16). In some circumstances, the frequency of cheaters can become high enough to cause collapse of the population (14, 15). One mechanism whereby *P. aeruginosa* restricts infiltration by cheaters is policing mediated by the RhlR-induced production of cyanide (CN<sup>-</sup>) (15). RhlR mutants are more sensitive to CN<sup>-</sup> than the wild type, and both CN<sup>-</sup>-synthesis mutants and RhlR mutants are particularly vulnerable to cheating (15).

LasR mutations are common among *P. aeruginosa* isolated from the lungs of chronically infected CF patients (17–22). However, unlike strain PAO1, many LasR mutants from chronic infections have an active RhlIR QS system and display QS-regulated phenotypes when grown under laboratory conditions (18, 22). It is likely that the apparent differences in QS regulatory

## Significance

Mutations in the master quorum-sensing signal receptor LasR are common in *Pseudomonas aeruginosa* isolated from chronically infected lungs of cystic fibrosis patients. In many of these LasR mutant isolates, a quorum-sensing system involving the transcription factor RhlR has escaped dependence on LasR. We have modeled the escape of RhlR activity from LasR regulation in laboratory evolution experiments. We gain insights into the genetic basis of the escape and show that an escape variant has a fitness advantage over a RhlR-null derivative. We also propose a hypothesis for the course of *P. aeruginosa* quorum-sensing evolution in chronic cystic fibrosis lung infections. Our work points to RhlR as a potential target for therapeutic development.

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networks of clinical isolates in comparison with PAO1 reflect adaptation of *P. aeruginosa* during the long-term infections. We are interested in understanding why some clinical isolates from CF lungs maintain an active QS system in a LasR-null background, and we want to identify laboratory conditions that would give rise to such a phenotype. We discovered that the presence of C4-HSL or C4-HSL-producing bacteria in cultures of a LasR-null mutant led to a rapid emergence of LasR mutant variants capable of growth on casein. These variants had characteristics similar to those of many CF clinical isolates. During preparation of our manuscript, Oshri et al. (23) reported that LasR-null mutants with an active RhlIR system emerged in casein broth without added C4-HSL but only after a prolonged incubation of 4 to 6 wk. We propose a course of evolution for *P. aeruginosa* QS during CF lung infections, whereby once LasR mutants emerge among populations capable of C4-HSL production, they can rapidly evolve an active LasR-independent RhlIR QS system. We also present experiments leading to a plausible explanation for why RhlIR mutants are rare in the context of CF lung infections.

## Results

### Reprogramming the QS Circuit: A LasR-Independent RhlIR System.

The observation that many *P. aeruginosa* LasR-null isolates obtained from chronically infected CF lungs have a functioning RhlIR system (18, 22) suggests that regaining or maintaining expression of at least a subset of the QS regulon is advantageous in the context of a long-term infection. Although LasR-null mutants are not expected to activate expression of QS-regulated genes, in the CF lung they would be exposed to the QS signals produced by neighboring cells with an active LasIR QS system. Production of biologically relevant levels of C4-HSL has been reported for clinical isolates from CF lungs (18, 22, 24). Given the key role of C4-HSL in RhlR gene regulation, we asked whether C4-HSL could promote emergence of a PAO1 LasR mutant with an activated RhlIR QS system, under conditions that require QS for growth. We conducted a laboratory evolution experiment, in which we grew a LasR mutant with or without C4-HSL in casein broth, a minimal medium which contains casein as the sole carbon and energy source. Growth in casein broth requires QS-regulated production of the extracellular protease elastase. Expression of the elastase gene *lasB* in PAO1 is activated primarily by LasR, with RhlR playing a lesser role (25). Wild-type bacteria readily grow in casein broth, but LasR mutants do not. However, when we added 30  $\mu$ M C4-HSL to a monoculture of LasR mutants at the time of inoculation and again 4 d later, growth was evident in less than a week (*SI Appendix, Fig. S1*).

To test whether growth of the LasR mutant in casein broth was conditional or a fixed heritable trait, we transferred the growing cultures to fresh casein broth without added C4-HSL and continued to monitor growth. Growth was observed within a few days and continued with subsequent transfers and no additional C4-HSL. We saw a similar outcome with a PAO1 LasI mutant (*SI Appendix, Fig. S1*). Growth was not observed over a period of 4 wk for either the LasR or the LasI mutant when C4-HSL was not added to the casein broth (*SI Appendix, Fig. S1*). Our observations are consistent with a recent report that in casein broth without added C4-HSL, growth of LasR-null *P. aeruginosa* was evident after an incubation period of over 1 mo (23).

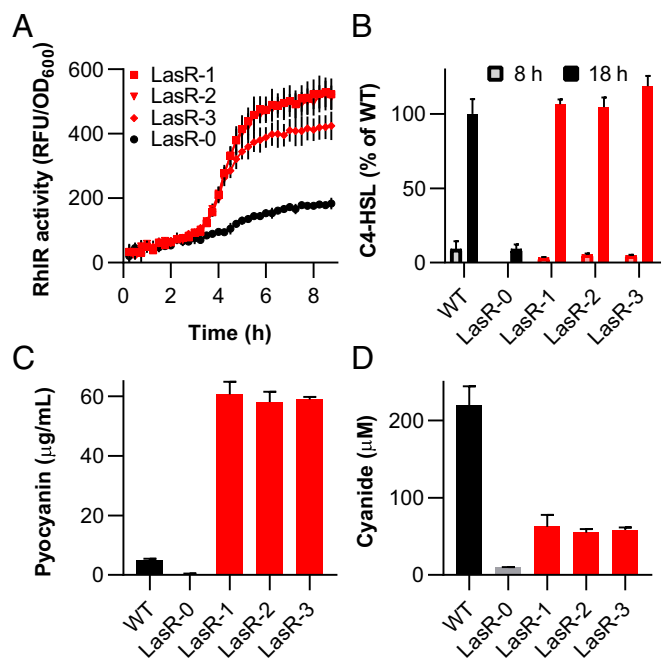
After several transfers, we used a skim milk agar plate assay to identify representative protease-secreting isolates from each of three biological replicates. We verified that these isolates were able to grow on casein as the sole carbon and energy source (*SI Appendix, Fig. S2*). It was possible that in contrast to wild-type PAO1, elastase production in the selected isolates did not require QS. To address this possibility, we added an AHL-degrading enzyme, AiiA lactonase (26), to casein broth cultures. Growth of the evolved variants was impaired by AiiA (*SI Appendix, Fig. S2*), indicating that protease production was regulated by an AHL-responsive factor.

### Reprogrammed LasR Mutants Have a Functioning RhlIR QS System.

We hypothesized that the LasR mutant variants, which grow in casein broth, had a functioning RhlIR system. To test this hypothesis, we transformed several such variants with a reporter plasmid, which contains the RhlR-responsive *rhlA* promoter fused to *gfp* (18). After 8 h of growth in LB-Mops broth, these variants displayed GFP fluorescence well above that of the parent LasR mutant (Fig. 1A). This result indicates that the reprogrammed variants had an active RhlIR QS system despite having a LasR-null mutation. These variants also produced C4-HSL and CN<sup>-</sup>, unlike the parent LasR mutant strain. Furthermore, when grown under phosphate limitation, these isolates produced pyocyanin at levels more than 10-fold higher than those of the wild-type PAO1 (Fig. 1B–D), which is likely due to the loss of repression by the LasR-dependent negative regulator RsaL (27). The evolved strains displayed characteristics similar to many of the LasR-null isolates from chronically infected lungs of CF patients described by Feltner et al. (18).

### Mutations in *mexT* Lead to an Active RhlIR QS System in the LasR Mutant.

To understand the genetic changes responsible for the reprogrammed QS phenotype, we sequenced the genomes of the parent LasR mutant, three protease-positive isolates, and three protease-negative isolates from the casein broth evolution experiments. We identified one gene, *mexT* (PA2492), which was mutated or deleted in all three protease-producing isolates but not in the non-protease-producing isolates or the parent strain (Table 1). Two of the protease-producing isolates had unique nonsynonymous point mutations in the *mexT* coding sequence, and the third had a 19.7-kb deletion, which included all of *mexT*



**Fig. 1.** Characteristics of three protease-producing LasR mutant variants (LasR-1, LasR-2, and LasR-3) derived from the LasR mutant (LasR-0). (A) RhlR activity of each variant and their nonprotease producing parent LasR-0, measured as GFP fluorescence and reported as relative fluorescence units (RFU). All strains contained the  $P_{rhlA}$ -*gfp* fusion plasmid pProbe-GT- $P_{rhlA}$ -*gfp*. (B) Relative levels of C4-HSL in culture fluid after 8 or 18 h of cell growth. Values are relative to those produced by *P. aeruginosa* PAO1 (WT) at 18 h. (C) Pyocyanin production by *P. aeruginosa* PAO1, the parent LasR mutant, and LasR mutant variants. (D) Cyanide levels of cultures. Data in A are means of triplicate experiments, and data in B–D are means of duplicate experiments. In all panels, error bars represent the SEM.

**Table 1. Genetic changes in protease-producing variants of PAO1 LasR and LasI mutants**

Variant*	<i>mexT</i> mutation	MexT change	Other affected genes
<b>LasR mutants</b>			
LasR-1	T353A	L118Q	None
LasR-2	A122G	S41G	None
LasR-3	Δ(2793006-2812975)	Δ <i>mexT</i>	PA2475, <i>dsbG</i> , PA2477-PA2490, <i>mexS</i> , <i>mexE</i> , <i>mexF</i>
LasR130-1	Δ(2808103-2808997)	Truncation	NA <sup>†</sup>
LasR130-2	Δ(A768)	Frameshift	NA <sup>†</sup>
LasR130-3	A559C	T560P	NA <sup>†</sup>
LasR131-1	A55C	T19P	NA <sup>†</sup>
LasR131-2	G31C, C478G	V11L, L160V	NA <sup>†</sup>
LasR131-3	NP <sup>‡</sup>	NP <sup>‡</sup>	NA <sup>†</sup>
<b>LasI mutants</b>			
LasI-1	A55C	T19P	<i>psdR</i>
LasI-2	A55C	T19P	<i>psdR</i>
LasI-3	A55C	T19P	<i>psdR</i> , <i>fleQ</i>

\*Variants LasR-1–3 were isolated from casein broth inoculated with the LasR mutant only, LasR130-1–3 and LasR131-1–3 were isolated from casein broth coinoculated with the LasR mutant and clinical *P. aeruginosa* isolates E130 or E131, and LasI-1–3 were isolated from casein broth inoculated with the LasI mutant. Further details about the variants can be found in [SI Appendix, Table S1](#).

<sup>†</sup>NA, other genes were not analyzed in these isolates.

<sup>‡</sup>NP, no product. We could not amplify *mexT* for sequencing.

along with the neighboring genes spanning PA2475 to PA2494. We also found *mexT* mutations in three protease-producing LasI mutant variants that we sequenced (Table 1) (28). *mexT* was also mutated in the slowly-evolved LasR<sup>−</sup> variants reported by Oshri et al. (23).

MexT is a transcription factor that affects expression of at least 40 genes (29), and its overexpression negatively affects known RhlR-regulated phenotypes, including C4-HSL, CN<sup>−</sup>, elastase, and pyocyanin production (30, 31). To confirm that the *mexT* mutations in the evolved isolates were involved in reprogramming QS so that RhlR was functioning in the absence of LasR, we deleted *mexT* from the PAO1 LasR-null mutant. We also deleted *psdR* because inactivation of this gene has been shown to improve growth on casein independently of QS and *psdR* mutations commonly arise early in evolution experiments on casein (16, 23). Surprisingly, mutations in *psdR* were present only in the analyzed evolved isolates of the LasI mutant but not the LasR mutant (Table 1). Consistent with the idea that MexT-inactivating mutations result in an altered QS hierarchy, the PAO1 LasR-PsdR-MexT triple mutant grew in casein broth, displayed RhlR gene activation measured with the P<sub>*rhIA-gfp*</sub> reporter, produced levels of C4-HSL comparable to wild-type PAO1, and produced high levels of pyocyanin (Fig. 2). Deletion of *rhlR* from the triple mutant abrogated these QS-dependent phenotypes ([SI Appendix, Fig. S3](#)). Thus, we concluded that inactivation of MexT results in reprogramming of the QS circuits such that RhlR QS is independent of LasR. As noted above, this is in agreement with Oshri et al. (23).

**A Functional RhlR QS System Provides a Competitive Advantage to Cells in Coculture.** When grown on casein as the sole carbon and energy source, LasR mutants, which do not incur the metabolic cost of QS, have a negative frequency-dependent fitness advantage over wild-type strain PAO1 in coculture (12, 13). For the same reason, a similar advantage could be expected for a LasR mutant in competition with an isogenic LasR-MexT mutant, which bears the cost of protease production for the coculture. To the contrary, we found that LasR-PsdR-MexT mutants outcompeted LasR-PsdR mutants when inoculated either at low or high initial frequency (Fig. 3A). The fitness advantage of the LasR-PsdR-MexT mutant could be due to either the *mexT* mutation itself or the activated RhlR system in the MexT mutant background. The latter possibility is attractive because previous work showed that the RhlR-dependent production of CN<sup>−</sup> by wild-type strain PAO1 provides a fitness advantage over LasR or

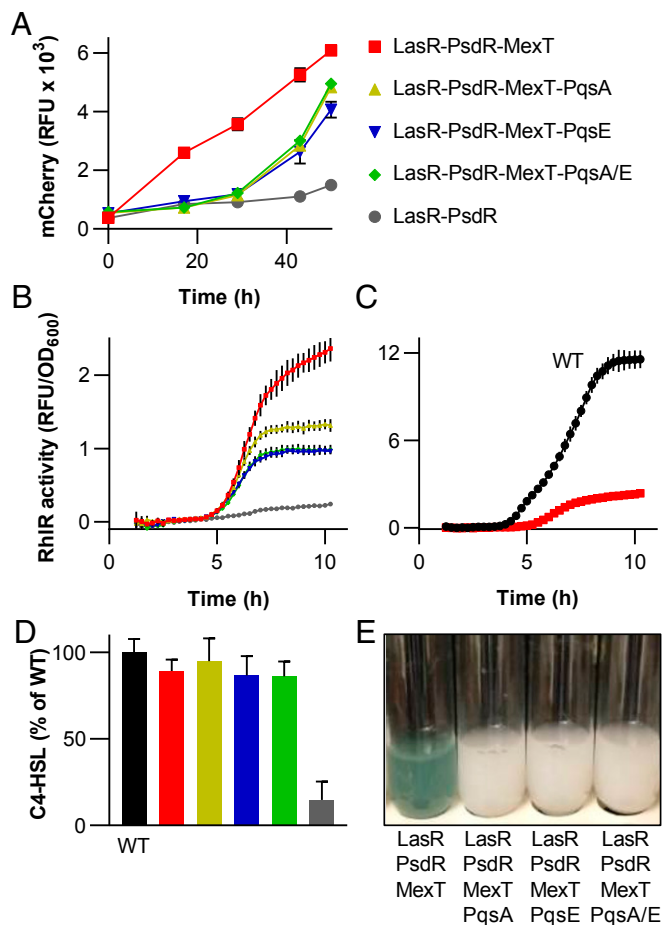
RhlR mutants (15). To distinguish between the two possible explanations, we performed competition experiments with a LasR-PsdR-MexT mutant and a LasR-PsdR-MexT-RhlR mutant. Although the LasR-PsdR-MexT mutant bears the costs associated with an active QS system, it still outcompeted the QS-null LasR-PsdR-MexT-RhlR mutant (Fig. 3B). This result is consistent with the idea that RhlR induces production of a self-produced stressor or toxin and corresponding resistance factors.

To test whether policing observed in the competition experiments was mediated by the RhlR-regulated production of CN<sup>−</sup> (15), we generated a CN<sup>−</sup>-deficient mutant, LasR-PsdR-MexT-HcnC, and competed it against either the LasR-PsdR or LasR-PsdR-MexT-RhlR mutants ([SI Appendix, Fig. S4](#)). In both competitions, the LasR-PsdR-MexT-HcnC mutant outcompeted the QS-null strain in a similar fashion as in the above experiments, indicating that the fitness advantage of the LasR-PsdR-MexT mutant cannot be explained by cyanide-mediated policing.

#### The Relationship Between PQS and RhlR Independence from LasR.

Overexpression of MexT has been shown to strongly decrease transcript levels of the *pqsABCDE* operon (29), which is involved in biosynthesis of PQS. The PQS system induces many RhlR-regulated phenotypes (9, 10). Thus, *mexT* mutations might lead to a functioning RhlR QS in LasR mutants by activation of *pqsABCDE* transcription. To test this hypothesis, we deleted, independently and together, *pqsA* and *pqsE* from the LasR-MexT-PsdR triple mutant. PqsA is an essential component of the PQS synthesis operon, and its deletion results in loss of PQS production (6). PqsE is an effector protein that modulates the activity of the PQS system (9), and it has been shown to function even in the absence of PQS or PqsR (11). Recently, it was shown to be involved in the synthesis of a putative alternative ligand for RhlR (32). As previously reported, deletion of either gene resulted in a loss of pyocyanin production (Fig. 2E) (7, 11, 32). However, growth on casein and RhlR gene activation, measured with the P<sub>*rhIA-gfp*</sub> reporter, were only partially reduced, with no additive effect by inactivation of both *pqsA* and *pqsE* (Fig. 2A and B). C4-HSL levels were not affected by either mutation (Fig. 2D). These results indicate that genes in the *pqsABCDE* operon contribute to but do not fully account for the observed RhlR-independent phenotype.

**LasR Mutants Can Evolve the Ability to Grow on Casein in Coculture with C4-HSL-Producing Clinical Isolates.** In chronic CF lung infections, the emergence of LasR mutants with an active RhlR



**Fig. 2.** The influence of *mexT*, *pqsA*, and *pqsE* mutations on RhlR activity in the *P. aeruginosa* LasR-PsdR mutant. (A) Growth of *P. aeruginosa* mutants in casein broth. Bacteria were tagged with *mCherry*, and fluorescence (RFU) was used as a proxy for growth, as described in *Materials and Methods*. (B) RhlR activity in the LasR-PsdR mutant and mutants derived from this strain, measured as GFP fluorescence (RFU). All strains contained the  $P_{rhlA}$ :*gfp* fusion plasmid pProbe-GT- $P_{rhlA}$ :*gfp*. (C) RhlR activity in strain PAO1 compared with RhlR activity in the LasR-PsdR-MexT mutant, measured as in B. (D) Relative concentrations of C4-HSL produced by the WT and mutants after 18 h. (E) Image of culture tubes after 2 d of growth of the indicated strains in casein broth. The LasR-PsdR-MexT mutant culture is blue green as a result of pyocyanin production. Data in A and D are means of duplicate experiments, and data in B and C are means of triplicate experiments. In all panels, error bars represent the SEM.

system may be encouraged by other bacteria that produce C4-HSL. Although it cannot provide proof, one test of this hypothesis is to coinoculate a LasR mutant with a C4-HSL-producing clinical isolate and ask whether reprogrammed LasR mutants emerge. We therefore selected three clinical *P. aeruginosa* isolates, E92, E130, and E131, which secrete little elastase but produce more C4-HSL than does our wild-type strain PAO1 (18), and coinoculated casein broth with each of the isolates along with our *mCherry*-marked PAO1 LasR mutant (Fig. 4). Reprogrammed PAO1 LasR mutants emerged in cocultures with E131 and 132 but not E92. We did not investigate why such variants did not arise during coinoculation of the LasR mutant with E92. After two transfers of the cocultures with E130 and E131, we identified protease-positive isolates on skim milk agar plates (*SI Appendix*, Fig. S5) and verified that these were from the PAO1 LasR mutant lineage by measuring *mCherry* fluorescence of the isolates. Sequencing of *mexT* revealed nonsynonymous mutations and deletions in five out of six selected isolates (Table 1). We were

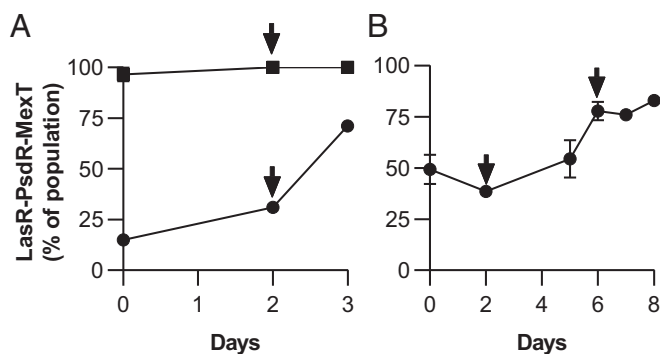
unable to amplify *mexT* in the sixth isolate, which suggests that there was a large deletion encompassing *mexT*. The results of these coinoculation experiments indicate that evolution of an active RhlIR QS system in a LasR-null *P. aeruginosa* strain could be driven by cooccurrence with some but not all C4-HSL producing bacteria.

## Discussion

*P. aeruginosa* has a complicated QS network, which likely plays a significant role in its ability to inhabit many environments (3). QS appears to be important for *P. aeruginosa* fitness. For example, QS-null mutants are attenuated for virulence in acute animal infection models (4, 5). It is therefore notable that LasR mutants commonly arise in chronic CF lung infections (17–21). The presence of LasR-null, RhlR-active isolates in chronically infected CF lungs (18, 22) suggests that this environment exerts pressure on LasR mutant cells to restore at least part of their QS regulon through the activation of the RhlIR circuit. Here we demonstrate that when a simple selective pressure is applied to a LasR-null mutant of strain PAO1, it can rapidly evolve a LasR-independent, active RhlIR QS system (Fig. 4 and *SI Appendix*, Fig. S1). Our results suggest that in a mixed culture, production of C4-HSL by a QS-proficient strain can facilitate the alteration of the QS hierarchy. It is not hard to imagine that similar interactions could take place in the context of a chronic *P. aeruginosa* infection. The reprogrammed LasR-deficient, RhlR-active variant out-competed the parental LasR mutant as well as the RhlR mutant of rewired variants (Fig. 3). The competitive advantage of the LasR-PsdR-MexT strain over the QS-null strains could not be explained by cyanide-mediated policing (*SI Appendix*, Fig. S4), which suggests that other policing mechanisms may contribute to the stabilization of QS-regulated cooperation in *P. aeruginosa*.

The rewiring of QS circuits such that RhlIR functions in a LasR-null background is caused by inactivation of the LysR-type transcriptional regulator MexT (Fig. 2 and ref. 23). Mutations in *mexT* have been reported among isolates obtained from chronically infected CF lungs (17) and from other *P. aeruginosa* infections (33, 34). Among the genes activated by MexT are those in the operon that encodes the efflux pump MexEF-OprN (29). Overexpression of MexT and MexEF-OprN leads to the attenuation of known RhlR-regulated phenotypes that include the production of C4-HSL, elastase, CN<sup>-</sup>, and pyocyanin (30, 31). Interestingly, although overexpression of MexEF-OprN results in lower levels of PQS (29, 30, 35), our results indicate that activation of the RhlIR QS system by inactivating mutations in *mexT* is only partially mediated through the PQS system (Fig. 2). This observation suggests that one or more other genes in the MexT regulon influence RhlIR QS. It is unlikely that MexT directly affects the expression of *rhlR* or *rhlI*, because neither of these genes contains the conserved MexT binding motif in the promoter region (29). Furthermore, inactivation of MexEF-OprN in a LasR-null background results in many of the same phenotypes that are observed for the inactivation of MexT (*SI Appendix*, Fig. S6, ref. 23). Finally, in our competition experiments (Fig. 3), the decrease in frequency of the RhlR-null mutant (Fig. 3B) was slower than that of the LasR-null mutant (Fig. 3A). Because the RhlR mutant also contains a *mexT* deletion, it is possible that the enhanced resilience of this mutant in competition is attributable to a QS-independent benefit to cells resulting from inactivation of MexT, as proposed by Oshri et al. (23). This effect could be either direct or indirect because the MexT regulon includes several genes coding for proteins of unknown function (29).

Our experiments and those of Oshri et al. (23) indicate that RhlIR QS system can be activated in the absence of an active LasR, and this requires only a mutation in *mexT*. Based on earlier studies (12, 13, 21), we propose that in the CF lung, and in similar environments, LasR mutants arise because they are either social cheaters on QS-intact bacteria, have a nutritional advantage over the wild type, or both. However, because some



**Fig. 3.** Competition between the LasR-PsdR-MexT mutant and QS mutants that cannot grow in casein broth by themselves. (A) Competition experiments with the LasR-PsdR parent. The competitions were initiated with either a low (circles) or high (squares) frequency of LasR-PsdR-MexT. (B) Competition with the LasR-PsdR-MexT-RhlR mutant. Arrows indicate a transfer to fresh casein broth (inoculum 2% vol/vol). Data are the means of duplicate experiments, and error bars represent the SEM.

QS-regulated products are important in CF lung infections, a variant that uses RhlR as the primary AHL QS regulator emerges from the QS-null subpopulation. Evolution of such strains is accelerated by C4-HSL, which can be provided by neighboring cells. Our competition experiments provide a plausible explanation for why RhlR mutants are rare among CF isolates. That is, cells with an active RhlR have fitness advantage over RhlR mutants. Our data indicate that even though LasR is often considered to be at the apex of the *P. aeruginosa* hierarchy, this hierarchy can be easily reprogrammed such that the PQS and RhlR systems are independent of LasR through a surprisingly simple genetic change. Although many questions remain, our work and other recent publications [accompanying article Chen et al. (36) and refs. 23, 32, and 37] point toward RhlR QS as a potential anti-QS therapeutic target.

## Materials and Methods

**Bacteria and Growth Conditions.** Bacteria used are described in *SI Appendix, Table S1*. *P. aeruginosa* was grown in Luria-Bertani broth buffered with 50 mM 3-(*N*-morpholino) propanesulfonic acid, pH 6.8 (LB-Mops broth), casein broth (38) containing 1% sodium caseinate as the sole source of carbon and energy, casamino acid broth (38) with 1% casamino acids as the sole source of carbon and energy, or *Pseudomonas* P broth (20 g/L pancreatic digest of gelatin, 1.4 g/L magnesium chloride, 10 g/L potassium sulfate) (39), as indicated. *Escherichia coli* was grown in LB broth. For colony growth we used LB agar (1.5% agar) or milk agar plates (13), as indicated. Liquid cultures were grown at 37 °C with shaking. Where indicated, gentamicin was included in media at a concentration of 100 µg per mL (Gm100).

**Construction of *P. aeruginosa* Mutants.** In all experiments, we used strain *P. aeruginosa* PAO1-UW (40). However, our strain differs from the originally sequenced strain by ~20 SNPs and lacks an inactivating 8-bp insertion (41) at the *mexT* locus that is present in the sequenced strain. Mutants with deletions of *lasR*, *lasI*, *mexT*, *mexEF*, *psdR*, *rhlR*, *pqsA*, and *pqsE* were derived from PAO1-UW by a homologous recombination-based two-step allelic exchange approach (42). Briefly, 300–500 bp of DNA flanking the gene of interest were PCR-amplified and cloned in pEXG2 by using *E. coli*-mediated DNA assembly (43) or standard restriction enzyme-based techniques. The primers used to generate the pEXG2 knockout plasmids are listed in *SI Appendix, Table S2*. *E. coli* S17-1 was used to deliver knockout plasmids to *P. aeruginosa* via conjugation. Merodiploids were selected by plating on *Pseudomonas* Isolation agar containing Gm100, and deletion mutants were then selected on LB agar containing 10–15% sucrose and no sodium chloride. All deletion mutations were confirmed by PCR of genomic DNA.

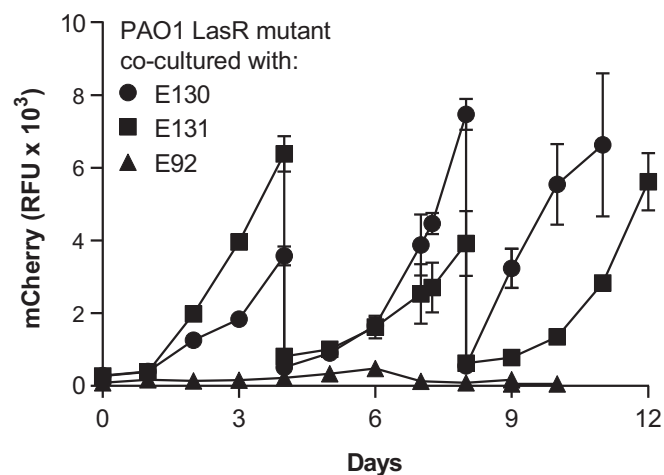
Because growth in casein broth cannot be measured using optical density, we used mCherry fluorescence of the LasR and LasI mutant strains as a proxy for growth. We integrated a constitutively expressed mCherry at the *att* site using the mini-Tn7 insertion system (44). Briefly, *P. aeruginosa* mutants were

transformed with mCherry-containing pUC18-mini-Tn7-Gm and pTNS2 plasmids. Gentamicin-resistant transformants were selected on LB agar containing Gm100. Integration of the mCherry-containing cassette was confirmed by PCR of genomic DNA. To excise the gentamicin resistance marker, we transformed strains with pFLP2 and selected transformants on LB agar containing 5% sucrose and no sodium chloride. Marker excision was confirmed by PCR. mCherry was present in all PAO1-derived strains. Expression of mCherry does not have a measurable metabolic cost when strains with and without mCherry are grown in LB-Mops.

**Evolution Experiments and Growth in Casein Broth.** To initiate evolution experiments, we inoculated 3 mL of casein broth in 18-mm tubes with either the LasR mutant or the LasI mutant. Inocula were 150 µL of an overnight culture grown in LB-Mops broth, which corresponded to about  $6\text{--}9 \times 10^8$  colony forming units (CFU). We added 30 µM C4-HSL (Cayman Chemical) to three of six tubes at the start of the experiment and again at day 4. When an increase in mCherry fluorescence was observed, we transferred 300 µL to 3 mL fresh casein broth. After several transfers, we incrementally decreased transfer volumes to 150, 100, 50, and 30 µL. Coculture evolution experiments were performed in a similar fashion. We added 60 µL of an overnight culture of each strain to 3 mL casein broth, and mCherry fluorescence was used to monitor growth of the LasR mutant. For the first and second passages, we transferred 300 and 150 µL of the culture to 3 mL casein broth, respectively.

To identify protease-producing variants, we isolated colonies on LB agar plates and then patched the colonies on skim milk agar plates (13). After about 18 h, the presence of a clear halo around a colony indicated protease production. To grow selected isolates on casein, we added 60 µL of an overnight LB-Mops broth culture to 3 mL casein broth. For experiments with *Bacillus thuringiensis* lactonase AiiA, we added either AiiA (100 µg lactonase per mL culture) or only buffer to the casein broth at the time of inoculation. AiiA was purified as described elsewhere (45). To monitor mCherry fluorescence, we transferred 150 µL to a 96-well plate and measured fluorescence intensity by using a BioTek Synergy H1 microplate reader.

**Competition Experiments.** Overnight LB-Mops broth cultures were used to inoculate 3 mL of casein broth. A 1% inoculum (vol/vol) of the LasR-PsdR-MexT mutant was used, and competing bacteria were inoculated at 0.1, 1, or 10% (vol/vol). We performed transfers of 2% (vol/vol), as indicated. To determine cell yields, we obtained CFU on LB agar and patched 100–120 colonies on milk agar plates. Colonies surrounded by a clear halo were scored as LasR-PsdR-MexT mutants.



**Fig. 4.** Emergence of PAO1 LasR mutant variants from coinoculation of PAO1 LasR mutant with clinical *P. aeruginosa* isolates in casein broth. PAO1 LasR mutant was tagged with mCherry and its growth was measured by monitoring mCherry fluorescence (RFU), as described in *Materials and Methods*. The clinical isolates were E92 (triangles), E130 (circles), and E131 (squares). Cocultures with E130 and E131 were transferred on days 4 and 8; those with E92 were transferred on days 6 and 9 (inoculum 2% vol/vol). Data are means of duplicate experiments, and error bars represent the SEM.

**Assays for RhlR Activity, C4-HSL, CN<sup>-</sup>, and Pyocyanin.** We measured RhlR activity in cells grown in LB-Mops broth as previously described, by using a reporter plasmid containing a P<sub>rhlA</sub>-gfp fusion (18). We used a previously described bioassay to measure C4-HSL (46). We measured CN<sup>-</sup> concentration as follows. Cells were grown with shaking (200 rpm) in 45 mL of LB-Mops broth for 17 h. Cells were removed from the culture fluid by centrifugation, and 450  $\mu$ L of 10 N sodium hydroxide was added to the fluid. The CN<sup>-</sup> in the base-treated fluid was then measured with a CN<sup>-</sup> electrode (Cole-Parmer). We prepared a CN<sup>-</sup> standard curve by using freshly prepared potassium cyanide in LB-Mops containing 1% (vol/vol) of 0.1 N sodium hydroxide. For pyocyanin measurements, we grew cells in *Pseudomonas* P broth for 20 h. Inocula were from overnight LB-Mops cultures (0.1% vol/vol). We added 2 mL chloroform to the cultures and vortexed for 30 s. After removal of the aqueous phase we added 800  $\mu$ L of 0.2 N hydrochloric acid and then measured absorbance of the aqueous fraction at 520 nm and multiplied the value by 17.072, as described elsewhere (47).

**Genome Variant Analysis.** DNA was isolated from bacteria grown in LB-Mops broth by using a Gentra Puregen kit (Qiagen). We used Illumina MiSeq to complete whole-genome sequencing with paired 150-bp reads and StrandNGS version 3.1 (Strand Life Sciences) to align the reads to the published sequence of *P. aeruginosa* PAO1 (accession no. NC\_002516) and to identify genetic polymorphisms including point mutations, deletions, insertions, and inversions (48). Genomic sequences have been deposited at NCBI (BioProject PRJNA514363).

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